Graduate School of
Systemic Neurosciences
LMU Munich

Molecular mechanisms of EphB-ephrinB endocytosis in neural cells

Jorg Körner

München 2015

Molecular mechanisms of EphB-ephrinB endocytosis in neural cells

Dissertation of the
Graduate School of Systemic Neurosciences
Ludwig-Maximilians-Universität, München

Jorg Körner

Eingereicht am 05.November 2015

Supervisor: Prof. Rüdiger Klein

2nd reviewer: Dr. Ilona Grunwald Kadow

External reviewer: David Wilkinson, PhD

Date of Oral Defense: 29th January, 2016

Table of contents

List of Figures and Tables

List of Abbreviations

°C	Degrees celsius
α	Anti
aa	Amino acid
ABR	Active breakpoint cluster region-related protein
ADAM	A-Disintegrin-And-Metalloprotease
ALS	Amyotrophic lateral sclerosis
ALS2	Alsin
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
BARS	BrefeldinA-ADP ribosylated substrate
BCR	Breakpoint cluster region-related protein
BDNF	Brain-derived neurotrophic factor
BSA	Bovine Serum Albumine
CC	Coiled-coil
CCP	Clathrin-coated pit
Cdc42	Cell division cycle 42
CLIC/GEEC	Clathrin-independent carrier/ GPI-anchored protein-enriched early endocytic compartment
CMB	CellMask™ Blue
CME	Clathrin-mediated endocytosis
CMV	Cytomegalovirus
C-terminus	Carboxy terminus
CTG	CellTracker™ green
DAPI	4',6-diamidino-2-phenylindole
Dbl	Diffuse B-cell lymphoma oncogene
DH	Dbl homology
DHR	DOCK homology region
DIV	Day(s) in vitro
DMEM	Dulbecco's modified Eagle's medium
DNA	Desoxyribonucleic Acid
DOCK	Dedicator of cytokinesis protein
EDTA	Ethylenediaminetetraacetic acid
EEA1	Early endosome antigen 1
EGF	Epidermal Growth Factor
EGFP	Enhanced green fluorescent protein
EGFR	Epidermal Growth Factor Receptor
ELMO	Engulfment and cell motility protein
Ephrin	Eph receptor interacting protein
Eph	erythropoietin-producing hepatocellular carcinoma receptor
FARP2	FERM, RhoGEF and pleckstrin domain-containing protein 2
FBS	Fetal bovine serum

Fc	Fragment crystallizable region
Fig.	Figure
FN	Fibronectin Type III domain
FRET	Förster resonance energy transfer
GAP	GTPase-activating protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDI	Guanine dissociation inhibitor
GDNF	Glia cell line-derived neurotrophic factor
GDP	Guanine di-phosphate
GEF	Guanine exchange factor
GFP	Green fluorescent protein
GIT1	G protein-coupled receptor kinase-interactor 1
GPI	Glycosylphosphatidylinositol
GPCR	G protein-coupled receptor
GRAF1	GTPase regulator associated with focal adhesion kinase-1
GRIP	Glutamate receptor interacting protein
GTP	Guanine tri-phosphate
HMHA1	Human Minor Histocompatibility Antigen1
HRP	Horseradish peroxidase
IF	Immunofluorescence
Ig	Immunoglobulin
ITSN	Intersectin
JAK	Janus kinase
JM	Juxtamembrane
LBD	Ligand binding domain
LTP	Long-term potentiation
MT1-MMP	Membrane type-1 matrix metallo-proteinase
Net1	Neuroepithelial cell-transforming gene 1 protein
NMDA	N-methyl-D-aspartate
ns	Non statistically significant
N-terminus	Amino-terminus
OCRL-1	Oculocerebrorenal syndrome of Lowe
oligo	Oligonucleotide
OPHN1	Oligophrenin1
PAK	α-p-21-activated kinase
PBS	Phosphate-buffered Saline
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptor
PDZ	Psd-95, Dlg and ZO1
PFA	Paraformaldehyde
PH	Pleckstrin homology
PIK3	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIK3R2	Phosphatidylinositol 3-kinase regulatory subunit beta
PIP_2	Phosphatidylinositol 4,5-bisphosphate

PIX	PAK-interacting exchange factor
Rac1	Ras-related C3 botulinum toxin substrate 1
Ras	Rat sarcoma viral oncogene homologue
RBD	Receptor binding domain
RET	RET proto oncogene
RGC	Retinal ganglion cell
Rho	Ras-homologue
RNA	Ribonucleic Acid
RTK	Receptor Tyrosine Kinase
RT-PCR	Reverse Transcriptase PCR
ROCK	Rho-associated coiled-coil-containing protein kinase
SAM	Sterile- motif
SEM	Standard error of the mean
SFK	Src-family kinase
SH	Src homology
SHIP2	SH2 domain containing inositol 5-phosphatase 2
siRNA	Short interfering RNA
Src	Sarcoma virus transforming gene product
Stef	SIF and Tiam1-like exchange factor
Tiam1	T-lymphoma invasion and metastasis-inducing protein1
TM	Transmembrane
TrkB	Tropomyosin receptor kinase B
V	Volume
VEGFR	Vascular endothelial growth factor receptor
WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP-family verprolin homologous protein
WB	Western blot

Abstract

Eph Receptors (Eph) belong to the largest group of receptor tyrosine kinases. Upon binding their membrane-tethered ligands, the ephrins, they regulate a great number of physiological processes including axon guidance and synapse formation. A unique feature of the Eph-ephrin interaction is the induction of bidirectional signalling into both Eph (forward) and ephrin (reverse) expressing cells. In this manner Ephs and ephrins can trigger repulsive guidance responses in a bidirectional manner.

An important step in Eph-ephrin signal transduction is the removal of Eph-ephrin complexes from the cell surface to turn the initial contact between two opposing cells into repulsion. This can be achieved by trans-endocytosis of Eph-ephrin complexes, during which the entire receptor-ligand complex including patches of cell membrane is taken up by either cell. The molecular mechanisms regulating this process, especially in the reverse direction, are poorly understood. Previous studies have often relied on stimulation with soluble proteins, which may differ greatly from a more physiologically relevant, cell-contact-mediated interaction of membrane-tethered Ephs and ephrins. Therefore this study sought to determine the key players in Eph-ephrin reverse endocytosis using a functional assay that depends on cell-cell contact. I conducted a systematic analysis of the Rho GTPase subfamilies (Rac, Rho, Cdc42) to decipher their respective involvement in different modes of Eph-ephrin endocytosis (forward/reverse, soluble/cell-cell). This study revealed that the Rac subfamily of GTPases (Rac1, Rac3, RhoG) is required for EphB trans-endocytosis from the EphB-expressing cell into the opposing ephrinB+ cell, but not for endocytosis of a soluble EphB protein. In addition to the experiments in human cell lines, co-culture of primary murine neurons with EphB-expressing cells implicated the same Rac-dependent mechanism for Eph-ephrin trans-endocytosis in a physiologically relevant setting. While I found no regulatory role for Rho GTPases of the Cdc42 subfamily (Cdc42, RhoQ and RhoU), knockdown of RhoA subfamily members (RhoA, RhoB) led to an increase in endocytosis of soluble EphB2 into ephrinB+ cells, but no change in EphB trans-endocytosis. An image-based siRNA screen of the Rho family guanine exchange factors (GEFs) and GTPase activating proteins (GAPs) revealed the Rac-specific GEF Tiam2 as a key upstream regulator of Rac in EphB trans-endocytosis upon EphB-ephrinB

engagement of two opposing cells. Furthermore the closely related Tiam1 is also required for trans-endocytosis in some cellular contexts. Analogously to the specific requirement of Rac activity in EphB trans-endocytosis, Tiam family proteins are dispensable for endocytosis of soluble EphB2 into ephrinB+ cells.

In summary, the work presented here provides new insights into the molecular regulation of Eph-ephrin endocytosis, which is relevant in physiological contexts, and outlines significant differences between endocytosis mediated by cell contact and endocytosis after stimulation with soluble proteins.

1 Introduction

An important step in evolution was the occurrence of multicellular life forms, since organisms consisting of more than one cell have the significant advantage of being able to divide the numerous tasks necessary for survival and procreation between different specialised cell types. However, with the transition to multicellularity come new challenges. In order to co-ordinate their behaviour and functions, multicellular organisms not only need to interact with cues from the external world, but the various cells also need to be able to communicate with each other to regulate their activities. This is especially true in order to achieve a meaningful organisation of cells within a multicellular organism. A process which requires the spatial distribution of specific cell types to be tightly regulated commencing from the development of the organism. While this task already requires an astonishing level of complexity, even in simple organisms such as algae, sponges or jellyfish, the complexity scales up even more when considering the development of the vertebrate nervous system. Here, billions of neurons need to form up to a thousand synaptic connections each onto their correct partner cells, which in some cases need to cover distances of several meters, for example, in the spinal cord of giraffes (Kandel 2013).

To communicate successfully with surrounding cells and the external world, cells have developed an intricate system of sensory proteins, which enables them to sense, process and convert signals coming from outside sources. These external cues can come in a great variety of forms, ranging from soluble molecules over elements of the extracellular matrix to molecules bound on the membranes of other cells. With a few exceptions, such as intracellular hormone receptors, sensory proteins of the cells are receptors that are present on the surface of the cell membrane. Given the diversity of possible interactors, these receptors also appear in multiple forms, all with their specific ligands and a specific intracellular machinery connected to translate the signal into the cell (Alberts 2015). These signalling events triggered by the activation of the receptor through its ligand can be very direct, such as changing membrane permeability for certain ions, or involve a whole cascade of intracellular signalling molecules. Accordingly, the cellular response can be

short-term, for example changing its morphology through rearrangement of the cytoskeleton, or long-term, as in the case of changes in gene expression.

There are three main families of cell surface receptors. The first are ligand-gated ion channels, which increase membrane permeability for certain ions after activation through their ligand. They are especially important in the nervous system, where, for example, glutamate-gated cation channels such as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) or N-methyl-D-aspartate (NMDA) are important for the signal transduction at synapses. Upon binding of the ligand, the G-protein dissociates from the receptor and separates into its subunits, which in turn act on different signalling molecules in the cell. The third large group of receptors are the enzyme-linked receptors, which in most cases contain only a single transmembrane domain. Many of the enzyme-linked receptors are protein kinases, which exert their function through the phosphorylation of target proteins, thus altering their biochemical properties. Among the enzyme-linked receptors, the Receptor-Tyrosine-Kinases (RTKs) are the most intensively studied and well characterised subfamily, and have many varying functions including control of cell division and the cell cycle, cell- cell communication, cell motility and cell survival (Alberts 2015). A group of RTKs that has been identified to play a prominent role during various aspects of development are the erythropoietin-producing hepatocellular carcinoma receptors (Eph) with their corresponding eph-receptor-interacting protein (ephrin) ligands. They are the main focus of this study and thus are introduced in more detail in the next chapter.

1.1 Eph receptors and ephrin ligands

Eph receptors and their corresponding ephrin ligands play crucial roles in a great variety of different physiological and pathological processes. While traditionally mainly implicated in many developmental processes such as cell migration, cell sorting and tissue-border formation (reviewed in (Batlle & Wilkinson 2012, Klein 2012); it has become increasing clear that the Eph-ephrin system is also essential for many functions in the developed body in both health and disease (Pasquale 2008, Klein 2009, Pasquale 2010).

Given their broad and essential roles, the focus of a large body of work has been to elucidate the structural properties of Ephs and ephrins, the molecular mechanisms regulating their signalling, and how they affect normal and pathological physiology, as described below.

1.1.1 Domain topology

Ephs are the largest family of RTKs. They are divided in two subclasses, EphAs and EphBs, and each subclass consists of several distinct members, whose number varies between different classes of organisms. For example, in mammals there are 9 A-type and 6 B-type Eph receptors, as well as five ephrinA and three ephrinB ligands (Eph Nomenclature Committee 1997), whereas in *Caenorhabditis elegans* there is only one Eph receptor and four different types of ephrins (George et al. 1998, Wang et al. 1999), and in *Drosophila melanogaster* only a single Eph-ephrin pair has been identified (Scully et al. 1999, Bossing & Brand 2002). The subdivision of Eph receptors into A- and B-type depends on their binding preference for either A-type or B-type ephrins, respectively. EphrinAs are tethered to the plasma membrane via a glycosylphosphatidylinositol (GPI) – anchor, while ephrinBs contain a single transmembrane (TM) domain and a highly conserved cytoplasmic tail.

Despite their distinct binding preferences, EphAs and EphBs share the same principle domain organisation (Fig. 1). The extracellular part of the receptor consists of a globular ligand binding domain (LBD), a cysteine-rich region, which itself contains a sushi domain and an EGF-like domain, and two fibronectin type-III repeats (FNIII). The TM segment of the receptor entails a single-pass α-helical domain. The juxtamembrane (JM) region of the

receptor is followed by a catalytically active kinase domain, a sterile-α motif (SAM) and a Psd-95, Dlg and ZO1 (PDZ) domain (Pasquale 2008).

The extracellular domain topology of ephrinAs and ephrinBs is highly conserved and consists of a globular receptor binding domain (RBD) and a short linker of about 40 amino acids (aa) in length. EphrinAs are tethered to the plasma membrane by a GPI-anchor and lack any intracellular domains, whereas the ephrinBs are TM proteins, whose cytoplasmic tail contains a PDZ binding motif as well as five key tyrosine residues that are phosphorylated upon activation (Kalo et al. 2001, Song et al. 2002, Pasquale 2008) (Fig.1).

1.1.2 Signalling from Ephs and ephrins

Ephs and ephrins are traditionally thought to reside in the membranes of opposing cells and trigger a signalling response upon coming into contact via their globular RBDs and LBDs, respectively (Labrador et al. 1997, Lackmann et al. 1997, Lackmann et al. 1998, Himanen et al. 2001). Nonetheless, more recently, several cases have been described for a functional role of *cis*-interaction of Eph receptors and ephrins being expressed in the same cell, in which the ephrins can attenuate the signalling response of the Eph receptor (Carvalho et al. 2006, Kao & Kania 2011).

A unique feature of the Eph-ephrin system is its ability to initiate bidirectional signalling (Holland et al. 1996, Bruckner et al. 1997, Kalo et al. 2001, Yu & Bargmann 2001, Davy et al. 2004). Signals into the Eph-expressing cell are referred to as forward signalling, while signalling in the ephrin-expressing cell is called reverse signalling. Reverse signals can either be mediated through co-receptors, as in the case of ephrinAs (Kramer et al. 2006, Beg et al. 2007, Lim et al. 2008b, Bonanomi et al. 2012) or through signalling molecules binding to the cytoplasmic tail of ephrinBs (Bruckner et al. 1999, Cowan & Henkemeyer 2001, Palmer et al. 2002). Interestingly, downstream targets of Eph-ephrin signalling differ significantly between the forward and reverse directions (Jorgensen et al. 2009). Typically Ephs and ephrins are expressed in a complementary pattern and mediate a repulsive signal, leading to morphological changes of the cells, thus enabling processes like cell separation or growth cone collapse in neurons (Drescher et al. 1995, Gale et al. 1996, Mellitzer et al. 1999, Mann et al. 2003). Nonetheless a growing number of physiological situations has emerged, where Eph-ephrin signalling is primarily adhesive, especially in the context of

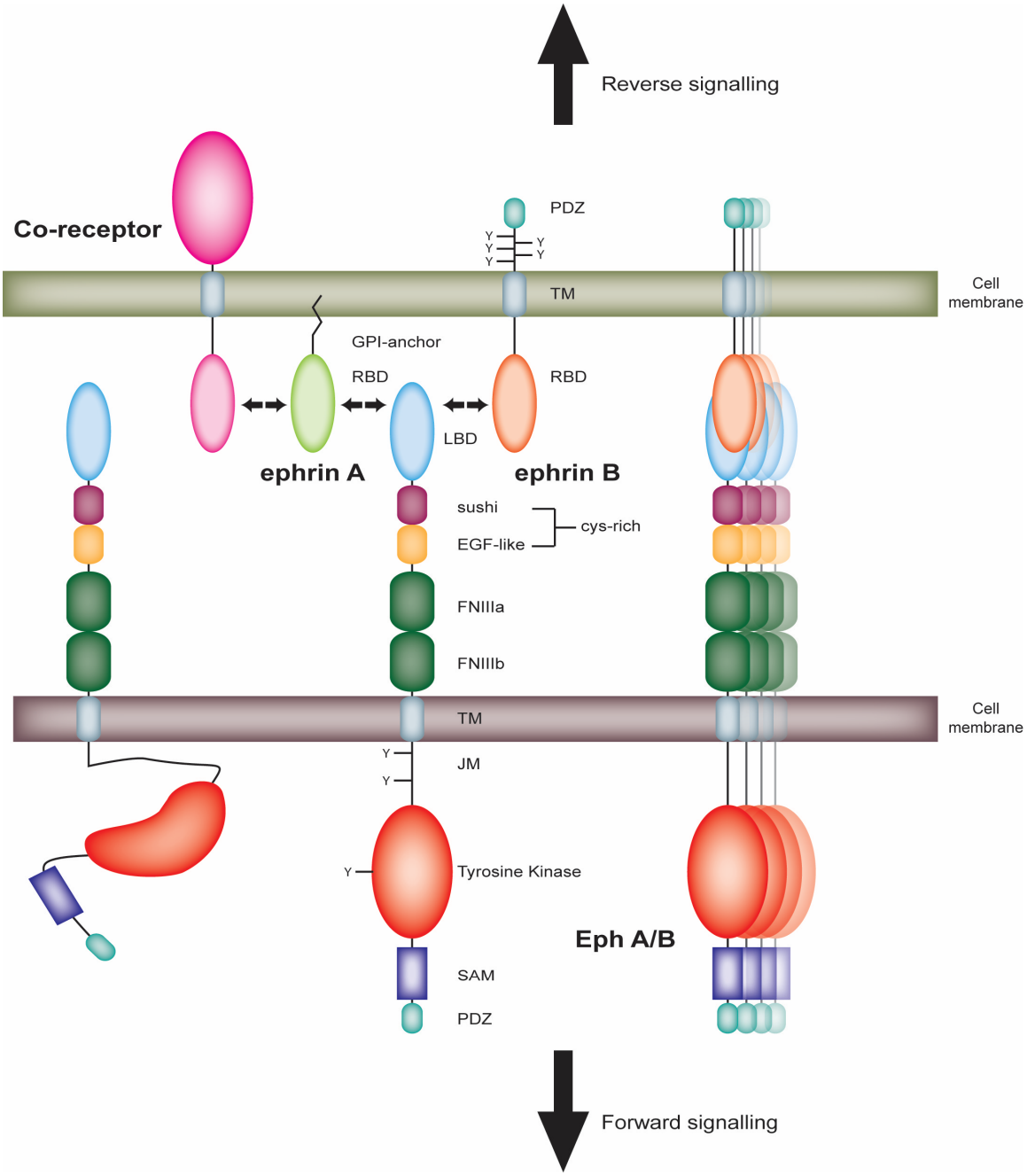


Figure 1. Domain topology of Eph receptors and ephrins

Eph receptor in its catalytically inactive, auto-inhibited state (left), its disinhibited confirmation after binding by ephrinAs or ephrinBs (middle) and in its active, clustered state (right). Putative co-receptor for ephrinA shown in pink. Active state only shown for ephrinB reverse signaling. Known tyrosine phosphorylation sites are indicated by Y. TM = transmembrane; JM = juxtamembrane; LBD = ligand binding domain; RBD = receptor binding domain; EGF-like = epidermal growth factor-like; PDZ = Psd-95, Dlg and ZO1; SAM = Sterile-□ motif; FNIII = fibronectin-type III repeat; cys-rich = cysteine rich. Scheme adapted from (Himanen & Nikolov 2003, Klein 2012)

synaptic plasticity, but also in some axon guidance decisions (Davy & Robbins 2000, Holmberg et al. 2000, Poliakov et al. 2004, Egea & Klein 2007, Klein 2009).

1.1.2.1 Eph forward signalling

Many RTKs become catalytically active after ligand binding leads to a dimerization of the receptor. Ephs differ from this pattern, as they require higher-order cluster formation to initiate signalling (Fig. 1) (Stein et al. 1998, Himanen et al. 2001, Smith et al. 2004, Himanen et al. 2010). After the initial hetero-dimer between Eph and ephrin is formed, a second receptor-ligand pair is recruited, forming a hetero-tetramer before higher-order clusters involving larger numbers of receptors can develop (Smith et al. 2004, Seiradake et al. 2010). In the case of EphB2-ephrinB2 signalling, the hetero-tetramer of two receptor and ligand molecules each has been reported to be sufficient to induce bidirectional signalling (Himanen et al. 2001). Recent publications have highlighted the fact that Eph-ephrin clusters can elicit different signalling responses depending on their composition. One study showed that the number of higher-order complexes versus the number of dimers in EphB2 clusters determines the strength of the cellular signalling response in case of cell collapse (Schaupp et al. 2014). Additionally, differences in the structural properties of Ephs can be responsible for distinguishing between adhesive and repulsive responses upon stimulation with the same ligand, as is the case for EphA2 and EphA4 upon ephrinA5 stimulation (Seiradake et al. 2013).

The clustering of Ephs can either be induced by membrane-tethered ephrins or by ephrin ectodomains that have been pre-clustered and are applied in solution (Davis et al. 1994), a fact that has been heavily utilised in subsequent functional studies of the Eph-ephrin system (Tanaka et al. 2004, Cowan et al. 2005, Sahin et al. 2005, Tolias et al. 2007, Um et al. 2014). However, in how far stimulation with pre-clustered ectodomains elicits the same signalling responses as cell contact-mediated stimulation is not clear, as the two approaches lead to distinct downstream phosphorylation patterns (Jorgensen et al. 2009). The clusters can propagate by including a growing number of molecules after their initial formation and there is also evidence that Ephs can be recruited into signalling clusters independent of ephrin binding (Wimmer-Kleikamp et al. 2004). This ephrin-independent recruitment of Ephs could potentially be explained by homotypic interactions between Ephs mediated by

their extracellular LBDs, sushi and/or FNII domains, as well as their cytoplasmic SAM domain (Lackmann et al. 1998, Himanen & Nikolov 2003, Himanen et al. 2010, Seiradake et al. 2010).

Within their respective subclasses, Ephs are able to bind promiscuously to several ephrins and vice versa, but the binding affinities can vary greatly between different ligand-receptor pairs (Brambilla et al. 1995, Brambilla et al. 1996, Gale et al. 1996). However, more and more evidence has also highlighted the role of cross-subclass binding, as in the examples of EphA4, which can bind to ephrinB2 or ephrinB3 in addition to ephrinAs (Smith et al. 1997, Kullander et al. 2001a, Kullander et al. 2001b, Qin et al. 2010), or EphB2 that can bind ephrinA5 (Himanen et al. 2004). This inter-class binding adds a further level of complexity to the Eph-ephrin system, thus enabling cells expressing a single type of receptor to receive and integrate important signalling cues from environments expressing different types of ligands.

Activity of the Eph receptor kinase domain is required for repulsive signalling in the forward direction (Holmberg et al. 2000). After ligand-induced cluster formation, Ephs transition from a state that is only weakly catalytically active to high kinase activity in a multi-step process (Binns et al. 2000, Kullander et al. 2001b, Wybenga-Groot et al. 2001). Firstly, ligand binding and cluster formation lead to phosphorylation of tyrosine residues in the JM region, which induces a conformational change that releases the auto-inhibition of the kinase domain by electrostatic repulsion (Fig.1) (Wybenga-Groot et al. 2001). Secondly, after initial phosphorylation of tyrosines in the JM region, Ephs can autophosphorylate several conserved tyrosines within the kinase domain, which further increases catalytic activity (Kalo & Pasquale 1999, Binns et al. 2000). Additionally, after initial phosphorylation events in the JM region, Ephs can be further phosphorylated by members of the sarcoma virus transforming gene product (src) family of kinases (SFK) (Ellis et al. 1996, Zisch et al. 1998). The phosphorylation-induced conformational changes also allow the docking of src-homology 2 (SH2) domain containing proteins. These interactions with SH2 domain containing proteins seem to be dispensable for the principle activation of Ephs and may instead rather play a role in fine-tuning or triggering additional, separate signalling responses (Zisch et al. 2000). Furthermore, the SAM and PDZ domains also play a role in Eph signalling. PDZ-containing interactors can stabilise Eph clusters as

scaffolding proteins or initiate distinct signalling pathways themselves (Hsueh & Sheng 1998, Torres et al. 1998). However, it is not yet completely understood whether, and to what extent, the interactions with SAM or PDZ domain-containing proteins are necessary for physiological Eph functions, as truncation of these domains does not abolish all signalling responses (Kullander et al. 2001b, Park et al. 2004). An interesting study by Egea and colleagues employing a mutated, constitutively catalytically active EphA4 further elucidated the importance of the kinase domain and highlighted the role of signalling modulation by interaction with the ephrin ligands. Their experiments show that while kinase activity independent of ligand binding is sufficient for axon guidance decisions like midline repulsion, correct formation of thalamo-cortical projections requires signalling modulation through ephrin ligands (Egea et al. 2005). While these results establish the importance of Eph receptor kinase function in forward signalling, the role of Ephs acting as ligands for ephrin reverse signalling is independent of their kinase activity (Kullander et al. 2001b).

1.1.2.2 Reverse signalling through ephrins

In contrast to the EphA and EphB receptors, which share many signalling properties, ephrinAs and ephrinBs differ significantly in their signalling mechanisms due to their very dissimilar structural architecture.

EphrinA signalling

Lacking a cytoplasmic domain and instead being linked to the plasma membrane by a GPI-anchor, the mechanisms by which ephrinAs translate their signal into the cell are particularly interesting. A growing body of evidence supports the theory that they mediate their function by recruiting co-receptors after being activated by Ephs. Among the receptors reported to function as co-receptors with ephrinAs are the Glia cell line-derived neurotrophic factor (GDNF) receptor, proto-oncogene tyrosine-protein kinase receptor RET (RET) (Kramer et al. 2006, Dudanova et al. 2010, Bonanomi et al. 2012), and the neurotrophin receptors p75 (Lim et al. 2008b) and tropomyosin receptor kinase B (TrkB) (Marler et al. 2008). The cross-talk of the neurotrophin and Eph-ephrin signalling systems is highly functionally relevant, as can be seen by the varied responses (axon branching

versus axon repulsion) downstream of ephrinA6 depending on its association with either p75 or TrkB (Poopalasundaram et al. 2011). Another proposed mechanism for ephrinA reverse signalling is that ephrinA clusters concentrate in special membrane compartments such as rafts or caveloae and trap SFKs such as fyn or other signalling molecules such as integrins within to enact their signalling (Davy et al. 1999, Davy & Robbins 2000).

EphrinB signalling

B-type ephrins contain a highly conserved cytoplasmic tail that encompasses five tyrosine residues, which can become phosphorylated upon binding to Eph receptors, as well as a PDZ domain (Bergemann et al. 1998, Torres et al. 1998, Bruckner et al. 1999, Song et al. 2002). As for Eph receptors, it has been proposed that ephrinBs undergo a conformational change in their cytoplasmic tail upon activation, thus facilitating the access of downstream signalling effectors (Song et al. 2002, Song 2003). Src family kinases are key mediators of ephrin phosphorylation (Kalo et al. 2001, Palmer et al. 2002, Foo et al. 2006, Georgakopoulos et al. 2006). Once phosphorylated, the cytoplasmic tail of ephrinBs can bind to SH2/SH3 adaptor proteins such as Grb4 that mediate downstream signalling events, for example, actin cytoskeleton rearrangements (Cowan & Henkemeyer 2001, Segura et al. 2007, Xu & Henkemeyer 2009).

In contrast to the Eph receptors, the PDZ domain in ephrinBs has been shown to be of crucial importance for several physiological functions. By binding protein tyrosine phosphatase PTP-BL, the PDZ domain triggers the dephosphorylation of the tyrosine residues in the cytoplasmic tail and thus enables fine tuning of the ephrinB signalling response, possibly also switching from phosphorylation-dependent to PDZ adaptor protein-mediated signalling (Palmer et al. 2002). Another study shows that for the remodelling of the vasculature mediated by ephrinB2 the PDZ domain is required, as mice expressing mutant ephrinB2 lacking the PDZ domain die within weeks after birth presenting severe malformation of lymphatic vasculature. In contrast, mice expressing ephrinB2 lacking all five cytoplasmic tyrosine residues exhibit only a very mild phenotype with minor malformations and no premature lethality (Makinen et al. 2005). PDZ-containing scaffolding proteins also mediate cross-talk to other signalling pathways as in the case of PDZ-RGS3, which attenuates signalling downstream of the heterotrimeric G-protein-

coupled receptor CXCR4 through its GTPase-activating properties (Lu et al. 2001). Interaction with the scaffolding proteins of the glutamate receptor interacting protein (GRIP) family helps recruit kinases to ephrin clusters (Bruckner et al. 1999) and also promotes glutamatergic synapse formation by stabilising α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in postsynaptic domains (Aoto et al. 2007, Essmann et al. 2008).

Together, these findings have emphasised the importance of reverse signalling in eliciting additional and distinct signalling responses, both through phosphorylation-dependent, and PDZ-dependent pathways.

1.1.2.3 Signal attenuation

An interesting question in Eph-ephrin signalling is how a high-affinity adhesive reaction between receptor and ligand is translated into cellular repulsion. While there is a growing number of physiological functions that require adhesive Eph-ephrin signalling, for example, the requirement of EphA splice isoforms lacking kinase-mediated repulsive signalling properties for neural tube formation (Holmberg et al. 2000), or the involvement of Eph receptors and ephrins in synaptic stability and plasticity (reviewed in Klein 2009); in the majority of cases the signal mediated by ephrins and Ephs is repulsive. In order to achieve repulsion between cells, it is not only necessary that the cytoskeletal architecture is re-arranged downstream of Eph-ephrin signalling, but also that the high-affinity ligand-receptor complex comprising molecules from opposing cell membranes is removed from the cell surface.

Two major pathways for removal of Eph-ephrin complexes from the cell surface have been discovered. The first is trans-endocytosis of entire Eph-ephrin complexes into the cell (Marston et al. 2003, Zimmer et al. 2003). This process is the main focus of this thesis and will be discussed in depth in section 1.3.3. The second mechanism to remove Eph-ephrin complexes from the cell surface is by proteolytic cleavage. The metalloprotease A-Disintegrin-And-metalloprotease 10 (ADAM10) has been reported to be able to cleave ephrinAs at their ectodomain *in trans* after being activated by EphAs and increase contact-mediated repulsion (Hattori et al. 2000, Janes et al. 2005, Janes et al. 2009). EphA2 can be cleaved by membrane type-1 matrix metallo-proteinase (MT1-MMP), triggering cell

repulsion in cancer cells (Sugiyama et al. 2013). EphA4 can be cleaved, but the physiological relevance for repulsive signalling of EphA4 cleavage is not completely understood as mice expressing cleavage-resistant EphA4 receptors show only a mild phenotype in axon guidance and EphA4 cleavage was independent of ephrinA activation (Gatto et al. 2014). Gatto and colleagues further observed altered levels of EphA4 expression and thus suggested a role for EphA4 cleavage in the fine-tuning between *cis* and *trans* signalling from EphA4 receptors. Addtionally, another study proposed EphA4 cleavage by γ-secretase to be dependent on synaptic activity (Inoue et al. 2009).

EphrinBs can be cleaved *in* c*is* by metalloproteinases and γ-secretases (Georgakopoulos et al. 2006, Tomita et al. 2006). The work by Georgakopoulos and colleagues further suggests that the cleavage of ephrinB2 does not terminate signalling, but rather that the generated intracellular fragment possesses distinct signalling capabilities and mediates the activation of src kinase downstream of ephrinB2 after activation by EphBs. Similarly, reports of the cleavage of EphBs have suggested physiological signalling properties for the cleaved intracellular fragments and implicated that cleavage is dependent on calcium influx and ligand binding (Litterst et al. 2007), and that the cleaved fragments are involved in regulating cell surface expression of NMDA receptors (Xu et al. 2009). However, whether these findings represent a general signalling mechanism mediated by cleaved fragments in EphB-ephrinB signalling remains unclear, especially, since another report shows EphB cleavage after binding to ephrinBs to be required in the more classical role of cell repulsion (Lin et al. 2008).

These studies show that cleavage of Eph receptors and/or ephrins fulfils several important physiological roles that might extend beyond enabling cell-cell detachment and signal attenuation.

1.1.3 Physiological functions of Eph-ephrin signalling

As alluded to already, the Eph-ephrin signalling system is involved in a plethora of physiological functions and plays important roles both in development and adult physiology, as well as in health and disease. By giving examples for the roles of Eph-ephrin signalling in the nervous system I would like to introduce some of the interesting features and common mechanisms of the Eph-ephrin system. Several excellent reviews exist that

examine the involvement of Eph-ephrin signalling in vascular development (Adams & Eichmann 2010, Klein 2012, Pitulescu & Adams 2014), cell differentiation and proliferation (Genander & Frisen 2010, Wilkinson 2014), the immune system (Wu & Luo 2005, Funk & Orr 2013), glucose homeostasis (Jain & Lammert 2009), bone maintenance (Edwards & Mundy 2008), as well as its role in neurodegenerative diseases (Cisse & Checler 2015) and cancer (Genander & Frisen 2010, Pasquale 2010), and I will thus refrain from discussing these functions in detail here.

1.1.3.1 Eph-ephrin signalling in axon guidance

Probably the most intensely studied and well-established role of Eph-ephrin signalling in the nervous system is its involvement in axon guidance during neuronal development. Already in the 1990s, a study by Bonhoeffer and colleagues established the ability of the Eph-ephrin system to initiate repulsive guidance responses by triggering axonal growth cone collapse (Drescher et al. 1995). Since then, a large number of subsequent studies have built upon this seminal work (some selected references Wahl et al. 2000, Mann et al. 2003, Sahin et al. 2005). Here, I will use two well-studied physiological settings, retinotopic map formation and guidance of motor neurons, to illustrate the physiological relevance of Eph-ephrin signalling in axon guidance.

Processing of sensory information often depends on the correct topographic mapping of sensory inputs onto higher brain centres. One example is the input of visual information through the retina. The spatial information of retinal activation patterns is crucial for its correct interpretation in higher brain centres. Therefore, the well-defined topographic organisation is retained in neurons projecting from the retina to the tectum. Axon guidance by the Eph-ephrin system is important for the correct formation of these topographic maps in order to relay information from the retina to higher brain centres. EphA-ephrinA bidirectional signalling is required to form correct retino-tectal projections and, depending on the exact localisation of expression of receptors and ligands, they can be either repulsive or attractive cues (Knoll et al. 2001, Knoll & Drescher 2002, Dufour et al. 2003, Marquardt et al. 2005). One mechanism important for topographic map formation is a differentiated EphA response resulting from gradients of EphA and ephrinA expression between the retinal origin and tectal target zones of neurons (Brown et al. 2000). Recent work has

shown that the gradient of ephrinA correlates with the target size of the topographic map established in the tectum (Tadesse et al. 2013). Topographic map formation also depends on the kinase function of EphA4 (Dufour et al. 2006). Marquardt and colleagues propose that contrasting repulsive and attractive signalling responses are regulated by EphAs and ephrinAs residing in the same neuron, but within distinct membrane domains (Marquardt et al. 2005). Another report suggests that eprhinAs reduce EphA-mediated repulsive signalling by acting on EphAs *in cis* (Carvalho et al. 2006). Reverse signalling from ephrinAs in topographic map formation seems to intersect with neurotrophic signalling and p75 has been implicated as the responsible co-receptor (Lim et al. 2008b, Grimbert & Cang 2012). In how far correct map formation also is dependent on neuronal activity in the retinal ganglion cells is still a matter of debate, with work supporting an important role of activity (Grimbert & Cang 2012) and other results suggesting neuronal activity is dispensable (Benjumeda et al. 2013).

Guidance of motor axons to their correct targeting zones in limbs also relies heavily on Eph-ephrin signalling. Motor neurons originating in the motor cortex need to cross the midline before forming the corticospinal tract and finally innervating targets on the contra-lateral side of their origin. This process requires EphA4 signalling in the axons, which are repelled by ephrinB3 expressed in cells on the midline of the spinal cord (Dottori et al. 1998, Coonan et al. 2001, Kullander et al. 2001a, Kullander et al. 2001b). These EphA4-expressing neurons form a component of the central pattern generator required for locomotion and the aberrant crossing of neurons in EphA4-knockout mice leads to a distinct hopping phenotype due to abnormal synchronous activation of limbs on both sides (Kullander et al. 2003). Three independent studies suggest that one key downstream player of EphA4 signalling in this context is the Rac GTPase activating protein (GAP) α2-chimaerin, as an α2-chimaerin knockout mouse phenocopies the EphA4 knockout (Beg et al. 2007, Iwasato et al. 2007, Wegmeyer et al. 2007). Correct formation of spinal motor circuits has been reported to consist of distinct clustering-dependent and independent EphA4 signalling mechanisms (Egea et al. 2005). Recent work from Paixao and colleagues has furthermore demonstrated that ephrinB3-EphA4 signalling is required for the correct formation of both descending and ascending axon tracts in the spinal cord (Paixao et al. 2013). Once motor neurons leave the spinal cord, interactions between ephrinAs and

EphA4 are crucial for neurons to reach their target areas in the limb musculature (Kramer et al. 2006, Dudanova et al. 2010, Kao & Kania 2011, Bonanomi et al. 2012, Dudanova et al. 2012). Reverse signalling by ephrinAs is required in this context, and RET functions as a co-receptor alongside ephrinAs linking contact-mediated Eph-ephrin interaction to signalling by the diffusible neurotrophic guidance cue GDNF (Kramer et al. 2006, Dudanova et al. 2010, Bonanomi et al. 2012). The studies from Dudanova and colleagues, as well as from Kao and colleagues, have highlighted an intricate interplay between repulsive EphA signalling, attractive ephrinA signalling and signal attenuation by *cis* interactions between ephrinAs and EphA receptors. All of these mechanisms are acting on the same neuron and are required for the precise spatio-temporal control of axon guidance from the spinal cord to the limb muscles (Kao & Kania 2011, Dudanova et al. 2012). Cleavage of EphAs is an additional mechanism to fine-tune this process (Gatto et al. 2014).

The Eph-ephrin system is well established as a key regulator of axon guidance in several physiological contexts. The examples given in this short overview illustrate that the role of Eph-ephrin signalling is far more complex than providing mere repulsive guidance cues by inducing growth cone collapse. Cross-talk between subclasses, regulation by downstream effectors, *cis* signalling and adhesive responses all contribute to the central role of Eph-ephrin signalling in orchestrating fine-tuned axonal guidance decisions. Despite the growing body of work, many details of the underlying molecular mechanism for Eph-ephrin-mediated axon guidance, especially regarding the process of cell detachment, remain to be unravelled.

1.1.3.2 Eph-ephrin signalling at synapses

Eph-ephrin signalling plays a different role at synapses, where it can mediate adhesive signals and is involved in the generation of synapses, as well as synaptic maintenance and plasticity.

Eph-ephrin signalling in synaptogenesis

Ephs can regulate the formation of excitatory synaptic connections by stabilising NMDA receptors at postsynaptic specialisations (Dalva et al. 2000). Another suggested mechanism for how Eph receptors contribute to synapse formation is by increasing motility of dendritic

filopodia (Kayser et al. 2008). Whether promotion of synaptogenesis by EphBs requires their kinase function is still a matter of debate. Recent work from Greenberg and colleagues using a chemical genetic approach suggests that the kinase function is not required for synaptogenesis (Soskis et al. 2012). However earlier work from the same group suggests that excitatory synapse formation is promoted by enhancing degradation of ephexin5 through its phosphorylation by EphB receptors (Margolis et al. 2010). Excitatory synapses often form on dendritic protrusions called spines and EphBs play important roles in forming these spines as a step leading up to synapse formation (Henkemeyer et al. 2003, Kayser et al. 2006).

While EphBs have been shown to play a role in postsynaptic development, ephrinBs have been implicated in presynaptic development (Kayser et al. 2006, Lim et al. 2008a, McClelland et al. 2009). This regulation of synaptic development can depend on interaction with syntenin (McClelland et al. 2009). Syntenin itself can bind and stabilise glutamatergic receptors at synapses (Hirbec et al. 2002). Nonetheless, other studies, found ephrinBs to also be involved in synapse formation on the postsynaptic side (Aoto et al. 2007, Segura et al. 2007, Xu et al. 2011). The postsynaptic involvement of ephrinB3 can also be mediated through syntenin, an interaction independent of phosphorylation of ephrinB3 (Xu et al. 2011). Other proteins implicated downstream of ephrinBs in synapse formation are GrB4, which in this case would argue for the requirement of ephrinB phosphorylation (Segura et al. 2007), and the PDZ domain interactor GRIP1 (Aoto et al. 2007).

Eph-ephrin signalling in neuronal plasticity

Eph-ephrin signalling is not limited to functions in the developing nervous system. An increasing body of work underlines the importance of the Eph-ephrin system for plasticity in the mature nervous system on both the levels of synapses as well as dendrites and spines. One important feature of plasticity on the synaptic level is the activity-dependent change in signalling properties and synaptic strength known as long-term potentiation (LTP) (Bliss & Lomo 1973, Bliss & Collingridge 1993). EphB receptors can phosphorylate NMDA receptors either directly (Dalva et al. 2000) or through src family kinases (Takasu et al. 2002), and thereby modulate synaptic plasticity. Further studies have identified the postsynaptic requirement of EphB receptors for NMDA-mediated LTP generation

(Grunwald et al. 2001, Contractor et al. 2002). EphB signalling in LTP is independent of its kinase function. Grunwald and colleagues showed that EphB receptors truncated at the carboxy-terminus were sufficient to rescue the EphB knockout phenotype (Grunwald et al. 2001). Potentially, the kinase-independent signalling is promoted by scaffolding proteins binding to the PDZ domain of EphB2 like GRIP1, which clusters glutamatergic receptors (Contractor et al. 2002).

Paralleling their requirement in synaptogenesis, ephrin molecules also play reciprocal roles in synaptic plasticity and can act on both the presynaptic and postsynaptic sides. Several studies showed the involvement of ephrinBs in LTP in the hippocampus (Contractor et al. 2002, Grunwald et al. 2004, Armstrong et al. 2006, Bouzioukh et al. 2007, Lim et al. 2008a). The effect of ephrinBs on LTP can be dependent on (Grunwald et al. 2004) or independent of the function of NMDA receptors (Armstrong et al. 2006, Lim et al. 2008a). LTP downstream of ephrinBs requires phosphorylation of the cytoplasmic tyrosine residues in contrast to long-term depression (LTD), which is phosphorylation independent (Bouzioukh et al. 2007). Another pathway by which ephrinBs can influence synaptic plasticity is by stabilising AMPA receptors at synapses, which requires the phosphorylation of a serine residue of ephrinB2 (Essmann et al. 2008). Interestingly, Eph-ephrin signalling can also influence LTP in a more indirect route, since an EphA4-ephrinA3 interaction between neuronal and glia cells regulates glutamate uptake into astrocytes and thereby the induction of LTP at adjacent synapses (Filosa et al. 2009).

In addition to changes at the synaptic level, plasticity in the mature nervous system is also mediated by morphological changes at the level of spines and dendrites (Yuste & Bonhoeffer 2001). Eph-ephrin signalling can exert these effects on the cytoskeletal architecture of cells, for example via signalling through Rho-family GTPases (see section 1.2.3 for more details), and is thus also able to effect morphological changes in neuronal plasticity. Experiments in cultured neurons have revealed that EphBs regulate the morphology of dendritic spines, for example through Cdc42 and its guanine exchange factor (GEF) intersectin (ITSN) (Irie & Yamaguchi 2002, Nishimura et al. 2006), or Rac1 and its GEFs Kalirin (Penzes et al. 2003) or Tiam1 (Tolias et al. 2005, Tolias et al. 2007). Ephrin reverse signalling can also facilitate dendritic remodelling in a phosphorylation- and PDZ-dependent manner through Grb4, PICK1 and syntenin downstream of eprinB3

(Xu et al. 2011). Similar to its relevance for LTP, neuron-glia cross-talk mediated by EphA4 and ephrinA3 is also involved in regulating dendritic spine morphology (Murai et al. 2003, Carmona et al. 2009). The effects of EphA4 on spine morphology can also be mediated by the regulation integrin signalling (Bourgin et al. 2007) or alternatively by activating phospholipase gamma and causing actin rearrangement through cofilin (Zhou et al. 2007). NMDA-dependent ephrinA2-signalling is required for maintaining spines and synapses, as ephrinA2 knockout mice show increased pruning of dendritic synapses (Yu et al. 2013).

The ability of the Eph-ephrin system to employ different downstream effectors, integrate information from several signalling pathways, and provide fine-tuned responses into opposing cells via bidirectional signalling allows the control of important elements of synaptic development and physiology. Still, several molecular details of how specific signalling responses can be achieved and how diverse pathways with contrasting effects downstream of Eph-ephrin signalling intersect remain to be deciphered.

As demonstrated by the examples from its physiological roles in the nervous system, the Eph-ephrin system can act as a versatile regulator of very distinct physiological responses. Understanding the molecular mechanisms regulating Eph-ephrin signalling is therefore a key element in gaining a deeper understanding of a large host of different physiological processes.

1.2 Rho-family GTPases

The Rho family of GTPases is a subgroup of the rat sarcoma viral oncogene homologue (Ras) superfamily of GTPases and itself consists of several subfamilies. The Rho GTPases are relatively small proteins (21 kDa) with their signature conserved GTPase domain residing in their N-terminus. They act as molecular switches and have been associated with several essential physiological functions including controlling cell morphology, polarisation, adhesion and migration, as well as vesicle trafficking (Ridley 2006, Iden & Collard 2008, Friedl & Gilmour 2009, Hall & Lalli 2010, Parsons et al. 2010). Rho family GTPases also play an important role in several diseases including neurodevelopmental disorders, neurodegeneration and cancer (Sahai & Marshall 2002a, Govek et al. 2005, Newey et al. 2005, Parri & Chiarugi 2010, Stankiewicz & Linseman 2014). Already early reports on the functions of Rho family GTPases linked their effects on cell morphology and motility to rearrangement of the actin cytoskeleton (Ridley & Hall 1992a, Ridley & Hall 1992b, Ridley et al. 1992). Subsequent studies revealed further roles for Rho family GTPases in controlling gene expression, mitosis, proliferation and secretion (Etienne-Manneville & Hall 2002, Jaffe & Hall 2002, Heasman & Ridley 2008). Although Rho family GTPase activity affects many intracellular processes, shaping the actin cytoskeleton has remained the focal point, and is particularly relevant in the context of the regulation of neurite morphology and endocytic processes (Ridley 2006, Hall & Lalli 2010).

Rho-family GTPases function as molecular switches. This ability arises from the distinct signalling properties they exhibit when in a guanine diphosphate (GDP)-bound or guanine triphosphate (GTP)-bound state. In their inactive, GDP-bound conformation, Rho family GTPases are mainly found in the cytosol and unable to bind their effector proteins (Jaffe & Hall 2005). When bound to GTP, Rho GTPases are in their active state and localised to membranes via a prenyl group connected to a conserved CAAX motif in their C-terminal part (Roberts et al. 2008). In their active state they are able to bind their respective effector proteins, often protein kinases such as PAK1 or Rho activated kinase (ROCK) (Bishop & Hall 2000). The transitioning between active and inactive states of Rho-family GTPases relies heavily on the interaction with three different classes of proteins: GEFs, GAPs, and guanine nucleotide-dissociation inhibitors (GDIs) (Jaffe & Hall 2005). GDIs can constrain

Rho GTPases in their inactive, GDP-bound state and regulate their subcellular location (Olofsson 1999). GEFs catalyse the exchange of GDP to GTP, thereby activating Rho GTPases (Hart et al. 1991, Schmidt & Hall 2002a), while GAPs greatly enhance the intrinsic GTPase activity of Rho GTPases, resulting in a transition to the inactive state (Garrett et al. 1989, Lamarche & Hall 1994, Tcherkezian & Lamarche-Vane 2007). The activity of both GEFs and GAPs can be regulated downstream of extracellular cues and they are therefore key regulators of Rho GTPase signalling and important mediators of cross-talk between different pathways (Bos et al. 2007). The physiological importance of GEFs and GAPs is further highlighted by the fact that both groups of proteins outnumber the 20 members of the Rho-family of GTPases several fold. The Rho GTPase cycle and the role of GEFs and GAPs is summarised in Figure 2A.

In this chapter I will provide a short overview of the different subfamilies of Rho GTPases and their relevant functions, a more detailed description of the properties and functions of GEFs and GAPs, as well as an overview of Rho family GTPase signalling downstream of Eph-ephrin complexes.

1.2.1 Rho GTPase subfamilies

Rho family GTPases are highly conserved in eukaryotes and can be found in organisms ranging from yeast to plants to animals. In mammals, a total of 20 Rho family GTPases have been described and they can be divided into 6-8 distinct subfamilies according to their sequence similarity (see also Fig.2B) (Burridge & Wennerberg 2004, Wennerberg & Der 2004, Heasman & Ridley 2008). Most studies so far have focussed on the three cardinal members of the Rho family GTPases: RhoA, Rac and Cdc42. Rho-family GTPases not falling into one of these three subfamilies are therefore often referred to as atypical Rho GTPases.

1.2.1.1 RhoA subfamily

The eponymous RhoA subfamily consists of RhoA, RhoB and RhoC. Traditionally RhoA has been linked to assembly of actin stress fibres and focal adhesions (Ridley & Hall 1992b, Nobes & Hall 1995). The effect on stress fibre assembly, however, does not rely on a large increase in actin polymerisation downstream of RhoA, but rather on myosin-induced

bundling of actin (Machesky & Hall 1997). To this end, RhoA activates ROCK, its most commonly studied downstream effector (Leung et al. 1995). One of the chief functions of ROCK signalling is phosphorylation of myosin, which thus facilitates the formation of stress fibres (Amano et al. 1996, Kimura et al. 1996). ROCK signalling downstream of RhoA can also affect the actin cytoskeleton more directly, for example through phosphorylation of Lim kinase, which phosphorylates the actin-binding protein cofilin, culminating in stress fibre formation. (Maekawa et al. 1999). RhoA subfamily proteins can also interact with actin nucleator proteins of the Dia family, which opens up possibilities for both shared and distinct signalling pathways with ROCK downstream of RhoA (Watanabe et al. 1999, Sahai & Marshall 2002b, Lammers et al. 2008).

Due to the high degree in sequence homology between the three subfamily members and their ability to bind the same effectors, the various Rho family members have overlapping and often redundant functions in some physiological contexts. RhoA and RhoB are both required for lamellipodia retraction in macrophages, with only double knockout mice showing a strong phenotype (Konigs et al. 2014). In differentiation of skin cells, RhoA-deficient mice displayed only a mild phenotype with increased cell spreading and defective cell-cell contacts *in vitro,* while RhoB expression was increased (Jackson et al. 2011). Additional pharmacological inhibition of RhoB and RhoC greatly exacerbated the phenotype, suggesting functional overlap between the RhoA subfamily members. Nonetheless, differences in the signalling properties of the different subfamily members have also emerged. RhoB differs in its subcellular location from RhoA and RhoC and seems to mediate different signalling responses, including regulation of vesicular trafficking (Adamson et al. 1992, Gampel et al. 1999, Michaelson et al. 2001). RhoC shows higher binding affinity to ROCK when compared with RhoA, enabling the two proteins to activate distinct signalling pathways within the same cell (Sahai & Marshall 2002b).

Physiologically, signalling from RhoA subfamily members is important for actin rearrangement in neuronal development and axon guidance, as it can mediate growth cone collapse and regulate neurite growth (Wahl et al. 2000, Shamah et al. 2001, Fournier et al. 2003, Sahin et al. 2005, Takeuchi et al. 2015).

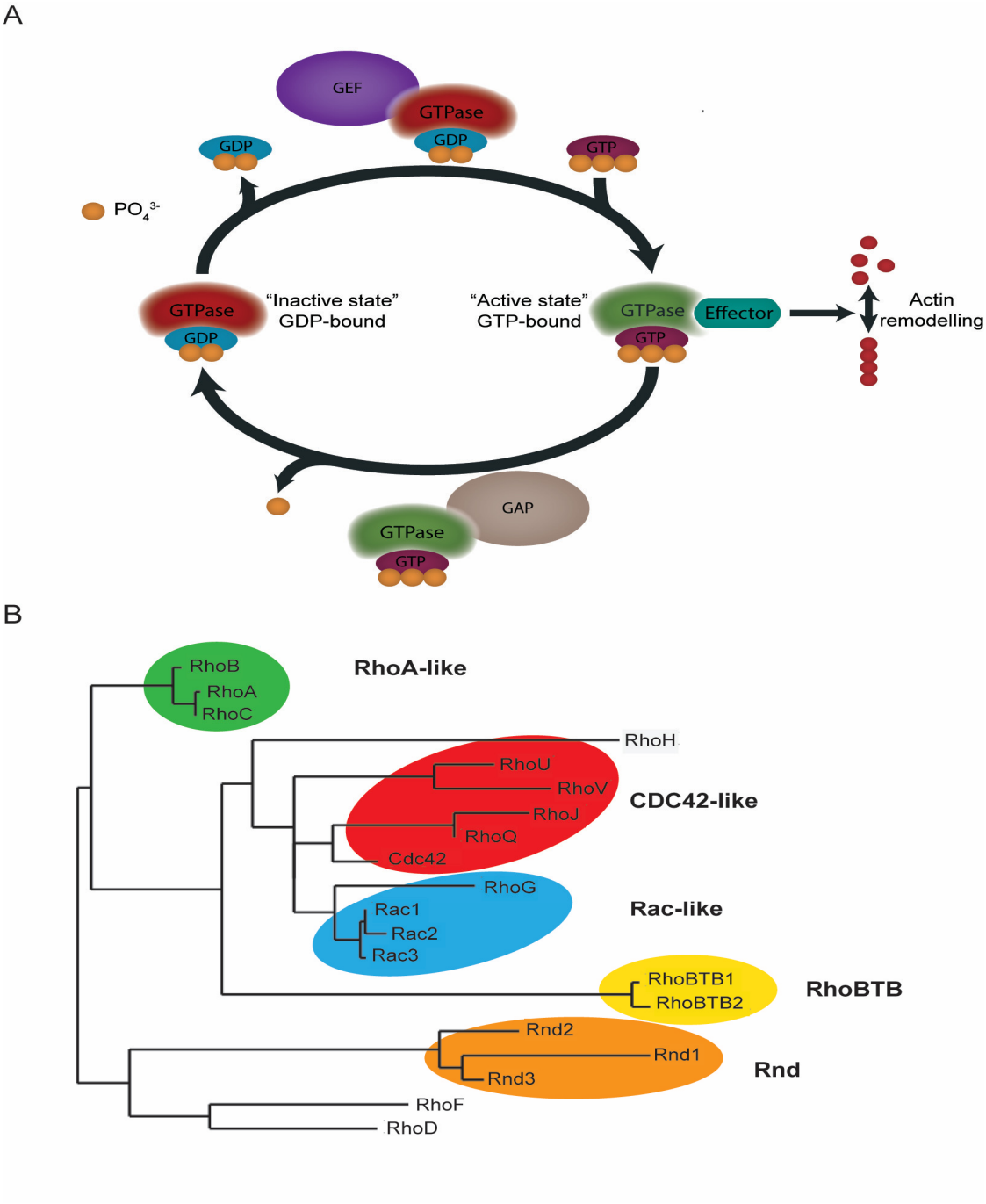


Figure 2. Rho family GTPases

(A) Rho family GTPase activity cycle. Rho family GTPases cycle between a GDP-bound, inactive and a GTP-bound, active state. The transition between these stages is mediated by guanine exchange factors (GEFs) and GTPase-activating proteins (GAPs). Active GTPases bind downstream effectors to mediate their signalling response, for example remodelling of the actin cytoskeleton. (B) Evolutionary relationship between different Rho family GTPases and their respective subfamilies according to sequence alignment with Phylogeny software (www.phylogeny.fr) (Dereeper et al. 2008).

1.2.1.2 Rac subfamily

The Rac subfamily consists of Rac1, Rac2, Rac3 and their distant relative RhoG (Figure 2 B). A further splice variant of Rac1, Rac1b, with very efficient intrinsic GTP-GDP exchange and therefore thought to be constitutively active, has also been described (Fiegen et al. 2004). Rac was first shown to be an important mediator in the formation of membrane ruffles and lamellipodia in response to extracellular signals such as growth factors (Ridley et al. 1992, Nobes & Hall 1995). Changes in the cytoskeleton induced by Rac result from an increased polymerisation of actin (Machesky & Hall 1997). Later, Rac subfamily members have also emerged as key regulators of endocytic processes including macropinocytosis (Dharmawardhane et al. 2000, West et al. 2000) and phagocytosis (Massol et al. 1998, Castellano et al. 2000, Chimini & Chavrier 2000, Hoppe & Swanson 2004). The contribution of Rac subfamily signalling to endocytic pathways, and more specifically, endocytosis of Eph-ephrin complexes will be explored in greater detail in chapter 1.3.

The separate Rac subfamily members vary in their expression patterns: While Rac1 is expressed ubiquitously, Rac2 is mainly found in hematopoietic cells (Roberts et al. 1999) and Rac3 is mainly expressed in the nervous system (Haataja et al. 1997). Rac1 and Rac2 have both overlapping and distinct signalling functions, as Rac1 and Rac2 single knockout mice show distinct phenotypes in hematopoietic cell regulation (Gu et al. 2003), while both Rac1 and Rac2 are required for B cell development (Walmsley et al. 2003). Rac1 and Rac3 have been found to have redundant functions in neuronal development (Corbetta et al. 2009). Finally, the function and role of RhoG signalling is still a matter of debate, but it has been proposed to act upstream of Rac1 and Cdc42, positively regulating their activity via interaction with GEFs of the DOCK family (Katoh & Negishi 2003, Hiramoto et al. 2006, Katoh et al. 2006, Kim et al. 2011, Franke et al. 2012). However, other studies suggest RhoG can signal in parallel to Cdc42 and Rac, making use of the same downstream effectors (Wennerberg et al. 2002).

One important downstream effector of Rac subfamily GTPases is the p21-activated kinase (PAK), a serine/threonine kinase (Manser et al. 1994, Knaus et al. 1998). Via activating PAK, Rac can induce changes in the actin cytoskeleton required for cell motility and

neurite morphology (Edwards et al. 1999, Sells et al. 1999, Nikolic 2008). However, Rac activity can also lead to actin rearrangement independent of PAK by a pathway involving the adaptor protein Nck and the Scar/WAVE (WASP-family verpolin homologous protein) complex (Eden et al. 2002).

Interestingly, activity of Rac subfamily GTPases is in many physiological situations opposed to the function of RhoA subfamily proteins and in some cases, leads to direct inhibition or downregulation of RhoA signalling (Sander et al. 1999, Nimnual et al. 2003). In turn, RhoA activity and signalling via ROCK has been shown to lead to a decrease in Rac activity, which can be mediated through activation of Rac-specific GAPs (Ohta et al. 2006). Furthermore, signalling through the same receptor can influence Rac and Rho activity in contrasting directions (Driessens et al. 2001, Hu et al. 2001, Sahin et al. 2005). More recently, RhoA, Rac1 and Cdc42 have been shown to act synergistically, but in a tightly orchestrated manner in the formation of cell protrusions: RhoA is required at the leading edge of the cell to initiate actin rearrangement, while Rac1 and Cdc42 show peak activity 2 μm from the cell edge and are active with a delay compared to RhoA. This shows a spatio-temporally controlled sequence of activity of the respective GTPases and the authors speculate that since RhoA and Rac1/Cdc42 activity are temporally and locally exclusive, cross-inhibition is important for this tight regulation (Machacek et al. 2009, Pertz 2010). It has thus become increasingly obvious that signalling pathways from different Rho subfamilies intersect and influence each other (Guilluy et al. 2011).

1.2.1.3 Cdc42 subfamily

The Cdc42 subfamily consists of five members: Cdc42, RhoQ (TC10), RhoJ (TCL), RhoU (Wrch) and RhoV (Wrch2/Chip) (Figure 2B). However, there is some debate whether RhoU and RhoV are actually a distinct subfamily, given that they show unique properties when compared to other Cdc42 subfamily proteins, including being potentially constitutively active (Shutes et al. 2006). Cdc42 activity induces long, filamentous processes called filopodia to be formed in cells (Nobes & Hall 1995). This function is shared by the other subfamily members, which can possibly be explained by their shared affinity towards some downstream effectors like PAK (Aronheim et al. 1998, Neudauer et al. 1998, Murphy et al. 1999, Tao et al. 2001, Aspenstrom et al. 2004). Nonetheless,

subsequent studies also revealed distinct signalling pathways and showed that Cdc42 subfamily members differ in their intracellular localisation, their sensitivity to regulation by GDIs and the respective downstream effects mediated (Michaelson et al. 2001, Murphy et al. 2001). Furthermore, despite the high level of sequence homology between RhoQ, RhoJ and Cdc42, GEFs seem to only marginally increase GTP-GDP exchange rates in RhoQ and RhoJ, while some of them are very potent activators of Cdc42 (Jaiswal et al. 2013a).

One key downstream effector of Cdc42 signalling to the actin cytoskeleton is Wiskott-Aldrich syndrome protein (WASP), which mediates actin polymerisation and is involved in the formation of Cdc42-induced filopodia (Symons et al. 1996). RhoQ and RhoJ, as well as RhoV can also induce filopodia formation via WASP (Aronheim et al. 1998, Abe et al. 2003). However, a WASP-independent pathway for inducing filopodia by Cdc42 has also been described (Peng et al. 2003). WASP mediates rearrangement of the actin cytoskeleton via the Arp2/3 complex, which induces actin branching (Stradal & Scita 2006). Another important pathway for Cdc42 subfamily GTPases uses PAK to regulate the actin cytoskeleton in an analogous fashion to Rac subfamily GTPases (Manser et al. 1995, Aronheim et al. 1998, Edwards et al. 1999).

Signalling from Cdc42 via the WASP signalling pathway has been shown to be involved in both regulation of neurite morphology (Wong et al. 2001, Irie & Yamaguchi 2002, Abe et al. 2003, Nishimura et al. 2006, Franke et al. 2012) and endocytic processes (Hussain et al. 2001, Qualmann & Kessels 2002, Sabharanjak et al. 2002, Chadda et al. 2007), and is therefore highly relevant in the context of this study.

1.2.1.4 Atypical Rho family GTPases

A more specialised subfamily of Rho GTPases are the Rnd proteins (Rnd1, Rd2, Rnd3/RhoE). Despite sequence homology with RhoA, the mechanism of action of the Rnd subfamily of proteins seems to differ significantly from the archetypical Rho family GTPases (Foster et al. 1996, Fiegen et al. 2002, Garavini et al. 2002, Chardin 2006). Rnd proteins cannot act as molecular switches, since they are GTPase-deficient and are constitutively active in their GTP-bound form (Foster et al. 1996, Fiegen et al. 2002, Garavini et al. 2002). Nonetheless, Rnd proteins have been shown to regulate the actin

cytoskeleton (Nobes et al. 1998, Aspenstrom et al. 2004). They can act as RhoA-antagonists by activating p190RhoGAP and their activity is regulated by a feedback-loop involving RhoA-activated kinase (ROCK) (Wennerberg et al. 2003, Riento et al. 2005, Goh & Manser 2012). As RhoA-antagonists, they have been shown to play a role in axon guidance, for example downstream of semaphorin signalling (Oinuma et al. 2004a, Oinuma et al. 2004b, Pacary et al. 2011).

Another pair of unusual Rho-family GTPases are RhoD and Rif, in that they seem not to be regulated by GEFs, like most other Rho-family GTPases (Jaiswal et al. 2013a). This may be because RhoD and Rif display a very high intrinsic exchange rate from GDP to GTP and are proposed to thus be constitutively active, similar to the splice variant Rac1b (Fiegen et al. 2004, Jaiswal et al. 2013b).Functionally, RhoD and Rif have been shown to induce rearrangement of the actin cytoskeleton and be involved in vesicular transport, among other functions (Murphy et al. 1996, Gasman et al. 2003, Aspenstrom et al. 2004, Gad & Aspenstrom 2010).

RhoH is an atypical Rho GTPase and just like the Rnd subfamily, it lacks a key residue in its GTPase domain, rendering it incapable of GTP hydrolysis (Li et al. 2002). While RhoH is not involved in rearrangement of the actin cytoskeleton, in contrast to most other members of the Rho family GTPases (Aspenstrom et al. 2004), it has been implicated to play an important role in some cancers (Preudhomme et al. 2000). A likely mechanism for its function is binding and inhibition of other Rho family GTPases (Li et al. 2002).

The Rho BTB subfamily, which consists of three members (RhoBTB1-3) differs from other Rho family GTPases in that they contain additional domains in their C-terminus, lack the CAAX-motif important for membrane localisation, and are also unable to rearrange the actin cytoskeleton (Rivero et al. 2001, Aspenstrom et al. 2004). Furthermore, RhoBTB3 has an atypical GTPase domain and is thought to bind ATP instead of GTP (Espinosa et al. 2009).

A final subfamily of Rho GTPases that shows no effects on the cytoskeleton is the Miro subfamily, consisting of members Miro1 and Miro2, which have been shown to localise to mitochondria and to be involved in regulation of apoptosis (Fransson et al. 2003, Aspenstrom et al. 2004). However, due to large sequence divergence from all other Rho

family GTPases, it is questionable whether they should be included in the Rho family at all (Fransson et al. 2003).

Despite this great variety of GTPases, research so far has mainly focussed on just three proteins: RhoA, Rac1 and Cdc42. Given that these three subfamilies have been implicated already in Eph-ephrin signalling and endocytic processes (see below) and the fact that their regulatory mechanisms are accessible for experimental manipulation, we also focussed on their role in EphB-ephrinB endocytosis in this study. When studying the effects of Rho GTPases it will be important to bear in mind that there is a degree of physiological redundancy within different subfamilies of Rho GTPases, as well as regulatory cross-talk within and between subfamilies.

1.2.2 Regulation of Rho subfamily GTPases by GEFs and GAPs

1.2.2.1 Regulation through GEFs

There are two major families of GEFs for Rho family GTPases in eukaryotes: the Diffuse B-cell lymphoma oncogene (Dbl) family and the Dedicator of Cytokinesis (DOCK)-family.

The Dbl family all share the eponymous Dbl Homology (DH) domain that is crucial for mediating guanine nucleotide exchange (Hart et al. 1991). For most Dbl family GEFs, the DH domain is paired with a pleckstrin homology (PH) domain, involved in binding the target GTPase (Rossman et al. 2002), or membrane localisation of GEFs through lipid binding (Ferguson et al. 1995, Lemmon & Ferguson 2000, Razzini et al. 2000). There are 74 proteins containing a DH encoded by the human genome, but not all of them display GEF activity towards Rho-family GTPases (Jaiswal et al. 2013a). In addition to the signature DH-PH tandem domains, GEFs can contain a great variety of domains involved in signalling or protein-protein interaction, amongst them SH2, SH3, GEF domains for other GTPases such as Ras, PDZ or Kinase domains. This assortment of diverse interaction sites enables GEFs to integrate signals from, and confer signals to a host of different signalling pathways (Schmidt & Hall 2002a).

Specificity of GEFs for distinct Rho-family GTPases covers a broad spectrum. A comprehensive study by Jaisawal and colleagues tested several GEFs for their catalytic activity towards a large number of Rho-family GTPases *in vitro*. Their results suggested that while some Dbl family GEFs do not show any activity towards the tested GTPases, others are active on several of them, for example, Prex1 that confers activity to members of all three cardinal Rho subfamilies, some are subfamily specific, for example, p190GEF for the RhoA subfamily; and yet others are highly specific for a single GTPase, for example, ITSN for Cdc42 (Jaiswal et al. 2013a). The study further revealed that the catalytic efficiency with which GEFs mediate GDP to GTP-exchange in GTPases can vary greatly, as it ranges from 5-fold to 60,000-fold increase over baseline intrinsic exchange activity (Jaiswal et al. 2013a). These facts suggest that different GEFs can affect Rho GTPase activity at different time scales and with different efficiencies. Interestingly, the results reported in this comprehensive study sometimes contradict previous reports in the literature regarding the specificity of some GEFs. For example, Prex1 had previously been described as a Rac-specific GEF (Welch et al. 2002), whereas Jaiswal and colleagues show that it also has significant activity towards members of the RhoA and Cdc42 subfamilies. However, some of these discrepancies can potentially be explained by this study relying solely on *in vitro* studies, while posttranslational modifications, interaction with GDIs, or subcellular compartmentalisation *in vivo* may significantly change the activity of GEFs, as can be observed in the case of ephexin changing its specificity upon phosphorylation (Sahin et al. 2005).

GEF activity can be regulated in several ways (Schmidt & Hall 2002a). A very important regulatory mechanism for GEFs is phosphorylation by protein kinases (Patel & Karginov 2014). For example, the Vav family of Rho GEFs has been shown to be present in the cell in an auto-inhibited confirmation that is relieved upon tyrosine phosphorylation by SFKs (Aghazadeh et al. 2000, Yu et al. 2010). Combined with the possibility of Vav proteins being de-phosphorylated by phosphatases (Stebbins et al. 2003, Sastry et al. 2006), the phosphorylation status of Vav provides tight and transient spatio-temporal control for its activity. While phosphorylation by tyrosine kinases correlates with higher GEF activity, phosphorylation of serine or threonine residues can be either inhibitory or activating. One example of this is GEF-H1 (ArhGEF2) that can be activated upon phosphorylation of a

threonine residue by Extracellular signal-Regulated Kinase (ERK), while a different phosphorylation event mediated by ERK on a serine residue inhibits its function, as does serine phosphorylation by PAK (Zenke et al. 2004, Fujishiro et al. 2008, von Thun et al. 2013). Finally, a remarkable effect of phosphorylation is observed in ephexin (NGEF), downstream of EphA4 receptor signalling, where the preference of GEF activity switches from Rac1/Cdc42 to RhoA upon phosphorylation (Sahin et al. 2005).

Aside from phosphorylation, there are multiple other mechanisms regulating GEF activity. One such pathway involves GPCRs. Synaptic GPCR Brain-specific Angiogenesis Inhibitor 1 (BAI1) is required for restricting Rac-specific GEF Tiam1 to synapses in synaptogenesis by direct interaction, thereby leading to the localised Rac activation necessary for establishing polarity (Duman et al. 2013). GPCR signalling is not only required for correct localisation of GEFs, but can also directly increase GEF activity. Gα activates p63GEF (GEFT) by directly binding to the PH domain, which leads to a release of auto-inhibition (Rojas et al. 2007). Gβγ and phosphatidylinositol signalling can directly and synergistically increase GEF-activity of Prex1, which enables Prex1 to act as a coincidence detector of these two signalling pathways in neutrophils (Welch et al. 2002). A unique feature of GPCR signalling via GEFs can be observed with Trio, a GEF containing two separate DH-PH domains showing distinct substrate specificity towards RhoA and Rac1/Cdc42, respectively (Debant et al. 1996). The RhoA-specific DH-PH domain of Trio shares sequence similarity with the DH-PH domain from p63GEF and accordingly, Gα-interaction through the same molecular mechanism leads to increased GEF activity towards RhoA and an associate shift away from activity towards Cdc42 and Rac1 (Rojas et al. 2007).

The DOCK family of GEFs has been discovered more recently and displays different characteristics when compared to Dbl family GEFs (Cote & Vuori 2002). Since they lack the DH domain, DOCKs are often referred to as atypical GEFs. Instead of the DH domain, DOCK proteins share two separate DOCK homology regions, DHR1 and DHR2, from which they mediate lipid binding and induce GEF activity (Laurin & Cote 2014). The DOCK family consists of 11 members and interestingly, they all show specificity for either Rac or Cdc42 GTPases and are not active towards RhoA (Cote & Vuori 2002, Cote &

Vuori 2006). DOCK-family GEFs are also different from Dbl family GEFs in that at least some members rely on interaction with members of the Engulfment and cell motility protein (ELMO) family in order to exhibit nucleotide exchange activity. This interaction, first described between Dock1 (also known as DOCK180) and ELMO1, leads to Rac activation (Wu et al. 2001, Brugnera et al. 2002, Grimsley et al. 2004, Lu et al. 2004). Dock4 has also been shown to require interaction with an ELMO protein to influence cell migration via Rac activation (Hiramoto et al. 2006) and an ELMO2-Dock3 complex is required for neurite outgrowth downstream of neurotrophic factor signalling (Namekata et al. 2012). However, it is not yet completely understood whether all DOCK proteins require complex formation with ELMOs to exhibit GEF activity. Furthermore, activation of Rac by the DOCK-ELMO complex on is regulated by another GTPase, RhoG, which seems to be important for translocation of the DOCK-Elmo complex to the cell membrane (Katoh & Negishi 2003, Hiramoto et al. 2006, Katoh et al. 2006, Namekata et al. 2012). A second regulatory mechanism for DOCK proteins is the formation of dimers. Homodimers of Dock9 or Dock1 have been reported to display increased GEF activity (Meller et al. 2004). Further research has also provided evidence of heterodimers between Dock1 and Dock5 and suggested ELMO proteins act as scaffold proteins for DOCK-family dimers (Patel et al. 2011). Recently DOCK proteins, and the DOCK-ELMO-RhoG signalling axis in particular, have emerged as important players in regulating neurite morphology, complexity and spine formation through effects on the actin cytoskeleton (Kim et al. 2011, Franke et al. 2012, Namekata et al. 2012). Also phagocytic uptake of apoptotic cells in *C. elegans* also employs a DOCK-ELMO complex downstream of RhoG, which is in turn activated by the Dbl-GEF Trio (deBakker et al. 2004).

In conclusion, it has become evident that GEFs are not only key regulators of GTPase activity, but due to their ability to interact with and be regulated by different signalling pathways also constitute a versatile mediator of signalling cross-talk.

1.2.2.2 Regulation through GAPs

GAPs are highly conserved in eukaryotes and can be found in organisms ranging from yeast to humans (Peck et al. 2002). Between 60 and 70 different proteins containing a Rho GAP domain are encoded in the human genome but not all of them have been functionally

described yet (Tcherkezian & Lamarche-Vane 2007). Initially thought of as mere signal terminators of Rho GTPase signalling, by now many pivotal roles for GAP proteins have been described, including intersection with other signalling pathways as well as signalling functions not mediated by the Rho GAP domain.

The key feature GAP proteins have in common is their signature Rho GAP domain, which enhances the intrinsic GTPase activity of Rho GTPases upon binding by the GAP to the GTPase (Peck et al. 2002). A conserved arginine residue within the Rho GAP domain is essential for its catalytic function, but not mandatory for the initial binding between GAP and GTPase (Graham et al. 1999). A notable exception to this rule is the Rac-specific GAP oculocerebrorenal syndrome of Lowe (OCRL-1), which displays low GAP activity despite lacking the conserved arginine residue in its GAP domain (Faucherre et al. 2003). Apart from the common RhoGAP domain, GAPs may contain a great variety of different interaction domains including PDZ, SAM, SH2, SH3, BAR and DH-PH domains, thus enabling them to make use of, and contribute to, several distinct signalling mechanisms and pathways (Peck et al. 2002, Tcherkezian & Lamarche-Vane 2007). An example highlighting the importance of GAPs independent of their GAP activity can be found in TCGAP, which regulates insulin-dependent glucose uptake via interactions with RhoQ despite not being catalytically active *in cellulo* (Chiang et al. 2003).

There are significant differences between GAPs regarding their specificity towards single Rho family GTPases or subfamilies. Studies have shown that some GAPs are specifically active towards single GTPases or at least GTPase subfamilies, for example p190GAP for RhoA (Ridley et al. 1993) and ARHGAP15 for Rac (Seoh et al. 2003). Some GAPs display an intermediate level of specificity and are active towards members of two of the main Rho subfamilies, but not towards the third. For example, CdGAP is active towards Cdc42 and Rac1, but not towards RhoA (Tcherkezian et al. 2006). Other GAPs, however, show a broad range of activity towards several GTPases, for example RICS and OPHN1 that both show activity towards Cdc42, Rac1 and RhoA (Billuart et al. 1998, Nakamura et al. 2002, Fauchereau et al. 2003, Moon et al. 2003). Identifying the specificity of GAPs towards single GTPases is challenging, as studies often only test the activity towards their GTPase of interest, or towards Cdc42, Rac1 and RhoA (being the most-studied Rho GTPases), and very few comprehensive studies exist. Furthermore, there seem to be significant differences

between the specificity GAPs display *in vitro* compared to observations *in vivo* (Tcherkezian & Lamarche-Vane 2007). Often there are also conflicting reports about the specificity of certain GAPs, as is the case for Slit/ROBO GAP1 (SrGAP1), which was originally reported to interact with Cdc42 and RhoA, but not Rac1 *in vivo* (Wong et al. 2001), but a more recent study reported activity mainly towards Rac1 (Yamazaki et al. 2013). Potentially, these contradictory findings can be explained by post-translational modifications, which can influence specificity in different cellular contexts ((Minoshima et al. 2003).

A subset of GAPs, but not all of them, show tissue specific expression. One example is RICS (also known as Grit), which is only expressed in the brain, where it contributes to neurite extension downstream of TrkA (Nakamura et al. 2002, Moon et al. 2003).

Regulation of GAP activity is often mediated through phosphorylation by protein kinases. Kinase-regulated pathways include signalling by SFKs fyn and src (Roof et al. 1998, Liu et al. 2006), signalling downstream of synaptic activity through calmodulin-dependent protein kinase II (CamKII) (Okabe et al. 2003) and cytokinetic signalling through Aurora B (Minoshima et al. 2003). GAPs are also involved in mitogenic signalling. CdGAP is phosphorylated at an important regulatory site by ERK1 (Tcherkezian et al. 2005) and another GAP, RhoA-specific Gmip, interacts with Ras GTPases, themselves important players in mitogenic signalling (Aresta et al. 2002). As with GEFs, phosphorylation can have very varied effects on GAP activity: it can lead to an increase in GAP activity (Roof et al. 1998, Ohta et al. 2006), it can inhibit activity (Okabe et al. 2003, Tcherkezian et al. 2005) or very interestingly, lead to a change in substrate specificity, as for example in the case of male germ cell Rac GAP (MgcRacGAP), which switches its specificity from Rac1 and Cdc42 to RhoA after phosphorylation (Minoshima et al. 2003). A similar switch has also been reported for SrGAP1 downstream of Roundabout receptor (ROBO) signalling, increasing its activity towards Cdc42, while downregulating GAP activity towards RhoA (Wong et al. 2001). GAPs are also regulated downstream of Eph-ephrin signalling, as detailed in the section below.

GAPs also provide a target for cross-talk between different subfamilies of Rho GTPases. Filamin A –associated Rho GAP (FilGAP) is a Rac GAP that gets phosphorylated

downstream of ROCK leading to an increase in its activity (Ohta et al. 2006). CdGAP experiences inhibition of its Rac GAP activity when bound by ITSN1, a Cdc42-GEF (Jenna et al. 2002). Bcr contains both a Rho GAP and a Rho GEF domain and has been shown to simultaneously inhibit Rac1 and promote Cdc42 signalling *in vivo* (Ridley et al. 1993, Korus et al. 2002). Finally, GAPs can also be downregulated by degradation through the proteasome after ubiquitination (Su et al. 2003).

These examples underline the importance of regulation of GTPase activity by GAPs and highlight that GAPs play just as central a role as GEFs in integrating information from different signalling pathways.

1.2.3 Eph-ephrin signalling through Rho-family GTPases

Many important functions of Eph-ephrin signalling rely heavily on processes involving rearrangement of the cytoskeleton. Therefore it is not surprising that Rho-family GTPases and their regulating GEFs and GAPs have emerged as key effectors in Eph-ephrin signalling.

Growth cone collapse in neurons can be initiated by RhoA signalling (Lehmann et al. 1999, Dergham et al. 2002). Experiments with retinal ganglion cells (RGCs) have shown that RhoA-dependent growth cone collapse is triggered downstream of EphA activation by ephrinA5 (Wahl et al. 2000). This signalling pathway uses ROCK downstream of RhoA to induce actin-myosin-based contraction and also seems to negatively regulate Rac1 signalling. Another study, however, showed Rac1 signalling downstream of ephrinA2 in RGCs to be crucial for correct formation of retino-tectal projections (Jurney et al. 2002). The role of Rac1 in growth cone collapse downstream of ephrinA-EphA signalling is linked to its ability to promote endocytic uptake (Fournier et al. 2000, Jurney et al. 2002). Recently the RhoA-ROCK pathway has also been shown to be responsible for growth cone collapse downstream of ephrinB reverse signalling induced by ectodomains of EphB2 (Takeuchi et al. 2015). A very interesting feature of EphA forward signalling mediated by RhoA is the effect EphA activation has on the RhoGEF ephexin (NGEF) in neurons. As described previously, phosphorylation of ephexin by EphA receptors leads to a change in its catalytic specificity - decreasing its ability to activate Cdc42 and Rac1, while increasing its activity towards RhoA - thereby activating the RhoA-ROCK pathway and leading to growth cone

collapse (Shamah et al. 2001, Sahin et al. 2005). Another GEF that has increased activity towards RhoA after phosphorylation by EphA4 is ArhGEF15. It is expressed in vascular smooth muscle cells and modulates vascular contractility by contributing to actin stress fibre formation (Ogita et al. 2003). Finally, a shift in balance away from Cdc42 and towards RhoA activity downstream of ephrinA-EphA signalling is also responsible for inhibiting chemotaxis in T-lymphocytes, but whether this signalling pathway also operates through phosphorylation of a GEF is not yet known (Sharfe et al. 2002).

The function of Rho family GTPases is also important for the role of Eph-ephrin signalling in the formation of the motor system. EphrinB3-EphA4 signalling in the spinal cord is important for the correct formation of central pattern generators. Correct axon guidance decisions for the development of this locomotor circuit require growth cone collapse mediated by α2-chimaerin, a Rac GAP, which gets activated upon binding to active EphA4 clusters (Beg et al. 2007, Iwasato et al. 2007, Wegmeyer et al. 2007). This interaction is potentially facilitated by the adaptor protein Grb4 (also known as Nck2), as mice deficient for Nck1 and Grb4 show the same phenotype as α2-chimaerin or EphA4 knockout mice (Fawcett et al. 2007).

In contrast to α2-chimaerin-induced inhibition of Rac1 activity causing growth cone collapse in spinal motor neurons, in RGCs growth cone collapse and correct axon guidance occurs after activated Ephs phosphorylate and thereby activate Vav family GEFs, which, in turn, activate Rac (Cowan et al. 2005). Moreover, the work of Cowan and colleagues presents a potential explanation for the seemingly opposing roles of Eph-Rac signalling plays in growth cone collapse: the authors link Vav and Rac function to endocytosis of Eph-ephrin clusters, which is thought to be required for cell detachment. The endocytic uptake possibly requires localised activation of Rac, whereas the collapse of the actin cytoskeleton of the growth cone in general requires a reduction in Rac activity. As the mechanisms of Eph-ephrin endocytosis are seminal to this dissertation, they will be discussed in greater depth in section X.

Rac activity has also been implicated in repulsive guidance and axon pruning downstream of reverse signalling by ephrinB3. Effective signalling requires phosphorylation of ephrinB3, which enables Grb4 to bind to its cytoplasmic tail. Grb4 in turn can bind Dock1

and the Rac downstream effector PAK and together, this signalling cascade regulates the actin dynamics necessary for growth cone collapse (Xu & Henkemeyer 2009).

For the effects on dendrite morphology mediated by Eph-eprin signalling, Rac and Cdc42 signalling downstream of EphB2 has been reported to be important. A signalling axis involving EphB2, Cdc42, the Cdc42-specific GEF ITSN and neuronal Wiskott-Aldrich syndrome protein (N-WASP), a regulator of actin polymerisation, has been shown to be required for the formation and maturation of dendritic spines in cultured hippocampal neurons (Irie & Yamaguchi 2002, Nishimura et al. 2006). Experiments indicated that Cdc42 activity is required for spine formation and that ITSN displayed greater GEF activity when bound by EphB2 or N-WASP. A complex of EphB2, N-WASP and ITSN resulted in an even larger increase in GEF activity, which suggests a synergistic effect of N-WASP and EphB2 binding on ITSN. Another GEF involved in dendrite morphogenesis is Kalirin (Penzes et al. 2000, Penzes et al. 2001). Kalirin localises to EphB2 clusters activated by ephrinB1 and is subsequently phosphorylated (Penzes et al. 2003). Rac activity is increased by Kalirin and in turn, leads to an increase in the activity of its downstream effector PAK, which mediates the localised actin rearrangement required for dendritic spine morphogenesis (Penzes et al. 2003). Another pathway employed by both EphA and EphB activated by ephrinBs to regulate dendrite morphogenesis uses the Rac-specific GEF Tiam1 to induce Rac activity (Tanaka et al. 2004, Tolias et al. 2007). Tiam1 is recruited to, and phosphorylated by ephrinB-induced EphB2 receptor clusters, and this activation is necessary for spine development (Tolias et al. 2007). The presence of NMDA receptors at EphB2 clusters and the previous established link between synaptic activity and Tiam1 signalling suggests that there is a connection between synaptic activity and EphB2-regulated dendrite morphogenesis (Tolias et al. 2005, Tolias et al. 2007). More recent work proposed that Tiam1 forms a complex with the Rac GAP Bcr in this context, which allows precise and tight spatio-temporal regulation of Rac activity to regulate synaptogenesis downstream of EphB receptors (Um et al. 2014). A finely tuned balance in Rac activity is required, as inhibition of Bcr function leads to both aberrant synaptic overgrowth due to increased Tiam1-induced Rac activity and increased spine-loss induced by ephrinB1 due to increased internalisation of ephrinB1-EphB clusters. Tiam1 has also been implicated in the endocytosis of EphAs and it has been proposed to mediate signalling from Eph receptor

clusters residing in endosomal compartments (Yoo et al. 2010, Boissier et al. 2013). A detailed description of the mechanisms of Eph-ephrin endocytosis is provided in chapter 1.3.3. A different role for Tiam1 activity downstream of ephrinB2 reverse signalling has been reported by Adams and colleagues for vascular smooth muscle cells. Here, ephrinB2 reverse signalling regulates Tiam1 activity, as well as localisation towards the cell membrane and sites of platelet-derived growth factor receptor (PDGFR) internalisation. Both PDGFR internalisation and Tiam1-induced Rac activity are required for correct vessel wall formation (Nakayama et al. 2013).

Spine morphogenesis and synapse formation can also be mediated by ephrinB reverse signalling. Experiments in cultured rat hippocampal neurons have shown a signalling pathway involving Grb4, GIT1, αPix, Rac and PAK to be required for the formation of dendritic spines and synapses downstream of ephrinBs (Zhang et al. 2005, Segura et al. 2007). Phosphorylation of ephrinBs is required, which in turn leads to localisation of GIT1 to synaptic compartments and binding of Grb4 (Segura et al. 2007). The GIT1-Grb4 complex recruits the GEF αPix, which activates Rac1 and thereby PAK. This signalling cascade leads to an increase in phosphorylation of myosin light chain II (MLC2), which is required for the effects on dendrite morphology (Zhang et al. 2005).

In conclusion, Rho-family GTPases and their regulatory GEFs and GAPs are well-established players in Eph-ephrin signalling and are instrumental for actin rearrangement-driven changes in cell morphology and motility, for example in growth cone collapse and spine morphology. Furthermore, they seem to be pivotal elements in Eph-ephrin endocytosis, however, their precise contribution still remains to be deciphered. The large number of GEFs and GAPs and their overlapping specificities also provide a high level of redundancy in the system, as can be seen in the role of Eph-ephrin signalling in dendrite morphogenesis, where both a Tiam1-Rac and an ITSN-Cdc42 pathway have been described. Studying the involvement of GEFs and GAPs in Eph-ephrin signalling therefore needs to address the potential redundant roles of GTPases and their regulators.

1.3 Endocytosis

The term endocytosis derives from the Greek words endos (into) and cytos ("hollow vessel" = cell) and is used to describe the cellular processes that mediate the up-take of molecules, membranes, liquids, or larger components into a cell. Being able to internalise external material into a cell is of vital importance for many physiological functions. Primarily thought to be required for the regulation of signalling responses and, especially, for the downregulation of signalling by the internalisation of cell surface receptors (Doherty & McMahon 2009, McMahon & Boucrot 2011), endocytosis is also important in the elimination of pathogens (Flannagan et al. 2012), in mediating changes of cellular morphology (Donaldson et al. 2009, Tojima et al. 2011), and in synaptic signalling (Royle & Lagnado 2010). Furthermore, endocytic pathways are also used by viruses and other pathogens to infect cells and are therefore important in the context of several diseases (Marsh & Helenius 2006, Maxfield 2014).

Internalisation of cargo serves several purposes and can also result in differing outcomes. Once internalised, vesicles from several endocytic pathways are considered to first localise to the early endosomal compartment. From the early endosomal compartment different endocytic routes exist: either cargo is returned to the plasma membrane via recycling endosomes, or it is sent for degradation via late endosomal and lysosomal compartments (Alberts 2015). The trafficking between these different endosomal compartments is tightly regulated by small GTPases of the Rab family (Wandinger-Ness & Zerial 2014). Different Rab GTPases are associated with distinct endosomal compartments. The best characterised ones are: Rab5 with the early endosome, Rab7 with the late endosome and Rab11 with the recycling endosome.

After the discovery of recycling from endosomal compartments had challenged the original notion that endocytosis is only a mechanism for restricting and terminating signalling from cell surface receptors, further evidence for a more diverse physiological function of endocytosis came from the observation that some receptors remain active and continue signalling from inside the endosomal compartment after internalisation. Initially reports for epidermal growth factor receptor (EGFR) signalling responses showed that the receptor stays active and phosphorylated and bound to its downstream effectors on endosomes (Di

Guglielmo et al. 1994, Baass et al. 1995, Burke et al. 2001). By now so-called signalling endosomes have also been described to occur after activation of several other RTKs including TrkA, TrkB and p75NTR (Grimes et al. 1996, Howe et al. 2001, Barker et al. 2002, Shao et al. 2002, Saxena et al. 2005).

Endocytosis is thus a diverse process, which is not only important in many physiological contexts, but can also serve multiple purposes depending on the trafficking of cargo downstream of the initial internalisation event.

1.3.1 Endocytic pathways

The term endocytosis encompasses a host of different processes, which differ significantly in their molecular mechanism and the kind of cargo they internalise. The distinction between these pathways is based on differences in ultrastructural appearance as revealed by electron microscopy, the type of cargo internalised, and the molecular mechanisms involved. Here I will provide a short overview of the most commonly described endocytic pathways.

1.3.1.1 Clathrin-mediated endocytosis

Perhaps the most intensively studied endocytic pathway is clathrin-mediated endocytosis (CME). In most cases CME is required for the uptake of cell surface receptors after binding to extracellular ligands, which is also the reason it was formerly called receptor-mediated endocytosis. In brief, a host of adaptor proteins can recruit activated receptors and initiate the formation of clathrin-coated pits (CCP) (Sorkin 2004, Schmid et al. 2006). Specific adaptor proteins are required for the recruitment of specific endocytic cargos within this process (Motley et al. 2003). Clathrin itself has the structure of a triskelion and forms a lattice around the endocytic pit inducing membrane curvature (Roth & Porter 1964, Schmid & McMahon 2007, McMahon & Boucrot 2011). While the clathrin-coat is sufficient to induce membrane curvature, scaffolding proteins of the epsin and/or BAR families are required to stabilise the curvature in order to form vesicles (Ford et al. 2002, Peter et al. 2004). For the budding of a vesicle from a CCP, activity of the small GTPase dynamin is required, which leads to the scission of the vesicle at its neck connecting it to the plasma membrane (De Camilli et al. 1995, Praefcke & McMahon 2004). GTPases of the Rho

family have been shown to inhibit CME when constitutively active (Lamaze et al. 1996), however it is questionable how physiologically relevant this regulation is, as it is not in the focus of the extensive recent literature on clathrin-mediated endocytosis (Ridley 2006, Schmid & McMahon 2007, Doherty & McMahon 2009, McMahon & Boucrot 2011).

1.3.1.2 Caveolae-mediated endocytosis

Another pathway important for the internalisation of cell surface receptors is caveolae-mediated endocytosis. Caveolae are specialised membrane compartments rich in lipids and the eponymous protein caveolin that is capable of forming invaginations in the cell membrane (Parton & Simons 2007). From these invaginations, endocytic cargo-containing vesicles can be cleaved off. This process requires dynamin activity (Henley et al. 1998). Endocytic vesicles deriving from caveolae are more stable than clathrin-coated vesicles and first reside in compartments termed caveosomes after internalisation (Tagawa et al. 2005). Nonetheless, subsequently they are trafficked through typical endosomal compartments, such as the early endosome, as is the case for several other endocytic pathways (Pelkmans et al. 2004). Their stable nature led to the hypothesis that caveolae function as signalling platforms (Krajewska & Maslowska 2004). Furthermore, recruitment of receptors to caveolae can prohibit their internalisation through other pathways, as in the case of EGFR (Park et al. 2000). A process closely related to caveolae-mediated endocytosis is flotillin-dependent endocytosis, however endocytic cargo and flotillin are localised to distinct membrane compartments outside of caveolae (Glebov et al. 2006, Frick et al. 2007).

1.3.1.3 Other clathrin-independent pathways

There is a host of further so-called clathrin-independent endocytic pathways and the molecular distinction between them is not always clear and varies between studies and authors (Sandvig et al. 2008, Doherty & McMahon 2009, Donaldson et al. 2009). One of these is the clathrin-independent carrier/GPI-anchored protein-enriched early endocytic compartment (CLIC/GEEC) pathway. Endocytosis through the CLIC/GEEC pathway mediates uptake of GPI-anchored proteins and extracellular fluids and is tightly regulated by Cdc42 and two GAPs: GTPase regulator associated with focal adhesion kinase-1

(GRAF1) and ARHGAP10 (Sabharanjak et al. 2002, Kumari & Mayor 2008, Lundmark et al. 2008, Doherty & Lundmark 2009). Internalisation through the CLIC/GEEC pathway can lead to either recycling back to the membrane or lysosomal degradation (Fivaz et al. 2002, Sabharanjak et al. 2002). A separate clathrin- and caveolae-independent pathway requires the activity of Rac1 and its downstream effector PAK for the internalisation of interleukin receptors or nicotinic acetylcholine receptors, respectively (Grassart et al. 2008, Kumari et al. 2008). As there is conflicting evidence about whether dynamin is required for the internalisation of endocytic cargo, it is not clear whether these two reports refer to an identical pathway or whether Rac and PAK, through their effects on the actin cytoskeleton, are involved in different endocytic routes. This notion is supported by their requirement for macropinocytosis (see below). Another clathrin-independent pathway is characterised by the requirement of the small GTPase Arf6 and its independence from dynamin activity (Naslavsky et al. 2004, D'Souza-Schorey & Chavrier 2006). Internalisation via this Arf6-dependent endocytic pathway is tightly linked to trafficking of endocytic cargos through recycling compartments and back to the plasma membrane (Donaldson et al. 2009). Strikingly, despite being required for a clathrin-independent internalisation pathway, Arf6 has also been shown to have a regulatory role in CME by binding to clathrin and recruiting adaptor proteins (Paleotti et al. 2005, Tanabe et al. 2005).

1.3.1.4 Macropinocytosis and Phagocytosis

Macropinocytosis is a pathway by which larger patches of membrane are taken up. It involves the formation of membrane ruffles, which share a similarity in their composition to lipid rafts (Manes et al. 2003). Through this process, large numbers of RTKs can be internalised at the same time (Orth et al. 2006, Orth & McNiven 2006). There are several proteins implicated in this process. The pinching off of macropinosomes from the cell membrane has been reported to not be regulated by dynamin, as in many other endocytic processes, but rather employs the activity of ATPase Pincher/EHD4 or brefeldinA-ADP ribosylated substrate (BARS) (Shao et al. 2002, Liberali et al. 2008). Rac1 is critical for the formation of dorsal membrane ruffles and thus for macropinocytosis (Ridley et al. 1992, West et al. 2000). Recruitment of Rac to sites of macropinosome formation requires cholesterol and potentially the activity of the small GTPase Arf6 (Grimmer et al. 2002,

Cotton et al. 2007). Several protein kinases are involved in macropinocytosis, but the complete regulatory network has not yet been deciphered. The downstream effector of Rac1, PAK, plays a central role in the regulation of macropinocytosis (Dharmawardhane et al. 2000). One of its important functions is the phosphorylation of BARS, which is required for BARS-mediated membrane fission to occur (Liberali et al. 2008). Furthermore, constitutive phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) activity is sufficient to induce membrane ruffling and macropinocytosis (Amyere et al. 2000). The same is true for SFK v-Src (Veithen et al. 1996). However, the precise contribution of these kinases to the macropinocytosis pathway is not clear yet.

Phagocytosis is the uptake of large particles, particularly pathogens and cell debris, by specialised cells in the immune system (Flannagan et al. 2012). This 'eating' of large particles (from Greek "phagein" = to devour) requires large scale remodelling of the cytoskeleton architecture and the function of Rho family GTPases (Massol et al. 1998, Chimini & Chavrier 2000, Hoppe & Swanson 2004). Activation of Rac1, Rac2 and Cdc42 in a precisely orchestrated location and temporal sequence is required for the formation of the phagocytic cup and for subsequent internalisation to ensue (Hoppe & Swanson 2004). A special form of phagocytosis is the uptake of entire living cells into another cell, called entosis, which requires RhoA activity and is thought to occur in many forms of cancer (Overholtzer et al. 2007).

1.3.1.5 Trans-endocytosis of transmembrane molecules

In rare cases, uptake of receptor-ligand complexes, in which both proteins are membrane-bound, is observed. The transmembrane ligand bride of sevenless in *Drosophila melanogaster* is internalised into cells expressing the sevenless receptor tyrosine kinase (Cagan et al. 1992). Sonic hedgehog is internalised into cells expressing its receptor in a dynamin-dependent process, as revealed by experiments in an avian system (Incardona et al. 2000). Another transmembrane protein internalised in a dynamin-dependent process is notch, however, this requires catalytic cleavage of its ectodomain (Parks et al. 2000). A final example of endocytosis of transmembrane proteins into another cell are the Eph receptors and their ephrin ligands (Marston et al. 2003, Zimmer et al. 2003). This will be discussed in greater depth in the following chapter.

In how far the different endocytic pathways described above are completely separate and functionally distinct is not completely understood. Given the overlapping molecular mechanisms of the different pathways it is possible that there is cross-talk and cross-regulation between different endocytic routes. An interesting question is to what extent cargos such as specific receptors are associated with a specific endocytic pathway. Extensive work on EGFR has provided some insight into how the same protein can be taken up by multiple pathways and which effects this has on its signalling and function. Depending on the concentration of ligand used to stimulate EGFP-expressing cells, EGFRs are internalised by a clathrin-dependent (at low doses of EGF) or independent (at high doses) pathway (Sigismund et al. 2005, Orth et al. 2006). Interestingly, the various endocytic pathways also lead to distinct fates of the internalised EGFR. When endocytosed through CME, EGFR can be recycled back to the plasma membrane or continue signalling from endosomal compartments, while endocytosis through clathrin-independent mechanisms leads to ubiquitination of the receptor and subsequent degradation (Sigismund et al. 2008).

The variety and complexity of different endocytic pathways underscores the biological importance for cells to be able to internalise external or membrane-bound substances. As more and more characteristics of individual pathways are deciphered, attributing an endocytic pathway of a protein of interest to one of the established pathways can potentially reveal additional information about its signalling characteristics. However, given that not all boundaries between different pathways are clearly defined and the molecular mechanisms, at least to some extent, overlap, there might be more of a spectrum of interlinked endocytic mechanisms than clearly defined pathways.

1.3.2 Role of the actin cytoskeleton in endocytosis

Since all endocytic processes require morphological changes of the cell membrane such as the formation of invaginations or protrusion around endocytic sites, it is not surprising that rearrangement of the cytoskeleton has emerged as a central element in the control of several endocytic pathways (Girao et al. 2008). Actin polymerisation drives the membrane extensions required for the engulfment of cargos and also provides an anchor for the force required to allow vesicle budding by membrane scission. The processes required for the

uptake of larger cargo or large patches of membrane, such as in macropinocytosis (Merrifield et al. 1999, Grimmer et al. 2002) and phagocytosis (Swanson et al. 1999, Chimini & Chavrier 2000), especially rely heavily on actin rearrangement. To which extent the actin cytoskeleton plays a role in CME is still a matter of debate. While there is evidence that CME is not dependent on changes in the actin cytoskeleton (Lamaze et al. 1996, Fujimoto et al. 2000), other reports show more efficient uptake of CCPs at sites of local actin reassembly at least in some physiological contexts (Gottlieb et al. 1993, Merrifield et al. 2002). A very recent report using super-resolution live microscopy shed more light on the role of actin in CME. In their system, some CCPs co-localised with polymerised actin while others did not. Internalisation occurred in the absence of co-localisation, but the rate of uptake was higher when CCPs co-localised with polymerised actin (Li et al. 2015).

The actin cytoskeleton is thus a pivotal element in the control of many endocytic processes and likely a key mediator of endocytosis of Eph-ephrin complexes (see below).

1.3.3 Endocytosis of Eph-ephrin complexes

Endocytosis plays an important role in Eph-ephrin signalling. In order to switch from an initially adhesive response to a repulsive one, the interlocked Eph-ephrin complexes linking opposing cells must be removed from the cell surface. One mechanism to achieve this is by cleavage of either the Eph receptor or the ephrin thus allowing cell detachment, as described in section 1.1.2.3 and first shown in the reports by Hattori and colleagues and Janes and colleagues (Hattori et al. 2000, Janes et al. 2005). The other option for removing Eph-ephrin complexes to allow cell detachment is endocytosis.

In 2003 both Zimmer and colleagues and Marston and colleagues established that in parallel with cell detachment, the entire EphB-ephrinB complex was endocytosed into either the receptor or ligand expressing cell, thus mirroring bidirectional signalling capability (Marston et al. 2003, Zimmer et al. 2003). In fact, in addition to proving the uptake of full-length proteins into the opposing cell by antibody staining against their intracellular domains, experiments with membrane stains revealed that the endocytosed vesicles also contain portions of the membrane of the opposing cell (Marston et al. 2003). Therefore, the process has been termed trans-endocytosis. Both studies show that the intracellular domains and, in the case of EphBs, their kinase function is required for

endocytosis to occur. Intracellular truncation of either the receptor or the ligand disrupts bidirectionality and shifts the direction of endocytosis into the opposite cell. If both elements are truncated, endocytosis and cell detachment are inhibited, and the cells adhere strongly to each other (Zimmer et al. 2003). In the unperturbed system with full-length receptors and ligands, the direction into which more endocytosis occurs depends on the cellular context and also on the sequence in which the two different cell types have been seeded, suggesting that the state of attachment plays a role. Interestingly, Marston and colleagues also showed that Ephs stay phosphorylated after endocytosis, which could allow for Eph receptor signalling from endosomal compartments. The two studies also provide some insight into the molecular mechanisms underlying the trans-endocytosis process, at least in the forward direction. As expected for a process involving reorganisation of cellular membranes to the extent needed for internalising whole Eph-ephrin clusters, including patches of membrane from the opposing cell, actin dynamics are essential for trans-endocytosis to occur, as revealed by treatment with the actin-depolymerising drug cytochalasin D. The importance of actin reorganisation was further underlined by the findings that both activity of the actin-binding Arp 2/3 complex, as well as activity of Rac1 is required for ephrinB trans-endocytosis into EphB+ cells. Interestingly, no co-localisation of internalised EphB with clathrin-coated pits or caveolin was observed, suggesting that ephrinB trans-endocytosis into EphB+ cells does not use the CME or caveolae-dependent internalisation pathways (Marston et al. 2003). Finally, the internalisation of Eph-ephrin clusters is also dependent on the GTPase dynamin, which most likely regulates vesicle scission from the membrane. Zimmer and colleagues also showed the physiological relevance of trans-endocytosis for axon guidance decisions. Not only does trans-endocytosis occur between EphB-expressing cells and neurons endogenously expressing ephrinBs in culture, but it is also required for contact-mediated growth cone collapse. Furthermore, when cells expressing truncated Ephs, which restrict endocytosis to the reverse direction, were compared to cells expressing the full-length protein, allowing bidirectional endocytosis, the latter were found to induce growth cone collapse more effectively. Later work by Lauterbach and Klein indicated that EphB trans-endocytosis into ephrinB+ cells is important in astrocyte-neuron communication (Lauterbach & Klein 2006). Taken together, these papers highlight the importance of the trans-endocytosis

process for EphB-ephrinB signalling, in particular, its relevance for transforming initial adhesion into repulsion. Further elucidation of the molecular mechanisms governing this process will enable a better understanding of the function of Eph-ephrin signalling in many relevant physiological contexts, such as axon guidance and tissue border formation.

After the initial work presented in these papers, several other studies further elucidated the molecular mechanisms of Eph-ephrin endocytosis. However, these studies did not examine endocytosis in a cell-cell contact-mediated setting, but rather made use of the fact that signalling responses can also be elicited by treating cells with pre-clustered soluble receptor or ligand ectodomains. In how far the uptake of clusters induced by soluble ectodomains uses the same molecular mechanisms is not clear, especially in light of recent work that has shown significant differences in phosphorylation patterns downstream of cell-contact induced Eph-ephrin signalling when compared to that induced by soluble ectodomains (Jorgensen et al. 2009). Still, at least some of the requisite proteins described by the Marston and Zimmer studies have also been implicated in regulating endocytosis of soluble ephrin ectodomains into Eph+ cells. For example, Rac1 was shown to be involved in uptake of EphA and EphB receptors after stimulation with soluble ephrin ectodomains (Cowan et al. 2005, Tolias et al. 2007, Yoo et al. 2010, Um et al. 2014). Moreover, Cowan and colleagues discovered that growth cone collapse downstream of EphA receptors in retinal ganglion cells is dependent on the Rac-GEF Vav. Vav-induced Rac activity leads to endocytosis of Eph-ephrin complexes, which is required for growth cone collapse. Without Vav-mediated growth cone collapse, retinogeniculate projection neurons show aberrant guidance and wiring. While endocytosis of ephrinA1—EphA4 receptor clusters is inhibited in neurons cultured from Vav2/3 knockout mice, uptake of transferrin, a protein endocytosed via CME, is not altered, suggesting uptake of ephrinA1-EphA4 clusters is independent of CME (Cowan et al. 2005).

The Rac subfamily-specific GEF Tiam1 has been implicated as a regulator of endocytosis of both EphAs and EphBs after stimulation with soluble ephrin ectodomains. Initial work by Tolias and colleagues revealed an interaction between Tiam1 and EphB receptors in dendritic spine development. Upon stimulation with ephrinB1 Tiam1 is recruited to EphB2 clusters, where it is phosphorylated (Tolias et al. 2007). Phosphorylation of Tiam1 then increases its GEF activity towards Rac (Servitja et al. 2003, Miyamoto et al. 2006). Tiam1

can also interact with EphAs via their JM region (Yoo et al. 2010, Boissier et al. 2013). This interaction is important for the ephrinA5-induced internalisation of EphA8 clusters (Yoo et al. 2010). Co-localisation with clathrin heavy chain molecules and transferrin suggests that EphA8 is internalised via the clathrin-mediated pathway after stimulation with soluble ephrinA5-FC. Subsequent work by the same group provides evidence that a mutated EphA8 receptor lacking the Tiam1 interaction domain in the JM region is able to negatively affect endocytosis of other EphA receptors and expression of this internalisation-deficient mutant leads to aberrant retinocollicular topographic mapping (Yoo et al. 2011).

Tiam1 is also involved in the internalisation of EphB receptors upon stimulation with ephrin ectodomains (Um et al. 2014). For the role of EphB receptors in spine development and synapse maturation, a fine-tuned balance in Rac activity needs to be achieved, since on one hand, Rac activity is required for spine growth and synapse development, while on the other hand, excessive Rac activity leads to excessive endocytosis and spine loss. This balance is achieved by a regulatory complex formed between Tiam1 and Bcr, which has GAP activity towards Rac. This regulatory complex can bind to EphB2 and in the inactive state of the receptor, the inhibitory function of Bcr on Rac activity is dominant. Upon stimulation with ephrinB1, however, a disruption of the Tiam1-Bcr complex and a transient increase of Tiam1 phosphorylation and Rac activity was observed. These events are required for ephrinB1-induced spine and synapse formation. Interestingly, experiments using neuronal cultures from Bcr knockout mice, or with neurons expressing dominant negative isoforms of Bcr, indicate that despite being a negative regulator of Rac activity, Bcr function is still required for ephrinB1-induced spine growth, as stimulation with ephrinB1 ectodomains in these genetic backgrounds led to a loss of spines and synapses. One explanation for these striking results is the effect Bcr exerts on the endocytosis of EphB2s. In Bcr knockout mice the level of internalisation of EphB2s after ephrinB1 stimulation is greatly elevated, which potentially attenuates the signalling required for spine growth and synapse development mediated by EphB2-activated Tiam1 and Rac. In addition to the requirement of Rac, Tiam1 and the regulating function of BCR, work by Um and colleagues also implies that activity of the GTPase dynamin and the CME pathway

are required for endocytosis of EphB2 receptors upon stimulation with ephrinB ectodomains.

Interestingly, additional research into the role of Tiam1 in Eph-ephrin signalling and endocytosis suggests continuous signalling of EphA-ephrinA complexes from endosomal compartments (Boissier et al. 2013). This study confirmed the earlier observation that Ephs remain phosphorylated after internalisation and also revealed that Tiam1 remains bound to Ephs in endosomes. However, the study does not provide direct evidence of EphA signalling from the endosome, so whether the retained phosphorylation and interaction of Eph with Tiam1 at the endosome has physiological relevance still remains to be clarified. Furthermore, Boissier and colleagues also shed some light on the intracellular trafficking of internalised EphAs, which can undergo either lysosomal degradation (about 2/3 of internalised clusters) or they can be recycled to the plasma membrane (1/3). Further evidence for degradation through the lysosome of internalised Eph receptors comes from studies linking EphA2 and EphB1 to the ubiquitin ligase Cbl and subsequent degradation (Walker-Daniels et al. 2002, Sharfe et al. 2003, Fasen et al. 2008). After stimulation with ephrinA1 or ephinB1 respectively, Ephs phosphorylate Cbl, which in turns ubiquitinates the receptors and primes them for degradation. A recent study provides further evidence for this by showing that ubiquitination of EphA2s shifts their trafficking away from processing through the recycling endosome to degradation in the lysosome (Sabet et al. 2015).

While signalling from Rac family GEFs positively regulates Eph receptor endocytosis, negative regulators have also been identified. SH2 domain containing inositol 5-phosphatase 2 (SHIP2) interacts with EphA2 via its SAM domain and negatively regulates the activity of PI3K, which in turn is an activator of Rac activity (Zhuang et al. 2007). Overexpression of SHIP2 leads to a reduction of ephrinA1-induced EphA2 endocytosis, while knockdown of SHIP2 by siRNA results in an increase of Rac activity and consequently, an increase of endocytosis.

Endocytosis of EphAs is not regulated only by small GTPases of the Rho family, but also by GTPases of the Rab family. Internalisation of EphA4 in cultured cells and amygdala neurons stimulated by pre-clustered ephrinB3 ectodomains requires the Rab5GEF activity

of Rin1 and internalised clusters traffic through Rab5-positive compartments (Deininger et al. 2008).

Both EphAs and EphBs interact with caveolin and localise to caveolae upon activation by their ligands, however it is unclear whether this results in internalisation through the caveolae-dependent endocytic route and whether this is a cell type-specific observation or a general feature of Eph-ephrin signalling (Vihanto et al. 2006) This finding is also in contrast to the report from Marston an colleagues that could not detect a co-localisation of cell contact-induced EphB clusters with caveolae (Marston et al. 2003).

Only a small number of studies have investigated the mechanisms of endocytosis into ephrin-expressing cells. One study suggests that ephrinBs can be internalised via clathrin-mediated endocytosis, since potassium depletion (a method used for studying CME (Larkin et al. 1983)) and abrogation of dynamin function inhibited uptake of ephrinB1 into the cell upon stimulation with EphB1 ectodomains (Parker et al. 2004). Work by Mann and colleagues shows that endocytosis of soluble EphB2 ectodomains into ephrinB expressing cells occurs in the context of growth cone collapse, but the endocytic machinery required is not described (Mann et al. 2003). As ephrinB1 has been shown to interact with Tiam1 and induce an increase in Rac activity, it is possible that endocytosis into ephrinB+ cells is also mediated via Tiam1 and Rac, though so far no direct evidence for this has been reported (Tanaka et al. 2004).

In conclusion, the molecular mechanisms underlying Eph-ephrin endocytosis are not yet completely understood. For forward endocytosis into Eph-expressing cells the endocytic machineries seem to overlap between ephrin trans-endocytosis and stimulation with soluble ephrin ectodomains, as well as between EphA+ and EphB+ cells, as all of these processes require Rac activity. However, in how far trans-endocytosis and endocytosis upon stimulation with soluble ephrin are completely comparable remains questionable as is indicated by the conflicting evidence on the involvement of the CME pathway. Nonetheless, it is conceivable that the GEFs and GAPs regulating endocytosis of Ephs stimulated with soluble ephrin ectodomains are also involved in the regulation of trans-endocytosis, since they can influence Rac activity. Even less is known about the mechanism of reverse endocytosis into ephrin+ cells. So far only very few studies

addressed the topic of its molecular mechanisms and if so, not in a systematic fashion. Furthermore, whether ephrinA-expressing cells experience reverse endocytosis at all is not known to date. Figure 3 summarises the current knowledge of Eph-ephrin endocytosis.

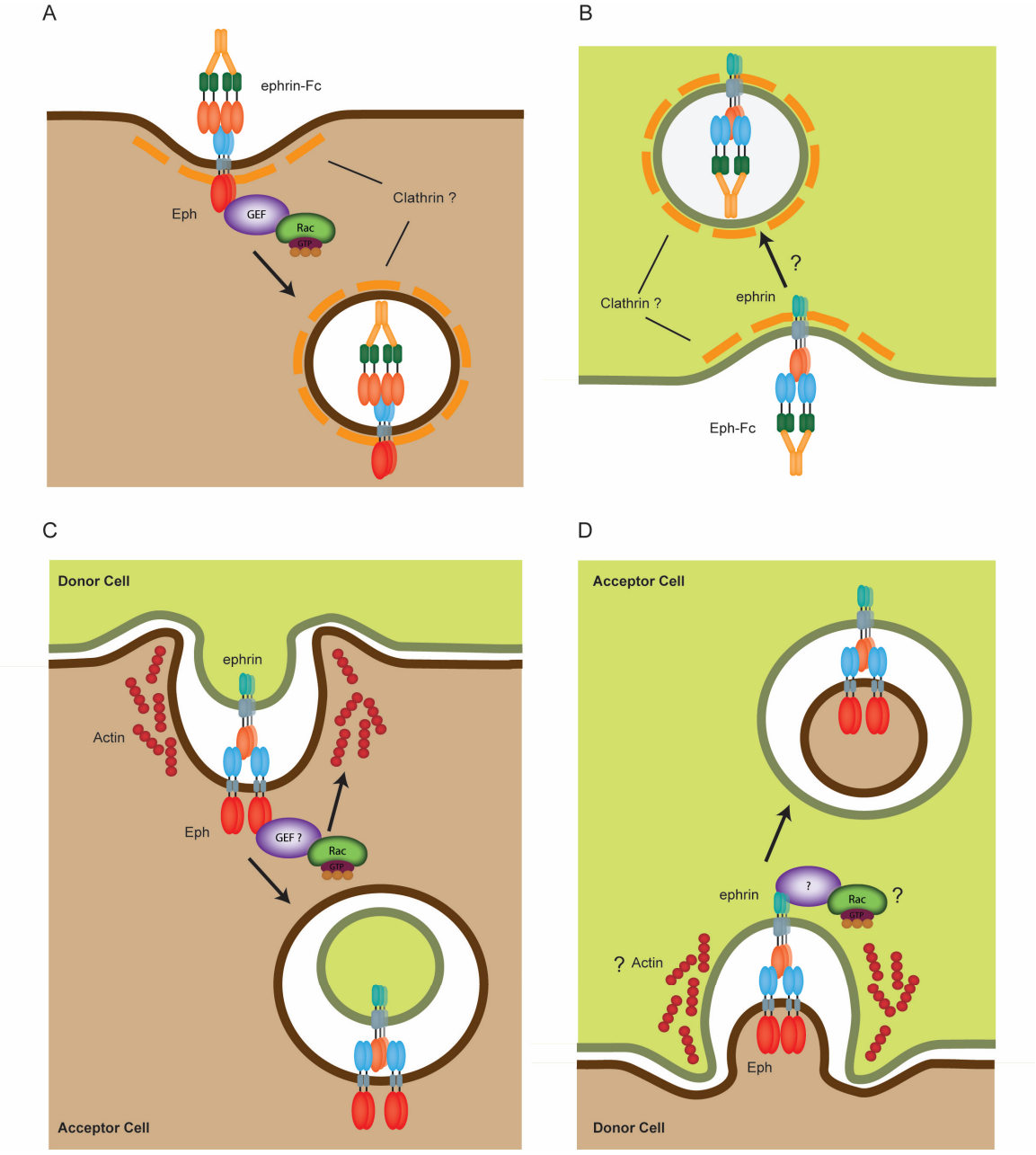


Figure 3. Endocytosis of Eph-ephrin complexes

(A) Forward endocytosis of Eph-ephrin complexes induced by soluble pre-clustered ephrin ectodomains. GEFs activate Rac downstream of Eph-receptors. Tiam1 and Vav2/3 have been implicated as GEFs mediating this process. Evidence for the involvement of clathrin exists. (B) Reverse endocytosis of Eph-ephrin complexes induced by soluble pre-clustered Eph receptors. Evidence for the involvement of clathrin exists. (C) Ephrin trans-endocytosis into Eph+ cell (forward

direction). Rac activity and actin reorganisation required. Identity of GEF in this context still unknown. (D) Eph trans-endocytosis into ephrinB+ cells (reverse direction). Rac activity and actin reorganisation postulated according to reports for the forward direction.

1.3.4 Endocytosis regulated by Eph-ephrin signalling

While endocytosis of Eph-ephrin complexes plays a central role in Eph-ephrin signalling, the Eph-ephrin system in turn can regulate the endocytosis of other molecules.

In axon guidance, collapse and steering of growth cones are pivotal events that both require gross changes in membrane surface area, which can be mediated by endocytosis (Tojima et al. 2011). This process can be regulated by Eph-ephrin signalling. In chick retinal axons, stimulation with ephrinA2 induces endocytosis required for growth cone collapse by activating Rac1 (Jurney et al. 2002).

At synapses, Eph-ephrin signalling can regulate endocytosis of AMPA receptors and thus influence synaptic maturation and plasticity (Irie et al. 2005, Essmann et al. 2008). Remarkably, forward and reverse signalling seem to have opposing effects on the internalisation of AMPA receptors. Irie and colleagues report that EphB2s can associate with the phosphatase synaptojanin and phosphorylate it. Phosphorylation inhibits the phosphatase function of synaptojanin and leads to elevated levels of phosphatidylinositol 4,5-bisphosphate (PIP_2). This in turn leads to an increase in CME and, particularly, to an increase in the internalisation of AMPA receptors. In contrast to this, postsynaptic ephrinB reverse signalling leads to a stabilisation of AMPA receptors at the cell membrane (Essmann et al. 2008). In neurons from ephrinB2 knockout mice, increased AMPA internalisation results in reduced synaptic transmission and synapse loss. Mechanistically the stabilising function of ephrinBs requires phosphorylation of a serine residue and an interaction with scaffolding proteins like GRIP1.

In the vascular system, ephrinB2 reverse signalling can regulate internalisation of vascular endothelial growth factor receptors (VEGFRs) and PDGFRs (Sawamiphak et al. 2010, Nakayama et al. 2013). Guidance of tip cells is as essential for vascular sprouting and development as axon guidance is for neuronal development. VEGFRs help orient tip cells by detecting VEGF gradients. VEGFR-mediated guidance requires the internalisation of the receptor in order to be effective. EphrinB2 reverse signalling, which requires the PDZ

domain of ephrinB2, is necessary for the internalisation of VEGFR2 (Sawamiphak et al. 2010), whereas, in vascular smooth muscle cells, ephrinB2 reverse signalling controls PDGFRbeta internalisation in the opposite fashion (Nakayama et al. 2013). Cells cultured from ephrinB2 mutant mice display increased endocytosis of PDGFRbeta, since the receptor gets redistributed from caveolae to clathrin-coated membrane components. Simultaneously, signalling downstream of PDGFRbeta through MAP kinase and JNK is increased, while signalling through Tiam1 and Rac is reduced, which results in decreased proliferation. Unfortunately, the study does not comment on any link between Tiam1/Rac signalling and endocytosis.

These studies show that regulation of endocytic processes by Eph-ephrin signalling is an important facet of Eph-ephrin function. The studying of the role of Eph-ephrin controlled endocytosis is thus complicated by the fact that the same or overlapping signalling molecules and mechanisms are involved in downstream signalling of Eph-ephrin complexes, the regulation of Eph-ephrin endocytosis itself, or the endocytosis of other proteins regulated by Eph-ephrin signalling. This makes it difficult to distinguish between direct effects on Eph-ephrin endocytosis and indirect effects by interfering with common endocytic pathways for any studied molecule.

1.4 Aims of the study

The Eph-ephrin signalling system is essential for a plethora of physiological functions, especially during development, as well as playing an important role in several diseases, including cancer. Understanding the precise molecular regulation of the Eph-ephrin system is therefore of great interest. An important element of signalling from Eph-ephrin complexes, especially with regards to a switch from mediating an adhesive to a repulsive signal, is the removal of Eph-ephrin complexes from the cell membrane. In addition to cleavage of ephrins (Hattori et al. 2000) or Ephs (Gatto et al. 2014), the key mechanism for removing Eph-ephrin complexes form the cell surface is internalisation by trans-endocytosis (Marston et al. 2003, Zimmer et al. 2003). Internalisation can occur both into the receptor-expressing cell (forward direction) or the ligand-expressing cell (reverse direction).

Despite years of study of the underlying molecular mechanisms of this process, it is still far from being completely understood (see Fig 3). Thus far, research has mainly focussed on forward direction signalling, while little is known about the regulation of the reverse direction. One candidate group of proteins are the Rho family of small GTPases and their regulating GEFs and GAPs, as their requirement in various endocytic pathways has not only been well established (Qualmann & Mellor 2003, Doherty & McMahon 2009), but they have also already been implicated in EphB trans-endocytosis into ephrinB+ cells (Marston et al. 2003).

Another potentially limiting factor of many studies conducted to date is their use of stimulation with soluble recombinant ligand or receptor ectodomains, whereas in the physiological situation, both elements are membrane-tethered and the trans-endocytosis process involves entire complexes of full-length proteins, including patches of membrane from the opposing cell. It is very conceivable that the internalisation of pre-clustered soluble ectodomains makes use of a distinct endocytic machinery compared to the trans-endocytosis occurring in a cell-cell contact-mediated situation.

This study therefore comprises four central aims:

1. Deciphering the contribution of different Rho-family GTPases in the regulation of EphB trans-endocytosis into ephrinB+ cells by systematic experimental analysis.
2. Identifying key upstream regulators of EphB trans-endocytosis among the Rho-family GEFs and GAPs using an image-based siRNA screen.
3. Comparing the molecular mechanisms required for EphB trans-endocytosis into ephrinB+ cells to those implicated in ephrinB trans-endocytosis into EphB+ cells, and verifying the physiological significance of our findings in primary neuronal cultures.
4. Clarifying the physiological relevance of using soluble recombinant ectodomains to study Eph-ephrin endocytosis by analysing whether internalisation of soluble ectodomain-induced Eph-ephrin complexes differs in the endocytic machinery used from the trans-endocytosis of membrane-tethered complexes.

Understanding these key points of the molecular mechanisms of Eph-ephrin endocytosis will be important in unravelling how the Eph-ephrin system shapes intercellular interactions and communication in developmental and disease contexts.

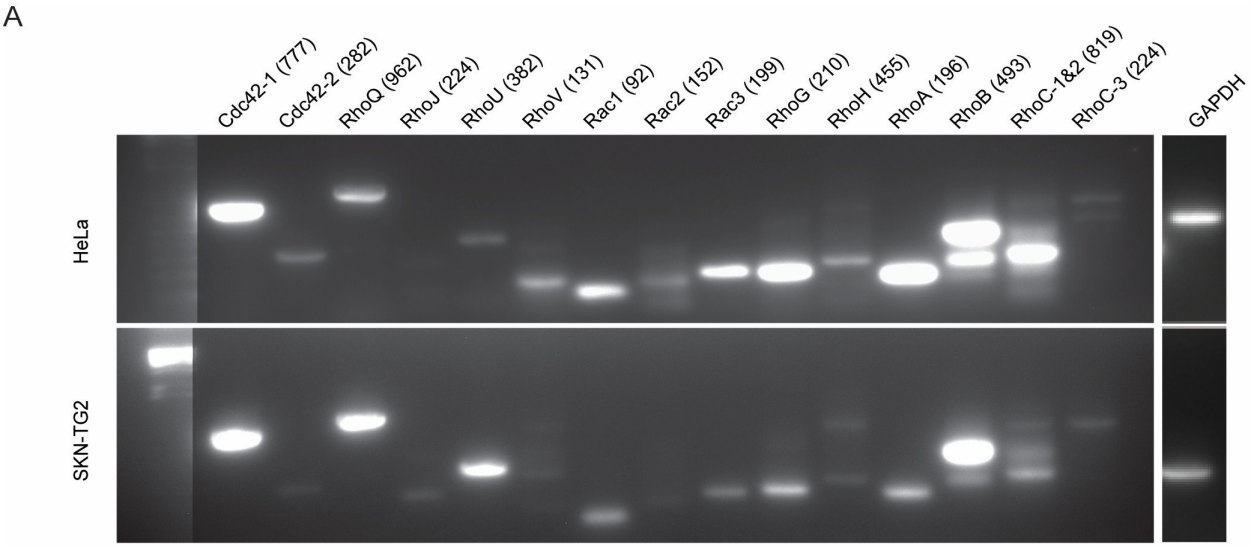
2 Results

2.1 Establishing tools for the investigation of EphB trans-endocytosis into ephrinB+ cells

2.1.1 HeLa and SKN cells express a wide variety of Rho-family GTPases

The general mechanisms of Eph-ephrin endocytosis can be studied in cultured cells originally derived from human cell lines. These cells are easily accessible using light microscopy and a considerable toolbox of genetic techniques, such as over-expression of selected proteins or knockdown of protein expression by siRNA transfection, can be easily utilised. As SKN neuroblastoma cells endogenously express ephrinB1 and ephrinB2 (T. Gaitanos, unpublished results), and since we are particularly interested in the mechanisms of EphB-ephrinB reverse endocytosis, we chose to employ them as the principal cell line to perform our experiments in. For easier identification of the SKN cells, we used a subclone stably over-expressing red fluorescent protein (RFP) tagged to histone 2B (H2B-RFP), which resulted in a fluorescently labelled nucleus. We additionally conducted experiments in HeLa cells, a human cell line derived from cervical cancer cells, to confirm that results obtained from SKN cells were not cell line-specific, but represented general mechanisms in Eph-ephrin endocytosis. Both HeLa and SKN cells are derived from cancer cells, which are known to express a variety of Rho-family GTPases (Sahai & Marshall 2002a), and there is the potential of physiological redundancy, if several members of the same subfamily are co-expressed. Therefore we used reverse transcriptase polymerase chain reaction (RT-PCR) to identify which GTPases are expressed. We focused on the RhoA, Rac and Cdc42 subfamilies, given their well-established role in endocytic processes and the atypical characteristics of the other Rho family GTPases (see Introduction section 1.2.1). Figure 4 depicts results from the RT-PCR with an image from the gel loaded with the RT-PCR products (Fig. 4A). As both the primers for RhoJ and RhoC resulted in PCR products of the wrong size, we repeated the RT-PCR for these genes with different primer sets (Fig. 4B). The table in Figure 4C shows that many of the tested GTPases are expressed in both SKN and HeLa cells. SKN cells express RhoA and RhoB, the Rac subfamily members Rac1, Rac3 and RhoG, and the Cdc42 subfamily members Cdc42, RhoQ and

RhoU. HeLa cells additionally express Rac2 from the Rac subfamily and RhoV from the Cdc42 subfamily. This high number of different GTPases expressed in SKN and HeLa cells could potentially pose a challenge for studying their involvement in Eph-ephrin endocytosis and the possibility of their physiological redundancy was thus addressed in our experiments.



C

Subfamily	Gene	Expression	
		SKN	HeLa
Cdc42	Cdc42	√, ½	√, ½
	RhoQ	√	√
	RhoJ	—	—
	RhoU	√	½
	RhoV	—	√
Rac1	Rac1	√	√
	Rac2	—	√
	Rac3	√	√
	RhoG	√	√
RhoA	RhoA	√	√
	RhoB	√	√
	RhoC	—	—

Figure 4. Expression of Rho family GTPases in SKN and HeLa cells

(A) Image from gels loaded with RT-PCR products generated with the primers labelled on top. Splice isoforms labelled after the gene name where required. GAPDH was used as a control for the effectiveness of the RT-PCR. Top panel depict results from HeLa cells, bottom panel from SKN cells. (B) Image from the repetition of RT-PCR with inconclusive results from (A) with different primer sets. (C) Table of the expression of the Rho family members in SKN and HeLa cell. All data in this figure were generated by T. Gaitanos.

2.1.2 Establishing siRNA knockdown as a tool for studying the involvement of Rho-family GTPases in Eph-ephrin endocytosis

A useful way to study the functional requirements of proteins is knockdown using siRNA. We confirmed the effectiveness of the siRNA knockdown of Rho family GTPases by Western blot analysis. Since SKN cells express several members of the respective GTPase subfamilies (see Fig. 4), we tested siRNA oligos for each of the expressed GTPases. Protein expression was detected from total cell lysates using specific antibodies against the protein being knocked down, or in the cases of over-expression, tagged proteins with anti-GFP or anti-FLAG antibodies, respectively. Tubulin or GAPDH were used as loading controls.

20 nM was chosen as the maximum working concentration for the siRNA, since it provided a good balance between knockdown efficiency and cell toxicity (T. Gaitanos, unpublished results). None of the commercially available antibodies for the GTPases RhoQ and RhoU detected a specific band at the correct molecular weight. Therefore I first over-expressed tagged versions of the proteins, RhoQ-GFP or FLAG-RhoU, and then knocked down expression with siRNA. Representative blots show that knockdown of Cdc42 was dose-dependent and highly effective at 20 nM oligo concentration (Fig. 5A). For RhoQ and RhoU, several oligos were tested and those resulting in the most effective knockdown (oligo number 19 for RhoQ and number 45 for RhoU) were used for subsequent experiments (Fig. 5B and C). Knockdown of Rac subfamily members was not as efficient as for Cdc42 subfamily GTPases. Treatment with Rac1-specific siRNA oligos or the combination of Rac1- and Rac3-specific oligos did not lead to a very effective knockdown of Rac1, as revealed by the amount of protein remaining in the representative blot with anti-Rac1 antibody (Fig. 5D, upper panel). Further, knockdown of Rac3 was not one hundred percent effective, as revealed by detection using an anti-Rac3 antibody (Fig. 5D, lower panel). However, the remaining amount of protein detected could also be due to cross-reactivity of the anti-Rac3 antibody with Rac1 protein, as suggested by the visible decrease in antibody-binding observed in the cells treated with a Rac1-specific oligo. Knockdown of RhoG, on the other hand, was very effective with both of the RhoG-specific oligos tested, as shown in the representative blot (Fig. 5E). For the RhoA subfamily proteins, we tested the protein-specific oligos at half the maximum concentration (10 nM) mixed with scramble oligos in order to resemble the condition of the double knockdown,

where the two oligos were also used at 10 nM each. The two representative blots in figure 5F clearly revealed effective knockdown of both RhoA (upper panel) and RhoB (lower panel) in the single as well as in the double knockdown conditions:

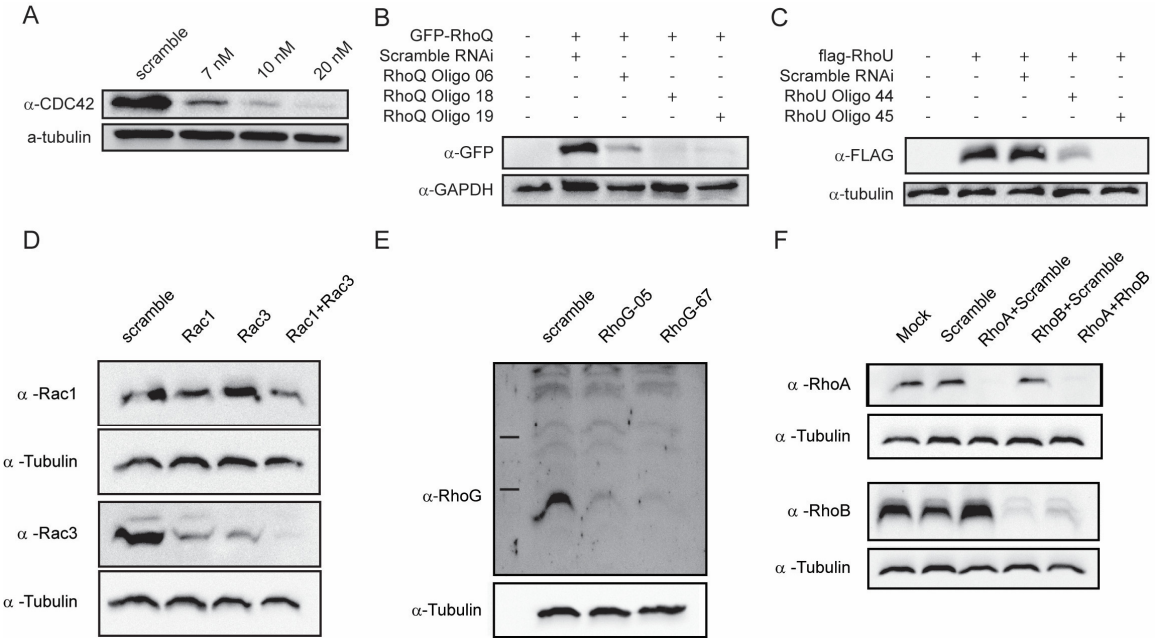


Figure 5. siRNA knockdown of Rho family GTPases

(A) Western Blot of siRNA knockdown of Cdc42 in SKN cells. Lysates from SKN cells treated with either 20 nM of scramble oligo or 7, 10, 20 nM of a Cdc42-specific oligo and blotted with anti-Cdc42 antibody. Anti-tubulin was used as a loading control. (B) Western Blot of siRNA knockdown of RhoQ in SKN cells overexpressing a RhoQ-GFP construct. Lysates from cells treated with either 20 nM of scramble oligo or three different RhoQ-specific oligos (numbered 06, 18 and 19) and blotted with anti-GFP antibody. Anti-GAPDH was used as a loading control. (C) Western Blot of siRNA knockdown of RhoU in SKN cells overexpressing a RhoU-FLAG construct. Lysates from cells treated with either 20 nM of scramble oligo or two different RhoU-specific oligos (numbered 44 or 45) and blotted with anti-FLAG antibody. Anti-tubulin was used as a loading control. (D) Western Blot of siRNA knockdown of Rac1 and/or Rac3 in SKN cells. Lysates from cells treated with either 20 nM of scramble, Rac1-specific or Rac3-specific oligo, or a combination of 10 nM each of the Rac1- and Rac3-specific oligos and blotted with anti-Rac1 antibody (upper panel) or anti-Rac3 antibody (lower panel). Anti-tubulin was used as a loading control. (E) Western Blot of siRNA knockdown of RhoG in SKN cells. Lysates from cells treated with either 20 nM of scramble oligo or two different RhoG-specific oligos (numbered 05 and 67) and blotted with anti-RhoG antibody. Anti-tubulin was used as a loading control. (F) Western Blot of siRNA knockdown of RhoA and/or RhoB in SKN cells. Lysates from cells treated with either 20 nM of scramble oligo, or a combination of 10 nM of scramble oligo and 10 nM of either RhoA-specific or RhoB-specific oligo (lanes 3 and 4, respectively), or a combination of 10 nM each of the RhoA- and RhoB-specific oligos and blotted with anti-RhoA antibody (upper panel) or anti-RhoB antibody (lower panel). Anti-tubulin was used as a loading control. Representative blots are shown in all panels. Data in panels (D)-(F) were generated by T. Gaitanos.

2.1.3 siRNA knockdown or pharmacological inhibition of Rho family GTPases do not alter surface expression of ephrinBs

To study the endocytosis of Eph-ephrin complexes, it is important that the amount of available ephrin on the cell surface is unchanged between different experimental conditions in order to avoid potentially skewed results. Therefore, I tested the surface expression of ephrinBs under siRNA treatment or pharmacological inhibition of Rac family members with EHT1864 (Shutes et al. 2007) by incubating SKN H2B-RFP cells with 2 µg/ml of pre-clustered EphB2-Fc tagged with a fluorescently labelled antibody. Incubation was limited to 2 min at room temperature before transferring cells on ice to prevent endocytosis. After fixation, I stained for clusters on the cell surface in non-permeabilising conditions with a fluorescently labelled secondary antibody against Fc (Fig. 6A). Images were analysed with CellProfiler™ software (Carpenter et al. 2006) and the number of surface clusters per cell between the different conditions were compared (for further details, see Materials and Methods section 4.2.12). To serve as a positive control, ephrinB itself was knocked down in SKN cells, since in cells depleted of ephrinBs, no binding of EphB2-Fc at the surface should be expected. Accordingly, surface cluster numbers were significantly reduced in cells depleted of both ephrinB1 and ephrinB2 (5.52 clusters/cell) when compared to the scramble control (27.63 clusters/cell), while knockdown of ephrinB2 alone gave an intermediate response (12.68 clusters/cell) as the cells were still expressing ephrinB1 (Fig. 6B). Remaining vesicles in the ephrinB knockdown control were potentially due to a less than 100% effectiveness of the transfected siRNA. As an additional control, cells were stimulated with hFc instead of Eph2-Fc, which form almost no surface clusters as expected. The observed average value of 0.2 clusters per cell treated with hFc very likely reflected unspecific debris or artefacts from the automated analysis with CellProfiler™ software. Importantly, neither knockdown of all expressed members of the Cdc42, Rac or RhoA subfamilies by combination of siRNA oligos (Fig. 6B and D), nor inhibition of Rac with EHT1864 (Fig. 6C and E) affected the levels of ephrinBs expressed on the surface of SKN cells significantly.

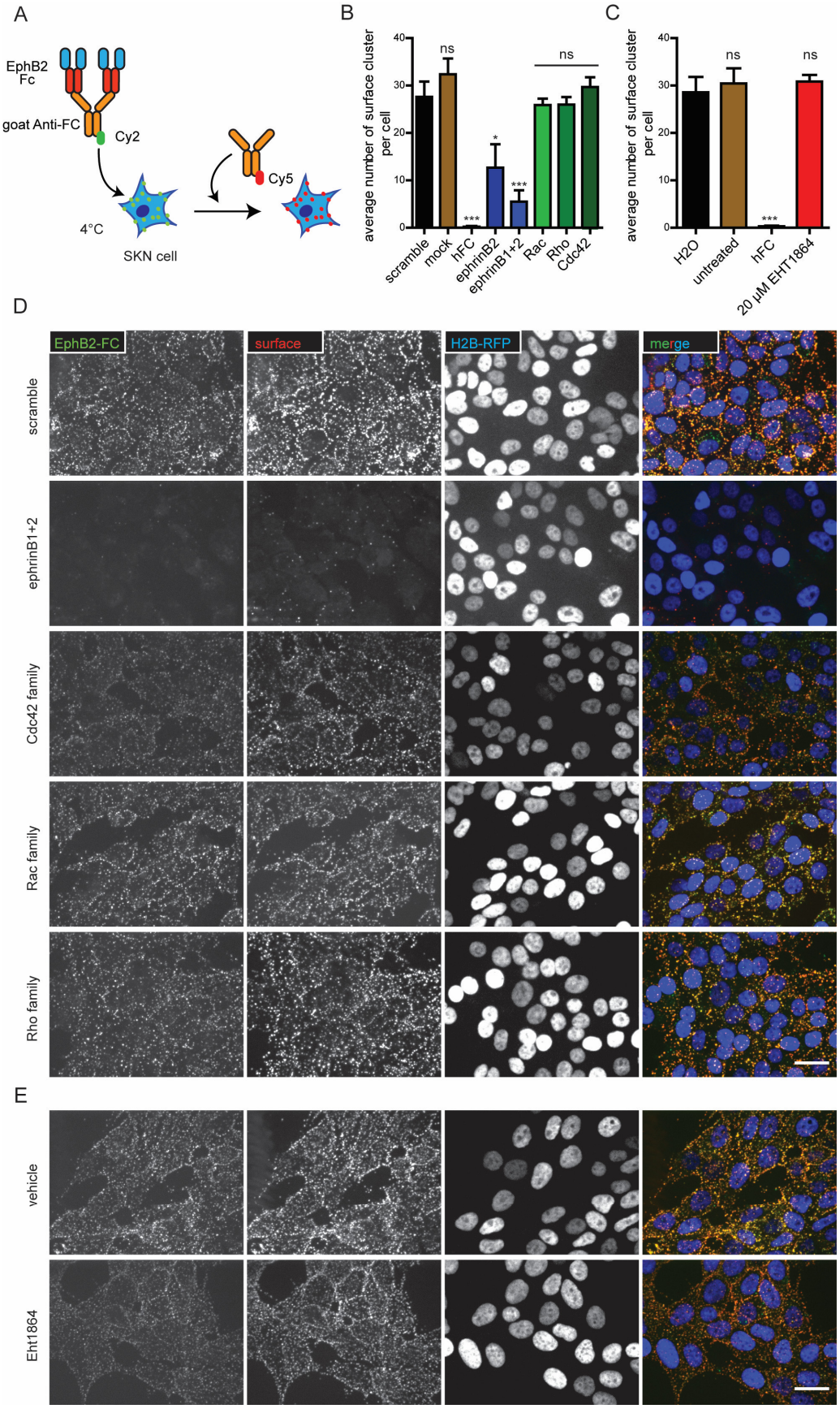

A
EphB2 Fc
goat Anti-FC
Cy2
4°C
SKN cell
Cy5
B
average number of surface cluster per cell
ns
ns
*
***
***
scramble
mock
hFC
ephrinB2
ephrinB1+2
Rac
Rho
Cdc42
C
average number of surface cluster per cell
ns
ns
***
H2O
untreated
hFC
20 µM EHT1864
D
EphB2-FC
surface
H2B-RFP
merge
scramble
ephrinB1+2
Cdc42 family
Rac family
Rho family
E
vehicle
Eht1864

Figure 6. Surface expression of ephrinBs

(A) Schematic representation of the experimental setup. SKN H2B-RFP cells treated with siRNA or Rac inhibitor EHT1864 were stimulated with EphB2-Fc pre-clustered with a Cy2-conjugated antibody (green dots) on ice for 5 min. Cells were subsequently fixed and stained with a dyLight649-conjugated antibody against Fc to visualise surface clusters (red dots). (B) Quantification of the average number of surface clusters per cell for SKN cell treated with mock transfection, scramble siRNA, ephrinB1 and ephrinB2 siRNA or a combination of Rho-subfamily siRNAs. As an absolute control stimulation with hFc instead of EphB2-Fc was used. CellProfiler™ was used to analyse images, n=3 independent experiments each performed in triplicate. (C) Quantification of average number of surface clusters per cell for cells untreated, treated with vehicle control or treated with 20 µM EHT1864. Again quantification was performed with CellProfiler™ and hFc stimulation was used as an absolute control. n=4 independent experiments, each performed in triplicate. For both B and C data represented by mean +/- SEM. Statistical significance was tested with ANOVA followed by Dunnett's post hoc test. (*<0.05, ***<0.005) (D) Example images for cells treated with scramble or a pool of ephrinB1 and ephrinB2 oligos, or simultaneous knockdown of all the expressed members the three Rho subfamilies. Total amount of EphB2-Fc in green, surface EphB2-Fc in red (yellow in the overlay), the H2B-RFP nuclei in blue and the merged image of the three channels are shown. (E) Example images for SKN cell treated with either vehicle or 20 µM EHT1864. Channels as in (D). Scale bar represents 20 µm.

2.1.4 siRNA knockdown of Rho family GTPases does not inhibit motility of SKN cells

One of our primary goals was to understand the trans-endocytosis mechanism of Eph-ephrin complexes in the context of cell-cell contact and repulsion. To adequately study this physiological process, we relied on experiments using co-culture systems, in which cells expressing ephrinBs or EphB receptors come into contact with each other. Given the well-established effects of Rho family GTPases on the actin cytoskeleton and cell motility (Parri & Chiarugi 2010), I tested whether siRNA knockdown of entire subfamilies of Rho GTPases interfered with cell motility and the ability of SKN cells to contact other cells. Live-cell imaging for extended periods of time (up to 6 h) revealed that SKN cells treated with siRNA against Rho family GTPases remain mobile and retain their ability to come into contact with other cells, while confirming the expected changes in cell morphology (Fig. 7). Experiments also demonstrated that over a period of 60 minutes, cells were able to form new contacts with other cells and detach from each other. We therefore chose 80 min as the duration for our co-culture trans-endocytosis experiments, as it allowed cells sufficient time to settle, come into contact with neighbouring cells, and engage in Eph-ephrin signalling and trans-endocytosis.

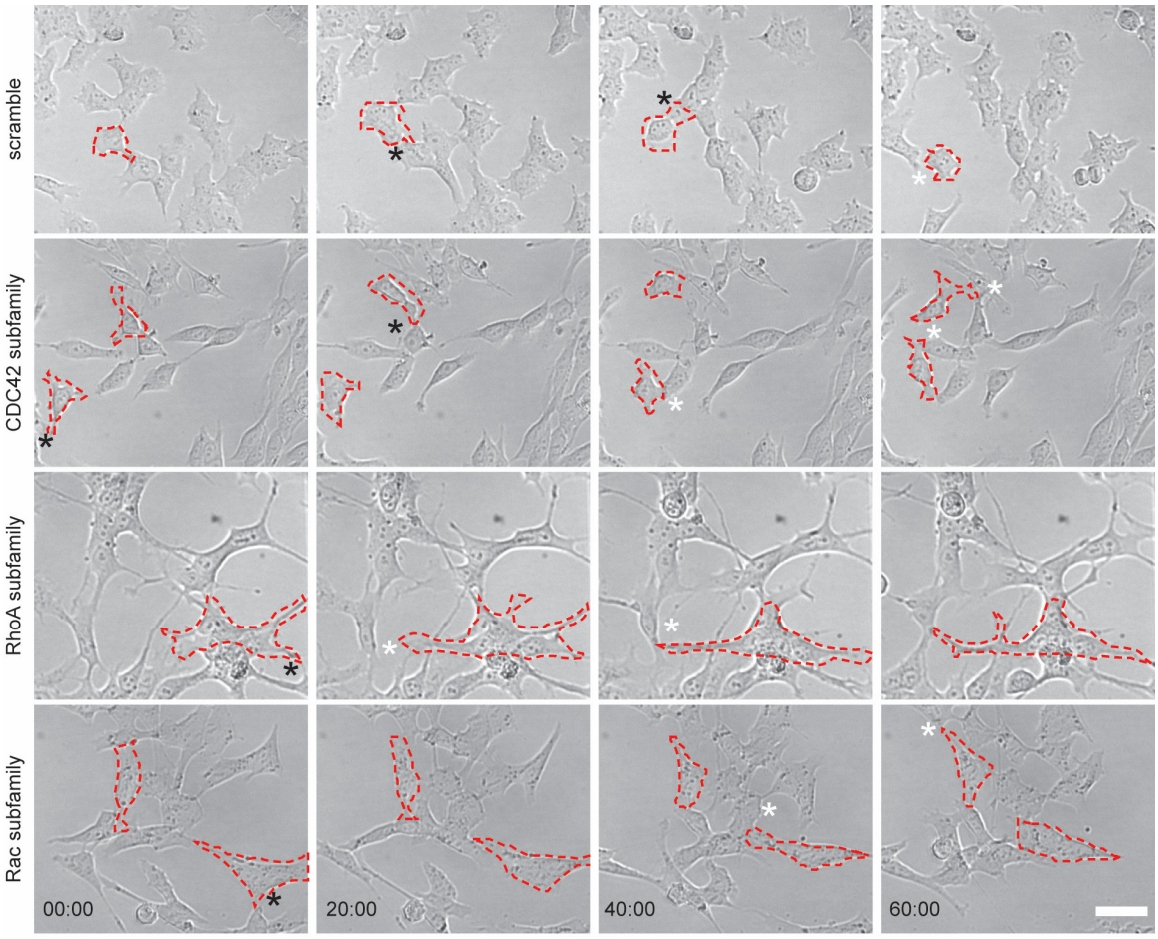


Figure 7. Live-cell imaging of motility of siRNA-treated SKN cells

Cells were seeded into 8-well live-cell imaging chambers and treated with siRNA as labelled. Live-cell imaging was performed over 6 h at 5 min intervals. 4 frames taken 20 min apart are shown for each treatment. Red dashed outline highlights cells of interest. Cell contacts lost in the next frame are marked by a black asterisk, contacts newly formed in this frame are marked by a white asterisk. Scale bar represents 20 µm.

2.2 Deciphering the role of Rho family GTPases in Eph-Ephrin endocytosis

2.2.1 Rac subfamily GTPases are required for EphB trans-endocytosis into ephrinB+ cells

The trans-endocytosis process of Eph-ephrin complexes containing both full-length ligands and receptors was originally described in two studies published 10 years ago (Marston et al. 2003, Zimmer et al. 2003). The work of Marston and colleagues already showed that trans-endocytosis in the forward direction is blocked in cells expressing a dominant negative version of Rac. For the reverse direction, however, the molecular mechanisms are

unknown. We therefore tested whether Rac subfamily GTPases are also required for EphB trans-endocytosis into ephrinB+ cells.

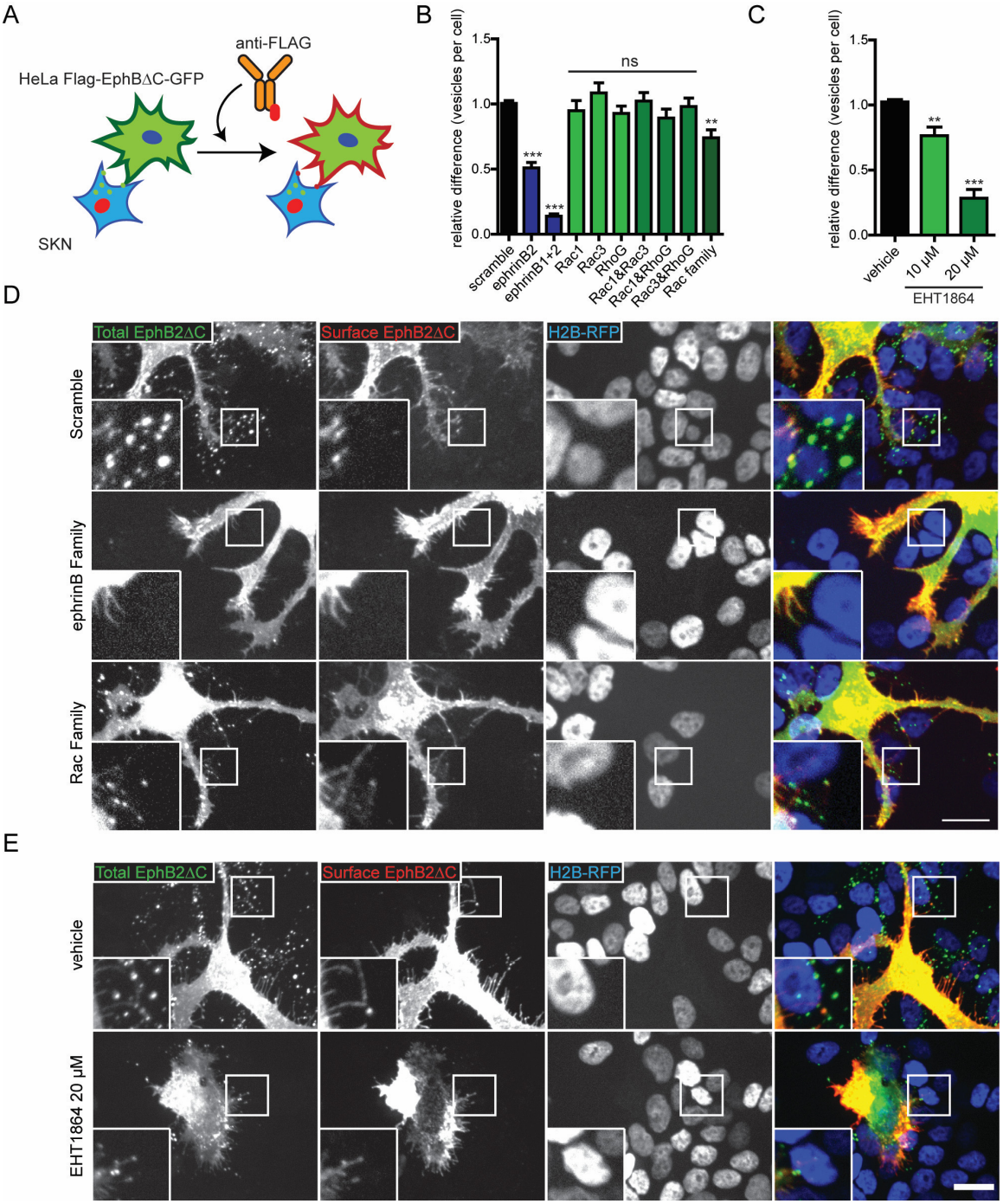
To this end, we performed co-culture experiments with ephrinB+ SKN H2B-RFP (acceptor cells) cells previously treated with siRNA against Rac proteins and HeLa cells expressing a FLAG-EphB2ΔC-GFP construct (donor cells). Since the C-terminally truncated form of the EphB2 receptor expressed in the donor cells is defective for endocytosis in the forward direction (Zimmer et al. 2003), trans-endocytosis will only occur in the reverse direction in this experimental setup, i.e. from the HeLa cell into SKN cells. The FLAG epitope on the extracellular domain of the EphB2 receptor enables staining of Eph-ephrin complexes located on the cell surface. The difference between the total number of Eph-ephrin clusters in SKN cells and the number of clusters located on the cell surface represents the number of internalised clusters (Fig. 8A). To dissect the potential individual contributions of the different Rac subfamily GTPases and account for possible redundancies between them, we used oligos against Rac1, Rac3 and RhoG separately, as well as all possible combinations of any two Rac subfamily GTPases and a knockdown of all three of them. As a negative control, cells were treated with scramble oligos and as a positive control, siRNA against ephrinB2 or against both ephrinB2 and ephrinB1 was used to prevent cluster formation and thus internalisation. After siRNA treatment for 72 h, SKN cells were co-cultured with HeLa cells expressing FLAG-EphB2ΔC-GFP for 80 min in order for cell contact, cluster formation and trans-endocytosis to occur. Cells were then fixed and stained against the FLAG epitope to visualise surface clusters. For each experiment, each condition was tested in triplicate in 96-well plates and for each well, 10 images were taken (Fig. 8D). Image analysis was performed semi-automatically using CellProfiler^{TM} and is described in detail in section 4.2.12 and Fig. 23..

Knockdown of ephrinBs reduced trans-endocytosis of Eph-ephrin complexes as expected and thus validated the experimental approach. In contrast knockdown of single Rac subfamily members or a combination of knockdowns of any two Rac subfamily members did not result in significant changes in the number of internalised Eph-ephrin clusters when compared to the negative control (Fig. 8B). However, when Rac1, Rac3 and RhoG were targeted by siRNA oligos simultaneously, the number of internalised clusters was

significantly decreased in comparison to treatment with scramble oligos. The observed decrease in trans-endocytosis was only partial, though (reduced to 75 % of control levels, Fig. 8B), which could potentially be explained by ineffectiveness of the siRNA oligos used (compare Fig. 5D). These results indicate that Rac subfamily GTPases are required for the efficient trans-endocytosis of EphB into ephrinB+ SKN cells, and that Rac1, Rac3 and RhoG are physiologically redundant in this context.

Figure 8. Rac subfamily GTPases are required for EphB trans-endocytosis into ephrinB+ SKN cells

(A) Schematic representation of the experimental setup. HeLa cells expressing FLAG-EphB2ΔC-GFP in green and SKN H2B-RFP cells in blue. After co-culture and reverse trans-endocytosis into SKN cells, surface EphB2ΔC is stained with anti-FLAG antibody. (B) Quantification of average number of internalised clusters per cell in the siRNA assay performed with CellProfiler™ including the data for the knockdown of the individual members of the Rac subfamily as well as for all different combinations of oligos. Data shown as mean of the individual means normalised to the median of the scramble control. Repeated measures ANOVA with Dunnett's post-hoc test was used to test for significance. **=p<0.01, ***=p<0.005 (n= 8 independent experiments) (C) Quantification of average number of internalised clusters per cell in the Rac inhibitor assay performed with CellProfiler™ including data for both 10 µM and 20 µM EHT1864. Data shown as mean of the individual means normalised to the median of the vehicle control. Repeated measures ANOVA with Dunnett's post-hoc test was used to test for significance. **=p<0.01, ***=p<0.005 (n= 4 independent experiments) (D) Co-culture of SKN-H2B-RFP cells with HeLa cells overexpressing EphB2ΔC. SKN H2B-RFP cells were treated with siRNA (either as scramble sequence, or pools of oligos targeting ephrinBs 1 and 2, or Racs 1, 3 and RhoG; top, middle and bottom rows, respectively) for 72 h prior to 80 min co-culture with HeLa cells expressing FLAG-EphB2ΔC-GFP. Cells were then fixed on ice and probed against the FLAG tag without permeabilisation (surface EphB2ΔC, shown in red). Internalised clusters were determined as total EphB2ΔC (green) puncta distinct from surface EphB2ΔC (appears as yellow) within the vicinity of the SKN nuclei (RFP channel, shown in blue). (E) Example image for co-culture of SKN H2B-RFP cells treated with vehicle (top panels) or 20 µM EHT1864 prior to seeding of HeLa-EphB2ΔC-GFP/FLAG cells. Cells were then fixed on ice and probed against the FLAG tag without permeabilisation (Surface EphB2ΔC, shown in red). Internalised clusters were determined as total EphB2ΔC (green) puncta distinct from surface EphB2ΔC (appears as yellow) within the vicinity of the SKN nuclei (RFP channel, shown in blue). Scale bars represent 20 µm. All experiments in this figure performed by T. Gaitanos.



In order to corroborate these findings, we pharmacologically inhibited Rac activity by treatment with the specific Rac inhibitor EHT1864 in the same experimental setup. Instead of transfection with siRNA oligos, SKN cells were treated with vehicle control or EHT1864 at a concentration of either 10 µM (images not shown) or 20 µM for 4 h before

co-culturing with HeLa cells (Fig. 8E). Confirming results from our siRNA experiments, treatment with EHT1864 led to a reduction in the number of internalised Eph-ephrin clusters in comparison with the control condition in a dose-dependent manner (Fig. 8C). The effect on trans-endocytosis was stronger than that observed in siRNA experiments (reduced to 25% of control levels with 20 µM EHT1864, Fig. 8C), suggesting that inhibitor treatment leads to a more effective reduction of Rac activity than siRNA-mediated knock down of protein expression.

We wanted to rule out that the requirement of Rac subfamily GTPases in EphB trans-endocytosis into ephrinB+ cells is a cell type-specific effect and thus conducted experiments with HeLa cells as both donor and acceptor cells. Since HeLa cells express Rac2 in addition to the other three Rac subfamily members expressed in SKN cells, and knockdown of a fourth protein by siRNA is technically very challenging, we chose to also use EHT1864 for experiments in HeLa cells. HeLa cells expressing ephrinB1-mCherry (as acceptor cells) were treated with EHT1864 as described above and co-cultured with HeLa cells expressing a FLAG-EphB2ΔC-GFP construct (Fig. 9A). Inhibition of Rac activity with EHT1864 resulted in a significant reduction in the number of internalised EphB-ephrinB clusters compared to vehicle-treated control at concentrations of both 10 µM and 20 µM (Fig. 9B and C), as determined by manual counting of images acquired and analysed blindly. These results confirm the findings in SKN cells and indicate that the requirement of Rac activity for EphB trans-endocytosis into ephrinB+ cells represents a general mechanism.

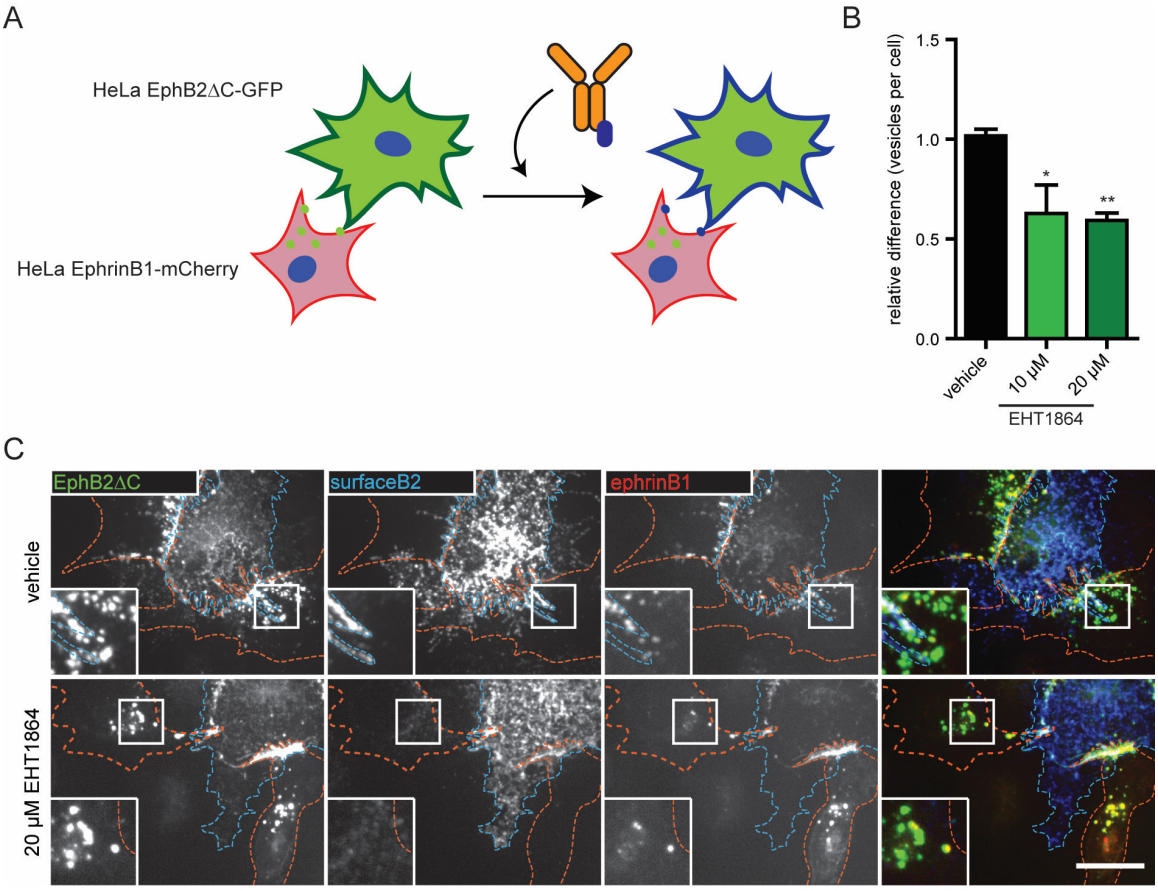


Figure 9. Rac subfamily GTPases are required for EphB trans-endocytosis into ephrinB-expressing HeLa cells

(A) Schematic representation of the experimental setup. HeLa cells expressing FLAG-EphB2ΔC-GFP (donor cells) in green and HeLa cells expressing ephrinB1-mCherry (acceptor cells) in red. After co-culture and reverse trans-endocytosis into acceptor cells the remaining EphB2ΔC on the surface is stained with anti-FLAG antibody. (B) Quantification of average number of internalised clusters per cell in the Rac inhibitor assay scored manually including data for both 10 µM and 20 µM EHT1864. Data shown as mean of the individual means normalised to the median of the vehicle control. All experiments performed blind. Repeated measures ANOVA with Dunnett's post-hoc test was used to test for significance. *=$p<0.05$, **=$p<0.01$ (n= 4 independent experiments) (C) Co-culture of HeLa cells expressing ephrinB1-mCherry (red dashed outline) with HeLa cells overexpressing EphB2ΔC (blue dashed outline). HeLa cells expressing ephrinB1-mCherry were treated with vehicle control (top panel) or 20 µM EHT1864 (bottom panel) for 4 h prior to 80 min co-culture with HeLa cells expressing FLAG-EphB2ΔC-GFP. Cells were then fixed on ice and probed against the FLAG tag without permeabilisation (surface EphB2ΔC, shown in blue). EphirnB1-mCherry expression is shown in red. Internalised vesicles were determined as total EphB2ΔC (green) puncta distinct from surface EphB2ΔC (appears as white) within the ephrinB1-mCherry expressing cell (red dashed outline). Scale bar represents 10 µm. All experiments in this figure performed by T. Gaitanos.

2.2.2 Rac activity is required for EphB trans-endocytosis into ephrinB+ primary cortical neurons

Trans-endocytosis of EphB receptors from opposing cells into ephrinB positive neurons is crucial for allowing efficient cell detachment during the growth cone collapse response (Zimmer et al. 2003). I therefore wanted to investigate if Rac activity is also required for EphB trans-endocytosis in cultured neurons. Primary cortical neurons endogenously express ephrinBs and are thus a very good experimental model (Tanaka et al. 2004). I cultured cortical neurons derived from E15.5 mouse embryos overnight in live cell imaging chambers. Neurons were stained with CellTracker Green and treated with vehicle control or several concentrations of Rac inhibitor EHT1864 prior to co-culturing with HeLa (donor) cells transiently over-expressing an EphB2ΔC-mCherry construct. Live cell imaging was performed on a spinning disk confocal microscope equipped with an incubation chamber to maintain an environment of 5% CO_2 and 37 °C. Several positions where EphB2ΔC-mCherry expressing HeLa cells settled next to a healthy neuron were selected and imaged every three minutes for a total duration of three hours. Resulting videos were analysed manually by observing sites of contact between neuron and HeLa cell and determining, whether internalisation of EphB2ΔC-mCherry-containing complexes into the neurons occurred. To ensure proper detachment had occurred internalisation was determined as a vesicle detaching from HeLa cell protrusions for at least three consecutive frames. Treatment with 20 µM EHT1864 led to growth cone collapse in the majority of neurons (data not shown), thus cells were treated with concentrations of 2.5 µM, 5 µM and 10 µM. Figure 10A shows frames taken from example movies of vehicle-treated neurons exhibiting trans-endocytosis of EphB2ΔC-mCherry-containing complexes (top two panels), and prolonged cell adhesion without internalisation of EphB2ΔC-mCherry-containing complexes in neurons treated with EHT1864 (bottom panel). Quantification of the average percentage of contact sites with internalisation events revealed a significant reduction of trans-endocytosis of EphB2ΔC-mCherry-containing complexes into neurons treated with EHT1864 at all tested concentrations (Fig. 10B). I therefore conclude that Rac activity is required for EphB trans-endocytosis into ephrinB+ neurons and the mechanism is potentially the same as the one employed by HeLa and SKN cells.

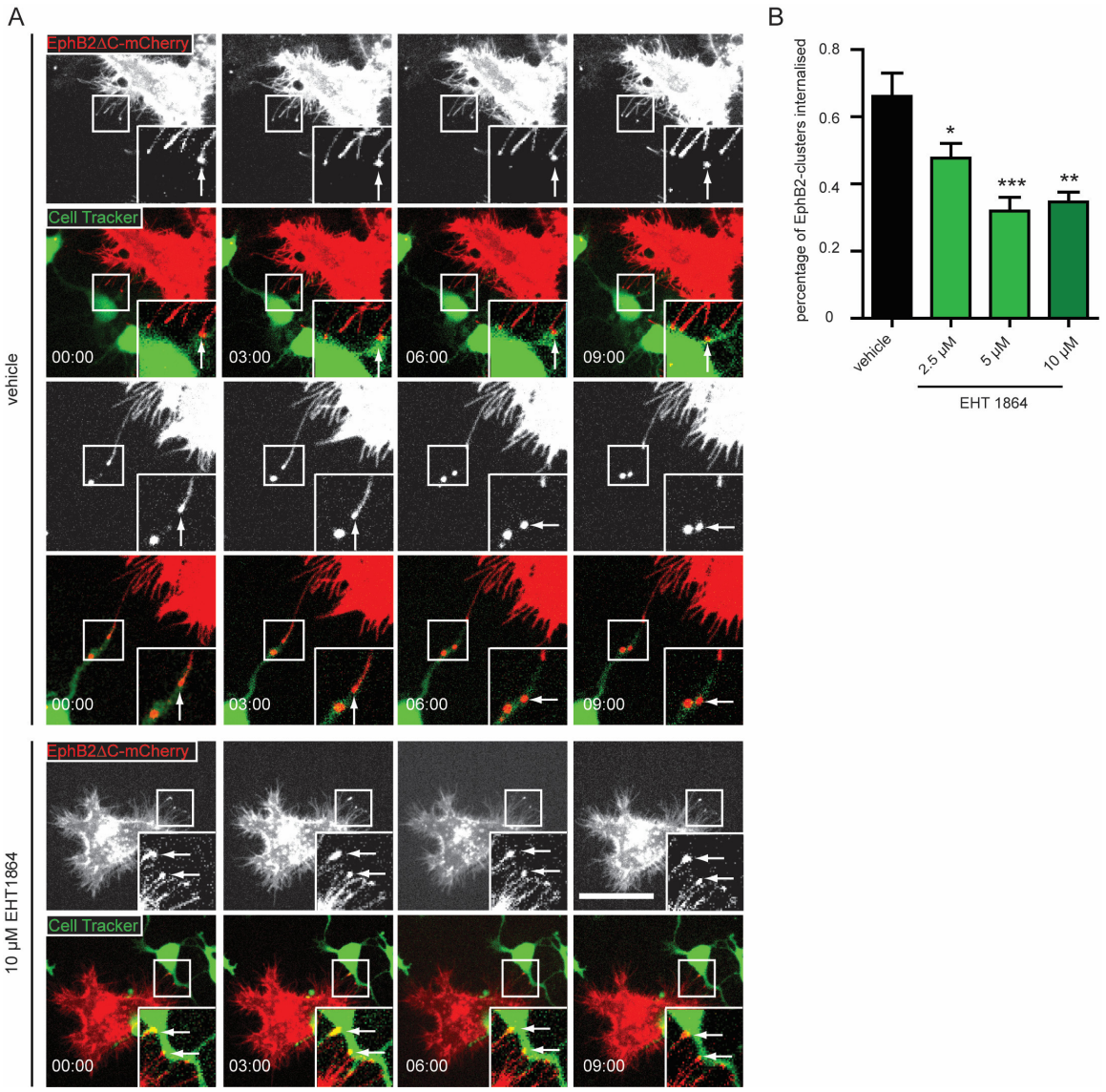


Figure 10. Live-cell imaging of EphB trans-endocytosis into ephrinB+ cortical neurons

(A) Neurons were grown in 8-well live-cell imaging chambers overnight and were stained with CellTracker™ Green (green in overlay). Subsequently they were treated with either vehicle control (top two panels) or 10 µM EHT1864 for 4h, before being co-cultured with HeLa cells over-expressing EphB2ΔC-mCherry (upper rows as single channel, red in overlay). Live-cell imaging was performed over 3 hours at 3 min intervals. 4 subsequent frames are shown as examples for each treatment. Arrows in the magnified inserts indicate sites of cell contact and cluster formation. Note that in the two examples for the control detachment of the Eph-ephrin cluster occurs, while the cell contact remains without internalisation in the Rac inhibitor treated cells. Scale bar represents 20 µm. (B) Quantification of the percentage of internalisation occurring at contact sites scored manually with MetaMorph™ software. Data for all three tested concentrations (2.5 µM, 5 µM and 10 µM) shown and vehicle control shown as average of n=4 independent experiments +/- SEM. Repeated measures ANOVA followed by Dunnett's post-hoc test was performed to test for significance. *=p<0.05, **=p<0.01, ***=p<0.005

2.2.3 Rac subfamily GTPases are not required for endocytosis of soluble EphB2 ectodomains into ephrinB-expressing cells

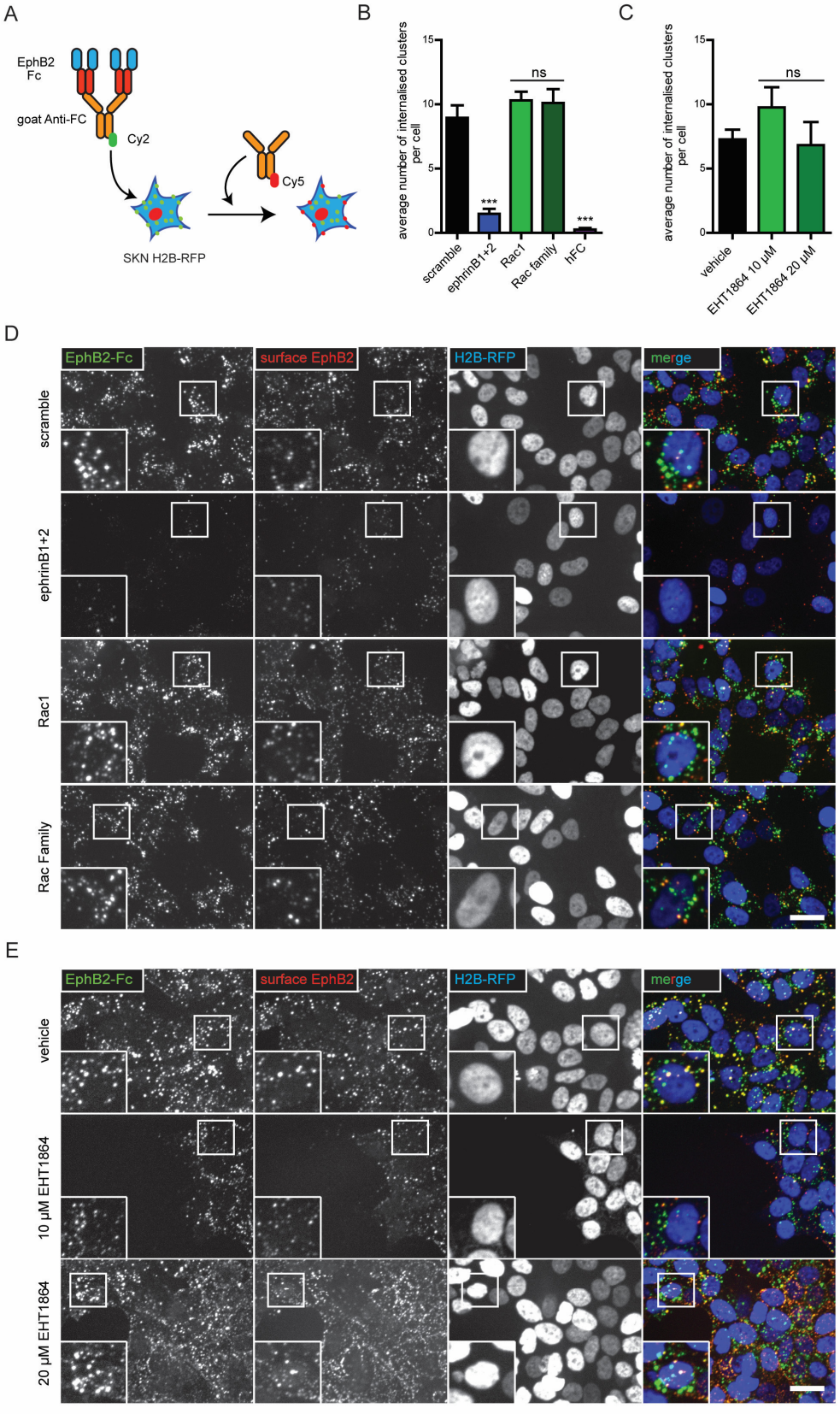
With very few exceptions (Marston et al. 2003, Zimmer et al. 2003, Lauterbach & Klein 2006) all studies of Eph-ephrin endocytosis use pre-clustered soluble ligand or receptor proteins to elicit internalization, despite the fact that these proteins are typically membrane-bound *in vivo* (Parker et al. 2004, Cowan et al. 2005, Yoo et al. 2010). Having established the importance of Rac subfamily members for EphB trans-endocytosis into ephrinB+ cells, I next examined whether the same mechanisms are employed for the reverse endocytosis of soluble EphB ectodomains. I treated SKN H2B-RFP cells with siRNA oligos against Rac1 alone or with a combination of oligos against the three Rac subfamily members expressed in SKN cells - Rac1, Rac3 and RhoG. The siRNA-treated cells were incubated with 2 µg/ml of a fusion protein of the ectodomain of EphB2 and the human Fc fragment (EphB2-Fc) pre-clustered with a Cy2-conjugated antibody at 37 °C for 30 minutes to allow endocytosis to occur. Subsequently, cells were fixed and stained against Fc without permeabilisation to visualise EphB2-ephrinB clusters that had not been internalised (Fig. 11A). Scrambled siRNA oligo, combined knockdown of ephrinB1 and ephrinB2, and incubation with pre-clustered hFc instead of EphB2-Fc were used as controls. As expected, only very few endocytosed clusters were observed in knockdown of ephrinBs (Fig. 11D, second row of images, and B). Treatment with hFc showed that endocytosis was dependent on the presence of the EphB ectodomain and not of the Fc portion of the fusion protein (Fig. 11B). When compared to the scramble control, neither treatment with Rac1 oligo alone, nor knockdown of the entire Rac subfamily, showed any significant difference in the amount of internalised EphB2-ephrinB clusters (Fig. 11B and D).

Since knockdown of Rac subfamily proteins by siRNA has been shown to be only partial (Fig. 5D) and treatment with EHT1864 had resulted in a stronger effect on EphB trans-endocytosis into ephrinB+ cells (Fig. 8B and C and Fig. 9B), I wanted to confirm the siRNA results by pharmacological inhibition of Rac subfamily GTPases. I treated SKN cells with either H_2O or EHT1864 (at 10 µM or 20 µM) for 4 h before incubating them with pre-clustered EphB2-Fc. There was no significant difference in the average number of internalised EphB-ephrinB clusters between vehicle treatment and treatment with EHT1864 at the tested concentrations (Fig. 11C and E). These data support the results from

the siRNA knockdown experiments and argue that Rac subfamily GTPases are not required for endocytosis of soluble EphB2 ectodomains into ephrinB+ SKN cells.

Figure 11. Rac subfamily GTPases are not required for endocytosis of soluble EphB2 ectodomains into ephrinB+ SKN cells

(A) Schematic representation of experimental setup for the assay with soluble pre-clustered EphB2-Fc. (B) Quantification of average number of internalised clusters per SKN cell transfected with siRNA performed with CellProfiler™ including the data for the hFC-treated control. One-way ANOVA with Dunnett's post-hoc test was used to test for significance. ***=p<0.005 (n= 4-6 independent experiments). (C) Quantification of average number of internalised clusters per SKN cell treated with vehicle/EHT1864 performed with CellProfiler™. One-way ANOVA with Dunnett's post-hoc test was used to test for significance (n= 6-8 independent experiments). (D) SKN H2B-RFP cells (nuclei in blue) were transfected with siRNA (scramble oligo, oligos against ephrinB1 and ephrinB2, Rac1 or a combination of oligos against Rac1, Rac3 and RhoG respectively) then incubated with EphB2-Fc pre-clustered with a Cy2-conjugated antibody (green) for 30 min, fixed without permeabilisation and subsequently stained with a dyLight649-conjugated antibody against Fc to visualise surface clusters (red/yellow in overlay). Scale bar equals 20 µm. (E) SKN H2B-RFP (nuclei in blue) treated with vehicle or Rac-inhibitor EHT1864 at 10 µM or 20 µM concentration, then incubated with EphB2-Fc pre-clustered with a Cy2-conjugated antibody (green) for 30 min, fixed without permeabilisation and subsequently stained with a dyLight649-conjugated antibody against Fc to visualise surface clusters (red/ yellow in overlay). Scale bar equals 20 µm.


A
EphB2 Fc
goat Anti-FC
Cy2
Cy5
SKN H2B-RFP
B
average number of internalised clusters per cell
ns
***
***
scramble
ephrinB1+2
Rac1
Rac family
hFC
C
average number of internalised clusters per cell
ns
vehicle
EHT1864 10 µM
EHT1864 20 µM
D
EphB2-Fc
surface EphB2
H2B-RFP
merge
scramble
ephrinB1+2
Rac1
Rac Family
E
EphB2-Fc
surface EphB2
H2B-RFP
merge
vehicle
10 µM EHT1864
20 µM EHT1864

To further support the findings that Rac subfamily GTPases are not required for reverse endocytosis of soluble EphB2 receptors, I also carried out experiments in HeLa cells transiently expressing ephrinB1-mCherry. Furthermore, working with HeLa cells also allowed me to compare the results with forward endocytosis of soluble ephrinB2 by using cells transiently over-expressing EphB2-mCherry. In principle, the experiments were conducted analogously to the experiments using SKN cells. HeLa cells expressing either EphB2-mCherry or ephrinB1-mCherry were pre-treated with vehicle control or EHT1864 at 10 μM (images not shown) or 20 μM concentrations for 4 h before being incubated with 2 μg/ml of ephrinB2-Fc or EphB2-Fc pre-clustered with a Cy2-conjugated antibody. After an incubation period of 30 min, cells were fixed with PFA and stained with a secondary antibody against Fc without permeabilisation to visualise surface clusters (Fig. 12A and B). Incubation with hFc instead of the ephrinB2 or EphB2 fusion proteins was used as an absolute control and showed only minimal internalisation or surface binding (data not shown). For reverse endocytosis, treatment with either 10 μM or 20 μM EHT1864 led to no significant difference in the number of internalised Eph-ephrin clusters compared to the vehicle control (Fig. 12C). In contrast, EHT1864 treatment led to a significant reduction in the amount of internalised Eph-ephrin clusters in the forward direction at concentrations of both 10 μM and 20 μM (Fig. 12D). These results are in line with previous reports proposing the involvement of Rac in forward endocytosis of Eph receptor clusters induced by soluble ephrins (Cowan et al. 2005, Yoo et al. 2010, Um et al. 2014). The data also show that the Rac inhibitor EHT1864 successfully generates an effect under our experimental conditions and that the lack of an effect of EHT1864 on reverse endocytosis is not due to inefficient inhibition of Rac family GTPases.

In conclusion, the data indicate that the mechanisms of endocytosis of soluble EphB and ephrinB ectodomains are different: uptake of ephrinB into EphB+ cells requires Rac activity, whereas uptake of EphB into ephrinB does not. Furthermore, the mechanism of ephrinB-mediated uptake of EphB critically depends on membrane tethering of EphB: trans-endocytosis of full-length EphB from an opposing cell into the ephrinB+ cell requires Rac activity, whereas uptake of soluble EphB ectodomain into the ephrinB+ cell does not.

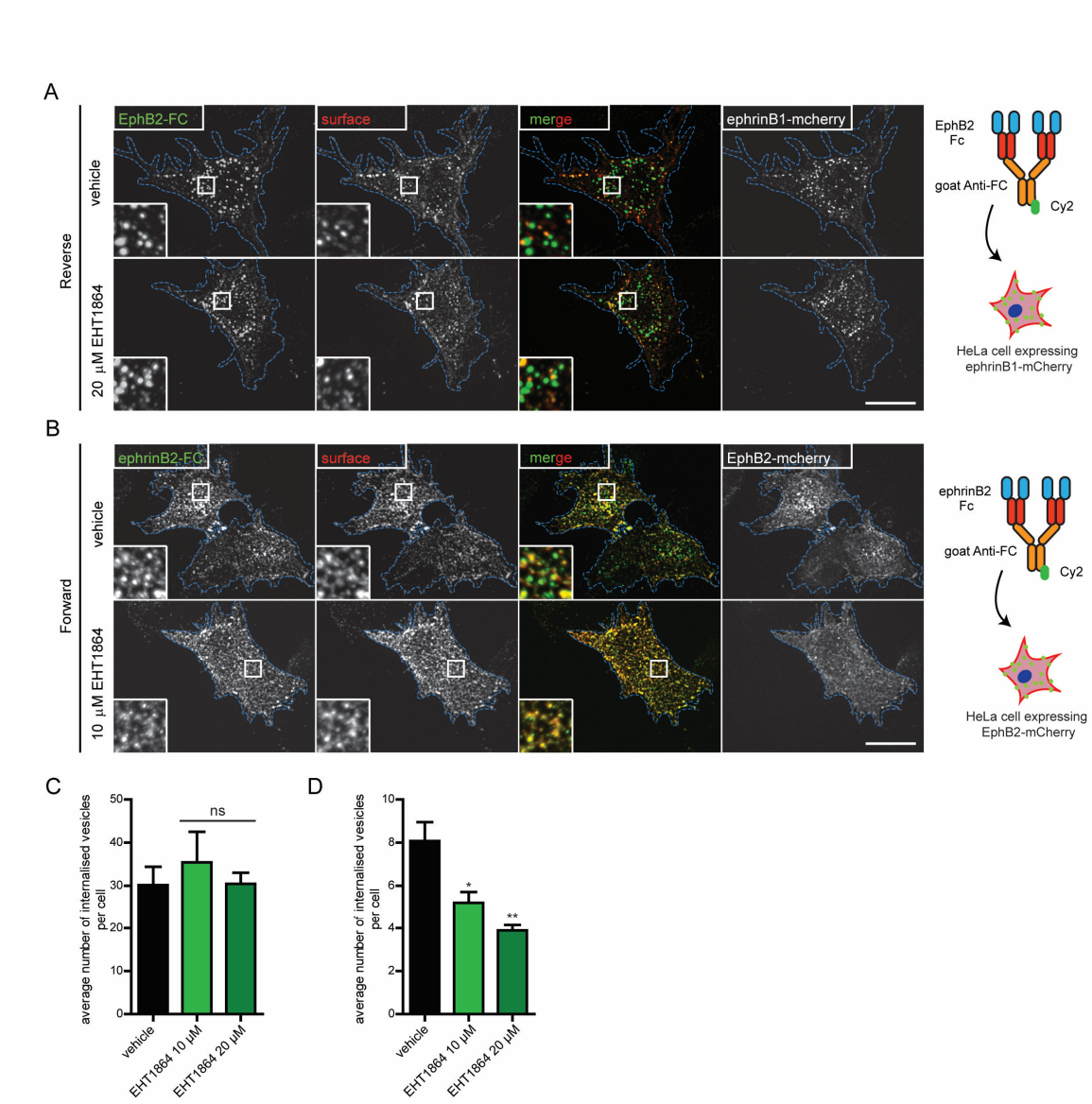


Figure 12. Different requirements for Rac in forward and reverse endocytosis of soluble Eph/ephrin ectodomains in HeLa cells

(A) HeLa cells expressing ephrinB1-mCherry (dashed outline) stimulated with EphB2–Fc (reverse endocytosis). HeLa cells expressing ephrinB1-mCherry were treated with vehicle control (top panel) or EHT1864 at 20 µM concentration (bottom panel) for 4 h prior to 30 min stimulation with EphB2-Fc pre-clustered with a Cy2-conjugated antibody (green) for 30 min. Cells were then fixed on ice and stained with a dyLight649-conjugated antibody against Fc to visualise surface clusters (red/ yellow in overlay). Scale bar represents 10 µm. (B) HeLa cells expressing EphB2-mCherry (dashed outline) stimulated with ephrinB2–Fc (forward endocytosis). HeLa cells expressing EphB2-mCherry were treated with vehicle control (top panel) or EHT1864 at either 10 µM or 20 µM concentration (example images only for 20 µM, bottom panel) for 4 h prior to 30 min stimulation with ephrinB2-Fc pre-clustered with a Cy2-conjugated antibody (green) for 30 min. Cells were then fixed on ice and stained with a dyLight649-conjugated antibody against Fc to visualise surface clusters (red/ yellow in overlay). Note the lack of internalise vesicles in the EHT-treated cell as

compared to the control). Scale bar represents 10 µm. (B). (C) Quantification of average number of internalised clusters per HeLa cell treated with vehicle or EHT1864 in reverse essay. Counting performed manually and experiments were performed blind. One-way ANOVA with Dunnett's post-hoc test was used to test for significance. (n= 3-5 independent experiments). (D) Quantification of average number of internalised clusters per HeLa cell treated with vehicle or EHT1864 in forward assay. Counting performed manually and experiments were performed blind. One-way ANOVA with Dunnett's post-hoc test was used to test for significance *=p<0.05, **=p<0.01. (n= 3-5 independent experiments).

2.2.4 Cdc42 subfamily GTPases are not required for endocytosis of EphB2 into ephrinB+ cells

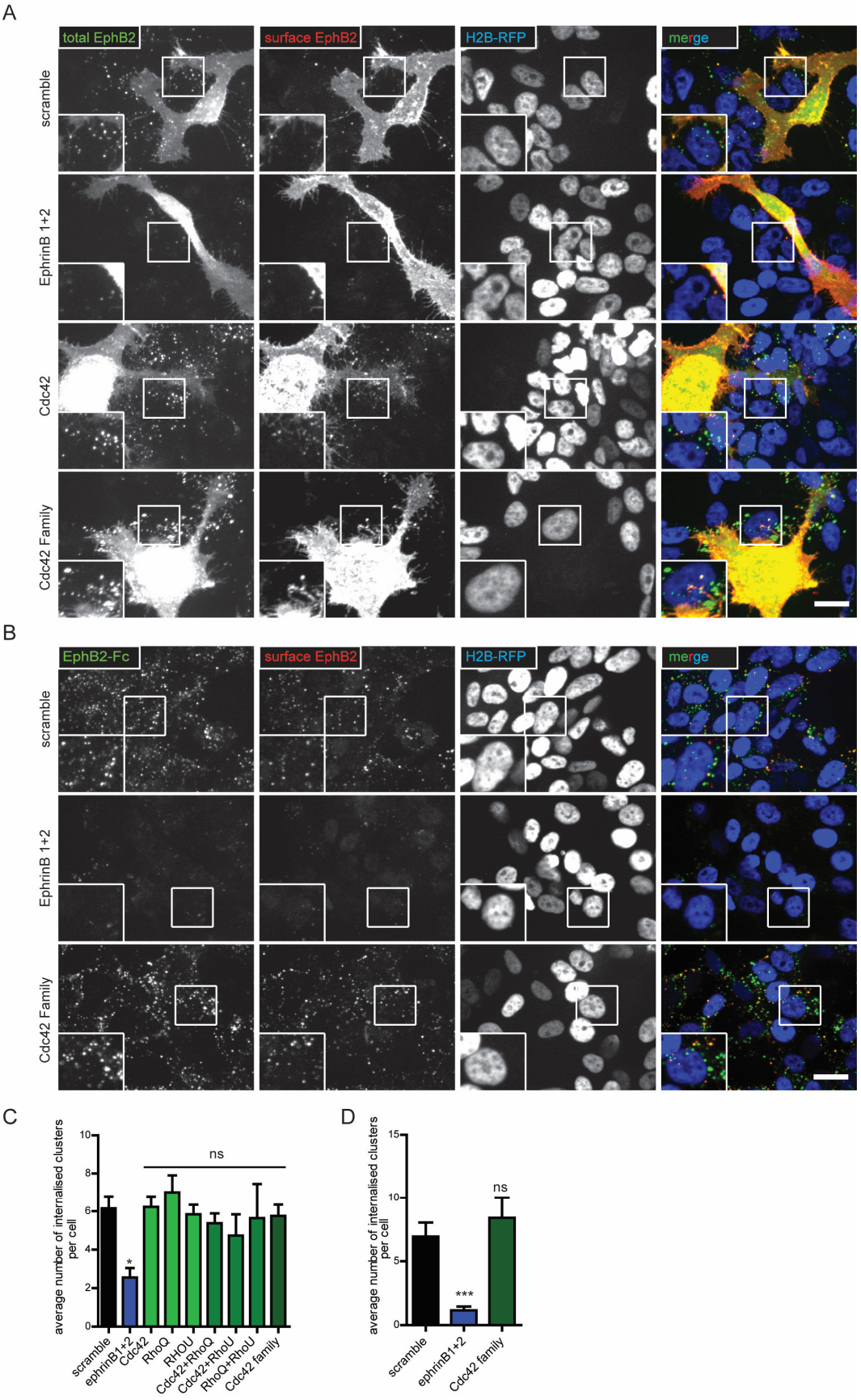
Cdc42 and the members of its subfamily have already been implicated in the context of endocytosis (Sabharanjak et al. 2002, Chadda et al. 2007), and also as downstream effectors of Eph-ephrin signalling (Irie & Yamaguchi 2002, Nishimura et al. 2006). They are therefore good candidates for being regulators of EphB trans-endocytosis into ephrinB+ cells. SKN cells express three members of the Cdc42 subfamily of Rho GTPases: Cdc42, RhoQ and RhoU (Fig. 4). To test the role of Cdc42 subfamily members in EphB trans-endocytosis, I used the trans-endocytosis assay co-culturing siRNA-treated SKN cells with HeLa cells expressing an EphB2ΔC-GFP construct as described before. In addition to the control conditions described above, knockdown of the expressed Cdc42 subfamily members individually, as well as all possible combinations of the three members, was performed (Fig. 13A). While knockdown of ephrinBs reduced the number of endocytosed EphB-ephrinB clusters as expected, neither knockdown of single Cdc42 subfamily GTPases nor any combination of knockdowns showed a significant change in the number of endocytosed clusters compared to scramble control condition (Fig. 13C).

As the data for the Rac subfamily GTPases suggested that different endocytic processes are employed in the trans-endocytosis assay and for the internalisation of soluble pre-clustered Eph ectodomains I also tested whether the Cdc42 subfamily members are involved in endocytosis of soluble EphB2 ectodomains into ephrinB+ cells. Combined siRNA knockdown of Cdc42, RhoQ and RhoU was performed in SKN cells, which were subsequently stimulated with pre-clustered EphB2-Fc (Fig. 13B). Compared with scramble control oligos, treatment with oligos against Cdc42 subfamily GTPases showed no significant difference in the number of endocytosed EphB-ephrinB clusters (Fig. 13D).

Taking into consideration results from both the trans-endocytosis assay and the assay with soluble EphB ectodomains, our data suggest that members of the Cdc42 subfamily of Rho GTPases do not play a key role for EphB-endocytosis into ephrinB+ cells.

Figure 13. Knockdown of Cdc42 subfamily GTPases in trans-endocytosis assay and assay with soluble EphB2 ectodomains

(A) SKN H2B-RFP cells (nuclei in blue) transfected with siRNA (scramble oligo, pool of oligos targeting ephrinB1+ephrinB2, or oligo targeting Cdc42 alone or a pool targeting Cdc42, RhoQ and RhoU, respectively) then co-cultured with FLAG-EphB2ΔC-GFP-expressing HeLa cells (green) for 80 min. Subsequently, cells were fixed and stained against the FLAG tag with a dyLight649-conjugated secondary antibody to visualise surface clusters (red). Scale bar equals 20 µm. (B) SKN H2B-RFP cells (nuclei in blue) treated with siRNA (scramble oligo, or a pool of oligos against ephrinB1 and ephrinB2 or Cdc42, RhoQ and RhoU respectively) then incubated with EphB2-Fc pre-clustered with a Cy2-conjugated antibody (green) for 30 min. Cells were subsequently fixed and stained with a dyLight649-conjugated antibody against Fc to visualise surface clusters (red/appear yellow in merged image). Scale bar equals 20 µm. (C) Quantification of average number of internalised clusters per cell in the cell-cell assay performed with CellProfiler™ including the data for the knockdown of the individual members of the Cdc42 subfamily as well as for all different combinations of oligos. One-way ANOVA with Dunnett's post-hoc test was used to test for significance. *=$p<0.05$ (n= 3 independent experiments). (D) Quantification of average number of internalised clusters per cell in the soluble assay performed with CellProfiler™. Repeated measures ANOVA with Dunnett's post-hoc test was used to test for significance. ***=$p<0.005$ (n= 6 independent experiments).


A
total EphB2
surface EphB2
H2B-RFP
merge
scramble
EphrinB 1+2
Cdc42
Cdc42 Family
B
EphB2-Fc
surface EphB2
H2B-RFP
merge
scramble
EphrinB 1+2
Cdc42 Family
C
average number of internalised clusters per cell
ns
*
scramble
ephrinB1+2
Cdc42
RhoQ
RhOU
Cdc42+RhoQ
Cdc42+RhoU
RhoQ+RhoU
Cdc42 family
D
average number of internalised clusters per cell
ns
***
scramble
ephrinB1+2
Cdc42 family

2.2.5 Knockdown of RhoA subfamily GTPases does not significantly alter EphB trans-endocytosis into ephrinB+ cells

The third major subfamily of Rho GTPases is the RhoA subfamily, of which RhoA, RhoB, are expressed in both SKN and HeLa cells (Fig. 4). While RhoA has been shown to be important for signalling events downstream of Eph-ephrin interactions, especially in the context of growth cone collapse (Shamah et al. 2001, Sahin et al. 2005, Takeuchi et al. 2015), there have been no direct links so far to the endocytosis of Eph-ephrin complexes.

To study the involvement of Rho subfamily GTPases in EphB trans-endocytosis, we first performed siRNA experiments in the trans-endocytosis assay using SKN cells co-cultured with HeLa cells expressing EphB2ΔC-GFP as described above. RhoA and RhoB were knocked down individually, as well as in combination. In Figure 14 both the example images (Fig. 14A) and the quantification (Fig. 14B) revealed that there is no significant difference between knockdown of Rho family proteins and scramble oligos, while knockdown of ephrinBs significantly reduced the amount of EphB trans-endocytosis into ephrinB+ SKN cells. We also performed experiments with HeLa cells as both donor and acceptor cells to further confirm that RhoA subfamily GTPases are not relevant in the context of EphB trans-endocytosis. As acceptor cells HeLa cells transiently expressing ephrinB1-mCherry were treated with siRNA against RhoA, RhoB, a combination of the two, or with scramble oligos. They were co-cultured with HeLa cells expressing EphB2ΔC-GFP to induce EphB trans-endocytosis into the acceptor cells. RhoA single and RhoA and RhoB double knockdowns led to a slight increase in the number of internalised Eph-ephrin complexes per cell in compared to scramble oligo treatment (47.11 and 47.87, internalised vesicles/cell compared to 42.37 internalised vesicles/cell). However, statistical testing with $\alpha = 0.05$ revealed these results to be non-significant (Fig. 14C).

Since the experiments using SKN cells showed no change in the number of internalised Eph-ephrin clusters, and the observed difference in HeLa cells is not statistically significant, these results indicate that RhoA subfamily GTPases are not involved in the regulation of EphB trans-endocytosis into ephrinB+ cells. This conclusion is further supported by the high effectiveness of siRNA knockdown of RhoA subfamily GTPases, which excludes insufficient knockdown as an explanation for the lack of an effect.

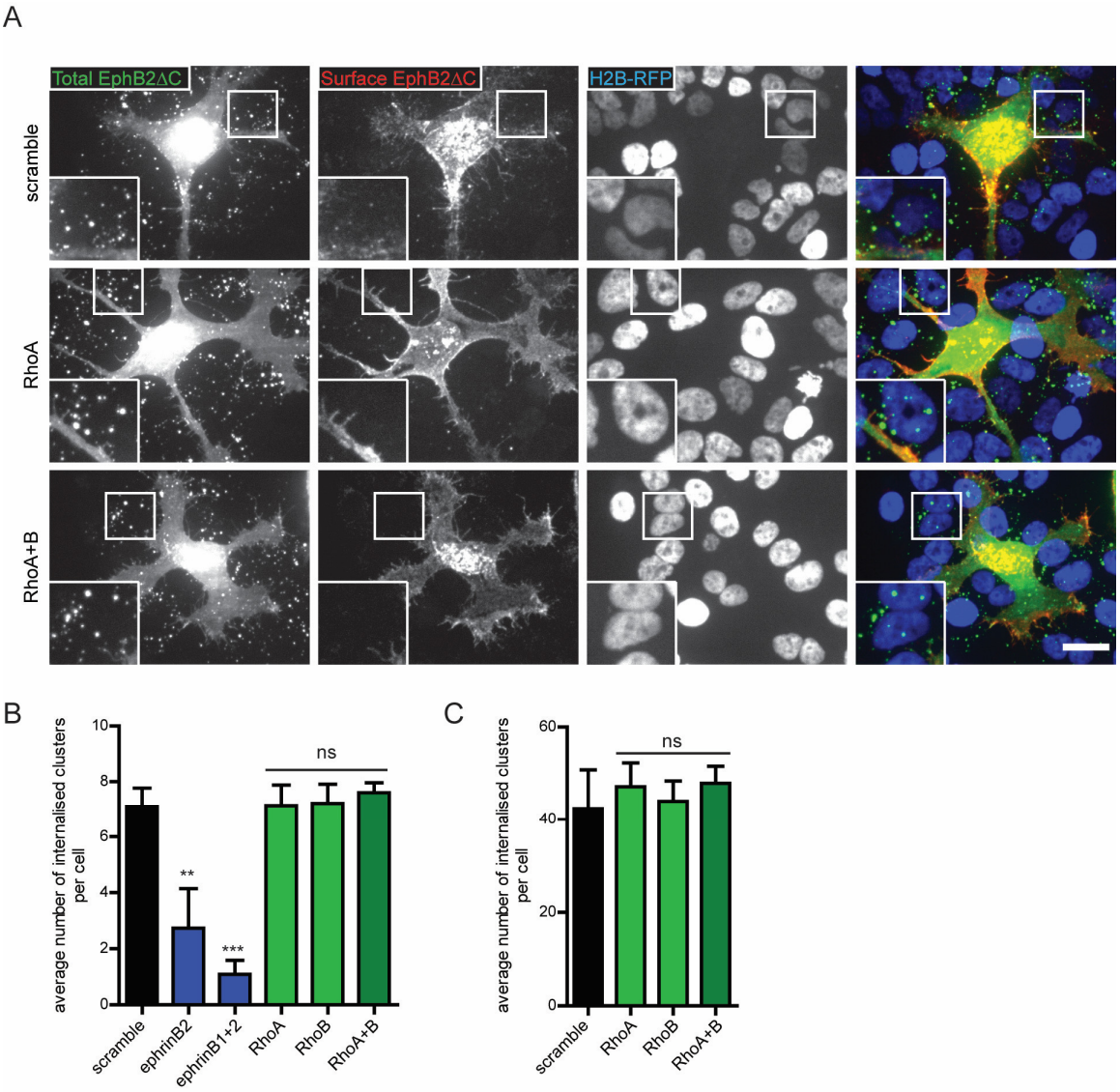


Figure 14. Knockdown of RhoA subfamily GTPases in EphB trans-endocytosis into ephrinB+ cells

(A) SKN H2B-RFP cells (nuclei in blue) transfected with siRNA (scramble oligo, oligo against RhoA, or a pool of oligos against RhoA and RhoB) were then co-cultured with FLAG-EphB2ΔC-GFP-expressing HeLa cells (green) for 80 min. Subsequently cells were fixed and stained against the FLAG tag with a dyLight649-conjugated secondary antibody to visualise surface clusters (red). Scale bar equals 20 µm. (B) Quantification of average number of internalised clusters per cell in the cell-cell assay in SKN performed with CellProfilerTM including the data for the individual knockdown of RhoB and the ephrinB controls. One-way ANOVA with Dunnett's post-hoc test was used to test for significance. **=p<0.01, ***=p<0.005 (n= 3-4 independent experiments). (D) Quantification of average number of internalised clusters per cell in the cell-cell assay in HeLa cells scored manually. All experiments performed blind. Repeated measures ANOVA with Dunnett's post-hoc test was used to test for significance (n= 3 independent experiments). All experiments in this figure performed by T. Gaitanos.

2.2.6 Knockdown of RhoA subfamily GTPases enhances the endocytosis of soluble EphB2 ectodomains into ephrinB+ cells

Although knocking down RhoA and RhoB in either SKN or HeLa cells showed no significant change in the EphB trans-endocytosis into ephrinB+ cells, I was also interested to see whether the same is true for endocytosis of soluble EphB ectodomains. Therefore, I knocked down RhoA, RhoB or a combination of the two proteins in SKN cells, which were subsequently incubated with pre-clustered EphB2-Fc as described before (Fig. 15A). Strikingly, I observed a very strong increase in the amount of internalised EphB-ephrinB clusters after knocking down RhoA subfamily GTPases when compared to scramble control oligos (Fig. 15C). For treatment with either siRNA for RhoA or the combination of oligos for RhoA and RhoB, the increase was almost two-fold, while for a knockdown of RhoB alone the increase was 1.5-fold. Since the combined knockdown of RhoA and RhoB did not result in a stronger effect than knockdown of RhoA alone, these results imply that RhoA is the main mediator of this physiological function.

These results further indicate that RhoA subfamily GTPases limit the rate of endocytosis of soluble EphB2 ectodomains into ephrinB+ cells. However, a potential alternate explanation for the increase in internalised EphB-ephrinB complexes, could be that RhoA subfamily GTPases regulate their downstream trafficking. Indeed, RhoB, and to a lesser extent RhoA, have been reported to regulate endocytic trafficking (Gampel et al. 1999, Fernandez-Borja et al. 2005, Rondanino et al. 2007, Stirling et al. 2009). Disturbing RhoB function interferes with transition from early endosomal compartments to later stages of endocytic processing such as multivesicular bodies, and results in smaller endocytic vesicles (Fernandez-Borja et al. 2005). A blocked fusion of early endosomes with later endocytic compartments could explain the higher number of endocytic vesicles observed in my experiments. Curiously, I also observed that the endocytic vesicles are also significantly smaller on average in the knockdown of RhoA and the combined knockdown of RhoA and RhoB compared to control (Fig. 15E), providing further evidence for this hypothesis.

In order to confirm the role of RhoA subfamily proteins in the endocytosis of soluble EphB2 ectodomains, I transfected HeLa cells with ephrinB1-mCherry and treated them

with siRNA for RhoA subfamily proteins or scramble control oligo (Fig. 15B). While knockdown of RhoA subfamily proteins resulted in an increase in the number of endocytosed Eph-ephrin clusters (27.98 for RhoA, and 29.24 for RhoA and RhoB, compared to 21.3 internalised vesicles/cell for scramble control), statistical testing with $\alpha = 0.05$ as significance level revealed the difference to be non-significant (Fig. 15D). The observed increase in endocytosis in HeLa cells (25-50 % for RhoA single and RhoA and RhoB double knockdown, 10-20% for RhoB single knockdown compared to control levels) is furthermore very modest compared to the substantial increase observed in SKN cells (150-200% compared to control levels). A potential explanation for this discrepancy can be found in the distinct experimental setups. Given that ephrinB1-mCherry needs to be over-expressed in HeLa cells, there is more ephrinB available on the cell surface compared to SKN cells, which endogenously express ephrinBs. The higher level of ephrinB expression on the cell surface leads to more binding opportunities for EphB2-Fc and in consequence, to more clusters forming and being internalised than under control conditions (8.1 clusters/cell in SKN cells, 21.3 clusters/cell in HeLa cells). The endocytic machinery of the HeLa cell is possibly already working close to its maximum capacity and therefore removal of RhoA subfamily proteins cannot exert as substantial an effect on the amount of endocytosis as it does in SKN cells. This could result in the difference between scramble oligo treatment and treatment with siRNA against RhoA subfamily members being non-significant in the experiments with HeLa cells.

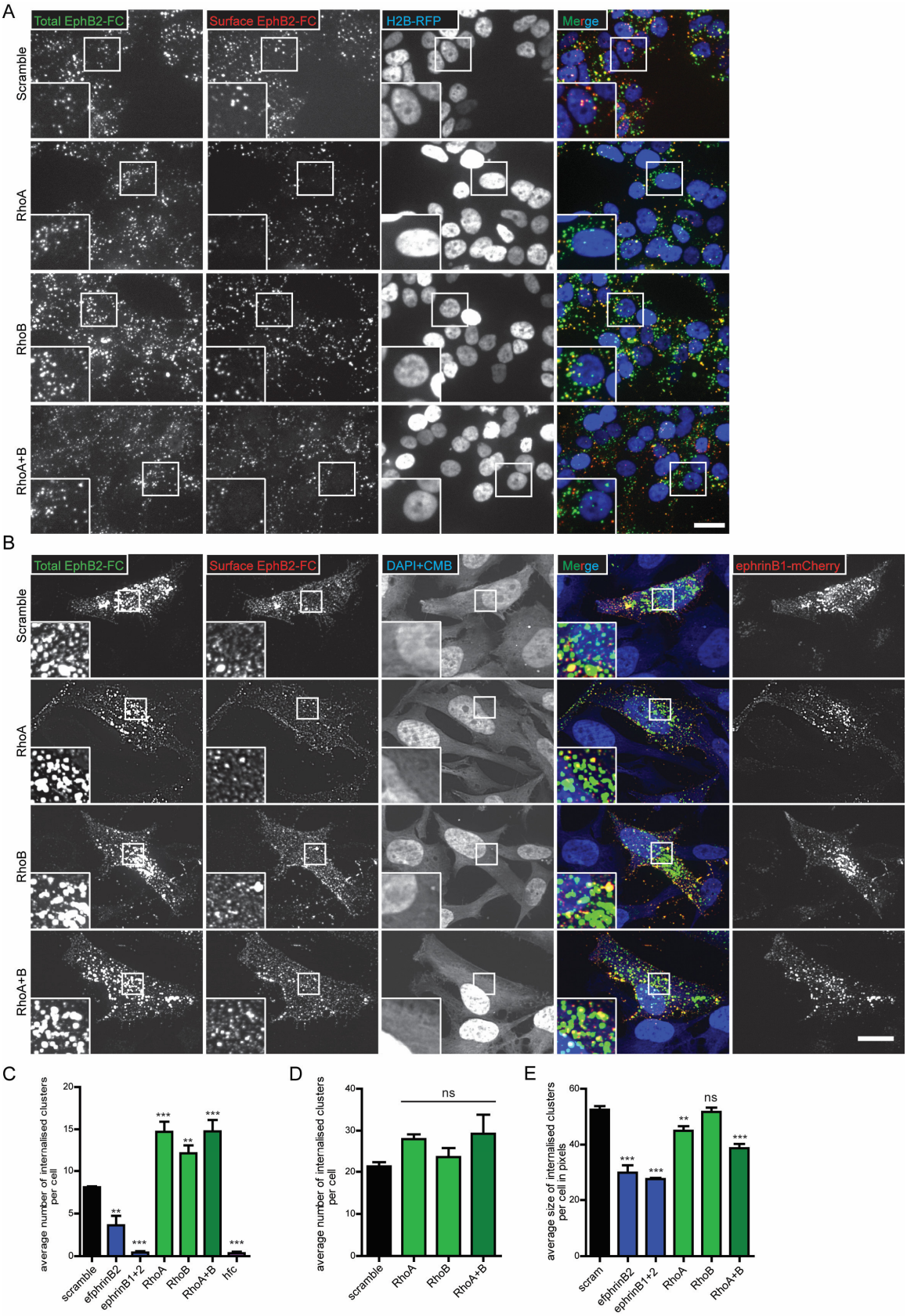

A
Total EphB2-FC
Surface EphB2-FC
H2B-RFP
Merge
Scramble
RhoA
RhoB
RhoA+B
B
Total EphB2-FC
Surface EphB2-FC
DAPI+CMB
Merge
ephrinB1-mCherry
Scramble
RhoA
RhoB
RhoA+B
C
average number of internalised clusters per cell
scramble
efphrinB2
ephrinB1+2
RhoA
RhoB
RhoA+B
Ifc
D
average number of internalised clusters per cell
ns
scramble
RhoA
RhoB
RhoA+B
E
average size of internalised clusters per cell in pixels
ns
scram
efphrinB2
ephrinB1+2
RhoA
RhoB
RhoA+B

Figure 15. Knockdown of RhoA subfamily GTPases in endocytosis of EphB2 ectodomains into ephrinB+ cells

(A) SKN H2B-RFP cells (nuclei in blue) treated with siRNA (scramble oligo, oligo against RhoA, RhoB or a pool of oligos against RhoA and RhoB respectively) then incubated with EphB2-Fc pre-clustered with a Cy2-conjugated antibody (green) for 30 min. Cells were subsequently fixed and stained with a dyLight649-conjugated antibody against Fc to visualise surface clusters (red/yellow in overlay). Scale bar equals 20 µm. (B) HeLa cells transiently overexpressing ephrinB1-mCherry (panel on the far right) treated with siRNA (scramble oligo, oligo against RhoA, RhoB or a combination of oligos against RhoA and RhoB respectively) then incubated with EphB2-Fc pre-clustered with a Cy2-conjugated antibody (green) for 30 min. Cells were subsequently fixed and stained with a dyLight649-conjugated antibody against Fc to visualise surface clusters (red/yellow in overlay). Nuclei were stained with DAPI and cell bodies were stained by CellMask Blue (blue). Scale bar equals 20 µm. (C) Quantification of average number of internalised clusters per SKN cell performed with CellProfiler™ including the data for the knockdown of ephrinB2 and ephrinB1 and B2, as well as the incubation with hFc instead of EphB2-Fc. Repeated measures ANOVA with Dunnett's post-hoc test was used to test for significance. **=p<0.01, ***=p<0.005 (n= 3 independent experiments). (D) Quantification of average number of internalised clusters per HeLa cell in the soluble assay performed on blinded samples manually with ImageJ. Repeated measures ANOVA with Dunnett's post-hoc test was used to test for significance. p=0.0765 (n= 4 independent experiments). (E) Quantification of average size (in pixels) of internalised clusters in SKN cells performed with CellProfiler™. Repeated measures ANOVA with Dunnett's post-hoc test was used to test for significance. **=p<0.01, ***=p<0.005 (n= 3 independent experiments)

2.3 Image-based siRNA screen of Rho GEFs and GAPs for their regulative function in EphB trans-endocytosis into ephrinB+ cells

Rho family GTPases are regulated in their function by GEFs and GAPs. To further explore the regulation of EphB trans-endocytosis into ephrinB+ cells we performed an image-based siRNA screen for Rho-family GEFs and GAPs. We bioinformatically identified human Rho-family GEFs and GAPs by searching for proteins containing either a RhoGAP, a DH, or a DOCK domain. Our search resulted in 61 GAPs, 11 DOCK-family GEFs and 67 Dbl-family GEFs. For each gene we used four different siRNA oligos with non-overlapping sequences. The scheme in Figure 16A provides an overview of the procedure and workflow of the screen. In principle, we used the same cell-cell assay as with the siRNA experiments with Rho-family GTPases. SKN H2B-RFP cells were seeded into 96-well plates and reverse transfected with siRNA oligos. The siRNA library consisted of 4 individual oligos per target gene, distributed in individual wells amongst 13 plates. Each plate also contained a set of control conditions: one well with siRNA oligo against kiff11 as a control for successful transfection (knockdown of kiff11 leads to an inhibition of mitosis and thus, cell

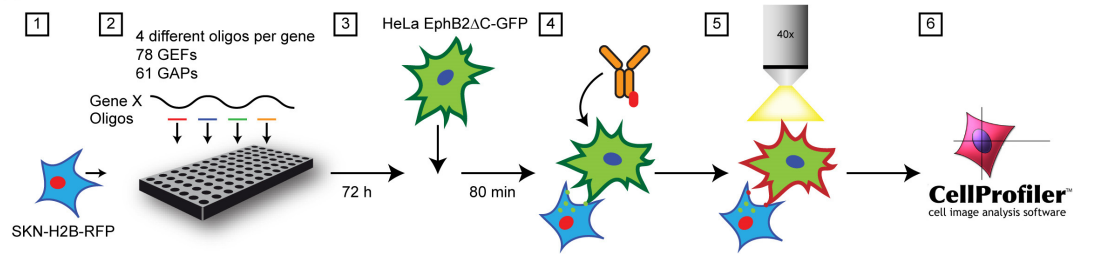
death); six wells of scramble oligos as negative controls; two wells with oligos against ephrinB2 as a positive control, resulting in partial inhibition of endocytosis; and three wells with a combination of oligos against both ephrinB1 and ephrinB2, resulting in an almost complete abolition of EphB2 uptake. Two runs with the full set of oligos were performed. The co-culture assay and analysis of images with CellProfiler™ software was performed as described before and in section 4.2.12. For each well the percentage of cells with more than a given number of vesicles was calculated. The number of vesicles for this cut-off was adjusted for each plate so that the negative controls showed a value of close to 40% of cells above the cut-off, to normalise for inter-plate variations in the levels of endocytosis. The value of 40% was chosen, since it allowed for very good discrimination against the two positive controls (Fig. 16B and C). From these cut-off values, z-scores were calculated for each oligo as described in Figure 16E. We regarded all results with z-scores below -2 as a hit for a decrease in endocytosis and all results with a z-score above 2.5 as a hit for an increase in endocytosis. The false positive rates for negative controls with these cut-offs were 2.9% for a decrease in endocytosis and 0.7% for an increase in endocytosis. Furthermore, 12.3% of ephrinB2 single depletions and 1.9% of ephrinB1 and ephrinB2 double knockdowns resulted in a false negative result (Fig. 16F). Figure 16 D shows a graph depicting the z-scores of all oligos tested. With z-score cut-offs at -2 and 2.5 the large majority of oligos from the siRNA library (95%) resulted in no significant change in the EphB trans-endocytosis into ephrinB+ cells. A list of oligos targeting GEF proteins with z-scores above and below the cut-offs is displayed in Figure 16G. Among the proteins for which siRNA oligo treatment resulted in a decrease of Eph-ephrin endocytosis, only for Dock11 more than one oligo fell below the -2 cut-off. However, these results represented three separate oligos, and none of them showed an effect below cut-off in repeat runs. Hence, we extended the number of oligos taken into consideration by including those barely missing the cut-off with a z-score of -1.9. Both ITSN1 and ELMO3 had one oligo each with a z-score of -1.9, in addition to one oligo scoring below the original cut-off value. For ITSN1, these two values derived from the same oligo in the two separate runs, suggesting a robust response. The oligos resulting in an increase of endocytosis did not give a clear result, as no candidate showed a z-score above the cut-off value in both

runs. Thus, we focused our follow-up analysis first on candidates leading to a decrease in endocytosis.

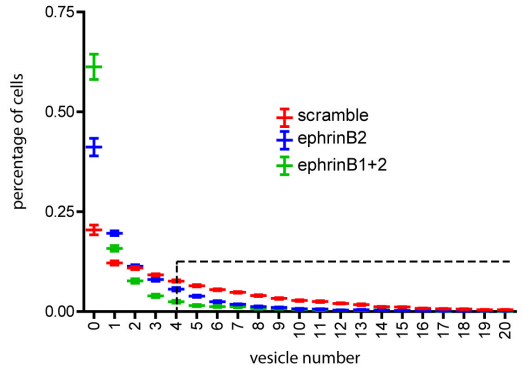
Figure 16. Image-based siRNA screen of Rho family GEFs/GAPs for regulators of EphB trans-endocytosis into ephrinB+ cells

(A) Schematic representation of the experimental setup. 1. SKN H2B-RFP cells (acceptor cells) were seeded into 96-well plates. 2. Transfection with siRNA oligos. 78 GEFs and 61 GAPs in total were tested. For each gene four different oligos were used. Incubation for 72 h. Each plate also contained 4 scramble oligo wells (negative control), 3 ephrinB2 single depletion wells, 2 ephrinB1+ephrinB2 double depletion wells (positive controls), and a single Kiff11 well (to ensure knockdown worked, not analysed). 3. HeLa cells over-expressing FLAG-EphB2ΔC-GFP (donor cells) were seeded on top of the SKN cells. Incubation for 80 min. 4. Cells were fixed and stained against the FLAG epitope to visualise surface clusters. 5. Imaging with a 40x objective at a Zeiss Spinning Disk microscope. 6. Semi-automated analysis with CellProfile™. (B) Frequency distribution of the number of internalised Eph-ephrin clusters averaged for the three control conditions from all screen data. Scramble in red, ephrinB2 single knockdown in blue and ephrinB1+2 double knockdown in green. Dotted line at 4 vesicles represents the cut-off of a total cumulative value of 40% in scramble controls used for analysing the screen data. (C) Quantification of control data with a cut-off of the number of internal vesicles resulting in a value close to 40% for scramble controls. Statistical significance was tested with ANOVA with Bonferroni´s correction for multiple comparisons. *=$p<0.05$, ***=$p<0.005$ (D) All z-scores for both siRNA libraries and controls blotted. Z-score analysis as normalisation to average of scramble controls from the whole screen. (E) Formula for calculation of z-scores. (F) Fail rates of controls according to analysis from (D). (G) List of candidate GEFs resulting in a significant difference in the number of internalised vesicles (z-score <-1.9 or >2.5). Genes leading to a decrease in endocytosis on the left, those resulting in an increase in endocytosis on the right. Colours highlight genes, for which several oligos showed a significant difference. Specificity for GTPase subfamilies is indicated as taken from Uniprot website (www.uniprot.org) and independent literature search.

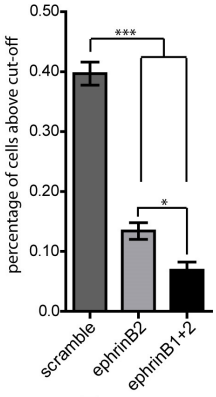
A



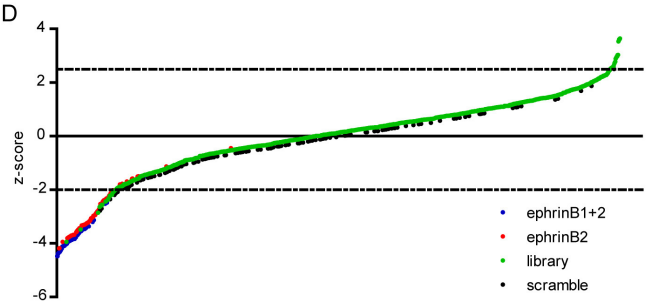
B



C



D



E

$$\text{z-score} = \frac{\text{value - average of all controls from the screen}}{\text{SD of all controls from the screen}}$$

F

Fail Rate of Controls

Scramble (false positive)

decrease (< -2): 2.9%
increase (> 2.5): 0.7%

Positive controls (false negative, > -2)

ephrinB2: 12.3%
ephrinB1+2: 1.9%

G

oligo/run	gene	z-score	RhoA	Rac	Cdc42
A/2	ITSN1	-3.9	-	-	√
A/1	ELMO3	-3.5	-	√	-
A/1	FARP2	-2.6	-	√	√
A/2	DOCK11	-2.6	-	-	√
B/1	DOCK11	-2.4	-	-	√
C/1	DOCK11	-2.2	-	-	√
A/2	NGEF	-2.2	√	√	-
A/1	DOCK5	-2.2	√	√	-
A/2	PLEKHG3	-2.1	-	-	√
A/2	DOCK3	-2.1	-	√	-
A/2	DOCK4	-2.0	-	√	-
A/2	DOCK10	-2.0	-	-	√
A/2	FARP1	-2.0	-	√	√
B/1	ELMO3	-1.9	-	√	-
A/1	ITSN1	-1.9	-	-	√
C/2	DOCK11	-1.9	-	-	√
A/2	VAV1	-1.9	-	√	-

oligo/run	gene	z-score	RhoA	Rac	Cdc42
A/2	ARHGEF10	2.5	√	-	-
A/2	ARHGEF18	2.5	√	√	-
A/2	ECT2	2.6	√	√	√
A/1	ARHGEF3	2.6	√	-	-
A/2	NET1	2.8	√	-	-
A/2	RASGRF1	3.0	-	√	-
A/2	ARHGEF19	3.0	√	-	-
A/2	ARHGEF10L	3.6	√	-	-
A/2	ARHGEF2	3.6	√	√	-

2.3.1 ITSN1 cannot be confirmed as a regulator of EphB trans-endocytosis into ephrinB+ cells

The single strongest result from the screen was the effect of one oligo for ITSN1, which markedly decreased the amount of observed internalisation of Eph-ephrin complexes into ephrinB+ cells (z-scores -3.9 and -1.9, Fig. 17B). Interestingly, ITSN1, a Cdc42-specific GEF, has already been implicated as a mediator of Eph-ephrin signalling in the context of dendritic spine development (Irie & Yamaguchi 2002, Nishimura et al. 2006). However, no direct link had yet been discovered to the endocytosis of Eph-ephrin complexes and studies so far have only implicated ITSN1 in forward signalling. I therefore chose ITSN1 as the first candidate for subsequent follow-up experiments. In order to confirm the requirement of ITSN1 in EphB trans-endocytosis into ephrinB+ cells, I repeated the experiments using the same setup that was employed for the screen, but increased the number of images taken per condition in order to achieve greater accuracy in the analysis. Since at least one oligo for ITSN2 also showed a trend to decrease trans-endocytosis of EphB2 receptors (z-score -1.53 and -0.24, Fig. 17B), and the high sequence similarity between ITSN1 and ITSN2 (Tsyba et al. 2011) suggests a potential redundancy between these two proteins, I also included ITSN2 and a combination of oligos for ITSN1 and ITSN2 in the experiment. Surprisingly, follow-up experiments could not confirm the role of ITSN1 as a regulator of EphB trans-endocytosis into ephrinB+ cells, as there was no significant difference between the average number of internalised EphB-ephrinB complexes in cells treated with ITSN1 oligo and cells treated with scramble oligo (Fig. 17A and C). Furthermore, knockdown of ITSN2 did not result in a significant difference in the amount of endocytosis either. The combined knockdown of ITSN1 and ITSN2 showed a trend towards a reduced number of endocytic events, however statistical analysis using $\alpha = 0.05$ as a significance level revealed this difference to be non-significant.

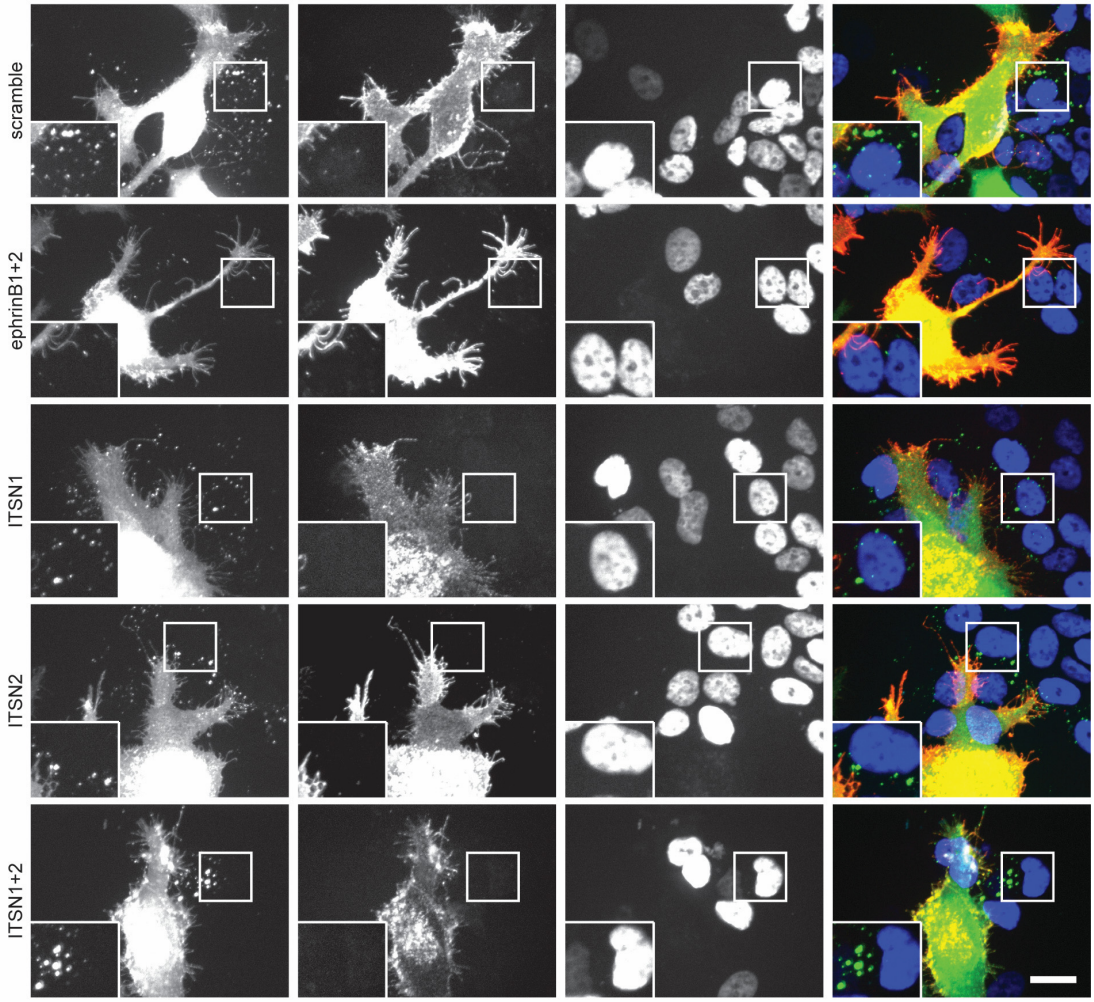
There are several potential explanations for these results. Of course, the most obvious being that the results from the screen were false positives and ITSN proteins are not required for Eph-ephrin reverse endocytosis. However, it could also be possible that ITSN proteins are involved in regulating the trans-endocytosis of Eph-ephrin complexes, but that they are just one component in a highly complex endocytic machinery and that their role can also be fulfilled by other proteins. In this case, the physiological redundancy could overcome the

effect a knockdown of ITSN proteins has on the regulation of endocytosis of Eph-ephrin complexes. I tried to account for at least the possibility of redundancy between ITSN1 and ITSN2 by combining the knockdown of both proteins, which, despite showing a trend towards fewer endocytic events per cell, did not give a significant result. Still it is possible that proteins other than members of the ITSN family can substitute for their potential role in Eph-ephrin endocytosis in the absence of ITSN. This possibility was addressed by additional experiments (see below). Finally, it is also conceivable that the effectiveness of the siRNA transfection and the resulting knockdown varied between experiments and that the knockdown effectiveness in the screen experiments was by chance higher than in the subsequent follow-up experiments, thus leading to a stronger effect.

Figure 17. ITSN proteins are not required for EphB trans-endocytosis into ephrinB+ cells

(A) SKN H2B-RFP cells (nuclei in blue) were transfected with siRNA (scramble oligo, combination of oligos against ephrinB1 and ephrinB2, oligos against ITSN1, ITSN2 or combination of oligos against ITSN1 and ITSN2) then co-cultured with FLAG-EphB2ΔC-GFP-expressing HeLa cells stained with CTG (green) for 80 min. Subsequently cells were fixed without permeabilisation and stained against the FLAG tag with a dyLight649-conjugated secondary antibody to visualise surface clusters (red). Scale bar equals 20 µm. (B) z-scores for used oligos from siRNA screen. (C) Quantification of average number of internalised clusters per cell performed with CellProfiler^{TM}. Repeated measures ANOVA with Dunnett's post-hoc test was used to test for significance. **= $p<0.01$ (n= 4 independent experiments).

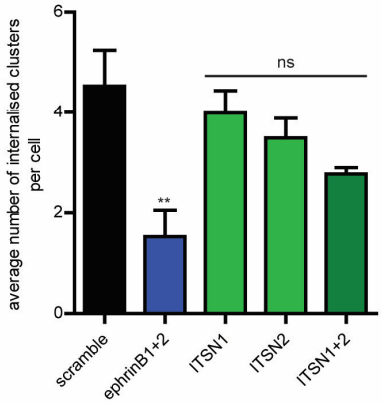
A



B

oligo/run	gene	z-score
A/2	ITSN1	-3.9
A/1	ITSN1	-1.9
A/2	ITSN2	-1.5
A/1	ITSN2	-0.2

C

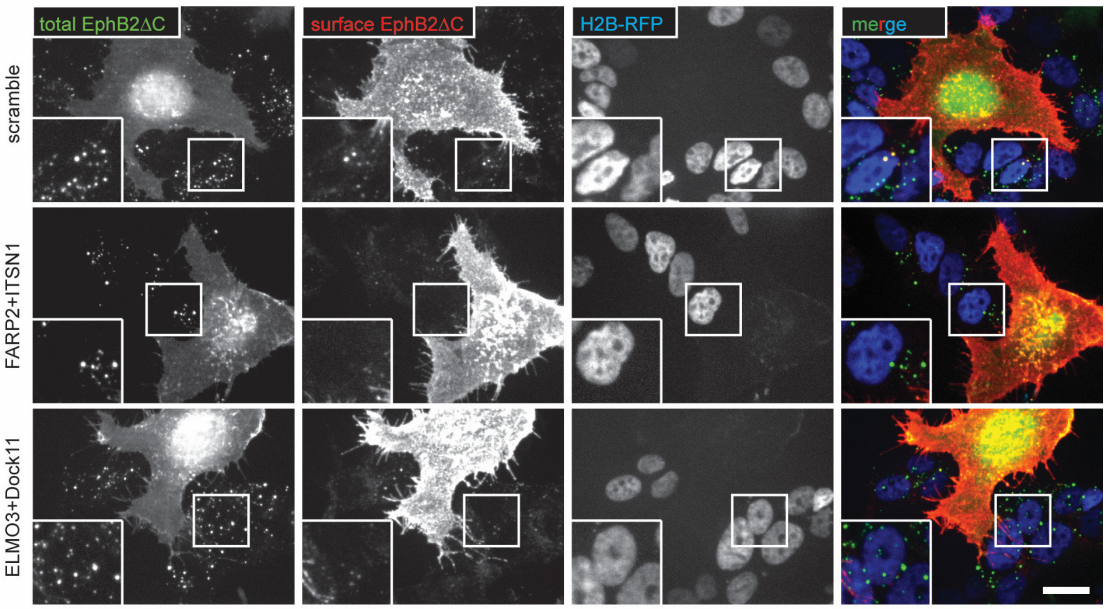


2.3.2 Combining siRNA against several candidates from the screen does not consistently inhibit EphB trans-endocytosis into ephrinB+ cells

As the initial attempts to confirm the strongest candidate from the siRNA screen, ITSN1, were not successful, I considered several explanations. One possible explanation is that due to redundancy on both the level of the Rho GTPases, as well as at the level of the GEFs and GAPs, it is difficult to obtain consistent results. I therefore addressed whether combining siRNA knockdown of two proteins that showed a decrease in endocytosis in the screen could overcome this redundancy. Two different combinations of oligos, both employing a different line of reasoning, were tested. Firstly, our analysis of the screen revealed that GEFs, whose knockdown leads to a decrease in EphB trans-endocytosis into ephrinB+ cells, are mainly active on GTPases of the Rac and Cdc42 subfamilies, and not the RhoA subfamily (Fig. 16G). According to the literature, ITSN1 is mainly active on Cdc42, while FARP2 exhibits GEF activity towards both Cdc422 and Rac (Jaiswal et al. 2013a). For both of these proteins siRNA oligos scored below the z-score cut-off (Fig. 18B). I therefore combined the oligos for these two genes in order to inhibit Rac and Cdc42 activity at the same time. Furthermore, the atypical DOCK subfamily of GEFs has been proposed to only exhibit its GEF function when acting in a complex with proteins of the ELMO family (Laurin & Cote 2014). Since both knockdown of a member of the DOCK subfamily of GEFs (Dock11), and knockdown of ELMO3 showed a decrease in the endocytosis of EphB2, we also tested a combination of oligos for these two genes. The same general experimental setup as for the screen was utilised: HeLa cells stably expressing the EphB2ΔC-GFP construct as donor cells and SKN H2B-RFP treated with siRNA oligos as acceptor cells. The combination of oligos was used at a concentration of 10 nM for each oligo (20 nM final concentration), while knockdown of single genes was performed using a concentration of 20 nM of each oligo. EphB trans-endocytosis was reduced by siRNA treatment for ephrinB2 or both ephrinB1 and ephrinB2 in SKN cells (images not shown). Treatment with siRNA for any of the four target genes alone (images not shown), or with a combination of oligos for Dock11 and ELMO3 or FARP2 and ITSN1 did not significantly change the number of internalised Eph-ephrin clusters per cell when compared to treatment with scramble oligo (Fig. 18A and C).

Taking into consideration all the results from the follow-up analysis which could not consistently confirm any of the candidates, we concluded that identifying hits from the screen solely by examining strong results from a single oligo did not provide reliable candidates for the regulation of EphB trans-endocytosis into ephrinB+ cells. While there are still potential explanations for the discrepancy between the screen results and follow-up experiments, for example, variations in knockdown effectiveness between experiments, or a level of redundancy between GEF proteins that cannot be overcome by the combined knockdown of only two different proteins; we decided to re-evaluate the criteria for identifying candidate genes from the screen data.

A



B

oligo/run	gene	z-score	RhoA	Rac	CDC42
A/2	ITSN1	-3.9	-	-	√
A/1	ELMO3	-3.5	-	√	-
A/1	FARP2	-2.6	-	√	√
A/2	DOCK11	-2.6	-	-	√
B/1	DOCK11	-2.4	-	-	√
C/1	DOCK11	-2.2	-	-	√

C

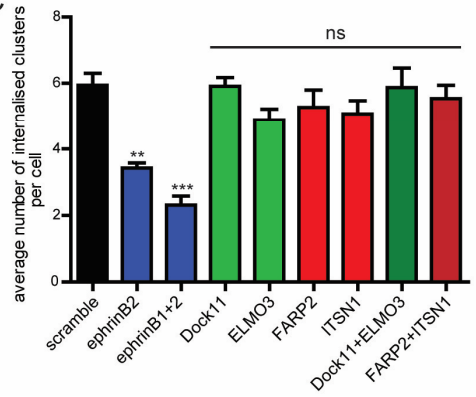


Figure 18. Combined siRNA knockdown of candidate regulators of EphB trans-endocytosis into ephrinB+ cells from siRNA screen

(A) SKN H2B-RFP cells (nuclei in blue) were treated with siRNA (scramble oligo or combination of oligos against FARP2 and ITSN1 or Dock11 and ELMO3), then co-cultured with FLAG-EphB2ΔC-GFP-expressing HeLa cells (green) for 80 min. Subsequently cells were fixed without permeabilisation and stained against the FLAG tag with a dyLight649-conjugated secondary antibody to visualise surface clusters (red/appear yellow in merge). Scale bar equals 20 µm. (B) z-scores for used oligos from siRNA screen. (C) Quantification of average number of internalised clusters per cell performed with CellProfiler™ including the values of positive controls (ephrinB2 and ephrinB1+2) as well as for the knockdown of Dock11, ELMO3, FARP2 and ITSN1 alone. Repeated measures ANOVA with Dunnett's post-hoc test was used to test for significance. **= $p<0.01$, ***=$p<0.005$ (n= 3 independent experiments)

2.4 Analysis of GEF/GAP Screen focussing on consistency over strength of responses

As my follow-up experiments could not confirm any candidate proteins obtained from our initial evaluation of the screen data as key regulators of EphB trans-endocytosis into ephrinB+ cells, we explored alternative criteria for analysing the screen. Our initial approach focused on strong z-scores, preferably in two runs for the same oligo, regardless of whether the other oligos against the same gene also showed an effect or not. The reasoning behind that being that potentially not all siRNA oligos actually result in an effective knockdown of the target protein. The alternate approach we subsequently employed was to search for consistent responses in the average of all 4 oligos tested, while being more lenient with the cut-off of z-scores which were regarded as a hit (-1.25 instead of -2, and 1.75 instead of 2.5). Furthermore, to account for high inter-plate variability in the results, z-scores were calculated for each plate individually instead of taking the average of the controls from the entire screen (Fig. 19B). Figure 19A illustrates all z-scores calculated this way and Table 1 and Table 2 show the z-scores listed for all tested proteins. With these criteria, 8% of negative controls showed a significant response, while 1% of ephrinB2 and 0% of ephrinB1 and ephrinB2 combined knockdowns failed to show a significant reduction in trans-endocytosis (Fig. 19C).

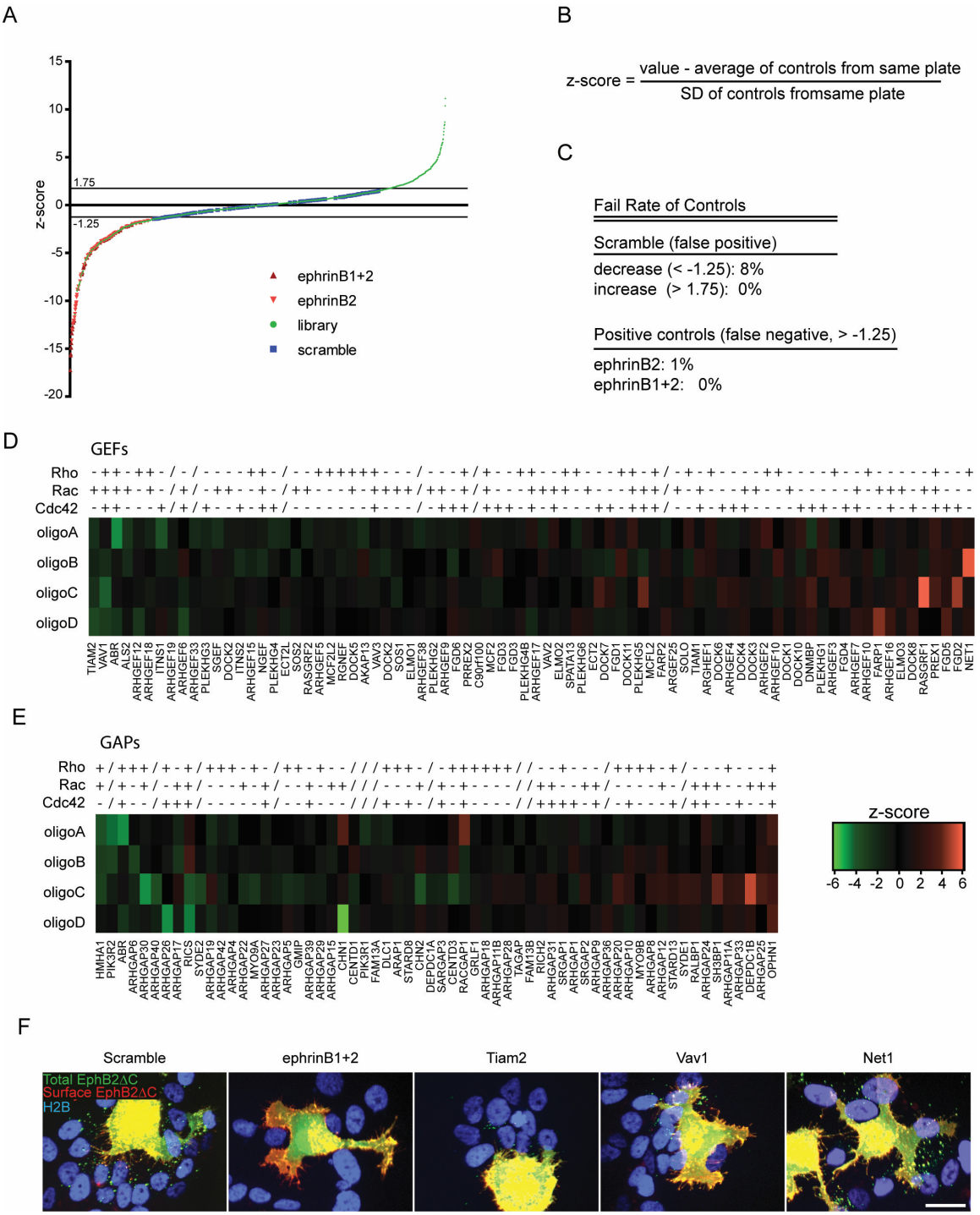
In Figure 19D and E, the tested GEFs and GAPs are displayed, with the average of the two runs for each oligo depicted in a colour code and ranked from left to right according to their overall average z-scores from lowest to highest. Also with this new approach, the majority of tested oligos did not result in a significant alteration of EphB trans-endocytosis into ephrinB+ cells. The candidates identified, however, differed from the previous analysis (compare Fig. 16). Figure 19F shows example images for the two strongest hits that decrease endocytosis (Tiam2 and Vav1), as well as for the strongest candidate that increased the amount of internalised Eph-ephrin complexes (Net1). Tiam2 itself has not yet been mentioned in the context of Eph-ephrin endocytosis, but it shares high sequence homology with Tiam1 (Matsuo et al. 2003), which in turn is a known player in endocytosis of soluble ephrinA ectodomains into EphA+ cells (Yoo et al. 2011, Boissier et al. 2013, Um et al. 2014). Vav1 is a close homologue of Vav2 and Vav3 (Fujikawa et al. 2003,

Pearce et al. 2004), which have already been described to also regulate endocytosis of soluble ephrinA ectodomains into EphA+ cells (Cowan et al. 2005).

The fact that the GEF showing the strongest increase in trans-endocytosis, Net1, is known to be specific for RhoA subfamily GTPases (Alberts & Treisman 1998, Srougi & Burridge 2011) is remarkable for two reasons. Firstly, despite no significant effect being observed for RhoA subfamily knockdown in EphB trans-endocytosis into SKN cells, the trend of an increase in endocytosis into ephrinB1-expressing HeLa cells (Fig. 14), and even more so, the strong increase in endocytosis observed in experiments using soluble EphB2 ectodomains (Fig. 15), suggest a possible role of RhoA subfamily GTPases as negative regulators of Eph-ephrin endocytosis. Secondly, given that the strongest candidates of the GEFs resulting in a decrease of trans-endocytosis are either Rac subfamily specific (Tiam2 (Jaiswal et al. 2013a)) or have been shown for to be active towards Rac in their physiological functions (Vav1(Villalba et al. 2001)), the screen data also suggest a potential antagonism between Rac subfamily members and RhoA subfamily members in the regulation of Eph-ephrin endocytosis. Similar antagonism between these two signalling pathways has been reported in several other physiological contexts (Guilluy et al. 2011)

Figure 19. Analysis of GEF/GAP screen emphasising consistency

(A) All z-scores for both siRNA libraries and controls plotted. Z-score analysis as normalization to average of controls per plate at 40% cut-off. (B) Formula for calculation of z-scores. (C)Fail rates of controls according to analysis from (A). (D), (E) Heat map for average values of the two independent runs of the screen showing all 4 oligos per gene for all GEFs or GAPs. Specificity for GTPase is indicated, taken from Uniprot website (www.uniprot.org) and independent literature search. / indicates unknown specificity. Genes are ranked from the average of all 4 oligos. Values are shown by colour according to the intensity profile in lower right. (F) Example images for scramble control, ephrinB1+2 control and top ranked GEF hits (Tiam2 and Vav1 for decrease in endocytosis, Net1 for increase). Only merged overlay images are shown, SKN H2B-RFP nuclei in blue, total EphB2ΔC-GFP in green, surface EphB2ΔC-GFP in red (appears as yellow in the overlay). Scale bar represents 20 µm.


A
15
10
5
1.75
0
-1.25
-5
-10
-15
-20
z-score
ephrinB1+2
ephrinB2
library
scramble
B
z-score = value - average of controls from same plate / SD of controls fromsame plate
C
Fail Rate of Controls
Scramble (false positive)
decrease (< -1.25): 8%
increase (> 1.75): 0%
Positive controls (false negative, > -1.25)
ephrinB2: 1%
ephrinB1+2: 0%
D
GEFs
Rho
Rac
Cdc42
oligoA
oligoB
oligoC
oligoD
TIAM2 VAV1 ABR ALS2 ARHGEF12 ARHGEF18 ITSN1 ARHGEF19 ARHGEF6 ARHGEF33 PLEKHG3 SGEF DOCK2 ITSN2 ARHGEF15 NGEF PLEKHG4 ECT2L SOS2 RASGRF2 ARHGEF5 MCF2L2 RGNEF DOCK5 AKAP13 VAV3 DOCK2 SOS1 ELMO1 ARHGEF38 PLEKHG2 ARHGEF9 FGD6 PREX2 C90rf100 MCF2 FGD3 FGD3 PLEKHG4B ARHGEF17 VAV2 ELMO2 SPATA13 PLEKHG6 ECT2 DOCK7 FGD1 DOCK11 PLEKHG5 MCFL2 FARP2 ARGEF25 SOLO TIAM1 ARGHEF1 DOCK6 ARHGEF4 DOCK3 ARHGEF2 ARHGEF10 DOCK1 DOCK10 DNMBP PLEKHG1 ARHGEF3 FGD4 ARHGEF7 ARHGEF10 FARP1 ARHGEF16 ELMO3 DOCK8 RASGRF1 PREX1 FGD5 FGD2 NET1
E
GAPs
Rho
Rac
Cdc42
oligoA
oligoB
oligoC
oligoD
HMHA1 PIK3R2 ABR ARHGAP6 ARHGAP30 ARHGAP40 ARHGAP26 ARHGAP17 RICS SYDE2 ARHGAP19 ARHGAP42 ARHGAP44 ARHGAP22 MYO9A ARHGAP27 ARHGAP23 ARHGAP5 GMIP ARHGAP39 ARHGAP29 ARHGAP15 CHN1 CENTD1 PIK3R1 FAM13A DLC1 ARAP1 STARD8 CHN2 DEPDC1A SARGAP3 CENTD3 RACGAP1 GRLF1 ARHGAP18 ARHGAP11B ARHGAP28 TAGAP FAM13B RICH2 ARHGAP31 SRGAP1 ARHGAP1 SRGAP2 ARHGAP9 ARHGAP36 ARHGAP20 ARHGAP10 MYO9B ARHGAP8 ARHGAP12 STARD13 SYDE1 RALBP1 ARHGAP24 SH3BP1 ARHGAP11A ARHGAP33 DEPDC1B ARHGAP25 OPHN1
z-score
-6 -4 -2 0 2 4 6
F
Scramble
ephrinB1+2
Tiam2
Vav1
Net1
Total EphB2ΔC
Surface EphB2ΔC
H2B

.

Table 1. siRNA screen for regulators of EphB trans-endocytosis – all z-scores from GEFs

All GEFs tested in the siRNA screen listed in alphabetical order. Columns A1-D1 show the individual z-scores for each of the four oligos per gene for the first run, columns A2-D2 show the z-scores for the repeat run, and columns A-D show the average z-score from both runs. Same oligos are depicted in the same colour. The last column shows the average of all four oligos.

Gene	A1	B1	C1	D1	A2	B2	C2	D2	A	B	C	D	avg
ABR	-6.12	-5.03	-1.78	-5.25	-3.17	4.00	3.21	0.48	-4.64	-0.51	0.71	-2.39	**-1.71**
AKAP13	-2.81	4.32	-1.31	1.34	-1.37	-0.80	-0.18	-1.06	-2.09	1.76	-0.74	0.14	**-0.23**
ALS2	-0.54	-0.47	0.58	0.14	-4.44	-3.45	0.31	-0.82	-2.49	-1.96	0.44	-0.34	**-1.09**
ARHGEF1	2.69	0.81	3.08	-0.82	1.86	-3.47	0.42	0.01	2.27	-1.33	1.75	-0.40	**0.57**
ARHGEF10	-0.02	1.28	0.58	3.51	0.24	2.02	2.20	0.54	0.11	1.65	1.39	2.02	**1.29**
ARHGEF10L	-0.66	2.67	0.18	3.10	0.45	3.10	-2.27	0.72	-0.10	2.89	-1.04	1.91	**0.91**
ARHGEF11	3.21	3.32	-0.11	1.10	0.73	-0.16	-0.80	-1.83	1.97	1.58	-0.45	-0.37	**0.68**
ARHGEF12	-1.10	-0.11	0.59	-4.71	-0.42	1.48	-1.21	-0.49	-0.76	0.69	-0.31	-2.60	**-0.75**
ARHGEF15	-0.32	-1.89	0.24	-1.16	-0.45	-0.65	1.32	-0.29	-0.39	-1.27	0.78	-0.72	**-0.40**
ARHGEF16	3.52	2.30	0.50	0.72	-1.91	-1.09	1.81	4.99	0.81	0.61	1.16	2.85	**1.36**
ARHGEF17	-1.28	0.38	0.25	2.27	0.14	-0.27	0.00	-0.05	-0.57	0.05	0.12	1.11	**0.18**
ARHGEF18	-0.81	-1.17	-0.94	-2.68	-2.22	0.76	-0.74	1.96	-1.52	-0.20	-0.84	-0.36	**-0.73**
ARHGEF19	-0.35	-2.60	-0.94	-4.12	-1.07	0.65	0.38	2.42	-0.71	-0.97	-0.28	-0.85	**-0.70**
ARHGEF2	7.34	0.25	1.36	1.41	-1.52	-1.06	0.98	-1.69	2.91	-0.41	1.17	-0.14	**0.88**
ARHGEF25	-0.02	1.32	-0.21	2.09	-0.60	0.58	1.85	-0.63	-0.31	0.95	0.82	0.73	**0.55**
ARHGEF3	0.53	4.52	0.71	0.84	-2.69	0.72	4.50	-0.16	-1.08	2.62	2.61	0.34	**1.12**
ARHGEF33	-2.72	0.28	-1.05	-0.23	-1.15	0.17	0.64	-0.95	-1.93	0.22	-0.21	-0.59	**-0.63**
ARHGEF38	-2.38	-0.53	0.62	1.03	-1.15	0.47	0.16	0.36	-1.76	-0.03	0.39	0.70	**-0.18**
ARHGEF4	-0.69	2.78	-0.02	-0.74	-1.34	-0.79	4.04	2.96	-1.01	0.99	2.01	1.11	**0.78**
ARHGEF5	0.57	0.73	0.31	-0.71	-1.77	-2.04	0.03	0.54	-0.60	-0.66	0.17	-0.08	**-0.29**
ARHGEF6	-0.12	3.19	-0.17	-0.57	0.29	0.47	-2.52	-5.66	0.08	1.83	-1.34	-3.11	**-0.64**
ARHGEF7	2.11	1.28	0.51	1.68	0.63	0.46	0.69	2.86	1.37	0.87	0.60	2.27	**1.28**
ARHGEF9	-1.74	0.29	0.41	0.20	0.32	-0.33	0.60	-0.09	-0.71	-0.02	0.51	0.05	**-0.04**

Gene	A1	B1	C1	D1	A2	B2	C2	D2	A	B	C	D	avg
C9orf100	3.16	1.19	0.25	-0.45	0.06	-1.96	-0.72	-0.48	1.61	-0.38	-0.24	-0.47	**0.13**
DNMBP	-0.32	0.62	-1.80	-1.20	-4.59	2.10	8.42	5.40	-2.46	1.36	3.31	2.10	**1.08**
DOCK1	0.87	1.55	1.25	1.25	1.87	0.61	0.94	-0.33	1.37	1.08	1.10	0.46	**1.00**
DOCK10	3.01	-0.04	3.78	3.09	1.73	-3.09	-0.06	-0.22	2.37	-1.56	1.86	1.44	**1.03**
DOCK11	3.65	-0.40	4.70	2.43	0.19	-2.89	-0.88	-3.85	1.92	-1.64	1.91	-0.71	**0.37**
DOCK2	-0.83	-0.09	-1.45	0.70	-0.63	-0.71	-0.19	-0.47	-0.73	-0.40	-0.82	0.11	**-0.46**
DOCK2	0.58	-1.40	0.82	1.87	-1.83	-2.00	0.01	0.24	-0.63	-1.70	0.42	1.06	**-0.21**
DOCK3	2.61	1.34	1.66	1.51	0.63	0.86	0.18	-1.83	1.62	1.10	0.92	-0.16	**0.87**
DOCK4	2.81	1.65	2.74	2.11	-1.44	-1.79	-0.26	0.94	0.69	-0.07	1.24	1.53	**0.85**
DOCK5	-0.90	-1.30	1.40	0.80	-1.18	0.00	0.04	-0.76	-1.04	-0.65	0.72	0.02	**-0.24**
DOCK6	-0.26	1.91	-1.54	0.77	1.38	2.47	0.29	-0.26	0.56	2.19	-0.63	0.25	**0.59**
DOCK7	1.64	-3.01	-1.15	1.38	0.61	1.02	0.50	1.43	1.13	-0.99	-0.33	1.41	**0.30**
DOCK8	-0.21	1.33	1.25	3.57	1.17	1.42	1.31	1.49	0.48	1.38	1.28	2.53	**1.42**
DOCK9	-2.42	1.31	4.25	1.49	0.74	0.11	0.50	0.18	-0.84	0.71	2.37	0.83	**0.77**
ECT2	-0.18	0.24	-0.08	1.40	-2.54	1.23	0.04	1.79	-1.36	0.73	-0.02	1.60	**0.24**
ECT2L	-1.44	-0.03	1.74	-1.43	-1.75	0.38	0.51	-0.89	-1.60	0.18	1.13	-1.16	**-0.36**
ELMO1	0.16	1.40	0.56	-0.55	-1.40	-1.49	-0.47	0.12	-0.62	-0.04	0.05	-0.22	**-0.21**
ELMO2	-1.94	0.74	0.71	0.73	-0.76	1.98	-0.15	0.41	-1.35	1.36	0.28	0.57	**0.22**
ELMO3	1.90	1.20	3.06	1.29	0.45	1.29	1.17	0.51	1.17	1.24	2.12	0.90	**1.36**
FARP1	-0.20	-1.75	-2.24	2.49	2.90	-0.98	3.84	6.55	1.35	-1.36	0.80	4.52	**1.33**
FARP2	0.78	-0.53	1.56	-2.32	-1.47	-3.38	6.84	2.74	-0.35	-1.95	4.20	0.21	**0.53**
FGD1	0.43	1.54	0.87	-1.14	-0.01	-4.00	5.95	-0.78	0.21	-1.23	3.41	-0.96	**0.36**
FGD2	0.28	0.01	1.91	0.06	3.60	2.24	7.67	1.87	1.94	1.12	4.79	0.96	**2.20**
FGD3	0.39	1.23	-0.76	-1.59	-1.29	3.74	-1.47	1.09	-0.45	2.49	-1.11	-0.25	**0.17**
FGD4	0.02	0.02	1.70	-0.87	0.11	2.31	3.70	2.36	0.07	1.16	2.70	0.74	**1.17**
FGD5	1.24	1.50	-0.84	0.32	0.88	2.49	4.24	6.90	1.06	2.00	1.70	3.61	**2.09**
FGD6	0.31	0.33	-1.43	-1.86	-2.29	-4.60	4.02	5.21	-0.99	-2.14	1.29	1.68	**-0.04**
ITSN1	-3.56	-1.10	1.36	2.36	-3.99	-1.10	-0.08	0.33	-3.78	-1.10	0.64	1.34	**-0.72**

Gene	A1	B1	C1	D1	A2	B2	C2	D2	A	B	C	D	avg
ITSN2	-0.75	-2.84	0.14	1.38	-0.53	-1.16	0.90	-0.36	-0.64	-2.00	0.52	0.51	**-0.40**
MCF2	-1.65	0.33	-2.49	-0.43	0.68	2.21	0.44	2.21	-0.49	1.27	-1.03	0.89	**0.16**
MCF2L (ARHGEF14)	3.78	-0.41	0.54	-0.04	1.27	-1.24	0.30	-0.56	2.53	-0.82	0.42	-0.30	**0.46**
MCF2L2	0.25	0.60	-1.19	-0.33	-1.91	-1.12	0.01	1.59	-0.83	-0.26	-0.59	0.63	**-0.26**
NET1	-1.64	1.11	-0.97	0.53	4.46	10.36	3.31	3.03	1.41	5.73	1.17	1.78	**2.52**
NGEF	-0.48	-1.19	-0.31	1.71	-1.14	-2.63	-0.12	0.97	-0.81	-1.91	-0.22	1.34	**-0.40**
PLEKHG1	-1.37	0.35	-0.50	0.24	6.01	5.27	0.05	-1.32	2.32	2.81	-0.22	-0.54	**1.09**
PLEKHG2 (CLG)	-1.60	1.31	-0.32	0.43	-0.56	-0.37	-0.71	0.46	-1.08	0.47	-0.52	0.44	**-0.17**
PLEKHG3 (KIAA0599)	-0.63	-2.48	-0.40	1.14	-2.31	0.47	-0.45	0.16	-1.47	-1.01	-0.43	0.65	**-0.56**
PLEKHG4	-1.75	0.76	-0.03	0.74	-2.07	-1.69	0.03	0.90	-1.91	-0.47	0.00	0.82	**-0.39**
PLEKHG4B	-1.25	1.25	0.05	0.57	0.62	-0.39	0.08	0.49	-0.32	0.43	0.07	0.53	**0.18**
PLEKHG5	-0.61	-0.54	-0.11	-0.45	2.15	6.25	-1.72	-1.59	0.77	2.86	-0.91	-1.02	**0.42**
PLEKHG6 (FLJ10665)	-0.69	0.37	0.29	0.83	1.08	0.57	-1.00	0.46	0.20	0.47	-0.35	0.64	**0.24**
PREX1	0.24	-0.85	2.03	1.27	4.99	5.20	4.70	-1.23	2.61	2.17	3.36	0.02	**2.04**
PREX2	-0.10	-1.71	-1.33	1.13	-0.82	1.53	0.81	1.17	-0.46	-0.09	-0.26	1.15	**0.08**
RASGRF1	-1.71	-1.44	1.83	0.14	4.02	-0.56	11.14	-0.38	1.15	-1.00	6.48	-0.12	**1.63**
RASGRF2	-1.72	0.76	0.74	-0.21	-1.22	-0.85	0.09	-0.08	-1.47	-0.04	0.41	-0.15	**-0.31**
RGNEF	0.67	-0.51	0.97	-0.11	-1.67	-1.67	0.20	0.21	-0.50	-1.09	0.59	0.05	**-0.24**
SGEF	-4.93	2.94	-4.59	-2.91	0.58	-0.93	3.62	2.32	-2.17	1.00	-0.48	-0.29	**-0.49**
Solo	-0.28	-0.41	-2.39	1.88	-2.14	3.54	1.76	2.53	-1.21	1.56	-0.31	2.20	**0.56**
SOS1	-0.63	0.55	-0.59	0.72	1.65	-1.22	-2.28	0.10	0.51	-0.33	-1.44	0.41	**-0.21**
SOS2 (FLJ25596)	-0.02	-1.74	-0.23	0.29	-0.14	-1.96	-0.36	1.55	-0.08	-1.85	-0.30	0.92	**-0.33**
SPATA13	0.17	1.55	-0.03	1.15	3.15	-2.07	0.17	-2.24	1.66	-0.26	0.07	-0.55	**0.23**
TIAM1 (FLJ36302)	1.11	0.76	1.38	1.07	-0.85	0.52	-0.20	0.78	0.13	0.64	0.59	0.93	**0.57**
TIAM2	-1.17	0.38	-1.36	-1.69	-3.30	-3.34	-3.03	-4.95	-2.24	-1.48	-2.20	-3.32	**-2.31**
VAV1	1.53	-0.15	-1.16	-0.36	0.11	-4.05	-6.96	-5.21	0.82	-2.10	-4.06	-2.79	**-2.03**
VAV2	-0.55	0.14	-0.08	-1.43	3.54	0.13	2.10	-2.19	1.50	0.14	1.01	-1.81	**0.21**
VAV3	1.33	-0.95	-0.47	-0.28	-0.41	-1.34	-0.76	1.05	0.46	-1.15	-0.61	0.38	**-0.23**

Table 2. siRNA screen for regulators of EphB trans-endocytosis – all z-scores from GAPs

All GAPs tested in the siRNA screen listed in alphabetical order. Columns A1-D1 show the individual z-scores for each of the four oligos per gene for the first run, columns A2-D2 show the z-scores for the repeat run, and columns A-D show the average z-score from both runs. Same oligos are depicted in the same colour. The last column shows the average of all four oligos.

Gene	A1	B1	C1	D1	A2	B2	C2	D2	A	B	C	D	avg
ABR	-6.12	-5.03	-1.78	-5.25	-3.17	4.00	3.21	0.48	-4.64	-0.51	0.71	-2.39	**-1.71**
ARAP1	-0.73	0.09	1.93	0.67	1.14	1.60	-3.60	-1.58	0.20	0.84	-0.83	-0.45	**-0.06**
ARHGAP1	0.83	1.64	0.18	0.72	1.30	1.98	-2.56	-0.36	1.06	1.81	-1.19	0.18	**0.46**
ARHGAP10	-1.58	3.46	3.34	-0.60	0.97	0.30	1.24	-1.95	-0.30	1.88	2.29	-1.28	**0.65**
ARHGAP11A	0.53	-0.49	4.75	2.11	0.08	1.24	-0.10	0.96	0.30	0.38	2.32	1.53	**1.13**
ARHGAP11B	-0.09	0.11	3.36	0.28	-0.54	-1.05	-0.44	0.18	-0.31	-0.47	1.46	0.23	**0.23**
ARHGAP12	-0.79	3.94	3.68	-0.52	0.09	-0.13	0.98	-1.15	-0.35	1.91	2.33	-0.84	**0.76**
ARHGAP15	0.10	-1.94	0.25	1.76	-1.10	-0.22	-0.59	0.20	-0.50	-1.08	-0.17	0.98	**-0.19**
ARHGAP17	-2.84	-1.36	2.70	-0.03	-0.26	-3.58	0.51	-2.11	-1.55	-2.47	1.61	-1.07	**-0.87**
ARHGAP18	0.15	-1.26	-0.51	-0.99	-0.37	2.32	2.05	0.03	-0.11	0.53	0.77	-0.48	**0.18**
ARHGAP19	-7.95	-4.23	2.81	-2.78	3.71	2.03	1.65	-1.24	-2.12	-1.10	2.23	-2.01	**-0.75**
ARHGAP20	-0.99	0.63	5.85	1.24	-1.23	-1.43	0.41	0.58	-1.11	-0.40	3.13	0.91	**0.63**
ARHGAP22	0.27	0.09	4.57	1.63	-0.35	-0.88	-8.80	-1.81	-0.04	-0.39	-2.12	-0.09	**-0.66**
ARHGAP23	0.47	1.03	-1.39	1.18	-0.45	-2.48	-1.38	-0.62	0.01	-0.73	-1.38	0.28	**-0.46**
ARHGAP24	2.67	2.97	2.31	0.82	1.70	-0.20	0.29	-2.08	2.18	1.38	1.30	-0.63	**1.06**
ARHGAP25	2.58	4.11	5.54	0.49	0.26	-0.01	1.64	-0.25	1.42	2.05	3.59	0.12	**1.79**
ARHGAP26	0.42	2.49	1.70	-0.71	0.60	-3.67	-1.49	-8.62	0.51	-0.59	0.10	-4.66	**-1.16**
ARHGAP27	-1.25	-1.06	3.75	0.02	-0.33	0.38	-5.57	0.36	-0.79	-0.34	-0.91	0.19	**-0.46**
ARHGAP28	-0.36	-1.97	6.22	0.77	0.52	0.36	-3.78	0.10	0.08	-0.81	1.22	0.43	**0.23**
ARHGAP29	0.03	-2.14	0.87	0.32	-0.15	1.01	-3.19	0.78	-0.06	-0.57	-1.16	0.55	**-0.31**
ARHGAP30	0.01	-1.28	-2.17	-1.01	-0.79	0.72	-7.14	0.25	-0.39	-0.28	-4.65	-0.38	**-1.43**
ARHGAP31	-0.08	-2.38	2.40	1.72	-0.74	0.63	0.71	0.93	-0.41	-0.88	1.55	1.32	**0.40**
ARHGAP33	0.46	0.46	2.74	1.18	1.08	0.64	2.29	1.41	0.77	0.55	2.51	1.30	**1.28**
ARHGAP36	-0.41	0.02	-0.95	3.11	0.39	1.47	-0.46	0.97	-0.01	0.74	-0.71	2.04	**0.52**

Gene	A1	B1	C1	D1	A2	B2	C2	D2	A	B	C	D	avg
ARHGAP39	-0.90	-1.73	6.35	1.77	0.16	2.08	-8.31	-1.96	-0.37	0.18	-0.98	-0.09	**-0.32**
ARHGAP4	-1.40	-0.46	0.97	0.66	-1.51	0.16	-1.06	-2.98	-1.45	-0.15	-0.05	-1.16	**-0.70**
ARHGAP40	0.65	-1.13	-4.99	-1.81	-1.06	1.46	-2.15	-0.81	-0.20	0.17	-3.57	-1.31	**-1.23**
ARHGAP42	-0.15	-1.34	-1.45	-0.68	-1.34	0.79	-1.06	-0.41	-0.74	-0.28	-1.25	-0.55	**-0.70**
ARHGAP5	0.14	-1.68	3.74	2.45	-0.30	0.52	-8.08	0.22	-0.08	-0.58	-2.17	1.33	**-0.37**
ARHGAP6	-1.88	-0.53	1.61	-0.08	1.58	-5.52	-4.65	-2.17	-0.15	-3.02	-1.52	-1.13	**-1.46**
ARHGAP8	0.22	0.10	4.26	0.03	-0.92	2.08	-0.10	-0.04	-0.35	1.09	2.08	0.00	**0.70**
ARHGAP9	1.99	2.68	-0.38	2.11	-0.57	-1.86	0.02	-0.02	0.71	0.41	-0.18	1.05	**0.50**
CENTD1	1.39	2.40	1.01	-1.49	-0.76	1.49	-4.63	-0.85	0.31	1.94	-1.81	-1.17	**-0.18**
CENTD3	3.64	5.00	-0.75	1.37	-0.64	-0.47	-5.75	-1.18	1.50	2.27	-3.25	0.10	**0.15**
CHN1	4.41	-2.82	0.02	-8.27	3.36	3.24	2.24	-3.69	3.88	0.21	1.13	-5.98	**-0.19**
CHN2	-0.25	2.41	0.00	2.06	0.74	1.79	-7.25	0.84	0.25	2.10	-3.63	1.45	**0.04**
DEPDC1A	-0.53	2.02	-0.74	2.28	0.38	-0.04	-1.83	-0.88	-0.08	0.99	-1.29	0.70	**0.08**
DEPDC1B	-0.89	-0.18	4.79	3.05	0.93	0.21	5.72	0.72	0.02	0.01	5.25	1.88	**1.79**
DLC1	-1.64	-0.95	2.75	0.62	-1.41	-0.14	-0.24	0.34	-1.52	-0.54	1.25	0.48	**-0.08**
FAM13A	-1.81	-0.80	3.28	-0.53	-0.07	-0.39	-2.21	1.74	-0.94	-0.60	0.53	0.60	**-0.10**
FAM13B	0.72	-1.94	2.17	0.78	-0.81	1.27	-1.01	1.10	-0.05	-0.33	0.58	0.94	**0.29**
GMIP	0.21	-1.15	-0.97	2.07	-0.62	0.95	-2.67	-0.60	-0.20	-0.10	-1.82	0.73	**-0.35**
GRLF1	0.01	-1.05	-0.69	2.47	-0.50	1.89	-0.64	-0.08	-0.24	0.42	-0.67	1.20	**0.18**
HMHA1	-5.39	-7.42	-8.23	-1.73	-2.26	1.72	2.23	1.08	-3.83	-2.85	-3.00	-0.33	**-2.50**
MYO9A	0.45	1.64	-1.25	-0.21	-0.24	-0.32	-3.86	0.05	0.10	0.66	-2.55	-0.08	**-0.47**
MYO9B	0.07	2.18	-1.86	0.05	0.46	0.58	2.20	1.54	0.26	1.38	0.17	0.79	**0.65**
OPHN1	-1.76	-2.20	-1.89	2.08	8.31	5.25	8.03	5.28	3.28	1.53	3.07	3.68	**2.89**
PIK3R1	-0.89	-0.23	-0.30	0.36	-3.87	1.87	0.40	1.58	-2.38	0.82	0.05	0.97	**-0.13**
PIK3R2	-1.64	-0.47	-0.63	-2.26	-6.88	-5.72	-1.71	3.27	-4.26	-3.09	-1.17	0.51	**-2.00**
RACGAP1	-0.20	-0.01	-4.99	-0.33	8.68	-3.45	1.00	0.60	4.24	-1.73	-2.00	0.14	**0.16**
RALBP1	-0.54	-0.37	3.18	1.59	0.61	0.53	1.73	1.39	0.03	0.08	2.46	1.49	**1.02**
RICH2	-1.22	-0.29	1.55	2.05	-0.58	-0.03	1.37	-0.43	-0.90	-0.16	1.46	0.81	**0.30**

Gene	A1	B1	C1	D1	A2	B2	C2	D2	A	B	C	D	avg
RICS	0.08	4.39	-8.45	-11.43	3.24	2.09	0.83	2.56	1.66	3.24	-3.81	-4.43	**-0.84**
SH3BP1	-0.19	-1.17	6.52	2.50	-0.71	1.33	1.79	-1.49	-0.45	0.08	4.15	0.50	**1.07**
SRGAP1	-0.42	0.68	2.12	1.19	-0.62	0.56	-1.62	1.30	-0.52	0.62	0.25	1.25	**0.40**
SRGAP2	-1.66	1.87	-0.27	-0.96	-0.83	-0.46	4.55	1.51	-1.25	0.71	2.14	0.28	**0.47**
SRGAP3	-0.26	-0.47	-3.71	1.17	0.50	2.00	0.85	0.87	0.12	0.77	-1.43	1.02	**0.12**
STARD13	-0.43	-1.30	1.40	-0.05	1.03	1.88	2.59	1.97	0.30	0.29	2.00	0.96	**0.89**
STARD8	-0.32	-0.96	0.44	-0.47	0.76	-0.28	-1.33	1.72	0.22	-0.62	-0.44	0.63	**-0.05**
SYDE1	-0.67	-0.08	0.97	1.10	-0.45	1.00	4.13	1.69	-0.56	0.46	2.55	1.39	**0.96**
SYDE2	0.23	0.05	-2.02	-1.52	-0.31	0.94	-4.28	0.79	-0.04	0.49	-3.15	-0.37	**-0.77**
TAGAP	-0.70	-0.18	1.48	-0.31	-0.74	0.38	1.01	1.01	-0.72	0.10	1.25	0.35	**0.25**

2.4.1 Tiam2 and Tiam1 are regulators of EphB trans-endocytosis into ephrinB+ cells

Tiam2, also known as Stef, is a GEF for Rac subfamily GTPases and shares high sequence similarity with Tiam1 (Matsuo et al. 2002, Terawaki et al. 2010). In a study by Tanaka and colleagues preliminary data indicated that Tiam2 is able to bind to ephrinB1 in similar fashion to Tiam1 (Tanaka et al. 2004). Our screen data revealed that across all four siRNA oligos used against Tiam2, the number of internalised Eph-ephrin clusters was reduced when compared to scramble control condition. Knockdown of Tiam1, on the other hand, which had already been implicated in endocytosis of soluble ephrinA ectodomains into EphA+ cells (Yoo et al. 2010, Um et al. 2014), did not show any effect on EphB trans-endocytosis into SKN cells. In order to confirm the involvement of Tiam2 in EphB trans-endocytosis into ephrinB+ cells, we repeated the experiments in the same setup used for the screen, but for each experiment the treatments were performed in triplicate to improve the validity of the results. Since Tiam1 had been previously shown to be involved in endocytosis of soluble ephrin ectodomains, we decided to also re-test Tiam1. These follow-up experiments confirmed the results from the screen. Knockdown of Tiam2 led to a significant reduction of EphB trans-endocytosis into SKN cells, while knockdown of Tiam1 resulted in no detectable effect (Fig. 20B).

Next, we wanted to establish, whether Tiam2 was also required for efficient EphB trans-endocytosis into other ephrinB+ cell lines. We therefore conducted co-culture experiments using two types of HeLa cells transiently expressing ephrinB1-mCherry and EphB2ΔC-GFP respectively. In addition to the siRNA knockdown of Tiam2, we again included knockdown of Tiam1 in the experimental conditions (Fig. 20A, top panel). Furthermore, to account for potential redundancy between the closely related Tiam proteins, we also tested a combined knockdown using oligos against both Tiam1 and Tiam2 (images not shown). We observed a significant decrease in trans-endocytosis upon knockdown of Tiam2 in HeLa cells, replicating the findings from SKN cells (Fig. 20C). In contrast to the results from SKN cells, knockdown of Tiam1 also resulted in a significant decrease of EphB trans-endocytosis into ephrinB1-expressing HeLa cells. A combination of oligos against Tiam2 and Tiam1 led to a significant reduction in trans-endocytosis as well, however, the combined effect was no larger than the effect of single knockdowns of either

Tiam2 or Tiam1. The results from the combined knockdown could suggest a combinatorial requirement of Tiam1 and Tiam2 for EphB trans-endocytosis into ephrinB1-expressing HeLa cells instead of functional redundancy between the two. Another potential explanation for not observing an increase in the effect of the combined knockdown as compared to the single knockdowns, could be the fact that in the combined knockdown only half the concentration of each single oligo is used, potentially decreasing knockdown effectiveness and thus weakening the effect.

Tiam1 had already been reported to be a regulator of Eph-ephrin endocytosis, however these experiments were performed with soluble pre-clustered ephrinA ectodomains into EphA+ cells and the role of Tiam2 was not addressed (Yoo et al. 2010, Um et al. 2014). Thus, to determine whether reverse and forward trans-endocytosis of Eph-ephrin complexes make use of the same molecular machinery, we performed co-culture experiments with HeLa cells transiently transfected with either EphB2-mCherry or ephrinB1ΔC-GFP, thus only allowing endocytosis into the EphB2-expressing cell (forward direction). We tested the same siRNA treatments as in the reverse direction (Fig. 20A, bottom panel). Indeed, knockdown of Tiam1, Tiam2, or both proteins combined caused a reduction in the number of Eph-ephrin complexes internalised into HeLa cells. As in the reverse direction, combined knockdown of Tiam1 and Tiam2 did not result in a more pronounced effect on the number of internalised vesicles (Fig. 20D).

Taken together, these results suggest that the molecular mechanism for trans-endocytosis of Eph-ephrin complexes is either the same or, at least shares overlapping molecular players in the forward and reverse direction. In both directions, activity of Rac subfamily GTPases is required, which is very likely induced by GEFs of the Tiam family.

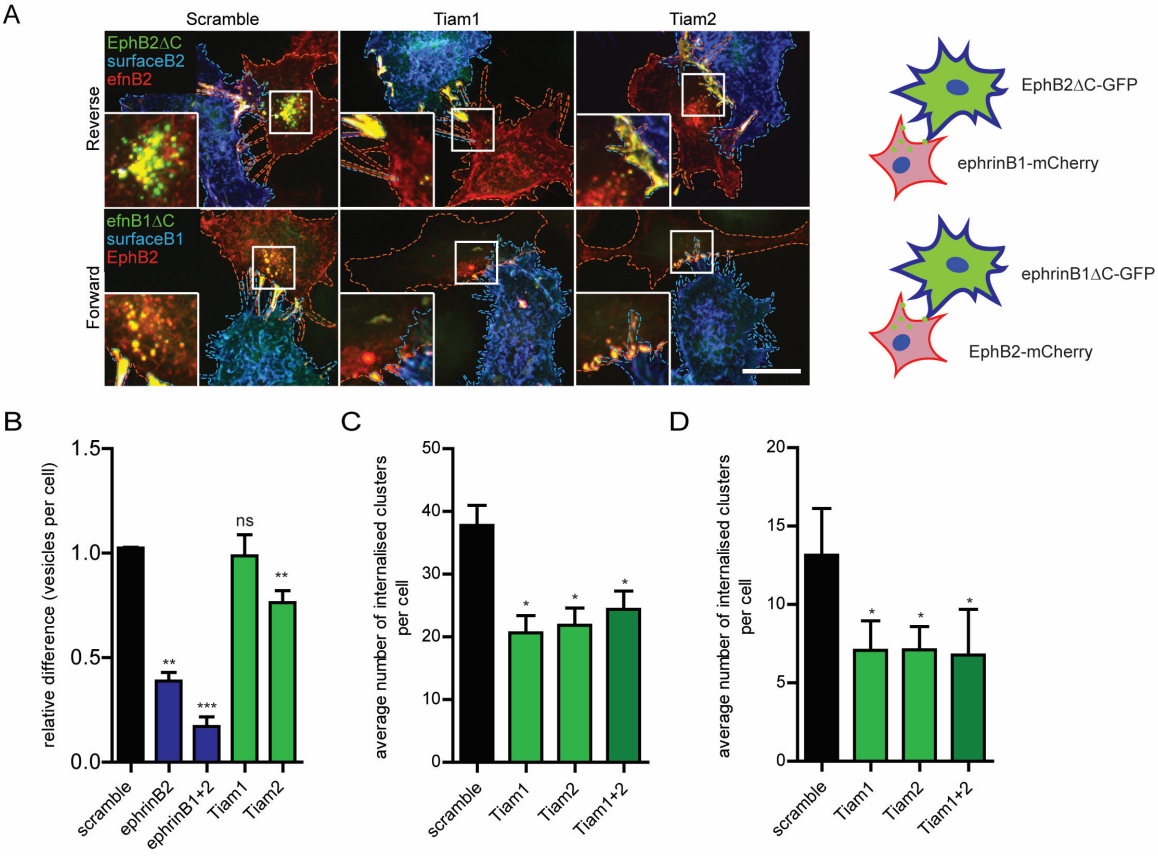


Figure 20. Tiam proteins in trans-endocytosis of EphB-ephrinB clusters

(A) Overlay images of reverse trans-endocytosis assay (top panel) with HeLa cells expressing ephrinB1-mCherry (red/red dashed outline) co-cultured with HeLa cells expressing FLAG-EphB2ΔC-GFP (green/blue dashed outline). Surface EphB2 stained with secondary antibody (blue/appears white in the overlay). Treatment with scramble, Tiam1 or Tiam2 siRNA oligos. Overlay images of forward trans-endocytosis assay (bottom panel) with HeLa cells expressing EphB2-mCherry (red/red dashed outline) co-cultured with HeLa cells expressing FLAG-ephrinB1ΔC-GFP (green/blue dashed outline). Surface ephrinB1 stained with secondary antibody (blue/appears white in the overlay). Treatment with scramble, Tiam1 or Tiam2 siRNA oligos. (B) Quantification of average number of internalised clusters per cell normalised to the median of the scramble controls in assay with SKN cells performed with CellProfiler™. Results for each gene and the combination of Tiam1 and Tiam2 oligos at 20 nM total final concentration are shown. Data shown as mean of the individual means normalised to the median of the scramble control. Repeated ANOVA with Dunnett's post-hoc test was used to test for significance. (n=5 independent experiments) **= $p<0.01$, ***=$p<0.005$. (C) Quantification of average number of internalised clusters per HeLa cell in reverse assay. Counting performed manually and experiments were performed blind. Repeated measures ANOVA with Dunnett's post-hoc test was used to test for significance. (n=4 independent experiments). *= $p<0.05$ (D) Quantification of average number of internalised clusters per HeLa cell in forward assay. Counting performed manually and experiments were performed blind. Repeated measures ANOVA with Dunnett's post-hoc test was used to test for significance. (n=3 independent experiments), *= $p<0.05$. Experiments performed by T. Gaitanos.

2.4.2 Tiam family proteins are not required for the endocytosis of soluble EphB2 ectodomains into ephrinB+ cells

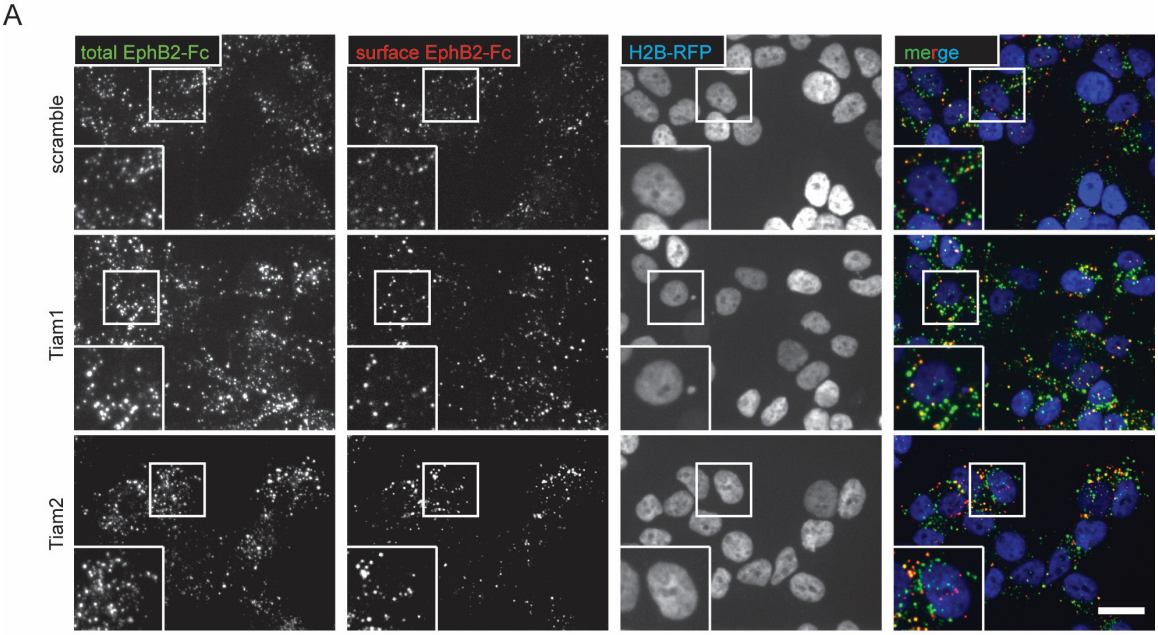
Given the differential requirement for Rac subfamily GTPases for endocytosis of membrane-tethered and soluble EphBs, I investigated whether the same is true for the Tiam family proteins, given their role as Rac-specific GEFs. I knocked down the expression of Tiam1 and Tiam2 in SKN cells using siRNA either individually or in combination and stimulated the cells with soluble EphB ectodomains described before (Fig. 21A). Neither individual knockdown of either Tiam1 or Tiam2, nor combined knockdown of both proteins at the same time (images not shown), resulted in a significant change in the number of internalised Eph-ephrin clusters (Fig. 21B).

The differential requirement for Tiam family proteins between the trans-endocytosis assay and the stimulation with soluble EphB ectodomains fit nicely with the observation that also Rac subfamily GTPases are only required for EphB trans-endocytosis into ephrinB+ cells, when the receptor is membrane-tethered, but not upon stimulation with soluble EphB ectodomains.

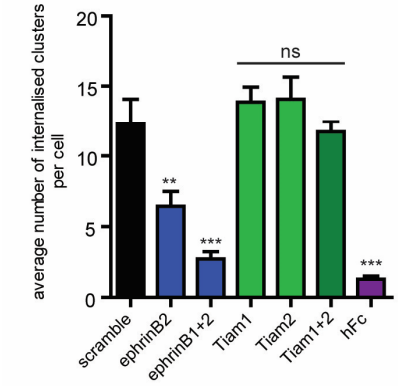
Figure 21. Tiam proteins are not required for endocytosis of soluble EphB2 ectodomains into ephrinB+ cells

(A) SKN H2B-RFP cells (nuclei in blue) treated with siRNA (scramble oligo or oligos against Tiam or Tiam2) then incubated with EphB2-Fc pre-clustered with a Cy2-conjugated antibody (green) for 30 min. Cells were subsequently fixed without permeabilisation stained with a dyLight649-conjugated antibody against Fc to visualise surface clusters (red/appear red in overlay). Scale bar equals 20 µm. (B) Quantification of average number of internalised clusters per cell performed with CellProfilerTM. Results for one oligo for each gene and the combination of Tiam1 and Tiam2 oligos at 20 nM total final concentration are shown. As a positive control results for a knockdown of ephrinB2 or ephrinB1 and ephrinB2 combined are shown. As absolute control cells were stimulated with pre-clustered hFc. One-way ANOVA with Dunnett's post-hoc test was used to test for significance. **=$p<0.01$, ***=$p<0.005$

A


total EphB2-Fc
surface EphB2-Fc
H2B-RFP
merge
scramble
Tiam1
Tiam2

B


average number of internalised clusters per cell
20
15
10
5
0
ns
**
***
***
scramble
ephrinB2
ephrinB1+2
Tiam1
Tiam2
Tiam1+2
hFc

3 Discussion

3.1 Molecular mechanisms of Eph-ephrin endocytosis

The findings of this study regarding the molecular regulation of Eph-ephrin endocytosis are summarised in the cartoon in Figure 22. In brief, trans-endocytosis of Eph-ephrin complexes in both the forward (Fig. 22C) and reverse (Fig. 22D) directions requires Tiam-induced Rac activity, probably in order to drive polymerisation of the actin cytoskeleton to allow for the pinching off of whole receptor-ligand complexes including pieces of the opposing membrane. Rac activity controls EphB trans-endocytosis into ephrinB+ SKN cells (Fig. 8), HeLa cells (Fig. 9), and primary cortical neurons (Fig. 10). These findings show that the general mechanism described in this study is not cell-type-specific and relevant in physiological contexts, where Eph-ephrin signalling is important.

For endocytosis of Eph-ephrin complexes induced by soluble ephrin ectodomains (forward direction), Tiam-induced Rac activity is also required. However, previous work also suggests that these complexes are internalised via Clathrin-mediated endocytosis (CME) (Yoo et al. 2010), which can represent either an overlapping or parallel pathway (Fig. 22A). The reverse endocytosis of Eph-ephrin complexes induced by stimulation with soluble EphB ectodomains does not require Rac or Tiam activity. Curiously, we also found that RhoA subfamily GTPases inhibit or at least slow down the rate of internalisation (Fig. 22B). While also for reverse endocytosis induced by stimulation with soluble Eph ectodomains evidence implicating the importance of the CME pathways exists (Parker et al. 2004), the exact molecular mechanism underlying this endocytic route still remains to be elucidated.

In the following chapters I will discuss the results from this study in more detail, as well as provide an outlook on possible future directions of research building on this work.

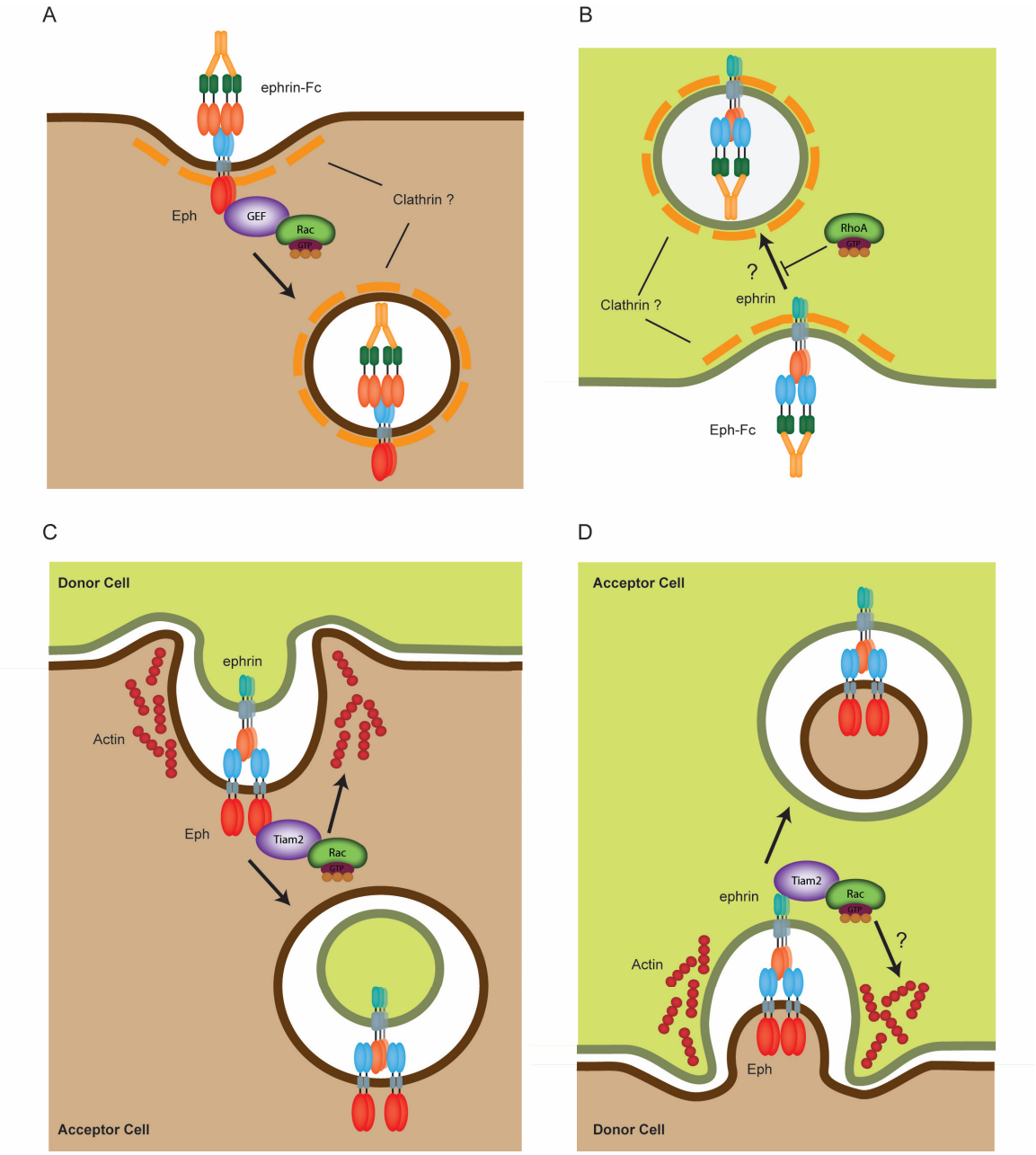


Figure 22. Updated models for endocytosis of Eph-ephrin complexes

(A) Forward endocytosis of Eph-ephrin complexes induced by soluble pre-clustered ephrin ectodomains. GEFs activate Rac downstream of Eph-receptors. Tiam1 and Vav2/3 have been implicated as GEFs mediating this process. Evidence for the involvement of clathrin exists. (B) Reverse endocytosis of Eph-ephrin complexes induced by soluble pre-clustered Eph ectodomains. Evidence for the involvement of clathrin exists. Activity of RhoA subfamily GTPases limits endocytic uptake of Eph-ephrin complexes. (C) Ephrin trans-endocytosis into Eph+ cell (forward direction). Rac activity and actin reorganisation required Rac activity is induced by Tiam1 and/or Tiam2. (D) Eph trans-endocytosis into ephrinB+ cells (reverse direction). Rac activity is required and is induced by Tiam2 and in some contexts also by Tiam1. Evidence for actin polymerisation at sites of EphB trans-endocytosis into ephrinB+ cells exists.

3.1.1 Which endocytic pathway does EphB-ephrinB internalisation take?

As discussed in the introduction (section 1.3.1), endocytic processes can be distinguished by their underlying molecular machineries (Doherty & McMahon 2009). Our findings suggest that Eph-ephrin complexes are internalised via different endocytic pathways, depending on whether they originate from stimulation with soluble fusion proteins or from cell contact-mediated Eph-ephrin signalling.

The original work from Marston and co-workers, as well as Zimmer and colleagues showed that trans-endocytosis of Eph-ephrin complexes does not occur via the clathrin-mediated pathway of endocytosis (CME), at least in the forward direction, as EphB-ephrinB complexes internalised by trans-endocytosis do not co-localise with clathrin-coated pits (Marston et al. 2003, Zimmer et al. 2003). Since also no co-localisation of Eph-ephrin clusters with caveolin could be detected, Eph-ephrin trans-endocytosis should also be independent of the caveolae- mediated endocytic pathway.

Among the well-described endocytic pathways, macropinocytosis and phagocytosis are reliant on Rac activity (Doherty & McMahon 2009). These two pathways both involve the uptake of relatively large cargo: either only to some extent discriminatively from the environment (macropinocytosis) or very selective uptake of pathogens or apoptotic bodies that have been primed for internalisation by specialised cells (phagocytosis). Apart from being limited to specialised cells in the immune system, phagocytosis has been shown to require the orchestrated activity of several Rho family GTPases (Massol et al. 1998, deBakker et al. 2004, Flannagan et al. 2012) and should thus be sensitive to disturbance of additional Rho subfamilies other than only Rac. As our experiments, however, only show the requirement of Rac subfamily GTPases and suggest that other Rho subfamilies are dispensable for EphB trans-endocytosis into ephrinB+ cells, and given that the endocytic uptake of Eph-ephrin complexes is not limited to the specialised cells traditionally thought to exhibit phagocytosis, it can be postulated that EphB trans-endocytosis into ephrinB+ cells is not mediated by canonical phagocytosis. Whereas macropinocytosis was originally thought to be involved mainly in the indiscriminate uptake of extracellular fluid and its content (hence the Greek origin of the name: “large cell drinking”) (Swanson & Watts 1995), it has later emerged that macropinocytosis also regulates the selective internalisation

of large patches of activated RTKs (Orth et al. 2006). Macropinocytosis is characterised by the formation of dorsal membrane ruffles, which requires the activity of Rac subfamily GTPases (Rac1 and RhoG) and their downstream effector PAK to initiate the reorganisation of the actin cytoskeleton required (Swanson & Watts 1995, Dharmawardhane et al. 2000, West et al. 2000, Ellerbroek et al. 2004, Doherty & McMahon 2009). EphB trans-endocytosis into ephrinB+ cells also requires the uptake of large clusters and corresponding patches of membrane and is regulated by Rac subfamily GTPases, as shown in this study. Therefore, it is possible that Eph-ephrin trans-endocytosis uses the macropinocytic pathway. To clarify whether Eph-ephrin trans-endocytosis truly goes through canonical macropinocytosis or whether it simply shares some molecular components, further work will be required. Firstly, studies with siRNA or pharmacological inhibition of PAK (Rudolph et al. 2015) could elucidate whether PAK is required downstream of Rac signalling in this context. Secondly, a unique feature of macropinocytosis is its susceptibility to inhibitors of Na^+/H^+ exchange, such as amiloride (West et al. 1989, Veithen et al. 1996, Koivusalo et al. 2010). Thus, inhibition of Eph-ephrin trans-endocytosis with amiloride might indicate that this process resembles macropinocytosis.

As a preliminary conclusion, I suggest that Eph-ephrin complexes between two opposing cells may be internalised via a macropinocytosis-like pathway.

Experiments performed using stimulation with soluble ectodomains in both the forward and reverse direction have provided some evidence for an involvement of the CME-pathway, as internalised Eph-ephrin complexes co-localised with markers of the CME-pathway (Parker et al. 2004, Yoo et al. 2010). Nonetheless, the role of CME is still disputed, as several studies also postulate that Rac activity is necessary for internalisation into Eph-expressing cells after stimulation with soluble ephrin ectodomains (Cowan et al. 2005, Yoo et al. 2010, Um et al. 2014). Rac activity is traditionally not considered to be obligatory for CME to occur (McMahon & Boucrot 2011).Furthermore Cowan and colleagues also showed that upon knockout of Vav2 and Vav3, which led to an inhibition of EphA endocytosis induced by soluble ephrinA ectodomains, the general CME pathway was still intact. Parker and colleagues reported that ephrin reverse endocytosis after stimulation with soluble EphB ectodomains is mediated by CME (Parker et al. 2004). They based their

claims on a combination of indirect observations (co-localisation with CME-associated proteins after internalisation), and experiments not specific for CME (transfection of a dominant negative version of dynamin, potassium depletion). Therefore more conclusive experiments, for example with direct siRNA knockdown of components of the clathrin-machinery, would be required to confirm that reverse endocytosis of Eph-ephrin complexes induced by soluble Eph ectodomains uses CME. However, as our results only indicate that Rac activity is not required for endocytosis of EphB ectodomains into ephrinB+ cells, they do not contradict the findings of Parker and colleagues. Additionally, we found in our study that depletion of RhoA subfamily GTPases leads to an increase in endocytosis of soluble EphB ectodomains into ephrinB+ cells. Since some evidence exists suggesting RhoA activity can negatively affect CME in some physiological contexts (Lamaze et al. 1996, Kaneko et al. 2005, Khelfaoui et al. 2009), these data are in agreement with the hypothesis that Eph-ephrin reverse endocytosis after stimulation with soluble Eph ectodomains occurs via CME. However, it should be noted that the literature on the role of RhoA subfamily GTPases in CME is contradictory, as positive regulation has also been reported (Malaval et al. 2009, Stirling et al. 2009).

An interesting feature of endocytosis induced by soluble ephrin or Eph ectodomains is the differential requirement of Rac activity between the forward and reverse directions. The evidence indicating CME is responsible for endocytosis of soluble ephrin ectodomains into Eph+ cells is not very strong, as it only derives from co-localisation studies of CME-associated proteins with Ephs after internalisation (Yoo et al. 2010). Such co-localisation after internalisation could, however, also arise from trafficking of Eph-ephrin complexes internalised through clathrin-independent pathways into shared endosomal compartments. On the other hand, the requirement of Rac activity for endocytosis of soluble ephrin ectodomains into Eph+ cells is fairly strong and has been replicated in several independent publications (Cowan et al. 2005, Yoo et al. 2010, Yoo et al. 2011, Um et al. 2014), as well as in our study presented here (Fig. 12). Furthermore, additional experiments in our group revealed differential activation of Rac upon stimulation with either soluble pre-clustered ephrin or Eph ectodomains with a Förster resonance energy transfer (FRET)-based probe for Rac activity (Itoh et al. 2002), emphasising that Rac is more strongly activated downstream of EphB receptors than ephrinBs (T. Gaitanos, unpublished data). There are

several other conceivable explanations for the seemingly conflicting evidence regarding the involvement of both Rac activity and CME in endocytosis of soluble ephrin ectodomains into Eph+ cells. Not only Rac activity, but also explicitly the GEFs Tiam1, Vav2 and Vav3 have previously been implicated in Eph-ephrin endocytosis (Cowan et al. 2005, Yoo et al. 2010, Um et al. 2014), and the same signalling axis has been shown to regulate Eph-ephrin trans-endocytosis in both directions in this study. Therefore, it is possible that trans-endocytosis and endocytosis of soluble ephrin ectodomains into Eph+ cells share the same macropinocytosis-like pathway and CME is of subsidiary relevance. Still, there is also another potential explanation. The role of actin reorganisation in CME is still a matter of debate (Mooren et al. 2012). However, there are reports that indicate that endocytosis of the vesicular stomatitis virus occurs through CCPs and requires polymerisation of actin for its full-length form, while an artificially truncated version of the viral particle can be internalised via CME independently of actin (Cureton et al. 2009, Cureton et al. 2010). Therefore, a difference in the size of Eph-ephrin clusters induced between forward and reverse signalling could be a potential explanation for the variance in the requirement of Rac activity for endocytosis. Once the initial cluster formation has been triggered by stimulation with ephrin, clusters of Eph receptors can propagate in size due to *cis* interactions independent of ephrin-binding (Wimmer-Kleikamp et al. 2004). However, no such propagation mechanism has been described for ephrins thus far. Hence, it is conceivable that Eph-ephrin clusters formed by stimulation with soluble ephrin ectodomains are larger than those formed after stimulation with soluble Eph ectodomains. Hence, it is possible that their internalisation requires an "extended" form of CME, which is also dependent on reorganisation of the actin cytoskeleton triggered by Rac activity. Finally, it is also possible that two separate pathways, one Rac-dependent, the other clathrin-dependent, govern the internalisation of soluble ephrin ectodomains into Eph+ cells. It would therefore be very informative to inhibit both pathways at the same time.

Taking both the results presented in this study and previous literature into consideration, trans-endocytosis of Eph-ephrin complexes can be linked to a macropinocytosis-(like) pathway, while the paradigm for endocytosis of clusters induced by soluble proteins is more nuanced and potentially involves CME as either the main, or at least one of several pathways. The evidence so far is not conclusive enough to firmly assign the endocytic

processes for Eph-ephrin complexes to any specific pathway. To further dissect the contribution of the individual endocytic pathways, careful analysis with manipulation of known molecular markers for the separate pathways should be conducted. Moreover, the possibility that Eph-ephrin endocytosis is governed by several pathways in parallel, or by pathways that are novel in their precise composition of regulating proteins and only partially overlap with well-characterised pathways, cannot be ruled out at this stage. Also the question in how far the endocytic pathways differ between forward and reverse directions and between EphA-ephrinA and EphB-ephrinB signalling was not completely answered yet. Here we show that for the trans-endocytosis with two opposing cells in contact the molecular regulators identified are the same for forward and reverse direction, while endocytosis after stimulation with soluble ectodomains differs in their requirement of Rac activity. Regarding the differences between the EphA and EphB systems our findings indicate that at least in the forward direction EphA and EphB endocytosis shares a common regulatory mechanism via Rac activity. While it should be noted that this assumption for EphAs is based primarily on data from studies with soluble ectodomains, it would also be interesting to examine whether trans-endocytosis also occurs in EphA-ephrinA signalling, or whether this system only relies on cleavage for cell detachment (Hattori et al. 2000, Janes et al. 2005). Finally, another intriguing question is which molecular mechanisms govern the decision into which cell trans-endocytosis occurs under physiological conditions with both cells coming into contact expressing either wild type Eph or ephrin.

3.1.2 Physiological relevance of Eph-ephrin endocytosis triggered by stimulation with soluble proteins

In physiological situations both Ephs and ephrins are membrane-tethered when they engage in signalling. Therefore, it is reasonable to assume that the trans-endocytosis of Eph-ephrin clusters we observe in experiments during cell-contact mediated signalling more truthfully represents the endocytic process *in vivo*. Nonetheless, it is possible that the mechanisms for endocytosis upon stimulation with soluble ephrin or Eph ectodomains are also relevant in physiological settings. As Ephs and ephrins have been shown to be cleaved by metalloproteases (Hattori et al. 2000, Georgakopoulos et al. 2006, Lin et al. 2008, Inoue et

al. 2009, Gatto et al. 2014), it is possible that their shed ectodomains can bind to receptors or ligands as soluble proteins and elicit signalling, albeit weakly. If this were the case, then endocytosis of Eph-ephrin complexes would probably be regulated by the two distinct mechanisms in the forward and reverse direction described in this study (Fig. 22A and B). However, so far cleavage of ephrins and Eph receptors has been thought of as an alternative mechanism for allowing cell detachment after initial adhesion due to receptor-ligand interactions, or a mechanism to fine-tune signalling responses by decreasing sensitivity, while no evidence for a signal-inducing role of the shed ectodomains has been found.

Recent work in our laboratory has shown that both Ephs and ephrins are secreted in exosomes, which retain their signalling capabilities (J. Gong, unpublished data). Exosome-induced ephrin signalling probably involves endocytosis and it would be interesting to investigate which endocytic mechanism is involved. It is possible that endocytosis of Eph-positive exosomes resembles Eph trans-endocytosis between two opposing cells, since the membranous nature of the exosome is more similar to a living cell than a soluble fusion protein. Further work will be required to assess the requirement of Rac activity and actin polymerization in this context.

In conclusion, given our current understanding of Eph-ephrin signalling, the trans-endocytosis process orchestrated by Rac activity proposed in this study is highly relevant for the physiological role of Eph-ephrin signalling in mediating cell repulsion, while the divergent pathway for endocytosis of complexes induced by soluble proteins is of lesser importance unless a physiological role of soluble Eph or ephrin ectodomains is discovered. Therefore, it would be advisable that future studies of EphB endocytosis into ephrinB+ cells are carried out in cell contact-mediated settings to obtain physiologically relevant results.

3.2 The role of Rho family GTPases in Eph-ephrin endocytosis

3.2.1 Redundancy between Rac subfamily members in the regulation of EphB trans-endocytosis into ephrinB+ cells

Our experiments in SKN cells, HeLa cells and cortical neurons all show the requirement of Rac activity for efficient EphB trans-endocytosis into ephrinB+ cells. Interestingly, only the simultaneous, combined siRNA knockdown of all three Rac subfamily members expressed in the SKN cells used in this study (Rac1, Rac3, and RhoG) resulted in a significant reduction of EphB trans-endocytosis, indicating physiological redundancy between them. Redundancy between Rac1 and Rac3 has already been established in the literature (Corbetta et al. 2009). In contrast, despite its sequence similarity, RhoG has been suggested to activate Rac signalling instead of being redundant to it (Katoh & Negishi 2003, Hiramoto et al. 2006, Katoh et al. 2006). Still, others have found that RhoG has both distinct and shared signalling pathways with other Rac subfamily GTPases (Wennerberg et al. 2002). In this study, we carefully dissected the individual contributions of the separate Rac subfamily members by performing individual siRNA knockdowns, a combination of knockdowns of any two proteins, as well as all three at once. Neither depletion of any single Rac subfamily GTPase, nor the combined knockdown of any two subfamily members significantly reduced EphB trans-endocytosis into ephrinB+ cells. An effect was only observed when Rac1, Rac3, and RhoG were knocked down simultaneously. These results suggest that for EphB trans-endocytosis into ephrinB+ cells the three Rac subfamily GTPases are physiologically redundant. Marston and colleagues had previously implicated Rac1 in the regulation of Eph-ephrin trans-endocytosis in the forward direction by transfecting cells with a dominant negative mutant of Rac1 (Marston et al. 2003). These experiments do not rule out the possibility that in the forward direction activity of different Rac subfamily members is also physiologically redundant, since the dominant negative mutant potentially inhibits all family members by sequestering shared regulators and effectors.

Notably, despite a significant decrease in the amount of internalised Eph-ephrin complexes in cells treated with siRNA against Rac subfamily members or the Rac inhibitor EHT1864, EphB trans-endocytosis is not completely abolished (Fig. 8). This is either due to the

treatments not sufficiently inhibiting Rac function, or it could be due to the possibility that EphB trans-endocytosis into ephrinB+ cells can also be mediated by an alternative Rac-independent mechanism.

3.2.2 The role of Cdc42 signalling in Eph-ephrin endocytosis

The results in this study indicate that activity of the Cdc42 subfamily of Rho GTPases is not involved in both EphB trans-endocytosis and endocytosis of soluble EphB ectodomains into ephrinB+ cells (Fig. 13). However, these results do not exclude the possibility that Cdc42 plays a minor role in EphB trans-endocytosis, which is masked by redundancy from Rac subfamily GTPases. Since both the knockdown of Rac subfamily members and pharmacological inhibition of Rac activity with EHT1864 did not completely block EphB trans-endocytosis and Rac and Cdc42 signalling has been reported to be redundant in certain physiological contexts (Izumi et al. 2004, Watanabe et al. 2004), it is conceivable that Cdc42 subfamily GTPases contribute to EphB trans-endocytosis into ephrinB+ cells in the absence of Rac activity. One way to address this possibility could be a combination of pharmacological inhibition and siRNA knockdown to target both subfamilies simultaneously.

Initial analysis of the GEF/GAP screen resulted in the Cdc42-specific GEF ITSN1 as a candidate for regulating EphB trans-endocytosis into ephrinB+ cells. However, both follow-up experiments and re-analysis of the screen data with a higher emphasis on consistency did not confirm ITSN1 as a regulator of EphB trans-endocytosis. Nonetheless, ITSN1 has previously been shown to be an important signalling component downstream of EphB receptors in the regulation of dendritic development (Irie & Yamaguchi 2002, Nishimura et al. 2006). Moreover, ITSN1 has also been implicated in the regulation of axon guidance at the cortical midline (Sengar et al. 2013), a feature it shares with Eph-ephrin reverse signalling (Kullander et al. 2001a, Mendes et al. 2006, Otal et al. 2006), which could imply that ITSN1 is also involved in Eph-ephrin signalling in this context. However, these results implicate ITSN1 downstream of Eph-ephrin forward signalling, while I examined the regulation of EphB reverse trans-endocytosis into ephrinB+ cells. Therefore I cannot exclude that ITSN1 is involved in the regulation of ephrin trans-endocytosis into Eph+ cells. It is also possible that ITSN1 fulfils functions not linked to

regulating Eph-ephrin endocytosis. Due to its nature as a multi-domain protein it could be required for the regulation of the endocytosis of target proteins triggered by Eph-ephrin signalling, or for the stabilisation of protein complexes as a scaffolding protein, as it has been described in other physiological scenarios (Tsyba et al. 2011, Wong et al. 2012).

3.2.3 The role of RhoA subfamily GTPases in Eph endocytosis into ephrinB+ cells

RhoA is well established as a downstream effector of Eph-ephrin signalling, especially in the contexts of growth cone collapse and cell retraction (Shamah et al. 2001, Sahin et al. 2005, Groeger & Nobes 2007, Takeuchi et al. 2015). So far, however, RhoA-like GTPases have not been described in the context of endocytosis of Eph-ephrin complexes. The results of this study reveal a complex picture for their involvement in Eph-ephrin reverse endocytosis.

Experiments with soluble EphB2 ectodomain stimulation in SKN cells showed a very strong phenotype for the knockdown of RhoA subfamily GTPases. The observed increase in the amount of Eph-ephrin complexes internalised after EphB2-Fc stimulation was highly significant in both the knockdown of RhoA or RhoB individually, or in a combination of the two. (Fig. 15C). Remarkably, the effect was stronger for the RhoA single and RhoA and RhoB double knockdown than for the RhoB single knockdown. Surprisingly, analogous experiments in HeLa cells did not result in a statistically significant increase in endocytosis, although a trend in the same direction was observed (Fig. 15D). This discrepancy either reveals a cell type-specific effect for RhoA subfamily activity on endocytosis of EphB ectodomains into ephrinB+ cells, or it could be explained by the circumstances of the artificial overexpression system used for the experiments in HeLa cells, with the sheer amount of available ephrin protein at the cell surface masking any effect resulting in an increase of endocytic events.

One possible explanation for the effect of the depletion of RhoA subfamily GTPases on endocytosis of soluble EphB ectodomains into ephrinB+ cells could be derived from their role in inhibiting CME. Previous work by Parker and colleagues suggests that endocytosis of EphB ectodomains into ephrinB+ cells is mediated by CME (Parker et al. 2004). RhoA activity, often conferred through its effector ROCK, has been shown to negatively regulate CME in several cellular contexts (Lamaze et al. 1996, Kaneko et al. 2005, Khelfaoui et al.

2009). It should be noted, however, that there are also reports providing evidence for a positive role of RhoA signalling in stimulating CME (Malaval et al. 2009, Stirling et al. 2009). Our findings are in agreement with the first line of evidence and would thus argue for a direct involvement of RhoA subfamily GTPases in negatively regulating endocytosis of soluble EphB ectodomains into ephrinB+ cells.

An alternative explanation for the role of RhoA subfamily GTPases in endocytosis of EphB ectodomains into ephrinB+ cells is that they do not directly inhibit the internalisation, but rather are required for subsequent endocytic trafficking. RhoB is a well-established regulator of endosomal trafficking that facilitates the transition of early endosomes towards the lysosomal or recycling compartments, while not effecting endocytic uptake per se (Gampel et al. 1999, Fernandez-Borja et al. 2005, Rondanino et al. 2007). Endosomes in cells with aberrant RhoB function are smaller than those under control condition and are observed in unusual cellular locations (Fernandez-Borja et al. 2005). Also in my experiments the endocytosed vesicles in SKN cells stimulated with soluble EphB2 ectodomains are significantly smaller upon knockdown of RhoA or a combined knockdown of RhoA and RhoB (Fig. 15E). This could indicate that the RhoA subfamily phenotype, at least to some extent, originates from a defect in endosomal trafficking. If the effect of RhoA subfamily depletion in Eph-ephrin endocytosis was due to aberrant endosomal trafficking, one would expect that knockdown of RhoB results in a stronger effect as compared to RhoA. My data, curiously, presents the opposite result. This should not be seen as a definitive argument against the hypothesis of an effect on endosomal trafficking, as also some evidence for a role of RhoA itself in endosomal trafficking has been reported (Nishimura et al. 2002, Stirling et al. 2009). Additionally, the preferential requirement of RhoA over RhoB might be a peculiarity of the Eph-ephrin system.

On the basis of the presented data, several plausible explanations for the role of RhoA subfamily GTPases in endocytosis of soluble EphB ectodomains into ephrinB+ cells can be given. In order to clarify the mechanisms involved and test for the role of endosomal trafficking, the interaction of Eph-ephrin endosomes with members of the Rab family GTPases could be studied, which are known regulators of endocytic trafficking events (Wandinger-Ness & Zerial 2014). An alternative or complimentary route of inquiry could

be the visualisation of the steps of endocytic processing using super resolution live cell-imaging.

In contrast to the results from the soluble assay, the trans-endocytosis assay with SKN cells resulted in no change in the amount of internalised complexes when compared to the control condition. Still, experiments in HeLa cells again displayed a trend towards an increase in endocytosis, which tested as statistically insignificant (Fig. 14). Interestingly, the trend for an increase in EphB trans-endocytosis into ephrinB-expressing HeLa cells was restricted to RhoA knockdown or a combination of RhoA and RhoB, while RhoB depletion alone showed trans-endocytosis exactly at control levels. This pattern is remarkably similar to the results obtained in the assay with soluble ectodomains (Fig. 15), but of course, without being statistically significant. One piece of evidence supporting some relevance of RhoA activity, also for EphB trans-endocytosis, can be drawn from the results of the GEF/GAP screen. The GEF showing the strongest increase in endocytosis when depleted was Net1. Net1 is a RhoA-specific GEF, which has been found to co-localise with RhoA in the nucleus (Alberts & Treisman 1998, Schmidt & Hall 2002b). So far no reports linking Net1 to endocytosis exist. Interestingly, one study suggests that Rac1 activity can increase Net1 GEF activity, thus suggesting a positive reinforcement from Rac to RhoA signalling (Carr et al. 2013), whereas an earlier study described Rac and PAK-dependent down-regulation of Net1 activity, which is more in line with the usual counteractive role of Rac and RhoA signalling (Alberts et al. 2005). Taken together with the well-established antagonistic role of RhoA and Rac signalling (Guilluy et al. 2011), these findings could imply that RhoA signalling can have a minor inhibiting effect on EphB trans-endocytosis into ephrinB+ cells. However, the evidence for this hypothesis is diminished by results from knockdown of RhoGAP OPHN1 in our screen. OPHN1 regulates RhoA activity *in vivo* and is reported to release the inhibitory effect of RhoA signalling on CME of synaptic receptors, which is required for proper neural development (Fauchereau et al. 2003, Govek et al. 2004, Khelfaoui et al. 2009). According to the hypothesis of RhoA as a negative regulator of EphB trans-endocytosis into ephrinB+ cells, depletion of OPHN1 should increase RhoA activity and thus lead to a decrease in trans-endocytosis. Our screen data reveals the exact opposite effect, with knockdown of OPHN1 resulting not only in an increase in trans-endocytosis, but it being one of the strongest

candidates (Fig. 19). A suitable explanation for this conflicting result could be that OPHN1 also possesses GAP activity for Rac1 and Cdc42, at least *in vitro* (Billuart et al. 1998), which could be the mechanism of action preferentially active in the context of EphB trans-endocytosis. In any case, more work, also addressing the validity of the tools used (for example, confirmation of siRNA knockdown effectiveness), would need to be conducted before final conclusions on the role of RhoA subfamily GTPases in trans-endocytosis of Eph-ephrin complexes can be drawn.

Another potential explanation for the discrepancy between the results from the trans-endocytosis assay and the assay with soluble EphB ectodomains could be that the two assays operate at different levels of sensitivity. Since trans-endocytosis can only occur at sites of cell contact, this could be a rate-limiting factor and mask effects a putative disinhibition of trans-endocytosis by knockdown of RhoA subfamily GTPases could exert. In contrast when saturated with soluble EphB ectodomains no such limitations exist and a knockdown of RhoA subfamily GTPases leads to an increase in endocytosis.

In this study we did not investigate the role of RhoA subfamily GTPases in endocytic processes in the forward direction. As the trans-endocytosis of Eph-ephrin complexes in both directions seem to share the same endocytic pathway and knockdown of RhoA subfamily GTPases did not significantly alter the amount of endocytosis observed for EphB trans-endocytosis into ephrinB+ cells, it would be reasonable that the same is true in the forward direction. Nonetheless, experiments confirming this assumption would give further support to the hypothesis that trans-endocytosis of Eph-ephrin complexes uses the same endocytic machinery in both the forward and reverse directions. Considering the differential requirements for Rac activity in the soluble endocytosis assays with either ephrin or Eph ectodomains, and that CME has been implicated in the forward direction (Yoo et al. 2010), it would be intriguing to address the question of whether RhoA subfamily GTPases also exhibit an inhibitory function towards endocytosis of soluble ephrin ectodomains into Eph+ cells.

Taken together, these results suggest that RhoA subfamily GTPases negatively regulate Eph-ephrin reverse endocytosis upon stimulation with soluble EphB2 ectodomains, while they do not have a crucial function in EphB trans-endocytosis into ephrinB+ cells. Given

the complex nature of the role that RhoA subfamily GTPases play in endocytic processes, further experimentation is required to elucidate the exact molecular mechanism of RhoA function in this context, and at which state of the endocytic process RhoA subfamily members exert their effect.

3.3 Polymerisation of actin in Eph-ephrin trans-endocytosis

Endocytic processes for the uptake of large cargoes are in most cases dependent on the rearrangement of the actin cytoskeleton (Girao et al. 2008, Doherty & McMahon 2009). Moreover, for ephrinB trans-endocytosis into Eph+ cells, the requirement of the actin cytoskeleton has already been demonstrated (Marston et al. 2003).

The work in this study, which implicates the activity of Rac-family GTPases, well-known regulators of the actin cytoskeleton in endocytic contexts, in the control of EphB trans-endocytosis into ephrinB+ cells indicates that the requirement for actin reorganisation is shared by the forward and reverse pathways. Supporting this hypothesis, recent results from our group directly link actin reorganisation to EphB trans-endocytosis into ephrinB+ cells (Gaitanos, unpublished data). SKN cells over-expressing LifeAct, a small peptide marker that allows the visualisation of F-actin (Riedl et al. 2008), were co-cultured with HeLa cells expressing EphB2ΔC-GFP. Live cell-imaging revealed strong co-localisation of polymerised actin with EphB2-containing clusters at sites of cell contact, as well as during the initial steps of internalisation of clusters including their pinching off from the membrane. Strikingly, co-localisation with polymerised actin is lost rapidly after internalisation (within 3 min). As a control experiment, cells were treated with EHT1864, which resulted in drastically reduced dynamics of the actin cytoskeleton and subsequently no co-localisation of polymerised actin with EphB2-containing clusters at sites of cell contact. These results indicate that rearrangement of the actin cytoskeleton is induced by Rac activity and occurs during the early steps of internalisation in EphB trans-endocytosis into ephrinB+ cells, but is dispensable for further endocytic trafficking.

Interestingly, the time scale for Tiam1 and Rac activation downstream of EphA signalling observed by Boissier and co-workers fits very nicely with timing observed in the LifeAct experiments, as they reported a peak in activation 3-5 min after stimulation (Boissier et al.

2013). Even though these results were obtained in a different experimental setup (forward direction and stimulation with soluble ectodomains), it still supports the idea that Tiam-induced Rac activity drives the actin polymerisation observed during the initial steps of Eph-ephrin internalisation.

It is difficult to further clarify the precise nature of the interaction of the actin cytoskeleton with the trans-endocytosis of Eph-ephrin complexes, because of the limitations in conventional light microscopy regarding spatial resolution. Electron microscopy or similar approaches allowing for a higher resolution are also inadequate for studying such short-lived and transient processes. Therefore, to gain a better understanding of which steps actin is required for during the trans-endocytic process, it would be very insightful to apply live cell imaging with super-resolution techniques. A recent example of the benefits of such an approach is the work by Li and colleagues that provided valuable insights into the molecular interaction of actin with endocytic processes at a resolution not previously attainable (Li et al. 2015).

In summary, the recent discoveries in our group support the central role of actin in the trans-endocytosis process proposed by both previous work and the findings in this study. Recent technological advances might open novel avenues to decipher the exact mechanism of actin contribution to the trans-endocytosis of Eph-ephrin complexes.

3.4 Regulation of Eph-ephrin endocytosis by Rho GEFs and GAPs

3.4.1 Analysing the data from the siRNA screen based on consistency over strength of results yields more accurate candidates

In this study I presented two separate ways of analysing the data obtained from the image-based siRNA screen for the function of Rho family GEFs and GAPs in EphB trans-endocytosis into ephrinB+ cells. Initially, we scored the effect of each siRNA oligo compared to the average of all negative controls from the whole screen. We then selected candidates on the basis of a single oligo showing a strong response in average over the two separate repetitions conducted (Fig. 16). However, the follow-up analysis of the strongest candidate determined this way, ITSN1 (Fig. 17), as well as a combination of oligos for different candidate genes to account for potential redundancy (Fig. 18), could not confirm

the results. In a second approach, we normalised the effect of each oligo not against the combined negative controls from the whole screen, but against the negative controls tested in the same plate in order to account for potential inter-plate variations in the baseline amount of endocytosis observed. Furthermore, we then examined the average from all four separate oligos for each gene to safeguard against potential false positive results derived from outliers or off-target effects of specific single oligos (Fig. 19). With this approach we identified Tiam2 as the candidate with the strongest effect on EphB trans-endocytosis into ephrinB+ cells and subsequent experiments replicated these results. Moreover, the importance of Tiam2 was also confirmed by additional experiments in HeLa cells (Fig. 20). The downside of the analysis taking the average over all tested siRNA oligos for a given protein is that it can mask potential effects and lead to false negative results, if some oligos do not result in a sufficient knockdown of the protein of interest. However, as our initial approach relying on single oligos proved unsuccessful in obtaining verifiable candidates, we were willing to accept this limitation.

We therefore found, at least for the presented study, basing the analysis of screen data on the consistency of responses to be superior to an analysis based on the size of an effect in single observations.

3.4.2 Regulation of Eph-ephrin trans-endocytosis by Tiam1/2

In this study we found that the Rac activity required for trans-endocytosis of Eph-ephrin complexes is induced by GEFs of the Tiam family. Already identified as a hit in our image-based siRNA screen, subsequent experiments confirmed the requirement of Tiam2 for efficient EphB trans-endocytosis into ephrinB+ SKN and HeLa cells (Fig. 20). Interestingly, knockdown of Tiam1 did not result in a significant change in the amount of EphB trans-endocytosis into SKN cells, but it showed a comparable effect to Tiam2 knockdown in HeLa cells (Fig. 20B and C). A combined knockdown of the two proteins also reduced trans-endocytosis in HeLa cells, but the effect was no larger than that seen in the single knockdowns. The close similarity between Tiam1 and Tiam2 suggests the possibility of physiological redundancy between the two (Matsuo et al. 2003). However, if physiological redundancy between Tiam1 and Tiam2 was assumed, one would expect a larger effect in the double knockdown. There are three possible explanations for the

absence of this expected increase in our observations: firstly, the reduced concentration of each specific siRNA oligo used in the combined knockdown could lead to an inefficient ablation of protein expression, and the remaining level of Tiam proteins decreases the observed effect; or, secondly, Tiam1 and Tiam2 are not physiologically redundant, but are co-operating in regulating trans-endocytosis of Eph-ephrin complexes in some cellular contexts, but not in others. Finally it is also possible that the expression levels of Tiam1 differ between SKN and HeLa cells and assuming a high expression of Tiam in SKN cells the knockdown with siRNA could not be sufficient Additional experiments in other cell lines, and, especially, in neuronal cells could help clarify the individual contribution of the two Tiam proteins to the endocytosis of Eph-ephrin complexes.

Previous work has established a role for Tiam1 in the regulation of endocytosis into Eph+ cells induced by stimulation with soluble ephrin ectodomains (Yoo et al. 2010, Boissier et al. 2013, Um et al. 2014). In agreement with these results, we also show a requirement for Tiam family proteins in ephrinB trans-endocytosis into EphB-expressing HeLa cells (Fig. 20C).

An unanswered question is how Eph-ephrin signalling is linked to the Tiam proteins molecularly. Previous work by Tanaka and colleagues has shown that both Tiam1 and Tiam2 bind to ephrinB1 via their N-terminal PH-CC-Ex domain (Tanaka et al. 2004). The binding and co-localisation with ephrinB1 of Tiam1 is increased after stimulation with soluble EphB2-Fc fusion proteins and leads to a rise in Rac activity. Evidence for the interaction of Tiam2 with ephrinBs in the context of EphB trans-endocytosis into ephrinB+ cells was provided by recent work from our group, which has demonstrated co-localisation of EphB-ephrinB clusters with Tiam2 (T. Gaitanos, unpublished data). However, which protein domains are involved in the potential interaction remains unknown. Overexpression of the Tiam PH-CC-Ex domain alone has shown a dominant negative effect in several studies (Tanaka et al. 2004, Tolias et al. 2007, Terawaki et al. 2010). Thus, overexpressing the Tiam PH-CC-Ex domain in trans-endocytosis experiments could elucidate whether binding via this domain is required for regulation of EphB-ephrinB trans-endocytosis by Tiam family proteins. Furthermore, it will also be valuable to map the exact interaction site within ephrinB molecules, since it might be possible to generate specific point mutations that interrupt Tiam-ephrinB binding. This could potentially result in a

version of ephrinB that is endocytosis-deficient, while unperturbed in its other signalling capabilities, which would be a very valuable tool in dissecting the individual contributions of endocytosis and other signalling mechanisms employed downstream of ephrinBs.

While several studies provide evidence for enhancement of Tiam1 activity after tyrosine phosphorylation (Servitja et al. 2003, Miyamoto et al. 2006, Tolias et al. 2007), Tanaka and colleagues found only weak phosphorylation of Tiam1 downstream of ephrinB1, even though they report a concomitant increase in Rac activity (Tanaka et al. 2004). In Eph-ephrin forward signalling, Tiam1 is directly phosphorylated by Eph receptors (Tanaka et al. 2004, Tolias et al. 2007, Boissier et al. 2013). Since ephrin ligands lack kinase activity of their own, phosphorylation of Tiam proteins in the reverse direction would require an intermediary kinase. Tiam1 can be phosphorylated by SFKs (Servitja et al. 2003) and SFKs are activated downstream of ephrinBs (Palmer et al. 2002, Georgakopoulos et al. 2006). Thus, SFKs are possible candidates to mediate phosphorylation of Tiam1 and Tiam2 downstream of ephrinBs, if phosphorylation is required for their function in regulating EphB-ephrinB endocytosis. Visualising and possibly interfering with the phosphorylation status of Tiam proteins, for example, by immunofluorescence with phospho-specific antibodies, or the transfection of phospho-mimetic or phosphorylation-deficient point mutants of Tiam proteins, would help elucidate the mechanisms of Tiam proteins in Eph-ephrin trans-endocytosis. A complimentary approach could clarify whether SFKs are involved by employing siRNA-mediated knockdown or treatment with pharmacological inhibitors.

Results from my experiments could not detect a requirement for Tiam1, Tiam2, or Rac activity for reverse endocytosis of soluble EphB ectodomains into ephrinB+ cells (Fig. 21). As the experiments by Tanaka and colleagues were also performed with stimulation by soluble EphB2 ectodomains and led to an increase in Rac activity induced by Tiam1 (Tanaka et al. 2004), these results could be viewed as contradictory to my findings. The two most plausible explanations for these contrasting results would be that either Tiam1-mediated Rac activity downstream of ephrinBs might serve a purpose other than regulating endocytosis, or it represents a cell type-specific phenomenon.

In summary, our work builds on the existing literature linking Tiam family proteins to Eph-ephrin signalling and firmly establishes their role as regulators of Eph-ephrin trans-endocytosis. Further work will be necessary to decipher the exact molecular mechanisms governing the interaction between Tiam proteins and Eph-ephrin complexes in this context.

3.4.3 The siRNA screen of Rho family GEFs and GAPs has provided further interesting candidates for the regulation of Eph-ephrin trans-endocytosis

Our image-based siRNA screen revealed several candidate genes that could regulate EphB trans-endocytosis into ephrinB+ cells. In line with our findings that EphB trans-endocytosis requires Rac activity, three out of the four strongest hits that decreased endocytosis upon siRNA knockdown were GEFs that show activity towards Rac (Tiam2, Vav1, Als2). Our follow-up experiments confirmed Tiam2 as a regulator of EphB trans-endocytosis into ephrinB+ cells, thus demonstrating the reliability of the results obtained from the screen. As depletion of Tiam proteins does not result in a complete inhibition of Eph-ephrin trans-endocytosis, it is possible that other GEFs take over its role in increasing Rac activity and promoting actin polymerisation downstream of Eph-ephrin signalling to some extent.

In our screen, among the GEFs whose depletion led to a decrease in trans-endocytosis, Vav1 showed the second strongest result when comparing the average z-scores of all 4 tested siRNA oligos. Vav1 is one of three closely related GEFs making up the Vav family, the other two being Vav2 and Vav3 (Bustelo 2014). Vav family GEFs exhibit activity towards GTPases from the RhoA, Rac1 and Cdc42-like subfamilies, but their catalytic activity is several folds higher towards Rac1 as compared to other GTPases, at least for Vav2 (Jaiswal et al. 2013a). This is in agreement with the majority of the literature on Vav-family proteins describing the relevance of their signalling through Rac (Bustelo 2014). The Vav-family GEFs differ in their expression patterns, as Vav2 and Vav3 are expressed ubiquitously, while Vav1 is mainly found in the hematopoietic system in the healthy body and is upregulated during many types of cancer (Bustelo 2000, Katzav 2015). Interestingly, Vav family GEFs have previously been implicated in Eph-ephrin signalling and endocytosis (Cowan et al. 2005). Vav2 interacts with, and is phosphorylated downstream of, EphA receptors and experiments with neuronal cultures from Vav2/Vav3 double knockout mice demonstrated the requirement of Vav family members for Eph-ephrin

signalling-induced growth cone collapse and the internalisation of Eph-ephrin complexes. However, the requirement for endocytosis was only tested by stimulation with soluble ephrinA ectodomains and not in a cell contact-mediated context. Unfortunately, the study also did not compare results from the double knockout to single knockout of Vav2 and/or Vav3, so it is not possible to infer whether the two proteins are redundant. In general, evidence for both redundant and non-redundant functions of Vav-family GEFs exists (Fujikawa et al. 2003, Pearce et al. 2004, Bustelo 2014). Knockdown of Vav2 and Vav3 did not result in significant changes to EphB trans-endocytosis into ephrinB+ cells in our screen. A possible explanation for these findings is, of course, the possibility that SKN cells do not express Vav2 or Vav3. Further evidence for mechanistic redundancy between Vav proteins and Tiam, as could be the case in the trans-endocytosis of Eph-ephrin complexes, derives from the work of Servitja and colleagues (Servitja et al. 2003). They show that both Vav2 and Tiam1 are phosphorylated downstream of c-src and in turn increase Rac activity. While in their study, phosphorylation of either GEF was dependent on the type of stimulation by different pathways, it is conceivable that both could also be activated by a common pathway, and assume the function of each other, especially, in the absence of one of the proteins. Furthermore, the fact that both proteins get phosphorylated by src fits very well in the context of Eph-ephrin trans-endocytosis, since SFKs are key signalling mediators in both Eph-ephrin forward and reverse signalling (Ellis et al. 1996, Holland et al. 1996, Zisch et al. 1998, Davy et al. 1999, Palmer et al. 2002). Therefore, future experiments, firstly confirming the relevance of Vav family GEFs, and secondly, exploring the possible redundancy between Vav and Tiam family GEFs, for example, by simultaneous siRNA knockdown, could provide valuable information on the molecular mechanisms governing Eph-ephrin trans-endocytosis.

A third candidate GEF that showed a decrease in trans-endocytosis upon siRNA knockdown is Alsin (ALS2). Its name derives from its implication in motor neuron diseases such as amyotrophic lateral sclerosis (ALS) (Hadano et al. 2001, Yang et al. 2001). ALS2 contains GEF domains for and binding affinity towards both Rab5 and Rac1 (Topp et al. 2004). In how far ALS2 actually displays GEF activity towards Rac, however, is still a matter of debate, as studies showing activity towards Rac1 (Otomo et al. 2008), and those showing no increase in Rac activity resulting from ALS2 overexpression have both been

published (Topp et al. 2004, Tudor et al. 2005). Their possible regulatory function in EphB trans-endocytosis into ephrinB+ cells is supported by their role in Rac-dependent macropinocytosis and endocytic trafficking, as well as in axonal growth dynamics (Tudor et al. 2005, Devon et al. 2006, Hadano et al. 2007, Kunita et al. 2007, Otomo et al. 2008). The discrepancy between the involvement of ALS2 in Rac-dependent macropinocytosis and its lack of catalytic activity towards Rac1 reported in some studies can be resolved by the role of ALS2 suggested in endocytic trafficking. In particular, ALS localises to active Rac1 at macropinocytic sites and mediates the fusion of macropinosomes with early endosomes via its Rab5GEF activity (Devon et al. 2006, Kunita et al. 2007, Otomo et al. 2008, Otomo et al. 2011). Nonetheless, the fact that another study showed increased Rac and PAK activity stimulated by ALS2 allows for the possibility that ALS2 directly regulates macropinocytosis, at least in some physiological contexts (Tudor et al. 2005). This idea has been supported by the finding that the function of ALS2 is probably cell-type specific, since macropinocytosis was decreased in ALS2-deficient neurons, but not fibroblasts (Otomo et al. 2008).Although the exact contribution of ALS2 to macropinocytosis has not yet been completely unravelled, it represents an interesting candidate for the regulation of Eph-ephrin trans-endocytosis. ALS2 could fulfil this role by either acting as an additional, potentially redundant, RacGEF required for the initiation of Rac activity and trans-endocytosis, or by affecting the subsequent trafficking of endosomes. In order to further decipher the precise role of ALS2 in Eph-ephrin trans-endocytosis, it would be necessary to confirm the original results of our screen and subsequently dissect out potential redundancies, as already suggested for the Vav family GEFs, by performing simultaneous siRNA knockdowns. Additionally, given its role as a Rab5GEF, and in light of previous work implicating Rab5 in the endocytosis of EphA receptors (Deininger et al. 2008), further analysis of the contribution of Rab5 to the trans-endocytosis process could prove insightful.

Of the four strongest candidates among the GEFs resulting in a decrease of endocytosis, I have so far omitted Active breakpoint cluster region-related protein (Abr) from the discussion, although it showed the third strongest effect when z-scores were averaged over all 4 tested oligos. Abr is a complex protein containing both a GAP and a GEF domain (Tan et al. 1993) and the literature on the specificity and relevance of the GEF and GAP

domains of Abr is very contradictory. Initial reports showed a broad specificity for the GEF domain with activity towards Cdc42, RhoA, and Rac subfamily members with highest catalytic activity on Cdc42, whereas GAP activity was limited to Cdc42 and the Rac subfamily GTPases (Chuang et al. 1995). In contradiction to these observations a later study, systematically analysing Rho family GEFs, found no catalytic GEF activity of Abr *in vitro* (Jaiswal et al. 2013a). In the more physiological context of single cell wound healing, however, Abr increases RhoA activity, suggesting that Abr is an active GEF (Vaughan et al. 2011). In contrast, GAP activity on Rac and/or Cdc42 has been well documented by several independent studies (Cho et al. 2007, Oh et al. 2010, Vaughan et al. 2011). Additionally, Abr shares high sequence similarity with Bcr (Chuang et al. 1995), another GEF and GAP domain-containing protein recently described to be important in Eph-ephrin signalling in the context of synapse development (Um et al. 2014). Abr and Bcr have both overlapping and distinct signalling functions (Cho et al. 2007, Cunnick et al. 2009), and many studies of their physiological function use mice deficient for both proteins (Cho et al. 2007, Cunnick et al. 2009, Um et al. 2014). Expression of a GAP-deficient Bcr variant and experiments with Bcr and Abr double deficient cells resulted in an increase in endocytosis of Eph stimulated by soluble ephrin ectodomains in the work by Um and colleagues (Um et al. 2014). These results are in disagreement to our findings of a decrease in EphB trans-endocytosis into ephrinB+ cells upon knockdown of Abr. However, these differences could derive from the quite varied GEF activities reported for Abr and might be cell-type specific. While we did not follow-up on the possible relevance of Abr in this study, future experiments analysing the role of Abr and possibly also Bcr in Eph-ephrin trans-endocytosis could prove very informative, but will have to address carefully the respective contribution of its GEF and GAP functions.

So far, I have mainly discussed candidates from the screen containing GEF domains and neglected the candidate proteins for regulation of EphB trans-endocytosis into ephrinB+ cells among the GAPs. Apart from Abr, which is discussed above, the depletion of two GAPs resulted in a significant reduction in the amount of internalised Eph-ephrin complexes: Human Minor Histocompatibility Antigen1 (HMHA1) and Phosphatidylinositol 3-kinase regulatory subunit beta (PIK3R2). HMHA1 has only recently been shown to exhibit broad GAP activity towards RhoA, Cdc42 and Rac1 *in*

vitro, and to decrease RhoA and, especially, Rac1 activity resulting in changes in actin dynamics in mammalian cell lines (de Kreuk et al. 2013). There are no reports about the GAP activity of PIK3R2 to date. PIK3R1, a very close homologue, however, displays GAP activity towards Rab5, as well as towards Rac and Cdc42, but to the Rho GTPases only at a lesser extent and only *in vitro* (Chamberlain et al. 2004, Runyan et al. 2012). These studies demonstrated that PIK3R1 modulates endocytic trafficking via the interaction with Rab5. Interestingly, a large scale proteomic screen conducted very recently revealed an interaction between ephrinB2 and PIK3R2 (Huttlin et al. 2015). In light of this finding, PIK3R2 constitutes a valid candidate for further follow-up analysis.

Our screen also identified a candidate GEF (Net1) and a candidate GAP (OPHN1), whose depletion resulted in an increase in EphB trans-endocytosis into ephrinB+ cells. As we were lacking a reliable positive control leading to an increase in endocytosis, we were not certain which magnitudes of effect to expect, which complicated evaluation of the screen data. Furthermore, the fact that Net1 is a GEF specific for RhoA subfamily GTPases (Alberts & Treisman 1998), while OPHN1, although it shows a broad tuning of GAP activity *in vitro* (Billuart et al. 1998), mediates its physiological function via inhibition of RhoA signalling (Govek et al. 2004, Khelfaoui et al. 2009), appears contradictory, as this suggests they should have opposing functions. In one of these studies it was also specifically proposed that the GAP activity of OPHN1 counteracts the inhibitory effect on endocytosis enacted by RhoA signalling (Khelfaoui et al. 2009), which would imply that siRNA knockdown of OPHN1 leads to a decrease of endocytosis instead of the observed increase. In light of these conflicting results, and the difficulties in evaluating candidates resulting in an increase of endocytosis, we focussed our follow-up analysis first on proteins whose depletion resulted in a decrease of endocytosis. Nonetheless, future work on the mentioned candidates could explain the contradicting result of a RhoA-specific GEF and GAP showing the same effect, and improve our understanding of the regulation of Eph-ephrin trans-endocytosis.

We focussed our follow-up analysis to a single candidate, Tiam2, which we successfully confirmed as a key regulator of Eph-ephrin trans-endocytosis. With this proof-of-principle validating our screen approach, it stands to reason that analysis of the other candidate proteins found in the screen will advance our understanding of the regulation of Eph-ephrin

trans-endocytosis and potentially also reveal redundancy or cross-talk between different Rho GTPase-regulating proteins.

3.5 Physiological role of Eph-ephrin endocytosis

3.5.1 Trafficking of Eph-ephrin complexes and the "signalling endosome" hypothesis

The discovery that Eph receptors remain phosphorylated after internalisation allows for the possibility of sustained Eph-ephrin signalling from the endosomal compartment (Marston et al. 2003). The idea of "signalling endosomes" derives from original work with EGFRs, which has proven that the signalling machinery stays attached to EGFRs on endosomes and that continued signalling from endosomal compartments is crucial for effective EGF signalling (Di Guglielmo et al. 1994, Bergeron et al. 1995, Burke et al. 2001). Several other cell surface receptors have since been shown to exhibit continued signalling after internalisation, including neurotrophic factor receptors TrkA, TrkB and p75NTR, as well as the NoGo receptor (Grimes et al. 1996, Heerssen et al. 2004, Saxena et al. 2005, Joset et al. 2010). Not only does signalling from endosomes extend signalling responses from cell surface receptors, but it can also serve to localise signalling by trafficking of endosomes to specific cellular locations, such as in the vicinity of the cell membrane for Rac1 during cell migration (Palamidessi et al. 2008), or to the nucleus for changes in transcription by retrograde transport of neurotrophic factor receptors (Riccio et al. 1997).

In several described cases, the formation of signalling endosomes has been linked to macropinocytosis-like endocytic events that are regulated by Rac (Valdez et al. 2007, Joset et al. 2010). Additionally, membrane scission for the formation of signalling endosomes can be controlled by the ATPase Pincher and not by dynamin as in many other endocytic processes (Shao et al. 2002, Joset et al. 2010). However, whether this is a general rule, and whether dynamin might not still be involved in the formation of some signalling endosomes, is still a matter of debate (Winckler & Yap 2011). As Eph-ephrin trans-endocytosis seems to resemble at least some aspects of the endocytic process described in these works and also requires Rac activity, it would be interesting to investigate whether the pathways are the same. Experiments with a dominant negative version of Pincher, as

described by Shao and colleagues (Shao et al. 2002), or, alternatively, knockdown with siRNA in an Eph-ephrin trans-endocytosis assay could shed some light on this question.

After the initial finding that Eph receptors remain phosphorylated after internalisation (Marston et al. 2003), work by Boissier and colleagues expanded on the notion that Eph-ephrin signalling could continue after endocytosis (Boissier et al. 2013). In their experiments with EphA2-expressing cells stimulated with soluble ephrinA1 ectodomains they observed association of Tiam1 with activated EphA2 after internalisation and concluded that this was evidence for Eph-ephrin signalling through Tiam1 and Rac from the endosomal compartment. Closer examination of their data, however, suggests that since peak activity of Tiam1 and Rac after stimulation with ephrinA1 occurred during the earliest observed time points, these findings only confirm the importance of Tiam-induced Rac activity in the endocytosis of Eph-ephrin complexes and are not necessarily substantial evidence for signalling from endosomes. Nonetheless, while the evidence presented in the study by Boissier and colleagues does not provide conclusive evidence for Eph-ephrin signalling from the endosomal compartment, it would be sensible to expand on their work and test whether interference with endosomal trafficking affects the signalling. Another unanswered question is in how far there is also the potential for signalling from the endosomal compartment in the reverse direction. EphrinBs have been shown to be de-phosphorylated shortly after their activation and could thus lose their signalling capacity by the time they arrive in endosomal compartments (Palmer et al. 2002). On the other hand, several features of ephrinB reverse signalling are independent of tyrosine phosphorylation, for example, the interaction with PDZ-binding proteins (Bruckner et al. 1999, Makinen et al. 2005). Hence, also the reverse direction of Eph-ephrin endocytosis merits investigation into whether signalling from endosomes takes place. A first step could be the extraction of endosomes followed by biochemical analysis of binding partners of Eph-ephrin clusters analogously to the work by Boissier and colleagues for the forward direction (Boissier et al. 2013).

Continued signalling from endosomal compartments only presents one possibility for the fate of Eph-ephrin complexes after internalisation. The two further alternative trafficking pathways, which are not necessarily mutually exclusive of continued signalling, are degradation and recycling back to the plasma membrane. Work by Boissier and colleagues

also quantified the relative amount of Eph-ephrin complexes routed toward degradation in lysosomes (two thirds) and recycled to the plasma membrane (one third) (Boissier et al. 2013). Several other studies also described the trafficking of Eph receptors into lysosomes, which was reported to be dependent on their ubiquitination by Cbl (Walker-Daniels et al. 2002, Sharfe et al. 2003, Fasen et al. 2008, Sabet et al. 2015). Unfortunately, these studies were performed solely using stimulation with soluble ephrin ectodomains and only focussed on the forward direction. Since there are very distinct downstream signalling patterns between forward and reverse direction, as well as cell-contact or soluble protein-induced signalling (Jorgensen et al. 2009), and since this study has further expanded on some of these differences in the context of endocytosis, it is not obvious, whether the same endocytic fate is shared between Eph-ephrin complexes in these distinct scenarios. Further work unravelling the endocytic fate of Eph-ephrin complexes is therefore needed. One possible approach in this context would be studying the role of Rab family GTPases downstream of Eph-ephrin signalling.

3.5.2 Endocytosis and growth cone collapse

One pivotal role of Eph-ephrin signalling is its function in axon guidance during neuronal development. In contexts ranging from retinotopic map formation to midline guidance in spinal projections of the motor system, Eph-ephrin signalling provides repulsive guidance cues crucial for the correct development of the nervous system (see also section 1.1.3). The repulsive signal mediated by the Eph-ephrin interaction originates from the induction of growth cone collapse, which allows the axon to re-sprout and continue growing in a different direction (Yu & Bargmann 2001, Egea & Klein 2007). Since the interaction between Ephs and ephrins initially leads to high affinity adhesion between the two cells in contact, Eph-ephrin complexes must be removed from the cell surface to allow cell detachment to occur. Trans-endocytosis of Eph-ephrin complexes enables cell detachment as a general mechanism and is particularly relevant in the context of growth cone collapse (Marston et al. 2003, Zimmer et al. 2003). The endocytosis of Eph-ephrin complexes required for efficient growth cone collapse has been shown to be governed by Rac, at least for signalling in the forward direction (Marston et al. 2003, Cowan et al. 2005). Additionally, the growth cone collapse response downstream of Eph-ephrin signalling in

both directions requires the activity of RhoA (Wahl et al. 2000, Sahin et al. 2005, Takeuchi et al. 2015). This differential requirement of both Rac and RhoA for growth cone collapse is interesting given that the two GTPases often fulfil contrasting physiological functions and their signalling pathways can inhibit each other (Guilluy et al. 2011).

Here, we show that EphB trans-endocytosis into ephrinB+ cells also requires Rac activity and that this mechanism is also employed by neurons (Fig. 10). Apart from its role in regulating the trans-endocytosis of Eph-ephrin complexes, Rac1 activity downstream of Eph-ephrin signalling has also been shown to induce endocytosis of large patches of the plasma membrane, which is required for growth cone collapse in addition to the rearrangement of the actin cytoskeleton (Jurney et al. 2002).

The question now arises of how the Rac-mediated regulation of endocytic processes by Eph-ephrin signalling is related to the requirement of RhoA activity in Eph-ephrin-induced growth cone collapse. The data presented in this thesis combined with findings from the extensive literature on Eph-ephrin signalling in growth cone collapse allow for the following model integrating the separate elements. Growth cone collapse induced by Eph-ephrin signalling consists of two or three distinct steps differentially regulated by Rho GTPases. To allow the cell detachment necessary for growth cone collapse, Eph-ephrin complexes need to be internalised, a process that is controlled by Rac activity. Additionally, Rac activity downstream of Eph-ephrin signalling also leads to the endocytosis of plasma membrane patches, reducing the surface of the growth cone. The third step is disassembly of the filamentous actin structures making up the cytoskeleton, which is orchestrated by Eph-ephrin signalling-induced RhoA activity. Possibly, the activation of RhoA can occur from Eph-ephrin complexes residing in endosomal compartments, and these signalling endosomes could control locally restricted RhoA activity, in a similar fashion to what has been reported for Rac signalling from endosomes in cell migration (Palamidessi et al. 2008). This hypothesis for the spatio-temporal control of actin reorganisation requiring a shift from Rac signalling to a RhoA-based signalling response could be mediated by proteins already implicated in Eph-ephrin induced growth cone collapse. The GEF ephexin is phosphorylated by Eph receptors, which changes its specificity by increasing activity towards RhoA, while decreasing its activity towards Rac and Cdc42 (Shamah et al. 2001, Sahin et al. 2005). Thus, it represents a valid candidate to

mediate a shift from Rac-induced endocytosis to RhoA-induced actin disassembly in the course of growth cone collapse. A second GTPase regulating protein shown to be important for growth cone collapse and axon guidance downstream of EphA4 signalling is the RacGAP α–chimaerin (Beg et al. 2007, Wegmeyer et al. 2007). Since it localises to activated Eph receptors, it could potentially mediate a down-regulation of Rac activity after its initial requirement for the endocytosis of Eph-ephrin complexes. The down-regulation of Rac activity then in turn could lead to a disinhibition of RhoA signalling (Guilluy et al. 2011). Another possibility for switching signalling properties has been described for ephrinB reverse signalling. Here recruitment of SFK to activated Eph-ephrin clusters first leads to phosphorylation-dependent signalling events, while subsequent dephosphorylation by PTP-BL switches the response to PDZ-dependent signalling (Palmer et al. 2002). Furthermore, a study of the role of Eph-ephrin signalling in dendritic spine development and synapse formation has shown an example where tight regulation of Rac activity by a complex consisting of Tiam1 and Bcr, a Rac-specific GEF and GAP respectively, is required to mediate Eph-ephrin function (Um et al. 2014).

A very similar model has been proposed for the regulation of repulsive axon guidance by the NoGo receptor (Joset et al. 2010). Also in this case, endocytosis of activated receptors requires Rac activity and the induction of growth cone collapse depends on both endocytosis of the receptors and localised activation of RhoA signalling from NoGo receptors in the endosomal compartment.

This proposed model for sequential activation of Rac and Rho subfamily GTPases by Eph-ephrin complexes in mediating growth cone collapse in a multi-step process requiring localised signalling from endosomes is, of course, at this point still highly speculative, although it is solidly grounded in the findings of this thesis and the existing literature. Providing evidence for this model being physiologically relevant will be challenging, since the overlapping roles and functions of Rho family GTPases and their regulators are difficult to decipher. In order to gain further insights into the precise spatio-temporal regulation of and the cross-talk between Rho family GTPases downstream of Eph-ephrin signalling, experimental approaches suited to both visualise and perturb Rho GTPase activity at a very high spatio-temporal resolution are required. The challenges of achieving this level of

understanding of Rho GTPase signalling networks are summarised in an informative commentary (Pertz 2010). One way to overcome the challenge of affecting several physiological processes simultaneously due to interference with Rho GTPase activity is to target regulating proteins specific for the function of interest. By identifying a Rac-specific GEF, Tiam2, to be required for Eph-ephrin trans-endocytosis this study provides one candidate for such specific regulation.

3.6 Conclusion and Outlook

The presented thesis puts forward the first comprehensive analysis of the regulation of EphB trans-endocytosis into ephrinB+ cells by Rho family GTPases. We describe an endocytic pathway that is regulated by Rac subfamily GTPases and their activation by GEFs of the Tiam family, which requires reorganisation of the actin cytoskeleton. Tiam-induced Rac activity is also required for ephrinB trans-endocytosis into EphB+ cells. The physiological relevance of this pathway is underlined by the observation that cortical neurons require the same molecular regulation for EphB trans-endocytosis. While the forward endocytosis of Eph-ephrin complexes induced by soluble ephrins ectodomains probably uses the same endocytic machinery as the trans-endocytosis observed upon cell-cell contact, reverse endocytosis triggered by soluble EphB ectodomains uses a different mechanism that is affected by the activity of RhoA, but not Rac subfamily GTPases.

The distinct molecular pathways employed between EphB trans-endocytosis after cell contact and reverse endocytosis after stimulation with soluble EphB ectodomains, indicates that future investigations into the trans-endocytosis of Eph-ephrin complexes should be conducted in settings that replicate the contact between Eph- and ephrin-expressing cells in order to remain as close as possible to the relevant physiological processes.

The results of the siRNA screen could still be further exploited. Since the follow-up analysis of the strongest candidate, Tiam2, confirmed its significance in the regulation of the trans-endocytosis of Eph-ephrin complexes, it is plausible to assume that further investigations based on the results of the screen will yield additional insights into the molecular regulation of Eph-ephrin endocytosis.

One goal for the analysis of Eph-ephrin signalling is the uncoupling of the endocytosis of Eph-ephrin complexes from other downstream signalling events. One challenge that must be overcome is the fact that both the trans-endocytosis of Eph-ephrin complexes, as well as the cellular responses traditionally associated with many physiological functions of Eph-ephrin signalling, such as growth cone collapse or cell rounding, require reorganisation of the actin cytoskeleton. Therefore the use of experimental techniques allowing for very tight spatio-temporal manipulation and analysis would be prudent. Approaching this challenge on the levels of the specific GEFs identified in this study might prove beneficial, as compared to approaches affecting, for example, the actin cytoskeleton as a whole or the Rho family GTPases themselves, since they are bound to have a more wide-spread physiological effect.

Taking all of this into consideration, this study represents a valuable expansion of our knowledge of the Eph-ephrin signalling system by describing a molecular regulatory mechanism of Eph-ephrin endocytosis, which is relevant in physiological contexts. Additionally, it provides a solid foundation for further analysis of the endocytic processes in the Eph-ephrin system and their contribution to signalling responses. Further unravelling these mechanisms will greatly benefit our understanding of the Eph-ephrin system and its many important functions during development and diseases processes.

4 Materials and Methods

4.1 Materials

4.1.1 Chemicals, reagents and kits

All chemicals and reagents were purchased from GE Healthcare GmbH (Solingen, Germany), Life Technologies (Carlsbad, United States), Merck KgaA, (Darmstadt, Germany), Sigma-Aldrich Chemie GmbH (München, Germany), Roche Diagnostics GmbH (Mannheim, Germany) and VWR International LLC (Radnor, United States), unless stated otherwise in the methods section. Water used for buffers, solutions and reactions mixes was filtered using a Milli-Q-Water System (Merck KgaA, Darmstadt, Germany) and autoclaved afterwards. Plasmid preparations were done using the Macherey-Nagel NucleoBond® Xtra Maxi kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany).

4.1.2 Buffers

PBS (phosphate-buffered saline), pH 7.3

137 mM NaCl

2.7 mM KCl

4.3 mM $Na_2HPO_4 \cdot 7H_2O$

1.4 mM KH_2PO_4

Lysis buffer for Western blotting

50 mM Tris, pH 7.4

150 mM NaCl

2mM EDTA

1% TritonX - 100 (vol/vol)

Protease inhibitor cocktail (Roche)

Phosphatase inhibitor cocktail (Roche)

6x Protein loading buffer (reducing)

300 mM Tris - HCl, pH 6.8

600 mM Dithiothreitol (DTT)

12% SDS

0.6% BromoPhenolBlue

60% Glycerol

4.1.3 Medium for cell lines

DMEM (Gibco, Life Technologies)

4.5 g/l D-Glucose

1% L-Glutamine

10% Fetal Bovine Serum (FBS) (GE Healthcare)

1% Penicillin/Streptomycin (Life Technologies)

OptiMEM (Gibco, Life Technologies)

4.5 g/l D-Glucose

1% L-Glutamine

10% FBS (GE Healthcare)

1% Penicillin/Streptomycin (Life Technologies)

Dissection medium

HBSS (Gibco, Life Technologies)

1% Penicillin/Streptomycin (Life Technologies)

10 mM HEPES

10 mM $MgSo_4$

Neuronal maintenance medium

Neurobasal medium (Gibco, Life Technologies)

1x B27 supplement (Life Technologies)

10 mM L-Glutamine (GE Healthcare)

1% Penicillin/Streptomycin (Life Technologies)

4.1.4 Oligonucleotides

A full list of all oligonucleotide sequences used in the siRNA screen are available upon request. Oligos for the screen library were purchased from either Life Technologies or the Max-Planck-institute of Molecular Cell Biology and Genetics, Dresden. Specific oligos listed below were purchased from Life Technologies.

siRNA oligonucleotides

ephrinB1	5’ – GAAGGGCUUGGUGAUCUAUCCGAAA – 3’
ephrinB2	5’ – ACUAUACCCACAGAUAGGAGACAAA – 3’
RhoA	5’ – GCCUGUGGAAAGACAUGCUUGCUCA – 3’
RhoB	5’ – ACACCGACGUCAUUCUCAUGUGCUU – 3’
Rac1	5’ – CCGGUGAAUCUGGGCUUAUGGGAUA – 3’
Rac3	5’ – CCUCCGCGACGACAAGGACACCAUU– 3’
RhoG 05	5’ – CAGGAGGAGUAUGACCGCCUCCGUA – 3’
RhoG 67	5’ – UCGUCAUCUGUUUCUCCAUUGCCAG – 3’
Cdc42	5’ – CACAACAAACAAAUUUCCAUCGGAA – 3’
RhoQ 06	5’ – GGUCCCUAAGUGAAAGGCUCUGCUU – 3’
RhoQ 18	5’ – CCAAUGACCGAUGUCUUCCUUAUAU – 3’
RhoQ 19	5’ – AAGAGGAGUGGGUACCGGAACUUAA – 3’
RhoU 44	5’ – UCAGUGAUGCCGGAGAUGAAAUGGG – 3’
RhoU 45	5’ – CCUCAUUGAGUUGGACAAAUGCAAA – 3’
ITSN1	5’ – CCUUUGAAUCCAGAAGCCAUGAUGA – 3’
ITSN2	5’ – CAACACACAGCAGUUAGCCCUUGAA – 3’
FARP2	5’ – UCGGAAAUAGGAGAUUACGAUGAAA – 3’
ELMO3	5’ – AGGUGGUGUGCUACGUGAACAUGAA – 3 ‘
Dock11	5’ – CACCCGAAUCUUACAUUCAUGGAAU – 3’
Tiam1	5’ – CAGCACAACCCUGACUGCGACAUUU – 3’
Tiam2	5’ – GGGAGAACUUCAGGCGUCACAUAAA – 3’

4.1.5 Plasmids

Insert	Backbone	Tag	Reference
EphB2ΔC	pcDNA 3.1	FLAG (N-terminal) GFP (C-terminal)	J. Gong, A. Schaupp
EphB2ΔC	pcDNA 3.1	FLAG (N-terminal) mCherry (C-terminal)	J. Gong, A. Schaupp
EphB2	pcDNA 3.1	FLAG (N-terminal) mCherry (C-terminal)	J. Gong, A. Schaupp
ephrinB1ΔC	pcDNA 3.1	HA (N-terminal) mCherry (C-terminal)	J. Gong, A. Schaupp
ephrinB1	pcDNA 3.1	HA (N-terminal) mCherry (C-terminal)	J. Gong, A. Schaupp
RhoQ	pEGFP-C	GFP (N-terminal)	Addgene Plasmid ID 23232, (Roberts et al. 2008)
RhoU	pCMV6-Entry	Myc (C-terminal) FLAG (C-terminal)	Origene (PS100001)

4.1.6 Primary antibodies

Antigen	Species	Supplier	Dilution	Application
α-tubulin	mouse	Sigma	1:5000	WB
Cdc42	rabbit	Abcam (Cambridge, United Kingdom)	1:500	WB
FLAG	rabbit	Sigma	1:1000	IF,WB
GAPDH	rabbit	Sigma	1:1000	WB
GFP (Jl-8)	mouse	Clontech Laboratories, Inc. (Mountain View, United States)	1:1000	WB

4.1.7 Secondary antibodies

All secondary antibodies were purchased from Jackson Immunoresearch (West Grove, United States). For Western blots, Horseradish Peroxidase (HRP)-coupled secondary antibodies and for immunostainings, fluorescently labelled secondary antibodies were used. Antibodies were diluted 1:5000 for Western blotting and 1:1000 for staining of cells.

4.2 Methods

4.2.1 Cell culture

All experiments used either HeLa cells, HeLa cells stably expressing EphB2ΔC-GFP (previously generated in our group) or SKN cells expressing histone2B tagged with red fluorescent protein (SKN H2B-RFP) (generated by T. Gaitanos). In general, cells were cultured in Falcon dishes according to ATCC's (American Type Culture Collection) recommendations concerning splitting ratios and media requirements. HeLa cells were cultured in DMEM, which was supplemented with the selective antibiotic Geneticin in the case of the cells stably expressing EphB2ΔC-GFP. SKN cells were cultivated in OptiMEM.

4.2.2 Plasmid transfections

Cell lines were transfected using Lipofectamine 2000 reagent (Life Technologies) according to the instructions provided by the manufacturer. Briefly, cells were transfected at a confluency of 50-70%. On the day of transfection, Lipofectamine 2000 was added to OptiMEM not containing serum or antibiotics. After 5 min incubation at room temperature (RT), DNA was added and the transfection mix was incubated at RT for 15 to 30 min and then added directly into the cell medium. Cells were left in the incubator for 24 h to 48 h for protein expression depending on the assay. A transfection reagent:DNA ratio of 3:1 (v/mass) was used for all experiments. 0.5 to 1 µg of total DNA was used to transfect one well of a 6-well plate, 1 µg to 2µg were used to transfect one 6 cm dish.

4.2.3 siRNA transfections

Cells were transfected with siRNA oligos using Lipofectamine RNAiMax (Life Technologies) transfection reagent. Unless otherwise stated cells were reverse transfected

at the moment of seeding. Lipofectamine RNAiMax was added to an appropriate volume of OptiMEM not containing serum or antibiotics so that the final dilution was 1:1000 for 5 min at RT. siRNA oligos were pre-incubated with transfection reagent for 30 min at RT. If not otherwise stated siRNA was used at a final working concentration of 20 nM. Cells were incubated for either 48 h (HeLa cells) or 72 h (SKN cells) at 37 °C for effective knockdown to occur.

4.2.4 Inhibitor treatment

Rac-specific inhibitor EHT1864 (Tocris Bioscience, Bristol, United Kingdom) was diluted in H_2O for storage at 4 °C. At the day of experiment a pre-dilution in serum-free OptiMEM was prepared and added straight to the respective culture medium to obtain the final concentrations specified in each experiment. Cells were incubated with EHT1864 for 4 h at 37 °C before subsequent experiments were performed. As a control cell were treated with the equivalent volume of H_2O in parallel.

4.2.5 Western blots

Cell lysates were prepared using a standard protocol in Lysis Buffer (see above) complemented with protease and phosphatase inhibitor cocktails (Roche). Protein concentration was measured using a DC protein assay (BioRad Hercules, United States). Samples were prepared in SDS sample buffer and equal amount of samples (20-50 µg) were loaded onto SDS-PAGE gels for separation with 4% acrylamide stacking gel and 7.5% - 12% acrylamide running gel depending on size of protein of interest. After separation proteins were blotted onto a protran nitrocellulose membrane (GE Healthcare) using a semi-dry blot chamber (Trans-blot SD, BioRad) and 60 mA per gel for 1 - 2 h. Successful transfer of protein was confirmed by PonceauS staining. Membranes were blocked in 5% non-fat milk in PBS with 0.1% Tween (PBT) for 30 min at room temperature. If not stated otherwise, membranes were incubated overnight with primary antibody solution (diluted in 5% non-fat milk in PBT, complemented with 0.02% $NaNH_3$ at 4°C. Membranes were washed in large quantities of PBT for at least 30 min with at least one change of PBT before applying secondary antibodies (HRP-coupled, specific for the respective species of the primary antibody, Jackson Immunoresearch) for 1 h at room

temperature. After another washing step (30 min, PBT) protein bands were detected with either Amersham ECL Western Blot reagent (GE Healthcare) or SuperSignal Femto Maximum Sensitivity Substrate (Life Technologies) in the Fusion FX7 chemiluminescence imaging chamber (PeqLab GmbH, Erlangen, Germany). Either α–tubulin or GAPDH were used as loading controls and membranes were incubated with primary antibody solution for 1-2 h, followed by PBT washes, secondary antibody and detection as described above. Representative blots are shown. If blots were quantified, the gel analysis feature of ImageJ (http://rsb.info.nih.gov/ij/index.html) was used for densiometric measurements.

4.2.6 Image acquisition

Imaging was performed on a Zeiss Axioobserver Z1 inverted microscope (Zeiss, Göttingen, Germany) equipped with a CSU-X1 spinning disc confocal unit (Yokogawa Electric, Tokyo, Japna) controlled by VisiView software (Visitron Systems, Puchheim, Germany) and a temperature-controlled CO_2 incubation chamber when required (Pecon GmbH, Erbach, Germany). Illumination was provided by Lasers of 405 nm, 488 nm, 561 nm or 640 nm wavelength for spinning disc confocal imaging (Visitron Systems,) or by an X-cite lamp (Excelitas Technologies GmbH & Co. KG, Wiesbaden, Germany) for epifluorescence imaging. All fixed samples were imaged using the confocal option. All live cell-imaging was performed with the incubation chamber set to maintain 37 °C ambient temperature and CO_2 concentration of 5 %. For the live-cell imaging of SKN cells a 20x air objective was used. Imaging of experiments in 96-well plates, as well as the neuron trans-endocytosis experiments were imaged with a 40x air objective. Experiments with HeLa cells on coverslips were imaged with a 63x oil immersion objective.

4.2.7 Endocytosis assay with soluble Eph or ephrin ectodomains

SKN-H2B-RFP cells or HeLa cells were cultured in 96-well plates (Greiner Bio One GmbH, Frickenhausen, Germany) or on cover slips in 6-well plates in OptiMEM or DMEM complemented as above at 37 °C and 5% CO_2. HeLa cells were transfected with either EphB2-mCherry (for forward direction experiments) or ephrinB1-mcherry (for reverse direction experiments) as described above. Cells were starved in serum-free OptiMEM overnight before stimulation. Fusion proteins of the ectodomain of either EphB2 (for

reverse direction experiments) or ephrinB2 (for forward direction experiments) fused to human Fc (R&D Systems, Minneapolis, United States) were pre-clustered with dylight488 dye-coupled anti-Fc antibodies (Jackson Immunoresearch) in volume ratio 5:1 for 45 min at room temperature. Human IgG-Fc (Jackson Immunoresearch) was used as a negative control and prepared in the same fashion. Cells were incubated with the clustered proteins at a final concentration of 2 µg/ml for 30 min at 37 °C. Cells were then fixed immediately on ice with 4% PFA (complemented with 4% sucrose). 3 washing steps with PBS were followed by blocking with 3% Bovine Serum Albumin (BSA) (Sigma Aldrich) for 30 min at room temperature. Staining for surface clusters was performed with a 1:1000 dilution of dyLight649 anti-human Fc antibodies (Jackson Immunoresearch) in 3% BSA at room temperature. HeLa cells were furthermore permeabilised with 0.1% Triton in PBS for 5 min and stained with CellMask^{TM} Blue (1:2000) and DAPI (1:5000) dissolved in PBS for 10 min. Afterwards cells were washed three times with PBS and, in case of experiments on cover slips, mounted with ProLong Gold antifade reagent (Life Technologies) and stored in the dark at 4 °C until imaging. Imaging of SKN experiments in 96-well plates was performed with a 40x air objective. For each well 10-20 positions were selected and stacks of 8 planes with 1 µm step size were taken. Experiments with HeLa cells on coverslips were imaged with a 63x oil immersion objective. For each cover slip 10-20 positions were selected and stacks of 15 planes with 0.5 µm as step size were taken. Image analysis is described below.

4.2.8 Assay to determine cell surface expression of ephrinBs

The assay was adapted from that above. SKN cells in 96-well plates were stimulated with EphB2-Fc, which had been pre-clustered with a dylight488-coupled antibody for 45 min at room temperature. In contrast to the endocytosis assay, stimulation took place at room temperature for only 2 min before cells were transferred to ice to prevent internalisation. Subsequent staining and image acquisition was performed as above.

4.2.9 Trans-endocytosis assay

SKN-H2B-RFP cells or HeLa cells transfected with either EphB2-mCherry (for forward direction experiments) or ephrinB1-mCherry (for reverse direction experiments) as

described above, were used as acceptor cells. SKN cells were cultured in 96-well plates (Greiner Bio One) in OptiMEM, while HeLa cells were cultured on cover slips in 6-well plates in DMEM. HeLa cells stably expressing EphB2ΔC-GFP or HeLa cells transiently transfected with either EphB2ΔC-GFP (for reverse direction experiments) or ephrinB1ΔC-GFP (for forward direction experiments) were used as donor cells. Acceptor cells were starved in serum-free OptiMEM overnight before stimulation. Donor cells were gently dissociated from the surface of the cell culture dish by treatment with 2 mM EDTA in PBS. Donor cells were seeded on top of acceptor cells and incubation for 80 min at 37 °C allowed for trans-endocytosis to occur. Cells were fixed with ice cold 4% PFA, 4% sucrose for 15 min, before 3 washes with PBS and blocking with 3% BSA in PBS for 30 min at RT. Staining for surface clusters was performed with a 1:1000 dilution of anti-FLAG antibodies in 3% BSA at room temperature for 1 h. After 3 washes with PBS FLAG signal was detected with dylight649-coupled secondary anti-rabbit antibodies for 1 h at RT. HeLa cells were additionally permeabilised with 0.1% Triton in PBS for 5 min and stained with CellMask™ Blue (1:2000) and DAPI (1:5000) dissolved in PBS for 10 min. Cells were then washed three times with PBS and, in case of experiments on cover slips, mounted with ProLong Gold antifade reagent (Life Technologies). Experiments were stored in the dark at 4°C until imaging. Imaging of SKN experiments in 96-well plates was performed with a 40x air objective. For each well 10-20 positions were selected and stacks of 8 planes with 1 µm step size were taken. Experiments with HeLa cells on coverslips were imaged with a 63x oil immersion objective. For each cover slip 10-20 positions were selected and stacks of 15 planes with 0.5 µm as step size were taken. Image analysis is described below.

4.2.10 Image-based siRNA screen of Rho GEFs and GAPs

A list of all human proteins containing either a Dbl-homology or DOCK homology domain (GEFs), as well as those containing a RhoGAP domain (GAPs) was generated by bioinformatic analysis of the UniProt database (www.uniprot.org). For every gene a set of four separate siRNA oligonucleotides with non-overlapping sequences was purchased. siRNA was applied to SKN cells in 96-well plates as described above. Each unique oligo was tested in a separate well and the whole set of experiments was repeated once. The trans-endocytosis assay was performed as described above and image acquisition and

analysis with CellProfiler™ software is detailed below. From the resulting quantification of the number of internalised clusters per cell several cut-offs representing a minimum number of internalised clusters were calculated. Subsequently, the percentage of cells containing more than the given number of internalised cluster was calculated for each cut-off and each treatment. Then the cut-off for which the negative scramble control was closest to a value of 40% cells above cut-off was taken for further analysis. From these values z-scores were calculated by subtracting the average control value from the observed value and dividing the result by the standard deviation of the controls. The average of the controls was either calculated from all values of the entire screen or only from the controls of a specific plate. Genes were ranked according to their z-scores and candidates identified by either examining results for single oligos or by calculating the average over the z-scores for all 4 oligos.

4.2.11 Trans-endocytosis assay with primary cortical neurons

Primary dissociated cultures of cortical neurons were generated from E 15.5 mouse embryos of wild type CD1 mice. On the day of the experiment mice were sacrificed and embryos transferred into PBS on ice. Brains were removed, followed by microdissection of the cortices in dissection medium on ice. Cortices from several embryos were dissociated in trypsin at 37 °C for 30 min, followed by further mechanical dissociation. Neurons were seeded into 8-well imaging chambers (Ibidi, Planegg, Germany) coated with Poly-D-lysin (1 mg/ml) and laminin (5 µg/ml) at 10^5 cells/well and incubated overnight at 37 °C and 5% CO_2. Neurons were stained with 1 µM CellTracker Green in in serum free Neurobasal medium for 30 min. Staining solution was then aspirated and replaced by neuronal maintenance medium. Subsequently, EHT1864 diluted in H_2O was added to the medium to produce final concentrations of either 2.5 µM, 5 µM or 10 µM. The control wells were treated with a volume of H_2O equal to the one used for the 10 µM EHT1864 treatment. Neurons were incubated at 37 °C and 5% CO_2 for 4 h before being transferred to a microscope equipped with a temperature-controlled CO_2 incubation chamber (Pecon/VisiTron systems). After acclimatisation to the imaging chamber HeLa cells over-expressing EphB2ΔC-mCherry were seeded on top of the cortical neurons at $2x10^4$ cells/well. HeLa cells were allowed to settle for several minutes before imaging. Imaging

was performed with a 40x air objective and images of a single focal plane with brightfleld illumination, as well as illumination with the 488 nm (CellTracker Green in neurons) and 561 nm (EphB2ΔC-mCherry) lasers were taken at each position at three minute intervals for a total duration of 3 h. Experiments were analysed with MetaMorph™ (Molecular Devices) software. Contact points between neurons and HeLa cell over-expressing EphB2ΔC-mCherry were identified and subsequently scored for whether internalisation occurred or not. For being scored an internalised cluster had to be visible within in the neuron and properly detached from any protrusions of the HeLa cell for at least three consecutive frames

4.2.12 Image analysis with CellProfiler™

All endocytosis experiments performed in SKN cells were analyses with CellProfiler™ software (Carpenter et al. 2006). The pipeline for analysing trans-endocytosis experiments is depicted in Figure 23 and the key steps are shown with example images. First, maximum projection images of the image stacks for the three separate channels are loaded. The 561 nm channel contained the signal from the H2B-RFP labelled SKN nuclei, the 488 nm channel contained the signal from the total EphB2DC-GFP, and the 641 nm channel contained the signal from the anti-FLAG staining for visualising surface EphB2 receptors. Images were first corrected for differences in background illumination signal by subtracting a blank image - taken with the same imaging setup - from the image of interest and then thresholded to further reduce signal noise. From the 561 nm channel the software identified the outlines of the SKN nuclei. In the next step the boundaries of the nuclei were expanded by 50 pixels to get an estimate of individual SKN cells (acceptor cells). The outline of the HeLa cell was identified from the 488 nm channel (donor cell). To exclude SKN cells from the analysis that were too far from the HeLa cells to experience cell contact-induced trans-endocytosis, only SKN cells in the vicinity of the HeLa cells were retained. These SKN cells were then used as a mask for the identification of total EphB2ΔC clusters. Subsequently, the total EphB2ΔC clusters were used as a mask for the identification of surface clusters in the 641 nm channel. For each cell and cluster identified the software measured several features including size and shape. Furthermore the clusters were also related to which SKN cell they had been identified in. The number of identified

cluster for each cell was then displayed on an image of the cell outlines. Additionally, all data on the identified objects was exported to excel files for subsequent analysis.

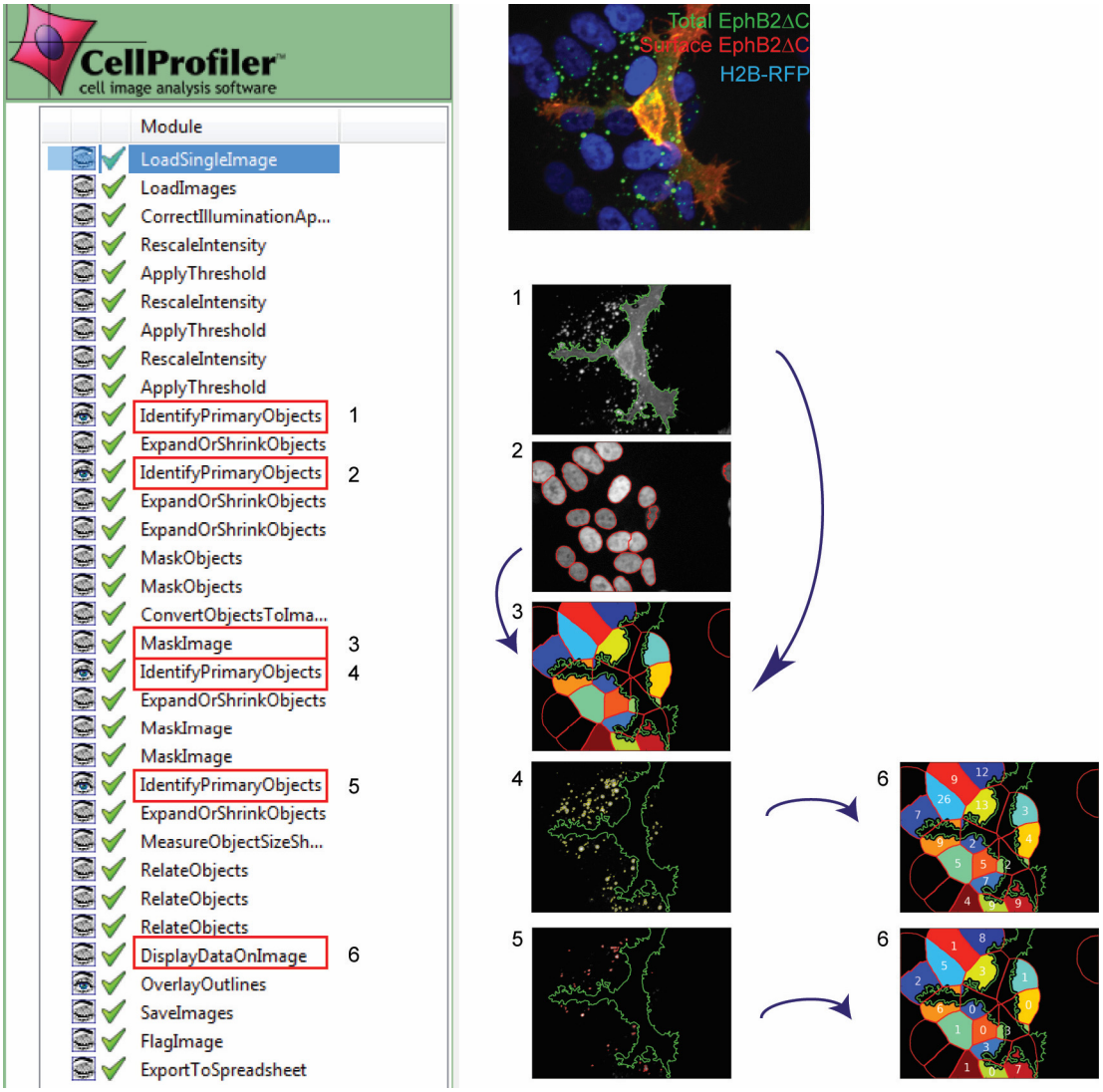
Analysis of experiments with soluble EphB ectodomains was performed with a similar pipeline. Instead of identifying the donor cells and restricting analysis to SKN acceptor cells in the vicinity, all SKN cells are identified and taken as a mask to then identify total and surface clusters.

4.2.13 Analysis of experiments with HeLa cells in ImageJ

Images from endocytosis experiments in HeLa cells were deconvolved with MetaMorph™ (Molecular Devices) software. Subsequent analysis was performed manually in Fiji (Schindelin et al. 2012) on blinded images. Overlay images of the maximum projections of image stacks for the three channels of total EphB2 (or ephrinB2 respectively), surface staining and CellMask Blue/DAPI were generated. Internalised vesicles were identified as clusters devoid of surface staining within a cell.

Figure 23. Analysis of trans-endocytosis assay with CellProfilerTM

The image on the left shows an overview of all steps in the CellProfiler™ pipeline. Important steps are highlighted with red boxes and depicted by images on the right. The input images consist of three channels: SKN-H2B-RFP makes up the blue channel, total EphB2ΔC-GFP signal the green channel and surface EphB2ΔC-GFP detected by anti-FLAG staining the red channel. The example image on the top shows an overlay with all three channels merged. For subsequent analysis both the outline of the HeLa cell (1) and the outline of the SKN nuclei (2) are detected. For the subsequent analysis only the SKN cells (coloured objects) in the vicinity of the HeLa cell (green outline) are taken into account (3). In those selected SKN cells the total EphB2ΔC-GFP clusters are identified (4). Surface clusters are identified from the total clusters that also show co-localising signal in the red channel (5). The respective number of both total and surface clusters can then be displayed on each cell (6).



4.2.14 Statistical analysis

Data were analysed using Prism (GraphPad) software. Data are shown as average of the mean +/- standard error of the mean (SEM) unless stated otherwise. Statistical significance was tested for with ANOVA and Dunnett's post hoc test or with repeated measures ANOVA followed by Dunnett's post hoc test where appropriate. Significance levels are denoted with asterisks ($P<0.05$=*, $P<0.01$=**, $P<0.005$=***).

5 Bibliography

Abe, T., M. Kato, H. Miki, T. Takenawa and T. Endo (2003). "Small GTPase Tc10 and its homologue RhoT induce N-WASP-mediated long process formation and neurite outgrowth." J Cell Sci **116**(Pt 1): 155-168.

Adams, R. H. and A. Eichmann (2010). "Axon guidance molecules in vascular patterning." Cold Spring Harb Perspect Biol **2**(5): a001875.

Adamson, P., H. F. Paterson and A. Hall (1992). "Intracellular localization of the P21rho proteins." J Cell Biol **119**(3): 617-627.

Aghazadeh, B., W. E. Lowry, X. Y. Huang and M. K. Rosen (2000). "Structural basis for relief of autoinhibition of the Dbl homology domain of proto-oncogene Vav by tyrosine phosphorylation." Cell **102**(5): 625-633.

Alberts, A. S., H. Qin, H. S. Carr and J. A. Frost (2005). "PAK1 negatively regulates the activity of the Rho exchange factor NET1." J Biol Chem **280**(13): 12152-12161.

Alberts, A. S. and R. Treisman (1998). "Activation of RhoA and SAPK/JNK signalling pathways by the RhoA-specific exchange factor mNET1." EMBO J **17**(14): 4075-4085.

Alberts, B. (2015). Molecular biology of the cell. New York, NY, Garland Science, Taylor and Francis Group.

Amano, M., M. Ito, K. Kimura, Y. Fukata, K. Chihara, T. Nakano, Y. Matsuura and K. Kaibuchi (1996). "Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase)." J Biol Chem **271**(34): 20246-20249.

Amyere, M., B. Payrastre, U. Krause, P. Van Der Smissen, A. Veithen and P. J. Courtoy (2000). "Constitutive macropinocytosis in oncogene-transformed fibroblasts depends on sequential permanent activation of phosphoinositide 3-kinase and phospholipase C." Mol Biol Cell **11**(10): 3453-3467.

Aoto, J., P. Ting, B. Maghsoodi, N. Xu, M. Henkemeyer and L. Chen (2007). "Postsynaptic ephrinB3 promotes shaft glutamatergic synapse formation." J Neurosci **27**(28): 7508-7519.

Aresta, S., M. F. de Tand-Heim, F. Beranger and J. de Gunzburg (2002). "A novel Rho GTPase-activating-protein interacts with Gem, a member of the Ras superfamily of GTPases." Biochem J **367**(Pt 1): 57-65.

Armstrong, J. N., M. J. Saganich, N. J. Xu, M. Henkemeyer, S. F. Heinemann and A. Contractor (2006). "B-ephrin reverse signaling is required for NMDA-independent long-term potentiation of mossy fibers in the hippocampus." J Neurosci **26**(13): 3474-3481.

Aronheim, A., Y. C. Broder, A. Cohen, A. Fritsch, B. Belisle and A. Abo (1998). "Chp, a homologue of the GTPase Cdc42Hs, activates the JNK pathway and is implicated in reorganizing the actin cytoskeleton." Curr Biol **8**(20): 1125-1128.

Aspenstrom, P., A. Fransson and J. Saras (2004). "Rho GTPases have diverse effects on the organization of the actin filament system." Biochem J **377**(Pt 2): 327-337.

Baass, P. C., G. M. Di Guglielmo, F. Authier, B. I. Posner and J. J. Bergeron (1995). "Compartmentalized signal transduction by receptor tyrosine kinases." Trends Cell Biol **5**(12): 465-470.

Barker, P. A., N. K. Hussain and P. S. McPherson (2002). "Retrograde signaling by the neurotrophins follows a well-worn trk." Trends Neurosci **25**(8): 379-381.

Batlle, E. and D. G. Wilkinson (2012). "Molecular mechanisms of cell segregation and boundary formation in development and tumorigenesis." Cold Spring Harb Perspect Biol **4**(1): a008227.

Beg, A. A., J. E. Sommer, J. H. Martin and P. Scheiffele (2007). "alpha2-Chimaerin is an essential EphA4 effector in the assembly of neuronal locomotor circuits." Neuron **55**(5): 768-778.

Benjumeda, I., A. Escalante, C. Law, D. Morales, G. Chauvin, G. Muca, Y. Coca, J. Marquez, G. Lopez-Bendito, A. Kania, L. Martinez and E. Herrera (2013). "Uncoupling of EphA/ephrinA signaling and spontaneous activity in neural circuit wiring." J Neurosci **33**(46): 18208-18218.

Bergemann, A. D., L. Zhang, M. K. Chiang, R. Brambilla, R. Klein and J. G. Flanagan (1998). "Ephrin-B3, a ligand for the receptor EphB3, expressed at the midline of the developing neural tube." Oncogene **16**(4): 471-480.

Bergeron, J. J., G. M. Di Guglielmo, P. C. Baass, F. Authier and B. I. Posner (1995). "Endosomes, receptor tyrosine kinase internalization and signal transduction." Biosci Rep **15**(6): 411-418.

Billuart, P., T. Bienvenu, N. Ronce, V. des Portes, M. C. Vinet, R. Zemni, H. Roest Crollius, A. Carrie, F. Fauchereau, M. Cherry, S. Briault, B. Hamel, J. P. Fryns, C. Beldjord, A. Kahn, C. Moraine and J. Chelly (1998). "Oligophrenin-1 encodes a rhoGAP protein involved in X-linked mental retardation." Nature **392**(6679): 923-926.

Binns, K. L., P. P. Taylor, F. Sicheri, T. Pawson and S. J. Holland (2000). "Phosphorylation of tyrosine residues in the kinase domain and juxtamembrane region regulates the biological and catalytic activities of Eph receptors." Mol Cell Biol **20**(13): 4791-4805.

Bishop, A. L. and A. Hall (2000). "Rho GTPases and their effector proteins." Biochem J **348 Pt 2**: 241-255.

Bliss, T. V. and G. L. Collingridge (1993). "A synaptic model of memory: long-term potentiation in the hippocampus." Nature **361**(6407): 31-39.

Bliss, T. V. and T. Lomo (1973). "Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path." J Physiol **232**(2): 331-356.

Boissier, P., J. Chen and U. Huynh-Do (2013). "EphA2 Signaling Following Endocytosis: Role of Tiam1." Traffic.

Bonanomi, D., O. Chivatakarn, G. Bai, H. Abdesselem, K. Lettieri, T. Marquardt, B. A. Pierchala and S. L. Pfaff (2012). "Ret is a multifunctional coreceptor that integrates diffusible- and contact-axon guidance signals." Cell **148**(3): 568-582.

Bos, J. L., H. Rehmann and A. Wittinghofer (2007). "GEFs and GAPs: critical elements in the control of small G proteins." Cell **129**(5): 865-877.

Bossing, T. and A. H. Brand (2002). "Dephrin, a transmembrane ephrin with a unique structure, prevents interneuronal axons from exiting the Drosophila embryonic CNS." Development **129**(18): 4205-4218.

Bourgin, C., K. K. Murai, M. Richter and E. B. Pasquale (2007). "The EphA4 receptor regulates dendritic spine remodeling by affecting beta1-integrin signaling pathways." J Cell Biol **178**(7): 1295-1307.

Bouzioukh, F., G. A. Wilkinson, G. Adelmann, M. Frotscher, V. Stein and R. Klein (2007). "Tyrosine phosphorylation sites in ephrinB2 are required for hippocampal long-term potentiation but not long-term depression." J Neurosci **27**(42): 11279-11288.

Brambilla, R., K. Bruckner, D. Orioli, A. D. Bergemann, J. G. Flanagan and R. Klein (1996). "Similarities and differences in the way transmembrane-type ligands interact with the Elk subclass of Eph receptors." Mol Cell Neurosci **8**(2-3): 199-209.

Brambilla, R., A. Schnapp, F. Casagranda, J. P. Labrador, A. D. Bergemann, J. G. Flanagan, E. B. Pasquale and R. Klein (1995). "Membrane-bound LERK2 ligand can signal through three different Eph-related receptor tyrosine kinases." EMBO J **14**(13): 3116-3126.

Brown, A., P. A. Yates, P. Burrola, D. Ortuno, A. Vaidya, T. M. Jessell, S. L. Pfaff, D. D. O'Leary and G. Lemke (2000). "Topographic mapping from the retina to the midbrain is controlled by relative but not absolute levels of EphA receptor signaling." Cell **102**(1): 77-88.

Bruckner, K., J. Pablo Labrador, P. Scheiffele, A. Herb, P. H. Seeburg and R. Klein (1999). "EphrinB ligands recruit GRIP family PDZ adaptor proteins into raft membrane microdomains." Neuron **22**(3): 511-524.

Bruckner, K., E. B. Pasquale and R. Klein (1997). "Tyrosine phosphorylation of transmembrane ligands for Eph receptors." Science **275**(5306): 1640-1643.

Brugnera, E., L. Haney, C. Grimsley, M. Lu, S. F. Walk, A. C. Tosello-Trampont, I. G. Macara, H. Madhani, G. R. Fink and K. S. Ravichandran (2002). "Unconventional Rac-GEF activity is mediated through the Dock180-ELMO complex." Nat Cell Biol **4**(8): 574-582.

Burke, P., K. Schooler and H. S. Wiley (2001). "Regulation of epidermal growth factor receptor signaling by endocytosis and intracellular trafficking." Mol Biol Cell **12**(6): 1897-1910.

Burridge, K. and K. Wennerberg (2004). "Rho and Rac take center stage." Cell **116**(2): 167-179.

Bustelo, X. R. (2000). "Regulatory and signaling properties of the Vav family." Mol Cell Biol **20**(5): 1461-1477.

Bustelo, X. R. (2014). "Vav family exchange factors: an integrated regulatory and functional view." Small GTPases **5**(2): 9.

Cagan, R. L., H. Kramer, A. C. Hart and S. L. Zipursky (1992). "The bride of sevenless and sevenless interaction: internalization of a transmembrane ligand." Cell **69**(3): 393-399.

Carmona, M. A., K. K. Murai, L. Wang, A. J. Roberts and E. B. Pasquale (2009). "Glial ephrin-A3 regulates hippocampal dendritic spine morphology and glutamate transport." Proc Natl Acad Sci U S A **106**(30): 12524-12529.

Carpenter, A. E., T. R. Jones, M. R. Lamprecht, C. Clarke, I. H. Kang, O. Friman, D. A. Guertin, J. H. Chang, R. A. Lindquist, J. Moffat, P. Golland and D. M. Sabatini (2006). "CellProfiler: image analysis software for identifying and quantifying cell phenotypes." Genome Biol **7**(10): R100.

Carr, H. S., C. A. Morris, S. Menon, E. H. Song and J. A. Frost (2013). "Rac1 controls the subcellular localization of the Rho guanine nucleotide exchange factor Net1A to regulate focal adhesion formation and cell spreading." Mol Cell Biol **33**(3): 622-634.

Carvalho, R. F., M. Beutler, K. J. Marler, B. Knoll, E. Becker-Barroso, R. Heintzmann, T. Ng and U. Drescher (2006). "Silencing of EphA3 through a cis interaction with ephrinA5." Nat Neurosci **9**(3): 322-330.

Castellano, F., P. Montcourrier and P. Chavrier (2000). "Membrane recruitment of Rac1 triggers phagocytosis." J Cell Sci **113 (Pt 17)**: 2955-2961.

Chadda, R., M. T. Howes, S. J. Plowman, J. F. Hancock, R. G. Parton and S. Mayor (2007). "Cholesterol-sensitive Cdc42 activation regulates actin polymerization for endocytosis via the GEEC pathway." Traffic **8**(6): 702-717.

Chamberlain, M. D., T. R. Berry, M. C. Pastor and D. H. Anderson (2004). "The p85alpha subunit of phosphatidylinositol 3'-kinase binds to and stimulates the GTPase activity of Rab proteins." J Biol Chem **279**(47): 48607-48614.

Chardin, P. (2006). "Function and regulation of Rnd proteins." Nat Rev Mol Cell Biol **7**(1): 54-62.

Chiang, S. H., J. Hwang, M. Legendre, M. Zhang, A. Kimura and A. R. Saltiel (2003). "TCGAP, a multidomain Rho GTPase-activating protein involved in insulin-stimulated glucose transport." EMBO J **22**(11): 2679-2691.

Chimini, G. and P. Chavrier (2000). "Function of Rho family proteins in actin dynamics during phagocytosis and engulfment." Nat Cell Biol **2**(10): E191-196.

Cho, Y. J., J. M. Cunnick, S. J. Yi, V. Kaartinen, J. Groffen and N. Heisterkamp (2007). "Abr and Bcr, two homologous Rac GTPase-activating proteins, control multiple cellular functions of murine macrophages." Mol Cell Biol **27**(3): 899-911.

Chuang, T. H., X. Xu, V. Kaartinen, N. Heisterkamp, J. Groffen and G. M. Bokoch (1995). "Abr and Bcr are multifunctional regulators of the Rho GTP-binding protein family." Proc Natl Acad Sci U S A **92**(22): 10282-10286.

Cisse, M. and F. Checler (2015). "Eph receptors: new players in Alzheimer's disease pathogenesis." Neurobiol Dis **73**: 137-149.

Contractor, A., C. Rogers, C. Maron, M. Henkemeyer, G. T. Swanson and S. F. Heinemann (2002). "Trans-synaptic Eph receptor-ephrin signaling in hippocampal mossy fiber LTP." Science **296**(5574): 1864-1869.

Coonan, J. R., U. Greferath, J. Messenger, L. Hartley, M. Murphy, A. W. Boyd, M. Dottori, M. P. Galea and P. F. Bartlett (2001). "Development and reorganization of corticospinal projections in EphA4 deficient mice." J Comp Neurol **436**(2): 248-262.

Corbetta, S., S. Gualdoni, G. Ciceri, M. Monari, E. Zuccaro, V. L. Tybulewicz and I. de Curtis (2009). "Essential role of Rac1 and Rac3 GTPases in neuronal development." FASEB J **23**(5): 1347-1357.

Cote, J. F. and K. Vuori (2002). "Identification of an evolutionarily conserved superfamily of DOCK180-related proteins with guanine nucleotide exchange activity." J Cell Sci **115**(Pt 24): 4901-4913.

Cote, J. F. and K. Vuori (2006). "In vitro guanine nucleotide exchange activity of DHR-2/DOCKER/CZH2 domains." Methods Enzymol **406**: 41-57.

Cotton, M., P. L. Boulay, T. Houndolo, N. Vitale, J. A. Pitcher and A. Claing (2007). "Endogenous ARF6 interacts with Rac1 upon angiotensin II stimulation to regulate membrane ruffling and cell migration." Mol Biol Cell **18**(2): 501-511.

Cowan, C. A. and M. Henkemeyer (2001). "The SH2/SH3 adaptor Grb4 transduces B-ephrin reverse signals." Nature **413**(6852): 174-179.

Cowan, C. W., Y. R. Shao, M. Sahin, S. M. Shamah, M. Z. Lin, P. L. Greer, S. Gao, E. C. Griffith, J. S. Brugge and M. E. Greenberg (2005). "Vav family GEFs link activated Ephs to endocytosis and axon guidance." Neuron **46**(2): 205-217.

Cunnick, J. M., S. Schmidhuber, G. Chen, M. Yu, S. J. Yi, Y. J. Cho, V. Kaartinen, P. Minoo, D. Warburton, J. Groffen and N. Heisterkamp (2009). "Bcr and Abr cooperate in negatively regulating acute inflammatory responses." Mol Cell Biol **29**(21): 5742-5750.

Cureton, D. K., R. H. Massol, S. Saffarian, T. L. Kirchhausen and S. P. Whelan (2009). "Vesicular stomatitis virus enters cells through vesicles incompletely coated with clathrin that depend upon actin for internalization." PLoS Pathog **5**(4): e1000394.

Cureton, D. K., R. H. Massol, S. P. Whelan and T. Kirchhausen (2010). "The length of vesicular stomatitis virus particles dictates a need for actin assembly during clathrin-dependent endocytosis." PLoS Pathog **6**(9): e1001127.

D'Souza-Schorey, C. and P. Chavrier (2006). "ARF proteins: roles in membrane traffic and beyond." Nat Rev Mol Cell Biol **7**(5): 347-358.

Dalva, M. B., M. A. Takasu, M. Z. Lin, S. M. Shamah, L. Hu, N. W. Gale and M. E. Greenberg (2000). "EphB receptors interact with NMDA receptors and regulate excitatory synapse formation." Cell **103**(6): 945-956.

Davis, S., N. W. Gale, T. H. Aldrich, P. C. Maisonpierre, V. Lhotak, T. Pawson, M. Goldfarb and G. D. Yancopoulos (1994). "Ligands for EPH-related receptor tyrosine kinases that require membrane attachment or clustering for activity." Science **266**(5186): 816-819.

Davy, A., J. Aubin and P. Soriano (2004). "Ephrin-B1 forward and reverse signaling are required during mouse development." Genes Dev **18**(5): 572-583.

Davy, A., N. W. Gale, E. W. Murray, R. A. Klinghoffer, P. Soriano, C. Feuerstein and S. M. Robbins (1999). "Compartmentalized signaling by GPI-anchored ephrin-A5 requires the Fyn tyrosine kinase to regulate cellular adhesion." Genes Dev **13**(23): 3125-3135.

Davy, A. and S. M. Robbins (2000). "Ephrin-A5 modulates cell adhesion and morphology in an integrin-dependent manner." EMBO J **19**(20): 5396-5405.

De Camilli, P., K. Takei and P. S. McPherson (1995). "The function of dynamin in endocytosis." Curr Opin Neurobiol **5**(5): 559-565.

de Kreuk, B. J., A. Schaefer, E. C. Anthony, S. Tol, M. Fernandez-Borja, D. Geerts, J. Pool, L. Hambach, E. Goulmy and P. L. Hordijk (2013). "The human minor histocompatibility antigen 1 is a RhoGAP." PLoS One **8**(9): e73962.

deBakker, C. D., L. B. Haney, J. M. Kinchen, C. Grimsley, M. Lu, D. Klingele, P. K. Hsu, B. K. Chou, L. C. Cheng, A. Blangy, J. Sondek, M. O. Hengartner, Y. C. Wu and K. S. Ravichandran (2004). "Phagocytosis of apoptotic cells is regulated by a UNC-73/TRIO-MIG-2/RhoG signaling module and armadillo repeats of CED-12/ELMO." Curr Biol **14**(24): 2208-2216.

Debant, A., C. Serra-Pages, K. Seipel, S. O'Brien, M. Tang, S. H. Park and M. Streuli (1996). "The multidomain protein Trio binds the LAR transmembrane tyrosine phosphatase, contains a protein kinase domain, and has separate rac-specific and rho-specific guanine nucleotide exchange factor domains." Proc Natl Acad Sci U S A **93**(11): 5466-5471.

Deininger, K., M. Eder, E. R. Kramer, W. Zieglgansberger, H. U. Dodt, K. Dornmair, J. Colicelli and R. Klein (2008). "The Rab5 guanylate exchange factor Rin1 regulates endocytosis of the EphA4 receptor in mature excitatory neurons." Proc Natl Acad Sci U S A **105**(34): 12539-12544.

Dereeper, A., V. Guignon, G. Blanc, S. Audic, S. Buffet, F. Chevenet, J. F. Dufayard, S. Guindon, V. Lefort, M. Lescot, J. M. Claverie and O. Gascuel (2008). "Phylogeny.fr: robust phylogenetic analysis for the non-specialist." Nucleic Acids Res **36**(Web Server issue): W465-469.

Dergham, P., B. Ellezam, C. Essagian, H. Avedissian, W. D. Lubell and L. McKerracher (2002). "Rho signaling pathway targeted to promote spinal cord repair." J Neurosci **22**(15): 6570-6577.

Devon, R. S., P. C. Orban, K. Gerrow, M. A. Barbieri, C. Schwab, L. P. Cao, J. R. Helm, N. Bissada, R. Cruz-Aguado, T. L. Davidson, J. Witmer, M. Metzler, C. K. Lam, W. Tetzlaff, E. M. Simpson, J. M. McCaffery, A. E. El-Husseini, B. R. Leavitt and M. R. Hayden (2006). "Als2-deficient mice exhibit disturbances in endosome trafficking associated with motor behavioral abnormalities." Proc Natl Acad Sci U S A **103**(25): 9595-9600.

Dharmawardhane, S., A. Schurmann, M. A. Sells, J. Chernoff, S. L. Schmid and G. M. Bokoch (2000). "Regulation of macropinocytosis by p21-activated kinase-1." Mol Biol Cell **11**(10): 3341-3352.

Di Guglielmo, G. M., P. C. Baass, W. J. Ou, B. I. Posner and J. J. Bergeron (1994). "Compartmentalization of SHC, GRB2 and mSOS, and hyperphosphorylation of Raf-1 by EGF but not insulin in liver parenchyma." EMBO J **13**(18): 4269-4277.

Doherty, G. J. and R. Lundmark (2009). "GRAF1-dependent endocytosis." Biochem Soc Trans **37**(Pt 5): 1061-1065.

Doherty, G. J. and H. T. McMahon (2009). "Mechanisms of endocytosis." Annu Rev Biochem **78**: 857-902.

Donaldson, J. G., N. Porat-Shliom and L. A. Cohen (2009). "Clathrin-independent endocytosis: a unique platform for cell signaling and PM remodeling." Cell Signal **21**(1): 1-6.

Dottori, M., L. Hartley, M. Galea, G. Paxinos, M. Polizzotto, T. Kilpatrick, P. F. Bartlett, M. Murphy, F. Kontgen and A. W. Boyd (1998). "EphA4 (Sek1) receptor tyrosine kinase is required for the development of the corticospinal tract." Proc Natl Acad Sci U S A **95**(22): 13248-13253.

Drescher, U., C. Kremoser, C. Handwerker, J. Loschinger, M. Noda and F. Bonhoeffer (1995). "In vitro guidance of retinal ganglion cell axons by RAGS, a 25 kDa tectal protein related to ligands for Eph receptor tyrosine kinases." Cell **82**(3): 359-370.

Driessens, M. H., H. Hu, C. D. Nobes, A. Self, I. Jordens, C. S. Goodman and A. Hall (2001). "Plexin-B semaphorin receptors interact directly with active Rac and regulate the actin cytoskeleton by activating Rho." Curr Biol **11**(5): 339-344.

Dudanova, I., G. Gatto and R. Klein (2010). "GDNF acts as a chemoattractant to support ephrinA-induced repulsion of limb motor axons." Curr Biol **20**(23): 2150-2156.

Dudanova, I., T. J. Kao, J. E. Herrmann, B. Zheng, A. Kania and R. Klein (2012). "Genetic evidence for a contribution of EphA:ephrinA reverse signaling to motor axon guidance." J Neurosci **32**(15): 5209-5215.

Dufour, A., J. Egea, K. Kullander, R. Klein and P. Vanderhaeghen (2006). "Genetic analysis of EphA-dependent signaling mechanisms controlling topographic mapping in vivo." Development **133**(22): 4415-4420.

Dufour, A., J. Seibt, L. Passante, V. Depaepe, T. Ciossek, J. Frisen, K. Kullander, J. G. Flanagan, F. Polleux and P. Vanderhaeghen (2003). "Area specificity and topography of thalamocortical projections are controlled by ephrin/Eph genes." Neuron **39**(3): 453-465.

Duman, J. G., C. P. Tzeng, Y. K. Tu, T. Munjal, B. Schwechter, T. S. Ho and K. F. Tolias (2013). "The adhesion-GPCR BAI1 regulates synaptogenesis by controlling the recruitment of the Par3/Tiam1 polarity complex to synaptic sites." J Neurosci **33**(16): 6964-6978.

Eden, S., R. Rohatgi, A. V. Podtelejnikov, M. Mann and M. W. Kirschner (2002). "Mechanism of regulation of WAVE1-induced actin nucleation by Rac1 and Nck." Nature **418**(6899): 790-793.

Edwards, C. M. and G. R. Mundy (2008). "Eph receptors and ephrin signaling pathways: a role in bone homeostasis." Int J Med Sci **5**(5): 263-272.

Edwards, D. C., L. C. Sanders, G. M. Bokoch and G. N. Gill (1999). "Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics." Nat Cell Biol **1**(5): 253-259.

Egea, J. and R. Klein (2007). "Bidirectional Eph-ephrin signaling during axon guidance." Trends Cell Biol **17**(5): 230-238.

Egea, J., U. V. Nissen, A. Dufour, M. Sahin, P. Greer, K. Kullander, T. D. Mrsic-Flogel, M. E. Greenberg, O. Kiehn, P. Vanderhaeghen and R. Klein (2005). "Regulation of EphA 4 kinase activity is required for a subset of axon guidance decisions suggesting a key role for receptor clustering in Eph function." Neuron **47**(4): 515-528.

Ellerbroek, S. M., K. Wennerberg, W. T. Arthur, J. M. Dunty, D. R. Bowman, K. A. DeMali, C. Der and K. Burridge (2004). "SGEF, a RhoG guanine nucleotide exchange factor that stimulates macropinocytosis." Mol Biol Cell **15**(7): 3309-3319.

Ellis, C., F. Kasmi, P. Ganju, E. Walls, G. Panayotou and A. D. Reith (1996). "A juxtamembrane autophosphorylation site in the Eph family receptor tyrosine kinase, Sek, mediates high affinity interaction with p59fyn." Oncogene **12**(8): 1727-1736.

Eph Nomenclature Committee (1997). "Unified nomenclature for Eph family receptors and their ligands, the ephrins. Eph Nomenclature Committee." Cell **90**(3): 403-404.

Espinosa, E. J., M. Calero, K. Sridevi and S. R. Pfeffer (2009). "RhoBTB3: a Rho GTPase-family ATPase required for endosome to Golgi transport." Cell **137**(5): 938-948.

Essmann, C. L., E. Martinez, J. C. Geiger, M. Zimmer, M. H. Traut, V. Stein, R. Klein and A. Acker-Palmer (2008). "Serine phosphorylation of ephrinB2 regulates trafficking of synaptic AMPA receptors." Nat Neurosci **11**(9): 1035-1043.

Etienne-Manneville, S. and A. Hall (2002). "Rho GTPases in cell biology." Nature **420**(6916): 629-635.

Fasen, K., D. P. Cerretti and U. Huynh-Do (2008). "Ligand binding induces Cbl-dependent EphB1 receptor degradation through the lysosomal pathway." Traffic **9**(2): 251-266.

Fauchereau, F., U. Herbrand, P. Chafey, A. Eberth, A. Koulakoff, M. C. Vinet, M. R. Ahmadian, J. Chelly and P. Billuart (2003). "The RhoGAP activity of OPHN1, a new F-actin-binding protein, is negatively controlled by its amino-terminal domain." Mol Cell Neurosci **23**(4): 574-586.

Faucherre, A., P. Desbois, V. Satre, J. Lunardi, O. Dorseuil and G. Gacon (2003). "Lowe syndrome protein OCRL1 interacts with Rac GTPase in the trans-Golgi network." Hum Mol Genet **12**(19): 2449-2456.

Fawcett, J. P., J. Georgiou, J. Ruston, F. Bladt, A. Sherman, N. Warner, B. J. Saab, R. Scott, J. C. Roder and T. Pawson (2007). "Nck adaptor proteins control the organization of neuronal circuits important for walking." Proc Natl Acad Sci U S A **104**(52): 20973-20978.

Ferguson, K. M., M. A. Lemmon, J. Schlessinger and P. B. Sigler (1995). "Structure of the high affinity complex of inositol trisphosphate with a phospholipase C pleckstrin homology domain." Cell **83**(6): 1037-1046.

Fernandez-Borja, M., L. Janssen, D. Verwoerd, P. Hordijk and J. Neefjes (2005). "RhoB regulates endosome transport by promoting actin assembly on endosomal membranes through Dia1." J Cell Sci **118**(Pt 12): 2661-2670.

Fiegen, D., L. Blumenstein, P. Stege, I. R. Vetter and M. R. Ahmadian (2002). "Crystal structure of Rnd3/RhoE: functional implications." FEBS Lett **525**(1-3): 100-104.

Fiegen, D., L. C. Haeusler, L. Blumenstein, U. Herbrand, R. Dvorsky, I. R. Vetter and M. R. Ahmadian (2004). "Alternative splicing of Rac1 generates Rac1b, a self-activating GTPase." J Biol Chem **279**(6): 4743-4749.

Filosa, A., S. Paixao, S. D. Honsek, M. A. Carmona, L. Becker, B. Feddersen, L. Gaitanos, Y. Rudhard, R. Schoepfer, T. Klopstock, K. Kullander, C. R. Rose, E. B. Pasquale and R. Klein (2009). "Neuron-glia communication via EphA4/ephrin-A3 modulates LTP through glial glutamate transport." Nat Neurosci **12**(10): 1285-1292.

Fivaz, M., F. Vilbois, S. Thurnheer, C. Pasquali, L. Abrami, P. E. Bickel, R. G. Parton and F. G. van der Goot (2002). "Differential sorting and fate of endocytosed GPI-anchored proteins." EMBO J **21**(15): 3989-4000.

Flannagan, R. S., V. Jaumouille and S. Grinstein (2012). "The cell biology of phagocytosis." Annu Rev Pathol **7**: 61-98.

Foo, S. S., C. J. Turner, S. Adams, A. Compagni, D. Aubyn, N. Kogata, P. Lindblom, M. Shani, D. Zicha and R. H. Adams (2006). "Ephrin-B2 controls cell motility and adhesion during blood-vessel-wall assembly." Cell **124**(1): 161-173.

Ford, M. G., I. G. Mills, B. J. Peter, Y. Vallis, G. J. Praefcke, P. R. Evans and H. T. McMahon (2002). "Curvature of clathrin-coated pits driven by epsin." Nature **419**(6905): 361-366.

Foster, R., K. Q. Hu, Y. Lu, K. M. Nolan, J. Thissen and J. Settleman (1996). "Identification of a novel human Rho protein with unusual properties: GTPase deficiency and in vivo farnesylation." Mol Cell Biol **16**(6): 2689-2699.

Fournier, A. E., F. Nakamura, S. Kawamoto, Y. Goshima, R. G. Kalb and S. M. Strittmatter (2000). "Semaphorin3A enhances endocytosis at sites of receptor-F-actin colocalization during growth cone collapse." J Cell Biol **149**(2): 411-422.

Fournier, A. E., B. T. Takizawa and S. M. Strittmatter (2003). "Rho kinase inhibition enhances axonal regeneration in the injured CNS." J Neurosci **23**(4): 1416-1423.

Franke, K., W. Otto, S. Johannes, J. Baumgart, R. Nitsch and S. Schumacher (2012). "miR-124-regulated RhoG reduces neuronal process complexity via ELMO/Dock180/Rac1 and Cdc42 signalling." EMBO J **31**(13): 2908-2921.

Fransson, A., A. Ruusala and P. Aspenstrom (2003). "Atypical Rho GTPases have roles in mitochondrial homeostasis and apoptosis." J Biol Chem **278**(8): 6495-6502.

Frick, M., N. A. Bright, K. Riento, A. Bray, C. Merrified and B. J. Nichols (2007). "Coassembly of flotillins induces formation of membrane microdomains, membrane curvature, and vesicle budding." Curr Biol **17**(13): 1151-1156.

Friedl, P. and D. Gilmour (2009). "Collective cell migration in morphogenesis, regeneration and cancer." Nat Rev Mol Cell Biol **10**(7): 445-457.

Fujikawa, K., A. V. Miletic, F. W. Alt, R. Faccio, T. Brown, J. Hoog, J. Fredericks, S. Nishi, S. Mildiner, S. L. Moores, J. Brugge, F. S. Rosen and W. Swat (2003). "Vav1/2/3-null mice define an essential role for Vav family proteins in lymphocyte development and activation but a differential requirement in MAPK signaling in T and B cells." J Exp Med **198**(10): 1595-1608.

Fujimoto, L. M., R. Roth, J. E. Heuser and S. L. Schmid (2000). "Actin assembly plays a variable, but not obligatory role in receptor-mediated endocytosis in mammalian cells." Traffic **1**(2): 161-171.

Fujishiro, S. H., S. Tanimura, S. Mure, Y. Kashimoto, K. Watanabe and M. Kohno (2008). "ERK1/2 phosphorylate GEF-H1 to enhance its guanine nucleotide exchange activity toward RhoA." Biochem Biophys Res Commun **368**(1): 162-167.

Funk, S. D. and A. W. Orr (2013). "Ephs and ephrins resurface in inflammation, immunity, and atherosclerosis." Pharmacol Res **67**(1): 42-52.

Gad, A. K. and P. Aspenstrom (2010). "Rif proteins take to the RhoD: Rho GTPases at the crossroads of actin dynamics and membrane trafficking." Cell Signal **22**(2): 183-189.

Gale, N. W., S. J. Holland, D. M. Valenzuela, A. Flenniken, L. Pan, T. E. Ryan, M. Henkemeyer, K. Strebhardt, H. Hirai, D. G. Wilkinson, T. Pawson, S. Davis and G. D. Yancopoulos (1996). "Eph receptors and ligands comprise two major specificity subclasses and are reciprocally compartmentalized during embryogenesis." Neuron **17**(1): 9-19.

Gampel, A., P. J. Parker and H. Mellor (1999). "Regulation of epidermal growth factor receptor traffic by the small GTPase rhoB." Curr Biol **9**(17): 955-958.

Garavini, H., K. Riento, J. P. Phelan, M. S. McAlister, A. J. Ridley and N. H. Keep (2002). "Crystal structure of the core domain of RhoE/Rnd3: a constitutively activated small G protein." Biochemistry **41**(20): 6303-6310.

Garrett, M. D., A. J. Self, C. van Oers and A. Hall (1989). "Identification of distinct cytoplasmic targets for ras/R-ras and rho regulatory proteins." J Biol Chem **264**(1): 10-13.

Gasman, S., Y. Kalaidzidis and M. Zerial (2003). "RhoD regulates endosome dynamics through Diaphanous-related Formin and Src tyrosine kinase." Nat Cell Biol **5**(3): 195-204.

Gatto, G., D. Morales, A. Kania and R. Klein (2014). "EphA4 receptor shedding regulates spinal motor axon guidance." Curr Biol **24**(20): 2355-2365.

Genander, M. and J. Frisen (2010). "Ephrins and Eph receptors in stem cells and cancer." Curr Opin Cell Biol **22**(5): 611-616.

Georgakopoulos, A., C. Litterst, E. Ghersi, L. Baki, C. Xu, G. Serban and N. K. Robakis (2006). "Metalloproteinase/Presenilin1 processing of ephrinB regulates EphB-induced Src phosphorylation and signaling." EMBO J **25**(6): 1242-1252.

George, S. E., K. Simokat, J. Hardin and A. D. Chisholm (1998). "The VAB-1 Eph receptor tyrosine kinase functions in neural and epithelial morphogenesis in C. elegans." Cell **92**(5): 633-643.

Girao, H., M. I. Geli and F. Z. Idrissi (2008). "Actin in the endocytic pathway: from yeast to mammals." FEBS Lett **582**(14): 2112-2119.

Glebov, O. O., N. A. Bright and B. J. Nichols (2006). "Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells." Nat Cell Biol **8**(1): 46-54.

Goh, L. L. and E. Manser (2012). "The GTPase-deficient Rnd proteins are stabilized by their effectors." J Biol Chem **287**(37): 31311-31320.

Gottlieb, T. A., I. E. Ivanov, M. Adesnik and D. D. Sabatini (1993). "Actin microfilaments play a critical role in endocytosis at the apical but not the basolateral surface of polarized epithelial cells." J Cell Biol **120**(3): 695-710.

Govek, E. E., S. E. Newey, C. J. Akerman, J. R. Cross, L. Van der Veken and L. Van Aelst (2004). "The X-linked mental retardation protein oligophrenin-1 is required for dendritic spine morphogenesis." Nat Neurosci **7**(4): 364-372.

Govek, E. E., S. E. Newey and L. Van Aelst (2005). "The role of the Rho GTPases in neuronal development." Genes Dev **19**(1): 1-49.

Graham, D. L., J. F. Eccleston and P. N. Lowe (1999). "The conserved arginine in rho-GTPase-activating protein is essential for efficient catalysis but not for complex formation with Rho.GDP and aluminum fluoride." Biochemistry **38**(3): 985-991.

Grassart, A., A. Dujeancourt, P. B. Lazarow, A. Dautry-Varsat and N. Sauvonnet (2008). "Clathrin-independent endocytosis used by the IL-2 receptor is regulated by Rac1, Pak1 and Pak2." EMBO Rep **9**(4): 356-362.

Grimbert, F. and J. Cang (2012). "New model of retinocollicular mapping predicts the mechanisms of axonal competition and explains the role of reverse molecular signaling during development." J Neurosci **32**(28): 9755-9768.

Grimes, M. L., J. Zhou, E. C. Beattie, E. C. Yuen, D. E. Hall, J. S. Valletta, K. S. Topp, J. H. LaVail, N. W. Bunnett and W. C. Mobley (1996). "Endocytosis of activated TrkA: evidence that nerve growth factor induces formation of signaling endosomes." J Neurosci **16**(24): 7950-7964.

Grimmer, S., B. van Deurs and K. Sandvig (2002). "Membrane ruffling and macropinocytosis in A431 cells require cholesterol." J Cell Sci **115**(Pt 14): 2953-2962.

Grimsley, C. M., J. M. Kinchen, A. C. Tosello-Trampont, E. Brugnera, L. B. Haney, M. Lu, Q. Chen, D. Klingele, M. O. Hengartner and K. S. Ravichandran (2004). "Dock180 and ELMO1 proteins cooperate to promote evolutionarily conserved Rac-dependent cell migration." J Biol Chem **279**(7): 6087-6097.

Groeger, G. and C. D. Nobes (2007). "Co-operative Cdc42 and Rho signalling mediates ephrinB-triggered endothelial cell retraction." Biochem J **404**(1): 23-29.

Grunwald, I. C., M. Korte, G. Adelmann, A. Plueck, K. Kullander, R. H. Adams, M. Frotscher, T. Bonhoeffer and R. Klein (2004). "Hippocampal plasticity requires postsynaptic ephrinBs." Nat Neurosci **7**(1): 33-40.

Grunwald, I. C., M. Korte, D. Wolfer, G. A. Wilkinson, K. Unsicker, H. P. Lipp, T. Bonhoeffer and R. Klein (2001). "Kinase-independent requirement of EphB2 receptors in hippocampal synaptic plasticity." Neuron **32**(6): 1027-1040.

Gu, Y., M. D. Filippi, J. A. Cancelas, J. E. Siefring, E. P. Williams, A. C. Jasti, C. E. Harris, A. W. Lee, R. Prabhakar, S. J. Atkinson, D. J. Kwiatkowski and D. A. Williams (2003). "Hematopoietic cell regulation by Rac1 and Rac2 guanosine triphosphatases." Science **302**(5644): 445-449.

Guilluy, C., R. Garcia-Mata and K. Burridge (2011). "Rho protein crosstalk: another social network?" Trends Cell Biol **21**(12): 718-726.

Haataja, L., J. Groffen and N. Heisterkamp (1997). "Characterization of RAC3, a novel member of the Rho family." J Biol Chem **272**(33): 20384-20388.

Hadano, S., C. K. Hand, H. Osuga, Y. Yanagisawa, A. Otomo, R. S. Devon, N. Miyamoto, J. Showguchi-Miyata, Y. Okada, R. Singaraja, D. A. Figlewicz, T. Kwiatkowski, B. A. Hosler, T. Sagie, J. Skaug, J. Nasir, R. H. Brown, Jr., S. W. Scherer, G. A. Rouleau, M. R. Hayden and J. E. Ikeda (2001). "A gene encoding a putative GTPase regulator is mutated in familial amyotrophic lateral sclerosis 2." Nat Genet **29**(2): 166-173.

Hadano, S., R. Kunita, A. Otomo, K. Suzuki-Utsunomiya and J. E. Ikeda (2007). "Molecular and cellular function of ALS2/alsin: implication of membrane dynamics in neuronal development and degeneration." Neurochem Int **51**(2-4): 74-84.

Hall, A. and G. Lalli (2010). "Rho and Ras GTPases in axon growth, guidance, and branching." Cold Spring Harb Perspect Biol **2**(2): a001818.

Hart, M. J., A. Eva, T. Evans, S. A. Aaronson and R. A. Cerione (1991). "Catalysis of guanine nucleotide exchange on the CDC42Hs protein by the dbl oncogene product." Nature **354**(6351): 311-314.

Hattori, M., M. Osterfield and J. G. Flanagan (2000). "Regulated cleavage of a contact-mediated axon repellent." Science **289**(5483): 1360-1365.

Heasman, S. J. and A. J. Ridley (2008). "Mammalian Rho GTPases: new insights into their functions from in vivo studies." Nat Rev Mol Cell Biol **9**(9): 690-701.

Heerssen, H. M., M. F. Pazyra and R. A. Segal (2004). "Dynein motors transport activated Trks to promote survival of target-dependent neurons." Nat Neurosci **7**(6): 596-604.

Henkemeyer, M., O. S. Itkis, M. Ngo, P. W. Hickmott and I. M. Ethell (2003). "Multiple EphB receptor tyrosine kinases shape dendritic spines in the hippocampus." J Cell Biol **163**(6): 1313-1326.

Henley, J. R., E. W. Krueger, B. J. Oswald and M. A. McNiven (1998). "Dynamin-mediated internalization of caveolae." J Cell Biol **141**(1): 85-99.

Himanen, J. P., M. J. Chumley, M. Lackmann, C. Li, W. A. Barton, P. D. Jeffrey, C. Vearing, D. Geleick, D. A. Feldheim, A. W. Boyd, M. Henkemeyer and D. B. Nikolov (2004). "Repelling class discrimination: ephrin-A5 binds to and activates EphB2 receptor signaling." Nat Neurosci **7**(5): 501-509.

Himanen, J. P. and D. B. Nikolov (2003). "Eph receptors and ephrins." Int J Biochem Cell Biol **35**(2): 130-134.

Himanen, J. P., K. R. Rajashankar, M. Lackmann, C. A. Cowan, M. Henkemeyer and D. B. Nikolov (2001). "Crystal structure of an Eph receptor-ephrin complex." Nature **414**(6866): 933-938.

Himanen, J. P., L. Yermekbayeva, P. W. Janes, J. R. Walker, K. Xu, L. Atapattu, K. R. Rajashankar, A. Mensinga, M. Lackmann, D. B. Nikolov and S. Dhe-Paganon (2010). "Architecture of Eph receptor clusters." Proc Natl Acad Sci U S A **107**(24): 10860-10865.

Hiramoto, K., M. Negishi and H. Katoh (2006). "Dock4 is regulated by RhoG and promotes Rac-dependent cell migration." Exp Cell Res **312**(20): 4205-4216.

Hirbec, H., O. Perestenko, A. Nishimune, G. Meyer, S. Nakanishi, J. M. Henley and K. K. Dev (2002). "The PDZ proteins PICK1, GRIP, and syntenin bind multiple glutamate receptor subtypes. Analysis of PDZ binding motifs." J Biol Chem **277**(18): 15221-15224.

Holland, S. J., N. W. Gale, G. Mbamalu, G. D. Yancopoulos, M. Henkemeyer and T. Pawson (1996). "Bidirectional signalling through the EPH-family receptor Nuk and its transmembrane ligands." Nature **383**(6602): 722-725.

Holmberg, J., D. L. Clarke and J. Frisen (2000). "Regulation of repulsion versus adhesion by different splice forms of an Eph receptor." Nature **408**(6809): 203-206.

Hoppe, A. D. and J. A. Swanson (2004). "Cdc42, Rac1, and Rac2 display distinct patterns of activation during phagocytosis." Mol Biol Cell **15**(8): 3509-3519.

Howe, C. L., J. S. Valletta, A. S. Rusnak and W. C. Mobley (2001). "NGF signaling from clathrin-coated vesicles: evidence that signaling endosomes serve as a platform for the Ras-MAPK pathway." Neuron **32**(5): 801-814.

Hsueh, Y. P. and M. Sheng (1998). "Eph receptors, ephrins, and PDZs gather in neuronal synapses." Neuron **21**(6): 1227-1229.

Hu, H., T. F. Marton and C. S. Goodman (2001). "Plexin B mediates axon guidance in Drosophila by simultaneously inhibiting active Rac and enhancing RhoA signaling." Neuron **32**(1): 39-51.

Hussain, N. K., S. Jenna, M. Glogauer, C. C. Quinn, S. Wasiak, M. Guipponi, S. E. Antonarakis, B. K. Kay, T. P. Stossel, N. Lamarche-Vane and P. S. McPherson (2001). "Endocytic protein intersectin-l regulates actin assembly via Cdc42 and N-WASP." Nat Cell Biol **3**(10): 927-932.

Huttlin, E. L., L. Ting, R. J. Bruckner, F. Gebreab, M. P. Gygi, J. Szpyt, S. Tam, G. Zarraga, G. Colby, K. Baltier, R. Dong, V. Guarani, L. P. Vaites, A. Ordureau, R. Rad, B. K. Erickson, M. Wuhr, J. Chick, B. Zhai, D. Kolippakkam, J. Mintseris, R. A. Obar, T. Harris, S. Artavanis-Tsakonas, M. E. Sowa, P. De Camilli, J. A. Paulo, J. W. Harper and S. P. Gygi (2015). "The BioPlex Network: A Systematic Exploration of the Human Interactome." Cell **162**(2): 425-440.

Iden, S. and J. G. Collard (2008). "Crosstalk between small GTPases and polarity proteins in cell polarization." Nat Rev Mol Cell Biol **9**(11): 846-859.

Incardona, J. P., J. H. Lee, C. P. Robertson, K. Enga, R. P. Kapur and H. Roelink (2000). "Receptor-mediated endocytosis of soluble and membrane-tethered Sonic hedgehog by Patched-1." Proc Natl Acad Sci U S A **97**(22): 12044-12049.

Inoue, E., M. Deguchi-Tawarada, A. Togawa, C. Matsui, K. Arita, S. Katahira-Tayama, T. Sato, E. Yamauchi, Y. Oda and Y. Takai (2009). "Synaptic activity prompts gamma-secretase-mediated cleavage of EphA4 and dendritic spine formation." J Cell Biol **185**(3): 551-564.

Irie, F., M. Okuno, E. B. Pasquale and Y. Yamaguchi (2005). "EphrinB-EphB signalling regulates clathrin-mediated endocytosis through tyrosine phosphorylation of synaptojanin 1." Nat Cell Biol **7**(5): 501-509.

Irie, F. and Y. Yamaguchi (2002). "EphB receptors regulate dendritic spine development via intersectin, Cdc42 and N-WASP." Nat Neurosci **5**(11): 1117-1118.

Itoh, R. E., K. Kurokawa, Y. Ohba, H. Yoshizaki, N. Mochizuki and M. Matsuda (2002). "Activation of rac and cdc42 video imaged by fluorescent resonance energy transfer-based single-molecule probes in the membrane of living cells." Mol Cell Biol **22**(18): 6582-6591.

Iwasato, T., H. Katoh, H. Nishimaru, Y. Ishikawa, H. Inoue, Y. M. Saito, R. Ando, M. Iwama, R. Takahashi, M. Negishi and S. Itohara (2007). "Rac-GAP alpha-chimerin regulates motor-circuit formation as a key mediator of EphrinB3/EphA4 forward signaling." Cell **130**(4): 742-753.

Izumi, G., T. Sakisaka, T. Baba, S. Tanaka, K. Morimoto and Y. Takai (2004). "Endocytosis of E-cadherin regulated by Rac and Cdc42 small G proteins through IQGAP1 and actin filaments." J Cell Biol **166**(2): 237-248.

Jackson, B., K. Peyrollier, E. Pedersen, A. Basse, R. Karlsson, Z. Wang, T. Lefever, A. M. Ochsenbein, G. Schmidt, K. Aktories, A. Stanley, F. Quondamatteo, M. Ladwein, K. Rottner, J. van Hengel and C. Brakebusch (2011). "RhoA is dispensable for skin development, but crucial for contraction and directed migration of keratinocytes." Mol Biol Cell **22**(5): 593-605.

Jaffe, A. B. and A. Hall (2002). "Rho GTPases in transformation and metastasis." Adv Cancer Res **84**: 57-80.

Jaffe, A. B. and A. Hall (2005). "Rho GTPases: biochemistry and biology." Annu Rev Cell Dev Biol **21**: 247-269.

Jain, R. and E. Lammert (2009). "Cell-cell interactions in the endocrine pancreas." Diabetes Obes Metab **11 Suppl 4**: 159-167.

Jaiswal, M., R. Dvorsky and M. R. Ahmadian (2013a). "Deciphering the molecular and functional basis of Dbl family proteins: a novel systematic approach toward classification of selective activation of the Rho family proteins." J Biol Chem **288**(6): 4486-4500.

Jaiswal, M., E. K. Fansa, R. Dvorsky and M. R. Ahmadian (2013b). "New insight into the molecular switch mechanism of human Rho family proteins: shifting a paradigm." Biol Chem **394**(1): 89-95.

Janes, P. W., N. Saha, W. A. Barton, M. V. Kolev, S. H. Wimmer-Kleikamp, E. Nievergall, C. P. Blobel, J. P. Himanen, M. Lackmann and D. B. Nikolov (2005). "Adam meets Eph: an ADAM substrate recognition module acts as a molecular switch for ephrin cleavage in trans." Cell **123**(2): 291-304.

Janes, P. W., S. H. Wimmer-Kleikamp, A. S. Frangakis, K. Treble, B. Griesshaber, O. Sabet, M. Grabenbauer, A. Y. Ting, P. Saftig, P. I. Bastiaens and M. Lackmann (2009). "Cytoplasmic relaxation of active Eph controls ephrin shedding by ADAM10." PLoS Biol **7**(10): e1000215.

Jenna, S., N. K. Hussain, E. I. Danek, I. Triki, S. Wasiak, P. S. McPherson and N. Lamarche-Vane (2002). "The activity of the GTPase-activating protein CdGAP is regulated by the endocytic protein intersectin." J Biol Chem **277**(8): 6366-6373.

Jorgensen, C., A. Sherman, G. I. Chen, A. Pasculescu, A. Poliakov, M. Hsiung, B. Larsen, D. G. Wilkinson, R. Linding and T. Pawson (2009). "Cell-Specific Information Processing in Segregating Populations of Eph Receptor Ephrin-Expressing Cells." Science **326**(5959): 1502-1509.

Joset, A., D. A. Dodd, S. Halegoua and M. E. Schwab (2010). "Pincher-generated Nogo-A endosomes mediate growth cone collapse and retrograde signaling." J Cell Biol **188**(2): 271-285.

Jurney, W. M., G. Gallo, P. C. Letourneau and S. C. McLoon (2002). "Rac1-mediated endocytosis during ephrin-A2- and semaphorin 3A-induced growth cone collapse." J Neurosci **22**(14): 6019-6028.

Kalo, M. S. and E. B. Pasquale (1999). "Multiple in vivo tyrosine phosphorylation sites in EphB receptors." Biochemistry **38**(43): 14396-14408.

Kalo, M. S., H. H. Yu and E. B. Pasquale (2001). "In vivo tyrosine phosphorylation sites of activated ephrin-B1 and ephB2 from neural tissue." J Biol Chem **276**(42): 38940-38948.

Kandel, E. R. (2013). Principles of neural science. New York, McGraw-Hill.

Kaneko, T., A. Maeda, M. Takefuji, H. Aoyama, M. Nakayama, S. Kawabata, Y. Kawano, A. Iwamatsu, M. Amano and K. Kaibuchi (2005). "Rho mediates endocytosis of epidermal growth factor receptor through phosphorylation of endophilin A1 by Rho-kinase." Genes Cells **10**(10): 973-987.

Kao, T. J. and A. Kania (2011). "Ephrin-mediated cis-attenuation of Eph receptor signaling is essential for spinal motor axon guidance." Neuron **71**(1): 76-91.

Katoh, H., K. Hiramoto and M. Negishi (2006). "Activation of Rac1 by RhoG regulates cell migration." J Cell Sci **119**(Pt 1): 56-65.

Katoh, H. and M. Negishi (2003). "RhoG activates Rac1 by direct interaction with the Dock180-binding protein Elmo." Nature **424**(6947): 461-464.

Katzav, S. (2015). "Vav1: A Dr. Jekyll and Mr. Hyde protein - good for the hematopoietic system, bad for cancer." Oncotarget **6**(30): 28731-28742.

Kayser, M. S., A. C. McClelland, E. G. Hughes and M. B. Dalva (2006). "Intracellular and trans-synaptic regulation of glutamatergic synaptogenesis by EphB receptors." J Neurosci **26**(47): 12152-12164.

Kayser, M. S., M. J. Nolt and M. B. Dalva (2008). "EphB receptors couple dendritic filopodia motility to synapse formation." Neuron **59**(1): 56-69.

Khelfaoui, M., A. Pavlowsky, A. D. Powell, P. Valnegri, K. W. Cheong, Y. Blandin, M. Passafaro, J. G. Jefferys, J. Chelly and P. Billuart (2009). "Inhibition of RhoA pathway rescues the endocytosis defects in Oligophrenin1 mouse model of mental retardation." Hum Mol Genet **18**(14): 2575-2583.

Kim, J. Y., M. H. Oh, L. P. Bernard, I. G. Macara and H. Zhang (2011). "The RhoG/ELMO1/Dock180 signaling module is required for spine morphogenesis in hippocampal neurons." J Biol Chem **286**(43): 37615-37624.

Kimura, K., M. Ito, M. Amano, K. Chihara, Y. Fukata, M. Nakafuku, B. Yamamori, J. Feng, T. Nakano, K. Okawa, A. Iwamatsu and K. Kaibuchi (1996). "Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase)." Science **273**(5272): 245-248.

Klein, R. (2009). "Bidirectional modulation of synaptic functions by Eph/ephrin signaling." Nat Neurosci **12**(1): 15-20.

Klein, R. (2012). "Eph/ephrin signalling during development." Development **139**(22): 4105-4109.

Knaus, U. G., Y. Wang, A. M. Reilly, D. Warnock and J. H. Jackson (1998). "Structural requirements for PAK activation by Rac GTPases." J Biol Chem **273**(34): 21512-21518.

Knoll, B. and U. Drescher (2002). "Ephrin-As as receptors in topographic projections." Trends Neurosci **25**(3): 145-149.

Knoll, B., S. Isenmann, E. Kilic, J. Walkenhorst, S. Engel, J. Wehinger, M. Bahr and U. Drescher (2001). "Graded expression patterns of ephrin-As in the superior colliculus after lesion of the adult mouse optic nerve." Mech Dev **106**(1-2): 119-127.

Koivusalo, M., C. Welch, H. Hayashi, C. C. Scott, M. Kim, T. Alexander, N. Touret, K. M. Hahn and S. Grinstein (2010). "Amiloride inhibits macropinocytosis by lowering submembranous pH and preventing Rac1 and Cdc42 signaling." J Cell Biol **188**(4): 547-563.

Konigs, V., R. Jennings, T. Vogl, M. Horsthemke, A. C. Bachg, Y. Xu, K. Grobe, C. Brakebusch, A. Schwab, M. Bahler, U. G. Knaus and P. J. Hanley (2014). "Mouse macrophages completely lacking Rho subfamily GTPases (RhoA, RhoB, and RhoC) have severe lamellipodial retraction defects, but robust chemotactic navigation and altered motility." J Biol Chem **289**(44): 30772-30784.

Korus, M., G. M. Mahon, L. Cheng and I. P. Whitehead (2002). "p38 MAPK-mediated activation of NF-kappaB by the RhoGEF domain of Bcr." Oncogene **21**(30): 4601-4612.

Krajewska, W. M. and I. Maslowska (2004). "Caveolins: structure and function in signal transduction." Cell Mol Biol Lett **9**(2): 195-220.

Kramer, E. R., L. Knott, F. Su, E. Dessaud, C. E. Krull, F. Helmbacher and R. Klein (2006). "Cooperation between GDNF/Ret and ephrinA/EphA4 signals for motor-axon pathway selection in the limb." Neuron **50**(1): 35-47.

Kullander, K., S. J. Butt, J. M. Lebret, L. Lundfald, C. E. Restrepo, A. Rydstrom, R. Klein and O. Kiehn (2003). "Role of EphA4 and EphrinB3 in local neuronal circuits that control walking." Science **299**(5614): 1889-1892.

Kullander, K., S. D. Croll, M. Zimmer, L. Pan, J. McClain, V. Hughes, S. Zabski, T. M. DeChiara, R. Klein, G. D. Yancopoulos and N. W. Gale (2001a). "Ephrin-B3 is the midline barrier that prevents corticospinal tract axons from recrossing, allowing for unilateral motor control." Genes Dev **15**(7): 877-888.

Kullander, K., N. K. Mather, F. Diella, M. Dottori, A. W. Boyd and R. Klein (2001b). "Kinase-dependent and kinase-independent functions of EphA4 receptors in major axon tract formation in vivo." Neuron **29**(1): 73-84.

Kumari, S., V. Borroni, A. Chaudhry, B. Chanda, R. Massol, S. Mayor and F. J. Barrantes (2008). "Nicotinic acetylcholine receptor is internalized via a Rac-dependent, dynamin-independent endocytic pathway." J Cell Biol **181**(7): 1179-1193.

Kumari, S. and S. Mayor (2008). "ARF1 is directly involved in dynamin-independent endocytosis." Nat Cell Biol **10**(1): 30-41.

Kunita, R., A. Otomo, H. Mizumura, K. Suzuki-Utsunomiya, S. Hadano and J. E. Ikeda (2007). "The Rab5 activator ALS2/alsin acts as a novel Rac1 effector through Rac1-activated endocytosis." J Biol Chem **282**(22): 16599-16611.

Labrador, J. P., R. Brambilla and R. Klein (1997). "The N-terminal globular domain of Eph receptors is sufficient for ligand binding and receptor signaling." EMBO J **16**(13): 3889-3897.

Lackmann, M., R. J. Mann, L. Kravets, F. M. Smith, T. A. Bucci, K. F. Maxwell, G. J. Howlett, J. E. Olsson, T. Vanden Bos, D. P. Cerretti and A. W. Boyd (1997). "Ligand for EPH-related kinase (LERK) 7 is the preferred high affinity ligand for the HEK receptor." J Biol Chem **272**(26): 16521-16530.

Lackmann, M., A. C. Oates, M. Dottori, F. M. Smith, C. Do, M. Power, L. Kravets and A. W. Boyd (1998). "Distinct subdomains of the EphA3 receptor mediate ligand binding and receptor dimerization." J Biol Chem **273**(32): 20228-20237.

Lamarche, N. and A. Hall (1994). "GAPs for rho-related GTPases." Trends Genet **10**(12): 436-440.

Lamaze, C., T. H. Chuang, L. J. Terlecky, G. M. Bokoch and S. L. Schmid (1996). "Regulation of receptor-mediated endocytosis by Rho and Rac." Nature **382**(6587): 177-179.

Lammers, M., S. Meyer, D. Kuhlmann and A. Wittinghofer (2008). "Specificity of interactions between mDia isoforms and Rho proteins." J Biol Chem **283**(50): 35236-35246.

Larkin, J. M., M. S. Brown, J. L. Goldstein and R. G. Anderson (1983). "Depletion of intracellular potassium arrests coated pit formation and receptor-mediated endocytosis in fibroblasts." Cell **33**(1): 273-285.

Laurin, M. and J. F. Cote (2014). "Insights into the biological functions of Dock family guanine nucleotide exchange factors." Genes Dev **28**(6): 533-547.

Lauterbach, J. and R. Klein (2006). "Release of full-length EphB2 receptors from hippocampal neurons to cocultured glial cells." J Neurosci **26**(45): 11575-11581.

Lehmann, M., A. Fournier, I. Selles-Navarro, P. Dergham, A. Sebok, N. Leclerc, G. Tigyi and L. McKerracher (1999). "Inactivation of Rho signaling pathway promotes CNS axon regeneration." J Neurosci **19**(17): 7537-7547.

Lemmon, M. A. and K. M. Ferguson (2000). "Signal-dependent membrane targeting by pleckstrin homology (PH) domains." Biochem J **350 Pt 1**: 1-18.

Leung, T., E. Manser, L. Tan and L. Lim (1995). "A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes." J Biol Chem **270**(49): 29051-29054.

Li, D., L. Shao, B. C. Chen, X. Zhang, M. Zhang, B. Moses, D. E. Milkie, J. R. Beach, J. A. Hammer, 3rd, M. Pasham, T. Kirchhausen, M. A. Baird, M. W. Davidson, P. Xu and E. Betzig (2015). "ADVANCED IMAGING. Extended-resolution structured illumination imaging of endocytic and cytoskeletal dynamics." Science **349**(6251): aab3500.

Li, X., X. Bu, B. Lu, H. Avraham, R. A. Flavell and B. Lim (2002). "The hematopoiesis-specific GTP-binding protein RhoH is GTPase deficient and modulates activities of other Rho GTPases by an inhibitory function." Mol Cell Biol **22**(4): 1158-1171.

Liberali, P., E. Kakkonen, G. Turacchio, C. Valente, A. Spaar, G. Perinetti, R. A. Bockmann, D. Corda, A. Colanzi, V. Marjomaki and A. Luini (2008). "The closure of Pak1-dependent macropinosomes requires the phosphorylation of CtBP1/BARS." EMBO J **27**(7): 970-981.

Lim, B. K., N. Matsuda and M. M. Poo (2008a). "Ephrin-B reverse signaling promotes structural and functional synaptic maturation in vivo." Nat Neurosci **11**(2): 160-169.

Lim, Y. S., T. McLaughlin, T. C. Sung, A. Santiago, K. F. Lee and D. D. O'Leary (2008b). "p75(NTR) mediates ephrin-A reverse signaling required for axon repulsion and mapping." Neuron **59**(5): 746-758.

Lin, K. T., S. Sloniowski, D. W. Ethell and I. M. Ethell (2008). "Ephrin-B2-induced cleavage of EphB2 receptor is mediated by matrix metalloproteinases to trigger cell repulsion." J Biol Chem **283**(43): 28969-28979.

Litterst, C., A. Georgakopoulos, J. Shioi, E. Ghersi, T. Wisniewski, R. Wang, A. Ludwig and N. K. Robakis (2007). "Ligand binding and calcium influx induce distinct ectodomain/gamma-secretase-processing pathways of EphB2 receptor." J Biol Chem **282**(22): 16155-16163.

Liu, H., T. Nakazawa, T. Tezuka and T. Yamamoto (2006). "Physical and functional interaction of Fyn tyrosine kinase with a brain-enriched Rho GTPase-activating protein TCGAP." J Biol Chem **281**(33): 23611-23619.

Lu, M., J. M. Kinchen, K. L. Rossman, C. Grimsley, C. deBakker, E. Brugnera, A. C. Tosello-Trampont, L. B. Haney, D. Klingele, J. Sondek, M. O. Hengartner and K. S. Ravichandran (2004). "PH domain of ELMO functions in trans to regulate Rac activation via Dock180." Nat Struct Mol Biol **11**(8): 756-762.

Lu, Q., E. E. Sun, R. S. Klein and J. G. Flanagan (2001). "Ephrin-B reverse signaling is mediated by a novel PDZ-RGS protein and selectively inhibits G protein-coupled chemoattraction." Cell **105**(1): 69-79.

Lundmark, R., G. J. Doherty, M. T. Howes, K. Cortese, Y. Vallis, R. G. Parton and H. T. McMahon (2008). "The GTPase-activating protein GRAF1 regulates the CLIC/GEEC endocytic pathway." Curr Biol **18**(22): 1802-1808.

Machacek, M., L. Hodgson, C. Welch, H. Elliott, O. Pertz, P. Nalbant, A. Abell, G. L. Johnson, K. M. Hahn and G. Danuser (2009). "Coordination of Rho GTPase activities during cell protrusion." Nature **461**(7260): 99-103.

Machesky, L. M. and A. Hall (1997). "Role of actin polymerization and adhesion to extracellular matrix in Rac- and Rho-induced cytoskeletal reorganization." J Cell Biol **138**(4): 913-926.

Maekawa, M., T. Ishizaki, S. Boku, N. Watanabe, A. Fujita, A. Iwamatsu, T. Obinata, K. Ohashi, K. Mizuno and S. Narumiya (1999). "Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase." Science **285**(5429): 895-898.

Makinen, T., R. H. Adams, J. Bailey, Q. Lu, A. Ziemiecki, K. Alitalo, R. Klein and G. A. Wilkinson (2005). "PDZ interaction site in ephrinB2 is required for the remodeling of lymphatic vasculature." Genes Dev **19**(3): 397-410.

Malaval, C., M. Laffargue, R. Barbaras, C. Rolland, C. Peres, E. Champagne, B. Perret, F. Terce, X. Collet and L. O. Martinez (2009). "RhoA/ROCK I signalling downstream of the P2Y13 ADP-receptor controls HDL endocytosis in human hepatocytes." Cell Signal **21**(1): 120-127.

Manes, S., R. Ana Lacalle, C. Gomez-Mouton and A. C. Martinez (2003). "From rafts to crafts: membrane asymmetry in moving cells." Trends Immunol **24**(6): 320-326.

Mann, F., E. Miranda, C. Weinl, E. Harmer and C. E. Holt (2003). "B-type Eph receptors and ephrins induce growth cone collapse through distinct intracellular pathways." J Neurobiol **57**(3): 323-336.

Manser, E., C. Chong, Z. S. Zhao, T. Leung, G. Michael, C. Hall and L. Lim (1995). "Molecular cloning of a new member of the p21-Cdc42/Rac-activated kinase (PAK) family." J Biol Chem **270**(42): 25070-25078.

Manser, E., T. Leung, H. Salihuddin, Z. S. Zhao and L. Lim (1994). "A brain serine/threonine protein kinase activated by Cdc42 and Rac1." Nature **367**(6458): 40-46.

Margolis, S. S., J. Salogiannis, D. M. Lipton, C. Mandel-Brehm, Z. P. Wills, A. R. Mardinly, L. Hu, P. L. Greer, J. B. Bikoff, H. Y. Ho, M. J. Soskis, M. Sahin and M. E. Greenberg (2010). "EphB-mediated degradation of the RhoA GEF Ephexin5 relieves a developmental brake on excitatory synapse formation." Cell **143**(3): 442-455.

Marler, K. J., E. Becker-Barroso, A. Martinez, M. Llovera, C. Wentzel, S. Poopalasundaram, R. Hindges, E. Soriano, J. Comella and U. Drescher (2008). "A TrkB/EphrinA interaction controls retinal axon branching and synaptogenesis." J Neurosci **28**(48): 12700-12712.

Marquardt, T., R. Shirasaki, S. Ghosh, S. E. Andrews, N. Carter, T. Hunter and S. L. Pfaff (2005). "Coexpressed EphA receptors and ephrin-A ligands mediate opposing actions on growth cone navigation from distinct membrane domains." Cell **121**(1): 127-139.

Marsh, M. and A. Helenius (2006). "Virus entry: open sesame." Cell **124**(4): 729-740.

Marston, D. J., S. Dickinson and C. D. Nobes (2003). "Rac-dependent trans-endocytosis of ephrinBs regulates Eph-ephrin contact repulsion." Nat Cell Biol **5**(10): 879-888.

Massol, P., P. Montcourrier, J. C. Guillemot and P. Chavrier (1998). "Fc receptor-mediated phagocytosis requires CDC42 and Rac1." EMBO J **17**(21): 6219-6229.

Matsuo, N., M. Hoshino, M. Yoshizawa and Y. Nabeshima (2002). "Characterization of STEF, a guanine nucleotide exchange factor for Rac1, required for neurite growth." J Biol Chem **277**(4): 2860-2868.

Matsuo, N., M. Terao, Y. Nabeshima and M. Hoshino (2003). "Roles of STEF/Tiam1, guanine nucleotide exchange factors for Rac1, in regulation of growth cone morphology." Mol Cell Neurosci **24**(1): 69-81.

Maxfield, F. R. (2014). "Role of endosomes and lysosomes in human disease." Cold Spring Harb Perspect Biol **6**(5): a016931.

McClelland, A. C., S. I. Sheffler-Collins, M. S. Kayser and M. B. Dalva (2009). "Ephrin-B1 and ephrin-B2 mediate EphB-dependent presynaptic development via syntenin-1." Proc Natl Acad Sci U S A **106**(48): 20487-20492.

McMahon, H. T. and E. Boucrot (2011). "Molecular mechanism and physiological functions of clathrin-mediated endocytosis." Nat Rev Mol Cell Biol **12**(8): 517-533.

Meller, N., M. Irani-Tehrani, B. I. Ratnikov, B. M. Paschal and M. A. Schwartz (2004). "The novel Cdc42 guanine nucleotide exchange factor, zizimin1, dimerizes via the Cdc42-binding CZH2 domain." J Biol Chem **279**(36): 37470-37476.

Mellitzer, G., Q. Xu and D. G. Wilkinson (1999). "Eph receptors and ephrins restrict cell intermingling and communication." Nature **400**(6739): 77-81.

Mendes, S. W., M. Henkemeyer and D. J. Liebl (2006). "Multiple Eph receptors and B-class ephrins regulate midline crossing of corpus callosum fibers in the developing mouse forebrain." J Neurosci **26**(3): 882-892.

Merrifield, C. J., M. E. Feldman, L. Wan and W. Almers (2002). "Imaging actin and dynamin recruitment during invagination of single clathrin-coated pits." Nat Cell Biol **4**(9): 691-698.

Merrifield, C. J., S. E. Moss, C. Ballestrem, B. A. Imhof, G. Giese, I. Wunderlich and W. Almers (1999). "Endocytic vesicles move at the tips of actin tails in cultured mast cells." Nat Cell Biol **1**(1): 72-74.

Michaelson, D., J. Silletti, G. Murphy, P. D'Eustachio, M. Rush and M. R. Philips (2001). "Differential localization of Rho GTPases in live cells: regulation by hypervariable regions and RhoGDI binding." J Cell Biol **152**(1): 111-126.

Minoshima, Y., T. Kawashima, K. Hirose, Y. Tonozuka, A. Kawajiri, Y. C. Bao, X. Deng, M. Tatsuka, S. Narumiya, W. S. May, Jr., T. Nosaka, K. Semba, T. Inoue, T. Satoh, M. Inagaki and T. Kitamura (2003). "Phosphorylation by aurora B converts MgcRacGAP to a RhoGAP during cytokinesis." Dev Cell **4**(4): 549-560.

Miyamoto, Y., J. Yamauchi, A. Tanoue, C. Wu and W. C. Mobley (2006). "TrkB binds and tyrosine-phosphorylates Tiam1, leading to activation of Rac1 and induction of changes in cellular morphology." Proc Natl Acad Sci U S A **103**(27): 10444-10449.

Moon, S. Y., H. Zang and Y. Zheng (2003). "Characterization of a brain-specific Rho GTPase-activating protein, p200RhoGAP." J Biol Chem **278**(6): 4151-4159.

Mooren, O. L., B. J. Galletta and J. A. Cooper (2012). "Roles for actin assembly in endocytosis." Annu Rev Biochem **81**: 661-686.

Motley, A., N. A. Bright, M. N. Seaman and M. S. Robinson (2003). "Clathrin-mediated endocytosis in AP-2-depleted cells." J Cell Biol **162**(5): 909-918.

Murai, K. K., L. N. Nguyen, F. Irie, Y. Yamaguchi and E. B. Pasquale (2003). "Control of hippocampal dendritic spine morphology through ephrin-A3/EphA4 signaling." Nat Neurosci **6**(2): 153-160.

Murphy, C., R. Saffrich, M. Grummt, H. Gournier, V. Rybin, M. Rubino, P. Auvinen, A. Lutcke, R. G. Parton and M. Zerial (1996). "Endosome dynamics regulated by a Rho protein." Nature **384**(6608): 427-432.

Murphy, G. A., S. A. Jillian, D. Michaelson, M. R. Philips, P. D'Eustachio and M. G. Rush (2001). "Signaling mediated by the closely related mammalian Rho family GTPases TC10 and Cdc42 suggests distinct functional pathways." Cell Growth Differ **12**(3): 157-167.

Murphy, G. A., P. A. Solski, S. A. Jillian, P. Perez de la Ossa, P. D'Eustachio, C. J. Der and M. G. Rush (1999). "Cellular functions of TC10, a Rho family GTPase: regulation of morphology, signal transduction and cell growth." Oncogene **18**(26): 3831-3845.

Nakamura, T., M. Komiya, K. Sone, E. Hirose, N. Gotoh, H. Morii, Y. Ohta and N. Mori (2002). "Grit, a GTPase-activating protein for the Rho family, regulates neurite extension through association with the TrkA receptor and N-Shc and CrkL/Crk adapter molecules." Mol Cell Biol **22**(24): 8721-8734.

Nakayama, A., M. Nakayama, C. J. Turner, S. Hoing, J. J. Lepore and R. H. Adams (2013). "Ephrin-B2 controls PDGFRbeta internalization and signaling." Genes Dev **27**(23): 2576-2589.

Namekata, K., H. Watanabe, X. Guo, D. Kittaka, K. Kawamura, A. Kimura, C. Harada and T. Harada (2012). "Dock3 regulates BDNF-TrkB signaling for neurite outgrowth by forming a ternary complex with Elmo and RhoG." Genes Cells **17**(8): 688-697.

Naslavsky, N., R. Weigert and J. G. Donaldson (2004). "Characterization of a nonclathrin endocytic pathway: membrane cargo and lipid requirements." Mol Biol Cell **15**(8): 3542-3552.

Neudauer, C. L., G. Joberty, N. Tatsis and I. G. Macara (1998). "Distinct cellular effects and interactions of the Rho-family GTPase TC10." Curr Biol **8**(21): 1151-1160.

Newey, S. E., V. Velamoor, E. E. Govek and L. Van Aelst (2005). "Rho GTPases, dendritic structure, and mental retardation." J Neurobiol **64**(1): 58-74.

Nikolic, M. (2008). "The Pak1 kinase: an important regulator of neuronal morphology and function in the developing forebrain." Mol Neurobiol **37**(2-3): 187-202.

Nimnual, A. S., L. J. Taylor and D. Bar-Sagi (2003). "Redox-dependent downregulation of Rho by Rac." Nat Cell Biol **5**(3): 236-241.

Nishimura, T., T. Yamaguchi, A. Tokunaga, A. Hara, T. Hamaguchi, K. Kato, A. Iwamatsu, H. Okano and K. Kaibuchi (2006). "Role of numb in dendritic spine development with a Cdc42 GEF intersectin and EphB2." Mol Biol Cell **17**(3): 1273-1285.

Nishimura, Y., K. Itoh, K. Yoshioka, K. Ikeda and M. Himeno (2002). "A role for small GTPase RhoA in regulating intracellular membrane traffic of lysosomes in invasive rat hepatoma cells." Histochem J **34**(5): 189-213.

Nobes, C. D. and A. Hall (1995). "Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia." Cell **81**(1): 53-62.

Nobes, C. D., I. Lauritzen, M. G. Mattei, S. Paris, A. Hall and P. Chardin (1998). "A new member of the Rho family, Rnd1, promotes disassembly of actin filament structures and loss of cell adhesion." J Cell Biol **141**(1): 187-197.

Ogita, H., S. Kunimoto, Y. Kamioka, H. Sawa, M. Masuda and N. Mochizuki (2003). "EphA4-mediated Rho activation via Vsm-RhoGEF expressed specifically in vascular smooth muscle cells." Circ Res **93**(1): 23-31.

Oh, D., S. Han, J. Seo, J. R. Lee, J. Choi, J. Groffen, K. Kim, Y. S. Cho, H. S. Choi, H. Shin, J. Woo, H. Won, S. K. Park, S. Y. Kim, J. Jo, D. J. Whitcomb, K. Cho, H. Kim, Y. C. Bae, N. Heisterkamp, S. Y. Choi and E. Kim (2010). "Regulation of synaptic Rac1 activity, long-term potentiation maintenance, and learning and memory by BCR and ABR Rac GTPase-activating proteins." J Neurosci **30**(42): 14134-14144.

Ohta, Y., J. H. Hartwig and T. P. Stossel (2006). "FilGAP, a Rho- and ROCK-regulated GAP for Rac binds filamin A to control actin remodelling." Nat Cell Biol **8**(8): 803-814.

Oinuma, I., Y. Ishikawa, H. Katoh and M. Negishi (2004a). "The Semaphorin 4D receptor Plexin-B1 is a GTPase activating protein for R-Ras." Science **305**(5685): 862-865.

Oinuma, I., H. Katoh and M. Negishi (2004b). "Molecular dissection of the semaphorin 4D receptor plexin-B1-stimulated R-Ras GTPase-activating protein activity and neurite remodeling in hippocampal neurons." J Neurosci **24**(50): 11473-11480.

Okabe, T., T. Nakamura, Y. N. Nishimura, K. Kohu, S. Ohwada, Y. Morishita and T. Akiyama (2003). "RICS, a novel GTPase-activating protein for Cdc42 and Rac1, is involved in the beta-catenin-N-cadherin and N-methyl-D-aspartate receptor signaling." J Biol Chem **278**(11): 9920-9927.

Olofsson, B. (1999). "Rho guanine dissociation inhibitors: pivotal molecules in cellular signalling." Cell Signal **11**(8): 545-554.

Orth, J. D., E. W. Krueger, S. G. Weller and M. A. McNiven (2006). "A novel endocytic mechanism of epidermal growth factor receptor sequestration and internalization." Cancer Res **66**(7): 3603-3610.

Orth, J. D. and M. A. McNiven (2006). "Get off my back! Rapid receptor internalization through circular dorsal ruffles." Cancer Res **66**(23): 11094-11096.

Otal, R., F. Burgaya, J. Frisen, E. Soriano and A. Martinez (2006). "Ephrin-A5 modulates the topographic mapping and connectivity of commissural axons in murine hippocampus." Neuroscience **141**(1): 109-121.

Otomo, A., R. Kunita, K. Suzuki-Utsunomiya, J. E. Ikeda and S. Hadano (2011). "Defective relocalization of ALS2/alsin missense mutants to Rac1-induced macropinosomes accounts for loss of their cellular function and leads to disturbed amphisome formation." FEBS Lett **585**(5): 730-736.

Otomo, A., R. Kunita, K. Suzuki-Utsunomiya, H. Mizumura, K. Onoe, H. Osuga, S. Hadano and J. E. Ikeda (2008). "ALS2/alsin deficiency in neurons leads to mild defects in macropinocytosis and axonal growth." Biochem Biophys Res Commun **370**(1): 87-92.

Overholtzer, M., A. A. Mailleux, G. Mouneimne, G. Normand, S. J. Schnitt, R. W. King, E. S. Cibas and J. S. Brugge (2007). "A nonapoptotic cell death process, entosis, that occurs by cell-in-cell invasion." Cell **131**(5): 966-979.

Pacary, E., J. Heng, R. Azzarelli, P. Riou, D. Castro, M. Lebel-Potter, C. Parras, D. M. Bell, A. J. Ridley, M. Parsons and F. Guillemot (2011). "Proneural transcription factors regulate different steps of cortical neuron migration through Rnd-mediated inhibition of RhoA signaling." Neuron **69**(6): 1069-1084.

Paixao, S., A. Balijepalli, N. Serradj, J. Niu, W. Luo, J. H. Martin and R. Klein (2013). "EphrinB3/EphA4-mediated guidance of ascending and descending spinal tracts." Neuron **80**(6): 1407-1420.

Palamidessi, A., E. Frittoli, M. Garre, M. Faretta, M. Mione, I. Testa, A. Diaspro, L. Lanzetti, G. Scita and P. P. Di Fiore (2008). "Endocytic trafficking of Rac is required for the spatial restriction of signaling in cell migration." Cell **134**(1): 135-147.

Paleotti, O., E. Macia, F. Luton, S. Klein, M. Partisani, P. Chardin, T. Kirchhausen and M. Franco (2005). "The small G-protein Arf6GTP recruits the AP-2 adaptor complex to membranes." J Biol Chem **280**(22): 21661-21666.

Palmer, A., M. Zimmer, K. S. Erdmann, V. Eulenburg, A. Porthin, R. Heumann, U. Deutsch and R. Klein (2002). "EphrinB phosphorylation and reverse signaling: regulation by Src kinases and PTP-BL phosphatase." Mol Cell **9**(4): 725-737.

Park, E. K., N. Warner, Y. S. Bong, D. Stapleton, R. Maeda, T. Pawson and I. O. Daar (2004). "Ectopic EphA4 receptor induces posterior protrusions via FGF signaling in Xenopus embryos." Mol Biol Cell **15**(4): 1647-1655.

Park, W. Y., J. S. Park, K. A. Cho, D. I. Kim, Y. G. Ko, J. S. Seo and S. C. Park (2000). "Up-regulation of caveolin attenuates epidermal growth factor signaling in senescent cells." J Biol Chem **275**(27): 20847-20852.

Parker, M., R. Roberts, M. Enriquez, X. Zhao, T. Takahashi, D. Pat Cerretti, T. Daniel and J. Chen (2004). "Reverse endocytosis of transmembrane ephrin-B ligands via a clathrin-mediated pathway." Biochem Biophys Res Commun **323**(1): 17-23.

Parks, A. L., K. M. Klueg, J. R. Stout and M. A. Muskavitch (2000). "Ligand endocytosis drives receptor dissociation and activation in the Notch pathway." Development **127**(7): 1373-1385.

Parri, M. and P. Chiarugi (2010). "Rac and Rho GTPases in cancer cell motility control." Cell Commun Signal **8**: 23.

Parsons, J. T., A. R. Horwitz and M. A. Schwartz (2010). "Cell adhesion: integrating cytoskeletal dynamics and cellular tension." Nat Rev Mol Cell Biol **11**(9): 633-643.

Parton, R. G. and K. Simons (2007). "The multiple faces of caveolae." Nat Rev Mol Cell Biol **8**(3): 185-194.

Pasquale, E. B. (2008). "Eph-ephrin bidirectional signaling in physiology and disease." Cell **133**(1): 38-52.

Pasquale, E. B. (2010). "Eph receptors and ephrins in cancer: bidirectional signalling and beyond." Nat Rev Cancer **10**(3): 165-180.

Patel, M. and A. V. Karginov (2014). "Phosphorylation-mediated regulation of GEFs for RhoA." Cell Adh Migr **8**(1): 11-18.

Patel, M., A. Pelletier and J. F. Cote (2011). "Opening up on ELMO regulation: New insights into the control of Rac signaling by the DOCK180/ELMO complex." Small GTPases **2**(5): 268-275.

Pearce, A. C., Y. A. Senis, D. D. Billadeau, M. Turner, S. P. Watson and E. Vigorito (2004). "Vav1 and vav3 have critical but redundant roles in mediating platelet activation by collagen." J Biol Chem **279**(52): 53955-53962.

Peck, J., G. t. Douglas, C. H. Wu and P. D. Burbelo (2002). "Human RhoGAP domain-containing proteins: structure, function and evolutionary relationships." FEBS Lett **528**(1-3): 27-34.

Pelkmans, L., T. Burli, M. Zerial and A. Helenius (2004). "Caveolin-stabilized membrane domains as multifunctional transport and sorting devices in endocytic membrane traffic." Cell **118**(6): 767-780.

Peng, J., B. J. Wallar, A. Flanders, P. J. Swiatek and A. S. Alberts (2003). "Disruption of the Diaphanous-related formin Drf1 gene encoding mDia1 reveals a role for Drf3 as an effector for Cdc42." Curr Biol **13**(7): 534-545.

Penzes, P., A. Beeser, J. Chernoff, M. R. Schiller, B. A. Eipper, R. E. Mains and R. L. Huganir (2003). "Rapid induction of dendritic spine morphogenesis by trans-synaptic ephrinB-EphB receptor activation of the Rho-GEF kalirin." Neuron **37**(2): 263-274.

Penzes, P., R. C. Johnson, M. R. Alam, V. Kambampati, R. E. Mains and B. A. Eipper (2000). "An isoform of kalirin, a brain-specific GDP/GTP exchange factor, is enriched in the postsynaptic density fraction." J Biol Chem **275**(9): 6395-6403.

Penzes, P., R. C. Johnson, R. Sattler, X. Zhang, R. L. Huganir, V. Kambampati, R. E. Mains and B. A. Eipper (2001). "The neuronal Rho-GEF Kalirin-7 interacts with PDZ domain-containing proteins and regulates dendritic morphogenesis." Neuron **29**(1): 229-242.

Pertz, O. (2010). "Spatio-temporal Rho GTPase signaling - where are we now?" J Cell Sci **123**(Pt 11): 1841-1850.

Peter, B. J., H. M. Kent, I. G. Mills, Y. Vallis, P. J. Butler, P. R. Evans and H. T. McMahon (2004). "BAR domains as sensors of membrane curvature: the amphiphysin BAR structure." Science **303**(5657): 495-499.

Pitulescu, M. E. and R. H. Adams (2014). "Regulation of signaling interactions and receptor endocytosis in growing blood vessels." Cell Adh Migr **8**(4): 366-377.

Poliakov, A., M. Cotrina and D. G. Wilkinson (2004). "Diverse roles of eph receptors and ephrins in the regulation of cell migration and tissue assembly." Dev Cell **7**(4): 465-480.

Poopalasundaram, S., K. J. Marler and U. Drescher (2011). "EphrinA6 on chick retinal axons is a key component for p75(NTR)-dependent axon repulsion and TrkB-dependent axon branching." Mol Cell Neurosci **47**(2): 131-136.

Praefcke, G. J. and H. T. McMahon (2004). "The dynamin superfamily: universal membrane tubulation and fission molecules?" Nat Rev Mol Cell Biol **5**(2): 133-147.

Preudhomme, C., C. Roumier, M. P. Hildebrand, E. Dallery-Prudhomme, D. Lantoine, J. L. Lai, A. Daudignon, C. Adenis, F. Bauters, P. Fenaux, J. P. Kerckaert and S. Galiegue-Zouitina (2000). "Nonrandom 4p13 rearrangements of the RhoH/TTF gene, encoding a GTP-binding protein, in non-Hodgkin's lymphoma and multiple myeloma." Oncogene **19**(16): 2023-2032.

Qin, H., R. Noberini, X. Huan, J. Shi, E. B. Pasquale and J. Song (2010). "Structural characterization of the EphA4-Ephrin-B2 complex reveals new features enabling Eph-ephrin binding promiscuity." J Biol Chem **285**(1): 644-654.

Qualmann, B. and M. M. Kessels (2002). "Endocytosis and the cytoskeleton." Int Rev Cytol **220**: 93-144.

Qualmann, B. and H. Mellor (2003). "Regulation of endocytic traffic by Rho GTPases." Biochem J **371**(Pt 2): 233-241.

Razzini, G., A. Brancaccio, M. A. Lemmon, S. Guarnieri and M. Falasca (2000). "The role of the pleckstrin homology domain in membrane targeting and activation of phospholipase Cbeta(1)." J Biol Chem **275**(20): 14873-14881.

Riccio, A., B. A. Pierchala, C. L. Ciarallo and D. D. Ginty (1997). "An NGF-TrkA-mediated retrograde signal to transcription factor CREB in sympathetic neurons." Science **277**(5329): 1097-1100.

Ridley, A. J. (2006). "Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking." Trends Cell Biol **16**(10): 522-529.

Ridley, A. J. and A. Hall (1992a). "Distinct patterns of actin organization regulated by the small GTP-binding proteins Rac and Rho." Cold Spring Harb Symp Quant Biol **57**: 661-671.

Ridley, A. J. and A. Hall (1992b). "The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors." Cell **70**(3): 389-399.

Ridley, A. J., H. F. Paterson, C. L. Johnston, D. Diekmann and A. Hall (1992). "The small GTP-binding protein rac regulates growth factor-induced membrane ruffling." Cell **70**(3): 401-410.

Ridley, A. J., A. J. Self, F. Kasmi, H. F. Paterson, A. Hall, C. J. Marshall and C. Ellis (1993). "rho family GTPase activating proteins p190, bcr and rhoGAP show distinct specificities in vitro and in vivo." EMBO J **12**(13): 5151-5160.

Riedl, J., A. H. Crevenna, K. Kessenbrock, J. H. Yu, D. Neukirchen, M. Bista, F. Bradke, D. Jenne, T. A. Holak, Z. Werb, M. Sixt and R. Wedlich-Soldner (2008). "Lifeact: a versatile marker to visualize F-actin." Nat Methods **5**(7): 605-607.

Riento, K., N. Totty, P. Villalonga, R. Garg, R. Guasch and A. J. Ridley (2005). "RhoE function is regulated by ROCK I-mediated phosphorylation." EMBO J **24**(6): 1170-1180.

Rivero, F., H. Dislich, G. Glockner and A. A. Noegel (2001). "The Dictyostelium discoideum family of Rho-related proteins." Nucleic Acids Res **29**(5): 1068-1079.

Roberts, A. W., C. Kim, L. Zhen, J. B. Lowe, R. Kapur, B. Petryniak, A. Spaetti, J. D. Pollock, J. B. Borneo, G. B. Bradford, S. J. Atkinson, M. C. Dinauer and D. A. Williams (1999). "Deficiency of the hematopoietic cell-specific Rho family GTPase Rac2 is characterized by abnormalities in neutrophil function and host defense." Immunity **10**(2): 183-196.

Roberts, P. J., N. Mitin, P. J. Keller, E. J. Chenette, J. P. Madigan, R. O. Currin, A. D. Cox, O. Wilson, P. Kirschmeier and C. J. Der (2008). "Rho Family GTPase modification and dependence on CAAX motif-signaled posttranslational modification." J Biol Chem **283**(37): 25150-25163.

Rojas, R. J., M. E. Yohe, S. Gershburg, T. Kawano, T. Kozasa and J. Sondek (2007). "Galphaq directly activates p63RhoGEF and Trio via a conserved extension of the Dbl homology-associated pleckstrin homology domain." J Biol Chem **282**(40): 29201-29210.

Rondanino, C., R. Rojas, W. G. Ruiz, E. Wang, R. P. Hughey, K. W. Dunn and G. Apodaca (2007). "RhoB-dependent modulation of postendocytic traffic in polarized Madin-Darby canine kidney cells." Traffic **8**(7): 932-949.

Roof, R. W., M. D. Haskell, B. D. Dukes, N. Sherman, M. Kinter and S. J. Parsons (1998). "Phosphotyrosine (p-Tyr)-dependent and -independent mechanisms of p190 RhoGAP-p120 RasGAP interaction: Tyr 1105 of p190, a substrate for c-Src, is the sole p-Tyr mediator of complex formation." Mol Cell Biol **18**(12): 7052-7063.

Rossman, K. L., D. K. Worthylake, J. T. Snyder, D. P. Siderovski, S. L. Campbell and J. Sondek (2002). "A crystallographic view of interactions between Dbs and Cdc42: PH domain-assisted guanine nucleotide exchange." EMBO J **21**(6): 1315-1326.

Roth, T. F. and K. R. Porter (1964). "Yolk Protein Uptake in the Oocyte of the Mosquito Aedes Aegypti. L." J Cell Biol **20**: 313-332.

Royle, S. J. and L. Lagnado (2010). "Clathrin-mediated endocytosis at the synaptic terminal: bridging the gap between physiology and molecules." Traffic **11**(12): 1489-1497.

Rudolph, J., J. J. Crawford, K. P. Hoeflich and W. Wang (2015). "Inhibitors of p21-activated kinases (PAKs)." J Med Chem **58**(1): 111-129.

Runyan, C. E., Z. Liu and H. W. Schnaper (2012). "Phosphatidylinositol 3-kinase and Rab5 GTPase inversely regulate the Smad anchor for receptor activation (SARA) protein independently of transforming growth factor-beta1." J Biol Chem **287**(43): 35815-35824.

Sabet, O., R. Stockert, G. Xouri, Y. Bruggemann, A. Stanoev and P. I. Bastiaens (2015). "Ubiquitination switches EphA2 vesicular traffic from a continuous safeguard to a finite signalling mode." Nat Commun **6**: 8047.

Sabharanjak, S., P. Sharma, R. G. Parton and S. Mayor (2002). "GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway." Dev Cell **2**(4): 411-423.

Sahai, E. and C. J. Marshall (2002a). "RHO-GTPases and cancer." Nat Rev Cancer **2**(2): 133-142.

Sahai, E. and C. J. Marshall (2002b). "ROCK and Dia have opposing effects on adherens junctions downstream of Rho." Nat Cell Biol **4**(6): 408-415.

Sahin, M., P. L. Greer, M. Z. Lin, H. Poucher, J. Eberhart, S. Schmidt, T. M. Wright, S. M. Shamah, S. O'Connell, C. W. Cowan, L. Hu, J. L. Goldberg, A. Debant, G. Corfas, C. E. Krull and M. E. Greenberg (2005). "Eph-dependent tyrosine phosphorylation of ephexin1 modulates growth cone collapse." Neuron **46**(2): 191-204.

Sander, E. E., J. P. ten Klooster, S. van Delft, R. A. van der Kammen and J. G. Collard (1999). "Rac downregulates Rho activity: reciprocal balance between both GTPases determines cellular morphology and migratory behavior." J Cell Biol **147**(5): 1009-1022.

Sandvig, K., M. L. Torgersen, H. A. Raa and B. van Deurs (2008). "Clathrin-independent endocytosis: from nonexisting to an extreme degree of complexity." Histochem Cell Biol **129**(3): 267-276.

Sastry, S. K., Z. Rajfur, B. P. Liu, J. F. Cote, M. L. Tremblay and K. Burridge (2006). "PTP-PEST couples membrane protrusion and tail retraction via VAV2 and p190RhoGAP." J Biol Chem **281**(17): 11627-11636.

Sawamiphak, S., S. Seidel, C. L. Essmann, G. A. Wilkinson, M. E. Pitulescu, T. Acker and A. Acker-Palmer (2010). "Ephrin-B2 regulates VEGFR2 function in developmental and tumour angiogenesis." Nature **465**(7297): 487-491.

Saxena, S., C. L. Howe, J. M. Cosgaya, P. Steiner, H. Hirling, J. R. Chan, J. Weis and A. Kruttgen (2005). "Differential endocytic sorting of p75NTR and TrkA in response to NGF: a role for late endosomes in TrkA trafficking." Mol Cell Neurosci **28**(3): 571-587.

Schaupp, A., O. Sabet, I. Dudanova, M. Ponserre, P. Bastiaens and R. Klein (2014). "The composition of EphB2 clusters determines the strength in the cellular repulsion response." J Cell Biol **204**(3): 409-422.

Schindelin, J., I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J. Y. Tinevez, D. J. White, V. Hartenstein, K. Eliceiri, P. Tomancak and A. Cardona (2012). "Fiji: an open-source platform for biological-image analysis." Nat Methods **9**(7): 676-682.

Schmid, E. M., M. G. Ford, A. Burtey, G. J. Praefcke, S. Y. Peak-Chew, I. G. Mills, A. Benmerah and H. T. McMahon (2006). "Role of the AP2 beta-appendage hub in recruiting partners for clathrin-coated vesicle assembly." PLoS Biol **4**(9): e262.

Schmid, E. M. and H. T. McMahon (2007). "Integrating molecular and network biology to decode endocytosis." Nature **448**(7156): 883-888.

Schmidt, A. and A. Hall (2002a). "Guanine nucleotide exchange factors for Rho GTPases: turning on the switch." Genes Dev **16**(13): 1587-1609.

Schmidt, A. and A. Hall (2002b). "The Rho exchange factor Net1 is regulated by nuclear sequestration." J Biol Chem **277**(17): 14581-14588.

Scully, A. L., M. McKeown and J. B. Thomas (1999). "Isolation and characterization of Dek, a Drosophila eph receptor protein tyrosine kinase." Mol Cell Neurosci **13**(5): 337-347.

Segura, I., C. L. Essmann, S. Weinges and A. Acker-Palmer (2007). "Grb4 and GIT1 transduce ephrinB reverse signals modulating spine morphogenesis and synapse formation." Nat Neurosci **10**(3): 301-310.

Seiradake, E., K. Harlos, G. Sutton, A. R. Aricescu and E. Y. Jones (2010). "An extracellular steric seeding mechanism for Eph-ephrin signaling platform assembly." Nat Struct Mol Biol **17**(4): 398-402.

Seiradake, E., A. Schaupp, D. del Toro Ruiz, R. Kaufmann, N. Mitakidis, K. Harlos, A. R. Aricescu, R. Klein and E. Y. Jones (2013). "Structurally encoded intraclass differences in EphA clusters drive distinct cell responses." Nat Struct Mol Biol **20**(8): 958-964.

Sells, M. A., J. T. Boyd and J. Chernoff (1999). "p21-activated kinase 1 (Pak1) regulates cell motility in mammalian fibroblasts." J Cell Biol **145**(4): 837-849.

Sengar, A. S., J. Ellegood, A. P. Yiu, H. Wang, W. Wang, S. C. Juneja, J. P. Lerch, S. A. Josselyn, R. M. Henkelman, M. W. Salter and S. E. Egan (2013). "Vertebrate intersectin1 is repurposed to facilitate cortical midline connectivity and higher order cognition." J Neurosci **33**(9): 4055-4065.

Seoh, M. L., C. H. Ng, J. Yong, L. Lim and T. Leung (2003). "ArhGAP15, a novel human RacGAP protein with GTPase binding property." FEBS Lett **539**(1-3): 131-137.

Servitja, J. M., M. J. Marinissen, A. Sodhi, X. R. Bustelo and J. S. Gutkind (2003). "Rac1 function is required for Src-induced transformation. Evidence of a role for Tiam1 and Vav2 in Rac activation by Src." J Biol Chem **278**(36): 34339-34346.

Shamah, S. M., M. Z. Lin, J. L. Goldberg, S. Estrach, M. Sahin, L. Hu, M. Bazalakova, R. L. Neve, G. Corfas, A. Debant and M. E. Greenberg (2001). "EphA receptors regulate growth cone dynamics through the novel guanine nucleotide exchange factor ephexin." Cell **105**(2): 233-244.

Shao, Y., W. Akmentin, J. J. Toledo-Aral, J. Rosenbaum, G. Valdez, J. B. Cabot, B. S. Hilbush and S. Halegoua (2002). "Pincher, a pinocytic chaperone for nerve growth factor/TrkA signaling endosomes." J Cell Biol **157**(4): 679-691.

Sharfe, N., A. Freywald, A. Toro, H. Dadi and C. Roifman (2002). "Ephrin stimulation modulates T cell chemotaxis." Eur J Immunol **32**(12): 3745-3755.

Sharfe, N., A. Freywald, A. Toro and C. M. Roifman (2003). "Ephrin-A1 induces c-Cbl phosphorylation and EphA receptor down-regulation in T cells." J Immunol **170**(12): 6024-6032.

Shutes, A., A. C. Berzat, E. J. Chenette, A. D. Cox and C. J. Der (2006). "Biochemical analyses of the Wrch atypical Rho family GTPases." Methods Enzymol **406**: 11-26.

Shutes, A., C. Onesto, V. Picard, B. Leblond, F. Schweighoffer and C. J. Der (2007). "Specificity and mechanism of action of EHT 1864, a novel small molecule inhibitor of Rac family small GTPases." J Biol Chem **282**(49): 35666-35678.

Sigismund, S., E. Argenzio, D. Tosoni, E. Cavallaro, S. Polo and P. P. Di Fiore (2008). "Clathrin-mediated internalization is essential for sustained EGFR signaling but dispensable for degradation." Dev Cell **15**(2): 209-219.

Sigismund, S., T. Woelk, C. Puri, E. Maspero, C. Tacchetti, P. Transidico, P. P. Di Fiore and S. Polo (2005). "Clathrin-independent endocytosis of ubiquitinated cargos." Proc Natl Acad Sci U S A **102**(8): 2760-2765.

Smith, A., V. Robinson, K. Patel and D. G. Wilkinson (1997). "The EphA4 and EphB1 receptor tyrosine kinases and ephrin-B2 ligand regulate targeted migration of branchial neural crest cells." Curr Biol **7**(8): 561-570.

Smith, F. M., C. Vearing, M. Lackmann, H. Treutlein, J. Himanen, K. Chen, A. Saul, D. Nikolov and A. W. Boyd (2004). "Dissecting the EphA3/Ephrin-A5 interactions using a novel functional mutagenesis screen." J Biol Chem **279**(10): 9522-9531.

Song, J. (2003). "Tyrosine phosphorylation of the well packed ephrinB cytoplasmic beta-hairpin for reverse signaling. Structural consequences and binding properties." J Biol Chem **278**(27): 24714-24720.

Song, J., W. Vranken, P. Xu, R. Gingras, R. S. Noyce, Z. Yu, S. H. Shen and F. Ni (2002). "Solution structure and backbone dynamics of the functional cytoplasmic subdomain of human ephrin B2, a cell-surface ligand with bidirectional signaling properties." Biochemistry **41**(36): 10942-10949.

Sorkin, A. (2004). "Cargo recognition during clathrin-mediated endocytosis: a team effort." Curr Opin Cell Biol **16**(4): 392-399.

Soskis, M. J., H. Y. Ho, B. L. Bloodgood, M. A. Robichaux, A. N. Malik, B. Ataman, A. A. Rubin, J. Zieg, C. Zhang, K. M. Shokat, N. Sharma, C. W. Cowan and M. E. Greenberg (2012). "A chemical genetic approach reveals distinct EphB signaling mechanisms during brain development." Nat Neurosci **15**(12): 1645-1654.

Srougi, M. C. and K. Burridge (2011). "The nuclear guanine nucleotide exchange factors Ect2 and Net1 regulate RhoB-mediated cell death after DNA damage." PLoS One **6**(2): e17108.

Stankiewicz, T. R. and D. A. Linseman (2014). "Rho family GTPases: key players in neuronal development, neuronal survival, and neurodegeneration." Front Cell Neurosci **8**: 314.

Stebbins, C. C., C. Watzl, D. D. Billadeau, P. J. Leibson, D. N. Burshtyn and E. O. Long (2003). "Vav1 dephosphorylation by the tyrosine phosphatase SHP-1 as a mechanism for inhibition of cellular cytotoxicity." Mol Cell Biol **23**(17): 6291-6299.

Stein, E., A. A. Lane, D. P. Cerretti, H. O. Schoecklmann, A. D. Schroff, R. L. Van Etten and T. O. Daniel (1998). "Eph receptors discriminate specific ligand oligomers to determine alternative signaling complexes, attachment, and assembly responses." Genes Dev **12**(5): 667-678.

Stirling, L., M. R. Williams and A. D. Morielli (2009). "Dual roles for RHOA/RHO-kinase in the regulated trafficking of a voltage-sensitive potassium channel." Mol Biol Cell **20**(12): 2991-3002.

Stradal, T. E. and G. Scita (2006). "Protein complexes regulating Arp2/3-mediated actin assembly." Curr Opin Cell Biol **18**(1): 4-10.

Su, L., J. M. Agati and S. J. Parsons (2003). "p190RhoGAP is cell cycle regulated and affects cytokinesis." J Cell Biol **163**(3): 571-582.

Sugiyama, N., E. Gucciardo, O. Tatti, M. Varjosalo, M. Hyytiainen, M. Gstaiger and K. Lehti (2013). "EphA2 cleavage by MT1-MMP triggers single cancer cell invasion via homotypic cell repulsion." J Cell Biol **201**(3): 467-484.

Swanson, J. A., M. T. Johnson, K. Beningo, P. Post, M. Mooseker and N. Araki (1999). "A contractile activity that closes phagosomes in macrophages." J Cell Sci **112 (Pt 3)**: 307-316.

Swanson, J. A. and C. Watts (1995). "Macropinocytosis." Trends Cell Biol **5**(11): 424-428.

Symons, M., J. M. Derry, B. Karlak, S. Jiang, V. Lemahieu, F. McCormick, U. Francke and A. Abo (1996). "Wiskott-Aldrich syndrome protein, a novel effector for the GTPase CDC42Hs, is implicated in actin polymerization." Cell **84**(5): 723-734.

Tadesse, T., Q. Cheng, M. Xu, D. J. Baro, L. J. Young and S. L. Pallas (2013). "Regulation of ephrin-A expression in compressed retinocollicular maps." Dev Neurobiol **73**(4): 274-296.

Tagawa, A., A. Mezzacasa, A. Hayer, A. Longatti, L. Pelkmans and A. Helenius (2005). "Assembly and trafficking of caveolar domains in the cell: caveolae as stable, cargo-triggered, vesicular transporters." J Cell Biol **170**(5): 769-779.

Takasu, M. A., M. B. Dalva, R. E. Zigmond and M. E. Greenberg (2002). "Modulation of NMDA receptor-dependent calcium influx and gene expression through EphB receptors." Science **295**(5554): 491-495.

Takeuchi, S., H. Katoh and M. Negishi (2015). "Eph/ephrin reverse signalling induces axonal retraction through RhoA/ROCK pathway." J Biochem.

Tan, E. C., T. Leung, E. Manser and L. Lim (1993). "The human active breakpoint cluster region-related gene encodes a brain protein with homology to guanine nucleotide exchange proteins and GTPase-activating proteins." J Biol Chem **268**(36): 27291-27298.

Tanabe, K., T. Torii, W. Natsume, S. Braesch-Andersen, T. Watanabe and M. Satake (2005). "A novel GTPase-activating protein for ARF6 directly interacts with clathrin and regulates clathrin-dependent endocytosis." Mol Biol Cell **16**(4): 1617-1628.

Tanaka, M., R. Ohashi, R. Nakamura, K. Shinmura, T. Kamo, R. Sakai and H. Sugimura (2004). "Tiam1 mediates neurite outgrowth induced by ephrin-B1 and EphA2." EMBO J **23**(5): 1075-1088.

Tao, W., D. Pennica, L. Xu, R. F. Kalejta and A. J. Levine (2001). "Wrch-1, a novel member of the Rho gene family that is regulated by Wnt-1." Genes Dev **15**(14): 1796-1807.

Tcherkezian, J., E. I. Danek, S. Jenna, I. Triki and N. Lamarche-Vane (2005). "Extracellular signal-regulated kinase 1 interacts with and phosphorylates CdGAP at an important regulatory site." Mol Cell Biol **25**(15): 6314-6329.

Tcherkezian, J. and N. Lamarche-Vane (2007). "Current knowledge of the large RhoGAP family of proteins." Biol Cell **99**(2): 67-86.

Tcherkezian, J., I. Triki, R. Stenne, E. I. Danek and N. Lamarche-Vane (2006). "The human orthologue of CdGAP is a phosphoprotein and a GTPase-activating protein for Cdc42 and Rac1 but not RhoA." Biol Cell **98**(8): 445-456.

Terawaki, S., K. Kitano, T. Mori, Y. Zhai, Y. Higuchi, N. Itoh, T. Watanabe, K. Kaibuchi and T. Hakoshima (2010). "The PHCCEx domain of Tiam1/2 is a novel protein- and membrane-binding module." EMBO J **29**(1): 236-250.

Tojima, T., J. H. Hines, J. R. Henley and H. Kamiguchi (2011). "Second messengers and membrane trafficking direct and organize growth cone steering." Nat Rev Neurosci **12**(4): 191-203.

Tolias, K. F., J. B. Bikoff, A. Burette, S. Paradis, D. Harrar, S. Tavazoie, R. J. Weinberg and M. E. Greenberg (2005). "The Rac1-GEF Tiam1 couples the NMDA receptor to the activity-dependent development of dendritic arbors and spines." Neuron **45**(4): 525-538.

Tolias, K. F., J. B. Bikoff, C. G. Kane, C. S. Tolias, L. Hu and M. E. Greenberg (2007). "The Rac1 guanine nucleotide exchange factor Tiam1 mediates EphB receptor-dependent dendritic spine development." Proc Natl Acad Sci U S A **104**(17): 7265-7270.

Tomita, T., S. Tanaka, Y. Morohashi and T. Iwatsubo (2006). "Presenilin-dependent intramembrane cleavage of ephrin-B1." Mol Neurodegener **1**: 2.

Topp, J. D., N. W. Gray, R. D. Gerard and B. F. Horazdovsky (2004). "Alsin is a Rab5 and Rac1 guanine nucleotide exchange factor." J Biol Chem **279**(23): 24612-24623.

Torres, R., B. L. Firestein, H. Dong, J. Staudinger, E. N. Olson, R. L. Huganir, D. S. Bredt, N. W. Gale and G. D. Yancopoulos (1998). "PDZ proteins bind, cluster, and synaptically colocalize with Eph receptors and their ephrin ligands." Neuron **21**(6): 1453-1463.

Tsyba, L., O. Nikolaienko, O. Dergai, M. Dergai, O. Novokhatska, I. Skrypkina and A. Rynditch (2011). "Intersectin multidomain adaptor proteins: regulation of functional diversity." Gene **473**(2): 67-75.

Tudor, E. L., M. S. Perkinton, A. Schmidt, S. Ackerley, J. Brownlees, N. J. Jacobsen, H. L. Byers, M. Ward, A. Hall, P. N. Leigh, C. E. Shaw, D. M. McLoughlin and C. C. Miller (2005). "ALS2/Alsin regulates Rac-PAK signaling and neurite outgrowth." J Biol Chem **280**(41): 34735-34740.

Um, K., S. Niu, J. G. Duman, J. X. Cheng, Y. K. Tu, B. Schwechter, F. Liu, L. Hiles, A. S. Narayanan, R. T. Ash, S. Mulherkar, K. Alpadi, S. M. Smirnakis and K. F. Tolias (2014). "Dynamic control of excitatory synapse development by a Rac1 GEF/GAP regulatory complex." Dev Cell **29**(6): 701-715.

Valdez, G., P. Philippidou, J. Rosenbaum, W. Akmentin, Y. Shao and S. Halegoua (2007). "Trk-signaling endosomes are generated by Rac-dependent macroendocytosis." Proc Natl Acad Sci U S A **104**(30): 12270-12275.

Vaughan, E. M., A. L. Miller, H. Y. Yu and W. M. Bement (2011). "Control of local Rho GTPase crosstalk by Abr." Curr Biol **21**(4): 270-277.

Veithen, A., P. Cupers, P. Baudhuin and P. J. Courtoy (1996). "v-Src induces constitutive macropinocytosis in rat fibroblasts." J Cell Sci **109 (Pt 8)**: 2005-2012.

Vihanto, M. M., C. Vindis, V. Djonov, D. P. Cerretti and U. Huynh-Do (2006). "Caveolin-1 is required for signaling and membrane targeting of EphB1 receptor tyrosine kinase." J Cell Sci **119**(Pt 11): 2299-2309.

Villalba, M., K. Bi, F. Rodriguez, Y. Tanaka, S. Schoenberger and A. Altman (2001). "Vav1/Rac-dependent actin cytoskeleton reorganization is required for lipid raft clustering in T cells." J Cell Biol **155**(3): 331-338.

von Thun, A., C. Preisinger, O. Rath, J. P. Schwarz, C. Ward, N. Monsefi, J. Rodriguez, A. Garcia-Munoz, M. Birtwistle, W. Bienvenut, K. I. Anderson, W. Kolch and A. von Kriegsheim (2013). "Extracellular signal-regulated kinase regulates RhoA activation and tumor cell plasticity by inhibiting guanine exchange factor H1 activity." Mol Cell Biol **33**(22): 4526-4537.

Wahl, S., H. Barth, T. Ciossek, K. Aktories and B. K. Mueller (2000). "Ephrin-A5 induces collapse of growth cones by activating Rho and Rho kinase." J Cell Biol **149**(2): 263-270.

Walker-Daniels, J., D. J. Riese, 2nd and M. S. Kinch (2002). "c-Cbl-dependent EphA2 protein degradation is induced by ligand binding." Mol Cancer Res **1**(1): 79-87.

Walmsley, M. J., S. K. Ooi, L. F. Reynolds, S. H. Smith, S. Ruf, A. Mathiot, L. Vanes, D. A. Williams, M. P. Cancro and V. L. Tybulewicz (2003). "Critical roles for Rac1 and Rac2 GTPases in B cell development and signaling." Science **302**(5644): 459-462.

Wandinger-Ness, A. and M. Zerial (2014). "Rab proteins and the compartmentalization of the endosomal system." Cold Spring Harb Perspect Biol **6**(11): a022616.

Wang, X., P. J. Roy, S. J. Holland, L. W. Zhang, J. G. Culotti and T. Pawson (1999). "Multiple ephrins control cell organization in C. elegans using kinase-dependent and -independent functions of the VAB-1 Eph receptor." Mol Cell **4**(6): 903-913.

Watanabe, N., T. Kato, A. Fujita, T. Ishizaki and S. Narumiya (1999). "Cooperation between mDia1 and ROCK in Rho-induced actin reorganization." Nat Cell Biol **1**(3): 136-143.

Watanabe, T., S. Wang, J. Noritake, K. Sato, M. Fukata, M. Takefuji, M. Nakagawa, N. Izumi, T. Akiyama and K. Kaibuchi (2004). "Interaction with IQGAP1 links APC to Rac1, Cdc42, and actin filaments during cell polarization and migration." Dev Cell **7**(6): 871-883.

Wegmeyer, H., J. Egea, N. Rabe, H. Gezelius, A. Filosa, A. Enjin, F. Varoqueaux, K. Deininger, F. Schnutgen, N. Brose, R. Klein, K. Kullander and A. Betz (2007). "EphA4-dependent axon guidance is mediated by the RacGAP alpha2-chimaerin." Neuron **55**(5): 756-767.

Welch, H. C., W. J. Coadwell, C. D. Ellson, G. J. Ferguson, S. R. Andrews, H. Erdjument-Bromage, P. Tempst, P. T. Hawkins and L. R. Stephens (2002). "P-Rex1, a PtdIns(3,4,5)P3- and Gbetagamma-regulated guanine-nucleotide exchange factor for Rac." Cell **108**(6): 809-821.

Wennerberg, K. and C. J. Der (2004). "Rho-family GTPases: it's not only Rac and Rho (and I like it)." J Cell Sci **117**(Pt 8): 1301-1312.

Wennerberg, K., S. M. Ellerbroek, R. Y. Liu, A. E. Karnoub, K. Burridge and C. J. Der (2002). "RhoG signals in parallel with Rac1 and Cdc42." J Biol Chem **277**(49): 47810-47817.

Wennerberg, K., M. A. Forget, S. M. Ellerbroek, W. T. Arthur, K. Burridge, J. Settleman, C. J. Der and S. H. Hansen (2003). "Rnd proteins function as RhoA antagonists by activating p190 RhoGAP." Curr Biol **13**(13): 1106-1115.

West, M. A., M. S. Bretscher and C. Watts (1989). "Distinct endocytotic pathways in epidermal growth factor-stimulated human carcinoma A431 cells." J Cell Biol **109**(6 Pt 1): 2731-2739.

West, M. A., A. R. Prescott, E. L. Eskelinen, A. J. Ridley and C. Watts (2000). "Rac is required for constitutive macropinocytosis by dendritic cells but does not control its downregulation." Curr Biol **10**(14): 839-848.

Wilkinson, D. G. (2014). "Regulation of cell differentiation by Eph receptor and ephrin signaling." Cell Adh Migr **8**(4): 339-348.

Wimmer-Kleikamp, S. H., P. W. Janes, A. Squire, P. I. Bastiaens and M. Lackmann (2004). "Recruitment of Eph receptors into signaling clusters does not require ephrin contact." J Cell Biol **164**(5): 661-666.

Winckler, B. and C. C. Yap (2011). "Endocytosis and endosomes at the crossroads of regulating trafficking of axon outgrowth-modifying receptors." Traffic **12**(9): 1099-1108.

Wong, K., X. R. Ren, Y. Z. Huang, Y. Xie, G. Liu, H. Saito, H. Tang, L. Wen, S. M. Brady-Kalnay, L. Mei, J. Y. Wu, W. C. Xiong and Y. Rao (2001). "Signal transduction in neuronal migration: roles of GTPase activating proteins and the small GTPase Cdc42 in the Slit-Robo pathway." Cell **107**(2): 209-221.

Wong, K. A., J. Wilson, A. Russo, L. Wang, M. N. Okur, X. Wang, N. P. Martin, E. Scappini, G. K. Carnegie and J. P. O'Bryan (2012). "Intersectin (ITSN) family of scaffolds function as molecular hubs in protein interaction networks." PLoS One **7**(4): e36023.

Wu, J. and H. Luo (2005). "Recent advances on T-cell regulation by receptor tyrosine kinases." Curr Opin Hematol **12**(4): 292-297.

Wu, Y. C., M. C. Tsai, L. C. Cheng, C. J. Chou and N. Y. Weng (2001). "C. elegans CED-12 acts in the conserved crkII/DOCK180/Rac pathway to control cell migration and cell corpse engulfment." Dev Cell **1**(4): 491-502.

Wybenga-Groot, L. E., B. Baskin, S. H. Ong, J. Tong, T. Pawson and F. Sicheri (2001). "Structural basis for autoinhibition of the Ephb2 receptor tyrosine kinase by the unphosphorylated juxtamembrane region." Cell **106**(6): 745-757.

Xu, J., C. Litterst, A. Georgakopoulos, I. Zaganas and N. K. Robakis (2009). "Peptide EphB2/CTF2 generated by the gamma-secretase processing of EphB2 receptor promotes tyrosine

phosphorylation and cell surface localization of N-methyl-D-aspartate receptors." J Biol Chem **284**(40): 27220-27228.

Xu, N. J. and M. Henkemeyer (2009). "Ephrin-B3 reverse signaling through Grb4 and cytoskeletal regulators mediates axon pruning." Nat Neurosci **12**(3): 268-276.

Xu, N. J., S. Sun, J. R. Gibson and M. Henkemeyer (2011). "A dual shaping mechanism for postsynaptic ephrin-B3 as a receptor that sculpts dendrites and synapses." Nat Neurosci **14**(11): 1421-1429.

Yamazaki, D., T. Itoh, H. Miki and T. Takenawa (2013). "srGAP1 regulates lamellipodial dynamics and cell migratory behavior by modulating Rac1 activity." Mol Biol Cell **24**(21): 3393-3405.

Yang, Y., A. Hentati, H. X. Deng, O. Dabbagh, T. Sasaki, M. Hirano, W. Y. Hung, K. Ouahchi, J. Yan, A. C. Azim, N. Cole, G. Gascon, A. Yagmour, M. Ben-Hamida, M. Pericak-Vance, F. Hentati and T. Siddique (2001). "The gene encoding alsin, a protein with three guanine-nucleotide exchange factor domains, is mutated in a form of recessive amyotrophic lateral sclerosis." Nat Genet **29**(2): 160-165.

Yoo, S., Y. Kim, H. Noh, H. Lee, E. Park and S. Park (2011). "Endocytosis of EphA receptors is essential for the proper development of the retinocollicular topographic map." EMBO J **30**(8): 1593-1607.

Yoo, S., J. Shin and S. Park (2010). "EphA8-ephrinA5 signaling and clathrin-mediated endocytosis is regulated by Tiam-1, a Rac-specific guanine nucleotide exchange factor." Mol Cells **29**(6): 603-609.

Yu, B., I. R. Martins, P. Li, G. K. Amarasinghe, J. Umetani, M. E. Fernandez-Zapico, D. D. Billadeau, M. Machius, D. R. Tomchick and M. K. Rosen (2010). "Structural and energetic mechanisms of cooperative autoinhibition and activation of Vav1." Cell **140**(2): 246-256.

Yu, T. W. and C. I. Bargmann (2001). "Dynamic regulation of axon guidance." Nat Neurosci **4 Suppl**: 1169-1176.

Yu, X., G. Wang, A. Gilmore, A. X. Yee, X. Li, T. Xu, S. J. Smith, L. Chen and Y. Zuo (2013). "Accelerated experience-dependent pruning of cortical synapses in ephrin-A2 knockout mice." Neuron **80**(1): 64-71.

Yuste, R. and T. Bonhoeffer (2001). "Morphological changes in dendritic spines associated with long-term synaptic plasticity." Annu Rev Neurosci **24**: 1071-1089.

Zenke, F. T., M. Krendel, C. DerMardirossian, C. C. King, B. P. Bohl and G. M. Bokoch (2004). "p21-activated kinase 1 phosphorylates and regulates 14-3-3 binding to GEF-H1, a microtubule-localized Rho exchange factor." J Biol Chem **279**(18): 18392-18400.

Zhang, H., D. J. Webb, H. Asmussen, S. Niu and A. F. Horwitz (2005). "A GIT1/PIX/Rac/PAK signaling module regulates spine morphogenesis and synapse formation through MLC." J Neurosci **25**(13): 3379-3388.

Zhou, L., S. J. Martinez, M. Haber, E. V. Jones, D. Bouvier, G. Doucet, A. T. Corera, E. A. Fon, A. H. Zisch and K. K. Murai (2007). "EphA4 signaling regulates phospholipase Cgamma1 activation, cofilin membrane association, and dendritic spine morphology." J Neurosci **27**(19): 5127-5138.

Zhuang, G., S. Hunter, Y. Hwang and J. Chen (2007). "Regulation of EphA2 receptor endocytosis by SHIP2 lipid phosphatase via phosphatidylinositol 3-Kinase-dependent Rac1 activation." J Biol Chem **282**(4): 2683-2694.

Zimmer, M., A. Palmer, J. Kohler and R. Klein (2003). "EphB-ephrinB bi-directional endocytosis terminates adhesion allowing contact mediated repulsion." Nat Cell Biol **5**(10): 869-878.

Zisch, A. H., M. S. Kalo, L. D. Chong and E. B. Pasquale (1998). "Complex formation between EphB2 and Src requires phosphorylation of tyrosine 611 in the EphB2 juxtamembrane region." Oncogene **16**(20): 2657-2670.

Zisch, A. H., C. Pazzagli, A. L. Freeman, M. Schneller, M. Hadman, J. W. Smith, E. Ruoslahti and E. B. Pasquale (2000). "Replacing two conserved tyrosines of the EphB2 receptor with glutamic acid prevents binding of SH2 domains without abrogating kinase activity and biological responses." Oncogene **19**(2): 177-187.

Appendix

Acknowledgements

First and foremost I have to thank my parents for their unconditional love, constant support and all the freedoms they granted me to pursue my dreams. I am also deeply grateful to Angelika Maier for her support, her patience, her understanding, and for never failing to put a smile on my face.

I want to thank my supervisor, Prof. Rüdiger Klein, for giving me the opportunity to work in a great lab. I am grateful for all the help in seeing the project through its ups and downs with valuable advice and support.

This work would not have been possible without Thomas Gaitanos, who took me on as his padawan and made it his task to show me the ropes of cell biology. He was responsible for teaching me an enormous share of the new things I learned during my PhD and was always there to answer any question I could come up with. I have been truly lucky to share this project with him, as he has not only been a great mentor, but also a true friend.

Furthermore, I would like to thank the members of my Thesis Committee, Dr. Ilona Kadow and Prof. Stephan Kröger, for their encouragement and valuable discussions.

A valuable resource, not only for discussions in our subgroup meetings, but also for sharing reagents and constructs has been Dr. Jingyi Gong, and I am very grateful for all her help. In dealing with the screen data we received support and ideas from Dr. Christian Kuffer and I would like to thank him for his contributions. I would also like to thank the members of the Klein lab, who taught me to finally do some neurobiology, especially Katharina.

I sincerely appreciate all the help and support by the technicians of the Klein lab. Louise, Jana, Pilar and Gönül have made life so much easier by always knowing where to find things and making sure we are always well stocked.

I would like to acknowledge all present and former members of the Klein lab that have made my time at the institute so thoroughly enjoyable. Special thanks to Pontus for always being up for some powder action, to Louise for providing good humour and a lot of culinary

delicacies over the years, to Sonia for being so passionate, to Graziana for being always right apart from when playing QuizClash, to Marion for bringing some French flair to the lab, to Annelies for being the Hulk, and to Dani for being a constant source of random science and non-science facts.

I have thoroughly enjoyed being a member of the Graduate School of Systemic Neurosciences and would like to express my appreciation for making the PhD time ever so more enjoyable by providing insightful courses, excursions and opportunities to socialise with a very talented and interesting group of young people. I also want to thank the PhD community of the MPI Neurobiology for a lot of memorable experiences.

I would like to also thank the mountains of the Alps, the weather gods, my trusted Nat and all people joining on early morning trips into the snow for allowing me to put things into perspective during a good powder run. Furthermore I would like to thank the moody diva from the river Main for providing a constant source of stress and those few magic moments, especially, those days in Israel.

A group of people have been very influential for me in forming my decision to pursue not only an education in biomedical sciences, but also focus on the molecular working of the most fascinating of organs: the brain. I would therefore like to thank Dr. Gerhard Neuhäusel, Dr Joachim Altschmied, Prof. Martin Heisenberg, Prof. Stephan Sigrist, Dr. David Owald and Prof. Gero Miesenböck for setting me on the right path.

Last, but certainly not least, a very large thank you to Thomas and Louise Gaitanos for their invaluable support in critically (and rapidly) reading my thesis and discussing the finer notions of English grammar with me.

Curriculum vitae

Jorg Körner

Born 11th December 1985 in Gelnhausen, Germany

Education and Work experience

10/2010 -	**PhD candidate**, Graduate School of Systemic Neurosciences, Ludwig-Maximilians-Universität München
	Project work performed at Max-Planck-Institute of Neurobiology under supervision of Prof. Rüdiger Klein
09/2009 - 08/2010	**Project Manager**, European Youth Parliament Germany
09/2008 – 09/2009	**MSc in Neuroscience**, University of Oxford
	Research Projects with Prof. Gero Miesenböck and Prof. Nigel Emptage
10/2005 – 08/2008	**BSc in Biomedicine**, Julius-Maximilians-Universität Würzburg
	BSc Thesis project with Prof. Stephan Sigrist
08/1996 – 06/2005	**Abitur**, Kopernikusschule Freigericht, European School of the State of Hesse

Publications

V Burlakov, R Taylor, **J Koerner**, NJ Emptage; *Analysis of microscopic parameters of single particle trajectories in neurons*, Biophysical Journal 2010

D Owald, W Fouquet, …, **J Koerner**, …, SJ Sigrist; *A Syd-1 homologue regulates pre- and postsynaptic maturation in Drosophila,* Journal of Cell Biology, 2010

Eidesstattliche Versicherung/Affidavit

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation „Molecular mechanisms of EphB-ephrinB endocytosis in neural cells“ selbstständig angefertigt habe, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

I hereby confirm that the dissertation „Molecular mechanisms of EphB-ephrinB endocytosis in neural cells“ is the result of my own work and that I have only used sources or materials listed and specified in the dissertation.

München, den /Munich, Date Unterschrift/ Signature

List of author contributions

The presented study derives from a project performed in close co-operation with **Dr. Thomas Gaitanos** at the Max-Planck-Institute of Neurobiology. To ensure the completeness of the scientific literature the whole study comprised of contributions by **Jorg Körner and Dr. Thomas Gaitanos** is presented.

Jorg Körner generated all data and prepared all figures if not stated otherwise.

Dr. Thomas Gaitanos generated the data for Fig. 4, Fig. 5D-F, Fig.8, Fig.9, Fig. 14 and Fig. 20.

The experiments for the Rho family GEF/GAP screen (Fig. 16 and Fig. 19) were performed by both **Jorg Körner and Dr. Thomas Gaitanos** and an individual contribution is not attributable.

Signature of supervisor: Signature of contributing author: Signature of student: