Ligand-induced downregulation of the kinase-dead EphB6 receptor

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By

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ABSTRACT

Ligand-induced internalisation and subsequent downregulation of receptor tyrosine kinases (RTKs) serve to determine biological outputs of their signalling. Intrinsically kinase-deficient RTKs control a variety of biological responses, however, the mechanism of their downregulation is not well understood and its analysis is focused exclusively on the ErbB3 receptor.

The Eph group of RTKs is represented by the EphA and EphB subclasses. Each bears one kinase-inactive member, EphA10 and EphB6, respectively, suggesting an important role for these molecules in the Eph signalling network. While EphB6 effects on cell behaviour have been assessed, the mechanism of its downregulation remains elusive.

Our work reveals that EphB6 and its kinase-active relative, and signaling partner, EphB4, are downregulated in a similar manner in response to their common ligand, ephrin-B2. Following stimulation, both receptors are internalised through clathrin-coated pits and are degraded in lysosomes. Their targeting for lysosomal degradation relies on the activity of an early endosome regulator, the Rab5 GTPase, as this process is inhibited in the presence of a Rab5 dominant-negative variant. EphB6 also interacts with the Hsp90 chaperone and EphB6 downregulation is preceded by their rapid dissociation. Moreover, the inhibition of Hsp90 results in EphB6 degradation, mimicking its ligand-induced downregulation. These processes appear to rely on overlapping mechanisms, since Hsp90 inhibition does not significantly enhance ligand-induced EphB6 elimination.

Taken together, our observations define a novel mechanism for intrinsically kinase-deficient RTK downregulation and support an intriguing model, where Hsp90 dissociation acts as a trigger for ligand-induced receptor removal.

ACKNOWLEDGMENTS

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DEDICATION

I would like to dedicate this thesis to my husband Chris, and my children, Odin and Emmaline. Thank-you for all your patience and understanding throughout the marathon that is a PhD program. I couldn't have done it without you.

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LIST OF ABBREVIATIONS

Abbreviation	Name
AML	acute myeloid leukemia
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
AP-2	adaptor protein 2
APPL	adaptor protein, phosphotyrosine interacting, PH domain, and leucine zipper-containing
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
BACE	beta-secretase
BAR	Bin/Amphiphysin/Rvs
CAM	chorioallontoic membrane
CCK4	colon carcinoma kinase 4
CCP	clathrin-coated pit
CHC	Clathrin heavy chain
CHIP	C-terminus of Hsc70 Interacting Protein
CLIC	Clathrin-Independent Carriers
CRD	Cysteine rich domain
CtBP1/BARS	C-terminal-binding protein-1/brefeldinA-ADP ribosylated substrate

DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
cDNA	complementary DNA
eB2	Ephrin-B2
EDTA	Ethylenediaminetetraacetic acid
EEA1	early endosomal antigen 1
EGFP	enhanced green fluorescent protein
EGFR	Epidermal growth factor recetpor
EH	Eps homology
ELMO2	Engulfment And Cell Motility 2
EMT	ephithelial-mesenchymal transition
Epsin	Eps 15 interacting protein
ESCRT	endosomal-sorting complex required for transport
FCHo1 and FCHo2	F-BAR domain-containing Fer/Cip4 homology domain-only proteins 1 and 2
FGFR	Fibroblast growth factor receptor
FITC	Fluorescein isothiocyanate
FLT3	FMS-like tyrosine kinase-3
FN1 and FN2	Fibronectin-type III domains 1 and 2
GA	geldanamycin
GAP	GTPase activating protein
GBM	glioblastoma

GEEC	GPI-Enriched Endocytic Compartments
GEF	guanine exchange factor
GPI	glycosylphosphatidylinositol
GRAF1	GTPase regulator associated with focal adhesion kinase-1
GTP	Guanine triphosphate
GTPases	guanine-triphosphatases
HBMEC	human brain microvascular endothelial cells
HCRP1	hepatocellular carcinoma related protein 1
HEK-293	Human embryonic kidney-293
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	hepatocyte growth factor
hIgG	Human IgG
HNSCC	head and neck squamous cell carcinomas
HOPS	homotypic fusion and vacuole protein sorting
Hsp90	heat shock protein 90
HUVEC	Human Umbilical Vein Endothelial Cells
IGF-IR/IGFR	Insulin-like growth factor receptor
IL-2Rb	interleukin-2b receptor
IR	Insulin receptor

LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MCF7	Michigan Cancer Foundation-7
MOI	multiplicity of infection
mTOR	mammalian target of rapamycin
MVB	multi-vesicular bodie
NMDAR	N-methyl-D-aspartate receptor
NSCLC	non-small cell lung cancer
NSF	*N*-ethylmaleimide sensitive factor
P.C.	Positive control
PAK1	p21 protein (Cdc42/Rac)-activated kinase 1
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGFR	platelet-derived growth factor receptor
PDZ	Postsynaptic density 95-Discs large-Zonula occludentes-1
PH	Pleckstrin homology
PI3K	Phosphatidylinositol-3-kinase
PICK1	Protein interacting with C kinase 1
PIP_2	phosphatidylinositol-4,5-bis-phosphate
PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate
PMSF	phenylmethylsulfonyl fluoride
PP2A	protein phosphatase 2A

PSD	post-synaptic density
PTB	Phosphotyrosine binding
PTEN	phosphatase and tensin homolog
PTK7	Protein Tyrosine Kinase 7
RACK1	Receptor for activated C kinase 1
RCP	Rab coupling protein
RGC	Retinal ganglion cells
RINL	**R**as and Rab **in**teractor-**l**ike
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RTK	Receptor tyrosine kinase
S.C.	Solvent control
SAM	Sterile alpha motif
SD	Standard deviation
SDS-PAGE	sodium dodecyl sulfate- polyacrylamide gel electrophoresis
SH2	Src-homology 2
SHC	Src Homology 2 Domain-Containing
shRNA	Short hairpin ribonucleic acid
siRNA	short interfering RNA
SM	Sec1/Munc18-like
SNARE	soluble NSF attachment protein receptor
SP	sodium pyruvate

STAM	signal-transducing adaptor molecule
STAT3	Signal transducer and activator of transcription 3
TCR	T-cell receptor
TGF-b	transforming growth factor-b
TKB	tyrosine kinase-binding
UBAP-1	ubiquitin-associated protein 1
UIM	ubiquitin interacting motif
VEGF	vascular endothelial growth factor
Vps	Vacuolar protein sorting
VSMC	Vascular smooth muscle cell
WASp	Wiskott–Aldrich Syndrome protein
WAVE	WASp family Verprolin-homologous
WT	Wild-type

CHAPTER ONE

REVIEW OF THE LITERATURE

1.1. Introduction

Molecular mechanisms controlling receptor downregulation that is assured by its internalisation, trafficking and eventually, proteolytic degradation, actively modulate the responses that cell-surface receptors generate following ligand stimulation, and have a profound influence on cell behaviour (Casaletto and McClatchey, 2012). Ligand-induced receptor internalisation is a multipurpose process that gates routes towards both receptor degradation and signal abrogation, as well as to efficient activation of certain signalling pathways, including the Ras-MAPK (mitogen-activated protein kinase) cascade (Haugh et al., 1999a). In agreement, imbalanced downregulation of a number of receptor tyrosine kinases (RTKs) through stimulation-triggered internalisation and degradation has been linked to oncogenic transformation (Abella and Park, 2009; Casaletto and McClatchey, 2012; Mosesson et al., 2008).

RTKs are single-pass transmembrane proteins that are involved in transducing signals from the exterior of the cell to the interior, and are essential to maintaining a proper relationship between the cell and its environment. Following binding to an extracellular ligand they become activated, resulting in the phosphorylation of targets within the cellular interior, allowing for the transmission of the signal from the outside to the inside of the cell. These signals generally lead to cell growth, proliferation, survival, movement, or apoptosis. The ligand-induced downregulation of many RTKs, including that of the EGFR (epidermal growth factor receptor) family (Roepstorff et al., 2008), IGF-IR (insulin-like growth factor receptor I)(Mao et al., 2011), and Met receptor

(Clague, 2011), have been described. However, despite the fact that the RTK family contains five intrinsically kinase-inactive members (Aasheim et al., 2005b; Gurniak and Berg, 1996; Katso et al., 1999; Mossie et al., 1995; Sierke et al., 1997) that are known to mediate important biological functions including T-cell regulation and aspects of embryonic development (Baselga and Swain, 2009; Clark et al., 2012; Truitt and Freywald, 2011), our current understanding of the mechanism of their ligand-induced downregulation is incomplete and is limited to reports focused exclusively on a member of the EGFR group, the ErbB3 receptor. Furthermore, the current reports on ErbB3 downregulation are often contradictory, with some studies proposing that it is downregulation deficient, and differs from kinase-active ErbB receptors in the mechanistic aspects of its downregulation, while others studies suggest ErbB3 downregulation proceeds efficiently and in much the same manner as what has been described for EGFR (Cao et al., 2007; Sak et al., 2013). Two of the five known kinase-dead RTKs belong to the Eph group of RTKs, EphB6 and EphA10 (Truitt and Freywald, 2011), and currently nothing is known of their downregulation. Of these, EphB6, in particular, is gaining increasing attention, as it not only plays an important role in normal biology (Wu and Luo, 2005), but also in malignancy, where EphB6 presence appears to act as a suppressor of invasive behavior in several solid tumor types including breast, lung, and skin cancers (Bailey and Kulesa, 2014; Bulk et al., 2012; Truitt et al., 2010). As ligand-induced receptor trafficking has been shown to have a profound impact on the responses generated following RTK activation (Andersson, 2012), it is important to understand the mechanisms underlying EphB6 downregulation, as these may play a role in mediating EphB6 effects. Furthermore, the observations of ErbB3 downregulation are

as of yet unclear, and it is unknown if they will be relevant to other kinase dead receptors, therefore understanding the mechanisms of EphB6 downregulation will bring some clarity to this area.

The following literature review will discuss RTKs, focusing in particular on the Eph receptor family, with an emphasis on EphB6. I will also review current understandings of the mechanisms used by cells to facilitate ligand-induced RTK and Eph downregulation, and briefly describe the mechanisms of ligand-induced downregulation of the only other kinase-dead receptor for which it has been explored, ErbB3.

1.2. Receptor Tyrosine Kinases

RTKs are essential in modulating a wide variety of cellular responses to external stimuli, including cell cycle, survival, differentiation, motility and metabolism (Schartl et al., 2015). To date, there are 58 described RTKs expressed in humans (Schartl et al., 2015). In general, RTKs are monomeric cell-surface single-pass transmembrane proteins, however, the insulin receptor (IR/IGFR) family is an exception to this rule and consists of two extracellular α-subunits, and two transmembrane β-subunits, all connected through disulphide bonds (Lee and Pilch, 1994). RTKs possess an extracellular ligand-binding domain, and an intracellular domain with intrinsic kinase activity. There are several sub-families of RTKs, grouped based on similarities within their extracellular domains, and the ligands for each RTK group tend to possess high levels of sequence similarity as well (Schartl et al., 2015). Close to 20 groups of RTKs have been identified, including EGFRs (ErbBs), platelet-derived growth factor receptors (PDGFRs), fibroblast growth factor receptors (FGFRs), and the Ephs (named from the **e**rythropoietin-**p**roducing

hepatocellular carcinoma cell line they were first identified in)(**Table 1.1**)(Schartl et al., 2015). Activation of these receptors generally requires ligand-induced dimerization

Table 1.1. RECEPTOR TYROSINE KINASES IN HUMANS.

Family Name	Number of members (humans)	Receptor names
EGF/ErbB	4	EGFR/ErbB1/Her, ErbB2/Her2, ErbB3/Her3, ErbB4/Her4
IR & IGFR	3*	IRA, IRB & IGFIR*
NTRK	3	TRKA, TRKB, TRKC
FGFR	4	FGFR1, FGFR2, FGFR3, FGFR4
VEGFR	3	VEGFR1, VEGFR2, VEGFR3
PDGFR	5	PDGFRa, PDGFRb, FMS, FLT3, KIT
TAM	3	TYRO, AXL, MERTK
DDR	2	Ddr1, ddr2
Eph	15	EphA1, EphA2, EphA3, EphA4, EphA5, EphA6, EphA7, EphA8, EphA10, EphB1, EphB2, EphB3, EphB4, EphB6
Ror	2	Ror1, ror2
Tie	2	Tie1, Tie2
Met	2	Met, Mst1r
Alk	2	Alk, Ltk
Musk	1	Musk
Ptk7	1	Ptk7
Ret	1	Ret
Ros	1	Ros
Ryk	1	Ryk
Nok	1	Styk1

*As the receptor functions as a dimer of two chains, combinations between all three listed types are possible, eg. IRA/IGFIR or IRA/IRB.

between sub- family members, and their consequent cross-phosphorylation (Hubbard and Miller, 2007; Schlessinger, 2014). Subsequently, numerous signalling pathways are initiated, depending both on the RTK type, and on the characteristics of the expressing cell. The phosphorylated tyrosine residues of the activated receptors serve as docking sites for intracellular proteins containing Src homology 2 (SH2) domains, or phosphotyrosine-binding domains (PTB). These proteins in turn may be phosphorylated by the activated receptors to facilitate downstream signalling, or may serve as scaffolds to assist in the formation of signalling complexes (Schlessinger, 2014). Major pathways activated by RTKs include the proliferative Ras/MAPK pathway (Zhang and Liu, 2002), and the phosphatidylinotsitol-3-kinase (PI3K)/Akt pathway that regulates cell survival (Song et al., 2005).

1.3. Eph Receptors

1.3.1. The Eph Receptor Family

The *eph* gene was initially identified in the late 1980's by Hirai et al. during a screen of the human genome for tyrosine kinase domains (Hirai et al., 1987). Today, sixteen Eph receptor tyrosine kinases have been described, including ten EphA (EphA1-EphA10) and six EphB receptors (EphB1-EphB6), and nearly all of them, with the exception of EphA9 and EphB5, are expressed in humans (Pasquale, 2005). The classification of a receptor as either EphA or EphB is based predominantly on sequence similarities within the two sub-classes, but also upon which type of ligand they bind to, the glycosylphosphatidylinositol anchored (GPI-anchored) ephrin-A class, or the transmembrane ephrin-B class (Pasquale, 2005). Eph-ligand binding tends to be promiscuous within a class, where EphA receptors are able to bind to multiple ephrin-As, and EphBs to multiple ephrin-Bs,

but is generally restricted from cross-class interactions (Lisabeth et al., 2013). However, there are exceptions to this rule as EphA4 is able to bind to ephrin-B2 and ephrin-B3 (Guo and Lesk, 2014), and EphB2 can be activated by ephrin-A5 (Himanen et al., 2004) (**Figure 1.1**).

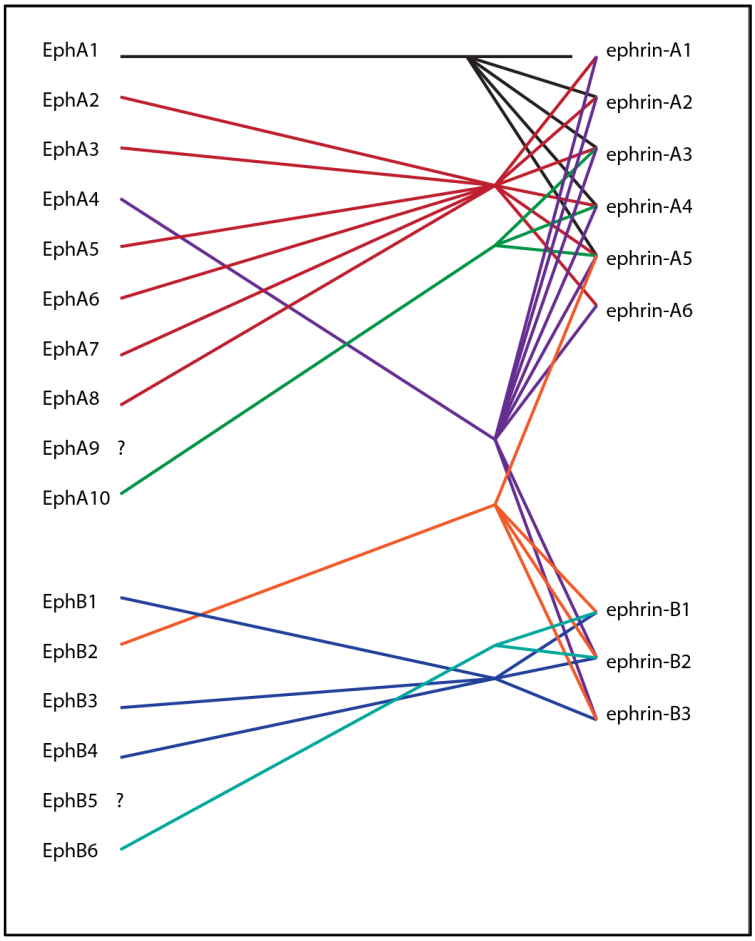

Figure 1.1 Eph receptor-ephrin interactions.
Eph receptors bind promiscuously to ligands within their own class, but are generally restricted from cross-class interactions, save a few exceptions, as illustrated above.

1.3.2. Eph Structure

The first crystal structure of an Eph receptor was described in 1998, and revealed important information on the topology of the EphB2 ligand binding domain, including probable regions responsible for ligand binding (Himanen et al., 1998). Since then, the entirety of the Eph receptor domain structures have been solved (Seiradake et al., 2010), and the current structural model for Eph receptors contains an extracellular ligand-binding domain, a cysteine-rich domain (Seiradake et al., 2010), 2 fibronectin-type-III-like repeats (Himanen, 2012), a hydrophobic transmembrane domain, juxtamembrane domain, kinase domain, sterile alpha motif (SAM) domain (Stapleton et al., 1999), and Postsynaptic density 95-Discs large-Zonula occludentes-1 (PDZ)-binding motif (Hock et al., 1998) (**Figure 1.2, 1.3**). Each domain is able to contribute to specific functions, as described below.

1.3.2.1. Ligand-binding domain

The ligand-binding domain is a globular domain located at the N-terminus of the receptor. There are two regions of the ligand binding domain that participate in the interaction with ephrins, an external surface area that facilitates polar interactions between the two, and a high-affinity binding pocket that associates with the ligand through non-polar interactions (Himanen et al., 2001). Crystallography studies on the binding between EphB2-ephrin-B2 (Himanen et al., 2001), EphB4-ephrin-B2 (Chrencik et al., 2006), and EphA2-ephrin-A1 structure (Himanen et al., 2009) have provided some insight into the general observation that Eph receptors tend to only bind to either ephrin-As or ephrin-Bs. Specifically, it appears that several residues involved in EphA-ephrin-A

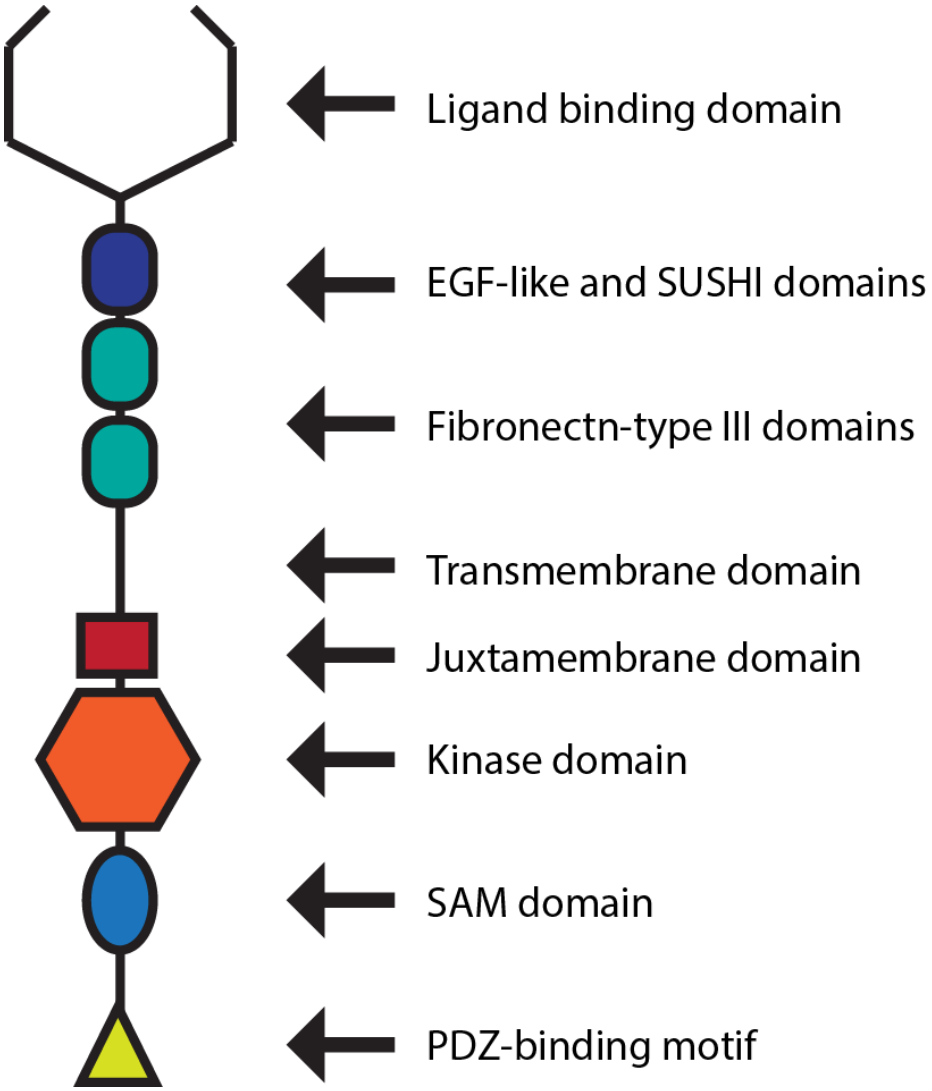

Figure 1.2. Eph receptor structure.
Eph receptors are single-pass transmembrane proteins, with an extracellular ligand-binding domain and an intracellular kinase domain, and also possess several other domains involved in Eph-Eph interactions and associations with downstream signaling effectors, as shown.

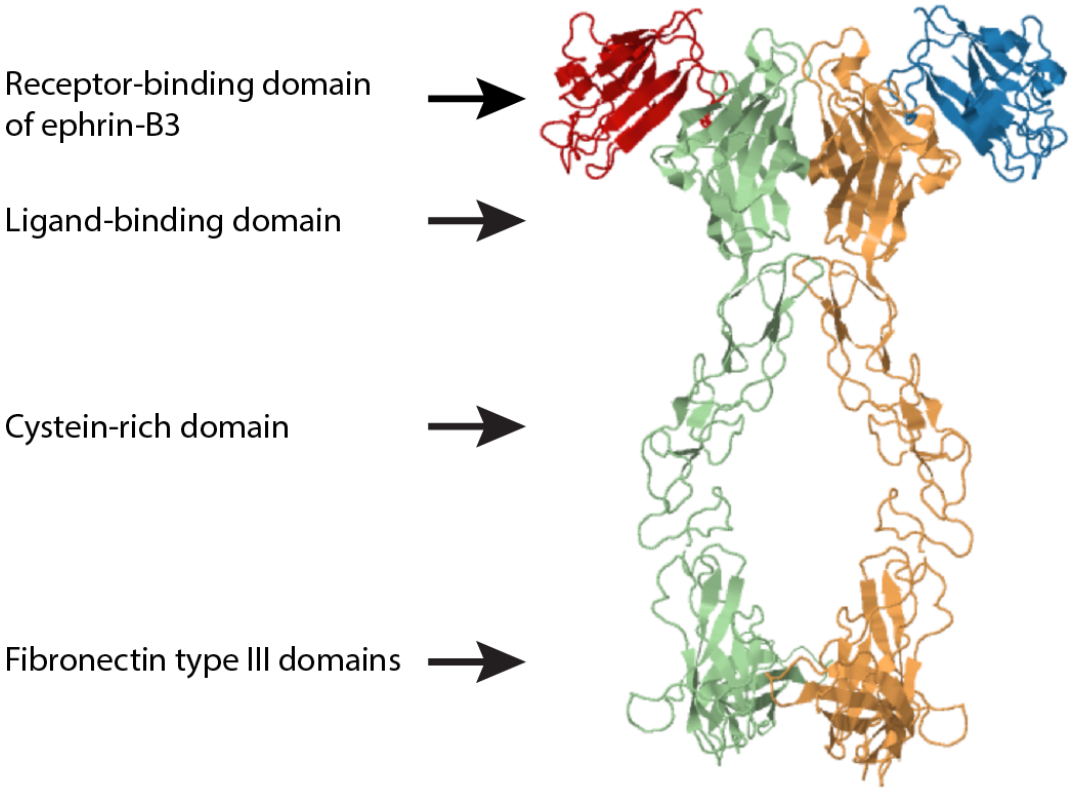

Figure 1.3. Crystal structure of EphA4 ectodomain bound to ephrin-B3.
EphA4 receptor is shown in green and orange, ephrin-B3 is shown in red ad blue. Note the interactions between the ligand-binding domains, cysteine-rich domains, as well as the fibronectin-type III domains. Crystal structure generated by Seiradake et al. (Seiradake et al., 2013).

or EphB-ephrin-B binding possess biochemically opposing characteristics in the corresponding residues of the other class. For example, in EphA2-ephrin-A2 interactions, there are non-polar interactions between a phenylalanine of ephrin-A2 and an alanine of EphA2, where the equivalent residues for ephrin-B2 and EphB4 are the polar residues asparagine and serine respectively (Himanen et al., 2009). The sizes of the amino-acid side chains in the Eph-ephrin interaction region also appear to play a significant role in determining class specificity, and it has been found that bulky-side chains may replace small ones, and vice versa, in corresponding residues within the binding regions between EphA and EphB receptors (Guo and Lesk, 2014). The binding between classes differs slightly as well, as EphB-ephrin-B binding is a dynamic process requiring shifts within the ligand-binding domain, whereas EphA-ephrin-A binding occurs in a more "lock-and-key" fashion (Himanen et al., 2009) (**Figure 1.4**). Eph-ephrin dimers are formed by an ephrin binding to the high-affinity ligand-binding pocket of the ligand-binding domain, while the second, lower-affinity region, is able to interact with a second ephrin, and in this way promote the stable interaction between two ligand-bound Ephs (Himanen, 2012) (**Figure 1.5**). Eph receptors are also able to form higher order oligomers, where Eph-ephrin tetramers combine to form larger complexes, and the propagation of oligomer formation following ligand-binding involves clustering of Eph receptors through interactions between their ligand-binding domains, as well as their cysteine-rich domains. Interestingly, following initial nucleation of clustering by ligand, Eph-Eph receptor

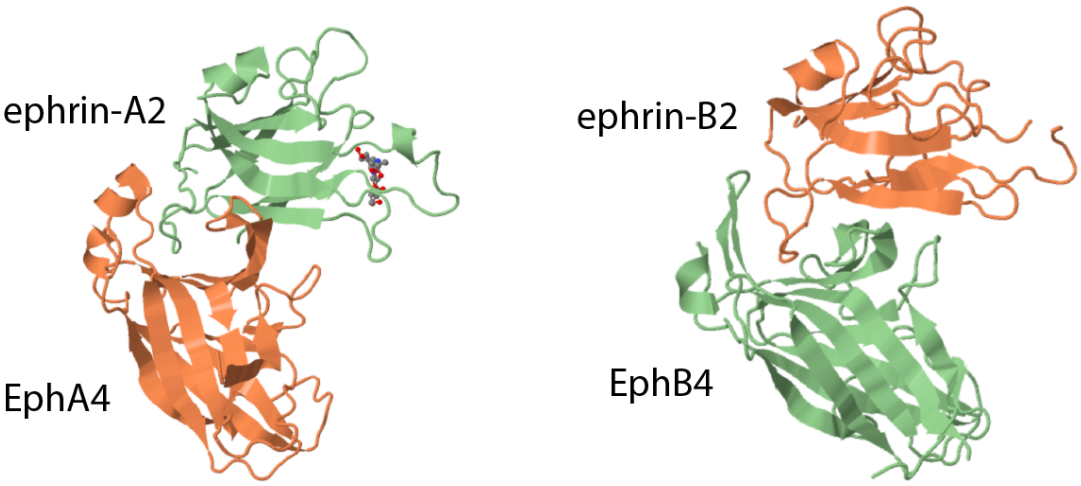

Figure 1.4. Crystal structures of ephrin-Eph binding for EphA and EphB receptors. Crystal structure for EphA4-ephrin-A2 interaction was solved by Bowden et al. (Bowden et al., 2009). Crystal structure for EphB4-ephrin-B2 interaction was solved by Chrencik at al. (Chrencik et al., 2006).

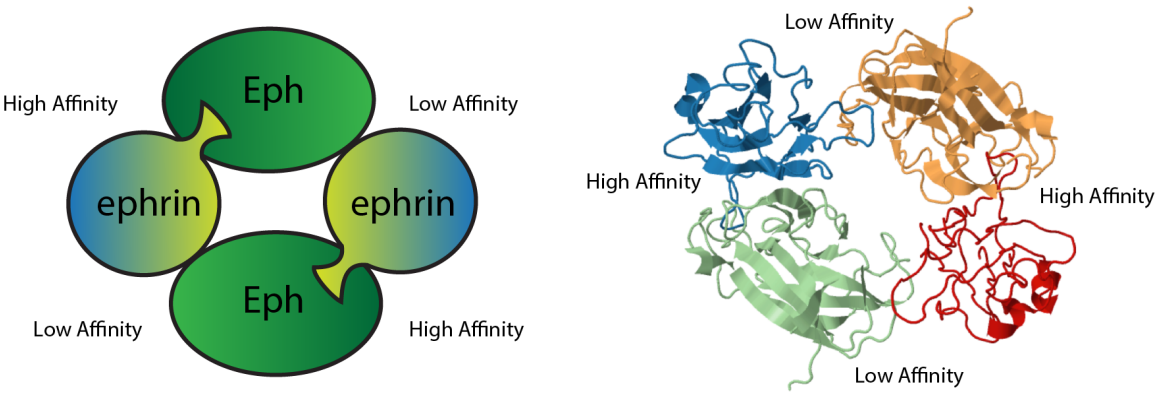

Figure 1.5. Eph-ephrin tetramer formation.
Eph receptors are able to interact with their ligands through both a high-affinity and low affinity binding interface, and this association brings Eph receptors together in a stable association with correct positioning for cross-phosphorylation. Image adapted from Murai and Pasquale (Murai and Pasquale, 2003). Crystal structure of EphB2-ephrin-B2 tetramer solved by Himanen et al. (Himanen et al., 2001). EphB2 is shown in green and orange, ephrin-B2 is shown in blue and red.

interactions contributing to the higher order oligomers can occur in a ligand independent manner (Wimmer-Kleikamp et al., 2004).

1.3.2.2. Cysteine-rich domain, Fibronectin-type III Like Domains, and the transmembrane helix

Immediately following the ligand-binding domain is the cysteine-rich region (CRD) of the receptor that was recently identified to structurally form a Sushi domain and an EGF-like domain (Seiradake et al., 2010). This region provides an interaction surface

between Eph receptors, and, as mentioned, appears to be important to ligand-induced receptor clustering (Himanen et al., 2010; Seiradake et al., 2010; Wimmer-Kleikamp et al., 2004).

Following the CRD, and immediately preceding the transmembrane domain, all Eph receptors possess two fibronectin-type III domains (FN1 and FN2), structural repeats of around 90 amino acids often found in fibronectin, and some cell surface proteins (Pankov and Yamada, 2002). These domains have been observed to assist in stabilising Eph-Eph interactions (Nikolov et al., 2014), but also to interact with ephrins in *cis* (within the same cell membrane) (Carvalho et al., 2006; Seiradake et al., 2010). Functionally, Eph-ephrin *cis* interactions are proposed to regulate the ability of Eph receptors to bind to and be activated by ephrins in *trans* (Carvalho et al., 2006), thus acting as an inhibitory mechanism to Eph activation.

The Eph receptor transmembrane domain is a short, hydrophobic alpha-helix (Bocharov et al., 2010), and aside from anchoring the receptor in the cell membrane, studies have also suggested that this region plays an important role in Eph-Eph interactions, stabilizing the receptor complex after ligand binding (Artemenko et al., 2008). However, its role may be more complex still, as EphA2 transmembrane domains are able to form dimers even in the absence of ligand (Bocharov et al., 2010), and there is structural evidence indicating that ligand-binding alters the interaction between the two transmembrane domains. Functionally, this effect is proposed to assist in ensuring receptor activation, potentially by promoting proper relative kinase domain orientation (Bocharov et al., 2010; Sharonov et al., 2014). In agreement, mutations within this region that inhibit its ligand-induced structural changes reduce the level of Eph receptor

activation following ligand binding (Sharonov et al., 2014). While this observation is as of yet exclusive to EphA2, it is possible that the transmembrane domains of other Eph receptors function in a similar manner, especially as other Eph receptor transmembrane domains have also been observed to interact (Bocharov et al., 2008).

1.3.2.3. Juxtamembrane domain

The Eph receptor juxtamembrane domain plays an important regulatory role in Eph receptor signalling as well as in signal propagation. Structural and biochemical analysis of the Eph receptor juxtamembrane domain determined that it is important to autoinhibition of kinase activity (Binns et al., 2000; Wybenga-Groot et al., 2001). This region contains two invariant tyrosine residues within a highly conserved motif of ten amino acids, and mutation of these residues severely compromises the kinase activity of the receptor in response to ligand treatment (Binns et al., 2000). When these residues are not phosphorylated, the conformation of the juxtamembrane domain is such that it disrupts the active site of the kinase domain, limiting its kinase activity, while these steric inhibitions are removed when the two regulatory tyrosines are phosphorylated (Wybenga-Groot et al., 2001) (**Figure 1.6**). Once phosphorylated these residues also play a major role in signal propagation as they are able to interact with a wide range of potential signal propagators, including the regulatory subunit of PI3K, p85 (Pandey et al., 1994), the adaptor protein Nck (Stein et al., 1998), and Src family kinases (Zisch et al., 1998).

1.3.2.4. Kinase domain

The Eph receptor kinase domain shares its major structural features will all other members of the RTK family. It is a two lobed structure, having a smaller N-terminal lobe

relative to the C-terminal lobe, with the two regions joined by a short linker. The kinase domain contains several highly conserved subdomains that are essential to its enzymatic activity, numbered I through XI, and within these there are key amino acids consistent between almost all tyrosine kinases, that are required for the kinase domain to function (Hanks et al., 1988; Hubbard and Till, 2000). Examples include the invariant aspartic

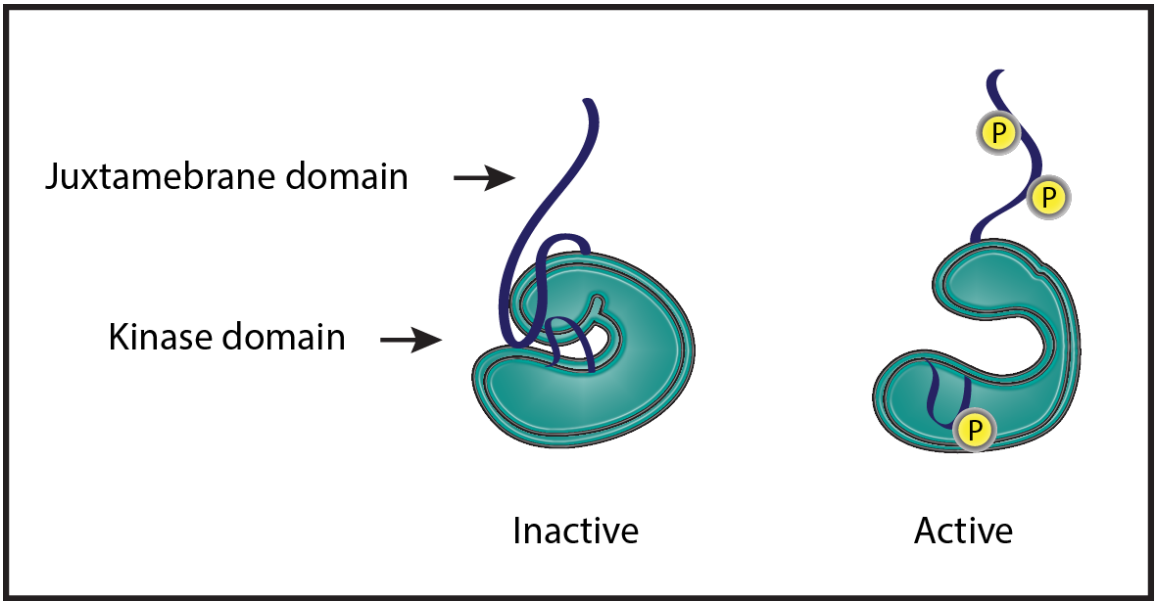

Figure 1.6. Auto-inhibition of the kinase domain.
In the absence of ligand, the juxtamembrane sterically inhibits the kinase domain, limiting the accessibility of its active site to ATP and target substrates, through interactions with both the N- and C-terminal lobes. Following ligand binding two tyrosine residues in the juxtamembrane domain are phosphorylated, relieving the steric inhibition, and allowing for the phosphorylation of a tyrosine in the kinase-domain activation loop, fully activating the receptor. Image adapted from Hubbard (Hubbard, 2004).

acid found in subdomain VI that is involved with ATP interactions, an invariant lysine residue, required for transfer of the phosphate group, located in subdomain II, and the consensus sequence Alanine-Proline-Glutamate in subdomain VIII (part of the activation loop), that is required for catalytic activity (Hanks et al., 1988). A more unique feature ofthe Eph receptor kinase domain is that it is auto-inhibited by the juxtamembrane domain, and also by the more classical example of kinase-domain steric inhibition, mediated by the kinase domain activation-loop, both of which inhibit ATP and substrate binding when in their unphosphorylated configurations (Wybenga-Groot et al., 2001). Phosphorylation of tyrosines within the Eph receptor's activation loop, as well the two residues within the juxtamembrane domain, allow the kinase domain to assume a more "open" conformation, facilitating substrate binding and kinase activity (Binns et al., 2000) (**Figure 1.6**).

1.3.2.5. The SAM domain and PDZ-binding motif

The crystal structure of the Eph SAM domain was solved during the early period of discovery, and revealed two potential self-interacting domains (Stapleton et al., 1999; Thanos et al., 1999). However, due to their observed low level of affinity, it is now believed that these domains are only weakly involved in stabilizing interactions between Eph receptors (Behlke et al., 2001). Rather, the primary function of SAM domains appears to be in assisting in signal propagation through the interaction with downstream effectors. Among these include the regulatory subunit of PI3K, p85, and the cytoskeletal regulator, Vav3 (Fang et al., 2008). The SAM domain has also been observed to interact with proteins that may affect receptor stability on the cell membrane (Kajiho et al., 2012; Kim et al., 2010).

The PDZ-binding motif interacts with downstream effectors containing a PDZ domain, including AF6 (Hock et al., 1998), a protein that associates with components of the actin cytoskeleton (Kooistra et al., 2007), as well as the to Ras family GTPases (guanine-triphosphatases) (Linnemann et al., 1999). Other examples of proteins that interact with the PDZ-binding motif include PICK1 (Protein interacting with C kinase 1) and Grip. Of these, PICK1 at least may help contribute to Eph receptor signaling, as it was observed to promote Eph receptor clustering (Torres et al., 1998). However, the biological relevance of the PDZ-binding motif is not yet fully clear, as at least one study shows this domain is dispensable for normal Eph biological activity (Dufour et al., 2006).

1.3.3. Eph-Ephrin Binding and Receptor Activation

Using crystallography, it has been determined that ligand binding to Eph receptors allows for the formation of stable Eph-ephrin dimers, and tetramers, (Himanen et al., 2001), where at least two Ephs simultaneously interact with two ephrins to initiate the activating phosphorylation of the receptors (Himanen et al., 2001; Himanen et al., 2010; Janes et al., 2012). While Eph receptors are able to form transient interactions with each other through multiple domains in the absence of ligand (Himanen et al., 2010), Eph-ephrin binding is believed to stabilize Eph receptor interactions, and allow for the initiation of Eph receptor oligomerisation following ligand interaction (Himanen et al., 2001; Himanen et al., 2010; Seiradake et al., 2010), and to also maintain Eph receptors in an orientation that promotes their ability cross-phosphorylate and activate each other (Seiradake et al., 2010) (**Figure 1.7 A&B**). While Eph receptor dimers may be sufficient for Eph receptor signalling (Himanen et al., 2001), it is now believed that Eph receptors

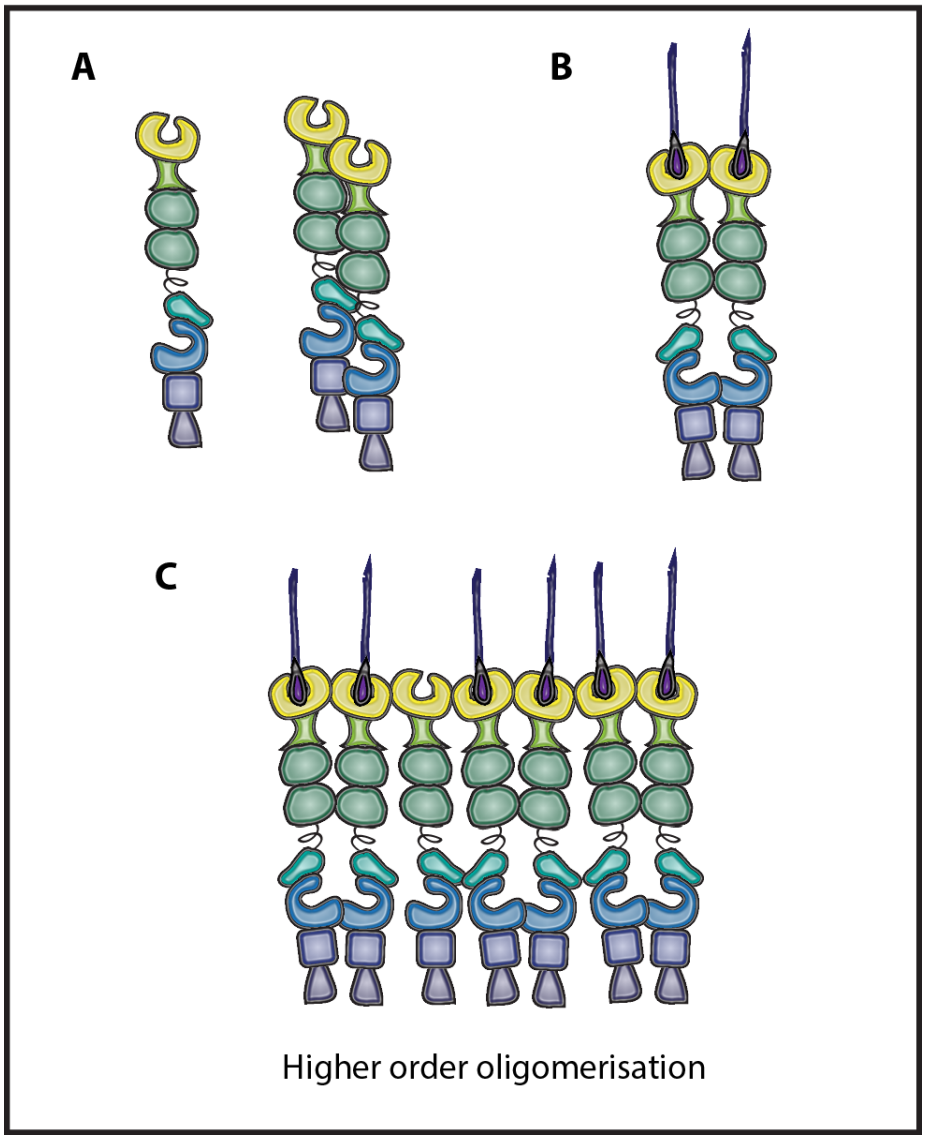

Figure 1.7. Ligand induced Eph receptor oligomerisation.
A) In the absence of ligand Eph receptors exist as monomers, or as transient interaction partners. The unliganded Eph receptor interactions have been observed to take place between the cysteine-rich domain and the kinase domain, among others, and do not result in Eph-Eph spatial relationships that are favorable to cross-phosphorylation. **B**) Ligands expressed on an adjacent cell bind to Eph receptors, inducing stable Eph-Eph interactions, and orient the receptors such that they are able to cross-phosphorylate each other. **C**) Following ligand binding, higher order Eph receptor oligomerisation often takes place, including both ligand-interacting and unliganded receptors.

also form higher level complexes, containing homo- and/or heterologous interactions between Ephs (Janes et al., 2011; Seiradake et al., 2010; Wimmer-Kleikamp et al., 2004) (**Figure 1.7 C**). Due to the ability of Ephs to form heterologous interactions (Janes et al., 2011), it is likely that the varying contributions of each receptor within a signalling complex allows for the fine-tuning of exact biological outputs. Some observations have even indicated that EphA and EphB receptors can interact with each other upon ligand activation of one or the other receptor class, and these cross-class interactions contribute to the overall signalling output (Janes et al., 2011).

1.3.4. Eph Receptor Signalling

Eph mediated effects are not restricted by the presence of ligand, and indeed, while many Eph receptor effects are ligand-initiated, others are strictly ligand independent. Through their active signalling, Eph receptors control a wide range of responses, including rearrangements of the actin cytoskeleton, cell attachment, repulsion, migration, proliferation and survival, in a variety of normal and malignant cell types (Pasquale, 2005, 2010). The ability of Eph signalling to influence the actin cytoskeleton is essential to facilitating their effects on cell movement, attachment, and repulsion (Klein, 2012). Eph receptors are able to influence actin dynamics by multiple mechanisms, including the ability to modulate the Rho family of GTPases, a protein family that largely coordinates the extension or collapse of branches of the actin cytoskeleton (Hanna and El-Sibai, 2013). Within this family, Rac1 is responsible for the formation of lamellipodia, Cdc42 is involved with filopodia formation, and RhoA has been implicated in the formation of stress fibers and grown cone collapse (Kozma et al., 1997) (**Figure 1.8**). Eph receptors

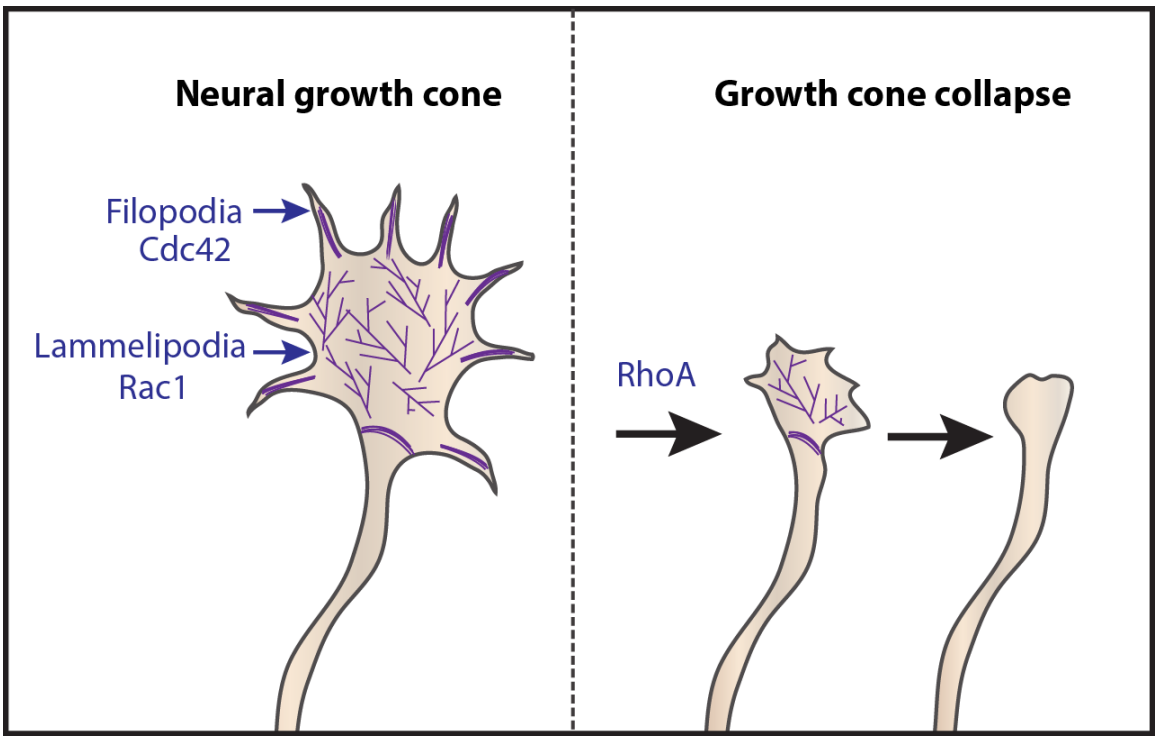

Figure 1.8. Cytoskeletal arrangements are coordinated by the Rho family. Neural growth cones depend on Rho family GTPases to help coordinate the actin skeleton and promote outgrowth. Filopodia are long finger like extensions that form focal adhesions with the substrate, and bundled actin filaments that rely on Cdc42 for their formation provide structural strength. Lammelipodia are regions of high actin polymerization activity, containing a network of branched actin that helps to push the leading edge of a cell forward, and depend on the activity of Rac1 for this process. In contrast, RhoA activity can lead to a disassembly of the actin structures in the growth cone and lead to growth cone collapse, an important process in the targeting of neuronal axons to their correct targets. Figure adapted from Pak et al. (Pak et al., 2008).

influence Rho GTPases, increasing or decreasing their activity, often acting through members of the Dbl family of guanine exchange factors (GEFs), ephexins (Shamah et al., 2001). For example, activation of EphA4 results in the phosphorylation of ephexin1, enhancing its activity towards RhoA in relation to Cdc42 and Rac1. This relative increase

in active RhoA compared to Rac1 and Cdc42 promotes growth cone collapse and retraction (Sahin et al., 2005) (**Figure 1.8**). Additionally, separate studies have indicated that ephrin initiated EphA4 signalling leads to Rac1 inactivation through a mechanism dependent on α-chimerin, a GTPase activating protein (GAP) for Rac1, providing a further Eph induced mechanism that shifts the balance in Rho family activities (Iwasato et al., 2007). In contrast to EphA4 signalling, EphB2 activation leads to the ubiquitination and degradation of ephexin5, inhibiting RhoA activity, and promoting growth cone extension and synapse formation (Margolis et al., 2010). Eph receptors are known to influence the cytoskeleton in the absence of ligand as well, as EphA2 interacts with ephexin4 independently of ligand-interaction, promoting the downstream activation of RhoG and recruitment of Rac GEFs, ELMO2 (Engulfment And Cell Motility 2) and Dock4. This allows for an increase in active Rac within the cell, and overall leads to enhanced breast cancer cell motility (Hiramoto-Yamaki et al., 2010).

In parallel to this, EphB3 activation is able to attenuate cell movement by directly reducing Akt activity (Li et al., 2012). In addition to the cyto-protective signalling Akt participates in, it is also able to regulate cell motility through several means, including altering cytoskeleton dynamics by phosphorylating the actin-interacting protein Girdin (Enomoto et al., 2005), modulation of intergrin recycling (Li et al., 2005), as well as other mechanisms (Jiang et al., 2009). EphB3 inhibits these activities by instigating the PP2A (protein phosphatase 2A)/RACK1 (Receptor for activated C kinase 1) dependent dephosphorylation of Akt, resulting in reduced cell motility (Li et al., 2012). EphA2 cross talk with Akt has also been found to modulate cell motlitlity, as it has been observed that in the absence of EphA2 ligand, active Akt phosphorylates Serine-897 of EphA2, and this

serves to promote cytoskeleton rearrangements and cell movement. In contrast to this, ligand-induced EphA2 kinase activity was found to actively block cell motility by inhibition of PI3K/Akt signaling and dephosphorylation of EphA2 Serine-897 (Miao et al., 2009). In contrast, some active Eph receptors have the ability to promote Akt signalling, as EphB receptor activation of the PI3K/Akt pathway has been observed in the spinal chords of mice, and is believed to be involved in nociception (pain perception) (Yu et al., 2012). Furthermore, our group has made observations in malignant T-cells, where activation of Akt by EphB receptors prevents Fas-induced apoptosis (Maddigan et al., 2011).

One mechanism utilized by Eph receptors to influence cell-cell adhesion is through the modulation of other cell surface proteins, including cadherins. Cadherins are involved in the formation of stable connections between cells, and are a crucial component of adherins junctions (Maitre and Heisenberg, 2013). In *Xenopus* embryos, activation of Eph receptors was shown to negatively regulate cadherin mediated cell-cell adhesion (Winning et al., 1996), which may be an important mechanism to facilitate Eph receptor mediated cell-cell repulsion, and in agreement, in a human cell line, Eph expression was found to reduce expression of cadherin17 (Bhushan et al., 2014). In contrast, in some situations, Eph receptors may also utilise cadherins to promote enhanced adhesion, as a separate group found that in a colorectal cancer cell line, DLD1, Eph activation led to an increased targeting of cadherin to the cell surface, and that this was important to the correct execution of Eph receptor effects (Cortina et al., 2007).

Not only do Eph receptors play key roles in regulating cell movement and attachment, they also act to promote or inhibit proliferation. As mentioned, activation of

the Ras/MAPK pathway frequently results in cellular proliferation, and EphA2 activation has been observed to inhibit Ras/MAPK signalling in multiple cell types, including prostate epithelial cells. Here, ephrin-A activated EphA2 is able to reduce overall levels of MAPK phosphorylation, and also attenuates EGF and PDGF enhancement of Ras/MAPK signalling (Miao et al., 2001). Ligand activation of EphA2 inhibits MAPK signaling in myoblasts as well, where it alters the output of IGF induced signals by reducing activation of the Ras/MAPK pathway through the activation of p120RasGAP (GTPase activating protein) (Minami et al., 2011). In contrast, EphA2 activation does not inhibit the IGF induced activation of the PI3K/Akt pathway, and this shift in overall signaling output was observed to enhance myoblast differentiation (Minami et al., 2011). Signalling appears to be cell-type dependent, as treatment of PC3, a PTEN (phosphatase and tensin homolog) deficient prostate cancer cell line with high levels of constitutive Akt activation, with an EphA2 ligand was observed to decrease both Erk1/2 and Akt phosphorylation (Yang et al., 2011). Furthermore, EphB4 appears to be able to activate, or inactivate the Ras/MAPK pathway depending on the cell type, as it has been observed to promote proliferation through MAPK activation in the breast cancer cell line, MCF7 (Michigan Cancer Foundation-7), but to inhibit growth and suppress Ras/MAPK signaling in the endothelial cell line, HUVEC (Human Umbilical Vein Endothelial Cells) (Xiao et al., 2012). Likewise, ephrin-B1-activated EphB2 decreases MAPK phosphorylation in glioma cell lines, by activating R-Ras, a Ras family member that does not initiate the MAPK signaling cascade (Hancock, 2003; Nakada et al., 2005; Self et al., 2001), while EphB2 signalling increases Ras and Erk2 phosphorylation in the colon carcinoma cell line, DLD1 (Riedl et al., 2005). In mice neuroepithelial cells, EphA3 and

EphA4 increase MAPK activity in response to ephrin-A1 treatment, but in a Ras independent manner, suggesting that non-classical activation of the MAPK signaling cascade can be initiated by Eph receptors as well (Aoki et al., 2004).

Eph receptors may also play a role in signalling pathways critical to regulating differentiation, as there are increasing observations of interactions between Eph activation and Wnt signalling. For example, EphA2 has been shown to contribute to the ephithelial-mesenchymal transition (EMT) in gastric cancer cells by enhancing activation of the Wnt/β-Catenin pathway (Huang et al., 2014). EphB6, on the other hand, may act to suppress EMT, as its expression was shown to reduce nuclear β-catenin, and to reduce β-catenin transcriptional activity (Toosi et al., In Revision), and this may contribute the less invasive phenotype observed when EphB6 is present (Truitt et al., 2010).

1.3.5. Eph Receptor Biological Functions

1.3.5.1. Ephs in embryogenesis

In agreement with the wide range of signalling pathways influenced by Eph receptors, they are actively involved in multiple biological processes. During embryo development Eph-ephrin interactions play crucial roles in segregation of tissues (Klein, 2012). The first observations of Eph mediated tissue separation came from studies of rhombomere segregation in the mouse embryo. Rhombomeres are sequential segments located in the developing hindbrain that ultimately give rise to the pons, cerebellum, and medulla (Gray et al., 2005). In the developing embryo, Eph receptors and their ligands are alternately expressed in sequential rhombomere segments (Becker et al., 1994;

Bergemann et al., 1995; Gilardi-Hebenstreit et al., 1992) (**Figure 1.9**), and experiments in the zebrafish have demonstrated the ability of active Eph and ephrin signalling to

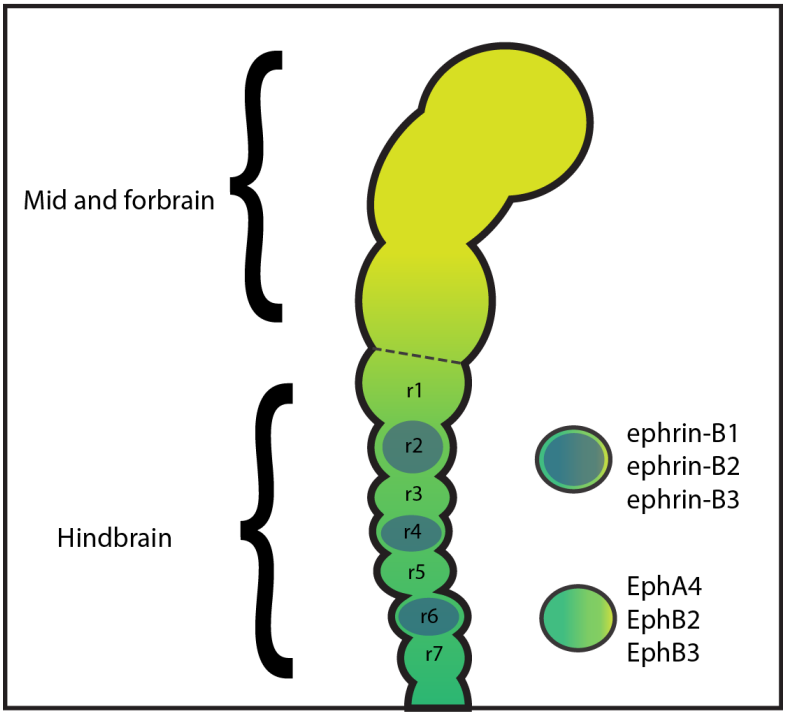

Figure 1.9. Rhombomere segregation in the developing embryonic brain. Eph receptors and their ligands are alternately expressed in odd (Ephs) or even (ephrins) numbered rhombomeres (r1-r7), are essential to the formation of boundaries between the segments. Figure adapted from McNeill (McNeill, 2000).

determine the preferential sorting of cells into either odd, or evenly numbered rhombomeres (Xu et al., 1999). Further support for the important role of Eph receptors in rhombomere segregation comes from observations that rhombomere formation and segregation is severely impaired when Eph expression is eliminated (Cooke et al., 2005).

It is now understood that Eph receptors play a major role in development of the nervous system, and are involved in various aspects of the process. Eph receptors are essential to mediating the correct migration of neural crest cells, an embryonic cell group that gives rise to the majority of the peripheral nervous system, as well as the most of structures of the vertebrate head (Simoes-Costa and Bronner, 2015). For example, neural crest cells migrating within pharyngeal arches, precursors to specific skeletal, muscle, venous, and nervous tissues within the head (Grevellec and Tucker, 2010), rely on signals from EphA4 and EphB1 to facilitate their correct patterning (Smith et al., 1997). Within somites, sections of embryonic mesodermic cells that give rise to the cartilaginous tissues of the spine, rib cage, and to the majority of skeletal muscle (Barrios et al., 2003), neural crest cell migration is limited to the anterior portion through active EphB signalling, maintaining their correct positioning to ultimately become sensory neurons (Krull et al., 1997). Eph receptors have also been implicated in the proper formation of the somites themselves, and in the resulting proper patterning and growth of the skeleton, including symmetrical fusion of the rib cage, digit formation, and craniofacial formation (Compagni et al., 2003; Davy et al., 2004).

Axons are frequently bundled together along the pathway between their source and their target, forming either nerves in the peripheral nervous system, or neural tracts within the brain. In 1992 the first observations of Eph involvement in axon bundling were

made, where it was found that chicken EphB2 was highly expressed in the bundled, or fasciculated regions, of unmyleniated axons in the developing hippocampus and cerebellum (Pasquale et al., 1992). Shortly after further evidence for Eph involvement in this process was found in rats, where it was observed that ephrin-A5 and EphA5 are highly expressed in the brain, and that their active signalling was required for the proper formation of axon bundles (Winslow et al., 1995). More recently, direct evidence of ephrin-B1 induced EphB2 signaling promoting axon bundling was obtained, where it was demonstrated that ephrin-B1 induced growth cone collapse in EphB2 expressing neurons, and acted as a negative guidance cue as well as a promoter of axon bundling (Luxey et al., 2013). Eph receptors are also important to axon defasciculation, the unbundling of axons that allows for their individual interaction with their correct targets (Van Vactor, 1998), as mice lacking EphB2 and EphB3 show abnormalities in this process (Chen et al., 2004).

An excellent example of Eph receptor involvement in mediating axon guidance can be found in the developing embryonic retina-brain interface. For example, in studies using mice embryos it was found that retinal axons exhibit repulsive behavior when they come into contact with EphB receptors, either by attenuating growth in the direction of the Eph receptor contact, or though growth cone collapse (Birgbauer et al., 2001), and similar observations have been made for EphA-ephrin-A interactions (Drescher et al., 1995). It is now understood that Eph-ephrin gradients play a crucial role in the correct formation of the retinal topographic map within the brain, serving to maintain spatial relationships between neurons of the superior colliculous and the retinal ganglion cells (Triplett and Feldheim, 2012). Retinal ganglion cells show an expression pattern of high

EphA expression in cells located temporally, in a declining gradient to low EphA expression in those cells located at the nasal side, coupled with high ephrin-A to low ephrin-A expression from the nasal to temporal side of the retina (Lambot et al., 2005). Likewise, EphB expression ranges from high to low along the ventral to dorsal axis, and the ephrin-B expression gradient is the reverse (Braisted et al., 1997). Within the neurons of the superior collilculous, there is a complementary expression pattern, although the axis shifts from vertical positioning, to a horizontal map that is rotated relative to the retinal positioning. During the formation of the topographic map, RGC axons grow towards the posterior of the superior colliculous. A well supported model for the function of EphAs in topographic modeling is that those RGCs with high expression of EphA receptors are positioned first, for as they enter the superior colliculous, and encounter the first neurons with ephrin-A expression (low ephrin-A), due to their high expression, have Eph-ephrin signaling cascades initiated in sufficient strength to cause growth cone collapse, and to prevent further outgrowth. As the axons move forward, they encounter higher ephrin concentrations, and this allows for cells with lower EphA expression to have their outgrowth inhibited as well, and this continues as such along the gradient of the anterior-posterior axis (Triplett and Feldheim, 2012). The mechanism of EphBs in mediating topographic mapping is less clear, but ephrin-B1 stimulation of EphB expressing RGCs appears to function both as an attractant and repellant, and is essential to correctly position cells along the lateral-medial axis, as EphB knock-out mice display correct anterior-posterior axonal positioning, but incorrect medial-lateral relationships (Harada et al., 2007; McLaughlin et al., 2003) (**Figure 1.10**).

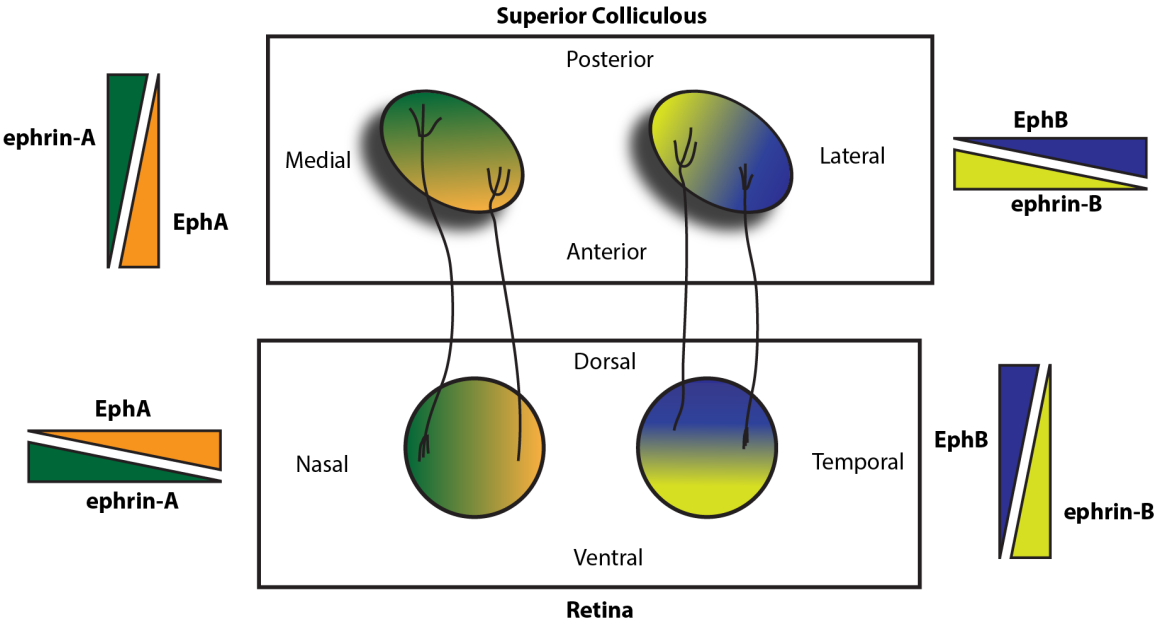

Figure 1.10. Eph receptors are involved in formation of the retinal topographic map.
Gradients of EphA and EphB receptors as well as their ligands play important roles in guiding axons from cell of retinal ganglion to their correct position within the superior colliculous. In general, neurons with high EphA expression target to areas with low ephrin-A expression, and those with low EphA expression target to areas with high ephrin-A expression along the anterior-posterior axis. RGCs with high EphB expression target to areas with high ephrin-B expression, and vice versa, along the medial-lateral axis. Figure adapted from Pasquale (Pasquale, 2005).

Eph receptors are essential signalling molecules for the correct guidance of spinal neuronal axons during development as well, where EphA4, in particular, has been demonstrated to play a regulatory role during axon development within the corticospinal

tract (Canty et al., 2006). In mice lacking EphA4, pronounced motor abnormalities are observed, including a "hopping" gait as opposed to the normal alternating limb movement (Kullander et al., 2003), and this was functionally observed to be the result of abnormal axonal crossing of the spinal chord midline (Kullander et al., 2003). This effect is mediated through repellent forward signalling into EphA4 expressing cells, triggered by their interaction with ephrin-B3 expressed at the midline of the spinal chord (Kullander et al., 2001). The necessity of EphA4 forward signalling was confirmed through experiments that showed a loss of ephrin-B3 or expression of a cytoplasmic domain deletion mutant of EphA4 resulted in the same physiological effects (Paixao et al., 2013; Yokoyama et al., 2001), while expression of a cytoplasmic deletion mutant of ephrin-B3 did not (Yokoyama et al., 2001). EphA4 has been implicated in regulating the correct temporal and spatial organization of axon branching within the spinal cord as well (Canty et al., 2006).

Synapses, specialised signalling junctions between neurons and other cells, are also dependent upon Eph receptors for their proper formation. Within dendrites, the information "receiving" arm of a neuron, structural protrusions called dendritic spines form synaptic connections with the transmitting cell, and the formation of these structures requires active signalling by EphB receptors (Ethell et al., 2001; Penzes et al., 2003). In agreement, in mice lacking EphB1, EphB2, and EphB3, fewer dendritic spines are formed, and those present exhibit structural abnormalities (Henkemeyer et al., 2003). Eph receptors may also be important to axon terminal positioning and formation, as EphA4 has been observed to localize to this region in neurons of the hippocampus (Tremblay et al., 2007).

Apart from the well established role of Eph receptors in the development of the nervous system, their involvement in the regulation of T-cell functionality and in thymus development is perhaps the most studied to date (Wu and Luo, 2005). The expression of several Ephs and ephrins have been detected in the thymus, and their individual expression levels are often localised to specific compartments. Among these include EphA7, ephrin-A1, and ephrin-A5 which are all expressed in the thymic cortex; EphA8, EphA1, EphA2, and EphA4 which are found in the medulla, and ephrin-A2 which is expressed throughout the thymus (Vergara-Silva et al., 2002). While less is known regarding the compartmental localisation of EphBs within the thymus, all EphB receptors and ephrins show some level of expression in this organ, with EphB6 expression being the most pronounced (Hafner et al., 2004). Of these, EphB2 and EphB3 are expressed on thymic epithelial cells (TECs) (Garcia-Ceca et al., 2009b), and EphB6 is found predominantly within the cortex (Hafner et al., 2004). Consistent with the ability of Eph receptors to mediate spatial patterning and cell motility, ephrin-B2 expression is essential for the proper migration of the developing thymus into the thoracic cavity during embryogenesis. When isolated thymus cells were treated with a soluble EphB4 fusion protein that blocks EphB signalling, they observed a similar inhibition of cell motility relative to those cells lacking ephrin-B2 expression, strongly indicating EphB-ephrin-B2 forward signalling in this process (Foster et al., 2010). EphB2 and EphB3 appear to be important to the proper structural organisation of the thymus, as morphological defects are observed when the expression of either is perturbed (Alfaro et al., 2011; Garcia-Ceca et al., 2009a). EphBs also appear to play a role in the survival of TECs, cells which are important to assisting in proper T-cell maturation (Takada et al., 2014), as disruption of

EphB signalling leads to increased apoptosis of embryonic TECs (Garcia-Ceca et al., 2013).

Eph receptors are also essential signaling molecules during formation of the vasculature. Eph receptors and ephrins are expressed on the endothelial and mesenchymal cells that organize during the process of vasculogeneis (Adams et al., 1999), and in agreement, are essential for proper formation of the heart and angiogenesis during embryonic development (Shin et al., 2001; Wang et al., 1998). The EphB4 receptor, and its ligand, ephrin-B2, are important to guiding mural and endothelial cells during the formation of vasculature (Fuller et al., 2003), and Eph-ephrin interactions play an essential part in the formation and separation of veins and arteries (Gerety et al., 1999; Shin et al., 2001; Wang et al., 1998). Indeed, EphB4 and ephrin-B2 serve as markers for venous or arterial cell types, respectively, in the developing embryo (Wang et al., 1998), where they play important roles in spatial organization and vessel formation (Fuller et al., 2003).

1.3.5.2. Ephs in the adult organism

Eph receptors continue to play a crucial part in the adult organism, able to assist in the maintenance of homeostasis, tissue repair, and spatial patterning (Pasquale, 2005). High levels of expression for multiple Eph receptors are found in the brain of the adult organism (Hafner et al., 2004; Hruska and Dalva, 2012), and in agreement, studies have demonstrated a significant role of Eph receptors in the adult brain, where they have been implicated in mediating synaptic plasticity, the ability of the brain to learn new things and adapt throughout life (Klein, 2009; Pasquale, 2010). One of the mechanisms underlying this process is the ability of neurons to undergo long-term potentiation (LTP). This

involves the formation of new synaptic connections between two neurons, as well as in an increase in the number of signal transmitting neural receptors, in response to high levels of repeated stimulus (Baudry et al., 2014). Overall, this allows for a higher signal to be generated in the receiving cell in the future, when the stimulus is repeated, even with lower levels of stimulus (Baudry et al., 2014). Two receptors involved in generating LTP are the N-methyl-D-aspartate receptor (NMDAR) and the α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor (Anwyl, 2009). Briefly, in order to generate LTP, AMPA receptors in the post-synaptic neuron need to be activated in a high enough level to allow for the subsequent activation of the NMDA receptors, and this facilitates NMDA initiation of LTP. EphB2 appears to play a role in regulating this, based on several observations. First, activation of EphB2 leads to its rapid association with NMDAR (Takasu et al., 2002), the interaction mediated through the extracellular domains of the proteins (Dalva et al., 2000), and EphB2 ligand-binding has been observed to cause NMDAR phosphorylation in a Src dependent manner (Takasu et al., 2002). Additionally, EphB activation has been observed to increase the effects of NMDAR signalling (Takasu et al., 2002). In mice lacking EphB2, there is a significant reduction in NMDAR localised in post-synaptic densities compared to those with EphB2 expression (Henderson et al., 2001), implying a role for EphB2 in recruiting NMDAR to this region, and importantly there is also a decrease in the observed NMDAR mediated LTP (Henderson et al., 2001). Furthermore, EphB2 is also able to interact with the AMPA receptor through its PDZ-binding motif, and this appears to assist in clustering and stabilisation of the AMPA receptor at the surface of the post-synaptic density (PSD), ensuring proper spatial localization for efficient activation (Kayser et al., 2006). In

contrast to EphB2-NMDAR interactions and effects, EphB2's effect on the AMPA receptor is independent of its kinase ability (Kayser et al., 2006). Thus, EphB2 is able to ensure correct targeting of synaptic signalling molecules to the post-synaptic density, as well as to enhance signaling, overall contributing to LTP and synaptic plasicity.

Although the ability of nerves to heal following damage is limited, some attempt at repair is often made, and Eph receptors have been observed to play an important role in promoting or inhibiting this process. For example, following optic nerve damage in mice, EphB3 expression is increased in macrophages that are recruited to the site of injury. It was demonstrated that the axons of ephrin-B3 expressing RGCs are attracted to EphB3, suggesting that it acts as a positive guidance cue during axon repair. In mice lacking EphB3, reduced axon sprouting and nerve regrowth was observed, strongly implicating EphB3-ephrin-B3 signalling as positive mediators of axon outgrowth following nerve damage in the adult organism (Liu et al., 2006). In contrast to this, EphA4 acts to inhibit axon repair in the spinal cord in mice following injury (Fabes et al., 2006), and mice lacking EphA4 show improved repair and return of function relative to mice with normal EphA4 expression (Goldshmit et al., 2004). Furthermore, positive results have been seen in mice where the use of peptides that block EphA4 activation lead to improved tissue repair and functional recovery, strongly implicating EphA4 forward signaling in axonal outgrowth inhibition (Goldshmit et al., 2011).

Eph receptors continue to function in the post-natal thymus as well, where they function predominantly as a component of the T-cell regulatory network. Eph receptors can act as guidance cues for immature thymocytes, demonstrated by the requirement for EphB2 expression for the proper migration of bone marrow hematopoietic progenitor

cells into thymic compartments, an important physical localization that promotes correct cellular differentiation and maturation (Stimamiglio et al., 2010). Within the thymus Eph receptors may help modulate T-cell selection, as both EphB receptors (Freywald et al., 2003), and EphA receptors (Freywald et al., 2006) are able to protect T-cells from TCR instigated apoptosis. The Eph-ephrin system also appears to play a role in T-cell motility, as ephrin-A1 stimulation of $CD4^+$ T-cells is able to modulate their chemotaxis in response to various cytokines (Aasheim et al., 2005a; Sharfe et al., 2002).

The presence of Ephs and ephrins in the cells of the circulatory system persists into adulthood (Shin et al., 2001), which suggests they play a role in maintaining these populations throughout the lifetime of the organism. Ephrin-B2 and EphB4 continue to serve as markers for arterial and venous vessels, respectively, in the adult organism (Gale et al., 2001), and changes in their expression are observed during the phenotypic shifts required during neovascularization in the adult organism (Kudo et al., 2007; Nunes et al., 2011). Additionally, Eph signaling appears to play a role in initiating neovascularization, working together with ephrin-B2. For instance, ephrin-B2 expression is upregulated in response to the angiogenesis-promoting vascular endothelial growth factor (VEGF) and FGF, and ephrin-B2 treatment has been demonstrated to initiate venous angiogenesis (Hayashi et al., 2005). Complementary to these findings, it has been shown that blockage of EphB4 activation is able to severely inhibit neovascularization, strongly supporting ephrin-B2-EphB4 signaling as a necessary element for initiation of this process (Su et al., 2013). Additionally, the human brain microvascular endothelial cell line (HBMEC) displayed impaired angiogenic behaviour in cell culture when EphA2 expression was

reduced, or a mutant of EphA2 lacking kinase activity was expressed, indicating other Eph receptors are also involved in regulating angiogenic progression (Zhou et al., 2011).

Eph receptors have also been implicated in actively regulating bone homeostasis (Matsuo and Otaki, 2012). Osteoblasts and osteoclasts promote bone formation, and bone resportion, respectively, and their activities are carefully balanced to maintain bone integrity. EphB4, ephrin-B2 and ephrin-B1 signals promote differentiation of osteoblasts, while inhibiting osteoclastogenesis, from osteoclast and osteoblast precursor cells, and thereby direct bone formation over resorption (Cheng et al., 2012; Zhao et al., 2006). Complementary to this, EphA2 and ephrin-A2 signalling acts to promote osteoclastogenesis, and to inhibit osteoblastogenesis, thereby promoting bone resporption (Irie et al., 2009). Together, these findings suggest that the modulation of the expression of Ephs and ephrins is an essential component in regulation of bone formation and degradation, and bone homeostasis.

While their function in the developing pancreas has not been explored, Eph receptors are expressed in this organ during embryogenesis (van Eyll et al., 2006), and continue to be expressed and play important roles in the pancreas of the adult organism. Here, Eph signalling has been observed to be involved in regulating insulin production. Cell-cell contact between β-cells of pancreatic islets results in constitutive forward signalling through EphA5, and this was found to suppress insulin production. Following exposure to glucose, cells rapidly dephosphorylate EphA5, leading to a shift in the balance between EphA5 signalling, and the signalling output from its ligand, ephrin-A5. Overall, this change in signaling output was observed to result in an increase in insulin production (Konstantinova et al., 2007).

1.3.5.3. Ephs in malignancies

Eph receptors also play an important role in human malignancies, including breast, colorectal, prostate, brain, skin, lung, hepatocellular, and gastric cancers (Xi et al., 2012). Their overall effect appears to be receptor and tissue type specific, as Eph receptors have been associated with both aggressive and non-aggressive cancers, and have been shown to have both pro and anti-malignant effects. In agreement with their role in mediating cell-cell and cell-substrate attachment and repulsion as well as motility, the observed impact of Eph receptors in malignancies is often related to the invasive and metastatic potential of the cancer (Kandouz, 2012). Invasive activity of a cancer cell is defined by its ability to invade the surrounding tissue, generally by altering the surrounding extracellular matrix, disengaging cell-matrix and cell-cell contacts at its point of origin, and promoting cell movement that occurs as a result of dynamic alterations to the actin cytoskeleton (Friedl and Alexander, 2011) (**Figure 1.11**). Eph receptors have been implicated in modifying the invasive behavior of cancer cells by altering the activity of enzymes involved in the degradation of the extracellular matrix, by inducing changes to the actin cytoskeleton associated with motility, and by altering the surface presence of proteins involved in cell anchorage (Campbell and Robbins, 2008; Gucciardo et al., 2014). In addition to having the ability to invade surrounding tissues, metastatic cancer cells must also have the ability to enter into either the blood or lymphatic system and travel to other organs in the body. For cancer cells originating from solid tumor types, such as those of the breast, lung, and skin, this requires that the metastatic cell is able to survive independent of the pro-survival signals normally generated by contact with the surrounding environment. A healthy cell will undergo a

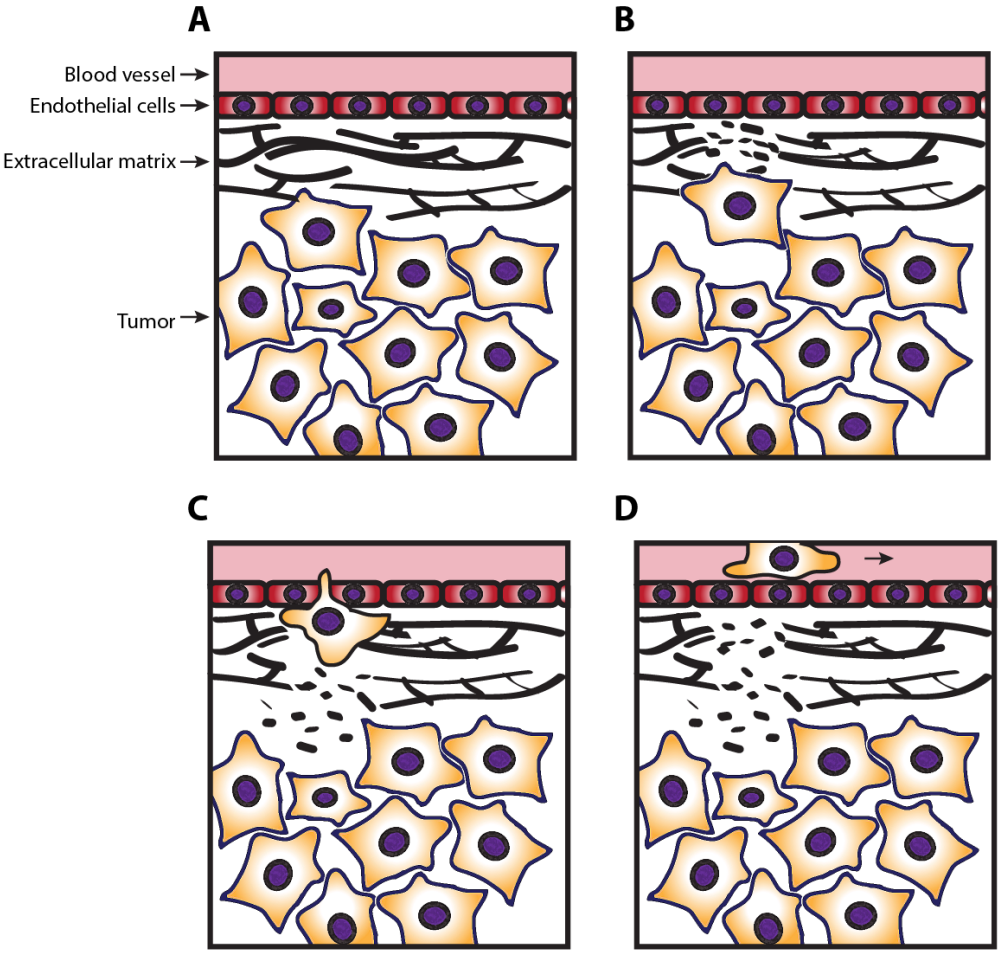

Figure 1.11. Cancer cell invasion.

A) Representation of a solid tumor mass and the surrounding tissue. **B**) An invasive cancer cell produces enzymes capable of degrading the extra cellular matrix, detaches from other cells of the tumor, as well as from its connections with the surround matrix, invading the surrounding tissue. **C**) Migrating cancer cells are able to invade the blood vessels by migrating between endothelial cells, breaking the connections between them as they do. **D**) Once in the blood stream, an invasive tumor can migrate to new tissues to form secondary tumors.

specialised form of apoptosis, anoikis, in the absence of these signals, where a metastatic cancer cell will not. Additionally, in order to form a secondary tumour in a distinct tissue from that in which the tumour initiated, a cancer cell must be able to invade the new tissue, adapt to the new environment, and to initiate proliferation in order to generate a secondary lesion (Chaffer and Weinberg, 2011). Eph receptors are able to modulate metastatic behaviour by facilitating changes in gene transcription that help cells evade anoikis, and exhibit greater plasticity allowing them to better adapt to foreign environments (Wang, 2011).

In non-small cell lung cancer (NSCLC), high levels of EphA2 expression were correlated to an increased risk of metastasis to the brain (Kinch et al., 2003), and biochemical analysis of the effect of EphA2 in lung cancer cell lines found that cells expressing EphA2 had increased invasive behaviour and resistance to apoptosis relative to EphA2-null cells (Faoro et al., 2010). Furthermore, an EphA2 mutation has been identified in several NSCLC patient samples, and this mutant exhibited constitutive kinase activity, an increased phosphorylation of EphA2 targets, including Src and cortactin, as well as increased cell-survival and invasive behaviour, relative to the wild-type receptor (Faoro et al., 2010). EphB3 has also been implicated in NSCLC, where its high expression has been linked to increased metastatic behaviour, through mechanisms independent of its kinase activity (Ji et al., 2011). Interestingly, ligands of EphB3, ephrin-B1 and ephrin-B2 are often downregulated concurrently with increased EphB3 expression in NSCLC, and it was found that ligand-induced EphB3 signalling inhibits metastasis, by acting on several regulators of the actin cytoskeleton (Ji et al., 2011).

As Eph receptors play critical roles in brain development, and show continued expression in the brain in adulthood, it is unsurprising that they would be implicated in its malignancies as well. In glioblastoma (GBM), a cancer of the glial, or support cells, of the brain, altered Eph expression is frequently observed. Most consistently, significantly increased EphA2 expression, relative to healthy tissue, is observed in patients with GBM (Wykosky et al., 2005). EphA2, in the absence of ligand, was found to promote invasive behavior in these cells, and this effect was dependent upon EphA2 cross-talk with Akt, which ligand treatment was observed to disrupt (Miao et al., 2014). Similar to the observations made for the EphB3 receptor in lung cancer, it was found that the expression of the EphA2 ligand, ephrin-A1, was not increased concurrently with the receptor, and ephrin-A1 treatment of GBM cells with high EphA2 expression resulted in both reduced cancer cell survival and invasive behavior (Binda et al., 2012; Wykosky et al., 2005). Other Eph receptors whose increased expressions in GBM are associated with more aggressive cancer phenotypes include EphA3 (Day et al., 2013), EphA7 (Wang et al., 2008), and EphB4 (Chen et al., 2013; Tu et al., 2012), with the receptors acting to promote proliferation, invasive behaviours, or tumour angiogenesis.

In contrast to high expression being linked to an unfavourable prognosis, in colorectal cancer, expression of several EphB receptors is associated with a less aggressive phenotype (Batlle et al., 2005). For instance, a high level of EphB2, which is expressed in normal epithelial cells of the intestine, is associated with prolonged survival in colorectal cancer patients (Jubb et al., 2005). A separate study found the expression levels of EphB2 decreased with increasing disease stage, and these reduced levels were associated with poorer survival and disease-free survival. Biochemical studies in

colorectal cancer cell lines demonstrated that induced expression of EphB2 in colorectal cancer cell lines resulted in reduced colony formation relative to cells lacking EphB2, and activation of the receptor by ephrin-B1 led to reduced cell adhesion and migration (Guo et al., 2006). Like EphB2, EphB1 expression is also reduced in more invasive colorectal cancers (Sheng et al., 2008), and studies examining the effects of EphB3 expression in colorectal cancer cell lines found that this receptor inhibited proliferation, anchorage-independent growth, and promoted the less invasive epithelial cell phenotype (Chiu et al., 2009). However, not all EphB receptors inhibit colorectal cancer cell growth and invasiveness, as the EphB4 receptor is upregulated in colorectal cancer, and its expression promotes cancer cell survival and metastatic behaviour (Kumar et al., 2009).

Eph receptors are important to prostate development (Lisle et al., 2013), and a wide number of them are deregulated in and contribute to prostate cancer oncogenesis (Lisle et al., 2013). Of the Eph receptors examined in this disease, the effects of EphA2 are among the most extensively studied, and as has been observed in glioblastoma, this receptor may have differential effects depending upon the presences or absence of ligand. EphA2 expression is frequently upregulated in prostate cancers, relative to normal tissue, and its levels of expression positively correlate to the stage of the disease (Zeng et al., 2003), while expression of its ligand, ephrin-A1, is decreased in aggressive prostrate cancer (Larkin et al., 2012). This suggests that EphA2 in aggressive prostate cancer would not be in a ligand-activated state, and fits well with the observations that in contrast to the situation when ligand in absent, ligand-stimulated EphA2 acts in a protective fashion by causing a decrease in cell proliferation (Miao et al., 2001). Ligand-treated EphA2 is also able to reduce invasive behaviour in prostate cancer cell lines, acting at least in part

through phosphatases that target the Akt/mTOR (mammalian target of rapamycin) pathway (Miao et al., 2000; Yang et al., 2011). However, the effects of EphA2 in prostate cancer are not fully clear, as a separate group found that cells expressing a kinase-deficient mutant of EphA2 were less invasive, and formed fewer metastatic lesions in mice, suggesting that EphA2 kinase activity may also be able to promote metastatic behavior. Nevertheless, the same group also found that stimulation of cells expressing wild-type (WT) EphA2 reduced their invasive behaviour, while having no impact on the invasive behaviour of kinase-dead or cytoplasmic domain deletion mutant EphA2 expressing cells (Taddei et al., 2009). Overall, it appears that the effects of EphA2 on prostate cancer cells are altered depending upon the presence of ligand, and generally ligand-induced activation of EphA2 appears to suppress invasive tendencies. EphB4 expression is also frequently increased in prostate cancer relative to normal tissue (Lee et al., 2005), and higher expression levels have been observed in higher-grade prostate cancer cells (Lee et al., 2005; Xia et al., 2005). Like EphA2, EphB4 appears to either suppress or enhance malignant behaviour depending on the presence of its ligand, ephrin-B2 (Rutkowski et al., 2012). EphB4 predominantly appears to promote malignant activities, as its expression was found to enhance anchorage independent growth, cell motility, and invasive behavior (Rutkowski et al., 2012; Xia et al., 2005). While one group found that all of these qualities were reversed by treatment with ephrin-B2 (Rutkowski et al., 2012), others have observed that EphB4-ephrin-B2 interaction promotes cell motility (Astin et al., 2010), or even acts to promote invasive behavior (Khan, 2009).

In breast cancer EphA2 contributes strongly to the malignant behavior of the tumour, acting to enhance angiogenesis and metastatic behavior (Brantley-Sieders et al., 2005), and in contrast to observations in other cancer types, ligand-induced EphA2 kinase activity enhances many of its pro-oncogenic effects (Fang et al., 2005). EphB4 is also expressed in a high percentage of breast malignancies, where it has been implicated in promoting breast cancer cell survival, invasive behaviour, and potentially angiogenesis (Kumar et al., 2006). However, as has been observed for other Eph receptors, these oncogenic effects were observed in the absence of ligand, and there are studies that demonstrate that active EphB4 signalling may inhibit malignant behaviour (Noren et al., 2006). In agreement, our group has shown that active EphB4 signalling, when in the presence of the kinase –dead EphB6 receptor, significantly reduces the invasive behavior of breast cancer cells, demonstrating that EphB4 signaling can be anti-malignant. However, the anti-invasive signaling was dependent upon EphB6 presence, and EphB4 was found to promote invasive behavior when acting alone (Truitt et al., 2010). Interestingly, EphB6 has also been shown to reduce EphA2 invasive signalling (Akada et al., 2014). Indeed, while the expression of other Eph receptors appears to have a highly inconsistent influence on the invasive behaviour and prognosis between cancer types, EphB6 expression is consistently associated with reduced metastatic potential in almost all malignancies it has been characterized in (Truitt and Freywald, 2011). This emphasizes the significant role kinase-deficient receptors can play, and highlights the need for a full understanding of the mechanisms they employ for their signaling and regulation.

1.4. Kinase-Dead RTKs

Despite being a part of the RTK family, five receptors lack a functional kinase domain due to various alterations of key amino acids. Among these is ErbB3, as well as two members of the Eph family, EphA10, and EphB6. The other two kinase-dead RTKs are Ryk and Protein Tyrosine Kinase 7 (PTK7, also known as colon carcinoma kinase 4, CCK-4) (Kroiher et al., 2001). Aside from EphA10, whose biological function has yet to be explored, all of these receptors are important to normal and/or cancer cell biology. For example, Ryk is involved in regulating cell motility, and has been observed to interact with Wnt signaling pathways (Green et al., 2014), as well as several Eph receptors (Halford et al., 2000; Kamitori et al., 2005; Trivier and Ganesan, 2002). PTK7 also influences Wnt signaling, and in normal tissues has been found to be important to determining cell polarity (Peradziryi et al., 2012). In cancer, PTK7 appears to promote cell motility and metastasis (Golubkov et al., 2014), and consistent with these findings, PTK7 is generally regarded as an indicator of high metastatic potential in several cancer types (Gartner et al., 2014; Jin et al., 2014; Shin et al., 2013). However, PTK7 effects in cancer may be context and type dependent, as its expression has also been correlated to an improved prognosis (Chen et al., 2014), and it has been observed to suppress proliferation and invasive behavior in lung cancer (Kim et al., 2014). ErbB3 is the most extensively studied of the kinase-dead receptors, due to its association with the highly oncogenic ErbB2. ErbB3 is overexpressed in multiple malignancies (Ma et al., 2014), and when signaling in a heterodimeric complex with ErbB2, is a potent activator of the PI3K/Akt signaling pathway, which has proven to be a key element of ErbB3's ability to promote drug resistance (Ma et al., 2014). In agreement, ErbB3 expression is frequently associated with a poor prognosis (Ledel et al., 2014; Park et al., 2014; Wu et al., 2014),

and as such, it is actively being studied as a drug target (Kol et al., 2014). In contrast to ErbB3, one of the kinase-deficient members of the Eph family, EphB6, is gaining increased attention as it has been found to suppress invasive and metastatic cancer in sever malignancies (Truitt and Freywald, 2011), and so well little is known regarding the other kinase-dead Eph receptor, EphA10, some light has begun to be shed on the functions of the EphB6 receptor.

1.4.1. EphB6

Both the EphA and EphB subclasses of Eph receptors contain an intrinsically kinase-dead member, EphA10 and EphB6, respectively (Aasheim et al., 2005b; Gurniak and Berg, 1996; Matsuoka et al., 1997), and as such, it is likely that both these kinase-inactive players have an important role in Eph receptor signalling network. The function of EphA10 is largely unknown, and its expression appears to be largely restricted to the testis (Aasheim et al., 2005b), however, the kinase deficient member of the EphB subclass, EphB6, is recognized as an important mediator of several important biological effects in normal and cancerous tissues (Truitt and Freywald, 2011). Murine EphB6 was first identified in 1996 during a PCR based screen for receptor tyrosine kinases. It was predicted to lack intrinsic kinase activity based on alterations to several residues in the highly conserved tyrosine-kinase domain, and was this was functionally determined to be true, at least in a bacterial fusion protein containing the EphB6 kinase domain (Gurniak and Berg, 1996). The human ortholog was identified shortly thereafter, and six amino acid substitutions within the kinase domain were identified (**Table 1.2**). These included the substitution of a glutamine residue in place of an invariant lysine residue in the ATP (adenosine triphosphate) binding site, as well as a serine in place of an invariant aspartic

Table 1.2. AMINO-ACID SUBSTITUIONS IN EPHB6 KINASE DOMAIN.

Conserved residue	EphB6 variant	Function of conserved residue
Lysine[72]	Cysteine[687]	Interacts with ATP, essential for phosphoryl-transfer.
Aspartate[166]	Serine[780]	Orientates and primes the substrate tyrosine for phosphorylation.
Glutamate[91]	Arginine[704]	Interacts with the invariant lysine and maintains its correct orientation for ATP-interaction.
Aspargine[171]	Serine[785]	Positioned within the catalytic loop, required for catalysis.
Aspartate[184]-Phenylalanine[185]-Glycine[186]	Arginine[798]-Leucine[799]-Glycine[800]	Interacts with ATP, promotes its functional orientation.

* Conserved residue numbers correspond to their locations in cyclic adenosine-monophosphate kinase α (Hanks et al., 1988; Matsuoka et al., 1997).

acid in the phosphor-transfer site, both of which would severely limit the functional properties of the domain. Like the murine receptor, fusion proteins containing the human EphB6 kinase domain were found to lack detectable kinase activity (Matsuoka et al., 1997). EphB6 is ubiquitously expressed, with the highest expression levels being found in the brain, pancreas, and thymus (Hafner et al., 2004; Matsuoka et al., 1997). While it lacks intrinsic kinase activity, EphB6 is able to interact with ephrin-B1 (Freywald et al., 2002), and ephrin-B2 (Munthe et al., 2000), and is able to generate active signalling in response to ligand- binding by undergoing phosphorylation in a Src- (Matsuoka et al., 2005), EphB1- (Freywald et al., 2002), or EphB4- (Truitt et al., 2010) dependent manner. Furthermore, although their role in EphB6 phosphorylation has yet to be examined, EphB6 has also been shown to interact with EphB2, as well as EphA2, and notably, to not associate with the other kinase-deficient Eph receptor, EphA10 (Fox and Kandpal, 2011). Due to its ability to interact with a wide range of Eph receptors, and its lack of intrinsic kinase ability, EphB6 has been proposed to modify the signalling output of its kinase-active signalling partners, and indeed this has been observed in several situations (Fox and Kandpal, 2011; Truitt et al., 2010).

1.4.2. EphB6 biological effects

Despite its lack of kinase activity, the EphB6 receptor is implicated in a number of biological responses in normal physiological conditions (Freywald et al., 2003; Luo et al., 2012; Luo et al., 2004) and in human malignancies (Fox and Kandpal, 2009; Maddigan et al., 2011; Tang et al., 2000; Truitt and Freywald, 2011; Truitt et al., 2010; Yu et al., 2010). EphB receptors are involved in signalling events important to hyperalgesia, an increased sensitivity to pain, and in neuropathic pain (Battaglia et al., 2003; Cao et al.,

2008; Slack et al., 2008; Song et al., 2008a; Song et al., 2008b), and EphB6 has recently been identified as a potential EphB receptor involved in modulating pain transmission signals, as it has been observed in induced colitis, a model used to study the effects of inflammatory pain and hyperalgesia (Laird et al., 2001), that EphB6 expression is greatly reduced in the spinal dorsal horn sensory neurons relative to those of healthy mice (King, 2014). However, the mechanisms leading to reduced EphB6 expression, as well as the alterations to pain signalling resulting from its loss have yet to be determined.

Another promising area where EphB6 appears to be involved is in the kidney, where EphB6 may be involved in regulating permeability. EphB6 is expressed in the cortex and outer medulla of the kidney, along with ephrin-B1, and this is in contrast to EphB2, which is expressed in the inner and outer medulla, but not the cortex. The specific effects of EphB6 signalling in this compartment have yet to be resolved, however, ephrin-B1 treatment of kidney cells expressing EphB2 and EphB6 resulted in increased Rho activity and decreased Rac activity, which led to cytoskeletal rearrangements and membrane retraction. The differential expression of the two receptors in distinct regions of the kidney suggest that they function to alternatively modulate the adhesive/permeability properties of these cells, and thus may contribute to the spatial organization of the kidney's uptake of water and solutes (Ogawa et al., 2006).

EphB6 has also been implicated in maintaining proper blood pressure levels. Vascular smooth muscle cells (VSMCs) are specialised cells found within the walls of blood vessels, whose primary function is to contract or relax in response to stimuli, increasing or decreasing the vessel diameter, and thus affecting blood pressure (Lacolley et al., 2012). EphB6, and its ligands ephrin-B1 and ephrin-B2, are expressed in VSMCs,

and a loss of EphB6 was found to result in alterations to contractility of the cells. EphB6 knock-out reduced arterial contractility in male mice, but increased it in females and castrated males, suggesting that EphB6 and testosterone work together to regulate VSMC contractility. Physiologically, mice exhibited increased blood pressure when lacking both EphB6 and testosterone, while the presence of either was able to partially compensate in the absence of the other, in part by regulating catecholamine production. Thus, EphB6 works in concert with testosterone to regulate vessel contractility to maintain normal blood pressure levels (Luo et al., 2012).

Although EphB6 expression is not limited to T-cells within the hematopoietic cells of the immune system, the role of EphB6 in these cells is currently the best described (Shimoyama et al., 2000). EphB6 activity is able to modify T-cell receptor (TCR) activation, and has been observed to impact both signaling intensity, as well as signaling output, and the presence or absence of EphB6 was also found to affect T-cell functionality (Freywald et al., 2003; Luo et al., 2001; Luo et al., 2002). Importantly, EphB6 colocalises with TCRs following their activation, even in the absence of EphB6 ligand, and this spatial localisation of the receptor may be required for the ability of EphB6 to influence TCR output (Luo et al., 2004; Luo et al., 2002). EphB6 is also highly expressed in monocytes, which may indicate that EphB6 plays a role in mediating chemotaxis in this cell population, however this has yet to be explored (Sakamoto et al., 2011).

While the role of EphB6 in normal physiology is only beginning to be fully explored, EphB6 has been more actively studied in malignancies, where it is becoming recognized for its apparent ability to suppress invasive and metastatic behaviour in several cancer

types (Truitt and Freywald, 2011). In agreement, EphB6 expression is frequently reduced in highly aggressive and metastatic cancers relative to normal tissues, or more benign tumors (Truitt and Freywald, 2011). Among those cancers for which reduced EphB6 cancer appears to coincide with a more aggressive phenotype is melanoma, as EphB6 expression is present in benign samples, reduced in cancerous lesions, and absent in melanoma metastases (Hafner et al., 2003). These observations were recapitulated in cell lines, as EphB6 was found to be present in a non-cancerous melanocyte cell line, as well as a poorly invasive melanoma cell line, while EphB6 expression was silenced in the highly invasive melanoma cell line, C8161 (Bailey et al., 2012). The loss of EphB6 expression in aggressive melanoma may indicate the loss of this receptor allows for a more invasive phenotype, and to explore this possibility, a study was performed to determine what impact EphB6 expression has on melanocyte migration and intravasation abilities. The chorioallontoic membrane (CAM) of the chick embryo has been found to serve as a useful tool to study metastatic behaviour of cancer cells, as it is highly vascularised, and due to its structural constraints, tumour cells must have the ability to enter its vasculature in order to form metastatic lesions distant from their point of origin (Deryugina and Quigley, 2008). When C8161 cells were seeded onto chick CAMs, ectopic expression of EphB6 was observed to significantly reduce the number of cells able to enter the vasculature, relative to their EphB6-null counterparts (Bailey and Kulesa, 2014). There was no observable difference in size of the primary tumor between the two groups, suggesting that EphB6 does not inhibit proliferation of melanoma cells, but rather their invasive and potential intravasative abilities (Bailey and Kulesa, 2014).

EphB6 also suppresses invasive behaviour in non-small cell lung cancer (NSCLC). In NSCLC cell lines, expression of wild-type EphB6 is able to strongly reduce cell migration and metastatic behaviour relative to cells lacking EphB6 (Bulk et al., 2012; Yu et al., 2010). In contrast, expression of an EphB6 mutant, identified in a patient sample of NSCLC, that is lacking three residues in its cytoplasmic domain (residues 915-917, aspartate-proline-glycine), did not produce the same anti-metastatic effects as the wild-type receptor, indicating that a 'functional' cytoplasmic domain is required for proper EphB6 effects despite the receptor's lack of kinase activity (Bulk et al., 2012). In agreement, EphB6 expression is frequently reduced in NSCLC, and this reduction is correlated to an increased risk of metastasis (Muller-Tidow et al., 2005; Yu et al., 2010). Experiments in the NSCLC cell line, A549, have provided evidence that EphB6 presence increases Erk2 phosphorylation, but reduces activation of one of Erk's targets, the transcription factor Elk-1 (Yu et al., 2009). As a result, the expression of genes initiated by the MAPK signalling cascade likely differ as a consequence of EphB6 signalling, which may contribute to EphB6 effects. As Elk-1 activation is known to increase the expression of multiple genes involved with invasive and metastatic behaviour (Kasza, 2013), and as Elk-1 is involved in active signalling cascades known to produce an invasive mesenchymal cellular phenotype (Hou et al., 2014), this possibility warrants further investigation.

Although the current data is almost exclusively limited to expression studies, EphB6 appears to also indicate a more favorable prognosis in neuroblastoma, as high EphB6 expression was found to strongly correlate to a lower tumor grade and higher survival incidence in neuroblastoma (Tang et al., 1999; Tang et al., 2000). In agreement, a

neuroblastoma cell line with induced EphB6 expression exhibited poorer ability to form colonies, and reduced tumor growth in mice (Tang et al., 2000).

In breast cancer, expression of EphB6 is frequently reduced relative to healthy surrounding tissue (Toosi et al., In Revision), and, in agreement, it has been found that invasive breast carcinoma cells have reduced EphB6 expression due to transcriptional silencing by methylation of its promoter region (Fox and Kandpal, 2006). As Eph receptors often form oligomeric complexes in response to ligand binding (Lisabeth et al., 2013), and since EphB6 has been shown to interact with both EphA and EphB receptors (Fox and Kandpal, 2011), EphB6 may act by modifying the signalling output of other Eph receptors that are implicated in invasive behaviour (Akada et al., 2014; Truitt et al., 2010). Indeed, our group has demonstrated that induced expression of EphB6 in breast cancer cell lines with high endogenous EphB4 expression leads to cytoskeletal rearrangements, as well as reduced invasive behaviour, relative to cells lacking EphB6. This effect was enhanced by treatment with an EphB6 ligand, ephrin-B2, and as EphB6 is unable to self-phosphorylate and relies on other molecules for its ligand-induced phosphorylation, this implies that some other kinase was important to mediating this effect. Further experiments determined that EphB6 phosphorylation following ephrin-B2 treatment was assured by the kinase- active EphB4 receptor, demonstrating a cross-talk between the two receptors. EphB4 was found to support invasiveness, when acting in the absence of EphB6, as knock-down of EphB4 in cells lacking EphB6 expression resulted in reduced invasive behavior. EphB4 also supported invasion when its expression was sufficiently high so as to overwhelm EphB6 signals, as overexpression of EphB4 in cells with EphB6 expression enhanced invasive behavior. However, as it was also determined

to be indispensable for EphB6-induced adhesive responses, it appears that the cross-talk between EphB4 and EphB6 serves to dampen pro-invasive EphB4 signalling, and that overall cell behavior is determined by the balance between the two receptors (**Figure 1.12**). Thus, while EphB4 has been reported to have both tumour suppressive (Noren et al., 2006) and tumour enhancing effects (Noren et al., 2004), our observations of EphB6 co-expression suppressing invasive behaviour of EphB4 positive breast cancer cell lines, in an EphB4 dependent manner, strongly supports the idea that EphB6 cross-talk with other Eph receptors can alter their biological output, and explains in part the duality exhibited by the EphB4 receptor (Truitt et al., 2010). Moreover, EphB6 has been observed to interact with and modify the output of other Eph receptors as well, as Akada et al (Akada et al., 2014) observed that in the breast cancer cell line MCF7, EphB6 and EphA2 interact with each other through their ligand-binding domains, in a ligand-independent manner. This interaction results in reduced phosphorylation of EphA2 on a serine residue, and also significantly reduces the ligand-independent interaction of EphA2 with ephexin4. As mentioned previously, an important characteristic needed for a cancer cell to exhibit invasive potential is the ability to escape anoikis, the apoptotic response to detachment from the cell-matrix and other cells (Frisch et al., 2013). As a result of EphB6 interaction with EphA2, the EphA2-ephexin4 initiated signalling cascade that protects cells from anoikis is significantly attenuated, and thus much higher levels of apoptosis were observed when EphB6 expressing cells were grown in non-adherent culture conditions relative to those lacking EphB6 (Akada et al., 2014). The anti-invasive impact of EphB6 presence in breast cancer may also be partly mediated through alterations in gene expression, as Kandpal has observed that ectopic expression of EphB6

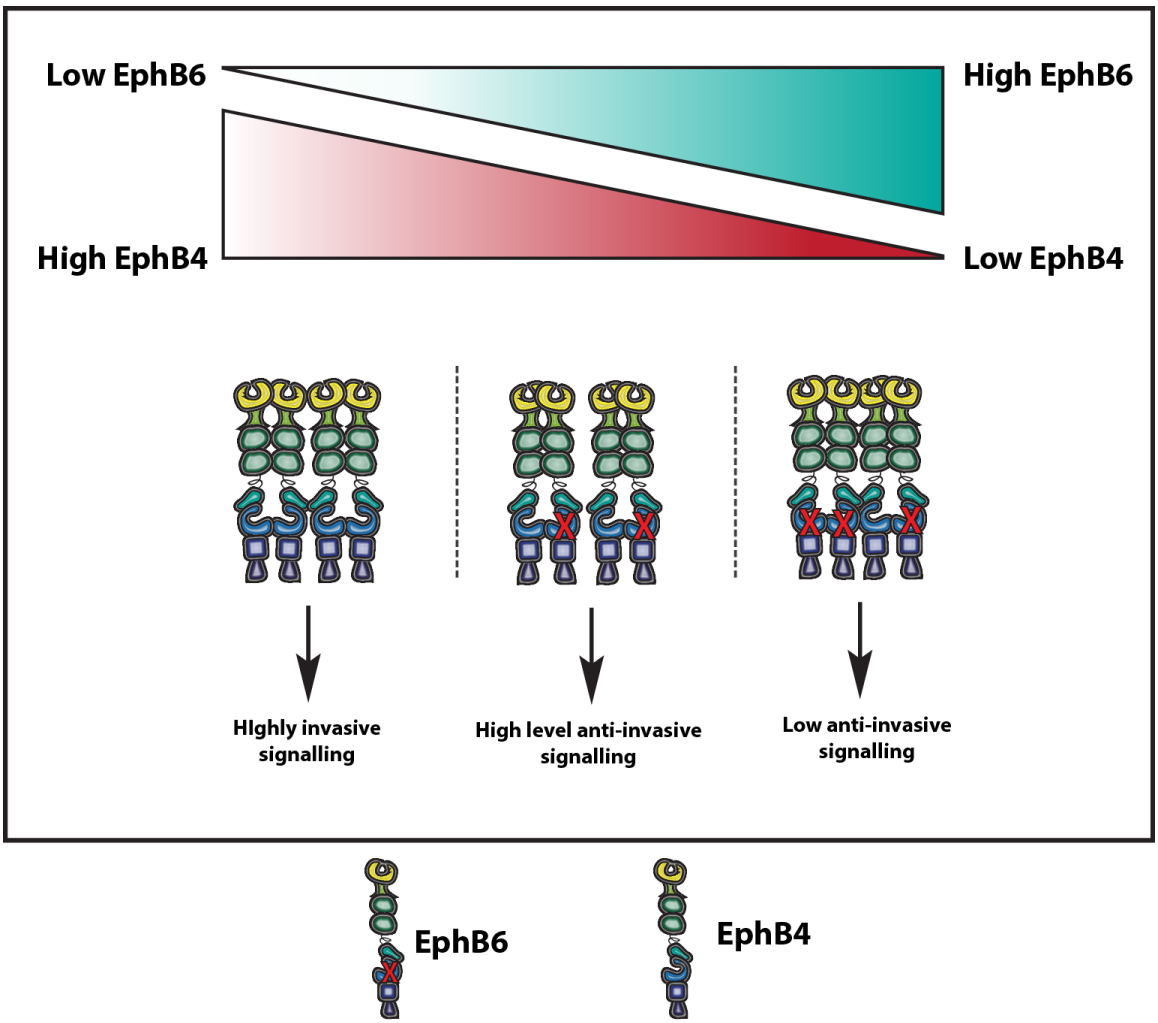

Figure 1.12. EphB6 suppresses pro-invasive signals of EphB4.
Active EphB4 signaling promotes invasive behavior in highly invasive breast cancer. Ectopic expression of EphB6 inhibits invasive behavior, by promoting cell spreading and attachment, through active EphB4-EphB6 signalling. Thus when EphB4 expression is high relative to EphB6, cells exhibit strongly invasive behavior, while EphB6 expression in equal or greater quantity to EphB4 results in anti-invasive signals that require EphB4 mediated phosphorylation for their initiation.

in MDA-MB-231 cells causes significant alterations to the expression of a number of genes (Kandpal, 2010), including matrix-metalloproteinases 7 and 19, and metalloproteinase 2 (Fox and Kandpal, 2009). Furthermore, our group has also made exciting observations regarding the impact of EphB6 on expression of numerous molecules involved in the EMT transition, an essential requirement for solid tumour cells to undergo metastasis (Toosi et al., In Revision).

In contrast to observations in solid tumour types, EphB6 has been identified as a possible marker for a poorer prognosis in chronic lymphocytic leukemia cells (Alonso et al., 2009). Complementary with these findings, our group has found that EphB6 is expressed in T-ALL cell lines, as well as in some patient samples, along with another EphB receptor, EphB3. We determined that EphB6 and EphB3 were able to act in a cyto-protective manner in these cells, preventing Fas receptor initiated apoptotic responses by stimulating Akt phosphorylation (Maddigan et al., 2011).

1.5. Ligand-Induced Downregulation

1.5.1. Downregulation Basics

The activation of receptors on the surface of the cellular membrane allows for the transmission of external information to the interior of the cell. In order to properly react to external cues, cells need to finely regulate their responses to signals they receive. The amplitude of a response and the duration of the signal generated by the activation of a receptor must reflect the environmental input for a cell to properly modulate processes and function as an effective component of a tissue, organ, or organism. Regulating the number of receptors present on the surface of a cell is one mechanism that allows for the control of signal intensity (Goh and Sorkin, 2013), and termination of the signals

propagated by activated receptors must take place in order to facilitate real-time responses to external stimuli. Often, this is achieved through ligand-induced receptor downregulation. Numerous receptor types employ this overall mechanic, including G-protein coupled receptors (von Zastrow, 2003), hormone receptors (Bouley et al., 2013), as well as RTKs (Lu and Hunter, 2009; Roepstorff et al., 2008), highlighting its effectiveness at addressing an essential process.

Following interaction with their ligands, activated receptors recruit signaling molecules within a cell to propagate the signal, and also to terminate their own signaling. Often, for cell surface receptors, the first phase in attenuating a signal is the internalisation of the activated receptor, frequently in a complex with the bound ligand. This is a dual-purpose step, as it not only acts as the first move towards receptor degradation, but can also bring the activated receptor into proximity with key molecules required for efficient signal transduction (Miaczynska et al., 2004b; Platta and Stenmark, 2011; Sorkin and von Zastrow, 2009), or alter the signals it generates relative to those initiated at the cell membrane (Sorkin and von Zastrow, 2009). Following internalisation, cessation of signaling can be achieved in one of two ways: the ligand-receptor complex can be dissociated, and any ligand-induced modifications of the receptor, such as phosphorylation, can be removed so that the receptor may be recycled back to the cell surface, ready to undergo another round of activation, or, alternatively, the receptor can be degraded. In the event that the ligand-receptor complex remains intact, the internalised receptor continues signaling from inside the cell, and may even generate a stronger response, as has been observed for EGFR (Taub et al., 2007; Vieira et al., 1996). In this situation, the receptor-ligand complex is eventually targeted for degradation, terminating

the signal (Sorkin and von Zastrow, 2009). Recycling of the receptor allows for rapid and frequent cycles of receptor activation, allowing a cell to respond to the same type of stimulus repeatedly in a short amount of time, degradation of the receptor, on the other hand, can leave a cell less able to respond to repeated stimuli of the same type until the cell-surface pool of the receptor has been replenished.

For some receptors, internalisation may not be necessary, and degradation can be initiated at the cell surface, where ligand binding induces cleavage of the activated receptor. This cleavage may serve to facilitate more than just removal of the cell-surface receptor, as is demonstrated by Notch signalling, where the proteolysis of the receptor itself is a means of signal transduction (Andersson et al., 2011).

1.5.2. Clathrin-coated Pits

As mentioned, in addition to the recruitment of molecules that are required for intracellular signal propagation, activated receptors also recruit the machinery required for their own signal attenuation, and frequently for RTKs this involves recruitment of clathrin and its associated machinery (Goh and Sorkin, 2013). First described in 1964 (Roth and Porter, 1964), clathrin-coated pits (CCP) have become the most widely explored mechanism of receptor internalisation. Clathrin is a protein heterohexamer found in the cytosol, composed of three heavy-chains and their three associated light chains. Together these peptides connect to form a structure called a triskelion, whose appearance looks much like the name suggests, consisting of a central connection point, and three bent arms (Young, 2007). Clathrin triskelions assemble together to form soccer ball like structures, with both five- and six-sided ring arrangements (Kirchhausen et al., 2014) (**Figure 1.13**).

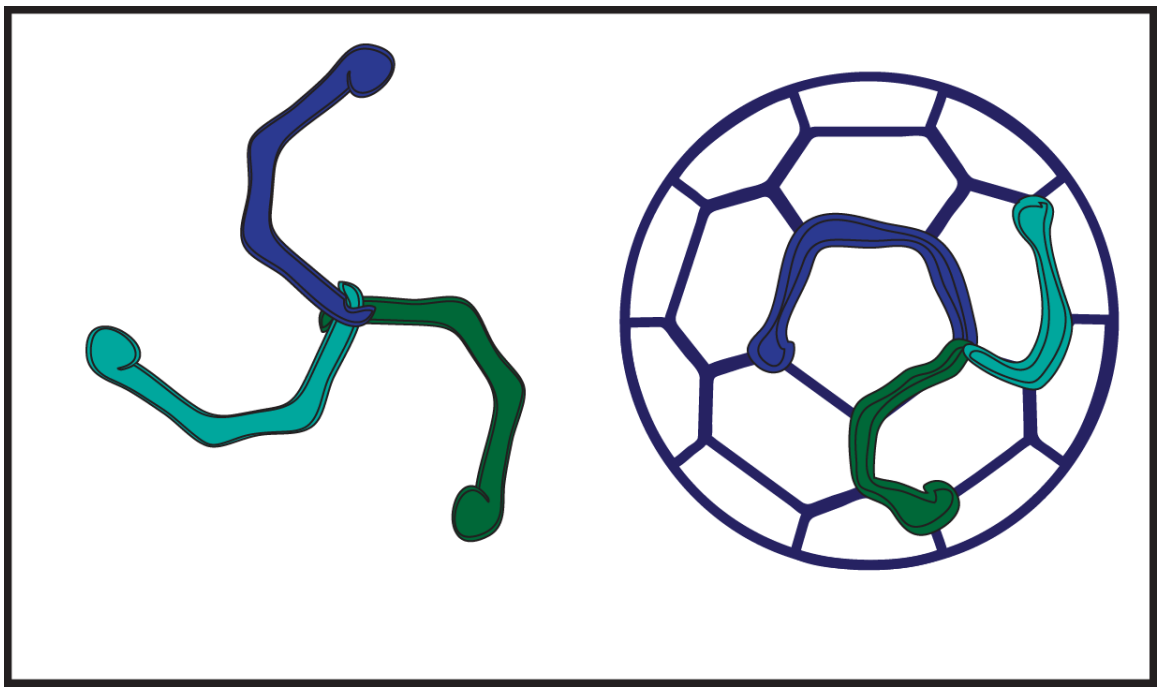

Figure 1.13. Clathrin triskelions.
Clathrin is made up of a trimer of pairs of light and heavy chains that connect to form a triskelion. Triskelions arrange to form five and six sided soccer ball like structures that help induce an inward curvature of the cell membrane. Image adapted from Harrison and Kirchhausen (Harrison and Kirchhausen, 2010).

While clathrin can spontaneously arrange into a polyhedral lattice *in vitro* under low pH conditions (Brodsky, 1988), in living cells, the proper formation of clathrin-coated pits in response to receptor activation requires the association of adaptor proteins (Kirchhausen et al., 2014). Adaptor proteins bind to membrane components, including activated receptors, and to clathrin, and thus are able to help both with the targeting of

cargo for internalisation, and in the recruitment of clathrin to the cell membrane (Kirchhausen et al., 2014). Perhaps the most important of these is the adaptor protein AP-2 (adaptor protein 2), that binds to phosphatidylinositol-4,5-bis-phosphate (PIP_2), a component of the cell membrane, and also to clathrin, target cargo (such as activated RTKs), and to numerous other proteins (accessory proteins) that promote CCP formation (Boucrot et al., 2010). In this way, AP-2 is able to assist in maintaining clathrin at the inner membrane, in cargo selection, and in promoting the further assembly of the clathrin lattice, and as such it is unsurprising that AP-2 is one of the most common proteins associated with clathrin-mediated endocytosis (Boucrot et al., 2010). However, while studies in AP-2 depleted cells show a significant reduction in overall levels of clathrin-mediated endocytosis, and while the endocytosis of some clathrin-targeted RTKs may be completely blocked, for others it remains unchanged, suggesting that despite its wide use, certain cargo are selected in AP-2 independent manners (Motley et al., 2003).

Following activation, numerous RTKs are ubiquitinated by E3 ligases, including EGFR (Piper and Lehner, 2011), and this modification may assist in their targeting for internalisation through CCPs by promoting their interaction with proteins involved in pit formation (Piper et al., 2014; Piper and Lehner, 2011). This is observed in the classical EGFR downregulation pathway, where the ubiquitination of the activated receptor is performed by the E3-ligase, c-Cbl (Waterman et al., 1999). Here, following receptor activation, c-Cbl interacts with the phosphorylated receptor with its tyrosine kinase-binding (TKB) domain (Thien and Langdon, 1997), and this promotes the phosphorylation and activation of c-Cbl by the receptor (Kassenbrock and Anderson, 2004). c-Cbl is then able to interact with a ubiquitin-carrying E2 enzyme, and mediates

the transfer and attachment of ubiquitin to a lysine residue of the receptor (Thien and Langdon, 2005). Several proteins involved in clathrin-pit formation have been shown to bind to ubiquitinated RTKs, as will be discussed below.

Accessory proteins are cytosolic proteins that interact with AP-2, or other adaptors, and help to provide additional cargo specificity, as well as to enhance lattice formation. For example, the AP-2 interacting protein Eps15 (Benmerah et al., 1998), is essential to proper assembly of the clathrin-coated pit (Benmerah et al., 1999; Tebar et al., 1996), and is also involved in the selection of cargo for internalisation. Eps15 is able to specifically bind to ubiquitinated RTKs through its UIM (ubiquitin interacting motif) (Polo et al., 2002), and in this way helps target them to CCPs for internalisation (Polo et al., 2002). Other major accessory proteins frequently involved in CCP formation, include FCHo1 and FCHo2 (F-BAR domain-containing Fer/Cip4 homology domain-only proteins 1 and 2) (Henne et al., 2010) (Umasankar et al., 2012), and the epsins (Eps 15 interacting proteins) (Horvath et al., 2007). The epsin family contains four members, epsin1, epsin2a, epsin2b, and epsin3, and except for epsin3, whose expression is restricted to motile keratinocytes, all of them are ubiquitously expressed (Horvath et al., 2007). Epsins, as their name suggests, were first identified due to their interaction with Eps15, and have since been shown to interact with AP-2 and clathrin as well. They appear to play a role in promoting membrane curvature during the formation of the growing pit (Ford et al., 2002), and as they also possess a UIM domain, they likely assist in ubiquitinated cargo selection as well (Hofmann and Falquet, 2001). FCHo1 and FCHo2 were initially believed to involved in the nucleation of CCPs (Henne et al., 2010), however more recent studies suggest that their predominant role is in assuring the

sustained growth of the clathrin-lattice following the initiation of its assembly (Umasankar et al., 2012). There are an immense number of other AP-2 accessory proteins, however their roles are more specific to certain receptors and cell-types, and thus are out of the scope of this review (Rodemer and Haucke, 2008).

The general mechanism of CCP mediated internalization for many RTKs is often prompted by activation of the receptor by ligand-binding. Following activation, the phosphorylated RTK recruits adaptor proteins such as AP-2, and accessory proteins such as Eps15, to the cell surface, and thus initiates the process of CCP formation. For the successful nucleation of a clathrin-coated pit at least two AP-2 molecules need to be bound to the inner membrane and to one clathrin triskelion (Cocucci et al., 2012). Eps15, epsins, and FCHo1/2 then work together to stabilize the forming clathrin structure, promote membrane curvature, and concentrate cargo, such as RTKs, in the growing pit (Cocucci et al., 2012), and are essential to promoting the growth of the CCP (Benmerah et al., 1999; Cocucci et al., 2012; Horvath et al., 2007). Following initiation, clathrin triskelions from the cytosolic pool are recruited to the growing pit at a constant rate (Ehrlich et al., 2004), and the continued assembly of clathrin along the inner membrane causes the membrane to curve inward, forming a round pit. Observations using confocal microscopy suggest that in CCPs containing RTKs, the pit diameter reaches a maximum of about 100 nm, and contains about 60 triskelions (Ehrlich et al., 2004). Once the clathrin lattice has fully assembled, a ball like invagination of the cell membrane is present, with a narrow opening at the outer periphery (**Figure 1.14**).

The large GTPase, dynamin (Ferguson and De Camilli, 2012), facilitates the separation of the CCP vesicle from the membrane (Ramachandran, 2011). The exact

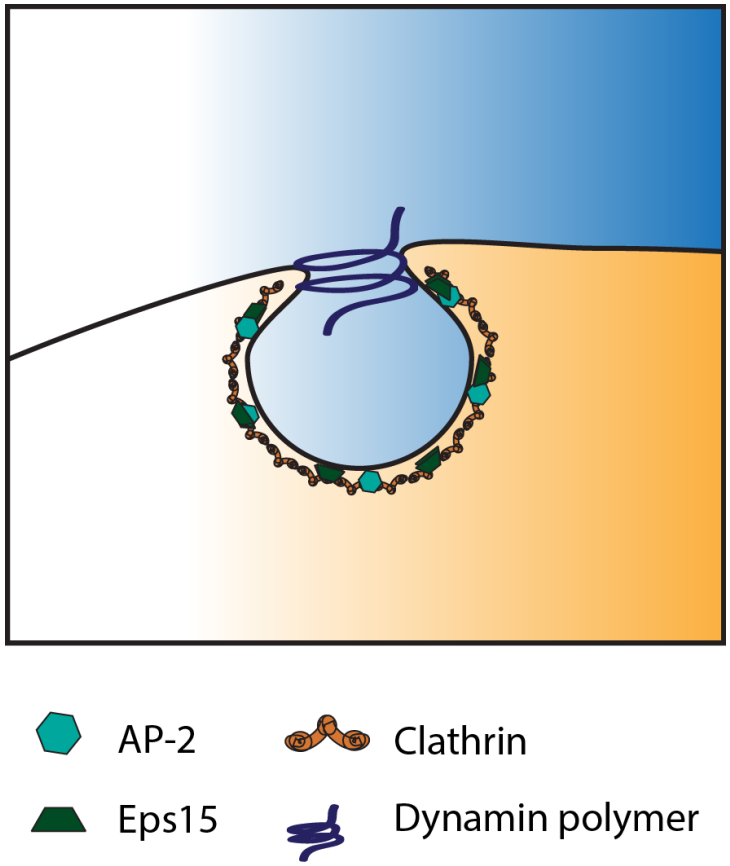

Figure 1.14. Structure of a clathrin-coated pit.
Following receptor activation, Clathrin is recruited to the inner membrane by interactions with numerous adaptor proteins. The assembly of the clathrin-lattice induces membrane curvature and pit formation. Ultimately, a vesicle is formed from the Clathrin-coated by pinching off of the membrane by the large GTPase, Dynamin.

mechanism underlying the ability of dynamin to promote membrane scission and release of the vesicle has yet to be fully elucidated, however, it involves the formation of a helical structure encircling the narrow opening of the pit, composed of multiple

connected units of dynamin (Ford et al., 2011). Dynamin hydrolysis of GTP (guanine triphosphate) induces a conformational change allowing for the constriction of the dynamin helix, and the membrane it is bound to (Ramachandran, 2011; Sweitzer and Hinshaw, 1998). Through this, and potentially other mechanical forces, the vesicle separates from the cell membrane. In order to facilitate fusion with other compartments of the endocytic pathway, the newly formed vesicle sheds its clathrin coat in an auxilin and Hsc70 dependent manner (Prasad et al., 1993; Ungewickell et al., 1995), and is subsequently targeted to the appropriate intracellular compartment. Generally, the cargo contained in the newly endocytosed vesicle will dictate how it will be trafficked, and many RTKs contain signaling motifs in their cytoplasmic domains which direct their sorting following endocytosis (Acconcia et al., 2009).

1.5.3. The Early Endosome

Following internalisation, many RTKs are targeted to the early endosome (Goh and Sorkin, 2013), a membranous structure characterized by the presence of markers such as the early endosomal antigen 1 (EEA1) (Mu et al., 1995) and Rab-5 (Bucci et al., 1992). The early endosome is highly dynamic, with tubule like protrusions, and several compartments of distinct lipid and protein composition within its limiting membrane (Jovic et al., 2010). Among these compartments are areas of high membrane activity, with regions where vesicles form and bud off from the early endosome, as well as areas of intraluminal vesicle formation and internalisation (Jovic et al., 2010). The pH of the early endosome is between 6.8-5.9, which is slightly lower than that of the surrounding cytoplasm (Maxfield and Yamashiro, 1987). This reduction in pH may help to facilitate the dissociation of ligand from receptor to enable the recycling of receptors back to the

cell surface, however, oftentimes, receptors continue signalling from this compartment (Miaczynska et al., 2004b).

For several RTKs, including EGFR, The GTPase Rab5, and its effectors, EEA1 (Simonsen et al., 1998), and rabenosyn-5 (Nielsen et al., 2000) help to initiate the fusion of the endocytotic vesicle with the early endosome (Nielsen et al., 2000). This dynamic process involves soluble NSF (*N*-ethylmaleimide sensitive factor) attachment protein receptor (SNARE) proteins, including syntaxin-6 and syntaxin-13 (Brandhorst et al., 2006), and the Sec1/Munc18-like (SM) protein, Vps45 (vacuolar protein sorting 45) (Nielsen et al., 2000), that are present on both the target and donor membranes. Phosphatidylinositol (3,4,5)-trisphosphate ($PIP_{3)}$ is actively enriched on the surface of early endosomes by several proteins, including PI3K isoforms and phosphatases (Zoncu et al., 2009), and this helps to assure the correct recruitment of effectors that bind to PIP_3 such as EEA1 and rabenosyn-5 (Mills et al., 1998; Nielsen et al., 2000). By binding to the SNARE proteins syntaxin-6 and syntaxin-13, and to Vps45, EEA1 and rabenosyn-5 facilitate the formation of a large component of the fusion complex on the surface of the early endosome and vesicle (Nielsen et al., 2000). Vesicle fusion is initiated when Rab-5, which is associated with a newly endocytosed vesicle, interacts with EEA1, rabenosyn-5, and other components of the tethering complex present on the early endosome, and concurrently Rab-5 on the early endosome interacts with components on the newly endocytosed vesicle. These interactions work together to stabilize the vesicle in close proximity to the early endosome (Barbieri et al., 1998; McBride et al., 1999; Mills et al., 1998; Mu et al., 1995; Nielsen et al., 2000; Stenmark, 2009), in a process commonly called tethering (Kummel and Ungermann, 2014). The SNARE proteins, which are

present on both the surface of the vesicle and the endosome, are then able bind to each other (Kummel and Ungermann, 2014). The N- to C-terminal "zippering" of the SNARE proteins to each other brings the two membranes in close proximity, destabilises them, and promotes vesicular fusion (Chen and Scheller, 2001) (**Figure 1.15**).

Following the fusion of the internalised vesicle with the early endosome, RTKs are sorted within its membrane, localising to compartments that facilitate their correct subsequent trafficking. At this point key sequences in their cytoplasmic domains, or structural modifications such as ubiquitination, play important roles in determining how cargo are sorted (Raiborg et al., 2003), and internalised receptors will begin to associate with proteins reflective of their ultimate fate (Seaman, 2008). For example, effectors such as Tsg101 are able to interact with ubiquitinated cargo, and target such proteins for late endosomes/ multi-vesicular bodies (MVBs) (Raiborg et al., 2003). Additionally, receptors destined for degradation may also localise to compartments with Rab-7 (Girard et al., 2014). For receptors which are to be recycled, there is an increased association with Rab4, and its effectors Rab coupling protein (RCP) (Lindsay et al., 2002) and rabaptin-4 (Deneka et al., 2003). Over time, some enriched compartments are able to bud from the early endosome, such as those bearing receptors to be recycled, while other receptors and proteins accumulate resulting in a refinement of cargo and endosome composition (Huotari and Helenius, 2011). Eventually, following fusion with multiple vesicles, and the targeting of cargo to correct compartments, the early endosome matures through the simultaneous loss of markers and characteristics of the early endosome such as Rab-5, and the acquisition of proteins such as Rab-11, the formation of intraluminal vesicles, a

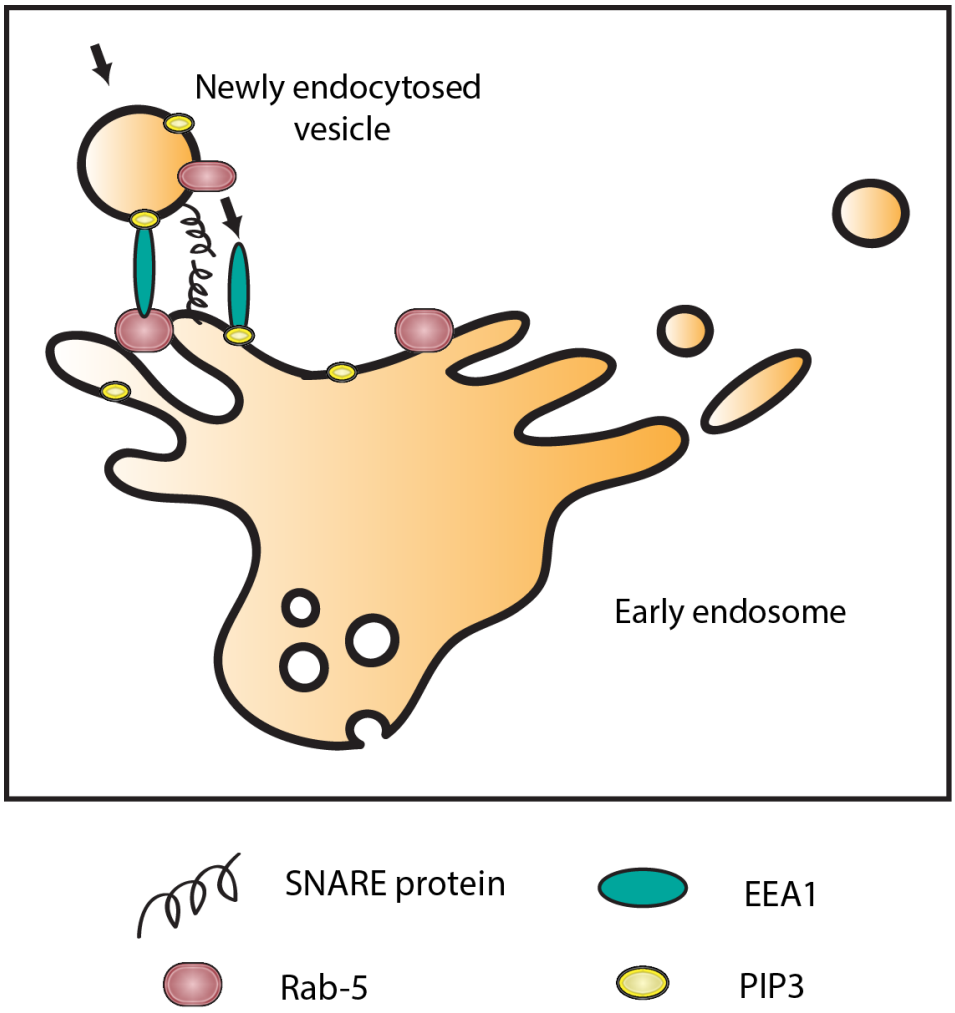

Figure 1.15. Endocytosed vesicles fuse with the early endosome.
Following their internalization, many vesicles containing RTKs are targeted to the early endosome, a sorting station for a variety of subcellular components. Fusion with the early endosome requires the tethering of the vesicle to the early endosome by Rab5 GTPase, and EEA1, as well as the activity of SNARE proteins for membrane fusion.

reduction in pH, and morphological changes including the loss of the tubular arms, to become the late endosome (Huotari and Helenius, 2011).

1.5.4. The Lysosomal Pathway

Those receptors destined for degradation are often sorted towards the lysosomal compartment, as is the case for the most studied instance of RTK downregulation, EGFR (Duan et al., 2003). Receptors, such as EGFR, when fated for degradation, accumulate within early endosomes, and there is a concurrent shift from lipids and proteins associated with the early endosome, to those associated with late endosomes, a process termed endosome maturation (Huotari and Helenius, 2011).

Ubiquitination is not only important for receptor interactions with proteins involved with internalisation (Piper et al., 2014), it is also essential for targeting of the many receptors to the degradative pathway (Eden et al., 2012). This modification facilitates EGFR interaction with Tsg101, Hrs, and other members of the endosomal-sorting complex required for transport (ESCRT) machinery, which are able to recognise and bind to ubiquitinated proteins through their UIM domains (Urbe et al., 2003). There are five ESCRT complexes that make up the ESCRT machinery, and receptors are trafficked through them in a sequential manner (Schmidt and Teis, 2012). ESCRT 0, which associates with endosomes through its interaction with PIP2, is composed of Hrs and STAM (signal-transducing adaptor molecule), both of which are able to bind to ubiquitinated proteins, and help to concentrate ubiquitinated cargo within the endosomal membrane (Mayers et al., 2011). ESCRT I, in turn, is recruited to the endosome through its interaction with ESCRT 0. ESCRT I is a tetramer containing Tsg101, Vps28, Vps37, and ubiquitin-associated protein 1 (UBAP-1). Tsg101 interacts with both ESCRT 0 as

well as the ubiquitinated proteins associated with it, and UBAP-1, as the name suggests, also interacts with the ubiquitinated cargo; these interactions are believed to help locally concentrate ubiquitinated proteins. The Vps28 subunit of ESCRT I acts to recruit the ESCRT II complex (Gill et al., 2007). ESCRT II is composed of Vps22, Vps25, and Vps36 (Babst et al., 2002b). ESCRT II also interacts with ubiquitinated cargo through the Vps36 subunit, and is believed to be responsible for nucleating the formation of the ESCRT III complex through the interaction between Vsp25 of ESCRT II and Vps20 of ESCRT III (Im et al., 2009). The ESCRT III complex, Vps2, Vps20, Vps24, and Snf7 (Babst et al., 2002a), together with ESCRT II, acts to initiate the formation of intraluminal vesicles. Unlike other ESCRT complexes, which are recruited to endosomal membranes as pre-formed complexes, ESCRT III components do not form stable complexes within the cytosol (Williams and Urbe, 2007), and the nucleation of ESCRT III assembly at the endosomal membrane requires the interaction between Vps20 and Vsp25 (Teis et al., 2008). The Vps20-Vps35 interaction promotes the subsequent recruitment of Snf7, and the formation of a multiple-Snf7 oligomeric filament that convexly distorts the membrane, assisting in the formation of the intraluminal vesicle (Teis et al., 2010). The elongation of the Snf7 filament is terminated when the final two components of the ESCRT III complex, Vps24 and Vps2, bind to its end, capping it (Saksena et al., 2009). Vps20, Snf7, and Vps24 are responsible for membrane budding and scission, while Vps2 acts to recruit the fifth and final component of the ESCRT machinery, Vps4, which disassembles the ESCRT III complex and allows it to be recycled for further rounds of vesicle formation (Babst et al., 1998; Saksena et al., 2009; Wollert et al., 2009). Thus, the ESCRT complexes 0, I, II, and III interact and mediate the

formation of intraluminal vesicles containing activated EGFR receptors, and other RTKs destined for degradation in lysosomes (Schmidt and Teis, 2012) (**Figure 1.16**). Once an accumulation of intraluminal vesicles has taken place, including those containing EGFR, the MVB, or late endosome, continues to acquire characteristics and proteins required for its fusion with lysosomes. The maturation of the MVB involves the loss of Rab-5, accumulation of Rab-7 (Rink et al., 2005), and a continued reduction in pH (reaching a pH of 6.0-4.8) (Maxfield and Yamashiro, 1987).

1.5.5. Mechanisms of Degradation

Late endosomes ultimately fuse with lysosomes to form endolysosomes (Kummel and Ungermann, 2014). Where Rab5 and EEA1 play key roles in tethering endocytic vesicles to the early endosome, the HOPS (homotypic fusion and vacuole protein sorting) complex is required for late endosome/lysosome fusion (Luzio et al., 2010).

Endolysosomes are a major compartment for macromolecular degradation, and RTKs that are contained in the intraluminal vesicles, as well as the membranes of the intraluminal vesicles themselves, are proteolytically cleaved by the acid hydrolases stored in the lysosomal compartment (Bonten et al., 2014; Katzmann et al., 2002) (**Figure 1.17**). Lysosomes contain up to 50 distinct hydrolases (Castino and Isidoro, 2008), and these enzymes are able to form complexes within the endolysosome to efficiently degrade a wide range of targets (Bonten et al., 2014). Due to their requirement for a low pH to function, with many exhibiting optimal activity at a pH close to 5, hydrolase activity is limited to the lysosomal compartment, which maintains an acidic state through the active function of proton pumps (DiCiccio and Steinberg, 2011). The degradation of a wide number of RTKs has been reported to take place in a lysosomal dependent manner,

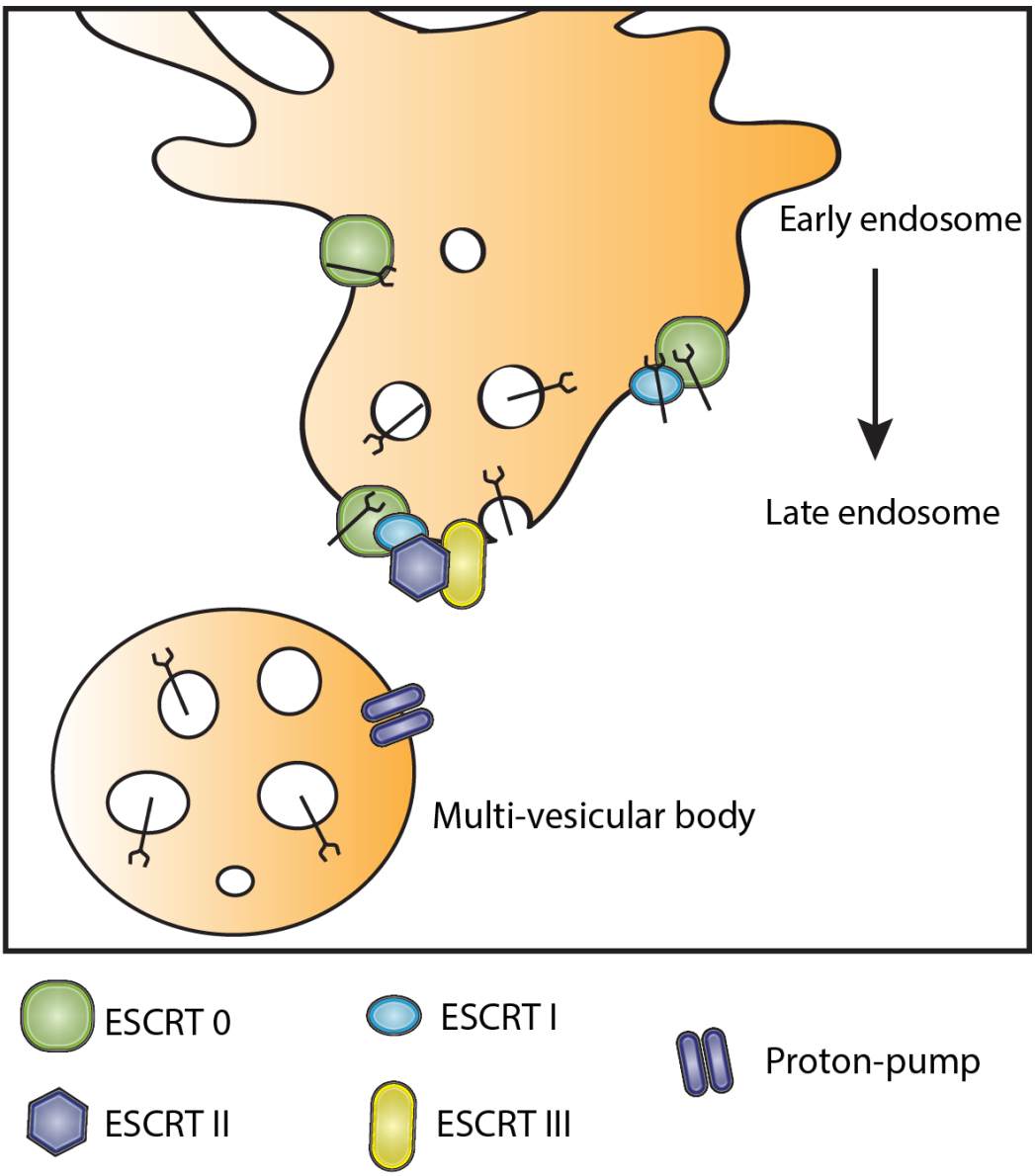

Figure 1.16. Formation of the multivesicular body.
Endocytosed receptors present in the early endosome can interact with several components of the ESCRT machinery, especially ESCRT 0 and ESCRT I, which serve to concentrate ubiquitinated cargo for internalization into intraluminal vesicles through ESCRT III activity. Figure adapted from Huotari and Helenius (Huotari and Helenius, 2011).

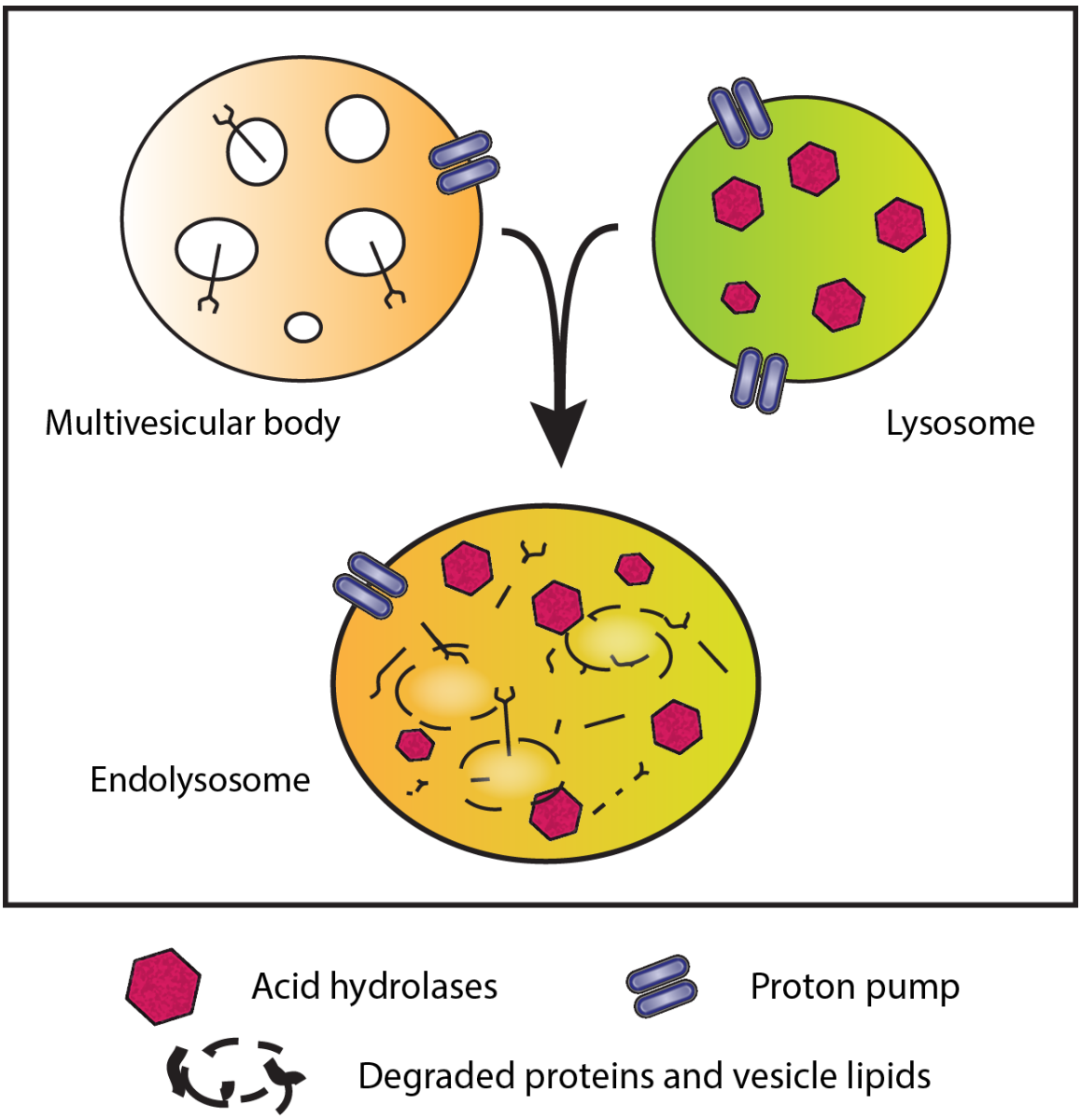

Figure 1.17. Formation of the endolysosome.
Late endosomes/multivesicular bodies fuse with lysosomes to form endolysosomes, a major compoenent of cellular degradative machinery. Following fusion internalized receptors and intraluminal vesicles are destroyed by hyrdrolases that were contained in the lysosome. Figure adapted from Huotari and Helenius (Huotari and Helenius, 2011).

including EGFR (Futter et al., 1996), and members of the PDGFR (Joly et al., 1995), FGFR (Wong et al., 2002), and VEGFR families (Han et al., 2014). While the lysosomal compartment is a major component of the degradative machinery of a cell, some activated receptors rely on extra-lysosomal proteasomal complexes for their degradation. The HGF (hepatocyte growth factor) receptor, Met, for example, can be poly-ubiquitinated following ligand stimulation, and its subsequent degradation can be blocked through the use of the proteasomal inhibitor, lactacystin (Jeffers et al., 1997), and similar observations have also made for the PDGFR (Mori et al., 1995). Additionally, rather than undergoing endocytosis, the ErbB4 receptor can be cleaved at the cell membrane by a metalloprotease, and the resulting ErbB4 C-terminal fragment is ubiquitinated and degraded by proteasomal complexes (Vecchi and Carpenter, 1997).

1.5.6. Alternative Mechanisms of Internalisation

1.5.6.1. Caveolae

Caveolae are flask shaped invaginations of the cell membrane that are rich in cholesterol and sphingolipids, and have been observed to internalize several RTKs following their activation (Di Guglielmo et al., 2003; Salani et al., 2010; Sigismund et al., 2005; Stan, 2005). The formation of these invaginations is dependent upon both the enrichment of these lipids, as well as on the presence of cavins, and caveolins (Hansen and Nichols, 2010) (**Figure 1.18**). There are three caveolin proteins identified to date, and while caveolin-1 and caveolin-2 are ubiquitously expressed, caveolin-3 is only expressed in muscle cells (Williams and Lisanti, 2004). Caveolin-1 is an integral membrane protein, and interacts with proteins, sphingolipids and cholesterol directly

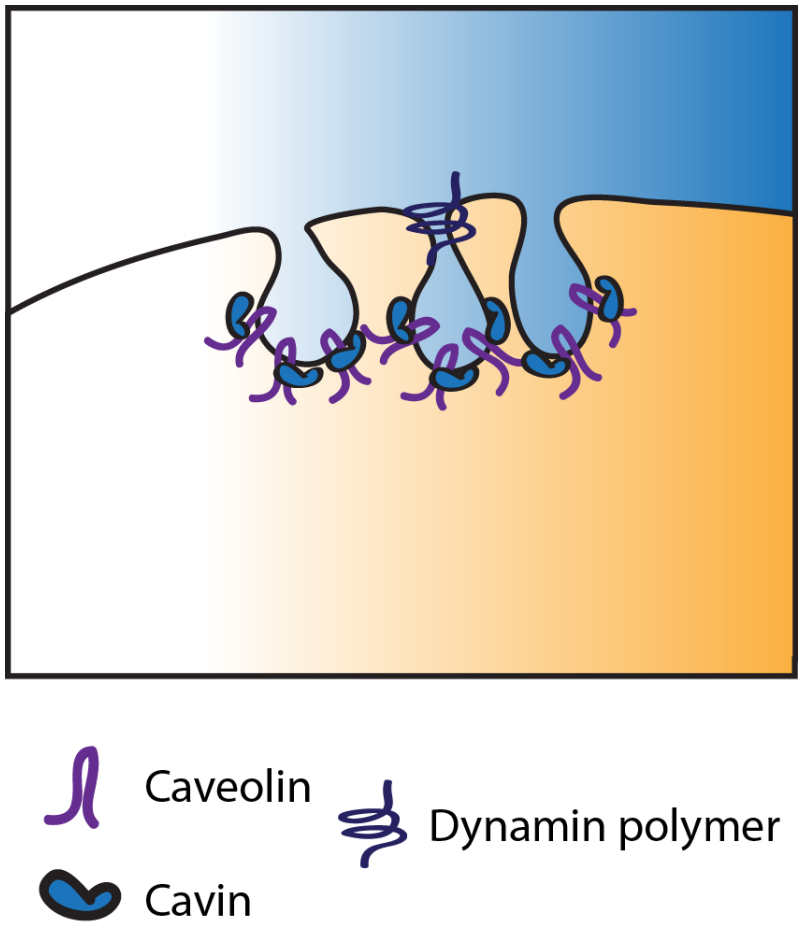

Figure 1.18. Structure of caveolae.
Caveolae are flask-like invaginations of the cell membrane formed in areas the cell membrane with high cholesterol content. Caveolin-1 and cavins induce and promote membrane curvature, and caveolar vesicles are separated from the cell membrane through dynamin activity.

(Fra et al., 1995; Murata et al., 1995). As such, it is likely that oligomers of caveolin-1 help to concentrate specific lipids and signalling molecules in rafts within the cell

membrane (Sargiacomo et al., 1995). Caveolin-1 is responsible for the recruitment of caveolin-2 to caveolae, and also appears to regulate the size of caveolar vesicles (Li et al., 1998). Overall, caveolin-1 is essential for the formation of caveolar flasks (Drab et al., 2001; Li et al., 1998), while caveolin-2 appears to play a stronger role in signalling than in caveolae formation (Razani et al., 2002; Sowa, 2011). In contrast to caveolins, cavins are cytosolic proteins, and appear to function as accessory proteins, stabilising the caveolar structure (Hayer et al., 2010).

While caveolae have been strongly implicated in facilitating the endocytosis of receptors such as the EGFR (Sigismund et al., 2005), the β-1-adrenergic receptor (Rapacciuolo et al., 2003), and the insulin receptor (Fagerholm et al., 2009), in response to their stimulation, it has also come into question if these structures truly undergo fission from the cell surface. Images acquired by electron microscopy show that when sliced parallel to the membrane, caveolae appear to form vesicles, however, samples that are prepared by perpendicular slicing show long tubule formations that are not separate from the peripheral membrane (Parton et al., 2002). This suggests the possibility that caveolae act predominantly as signalling structures, grouping components together, and facilitating efficient activation of certain pathways by ensuring their spatial organization (Harvey and Calaghan, 2012), and only play a minor role in endocytosis. Indeed, proper signalling of several receptors, including EphB1 (Vihanto et al., 2006) and EphB4 (Muto et al., 2011), has been found to be dependent upon their association with caveolin-1, verifying the important role caveolae play in signal regulation. However, the proper trafficking and degradation of the TGF-β (transforming growth factor-β) receptor (Di Guglielmo et al., 2003) and of IGF-1R (Fagerholm et al., 2009; Salani et al., 2010) is impaired when

caveolae-mediated internalisation is blocked, and other receptors have also been shown to internalize in a caveolin-dependent and clathrin-independent manner (Sigismund et al., 2005), strongly indicating that at least for some receptors, caveolae mediated internalisation is important as well.

Caveolar mediated internalisation is initiated when the activation of the RTK promotes its association with caveolin-1, localising it to caveolae. This may also result in the phosphorylation of caveolin-1, as is the case following IGF-IR activation by IGF, however the importance of this to internalization has not yet been fully explored (Fagerholm et al., 2009; Salani et al., 2010). As with CCP mediatied internalisation, there is strong evidence implicating dynamin in mediating caveolar vesicle scission from the membrane (Oh et al., 1998). Following their formation, and separation from the cell membrane, caveolar vesicle trafficking is dependent upon both actin and microtubules (Mundy et al., 2002).

Thus, while the importance of caveolae-mediated active internalisation of cell-surface receptors remains controversial, there is compelling evidence that caveolae play an important role in endocytosis for at least a few proteins, including EGFR (Sigismund et al., 2005), and like clathrin mediated internalisation, dynamin mediated pinching-off at the cell membrane has been implicated in their activity (Oh et al., 1998).

1.5.6.2. Lipid Rafts/ CLIC-GEEC

The Clathrin-Independent Carriers (CLICs), and GPI-Enriched Endocytic Compartments (GEECs) pathway is predominantly associated with the internalization of GPI-anchored proteins, however it has been linked to RTK endocytosis as well (Jahn et al., 2002; Vaidyanath et al., 2011). CLICs are cholesterol rich lipid-raft microdomains,

distinct from caveolae, that undergo endocytosis (Kirkham and Parton, 2005). CLIC mediated internalization can be initiated through active cellular signalling, and is dependent upon the action of members of the Rho family of GTPases (Doherty and Lundmark, 2009). CLICs are concentrated at the leading edge of cells, and are important to ensuring proper cell migration (Howes et al., 2010). Unlike the pit formed during clathrin mediated endocytosis, or the characteristic flask shape of caveolae, the CLIC mediated internalisation pathway involves the formation of long tubule like invaginations from the cell surface (Kirkham and Parton, 2005) (**Figure 1.19**). The separation of the vesicle from the cell membrane may not be dynamin dependent, as overexpression of a dominant negative dynamin mutant had no observable impact on the overall rate of endocytosis through this pathway (Sabharanjak et al., 2002). However, as a separate group found that lipid-raft endocytosis was dependent upon dynamin (Lundmark et al., 2008), its role in these structures is not yet clear. A Rho-GAP-domain-containing protein, GRAF1 (GTPase regulator associated with focal adhesion kinase-1), has been demonstrated to play an important role in regulating this pathway (Lundmark et al., 2008), as has the Rho family member, Cdc42 (Chadda et al., 2007; Sabharanjak et al., 2002). GRAF1 may play a dual role, both by acting as a Rho-GAP protein, thus affecting the actin cytoskeleton, and by enhancing membrane curvature through its Bin/Amphiphysin/Rvs (BAR) domain (Doherty and Lundmark, 2009). BAR domains are believed to be recruited to areas of positive membrane curvature (Galic et al., 2012), and to strongly enhance further curvature through physical interactions with the membrane, thus facilitating pit formation (Mim and Unger, 2012). As expected from the observations that Rho family members are involved in mediating CLIC endocytosis, actin

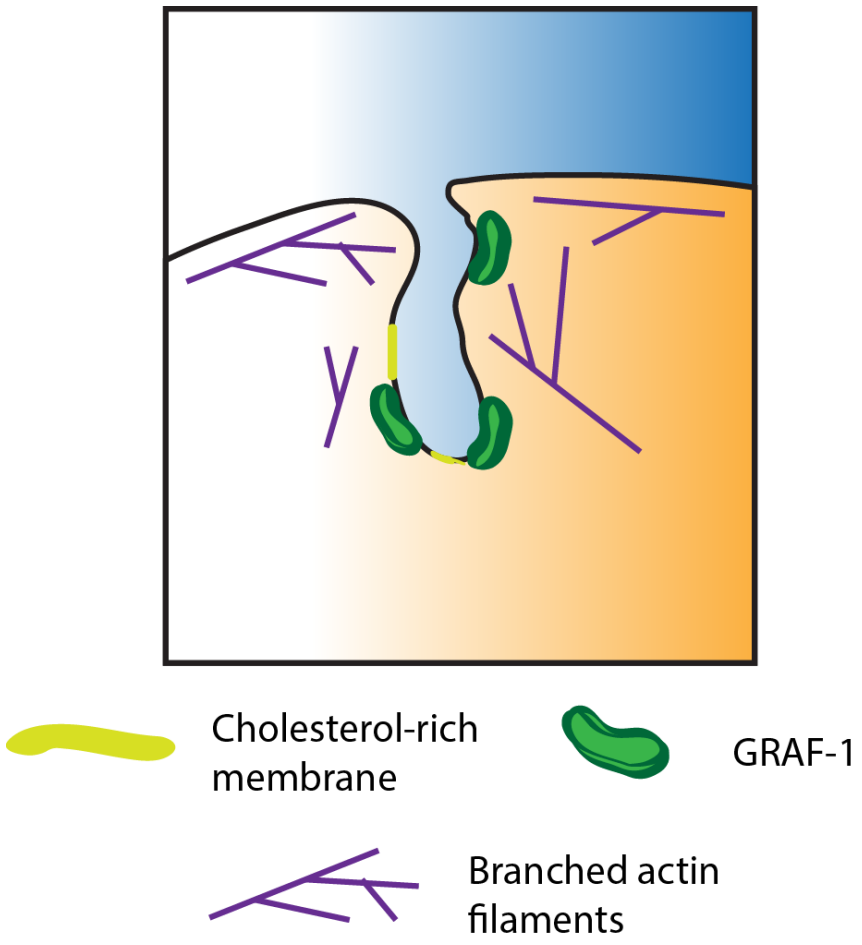

Figure 1.19. Structure of CLIC/GEEC membrane invagination.
Lipid-rich regions of membrane form long, tubule like invaginations, which are promoted both by structural rearrangements of the actin cytoskeleton, but also by the BAR domain containing GRAF-1, which also plays a role in membrane scission.

polymerisation is essential for CLIC mediated internalisation (Chadda et al., 2007). Similar to caveolae, CLIC endocytosis also requires membranous cholesterol (Chadda et

al., 2007). While it is not yet fully clear if all cargo internalized through CLICs end up in GEECs (Xu et al., 2013), CLIC internalized vesicles can be targeted to the early endosome as association with typical markers such as Rab5 and EEA1 has been observed (Kalia et al., 2006). ErbB2 was observed to be internalised in a clathrin-independent manner in response to Hsp90 inhibition (Barr et al., 2008), and it has been proposed that the CLIC/GEEC pathway may be responsible (Vaidyanath et al., 2011). Additionally, the RTK Ret is actively transported to GPI-enriched lipid rafts following its activation by ligand treatment, as is c-Kit, suggesting they too may be internalized through this compartment (Jahn et al., 2007; Jahn et al., 2002; Tansey et al., 2000).

1.5.6.3. Macropinocytosis

Macropinocytosis is a process that involves the internalisation of a large area of the cell membrane and extracellular fluids. While it is not normally associated to ligand-induced receptor internalisation, it has been implicated in bulk receptor removal (Kerr and Teasdale, 2009), and macropinocytosis is often initiated through RTK activation (Hamasaki et al., 2004; Lanzetti et al., 2004). The process of macropinocytosis involves the initiation of actin polymerization through the activation of the Rho family of GTPases (Egami et al., 2014) and phosphorylation of PIP_2 by PI3K (Hoeller et al., 2013). In turn, members of the Wiskott–Aldrich Syndrome protein (WASp) family and WASp family Verprolin-homologous (WAVE) proteins are activated and promote the Arp2/3 dependent nucleation of a new actin filament branch points (Kerr and Teasdale, 2009; Veltman et al., 2014). Additionally, PAK1 (p21 protein (Cdc42/Rac)-activated kinase 1) has been found to be essential for the initiation of this process (Dharmawardhane et al., 2000). The localized growth of the actin skeleton promotes protrusions of the cell

membrane, which eventually branch to form pockets, and fuse back to the cell membrane (Swanson, 2008) (**Figure 1.20**). Macropinocytosis is a dynamin- independent mechanism (Liberali et al., 2008). Interestingly, following vesicle formation, macropinosomes can follow routes common to canonical clathrin-mediated receptor endocytosis. For example, Rab5, a key protein in vesicular trafficking following clathrin-mediated endocytosis, appears to be important to macropinosome formation (Lanzetti et al., 2004; Schnatwinkel et al., 2004), and macropinosomes are consistently found to associate with markers of the classical clathrin-mediated internalization pathway such as EEA1 (Kerr et al., 2006). Furthermore, macropinosomes have been shown to mature, much like canonical vesicles of internalisation, and instead, membrane fission has been observed to be assured by C-terminal-binding protein-1/brefeldinA-ADP ribosylated substrate (CtBP1/BARS) (Kerr et al., 2006) and target to lysosomes (Racoosin and Swanson, 1993). In addition to CCP and caveolar mediated internalisation, EGFR has been observed to be internalised in a specialised form of macropinocytosis involving the formation of circular dorsal ruffles. These wave like structures are rich in F-actin and form in response to growth factor stimulation on the migratory surface of epithelial and mesenchymal cells (Krueger et al., 2003). Following stimulation with EGF, activated, EGF-bound EGFR phosphorylates PI3K at the cell and membrane, leading to an increase in PIP_3, and initiates WAVE/WASp and Arp2/3 mediated dorsal ruffle formation. EGFR and PIP_3 are concentrated in the dorsal ruffles, and are subsequently internalised (Orth et al., 2006). However, unlike some instances of macropinocytosis, dorsal ruffle mediated EGFR internalization appears to require dynamin, where it may function to promote membrane curvature (Orth et al., 2006).

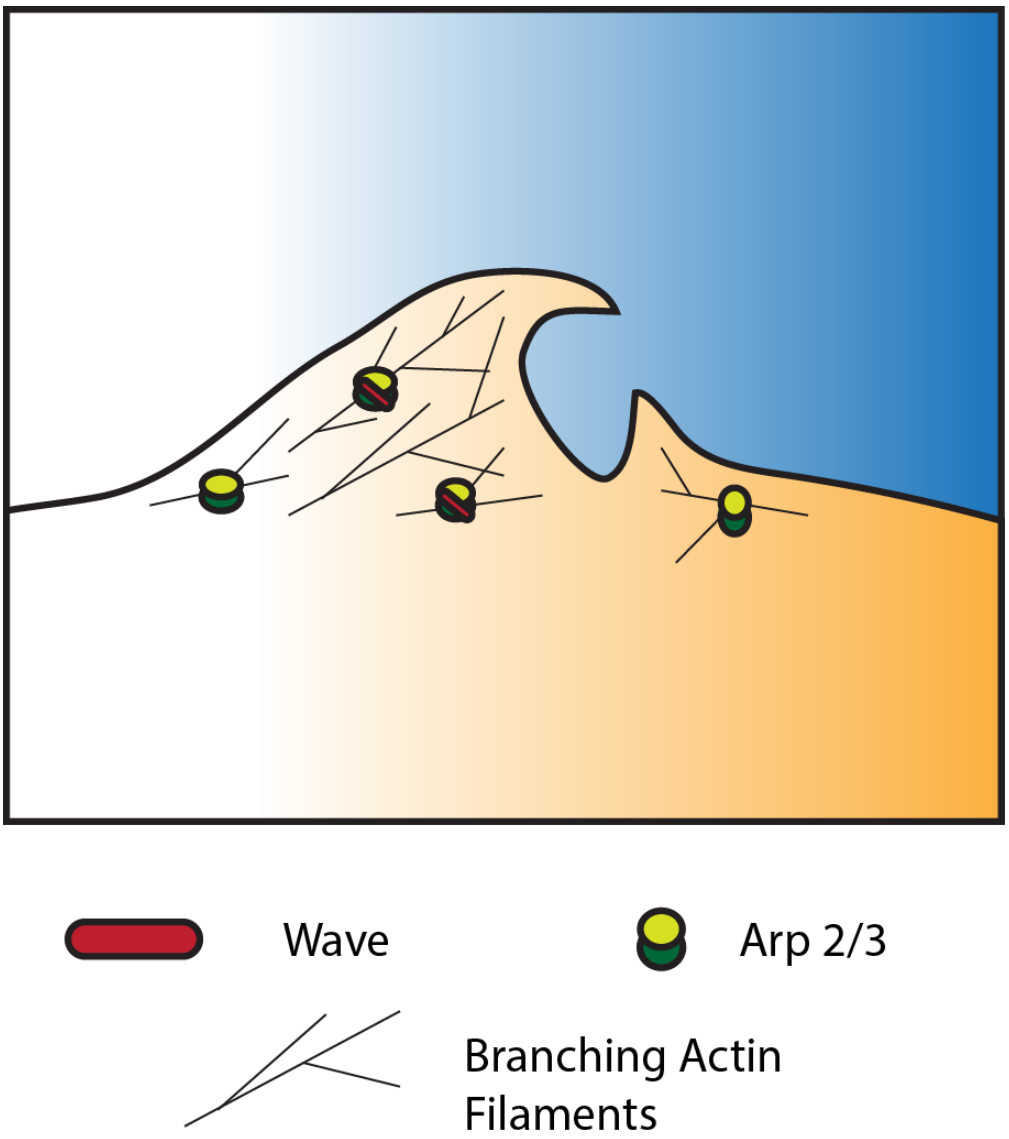

Figure 1.20. Formation of a macropinocytotic vesicle.
Following receptor activation, Rho family GTPases promote growth of the actin cytoskeleton at the leading edge of the cell. WAVE and WASp proteins work with Arp2/3 complexes to promote branching of the actin cytoskeleton, resulting in stable outgrowths of the cell membrane that ultimately fuse to engulf extracellular material and areas of cell membrane.

The RTK Met can also utilise circular dorsal ruffles for its internalisation, and will subsequently colocalise with components of the classical endosomal pathway including Rab5, EEA1, and components of the ESCRT machinery, including Hrs (Abella et al., 2010), further demonstrating that this process is not only able to internalise receptors, but can also act as the initial step in ligand-induced receptor downregulation. The RTK, TrkA, which is involved in neurotrophic signalling, is also endocytosed through macropinocytosis in response to activation, and this was observed to contribute to proper downstream signalling (Shao et al., 2002).

1.5.6.4. Endophilin-mediated internalization

One of the most recently described mechanisms of non-clathrin mediated endocytosis involves the BAR-domain containing protein, endophilin. While endophilin has been observed to associate with CCPs, its presence is dispensable for the formation and internalization of these structures (Taylor et al., 2011), and in agreement, it is only found in a fraction of CCPs (Taylor et al., 2011). Stimulation of EGFR with high levels of EGF was found to rapidly promote the formation of endophilin and EGFR positive vesicles, and knock-down of endophilin led to an accumulation of EGFR at the cell surface. Importantly, knock-down of clathrin did not affect the formation of endophilin positive vesicles in response to EGF. Like CCP mediated internalisation, endophilin mediated internalization requires dynamin activity (Boucrot et al., 2015). The same group also found that interleukin-2β receptor (IL-2Rβ) could also be internalized in an endophilin dependent manner, confirming observations made by separate team same who found that the IL-2Rβ appears to undergo endocytosis in a mechanism distinct from

clathrin, caveloae, or the CLIC/GLEEC pathway (Grassart et al., 2008). Furthermore, both studies found that this mechanism of internalization requires the activity of dynamin, Rac1, PAK1, and actin (Boucrot et al., 2015; Grassart et al., 2008).

1.6. Downregulation and Signaling

Internalisation of an activated receptor does not necessarily signify a termination of RTK signalling. Indeed, the opposite is often found to be true, where increased activation of target pathways can be achieved in the endosomal compartment (Sorkin and von Zastrow, 2009). For example, several studies have indicated that the full activation of the Ras/MAPK pathway following EGFR activation requires the internalisation of the receptor (Sigismund et al., 2008; Vieira et al., 1996), and in agreement, several proteins which complex with EGFR to initiate this signalling cascade, including SHC (Src Homology 2 Domain-Containing) and Grb2, were found to associate with EGFR at endosomes (Di Guglielmo et al., 1994). Furthermore, several studies have clearly demonstrated that EGFR is able to efficiently activate Ras from endosomes (Haugh et al., 1999a; Wang et al., 2002). These observations are not specific to EGFR, as the RTK also TrkA interacts with signalling effectors from endosomes (Grimes et al., 1996), and effectively leads to the activation of downstream targets (Howe et al., 2001), as does PDGFR (Wang et al., 2004).

While internalization does not indicate an end of signaling, it can signify a change in the signals that are generated. For example, while EGFR demonstrates a continued ability to activate the Ras/MAPK cascade following internalisation, endocytosis inhibits its ability to activate the phospholipase-C pathway, providing evidence that the signals generated by a receptor can be controlled by its spatial organisation (Haugh et al.,

1999b). Similar observations of VEGF receptor signalling further support this, as the cell-surface receptor can only poorly activate the Ras/MAPK pathway, whereas the internalised receptor exhibits efficient signalling leading to cell proliferation (Lampugnani et al., 2006). Additionally, the c-Met receptor also relies on endocytosis to guarantee efficient downstream effects are achieved, although in this instance it is the phosphorylation and nuclear translocation of STAT3 (Signal transducer and activator of transcription 3) following c-Met activation that requires receptor internalisation (Kermorgant and Parker, 2008). However, it should be noted that as some reports suggest that signalling is indeed terminated following internalisation, the impact of internalisation for signal activation is likely be cell-type, pathway, or situation dependent (Galperin and Sorkin, 2008; Johannessen et al., 2000).

In some cases, internalisation of receptors to the endosomal compartment appears to allow for their better association with scaffolding proteins that serve to bring together components of signalling pathways. For example, p-18, a MAPK scaffolding protein, is only observed in endosomes, and is absent from the peripheral cell membrane (Nada et al., 2009). P-18 acts by anchoring components of the MAPK signalling pathway to the RTK bearing membrane promoting their efficient activaiton (Nada et al., 2009). P-18, and other endosome specific scaffolds are necessary for proper activation of RTK downstream signals, as loss of their expression results in reduced signalling efficiency (Nada et al., 2009; Teis et al., 2002).

Some endosome-associated proteins are not only important to facilitating the formation of receptor signalling complexes, but also play a direct role signal transduction. Two such proteins are represented by the Rab-5 effectors, adaptor protein,

phosphotyrosine interacting, PH (Pleckstrin homology) domain, and leucine zipper-containing-1 (APPL-1) and APPL-2 (Urbanska et al., 2011). Rab-5, APPL-1, and APPL-2 positive vesicles are considered to be specialised signalling endosomes, and, likely due to the ability of APPL-1 and 2 to bind to both PI3K and Akt, show enhanced PI3K/Akt signalling relative to EEA1 positive endosomes (Schenck et al., 2008; Zoncu et al., 2009). In neuronal cells, APPL-1 has been observed to be required for proper TrkA signalling and activation of multiple pathways, including the PI3K/Akt pathway and Ras/MAPK pathway (Lin et al., 2006; Varsano et al., 2006). EGFR also associates with APPL-positive endosomes (Zoncu et al., 2009), and in agreement, a portion of its ligand, EGF, was found to rapidly internalise and colocalise with APPL-1 and APPL-2 (Miaczynska et al., 2004a). Interestingly, the authors found that the presence of EGF in APPL endosomes initiated the translocation of APPL-1 and APPL-2 to the cell nucleus, where they were observed to interact with components of a complex of proteins involved in chromatin remodeling, and that knock-down of APPL-1 or APPL-2 resulted in reduced proliferation. This suggests that APPLs may also directly transfer signals to the nucleus, and that this may be important to proliferative signaling initiated by some RTKs (Miaczynska et al., 2004a). Several observations have been made that suggest that APPL-positive endosomes "mature" to form more canonical early endosomes (Miaczynska et al., 2004a; Zoncu et al., 2009), including the finding that that EGF association with EEA1-positive endosomes increases as APPL-1 and APPL-2 translocate to the nucleus (Miaczynska et al., 2004a). Mechanistically, it has been suggested that APPL proteins compete with EEA-1 for binding to Rab-5, and it was demonstrated that as the endosome becomes enriched with PIP_3, possibly as a result of receptor driven-PI3K activation,

EEA-1 is consequently accumulated. This could allow EEA1 to potentially outcompete the APPL proteins for Rab5 interaction, mediating the conversion to the canonical early endosome (Zoncu et al., 2009). As APPL-positive endosomes have been shown to enhance signalling by EGFR (Zoncu et al., 2009), TrkA (Lin et al., 2006), and other non-RTKs (Hu et al., 2003; Mao et al., 2006), especially through the PI3K/Akt pathway, this method of receptor-initiated conversion from a highly active signalling endosome, to one with relatively reduced PI3K/Akt signalling potential could represent a second level of regulation of signalling ouput, allowing for a controlled short-term burst of high level, self-limiting receptor signalling. Importantly, the method of internalization appears to impact targeting to APPL-positive endosomes, as it was found that EGF channeled to these compartments was internalised in a dynamin independent manner. Therefore, the subset of EGFR receptors that are targeted to APPL-positive endosomes undergo endocytosis in a manner distinct from the canonical pathway, and thus the mechanism of internalization too is important to downstream signaling effects (Miaczynska et al., 2004a).

As suggested by the dynamin independent internalisation of EGF that targets to APPL-positive endosomes, the mechanism of receptor internalisation can alter the availability and potential signalling output of RTKs. For example, EGFR can be internalised in CCP-dependent and independent manners, and recent observations suggest that this results in its targeting for either recycling or degradation (Sigismund et al., 2008). EGFR has been found to be endocytosed through CCPs when stimulated with low levels of EGF (Sigismund et al., 2005), however, it utilises alternate mechanisms of internalisation when in the presence of high EGF (Sigismund et al., 2008; Sigismund et al., 2005).

According to observations by one group, when internalised through CCPs, EGFR is preferentially sorted for recycling, and thus is able to be activated again rapidly. In contrast, when a high concentration of ligand is present, EGFR is endocytosed in a CCP independent manner, and is efficiently targeted to MVBs and degraded (Sigismund et al., 2008) (**Figure 1.21**). Furthermore, it was found that EGFR recycling led to sustained signaling in comparison to that observed for the lysosomal-targeted receptor (Sigismund et al., 2008). While it should be noted that there are several other reports of CCP mediated EGFR internalization leading to receptor downregulation (Grovdal et al., 2004; Madshus and Stang, 2009), and as such these observations may be context specific, the ability of ligand concentration to alter receptor endocytosis, resulting in differential receptor sorting and signaling output is an excellent example of the impact trafficking has on RTK activity. Furthermore, as this model represents a useful mechanism to finely regulate cell surface RTK levels in response to ligand concentration in a very rapid manner, and it may be important to other RTKs as well. Thus, while it was initially believed that signalling ended upon endocytosis and entry into the endosomal pathway, it is now recognised that internalisation is integrally linked to signalling, and that there even exist specialized proteins and compartments within this pathway specifically for the production of efficient signaling responses. Ultimately, until the receptor is sorted into intraluminal vesicles, it has the opportunity to interact with a variety of downstream targets, many of which may be represented in higher concentrations at endsomes than at the cell membrane, and so receptor internalisation and trafficking represents an important component of active signalling.

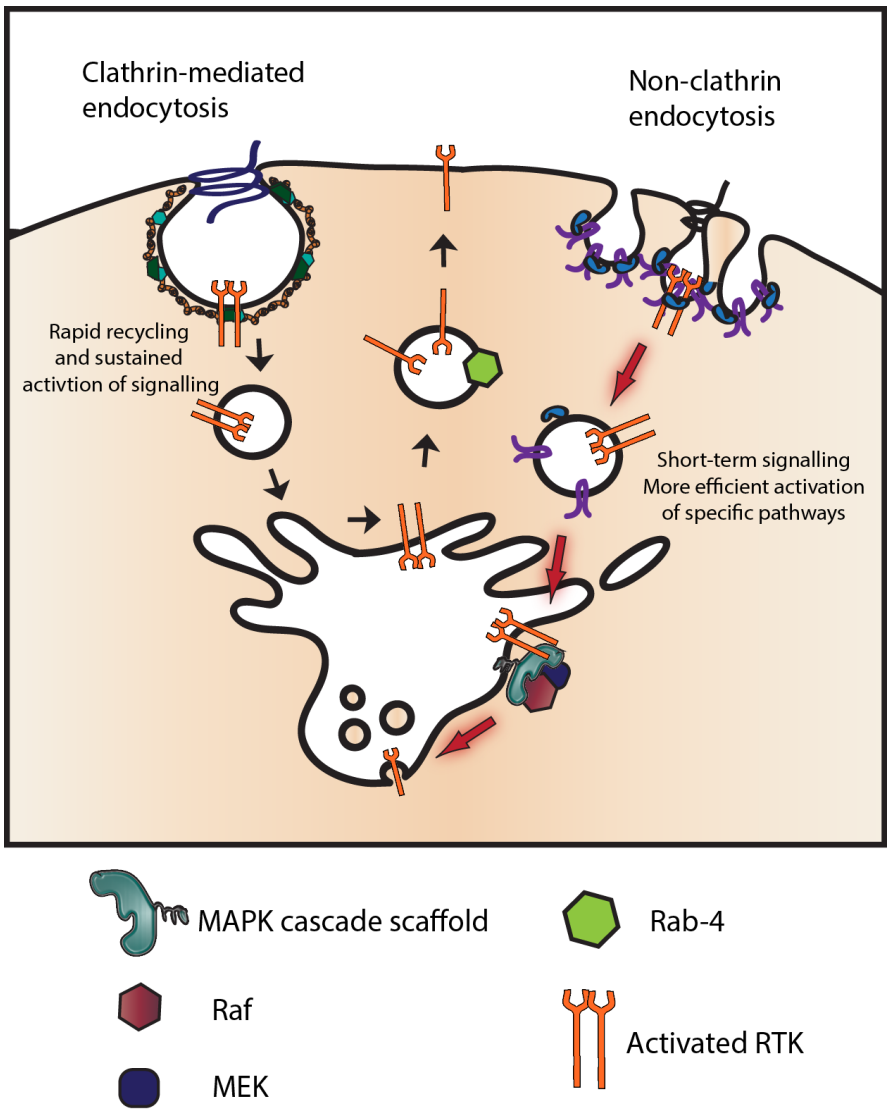

Figure 1.21. Example of the impact of trafficking on signaling output.
EGFR has been shown to be preferentially sorted to the recycling endosome following internalization through clathirin-coated pits. In contrast, EGFR that is internalized in a clathrin-independent manner has been observed to initiate increased signaling output through the MAPK pathway. Non-clathrin endocytosis has also been observed to be the primary mechanism of internalization for activated EGFR that is destined for degradation.

1.7. Downregulation and Malignancy

A striking example of the power of naturally inefficient RTK downregulation is presented by a member of the EGFR group (also referred to as the ErbB group), ErbB2. The high potency of ErbB2 receptor action is in part, assured by its ineffective internalisation and degradation, and by its ability to slow down ligand-induced downregulation of other ErbB receptors in heterodimers (Haslekas et al., 2005; Lenferink et al., 1998; Wang et al., 1999; Worthylake et al., 1999). This results in highly active and robust signalling, which in pathological situations, drives malignant cell behaviour in a variety of tumours, including those of breast, ovarian, gastric, and lung origins (Baselga and Swain, 2009; Parachoniak and Park, 2012). The oncogenic potential that stems from inefficient ErbB2 downregulation, and from its ability to modify the internalisation and degradation of its signalling partners gives a strong indication of the biological importance of modulating the balance in these processes.

Thus, disruption of Met receptor degradation, following activation by its ligand, HGF, leads to a sustained activation of the Ras-MAPK pathway and oncogenic transformation in non-small-cell lung cancer (Abella et al., 2005). Colony-stimulating factor-1 receptor mutations, which impair its internalisation and degradation, have been linked to myelodysplasia and acute myeloid leukemia (Ridge et al., 1990), and the mitogenic capacity of c-Kit receptor signalling is greatly enhanced in a mutant lacking a docking site for c-Cbl**,** a well-described regulator of receptor downregulation (Herbst et al., 1995).

Receptor downregulation can be impaired due to mutations of the receptor itself, as is the case for c-Kit, or, alternatively, can result from aberrations within the downregulation machinery. Cortactin is a protein that is involved in actin polymerisation at the cell periphery (Schafer et al., 2002), and has been linked to a regulatory role in both CCP dependent and independent endocytosis (Cao et al., 2003; Sauvonnet et al., 2005). Overexpression of cortactin has been observed in several cancer types, including invasive melanoma (Xu et al., 2010), breast cancer (Buday and Downward, 2007), head and neck squamous cell carcinomas (HNSCC) (Akervall et al., 1995), as well as colorectal cancer (Hirakawa et al., 2009), and is linked to increased metastatic behaviour (MacGrath and Koleske, 2012). This appears to be at least in part due to alterations to endocytosis of RTKs, as HNSCC cell lines with high expressions of cortactin exhibited reduced rates ligand-induced downregulation of EGFR compared to those with lower cortactin expression. Furthermore, when siRNA was used to reduce the levels of cortactin expression, the rate of EGFR downregulation correspondingly increased (Timpson et al., 2005).

Vps37, otherwise known as HCRP1 (hepatocellular carcinoma related protein 1), is a component of the ESCRT I machinery, and is required for EGFR degradation (Bache et al., 2004). In hepatocellular carcinoma the expression levels of Vps37 are significantly reduced relative to healthy tissue, and knock-down of Vps37 expression resulted in increased cancer cell proliferation (Xu et al., 2003). In agreement, reduced Vps37 expression is associated with a poorer prognosis in breast (Xu et al., 2014), oral, and oropharyngeal cancer (Perisanidis et al., 2013). Furthermore, in ovarian cancer high EGFR or ErbB2 coupled with low Vps37 was found to have a strong negative impact on

overall patient survival, while patients with high levels of Vps37 were found to have a significantly better prognosis regardless of EGFR and ErbB2 expression levels, suggesting that Vps37 induced degradation of these receptors is important to attenuating their oncogenic signalling (Wittinger et al., 2011). This is supported by the observations that knock-down of Vps37 directly inhibits EGFR degradation following its activation, leading to an increase Erk1/2 phosphorylation in ovarian cancer cell lines (Wittinger et al., 2011).

Point mutations of c-Cbl have been detected in samples from acute myeloid leukemia (AML) patients, and these mutants were found to be unable to bind to and ubiquitinate one of the known RTK drivers of this disease, FLT3 (FMS-like tyrosine kinase-3) (Abbas et al., 2008; Caligiuri et al., 2007; Sargin et al., 2007). The loss of c-Cbl mediated ubiquitination of FLT3 following its activation results in its impaired endocytosis as well as an increased duration of FLT3 signalling, and promoting cellular transformation (Sargin et al., 2007).

1.8. Current Knowledge of Eph Receptor Downregulation

As mentioned above, stimulation-induced receptor downregulation through internalization and subsequent targeted degradation play an important role in controlling cellular responses by both enhancing and suppressing cytoplasmic signaling (Platta and Stenmark, 2011). While these processes are well described for some receptor groups (Andersson, 2012; Parachoniak and Park, 2012; Platta and Stenmark, 2011; Sorkin and Goh, 2009), much less is understood about downregulation of Eph receptors. Currently, the available data on Eph receptor downregulation are not systematic, and imply that different Ephs may use very distinct mechanisms. As such, little can currently be

assumed regarding the downregulation of one receptor based upon observations made for a separate receptor, and any generalizations would likely be inaccurate. For example, ligand-induced downregulation of the EphA2 receptor is assured by its degradation in the lysosomal compartment (Boissier et al., 2013), while in contrast, EphA3 is internalised following its activation, but is not transported to lysosomes (Nievergall et al., 2012), and instead is degraded by proteasomal complexes (Sharfe et al., 2003). While EphA2 can be internalised through macropinocytosis (Ha et al., 2014), activation with ephrin-A1 increases its association with clathrin and dynamin, both of which are likely to be important to mediating ligand-induced receptor internalisation (Greene et al., 2014). Furthermore, EphA2 internalisation also appears to utilise members of the Rho family, as knock-down of the Rho family GEF, Tiam-1, leads to a reduction in EphA2 endocytosis (Boissier et al., 2013). EphA8 is ubiquitinated and internalised in response to ligand stimulation, and this appears to be regulated by interactions with the Rab5 GEF, RINL (**R**as and Rab **in**teractor-**l**ike), and the Anks family protein, Odin (Kajiho et al., 2012; Kim et al., 2010; Shin et al., 2007). EphA4 (Bouvier et al., 2010), EphA8 (Yoo et al., 2010) and EphB1 (Parker et al., 2004) are endocytosed through CCPs following ligand stimulation, and internalized EphB1 and EphA8 appear to be degraded in the lysosomal compartment inside the cell (Fasen et al., 2008; Kajiho et al., 2012). In contrast, EphB2 can be cleaved at the cell membrane by matrix-metalloproteases (Lin et al., 2008), and potentially other proteases, such as BACE (β-secretase) (Litterst et al., 2007). The C-terminal fragment of cleaved EphB2 is subsequently processed by the presenilin dependent γ-secretase complex (Lin et al., 2008; Litterst et al., 2007), overall highlighting

the vastly different mechanisms Eph receptors are able to utilise to mediate receptor internalisation and degradation following ligand stimulation (**Table 1.3**).

Table 1.3. KNOWN MEDIATORS OF EPH RECEPTOR DOWNREGULATION.

Receptor	Molecule	Effect	Source
EphA2	c-Cbl, proteasome	Interacts with EphA2 following activation. Acts as an E3-ligase, facilitating ubiquitination of EphA2, leading to EphA2 proteasomal degradation.	(Walker-Daniels et al., 2003; Walker-Daniels et al., 2002)
EphA4	Clathrin	Forms vesicles in response to EphA4 activation facilitating EphA4 internalisation.	(Bouvier et al., 2010)
EphA4	Rab5 and Rin1	Promote EphA4 internalisation following its activation.	(Deininger et al., 2008)
	γ-secretase	Cleaves EphA4 at the cell membrane.	(Inoue et al., 2009)
EphA8	Rac-1, Tiam-1, and Clathrin	Assist in the endocytosis of EphA8 following ephrinA interaction.	(Yoo et al., 2010)
	c-Cbl	Ubiquitinates EphA8 following ligand-binding, inducing its internalization and degradation	(Kim et al., 2010)
	Rab-5 and RINL	RINL acts as a GEF for Rab-5. Involved in facilitating EphA8 internalisation.	(Kajiho et al., 2012)
EphB1	Caveolin-1	EphB1 associates with caveolin-1 following activation and localizes to caveolae.	(Vihanto et al., 2006)
EphB1	c-Cbl and lysosomal compartment	c-Cbl ubiquitinates EphB1 following ligand activation, targeting EphB1 for lysosomal degradation.	(Fasen et al., 2008)
EphB2	γ-secretase	Cleaves EphB2 in response to ligand stimulation	(Litterst et al., 2007)
	Proteasome	Mediates EphB2 degradation following ligand-induced internalisaiton.	(Mann et al., 2003)

Receptor	Molecule	Effect	Source
EphB2	BACE	Cleaves EphB2 in response to ligand-stimualtion	(Litterst et al., 2007)
EphB4	Ena/Vasp	Proteins that are involved in regulating the actin cytoskeleton, implicated in EphB4 internalisation.	(Evans et al., 2007)
	Rac	Required for receptor internalization.	(Marston et al., 2003)

In this situation, it is not a surprise that despite the recent accumulation of data that highlight an important role for one of Eph receptors, EphB6, in both normal physiology and in malignancy (Fox and Kandpal, 2009; Freywald et al., 2003; Luo et al., 2012; Luo et al., 2004; Maddigan et al., 2011; Tang et al., 2000; Truitt and Freywald, 2011; Truitt et al., 2010; Yu et al., 2010), the molecular mechanism of its downregulation has never been investigated. Such an investigation is especially interesting in light of the unusual nature of this protein, which is kinase-inactive, despite its association with the Eph group of RTKs (Matsuoka et al., 1997), and due to the lack of available information on the ligand-induced downregulation of kinase dead receptors in general. Until now, downregulation of only one other kinase-deficient RTK, the ErbB3 receptor, has been dissected and yet even this remains not well understood, as multiple reports suggest that ErbB3 is internalisation- and downregulation- deficient, while some recent observations show that ErbB3 undergoes ubiquitination and is efficiently downregulated in response to ligand stimulation (Baulida et al., 1996; Cao et al., 2007; Chen et al., 1996; Daly et al., 1997; Sak et al., 2013; Waterman et al., 1998).

1.9. Ligand Induced Downregulation of Kinase-Dead RTKs

Initial studies into the downregulation of ErbB3 utilised a chimeric protein, composed of the EGFR extracellular domain fused to the intracellular domain of ErbB3. While it was found that the addition of EGF was able to rapidly induce phosphorylation of the ErbB3 chimera, a very low level of internalisation was observed relative to EGFR, and there was no significant alteration to the apparent rate of receptor degradation (Baulida et al., 1996). Additionally, experiments in breast cancer cells with endogenous ErbB3 expression consistently demonstrated a slower rate of ligand internalisation and reduced levels of cell-surface receptor removal for ligand-treated ErbB3, relative to EGFR (Baulida and Carpenter, 1997). Two other groups observed similar effects, where ligand treatment of ErbB3 resulted in its association with ErbB2 and phosphorylation, but failed to reduce overall ErbB3 protein levels (Chen et al., 1996; Daly et al., 1997). A more in depth study also found that ligand-induced ErbB3 endocytosis was relatively slow compared to EGFR, and that ErB3 was recycled back to the cell-surface following its internalisation, whereas EGFR was targeted for lysosomal degradation. The examination of ligand-binding affinities suggested that the ErbB3-ligand complex was more transient than that between EGF and EGFR, and thus rapid ligand dissociation following receptor internalisation was proposed to promote ErbB3 receptor recycling over degradation (Waterman et al., 1998). Additionally, it was also found that ligands of EGFR and ErbB3 were degraded by different mechanisms, where EGF appeared to rely on lysosomes for its degradation, while ErbB3 ligands did not (Waterman et al., 1998). In agreement, ErbB3 degradation following ubiquitination by Ndrp1 was shown to occur preferentially in proteasomal complexes rather than lysosomes (Qiu and Goldberg, 2002). However, more recent studies of ligand-induced ErbB3 degradation found that it in

contrast to previous observations, the receptor is indeed efficiently downregulated in response to ligand treatment, and that its degradation requires lysosomal activity (Cao et al., 2007). Further evidence for ligand-induced downregulation of ErbB3 was obtained by a separate group, who found that while ErbB3 is constitutively endocytosed in a clathrin-dependent manner at a relatively high rate compared to EGFR (Sak et al., 2012), ligand treatment significantly increased its internalisation, as well as its degradation (Sak et al., 2013). Interestingly, their observations suggest that while ErbB3 is downregulated efficiently in response to ligand, its interaction with ErbB2 has a potential to inhibit its ligand-induced downregulation (Sak et al., 2013), which may explain some of the discrepancies described for the ErbB3 receptor. Thus, while the ligand-induced downregulation of ErbB3 has been actively explored, the details are still unclear, and since the ligand-induced downregulation of any other RTK with an intrinsic deficiency in kinase activity has not been assessed, it is impossible to determine if these observations are relevant to other kinase-dead RTKs, or are unique to ErbB3.

As two of the five known kinase-deficient RTKs, EphA10 and EphB6, belong to the Eph group of receptors (Aasheim et al., 2005b; Gurniak and Berg, 1996; Matsuoka et al., 1997), it strongly suggests that they are likely to play important roles in modulating Eph receptor effects. Eph receptors play an essential role in a wide variety of biological functions, including embryo development and angiogenesis (Pitulescu and Adams, 2010), and have also been implicated in both positive and negative effects in malignancies (Xi et al., 2012). At present, little is known regarding the functions of EphA10, however, EphB6 is rapidly emerging as an important member of the Eph receptor group, influencing both normal biology and malignant behaviour in cancer cells (Truitt and

Freywald, 2011). Despite the important role that ligand-induced trafficking can play in receptor signalling, nothing is yet known about the downregulation of the kinase dead Eph receptors, EphA10, and EphB6. As EphB6 is known to be important to numerous processes, the mechanism of its ligand-induced downregulation is of particular interest, as this may be pivotal to EphB6 effects. Furthermore, an understanding of EphB6 downregulation will give a better general understanding of the ligand-induced downregulation of kinase-dead RTKs in general, as the current knowledge is limited to but one receptor, and the observations are frequently inconsistent.

1.10. Conclusion

Ligand-induced internalisation is an essential mechanism utilised by cells to attenuate signals generated following ligand activation, acting as a gateway to receptor degradation, and also as a means to target receptors to the correct compartments for their proper interaction with downstream effectors and targets. Despite Eph receptors being the largest known family of RTKs, playing essential roles in both normal and cancer biology, the mechanisms of their ligand-induced downregulation have yet to be fully described, and based upon current knowledge, there appears to be important distinctions between receptors. Of note, the Eph family contains two kinase dead members, EphA10 and EphB6, and of these, EphB6 has been demonstrated to be important to several biological functions, including T-cell and thymus biology, blood pressure regulation, potential roles in pain signaling and osmotic regulation, and also in cancer, where it appears to act as a metastasis suppressor. Ligand-induced downregulation has only been described for one other kinase-deficient receptor, ErbB3, however the observations are inconsistent, and it is unclear if other kinase-deficient receptors will share any commonalities in these

mechanisms. Therefore, knowledge of the ligand-induced downregulation of EphB6 will expand understanding of the mechanisms utilized by kinase-deficient receptors in general for this process. Furthermore, as the pathways and mechanisms utilised by RTKs for their downregulation have been shown to have a profound influence on their overall signalling output, knowledge of the factors involved in this process for EphB6 will provide important insights into how the activity of this receptor is controlled, and as EphB6 signaling is strongly anti-metastatic, could potentially offer opportunities for therapeutic interventions.

CHAPTER 2

HYPOTHESIS AND OBJECTIVES

Hypothesis

1. The only kinase-dead receptor for which ligand-induced downregulation is currently described is ErbB3, and it is internalised and degraded following ligand stimulation. Furthermore, EphB6 is phosphorylated by kinase-active signalling partners in response to ligand stimulation, thus enabling it to interact with downstream signalling effectors, and therefore potentially also with components of the downregulation machinery. Based on all this, I hypothesized that EphB6 is actively downregulated in response to ligand stimulation.

Objectives

1. To determine if EphB6 is actively downregulated in response to ligand-stimulation.

2. If EphB6 is actively downregulated, to explore the mechanisms and pathways used by EphB6 during ligand-induced downregulation, and to compare ligand induced downregulation of kinase-deficient EphB6 to that of its kinase active signaling partner, EphB4. If EphB6 is not actively downregulated, to fully examine the impact it has on the mechanisms and pathways of downregulation utilised by its kinase-active signaling partners.

CHAPTER 3

MATERIALS AND METHODS

3.1 Antibodies

Antibodies for Myc, Erk2, b-Tubulin, EphB4, Clathrin heavy-chain, EphB4, and Eps15 were from Santa Cruz Biotechnology, goat anti-human Fc (anti-hFc) was from Pierce Biotechnology, anti-EphB6 were from Santa Cruz, R&D Systems, and Sigma-Aldrich, anti-Hsp90 was from StressGen, and Goat F(ab')2 Anti-Human IgG (Fc) FITC was from Beckman Coulter. Ephrin-B2-Fc and fluorescein labelled anti-sheep IgG were purchased from R&D Systems. Secondary antibodies linked to IR-dyes for Western blotting (anti-goat, anti-rabbit, anti-rat and anti-mouse) were purchased from Mandel Scientific. Anti-rat Alexafluor 594 was purchased from Cell Signalling.

Table 3.1 LIST OF CHEMICALS AND SUPPLIERS

Chemical Name	Source
Acrylamide	**Fisher**
Agarose	**Fermentas**
Ammonium persulfate	**Sigma**
Bleach	**Lavo**
Bromophenol blue	**Sigma**
DMSO	**Fisher**
EDTA	**Fisher**
Ethanol	**Fisher**
Formaldehyde	**Polysciences**

Chemical Name	Source
Glacial Acetic acid	**EMD**
Glycerol	**Fisher**
Glycine	**Fisher**
HEPES	**Sigma**
Hydrogen chloride	**Fisher**
Igepal	**Sigma**
Isopropyl Alcohol	**Fisher**
LB Agar	**Sigma**
LB Broth	**Sigma**
Methanol	**Sigma**
Phenylmethanesulfonyl fluoride	**Sigma**
Phosphate Buffered Saline	**Fisher**
Ponceau	**Sigma**
Potassium phosphate	**EMD**
Saponin	**Sigma**
Sodium Azide	**Fluka**
Sodium chloride	**Fisher**
Sodium Dodecyl Sulfate	**Sigma**
Sodium hydroxide	**Fisher**
Sodium Orthovanadate	**Fisher**
Sodium phosphate dibasic anhydrous	**Fisher**
Trizma base	**Sigma**

Chemical Name	Source
Triton-X	**Sigma**
Tween	**Sigma**
β-Mercaptoethanol	**Sigma**

3.2 Expression Constructs and shRNA

The wild-type EphB6 receptor and EGFP (enhanced green fluorescent protein)-tagged EphB6 constructs were kindly provided by Dr. C.M. Roifman (Sick Children's Hospital, Toronto, ON). EphB4 and EphB4-EGFP constructs were purchased from OriGene Technologies, Inc. Myc-tagged EphB4 was generated by polymerase chain reaction (PCR) utilising the forward primer 5'-cgcggatcccgccatggagctccgggtgctgctctg-3' and the reverse primer 5'-ccggaattcttattaattcagatcctcttctgagatgagtttttgttcgtactgcggggccggtcctcctg-3'. Myc-tagged EphB6 and an EphB6 mutant, lacking the cytoplasmic domain (ΔEphB6) and a tyrosine to phenylalanine substitution mutant of EphB6 (EphB6 Y->F) were described previously (Freywald et al., 2003; Freywald et al., 2002; Truitt et al., 2010). Clathrin heavy chain (CHC) shRNA (short hairpin ribonucleic acid) lentiviral particles and control non-silencing shRNA were purchased from Santa Cruz. Dr. Anderson from the University of Saskatchewan provided HA-tagged Rab5 S34N. An Eps15 mutant, DIII, as well as a corresponding control peptide, were provided by Dr. Benmerah from the Institut Imagine, Paris, France.

3.3 Cell Culture and Generation of Stable Cell Lines

3.3.1 Electroporation

Stable cell lines of HEK-293 (human embryonic kidney-293) cells [American Type Culture Collection (ATCC)], expressing either Myc-tagged EphB4 (HEK-B4-M), EphB6 (HEK-B6), ΔEphB6 (HEK-ΔEphB6), EphB6(Y->F) (HEK-EphB6(Y->F)), EGFP-tagged EphB4 (HEK-B4-EGFP), or Myc-tagged EphB6 (HEK-B6-M) were generated by electroporation using 20 mg of DNA (70 ms, 140 V, ECM 830 electroporator; Harvard Apparatus Inc.). Cells were allowed to rest overnight and were then subjected to G418 (1mg/ml) (Calbiochem) selection for 30 days. Cells were lysed and screened for Eph receptor expression by Western blotting.

3.3.2 Cell Culture

All HEK-293 based cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, HyClone), 10% fetal bovine serum (FBS, Gibco), and 1 mM sodium pyruvate (SP, HyClone). MDA-MB-231 (ATCC) were cultured as HEK-293 with the addition of 1% Pen Strep (Gibco). Hcc-70 (ATCC) were cultured in Roswell Park Memorial Institute (RPMI)-1640 (HyClone) medium with 10% FBS and 0.5% Pen Strep. Cells were grown to 80-90% confluence prior to re-plating. Cells were kept at 37^{o}C and 5% CO_2 for expansion and during stimulations.

3.3.3 Lentiviral Transducdtion

Stable cell lines with CHC knock-down were generated based on the HEK-B6 or HEK-B4-M cell lines, using CHC shRNA lentiviral particles (Santa Cruz Biotechnology) according to the manufacturer's instructions. Briefly: cells at 50% confluence were

incubated with 10 MOI (multiplicity of infection) of lentiviral particles in the presence of 2 mg/ml Polybrene (Sigma) for 11 h. Cells were grown for an additional 24 h in normal growth media prior to selection with 5 mg/ml puromycin (VWR, Mississauga, ON).

3.3.4 Transient Transfections

Transient transfections of cells were performed using 3.7 ml of Metafectene Pro purchased from BionTex (San Diego, CA), and 3 mg of construct DNA for approximately 1 x 10^6 cells. Cells were incubated at 37^{o}C , 5% CO_2 for 5 h in the presence of the construct, Metafectene Pro and 400 ml OptiMEM (Invitrogen), and then returned to normal growth conditions.

3.4 Cell Stimulations

In all experiments involving stimulation, cells were treated with 1 μg/ml soluble ephrin-B2-Fc precomplexed with 1.5μg/ml anti-human-Fc in serum free media. Precomplexed human IgG (Sigma) was used as a specificity control for the Fc portion of the ephrin-B2-Fc (eB2) fusion protein. In some experiments, cells were preincubated at 37^{o}C with 80 μM dynasore (Santa Cruz) for 1 h (Garcia Lopez et al., 2009), 100 nM chloroquine for 2 h (Litterst et al., 2007), or 25 mM NH_4Cl for 1 h (Jia et al., 2009), as indicated in figure legends. Inhibitor presence was maintained throughout treatment with ephrin-B2. Geldanamycin (10 μM, Santa Cruz) was used with no prior incubation (Whitesell et al., 1994). Matching solvents were used as controls.

3.5 Flow Cytometry

3.5.1 Cell-Surface Expression

To confirm cell surface expression of EphB6 mutants, cells were collected with 2 mM EDTA, washed in serum free media, and incubated with anti-EphB6 or matching IgG control (R&D Systems) for 40 min on ice. Labelled cells were washed twice with serum free media, and incubated with FITC (Fluorescein isothiocyanate) conjugated secondary antibody for 30 min on ice in the dark, washed twice with serum free media, and suspended in PBS (phosphate buffered saline) for analysis.

3.5.2 Ligand Internalisation Assay

For the ligand internalisation assay cells were stimulated for 30 min with 1 μg/ml eB2, washed with acidic PBS (0.2 M Acetic acid, pH 3.0) for 5 min 3 times, collected with 2 mM EDTA (Ethylenediaminetetraacetic acid), and fixed with 1% formaldehyde in PBS, or fixed and permeabilised with 0.1% Triton-X-100 (Sigma). Cells were stained with anti-human IgG (Fc)-FITC.

In all experiments, staining was monitored by flow-cytometry using a Coulter Epics XL or a MACSQuant VYB (Miltenyi Biotec) Flow Cytometer. Results were analysed with the FlowJo software.

3.6 Cell Lysis, Immunoprecipitation and Western Blotting

3.6.1 Cell Lysis

Following stimulation cells were washed once in serum free media and once with PBS to remove excess ligand and ions. Cells were detached from the surface of the plate in the lysis buffer (0.1 M EDTA, 0.3 M Tris, 0.1 M NaCl, 6 mM PMSF

(phenylmethylsulfonyl fluoride) and 3 mM sodium ortho-vanadate), and transferred to a 1.5 ml tube. Following a 20 min incubation period on ice, cell lysates were centrifuged at 9500 x g for 15 min to remove the nuclear fraction and debris. The resulting supernatant was transferred to a fresh 1.5 ml tube and mixed with 2x loading buffer (0.5 M Tris, 0.5% SDS, 0.1% 2-mercaptoethanol).

3.6.2 Immunoprecipitation

For immunoprecipitation, cell lysates were prepared as described above, omitting the addition of the 2x loading buffer. Instead, 20 μl of Protein G Sepharose beads (GE Healthcare) and 3 μg of the required antibody were added, and samples were rotated at 4^{o}C overnight. Beads were washed three times in lysis buffer, and resuspended in 35 μl of 2x loading buffer.

3.6.3 Western Blotting

Samples were resolved by SDS-PAGE (sodium dodecyl sulfate- polyacrylamide gel electrophoresis), and transferred to a nitrocellulose membrane (Amersham) for Western blotting. The membrane was blocked with 7% non-fat dried milk (Bio-Rad; Mississauga, ON) and incubated with gentle agitation in primary antibodies overnight. Membranes were washed 3 times in PBS with 0.1% Tween (Sigma-Aldrich) and incubated with secondary antibodies in PBS with 5% milk and 0.1% Tween, with gentle agitation, for 1 h. Excess secondary antibody was removed by washing with PBS, and images were acquired using the LI-COR Odyssey imaging system. Densitometry analysis was performed using Carestream software (Carestream Health).

3.7 Confocal Microscopy

3.7.1 Live Cell Imaging

HEK-293 cells were plated on glass bottom culture dishes (MatTek) at low density (~10%), and allowed to adhere for 24 h. Seeded cells were transfected with EphB6-EGFP or EphB4-EGFP cDNAs (complementary DNA) and cultured 72 h to facilitate optimal expression of the constructs (Rice et al., 1991). To examine lysosomal colocalisation, transfected cells were stained for 30 min with 60 nM LysoTracker Red DND-99 (Invitrogen) in serum-free DMEM, and washed twice with DMEM prior to ephrin-B2 stimulation. To reduce background fluorescence, cells were imaged in phenol-red free DMEM (Fisher), and media pH was buffered with 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Sigma). Cells were visualized using an Olympus FV1000 confocal microscope, and live cells were imaged at 40X magnification. Experiments took place in a heated imaging chamber maintained at 37^{o}C, with images collected prior to, and every 1 or 2 min for 20 min following the addition of ephrin-B2.

3.7.2 Immunofluorescence Staining

To monitor Hsp90 association, HEK-293 cells were seeded at low density on glass coverslips (Fisher) pre-coated with poly-L-lysine (Trevigen), transfected with EphB6-EGFP or EphB4-EGFP construct, and stimulated 72 h post transfection. Cells were treated with ligand or hIgG for 20 min and fixed in 4% formaldehyde/PBS on ice for 20 min. To facilitate staining and reduce background, fixed cells were permeabilised and blocked for 1h at room temperature in blocking buffer (PBS containing 5% normal horse serum (Sigma), 1% bovine serum albumin (Sigma), and 0.1% saponin (Sigma)), and

incubated with an antibody of interest in blocking buffer for 48-72 h with gentle agitation at 4^{o}C. Incubation for 1 h with matching Alexafluor-conjugated antibodies was used for detection, and excess antibody was removed by washing 3 times with blocking buffer. ProLong Gold antifade reagent with DAPI (4',6-diamidino-2-phenylindole) (Invitrogen) was used as a mounting medium and provided nuclear staining. Cells were visualized using an Olympus FV1000 confocal microscope. Fixed cells were imaged using a 60X oil-immersion lens.

3.7.3 Image Processing

All images have been subjected to deconvolution using Auto-Deblur (AutoQuant X3, Media Cybernetics). Images were processed using the Image J software (McMaster Biophotonics) and Adobe Photoshop CS6 (Adobe Systems Inc.). 3D-reconstruction was performed using the IMARIS 7.4.2 software (Bitplane). Pearson's correlation coefficients were determined using FIJI software (2012).

3.8 Statistical Analysis

All experiments were performed at minimum three times. For experiments involving Western blotting, bands were quantified by densitometry, the average and standard deviation between all replicates was determined. For experiments involving confocal microscopy, colocalisation was assessed using Pearson's correlation coefficient, as this method is widely employed as means to measure the level of colocalisation between two fluorophores (Dunn et al., 2011). Pearson's correlation coefficient was determined using Image J software, and the values from at least 50 randomly selected

cells representing at least three independent experiments were averaged. P-values were calculated using the students t. test in GraphPad Prism software.

CHAPTER 4

RESULTS

4.1. The EphB6 Receptor is Actively Downregulated in Response to Ligand Stimulation

To assess the ligand-induced downregulation of the kinase-deficient EphB6 receptor, we compared it with the downregulation of its kinase-active signalling partner, EphB4 (Truitt et al., 2010). To do this, we generated cell lines with stable expression of EphB6 (HEK-B6) or EphB4 (HEK-B4-M) by transfecting human embryonic kidney cells, HEK-293, a cell line that is commonly used as a model for this type of investigation, and contains all necessary cellular machinery to evaluate downregulation (Cui et al., 2009; Gironacci et al., 2011; Stautz et al., 2012; Thompson and Whistler, 2011; Yancoski et al., 2012). A Myc-tagged version of EphB4 was used in these experiments to overcome a relatively low sensitivity of available anti-EphB4 antibodies at the onset of the study (**Fig. 4.1 A&B**). Cells were stimulated for up to 3 hours with a common ligand of the EphB6 and EphB4 receptors, ephrin-B2. As an ephrin-B2-Fc fusion protein was used for the stimulation, human IgG (hIgG) was applied as a specificity control for the human-Fc portion of this chimera. Following previously reported models related to other Eph receptors, all stimulations were performed at 1 μg/ml of ephrin-B2 (Fasen et al., 2008; Vihanto et al., 2006). Receptor downregulation was monitored by Western blotting. These experiments showed that kinase-dead EphB6 and kinase-active EphB4 are efficiently downregulated, with both receptors being degraded following their activation (**Fig. 4.2 A&B**). EphB6 downregulation was also monitored in MDA-MB-231 breast cancer cells with restored EphB6 expression, MDA-B6-M, which express Myc-tagged EphB6 (described in our previous work (Truitt et al., 2010)), and in a breast cancer cell line, HCC-70, that expresses this receptor endogenously (**Fig. 4.3 A&B**). These results indicate that our initial observations were not restricted to

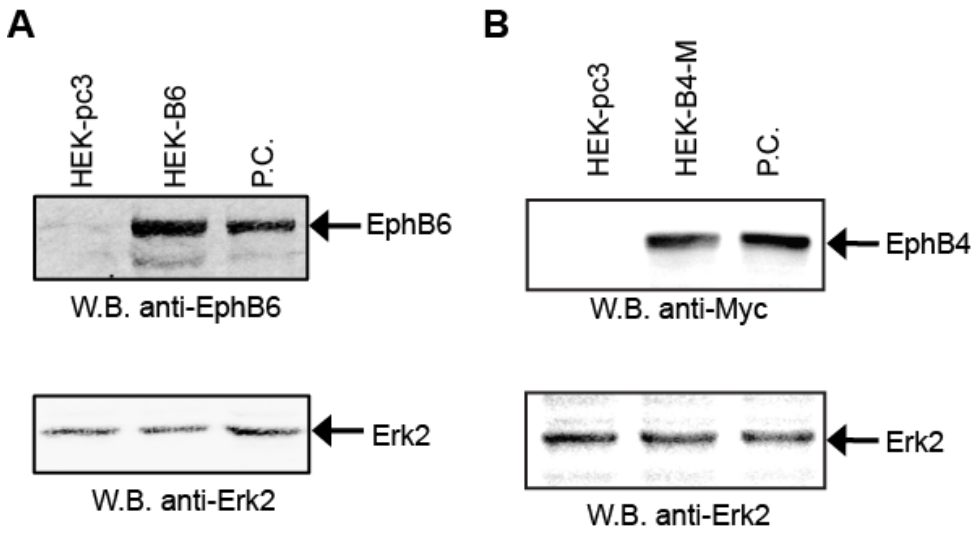

Figure 4.1. Expression of EphB6 and EphB4 in HEK-293 cells.

A and B) Human embryonic kidney cells (HEK-293), were electroporated with the pcDNA3 expression vector encoding EphB6 (HEK-B6) (***A***), or Myc-tagged EphB4 (HEK-B4-M) (***B***). Cells were cultured in G418 (1 mg/ml) selection medium for 30 days and receptor expression was analyzed by Western blotting with anti-EphB6 or anti-Myc. Cells mock-transfected with the empty pcDNA3 expression vector (HEK-pc3) were used as a specificity control. HEK-293 cells transiently transfected with EphB6 or Myc-tagged EphB4 cDNAs were used as a positive control (P.C.). Western blotting of different slices of the same membranes with anti-Erk2 was used as loading control.

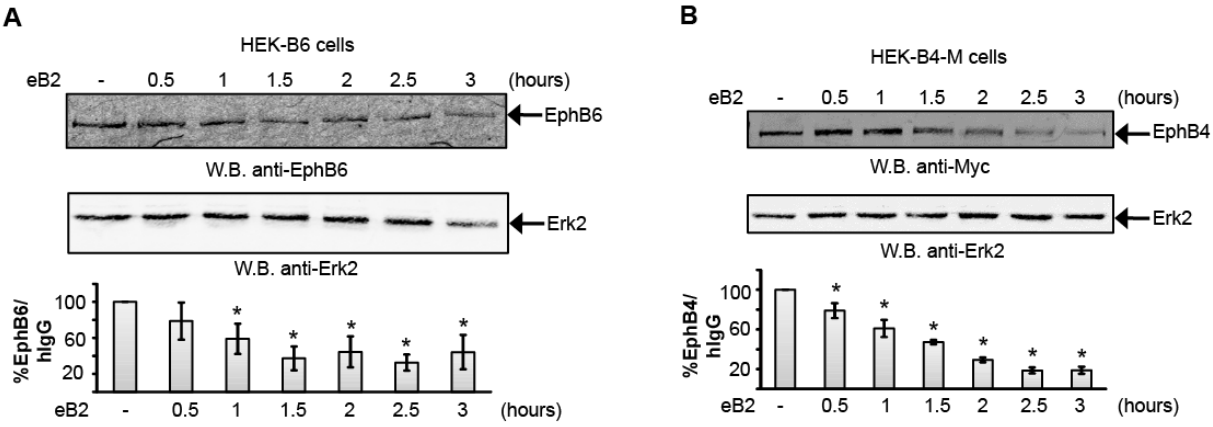

Figure 4.2. EphB6 and EphB4 are downregulated in response to ligand stimulation.
A) HEK-B6 cells were stimulated for the indicated time periods at 37^{o}C with 1 μg/ml of ephrin-B2-Fc (eB2). EphB6 downregulation was assessed by Western blotting with anti-EphB6 and gel loading was monitored by Western blotting with anti-Erk2. In all receptor downregulation experiments, unstimulated cells (-) were treated with human IgG (hIgG) for time periods matching the longest eB2 stimulation, as a specificity control for the Fc portion of the eB2 fusion protein. The results of Western blotting were quantitated by densitometry. EphB6 quantifications were normalized on matching Erk2 loading controls and plotted as a percentage relative to the unstimulated control (%EphB6/hIgG). **B**) HEK-B4-M cells were treated as in (***A***). EphB4 presence was monitored by Western blotting with anti-Myc and receptor downregulation was assessed and presented as in (***A***). All Western blot panels in this figure represent one of at least three independent experiments. Each graph summarises the analysis of at least three independent experiments, bars, SD. *, $P < 0.05$, Student's t test, for indicated points and corresponding controls as shown.

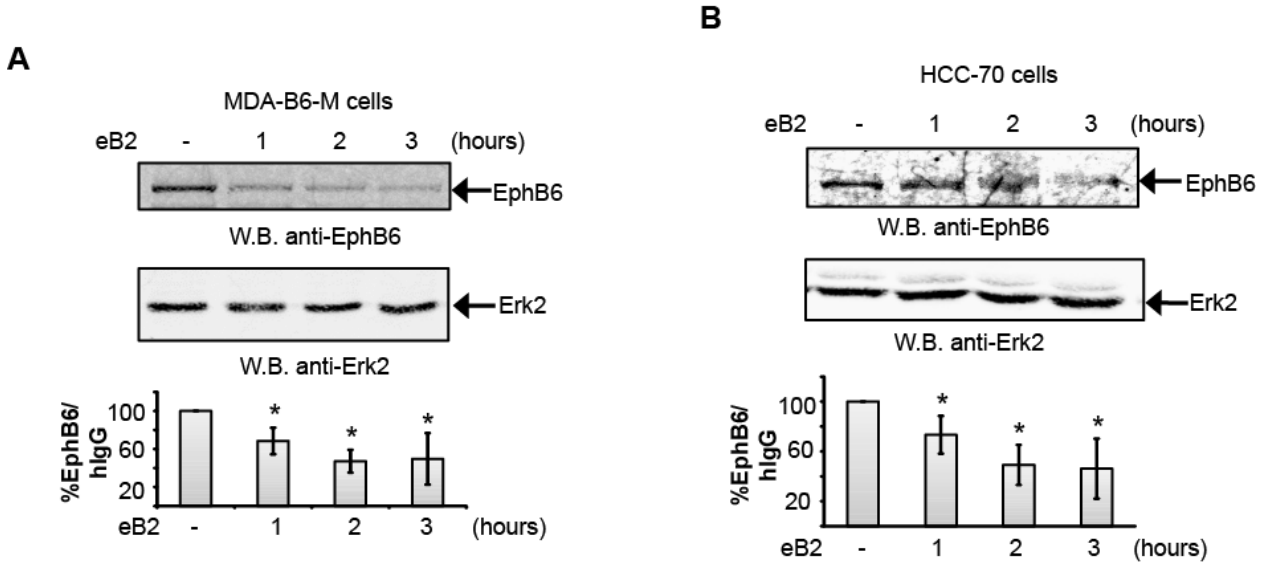

Figure 4.3. EphB6 is actively downregulated in response to ligand stimulation in breast cancer cells.

A) MDA-MB-231 cells with induced EphB6 expression (MDA-B6-M) (Truitt et al., 2010) were stimulated with eB2 and EphB6 downregulation was monitored and assessed as in (***Fig. 4.2 A***). **B**) HCC-70 breast cancer cells were treated with eB2 and EphB6 downregulation was monitored as in (***Fig. 4.2 A***). All Western blot panels represent one of at least three independent experiments. Each graph in this figure summarizes the analysis of three independent experiments, bars, SD. *, $P < 0.05$, Student's t test, for indicated points and corresponding controls. In all downregulation experiments, unstimulated cells were treated with hIgG as a specificity control.

HEK-293, and are relevant to the mechanism acting in other cell types, including human breast cancer cells.

One of the means by which the ErbB2 receptor imparts its biological effects is through a negative impact on the downregulation of its signalling partners (Haslekas et al., 2005; Lenferink et al., 1998; Wang et al., 1999; Worthylake et al., 1999). To determine if EphB6 also relies on this mode of action, we examined if EphB6 modulates EphB4 downregulation, as this receptor specifically has been observed to show altered responses in the presence of EphB6 (Truitt et al., 2010). EphB6 was transiently co-expressed in HEK-B4-M cells and ephrin-B2-induced EphB4 downregulation was monitored (**Fig. 4.4**) However, no consistent inhibition of EphB4 removal was observed, suggesting that EphB6 does not act by reducing the efficiency of downregulation of its signalling partners. Interestingly, we consistently could not observe downregulation of the EphB6 receptor in these experiments, most likely due to the extremely high level of continuous EphB6 production in the transient expression model. Overall, these data demonstrate that the kinase-dead EphB6 receptor is efficiently downregulated in response to ligand stimulation in multiple cell types, and indicate that it is unlikely to function by suppressing downregulation of its kinase-active relatives.

4.2. EphB6 Receptor Downregulation Depends on the Functional Properties of its Cytoplasmic Domain

As EphB4 acts as EphB6 partner and assures initiation of ligand-induced cytoplasmic signalling by this kinase-dead receptor (Truitt et al., 2010), we transiently transfected HEK-B6 cells with EphB4-encoding cDNA to examine if EphB4 presence would increase the rate of EphB6 downregulation. Surprisingly, no enhancement in ligand-initiated EphB6 elimination was observed in HEK-B6 cells co-expressing the EphB4 receptor (**Fig. 4.5)**.

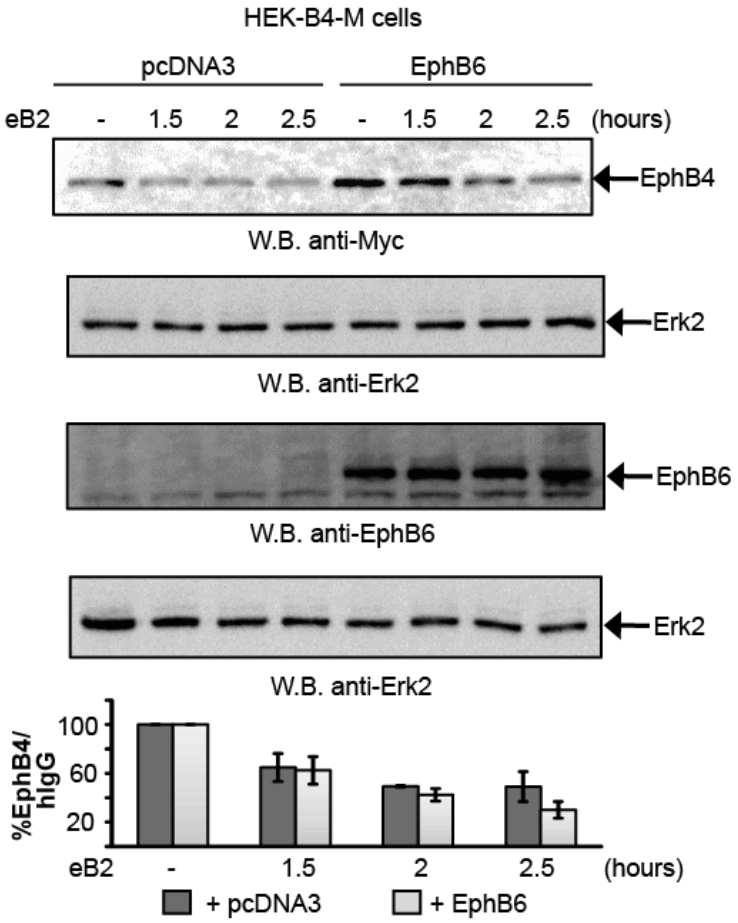

Figure 4.4. EphB6 does not alter the rate of EphB4 downregulation.
HEK-B4-M cells were transiently transfected with EphB6 or mock-transfected with the pcDNA3 expression vector and EphB4 downregulation was examined as in (***Fig. 4.2 A***). EphB6 expression was confirmed by Western blotting with anti-EphB6. The Western blot panels represent one of three independent experiments. The graph in this figure summarises the analysis of three independent experiments, bars, SD. *, $P < 0.05$, Student's t test, for indicated points and corresponding controls. Statistical analysis revealed no significant difference in the rates of eB2-induced EphB4 removal between pcDNA3- or EphB6- transfected cells at any time point monitored. Unstimulated cells were treated with hIgG as a specificity control.

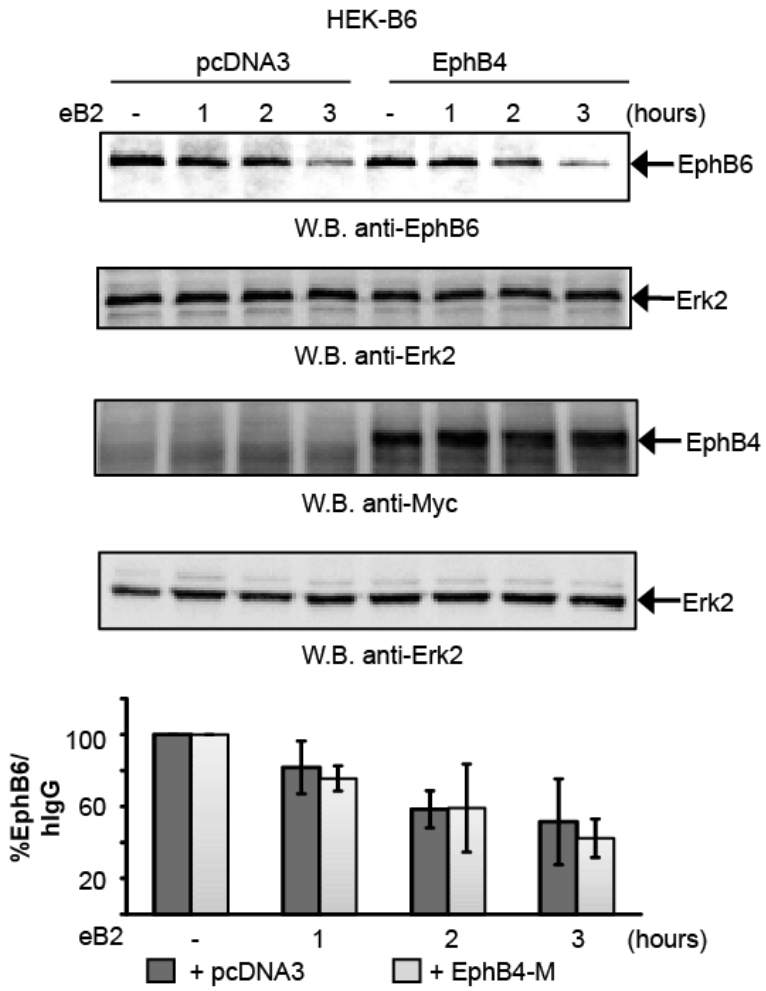

Figure 4.5. EphB4 presence does not alter the rate of EphB6 removal in response to ligand stimulation.

HEK-B6 cells were transfected with EphB4-encoding pcDNA3 or mock-transfected as indicated and EphB6 downregulation was analyzed as in (***Fig. 4.2 A***). EphB4 expression was confirmed with anti-EphB4. All Western blot panels in this figure represent one of three independent experiments. The graph summarises the analysis of three independent experiments, bars, SD. *, $P < 0.05$, Student's t test, for indicated points and corresponding controls as shown. Statistical analysis revealed no significant difference in the rates of eB2-induced EphB6 removal between pcDNA3- or EphB4- transfected cells at any time point monitored. Unstimulated cells were treated with hIgG as a specificity control.

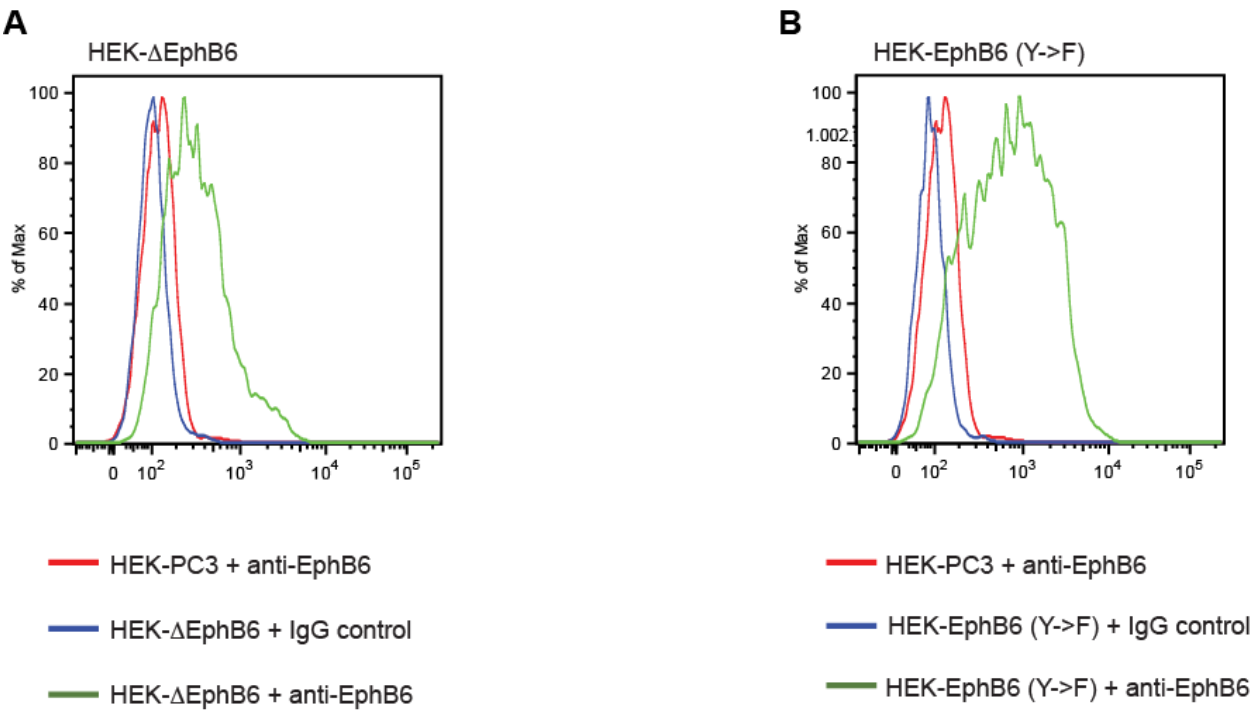

Figure 4.6. EphB6 mutants, ΔEphB6 and EphB6 (Y->F) are expressed on the cell surface. **A and B**) HEK-293 cells with stable expression of an EphB6 mutant lacking the cytoplasmic domain (***A***) (HEK-ΔEphB6) or a tyrosine-deficient EphB6 mutant with all cytoplasmic tyrosine residues mutated to phenylalanine (***B***) (HEK-EphB6 (Y->F)) were generated as in (***Fig. 4.1 A***). HEK-ΔEphB6 and HEK-EphB6 (Y->F) cells were stained with anti-EphB6 and FITC-conjugated secondary antibodies. Staining with a matching IgG control and staining of mock-transfected HEK-pc3 cells were used as specificity controls in each experiment. Cell debris were gated out and cell staining was analysed by flow cytometry. Panels represent one of three independent experiments.

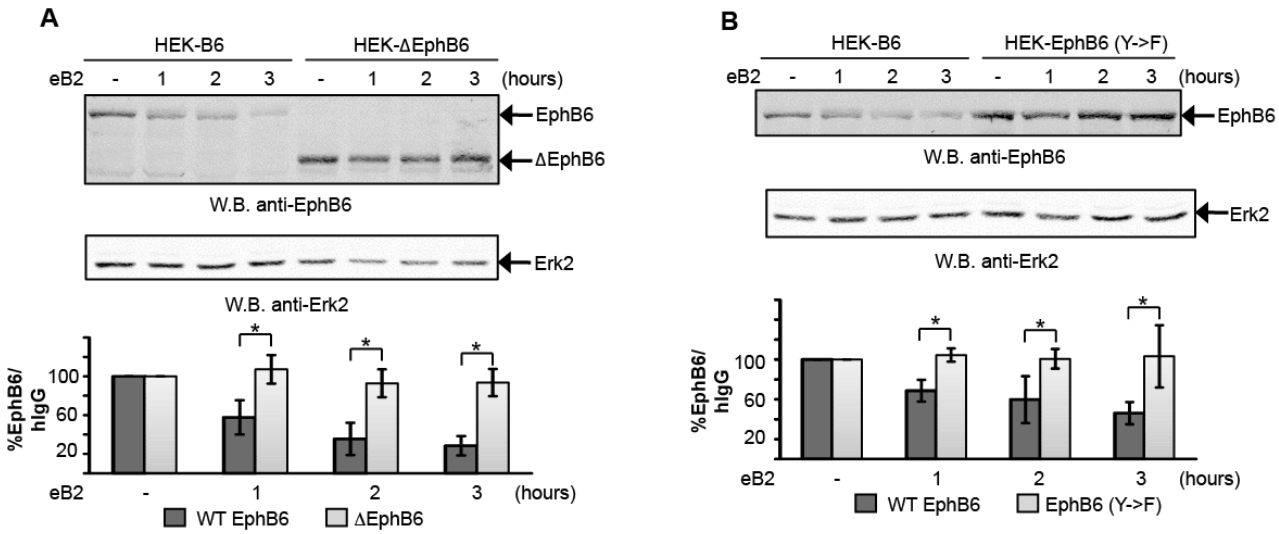

Figure 4.7. EphB6 downregulation depends on its functional cytoplasmic domain.
A) HEK-B6 and HEK-ΔEphB6 cells were stimulated with eB2 for the indicated time periods and receptor downregulation was monitored and assessed as in (***Fig. 4.2 A***). **B**) HEK-EphB6 (Y->F) downregulation was analyzed as in (***Fig. 4.2 A***). HEK-B6 cells were used as a control. Each graph summarises the analysis of three independent experiments, bars, SD. *, $P < 0.05$, Student's t test, for indicated points and corresponding controls, as shown. All Western blot panels in this figure represent one of three independent experiments. In all downregulation experiments, unstimulated cells were treated with hIgG as a specificity control.

This observation prompted us to examine the necessity of EphB6 interactions with cytoplasmic signalling molecules for its downregulation. To exclude the possibility that ligand-induced complexing of the EphB6 receptor on the cell membrane could be sufficient to trigger its removal, we took advantage of a previously described cytoplasmic domain deletion mutant of EphB6, ΔEphB6 (Truitt et al., 2010), which completely lacks the cytoplasmic portion. Although our flow cytometry analysis revealed that ΔEphB6 is successfully delivered to the cell membrane (**Fig. 4.6**), this mutation completely abolished EphB6 downregulation (**Fig. 4.7 A**), indicating that interactions with cytoplasmic molecules are likely to play a crucial role in EphB6 ligand-induced elimination. This observation is conceptually interesting, as a cytoplasmic domain deletion mutant of the kinase-dead ErbB3 receptor has been shown to undergo ligand-induced internalisation (Waterman et al., 1998).

To further clarify the importance of the fully-functional cytoplasmic domain for EphB6 downregulation, a previously generated EphB6 mutant with all tyrosine residues in its cytoplasmic domain substituted for phenylalanines (EphB6 Y->F) (Truitt et al., 2010), was expressed in HEK-293 cells (**Fig. 4.6**). Interestingly, no downregulation of this mutant was observed (**Fig. 4.7 B**), confirming that intact cytoplasmic signaling motifs are required for EphB6 downregulation.

4.3. EphB6 and EphB4 Downregulation is Preceded by Their Internalisation Through Clathrin-Coated Pits

To further follow the route leading to EphB6 and EphB4 downregulation, we examined if EphB6 and EphB4 are internalised prior to their degradation. HEK-B6 or HEK-B4-M cells were treated with ephrin-B2-Fc, washed with acidic PBS to remove receptor-bound ligand from the

cell surface (as previously described (Olwill et al., 2013)), fixed with formaldehyde, or fixed and permeabilised with Triton-X-100. To detect ligand co-internalized with the receptors, cells were stained with anti-Fc-FITC and analyzed by flow cytometry. A stronger staining was consistently observed in permeabilised cells, indicating that receptor-ligand complexes were efficiently internalised (**Fig. 4.8 A, B &C**). No internalisation-related staining could be observed in control mock-transfected cells (HEK-pc3), confirming that the observed response was specific to EphB6 and EphB4 receptors.

The final step of internalisation culminates in pinching off the endocytic vesicle from the cell membrane, and this step is frequently controlled by a large GTPase, dynamin (Ferguson and De Camilli, 2012). The requirement for dynamin action for EphB6 and EphB4 internalisation was assessed by using a dynamin inhibitor, dynasore, that blocks the GTPase activity of dynamin, that plays a crucial role in membrane scission (Macia et al., 2006; Thompson and McNiven, 2006). Indeed, degradation of both EphB6 and EphB4 was reduced when they were stimulated with ephrin-B2 in the presence of dynasore (**Fig. 4.9 A&B**), further confirming the importance of internalisation for the downregulation of these receptors. In some experiments, increased EphB6 receptor levels could be observed in the presence of dynasore, indicating that dynamin is also involved in mediating basal, ligand-independent EphB6 degradation.

In response to ligand stimulation, RTKs are often internalised through clathrin-coated pits (CCPs), structures formed on the inner surface of the cell membrane by a complex of proteins, including clathrin (Doherty and McMahon, 2009). However some Eph receptors, including EphB1 and EphA2, have been shown to interact with caveolin-1, a protein associated with another endocytic membrane structure, caveolae (Sainz-Jaspeado et al., 2013; Vihanto et al., 2006) . To examine if EphB6 and EphB4 preferentially use the CCP-mediated gateway, we took

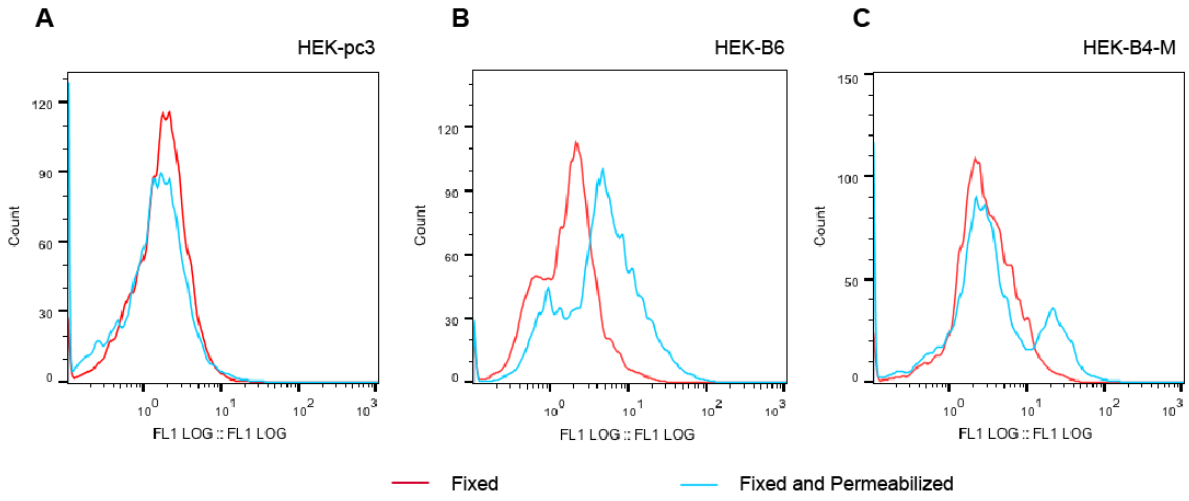

Figure 4.8. EphB6 and EphB4 are internalised in response to ligand stimulation.
A) HEK-pc3, **B**)HEK-B6 or **C**) HEK-B4-M cells were stimulated for 30 min with 1 μg/ml of eB2, washed with acidic PBS (pH 3.0) for 5 min 3 times, collected with 2 mM EDTA, and fixed with 1% formaldehyde in PBS (red) or fixed and permeabilized with 0.1% Triton-X-100 (blue). Cells were stained with FITC-labelled anti-human Fc antibody, and the ligand-receptor complex was detected by flow cytometry. Results were analyzed with the FlowJo software. All panels in this figure represent one of at least three independent experiments.

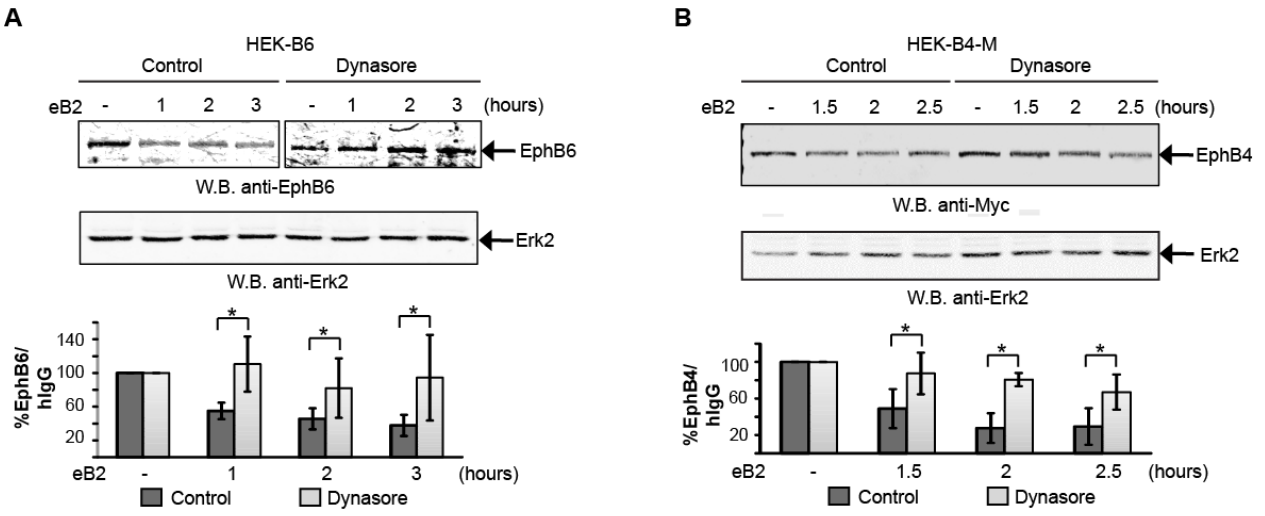

Figure 4.9. EphB6 and EphB4 ligand-induced downregulation depends on dynamin-activity.

A) HEK-B6 cells were pre-treated with 80 μM of a dynamin inhibitor, dynasore, or a matching volume of DMSO as a solvent control, for 1 h and stimulated with eB2 for the indicated time periods. EphB6 downregulation was assessed and presented as in (***Fig. 4.2 A***). **B**) HEK-B4-M cells were treated as in (***A***) and EphB4 downregulation was monitored as in (***Fig. 4.2 A***). All Western blot panels in this figure represent one of at least three independent experiments. Each graph summarises the analysis of at least three independent experiments, bars, SD. *, $P < 0.05$, Student's t test, for indicated points and corresponding controls, as shown. In all downregulation experiments, unstimulated cells were treated with hIgG as a specificity control.

advantage of a previously described dominant-negative mutant of the Eps15 adaptor protein, DIII, (Benmerah et al., 1998), as Eps15 is actively involved in CCP-dependent endocytosis (Benmerah et al., 1998; Benmerah et al., 2000; Parachoniak and Park, 2009). This mutant lacks all Eps homology (EH) domains that target it to CCPs and represents only the DIII domain responsible for binding to the clathrin adaptor protein, AP-2. DIII prevents wild-type Eps15 from interacting with AP-2, which is required for receptor internalisation through CCPs, and has been shown to block CCP formation (Benmerah et al., 1998; Benmerah et al., 2000). In agreement, the stimulation-initiated downregulation was strongly reduced for both EphB4 and EphB6, when this mutant was expressed (**Fig. 4.10 A-D**), strongly implicating CCP in the internalisation and subsequent downregulation of these receptors. In these experiments, the extent of DIII effects matched well the efficiency of its action reported by other groups (Benmerah et al., 1999). To further verify the role of CCP-mediated internalisation in EphB4 and EphB6 downregulation, we partially silenced the expression of the clathrin heavy chain (CHC) in HEK-B6 and HEK-B4-M cells with CHC-targeting shRNA, and as expected, this decrease in CHC expression greatly reduced ligand-triggered receptor removal (**Fig. 4.11 A-D**). Overall, these data imply that both EphB4 and EphB6 are internalized following ligand treatment, and rely on CCP-dependent internalisation for their downregulation.

4.4. Both EphB6 and EphB4 are Degraded in the Lysosomal Compartment

Following activation and internalisation, a significant portion of receptor molecules are destined for degradation, which is often performed in the lysosomal compartment (Clague and Urbe, 2010). We monitored EphB6 and EphB4 ligand-induced re-localisation into these organelles in

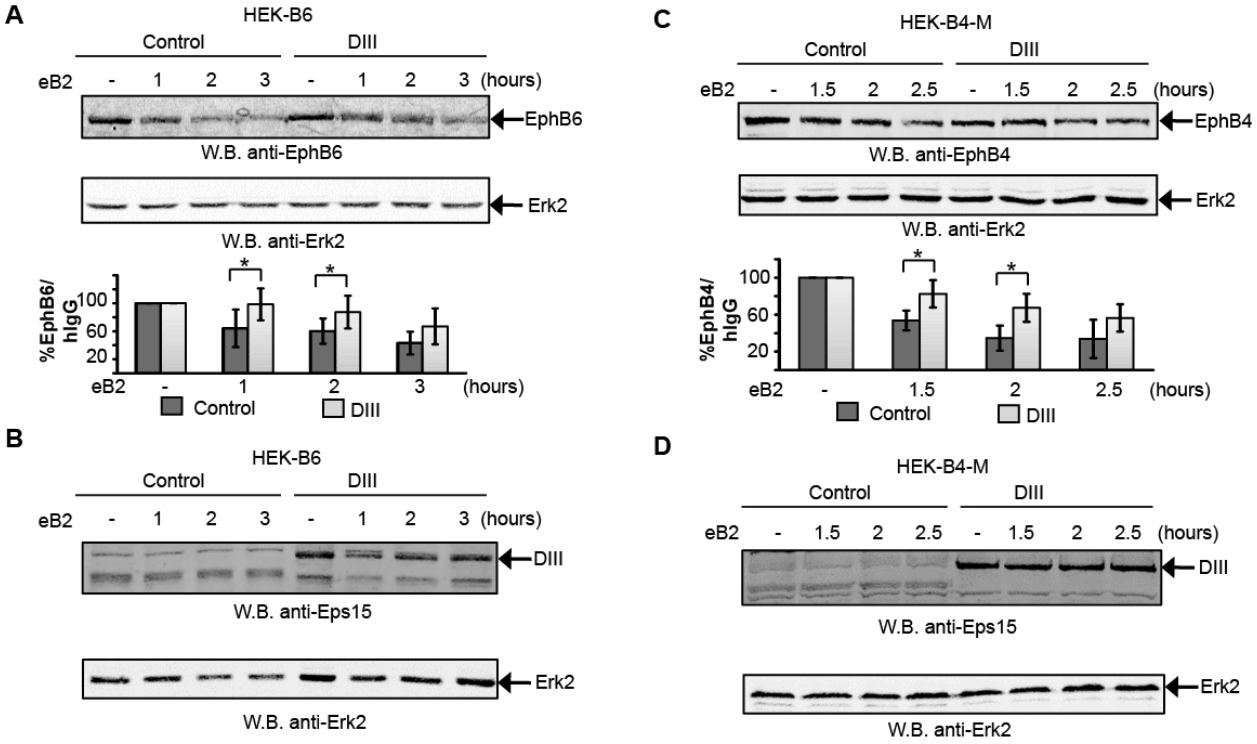

Figure 4.10. A dominant-negative mutant of Eps15 impairs EphB6 and EphB4 downregulation.

A) HEK-B6 cells were transfected with a dominant-negative mutant of Eps15 (DIII) or an irrelevant cDNA as a control and stimulated for the indicated time periods with eB2. EphB6 downregulation was monitored and assessed as in (***Fig. 4.2 A***). **B**) DIII expression in samples from (***A***) was examined by Western blotting with anti-Eps15; Western blotting with anti-Erk2 was used as a loading control. **C**) HEK-B4-M cells were transfected and treated as in (***A***), and EphB4 downregulation was analyzed and presented as in (***Fig. 4.2 A***). **D**) DIII expression in samples from panel (***C***) was assessed as in (***B***). Each graph summarises the analysis of at least three independent experiments, bars, SD. *, $P < 0.05$, Student's t test, for indicated points and corresponding controls as shown. In all downregulation experiments, unstimulated cells were treated with hIgG as a specificity control.

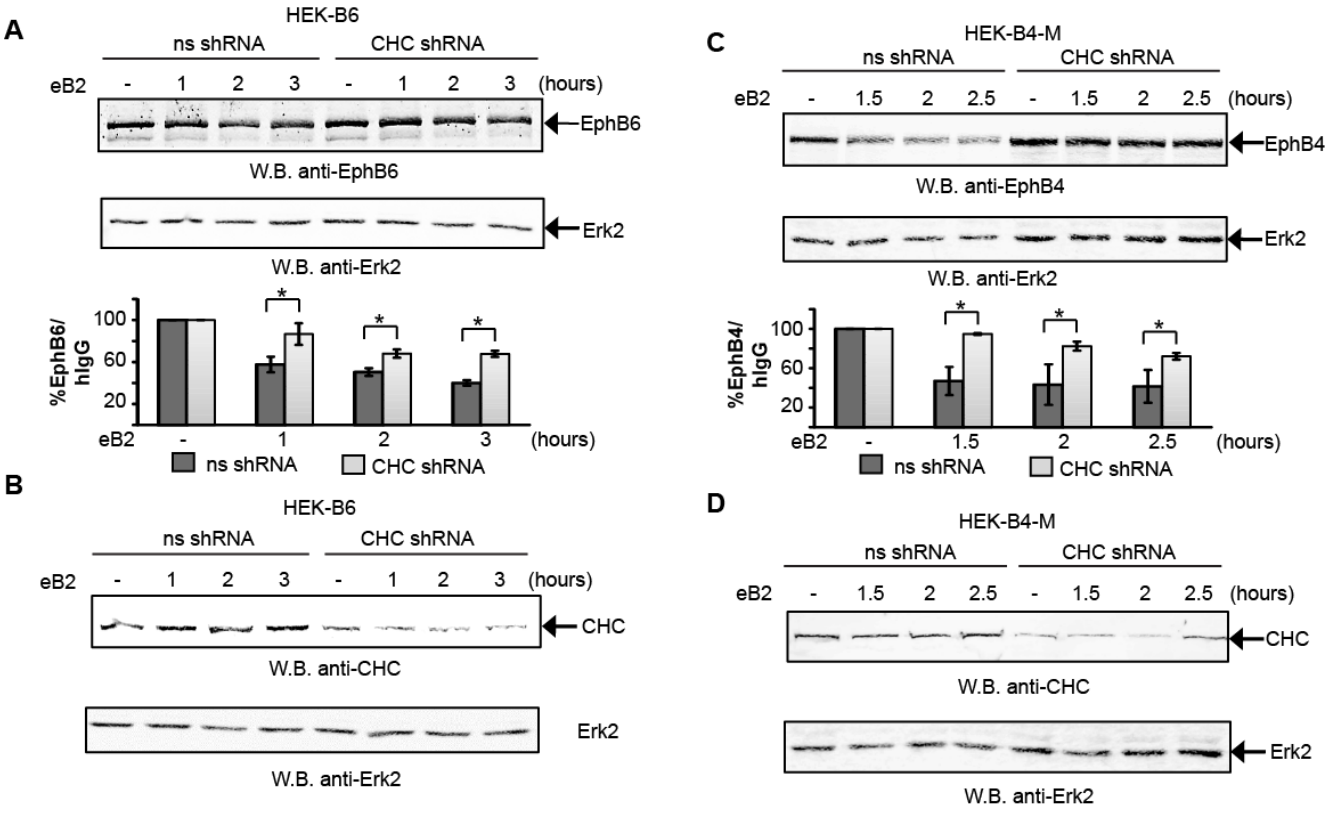

Figure 4.11. EphB6 and EphB4 downregulation is clathrin-dependent.

A) HEK-B6 cells were transduced with shRNA targeting clathrin heavy-chain (CHC shRNA), or non-silencing shRNA (ns shRNA) in the presence of 5 μg/ml of Polybrene overnight, and placed on puromycin selection (2 μg/ml) for 3 days. Selected cells were stimulated with eB2 for the indicated time periods and EphB6 downregulation was examined as in (***Fig. 4.2 A***). **B**) CHC expression in samples from panel (***A***) was monitored with anti-CHC. **C**) HEK-B4-M cells were transfected and treated as in (***A***) and EphB4 downregulation was assessed as in (***Fig. 4.2 A***). **D**) CHC levels for panel (***C***) were monitored as in (***B***). All Western blot panels in this figure represent one of at least three independent experiments. Each graph summarises the analysis of at least three independent experiments, bars, SD. *, $P < 0.05$, Student's t test, for indicated points and corresponding controls as shown. In all downregulation experiments, unstimulated cells were treated with hIgG as a specificity control.

cells stained with the lysosomal-specific dye, LysoTracker Red (Chazotte, 2011) (**Fig. 4.12 A&B, Fig. 4.13 & 4.14**). Interestingly, a strong increase in the localisation of EphB6 and EphB4 in lysosomes was observed following ephrin-B2 treatment, suggesting that ligand-induced receptor degradation likely happened in the lysosomal compartment. To assess this possibility, HEK-B6 and HEK-B4-M were treated with the lysosomal inhibitors, chloroquine and ammonium chloride (Amenta and Brocher, 1980; Poole et al., 1977). These chemicals prevent acidification of the lysosomes, and therefore, interfere with proper functioning of enzymes contained there (Wibo and Poole, 1974). The ligand-induced degradation of both EphB6 and EphB4 was strongly suppressed by these inhibitors, thereby confirming a central role for the lysosomal compartment in their downregulation (**Fig. 4.15 A-D**).

Trafficking of internalised receptors from the plasma membrane to lysosomes is in part, mediated by early endosomes that are actively regulated by the Rab5 GTPase (Huotari and Helenius, 2011). In agreement, EphB6 and EphB4 downregulation was greatly impaired in the presence of a Rab5 dominant-negative mutant, Rab5 S34N (Volpicelli et al., 2001) (**Fig. 4.16 A&B**), suggesting that the endosomal route is involved in targeting EphB6 and EphB4 for degradation.

In sum, our observations show that the kinase-deficient EphB6, and kinase-active EphB4 receptors are efficiently downregulated in response to ligand stimulation. Both receptors follow the same pathway in their ligand-induced internalisation, trafficking and degradation, relying on CCPs for internalisation, dynamin for membrane scission and on Rab5 for cytoplasmic trafficking, whereas their degradation is carried out in the lysosomal compartment.

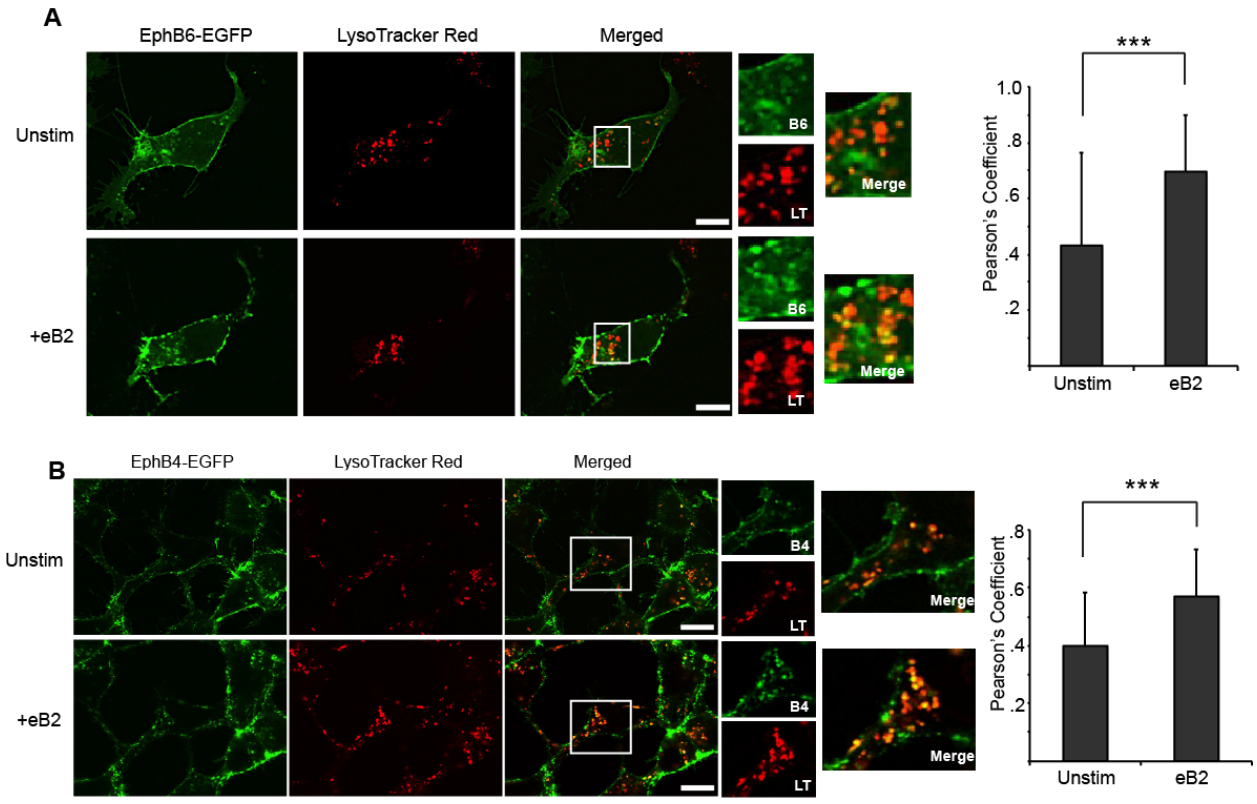

Figure 4.12. In response to ligand stimulation, EphB6 and EphB4 colocalise with the lysosomal compartment.

A) HEK-293 cells were seeded onto glass-bottomed plates, transfected with cDNA encoding an EphB6-EGFP fusion protein, and cultured for 72 h. Cells were stained with 60 nM LysoTracker Red DND99 for 30 min, washed twice with phenol red-free media, and stimulated at 37^{o}C for the indicated time periods. Cells were visualized at 40X magnification with an Olympus FV 1000 confocal microscope prior to, and at each minute following the addition of eB2 (1 μg/ml) for a total of 20 min. Images were deconvoluted using AutoQuant X3. Scale bar is equal to 10 μm. **B**) HEK-293 cells expressing EphB4-EGFP (HEK-B4-EGFP) were seeded, stained, treated, and visualized as in (***A***). In both A and B, graphs represent quantifications of colocalisation of LysoTracker Red with EphB6 or EphB4 using Pearson's correlation coefficient. Pearson's coefficient was determined using FIJI software, and values shown represent values obtained from at least 50 cells selected from 3 independent experiments for each receptor. ***, $P < 2 \times 10^{-6}$.

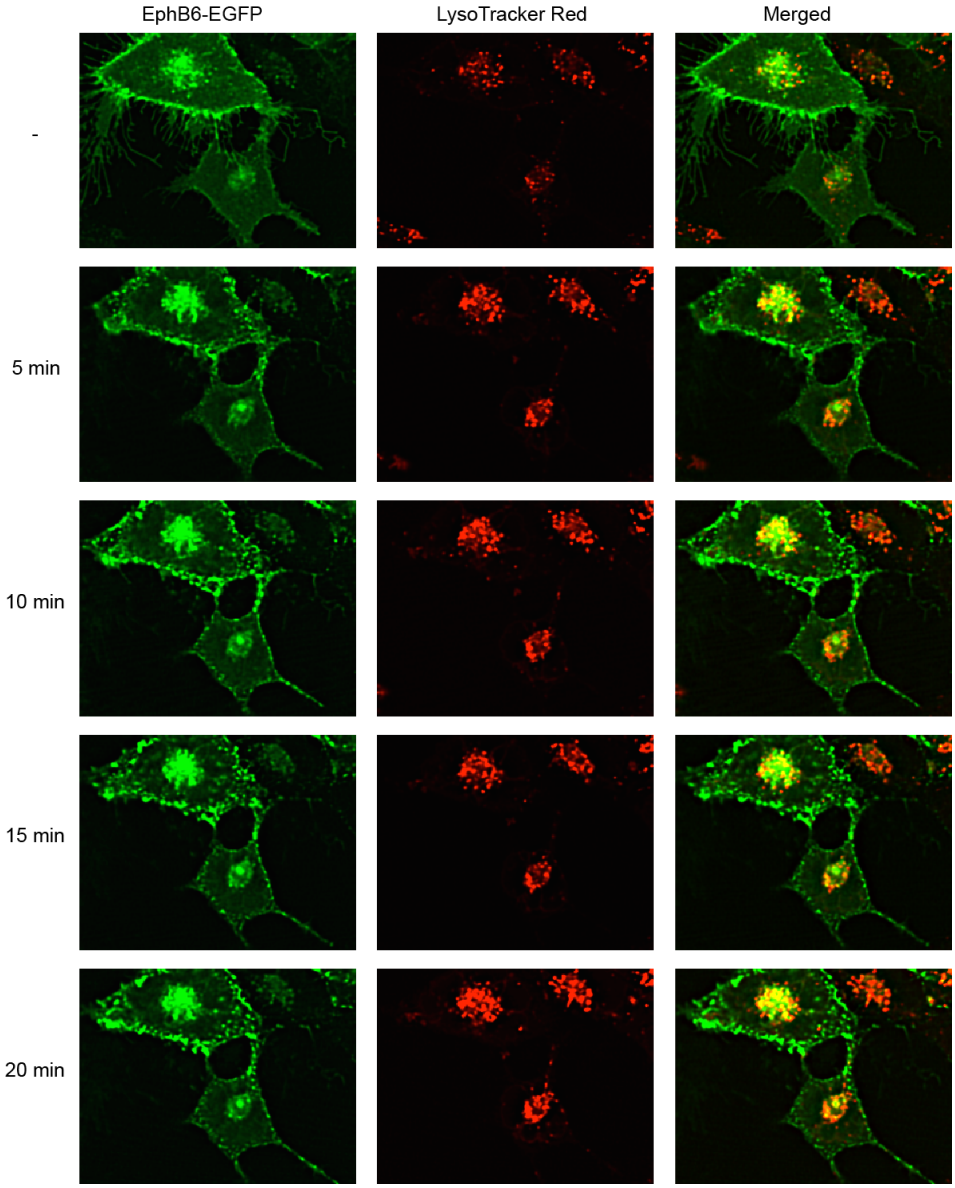

Figure 4.13. EphB6 colocalises with the lysosomal compartment following ephrin-B2 treatment.

HEK-293 cells were seeded onto glass-bottomed plates, transfected with cDNA encoding an EphB6-EGFP fusion protein, and cultured for 72 h. Cells were stained with 60 nM LysoTracker Red DND99 for 30 min, washed twice with phenol red-free media, and stimulated at 37^{o}C for the indicated time periods. Cells were visualized at 40X magnification with an Olympus FV 1000 confocal microscope prior to, and at each minute following the addition of eB2 (1mg/ml) for a total of 20 min. Images were deconvoluted using AutoQuant X3. Images represent one of three independent experiments.

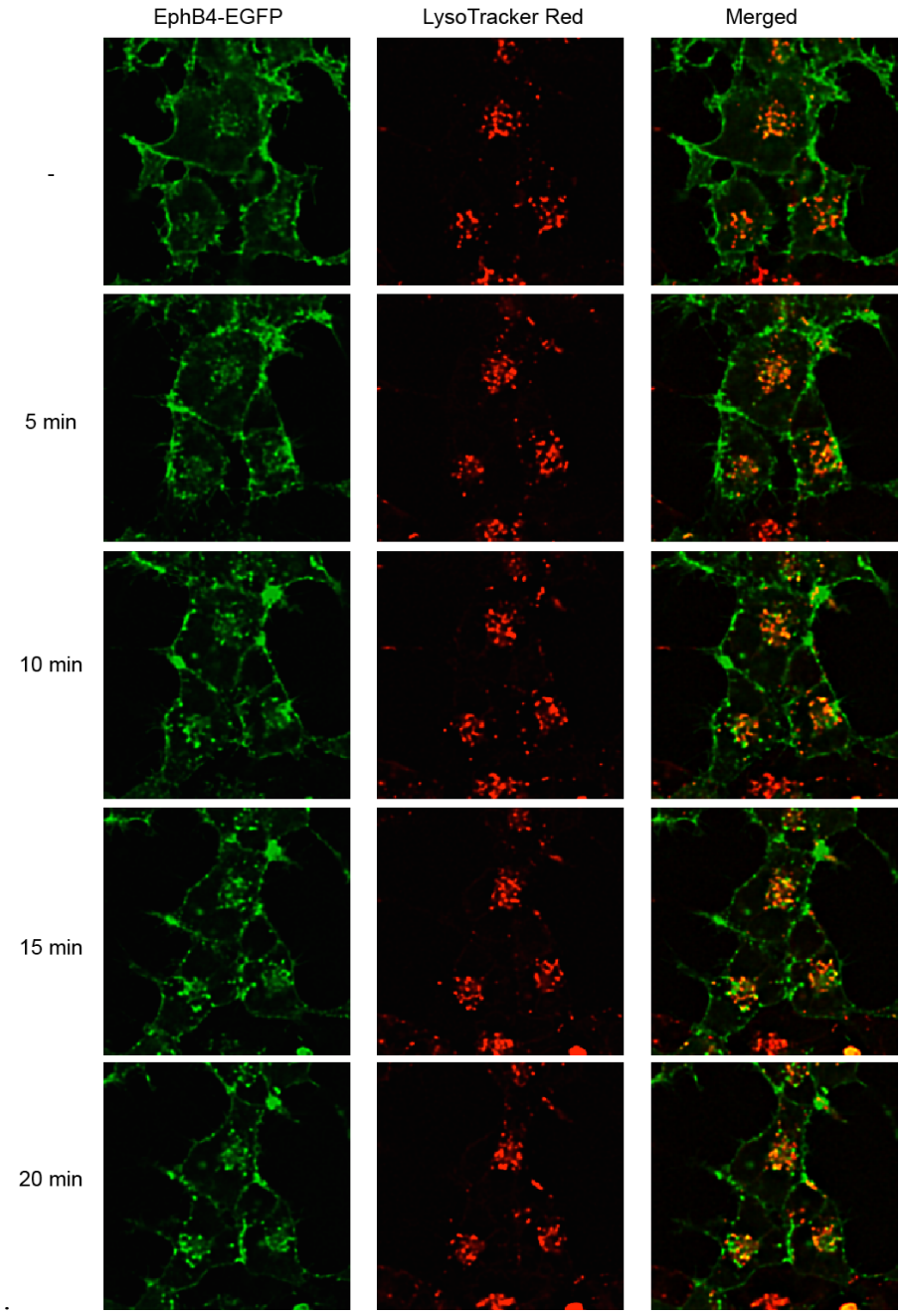

Figure 4.14. Ephrin-B2 promotes EphB4 colocalisation with the lysosomal compartment. HEK-B4-EGFP cells were seeded onto glass-bottomed plates, and cultured for 72 h. Cells were stained with 60 nM LysoTracker Red DND99 for 30 min, washed twice with phenol red-free media, and stimulated at 37^{o}C for the indicated time periods. Cells were treated and visualized as in (***Fig 4.12 A***). Images were deconvoluted using AutoQuant X3. Images represent one of three independent experiments.

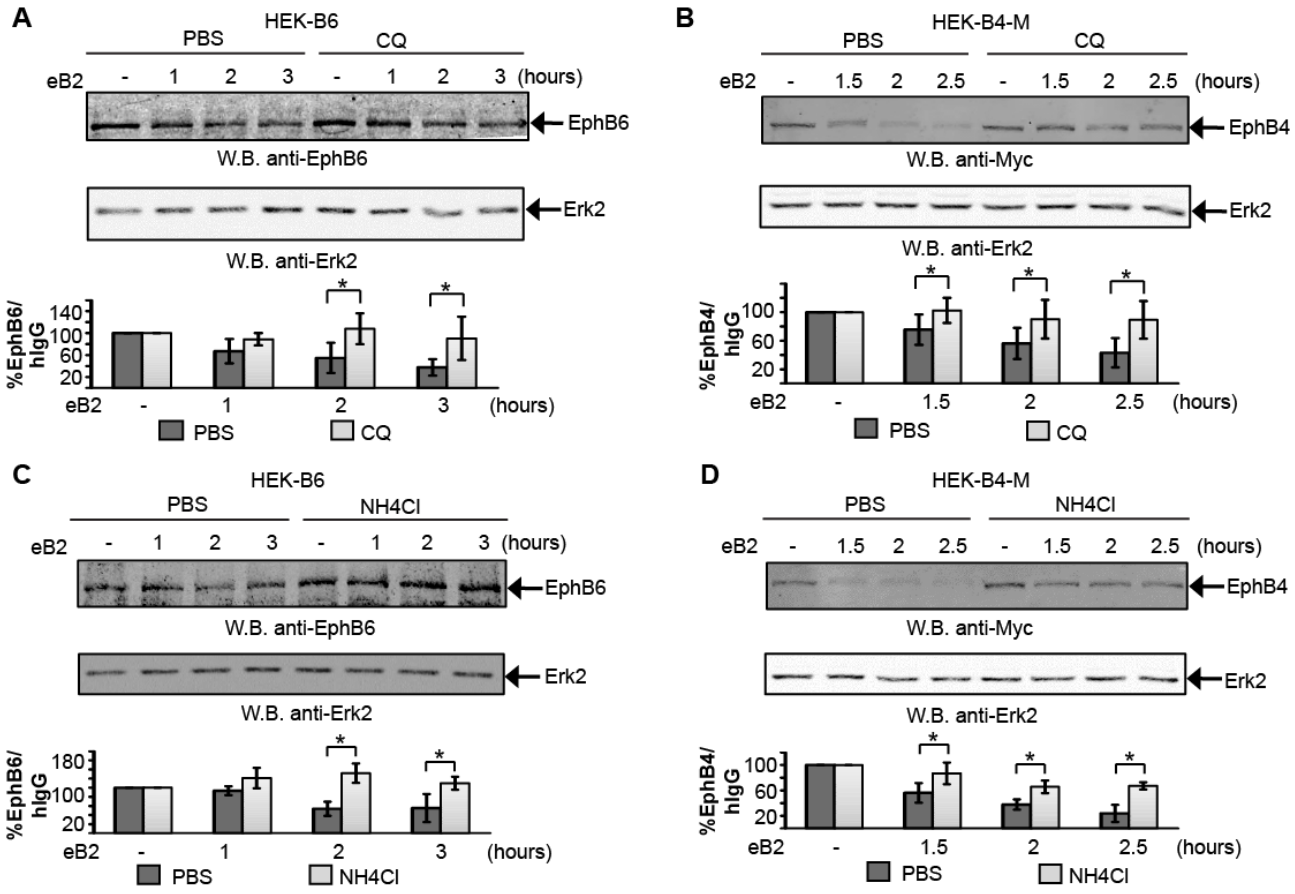

Figure 4.15. EphB6 and EphB4 are degraded in the lysosomal compartment in response to ligand stimulation.

A) HEK-B6 cells were pre-treated with 100 nM of a lysosome inhibitor, chloroquine (CQ), for 2 h and stimulated with eB2 for the indicated time periods. Receptor degradation was analyzed and presented as in (***Fig. 4.2 A***). **B**) HEK-B4-M cells were treated as in (**A**), and EphB4 degradation was monitored as in (***Fig. 4.2 A***). **C**) HEK-B6 cells were pre-treated with 25 mM of NH_4Cl for 1 h and stimulated with eB2. EphB6 degradation was monitored and presented as in (***Fig. 4.2 A***). **D**) HEK-B4-M cells were treated as in (**C**) and EphB4 degradation assessed as in (***Fig. 4.2 A***). All Western blot images in this figure represent one of at least three independent experiments. Each graph summarises the analysis of at least three independent experiments, bars, SD. *, $P < 0.05$, Student's t test, for indicated points and corresponding controls, as shown. In all downregulation experiments, unstimulated cells were treated with hIgG as a specificity control.

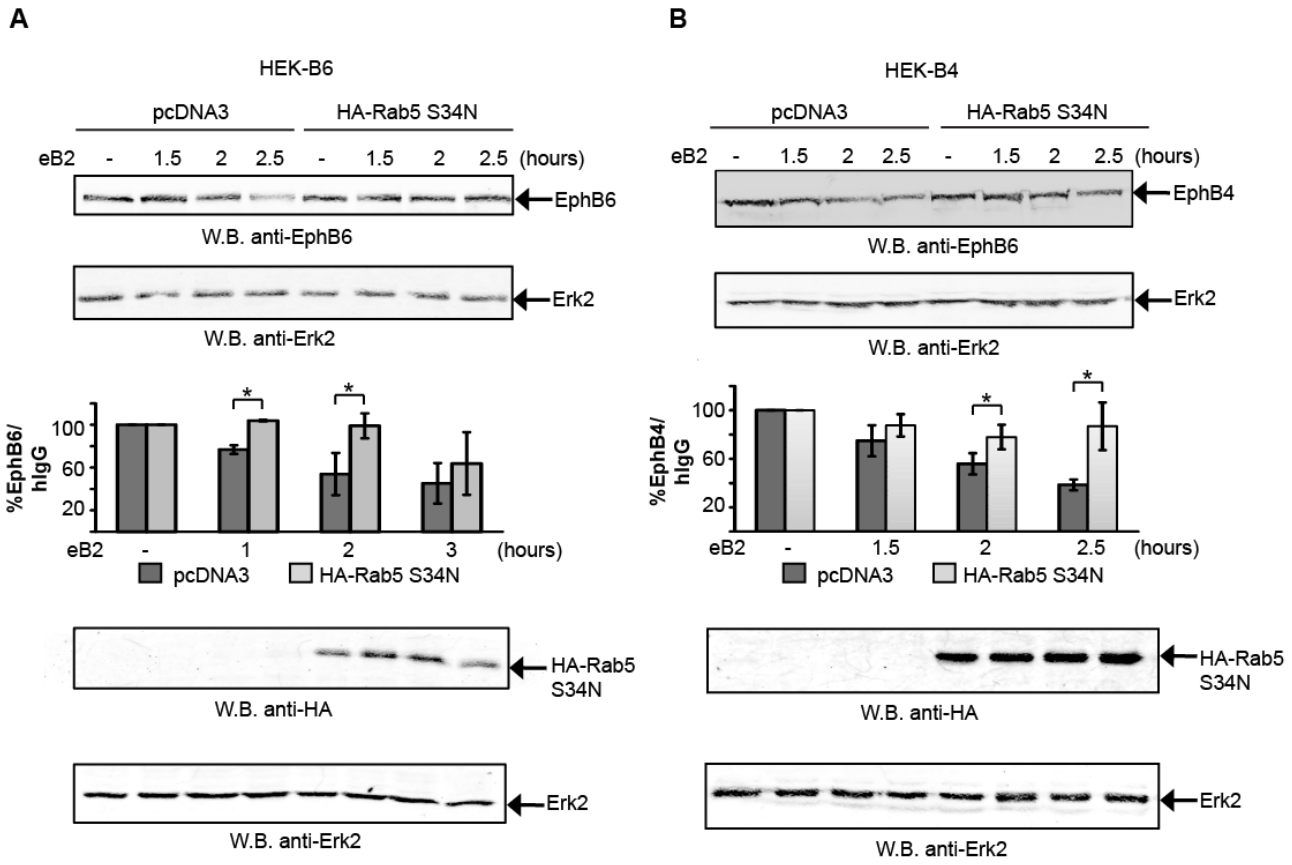

Figure 4.16. Rab5 is required for trafficking of EphB6 and EphB4 for degradation.
A) HEK-B6 cells were transfected with an HA-tagged dominant-negative mutant of Rab5, Rab5 S34N, or mock-transfected with pcDNA3 as a control and stimulated for the indicated time periods with eB2. EphB6 downregulation was monitored and assessed as in (***Fig. 2 A***). Expression of the mutant Rab5 was confirmed by Western blotting with anti-HA. **B**) HEK-B4-M cells were transfected and treated as in (***A***), and EphB4 degradation was assessed as in (***Fig. 2 A***). All Western blot panels in this figure represent one of at least three independent experiments. Each graph summarises the analysis of at least three independent experiments, bars, SD. *, $P < 0.05$, Student's t test, for indicated points and corresponding controls, as shown. In all downregulation experiments, unstimulated cells were treated with hIgG as a specificity control.

4.5. EphB6 stability is Supported by the Stimulation-Sensitive Interaction with Hsp90

Hsp90 (heat shock protein 90) is a well-documented chaperone responsible for the proper folding, stability, and turnover of a wide range of proteins (Saibil, 2013). In addition to its role in protein stability, Hsp90 has also been implicated in assisting in the maintenance of proteins at the cell surface and in ensuring the availability of receptors for ligand-binding (Sidera et al., 2008; Wayne et al., 2011; Xu et al., 2001; Xu et al., 2007). Our co-immunoprecipitation experiments showed that EphB6 also interacts with Hsp90, and that this interaction was rapidly disrupted by treatment with ephrin-B2. The effect could be readily observed after five minutes of stimulation (**Fig. 4.17 A**). Similar experiments were performed with the EphB4 receptor; however, we were unable to consistently detect the EphB4-Hsp90 interaction. To determine the localisation of the EphB6-Hsp90 complexes affected by ephrin-B2 stimulation, EphB6-EGFP-expressing HEK-293 cells were stimulated with ephrin-B2 for 20 minutes and stained with anti-Hsp90. Interestingly, a strong colocalisation of EphB6 with Hsp90 was observed at the cell membrane in unstimulated cells and this co-localisation was very significantly reduced upon ephrin-B2 treatment. While some minimal presence of cytoplasmic complexes was also observed, there was no major change in cytoplasmic EphB6-Hsp90 colocalisation in response to ephrin-B2 treatment (**Fig. 4.17 B, Fig. 4.18**). These novel observations suggest that Hsp90 and EphB6 interact mostly at the cell membrane and to a lesser degree inside the cell, and that the interaction at the membrane is terminated following activation of EphB6 by its ligand.

To examine if the absence of Hsp90 support was sufficient to initiate EphB6 degradation, we treated HEK-B6 cells with the Hsp90 inhibitor, geldanamycin (GA) (Miyata, 2005). This treatment triggered EphB6 downregulation at a rate that was similar to the rate of its ephrin-B2-

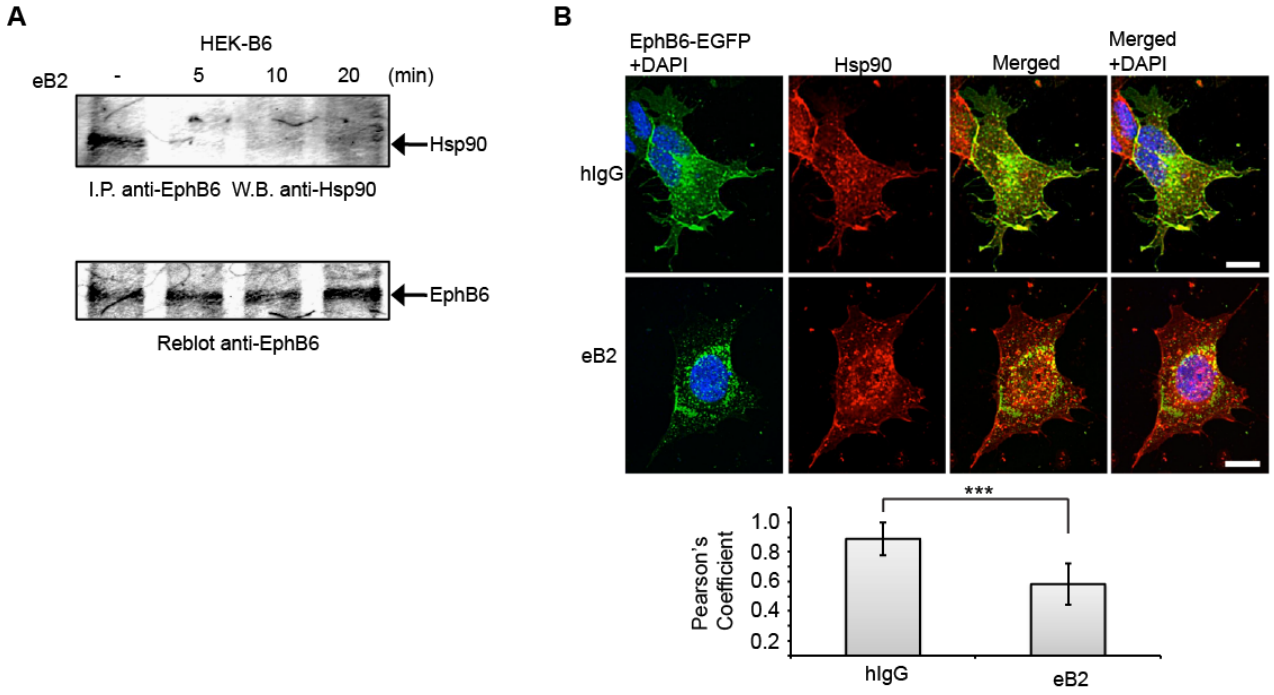

Figure 4.17: Ligand-treatment induces rapid EphB6-Hsp90 dissociation.

A) HEK-B6 cells were treated with eB2 for the indicated time periods, and EphB6 was immunoprecipitated with anti-EphB6. Precipitates were analyzed for Hsp90 presence by Western-blotting with anti-Hsp90. EphB6 immunoprecipitation was confirmed by re-blotting with anti-EphB6. **B**) HEK-293 cells were plated on glass coverslips and transfected with EphB6-EGFP cDNA. Cells were cultured for 72 h and stimulated with eB2 for 20 min. Stimulated cells were fixed in 4% formaldehyde, permeabilized with 0.1% saponin, and stained with anti-Hsp90 and anti-rat Alexafluor-594 (red) labelled antibodies. Stained cells were visualized with an Olympus FV-1000 confocal microscope at 60X magnification. Images were deconvoluted using AutoQuant X3. Scale bar, 10 μm. The graph represents quantifications of colocalisation of EphB6 with Hsp90 using Pearson's correlation coefficient. Values were obtained from 3 independent experiments. ***, $P < 2 \times 10^{-6}$. All Western blot and confocal microscopy images represent one of at least three independent experiments.

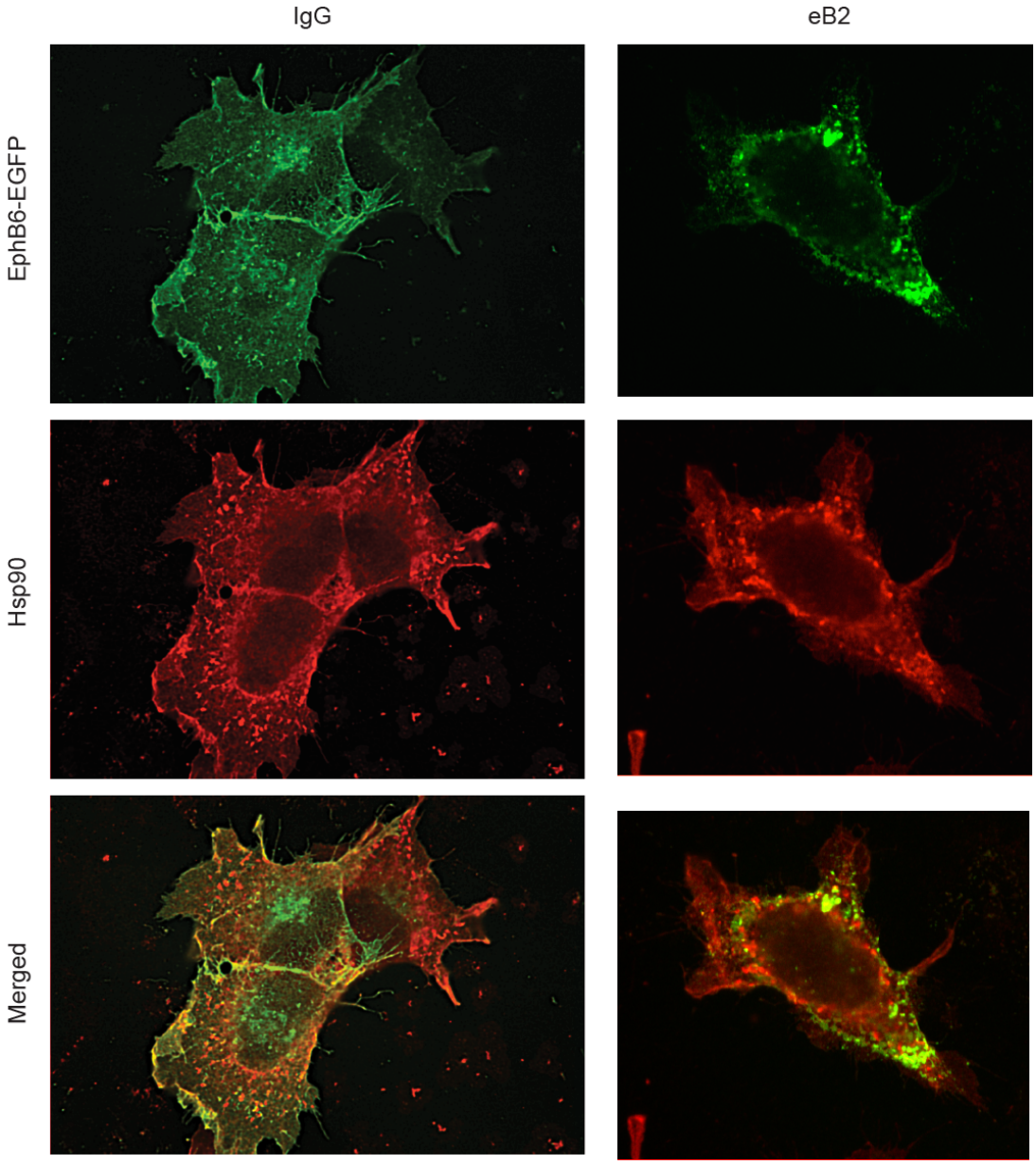

Figure 4.18. EphB6-Hsp90 colocalisation is abolished by ephrin-B2 treatment.
A) HEK-293 cells were plated on glass coverslips and transfected with EphB6-EGFP cDNA. Cells were cultured for 72 h and stimulated with eB2 for 20 min. Stimulated cells were fixed in 4% formaldehyde, permeabilized with 0.1% saponin, and stained with anti-Hsp90 and anti-rat Alexafluor-594 (red) labelled antibodies. Stained cells were visualized with an Olympus FV-1000 confocal microscope at 60X magnification. Images were deconvoluted using AutoQuant X3. Scale bar, 10 mm. Images represent one of three independent experiments.

initiated removal (**Fig. 4.19**). While GA application on its own resulted in strong EphB6 degradation, no consistent or statistically significant increase in the efficiency of ligand-induced receptor downregulation could be observed in response to simultaneous co-treatment with ephrin-B2 and GA (**Fig. 4.20 A&B**). This lack of a significant enhancing effect indicates that stimulation-induced EphB6 downregulation and GA-initiated degradation may rely on an overlapping molecular mechanism. Overall, these observations suggest an entirely new model, where the rapid disruption of the EphB6-Hsp90 complex in response to ligand treatment may represent a key event in the initiation of EphB6 stimulation-induced downregulation.

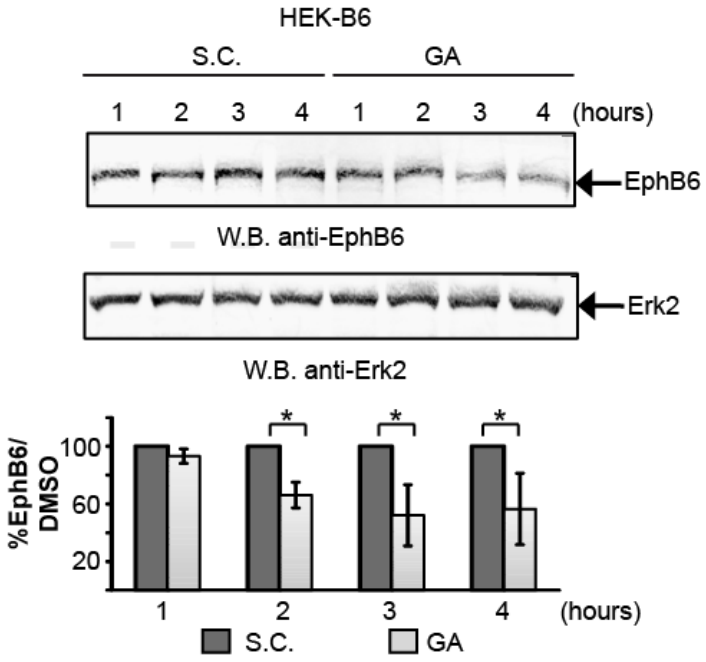

Figure 4.19. Treatment with an Hsp90 inhibitor induces EphB6 downregulation.
HEK-B6 cells were treated with 10 μg/ml of Hsp90 inhibitor, geldanamycin (GA) or the matching volume of solvent control (S.C.) for the indicated time periods. EphB6 levels were monitored by Western blotting with anti-EphB6. Results of Western blot quantification in GA-treated cells were normalized on Erk2 levels and presented in a graph as a percentage relative to matching solvent controls. All Western blot images represent one of at least three independent experiments.The graph in this figure summarises the analysis of three independent experiments, bars, SD. *, $P < 0.05$, Student's t test, between stimulated and control treated cells for the indicated time points.

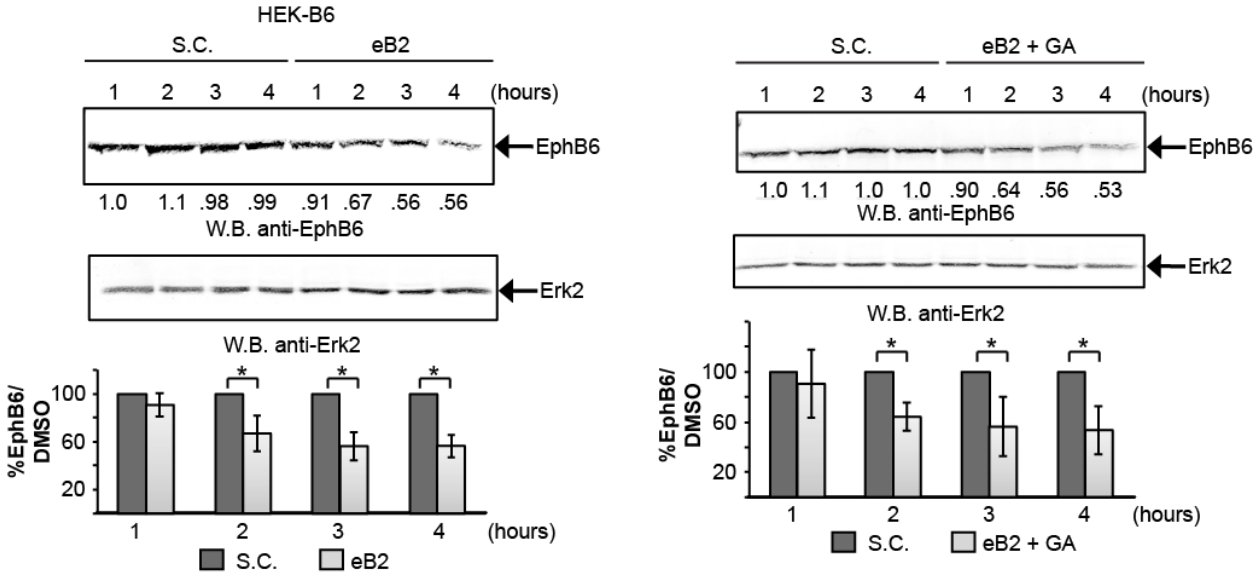

Figure 4.20. Hsp90 inhibition and ligand treatment induce EphB6 downregulation through overlapping pathways.

HEK-B6 cells were treated with eB2 in the presence of the solvent control, or eB2 with GA, or with the solvent control alone, for the indicated time periods, and EphB6 levels were monitored by Western blotting. EphB6 levels in eB2-stimulated cells were normalized as in (***Fig. 4.19***). Numbers representing this normalization are shown below Western blot images. Each graph in this figure summarises the analysis of three independent experiments, bars, SD. *, $P < 0.05$, Student's t test, between stimulated and control treated cells for the indicated time points. No statistically significant difference was observed between the rates of EphB6 downregulation in eB2 treated cells or GA treated cells, and cells co-treated with eB2 and GA at any time point measured. In all experiments involving eB2 stimulation, unstimulated cells were treated with hIgG as a specificity control. All Western blot and confocal microscopy images represent one of at least three independent experiments.

Chapter 5

DISCUSSION AND CONCLUSIONS

Stimulation-induced receptor downregulation through internalisation and subsequent targeted degradation play an important role in controlling cellular responses by both enhancing and suppressing cytoplasmic signalling (Platta and Stenmark, 2011). While these processes are well described for the EGFR receptor (Sorkin and Goh, 2009), much less is understood about downregulation of Eph receptors. Available data are not systematic and imply that different Ephs may use very distinct mechanisms. For example, ligand-induced downregulation of the EphA2 receptor relies on its degradation in the lysosomal compartment (Boissier et al., 2013), while in contrast, stimulation-initiated EphA3 elimination is mediated by proteasomal complexes (Sharfe et al., 2003) . Both EphA8 (Yoo et al., 2010) and EphB1 (Parker et al., 2004) are endocytosed through CCPs following ligand stimulation, and internalised EphB1 is degraded in the lysosomal compartment inside the cell (Fasen et al., 2008). Unlike these examples, EphB2 proteolysis in response to ligand stimulation is performed by the γ-secretase complex and is triggered by the initial ligand-induced cleavage of EphB2 by metalloproteases at the cell membrane (Litterst et al., 2007). In this situation, it is not a surprise that despite the recent accumulation of data that highlight an important role for one of Eph receptors, EphB6, in both normal physiology and in malignancy (Fox and Kandpal, 2009; Freywald et al., 2003; Luo et al., 2012; Luo et al., 2004; Maddigan et al., 2011; Tang et al., 2000; Truitt and Freywald, 2011; Truitt et al., 2010; Yu et al., 2010), the molecular mechanism of its downregulation has never been investigated. Such an investigation appears especially attractive in light of an unusual nature of this protein, which is kinase-inactive,

despite its association with the Eph group of RTKs (Matsuoka et al., 1997). Until now, downregulation of only one other kinase-deficient RTK, the ErbB3 receptor, has been dissected and yet, even this remains not well understood, as multiple reports suggest that ErbB3 is internalisation- and downregulation- deficient, while some recent observations show that ErbB3 undergoes ubiquitination and is efficiently downregulated in response to ligand stimulation (Baulida et al., 1996; Cao et al., 2007; Chen et al., 1996; Daly et al., 1997; Sak et al., 2013; Waterman et al., 1998).

The work presented within this thesis, attempts to systematically analyse the mechanism of ligand-induced downregulation of the kinase-dead EphB6 receptor, while comparing it with the downregulation of its kinase-active relative and signaling partner, EphB4 (Truitt et al., 2010), which also has not been fully assessed**.** It shows that similarly to the EphB4 receptor, EphB6 is actively downregulated in response to ligand stimulation and does not suppress EphB4 downregulation. Furthermore, despite the lack of intrinsic kinase ability, EphB6 removal occurs at a rate that closely resembles what is observed for EphB4. Likewise, following treatment with their common ligand, ephrin-B2, both receptors are internalised through clathrin-coated pits and both are eventually, targeted towards their degradation in the lysosomal compartment in a Rab5-dependent manner. Efficient receptor internalisation requires separation of the endocytic vesicles from the cell membrane into the cytoplasm and the dynamin GTPase is often responsible for this process (Ferguson and De Camilli, 2012). Matching our other observations, ligand-induced downregulation of both EphB6 and EphB4 proved to require dynamin activity.

Perhaps the most intriguing finding of these studies is the apparent role of Hsp90 in EphB6 downregulation. In the absence of stimulation, EphB6 consistently interacts with the Hsp90 chaperone that is known to bind to and stabilize few other RTKs, including EGFR and ErbB2 (Ahsan et al., 2012; Xu et al., 2001). According to our confocal microscopy data, Hsp90 stabilizes the EphB6 receptor predominantly at the cell surface, as the EphB6-Hsp90 interaction is compartmentalized mostly in this area. These observations are similar to what has been described for the ErbB2 receptor, as Hsp90 has also been shown to associate with this protein and to protect it from degradation (Chavany et al., 1996). Intriguingly, the membrane-associated EphB6-Hsp90 complex is rapidly disrupted following ligand application and this response precedes EphB6 degradation, implying that EphB6 downregulation could be triggered by modifications caused by ligand-induced Hsp90 dissociation. This possibility is strongly supported by our data, showing that inhibition of Hsp90 activity results in EphB6 elimination at a rate that closely resembles the rate of its ephrin-B2-triggered downregulation. Moreover, Hsp90 inhibition does not produce any consistent significant increase in ligand-induced EphB6 removal, suggesting that in both cases, receptor downregulation may be executed by the same molecular mechanism that is most likely initiated by the lack of Hsp90 activity. This is in contrast to EGFR behaviour, which has been reported to be downregulated much more efficiently when simultaneously treated with its ligand, EGF, and GA in comparison to each individual treatment alone (Pedersen et al., 2009).

This mode of action has not been reported previously in relation to ligand-induced receptor degradation, and it remains to be seen if it represents a frequent approach to downregulation of Eph receptors or RTKs in general, or if it is EphB6-specific.

Interestingly, the Eph group contains one more kinase-deficient member, the EphA10 receptor (Aasheim et al., 2005b). Its functional properties are still enigmatic and it would be curious to see if its downregulation is also governed by Hsp90.

At this stage, Hsp90 inhibitors are being tested in clinical trials for treating ErbB2-overexpressing breast cancer (Garcia-Carbonero et al., 2013). As previous observations indicate that the EphB6 receptor acts to suppress breast cancer aggressiveness (Fox and Kandpal, 2009; Truitt et al., 2010), our current data, showing that Hsp90 inhibition reduces EphB6 presence, suggest that this approach should be applied with caution to EphB6-expressing breast cancer tumours.

Overall, our work provides the first description of the route of ligand-induced downregulation of an intrinsically kinase-deficient member of the Eph group of RTKs, EphB6, and shows that EphB6 is removed in a manner that is very similar to the mode of downregulation of its kinase-active relative, the EphB4 receptor. Perhaps more importantly, our observations present the first example of Eph receptor interaction with the Hsp90 chaperone and reveal that stimulation-dependent EphB6 degradation is preceded by the disruption of its association with Hsp90, which likely acts as downregulation trigger (**Figure 5.1**). To our knowledge, this model has not been previously discussed and this mechanism may potentially represent a frequently used stage in ligand-induced RTK downregulation, shared by multiple family members on their way to lysosomal degradation.

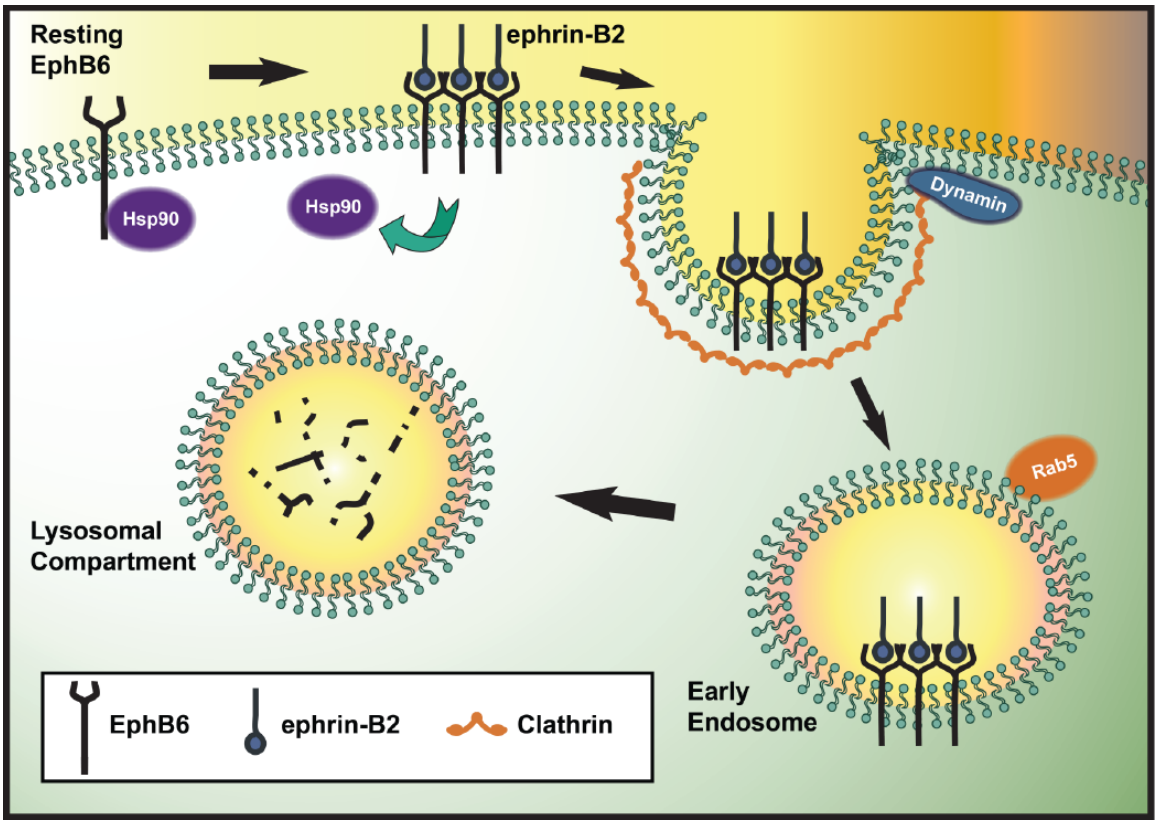

Figure 5.1. Proposed pathway of EphB6 ligand-induced downregulation.

Prior to ligand binding, EphB6 is associated with Hsp90 at the cell membrane. Ligand-binding initiates EphB6 receptor clustering and Hsp90 dissociation. EphB6 is recruited into CCPs and endosomal vesicle formation is finalized by pinching off at the cell membrane by dynamin. Internalised EphB6 receptors are trafficked within Rab5-positive early endosomes, and are eventually targeted to the lysosomal compartment for degradation.

Chapter 6

FUTURE WORK

6.1 Receptor recycling

Following internalization many receptors undergo recycling back to the cell surface as opposed to degradation. For example, EGFR, Met, and PDGFR have all been observed to undergo recycling following ligand-induced internalization (Hellberg et al., 2009; Parachoniak et al., 2011; Sigismund et al., 2008; Sigismund et al., 2005). It is likely that some internalized EphB6 molecules also undergo recycling, and this may play a role in the signalling output generated in response to activation. To determine if EphB6 undergoes recycling, cells can be simultaneously treated with ligand and labeled with anti-EphB6 at 37^{o}C, and the receptor allowed to internalize, or at 4^{o}C to prevent internalization as a control. An acidic wash at 4^{o}C can then be used to remove the antibody from remaining cell surface EphB6, and cells returned to normal growth conditions to allow any internalized receptor to recycle back to the surface. Fixation and staining with a fluorescently tagged secondary antibody targeting the EphB6 antibody and analysis by flow cytometry or confocal microscopy will then allow for the identification of recycled receptors (Fraile-Ramos et al., 2001; Mitchell et al., 2004). Alternatively, cell surface proteins, including EphB6, can be biotinylated, and cells subsequently treated with ephrin-B2 and receptors allowed to internalize. Remaining cell surface biotin can be stripped from the cell in reducing conditions at 4^{o}C, the cells returned to 37^{o}C to allow recycling, and a second round of biotin stripping performed. Biotinylated proteins can then be recovered using avadin beads. A comparison, through Western blot analysis, of the of the amount of biotinylated EphB6 found in cells that have undergone a second round of stripping following the recycling period, to those that have not, will give a general ratio of the amount of receptor that is being recycled (Hammond et al., 2003; Parachoniak et al., 2011). If EphB6 does undergo

recycling, fewer biotinylated EphB6 should be recovered and observed by Western blot analysis when cells undergo a second round of stripping.

Should it become apparent that EphB6 does indeed undergo recycling, the effects of this trafficking on EphB6 removal from the cell surface and downregulation could be assessed through the use of inhibitors of the recycling pathway, such as monensin, as well mutants of Rab GTPases involved in receptor recycling, such as Rab4 and Rab11. Recycling of EphB6 back to the cell surface should be greatly decreased upon use of recycling inhibitors, or the expression of the Rab mutants. It is possible that EphB6 that fails to recycle will remain in the early endosome, as has been observed for FGFR4 (Haugsten et al., 2014), or alternatively, EphB6 that is prevented from recycling may be rapidly targeted for degradation, in which case a significant increase in the observed rate of ligand-induced downregulation would be observed. To distinguish between these possibilities, EphB6 subcellular localization can be examined by the use of EGFP-EphB6 in conjunction with early endosome specific antibodies, such as anti-EEA1, as well as markers for later compartments, such as LysoTracker, and through Western blotting to examine total protein levels of the receptor, to clarify how EphB6 is trafficked when recycling is inhibited.

There are two distinct pathways of receptor recycling currently described, rapid recycling which takes place from the early endosome and is mediated largely by Rab4, as well as a slower-rate recycling which is observed from late endosomes/MVBs that relies on Rab11 (Grant and Donaldson, 2009; Sheff et al., 1999). The use of shRNAs specific to Rab4 or Rab11, and monitoring receptor recycling through the assay described above, as well as the analysis of receptor localization/degradation following Rab4 or Rab11 silencing will give a strong indication of which recycling pathway EphB6 predominantly utilizes. Additionally, stimulation and fixation

of EGFP-EphB6 expressing cells, and staining with antibodies for Rab4 or Rab11, and analysis by confocal microscopy would allow for the visualization of EphB6 colocalisation with specific recycling pathways.

For PDGFR-β, PKC has been observed to be important to mediating receptor recycling through the Rab4 recycling pathway, as PKC activation is able to significantly increase PDGFR-β recycling, and knock-down of PKC inhibits recycling and promotes degradation of this receptor (Hellberg et al., 2009). The involvement of PKC in EphB6 recycling could be examined through the use of shRNA to knock-down PKC expression, and the subsequent analysis of receptor recycling in response to ligand-stimulation using the recycling assay described above. Should PKC be important to mediating EphB6 recycling, its knock-down should result in a decrease in its cell surface levels. Furthermore, as receptor recycling can impact the level of observed downregulation/degradation, an examination of the overall rate and degree of EphB6 downregulation in response to ligand treatment in PKC-deficient cells would give further insight into the importance of PKC in mediating the balance between EphB6 recycling/degradation in response to ligand treatment. Here, Western blot analysis would be sufficient to determine total EphB6 protein levels following ligand-treatment, and should PKC be important to mediating EphB6 recycling, its knock down should result in an increase in the observed rate, and potentially the level, of EphB6 receptor degradation.

Met receptor recycling was shown to require the activity of GGA3 (Golgi-localized gamma-ear containing Arf-binding protein 3). It was found that GGA3 rapidly associates with Met following ligand stimulation, and promotes its localization to Rab4 positive endosomes. Moreover, knock-down of GGA3 resulted in a significant loss of receptor recycling and reduced surface levels (Parachoniak et al., 2011). Examination of EphB6 interaction with GGA3 through

co-immunoprecipitation and Western blot analysis, with and without ligand stimulation, would give some indication if GGA3 is also involved in EphB6 recycling. Staining of ligand treated EGFP-EphB6 expressing cells for GGA3 and analysis by confocal microscopy would further allow for the determination of whether or not EphB6 also associates with GGA3 following ligand-treatment, and give some indication where this association takes place. The use of shRNA for GGA3 would allow for the analysis of the impact of knock-down GGA3 on EphB6 trafficking, which could be assessed using experimental approaches relying on receptor biotinylation. If GGA3 is indeed involved in EphB6 recycling, its knock-down should cause a reduction in EphB6 recycling and potentially an increase in overall levels of EphB6 downregulation.

6.2 How Hsp90-dissociation induces EphB6 downregulation

The association of EphB6 with Hsp90 is perhaps one of the most important observations acquired through the course of my work. There are numerous reports in recent years suggesting a role for Hsp90 in either membrane stability of receptors, receptor activation, or both (Mahalingam et al., 2009), however, this is the first report linking Hsp90 behaviour to ligand-induced downregulation. At this stage it is unclear if these observations are EphB6-specific or not, and the potential involvement of Hsp90 dissociation in the downregulation of other Eph receptors is an interesting prospect which warrants further investigation. Furthermore, many Eph receptors have an unfavourable impact on the prognosis for several cancer types (Kandouz, 2012), so establishing whether or not targeting Hsp90 would also effectively induce their downregulation could lead to effective cancer treatments aimed at reducing overall Eph receptor levels in tumors. Co-immunoprecipitation of Hsp90 with other Eph receptors could be performed to determine if any other members of this RTK family interact with this chaperone. Analysis of

observed interactions following ligand stimulation would determine whether receptor activation induces Hps90 dissociation, as was observed for EphB6. The subcellular localisation of Eph-Hsp90 interaction, examined by confocal microscopy, will determine if it predominantly takes places at the cell surface or in intracellular compartments. Should the Eph-Hsp90 colocalisation occur at the cell surface, as was observed for EphB6, monitoring receptor downregulation following treatment with Hsp90 inhibitors using Western blot analysis will indicate whether or not other Eph receptors also utilize Hsp90 dissociation as a key step in their downregulation.

Our experiments strongly suggest that ephrin-B2 and GA induced downregulation of EphB6 both promote receptor downregulation through the same pathway; however, it is not yet fully clear how Hsp90 dissociation is initiating this process. Therefore, some investigation into the effectors underlying ligand-induced and Hsp90 inhibition-promoted downregulation is required. The E3 ligase CHIP (C-terminus of Hsc70 Interacting Protein) and Hsp70 are important to mediating ErbB2 downregulation induced through Hsp90 inhibition (Xu et al., 2002). Here, the induction of Hsp90 dissociation from ErbB2 promotes a concurrent increase in ErbB2 association with another molecular chaperone, Hsp70 and CHIP. The formation of the CHIP/Hsp70/ErbB2 complex results in CHIP-mediated ubiquitination of the receptor, leading to ErbB2 degradation (Xu et al., 2002; Zhou et al., 2003). Examining the role of CHIP and Hsp70 in ligand-induced and GA-triggered downregulation of EphB6 would determine if these proteins also mediate Hsp90 effects for other RTKs, and clarify how Hsp90 dissociation induces EphB6 downregulation. As mentioned, following inhibition of Hsp90, Hsp70 forms a complex with CHIP and ErbB2, and therefore, Hsp70 presence is likely required for targeting of the receptor for degradation, as has been observed for numerous other proteins that undergo CHIP mediated ubiquitination (Gao et al., 2010; Luo et al., 2010; Meacham et al., 2001). Co-

immunoprecipitation experiments examining the association of Hsp70 with EphB6 with, and without ligand stimulation, as well as in the presence or absence of GA, would indicate whether Hsp70 is also involved in the EphB6 degradation pathway. Should Hsp70 be involved, there will be an increase in Hsp70 immuoprecipitation with the receptor following ligand or GA treatment. Knock-down of Hsp70 levels by using shRNA would then cause a decrease in ligand- and GA-induced receptor downregulation, which can be assessed through Western blot analysis. Co-immunoprecipitation experiments of EphB6 with CHIP, as well as examining the effect of CHIP knock-down on EphB6 downregulation would indicate whether or not CHIP is involved in this process. In the co-immunoprecipitation experiments, an increase in association with EphB6 following ligand stimulation or GA treatment should be observed if it is indeed involved in EphB6 downregulation, and the knock-down of CHIP expression using shRNA should reduce the observed GA- and ephrin-B2-induced EphB6 downregulation. Furthermore, experiments monitoring EphB6 ubiquitination following ligand stimulation or GA treatment in cells with reduced and unmanipulated levels of CHIP expression would give evidence of whether or not CHIP is responsible for EphB6 ubiquitination in these situations. Here, cells with normal or reduced CHIP expression should be treated with ligand or GA, EphB6 immunoprecipitated, and analysed for ubiquitination by Western blotting. Should CHIP ubiquitinate EphB6, there should be a reduction in observed ubiquitination of the receptor following either ligand or GA treatment in cells with CHIP expression knocked down relative to cells with normal CHIP expression.

6.3 Negative impacts of Hsp90 inhibition

Finally, as indicated in the discussion, several inhibitors of Hsp90 are under active investigation for the treatment of ErbB2 positive breast cancers, however, as this treatment would concurrently reduce surface EphB6 levels, it could lead to an increase in the metastatic

potential of the cells. To clarify this, potential unfavorable aspects of Hsp90 inhibition should be analyzed in animal models of EphB6-positive breast cancer. This would involve the injection of breast cancer cells with high EphB6 expression into the mammary fat pads of immune-deficient mice, and monitoring the number of metastatic lesions that form in the presence or absence of treatment with Hsp90 inhibitor. By transfecting the EphB6-positive cancer cells with a luciferase-expressing vector, tumor growth and metastasis can be monitored in live mice by injecting them with D-luciferin and visualization of the resulting fluorescent areas with a bioluminescence imager. Metastases can be confirmed at the end of the experimental period by collection of organs and examination for tumor presence. If Hsp90-inhibition induced EphB6 removal does increase the invasive activities of the cancer cells, a higher number of metastatic lesions will be observed in the Hsp90 inhibitor treated mice. Should this prove to be the case, Hsp90 inhibition should be used with caution when EphB6 is known to be expressed by the tumor.

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