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Effects of Localized EphB2 Stimulation on Dendritic Filopodia of Hippocampal Neurons

Clifford Locke

University of Connecticut - Storrs, cblocke35@gmail.com

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Dendritic filopodia are thin, dynamic protrusions of developing neurons that are thought to search the area surrounding dendrites for pre-synaptic axons. Upon contact with axons, they are believed to transform into the mature glutamatergic post-synaptic compartments known as dendritic spines. Through synaptic plasticity, dendritic spines are suggested to play crucial roles in learning and memory formation. While glutamatergic activity is a major stimulus of dendritic spine formation, cell-cell recognition receptors also play significant roles.

Eph receptors are receptor tyrosine kinases that act as cell-cell contact receptors through binding their membrane-bound ephrin ligands. They are classified into EphA and EphB subtypes based on their preferential binding to either ephrinA or ephrinB ligands. EphB1, EphB2, and EphB3 are collectively necessary for dendritic spine formation in vivo. They are thought to be activated at axo-dendritic contacts and trigger direct changes in actin polymerization that drive the transition from filopodia to spines. While the relevant signaling pathways have been extensively studied, how EphB
signaling changes the motility and morphology of dendritic filopodia to form dendritic spines remains poorly understood.

To facilitate real-time monitoring of dendritic filopodia following EphB stimulation, a photoactivatable EphB2, optoEphB2, was developed. Since Eph receptor clustering is necessary for efficient downstream signaling, optoEphB2 employs the blue light-induced clustering of Cryptochrome 2. Photoactivation of optoEphB2 resulted in rapid tyrosine phosphorylation and signaling to SH2 domain proteins that are known to act downstream of EphB2. In fibroblasts and hippocampal growth cones, optoEphB2 activation resulted in collapse of protrusive structures, consistent with previous findings for EphB signaling.

Surprisingly, localized activation of EphB2 at dendritic filopodia promoted filopodia branching and plasma membrane expansion. Activation along the dendritic shaft led to formation of new filopodial protrusions in an Arg- and Arp2/3-dependent manner. In addition, local phosphatidylinositol(3,4,5)-triphosphate (PIP3) accumulation via phosphoinositide 3-kinase (PI3K) activation was necessary for protrusion formation and marked a key difference between dendrites and fibroblasts. These results provided new insights into the role of EphBs in dendritic filopodia, suggesting that they may increase filopodia density near sites of axo-dendritic contact. Differential regulation of PIP3 synthesis may represent an important underlying mechanism of the cell context-dependence that characterizes Eph receptor signaling.
Effects of Localized EphB2 Stimulation on Dendritic Filopodia of Hippocampal Neurons

Clifford Locke

B. S., University of Connecticut, 2010

A Dissertation
Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy at the University of Connecticut 2018
Effect of Localized EphB2 Stimulation on Dendritic Filopodia of Hippocampal Neurons

Presented by:
Clifford Locke, B.S.

Major Advisor: Ji Yu

Yi I. Wu

Ann E. Cowan

Bruce J. Mayer

Betty A. Eipper

University of Connecticut
2018
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**Chapter III: Local EphB2 Signaling Induces Branching of Dendritic Filopodia, Expansion of Filopodia Tips, and Promotes Dendritic Filopodia Formation**

**Abstract**

**Introduction**

**Methods**

- Antibodies, reagents, and plasmids
- Cell culture, transfections, and drug treatments
- Microscopy and image analysis

**Results**

- OptoEphB2 activation in dendritic filopodia induces actin polymerization that results in branching and plasma membrane expansion
- Local EphB2 signaling in dendritic shafts induces dynamic filopodia-like protrusions
- Differential regulation of PIP₃ synthesis underlies cell context-dependent effects of EphB2 signaling on the actin cytoskeleton
- Filopodia formation by optoEphB2 depends on Abelson family kinases

**Discussion**

**Figures**

**Movie Captions**

**Chapter IV: Discussion and Future Directions**

**Summary**

**Discussion and Future Directions**

- Development of an optogenetic method for Eph receptor activation
- Novel insights into dendritic spine morphogenesis
- Implications for understanding the cell context-dependence of EphB2 signaling

**Conclusion**

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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ADAM</td>
<td>A disintegrin and metalloproteinase</td>
</tr>
<tr>
<td>AMPAR</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor</td>
</tr>
<tr>
<td>Arp2/3</td>
<td>Actin-related proteins Arp2 and Arp3</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CA</td>
<td>Constitutively-active</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Calcium/calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>CIB1</td>
<td>Cryptochrome-interacting basic helix-loop-helix</td>
</tr>
<tr>
<td>CIBN</td>
<td>Truncated CIB1</td>
</tr>
<tr>
<td>CLICR</td>
<td>Clustering indirectly using Cryptochrome 2</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CRD</td>
<td>Cysteine-rich domain</td>
</tr>
<tr>
<td>Cry2</td>
<td>Cryptochrome 2</td>
</tr>
<tr>
<td>Cry2olig</td>
<td>Cryptochrome 2 with E490G mutation</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential interference contrast</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DPSS</td>
<td>Diode-pumped solid state</td>
</tr>
<tr>
<td>ECD</td>
<td>Extracellular domain</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>EM-CCD</td>
<td>Electron-multiplying charge-coupled device</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>Eph</td>
<td>Erythropoietin-producing human hepatocellular carcinoma</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment crystallizable region of immunoglobulin heavy chain</td>
</tr>
<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
</tr>
<tr>
<td>FKBP</td>
<td>FK506 binding protein</td>
</tr>
<tr>
<td>FNIII</td>
<td>Fibronectin type III</td>
</tr>
<tr>
<td>FP</td>
<td>Fluorescent protein</td>
</tr>
<tr>
<td>FRAP</td>
<td>Fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>G-actin</td>
<td>Globular (monomeric) actin</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosyl phosphatidylinositol</td>
</tr>
<tr>
<td>GRIP</td>
<td>Glutamate receptor-interacting protein</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>ICD</td>
<td>Intracellular domain</td>
</tr>
<tr>
<td>iSH2</td>
<td>Inter-SH2</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
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<tr>
<td>JM</td>
<td>Juxtamembrane</td>
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<tr>
<td>Abbr.</td>
<td>Term</td>
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<tr>
<td>KD</td>
<td>Kinase-dead</td>
</tr>
<tr>
<td>KLB</td>
<td>Kinase lysis buffer</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LARIAT</td>
<td>Light-activated reversible inhibition by assembled trap</td>
</tr>
<tr>
<td>LBD</td>
<td>Ligand-binding domain</td>
</tr>
<tr>
<td>LED</td>
<td>Light-emitting diode</td>
</tr>
<tr>
<td>LI</td>
<td>Light-insensitive</td>
</tr>
<tr>
<td>LIMK</td>
<td>LIM domain kinase</td>
</tr>
<tr>
<td>LINC</td>
<td>Light-induced co-clustering</td>
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<tr>
<td>LMW-PTP</td>
<td>Low molecular weight protein tyrosine phosphatase</td>
</tr>
<tr>
<td>LTD</td>
<td>Long-term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>mCh</td>
<td>mCherry fluorescent protein</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>mRFP</td>
<td>Monomeric red fluorescent protein</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NA</td>
<td>Numerical aperture</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization sequence</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-methyl D-aspartate receptor</td>
</tr>
<tr>
<td>NRTK</td>
<td>Non-receptor tyrosine kinase</td>
</tr>
<tr>
<td>PAK</td>
<td>p21-activated kinase</td>
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Trk  Tropomyosin-related kinase
N-WASP  Neural Wiskott-Aldrich Syndrome protein
VEGFR  Vascular endothelial growth factor receptor
WAVE  WASP-family verprolin homologous
WT  Wild-type
YFP  Yellow fluorescent protein
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CHAPTER I: Introduction

Attribution: This chapter was proof-read by Ji Yu and Yi Wu. Clifford Locke wrote the text, made the figures, and performed the experiments.
Motivation

Dendritic spines are the mushroom-shaped protrusions of neuronal dendrites that comprise most post-synaptic terminals of excitatory synapses in the central nervous system (CNS). Dendritic spines are supported by an actin cytoskeleton and contain a bulbous head that is connected to the dendritic shaft by a thin neck. This architecture allows for the compartmentalization of post-synaptic proteins and electrical activity\(^1,2\). Dendritic spines are believed to play important roles in learning and memory formation in humans, and their numbers and morphology are tightly regulated throughout development. Defects in dendritic spine formation, resulting in abnormal spine density or morphology, have been described in a number of developmental disorders\(^3\).

Many studies have suggested that dendritic spines derive from thin, dynamic protrusions known as dendritic filopodia\(^1,4\). The first study to suggest this model examined dendritic filopodia in cultured hippocampal neurons. It was demonstrated that dynamic filopodia formed stable contacts with axons and that, following the first week \textit{in vitro}, the density of dendritic filopodia decreased while that of dendritic spines increased\(^4\). Later electron microscopy studies of rat hippocampal slices showed synaptic vesicles, which contain neurotransmitters, near sites of contact between filopodia and axons\(^5\). It is thus thought that dendritic filopodia are dynamic protrusions that seek out axons, stabilize upon contact with them, and subsequently develop into post-synaptic dendritic spines. Dendritic spines may then undergo maturation and strengthening, marked by growth of the spine head, or may be lost. This dynamic nature is termed synaptic plasticity, while loss is often referred to as synaptic pruning, and these processes are thought to contribute to memory formation or loss\(^6\).
Changes in the actin cytoskeleton drive most aspects of dendritic spine formation from dendritic filopodia and subsequent plasticity. Changing the architecture of actin is necessary to form and enlarge the spine head and organize post-synaptic proteins, such as neurotransmitter receptors and important scaffolds. Signaling pathways that ultimately converge on the actin cytoskeleton in these contexts are known to originate from the actions of glutamate, the major excitatory neurotransmitter in the CNS. Glutamate binds post-synaptic ionotropic receptors, triggering an influx of extracellular calcium. Calcium-sensitive proteins may then transmit signals to the cytoskeleton. Generally, the result of repeated glutamatergic stimulation is spine head enlargement and recruitment of additional glutamate receptors, which strengthens the synapse.

While glutamate is the primary director of dendritic spine maturation, filopodia have been shown to form stable contacts with axons in the absence of glutamatergic activity. There is, therefore, a major role for cell-cell recognition molecules in the formation of dendritic spines. An important family of cell-cell contact receptors that drive the formation of dendritic spines is the Eph receptor family. Briefly, Eph receptors are members of the largest receptor tyrosine kinase (RTK) family in mammals, and are sub-classified into EphA (EphA1-A8, A10) and EphB (EphB1-B4, B6) subtypes. They become activated at cell-cell contacts upon binding their membrane-bound ligands, the ephrins, and subsequent receptor clustering. Their primary function is to guide migrating cells during developmental processes, and they therefore play important roles in gastrulation, cell positioning, tissue patterning, and organogenesis.

Prior studies have suggested that EphB signaling promotes the formation of dendritic spines in hippocampal neurons. These conclusions were drawn from an initial
observation that overexpression of kinase-dead EphB2 in cultured rat hippocampal neurons impaired dendritic spine development\textsuperscript{13}. Follow-up work examined the morphology of dendritic protrusions in cultured mouse hippocampal neurons and brain slices when various combinations of EphB1, EphB2, and EphB3 were genetically knocked out. It was found that knocking out two of these three EphB subtypes significantly reduced dendritic spine density \textit{in vitro}, and knocking out all three of EphB1-B3 reduced spine density \textit{in vivo}\textsuperscript{14}. Additionally, many studies used ephrinB ligands to stimulate EphB signaling in cultured hippocampal neurons and observed increased dendritic spine density and spine head size\textsuperscript{15}. These studies strongly suggested that signaling from EphB receptors was necessary for both proper spine formation and maturation, and that EphB1-B3 were at least partially redundant in these processes.

To understand the effect of EphB signaling on dendritic spine formation, a logical step is to understand how they affect dendritic spine precursor structures, dendritic filopodia. Indeed, by knocking down EphB2 in cultured hippocampal neurons in a time-specific fashion, it was found that the effect of EphB2 on spine formation was confined to the second week \textit{in vitro}, when filopodia are most abundant\textsuperscript{16}. Overexpression of EphB2 rescued dendritic spine loss in EphB1-B3 triple-knockout neurons only during this time frame as well\textsuperscript{16}. This suggested that EphB signaling must have specific effects on dendritic filopodia that promote their transition to dendritic spines.

Interestingly, neurons cultured from EphB1-EphB3 triple-knockout mice displayed normal filopodia density early in development\textsuperscript{16}, suggesting that EphB signaling did not affect filopodia formation. However, decreased filopodia motility was observed versus wild-type neurons\textsuperscript{16}. This would suggest that basal EphB activity is
important to maintain the motility of dendritic filopodia, a property that is important for their axon-searching function. Other studies examined the effects of EphB signaling by stimulating cultured neurons with ephrinB ligands. In addition to increasing spine formation, ligand stimulation also resulted in an overall increase in dendritic protrusion density, including spines and filopodia, and filopodia shortening\textsuperscript{14,17}. These outputs are consistent with a role for EphB signaling in increasing the number of dendritic spines. Filopodia shortening is expected during dendritic spine morphogenesis, immediately following contact formation, and increasing protrusion density would necessarily lead to more potential contact sites.

The results discussed above are consistent with an increase in dendritic spine density following activation of EphB signaling by ephrinB binding, but do not fully address how this signaling contributes to the transition from filopodia to spines. Genetically knocking out EphB1-B3 eliminates their basal activities, but does not fully address how their signaling affects filopodia motility or morphology at axo-dendritic contacts, where EphBs are presumably engaged by their ligands. Knocking out EphB isoforms may also affect the expression of other proteins, and the observed effects may have been the result of an abnormal pattern of protein expression. Since ephrinB ligand treatment increased protrusion density, one may expect that EphB1-B3 triple-knockout would reduce protrusion density, though no effect was observed. Protrusion density is inherently governed by both the rate of protrusion formation and loss. It is unclear if the increased protrusion density was secondary to increased formation of filopodia, some of which may have become spines, or stabilization of existing protrusions. Increased protrusion formation would not be supported by the separate finding of filopodia
shortening upon EphB stimulation; elongation would be expected to promote protrusion formation. Additionally, bath application of ligands does not fully reflect what may happen to single filopodia when EphBs are activated at individual contacts.

Therefore, the effects of EphB signaling on the motility and morphology of dendritic filopodia remain poorly understood. This biological question is the primary motivation behind this thesis, and answering it will improve our understanding of how EphBs transform filopodia into spines. A number of hypotheses can be made. Since EphBs are activated at cell-cell contacts, and filopodia must stabilize upon contact with axons to become spines, EphB signaling may initiate spine morphogenesis by directing contact stabilization. One would expect, then, reduced filopodia motility in response to EphB stimulation. EphB signaling at contacts along the dendritic shaft is also possible, since EphBs are expressed on the dendritic shaft\textsuperscript{18}. EphBs may direct the formation of protrusions from the dendritic shaft, which may then become spines. The known interactions between EphBs and glutamate receptors\textsuperscript{19,20} may suggest that EphBs have no direct effect on the actin cytoskeleton. Rather, their function may be to recruit glutamate receptors to filopodia, whose activity via calcium influx may then independently alter the actin cytoskeleton to direct the transition to mature dendritic spines.

Observing the effect of EphB signaling on the morphology and motility of dendritic filopodia would be facilitated by spatial and temporal control over EphB signaling. This would ensure that signaling originates from dendritic filopodia, which would better simulate the scenario of filopodia coming into contact with axons. Thus, the aims of this thesis were two-pronged: to develop a technique to spatially and temporally
stimulate EphB signaling, and then dynamically monitor responses of dendritic filopodia to localized stimulation.

This chapter provides a detailed overview of the topics covered in this thesis. I will begin with a discussion of Ephs and ephrins, covering the structural and molecular basis behind their signaling and functional consequences. A discussion of optogenetics and, specifically, the blue light-sensitive plant photoreceptor Cryptochrome 2 follows. Dendritic spine formation and the role of Eph receptors in this process will then be discussed. I will then give a summary of experimental results and the primary conclusions of this thesis, which will be discussed in detail in Chapters II and III.

**Eph Receptors and Ephrins**

Erythropoietin-producing human hepatocellular carcinoma (Eph) receptors comprise the largest family of receptor tyrosine kinases (RTKs) in humans. As their name suggests, the first Eph receptors were identified in 1987 as candidate oncogenes in an erythropoietin-producing hepatocellular carcinoma cell line. Since then, nine EphA (EphA1-A8, A10) and five EphB (EphB1-B4, B6) receptors have been characterized in humans. This classification into A and B subtypes is based on the receptors’ relative binding affinities for the five glycosyl phosphatidylinositol (GPI)-linked ephrinA (ephrinA1-A5) or three transmembrane ephrinB (ephrinB1-B3) ligands. It should be noted, however, that these binding interactions display some promiscuity. For instance, EphA4 binds ephrinBs, and ephrinA5 is capable of binding EphBs.

Both Ephs and ephrins are membrane-bound and are therefore activated at cell-cell contacts. Uniquely, both the receptors and ligands transmit downstream signals,
resulting in so-called “bidirectional” signaling. Colloquially, signaling from Eph receptors is termed “forward” signaling, and “reverse” signaling emanates from ephrins. Eph receptors and ephrins transduce cell-cell contacts into changes in cell migration, proliferation, and survival. They are crucial to normal cell positioning and tissue patternning during developmental processes, such as organogenesis, tissue boundary formation, topographic mapping, axon guidance, and synaptogenesis. As suggested by their discovery, Eph receptors are also important in cancer biology and are known to function as tumor suppressors and promoters, depending on the Eph receptor subtype and type of cancer.

The structure, function, and signaling pathways that define Eph receptors mirror those of other members of the RTK family. To fully appreciate their biology, an overview of RTKs is given. Specific features of Eph receptors will follow.

Receptor Tyrosine Kinases

Receptor tyrosine kinases (RTKs) are unique transmembrane receptors that regulate critical cell functions, including cell proliferation, differentiation, migration, and survival. A large number of RTK families exist and many, for example, the fibroblast growth factor receptor (FGFR) and epidermal growth factor receptor (EGFR) families, respond to growth factors. RTKs are crucial in developmental processes, and mutations or other dysfunctions of RTKs are associated with numerous developmental and neoplastic disease states. For example, many breast cancers are marked by overexpression of the EGFR family member ErbB2, and Kallman Syndrome, a
developmental disorder affecting the normal development of cranio-facial structures, is caused by a mutation in FGFR1.

Typically, RTKs are composed of extracellular domains that consist of a ligand-binding domain and other unique motifs, a transmembrane domain, and intracellular domains with catalytically-active tyrosine kinases. Most RTK intracellular domains assume an auto-inhibitory conformation in the inactive state. Typically, ligand-induced dimerization results in auto-phosphorylation events that relieve this auto-inhibitory configuration. Intracellular tyrosine kinase domains may then access their substrates, which include tyrosine residues on the receptors themselves that are phosphorylated in trans and other downstream signaling molecules.

Phosphotyrosines on the RTKs and their substrates serve as docking sites for proteins containing Src homology-2 (SH2) and phosphotyrosine binding (PTB) domains. The various SH2 and PTB domains selectively bind different phosphopeptides, which allows many RTKs to display specificity for particular effectors. These SH2/PTB domain-containing proteins may serve as adaptors or scaffolds to organize large macromolecular complexes, such as focal adhesions, and others contain enzymatic properties. Some, such as Src, Abl, and Arg, are tyrosine kinases themselves, known as non-receptor tyrosine kinases (NRTKs). Interactions other than SH2/PTB domain-phosphotyrosine interactions, such as PDZ (PSD-95, Discs large, Zona occludens-1) domain binding, Src homology-3 (SH3) domain binding to polyproline motifs, and plasma membrane lipid alterations, are also important in RTK signaling pathways. In many cases, these occur as secondary interactions with SH2/PTB
domain proteins that stabilize phosphotyrosine-mediated interactions. These other interactions also diversify the signaling complexes formed by SH2 domain adaptors.

Downstream signals from RTKs affect the cytoskeleton and expression of genes that regulate cell survival, metabolism, cell cycle progression, and differentiation. Major downstream signaling pathways include the Rho family of GTPases, the Ras/MAPK signaling cascade, and phosphoinositide 3-kinase (PI3K) signaling. Some receptors do not themselves contain tyrosine kinase activity, but resemble RTKs by associating with the Janus family of NRTKs (JAK), which activate the signal transducer and activator of transcription (STAT) family of transcription factors. The Rho and Ras GTPases and PI3K signaling are most relevant to this thesis and are discussed in further detail below.

Rho and Ras GTPases are signaling proteins with intrinsic GTPase activity that are active when GTP-bound and inactive when GDP-bound. Relevant to this discussion, the Rho GTPase family refers to the GTPases Rac1, Cdc42, and RhoA. The Ras GTPases consist of a variety of members, with emphasis here on the canonical H-Ras, N-Ras, and K-Ras isoforms, collectively referred to as “Ras” in many publications, and the Ras-related (R-Ras) proteins. RTKs typically regulate their activity by signaling guanine nucleotide exchange factors (GEFs), which activate GTPases by facilitating replacement of GDP with GTP, and GTPase-activating proteins (GAPs), which inhibit GTPase signaling activities by facilitating hydrolysis of GTP to GDP.

Most canonical Ras isoforms signal the Raf family of kinases, which then activate the mitogen-activated protein kinase (MAPK) cascade. This cascade is a string of kinases, beginning with activation of the Raf kinases, which then phosphorylate and
activate MAPK kinase (MEK), which then activates MAPK, also known as ERK. Phosphorylated ERK isoforms may translocate to the nucleus, where they increase the transcription of genes that promote cell growth and survival. Both canonical Ras and R-Ras proteins are capable of activating PI3K. However, R-Ras proteins typically do not interact with Raf kinases and activate the MAPK cascade. In addition to gene regulation, canonical Ras and R-Ras family members can activate or inhibit signaling of integrins, which are transmembrane receptors that bind extracellular matrix (ECM) proteins to influence cell adhesion and migration.

The Rho GTPases Rac1, Cdc42, and RhoA affect cell migration and motility by altering actin polymerization. RhoA is generally thought to direct cell retraction by signaling through Rho-associated protein kinase (ROCK) to activate myosin-based contractile forces. It may also signal the mDia family of formins, which increase actin polymerization. Rac1 and Cdc42 signal WAVE and N-WASP, respectively, which may activate the Arp2/3 complex and nucleate branched actin filaments. They also signal through p21-associated kinase (PAK) to inhibit acto-myosin contractile forces. They, therefore, mediate the formation of cellular protrusions. Lamellipodia are formed by inducing broad plasma membrane extensions through nucleation of a highly-branched actin network. Filopodia are formed by elongating parallel actin bundles. Tight spatial and temporal regulation of these Rho GTPases are necessary for proper cell motility, migration, and polarity.

Phosphoinositide-3-kinases (PI3K, Fig. 1.1) comprise a family of lipid kinases that phosphorylate the 3-hydroxyl group of inositol ring of phosphatidylinositol family lipids on the inner leaflet of the plasma membrane. Of special interest in RTK signaling are the
class I PI3Ks, which are composed of regulatory and catalytic subunits. Some regulatory subunits contain SH2 domains that bind phosphotyrosines on RTKs. These subunits derive from three genes: p85α, p55α, and p50α are splice isoforms of the PIK3R1 gene, p85β is the product of the PIK3R2 gene, and p55γ is the product of the PIK3R3 gene36. Each regulatory subunit contains two SH2 domains whose inter-SH2 domain binds the catalytic subunits p110α, p110β, and p110δ. The regulatory subunits hold the catalytic subunits in an inactive conformation, and recruitment to phosphotyrosines in RTK signaling complexes is one mechanism to relieve this inhibition and bring the catalytic subunits to their plasma membrane substrates36. In addition to regulatory subunit recruitment, Ras GTPases also activate class I PI3K activity through binding the catalytic subunit37–39.

Class I PI3Ks phosphorylate the plasma membrane lipid phosphatidylinositol(4,5)-bisphosphate (PIP₂) to form phosphatidylinositol(3,4,5)-triphosphate (PIP₃)36. These lipids serve crucial roles in regulating actin polymerization and gene expression. PIP₃ and, in some cases, PIP₂, can interact with lipid-binding domains, such as the pleckstrin homology (PH) domain, in a variety of proteins to activate their signaling. Notable examples include Akt/protein kinase B, many of the same adaptors that signal in RTK signaling complexes, and GEFs and GAPs that activate and inhibit Ras and Rho family GTPases36. Akt is a serine/threonine kinase that promotes cell survival. Its signaling promotes the expression of anti-apoptotic proteins and degradation of pro-apoptotic proteins. Akt also increases protein translation by signaling the mammalian target of rapamycin (mTOR)40. The PH domains of many GEFs and GAPs have been shown to bind PIP₃ and, in some cases, this binding enhances their
activation\textsuperscript{36}. Subsequent changes in Rho GTPase activity affect actin polymerization and cell migration\textsuperscript{32}, as outlined previously.

As previously stated, Eph receptors are very similar to other RTKs in their signaling activation and properties. They do, however, display many unique features that allow them to serve specific functions, such as their role in contact repulsion secondary to their activation at cell-cell contacts. Details are discussed for the remainder of this section.

\textit{Structural Basis of Eph Receptor Clustering and Forward Signaling}

Like other RTKs, Eph receptors undergo autophosphorylation upon ligand binding and transmit downstream signals through tyrosine phosphorylation of substrates and binding other adaptors. As previously discussed, Eph receptor ligands are membrane-bound and capable of transmitting downstream signals. Another unique feature of Eph receptors is the requirement of receptor clustering for efficient downstream signaling\textsuperscript{11} (Fig. 1.2). This was first demonstrated by Davis \textit{et al.}\textsuperscript{41}, who showed that only membrane-bound or antibody-clustered ephrin ligands were sufficient to activate Eph receptors. Another study showed that dimerized soluble ligands were sufficient to induce Eph receptor autophosphorylation, but larger aggregates were necessary for downstream effector recruitment and to observe expected changes in cell adhesion and migration\textsuperscript{42}. Studies that examined the effect of cluster size, by either antibody-mediated soluble ligand clustering\textsuperscript{42} or chemical cross-linking\textsuperscript{43}, demonstrated that Eph receptor aggregates larger than dimers represent the minimum signaling unit required for Eph receptor signaling. It was also suggested larger clusters transmit
stronger signals\textsuperscript{43}. Studies have shown that different types of Eph receptors, including both EphAs and EphBs, may co-exist in the same cluster, and signaling outputs depend on the relative amounts of each receptor present\textsuperscript{11,44}. Signaling pathways and functional consequences downstream of Eph receptor signaling are, thus, highly complex. The structural basis of ligand-receptor binding, Eph receptor activation, and clustering have been analyzed by a variety of structural studies.

This section will focus primarily on forward signaling, but a brief overview of ephrin reverse signaling is provided here. Both ephrinBs and ephrinAs consist of extracellular receptor-binding domains (RBDs, Fig. 1.2) and bind Ephs with high affinity. A surface plasmon resonance study showed that single ephrin-Eph interactions bind with a dissociation constant on the order of tens of nM\textsuperscript{45}. EphrinBs are transmembrane proteins with unstructured intracellular domains that contain C-terminal PDZ binding motifs and numerous conserved tyrosine residues that are phosphorylated upon activation (Fig. 1.2). It is thought that tyrosine phosphorylation of ephrinBs is carried out by other tyrosine kinases, especially the Src family\textsuperscript{46}. EphrinBs serve many of the same functions as Eph receptors through binding SH2 domain adaptors, such as Grb4, and PDZ domain proteins\textsuperscript{47}. EphrinAs are GPI-linked and entirely extracellular. They associate with co-receptors to transmit downstream signals. For instance, ephrinAs are known to bind and signal through the neurotrophin receptor p75 in retinal axons to direct growth cone steering during axon guidance\textsuperscript{48}.

The general structure of Eph receptors is shown in Figure 1.2. In the extracellular region, EphA and EphB receptors contain an N-terminal ligand-binding domain (LBD), followed by a cysteine-rich domain (CRD) that contains sushi and EGF-like motifs, and
two fibronectin-type III repeats\textsuperscript{10}. The juxtamembrane (JM) region immediately C-terminal to the transmembrane domain contains conserved phosphotyrosine motifs that relieve intracellular autoinhibition of the kinase domain upon phosphorylation\textsuperscript{49}. They are also major binding sites for SH2 domain-containing proteins, though many sites have been identified throughout the intracellular domain\textsuperscript{50–53}. The JM region is followed by the kinase domain, and the C-terminus of Eph receptors consists of a sterile-alpha motif (SAM) and a PDZ-binding motif (PBM).

Eph-ephrin signaling is believed to initiate from heterotetramers consisting of two ephrins and two Ephs. The structure of these heterotetramers was determined by X-ray crystallography of purified complexes containing the ephrinB\textsubscript{2} ECD and the EphB\textsubscript{2} LBD\textsuperscript{54}. The heterotetramers were formed by multivalent interactions that allowed each ephrin to simultaneously bind both Eph receptors, and vice versa, through two interfaces in the Eph LBD and ephrin ECD. One of these interfaces was, of course, composed of the Eph receptor ligand-binding pocket and the ephrin receptor binding domain, and the other was located outside of these regions. Other structural studies observed similar interactions\textsuperscript{55,56}. It is thought that these heterotetramers then arrange into higher-order clusters. Further crystallography studies with larger portions of the Eph receptors’ ECDs predicted residues in the CRD and LBD that mediate \textit{cis} interactions between receptors on the same cell surface\textsuperscript{55,56}. Thus, Ephs may initially bind ephrins in the heterotetramer arrangement and then use the CRD- and LBD-mediated interactions to expand into clusters.

Interestingly, the LBD- and CRD-mediated receptor-receptor interactions were observed in the absence of ligand binding. Overexpression of Eph receptors induced
ligand-independent receptor autophosphorylation that was abrogated by select point mutations in the CRD\textsuperscript{56}. These results suggested that clustering is an intrinsic property of the receptors themselves and may only depend on the concentration of receptors on the cell surface. It is possible that ephrin ligands only serve to increase the local concentration of receptors, or orient the receptors in an appropriate conformation, to promote clustering. This idea is supported by the observation that the Eph receptor ECD undergoes little conformational change after ligand binding\textsuperscript{56}. Additionally, immunofluorescence studies have shown that ephrins may exist in plasma membrane microdomains, and some studies suggest that ephrins associate with lipid rafts\textsuperscript{57,58}. These results suggest that ephrins are closely-packed to begin with. In fact, ephrin receptor-binding domains have been shown to homo-dimerize\textsuperscript{54}. Since Eph receptors can self-associate, it is also possible that both ephrins and Eph receptors exist in an equilibrium between monomers and dimers, and that ephrin dimers can cross-link Eph receptor dimers to result in clustering arrays.

Other domains of Eph receptors, and their effectors, have been shown to modulate Eph receptor clustering. The N-terminal fibronectin type III (FNIII) repeat was shown to contain a receptor-receptor binding interface in EphAs\textsuperscript{55}. Crystal structures of the SAM domain showed the potential for dimerization\textsuperscript{59}, suggesting a role for intracellular regulation of Eph receptor dimerization or clustering. Additionally, studies suggest that interactions between Eph receptors and PDZ domain proteins may promote or reduce Eph receptor clustering and activation. Overexpression of the PDZ domain protein PICK1 with EphB2 in COS7 cells induced EphB2 clustering\textsuperscript{60}. However, deletion of the SAM
and PBM of EphB2 was independently shown to enhance EphB2 clustering and activation\textsuperscript{43}.

Eph receptor clustering results in complex functional consequences. Ephs of different types, including those of different classes, may co-exist in the same signaling complexes. This was demonstrated by co-immunoprecipitation of, and co-localization of clusters containing, EphB2 and EphA3 receptors in HEK293 cells\textsuperscript{44}. Treatment with ephrinA or ephrinB ligands caused phosphorylation of both EphB2 and EphA3, suggesting cross-phosphorylation\textsuperscript{44}. Some data suggest that ephrin stimulation of cells often results in functional outputs that represent a composite of all Eph receptors expressed, and are dependent on the relative expression level of each receptor subtype\textsuperscript{11}. Competition between Eph receptors on the same cell has been shown. Astin \textit{et al.} (2010)\textsuperscript{61} used ephrinA5- and ephrinB2-coated microbeads to stimulate Eph signaling in a prostate cancer cell line. While ephrinA5 caused cell repulsion, ephrinB2 promoted cell migration, and was able to reverse cell repulsion caused by ephrinA5 when both ligands were present on the same beads\textsuperscript{61}.

Given the promiscuity of ligand binding and the heterogeneity of Eph receptor clusters, canonical signaling pathways for each receptor subtype remain elusive\textsuperscript{10}. Adding to this complexity is the cell context-dependence of Eph receptor signaling. An individual Eph receptor subtype may trigger diametrically-opposed responses in different cell types, and the mechanism behind this phenomenon is poorly understood. The following section will outline the regulation, major downstream pathways, and functional consequences of Eph receptor signaling.
**Functions and Mechanisms of Eph Receptor Signaling**

Eph receptors signal the actin cytoskeleton and affect protein expression to regulate cell adhesion, migration, and survival. Eph-ephrin signaling cascades typically mediate repulsive cues, and spatial gradients of Eph and ephrin expression across tissues are regulated over space and time to ensure that cells or cellular processes migrate to the right place at the right time\textsuperscript{10}. This interplay between repulsive cues and spatial regulation has been well-studied in topographic mapping of the central nervous system by Eph receptor-mediated axon guidance\textsuperscript{62-64}. EphA-expressing growth cones in the retinotectal system, for example, were shown to be directed from areas of high ephrinA expression to areas of low expression. This process ensures that axons from a given visual field in the retina reach their appropriate target neurons in the superior colliculus\textsuperscript{64}. As another example, Eph/ephrin signaling is important to establish boundaries between arterial and venous vascular structures\textsuperscript{65}.

Eph and ephrin signaling outputs are highly heterogeneous and cell context-dependent, and Eph receptor signaling sometimes functions to promote cell migration and cell-cell adhesion. For example, EphB4 promotes tumor growth in some breast and lung cancers\textsuperscript{66,67}, but was shown to have tumor-suppressing effects in colorectal cancer\textsuperscript{68}. EphrinA1/EphA2 forward signaling is pro-angiogenic by increasing endothelial cell migration\textsuperscript{69}, but inhibits the migration of prostate cancer and glioma cells\textsuperscript{70}. The cause of this cell context-dependence is unclear. Some studies of Eph receptor signaling to specific downstream effectors have addressed this question, and will be discussed below.

Functional consequences of Eph receptor signaling are primarily mediated by the Rho GTPases Rac1, Cdc42, and RhoA, the Ras family of GTPases, and PI3K\textsuperscript{10}. The
following sections will focus on how Eph/ephrin forward signaling affects the aforementioned pathways to confer cell-cell repulsion or adhesion, and cell survival or death, with illustrative examples. The nature of cell context-dependence will be discussed where appropriate. I will focus on EphB signaling pathways as illustrative examples over EphAs due to the nature of my thesis work.

*Ras Family GTPases*

Unlike many other RTKs, Eph receptors have been shown to induce cell process retraction and reduce cell adhesion and survival by inhibiting R-Ras and Ras/MAPK signaling\(^47\). In general, published literature on this topic does not differentiate between the canonical Ras isoforms H-Ras, K-Ras, and N-Ras, and, as such, the connotation “Ras” will indicate the canonical isoforms collectively and the term “R-Ras” will refer specifically to the R-Ras proteins. A common mechanism among Eph receptors to inhibit Ras and R-Ras is activation of p120RasGAP through binding its SH2 domain\(^47,71,72\). EphB2 induced repulsive responses in COS-1 cells and neurite retraction in EphB2-expressing neuroblastoma (NG-108) cells by activating p120RasGAP\(^50,71,72\). Since the Ras/MAPK pathway acts downstream of many RTKs, Eph receptor signaling can modulate outputs from other RTKs. For example, Eph receptor signaling was shown to inhibit MAPK activation by the platelet-derived growth factor receptor (PDGFR) and the vascular endothelial growth factor receptor (VEGFR), among others\(^73\).

EphB2 has also been shown to reduce cell adhesion by inhibiting R-Ras through p120RasGAP or direct tyrosine phosphorylation\(^72,74\). Inhibition of R-Ras by EphB2 was shown to reduce adhesion of cells to substrates containing integrin ligands\(^74\), suggesting
R-Ras inhibition as a means for Eph receptors to inhibit integrin-mediated adhesion. This aspect of EphB2/R-Ras signaling may be relevant to cancer biology. A study of glioma cell migration revealed that EphB2-mediated R-Ras tyrosine phosphorylation was associated with reduced adhesion and increased invasion\textsuperscript{75}. This study, however, characterized R-Ras tyrosine phosphorylation as activating, though there was no data to show an increase in GTP-bound R-Ras.

In some contexts, Eph receptors may activate Ras/MAPK signaling. Both EphA2 and EphB1 increased MAPK signaling through binding the SH2 domain adaptor Grb2 and members of the Shc family of SH2 domain adaptors, albeit with different functional outputs- EphA2-mediated signaling decreased ECM attachments, while EphB1 signaling promoted chemotaxis. In both cases, MAPK activation and Grb2 recruitment were dependent on engaging Shc-family adaptors\textsuperscript{76,77}. Grb2 is known to associate with the Ras-GEF SOS, and presumably this mechanism is responsible for MAPK activation\textsuperscript{76}.

Some studies have explored the mechanism underlying this cell context-dependence. Signaling from other RTKs may influence the effect of Eph receptors on Ras. For example, increased FGFR signaling was shown to switch the effect of EphB2 from activation to inhibition of MAPK signaling\textsuperscript{78}. Differences in downstream effectors have also been suggested to play a role. For example, a study of EphB4 signaling in human umbilical vein endothelial cells found that EphB4 inhibited Ras/MAPK signaling by engaging p120RasGAP. However, EphB4 increased Ras activity in MCF7 breast cancer cells in a manner that depended on protein phosphatase 2 (PP2A)\textsuperscript{79}. The respective effects on Ras activity were abrogated by knocking down p120RasGAP or PP2A.
Eph receptors may also activate or inhibit the Ras-related GTPase Rap1 in a cell context-dependent manner. Briefly, Rap1 signaling overlaps with the other Ras superfamily GTPases, but is uniquely involved in regulating cell-cell junctions, largely through its effector, afadin\textsuperscript{80}. In fact, afadin can bind Eph receptors as well\textsuperscript{81}. Eph receptors typically activate Rap through the GEF C3G, which associates with the SH2 adaptors Crk and CrkL\textsuperscript{10,82}. EphB signaling was shown to activate Rap through direct recruitment of Crk to increase membrane ruffling and cell adhesions in endothelial cells\textsuperscript{83}. In some contexts, however, Crk and CrkII are inhibited by recruitment of the NRTK Abl to Eph receptors, which deactivates Crk by phosphorylation and disrupts its effector binding\textsuperscript{67,82}. In neurons, EphA4 inhibits Rap through PBM-mediated recruitment of the Rap-GAP SPAR\textsuperscript{84}.

\textit{Rho Family GTPases}

Eph receptors may also initiate cell-cell repulsion by activation of RhoA and inhibition of Rac1 and Cdc42, resulting in acto-myosin contractility to inhibit protrusive structures and cell migration\textsuperscript{73,85}. EphAs are known to induce growth cone collapse, alter vascular smooth muscle contractility, and reduce T cell chemotaxis by activating RhoA through the ephexin family of RhoGEFs\textsuperscript{86,87}. In growth cone collapse, transient Rac1 inhibition is accomplished through recruitment of the Rac1 GAP α2-chimaerin through binding its SH2 domain\textsuperscript{88}. Both ephexin and α2-chimaerin are tyrosine phosphorylated by the Src family kinases in this context\textsuperscript{88,89}. However, growth cone collapse requires Rac1 activity to endocytose the Eph/ephrin complexes, and recruitment of the GEF Vav2 to EphA4 was suggested to play a role\textsuperscript{90}. EphBs are also known to activate RhoA to
induce growth cone collapse\textsuperscript{91,92}, synaptogenesis\textsuperscript{17}, and increase migration of invasive melanoma cells\textsuperscript{93}, though the specific GEFs involved are unknown.

Eph receptors are also known to promote cellular protrusions and cell migration by favoring Rac1/Cdc42 signaling over RhoA in some contexts. In addition to mediating endocytosis during growth cone collapse, Vav-mediated Rac1 activation is also important to induce endothelial cell migration in EphA-dependent angiogenesis\textsuperscript{94}. Another study showed PI3K upstream of Rac1 in this process, and Cdc42 activation was observed as well\textsuperscript{69}. In glioma cells, EphA4 overexpression was associated with increased migration and proliferation due to Rac1 and Cdc42 activation\textsuperscript{95}, though EphA4 showed interactions with FGFR in this context, and the effects may have been indirect. EphB signaling increased membrane ruffling and cell adhesions in endothelial cells by activating Rac1 via the GEF DOCK1 downstream of Crk\textsuperscript{83}. EphBs, as discussed in a later section, also interact with the Rac-GEFs kalirin-7 and Tiam1 and the Cdc42-GEF intersectin to promote dendritic spine formation in hippocampal neurons\textsuperscript{12}. An interaction between EphB2 and the Rac-GEF α-Pix\textsuperscript{96}, mediated by the adaptor protein Nck, has been demonstrated and also suggests Rac1 and Cdc42 activation by EphBs. In prostate cancer cells, EphAs and EphBs were shown to differentially regulate contact inhibition of locomotion through selective Rho GTPase activation\textsuperscript{61}. EphAs induced cell process retraction through RhoA, and EphBs increased cell migration through Cdc42.

*Phosphoinositide 3-kinase*

Eph receptors also display highly context-dependent regulation of class I PI3K activity to promote or suppress cell survival and migration. The general functions of Eph
receptors to mediate repulsive cues and inhibit Ras are consistent with inhibition of PI3K signaling. EphB2, for example, was shown to inhibit PI3K signaling pathways in multiple contexts. EphrinB1 stimulation of EphB2 and EphB4 in medulloblastoma cells decreased cell adhesion and levels of phosphorylated mTOR\textsuperscript{97}, indicating reduced PI3K/Akt signaling. A recent study of chemotaxis in MTLn3 breast cancer cells overexpressing EGFR showed down-regulation of PI3K activity by EphB2 that opposed EGFR-mediated chemotaxis\textsuperscript{98}. However, EphB signaling is known to activate PI3K in other contexts. Intrathecal and peripheral injections of ephrinB1-Fc in living mice induced hyperalgesia that was dependent on increased expression and activity of PI3K catalytic subunits\textsuperscript{99,100}. EphB2-mediated activation of $\alpha$-Pix through PI3K was demonstrated in 293T cells\textsuperscript{96}. Additionally, EphB2 was shown to inhibit Tau phosphorylation, which promotes the pathogenesis of Alzheimer’s Disease, in mature hippocampal neurons by activating PI3K signaling\textsuperscript{101}. 

Little is known about how PI3K is activated or inhibited downstream of EphBs. Inhibition may be mediated by inhibition of Ras family GTPases, which are known PI3K activators\textsuperscript{37}. Activation may occur through recruitment of regulatory subunits, suggested by an \textit{in vitro} interaction reported between phosphorylated EphB2 and the SH2 domain of p85 ($\alpha$ or $\beta$ not specified)\textsuperscript{50}. A study of EphB2 signaling in colonic crypt cells \textit{in vivo} showed that EphB2 promotes PI3K-dependent cell migration in an EphB2 kinase-independent manner\textsuperscript{102}. Kinase-independent signaling would suggest a mechanism independent of SH2 binding and, perhaps, constitutive association with a PI3K activator.

Other Eph receptors present a similarly complex story. EphA2, for example, was shown to promote vascular endothelial cell migration through PI3K following ephrinA1
stimulation. Similar treatment of glioma and prostate cancer cells, however, caused reduced cell migration secondary to PI3K inhibition. In the same study, overexpression of EphA2 was shown to increase migration in these cells in a ligand-independent manner, suggesting PI3K activation in the absence of kinase activity. These results are strikingly similar to the aforementioned observations of kinase-independent PI3K activation by EphB2 in colonic crypts, raising the possibility of a common mechanism. However, PI3K activation is largely thought to occur by regulatory subunit recruitment or Ras activation, both of which typically require RTK activation.

**Abelson family kinases**

The Abelson family kinases Abl and Abl2/Arg are non-receptor tyrosine kinases (NRTKs) that act as regulators of the cell cycle and actin cytoskeleton organization. Kinase activation is achieved by engagement of their SH2 domains, typically through binding RTKs, or SH3 domains. Arg is of particular interest because it is enriched in dendritic spines and is known to maintain dendritic spines in adult mice. The SH2 domains of Abl and Arg were shown in a yeast-two-hybrid assay to bind the phosphorylated juxtamembrane tyrosine residues of EphB2. Their results were confirmed by *in vitro* pull-down assays. This study also demonstrated a positive feedback loop between EphB2 and Arg, whereby EphB2 phosphorylates Arg, which may then phosphorylate EphB2. Interestingly, no follow-up work has been done to study this interaction further, though some studies have characterized Abl as an important effector of Eph receptors. Previous sections described Eph receptor-mediated inhibition of the adaptor Crk through Abl, which inhibited downstream Rho and Rap GTPases. In
intestinal crypts, EphB signaling promoted proliferation of epithelial cells through Abl-mediated activation of cyclinD1, a key regulator of the cell cycle\textsuperscript{102}. Abelson family kinases also interact with EphA4, as shown by the aforementioned yeast-two-hybrid study\textsuperscript{104}. This interaction is also of functional significance, because the Abelson family kinase inhibitor STI571 blocked retinal growth cone collapse downstream of EphA4 activation\textsuperscript{105}.

\textit{Catalytically-inactive Eph receptors}

Two members of the Eph receptor family, EphB6 and EphA10, are catalytically-inactive owing to mutations in conserved regions of their tyrosine kinase domains. Nonetheless, they contain their own signaling activities and contribute to the complexity and cell context-dependence of Eph receptor signaling\textsuperscript{106}. Studies, primarily of EphB6 signaling, have shown that catalytically-inactive Eph receptors associate with, and are tyrosine phosphorylated by, catalytically-active Eph receptors and other non-receptor tyrosine kinases. EphB1 and EphB4 bind and tyrosine phosphorylate EphB6, as do Src family kinases\textsuperscript{107–109}. EphB6 is thought to be tumor-suppressive, as studies in various forms of cancer showed an inverse relationship between EphB6 expression levels and tumor invasiveness, particularly in breast cancer\textsuperscript{106,110}. A mechanistic explanation for these observations was described in a study that showed competition between EphB6 and EphB4 in modulating the invasiveness of breast cancer cells. By signaling c-Cbl and Abl in an EphB4-dependent manner, EphB6 promoted breast cancer cell adhesion to fibronectin and reduced invasiveness in a Matrigel assay. This opposed the pro-invasive
effects of EphB4 and suggested that catalytically-inactive Eph receptors can associate with other Ephs to antagonize downstream signaling pathways.

Unlike EphB6, EphA10 is thought to act as a tumor promoter. Though the signaling pathways remain uncharacterized, its expression is up-regulated in multiple highly-invasive and metastatic cancers. Normally, EphA10 is silenced in most tissues except for the testes, and it is thought that up-regulation of gene expression in some cancers may play a role in increasing invasiveness.

Negative regulation of Eph receptor signaling

The primary mechanisms by which Eph/ephrin signaling is down-regulated are endocytosis of the receptors, proteolytic cleavage of Eph and ephrin extracellular domains, and de-phosphorylation of phosphotyrosines. Receptor internalization by endocytosis is a typical outcome of RTK signaling. Once internalized, receptors may be shunted to the endosomal pathway for degradation, or they can be recycled. Eph/ephrin complexes display trans-endocytosis, in which the Eph- or ephrin-expressing cell internalizes the entire Eph/ephrin complex. This process depends on Rac1 activity, and some data suggest that the Vav family of GEFs is specifically responsible for Rac1 activation that leads to trans-endocytosis. The ubiquitin ligase Cbl, which contains SH2 domains, is also known to bind Eph receptors and contribute to their internalization and degradation in some contexts.

In some cases, extracellular proteolytic cleavage of Ephs or ephrins precedes internalization. Members of the matrix metalloproteinase (MMP) family and ADAM10 (a disintegrin and metalloproteinase 10) have been shown to cleave both Ephs
and ephrins to down-regulate signaling\textsuperscript{112}. MMPs are secreted or cell membrane-bound proteases that typically act on ECM or other membrane-bound proteins\textsuperscript{112}. MMP family members have been shown to cleave members of the EphA and EphB subfamilies, and cleavage sites identified in EphA2 and EphB2 lie within the FNIII repeat domains\textsuperscript{92,113}. ADAM family proteases are membrane-bound and known to act on growth factor receptors, cell adhesion molecules, and other membrane-bound proteins\textsuperscript{112}. One member of the disintegrin and metalloproteinase (ADAM) family, ADAM10, has been shown to cleave ephrin ECDs in numerous contexts\textsuperscript{112}. Studies have shown that ADAM10 weakly associates with Eph receptors, and that this association strengthens with Eph receptor activation and allows it to cleave ephrin ECDs in \textit{trans}\textsuperscript{112}.

Proteolytic cleavage and/or internalization of the Eph/ephrin signaling complexes are thought to be necessary to convey repulsive cues in response to Eph/ephrin signaling\textsuperscript{47,92,111,112}. A study by Lin \textit{et al.} (2008)\textsuperscript{92} showed that retraction of HEK293 cells and growth cone collapse were inhibited by mutating EphB2 to render it resistant to proteolytic cleavage. Pharmacologic inhibition of MMP-2 and MMP-9 produced similar results. In another study, overexpression of dominant-negative Rac1 in human umbilical vein endothelial cells prevented \textit{trans}-endocytosis of ephrinB2/EphB4 complexes and cell retraction\textsuperscript{111}. However, it was unclear if abrogation of cell retraction was due to the lack of endocytosis or the reduced Rac1 activity. If endocytosis or proteolytic cleavage is required to render cell-cell separation, it would be interesting to know if the persistence of complexes leads to signaling that promotes cell-cell adhesions, or if signaling during endocytosis somehow imparts repulsive cues.
Like most other RTKs, tyrosine phosphorylation of Eph receptor ICDs is negatively regulated by a variety of tyrosine phosphatases. Major phosphatases that have been identified include low molecular weight protein tyrosine phosphatase (LMW-PTP), protein tyrosine phosphatase, receptor type O (PTP-RO), and protein tyrosine phosphatase 1B (PTP1B)\(^{37,114}\). PTP-RO is thought to selectively de-phosphorylate juxtamembrane tyrosine residues in both EphAs and EphBs\(^{115}\), suggesting a role in negative feedback of tyrosine kinase activity. Phosphatase activity may additionally play a role in directing adhesive or repulsive outcomes. A study of EphA3 signaling in pre-B leukemia cells suggested that increased endogenous activity of PTP1B changed the response from repulsion to adhesion\(^{116}\).

*Experimental techniques to cluster and activate Eph receptors*

The requirement for receptor clustering in Eph receptor signaling has led many groups to develop unique methods of inducing receptor clustering *in vitro*. Use of antibody-clustered soluble ephrin ligands has been the most widely-used technique. To accomplish clustering, the ECDs of ephrins are ligated to the crystallizable fragment (Fc) of human IgG. These chimeras are dimerized by Fc cross-linking. Pre-clustering of these dimeric ephrin-Fc chimeras by anti-Fc antibodies is done prior to treatment of cell cultures\(^{11}\). An early study demonstrated an array of ephrin cluster sizes with antibody-mediated clustering, with tetramers being the most effective to induce signaling\(^{42}\).

Multiple studies have demonstrated that freely-diffusing ephrins are also sufficient to activate Eph receptors. Co-culture studies of ephrin-expressing and Eph-expressing cells, or of cell lines that contain an appropriate Eph/ephrin combination, have
shown contact-dependent activation of Eph receptors\textsuperscript{60}. In addition, studies have also shown that lipid substrates functionalized with ephrin ECDs are also sufficient to activate Eph receptor signaling in cells plated on top of them\textsuperscript{85}. This may reflect the aforementioned observations that ephrins are capable of self-dimerization\textsuperscript{54}, and perhaps they may cluster on their own.

Both of the techniques described above have been used to provide a plethora of information about Eph receptor signaling. However, spatial and temporal regulation of signaling molecules are important to direct cell migration. The nature of Eph/ephrin activation at cell-cell contacts necessitates sub-cellular regions of activation. Thus, prior attempts have been made to achieve spatial and temporal control over Eph receptor clustering. A study of contact inhibition of locomotion in prostate cancer cells simulated the nature of cell-cell contacts by functionalizing microbeads, which were coated with anti-Fc antibodies, with dimeric ephrin-Fc chimeras\textsuperscript{61}. Since these microbeads are much smaller than cells, these provided good models of sub-cellular cell-cell contacts and imparted some spatial regulation of signaling. Chemical induction of Eph receptor clustering was recently developed by inserting a variable number (one to three) of FK506 binding protein (FKBP) domains between the transmembrane and juxtamembrane regions of EphB2 and treating with the cross-linking drugs AP20187 or AP1887\textsuperscript{43}. Depending on the number of FKBP domains inserted into the Eph receptor sequence, cross-linking produced clusters of different sizes. This tunability of cluster size allowed the group to demonstrate that larger Eph receptor clusters generate stronger signals, and to provide further evidence that aggregates larger than dimers are necessary for efficient
signaling. This technique allowed some degree of temporal control over signaling, though drug treatments often exceeded 20 minutes, and there was no spatial control.

Each of these techniques offered their own advantages in controlling Eph receptor signaling variables, though there is a role for additional techniques to combine multiple effects to achieve simultaneous spatial and temporal control. Optogenetic techniques, specifically those that induce protein-protein interactions, have been used to gain spatial and temporal control over related receptor systems and cell signaling pathways. Many applications have used the A. thaliana photoreceptor Cryptochrome 2, which will be described in the next section.

**Cryptochrome 2 in Optogenetics**

Optogenetics describes the use of genetically-encoded, light-sensitive proteins to control cellular signaling *in vitro* or *in vivo* using light, which is desirable to impart spatial and temporal control over signaling. Perhaps most famously, use of light-sensitive ion channels known as channelrhodopsins or halorhodopsins have been useful for *in vivo* applications in neuroscience, such as mapping and analysis of neural circuits. This discussion will focus on Cryptochrome 2, a blue light-sensitive photoreceptor from *Arabidopsis thaliana* that has been used to control protein-protein interactions with light.

**Cryptochrome 2 and CIB1 in optogenetics**

Cryptochrome 2 (Cry2) is a photoreceptor derived from *Arabidopsis thaliana* that undergoes a yet-uncharacterized blue light-induced conformational change that allows it
to bind the transcription factor CIB1\textsuperscript{119}. This property is imparted by its N-terminal photolyase homology region (PHR), which contains a flavin adenine dinucleotide (FAD) chromophore that is responsible for blue light absorption. The protein also contains a C-terminal nuclear localization sequence (NLS), which is mutated or truncated for optogenetic applications\textsuperscript{120}.

Blue light-induced Cry2-CIB1 binding has been used in mammalian cells to control protein translocation (Fig. 1.4, Movie 1.1) and protein-protein interactions using light. Optogenetic applications of the Cry2-CIB1 system were first demonstrated by Kennedy et al.\textsuperscript{120}, who modified CIB1 by truncation (CIBN) to remove DNA-binding domains. It was found that both truncated Cry2, leaving just the PHR, and full-length Cry2 (with a mutated NLS) bound CIBN upon blue light illumination. Recruitment of Cry2 to the plasma membrane was demonstrated by tagging CIBN with a C-terminal CAAX (Ras) domain (Fig. 1.4). Analysis revealed seconds-timescale Cry2 recruitment, with maximal plasma membrane signal \(\sim \)15 seconds following a single photoactivation pulse. The off-rate, however, was slow, as cytoplasmic recovery required \(\sim \)12.5 minutes\textsuperscript{120}. The functionality of these blue light-induced Cry2-CIBN interactions was demonstrated by blue light-induced gene expression using a split Gal4 promoter. The DNA-binding domain of the promoter was ligated to Cry2, and the activation domain was ligated to CIBN. A similar technique was used to achieve optogenetic control of Cre recombinase by splitting the enzyme\textsuperscript{120}.

Since this initial study, many groups have used the blue light-induced Cry2-CIBN binding to recruit proteins to the plasma membrane\textsuperscript{120} (Fig. 1.4, Movie 1.1) or control protein-protein interactions. In general, CIBN is tagged to the plasma membrane as
shown in Fig. 1.4, and Cry2 is fused to a protein of interest in the cytoplasm. Much attention has been paid to optogenetic manipulation of membrane lipids. For example, Idevall-Hagren et al.\textsuperscript{121} used a fusion of Cry2 and phosphoinositide-5-phosphatase to recruit the phosphatase to the plasma membrane and dephosphorylate PIP\textsubscript{2} to phosphatidylinositol-4-phosphate, PI(4)P. PIP\textsubscript{3} synthesis was also demonstrated by inducing plasma membrane recruitment of the inter-SH2 domain of the PI3K regulatory subunit (illustrated in Fig. 1.4). The iSH2 domain, in this case, is bound to endogenous PI3K catalytic subunits, which are activated upon plasma membrane recruitment. This technology was applied to show the effect of PI3K signaling on actin in axons and growth cones\textsuperscript{122}. Photoactivatable Akt, a downstream effector of PI3 kinase, was developed by ligating Akt to Cry2 and activating it by plasma membrane translocation\textsuperscript{123}.

Use of the Cry2-CIBN system has not been limited to membrane lipids. Recently, molecular motors were recruited to intracellular vesicles to control anterograde or retrograde transport and their intracellular distribution\textsuperscript{124}. By using the previously-described\textsuperscript{120} split galactosidase promoter, \textit{in vivo} optogenetic control of gene transcription was achieved in zebrafish\textsuperscript{125}. Optogenetic inhibition and sequestration of proteins have also been explored. Recruitment of RGS proteins to the plasma membrane achieved optogenetic inactivation of G protein-coupled receptors\textsuperscript{126}. Lee \textit{et al.}\textsuperscript{127} reported ligating CIB1 to a multimeric protein, in this case CaMKII, for blue light-controlled sequestration of a target protein ligated to Cry2. This technique was dubbed light-activated reversible inhibition by assembled trap (LARIAT) and was successfully applied to tubulin, Vav2, Tiam1, Rac1, RhoG, and Cdc42\textsuperscript{127,128}. 
Use of the Cry2-CIBN optogenetic module resulted in an innovative optogenetic toolkit for a wide variety of applications. The rapid timescale of Cry2-CIBN interactions and the ability to spatially define patterns of stimulation proved highly desirable to study cell signaling. However, these modules required overexpression of two artificial fusions, which may have affected baseline cell behavior or caused technical problems with transfection, especially in the case of large proteins. The next section will discuss the unique properties of Cryptochrome 2 that have permitted its use as a single-component module for optogenetic control of protein-protein interactions.

*Cryptochrome 2 clustering in optogenetics*

Recently, Cry2 was demonstrated to undergo blue light-induced clustering as a cytoplasmic Cry2-mCherry fusion (Fig. 1.4, Movie 1.2). This clustering response was rapid- in response to repeated pulses of blue light, Cry2 clusters appeared within 10 seconds and reached their half-maximal density in ~30 seconds. The recovery rate, however, was much slower, as clusters dissipated with a ~5.5-minute time constant (exponential decay). The significance of this clustering for optogenetics was demonstrated by controlling activation of β-catenin through clustering of an upstream protein, LRP6c, and activation of Rac1 and RhoA through clustering. These techniques represented single-component optogenetic activation of intracellular proteins, a primary advantage of using Cry2 alone instead of the Cry2-CIBN module. However, high concentrations of wild-type Cry2, or Cry2PHR, were necessary to observe efficient clustering. A later study reported that an E490G mutation in Cry2 (Cry2olig) enhanced blue light-induced clustering, though the half-time of cluster recovery was
extended from \(~5.5\) minutes to \(~23\) minutes in the cytoplasm\textsuperscript{130}.

In light of these discoveries, numerous applications that exploit this clustering property have been explored. An assay dubbed light-induced co-clustering (LINC) was developed in conjunction with Cry2olig to query protein interactions by ligating the “bait” to Cry2olig-mCherry and the “prey” to another fluorescent protein\textsuperscript{130}. In response to blue light, the Cry2-fused bait clusters and, if the bait and prey interact, binds the prey by avidity, causing co-localizing clusters. Fluorescence recovery after photobleaching was demonstrated in conjunction with LINC (LINC-FRAP) to study the kinetics of binding. In another study, activation of C-RAF was demonstrated by Cry2-mediated clustering, but was also shown to occur via Cry2-CIB1 interactions\textsuperscript{131}.

Most recently, Cry2 clustering has been used to optogenetically control signaling from transmembrane proteins. Receptor tyrosine kinases, for instance, are endogenously activated by ligand-induced dimerization and, in some cases, clustering. Blue light-induced activation of Trk receptors and FGFRs was achieved by ligating a Cry2-FP fusion to the C-terminus of these RTKs\textsuperscript{132,133}. Another strategy, called clustering indirectly using Cryptochrome 2 (CLICR), uses a two-component system to induce clustering and activation of transmembrane proteins (illustrated in Fig. 1.4). Cry2 resides in the cytoplasm, fused to a protein or protein domain that interacts weakly with the receptor of interest. Blue light-induced clustering, by avidity, then causes binding of these components and clustering of the transmembrane receptor. This was demonstrated using \(\beta\)-catenin signaling and receptor tyrosine kinases, the latter by a cytoplasmic Cry2-SH2 domain fusion\textsuperscript{134}.
Chapter II of this thesis reports the development of a Cry2-based optogenetic tool for Eph receptor signaling. This tool was used to study Eph receptors in the context of synaptogenesis at dendritic spines. The following section provides an overview of the biology and development of dendritic spine synapses, and current knowledge of Eph receptor, particularly EphB, signaling in this field.

**Dendritic Spines and Filopodia**

Dendritic spines are the mushroom-shaped protrusions of neuronal dendrites that comprise the post-synaptic compartments of most excitatory, glutamatergic synapses in the central nervous system (CNS)\(^1\). Structurally, dendritic spines contain a bulbous head that is attached to the dendritic shaft by a thin neck, and this structure is maintained by a dense actin cytoskeleton. This morphology allows spines to compartmentalize post-synaptic proteins and locally restrict post-synaptic signaling and electrical activity\(^2,135\)\(^.\) Post-synaptic molecules, including glutamate receptors, cell adhesion molecules, and important post-synaptic scaffolds, are concentrated at the post-synaptic density (PSD), which is directly opposed to the pre-synaptic axon\(^135\)\(^.\)

Over time, dendritic spines undergo a phenomenon termed synaptic plasticity, defined as changes in synaptic strength. Synaptic strength is modified by changes in spine head size and the density of glutamate receptors, which affects electrical conduction. Long-term potentiation (LTP) describes synaptic strengthening by spine head enlargement and increased glutamate receptor content at the PSD. Long-term depression (LTD) is the weakening or loss of a spiny synapses\(^135,136\)\(^.\) LTP and LTD are
thought to play important roles in memory formation and loss, respectively, *in vivo*. Indeed, studies performed *in vivo* have shown changes in spine turnover and morphology following sensory stimulation and motor learning\(^{137-139}\).

Dendritic spine density and shape are tightly regulated throughout development and adulthood in humans to ensure normal learning and cognition. Abnormalities in spine density and morphology are frequently associated with neurological disorders\(^3\). For instance, Alzheimer’s Disease, a neurodegenerative disorder in older adults characterized by progressive dementia, is associated with abnormal loss of dendritic spines as an individual ages. Fragile X syndrome, a genetic disorder that causes intellectual disability, is associated with an abnormally high density of spines that do not have well-formed spine heads\(^3\). Thus, studying the mechanisms of spine formation, maintenance, and plasticity is crucial to understanding the underlying pathogenesis of, and developing treatments for, many neurological diseases.

*Dendritic filopodia*

Dendritic spines are thought to derive from dendritic filopodia, the long, thin, actin-based protrusions present on the dendrites of developing neurons\(^{135}\). They are dynamic structures that have been observed to extend, retract, and bend on a timescale of minutes\(^4,140\). The idea of dendritic filopodia as spine precursor structures was first suggested by observations of filopodia in cultured hippocampal neurons by Ziv and Smith in 1996\(^4\). Some filopodia, though typically dynamic, were observed to stabilize upon contact with axons. Additionally, filopodia density was highest during the first week *in vitro* and decreased during the second week, while dendritic spine density
increased concurrently. Electron microscopy studies of brain slices by Fiala et al. two years later reported synaptic structures juxtaposed to contacts between dendritic filopodia and axons. Thus, conventional wisdom holds that dendritic filopodia are dynamic structures that explore the area surrounding dendrites in search of axons. A subset of filopodia becomes stabilized upon making contact, and these filopodia ultimately transform into dendritic spines.

Further studies of the actin structures and dendritic filopodia and spines provide more evidence supporting this model. At the cytoskeletal level, dendritic filopodia resemble dendritic spines more than “conventional” filopodia associated with growth cones or lamellipodia. Electron microscopy studies by Korobova and Svitkina (2010) showed that, unlike conventional filopodia, dendritic filopodia display some actin branching and do not contain the actin-bundling protein fascin. Not surprisingly, given the presence of actin branching, the Arp2/3 complex is present in dendritic filopodia, as are myosin and capping protein. This structure stood in stark contrast to the long, parallel bundles of actin typically observed in conventional filopodia, but compared well to the actin structure of dendritic spines. Spine heads contained highly-branched actin networks, with higher levels of the Arp2/3 complex and capping protein relative to the spine neck. Perhaps the machinery necessary to form the highly-branched dendritic spine head, including Arp2/3 and capping protein, is present on dendritic filopodia to “prime” them for spine formation.
**Dendritic spine morphogenesis from dendritic filopodia**

Mature dendritic spines are marked by a well-formed spine head and the presence of post-synaptic scaffolding proteins, such as PSD-95, and the NMDA- and AMPA-subtypes of glutamate receptors (NMDAR, AMPAR) in the PSD. Studies showing the similarity in actin structure between dendritic filopodia and spines underscore the importance of actin reorganization to dendritic spine formation and synaptic plasticity. Spine head formation and enlargement are directed by increased actin polymerization in spines. In particular, nucleation of highly-branched dendritic actin through activation of the Arp2/3 complex is important in the formation and expansion of spine heads. Additionally, actin directs the organization of post-synaptic proteins at the PSD and membrane recycling of AMPARs, whose density in the PSD is correlated with synaptic strength.

Certain patterns of neural activity are known to trigger synaptic plasticity. For the sake of simplicity, spiny synapses that receive frequent glutamatergic input undergo maturation, or LTP, to become stronger and longer-lasting. Glutamatergic activity is also thought to account for initial formation of the spine head. Glutamate binding to NMDARs, which are ionotrophic receptors, is thought to direct spine formation and LTP, in part, through an influx of extracellular calcium. This causes activation of calcium/calmodulin-dependent protein kinase II, a master regulator of signaling cascades in dendritic spines that direct actin reorganization via the Rho GTPases Rac1, Cdc42, and RhoA. This is accomplished through interacting with and phosphorylating GEFs.

Most signaling pathways that alter actin polymerization in dendritic spine morphogenesis and plasticity converge on the Rho GTPases Rac, Cdc42, and RhoA.
which also mediate filopodia formation and motility. Overexpression of dominant-negative Rac and Cdc42 mutants in cultured neurons reduced spine density and caused immature dendritic protrusion morphologies\textsuperscript{15,147}. Rac and Cdc42 are known to mediate dendritic actin nucleation through the activation of Arp2/3 via the nucleation-promoting factors WAVE1 and N-WASP, respectively\textsuperscript{135,146–148}. Their effector p21-associated kinase (PAK) also positively influences dendritic spine formation\textsuperscript{6}. RhoA-mediated inhibition of the actin-severing protein ADF/cofilin was shown to be critical for LTP induction\textsuperscript{135}. RhoA is also known to signal myosin IIb through the activity of Rho-associated protein kinase (ROCK). Inhibition of myosin IIb by blebbistatin caused replacement of spines by dendritic filopodia, suggesting an important role in maintaining spine morphology\textsuperscript{149}.

It was observed that only a subset of dendritic filopodia that come into contact with axons form stable contacts\textsuperscript{4}. A study\textsuperscript{8} that imaged a fluorescent calcium indicator in hippocampal slices revealed that stable contact formation between filopodia and axons was associated with a high frequency of local dendritic calcium transients. Surprisingly, both contact stabilization and calcium transients occurred in the presence of NMDAR inhibitors\textsuperscript{8}. Since contact seemed to be a triggering event for filopodia stabilization, these results suggested a role for cell-cell recognition molecules in not only inducing these calcium transients, but also for directing contact stabilization and dendritic spine formation. A role for Eph receptors\textsuperscript{12}, which were described in an earlier section, in dendritic spine formation was identified years before these results were published. This is discussed in the following section.
EphB signaling in dendritic spine morphogenesis

A synaptic function of EphBs was first suggested when the NMDAR subunit NR1 was shown to immunoprecipitate with EphB2 from whole rat cortex and ephrinB1-stimulated cultured cortical neurons\textsuperscript{20}. A later study established a role for EphBs in dendritic spine development by observing reduced dendritic spine density in hippocampal neurons overexpressing kinase-inactive EphB2. This study also showed that EphB2-mediated tyrosine phosphorylation of the proteoglycan syndecan-2 promoted spine formation\textsuperscript{13}. A follow-up study demonstrated severely impaired dendritic spine formation in dissociated primary hippocampal neurons and hippocampal slices from EphB1/B2/B3 triple-knockout (EphB TKO) mice\textsuperscript{14}. Interestingly, cultured neurons from single EphB (EphB1, B2, or B3) knockout mice showed no defects in spine formation or morphology, and double knockouts (different combinations of EphB1-B3) showed defects less severe than in triple knockouts. Imaging of hippocampal slices revealed that triple EphB knockout was necessary to reduce dendritic spine density \textit{in vivo}. These results suggested that the various EphB isoforms show some redundancy in synaptogenesis. Additionally, treatment of cultured neurons with pre-clustered ephrinB ligands increased dendritic spine density and spine head width, indicative of spine formation and maturation, respectively\textsuperscript{15}.

Studies of the signaling pathways responsible for EphB-induced dendritic spine morphogenesis have converged on regulation of the Rho GTPases Rac1, Cdc42, and RhoA. A summary is provided in Fig. 1.6. EphB2 has been shown to interact with the Rac1 GEFs Tiam1 and kalirin-7 to affect dendritic spine morphogenesis and maturation, respectively\textsuperscript{15,150}. Treatment of cultured hippocampal or cortical neurons with ephrinB
ligands caused recruitment of these GEFs to EphB2 clusters\textsuperscript{15,147,150}. These interactions depended on an intact EphB2 intracellular domain, but did not require the PDZ binding motif, despite the fact that Tiam1 contains a PDZ domain\textsuperscript{151} and kalirin-7 has a PBM that binds other synaptic PDZ domain adaptors\textsuperscript{152}. It was recently shown that Tiam1 exists in a complex with the Rac1-specific GAP Bcr, and EphB2 disrupts this complex to increase Rac1 activation\textsuperscript{153}. Interestingly, Tiam1 and kalirin-7 are both tyrosine phosphorylated following EphB stimulation, though kalirin-7 showed no change in activity with this modification\textsuperscript{15,150}. It is thus questionable whether or not EphB2 stimulation of dendrites and interactions with GEFs and GAPs directly leads to Rac1 activation. Use of the Raichu-Rac1 FRET sensor showed dendritic Rac1 activation with ephrinB ligand treatment\textsuperscript{153}. In another study, however, lysates of hippocampal neurons treated with ephrinB ligands showed no increase in active Rac1\textsuperscript{147}. Thus, the functional consequences of the interactions between EphB2 and Tiam1 or kalirin-7 are incompletely understood.

Biochemical analysis of hippocampal lysates demonstrated Cdc42 activity with ephrinB treatment\textsuperscript{147}. EphB2 was shown to recruit the Cdc42-specific GEF intersectin into a complex with Cdc42 and N-WASP\textsuperscript{147}, which then promotes F-actin nucleation. Intersectin-1 was shown to bind syndecan-2\textsuperscript{147}, suggesting a mechanistic link between the aforementioned interaction between EphB2 and syndecan-2 and dendritic spine morphogenesis. The adaptor protein Numb was also shown to bind intersectin-1 and complex with EphB2 and NMDARs\textsuperscript{147,148}, indicating that EphB2 may coordinate changes in actin polymerization with key electrophysiological elements of spine formation.

Signaling from EphB2 to RhoA in dendritic spine morphogenesis remains controversial. EphB2 signaling was shown to cause degradation of the RhoA-specific
GEF ephexin5 by UBE3A, thus promoting dendritic spine morphogenesis\textsuperscript{154}. Surprisingly, another study showed RhoA activation by ephrinB treatment of cultured hippocampal neurons\textsuperscript{17}. Further study demonstrated that EphB2 activates RhoA via a complex containing Grb2, Src, focal adhesion kinase, and paxillin, to support dendritic spine morphogenesis and maintenance\textsuperscript{17,155}. The GEF responsible for activation in this complex remained unknown. Presumably, degradation of ephexin5 by EphB2 did not preclude RhoA activation by additional mechanisms. Perhaps the specific GEFs and adaptors involved are important for maintaining the proper spatial or temporal regulation of RhoA.

In addition to regulating Rho GTPases, EphB2 signaling also affects the synaptic localization and function of the NMDA- and AMPA-type ionotropic glutamate receptors. EphrinB1-Fc treatment of cultured cortical neurons induced co-localization of EphB2 clusters with the NR1, NR2A, and NR2B subunits of NMDARs, and EphB2 co-precipitated from cortical lysates with NR1\textsuperscript{20}. EphB2-NR1 binding was shown to be mediated by the extracellular domains of the proteins\textsuperscript{20}. EphB2 uniquely causes tyrosine phosphorylation of the NR2B subunits, and increases current flow through and reduces desensitization of NR2B-containing, but not NR2A-containing, NMDARs in a kinase-dependent manner\textsuperscript{19}. Synaptic and surface expression of NMDARs were also increased by EphB2 stimulation\textsuperscript{19,20}. Given the established role of EphB2 in dendritic spine morphogenesis, it seems that EphB2 signaling at axo-dendritic contacts may help the initial localization of NMDARs to nascent synapses. Additionally, tyrosine phosphorylation of the NR2B subunit may potentiate the effects of glutamate, thereby facilitating spine head formation and LTP. EphB2 has also been shown to co-localize
with, and increase the synaptic targeting of, AMPARs. This interaction depends on the PDZ-binding motif of EphB2, through binding of the PDZ domain protein GRIP1. This supports a role for EphB2 in synapse formation as well as maturation, since AMPAR levels typically increase in synapses as they mature.

**EphB signaling in neuropathologies**

Given the association between dendritic spine morphogenesis and normal cognition and memory formation, it is not surprising that alterations in the EphB-Rho GTPase signaling axes are associated with psychiatric disorders and neurodevelopmental diseases. Behavioral deficiencies and impaired hippocampal LTP were observed in EphB2-KO mice, effects that were, interestingly, kinase-independent. Development of schizophrenia, a psychiatric illness characterized by psychosis and mood and cognitive defects, was associated with copy-number mutations of EphB1 and mutations of the EphB effector kalirin-7. UBE3A, which degrades tyrosine-phosphorylated ephexin5, is involved in numerous disorders. Angelman syndrome is caused by a loss of UBE3A function, and autism spectrum disorders are associated with duplication of the UBE3A gene. These links to EphB function are, however, indirect, though missense mutations in EphB1 showed weak but insignificant correlations with schizophrenia. This may be explained by the redundant functions of the EphB isoforms in synaptogenesis, such that simultaneous aberrations in multiple isoforms may be necessary to perturb function in a physiologically-significant manner.

A number of studies have linked EphB2 to the pathogenesis of Alzheimer’s Disease, which is a neurodegenerative disease of old age characterized by severe...
progressive dementia. It is associated with abnormally reduced synapse density, the accumulation of extracellular plaques consisting of oligomers of amyloid-β, derived from the amyloid precursor protein, and neurofibrillary tangles composed of phosphorylated tau protein\textsuperscript{160,161}. In a mouse model of Alzheimer’s Disease, reduced EphB2 expression is observed prior to disease manifestation\textsuperscript{162}. Overexpression of EphB2 rescued behavioral defects and NMDAR-dependent LTP in Alzheimer’s Disease model mice and prevents changes in cultured hippocampal neurons secondary to Aβ-oligomer treatment\textsuperscript{163}. Signaling to PI3K by EphBs reduced Tau phosphorylation, such that EphB2 was protective against AD progression\textsuperscript{101}. It is unclear if aberrations in EphB2 signaling are causal in, or the result of, AD progression, and further work is needed to clarify this role.

\textit{EphB signaling and dendritic filopodia}

An elegant study by Kayser et al. (2008)\textsuperscript{16} knocked down EphB2 in cultured cortical neurons during different stages in development (days \textit{in vitro}) and examined the effect on dendritic spine density in mature neurons. This study showed that the ability of EphB signaling to promote spinogenesis was restricted to DIV7-14, when filopodia are most abundant\textsuperscript{4,16}. Additionally, overexpression of EphB2 in EphB TKO neurons was only effective to rescue dendritic spine density when performed at 3 DIV, rather than at 10 DIV. These results suggested a role for EphB signaling in dendritic spine formation that was specific to the presence of dendritic filopodia. Therefore, observing how EphB signaling alters filopodia motility, morphology, or density is therefore important to understand how the filopodium-to-spine transition takes place.
A role for EphB signaling in dendritic filopodia formation is controversial. EphB TKO neurons did not show an increase or decrease versus wild-type neurons of dendritic filopodia density\textsuperscript{16}, indicating no effect on formation. Findings of filopodia shortening with ephrinB1-Fc treatment of cultured hippocampal neurons\textsuperscript{17} suggests, at least, that EphB signaling does not promote filopodia formation. Elongation would be expected from signaling that promotes filopodia formation. However, increased overall dendritic protrusion density was separately reported in cultured hippocampal neurons following ligand treatment\textsuperscript{15}. Since protrusion density is a product of both formation and retraction, EphB signaling may have induced further protrusion formation or stabilized extant protrusions. Only the increases in dendritic spine and synapse densities were explicitly quantified, such that an effect on filopodia formation could not be concluded.

The same study by Kayser et al. (2008)\textsuperscript{16} that demonstrated a temporally-regulated effect of EphB signaling on dendritic spine formation also quantified the effect of EphB TKO on the motility of dendritic filopodia. This group found that filopodia in EphB-TKO cortical neurons were less motile than those of wild-type neurons, and that motility in the knockouts was rescued by overexpression of CA-PAK\textsuperscript{16}. This study indicated that EphB signaling may promote filopodia motility, thereby increasing their axon-searching function. Rac1 and Cdc42, which are the major upstream regulators of PAK, may play important roles in this regulation. However, since the cultures were derived from EphB-TKO animals, all cells in the dissociated culture were devoid of EphB1-B3. Given that EphBs are RTKs, their knockdown likely affected the expression of other proteins. Therefore, the observed effects may have been indirect. Additionally, a function for EphBs in stabilizing dendritic protrusions would be more consistent with a
role in spine morphogenesis. Since EphBs are presumably activated at contacts, and filopodia stabilize at these contacts, one might expect a negative effect on filopodia motility. On this note, knockout of EphBs may reflect an indirect effect of EphB signaling, such as the impact on expression of other genes, but not necessarily what happens upon ligand engagement at contacts.

How EphB signaling to the Rho GTPases affects the motility and morphology of dendritic filopodia thus remains unclear. As mentioned in the opening section, this thesis seeks to clarify this problem. It is possible that EphBs direct the stabilization of filopodia upon contact with axons. They may serve to contribute to dendritic spine head formation. Perhaps, even, the observed effects on the cytoskeleton are indirect. My approach to this problem, and a summary of my findings, are described below.

**Overview**

As mentioned in the opening section, this thesis seeks to define the effect of EphB signaling on the motility and morphology of dendritic filopodia. To approach this problem, we sought to gain spatial and temporal control over EphB signaling. Even independently of their role in synaptogenesis, spatial and temporal regulation of signaling events is important to the biology of Eph receptors. As cell guidance molecules, Eph receptors must establish gradients of downstream signaling mediators in individual cells to direct changes in cell migration\textsuperscript{164,165}. To achieve this goal, we used the blue light-induced clustering of the *Arabidopsis* photoreceptor Cryptochrome \textsuperscript{2,129,130} to design an optogenetic tool, optoEphB2, to activate EphB2 signaling with spatial and temporal specificity. Our data showed rapid tyrosine phosphorylation, expected cellular
phenotypes, and engagement of expected downstream effectors with optoEphB2 photoactivation, confirming activation of EphB2 signaling.

We used patterned blue light illumination to target activation of optoEphB2 to dendritic filopodia and sub-cellular regions of dendrites to simulate the nature of axo-dendritic contact. Stimulation of filopodia resulted in branching and expansion of filopodial tips in conjunction with F-actin accumulation. Such results suggested formation of branched actin networks in filopodia, which would be expected in conjunction with spine head formation. Stimulation of the dendritic shaft showed localized formation of filopodia-like structures that was dependent on the Arp2/3 complex, which nucleates actin branches, and accumulation of the plasma membrane lipid phosphatidylinositol(3,4,5)-triphosphate (PIP₃), which plays important roles in actin polymerization. This suggests that EphB may direct formation of filopodia near sites of axo-dendritic contact, thereby up-regulating the local density of potential contacts that may develop into spines.

Functional outcomes of EphB signaling are highly cell type- and context-dependent. While our observations suggested a role for EphB2 in promoting dendritic protrusions, Eph receptors as a whole are much better known for promoting repulsive responses between cells. These responses would entail retraction of cell protrusions. Interestingly, prior studies and our data showed that plasma membrane PIP₃ content is reduced downstream of EphB2 signaling when retractions occur. Our results therefore also suggest that differential regulation of plasma membrane PIP₃ content contributes to the context-dependence of EphB2 signaling in different cell types.
Figures

Figure 1.1

Ligand RTK

\[ \text{Py} \text{p85} \]

\[ \text{p110} \]

Adaptor

\[ \text{p85} \]

\[ \text{p110} \]

PTEN

Ras

\[ \text{PI}(4,5)\text{P}_2 \]

\[ \text{PI}(3,4,5)\text{P}_3 \]

Akt

pAkt

GEF

GAP

Rho, Ras GTPases

Protein Synthesis

Cell Survival

Actin Cytoskeleton
Figure 1.1. PI3K activation and signaling downstream of receptor tyrosine kinases. Receptor tyrosine kinases (RTK) frequently activate class I PI3 kinases through recruitment of the regulatory subunit (p85 shown). This is typically accomplished by engagement of the regulatory subunit SH2 domains by RTK phosphotyrosines or through binding SH2 adaptors. Catalytic subunits (p110) are constitutively bound to regulatory subunits and are activated by conformational changes in the regulatory subunit and recruitment to their plasma membrane substrates. Ras GTPases are also known to increase PI3K activity through direct binding of Ras-binding domains in the catalytic subunits. The catalytic subunits phosphorylate phosphatidylinositol(4,5)-bisphosphate (PI(4,5)P₂, PIP₂) on the plasma membrane to generate phosphatidylinositol(3,4,5)-triphosphate (PI(3,4,5)P₃, PIP₃), which recruits downstream effectors via pleckstrin homology (PH) or other lipid-binding domains. Important effectors include Akt and GEFs and GAPs that activate or inhibit, respectively, the Rho GTPases. PIP₃ can be dephosphorylated to PIP₂ by PTEN, and is also dephosphorylated by many lipid phosphatases (not shown).
Figure 1.2

Changes in actin polymerization, cell migration, and cell proliferation
Figure 1.2. Structure and clustering of Eph receptors and ephrins. Eph receptors and ephrins are both membrane-bound molecules and are activated at cell-cell contacts. The two subtypes of ephrins, ephrinBs and ephrinAs, are structurally distinct. EphrinBs are transmembrane proteins with unstructured intracellular domains that contain conserved tyrosine residues (Y) and PDZ-binding motifs (PBM). These tyrosines become phosphorylated upon activation by other RTKs and non-receptor tyrosine kinases. EphrinAs are linked to the outer leaflet of the plasma membrane by glycosylphosphatidylinositol (GPI). The Eph receptor extracellular domain consists of the ligand-binding domain (LBD), a cysteine-rich domain (CRD) consisting of sushi and epidermal growth factor (EGF)-like motifs, and two fibronectin-type III (FNIII) repeats. The intracellular domain contains conserved tyrosine (Y) residues in the juxtamembrane (JM) region that are important for kinase regulation, a kinase domain, a sterile-alpha motif (SAM), and a PDZ-binding motif (PBM). Autophosphorylation (pY) of the juxtamembrane tyrosines relieves intracellular autoinhibition, permitting substrate phosphorylation by the kinase domain. While dimerization of Ephs is sufficient to induce tyrosine phosphorylation, clustering is necessary for efficient signal transduction. Residues in the LBD, CRD, and the N-terminal FNIII allow these domains to promote clustering through cis receptor-receptor interactions.
Depending on the cell context, Eph receptors can increase or decrease cell adhesion, migration, proliferation, and survival through many of the same pathways activated by other RTKs. These pathways most often converge on the Rho and Ras GTPases and PI3K. EphAs activate the ephexin family of GEFs in multiple contexts to activate RhoA and promote cell-cell repulsion. During growth cone collapse, α2-chimaerin is recruited to EphAs by its SH2 domain to inhibit Rac1. However, Rac1 signaling is necessary for endocytosis of receptor-ligand complexes during cell-cell repulsion, and Vav family GEFs are thought to activate Rac1 in these situations. Rac1 may also be activated through the GEFs DOCK1, associated with the SH2 adaptor Crk, and α-Pix, which binds the SH2 adaptor Nck. Nck may also signal PAK independently of Rac1 and Cdc42, which was shown in growth cone collapse downstream of EphBs. When engaged by Eph receptors, Crk also promotes adhesion and activates integrin signaling through the Ras family GTPase Rap via the Rap-GEF C3G. Signaling through the NRTK Abl can reduce cell adhesions by inhibiting Crk, dissociating it from C3G and p130Cas. Abl, however, can also promote adhesions and increase cell proliferation through activation of cyclinD1. Unlike other RTKs, Eph receptors are generally known to inhibit Ras and R-Ras, typically by SH2 domain-mediated recruitment of p120RasGAP. R-Ras inhibition can also occur by direct tyrosine phosphorylation. These activities reduce cell survival and migration. Activation of Ras may occur through recruitment of the SH2 adaptors Shc and Grb2, which bind the Ras-GEF SOS. Src family kinases serve a variety of functions downstream of Eph receptors. This figure displays Src-mediated tyrosine phosphorylation of ephexins and α2-chimaerin, which happens during EphA-mediated growth cone collapse, though SFKs are activated by Eph receptors in many systems and can phosphorylate a wide variety of downstream effectors. Reduction of phosphotyrosine can occur through recruitment of protein tyrosine phosphatases, including LMW-PTP, PTP1B, and PTP-RO. Multiple MMPs can cleave the ECDs of Ephs and ephrins, and ADAM10 can cleave the ECDs of ephrins, to down-regulate signaling and facilitate cell-cell repulsion.
Figure 1.4
**Figure 1.4. Cryptochrome 2 in Optogenetics.** (a) Schematic showing blue light-induced (blue arrows) binding between Cry2 and CIBN, a truncated mutant of the transcription factor CIB1. In this example, CIBN is tethered to the plasma membrane and blue light illumination recruits Cry2. (b) Confocal images of MCF7 cells co-transfected with Cry2-olig-mCherry and a CIBN-GFP fusion tagged to the plasma membrane with a C-terminal CAAX sequence (K-Ras). Blue light (488-nm laser used to image GFP) caused translocation of Cry2-olig to the plasma membrane. Total acquisition time was 20.4 s. Scale bar, 10 µm. (c) Schematic of blue-light induced (blue arrow) clustering of a Cry2-olig-mCherry fusion. (d) Confocal images of MCF7 cells transfected with Cry2-olig-mCherry, which clustered in response to blue light illumination (458-nm laser, scanning simultaneously, 48.4 s acquisition). Scale bar, 10 µm. (e) Optogenetic activation of PI3K. A CIBN-GFP-CAAX construct is tethered to the plasma membrane and co-expressed with a fusion between Cry2 and the inter-SH2 (iSH2) domain of the PI3K regulatory subunit. The iSH2 domain binds endogenous PI3K catalytic subunits (p110), which become activated upon blue light-induced plasma membrane recruitment. (f) Schematic of clustering indirectly using Cryptochrome 2 (CLICR). Cry2 is tethered to a specific downstream effector of a transmembrane receptor (shown as example), or other protein of interest. Blue light-induced clustering of these effectors causes binding to the receptor by avidity, thereby clustering and activating the receptor. Confocal images (b and d) were taken on a Zeiss LSM510 confocal microscope, using a 488-nm laser line to excite GFP and a 561-nm laser line to excite mCherry.
Figure 1.5. Dendritic filopodia make contact with axons and transform into dendritic spines. (a) Dendritic filopodia are actin-based protrusions that arise from actin patches on the dendritic shaft. (b) Dendritic filopodia extend, retract, bend, and sometimes branch in search of pre-synaptic axons. Some filopodia form stable contacts with axons. (c) Dendritic filopodia that form stable contacts with axons undergo reorganization of the actin cytoskeleton to form dendritic spines.
Figure 1.6
Figure 1.6. EphB signaling in dendritic spine morphogenesis. EphBs signal GEFs and GAPs that regulate the Rho GTPases Rac1, Cdc42, and RhoA to drive dendritic spine morphogenesis. Interactions have been shown between EphBs and the Rac-GEFs kalirin-7 and Tiam1, both of which are tyrosine phosphorylated by EphBs. The Rac-GAP Bcr also interacts with Tiam1, and EphB2 disrupts this interaction. EphB2, the Cdc42-GEF intersectin, and N-WASP form a signaling complex. Numb also binds EphB in complex with intersectin. Both Rac1 and Cdc42 activate the Arp2/3 complex, through WAVE1 and N-WASP, respectively, and PAK, to promote dendritic spine formation and maturation. Regulation of RhoA activity by EphBs in dendrites is more complicated. EphBs are known to tyrosine phosphorylate the Rho-GEF ephxin5, leading to its UBE3A-mediated ubiquitination and degradation. However, a signaling complex involving Grb2, Src, and focal adhesion kinase (FAK) was shown to activate RhoA downstream of EphBs via an unknown GEF. Downstream of RhoA, signaling through ROCK and myosin is thought to affect spine morphogenesis, whereas LIMK/cofilin signaling is thought to mediate spine stability. In addition, EphBs also interact with syndecan-2 and glutamate receptors. The ECDs of the NMDAR and EphB2 were shown to mediate binding, and EphBs were also tyrosine phosphorylate the NR2B subunit of NMDARs to alter their conductivity. EphBs recruit AMPARs through the PDZ domain scaffolding protein GRIP. Tyrosine phosphorylation of syndecan-2, a heparan sulfate proteoglycan that promotes dendritic spine formation, has also been shown. EphrinB3 was shown to interact with EphBs in cis to inhibit spine morphogenesis.
Movie Captions

Movie 1.1. Light-inducible plasma membrane recruitment of Cryptochrome 2. Movie shows Cry2olig-mCherry signal in MCF7 cells co-expressing CIBN-GFP-CAAX. Cells were imaged by confocal microscopy (Zeiss LSM510), co-scanning with a 488 nm (GFP excitation, Cry2 photoactivation) and 561 nm (mCherry excitation) lasers. Rapid translocation of Cry2 to the plasma membrane was observed with blue light illumination.

Movie 2.2. Light-inducible clustering of cytoplasmic Cryptochrome 2. Movie shows Cry2olig-mCherry signal in MCF7 cells. Cells were imaged by confocal microscopy (Zeiss LSM510), using a 561 nm laser to excite mCherry and simultaneously scanning with a 458 nm laser to photoactivate Cry2olig. Cry2olig showed rapid clustering in response to blue light.
CHAPTER II: Development of an Optogenetic Method for Eph Receptor Activation

Attribution: This chapter contains excerpts and figures from the manuscript “Effects of Localized EphB2 Activation on Dendritic Filopodia of Hippocampal Neurons” co-authored by Clifford Locke, Qingfen Yang, Kazuya Machida, Chandra Tucker, Yi Wu, and Ji Yu. Qingfen Yang assisted with cloning procedures. Kazuya Machida ran the phosphotyrosine blot (Fig. 2.2b) of whole cell lysates, performed the rosette assay, constructed Figure 2.3a, c, and d, and edited the relevant portions of the Methods section. Chandra Tucker provided the Cry2 mutant, Cry2olig, ahead of her manuscript on its development. Drs. Betty Eipper and Richard Mains provided embryonic rat hippocampi. The text was written by Clifford Locke and proof-read and edited by Ji Yu and Yi Wu. Clifford Locke wrote the text, constructed the figures that were not otherwise attributed, and performed all experimental work that was not otherwise attributed.
Abstract

Eph receptors comprise the largest family of receptor tyrosine kinases in mammals. Together with their membrane-bound ephrin ligands, they transduce cell-cell contacts into changes in cell migration that affect cell positioning and tissue patterning during developmental processes. Spatial and temporal regulation of Eph receptor signaling on the tissue and single cell levels is thus important to proper function, and current in vitro stimulation techniques do not permit this level of control. Here, we develop an optogenetic tool (optoEphB2) that allows for light-controlled reversible activation of EphB2 through blue light-induced clustering of the plant photoreceptor Cryptochrome 2. Biochemical analysis showed rapid blue light-induced tyrosine phosphorylation and engagement of SH2 domain-containing effectors typical of EphB2 signaling. OptoEphB2 also facilitated real-time monitoring of cellular responses to EphB2 signaling that was targeted to specific sub-cellular regions. We thus developed a method for spatial and temporal control over EphB2 signaling.

Introduction

Eph receptors comprise the largest receptor tyrosine kinase (RTK) family in mammals, consisting of nine EphA and five EphB receptors. They are named according to their relative binding affinities for either glycosyl phosphatidylinositol (GPI)-linked ephrin-A or transmembrane ephrin-B ligands, although it should be noted that receptor-ligand binding is promiscuous and ligand specificities are not absolute. Because both the receptors and their ligands are membrane-bound molecules, Eph receptors have a unique property among RTKs that their signaling activation in vivo typically requires
direct cell-cell contact. Additionally, Eph receptors rely on clustering for efficient signal transduction\(^41\). While Eph receptor dimerization is sufficient for tyrosine phosphorylation, efficient effector recruitment and resulting cellular phenotypes typically require higher-order associations\(^{42,43}\). During development, Ephs and ephrins sense cell-cell contacts and alter the actin cytoskeleton to guide cell migration and thereby control a host of important biological processes, including axon guidance\(^{64}\), tissue patterning\(^{65}\), angiogenesis\(^{167}\), and cell proliferation\(^{102}\). Dysfunction in Eph/ephrin signaling has been linked to many pathological processes, such as various forms of cancer and Alzheimer’s Disease\(^{24,168}\).

Proper cell guidance by Eph receptors and ephrins relies on precise spatial and temporal control of their signaling, which is necessary during development to position cells in the right place at the right time. On the tissue level, this is accomplished by spatial gradients of Ephs and ephrins, and has been well-described for the role of Eph receptors in axon guidance\(^{64}\). For example, Ephs are thought to act as repulsive cues to guide retinal axons to their appropriate targets in the visual system, and this is important for spatial mapping of visual fields in the central nervous system (CNS)\(^{64}\). In individual migrating cells, cell-cell contact typically occurs at sub-cellular regions. Subsequent directionality inherently requires establishing spatial gradients of activated receptors and downstream signals across the cell\(^{165}\).

Multiple techniques have been developed to cluster and activate Eph receptors \textit{in vitro}. In most studies, dimeric Fc-ephrin chimeras are pre-clustered by anti-Fc antibodies and then applied to cultured cells\(^{42}\). This technique is most commonly used, but does not impart spatial or temporal control over signaling. A chemical oligomerization system
was recently developed and used to tune cluster size and correlate it with signal strength. This system was designed with 1-3 FKBP domains inserted into the EphB2 ICD, which were cross-linked with the homodimerizers AP20187 or AP1887\textsuperscript{13}. This technique appears to provide temporal control, though most drug incubations were 20 minutes or longer, and spatial control was not demonstrated. Additionally, antibody-coated microbeads have been used to pre-cluster ephrins and study cell migration\textsuperscript{61}. This technique simulates sub-cellular contacts, but does not permit temporal control and is impractical for non-migratory cell types, such as neurons. Simultaneous spatial and temporal control over signaling would be invaluable to precisely model cell-cell contacts and study the dynamics of downstream signaling mediators.

Recent concurrent studies reported spatial and temporal control of Trk receptors\textsuperscript{132} and FGFR\textsuperscript{133}, other RTK family members, using the blue light-induced clustering of Cryptochrome 2 (Cry2). Cry2 is a photoreceptor derived from \textit{Arabidopsis thaliana} that clusters in response to blue light absorption by the flavin adenine dinucleotide (FAD) chromophore in its photolyase homology region (PHR)\textsuperscript{134}. Here, we report the development of optoEphB2, a genetically-encoded, photoactivatable EphB2. Blue light-induced clustering of optoEphB2 caused rapid tyrosine phosphorylation and recruitment of SH2 domain-containing effectors that typically bind endogenous EphB2 receptors. Retraction of cellular protrusions in fibroblasts and axonal growth cone collapse were also observed and agreed with studies that used ephrinB ligand stimulation. Signaling was reversible and repeatable, and spatial restriction of clustering and cellular phenotypes were demonstrated. We thus developed a versatile module for spatial and
temporal control over EphB2 signaling that can be applied in a multitude of cellular systems.

**Methods**

*Antibodies, reagents, and plasmids*

All chemicals used for the experiments were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. The mouse anti-phosphotyrosine antibody was purchased from Cell Signaling Technologies (Danvers, MA). The mouse anti-tubulin and rabbit anti-mCherry antibodies were purchased from Thermo Scientific (Waltham, MA). IRDye 680- and IRDye 800-labeled secondary antibodies were purchased from LI-COR (Lincoln, NE).

Cry2olig-mCherry (full-length Cry2 or its PHR with E490G mutation), light-insensitive Cry2-mCherry (Cry2 with D387A mutation), and wild-type Cry2-mCherry (full-length or PHR) were described previously\(^{120,129,130}\). Human EphB1 (plasmid #23930) and EphB6 (plasmid #23931) sequences were obtained from Addgene (Cambridge, MA). The human EphB2 sequence was obtained from DNASU (plasmid #80351). To create optoEphB2 plasmids, the Gateway cloning system (Invitrogen) was used. A Gateway cassette (Invitrogen) was first introduced into the Cry2-mCherry or Cry2olig-mCherry plasmids, N-terminal to the Cry2 sequences, to produce destination vectors. An entry vector containing the myristoylation signal peptide was generated by inserting the oligonucleotide sequence corresponding to the N-terminal signal peptide of c-Src (MGSNKSKPK) into pDONR223 (Invitrogen). Entry clones containing the ICD sequences of EphB1 (amino acids 564-984), EphB2 (amino acids 595-986), and EphB6
(amino acids 328-729), with N-terminal myristoylation, were then created using a standard PCR-based cloning procedure. Finally, expression clones for optoEphB1, optoEphB2, and optoEphB6 were made by LR recombination (LR Clonase, Invitrogen) of the corresponding Cry2-based destination vectors and entry clones. The kinase-dead optoEphB2 (KD-optoEphB2) construct was made by site-directed mutagenesis (K662M in full-length EphB2, K98M in optoEphB2). To generate optoEphB2 clones containing Venus, mCherry was excised and replaced with the yellow-fluorescent Venus sequence, which was amplified by PCR. A lentiviral vector carrying optoEphB2 (pLIX401-optoEphB2) was made by excising the whole optoEphB2 sequence and subcloning it into an inducible lentiviral expression vector, pLIX401 (Addgene plasmid #41390). The Arg-YFP plasmid was a gift from Anthony Koleske.

*Cell culture, transfection, and ephrinB1 Treatment*

All cells were kept in a humidified 37°C incubator with 5% CO₂. HEK293, HEK293FT, MCF7, MEF, and COS7 cells were maintained in Dulbecco’s Modified Eagle Medium (Gibco, Grand Island, NY or Lonza, Basel, Switzerland) with 10% fetal bovine serum (FBS, BioWest, Kansas City, MO) and penicillin/streptomycin (Gibco) at 100 U/ml penicillin and 100 µg/ml streptomycin. The medium used to maintain HEK293FT cells was DMEM/FBS supplemented with 500 µg/ml G418 (Gibco). Transient transfections were carried out using Lipofectamine 2000 (Invitrogen, Grand Island, NY) following the manufacturer’s protocol. To establish cells stably-expressing optoEphB2 and KD-optoEphB2, pseudo-lentiviral particles were prepared. Briefly, pLIX401-based DNA was co-transfected into HEK293FT cells by calcium phosphate
precipitation with the 3rd generation packaging plasmids pRRE, pMD2G, and pRSV (Addgene plasmid #12251, #12259, #12253). Viruses were precipitated from cell culture medium with PEG-it (400 g/L PEG 8000, 88 g/L NaCl) and concentrated by centrifugation. The MEF Tet-off cells (Clontech, Mountain View, CA) were infected with viral particles to produce the MEF-OptoEphB2 and MEF-KD-OptoEphB2 cells, which were maintained in DMEM/FBS media supplemented with 300 µg/ml G418.

Primary hippocampal neurons were plated and maintained as previously described\textsuperscript{170,171}. Briefly, hippocampi were dissected from E17-19 Sprague-Dawley rats and were mechanically dissociated. Some hippocampi were obtained from BrainBits, and other tissue was a generous gift from Drs. Betty Eipper and Richard Mains. Cells were plated onto plasma-cleaned 30-mm coverslips, that were coated with 0.05% poly-L-lysine, at 90,000-100,000 cells/dish. Neurons were maintained in Neurobasal (Gibco) with B27 (Gibco) at 1:50, GlutaMax (Gibco) at 1:400, and penicillin-streptomycin at 100 U/ml of penicillin and 100 µg/ml streptomycin. FBS at 2% and 25 µM glutamate were also added at time of plating. Transfections were done using Lipofectamine 2000 and BrainBits (Springfield, IL) Transfection Medium, following manufacturer’s protocol with some modification. Transfections of neurons were carried out 24-48 hours prior to experiments.

\textit{Biochemical assays}

For western blot assays of phosphotyrosine, MEFs expressing optoEphB2 or KD-optoEphB2 were treated with blue LED light ($\sim$10$^{-2}$ W/cm$^2$), or incubated in the dark, for 1 minute. Cells were lysed in modified kinase lysis buffer (KLB, 150 mM NaCl, 25 mM
Tris-HCl pH 7.4, 5 mM EDTA, 1 mM PMSF, 1% Triton-X-100, 10% glycerol, 0.1% sodium pyrophosphate, 10 mM β-glycerophosphate, 10 mM NaF, 5 μg/ml aprotinin, 50 μM pervanadate) as previously described\textsuperscript{172}. 0.1% SDS was added to the KLB to aid in solubilizing large optoEphB2 or KD-optoEphB2 clusters. Proteins from lysates were separated by gel electrophoresis on 4-15% gradient polyacrylamide gels (BioRad, Hercules, CA) in buffer containing 3.03 g/L Tris base, 14.4 g/L glycine, and 0.1% SDS. For loading into wells, 2-mercaptoethanol was added at 1:200. Samples were transferred to nitrocellulose membranes (General Electric Healthcare, Pittsburgh, PA) in buffer containing 3.03 g/L Tris base and 14.4 g/L glycine. Membranes were blotted with mouse anti-phosphotyrosine and rabbit anti-mCherry antibodies. Blots were visualized and quantified on an Odyssey IR scanner (LI-COR) using secondary antibodies labeled with IR dyes (rabbit IRDye 680 for mCherry, mouse IRDye 800 for pan-phosphotyrosine). The membrane was stripped in buffer containing 2% SDS, 62 mM Tris-HCl pH 6.8, and 0.7% 2-mercaptoethanol at 55°C, rinsed, and re-probed for anti-tubulin, which was visualized with a mouse IRDye 800 secondary antibody.

Immunoprecipitation of optoEphB2 was carried out by first measuring the concentration of protein in cell lysates by colorimetry with Bradford reagent using a BioTek Synergy HT plate reader. Cell lysate containing 200 μg of protein was mixed with 2-4 μg rabbit anti-mCherry antibody. Pulldown was done with 25 μL of protein A-coated magnetic bead suspension (Invitrogen). Beads were pulled down using magnets, washed with KLB (no SDS), denatured at 95°C for 10 minutes, in the same buffer used for gel electrophoresis plus 1:200 2-mercaptoethanol, and separated, transferred, and analyzed by the aforementioned western blotting procedure. For photoactivation, MEFs
in a 6-cm dish were illuminated with blue LED light bulb of 1W total optical power for 1 minute. Control cells were kept in the dark.

The dot-blot SH2 binding assay was performed as previously described\textsuperscript{172,173} using the lysates prepared for the phosphotyrosine assay. Briefly, aliquots of the MEF lysates, or of lysates from pervanadate-treated cells (positive control) or phosphatase-treated cells (negative control), were spotted on a membrane. These aliquots were incubated with purified glutathione-S-transferase (GST)-tagged SH2 domains and detected by immunoblotting with horseradish peroxidase (HRP)-labeled anti-GST antibodies, treating with an enhanced chemiluminescence (ECL) reagent (Perkin-Elmer), and imaging on a Kodak Image Station 4000MM Pro with Carestream MI SE software. The quantification shown in Fig. 2.3d represents the average intensity from triplicates, normalized to the maximum signal derived from any of the optoEphB2 or KD-optoEphB2 treatment conditions.

\textit{Microscopy and Image Analysis}

Most live cell imaging experiments were carried out on a Nikon (Tokyo, Japan) Ti-E inverted fluorescence microscope with a 60x TIRF objective (NA = 1.49, Nikon). Images were acquired with an iXon Ultra EM-CCD (Andor, Oxford Instruments, Abingdon, Oxfordshire, UK). The microscope was placed within a temperature-regulated imaging chamber and cells were maintained at 37°C during imaging. For imaging mammalian cell lines, cells were kept in DMEM/F12 (Gibco) containing 2% FBS and 20 mM HEPES (Gibco). For imaging neurons, the cells were kept in imaging medium containing 117 mM NaCl, 5 mM KCl, 1.25 mM NaH\textsubscript{2}PO\textsubscript{4}, 20 mM HEPES, 50
mM dextrose, 1 mM MgCl₂, 2 mM CaCl₂, and 100 mg/L BSA, at pH 7.2. Neurons were imaged in epifluorescence, using a 575-nm LED (Lumencor, Beaverton, OR) to excite mCherry and mRFP and a 515-nm LED (Lumencor) to excite Venus. MEFs were imaged in TIRF (total internal reflection fluorescence) mode. A 594-nm DPSS laser (CrystaLaser, Reno, NV) was used to excite mCherry, and Venus/YFP was excited with a 515-nm DPSS laser (CrystaLaser). TIRF and DIC imaging of optoEphB2 clustering in 293 and COS7 cells and ligand-mediated cell collapse in MEFs were carried out on an Olympus (Tokyo, Japan) IX81 TIRF microscope equipped with a 60x TIRF objective (NA = 1.49, Olympus) and a TE-cooled EM-CCD (PhotonMax, Princeton Instruments, Trenton, NJ). The 488-nm line of an argon ion laser (Melles Griot, Carlsbad, CA) was used to excite GFP and photoactivate optoEphB2, a 562-nm DPSS laser (CrystaLaser) was used to excite mCherry, and a 442-nm DPSS laser (CrystaLaser) was alternatively used for photo-activation of optoEphB2.

Spatial control of optoEphB2 on the Nikon Ti-E system was achieved using a Mosaic illumination system (Andor) coupled to a 440-nm LED (CoolLED, Andover, Hampshire, UK) on the Nikon Ti-E microscope, unless otherwise noted. The region of illumination (ROI) was expanded to cover the whole mosaic for illumination over the field of view. Photoactivation of sub-cellular regions and growth cones were accomplished by manually defining a ROI that encompassed the desired area. For growth cones, the entire growth cone area was covered, and the ROI was adjusted as necessary for growth cone migration throughout the acquisition. Images for whole-cell stimulation of MEFs were taken at 3 frames/min, with 50-ms pulses of photoactivation at 3.5% LED power delivered between frames. Growth cones were imaged at 6 frames/min.
with 100-ms pulses of photoactivation at 2% LED power. Alternatively, a 460-nm LED (Prizmatix, Givat-Shmuel, Israel), or the 488-nm or 442-nm lasers on the Olympus IX81 system, were used for photoactivation where noted.

Maximum intensity projections of growth cones were generated using ImageJ after background subtraction. Area was measured with manual thresholding, and the same threshold was used for multiple projections from any one cell. The growth cones were defined by the cellular area from the expansion point on the axonal shaft to every point touched by protrusive structures. Two growth cones from the optoEphB2 group were excluded due to problems defining the expansion point. MEF cell area was measured by measuring cellular area from the optoEphB2 signal after background subtraction and sharpening using the Unsharp Mask function. Octane\textsuperscript{174} was used to count optoEphB2 clusters.

**Results**

*Design of optoEphB2*

Eph receptors are typically activated *in vitro* by antibody-mediated clustering of soluble ephrin ligands prior to treatment\textsuperscript{11,41,42}. We hypothesized that light-induced clustering would be sufficient for this, given the role of clustering in Eph receptor signaling, and to ultimately achieve spatial and temporal control. Two concurrent studies\textsuperscript{132,133} demonstrated activation of Trk receptors and FGFR, two other members of the RTK family, by fusing Cry2 to the receptors’ C-terminus. We therefore ligated the PHR of Cry2, which will be referred to simply as Cry2, and mCherry to the C-termini of multiple Eph receptors (Table 2.1). However, confocal microscopy of transfected
HEK293 cells revealed that Cry2 fusions involving EphB2 and EphB1 gave poor plasma membrane localization, and large fluorescent puncta were detected in the cytoplasm (Fig. 2.1a). We speculated that these puncta were internalization vesicles secondary to receptor activation. This notion was supported by the fact that a construct consisting of EphB6, a catalytically-inactive EphB subtype, showed relatively more plasma membrane localization (Fig. 2.1a). It was possible that activation of Eph receptors was occurring secondary to interactions with endogenous ephrins on other cells. Alternatively, Himanen et al.\textsuperscript{56} demonstrated increased tyrosine phosphorylation of overexpressed Eph receptors. Mutation of extracellular domain (ECD) residues that are responsible for interactions between receptors on the same cell surface mitigated the tyrosine phosphorylation induced by overexpression\textsuperscript{56}.

We therefore eliminated the ECD and, instead, tagged the intracellular domains of the Eph receptors to the plasma membrane with an N-terminal myristoylation sequence derived from c-Src (Table 2.1). A similar design was reported for optoFGFR\textsuperscript{133}. This modification improved plasma membrane localization (Fig. 2.1b). However, photoactivation and imaging by total internal fluorescence (TIRF) microscopy showed that blue light-induced clustering was weak and inconsistent, insufficient for Eph receptor activation (Fig. 2.1c). We then incorporated a recently-identified mutant, Cry2olig (Cry2 E490G), which has a higher tendency to form high-order clusters\textsuperscript{130}. This was effective to produce robust blue light-induced clustering (Fig. 2.1c). Two mutants of optoEphB2 (Table 2.1), kinase-dead optoEphB2 (KD-optoEphB2) and a light-insensitive optoEphB2 (LI-optoEphB2), were also constructed to serve as controls. The kinase-dead construct
was made by introducing an arginine-to-methionine mutation in the ATP-binding pocket of the kinase domain (K98M in optoEphB2, K662M in full-length EphB2).

**Biochemical validation of optoEphB2**

The final design of optoEphB2 is shown in Figure 2.2a. To test if optically-induced optoEphB2 clustering resulted in receptor activation, we assayed for tyrosine phosphorylation in cell lysates of mouse embryonic fibroblasts (MEFs) stably expressing optoEphB2 or KD-optoEphB2. We found that optoEphB2-expressing MEFs subjected to blue LED light illumination for just one minute exhibited significantly higher overall tyrosine phosphorylation compared to cells left in the dark (Fig. 2.2b). In contrast, blue light produced no increase in tyrosine phosphorylation in cells expressing KD-optoEphB2 (Fig. 2.2a,b). The most significant increase in phosphorylation was observed near 135 kDa, consistent with the size of optoEphB2. Anti-phosphotyrosine blot analysis of immunoprecipitated optoEphB2 showed an approximately 29-fold increase of tyrosine phosphorylation (Fig. 2.2b) in blue light-illuminated samples. These results verified optoEphB2 kinase activation by blue light-induced clustering.

RTK phosphotyrosines typically serve as docking sites for Src homology 2 (SH2) domains of various adaptor proteins\(^\text{30}\). For further biochemical validation of optoEphB2, we screened for candidate SH2 domains that interacted with proteins in the whole cell lysate. To do this, we used a “rosette” assay as previously described\(^\text{172,173}\) (see Methods). Our results (Fig. 2.3) showed, in response to blue light illumination of optoEphB2, significantly increased binding of SH2 domains that had been previously shown to bind EphB2, including Abl\(^\text{104}\), Arg\(^\text{104}\), Crk\(^\text{175}\), RasGAP\(^\text{50,71}\), and Nck\(^\text{50}\). The
SH2 domains of CrkL, Cten, and Fes were also shown to bind the lysate, but have not been shown to interact with EphB2 in other studies. CrkL and EphA3, however, were shown to interact\textsuperscript{176}. It is likely that these SH2 domains are binding other tyrosine-phosphorylated proteins in the lysate, or that these are the result of non-specific binding. The SH2 domain of Src, whose binding of EphB2 is well-documented\textsuperscript{50,52,177}, did not bind the cell lysate in our experiment, but also failed to bind the positive control, and the result is therefore inconclusive. To confirm these results, optoEphB2 or KD-optoEphB2 and Arg were co-expressed in 3T3 cells and formed co-localizing clusters upon blue light illumination (Fig. 2.3b). These results show that SH2 domains that typically bind EphB2 are also binding phosphotyrosine motifs in our cell lysates, providing further validation of optoEphB2.

\textit{OptoEphB2 confers spatial and temporal control over EphB2 signaling}

We next asked if photoactivation of optoEphB2 produced the same cellular phenotypes as those caused by ligand-mediated activation. Previous studies have shown that a prominent cellular response to EphB activation is cell-cell repulsion, marked by local retraction of cell protrusions or cell collapse\textsuperscript{10}. Consistent with these previous findings, we found that photoactivation of optoEphB2 in MEFs quickly induced cell collapse (Fig 2.4a, Movie 2.1). OptoEphB2 clusters formed rapidly, with a time constant of 14.9 seconds (Fig. 2.4b), in response to repeated pulses of blue light. Collapse of protrusions commenced with a delay of ~2 minutes following the first photoactivation pulse (Fig. 2.4b). Quantification of cell area showed a greater than 60% overall reduction, on average, with optoEphB2 activation, with 4/6 cells tested losing greater
than 40% of cell area (Fig. 2.4b). In contrast, KD-optoEphB2 caused significantly less cell collapse, and none of the cells lost more than 40% of their area (Fig. 2.4b). Cell collapse was thus kinase-dependent as previously described\(^\text{10}\), and the KD-optoEphB2 results also suggested that collapse was not an effect of phototoxicity. Consistent with this finding, stimulation of MEFs expressing EphB2-EGFP with pre-clustered ephrinB1-Fc ligands, but not the Fc control, caused retraction of cellular protrusions (Fig. 2.4c).

The effects of optoEphB2 on cell morphology were also examined in HEK293 cells, in which EphB2 was previously shown to cause cell collapse\(^\text{175}\), and MCF7 cells, whose substrate attachment was reduced by ephrinB2 treatment\(^\text{178}\). OptoEphB2 photoactivation also resulted in total collapse of HEK293 cells and reduced membrane ruffling and lamellipodial protrusions in MCF7 cells, and these effects were not observed with KD-optoEphB2 (Fig. 2.4d,e). To further rule out any effects of Cry2 or phototoxicity, photoactivation of a myristoylated Cry2-mCherry fusion (myr-Cry2-mCherry) also did not cause HEK293 cell collapse. These results confirmed functional EphB2 signaling with blue light-induced optoEphB2 clustering.

Spatial and temporal control over signaling drives the development of optogenetic modules. When blue light illumination was restricted to sub-cellular regions of optoEphB2-expressing MEFs and MCF-7 cells using digital light patterning\(^\text{179}\), we found that both receptor clustering and cell retraction were spatially restricted to the region of illumination (ROI), while non-illuminated regions were unaffected (Fig. 2.5a,b, Movie 2.2). These results demonstrated the ability of spatially controlling EphB signaling with OptoEphB2. To test if optoEphB2 signaling can be reversed, we optically activated MEFs and monitored the morphology changes after the removal of blue light illumination.
(Fig. 2.5c, Movie 2.3). We found that within ~5 min, the receptor clusters dissipated and the cell started to re-expand by generating highly dynamic membrane protrusions (Fig. 2.5c, Movie 2.3). Furthermore, we found that the activation-deactivation cycles can be repeated multiple times (Fig. 2.5c). Therefore, optoEphB2 allows for reversible and repeatable activation of EphB2 signaling. This may be important to study the role of EphB2 signaling in cell segregation, for instance, to determine signaling functions once segregation is complete. The reversibility of the clustering of Cry2olig itself had been previously examined\textsuperscript{130}. Interestingly, we found that the clusters of optoEphB2 dissipated much faster than previously reported for cytoplasmic Cry2olig-mCherry\textsuperscript{130}. The difference may reflect a change in spatial dimensionality from the cytosol (3D) to the plasma membrane (2D), or may be related to EphB2 domain interactions or downstream signaling events.

**OptoEphB2 causes growth cone collapse in hippocampal neurons**

To test whether OptoEphB2 is functional in neurons, we examined if OptoEphB2 activation induces repulsive responses in axonal growth cones\textsuperscript{43,45,62,91,92}. Growth cones of DIV5 primary hippocampal neurons co-expressing OptoEphB2 or KD-optoEphB2 (fused to Venus in lieu of mCherry) with mCherry (volume marker) were illuminated with blue light. We acquired time-lapse images of the growth cones before and during photoactivation. Growth cone collapse and retraction were frequently observed with optoEphB2 photoactivation, but not with KD-optoEphB2 (Fig. 2.6a, Movie 2.4). To quantify these effects, we measured the total area explored by the growth cone before and after photoactivation (Fig. 2.6b). Because growth cone morphology is highly dynamic,
the area was measured from maximum intensity projections of the image stacks, compiled from the 5-minute period prior to, and the final 5 minutes of, blue light illumination. These projections plot the maximum intensity recorded at each pixel during the indicated 5-minute time frame and thereby account for each point that was touched by the growth cone. We found that photoactivation reduced the maximum projection area in 10/12 growth cones expressing optoEphB2 (Fig. 2.6c). In 9/10 of growth cones with reduced dynamics, a loss of greater than 20% was observed. With KD-optoEphB2, this magnitude of reduction was only observed in 3/8 cases, and 4/8 growth cones showed increased dynamic activity. Normalizing the final 5 minutes of photoactivation to the 5 minutes before, optoEphB2 reduced the area explored by growth cones to ~68% of the original value, on average, while KD-optoEphB2 caused a marginal (~3%) increase in dynamics (Fig. 2.6c). These results confirm the role of optoEphB2 in reducing hippocampal growth cone dynamics, in agreement with published results for EphB243.

To further understand this phenomenon, we classified growth cones into three categories: lamellipodial, filopodial or blunt (Fig. 2.6d), following criteria used by an earlier study180. Growth cones classified as “lamellipodial” contained predominantly broad membrane extensions. “Filopodial” growth cones contained only long filopodia or filopodia with small lamellipodial veils. “Blunt” growth cones contained no filopodia or lamellipodia. For highly dynamic growth cones, a classification was assigned to each frame of the time-lapse movie during the initial and final 5-minute segments, and the most frequent classification was chosen. Only growth cones that were lamellipodial or filopodial to start were considered for analysis. As shown in Fig. 2.6d, optoEphB2 activation promoted a filopodial or blunt morphology, observed in 5/14 and 3/14 growth cones.
cones, respectively, post-stimulation, compared to 2/14 filopodial growth cones prior. Of the 12 growth cones that initially displayed lamellipodial morphology, optoEphB2 caused 4 to switch to filopodial and 2 to blunt. Only 1 of 7 lamellipodial growth cones became filopodial with KD-optoEphB2 stimulation, and none became blunt. This suggests selective disassembly of dendritic actin networks in growth cones.

**Discussion**

Receptor tyrosine kinases, such as the Eph receptors, are often spatially and temporally regulated to achieve desired signaling outputs\(^{165}\). This is particularly important in cell migration, where asymmetric signaling over the cell is necessary to steer cells in the appropriate direction\(^ {165}\). Concurrent studies by the Heo group achieved spatial and temporal control over the Trk and FGF family of RTKs using optogenetics\(^ {132,133}\). Here, this technique is applied to Eph receptors, whose function in cell migration and requirement of clustering for signaling render Cry2-based clustering an ideal method for spatial and temporal control. Interestingly, our design required Cry2olig, as opposed to wild-type Cry2 as reported for previous applications, for efficient clustering on the cell surface. This held despite the fact that membrane-tagged wild-type Cry2 demonstrated efficient blue light-induced clustering (Fig. 2.4d). It is possible, however, that wild-type Cry2 produced clusters small enough as to not be visible. Or, the design of the construct itself may have prevented Cry2 interactions between molecules, perhaps by weak steric hindrance or limited degrees of freedom.

We used biochemical assays and microscopic imaging to ensure optoEphB2 behaved similarly to endogenous EphB2 in live cells. The SH2 screening assay revealed
that phosphotyrosine residues in photoactivated cell lysates were capable of binding the SH2 domains of Abl, Arg, Crk, CrkL, Cten, Fes, Nck, and RasGAP. Since we are using whole cell lysates, our assay does not confirm or refute direct binding of these domains to optoEphB2, since any of these SH2 domains may be binding to phosphotyrosine residues on proteins other than, or concurrently with, EphB2. Indeed, multiple proteins displayed increased tyrosine phosphorylation in response to optoEphB2 photoactivation (Fig. 2.2b), so a multitude of sites are available for binding. The literature shows evidence of direct binding between EphB2 and the SH2 domains of Abl, Arg, Crk, Nck, and RasGAP, and we thus suspect that they also bind optoEphB2 directly. We suspect indirect interactions for the CrkL, Fes, and Cten SH2 domains. However, we cannot make these conclusions outright.

In practice, other techniques used to conclude direct binding to EphB2 are also problematic, if less so. Prior EphB2-SH2 interaction assays frequently used pull-downs, which may show indirect binding and often involve overexpression of SH2 domains or use of GST-SH2 fusions, thereby artificially increasing SH2 domain concentrations. These assays are thus susceptible to non-specific interactions and false positives. Other in vitro screening techniques, such as yeast-two-hybrid, may not reflect conditions in living cells. As such, techniques using only the SH2 domains, including the rosette assay, may yield false negatives as well as false positives. So, although we cannot conclude direct binding with the rosette assay, we still conclude that the interactions observed are consistent with native EphB2 signaling.

Interactions between EphB2 and Grb2, Vav2, and Src have also been reported in the literature, though their SH2 domains did not show statistically significant
binding of optoEphB2 lysates. For Src, this is not concerning because its SH2 domain also showed relatively weak binding to our positive controls (Fig. 2.3c,d). The interaction with Grb2 was shown by immunoprecipitation from cultured hippocampal neurons, but involvement of the SH2 domain was not explored. Multiple other studies reported negative results for interactions between the Grb2 SH2 domain and EphB2\(^{50,182}\). An interaction between Vav2 and both EphA4 and EphB2 were demonstrated by immunoprecipitation, and deleting the Vav2 SH2 domain blocked its interaction with EphA4. This was not explicitly tested for EphB2, and its interaction with Vav2 was substantially weaker than it was for EphA4\(^{90}\), suggesting that this may not be an important interaction. Thus, despite the aforementioned problems with the rosette assay, there is substantial agreement between the SH2 domains that we detected and SH2 domain binding interactions reported in the literature. This leads us to believe that appropriate signaling pathways are being activated by optoEphB2.

As observed in many prior studies with ligand-induced EphB2 clustering, blue light-induced optoEphB2 clustering yielded a substantial (~30 fold) increase in tyrosine phosphorylation. Only 1 minute of blue light illumination was necessary for this response, suggesting robust temporal control over EphB2 signaling. The chemical dimerization system, based on cross-linking FKBP domains inserted into Eph receptor sequences, appeared to require drug incubations of at least 20 minutes to fully activate the receptors. Most studies use ephrinB1-Fc treatments in excess of tens of minutes to study Eph receptor signaling. For example, ephrinB1-Fc stimulation of COS1 cells, which express EphB2 endogenously, required longer than 15 minutes to observe an appreciable increase in tyrosine phosphorylation\(^{71}\). However, ligand stimulation has been
reported to act more quickly in some studies. COS1 cell process retraction was initiated within 5 minutes of ephrinB1-Fc treatment\textsuperscript{72}, for example. Neuroblastoma cells overexpressing EphB2 showed increased tyrosine phosphorylation 5 minutes following ligand stimulation\textsuperscript{50}. So, it is clear that, at least in some contexts, optoEphB2 offers a substantial advantage in temporal control.

The other major advantage of optoEphB2 is spatial control. This may only be achieved with soluble ligands by using special equipment to establish chemical gradients. Astin \textit{et al.}\textsuperscript{61} studied contact inhibition of locomotion in prostate cancer cells by conferring spatial control with ephrin-coated microbeads. While this technique was useful, it may not be extended to slowly- or non-migrating cells, and does not allow temporal control over stimulation. OptoEphB2 permits user-defined, on-demand regions of illumination to stimulate Eph receptors, representing a more efficient and customizable method.

We thus conclude that light-mediated activation of optoEphB2 provides all the advantages of the aforementioned techniques, while limiting any drawbacks. We demonstrated spatial control of signaling to user-defined regions of illumination and showed reversibility and repeatability of signaling, such that any rebound effects may be studied, where relevant. Since Cry2 clustering is tunable using light power and pulse duration and frequency\textsuperscript{183} this technique may, too, be used to tune cluster size and study cell responses. Additionally, transfection of multiple optoEph constructs allows for co-clustering multiple Ephs simultaneously. However, this optical technique is not without its flaws. The off-rate of Cry2 clustering is slow, thus analysis of short-lived Eph receptor stimulation (less than tens of minutes timescale) is not possible. Additionally,
the PDZ-binding motif is blocked by the Cry2-FP fusion, though our data suggest that expected cellular phenotypes and effector recruitment persist despite this modification. Overall, optoEphB2, and the other optoEphs, represent a versatile module for spatial and temporal control over Eph receptor signaling that may be applied in a variety of cell types.
Table 2.1. Nomenclature of constructs used to design and test an optogenetic tool for EphB2 clustering and signaling activation. KD, kinase-dead. LI, light-insensitive. Cry2 or Cry2olig indicate the corresponding PHR, unless otherwise noted. FP, fluorescent protein. Myr, myristoylation sequence derived from c-Src. EphR, Eph receptor. ICD, intracellular domain. K→M, arginine-to-methionine mutation in the ATP-binding pocket at position 662 of EphB2, or position 98 of optoEphB2. D→A indicates aspartate-to-alanine mutation in Cry2 at position 387, rendering it insensitive to blue light by disrupting FAD binding. This was done to full-length Cry2. *Versions containing full-length Cry2olig were also made.
Figures

Figure 2.1

(a) EphB1-Cry2-mCh  EphB2-Cry2-mCh  EphB6-Cry2-mCh

(b) OptoEphB2 (WT-Cry2)

(c) OptoEphB2 (WT-Cry2)  OptoEphB2 (Cry2-alkg)

Blue Light

*Blue Light*
**Figure 2.1. Design iterations for optoEphB2.** (a) Confocal microscopy images of HEK293 cells expressing full-length EphB1, EphB2, or EphB6 fused to Cry2-mCh. Most of the fluorescent signal appeared in punctate cytoplasmic structures with EphB2 and EphB1. EphB6 showed relatively improved plasma membrane localization. (b) Confocal images of HEK293 cells expressing optoEphB2 with wild-type Cry2 or Cry2olig. These constructs showed improved localization to the plasma membrane. Confocal images in (a) and (b) were acquired on a Zeiss LSM510 confocal microscope, using a 561-nm laser to excite mCherry. (c) TIRF microscopy images of optoEphB2 with wild-type (WT) Cry2 (COS7 cell) or Cry2olig (HEK293 cell) before and following blue light illumination. COS7 cells were given 500-ms pulses of 488-nm laser light at 5-second intervals for 85 seconds. HEK293 cells were given three 250-ms pulses of 488 nm light, 4.5 seconds apart. Scale bars, 10 µm.
Figure 2.2

(a) Diagram showing the effect of blue light on EphB2 ICD and Cry2 oligomers. Blue Light induces a conformational change leading to the activation of the tyrosine kinase domain (Tyr Phos).

(b) Western blot analysis showing the expression levels of α-pTyr, α-mCh, and α-Tubulin under different conditions: WCL, OptoEphB2, and KD-OptoEphB2. The graph represents the relative tyrosine phosphorylation (pTyr/mCh) under Blue Light conditions.
Figure 2.2. Optogenetic activation of EphB2 tyrosine kinase activity. (a) Illustration of optoEphB2 domain structure and the photoactivation process. Blue light illumination induces Cry2 clustering, which results in receptor autophosphorylation (Y, tyrosine and pY, phosphotyrosine) and downstream signaling. ICD, intracellular domain. FP, fluorescent protein. Cry2olig indicates full-length Cry2olig or the PHR of Cry2olig. (b) Left: western blot analysis of whole cell lysates collected from MEFs stably expressing optoEphB2 or KD-optoEphB2 that were illuminated by blue LED light (~10^-2 W/cm²), or left in the dark, for 1 minute. Right: quantification of optoEphB2 phosphorylation. Relative tyrosine phosphorylation was assayed in optoEphB2 immunoprecipitates and quantified by dividing the phosphotyrosine signal by the mCherry signal. Error bars show SEM (n=3).
Figure 2.3

(a) GST-Abl interaction with various proteins.

(b) Image showing interaction under different conditions.

(c) Table listing various proteins and their interactions.
**Figure 2.3.** OptoEphB2 signals SH2 domain proteins typical of EphB2. (a) Illustration showing the position of each sample on any given rosette in the SH2 screening assay (see Methods) performed on the MEF cell lysates. Representative results (Arg) are shown with relevant controls. (b) TIRF microscopy images of NIH3T3 cells co-expressing the indicated constructs prior to and following blue light illumination. Bottom row shows overlay. Scale bar, 10 µm. (c) Rosettes for all SH2 domains tested. (d) Quantification of rosette results. Values represent mean intensity of triplicates, measured by densitometry, normalized to the maximum value of all experiments. Error bars, SD. *p < 0.05 by two-way ANOVA. KD-L, KD-optoEphB2 treated with 1 minute of blue light. KD-D, KD-optoEphB2 left untreated (dark). WT-L, optoEphB2 treated with 1 minute of blue light. WT-D, optoEphB2 left untreated (dark). Error bars, SD.
Figure 2.4

(a) Pre-Illum. 40s Blue Light 2 min 10 min

(b) OptoEphB2 KD-OptoEphB2

(c) OptoEphB2 EGFP

(d) OptoEphB2 KD-OptoEphB2 Myr-Cry2oligPHR

(e) OptoEphB2 KD-OptoEphB2
Figure 2.4. OptoEphB2 activation causes reversible and repeatable cell collapse and retraction of cell protrusions. (a) Time-lapse TIRF images of optoEphB2 or KD-optoEphB2 in MEFs during blue light illumination (50-ms pulses, 3 pulses/min.). Black dotted lines trace initial cell area. (b) Top: Quantification of mean normalized MEF cell area in response to blue light illumination of optoEphB2. Area at each time point is normalized to the mean prior to photoactivation and averaged between cells. Error bars, SEM. Bottom: OptoEphB2 cluster density (gray triangles) in response to continuous pulses of blue light illumination. Each time point is normalized to the mean cluster density prior to photoactivation and averaged between cells. This curve was fit (dotted line) using an exponential to calculate the time constant ($\tau$) of 14.9 seconds. Error bars, SEM. (c) DIC and TIRF microscopy images of MEFs transiently transfected with EphB2-EGFP that were treated with pre-clustered ephrinB1-Fc chimeras (left) or human Fc fragments (right) for 15 minutes. TIRF image shows EphB2-EGFP signal prior to treatment. Dotted black lines trace initial cell area. (d) Left: TIRF images of optoEphB2, KD-optoEphB2, or myr-Cry2olig-mCherry in HEK293 cells, before and 3 minutes following blue light illumination (two 1-s pulses at 460 nm, gray triangles, 6 frames/min.). Right: Quantification of HEK293 cell area, normalized to the mean prior to blue light illumination, and averaged between cells. Error bars, SEM. (e) TIRF and DIC images of MCF7 cells expressing optoEphB2. TIRF images show optoEphB2 signal before and following photoactivation (100-ms pulses, 440 nm, 6 frames/min.). The kymographs correspond to the white dotted lines in the DIC images. Blue dotted lines indicate the start of photoactivation. Scale bars, (a), (c)-(e), 10 μm (unless otherwise noted).
Figure 2.5

(a) - Blue Light
   + Blue Light

(b) 0 min  2 min  4 min

(c) 0 min  3 min  8 min  13 min  18 min

Norm. Cell Area

Time (min)

0 min  2 min  4 min

0.5  1.0  1.1

0  10  20  30

5 min

10 µm
**Figure 2.5.** Local optoEphB2 photoactivation causes spatially-confined clustering and retraction of cell protrusions. (a) TIRF images of optoEphB2 in MCF7 cells before and after focal blue light illumination (white circle, 10-ms pulses at 440 nm, 6 frames/min, 25 min.). (b) Time-lapse fluorescence images of optoEphB2 (MEF), which was activated by blue light illumination (100-ms pulses, 6 pulses/min.) within the specified region of illumination (ROI, black circle). Time labels are relative to the start of photoactivation. (c) Time lapse TIRF images (top) of optoEphB2 (MEF) that was photoactivated (100-ms pulses, 10-s intervals) over the indicated one-minute time segments (blue, bottom), which were spaced at 10-minute intervals. Plot shows cell area over time, normalized to the average cell area prior to the first stimulation (2 minutes). Kymograph (bottom right) of the indicated region (dotted line) shows repeated formation and dissipation of clusters, as well as cell shrinkage and expansion. Scale bars, 10 µm.
Figure 2.6

(a) Maximum projections over time

(b) Pre-illumination (5 min) 10-15 min Blue light

(c) Norm Maximum Projection Area

(d) Lamellipodial Filopodial Blunt
Figure 2.6. OptoEphB2 photoactivation causes growth cone collapse and retraction. (a) Time lapse fluorescence images of mCherry (volume marker) in growth cones during photoactivation (100-ms pulses, 3 pulses/min.) of co-expressed optoEphB2 or KD-optoEphB2. Examples of growth cone collapse and retraction in response to optoEphB2 stimulation are shown. Time labels are relative to the start of photoactivation. (b) Maximum intensity projections of mCherry (volume) signal growth cones, constructed from 5-minute time segments before, and at the end of, blue light illumination. Illustration at top shows a timeline of the acquisition, indicating the relevant 5-minute intervals (gray). (c) Plot shows normalized maximum projection area for each growth cone. Maximum projection area for each growth cone was measured for the final 5 minutes of blue light illumination, and this value was normalized to the maximum projection area calculated prior to blue light illumination. Diamonds indicate means, error bars show standard deviations. *p < 0.05, t-test. (d) Analysis of growth cone morphology before and after blue light illumination. Growth cones were classified as lamellipodial, filopodial, or blunt (see text). Scale bars, (a), (b), (d), 10 μm.
Movie Captions

Movie 2.1. OptoEphB2 photoactivation causes cell collapse in fibroblasts. Blue light illumination (blue dot) of MEFs stably expressing optoEphB2 (Left) caused rapid cell collapse, which was not observed with KD-optoEphB2 (Right).

Movie 2.2. Spatial regulation of optoEphB2 stimulation. Focal (blue circle at 2:00) blue light illumination (blue dot) of optoEphB2 in MEFs results in spatially-restricted clustering and cell protrusion collapse, without perturbation of the remaining cell area.

Movie 2.3. OptoEphB2 clustering and signaling are reversible and repeatable. OptoEphB2 (MEF) is illuminated with blue light for 1 minute at 10-minute intervals. After each illumination period (blue dot), there is rapid collapse of cell protrusions followed by recovery of cell area. Repeated photostimulation causes additional collapse and recovery cycles.

Movie 2.4. OptoEphB2 photoactivation causes growth cone collapse and retraction. Movie shows mCherry (volume marker) signal in growth cones of DIV5 hippocampal neurons. Photoactivation (blue dot) of optoEphB2 for 15 minutes resulted in growth cone collapse (Left) and retraction (Center). Photoactivation of KD-optoEphB2 (Right) did not affect growth cone dynamics.
CHAPTER III: Local EphB2 Signaling Induces Branching of Dendritic Filopodia, Expansion of Filopodia Tips, and Promotes Dendritic Filopodia Formation

Attribution: This chapter contains excerpts from the manuscript “Effects of Localized EphB2 Activation on Dendritic Filopodia of Hippocampal Neurons” co-authored by Clifford Locke, Qingfen Yang, Kazuya Machida, Chandra Tucker, Yi Wu, and Ji Yu. Qingfen Yang assisted with cloning procedures. Chandra Tucker supplied the Cry2 mutant, Cry2olig, ahead of her manuscript on its development. Ji Yu performed the experiments and analysis that measured PIP3 levels in 3T3 cells and constructed Figure 3.4a-3.4d. Drs. Betty Eipper and Richard Mains supplied rat hippocampi. Anthony Koleske provided Arg constructs and GNF2. Ji Yu and Yi Wu proof-read and edited the text. Clifford Locke wrote the text, constructed all figures that were not otherwise attributed, and did all experimental work that was not otherwise attributed.
Abstract

Dendritic spines are believed to derive from dendritic filopodia by reorganization of the actin cytoskeleton and recruitment of post-synaptic molecules. Previous studies suggested that EphB signaling, initiated at contacts between axons and dendritic filopodia, plays a critical role in this transition. However, the exact effects of EphB signaling on the motility and morphology of dendritic filopodia have not been clearly defined. We used optoEphB2, an optogenetic module for spatial and temporal control over EphB2 signaling, to specifically stimulate EphB2 in dendritic filopodia and small regions of dendritic shafts. Presumably, this would model local signaling at axo-dendritic contacts. Using this strategy, we found that localized EphB signaling at dendritic filopodia promoted filopodia branching and plasma membrane expansion associated with F-actin accumulation. Activation along the dendritic shaft also promoted actin nucleation, but resulted in de novo formation of dynamic filopodia that was dependent on activation of the Arp2/3 complex. Furthermore, we show evidence that local EphB signaling resulted in an activation of phosphoinositide 3-kinase (PI3K), which marks a key difference between signaling in dendrites and in fibroblasts, in which EphB signaling causes retraction of cell protrusions. Combined, these results provided direct evidence of actin polymerization in filopodia following EphB stimulation and suggested that differential PI3K activity is important to adhesion versus repulsion downstream of EphB signaling in neurons.

Introduction

In the central nervous system, EphBs are known to promote synaptogenesis and dendritic spine development\textsuperscript{12}. Inhibition of EphB signaling via genetic deletion or over-
expression of dominant-negative constructs resulted in reduced spine density and
dysmorphic spines in hippocampal neurons\textsuperscript{13,14}. \textit{In vitro} activation of EphB receptors
with pre-clustered ephrinB-Fc ligands rapidly increased the number of synaptic spines in
cultured rat hippocampal neurons at DIV10\textsuperscript{15}. These results suggested that EphB
signaling at axo-dendritic contacts is a key event that drives the formation of dendritic
spines. Initial contacts likely form between axons and dendritic filopodia, which are thin
and transient actin-based protrusions on dendritic shafts that are most abundant in
immature neurons\textsuperscript{4,135}. Dendritic filopodia are highly motile and thought to actively “seek
out” axons in order to establish contacts, which then may subsequently lead to EphB
activation and development of spines\textsuperscript{5,12}. However, the exact effects of local EphB signal
activation on the morphology and dynamics of dendritic filopodia have never been
clearly defined, because \textit{in vitro} activation of EphB receptors using soluble ligands
inevitably induces signaling globally in all cell types and cellular compartments. In
contrast, EphB signals \textit{in vivo}, particularly at axo-dendritic contacts, are presumably
highly localized.

To examine dynamic responses of filopodia to local EphB signaling, we utilized
optoEphB2 to activate EphB2 signaling with precise spatial and temporal control. Signal
activation in dendritic filopodia of hippocampal neurons, in contradiction to previously-
reported results in fibroblasts and growth cones, promoted actin polymerization that
resulted in filopodia branching and membrane expansion. Interestingly, focal
illumination of dendritic shafts resulted in \textit{de novo} formation of filopodia-like
protrusions, associated with activation of PI3-kinase. Inhibition of Arp2/3 and Arg, an
upstream regulator of Arp2/3 signaling, also inhibited filopodia formation. While EphB2
signaling caused an accumulation of the plasma membrane lipid phosphatidylinositol(3,4,5)-triphosphate (PIP$_3$) in dendrites, a reduction of plasma membrane PIP$_3$ was associated with cell process retraction in 3T3 cells. These results suggested that differential regulation of PIP$_3$ synthesis may account for the differences seen between dendrites and other cell types. Our experimental results demonstrated the mechanistic link between local EphB signaling and actin polymerization and provided new insights into the role of EphB2 signaling in the dendritic filopodia-to-spine transition.

**Methods**

*Antibodies, reagents, and plasmids*

Cloning of optoEphB2 and other optoEphB constructs, as shown in Figure 2.2a, is described in the Methods section of Chapter II. The light-insensitive optoEphB2 construct, containing the Cry2 D387A mutation as previously described$^{129}$, was made by subcloning fragments that contained the mutation into optoEphB2. OptoEphB2 and KD-optoEphB2 containing Cry2olig (full-length) were also made by subcloning DNA fragments containing the C-terminal segment missing in the truncated versions$^{130}$. These clones containing Cry2olig were used for optoEphB2 photoactivation experiments in neurons. OptoEphB2-PBM was made by subcloning a DNA fragment containing the sequences for mCherry and the final 6 amino acids of EphB2 (IQSVEV) into optoEphB2, taking the place of mCherry. The Lifeact-mCherry (plasmid #54491) construct was obtained from Addgene. The PH$_{Akt}$-mRFP plasmid was previously described$^{121}$. The myr-mCherry plasmid was prepared by recombination between an entry vector
containing the myristoylation sequence and a destination vector containing mCherry (Addgene plasmid #31907). The Arg-YFP and kinase-dead Arg-YFP (ArgKD-YFP) sequences\(^{169,184}\) were gifts from Anthony Koleske. The kinase-dead Src-YFP (SrcKD-YFP) plasmid was a gift from Yi Wu.

**Cell culture, transfections, and drug treatments**

Protocols for extraction, culture, and transfection of hippocampal neurons are described in the Methods section of Chapter II. For treatment of neurons with LY294002 (Tocris) and DMSO, neurons transfected with optoEphB2 were initially photoactivated to confirm protrusion formation and PH\(_{Akt}\) accumulation. Cultures were then incubated in the dark for at least 20 minutes to allow optoEphB2 clusters to dissipate, and were then treated for 30 minutes with 50 \(\mu\)M LY294002 or a 1:200 dilution of DMSO, representing the dilution factor from a 10 mM stock of LY294002. Photoactivation was then repeated over the same region of illumination. Treatment with 5 \(\mu\)M cytochalasin D was initiated immediately following the first photoactivation and continued for 30 minutes. For CK-666 and GNF-2 (gift from Anthony Koleske) treatment in conjunction with dendritic shaft stimulation, neuronal cultures were pre-treated with 200 \(\mu\)M CK-666 or a 1:50 dilution of DMSO for 30 minutes, or 10 \(\mu\)M GNF-2 or 1:1000 DMSO for 1 hour, prior to photostimulation.

**Microscopy and image analysis**

Imaging conditions and photoactivation equipment are described in the Methods section of Chapter II. To illuminate filopodia, ROIs were defined along the length of the
dendrite, placed just far enough to avoid direct shaft stimulation, though spatial confinement was not perfect. For focal illumination of dendritic shafts, the mosaic was used to deliver blue light to a 40-pixel-diameter (~266 nm/pixel) circular region, unless otherwise noted. Images for all dendritic shaft stimulation experiments were acquired at 3 frames/min, unless otherwise noted, and filopodia stimulation was acquired at 6 frames/min. Photoactivation was delivered in 50-ms pulses between frames, at 1% LED power, unless otherwise noted.

Maximum intensity projections were generated after background subtraction and drift correction using the “BG Subtraction from ROI” and the “StackReg” plugins, respectively, in ImageJ. Images were sharpened using the “Unsharp Mask” function. Maximum projection areas were measured within the ROI, using the same threshold value for any given cell. Neurons were excluded if the dendritic shaft deformed within the ROI during the acquisition. Only protrusions with greater than 0.5-μm length were considered filopodia and analyzed for density or Lifeact content. Lifeact accumulation was quantified by the mean Lifeact-mCherry intensity along a linescan that was manually drawn along the center of a filopodium from base to tip. This was done for each filopodium adjacent to, or falling within, the ROI at the first and last frames of photoactivation. In the case of plasma membrane expansion, a region of interest was drawn around the protrusion and the mean signal was quantified. For each cell, the mean filopodium intensity for the final frame was normalized to that of the first frame, and values were averaged between cells. Filopodia were examined for branching throughout the photoactivation period, and only those filopodia within the ROI for greater than three imaging frames were included. PH_{Akt}-mRFP and mCherry accumulation were measured.
by normalizing the average intensity in the ROI, frame-by-frame, to the mean intensity within the ROI throughout the 5-minute period prior to photoactivation.

**Results**

*OptoEphB2 activation in dendritic filopodia induces actin polymerization that results in branching and plasma membrane expansion*

To understand effects of local EphB activation in dendritic filopodia, we expressed either optoEphB2 or KD-optoEphB2 in DIV9-11 hippocampal neurons and monitored cell morphology changes after signal activation. Cells were co-transfected with either myr-mCherry, used as a membrane marker, or Lifeact-mCherry, to monitor actin polymerization. Filopodia were stimulated by targeting blue light to a region of illumination (ROI) oriented lengthwise along, but offset from, the dendritic shaft, and imaging the optoEphB2 and KD-optoEphB2 signals showed confinement of clustering to filopodia (Fig. 3.1a). To our surprise, we found that stimulation of optoEphB2 in filopodia resulted in a ~2-fold increased probability of filopodia branch formation (Fig. 3.1b, Movie 3.1), in comparison to the KD-optoEphB2 control. In some protrusions, broadening of filopodia was observed, with formation of lamellipodia-like structures in a small number of cases (Fig. 3.1e, Movie 3.2). The next section describes filopodia formation following optoEphB2 clustering on the dendritic shaft. No changes in filopodia density were observed with stimulation at dendritic filopodia (Fig. 3.1c), further confirming the desired spatial regulation. Dendritic filopodia are supported by a dynamic actin cytoskeleton\textsuperscript{141}. Therefore, we suspected that the observed changes in filopodia morphology were caused by nucleation of actin filaments. To further characterize the
effect of EphB2 on actin cytoskeleton, we quantified Lifeact-mCherry signals in filopodia before and after blue light illumination. Significant accumulation of Lifeact signal (~57% increase) was observed with OptoEphB2 stimulation in filopodia (Fig. 3.1d,f, Movie 3.1), indicating increased F-actin concentration.

Local EphB2 signaling in dendritic shafts induces dynamic filopodia-like protrusions

EphB2 is expressed extensively along the dendritic shafts of hippocampal neurons. We therefore further examined whether EphB2 activation in the dendritic shaft may also affect the actin cytoskeleton and cell morphology. Localized blue light photoactivation (~10 µm diameter) was delivered to dendritic regions of DIV10-11 hippocampal neurons co-expressing mCherry, or Lifeact-mCherry, with either optoEphB2, KD-optoEphB2, or light-insensitive optoEphB2 (LI-optoEphB2, contains Cry2 with inactivating D387A mutation, see Table 2.1). We found that photoactivation of optoEphB2 locally induced formation of dynamic dendritic protrusions that resembled filopodia (Fig. 3.2a-c, Movie 3.3). This phenotype was not observed with KD-optoEphB2 (Fig. 3.2a,c) or LI-optoEphB2 (Fig. 3.2a), indicating that the effect requires both clustering and kinase activity, and is not an effect of phototoxicity. In neurons co-transfected with Lifeact-mCherry, Lifeact accumulated in a punctate distribution on the periphery of the dendrite in the ROI (Fig. 3.2b), indicating increased actin nucleation at the base of newly-formed filopodia.

To quantify these morphological changes in dendrites, we generated maximum intensity projection images over 5-minute segments of time-lapse images both before and during the 15 minutes of photoactivation (Fig. 3.2b). The cellular area of a maximum
intensity projection is affected by changes in the morphology and dynamics of the dendritic filopodia, thereby measuring the area explored by the filopodial protrusions. We found that the cellular area, including the area of dendritic shaft and the areas explored by filopodial protrusions, increased significantly (~25%) after optoEphB2 activation, and became progressively larger during the 15-minute photoactivation period (Fig. 3.2d). This occurred in a kinase-dependent manner, as KD-optoEphB2 did not produce such an effect. After stimulation and letting the cells sit in the dark for 20 min, we found that the number of dynamic filopodia was reduced to the pre-stimulation level. A second round of blue light stimulation promoted new filopodial growth, indicating that the stimulation is reversible and repeatable (Fig. 3.2e), as previously demonstrated in MEFs (see Fig. 2.4). Finally, we also found (Fig. 3.2f) that the induction of filopodia can be blocked by treatment with CK-666, an inhibitor of Arp2/3 complex, suggesting that, similar to stimulation in filopodia, EphB2 activation in dendritic shaft also induces branched actin nucleation, which in turn gives rise to formation of new filopodia.

Additional tests were performed on other EphB family receptors. We found that focal blue light illumination of hippocampal dendrites expressing optoEphB1 led to filopodia formation in a similar manner to optoEphB2 (Fig. 3.3), while stimulation of optoEphB6 did not produce filopodia formation (Fig. 3.3). EphB6 is different from all other EphB members in that it is the only member that does not have a functional kinase domain. Thus the results further verified that the kinase activity of EphBs is essential for the effects on actin cytoskeleton. Additionally, these observations are consistent with prior observations of functional redundancy among EphB isoforms in dendrites. Since the design of optoEphB2 blocks the C-terminus of the PBM, which is known to link
EphB2 to downstream effectors and PDZ scaffolds that additionally bind glutamate receptors, we wanted to confirm that our observations would hold with PBM-mediated interactions. We thus subcloned the six C-terminal amino acids of EphB2 (IQSVEV), containing the PBM (VEV), into the C-terminus of optoEphB2 (optoEphB2-PBM). We suspected that PDZ binding would be restored, given the ability of isolated PBMs to bind their respective PDZ domain partners\textsuperscript{186}. Additionally, overexpression of an EphB2-YFP fusion, in which YFP was inserted N-terminal to the PBM, was able to rescue spine formation in EphB1-B3 triple-knockout neurons\textsuperscript{16}. Local stimulation of optoEphB2-PBM recapitulated the filopodia formation seen with optoEphB2 (Fig. 3.3), further validating the physiologic relevance of our observations.

"Differential regulation of PIP\textsubscript{3} synthesis underlies cell context-dependent effects of EphB2 signaling on the actin cytoskeleton"

We observed that optoEphB2 photoactivation increased protrusive activity in dendrites and caused collapse of protrusive structures in growth cones and MEFs. This raises the question of how downstream EphB signals cause such differences in phenotype. Lin et al. recently studied EphB2-mediated CIL (contact inhibition of locomotion) in motile cells and identified down-regulation of phosphatidylinositol-(3,4,5)-triphosphate (PIP\textsubscript{3}) synthesis as a key downstream pathway\textsuperscript{98}. Therefore, we set out to investigate whether the effect on PIP\textsubscript{3} synthesis is cell type-specific. First, we sought to confirm that optoEphB activation also suppress PIP\textsubscript{3} synthesis in fibroblasts (Fig. 3.4a-d). Using a PIP\textsubscript{3} sensor, the pleckstrin homology domain of Akt (PH\textsubscript{Akt}) labeled with mRFP (PH\textsubscript{Akt-mRFP})\textsuperscript{121,174}, and total-internal-reflection fluorescence (TIRF)
microscopy, we measured the spatial distribution of PIP$_3$ in 3T3 cells (Fig. 3.4a). As expected, elevated PIP$_3$ signal was found at the leading edges of cell protrusions, which is evident from the intensity linescans (Fig. 3.4b) perpendicular to the edge of cell protrusions. Upon optoEphB2 activation, we observed a rapid (< 1 min) decrease of PH$_{Akt}$ intensity at the leading edge (Fig. 3.4b). Importantly, the reduction of PH$_{Akt}$ intensity preceded the retraction of cell protrusions. Retractions were initiated after 1 minute of stimulation (Fig. 3.4b). On average, we observed ~20% decrease in PH$_{Akt}$ intensity during the first minute of photoactivation (Fig. 3.4c) near the leading edge (1 µm from the cell edge). A smaller decrease was also observed at the interior of the cells (5 µm from cell edge), but the decrease was not statistically significant (Fig. 3.4d). Overall, these results confirm the recent study$^{98}$ and indicated that optoEphB2 elicits a similar pathway to EphB2.

Different results were observed in hippocampal dendrites using the same PIP$_3$ sensor. In DIV10-11 hippocampal neurons, we found coincidental PH$_{Akt}$ accumulation in the ROI (Fig. 3.4e-g, Movies 3.3-3.5) when we activated optoEphB2 and observed formation of filopodial protrusions. Epi-fluorescence microscopy was used here because TIRF microscopy was not suitable for imaging dendrites, which are not adherent to glass substrates. Therefore, the intensity of a volume marker, mCherry, was also measured to control for a potential artifact due to volume changes. We found that the average signal change is 29.8% for PH$_{Akt}$, compared to 5.0% for mCherry, after 15 minutes of blue light illumination (Fig. 3.4f), suggesting that volume change was not a significant factor and PIP$_3$ accumulation was primarily responsible for the PH$_{Akt}$ signal increase. Therefore,
regulation of PIP₃ synthesis downstream of EphB2 is cell type-dependent and may play a role in regulating adhesive versus repulsive responses downstream of EphB2 activation.

To further validate the role of PIP₃ synthesis in the observed signal outcome, we used a PI3K inhibitor, LY294002, to block PIP₃ synthesis. To minimize effects of cell-to-cell variability, we performed the comparison experiments on the same dendrite in a pairwise fashion. Specifically, we first performed focal photoactivation of optoEphB2 as described earlier to confirm the induction of actin polymerization and filopodia growth. The cells were then incubated in the dark for at least 20 minutes to disperse optoEphB2 clusters, followed by treatment with either LY294002 or DMSO (control) and re-stimulation of the same ROI (Figure 3.5a,b). Quantification of PHAkt intensity in the ROI showed that LY294002 treatment eliminated PHAkt accumulation, which was maintained in DMSO-treated controls (Fig. 3.5a,b, Movies 3.3 and 3.4). In addition, LY294002 treatment resulted in abrogation of filopodia formation (Fig 3.5a,b). Finally, to see whether the induced PIP₃ synthesis was upstream of F-actin synthesis, as opposed to be a consequence of it¹⁸⁷, we inhibited actin polymerization using cytochalasin D during the second round of stimulation (Fig. 3.5c, Movie 3.6). Yet, we still observed increased PIP₃ signal comparable to the first round of stimulation (Fig. 3.5c). Therefore, we conclude that PI3K activity was necessary for both the observed PIP₃ synthesis, and formation of optoEphB2-induced protrusions.

Filopodia formation by optoEphB2 depends on Abelson family kinases

Our previous experiments established a role for Arp2/3 activity in filopodia formation downstream of optoEphB2 and binding of phosphotyrosines in cell lysates to
the SH2 domain of Arg. Arg, also known as Abl2, is a non-receptor tyrosine kinase (NRTK) that enriches in dendritic spines and functions in dendritic spine stability and maintenance\textsuperscript{103}. Arg is known to activate the Arp2/3 complex through the actin-binding protein (ABP) cortactin, which plays an important role in dendritic spine formation\textsuperscript{188}. We thus hypothesized that Arg is mechanistically involved in regulating actin dynamics downstream of optoEphB2 in neurons. To test this hypothesis, DIV11-12 neurons were co-transfected with Arg-YFP and optoEphB2-mCherry and photostimulated as described for filopodia formation. After photoactivation, optoEphB2 and Arg signals co-localized in the ROI, suggesting an interaction (Fig. 3.6a). Co-expression of kinase-dead Arg, but not kinase-dead Src, with optoEphB2 reduced the area explored by filopodia following stimulation (Fig. 3.6b). Kinase-dead Src overexpression aids in controlling for non-specific SH2 binding, since the Arg and Src SH2 domains are known to bind the juxtamembrane tyrosines of EphB2\textsuperscript{50,104,177}. Additionally, treatment of neurons with the allosteric Arg inhibitor GNF-2 reduced the area explored by filopodia (Fig. 3.6c). We thus conclude that Arg is functionally linked to EphB2 in regulating actin dynamics in dendrites.

**Discussion**

A key advantage to using an optogenetic method of receptor stimulation is tight spatial and temporal control over signaling. We took advantage of this property to directly interrogate the effects of local EphB2 signaling in dendritic filopodia, thereby simulating the scenario of local contact formation with axons. Results from prior studies that addressed this question were inconclusive. Cortical neurons from EphB1-B3 triple
knockout mice did not show any defect in the density of dendritic filopodia\textsuperscript{16}, conflicting with reports of increased dendritic protrusion density following ephrinB1-Fc treatment of cultured hippocampal neurons\textsuperscript{15}. Triple-knockout neurons also displayed impaired filopodial motility\textsuperscript{16}, but this does not address the question of the effect of ligand-mediated stimulation on filopodia motility.

Our optogenetic technique expanded on prior work by allowing real-time observation of protrusion morphology with simultaneous stimulation of EphBs targeted to dendritic filopodia. We observed minutes-timescale increased actin polymerization in filopodia, ultimately resulting in branching and plasma membrane expansion. Given that filopodia and spines are supported by a dense actin network\textsuperscript{141}, such expansion of the membrane would suggest direct nucleation of dendritic actin networks by EphB signaling in filopodia. In fact, this model is more consistent with studies that suggested Rac1 activation and a Cdc42-N-WASP-Arp2/3 signaling complex downstream of EphB2 in dendrites\textsuperscript{15,147,148,150}. Dendritic spine heads contain dendritic actin networks, and as such, our findings may link EphB signaling to initiating spine head formation. Further detailed studies of actin in filopodia and spine head markers would be necessary to draw these conclusions.

Focal stimulation of EphB signaling along the dendritic shaft caused local formation of numerous filopodia-like protrusions. Previous reports suggested that EphB signaling increased dendritic spine and overall protrusion density\textsuperscript{15}, but did not specifically address the formation of dendritic filopodia. Our findings would thus suggest that EphB signaling may increase dendritic spine density by not only converting filopodia to spines, but also by generating new filopodia near sites of axo-dendritic
contact to serve as a “positive feedback” mechanism to form more spines. Given that filopodia formation was confined to areas of optoEphB2 clustering, it is possible that EphBs “hone” filopodia formation to sites of axo-dendritic contact.

Interestingly, the formation of filopodial protrusions was blocked by Arp2/3 inhibition, suggesting a role for dendritic actin nucleation. While dendritic filopodia are unique in containing some actin branching, they still predominantly contain linear actin networks, except for the triangular filopodia base. Our data showed accumulation of F-actin predominantly at the periphery of the dendritic shaft, suggesting that EphB signaling may promote the formation of branched actin patches to serve as new sites for filopodia formation. Additionally, neurons from EphB1-B3 triple-knockout mice, while failing to form spines in culture, formed F-actin patches on dendritic shafts, suggesting a shift in axo-dendritic contact sites from protrusions to the dendritic shafts. Since EphB2 is highly expressed along hippocampal dendritic shafts, promoting actin polymerization on the shaft likely has some functional role in dendrite or dendritic spine morphogenesis. EphB signaling may ensure that, even at axo-dendritic contacts along the dendritic shaft, proper spine formation ensues. This result may also suggest that the branching and plasma membrane expansion induced in dendritic filopodia also occur secondary to Arp2/3 complex activation. This provides more evidence for the nucleation of dendritic actin networks in filopodia.

One recent study of EphB signaling in neurons showed reduced Tau phosphorylation, and subsequent mitigation of Alzheimer’s Disease progression, by EphB2-mediated PI3K signaling. PI3K activation by EphBs was also implicated in activation of peripheral and central pain pathways. Thus, activation of PI3K by
EphBs in neurons is known, and our results provide the first evidence that connects PI3K signaling to the actin cytoskeleton in dendrites and dendritic protrusions downstream of EphB2. Given the known roles of EphBs and PI3K, independently, in promoting dendritic spine morphogenesis, these results are not surprising. PI3K is also known to increase the motility of dendritic filopodia downstream of RTKs, such as Trk receptors, that are activated by neurotrophins.

Our observations of PI3K activation may be relevant in light of Rho GTPase regulation by EphBs in neurons. We recently demonstrated that sub-cellular PI3K signaling had a strong impact on polarization of Rac1 activity in migrating cells. EphB signaling is also known to activate Rac1, and other Rho GTPases, in hippocampal dendrites. Therefore, it is plausible that PI3K activation may contribute to Rac1 activation observed downstream of EphB signaling. Perhaps, local accumulation of PIP_3 serves to spatially coordinate Rac1 activity at sites of axo-dendritic contact. This may occur through the recruitment of GEFs. The pleckstrin homology domains of Tiam1 and intersectin, and the sec14 domain of kalirin-7, have all been shown to interact with PIP_3.

Effects of Eph receptor signaling on the actin cytoskeleton are highly context-dependent. Initially, Eph receptors were thought to primarily mediate repulsive cues, which are correlated with the inhibition of actin polymerization. But later evidence, particularly in cancer and dendritic spine morphogenesis, indicated that Eph receptors mediate a much broader array of cell behaviors. This included promoting cell-cell or cell-matrix adhesion and cell migration. The underlying mechanisms of this cell context-dependence remain poorly understood. Our observations suggest that differential
regulation of PI3K, or plasma membrane PIP₃ content, contributes to this phenomenon. Our observations and prior studies⁹⁸ of EphB2 signaling suggest down-regulation of plasma membrane PIP₃ content in conjunction with retraction of cell protrusions. When we observed a protrusive phenotype in hippocampal dendrites, local accumulation of PIP₃ was observed. Activation of PI3K was found necessary for this accumulation and formation of filopodia-like structures.

Further studies are necessary to determine the underlying mechanisms of such differential regulation. Differences in protein expression and effector binding between cell types, or between axons and dendrites, are likely involved. This mechanism was suggested in a study of differential Ras/MAPK regulation by EphB4. In human umbilical vein endothelial cells, EphB4 inhibited the Ras/MAPK pathway through p120RasGAP. In MCF7 breast cancer cells, EphB4 promoted Ras/MAPK signaling in a PP2A-dependent manner, and p120RasGAP was not expressed⁷⁹. Since multiple Ras isoforms are involved in the regulation of PI3K³⁶, it is possible that differential regulation of Ras also underlies our observed context-dependent effects on PIP₃ synthesis. Down-regulation of Ras by EphB signaling is well-documented in conjunction with p120RasGAP⁷². Numerous potential pathways exist for EphBs to activate PI3K. In dendrites, a signaling complex of EphB, Src, focal adhesion kinase, and Grb2 formed following ephrinB ligand treatment¹⁷. In another context, EphB1 was shown to activate Ras through the Ras-GEF SOS by recruiting the adaptor Grb2 through Shc⁷⁶. Thus, SOS may be responsible for Ras activation and downstream PI3K signaling. GAB1 is another potential binding partner of Grb2 that is known to activate PI3K¹⁹⁷. EphB2 was shown to interact with the SH2 domain of a p85 regulatory subunit of PI3K in vitro, which would
result in PI3K activation\textsuperscript{36,50}. Differences in kinase-dependent and –independent signaling may also account for the discrepancies. An \textit{in vivo} study of EphB signaling in intestinal crypts showed kinase-independent activation of PI3K to promote cell migration\textsuperscript{102}, though a mechanism was not shown. Overexpression of kinase-dead EphB2 has been reported to impair spine development\textsuperscript{13,14}, supporting a role for a kinase-dependent mechanism and, perhaps, differential regulation of kinase-dependent downstream effectors in different systems.

Our results demonstrated a role for the non-receptor tyrosine kinase Arg in EphB-mediated protrusion formation. This interaction between EphB2 and Arg in neurons is not unexpected, as EphB2 and Arg have been shown to interact in a positive-feedback mechanism, and Arg is enriched in dendritic spines\textsuperscript{103,104}. However, EphB2, especially in the DIV10-11 age of neurons studied, has been primarily shown to drive the formation of dendritic spines\textsuperscript{16}. Arg, however, has been shown to promote dendrite and dendritic spine stability and maturation, since dendritic spines develop normally in Arg-knockout mice until adulthood\textsuperscript{198,199}. While EphBs likely play a role in spine stability as well, no studies have linked them to Arg in spine stability, let alone in spine formation. It should be noted that the SH2 domains of Abl and Arg are similar and that GNF-2 allosterically inhibits both. Our results may thus suggest a novel role for Arg, or Abl-family kinases in general, in the regulation of dendritic spine morphogenesis by EphBs.

The findings in this chapter thus suggested a new model by which EphBs may promote the formation of dendritic spines. Instead of simply supporting a transition from filopodia to spines, EphBs may play a role in the formation of new dendritic filopodia that can form additional contacts with axons. Our findings of differential PIP\textsubscript{3} synthesis
in protrusive versus repulsive responses should prompt future work on the underlying mechanism. Our findings have, thus, further elucidated important biological questions about the nature of EphB signaling in multiple cell types, and raised some interesting questions for future studies.
Figure 3.1
**Figure 3.1. OptoEphB2 activation in dendritic filopodia causes filopodia branching and plasma membrane expansion.** DIV8-11 neurons co-expressed optoEphB2 or KD-optoEphB2 and either myr-mCherry or Lifeact-mCherry. Blue light (50-ms pulses, 6 pulses/min) was delivered to specifically stimulate filopodia. (a) Top: Illustration showing orientation of blue light illumination to stimulate filopodia. Bottom: Fluorescence images of optoEphB2 and KD-optoEphB2, showing clustering in filopodia. Scale bar, 5 μm. (b) Percentage of filopodia that showed increased branching or showed plasma membrane expansion in response to photoactivation. n = 154 filopodia from 11 cells for optoEphB2, n = 191 filopodia from 7 cells for KD-optoEphB2. (c) Quantification of protrusion density in response to photoactivation of optoEphB2 or KD-optoEphB2. For each neuron, protrusion density along the ROI was measured at the start and end of blue light illumination. The end value was normalized to the start value, and the numbers shown represent the average of all cells. Error bars, SEM. N.S., no significance (t-test). (d) Quantification of Lifeact intensity in filopodia. Normalized intensity was calculated cell-by-cell by averaging the intensity in all filopodia along the ROIs before and after illumination, normalizing to the pre-illumination value, and averaging between cells. Error bars, SEM. *p < 0.05, t-test. (e) Time lapse images of the myr-mCherry signal in photostimulated filopodia. (f) Time lapse images of Lifeact-mCherry in filopodia during photoactivation. Time labels in (e) and (f) are relative to the start of photoactivation.
Figure 3.2

a) Pre-Illumination (5 min) 0-5 min 10-15 min 15-20 min
b) Maximum projections over time
Pre-Illumination (5 min) 0-5 min Blue light 5-10 min Blue light 10-15 min Blue light
c) OptoEphB2 - Venus + Lifeact-mCh

d) Graph showing changes in normalized mean projection area:
- OptoEphB2 (n=32)
- OptoEphB2-Venus (n=16)

e) Maximum projections over time
Pre-Illumination (5 min) 10-15 min Blue Light

f) Maximum Projections Over Time
5 min Pre-Illum. 10-15 min Blue Light

Graph showing cumulative probability:
- * CK666
- DMSO
**Figure 3.2.** Local optoEphB2 clustering induces dynamic filopodia-like protrusions on dendrites of hippocampal neurons in a manner that depends on Arp2/3 activity. (a) Left: Time lapse images of mCherry (volume marker) in DIV10-11 neurons during blue light illumination (50-ms pulses, 3 pulses/min.) of the indicated optoEphB2 construct over the ROI shown (white circles). Right: Fluorescence images of optoEphB2, or mutant, before and after photoactivation. Time labels are relative to the start of photoactivation. (b) Time lapse images of Lifeact-mCherry in a DIV11 neuron co-expressing optoEphB2 that was photoactivated over the ROI (white circle). (c) Maximum intensity projection images of mCherry (volume marker), from the dendritic segments shown in (a) that express optoEphB2 and KD-optoEphB2. Projection images were constructed from 5-minute time segments before and during photoactivation. (d) Quantification of increased filopodial protrusions. Dendritic areas within the ROI were measured from maximum intensity projections (e) and normalized to measurements before photoactivation. *p < 0.05, t-test, comparing optoEphB2 to KD-optoEphB2. (e) Maximum intensity projection images of mCherry (volume marker) in DIV11 neurons co-expressing optoEphB2. OptoEphB2 was photoactivated (Stimulation 1) over the indicated ROI (white). After 20 minutes of incubation in the dark, photoactivation was repeated (Stimulation 2). Protrusive activity was reduced to baseline then re-stimulated to a level similar to the first photoactivation. (f) Neurons co-expressing mCherry and optoEphB2 were pre-treated for 30 minutes with DMSO or 200 μM CK-666 (n=16 for each). The image panels show maximum intensity projections of the mCherry signal over the indicated timeframes. The Kolmogorov-Smirnov plot shows the cumulative probability of the normalized maximum intensity projection areas within the ROI. This was calculated by normalizing the value calculated from the final 5 minutes of photostimulation to that of the 5 minutes preceding photostimulation. Scale bars, (a)-(c), (e), (f), 5 μm.
Figure 3.3

[Image of a figure with multiple panels showing different conditions and time points, labeled OptoEphB1, OptoEphB6, and OptoEphB2-PBM]
Figure 3.3. Optogenetic stimulation of optoEphB1, optoEphB6, and optoEphB2-PBM. Time lapse fluorescence images of optoEphB1, optoEphB6, or optoEphB2-PBM in DIV11 neurons. OptoEphB1 and optoEphB6 were imaged and stimulated over the indicated ROI (blue circles) as previously described for optoEphB2 (see Methods). OptoEphB2-PBM was imaged at 6 frames/min and photoactivated (440-nm light, between frames) over an ROI (blue circle) ~8 µm in diameter. OptoEphB1 and optoEphB2-PBM stimulation resulted in formation of protrusions, while stimulation of optoEphB6 did not. Time labels are relative to start of photoactivation. Scale bar, 5 µm.
Figure 3.4

(a) PH-Akt-mRFP
0 sec
30 sec
60 sec
150 sec

(b) 
Ph-Akt intensity (AU)

Distance (μm)

(c) Leading edge PIP3
(d) Interior PIP3

(e) Intensity
Time of Illumination (min)

(f) Normalized tdTomato intensity

(g)
**Figure 3.4.** Differential regulation of PIP₃ synthesis downstream of EphB2 in dendrites and fibroblasts. (a) TIRF images of PHAkt-mRFP (PIP₃ probe) in a 3T3 cell co-expressing optoEphB2 and stimulated with blue light (442 nm laser). (b) Intensity linescan of PHAkt signal near the cell leading edge as indicated by the dashed line in (a). Each line (from the darkest to the lightest) indicates a different time point with 30 sec spacing. (c) Quantification of PHAkt sensor intensity at the leading edge (1 µm from cell edge). Time points represent 1 minute before (Before), the start of (- Blue Light), and after 1 min. of (+ Blue Light) blue light stimulation (n=6). *p < 0.05, t-test, comparing to “Before.” (d) Quantification of PHAkt intensity at the cell interior (5 µm from cell edge), using the same time points as in (c). (e) Time-lapse images of PHAkt-mRFP or mCh in dendrites of DIV10-11 neurons during photoactivation (ROI, white circles) of optoEphB2 and filopodia formation. (f) Quantification of PHAkt or mCherry accumulation within the ROI upon optoEphB2 activation and filopodia formation in neurons. Values represent mean fluorescence intensity within the ROI normalized to the mean prior to photoactivation and averaged between cells. Error bars, SEM. (g) Maximum intensity projections of PHAkt-mRFP in dendrites before and after optoEphB2 photoactivation over the ROI (white circles). Scale bars, (a), (e), (g), 5 µm.
Figure 3.5

(a) Maximum projections over time

(b) Maximum projections over time

(c) Maximum projections over time
Figure 3.5. OptoEphB2 activates PI3K in dendrites to induce PIP3 accumulation.
(a) Left: Fluorescence images of PH\textsubscript{Akt}-mRFP dendrites before and after two rounds of optoEphB2 stimulation (ROI, white). The top row shows the first stimulation. The neurons were then incubated in the dark and treated with the PI3K inhibitor LY294002. Re-stimulation is shown in the bottom row. Right: Quantification of PH\textsubscript{Akt}-mRFP intensity in the ROI, normalized to the mean signal prior to blue light illumination. Error bars, SEM (n=3).
(b) Same as in (a) except, that neurons were treated with DMSO (control) instead of LY294002. Error bars, SEM (n=3).
(c) Maximum intensity projection images of PH\textsubscript{Akt}-mRFP in a dendritic segment. OptoEphB2 was photoactivated (white circle; ~8 μm, 50-ms pulses, 10-s intervals), incubated in the dark, and re-illuminated following treatment with 5 μM Cytochalasin D, similar to (a) and (b). Plot on the right shows PH\textsubscript{Akt}-mRFP signal normalized to the mean prior to illumination for both stimulations. Scale bars, (a)-(c) 5 μm. All time labels are relative to the start of photoactivation.
Figure 3.6

The image shows a figure with four panels:

(a) Two panels showing the effect of blue light on Arg-YFP OptoEphB2-mCh Overlay.

(b) Two panels illustrating the time course of blue light exposure.

(c) A bar graph comparing the normalized maximum projection area between DMSO and GNF2 with 95% confidence intervals.

(d) A graph showing the cumulative probability of normalized maximum projection area over time for DMSO and GNF2.
**Figure 3.6.** OptoEphB2 promotes dendritic filopodia formation by signaling through the non-receptor tyrosine kinase Arg.  (a) Time lapse images of DIV10-11 hippocampal neurons co-expressing ArgKD-YFP and OptoEphB2, before and after blue light illumination (white circle; 50-ms pulses at 20-s intervals). (b) Maximum intensity projections of optoEphB2 signal in DIV10-11 neurons co-expressing ArgKD-YFP, SrcKD-YFP, or YFP, over the indicated time frames. Plot at right shows normalized maximum projection area, calculated by dividing the area over the final 5 minutes of photoactivation by that of the 5 minutes preceding photoactivation. Error bars, SEM. *p<0.01, t-test. (c) DIV11-12 neurons co-expressing optoEphB2 and mCherry were photostimulated after incubation with DMSO or 10 μM GNF-2 for 1 hour. GNF-2 treatment reduced the average normalized maximum intensity projection area, measured during the final 5 minutes of photoactivation. Error bars, SEM. *p<0.01, t-test. (d) Maximum intensity projections of mCherry signal in DIV11-12 neurons, co-expressing optoEphB2, that were pre-treated with DMSO or GNF2 as in (c). Photoactivation was delivered over the ROI shown (white). Scale bars, (a), (b), (d), 5 μm.
Movie Captions


Movie 3.2. OptoEphB2 activation in filopodia forms lamellipodia-like protrusions. Movie of myr-mCherry signal in DIV9 neurons, co-expressing optoEphB2, that shows formation of lamellipodia-like protrusions from filopodia following localized optoEphB2 stimulation (starts at 0:00).

Movie 3.3. Local stimulation of optoEphB2 in dendrites induces formation of filopodia-like protrusions. Movie of mCherry signal in DIV11 neurons co-expressing optoEphB2 (Left) or KD-optoEphB2 (Right). Following focal (blue circle at 5:00) blue light illumination (blue dot) of optoEphB2, numerous filopodia-like protrusions form.

Movie 3.4. Filopodial protrusions are associated with PI3K activation and PIP3 accumulation. Movie of PH\textsubscript{Akt}-mRFP signal in DIV11 neurons co-expressing optoEphB2. Blue light illumination (blue dot) of optoEphB2 caused accumulation of PH\textsubscript{Akt} (Left) in the ROI (blue circle at 5:00). After incubating in the dark and treating with 50 µM of the PI3K inhibitor LY294002, neurons were re-stimulated (Right) and both PH\textsubscript{Akt} accumulation and protrusion formation were abrogated.

Movie 3.5. Formation of filopodial protrusions is reversible and repeatable. Movie of PH\textsubscript{Akt}-mRFP signal in DIV11 neurons co-expressing optoEphB2. PH\textsubscript{Akt}-mRFP accumulation in the ROI (blue circle at 5:00) was observed (Left) with dendritic protrusion formation in response to focal optoEphB2 photoactivation (blue dot). Both accumulation and protrusion formation were repeatable upon re-stimulation after 30 minutes of DMSO treatment (Right).

Movie 3.6. PIP3 accumulation occurs in the absence of actin polymerization. PH\textsubscript{Akt}-mRFP accumulation in the ROI (blue circle at 0:00, then at 5:00 following cytochalasin D treatment) was observed with dendritic protrusion formation in response to focal optoEphB2 photoactivation (blue dot). During re-stimulation, treatment with 5 µM cytochalasin D blocked protrusion formation, but did not prevent repeated accumulation of PH\textsubscript{Akt}-mRFP.
CHAPTER IV: Discussion and Future Directions

Attribution: This chapter was proof-read by Ji Yu and Yi Wu. Clifford Locke wrote the text.
Summary

In neurons, EphB signaling was originally studied in the context of axon guidance\(^6\). It was found that EphBs, among other Eph receptors, acted as repulsive cues, and Eph/ephrin expression gradients targeted axons to their appropriate destinations\(^6\). A role for EphBs in dendritic spine formation was first described when overexpression of kinase-dead EphB2 caused impaired dendritic spine formation in cultured hippocampal neurons\(^13\). Genetic studies would later reveal that EphB1, EphB2, and EphB3 are collectively essential for the normal formation of dendritic spines\(^14\). Additionally, stimulation of cultured neurons with pre-clustered ephrinB ligands induced dendritic spine formation and spine head enlargement\(^15\).

Dendritic filopodia are thought to give rise to dendritic spines by forming stable contacts with axons, and subsequent reorganization of the actin cytoskeleton\(^4,20\). To better understand the role of EphBs in this process, previous studies addressed the effect of EphB signaling on the motility and morphology of dendritic filopodia. Cultured neurons from EphB1-B3 triple knockout (TKO) neurons showed reduced filopodia motility versus wild-type neurons\(^16\), though this did not address the effect of ligand binding. Filopodia density was unaffected in these neurons\(^16\), contrasting with another study that reported increased total protrusion density following stimulation of cultured hippocampal neurons with pre-clustered ephrinB ligands\(^15\). These results were, therefore, inconclusive regarding the effects of EphB signaling on filopodia at sites of axo-dendritic contact.

I became interested in the effect of EphB signaling on the morphology and motility of dendritic filopodia to better understand how EphBs affect the actin
cytoskeleton to induce spine morphogenesis. Given the role of clustering in EphB signaling, and the recent report of blue light-induced clustering of Cry2\textsuperscript{129}, I decided to use an optogenetic approach to model local axo-dendritic contacts and target stimulation to filopodia. Concurrent development of other blue light-controlled RTKs\textsuperscript{132,133} supported this idea. Such a technique would allow real-time monitoring of filopodia motility, morphology, and downstream signaling proteins following local EphB2 stimulation.

Chapter II reported the development of optoEphB2 for optogenetic control over EphB2 signaling. Blue light illumination of optoEphB2 resulted in rapid tyrosine phosphorylation and binding of SH2 domains to phosphotyrosine residues in cell lysates. The corresponding SH2 domain proteins mirrored those known to act downstream of EphB2. Expected cell-cell retraction phenotypes were demonstrated in MEF, HEK293, and MCF7 cells and axonal growth cones. Spatio-temporal control over optoEphB2 was confirmed by showing reversibility and repeatability of clustering and signaling, and confinement of both clustering and expected phenotypes to sub-cellular regions of illumination.

Chapter III focused on the effect of EphB signaling on actin in dendritic filopodia and along the dendritic shafts. With targeted optoEphB2 clustering, filopodia displayed increased actin polymerization and branching, some to the extent of showing plasma membrane expansion, indicative of a highly-branched, or dendritic, actin network. On the dendritic shaft, optoEphB2 clustering promoted the formation of filopodia-like structures, which depended on the accumulation of phosphatidylinositol(3,4,5)-triphosphate (PIP\textsubscript{3}) downstream of phosphoinositide-3 kinase (PI3K). Given the reduction of plasma membrane PIP\textsubscript{3} associated with EphB2-mediated cell collapse, differential regulation of
plasma membrane PIP₃ could represent a mechanism for the cell context-dependence of Eph receptor signaling.

This thesis reports the successful development of a photoactivatable Eph receptor that may be used in a variety of contexts. Activation of optoEphB2 in dendritic protrusions and along dendritic shafts improved our understanding of EphB signaling in the development of dendritic spines. A greater mechanistic understanding of the differences between EphB signaling in dendrites and other cell types was also achieved. My final comments on this thesis are given in this chapter.

Discussion and Future Directions

Development of an optogenetic method for Eph receptor activation

A primary goal of this thesis was to target EphB signaling to dendritic filopodia. Optogenetic clustering using Cry2 was an intriguing method, and our design was achieved after multiple iterations. After just one minute of blue light illumination, we achieved robust tyrosine phosphorylation and binding of multiple SH2 domains. Collapse of MEFs, localized cell process retraction, and growth cone collapse were observed with patterned blue light illumination of transfected cells, in agreement with previously observed phenotypes of EphB2 signaling. Our data suggest that we have a functional optogenetic module for spatial and temporal control over Eph receptor signaling.

Using an optogenetic tool for Eph receptor signaling will permit assays for the spatial and temporal regulation of downstream signals. This is especially important for
the Rho and Ras GTPases, which are important downstream effectors of Eph receptors. Their complex interplay in space and time is responsible for proper cell migration. Changes in Rho GTPase activity occur on the timescale of minutes, and their activities are confined to sub-cellular regions. Numerous probes have been developed to examine the spatial and temporal characteristics of Rho GTPases in a variety of processes, for instance, the formation of cellular protrusions\textsuperscript{201}. The next logical step is to examine how the spatial and temporal characteristics of upstream regulators, such as the Eph receptors, can affect these signaling patterns.

An optogenetic tool also opens the door for \textit{in vivo} work. Many studies of Eph receptors \textit{in vivo} employed knockout models. While these studies are informative, Eph receptors are RTKs that affect Ras/MAPK signaling and, therefore, expression of genes involved in cell survival, proliferation, and differentiation. These effects may confound experimental observations. Since Ephs and ephrins are so widely expressed and are involved in crucial developmental processes, knockouts are often lethal. For instance, it was reported that vascular problems in EphB2/B3 double-knockout mice caused a 30% lethality rate\textsuperscript{69}. Using an optogenetic tool permits targeted signal activation and, thus, stimulation of a particular organ to achieve spatial specificity.

\textit{Novel insights into dendritic spine morphogenesis}

Induction of actin branching suggests that EphB2 signaling can contribute to spine head formation on filopodia, though new spines were not observed, possibly due to the loss of extracellular domain-mediated interactions or the absence of a pre-synaptic membrane that may contribute \textit{trans}-cellular co-stimulatory signals that are also
necessary for dendritic spine development. Prior data indicated the necessity of EphB signaling in the development of dendritic spines\textsuperscript{14}. We thus speculate that EphB2 activity is necessary, but not sufficient, for the development of dendritic spines. Prior studies showed increased spine density via treatment with pre-clustered ephrinB ligands\textsuperscript{15}, suggesting that EphB stimulation was sufficient for additional spine formation. However, these spines may have formed at contacts with axons, a notion supported by immunofluorescence assays that showed an increased density of co-localizing puncta containing pre- and post-synaptic markers\textsuperscript{15}. If these spines formed at axonal contacts, it is likely that other cell-cell recognition molecules, and perhaps glutamatergic activity, supplemented EphB signaling to complete spine formation. The ability of EphA4, which is enriched at dendritic spines\textsuperscript{202}, to bind ephrinBs\textsuperscript{23} also sheds doubt on the sufficiency of EphB signaling alone to induce spines.

Since axons and dendrites can form local contacts on dendritic shafts, we examined the effect of local EphB signaling on dendritic shafts as well and observed formation of new filopodia-like protrusions. An early study of EphB signaling in dendritic spine morphogenesis described an increase in total protrusion density following ephrinB1-Fc ligand treatment of cultured hippocampal neurons\textsuperscript{15}. It was, however, unclear if EphB signaling induced the formation of new protrusions or served to stabilize extant filopodia and spines. The latter case would strongly suggest a function for EphB signaling in stabilizing filopodia upon contact with axons, a crucial initial step in dendritic spine morphogenesis\textsuperscript{9}. Our observations show spatially-restricted formation of filopodia-like protrusions, suggesting that EphB signaling up-regulates spine density by introducing additional putative synapses. Spatial restriction suggests that EphB signaling
at axo-dendritic contacts may help neurons “hone” filopodia formation to dendritic locations nearest axons. Future experiments may explore how spatial confinement of filopodia formation is achieved.

The design of optoEphB2 may also explain the discrepancy between our stimulation protocol and results of ligand-mediated stimulation. Removal of the extracellular domain was advantageous to reduce interactions with endogenous ephrins and cis interactions with other receptors, but EphB2 was shown to interact with NMDARs via its extracellular domain\textsuperscript{20}. The Cry2olig-mCherry fusion at the receptor’s C-terminus blocked the carboxy terminus of the PDZ-binding motif, which was shown to scaffold EphB2 together with AMPARs through GRIP\textsuperscript{156}. Structural studies have suggested that a free carboxy-terminus is necessary for PDZ binding, but this conclusion is controversial\textsuperscript{186,203}. Our data indicated that placing the PBM at the C-terminus of optoEphB2 did not have a significant impact on filopodia formation in dendrites. Given that isolated PBMs were shown to bind PDZ domains\textsuperscript{186}, we suspect that moving the PBM itself to the C-terminus of optoEphB2 would allow interactions with PDZ domains. A C-terminal YFP fusion of EphB2 did not affect the normal kinase activity or regulation of EphB2, and stimulation of NG108 cells overexpressing EphB2-GFP fusion caused the expected retraction of neurites\textsuperscript{182}. Additionally, overexpression of an EphB2-YFP fusion, in which YFP was inserted N-terminal to the EphB2 PBM, rescued dendritic spine morphogenesis in EphB1-B3 TKO neurons\textsuperscript{16,156}. This is similar to the design of optoEphB2-PBM. I thus suspect that the placement of the PBM in optoEphB2 did not significantly affect the validity of the experimental results, or the conclusions drawn from them. However, these results can only be interpreted as showing the effects of EphB
kinase activity on dendritic filopodia. Since previously-demonstrated interactions with GEFs did not involve the PBM\textsuperscript{15,147,150}, EphB kinase activity is likely the driving force in spine morphogenesis and these caveats do not detract from the significance of the findings.

Future studies may focus on how EphB2 recruits various synaptic proteins to filopodia to affect the actin cytoskeleton. For example, the Shank family of scaffolding proteins, which are enriched in spines, contain SAMs\textsuperscript{6} that may bind the SAM of EphB receptors to affect filopodia. Using the optogenetic tool to map the temporal characteristics of actin and effector recruitment may permit differentiation between effectors that are recruited to affect the actin cytoskeleton, and which effectors may then be recruited by actin polymerization \textit{per se}. Spatial restriction may also permit observation of differences between effects at filopodia, and effects on the dendritic shaft. An increase in filopodia density was not observed with stimulation of filopodia specifically, indicating that downstream signals were confined to filopodia, or that there were differences in the signaling pathways initiated between the dendritic shafts and filopodia. Studies on individual spines may also be performed to examine the effect of Eph receptor signaling on dendritic spine plasticity.

\textit{Implications for understanding the cell context-dependence of EphB2 signaling}

This thesis reports the first direct evidence of PI3K activation by EphB2 in dendrites. EphB signaling was previously shown to affect pain pathways and Alzheimer’s Disease pathogenesis through PI3K\textsuperscript{99–101}, though the localization of PI3K activation remained unknown. Our observation of PI3K signaling by EphB2 in dendrites
builds on the well-studied regulation of the Rho GTPases by EphB2 and the role of PI3K in mediating Rho GTPase signaling. Our lab recently published a paper showing the spatial regulation of Rac1 through PI3K activity\textsuperscript{174}. Prior studies have shown that PIP\textsubscript{3} can interact with the PH domains of the EphB2-interacting GEFs intersectin\textsuperscript{194} and Tiam1\textsuperscript{193} and the sec14 domain of kalirin-7\textsuperscript{195,196}. Thus, it is possible that EphB2-mediated PIP\textsubscript{3} synthesis may serve to spatially coordinate Rac1 and Cdc42 activation near sites of axo-dendritic contact to drive dendritic spine morphogenesis, and future experiments should address this question. Additionally, PIP\textsubscript{3} synthesis was well-localized to the region of illumination, especially considering the rapid diffusion of membrane lipids. The mechanism of such spatial restriction should be explored. Local accumulation of PIP\textsubscript{3} itself may be promoted, for instance, by the surrounding actin dynamics, as demonstrated in chemotaxis\textsuperscript{187}, or perhaps by rapid PIP\textsubscript{3} dephosphorylation or internalization. Use of an optogenetic tool, including EphB2, would be crucial to such studies for spatial regulation of upstream signals. Such work could be extended to spatial regulation in migratory cells to study, for example, how actin is spatially regulated to achieve contact inhibition of locomotion.

Our results in MEFs and growth cones contradicted our observations of increased protrusive activity and PI3K activation in dendrites. Observations of cell protrusion collapse and changing growth cone morphology from lamellipodial to filopodial strongly suggest collapse of dendritic actin networks. However, broad plasma membrane extensions were induced in filopodia, suggesting nucleation of dendritic actin networks. In the case of hippocampal neurons, these diametrically opposed phenotypes were found in the same cell type. Observing PIP\textsubscript{3} accumulation in dendrites, with depletion in
collapsing MEFs, suggests that Eph receptor-induced repulsion or adhesion depends, at least in part, on up- or down-regulation of plasma membrane PIP3.

Further studies are therefore necessary to elucidate the neuronal pathways between EphB2 and PI3K that result in activation or inhibition. Prior in vitro studies indicated an interaction between phosphorylated EphB2 and the SH2 domain of p85 (α or β not specified)50, which would result in PI3K activation. EphB2 may signal through Ras in a cell context-dependent manner to activate or inhibit PI3K36. EphB2 is known to inhibit Ras signaling through p120RasGAP in multiple contexts, though the SH2 adaptor Grb2 was recruited to EphB signaling complexes in hippocampal dendrites17,72. Grb2 may activate PI3K through Ras by recruiting the Ras-GEF SOS, as shown in other cell types downstream of EphB176. Grb2 may act independently of Ras by recruiting another adaptor, Gab1, that can activate PI3K197. Our SH2 domain profiling, however, did not detect binding of the SH2 domains of Grb2, p85α, p85β, or p55γ to phosphotyrosines downstream of optoEphB2 (Fig. 2.3c). These SH2 domains did bind the positive controls (Fig. 2.3c). Activation of PI3K via these pathways is, therefore, less likely. EphB2 was also shown to form a complex with the Rac-GEF α-Pix, Nck, and Pak, with PI3K activity necessary to activate α-Pix96. Although Nck did bind optoEphB2 in our assay, and is known to bind EphBs50, this does not explain the activation of PI3K. It is also possible that PI3K activation occurs through activation of Rac1 or Cdc42 as previously described in other contexts37,174.

In addition to understanding the pathways themselves, central to Eph biology is understanding the underlying mechanisms of cell context-dependence, and how that may affect the pathways selected in different cell types. A study of EphB4 signaling to Ras
supports the notion that differential expression of downstream effectors is responsible for cell context-dependence\textsuperscript{79}. EphB4 inhibited proliferation in human umbilical vein endothelial cells by down-regulating Ras through p120RasGAP, which was abrogated by p120RasGAP knockdown. However, EphB4 increased cell growth in MCF7 cells by up-regulating Ras activity in a manner that depended on the phosphatase PP2A. Other studies suggest that the signaling background provided by other receptors may determine the Eph receptor output. For instance, a study of cross-talk between EphB2 and FGFR showed that EphB2 inhibited Ras when FGFR was activated, but activated Ras when FGFR was not\textsuperscript{78}. Feedback control of EphBs by the MAPK cascade was described as the underlying mechanism. EphB signaling inhibited chemotaxis in MTLn3 cells that overexpressed EGFR\textsuperscript{98}, which perhaps resulted in a Ras-activating background as well.

To determine the underlying biology in hippocampal neurons, it will be necessary to examine the dendritic and axonal expression of potential EphB2 downstream targets. Perhaps p120RasGAP is lacking in dendrites, but is expressed in axons, allowing EphBs to up-regulate Ras through other adaptors and activate PI3K in dendrites. Down-regulation of Ras in growth cones by the typical mechanisms may explain growth cone collapse. Functional assays would identify proteins that may reverse the functional outcome of EphB2 signaling, discoveries that would mark a great advance in understanding Eph receptor biology.

OptoEphB2 provides a functional module to approach these questions of cell context-dependence, particularly in the case of polarized cell types. Neurons provide an example, because protein expression and signaling activity can differ greatly between axons and dendrites. However, migrating cells may also be polarized, especially during
chemotaxis, with different signaling activities at the leading and lagging edges. Our data and previous studies have shown that EphBs behave differently in the axonal and dendritic compartments of hippocampal neurons. Activation of other RTKs, such as FGFR, can influence the regulation of Ras/MAPK signaling by EphBs, through feedback mechanisms in the Ras/MAPK pathway\textsuperscript{78}. In Dictyostelium, spatial regulation of Ras and PI3K signaling, with activation at the leading edge, has been demonstrated to guide chemotaxis\textsuperscript{204,205}, providing an example of how spatial regulation of proteins at the cellular level affects cell migration. This raises the question of whether or not Eph receptors differentially mediate adhesion or repulsion at the leading or lagging edges of migrating cells. Immunofluorescence can be used to detect spatially-regulated binding between receptors and their effectors. However, optogenetics can be used to monitor recruitment of effectors over space and time while simultaneously observing downstream phenotypes. This advantage will allow future studies to probe the effects of Eph receptor signaling in different cell compartments, which will expand our understanding of the role that cell context plays in Eph receptor signaling outputs.

**Conclusion**

OptoEphB2 can be used for spatial and temporal control over an RTK that is crucial to normal spine development and other developmental processes. This thesis demonstrated the local morphologic effects of EphB2 signaling on the dendritic spine precursors, dendritic filopodia, and suggested an important signaling pathway in determining adhesion versus repulsion in response to EphB2 signaling. These observations improved the scientific understanding of EphB2 signaling in spine
formation, and opened an array of new questions to continue exploring the molecular mechanisms of the cell context-dependence of Eph receptor signaling.
REFERENCES


