Regulation of EphA4 Expression through the
APC-Mediated Ubiquitin-Proteasome Pathway

by

SHEN, Ying

A Thesis Submitted to
The Hong Kong University of Science and Technology
in Partial Fulfillment of the Requirements for
the Degree of Master of Philosophy
in Department of Biochemistry

July 2007, Hong Kong
Authorization

I hereby declare that I am the sole author of the thesis.

I authorize the Hong Kong University of Science and Technology to lend this thesis to other institutions or individuals for the purpose of scholarly research.

I further authorize the Hong Kong University of Science and Technology to reproduce the thesis by photocopying or by other means, in total or in part, at the request of other institutions or individuals for the purpose of scholarly research.

_Shen Ying_

SHEN, Ying
Regulation of EphA4 Expression through the APC-Mediated Ubiquitin-Proteasome Pathway

by

SHEN, Ying

This is to certify that I have examined the above MPhil thesis and have found that it is complete and satisfactory in all respects, and that any and all revisions required by the thesis examination committee have been made.

Nancy Y.
IP, Nancy Y., Professor
Supervisor

Nancy Y.
IP, Nancy Y., Professor
Head of Department

Department of Biochemistry
11 July 2007
Acknowledgement

I would like to express my sincere gratitude to Prof. Nancy Y IP for providing me the opportunity to conduct my MPhil study in her laboratory. She gave me a lot of useful advices not only on my research, but also on more general aspects of my career and personal life. Her enthusiasm and dedication towards science is a great inspiration to all of us and sets up a role model for us to treat science seriously and honestly.

Thanks also go to my supervisory committee members, Prof. Mingjie ZHANG and Dr. Zhenguo WU, who gave me constructive criticisms and helpful suggestions on my project.

I would also like to devote my sincere thanks to Dr. Amy FU for her encouragement and guidance on my studies, constantly helping me out through my difficulties. I also appreciate the help from Dr Ada FU and CHEN Yu on the spine experiments.

A very special thanks to Mr. Nelson HUNG, who I have been working with on the same project since my undergraduate final year project, and taught me most of my experimental techniques. He has been so kind and patient to me that I couldn’t have asked for a better teacher. His brilliance and hard work has brought numerous excitements to the project and made it a wonderful endeavor to work on.

Last but not least, I would like to thank all my other current and past colleagues in the laboratory for their day to day help on my lab works, especially Dr. WAN Jun, Dr. NG
Yu Pong, Dr. Fanny IP, Ms. SHI Lei, Ms. ZHAO Xiao Su, Ms. Grace WONG, Ms. WU Qian, Mr. Anthony CHEUNG, Ms. Winnie CHIEN, Ms. Cara KWONG, Ms. Busma BUTT. It has been a great pleasure to work with you.
Table of Contents

Authorization ii
Signature Page iii
Acknowledgement iv
Table of Contents vi
List of Figures and Tables xi
Abbreviations xiv
Abstract xvii

Chapter 1 Introduction 1

1.1 Ephrin-Eph signaling 1

1.1.1 Ephrins and Ephs 1

1.1.2 Ephrin-Eph signaling: Forward or Reverse 2

1.1.2.1 Forward signaling 2

1.1.2.2 Reverse signaling 5

1.1.3 Functions of Ephrin-Eph signaling 7

1.1.3.1 Early segmentation and morphogenesis 7

1.1.3.2 Axon guidance and remodeling 8

1.1.3.3 Synapse formation and plasticity 9

1.1.4 Regulation of Ephrin-Eph signaling 10

1.2 The ubiquitin-proteasome proteolytic system 11

1.2.1 The ubiquitination reaction 11

1.2.2 Mono Vs. poly-ubiquitination 12

1.2.3 Roles for the ubiquitin-proteasome system (UPS) in neuronal 13
functions

1.3 The Anaphase-promoting complex (APC) 15

1.3.1 Structure and subunits of the APC 16

1.3.2 Functions of the APC in the cell cycle 18

1.3.2.1 Sister-chromatid separation 18

1.3.2.2 Cyclin destruction 19

1.3.2.3 DNA replication 19

1.3.3 Functions of the APC in the nervous system 20

1.3.3.1 Axonal growth and patterning 20

1.3.3.2 Synaptic development and function 21

1.3.3.3 Neuronal survival 21

1.4 Aims of study 22

Chapter 2 Material and Methods 23

2.1 Materials 23

2.2 Tissue dissection 29

2.3 Primary neuron cultures 29

2.4 Cell lines 30

2.5 Polymerase chain reaction (PCR) 30

2.6 Agarose gel electrophoresis 31

2.7 DNA subcloning 31

2.8 Bacterial transformation of plasmid DNA 32

2.9 Bacterial DNA extraction 32

2.10 DNA sequencing 32
2.11 Transient transfection of 293 cells
2.12 Transient transfection of primary cell cultures
2.13 Ephrin-Fe treatment
2.14 Cell-surface biotinylation
2.15 Protein preparation from cell cultures and tissues
2.16 Immunoprecipitation
2.17 UBA (ubiquitin-associated domain) pull down
2.18 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)
2.19 Western blot analysis
2.20 Immunocytochemical analysis

Chapter 3 Results

3.1 EphA4 interacts with APC2
3.2 Cdh1 regulates EphA4 expression in mammalian cells
   3.2.1 Cdh1 interacts with EphA4 in mammalian cells
   3.2.2 Cdh1 down-regulates EphA4 expression in mammalian cells
   3.2.3 The down-regulation of EphA4 expression is dependent on the substrate binding domain of Cdh1
   3.2.4 The down-regulation of EphA4 expression is independent on the kinase activity of EphA4
   3.2.5 The interaction between EphA4 and Cdh1 is D-box independent
3.3 Ephrin-A1 stimulates proteasome-dependent degradation of EphA4
3.3.1 The expression of EphA4, APC2 and Cdh1 are developmentally regulated

3.3.2 EphA4 interacts with APC2 after Ephrin-A1 treatment in cortical neurons

3.3.3 Ephrin-A1 enhances the ubiquitination of EphA4 in hippocampal neurons

3.3.4 Surface expression of EphA4 is decreased in cortical neurons after ephrin-A1 treatment

3.3.5 Lactacystin treatment attenuates the ephrin-A1 mediated down-regulation of surface EphA4 in neurons

3.4 Cdh1 regulates dentritic spine density in hippocampal neurons

3.4.1 Knockdown of Cdh1 results in reduced dentritic spine density in hippocampal neurons

3.4.2 Overexpression of Cdh1 abolishes ephrin-A1 mediated dentritic spine retraction in hippocampal neurons

Chapter 4 Discussion

4.1 Interaction of EphA4 with APC2

4.2 Regulation of EphA4 by Cdh1

4.3 Developmental regulation of EphA4, APC2 and Cdh1

4.4 Ubiquitination of EphA4

4.5 Proteasomal degradation of EphA4

4.6 Possible model for APC<sup>Cdh1</sup>-mediated proteasomal degradation of EphA4
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.7</td>
<td>Cdh1 regulates spine density</td>
<td>74</td>
</tr>
<tr>
<td>4.8</td>
<td>Other possible functions for APC\textsuperscript{Cdh1}-mediated proteasomal degradation of EphA4</td>
<td>77</td>
</tr>
<tr>
<td>4.8.1</td>
<td>Axon guidance</td>
<td>77</td>
</tr>
<tr>
<td>4.8.2</td>
<td>Synaptic plasticity and regulation of Glutamate receptors</td>
<td>78</td>
</tr>
<tr>
<td>4.9</td>
<td>Summary and conclusion</td>
<td>80</td>
</tr>
<tr>
<td>References</td>
<td></td>
<td>81</td>
</tr>
</tbody>
</table>
List of Figures and Tables

Figure 1.1 Interactions between ephrins and Ephs 1
Figure 1.2 Structures of ephrins and Ephs 3
Figure 1.3 The mono-ubiquitin mediated endocytic pathway of membrane proteins 14
Figure 1.4 The ubiquitin-proteasome degradation pathway 14
Figure 1.5 APC substrate in the cell cycle 18
Figure 3.1 EphA4 interacts with APC2 in mammalian cells 39
Figure 3.2 EphA4 interacts with APC2 in the brain 39
Figure 3.3&3.4 EphA4 interacts with Cdh1 in mammalian cells 41
Figure 3.5 Cdh1 down-regulates the EphA4 protein expression in a dosage-dependent manner 43
Figure 3.6 Cdh1 down-regulates the surface expression of EphA4 in mammalian cells 43
Figure 3.7 The interaction between EphA4 and Cdh1 is dependent on the substrate binding domain of Cdh1 44
Figure 3.8 The down-regulation of EphA4 expression is dependent on the substrate binding domain of Cdh1 44
Figure 3.9 The interaction between EphA4 and Cdh1 is independent on the kinase activity of EphA4 46
Figure 3.10 The down-regulation of EphA4 expression is independent on the kinase activity of EphA4 47
Figure 3.11 The interaction between EphA4 and Cdh1 is D-box 47
The expression of EphA4, APC2 and Cdh1 are developmentally regulated.

EphA4 interacts with APC2 after Ephrin-A1 treatment in cortical neurons.

Ephrin-A1 enhances the ubiquitination of EphA4 in hippocampal neurons.

Surface expression of EphA4 is decreased in cortical neurons after ephrin-A1 treatment.


Treatment of a proteasome inhibitor lactacystin attenuated the ephrin-A1 mediated-down-regulation of surface EphA4 in hippocampal neurons.

Representative confocal images of neurons transfected with pSUPER or pSUPER-Cdh1 RNAi.

Quantitative analysis of spines in neurons before or after transfection with pSUPER-Cdh1 RNAi or pSUPER vector and ephrin-A1 stimulation.

Quantitative analysis of spines in neurons before or after transfection with pcDNA3-Cdh1 or pcDNA3 vector and ephrin-A1 stimulation.
Figure 4.1  Different types of protein ubiquitination 69
Figure 4.2  Model for APC-mediated proteasomal degradation of EphA4 72
Table 1.1  APC/C subunits and co-activators 17
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AChR</td>
<td>acetylcholine receptor</td>
</tr>
<tr>
<td>A–P</td>
<td>anterior-posterior</td>
</tr>
<tr>
<td>APC</td>
<td>anaphase-promoting complex</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>Cas</td>
<td>Crk-associated susbstrate</td>
</tr>
<tr>
<td>Cdk</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>CHIP</td>
<td>Hsp70 interacting protein</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>D–V</td>
<td>dorsal–ventral</td>
</tr>
<tr>
<td>Eph</td>
<td>Erythropoietin-producing hepatocellular</td>
</tr>
<tr>
<td>ephrin</td>
<td>Eph receptor interacting protein</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factors</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>GRIP</td>
<td>glutamate receptor interacting protein</td>
</tr>
<tr>
<td>HD</td>
<td>Huntington’s Disease</td>
</tr>
<tr>
<td>HECT</td>
<td>homologous to E6-AP carboxyl terminus</td>
</tr>
<tr>
<td>Id2</td>
<td>inhibitor of DNA binding 2</td>
</tr>
<tr>
<td>IR</td>
<td>isoleucine-arginine</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun NH2-terminal kinase</td>
</tr>
<tr>
<td>KD</td>
<td>kinase dead</td>
</tr>
<tr>
<td>LMP-PTP</td>
<td>low molecular weight protein-tyrosine phosphatase</td>
</tr>
<tr>
<td>MCM</td>
<td>mini-chromosome maintenance</td>
</tr>
<tr>
<td>MV Boeing</td>
<td>multivesicular body</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>N-WASP</td>
<td>Wiskott-Aldrich syndrome protein</td>
</tr>
<tr>
<td>ORC</td>
<td>origin recognition complex</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s Disease</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PDZ</td>
<td>PSD-95/Disc large/ZO-1</td>
</tr>
<tr>
<td>PICK1</td>
<td>protein interacting with C-kinase</td>
</tr>
<tr>
<td>PSD</td>
<td>post synaptic density</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>PTP-BL</td>
<td>protein tyrosine phosphatases-Basophil like</td>
</tr>
<tr>
<td>RGC</td>
<td>retinal ganglion cell</td>
</tr>
<tr>
<td>RGS</td>
<td>regulator of G-protein signaling</td>
</tr>
<tr>
<td>RING</td>
<td>really interesting new gene</td>
</tr>
<tr>
<td>SCF</td>
<td>Skip1/Cullin/F-box</td>
</tr>
<tr>
<td>SFK</td>
<td>Src family kinase</td>
</tr>
<tr>
<td>SHEP1</td>
<td>SH2 domain-containing Eph receptor-binding protein 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>TKB</td>
<td>tyrosine kinase-binding</td>
</tr>
<tr>
<td>TPR</td>
<td>tetratricopeptide repeats</td>
</tr>
<tr>
<td>Ub</td>
<td>ubiquitin</td>
</tr>
<tr>
<td>UPS</td>
<td>ubiquitin-proteasome system</td>
</tr>
</tbody>
</table>
Regulation of EphA4 Expression through the APC-Mediated Ubiquitin-Proteasome Pathway

by

SHEN Ying

Department of Biochemistry

The Hong Kong University of Science and Technology

Abstract

Ephrin-Eph signaling plays critical roles in diverse processes in both developing and mature nervous system. For example, EphA4 activation regulates the dendritic spine density in hippocampal neurons. However, the underlying mechanisms that regulate Eph expression and their intracellular fate are unclear. Using yeast-two hybrid screening, we have previously identified the interaction of EphA4 with APC2i, an isoform of APC2 which is one of the major components of anaphase promoting complex (APC). The APC was first identified to be a multi-subunit ubiquitin ligase that controls the degradation of cell cycle regulatory proteins. However, recent emerging studies suggest that the APC may control protein degradation in neurons. In particular, the APC activator Cdh1 regulates the development and survival of postmitotic neurons. In this study, we report that EphA4 protein expression may be regulated by the APC\textsuperscript{Cdh1}-mediated ubiquitin-proteasome degradation pathway. We showed that EphA4 interacts with different components of APC including APC2 and Cdh1. In addition, we showed that the surface as well as total expression of EphA4 is down-regulated by the overexpressed Cdh1 in
HEK293T cells. Furthermore, we found that ephrin-A1 treatment promote the ubiquitination of EphA4 in neurons, leading to a reduction in the surface expression of EphA4, which is attenuated by proteasome inhibitor. Together, our findings suggest that EphA4 expression in neurons is regulated by APC\textsuperscript{Cdh1}-mediated ubiquitin-proteasome degradation pathway, which may play a role in EphA4-mediated signaling in neurons.
Chapter 1 Introduction

1.1 Ephrin-Eph signaling

1.1.1 Ephrins and Ephs

Eph receptors constitute the largest family of receptor tyrosine kinases (RTK). They are activated by ephrins (Eph Receptor Interacting Proteins), which unlike the ligands of other RTKs, are also membrane-bound proteins. 15 Ephs and 9 ephrins have been identified in the vertebrate genome. Ephrins are classified into A and B subclasses. Ephrin As (A1-A6) are tethered to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor, while ephrin Bs (ephrinB1-B3) are transmembrane proteins. The Eph receptors are also divided into A and B sub-classes based on sequence homology and ligand affinity. A-type receptors (EphA1-A9) typically bind to most or all A-type ligands, and B-type receptors (EphB1-B6) bind to most or all B-type ligands, but there are a few exceptions: EphA4 is also activated by Ephrin-B2/B3, and EphB2 by ephrin-A5. (Kullander, 2002; Martinez, 2005; Aoto, 2006)

Figure 1.1 Interactions between ephrins and Ephs
The extracellular region of Ephs consists of a highly conserved N-terminal ligand-binding globular domain, a cysteine-rich region, followed by two fibronectin type III repeats. The intracellular region can be divided into four parts: a juxtamembrane domain containing two conserved tyrosine residues, a classical tyrosine kinase domain, a sterile-α-motif (SAM), and a type-II PDZ-binding motif. The oligomerization of Ephs mainly involves the cysteine-rich region, fibronectin type III repeats, and the SAM motif. The cytoplasmic tail of ephrinBs encompasses only around 80 amino acids, but is highly conserved with a type-II PDZ-binding motif at the C-terminus and several tyrosine phosphorylation sites. (Kullander, 2002; Martinez, 2005; Aoto, 2006)

1.1.2 Ephrin-Eph signaling: Forward and Reverse

In contrast to other RTKs that have soluble ligands, Eph receptors are only activated by clustered, membrane bound ligands. Therefore, contact between cells expressing Eph receptors and ephrin ligands is required for Eph receptor activation.

Another unique feature of ephrin-Eph signaling is bi-directional signaling, meaning that ephrins can also signal into their host cells upon contact with Ephs—a process called “reverse signaling” as opposed to “forward signaling” mediated by Ephs. (Kullander, 2002; Martinez, 2005)

1.1.2.1 Forward signaling

Upon ligand engagement, Eph signaling is initiated by autophosphorylation of juxtamembrane tyrosine residues and activation of the kinase domain. The most well
studied downstream signaling pathway is the modulation of actin cytoskeleton through activation of guanine nucleotide exchange factors (GEFs). GEFs are regulators of small Rho family GTPases that are important regulators of actin cytoskeleton (Luo, 2000). The best characterized members of the family include Rho, Rac and Cdc42. They act as molecular switches, cycling between an active GTP-bound state and an inactive GDP-bound state. Activation of RhoA promotes growth cone collapse, while Rac and Cdc42 promote the extension of filopodia and lamellipodia, respectively.
Three GEFs mediate the regulation of Rho GTPases by EphA receptors: Ephexin, Vav and Tiam1 (Cowan, 2005; Hunter, 2006; Shamah, 2001; Tanaka, 2004). Interestingly, binding of different EphA receptors to each mediator triggers opposite effects on axon motility. For example, activation of Ephexin by EphA receptors result in enhanced RhoA activity and, concomitantly, a down-regulation of Rac1 and Cdc42 activity, thereby promoting axonal growth cone collapse (Shamah, 2001). On the other hand, interaction of EphA2 with the Rac1-specific GEF Tiam1 mediates neurite outgrowth in cortical neurons (Tanaka M, 2004).

EphB receptors interact with a different set of RhoGEFs: intersectin and kalirins, two exchange factors for Cdc42 and Rac1, respectively (Irie, 2002; Penzes, 2003). EphB2 physically associates and activates intersectin in cooperation with neural Wiskott-Aldrich syndrome protein (N-WASP), which in turn activates Cdc42 and spine morphogenesis (Irie, 2002). Activation of the EphB receptor induces translocation of the Rho-GEF kalirin to synapses and activation of Rac1 and its effector PAK (Penzes, 2003). These novel signal transduction pathways may be critical for the regulation of the actin cytoskeleton controlling spine morphogenesis during development and plasticity.

Another actin modulating protein downstream of Eph forward signaling is SHEP1 (SH2 domain-containing Eph receptor-binding protein 1), which binds to the GTPases R-Rac and Rap1A (Dodelet, 1999), and down-regulates the Ras-MAPK pathway. Eph receptor activation also regulates membrane ruffling and cell migration by inhibiting the interaction between SHEP1 and the scaffolding protein Crk-associated substrate (Cas)
In addition, Eph receptors can also associate with the p120-Ras GTPase activating protein (p120-RasGAP) upon ligand binding through SH2-mediated interactions (Dail, 2006; Elowe et al., 2001; Kim, 2002). This interaction inhibits the Ras-MAPK pathway and plays important roles in growth cone collapse, cell repulsion and morphogenesis of capillary endothelium. The C-terminal PDZ-binding motif of Eph receptors mediates their interactions with many neuronal PDZ scaffolding proteins, such as glutamater receptor interacting protein (GRIP), protein interacting with C-kinase (PICK1) and AF-6 (Hock, 1998; Hoogenraad, 2005; Torres, 1998). In summary, forward signaling through Eph receptors induces changes to the local milieu either through modulating actin dynamics or through the localization of scaffolding molecules in lipid rafts.

1.1.2.2 Reverse signaling

Reverse signaling of the ephrin ligands is activated by Eph receptor binding. Activated ephrinB ligands are phosphorylated at several conserved tyrosine residues in the cytoplasmic domain by Src family kinase (SFK) members (Src and Fyn) (Georgakopoulos et al., 2006 and Palmer et al., 2002), PDGF (Bruckner et al., 1997) and FGF receptors (Yokote et al., 2005). Tyrosine phosphorylation of ephrins by SFKs leads to the recruitment of Grb4, a SH2 domain containing scaffolding protein that alters the actin cytoskeleton via the recruitment and activation of FAK (Cowan and Henkemeyer et al., 2001). Like Eph receptors, ephrin B ligands can also recruit PDZ-domain proteins through a C-terminal (YKV) PDZ-binding site. After tyrosine phosphorylation, ephrinB ligands recruit a phosphotyrosine phosphotase PTP-BL through their PDZ-binding
domain and are subsequently dephosphorylated (Palmer et al., 2002). In addition to PTP-BL, a number of other PDZ domain proteins, including GRIP, syntenin, PSD-95, PICK1 and regulator of G-protein signaling (PDZ-RGS-3), have been found to interact with ephrinB ligands (Bruckner et al., 1999; Lu et al., 2001; Meyer et al., 2004). So far, GRIP1/2, PDZ-RGS3 and PTP-BL are known to interact with the PDZ-binding motifs of ephrinBs endogenously (Bruckner et al., 1999, Lu et al., 2001 and Palmer et al., 2002). However, the physiological relevance of these interactions remains mostly unclear.

Beyond the signaling pathways associated with proteins directly interacting with the ephrinB C-terminus, a few novel signaling pathways have been recently discovered, perhaps through indirect interactions mediated by PDZ scaffolds. For example, a recent study has identified the MAP kinase c-Jun NH2-terminal kinase (JNK) as a crucial mediator of ephrin-B1 reverse signaling (Xu et al., 2003). In this case, JNK activation induced by this ligand leads to rounded morphology of cultured cells in a phosphotyrosine-independent manner (Xu et al., 2003).

Surprisingly, it was later discovered that ephrinA ligands, lacking a intracellular portion and the abilities to recruit signaling molecules, also have reverse signaling potential. The activation of ephrin-A2 or ephrin-A5 by one of their receptors, EphA3, results in a h1-integrin-dependent increase in cellular adhesion of ephrin-A-expressing cells to laminin (Huai and Drescher et al., 2001). Members of the Src family of protein tyrosine kinases could be involved in these effects (Davy and Robbins et al., 2000). EphrinAs are also found to interact independent of their ligand-binding domain with EphAs in \textit{cis}, which
prevents trans interaction and silences EphA forward signaling (Carvalho et al., 2006 and Yin et al., 2004).

1.1.3 Functions of Ephrin-Eph signaling

Eph receptors and ephrins mediate cell-cell contact-dependent signaling in diverse processes in both developing and mature nervous system. They act as repulsive or permissive primary messengers in axon guidance, topographic mapping, and neural patterning during early development of the nervous system. More recent studies have also revealed novel functions of Eph receptors in synapse formation and synaptic plasticity.

1.1.3.1 Early segmentation and morphogenesis

The Eph/ephrin system is crucially involved in segmentation processes operating in vertebrates; examples include the vertebrate hindbrain, where the ephrins and Ephs function in rhombomere-specific cell sorting (Cooke et al., 2002) and the somites, where the Eph/ephrin system appears to contribute to the segmentation of the paraxial mesoderm and to the division of the somites into anterior (EphA4-positive) and posterior (ephrinB2-positive) halves (Durbin, 1998&2000).

The role of ephrin/Eph interactions in cell migration has been studied in the early developmental stages of the nervous system. At this age, the trunk neural crest cells of chick and rodent migrate through the anterior but not posterior half of each somite (Bronner-Fraser et al., 1986; Rickmann et al., 1985). These specific destinations seem to be determined by expression levels of class B receptors and ligands. Thus, expression of
ephrin-B1 and ephrin-B2 in the posterior half of somites in chick and mouse, respectively, allows a repulsive signal for trunk neural crest cells, which express EphB receptors (C.E. Krull et al., 1997; Wang. Simila et al., 1997). Similarly, the repulsive signal promoted by ephrin-B2 and receptors EphA4 and EphB1 regulates the migration of branchial neural crest cells in *Xenopus* (Smith et al., 1997).

1.1.3.2 Axon guidance and remodeling
Ephs and ephrins are involved in the establishment of numerous topographic maps in many regions of the developing nervous system, including projections in olfactory system, connections from retina to optic tectum, hippocampus to septum, and thalamus to cortex (klein, 2004). These specific projection maps require a complementary and gradual expression of ephrin and Eph receptor gradients in origin and target regions of the neuronal connections. The visual system, specifically the retino-tectal projection, has been the prototype model to study the development of topographic maps. Retinal axon connections with the superior colliculus are topographically organized such that neighborhood relationships are preserved from the RGCs to their target cells. Ephrins and Ephs are critical components that help to set up topographic maps in all directions. EphrinAs and EphA receptors are required for anterior-posterior (A–P) topographic mapping (Wilkinson, 2000; Feldheim et al., 2004), whereas ephrinBs and EphB receptors are required for dorsal–ventral (D–V) mapping along the lateral–medial (L–M) axis of the midbrain target area (McLaughlin, 2003).

Besides topographic mapping, Ephs and ephrins also regulate the guidance of motor axons and commissural axons across the midline in the CNS and in the spinal cord, as
well as the remodeling of efferent axons that have reached their muscle target (Klein, 2004).

1.1.3.3 Synapse formation and plasticity

Ephrin/Eph signaling has received increasing attention for their roles in the formation and maturation of synapses in recent years. Initially, the expression of ephrins and Eph receptors was described at neuromuscular binding sites (Feng et al., 2000) and in pre- and post-synaptic domains of synaptic contacts in the CNS (Bruckner et al., 1999; Buchert et al., 1999; Martone et al., 1997; Torres et al., 1998). Later, it was confirmed that the ephrin/Eph system was directly related to the establishment and development of these neuromuscular (Lai et al., 2001) and central synapses (Dalva et al., 2000; Gerlai, 2002).

Furthermore, Eph receptors also regulate the structural and functional properties of synapses by regulating the formation and maintenance of dendritic spines. Genetic studies revealed a highly redundant requirement of three EphB receptors (EphB1–3) for dendritic spine morphogenesis in the hippocampus (Henkemeyer et al., 2003). Syndecan-2 (Ethell et al., 2001) and Rho family small GTPases (Penzes et al., 2001, 2003) have been identified as major players in EphB-mediated spine formation. Although EphA receptors do not seem to directly promote spine formation, recent studies suggest that they may regulate spine stability. Ephrin-A3/EphA4 signaling regulates hippocampal dendritic spine morphology through neuroglial cross-talk (Murai et al., 2003), and later it was proposed that Cdk5 and ephexin1 are involved in EphA4-mediated dendritic spine retraction in the mouse hippocampus (Fu, Chen & Ip et al., 2007).
In addition, EphB signaling participates in activity-dependent synaptic plasticity. It was demonstrated that the extracellular juxtamembrane region of EphB directly interacts with the NMDA (N-methyl-D-aspartate) receptor subunit NR1. EphB regulates NMDA receptors to promote NMDA-dependent plasticity, or is clustered via PDZ interactions to activate reverse signaling by presynaptic ephrinBs, thus promoting NMDA-independent forms of plasticity (Dalva et al., 2000; Takasu et al., 2002). EphB2 also controls AMPA-type glutamate receptor localization through PDZ binding domain interactions and triggers presynaptic differentiation via its ephrin binding domain (Kayser et al., 2006).

1.1.4 Regulation of Ephrin-Eph signaling

In contrast to the extensively studied Ephrin-Eph functions, the underlying mechanisms regulating their cell surface expression and intracellular fate are unclear. It is reported that ligand-induced receptor endocytosis of both EphAs and EphBs occurs upon cell-cell contact, converting an initial adhesive interaction into a repulsive event in growth cone collapse response (Zimmer, 2003; Marston, 2003; Parker, 2004; Cowan, 2005). In tumor cells and T cells, EphA receptors are also found to be endocytosed and down-regulated following ligand activation, and adaptor protein c-Cbl which could function as a RING finger E3 ubiquitin ligase is thought to mediate their degradation via the proteasome pathway (Walker-Daniels et al., 2003; Wang et al., 2002; Sharfe et al., 2003).

Another mechanism to terminate ephrin-Eph signaling is dephosphorylation by phosphatases. Upon ephrin stimulation, Eph receptors are activated through autophosphorylation of juxtamembrane tyrosine residues, which releases the kinase
domain from intramolecular inhibitory interactions with the juxtamembrane segment. Such conformational change favors binding of substrates and downstream signaling molecules. EphA2 was identified as a substrate of low molecular weight protein-tyrosine phosphatase (LMP-PTP) (Kiawa et al., 2002). Overexpression of LMP-PTP resulted in a reduced EphA2 expression and its kinase activity, suggesting a role for phosphatase in terminating ephrin-Eph signaling. This mechanism could be important for the malignant behavior of cancer cells (Kiawa et al., 2002), as well as offering an efficient negative feedback loop on ephrinA1/EphA2 mediated repulsive response (parri et al., 2005).

1.2 The ubiquitin-proteasome proteolytic system

1.2.1 The ubiquitination reaction

Ubiquitin is a 76-amino-acid polypeptide that is conjugated post-translationally to a wide variety of substrates. The conjugation of a ubiquitin to a protein substrate occurs via the sequential action of three enzymes: E1 Ub-activating enzyme, E2 Ub-conjugating enzyme, and E3 Ub ligase. Ubiquitin is conjugated to an active site cysteine residue in an E1 enzyme in an ATP-dependent manner, followed by transfer to an E2 ubiquitin-conjugating enzyme, also through a thiol–ester linkage. Although some E2s can ubiquitylate substrates directly, in most cases E3 ubiquitin ligases mediate substrate recognition, and are required for ubiquitin conjugation to substrates. The ubiquitination reaction results in the formation of an isopeptide bond between the carboxyl terminus glycine residue of ubiquitin (glycine 76) and the ε-amino group of a lysine residue within the substrate.
There is only one isoform of E1 in eukaryotic cells, with quite a few E2s and many different E3s that work in various combinations to ensure substrate specificity and correct timing of the ubiquitination reaction (d’Azzo A, 2005; Marmor, 2004). There are mainly two types of E3 ligases. The catalytic HECT (homologous to E6-AP carboxyl terminus) domain-containing proteins, such as Nedd4 family ligases, proceed through a thiolester: ubiquitin intermediate to transfer ubiquitin to the substrate. These E3s are usually single, large proteins. The adaptor RING (really interesting new gene)-type E3 ligases are by far the most abundant in nature. Despite having no intrinsic catalytic activity, it is assumed that RING E3s contribute to catalysis by serving as a scaffold that brings together the E2-active site and the substrate's acceptor lysine(s). Examples of this class include proteins containing RING finger domains, such as Cbl, and multiprotein complexes like SCF (Skip1/Cullin/F-box), as well as U-box-containing proteins, such as CHIP (Hsp70 interacting protein) (Weissman, 2001).

Ubiquitination is highly dynamic and exists as a balance between ubiquitinating and deubiquitinating processes, and ubiquitin can be removed by the action of de-ubiquitinating enzymes (Weissman, 2001).

1.2.2 Mono Vs. poly-ubiquitination

Multiple rounds of ubiquitination reaction on the same lysine residue conjugate a polymeric Ub chain onto substrate proteins, which targets them for degradation by the 26S proteasome complex that recognizes polymeric chains of at least four Ub
polypeptides. In mammalian cells, most short-lived cytosolic proteins are turned over by this mechanism (Fig 1.3).

Membrane proteins follow a pathway that not necessarily involves polyubiquitination and proteasomal degradation. Addition of an Ub molecule at one (monoubiquitination) or multiple (multiubiquitination) lysine residues of ion channels, receptors or junctional complexes leads to their regulated internalization and sorting to the endocytic compartment. Internalized membrane proteins are either recycled (returned to the plasma membrane) or degraded in the multivesicular body (MVB)-lysosome system (Fig 1.4). In addition to providing internalization or sorting signals, monoubiquitin tags regulate other biological processes, including histone modification, transcription, DNA repair and viral budding. (d’Azzo A, 2005; Marmor, 2004).

1.2.3 Roles for the ubiquitin-proteasome system (UPS) in neuronal functions

The UPS is well recognized for its roles in neurodegenerative diseases. Many late-onset neurodegenerative diseases are associated with the formation of intracellular aggregates by toxic proteins (Rubinsztein, 2006). These diseases are called proteinopathies. Many of the proteins that cause proteinopathies are partly dependent on the ubiquitin–proteasome pathway for their clearance. Not surprisingly, a number of proteinopathies have been associated with decreased proteasome activity in humans or in cell models, including sporadic Parkinson’s disease (PD) and Huntington’s disease (HD). The strongest evidence comes from human/mammalian mutations that involve components of this pathway and cause neurodegeneration. The most famous of these is the E3 ubiquitin
Marmor et al., 2004

Figure 1.3 The mono-ubiquitin mediated endocytic pathway of membrane proteins

Ciechanover et al., 2005

Figure 1.4 The ubiquitin-proteasome degradation pathway
ligase, parkin, in which autosomal recessive loss-of-function mutations cause PD. A number of parkin substrates have been identified, but at present it is not clear which is the major contributor to pathology in parkin deficiency (Rubinsztein, 2006).

Although initially noted in neuronal dysfunction and degeneration, more recent studies have revealed diverse roles for the UPS in neuronal growth and development, synaptic function and plasticity, and neuronal survival (Yi, 2007). For example, it was shown that axonal growth cones are primed for enhanced UPS signaling. Pharmacological inhibition of the proteasome could compromise both the formation of new growth cones and growth cone responsivity to extracellular guidance cues (Campbell and Holt et al., 2001; Verma et al., 2005). In terms of regulation of synaptic functions, it was suggested that proteasomal degradation at the presynaptic terminal may negatively regulate neurotransmitter release during times of intense synaptic activity (Willeumier et al., 2006). Furthermore, changing levels of neuronal activity cause reciprocal alterations in the protein composition of the PSD in a UPS-dependent manner (Ehlers, 2003), and these local changes in synapse composition seem to be facilitated by activity-dependent alterations in the distribution of proteasomes in neurons (Bingol and Schuman et al., 2006). In addition, the UPS has also been shown to regulate trafficking of postsynaptic receptors, including glutamate receptors, GABA receptors, and AChRs (Yi, 2007).

1.3 The Anaphase-promoting complex (APC)

The anaphase-promoting complex (APC) is a RING-type E3 ubiquitin ligase that has a particularly prominent role in regulating cell cycle progression.
1.3.1 Structure and subunits of the APC

To date, the APC is the most complicated member of the RING/cullin family of multisubunit E3s. It has at least 13 core subunits in yeast and 12 in human, with three additional activators in yeast and two in human (Table 1.1). So far, there’s limited knowledge about the structure and organization of the complex, as well as how these subunits work together to form a functional enzyme. The understanding of the individual subunits is mostly limited to the conserved structural motifs in their amino acid sequences (Acquaviva, 2006).

APC2 and APC11 form the catalytic core of the complex. They harbour a cullin and a RING-finger domain, respectively. In vitro, APC2/APC11 complex is sufficient to conduct substrate ubiquitination, but with little substrate specificity. Four subunits APC3, APC6, APC7 and APC8 contain randomly tetratricopeptide repeats (TPR), which is a common motif in multiprotein complexes to mediate protein-protein interaction. Those of APC3 and APC7 interact with the Isoleucine-arginine (IR) motifs of the activators, Cdc20 and Cdh1. Moreover, the four TPR subunits contain most of the phosphorylation sites present in the APC. Due to these reasons, they are thought to be essential for the activation of the APC. The functions of the rest subunits are even less understood. They are currently thought to serve mainly structural roles and to promote complex stability, although APC10 also seems to aid in substrate recognition (Castro, 2005; Acquaviva, 2006).
Table 1.1 APC/C subunits and co-activators (Peters, 2006)

<table>
<thead>
<tr>
<th>Subunits</th>
<th>Vertebrates</th>
<th>Drosophila melanogaster</th>
<th>Budding yeast</th>
<th>Structural motifs</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC1/TSG24</td>
<td>Shattered</td>
<td>Apc1</td>
<td>RPN1 and RPN2 homology*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>APC2</td>
<td>Morula</td>
<td>Apc2</td>
<td>Cullin homology</td>
<td>APC11 and DOC1 binding</td>
<td>-</td>
</tr>
<tr>
<td>CDC27/APC3</td>
<td>Makos</td>
<td>Cdc27</td>
<td>TPRs</td>
<td>CDH1 binding</td>
<td>-</td>
</tr>
<tr>
<td>APC4</td>
<td>-</td>
<td>Apc4</td>
<td>WD40 repeats</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>APC5</td>
<td>Idn</td>
<td>Apc5</td>
<td>TPRs</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CDC16/APC6</td>
<td>-</td>
<td>Cdc16</td>
<td>TPRs</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>APC7</td>
<td>-</td>
<td>-</td>
<td>TPRs</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CDC23/APC8</td>
<td>-</td>
<td>Cdc23</td>
<td>TPRs</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DOC1/APC10</td>
<td>-</td>
<td>Doc1/Apc10</td>
<td>Doc domain</td>
<td>Substrate recognition, processivity</td>
<td>-</td>
</tr>
<tr>
<td>APC11</td>
<td>Lemming</td>
<td>Apc11</td>
<td>RING-H2 finger</td>
<td>E2 recruitment, E3 activity</td>
<td>-</td>
</tr>
<tr>
<td>CDC26</td>
<td>-</td>
<td>Cdc26</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SWM1/APC13</td>
<td>-</td>
<td>Swm1/Apc13</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>Apc9</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>Mnd2</td>
<td>-</td>
<td>Ama1 inhibition</td>
<td>-</td>
</tr>
<tr>
<td>Co-activators</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDC20/p5502c</td>
<td>Fizzy</td>
<td>Cdc20</td>
<td>C-box, WD40 repeats and IR-tail</td>
<td>Substrate recognition</td>
<td>-</td>
</tr>
<tr>
<td>CDH1 A–D</td>
<td>Fizzy-related</td>
<td>Cdh1/Hct1</td>
<td>C-box, WD40 repeats and IR-tail</td>
<td>Substrate recognition</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>Rep</td>
<td>-</td>
<td>C-box, WD40 repeats and IR-tail</td>
<td>Substrate recognition</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>Ama1</td>
<td>-</td>
<td>C-box, WD40 repeats and IR-tail</td>
<td>Substrate recognition</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>Cortex</td>
<td>-</td>
<td>C-box, WD40 repeats and IR-tail</td>
<td>Substrate recognition</td>
<td>-</td>
</tr>
</tbody>
</table>

Apart from the core subunits, there are three additional activators. One of them called Ama1 is only found in the yeast and functions specifically in meiosis. Cdc20 and Cdh1 are the most important ones because they are conserved to all studied species. They transiently associate with the APC core during different phases of the mitosis cell cycle, and target it to different sets of substrates. The exact mechanism through which these proteins increase APC activity is not known. They all contain a conserved WD40 domain, which mediates substrate binding to the F-box proteins in the SCF. Therefore it is speculated that the activators conduct substrate recognition. In support of this hypothesis,
several studies have shown that different APC substrates can directly and specifically bind to Cdc20 or Cdh1 (Castro, 2005; Acquaviva, 2006).

1.3.2 Functions of the APC in the cell cycle

As its name indicates, the APC was first identified to promote metaphase to anaphase transition in mitosis. Actually, APC is essential for several key events in mitosis depending on its activitors to selectively recognize its substrates at the correct time, including the initiation of anaphase, exit from mitosis and the preparation for the next round of DNA replication (Fig 1.5).

Figure 1.5 APC substrate in the cell cycle

1.3.2.1 Sister-chromatid separation

To allow chromosome segregation, the cohesion that holds sister chromatids together first has to be dissolved. In metaphase, $\text{APC}^{\text{Cdc20}}$ initiates this process by destructing securin,
an inhibitor of the protease separase. Once activated, separase cleaves the Scc1 subunit of cohesin, a complex that holds sister chromatids together, and therefore dissolves cohesion between sister chromatids (Peters, 2006).

1.3.2.2 Cyclin destruction
Securin degradation is not the only requirement for the metaphase-anaphase transition, in part because mitotic cyclin-Cdk can inhibit separase. Destruction of cyclin A and cyclin B begins at metaphase and continues throughout mitosis and G1 phase. During metaphase to anaphase transition, APC\textsuperscript{Cdc20} mediate cyclin degradation to inhibit Cdk1 activity and as consequence to induce different cell processes such as sister chromatid separation, disassembly of the mitotic spindle, chromosome decondensation, cytokinesis and reformation of the nuclear envelope. In G1 phase, APC\textsuperscript{Cdhl}-dependent degradation of the cyclins prevents the accumulation of these proteins and premature entry into S phase (Castro, 2005; Acquaviva, 2006).

1.3.2.3 DNA replication
During S phase, APC regulate three proteins that control prereplication complex formation at the replication origins: Orc1, Cdc6 and geminin. The origin recognition complex (ORC) binds the origins of replication and serves as a platform for subsequent loading of additional factors such as Cdc6, Cdt1 and MCM proteins rendering DNA competent for replication. The levels of the ORC subunit Orc1 are controlled during the cell cycle. Orc1 persists from late G1 phase until late mitosis and it is degraded by APC\textsuperscript{Cdhl} at the exit from mitosis. Cdc6 binds to the ORC complex and mediates
subsequent recruitment of the MCM complex. Similarly, human Cdc6 is degraded in early G1 by APC$^{Cdh1}$. Geminin prohibits initiation of DNA replication at inappropriate times of the cell cycle by preventing MCM recruitment at the replication origins. In HeLa cells, geminin is absent during G1 and accumulates during S, G2 and M phases, due to its regulation by APC (Castro, 2005).

### 1.3.3 Functions of the APC in the nervous system

Nearly ten years after its discovery as a crucial cell-cycle regulated ligase, the anaphase-promoting complex (APC) is making a debut in neurobiology. At first, core components of APC including activator Cdh1 were found to be expressed in brain tissue (Gieffers et al., 1999). From then on, several studies have defined novel roles for APC in postmitotic neurons, including regulation of axonal growth and patterning, neuronal survival, as well as synaptic development and function.

#### 1.3.3.1 Axonal growth and patterning

Bonni and colleges demonstrated that Cdh1 knockdown in granule neurons of the developing cerebellum promotes the robust elongation of axons, suggesting that Cdh1 inhibits axon growth. In vivo, APC$^{Cdh1}$ appears to cell autonomously control the growth of granule neuron axons in a layer-specific manner, limiting their growth to the molecular layer in the classic parallel-fiber pattern (Konishi et al., 2004). Later, the group reported that APC$^{Cdh1}$ operates in the nucleus to inhibit axonal growth. They identified two nuclear targets of the APC$^{Cdh1}$ that promote axonal growth: transcriptional corepressor SnoN (Stegmuller et al., 2006) and Id2 (inhibitor of DNA binding 2) (Lasorella et al., 2006).
1.3.3.2 Synaptic development and function

Two studies have described novel functions of the APC at the synapse in the worm and fly, respectively. Perturbing APC function in postmitotic neurons in C. elegans increased the abundance of GLR-1 in the ventral nerve cord. Blockade of clathrin-mediated endocytosis suppressed the effects of the APC mutations, suggesting that the APC regulates some aspect of GLR-1 recycling. Phenotypically, APC mutants had locomotion defects consistent with increased synaptic strength (Juo and Kaplan et al., 2004). At Drosophila neuromuscular synapses, the APC seems to independently regulate synaptic growth and synaptic transmission. In neurons, the APC controls synaptic size via a downstream effector Liprin-alpha; in muscles, the APC regulates synaptic transmission also by controlling the concentration of a postsynaptic glutamate receptor (Roessel et al., 2004).

1.3.3.3 Neuronal survival

Aberrant expression of cell-cycle proteins has been reported to be an important mechanism leading to developmentally regulated neuronal loss and apoptosis in the adult brain (Becker and Bonni, 2004). For example, the Cdk1–cyclin B1 complex is reactivated in degenerating neurons of patients suffering from Alzheimer's disease (Vincent et al., 1997) and in experimental models of ischemia (Wen et al., 2004). In a study by Almeida and colleges, it was reported that Cdh1-APC is involved in preventing cell-cycle progression in terminally differentiated neurons. They showed that Cdh1 silencing rapidly triggered apoptotic neuronal death. They proposed a possible mechanism that Cdh1 is required to prevent the accumulation of cyclin B1 in terminally differentiated
neurons, and thereby prevent these neurons from entering an aberrant S phase that led to apoptotic cell death. They suggested that this mechanism provides an explanation for cyclin B1 reactivation that occurs in the brain of patients suffering from neurodegenerative diseases, such as Alzheimer's disease (Almeida et al., 2005).

1.4 Aims of study

The mechanisms underlying the regulation of the cell surface expression and intracellular fate of Eph receptors are unclear. In a yeast two-hybrid screening using the cytoplasmic region of EphA4 as the bait, a truncated isoform of APC2 (anaphase-promoting complex subunit 2) was identified to be an EphA4 interacting protein (Hung & Ip, unpublished observation). In this study, the implication of this interaction is further explored.

Because EphA4 interacts with the core subunit of the APC which is an E3 ubiquitin ligase, we would like to examine whether the APC regulates EphA4 expression via the ubiquitin-proteasome pathway. The APC together with its neuronal specific activator Cdh1 have been found to play emerging roles in the nervous system, therefore we will also explore the effect of the neuronal specific activator Cdh1 on the expression of EphA4.
# Chapter 2 Material and Methods

## 2.1 Materials

<table>
<thead>
<tr>
<th>Materials:</th>
<th>Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Animals</strong></td>
<td></td>
</tr>
<tr>
<td>Rat Embryo, Pup, Adult</td>
<td>Animal Care Centre, HKUST, HK</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Animal Care Centre, HKUST, HK</td>
</tr>
<tr>
<td><strong>Antibodies:</strong></td>
<td></td>
</tr>
<tr>
<td>Anti-APC2 (C-term) pAb</td>
<td>HKUST, Hong Kong, China</td>
</tr>
<tr>
<td>Anti-Cdc27(AF3.1) mAb</td>
<td>Sigma, St Louis, MO, USA</td>
</tr>
<tr>
<td>Anti-Cdh1 mAb</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>Anti-Cdc20 mAb</td>
<td>Santa Cruz, Biotech., Inc, CA, USA</td>
</tr>
<tr>
<td>Anti-EphA4 (sc921)</td>
<td>Santa Cruz, Biotech., Inc, CA, USA</td>
</tr>
<tr>
<td>Anti-HA mAb</td>
<td>Santa Cruz, Biotech., Inc, CA, USA</td>
</tr>
<tr>
<td>Anti-HA pAb</td>
<td>Santa Cruz, Biotech., Inc, CA, USA</td>
</tr>
<tr>
<td>Anti-His pAb</td>
<td>Santa Cruz, Biotech., Inc, CA, USA</td>
</tr>
<tr>
<td>Anti-His mAb</td>
<td>Santa Cruz, Biotech., Inc, CA, USA</td>
</tr>
<tr>
<td>Anti-Human Fc</td>
<td>Jackson ImmunoResearch Laboratories Inc., PA, USA</td>
</tr>
<tr>
<td>Anti-α tubulin mAb</td>
<td>Santa Cruz, Biotech., Inc, CA, USA</td>
</tr>
<tr>
<td>Anti-Ubiquitin mAb</td>
<td>Calbiochem-Novabiochem CN Biosciences, Inc, Germany</td>
</tr>
</tbody>
</table>
### Chemicals

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine triphosphate (ATP)</td>
<td>Sigma, St Louis, MO, USA</td>
</tr>
<tr>
<td>Agarose</td>
<td>Invitrogen™ Life Technologies, CA, USA</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Amresco Solon, Ohio, USA</td>
</tr>
<tr>
<td>Antipain</td>
<td>Roche Molecular Biochemicals, Germany</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>Roche Molecular Biochemicals, Germany</td>
</tr>
<tr>
<td>Benazamidine</td>
<td>Roche Molecular Biochemicals, Germany</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>Riedel-de Haën, Seelze, Germany</td>
</tr>
<tr>
<td>Calcium chloride (CaCl₂)</td>
<td>Amresco, Solon, Ohio, USA</td>
</tr>
<tr>
<td>Chemical Name</td>
<td>Supplier and Location</td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td>----------------------------------------------------------</td>
</tr>
<tr>
<td>Di-sodium ethylenediaminetetraacetatic acid (EDTA)</td>
<td>BDH, Poole, UK</td>
</tr>
<tr>
<td>Di-sodium hydrogen phosphate</td>
<td>BDH, Poole, UK</td>
</tr>
<tr>
<td>Dithiothreitol (DDT)</td>
<td>Sigma, St Louis, MO, USA</td>
</tr>
<tr>
<td>Doblecco’s modified Eagle’s medium (DMEM)</td>
<td>Invitrogen™ Life Technologies, CA, USA</td>
</tr>
<tr>
<td>Doblecco’s phosphate buffered saline (DPBS)</td>
<td>Invitrogen™ Life Technologies, CA, USA</td>
</tr>
<tr>
<td>Ethylenebis(oxyethylenenitrilo)tetracetic acid (EGTA)</td>
<td>Sigma, St Louis, MO, USA</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>Merck &amp; Co., Inc., NJ, USA</td>
</tr>
<tr>
<td>Formamide</td>
<td>Invitrogen™ Life Technologies, CA, USA</td>
</tr>
<tr>
<td>Glucose</td>
<td>Fisher Scientific, NJ, USA</td>
</tr>
<tr>
<td>Guanidium thioeyanate</td>
<td>Invitrogen™ Life Technologies, CA, USA</td>
</tr>
<tr>
<td>HEPES</td>
<td>Invitrogen™ Life Technologies, CA, USA</td>
</tr>
<tr>
<td>Iospropylthio- β-D-galactoside (IPTG)</td>
<td>Sigma, St Louis, MO, USA</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>Roche Molecular Biochemicals, Germany</td>
</tr>
<tr>
<td>Lithium chloride</td>
<td>Sigma, St Louis, MO, USA</td>
</tr>
<tr>
<td>Chemical Name</td>
<td>Supplier</td>
</tr>
<tr>
<td>---------------------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>Sigma, St Louis, MO, USA</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>Invitrogen™ Life Technologies, CA, USA</td>
</tr>
<tr>
<td>MOPS</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Paraformaldehyde (PFA)</td>
<td>Riedel-de Haën, Seelze, Germany</td>
</tr>
<tr>
<td>Phenylmethylsulfonyl fluoride (PMSF)</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Potassium acetate</td>
<td>Merck &amp; Co., Inc., NJ, USA</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>Riedel-de Haën, Seelze, Germany</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>USB Amersham Biosciences, Sweden</td>
</tr>
<tr>
<td>Sodium dodecyl sulphate (SDS)</td>
<td>USB Amersham Biosciences, Sweden</td>
</tr>
<tr>
<td>Sodium fluoride</td>
<td>Sigma, St Louis, MO, USA</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>BDH, Poole, UK</td>
</tr>
<tr>
<td>Sodium Orthovandate</td>
<td>Sigma, St Louis, MO, USA</td>
</tr>
<tr>
<td>Sucrose</td>
<td>Sigma, St Louis, MO, USA</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>USB Amersham Biosciences, USA</td>
</tr>
<tr>
<td>Trypsin</td>
<td>Invitrogen™ Life Technologies, CA, USA</td>
</tr>
<tr>
<td>Trypsin inhibitor</td>
<td>Roche Molecular Biochemicals, Germany</td>
</tr>
<tr>
<td>Tryptone</td>
<td>Oxoid, Unipath Ltd., UK</td>
</tr>
<tr>
<td>X-Gal (5-Bromo-4-Chloro-3-Indolyl-β-D-Galactoside)</td>
<td>Promega, Madison, WI, USA</td>
</tr>
<tr>
<td>Solutions</td>
<td>Supplier</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>Merck &amp; Co., Inc., NJ, USA</td>
</tr>
<tr>
<td>Acrylamide:bis-acrylamide 29:1 solution</td>
<td>Amresco, Solon, Ohio, USA</td>
</tr>
<tr>
<td>30% Acrylamide:bis-acrylamide</td>
<td>Bio-Rad Laboratories, Inc., CA, USA</td>
</tr>
<tr>
<td>B27 supplement</td>
<td>Invitrogen™ Life Technologies, CA, USA</td>
</tr>
<tr>
<td>Bio-Rad Protein Assay</td>
<td>Bio-Rad Laboratories, Inc., CA, USA</td>
</tr>
<tr>
<td>Chloroform</td>
<td>Merck &amp; Co., Inc., NJ, USA</td>
</tr>
<tr>
<td>Diethyl pyrocarbonate (DEPC)</td>
<td>Sigma, St Louis, MO, USA</td>
</tr>
<tr>
<td>Dimethylsulfoxide (DMSO)</td>
<td>Invitrogen™ Life Technologies, CA, USA</td>
</tr>
<tr>
<td>Ephrin-A1/Fc Chimera</td>
<td>R &amp; D System, Inc., MN, USA</td>
</tr>
<tr>
<td>Ethidium bromide (EtBr)</td>
<td>Invitrogen™ Life Technologies, CA, USA</td>
</tr>
<tr>
<td>Fetal bovine serum</td>
<td>Invitrogen™ Life Technologies, CA, USA</td>
</tr>
<tr>
<td>Glycerol</td>
<td>USB Amersham Biosciences, Sweden</td>
</tr>
<tr>
<td>Goat serum (GS)</td>
<td>Invitrogen™ Life Technologies, CA, USA</td>
</tr>
<tr>
<td>Hydrochloric acids (HCl)</td>
<td>Merck &amp; Co., Inc., NJ, USA</td>
</tr>
<tr>
<td>Isoamylalcohol</td>
<td>Riedel-de Haën, Seelze, Germany</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>Merck &amp; Co., Inc., NJ, USA</td>
</tr>
<tr>
<td>Material</td>
<td>Supplier</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Nonidet P40 substitute (NP40)</td>
<td>USB Amersham Biosciences, Sweden</td>
</tr>
<tr>
<td>Penicillin/Streptomycin</td>
<td>Invitrogen™ Life Technologies, CA, USA</td>
</tr>
<tr>
<td>Phenol</td>
<td>Invitrogen™ Life Technologies, CA, USA</td>
</tr>
<tr>
<td>Ponceau S solution</td>
<td>Sigma, St Louis, MO, USA</td>
</tr>
<tr>
<td>Triton-X 100</td>
<td>Sigma, St Louis, MO, USA</td>
</tr>
<tr>
<td>Trypsin</td>
<td>Gibco-BRL, NY, USA</td>
</tr>
<tr>
<td><strong>Kits</strong></td>
<td></td>
</tr>
<tr>
<td>Lipofectamine plus transfection kit</td>
<td>Invitrogen™ Life Technologies, CA, USA</td>
</tr>
<tr>
<td>QIAquick Gel Extraction Kit</td>
<td>Qiagen Inc., Chatswoth, CA, USA</td>
</tr>
<tr>
<td>QIAquick PCR Purification Kit</td>
<td>Qiagen Inc., Chatswoth, CA, USA</td>
</tr>
<tr>
<td>T7 DNA Polymerase Sequencing Kit</td>
<td>Amersham Biosciences Sweden.</td>
</tr>
<tr>
<td>Ubiquitinated protein enrichment kit</td>
<td>Calbiochem-Novabiochem CN Biosciences, Inc, Germany</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
</tr>
<tr>
<td>Prestained protein marker, broad range</td>
<td>Invitrogen™ Life Technologies, CA, USA</td>
</tr>
<tr>
<td>Molecular weight marker (Lambda DNA/Hind III fragments, 100bp ladder DNA)</td>
<td>Invitrogen™ Life Technologies, CA, USA</td>
</tr>
<tr>
<td>Nitrocellulose membrane</td>
<td>Micron Separations Inc., Westboro.</td>
</tr>
</tbody>
</table>
2.2 Tissue dissection

Brain and hindleg muscle of Sprague-Dawley rats (embryonic, postnatal stages and adult) were dissected. Dissected tissues were washed in $1 \times$ phosphate-buffered saline ($1 \times$ PBS; $0.8\%$ NaCl, $0.02\%$ KCl, $8 \text{mM} \text{Na}_2\text{HPO}_4$, $1.76 \text{mM} \text{KH}_2\text{PO}_4$, pH 7.4). The washed tissues were then frozen in liquid nitrogen and stored at -80°C for later use.

2.3 Primary neuron cultures

Rat cortical neuronal cultures were prepared from embryonic day18 rat cortex. After digested with trypsin in Hanks' Balanced Salt Solution without Ca$^{2+}$ and Mg$^{2+}$ (Invitrogen) at 37°C for 15 min, the enzyme reaction was stopped by addition of serum. The cells were centrifuged with 14000 rpm for 5 min and the pellet was resuspended in DMEM with 10 % horse serum and triturated for 10 times. The cells were then seeded on culture
plates coated with a 0.01 mg/ml solution of poly-L-lysine (Sigma). The medium is replaced by NEUROBASAL medium supplemented with 2% B27 after cell seeding for 20 min.

2.4 Cell lines
All cell lines were obtained from American Type Culture Collection (ATCC). HEK293T and COS7 cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (HIFBS) and penicillin/streptomycin, and incubated at 37°C supplied with 5% carbon dioxide.

2.5 Polymerase chain reaction (PCR)
PCR amplification was performed to obtain DNA fragments for cloning or overlapping PCR. For each reaction, 100 ng template plasmid was mixed with Buffer for Vent DNA polymerase, 0.2 mM dNTPs, 5’ and 3’ primer at 0.2 μM and 0.5 unit Vent DNA polymerase. The mixture was finally filled up to 50 μl with milli-Q water. The reaction was carried out in a thermocycler (Robocycler, Stratagene) with a program set as following:

(i) Pre-denaturing; 94°C, 10 min;

(ii) Denaturing: 94°C, 1 min;
Annealing: 55°C, 2 min;
Extending: 55°C, 2 or 3 min;
Repeat for 30-35 cycles;

(iii) Post-extending: 72°C, 10 min.
Overlapping PCR was performed to obtain a point mutation in DNA sequence by overlapping two DNA fragments. The reaction mixture is the same with the one described above, except that the template plasmid is replaced by 100 ng of the two DNA fragments each. The PCR products were analyzed by DNA Agarose gel electrophoresis

2.6 Agarose gel electrophoresis

DNA samples were diluted with 10× loading buffer (0.25% bromophenol blue, 0.25% cyanol FF, 15% Ficoll). Samples were electrophoresed on 1% agarose gel in 1× TBE with 0.5 µg/ml EtBr. Gel electrophoresis was performed at 130V for 40 min. A photograph of the gel was taken under UV illumination.

2.7 DNA subcloning

To subclone a DNA fragment into a specific vector, the desired DNA fragment was first obtained either by PCR or restriction digestion of another plasmid containing the fragment, followed by agarose gel electrophoresis. The target DNA band was excised with a blade under UV illumination. The gel containing the DNA fragment was weighed and purified using QIAquick Gel Extraction Kit following the supplier’s protocol.

The purified DNA fragment was digested with appropriate restriction endonucleases, and the vector was linearized with the same enzymes. The digestion products were subject to agarose gel electrophoresis followed by gel purification as previously described.

Finally, ligation reaction was carried out with the digested vector and target DNA
fragment. 50 ng of linearized vector was mixed with 3-fold in molar ratio of the digested DNA fragment. 2 mg of 10× ligation buffer and 1 unit of T4 DNA ligase were added into the ligation mix, which was made up to 20 µl with Milli-Q water. The mixture was incubated in a 14°C water bath for 16 h before transformation into the bacteria.

2.8 Bacterial transformation of plasmid DNA

The ligated plasmid DNA was transformed into *E. coli* cells (TG1) to obtain a large amount of the plasmid. 100 ml of the competent cells were added to the ligation mix and incubated on ice for 30-45 min. The DNA-cell mixture was heat shocked in a 42°C water bath for 1-2 min, followed by centrifugation at 14000 rpm for 30 sec. The supernatant was discarded, and the cell pellet was resuspended with the remaining LB medium and spreaded on a LB agar plate containing appropriate antibiotics for growth selection. The plate was incubated in a 37°C oven overnight to obtain bacterial colonies.

2.9 Bacterial DNA extraction

The mini-preparation of bacteria harbored plasmids was carried out under standard procedures described in Molecular Cloning by J.Sambrook et al, 1989. The plasmids prepared from 2ml bacterial culture were resuspended in 20µl RNaseA-containing H2O to remove RNA in sample. For large scale preparation, the DNA was purified from 200ml or 500 ml LB/antibiotics culture using QIAGEN plasmid Midi or Maxi kits according to the manufacturer’s protocols.

2.10 DNA sequencing
DNA autosequencing was performed in ABI PRISM™ 3100 Genetic Analyzer (Applied Biosystems, CA, USA). For each autosequencing reaction, 200 ng of DNA to be sequenced was mixed with 4 µl Terminator Ready Reaction Mix, 2 µl of 5× BigDye® sequencing buffer, 1 µl of sequencing primers (forward and reverse), and the mix was filled up to 20 µl with Milli-Q water. Sequencing reaction was performed in the GeneAmp® PCR System 9700 (Applied Biosystems, CA, USA), using a program of 96°C for 10 sec, 50°C for 5 sec, 60°C for 4 min, repeating for 25 cycles. The mixture was then added to 130 µl absolute ethanol and 2 µl 1.5 M sodium acetate (pH 4.6) with 250 mM EDTA. The mixture was kept at -20°C for at least 3 h for precipitation. After centrifugation at 14000 rpm for 30 min, the pellet obtained was washed with 70% ethanol and air dried, and then dissolved in 12 µl HiDi formamide and denatured at 95°C for 2 min. The sample was finally loaded on ABI PRISM™ 3100 Genetic Analyzer for sequencing.

2.11 Transient transfection of 293 cells

HEK293T cells were seeded at 70% confluence in 100 mm or 60 mm culture dish for transfection. 4-7 mg of plasmid DNA was transfected using lipofatamine-plus reagents following the supplier’s instruction. The cells were harvested after 24-48 h.

2.12 Transient transfection of primary cell cultures

Cortical neurons and hippocampal neurons were seeded on coverslips in 12-well dishes at a density of 2×10⁵ per coverslip. The coverslips were transferred to a new 12-well dish containing DMEM. The conditioned medium was incubated at 37°C incubator with 5%
CO₂. The cells were washed once with DMEM and incubated in 37°C incubator with 7.5% CO₂ for 1 h. For each coverslip, 6.7 μg DNA was mixed with 250 mM CaCl₂ and filled up to 50 μl with Milli-Q water. The DNA-CaCl₂ mixture was added dropwise to 50 μl 2× HBS (42 mM HEES, 0.27 M NaCl, 10 mM KCl, 0.2% glucose, 0.29 mM Na₂HPO₄) with vortex. After 30 min, the DNA-CaCl₂-HBS mixture was added evenly to the coverslips and then incubated in 37°C incubator with 7.5% CO₂ for 30 min to form precipitate. At last, the coverslips were washed three times with DMEM and transferred to the original conditioned medium. 24 h after transfection, the cells were ready to be harvested or fixed.

2.13 Ephrin-Fc treatment
Ephrin-A1–Fc (R&D Systems) and Fc (Jackson Immunoresearch Labs) were preclustered with goat or mouse antibody to human Fc (Jackson Immunoresearch Labs) in a ratio of 1:4.5 and incubated at room temperature for 60 min before use. The final concentration for ephrin-A1–Fc or Fc was 5 mg ml⁻¹ for dissociated neurons. The neurons are put back to 37°C incubator with 7.5% CO₂ for desired time points of ephrin treatment.

2.14 Cell-surface biotinylation
24 h after transfection, HEK 293T cells or cultured neurons were washed twice in ice-cold Dulbecco's phosphate buffer and subsequently incubated on ice for 30 min with 0.5 mg/ml EZ-link-sulfo-NHS-Biotin (Pierce). Biotin was removed, and the reaction was quenched by the addition of 5 mM glycine in DPBS. The cells were washed three times in ice-cold DPBS, and then scraped in 0.1 ml of radioimmunoprecipitation assay (RIPA)
buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM sodium phosphate, 2 mM EDTA, and 0.2% sodium vanadate) supplemented with protease inhibitor mixture and solubilized for 30 mins at 4°C. Nonsolubilized material was removed by centrifugation at 16,000 \text{x} g for 10 min. Biotinylated proteins were separated from nonbiotinylated proteins by incubation with UltraLink™ Plus Immobilized Streptavidin Gel (Pierce) for 1 h at room temperature. Beads were washed 5 times with 1 ml of the lysis buffer, and adsorbed proteins were eluted with SDS sample buffer.

### 2.15 Protein preparation from cell cultures and tissues

293T cells and cultured neurons were washed with ice-cold 1× PBS once. The cells were lysed at 4°C with rotation in lysis buffer A (20mM Tris, 150mM NaCl, 1mM EDTA, 5mM NaF) that contained 0.5% Nonidet P-40 plus protease inhibitors (5mM phenylmethysulfonyl fluoride, 5mM sodium orthovanadate, 1mM leupeptin, 1mM aprotinin, 1mM antipain, 1mM trypsin inhibitor). Brain tissues were homogenized in lysis buffer B (50 mM Tris, pH 8, 150 mM NaCl, 2 mM EGTA, 1 mM dithiothreitol, 1% Nonidet P-40, 0.25% sodium deoxycholate, 50 mM NaF) with various protease inhibitors. The lysed cells or tissues were centrifuged at 14000 rpm for 15 min to remove insoluble debris. The supernatants were collected and kept at -80°C for storage.

### 2.16 Immunoprecipitation

The protein lysates (500 µg-2 mg) prepared from cell cultures or tissues were incubated with corresponding antibodies (1 µg) at 4 °C overnight with rotation and then incubated with 50 µl of protein G-Sepharose at 4 °C for 1 h. The beads were washed with 1ml
corresponding lysis buffer three times. The attached protein(s) were then eluted by adding 50µl 2× SDS sample buffer and boiling at around 95°C for 5 minutes. The IP samples together with the corresponding lysates were analyzed by SDS-PAGE and western blot.

2.17 UBA (ubiquitin-associated domain) pull down

The UBA pull down experiments were performed using the Ubiquitinated Protein Enrichment Kit (Calbiochem) following the supplier’s protocol. Briefly, the cells were lysed in lysis buffer A or RIPA supplemented with protease inhibitors and 10mM N-ethylmaleimide (de-ubiquitinase inhibitor) immediately before use. 0.5-1 mg of cell lysates were added to ~40 µl of the affinity beads, and were incubated at 4°C for 2-4 h with rotation. The beads were washed 3 times with the same lysis buffer without protease inhibitors and NEM. The attached protein(s) were eluted by adding 50µl 2× SDS sample buffer and boiling at around 95°C for 5 minutes. After centrifugation for 1 min at 14000 rpm, the UBA pull down samples together with the corresponding lysates were analyzed by SDS-PAGE and western blot.

2.18 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-polyacrylamide gels were prepared according to Laemmli et al. (1970). Samples were loaded into the wells of the stacking gel and electrophoresed in running buffer (25mM Tris-HCl, pH 8.3, 192mM glycine, 0.1% SDS) at 20mA per gel for 2.5 h. The molecular sizes of the proteins were estimated by running the prestained molecular weight markers alongside the samples.
2.19 Western blot analysis

After SDS-PAGE, proteins on polyacrylamide gel were transferred onto the nitrocellulose membrane in 1× transfer buffer using Trans-Blot electrophoric transfer cell (BioRad, CA). The membrane was stained with Ponceau S solution (Sigma, MO) to visualize the proteins present on the membrane. Ponceau S solution was then washed away from the membrane by 1× Tris-Buffered saline with 0.1% Tween-20 (TBS-T). The membrane was blocked with 5% non-fat dry milk in TBS-T at room temperature for 1 h. Excess milk on the membrane was then washed away by TBS-T and incubated with primary antibody at 4°C overnight. The membrane was then incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. The signals were then developed using the Supersignal West Pico Chemiluminscent substrate system (Pierce).

2.20 Immunocytochemical analysis

Cultured hippocampal neurons were washed with PBS (Ca²⁺/Mg²⁺) twice and fixed with 4% PFA in PBS on ice for 20 min. The cells were blocked and permeabilized with 0.1% Triton X-100, 4% goat serum and 1% BSA for 20 min, and then incubated with specific primary antibodies at 4°C overnight. The next day, the cells were incubated with corresponding fluorescence conjugated secondary antibodies at room temperature for 1 h, followed by washing with PBS for three times, and were finally mounted with Mowiol anti-fade reagent. The cells were observed and photographed under confocal fluorescent microscope. The spine density was quantified using Metamorph software.
Chapter 3 Results

3.1 EphA4 interacts with APC2

In order to further study the functional roles of EphA4 at the synapse, a yeast two-hybrid screening was performed previously in our lab using the cytoplasmic domain of EphA4 as the bait to screen from a P12 mouse muscle cDNA library. One of the positive clones obtained was a truncated isoform of APC2 (Anaphase promoting complex subunit 2) (Hung & Ip, unpublished observation). The full length APC2 did not interact with EphA4 in the yeast. However, co-immunoprecipitation in HEK 293T cells showed that they do associate with each other in mammalian cells (Fig 3.1).

To check whether the association is physiological, we examined the interaction between EphA4 and APC2 in the rat brain. Since EphA4 is a receptor on the cell membrane, it is not surprising that it is predominantly detected in the membrane fraction, with very little in the cytosolic or the nuclear fractions as revealed in fractionation analysis (Hung & Ip, unpublished observation). On the other hand, APC2 is present abundantly in all three locations. Therefore we performed co-immunoprecipitation using P9 rat brain membrane fraction, and found that EphA4 could be co-immunoprecipitated with APC2, suggesting an in vivo association between the two proteins (Fig 3.2).

3.2 Cdh1 regulates EphA4 expression in mammalian cells

Because APC2 is one of the catalytic core subunit of the APC (Acquaviva, 2006), the interaction between EphA4 and APC2 raises the possibility that EphA4 might be a
Figure 3.1 EphA4 interacts with APC2 in mammalian cells. HEK293T cells were transfected with EphA4 and APC2 as indicated. Cell lysates were immunoprecipitated by EphA4 antibody, and immunoblotted with EphA4 and APC2 (home made, anti-C-term) antibodies, respectively.

Figure 3.2 EphA4 interacts with APC2 in the brain. P9 rat brain membrane fraction was immunoprecipitated by APC2 antibody and immunoblotted with EphA4 and APC2 antibodies.
substrate of the APC. The activity of the APC usually requires one of its activators that recognizes and brings specific sets of protein substrates to the APC core for the ubiquitination reaction (Castro A, 2005). Since Cdh1 seems to be the only activator expressed in neurons (Peters, 1999), and several studies have described novel functions of APC$^{\text{Cdh1}}$ in the brain (Roessel et al., 2004; Konishi et al., 2004; Almeida et al., 2005; Stegmuller et al., 2006; Lasorella et al., 2006), we examined the role of Cdh1 in regulating the expression of EphA4 in a hope to uncover the relationship between the APC and EphA4.

### 3.2.1 Cdh1 interacts with EphA4 in mammalian cells

APC activators are thought to mediate substrate recognition (Castro, 2005), so we first checked whether EphA4 interacts with the neuronal specific activator, Cdh1. Co-immunoprecipitation in HEK 293T cells showed that they interacted with each other in mammalian cells (fig 3.3&3.4). This confirms our hypothesis that EphA4 is a substrate of the APC.

### 3.2.2 Cdh1 down-regulates EphA4 expression in mammalian cells

If EphA4 could be ubiquitinated by APC$^{\text{Cdh1}}$, then the overloading of Cdh1 should accelerate the ubiquitination of EphA4 and in turn its degradation. Indeed, the expression of EphA4 was greatly down-regulated when co-expressed with Cdh1 in HEK 293T cells (fig 3.5). Further more, this down-regulation is dosage-dependent.

Since EphA4 is a cell membrane receptor, it is sensible to investigate the expression of
Figure 3.3&3.4 EphA4 interacts with Cdh1 in mammalian cells. HEK 293T cells were transfected with EphA4 together with Cdh1 as indicated. Cell lysates were co-immunoprecipitated with EphA4 or anti-HA antibodies and immunoblotted with antibodies as shown.
EphA4 on the cell surface apart from its total expression level. We performed biotinylation labeling assay to track the surface expression of EphA4, which turned out to be dramatically down-regulated in a similar manner as the total EphA4 expression (fig 3.6). This indicates that Cdh1 not only accelerates the degradation of EphA4, but also speeds up its internalization from the cell membrane.

3.2.3 The down-regulation of EphA4 expression is dependent on the substrate binding domain of Cdh1

The conserved WD40 domains in APC activators are the putative substrate binding domain implied by comparison between the structures of the SCF and APC (Castro, 2005). To confirm the down-regulation effect of Cdh1, we constructed a deletion mutant of the Cdh1 lacking the WD40 domain, and performed similar co-immunoprecipitation and biotinylation experiments as in section 3.2.2. In the absence of the WD40 domain, the interaction between Cdh1 and EphA4 is no longer detectable (fig 3.7). Furthermore, the decrease in the surface as well as the total expression of EphA4 stimulated by Cdh1 is largely abandoned by the deletion of the WD40 domain (fig 3.8). These results suggest that the WD40 domain is required for the interaction as well as the down-regulation of EphA4 by Cdh1, consistent with the hypothesis that WD40 domains mediate substrate binding.
Figure 3.5 Cdh1 down-regulates the EphA4 protein expression in a dosage-dependent manner. EphA4 together with increasing amounts of Cdh1 were transfected into HEK 293T cells. Total cell lysates were collected and subjected to Western blot analysis using EphA4 and Cdh1 antibodies.

Figure 3.6 Cdh1 down-regulates the surface expression of EphA4 in mammalian cells. EphA4 was co-transfected with Cdh1 into HEK 293T cells. The cells were biotin-labeled and lysed. Cell lysates were then collected and subjected to pull-down with streptavidin-conjugated agarose beads, followed by immunoblotting with EphA4 antibody. Western blot of unbound fractions for α-tubulin reveals equal input of the cell lysates for the pull down reaction.
Figure 3.7 The interaction between EphA4 and Cdh1 is dependent on the substrate binding domain of Cdh1. EphA4 was co-transfected with Cdh1 or its mutant lacking the substrate binding domain (WD40 domain) into HEK 293T cells. Cell lysates were co-immunoprecipitated with anti-HA antibody and immunoblotted with antibodies as shown.

Figure 3.8 The down-regulation of EphA4 expression is dependent on the substrate binding domain of Cdh1. EphA4 was co-transfected together with Cdh1 or Cdh1ΔWD40 into HEK 293T cells. The cells were subjected to biotinylation pull down assay as in Fig 3.6, followed by immunoblotting with EphA4 antibody.
3.2.4 The down-regulation of EphA4 expression is independent of the kinase activity of EphA4

The initiation of downstream signaling events of the Eph receptors generally requires the activity of the kinase domain, so we would like to see whether its own regulation by Cdh1 is kinase-dependent. We used the kinase dead (KD) mutant of EphA4 to perform similar co-immunoprecipitaion and biotinylation experiments in HEK 293T cells as in previous sections. The results showed that loss of the kinase activity affected neither the interaction with Cdh1 (fig 3.9) nor its down-regulation by Cdh1 (fig 3.10), indicating that at least in vitro, these events happen in a kinase-independent manner.

3.2.5 The interaction between EphA4 and Cdh1 is D-box independent

Most APC substrates contain conserved sequence motifs that are recognized by the APC activators and required for their ubiquitination. The most widespread motif is the destruction box (D-box) with the general sequence pattern RXXLXXXXN (where R is arginine, L is leucine, N is asparagine, and X is any amino acid) (Stegmuller, 2006).

By manually scanning through the protein sequence of EphA4, we identified two D-box like sequences: RDFL (665-668 a.a.) and RNIL (750-753 a.a.). To examine whether the interaction with Cdh1 is D-box directed, we mutated both possible D-boxes in EphA4 by changing R to L, and performed co- immunoprecipitaion experiment with Cdh1 (fig 3.11). We found the interaction unaffected by the mutations, indicating that the D-box-like sequences in EphA4 are not the target for Cdh1 recognition. It is not totally surprising because not all APC substrates depend on the D-box for destruction. There are several
target sequences for the APC other than the D-box, and probably even more remain yet to be identified. Possibly it is one of those other sequences that are recognized by Cdh1 in EphA4.

![Table and images](image)

Figure 3.9 The interaction between EphA4 and Cdh1 is independent of the kinase activity of EphA4. HEK293T cells were transfected with EphA4 or its kinase dead mutant together with Cdh1 as indicated. Cell lysates were immunoprecipitated by EphA4 antibody, and immunoblotted with EphA4 and Cdh1 antibodies, respectively.
Figure 3.10 The down-regulation of EphA4 expression by Cdh1 is independent of the kinase activity of EphA4. EphA4 or its kinase dead mutant were co-transfected together with Cdh1 into HEK 293T cells. The cells were subjected to biotinylation pull down assay as in Fig 3.6, followed by immunoblotting with EphA4 antibody.

<table>
<thead>
<tr>
<th></th>
<th>Biotin</th>
<th></th>
<th>Lysate</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>EphA4</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KD</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cdh1</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Figure 3.11 The interaction between EphA4 and Cdh1 is D-box independent. EphA4 or its D-box double mutant was co-transfected with into HEK 293T cells. Cell lysates were co-immunoprecipitated with EphA4 antibody and immunoblotted with antibodies as shown.

|        | EphA4 |  | IgG |  | Lysate |  |
|--------|-------|--------|----|--------|--------|
| EphA4  | -     | +      | -  | -      | +      |
| Dbox Mut| -     | -      | +  | +      | -      |
| Cdh1   | +     | +      | +  | +      | +      |

- α-tubulin

- Cdh1

- EphA4
3.3 Ephrin-A1 stimulates proteasome-dependent degradation of EphA4

3.3.1 The expression of EphA4, APC2 and Cdh1 are developmentally regulated

Having examined the regulation of EphA4 by APC<sup>Cdh1</sup> in vitro, we moved on to explore the situation in neurons. The first issue to consider is the choice of developmental stage to perform the experiments. So we checked the expression patterns of EphA4, APC2 and Cdh1 in cortical neurons by western blot analysis (Fig 3.12). Consistent with previous observations, the expression of EphA4 was slightly increased along development. The expression of APC2 and Cdh1 decreased as the neurons differentiate and deviate further away from cell cycle, but they still maintained a significant expression level at later stages, consistent with the notion that they might have functions in postmitotic neurons. Based on the above observations, we chose 14 DIV neurons to perform the experiments in this section, because all three proteins are expressed abundantly at that stage. Also, the neurons are relatively mature, so the manipulation of the APC apparatus wouldn’t have any cell cycle related effect.

3.3.2 EphA4 interacts with APC2 after Ephrin-A1 treatment in cortical neurons

First of all we examined the interaction between EphA4 and APC2 in neurons. 14 DIV cortical neurons treated with ephrin-A1, one of EphA4’s ligands, or Fc as control. Cell extracts from both treated and untreated neurons were subjected to immunoprecipitaion with APC2 antibody. No association was observed under basal conditions; However, after ephrin-A1 treatment, an endogenous association quickly appeared within 5 minutes, and grew stronger after 1 hr (fig. 3.13). This suggests that the recruitment of APC2 to EphA4 requires ligand stimulation.
Figure 3.12 The expression of EphA4, APC2 and Cdh1 are developmentally regulated in cortical neurons. Total cell lysates from cultured cortical neurons at 4, 7, 14, 21 DIV are collected and subjected to western blot analysis using antibodies as indicated.

Figure 3.13 EphA4 interacts with APC2 in cortical neurons upon ephrin-A1 treatment. Cortical neurons at 14 DIV were treated with clustered ephrin-A1 for the indicated time points. Cell lysates were immunoprecipitated with APC2 antibody, followed by immunoblotting with antibodies as shown.
3.3.3 Ephrin-A1 enhances the ubiquitination of EphA4 in hippocampal neurons

EphA receptors, EphA2 and EphA3 were shown to be ubiquitinated by a E3 ubiquitin ligase, c-Cbl upon ligand stimulation (Sharfe et al., 2003; Walker-Daniels et al., 2002; Wang et al., 2002). Since APC is also an E3 ubiquitin ligase, we wondered if the recruitment of APC2 to EphA4 induced by ephrin-A1 could lead to its ubiquitination. To examine the ubiquitination of EphA4, we applied the ubiquitinated protein enrichment kit (Calbiochem) which utilizes the ubiquitin-associated domains to pull down polyubiquitinated proteins from cell and tissue lysates (Fig 3.14). The ubiquitin-associated domains are common to a subset of proteins that are involved in ubiquitination/deubiquitination reactions or shuttling factors that translocate the ubiquitinated proteins to the 26S proteasome for degradation (Hicke, 2005).

In order to compare the level of ubiquitination before and after ephrin-A1 treatment, the neurons (hippocampal, 15 DIV) were pre-treated with lactacystin, a proteasome inhibitor, to prevent its possible degradation by the proteasome after ubiquitination. We showed that EphA4 was present in the UbA pull down fraction, indicating its ubiquitination (Fig 3.14 upper panel). The smears above EphA4 bands in the pull down fraction visualized by EphA4 antibody were likely to be poly-ubiquitinated EphA4. The amount of ubiquitinated EphA4 appeared to be increased after ephrin-A1 treatment compared with the Fc treated control, indicating that ephrin-A1 stimulates the ubiquitination of EphA4 (upper panel). In this case ephrin-A1 treatment should also lead to decreased EphA4 protein level under normal conditions. However, since the neurons are pre-treated with lactacystin to prevent proteasomal degradation, the down-regulation effect should be less
Figure 3.14 Ephrin-A1 enhances the ubiquitination of EphA4 in hippocampal neurons. Hippocampal neurons at 15 DIV were pre-treated with lactacystin for 1 hr before treated with clustered ephrin-A1 for 30 min. Cell lysates were subjected to pull down by UBA (ubiquitin associated domain) using Ubiquitinated protein enrichment kit (Calbiochem), followed by immunoblotting with EphA4 (upper and middle panels) and ubiquitin (lower panel) antibodies.
obvious. This assumption is consistent with our observation that the EphA4 protein level was only slightly decreased after ephrin-A1 treatment as revealed by EphA4 antibody (middle panel). The lower panel shows the amount of total ubiquitinated proteins in the pull down fraction which seems to be similar, indicating that the ephrin-A1 stimulated ubiquitination is specific to EphA4.

3.3.4 Surface expression of EphA4 is decreased in cortical neurons after ephrin-A1 treatment

Given that ephrin-A1 promotes both the recruitment of APC2 to EphA4 and its ubiquitination, we asked if the protein level of EphA4 is also responsive to ephrin-A1 treatment. There have been several publications reporting that both EphA and EphB receptors undergo endocytosis following ligand activation (Zimmer, 2003; Marston, 2003; Parker, 2004; Cowan, 2005). Therefore we investigated if ephrin-A1 would trigger the down-regulation of the surface expression of EphA4 using biotinylation labeling (Fig 3.15). We showed that after 24 hrs of ephrin-A1 treatment, the surface expression of EphA4 in cortical neurons (14 DIV) reduced dramatically, so did the total EphA4 expression, but to a lesser extent. This result supports the endocytosis model, and also implies that EphA4 is degraded after internalization.

3.3.5 Lactacystin treatment attenuates the ephrin-A1 mediated down-regulation of surface EphA4 in neurons

To find out whether the down-regulation stimulated by ephrin-A1 is attributable to proteasomal degradation, we performed similar experiments as in Fig 3.15, except that
Figure 3.15 Surface expression of EphA4 is decreased in cortical neurons after ephrin-A1 treatment. Cortical neurons at 14 DIV were treated with clustered ephrin-A1 for 24 hours. The neurons were subjected to biotinylation pull-down analysis as in Fig. 3.6, followed by immunoblotting to EphA4.

Figure 3.16 Treatment of a proteasome inhibitor lactacystin attenuated the ephrin-A1 mediated-down-regulation of surface EphA4 in cortical neurons.
Cortical neurons at 14 DIV were pre-treated with lactacystin or its vehicle DMSO for 1hr, followed by treatment with clustered ephrin-A1 for 30min. Biotinylation pull-down assay was performed to examine the surface expression of EphA4 (top panel). Expression of EphA4 in total lysates was depicted in the lower panels. Western blot for α-tubulin served as equal input control (unbound) and equal loading control (bottom panel).

Figure 3.17 Treatment of a proteasome inhibitor lactacystin attenuated the ephrin-A1 mediated-down-regulation of surface EphA4 in hippocampal neurons. The same experiment as in Fig.3.17 was done to hippocampal neurons at 15 DIV.
the neurons were pre-treated with lactacystin. Indeed, the surface expressions of EphA4 were greatly rescued by lactacystin treatment in both cortical (Fig 3.16) and hippocampal (Fig 3.17) neurons compared with the DMSO treated controls. Ephrin-A1 can no longer trigger the down-regulation of surface EphA4 when proteasomal degradation is blocked, suggesting that the 26S proteasome is involved in the degradation of EphA4 induced by Ephrin-A1.

Taken together, the above results suggest that ephrin-A1 triggers the APC-mediated proteasomal degradation of EphA4 in neurons, leading to its down-regulation on the cell surface.

3.4 Cdh1 regulates dentritic spine density in hippocampal neurons

In previous sections, we demonstrated the expression of EphA4 is regulated by APC^{Cdh1}-mediated proteasomal degradation pathway. The next question is the physiological significance of this regulation. Eph receptors are reported to regulate the structural and functional properties of synapses by regulating the formation and maintenance of dendritic spines. EphB receptors are required for spine morphogenesis (Henkemeyer et al., 2003; Ethell et al., 2001; Penzes et al., 2001, 2003), while EphA4 may play a role in more mature neurons to regulate spine density (Murai et al., 2003; Fu, Chen & Ip et al., 2007). Therefore, we explored the effect of Cdh1 on dentritic spine density in late stage hippocampal neurons. (* The experiments in this section were only performed once, so the results are only preliminary.*)
3.4.1 Knockdown of Cdh1 results in reduced dendritic spine density in hippocampal neurons

Hippocampal neurons were transfected with pSUPER vector or pSUPER-Cdh1 RNAi together with eGFP (for visualization of the cell morphology and the spines) at 17 DIV. Two days after transfection, the neurons were treated with pre-clustered ephrin-A1 or Fc as control for 24 hrs. Then the cells were fixed and photographed under confocal fluorescence microscope. The images taken were quantified for the dendritic spine densities.

Under normal conditions, ephrin-A1 would stimulate spine retraction leading to a drop around 10-20% in spine density (Murai et al., 2003; Fu, Chen & Ip et al., 2007). This is consistent with our observation when the neurons were transfected with the control vector (Fig 3.19). After Cdh1 knockdown, both Fc and ephrin-A1 treated neurons showed a dramatic reduction in spine density (Fig 3.18&19). However, no further reduction is detected after ephrin-A1 treatment. In conclusion, knockdown of Cdh1 caused a decrease in dendritic spine density under basal conditions which is no longer responsive to ephrin-A1 stimulation.

3.4.2 Overexpression of Cdh1 abolishes ephrin-A1 mediated dendritic spine retraction in hippocampal neurons

The other direction to investigate the effect of Cdh1 on spine behavior is to overexpress Cdh1. Therefore similar experiments were conducted as in the previous section with
replacement of pSUPER-Cdh1 and pSUPER vector by pcDNA3-Cdh1 and pcDNA3 vector for the transfection (Fig 3.20).

As usual, the neurons transfected with vector showed a reduction in spine density after ephrin-A1 treatment. After overexpression of Cdh1, the spine density was similar for the Fc control neurons; However, ephrin-A1 treatment did not result in significant decrease compared with the control neurons. In conclusion, the overexpression of Cdh1 had no effect on basal spine density, while appeared to abolish the decrease induced by ephrin-A1.

Taken together, the results in this section suggest that Cdh1 is involved in the regulation of dendritic spine density in mature hippocampal neurons. However, how it is related to the EphA4 mediated spine retraction remains unclear and needs to be further investigated.
Figure 3.18 Representative confocal images of neurons transfected with pSUPER vector (left panels) or pSUPER-Cdh1 RNAi (right panels). A segment of dendrite from each neuron is enlarged to illustrate the difference in spine density (lower panels).
Figure 3.19  Quantitative analysis of spines in neurons before or after transfection with pSUPER-Cdh1 RNAi or pSUPER vector and ephrin-A1 stimulation. Data is presented as the number of spines (protrusions) per 10 μm.
Figure 3. Quantitative analysis of spines in neurons before or after transfection with pcDNA3-Cdh1 or pcDNA3 vector and ephrin-A1 stimulation. Data is presented as the number of spines (protrusions) per 10 μm.
Chapter 4 Discussion

4.1 Interaction of EphA4 with APC2

The exact mechanism by which the APC recognize its substrates in a spatial and temporal specific manner is unclear. The activators are believed to be important for these events. However, substrate recognition by the APC is probably not dependent only on Cdc20 and Cdh1. During the embryonic *Drosophila* cell cycle, no Cdh1 is expressed but degradation of specific substrates is still temporally regulated. Moreover, substrates of Cdc20 or Cdh1 do not all disappear at the same time in mitosis – for example, APC-Cdc20 ubiquitinates cyclin A in prometaphase before cyclin B in metaphase (Castro, 2005). Other subunits of the APC might therefore be involved in substrate selection. Indeed, the cyclin B D-Box motif and the APC directly interact in *Xenopus* egg extracts (Yamano et al., 2004) and in *S. pombe* (Meyn et al., 2002). Substrates might thus cooperatively bind to Cdc20 or Cdh1 and the APC itself. So far the only core subunit that is described to mediate substrate recognition is APC10. Binding of Cdh1 with APC substrates is prevented in APC complexes purified from APC10 mutants, indicating that APC10 mediates substrate binding to Cdh1 and to the APC either direct or indirectly (Passmore et al., 2003).

Though no interaction between substrates and APC2 is reported, the possibility of APC2 to possess substrate-binding ability is not excluded. APC2 and APC11 form the enzymatic core of the complex. They harbour a cullin and a RING-finger domain, respectively. By analogy to the structure of the SCF, APC2 and APC11 should form a two-subunit catalytic core that binds the E2. *In vitro*, purified APC2/APC11 complex is
sufficient to catalyse ubiquitination in the presence of the UbcH10 enzyme (E2) (Castro, 2005). Although this reaction possesses little substrate specificity, it is likely for the core subunits to have certain kind of interaction with the substrates in order for the ubiquitination reaction to occur. As the starting point of this study, the interaction of EphA4 with APC2 could therefore hold a clue on its possible relationship with the APC as a whole.

Theoretically, co-expression of an ubiquitin ligase with its substrate would result in down-regulation of the substrate. For example, overexpression of Cbl in 293T cells causes a dose-dependent decrease in the level of EphA2 (Wang et al., 2002). In order to explore the possibility of EphA4 being a substate of the APC, we overexpressed APC2 together with EphA4 in HEK 293T cells, but no decrease in surface or total EphA4 expression was observed (data not shown). One explanation is that unlike the single-subunit E3 ligases such as Cbl and parkin, APC is a multi-subunit enzyme. Perfusion of one subunit out of the complex is not effective to promote the ubiquitination reaction. That’s why we turned our focus to Cdh1 which can enhance APC activity relatively independently on the other subunits.

4.2 Regulation of EphA4 by Cdh1
Cdh1 is the only APC activator that is expressed prominently in postmitotic neurons (Gieffers et al., 1999). Emerging studies have uncovered novel functions of the APC in the brain unrelated to the cell cycle mediated by Cdh1, including regulation of axonal growth and patterning (Bonnie et al., 2004&2006), neuronal survival (Almeida et al.,
2005), and synaptic development and function (Juo and Kaplan et al., 2004; Roessel et al., 2004). In this study, we demonstrated that Cdh1 interacts with EphA4 and regulates its expression in vitro.

The exact mechanism through which APC activators enhance APC activity is not known. One possibility is that they function as adaptors that bring the substrates close to the APC core by concomitantly interacting with the core subunits and specific substrates. On the one hand, their ability to stimulate in vitro ubiquitination of substrates is dependent on their own association to the APC. This interaction seems to be mediated through direct binding of the TPR subunits APC and APC7 with the IR C-terminal tails of Cdc20 and Cdh1, as well as the C-Box at the N-Terminus. On the other hand, they both contain a conserved WD40 domain, which mediates substrate binding in a subset of F-box subunits of the SCF. Comparison between structures of the SCF and APC suggests a putative role of these activators in substrate recognition. Indeed, several studies have shown that different APC substrates can directly and specifically bind to Cdc20 or Cdh1 (Castro, 2005). Our finding that Cdh1 interacts with EphA4 in a WD40 domain dependent manner is consistent with this model, supporting our hypothesis that EphA4 is a substrate of the APC<sup>Cdh1</sup>. Also, the down-regulation effect of Cdh1 on EphA4 can be explained by the adaptor model. While overexpression of one single subunit (APC2) fails to alter EphA4 expression, presence of extra activators (Cdh1) provides more chances for EphA4 to contact with the APC core, leading to boosted ubiquitination and in turn degradation. Naturally, this down-regulation depends on the interaction between Cdh1 and EphA4, and therefore also relies on the WD40 domain.
Another element thought to be important in activator-substrate interaction is the so called “degradation motifs” present in the substrates that are specifically recognized by Cdc20 and Cdh1. The first described degradation motif is the destruction box (D-box) with sequence pattern RXXLXXXXN (where R is arginine, L is Leucine, N is asparagines, and X is any amino acid) (Stegmuller, 2006). It is to date the most widespread degradation motif found in APC substrates. Another important motif named KEN box (KENXXRN, where K is lysine and E is glutamate) is recognized by Cdh1, but not Cdc20. Interestingly, Cdc20 also contains a KEN box and is itself an APC<sup>Cdh1</sup> substrate. By manually scanning through the protein sequence of EphA4, we identified two D-box like sequences: R<sub>DFL</sub> (665-668 a.a.) and R<sub>NIL</sub> (750-753 a.a.) but no KENbox. However, double mutation of the two D-boxes didn’t affect the association between EphA4 and Cdh1. It can be argued that other sequence motifs might be present in EphA4 to direct its recognition by Cdh1. In fact, several novel degradation motifs have been described in recent studies, such as the “GxENbox” and “Abox” (Castro, 2005). There are probably even more remain unidentified. Moreover, the conserved sequence motifs alone are generally not sufficient to promote APC-dependent destruction, and there could be other unknown features that allow the recognition of a target protein by the APC. Hence the D-box independent interaction between EphA4 and Cdh1 does not deny their correlation.

As an RTK, Eph receptors rely on their kinase activity to mediate down-streaming signaling events. Therefore, it is a little unexpected to find that the interaction and regulation of EphA4 by Cdh1 is kinase independent. Although the in vivo situation could be very different from the in vitro experiments we performed, the kinase activity of
EphA4 is unlikely to be crucial even in neurons because the kinase dead mutant failed to cause any significant effect on the down-regulation by Cdh1 what so ever. This can only mean that the regulation of the EphA4 itself takes place independently from the other signaling events it initiates. Obviously the down-regulation is not constitutive and requires ligand activation as shown in section 3.2. The problem is how it is dependent on ligand engagement only but not on the kinase activity. One possibility is that it only requires clustering of EphA4 but not kinase activation. Contact with ephrin ligands in neighboring cells initiates the clustering of EphA4 and thus conformational changes to the intracellular domain, which may expose certain region that interacts with APC$^{\text{Cdh1}}$. Another explanation involves compartmentalization. Both the recruitment of APC$^{\text{Cdh1}}$ to EphA4 and its endocytosis require ephrin stimulation, but it is uncertain which event happens first. It is feasible that EphA4 is first endocytosed upon ligand activation through some unknown mechanism into the cytoplasm where it contacts the APC$^{\text{Cdh1}}$ subsequently, so that any part of the EphA4 could be the site of contact with Cdh1, even the extracellular region. That’s why the kinase dead mutant overexpressed in vitro didn’t affect the interaction or down-regulation.

4.3 Developmental regulation of EphA4, APC2 and Cdh1

The temporal expression pattern of EphA4, APC2 and Cdh1 shown in Fig 3.12 is consistent with previous findings and their reported functions.

EphA4 and other Eph receptors together with their ephrin ligands are involved in many important steps throughout the development of the central nervous system, including cell
migration, axon guidance, topographic mapping and synapse formation. In the adult, they have also been implicated in plasticity and regulation of synaptic function (Klein, 2006). Therefore it is not surprising to find that EphA4 is expressed prominently at all examined stages in cortical neurons.

Interestingly, although the neurons have long exited mitosis and are fully differentiated after DIV 14, components of the APC like APC2 and Cdh1 still maintained a high expression level. In fact, it has been demonstrated that core subunits of APC and activator Cdh1 (but not Cdc20) are ubiquitously expressed in the nuclei of terminally differentiated neurons. It was also shown that the APC purified from brain contains all core subunits known from proliferating cells and is tightly associated with Cdh1. Further more, purified brain APC\textsuperscript{Cdh1} has a high ubiquitin ligase activity. On the basis of these results, it was profited that the functions of APC\textsuperscript{Cdh1} are not restricted to controlling cell-cycle progression but may include the ubiquitination of yet unidentified substrates in differentiated cells (Gieffers et al., 1999). This was proven in several later studies that defined novel functions for the APC\textsuperscript{Cdh1} in postmitotic neurons, including regulation of axonal growth and patterning (Bonni et al., 2004&2006), neuronal survival (Almeida et al., 2005), as well as synaptic development and function (Juo and Kaplan et al., 2004; Roessel et al., 2004).

4.4 Ubiquitination of EphA4

Ligand induced receptor ubiquitination and endocytosis has been widely reported for RTKs. These proteins are often constitutively internalized at a slow rate. Upon ligand
binding, the receptors are ubiquitinated and endocytosed to downregulate signaling. This irreversible mode of signal attenuation is critical for the appropriate extent and duration of signaling. After internalization, subsequent sorting steps determine whether the receptor is destined for degradation or recycled to the cell surface. The versatile E3 ubiquitin ligase Cbl is implicated in the ubiquitination of several RTKs, including receptors for EGF, platelet-derived growth factor (PDGF), colony-stimulating factor (CSF)-1 and HGF. Ligand-activated receptors autophosphorylate a specific tyrosine residue in their cytoplasmic tail, which is in turn recognized by the tyrosine kinase-binding (TKB) domain of Cbl. Cbl proteins contain a RING finger domain, which binds the E2-conjugating enzyme UbcH7 and mediates ubiquitin ligase activity (Marmor, 2004; d'Azio, 2005).

Cbl may not be the only E3 ubiquitin ligase capable of mediating RTK ubiquitination, and other E3 family ligases may function analogously. A recent report characterized the ligand-induced, Grb10-mediated coupling of the insulin-like growth factor receptor to Nedd4 (Vecchione et al., 2003). Another study found that Trk receptors are differentially ubiquitinated and degraded after binding to neurotrophins. Nedd4-2 binds specifically to the C-terminus of the TrkA receptor and induces the down-regulation of the receptor. The site of Nedd4-2 association is not conserved in TrkB and TrkC receptors. As a result, expression of Nedd4-2 affects the survival of NGF-dependent, but not BDNF-dependent neurons (Arevalo et al., 2006).

Ephrin-A1 stimulation was found to cause down-regulation of EphA2 and EphA3 from the cell surface and their degradation in T cells and tumor cells (Walker-Daniels et al.,
2003; Wang et al., 2002; Sharfe et al., 2003). c-Cbl is thought to mediate their degradation because ligand binding stimulates the association of the EphA receptors with c-Cbl and its phosphorylation. Furthermore, overexpression of c-Cbl leads to the down-regulation of the EphA2 and EphA3 receptors in vitro. In order to be degraded or endocytosed, the receptors need to be ubiquitinated by the E3 first. However, these studies did not investigate the ubiquitination status of the corresponding EphA receptors.

In the present study, we propose a similar regulation mechanism for EphA4 in neurons. We showed that Ephrin-A1 treatment triggers the internalization and down-regulation of EphA4. APC, another RING finger E3, is likely to mediate this down-regulation because ephrin-A1 stimulates the recruitment of APC2 to EphA4 in cortical neurons. Furthermore, we also investigated the ubiquitination of EphA4 induced by ligand binding. Previous studies in our lab showed that EphA4 is ubiquitinated in vitro (Hung & Ip, unpublished observation). In this study, we further demonstrated that not only is it ubiquitinated in neurons, but also that its ubiquitination is greatly enhanced by ligand stimulation (Fig 3.14). However, the specific type of this ubiquitination is not thoroughly investigated.

The different types of substrate ubiquitination are schematically represented in Fig 4.1. A protein substrate can be conjugated with one ubiquitin moiety at a certain lysine residue (monoubiquitination), or several single ubiquitin moieties at different lysine residues (multiubiquitination), or a single linked ubiquitin chain (polyubiquitination). Lysine 48 (K48)-linked polyubiquitination, composed of a minimal unit of four ubiquitin moieties,
targets proteins for degradation by the 26S proteasome, and occurs mainly on cytosolic and nuclear proteins. In addition, K48-linked polyubiquitination is implicated in the endoplasmic reticulum-associated degradation (ERAD) pathway, a quality control mechanism that targets misfolded proteins for retrotranslocation from the ER and proteasomal degradation. In contrast, chains linked through K29 and K63 mediate other, nonproteasomal cellular functions. Mono- and multi-ubiquitination play a role in the regulation of receptor endocytosis and targeting for lysosomal degradation, in addition to histone activity and DNA repair (Marmor, 2004).

Figure 4.1 Different types of protein ubiquitination (Marmor, 2004)

Most of the studied cases of membrane protein ubiquitination are monoubiquitination, which aids in their regulated internalization and sorting to the endocytic compartment, as well as the subsequent recycling or lysosomal degradation. The polyubiquitination of membrane proteins are rarely reported. However, because most known APC substrates are polyubiquitinatated and degraded by the 26 proteasome, we hereby try to demonstrate that EphA4 is actually polyubiquitinated. The evidence is shown in Fig 3.14. While the presence of an Ub-positive ladder for many membrane receptors visualized by Western
blotting has been interpreted as polyubiquitination, it could also be due to multiubiquitination at several different sites. However, this is unlikely the case in our study because the *ubiquitinated protein enrichment kit* (*Calbiochem*) applied in Fig 3.14 can pull down only polyubiquitinated proteins (*Calbiochem* cat.No. 662200 user protocol). This is because the UBA (ubiquitin-associated) domain has a much higher affinity and thus lower Kd (~100 fold) for polyubiquitin chain than for monoubiquitin moieties (Hicke, 2005).

### 4.5 Proteasomal degradation of EphA4

After showing that EphA4 is polyubiquitinated, we went on to prove that EphA4 is degraded by the proteasome. We demonstrated that proteasome inhibitor greatly attenuated the down-regulation of surface EphA4, indicating that the degradation of EphA4 depends on the proteasome (Fig 3.17&18). Further more, previous studies in our lab demonstrated that EphA4 physically associates with the 26S proteasome in neurons, and the association is enhanced by ligand stimulation in a time dependent manner (Hung & Ip, unpublished observation).

As mentioned previously, most membrane proteins are monoubiquitinated and degraded through the lysosome pathway. The role of the proteasome in the degradation of ubiquitinated membrane proteins remains debatable. The major evidence comes from biochemical inhibition of the proteasome which does block the internalization or degradation (or both) of several membrane proteins (d'Azzo, 2005). Although proteasome inhibitors like lactacystin, MG132 and ALLN may also inhibit the activity of some
lysosomal enzymes, the fact that they preferentially inhibit the degradation of certain membrane proteins suggests that both degradation pathways collaborate in the turnover of membrane proteins. A model supporting this hypothesis has been proposed to explain the proteasome-dependent regulation of the erythropoietin receptor (Walrafen et al., 2005). After monoubiquitination and internalization of the receptor, its intracellular portion is cleaved or processed by the proteasome (or both), while the remaining part travels to the lysosome through the MVB. This scheme would entail the sequential and selective action of one or more E3s that modify the initial Ub moiety on the cargo and hence determine its intracellular route. Finally, the stability of transmembrane proteins may depend on their regulated interaction with intracellular partners (e.g. kinases and cytoskeletal components) that may themselves be targeted for proteasomal degradation. In these instances, the effect of proteasome inhibitors on the stability of transmembrane proteins could be due to the indirect stabilization of their interacting partners (d'Azzo, 2005).

The regulation on EphA4 by the proteasome is most likely direct because EphA4 associates with the E3 ligase and the 26S proteasome itself. However, our study could not exclude the possibility of lysosomal degradation of EphA4 because we didn’t examine the effect of any lysosome inhibitor. The two pathways are not exclusive and probably collaborate with each other to regulate surface expression of EphA4 like other membrane proteins.

### 4.6 Possible model for APC<sup>Cdh1</sup>-mediated proteasomal degradation of EphA4
Based on the current findings and above discussion, we propose a model that roughly depicts the process of $\text{APC}^\text{Cdh1}$-mediated endocytosis and proteasomal degradation of EphA4 (Fig 4.2). Without ephrin ligands, EphA4 is dispersed on the cell membrane as single, inactive receptors. They are endocytosed and recycled at a slow rate and kept at a constant density on the cell surface. Upon contact with clustered ligands on neighbouring cells, EphA4 receptors themselves become clustered causing conformational changes and thereby activated through autophosphorylation. The clustered EphA4 recruit the $\text{APC}^\text{Cdh1}$ E3 machinery which decorates the receptors with a polyubiquitin chain. After internalization, the ubiquitinated EphA4 are then recognized and degraded into peptides by the 26S proteasome while the ubiquitin molecules are released and recycled. Assuming the rate of EphA4 production and insertion into the cell surface unchanged, the

![Figure 4.2 Model for APC-mediated proteasomal degradation of EphA4](image)

Figure 4.2 Model for APC-mediated proteasomal degradation of EphA4
net result of this series of ligand inducted events is a drop in the surface as well as total EphA4 expression.

It must be pointed out that the above model is obviously an oversimplified depiction of the actual mechanism regulating EphA4 expression. There are quite a few vague points in the description due to insufficient experimental data. The following questions are left unanswered:

1) Which event happens first, the recruitment of APC\textsuperscript{Cdh1} and the ubiquitination of EphA4 or its endocytosis? Previous studies have shown that monoubiquitination of the some RTKs begins at the membrane, but the ligase and the receptor remain associated throughout the endocytic compartments, and this proximity facilitates multiple rounds of Cbl-mediated monoubiquitination at different lysine residues (d'Azou, 2005). So it is possible that the ubiquitination and internalization probably happen simultaneously and are two independent events both triggered by ligand binding.

2) What triggers the recruitment of APC\textsuperscript{Cdh1} independently of the kinase activity of EphA4? As mentioned in section 4.2, the interaction and down-regulation of EphA4 by Cdh1 is kinase independent \textit{in vitro} yet depends on ligand activation in neurons. Thus it is important to find out what process induced by ephrin ligands other than kinase activation initiates the recruitment of APC\textsuperscript{Cdh1} to EphA4.

3) Is the lysosome pathway also involved in the regulation of EphA4 expression? As previously discussed, it is quite possible that both pathways collaborate in the regulation of membrane proteins according to past findings. While this study is so
far constrained to the proteasome pathway, it would be interesting to have a brief look at the role of the lysosome in the degradation of EphA4, for example by treating the neurons with a lysosome inhibitor alongside proteasome inhibitors as an additional control.

Apart from the above extra information needed to generate a complete picture, the existing model also needs further supporting experimental data. For one thing, although we demonstrated the interaction and down-regulation relationship between EphA4 and APC Cdh1, we didn’t directly prove that EphA4 can actually be ubiquitinated by APC Cdh1. The ultimate evidence can only be provided by in vitro ubiquitination assay if it shows that EphA4 could be ubiquitinated when mixed in vitro with APC Cdh1 and the corresponding E1, E2, as well as ubiquitin and ATP. Also, the polyubiquitination of EphA4 needs to be confirmed by, for example, polyubiquitin-specific antibodies.

4.7 Cdh1 regulates spine density

In the last part of the study, we investigated the possible functional connection between the regulation of EphA4 by APC Cdh1 with EphA4-mediated dendritic spine retraction in hippocampal neurons (21 DIV). We found that knockdown of Cdh1 caused a dramatic decrease in dendritic spine density under basal conditions which no longer responded to ephrin-A1 stimulation, while the overexpression of Cdh1 had no effect on basal spine density, but appeared to abolish the decrease induced by ephrin-A1 (Fig 3.18, 19, 20). These results suggest that Cdh1 is involved in the regulation of dendritic spine density in mature hippocampal neurons, but how it is related to the EphA4 mediated spine retraction is not clear.
Ephrin/Eph signaling has been reported to play roles in the formation and maturation of synapses in recent years. One aspect through which Eph receptors regulate the structural and functional properties of synapses is by regulating the formation and maintenance of dendritic spines. Spine morphology is regulated through the reorganization of the cytoskeleton, a mechanism that is modulated by guanine nucleotide exchange factors (GEFs) that activate small Ras-homologous (Rho) GTPases. Ephs bind distinct GEFs and regulate specific Rho GTPases. Different GTPases affect actin rearrangement and spine development in distinct ways. Rac1 and Cdc42 promote the development and maintenance of dendritic spines, whereas RhoA activation inhibits spine formation and promotes spine retraction (Kullander, 2002).

EphB (EphB1–3) receptors are involved in the development of spines at earlier stages through downstream effectors such as Syndecan-2 and Rho family small GTPases (Henkemeyer et al., 2003; Ethell et al., 2001; Penzes et al., 2001, 2003). The role of EphA signaling in spine morphogenesis is less well studied, and the only case reported so far is EphA4. Activation of EphA4 by ephrin-A3 localized on astrocytic processes that envelop spines was found to induce spine retraction, whereas inhibiting ephrin/EphA4 interactions distorted spine shape and organization in hippocampal slices. Furthermore, disrupting EphA4-forward signaling in hippocampal neurons leads to severe defects in spine morphogenesis, indicating that ephrin/EphA4 signaling is critical for spine morphology. Thus, a model was proposed in which transient interactions between the ephrin-A3 ligand and the EphA4 receptor regulate the structure of excitatory synaptic connections through neuroglial cross-talk (Murai et al., 2003). Later studies in our lab
showed that EphA4 mediates dendritic spine retraction in hippocampal neurons through Cdk5-dependent regulation of ephexin1 GEF activity towards RhoA (Fu, Chen & Ip et al., 2007). The study demonstrated that the activation of EphA4 resulted in the recruitment of Cdk5 to EphA4, leading to the tyrosine phosphorylation and activation of Cdk5. EphA4 and Cdk5 then enhanced the activation of ephexin1, a guanine-nucleotide exchange factor (GEF) that regulates activation of the small Rho GTPase RhoA, which in turn modulates actin cytoskeletal dynamics to control spine morphogenesis.

Based on the above information, the observations in this study can be explained. Overexpression of Cdh1 promotes the degradation of EphA4, leading to a decrease in its surface expression. The responsiveness of EphA4 signaling to ephrin stimulation is thus attenuated, including EphA4 mediated spine retraction, explaining why the spine density did not change after ephrin-A1 treatment. On the other hand, less Cdh1 allows more EphA4 to be accumulated on the cell surface, therefore ephrin stimulation should result in hyper-activated downstream response, leading to a higher percentage of spine retraction. The fact that the spine density is already low before ephrin treatment means that sufficient amount of Cdh1 is required to maintain the basal spine density. This can happen either through undiscovered EphA4 signaling pathway or other pathways that control the maintenance of spines under basal conditions. In order to verify the above hypotheses, further studies must be carried out in the future. For example, it would be interesting to see if Cdh1 regulates spine density through the EphA4-Cdk5/ephexin1-RhoA pathway.
4.8 Other possible functions for APC<sup>Cdh1</sup>-mediated proteasomal degradation of EphA4

Apart from regulation of dendritic spine morphogenesis, past studies revealing converging functions of APC<sup>Cdh1</sup> and EphA4 signaling in the brain offer a clue to other possible functions for the APC<sup>Cdh1</sup>-mediated proteasomal degradation of EphA4.

4.8.1 Axon guidance

Ephrin-Eph signaling regulates the guidance of motor axons and commissural axons across the midline in the CNS and in the spinal cord. Eph receptors, including EphA4 transduce short-range repulsive signals for axon guidance by modulating actin dynamics within growth cones. One reason why this might involve APC<sup>Cdh1</sup>-mediated proteasomal degradation of EphA4 is that it shares similar downstream signaling pathways with EphA4 mediated spine retraction: Ephrin-A stimulation of EphA4 modulates the activity of ephexin1 leading to RhoA activation, Cdc42 and Rac1 inhibition, resulting in growth cone collapse (Shamah et al., 2001; Sahin et al., 2005).

On the other hand, APC<sup>Cdh1</sup> has been reported to regulate axonal outgrowth and patterning in the mammalian brain (Bonni et al., 2004&2006). APC<sup>Cdh1</sup> appears to cell autonomously control the growth of granule neuron axons in a layer-specific manner, limiting their growth to the molecular layer in the classic parallel-fiber pattern. Two nuclear proteins that modulate gene transcription have been identified as the substrates of APC<sup>Cdh1</sup> to regulate axonal growth. Since Eph receptors regulate axon guidance, APC<sup>Cdh1</sup>
may provide a linkage between the extracellular cues to nuclear events that ultimately lead to morphological changes in the axon.

4.8.2 Synaptic plasticity and regulation of Glutamate receptors

EphB signaling participates in activity-dependent synaptic development and plasticity through modulation of glutamate receptor activity. EphrinB binding to EphB induces a direct interaction of EphB with NMDA-type glutamate receptors that occurs at the cell surface mediated by the extracellular regions of the two receptors and potentiates NMDA receptor-dependent influx of calcium, resulting in enhanced NMDA receptor-dependent gene expression (Dalva et al., 2000; Takasu et al., 2002). Further more, ephrinB reverse signaling into the presynaptic mossy fiber bouton is required for the induction of NMDA receptor-independent LTP at this synapse (Armstrong et al., 2006). In addition, EphB2 controls AMPA-type glutamate receptor localization through PDZ binding domain interactions and triggers presynaptic differentiation via its ephrin binding domain at excitatory synapses in the CNS (Kayser et al., 2006). These findings suggest a mechanism whereby activity-independent and activity-dependent signals converge to regulate the development and remodeling of synaptic connections. Although there hasn’t been any report about the involvement of EphA signaling in the regulation of glutamate receptors, it is possible for EphA receptors to share similar roles with EphBs given their highly conserved structures and functions.

Intriguingly, APC^Cdh1^-proteasome system is also connected to synaptic development and plasticity, especially through regulation of glutamate receptors. Pharmacological and genetic studies in organisms including Aplysia, C. elegans, flies and rodents have
suggested that the ubiquitin–proteasome machinery operates within both the presynaptic and the postsynaptic compartments, affecting diverse aspects of synapse development and function. Ubiquitin-dependent protein turnover controls neurotransmitter release presynaptically and neurotransmitter receptor abundance postsynaptically, and thereby strongly influences synaptic transmission, regulating synaptic plasticity and associated learning and memory tasks in diverse animal models (Stegmuller, 2005). In particular, ubiquitin promotes loss of GLR-1-containing synapses and produces behavioral changes consistent with reduced synaptic strength (Burbea et al., 2002). An important question arising from these studies is the identity of the specific ubiquitin ligases that act at synapses.

Two studies in the worm and fly have implied APC to be a strong candidate of the ubiquitin ligases that regulate synaptic plasticity. Perturbing APC function in postmitotic neurons in C. elegans increased the abundance of GLR-1 in the ventral nerve cord, suggesting that the APC regulates some aspect of GLR-1 recycling. Phenotypically, APC mutants had locomotion defects consistent with increased synaptic strength (Juo and Kaplan et al., 2004). At Drosophila neuromuscular synapses, the APC seems to independently regulate synaptic growth and synaptic transmission. In neurons, the APC controls synaptic size via a downstream effector Liprin-alpha; in muscles, the APC regulates synaptic transmission also by controlling the concentration of a postsynaptic glutamate receptor GluRIIa (Roessel et al., 2004). Although both GLR-1 and Drosophila GluRIIa each have a conserved destruction box motif, both studies reasoned that the glutamate receptors are unlikely to be a direct target of the APC, because these D-boxes
lie in their extracellular domains. Mammalian GluRs have been shown to bind a number of scaffolding protein, and several of these proteins undergo activity-dependent ubiquitination. Thus, it is possible that APC regulation is mediated by ubiquitination of a scaffolding protein associated with the GluRs, such as PICK1 and GRIP.

Taken together, the above information strongly suggests that EphA4 and APC$^{\text{Cdh1}}$ might work together to regulate activity-dependent synaptic development and plasticity by modulating the recycling and functional properties of GluRs.

**4.9 Summary and conclusion**

In this study, we propose a novel regulation pathway of EphA4 that involves the E3 ubiquitin ligase APC$^{\text{Cdh1}}$ and 26S proteasome. EphA4 associates with APC2, one of the catalytic core subunits of the APC. The neuronal specific activator Cdh1 also interacts with EphA4, and down-regulates the expression of EphA4 in mammalian cells. In neurons, ephrin-A1 stimulates the recruitment of APC2 to EphA4 and promotes its ubiquitination, leading to a decrease in surface EphA4 expression. Functionally, Cdh1 regulates dendritic spine density in hippocampal neurons, but the detailed mechanism and how this is related to EphA4 signaling are not clear. These findings support a model where ephrin binding triggers the APC$^{\text{Cdh1}}$-mediated ubiquitination of EphA4, followed by internalization and proteasomal degradation. This study shed light on the regulation mechanism of Eph signaling, as well as the growing functions of the APC$^{\text{Cdh1}}$ in postmitotic neurons. Future studies should focus on the further verification of the model, as well as the functional consequences of the regulation.
Reference


Buchert M, Schneider S, Meskenaite V, Adams MT, Canaani E, Baechi T, Moelling K and Hovens CM (1999) The junction-associated protein AF-6 interacts and


d’Azzo A, Bongiovanni A, Nastasi T (2005) E3 ubiquitin ligases as regulators of

intermediate, SHEP1, directly couples Eph receptors to R-Ras and Rap1A. J Biol
Chem. 274:31941-6.

Durbin L, Brennan C, Shiomi K, Cooke J, Barrios A, Shanmugalingam S, Guthrie B,
Lindberg R, Holder N (1998) Eph signaling is required for segmentation and

Wilson S, Holder N (2000) Anteroposterior patterning is required within
segments for somite boundary formation in developing zebrafish. Development
127:1703-1713.

Ehlers MD (2003) Activity level controls postsynaptic composition and signaling via the
ubiquitin-proteasome system. Nat Neurosci. 6:231-242

activated protein kinase pathway by the EphB2 receptor tyrosine kinase is

Ethell IM, Irie F, Kalo MS, Couchman JR, Pasquale EB and Yamaguchi Y (2001)
EphB/syndecan-2 signaling in dendritic spine morphogenesis. Neuron 31:1001–
1013.

Feldheim DA, Nakamoto M, Osterfield M, Gale NW, DeChiara TM, Rohatgi R,

Sanes JR (2000) Roles for ephrins in positionally selective synaptogenesis


