Cyclin Dependent Kinase 5 (Cdk5)
Cyclin Dependent Kinase 5 (Cdk5)
Preface

When cyclin-dependent kinase 5 (Cdk5) was identified 15 years ago, little was known about its physiological functions. While other cyclin-dependent kinases cooperate to control the cell cycle, this new addition to the family exceeds all expectations. As you will learn through the chapters in this book, although Cdk5 is structurally homologous to other Cdks, Cdk5 is unlike its family members in every way—a different set of activators, different localization of its activity, and importantly, its unexpected involvement in such a diverse plethora of biological actions. Through the extensive efforts in identifying interacting proteins and substrates of Cdk5/p35, it is now recognized that Cdk5 is implicated in almost every aspect of neuronal development and neural functions. Right from when the newly born neurons leave the ventricular zone for their long migratory journey to their final destination, Cdk5 activity is pivotal for proper neuronal migration. Indeed, newly generated neurons fail to display the “inside-out” organization in Cdk5-deficient brain. Upon reaching the target, neurons send out dendrites and axons to form functional connections with neighboring neurons. During this stage, Cdk5 is implicated in the regulation of neurite extension, synapse formation, and synaptic transmission. Cdk5 also plays a role in the control of apoptosis during development, which is important for the pruning and fine tuning of neural connections. As the neuronal network matures, Cdk5 activity remains essential and contributes to the regulation of synaptic plasticity. Indeed, Cdk5 is increasingly implicated in higher cognitive functions such as learning, memory formation, and drug addiction. More importantly, deregulation of Cdk5 activity is also associated with neuronal death in neurodegenerative diseases.

Through the years of research, it is now becoming clear that this kinase plays monumental role in essentially every facet of neuronal development and functions. It is also increasingly recognized that the action of Cdk5 is not limited to the nervous system. Cdk5 was also observed to play an important role at the neuromuscular junction, and in the control of insulin secretion in the pancreas. These findings provide novel grounds for investigating the function of Cdk5 in other systems. Indeed, given the involvement of Cdk5 in such diverse processes and its extensive crosstalk with other signaling pathways, it would not be surprising that Cdk5 is involved in yet unknown aspects of cellular functions.
We are very grateful to each and every contributor of this book for putting in extensive hours to bring these chapters into reality. We would also like to thank Miss Anna So and Dr. Zelda Cheung of the Hong Kong University of Science and Technology, whose efforts have made this book possible. Our collective effort will bring to the awareness of the scientific community the augmenting importance of this kinase as an essential regulator of neuronal development and functions. We believe that this book will also lay the groundwork of many more years of Cdk5 research to come.

Hong Kong, China
Cambridge, Massachusetts

Nancy Y. Ip
Li-Huei Tsai
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Contributors

Marco Angelo
Institute of Neurology, University College London, London, UK,
e-mail: m.angelo@ucl.ac.uk

Mathias Bähr
University of Göttingen, Department of Neurology, Robert-Koch-Str. 40,
37075 Göttingen, Germany
e-mail: mbaehr@gwdg.de

Christina Bark
The Rolf Luft Research Center for Diabetes and Endocrinology, Karolinska
Institutet, Stockholm, Sweden, Phone: 46-8-517 794 52, Fax: 46-8-517 794 50,
e-mail: christina.bark@ki.se

Per-Olof Berggren
The Rolf Luft Research Center for Diabetes and Endocrinology, Karolinska
Institutet, Stockholm, Sweden,
e-mail: per-olof.berggren@ki.se

Zelda H. Cheung
Department of Biochemistry, Hong Kong University of Science and
Technology, Clear Water Bay, Kowloon, Hong Kong, China,
e-mail: zelda@ust.hk

Sul-Hee Chung
Graduate Program in Neuroscience, Institute for Brain Science and
Technology, Inje University, 633-146 Gaeguem 2-dong, Busanjin-gu,
Busan, South Korea 614-735, Phone: 82-51-892-4185, Fax: 82-51-892-0059,
e-mail: sulchung@inje.ac.kr

Karen Duff
Department of Pathology, Taub Institute for Alzheimer’s Disease research,
Columbia University/NYS Psychiatric Institute, BB-513, Black Building,
650 W 168th St., New York 10032, USA, Phone: 212-305 8790,
e-mail: ked2115@columbia.edu
Andre Fischer
European Neuroscience Institute (ENI), Department for Experimental Neuropathology, Medical School University Goettingen, Max Planck Society, Germany,
e-mail: andre.fischer@mpi-mail.mpg.de

Peter K. Giese
Institute of Psychiatry, King’s College London, London, UK,
e-mail: peter.giese@iop.kcl.ac.uk

Yoshio Goshima
Department of Molecular Pharmacology & Neurobiology, Yokohama City University Graduate School of Medicine, 236-0004, Japan, Phone: 81-45-787 2593, Fax: 81-45-785 3645,
e-mail: goshima@med.yokohama-cu.ac.jp

Lisheng He
Department of Biochemistry, Hong Kong University of Science and Technology, Kowloon, Hong Kong, China,
e-mail: hlxaa@ust.hk

Karl Herrup
Department Cell Biology and Neuroscience, Nelson Biological Laboratories, Rutgers University, 604 Allison Road, Piscataway, NJ 08854, USA, Phone: 732-445-3306, Fax: 732-445-2165,
e-mail: herrup@biology.rutgers.edu

Carol D. Hicks
CNS Discovery, Pfizer Global Research and Development, Groton, CT 06340, USA

Shin-ichi Hisanaga
Molecular Neuroscience, Department of Biological Sciences, Graduate School of Science, Tokyo Metropolitan University, Hachioji, Tokyo 192-0397, Phone: 81-42-677 2577, Fax: 81-42-677 2559, Japan,
e-mail: hisanaga-shinichi@c.metro-u.ac.jp

Nancy Y. Ip
Department of Biochemistry, Biotechnology Research Institute and Molecular Neuroscience Center, Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong, China, Phone: 852-2358 7304, Fax: 852-2358 1552,
e-mail: boip@ust.hk

Koichi Ishiguro
Mitsubishi Kagaku Institute of Life Sciences, Machida, Tokyo 194-8511, Japan

Marko Jevsek
Institute of Physiology, Faculty of Medicine, University of Maribor, Maribor, Slovenia
Contributors

**Jyotshna Kanungo**
Laboratory of Neurochemistry, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20814, USA
e-mail: kanungoJ@mail.nih.gov

**Sashi Kesavapany**
National University of Singapore, Singapore 117597

**Niranjana D. Amin**
Laboratory of Neurochemistry, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20814, USA,
e-mail: bchsk@nus.edu.sg

**Ashok B. Kulkarni**
Functional Genomics Section, Laboratory of Cell and Developmental Biology, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, USA, Phone: 39-02-57489829/57489828, Fax: 39-02-57489851,
e-mail: andrea.musacchio@ifom-ieo-campus.it

**Lit-Fui Lau**
CNS Discovery, Pfizer Global Research and Development, MS 8220-4013, Eastern Point Road, Groton, CT 06340, USA, Phone: 860-715-1921,
Fax: 860-715-2349,
e-mail: lit-fui.lau@pfizer.com

**Slavena A. Mandic**
The Rolf Luft Research Center for Diabetes and Endocrinology, Karolinska Institutet, Stockholm, Sweden

**Zixu Mao**
Departments of Pharmacology and Neurology, Center for Neurodegenerative Disease, Emory University School of Medicine, 615 Michael St., Atlanta, GA 30322 USA,
e-mail: zmao@pharm.emory.edu

**Katrin Meuer**
University of Göttingen, Department of Neurology, Robert-Koch-Str. 40, 37075 Göttingen, Germany

**Andrea Musacchio**
Department of Experimental Oncology, European Institute of Oncology, Via Adamello 16, I-20139 Milan, Italy,
e-mail: andrea.musacchio@ifom-ieo-campus.it
Contributors

Fumio Nakamura  
Department of Molecular Pharmacology & Neurobiology, Yokohama City University Graduate School of Medicine, 236-0004, Japan

Gary Kar Ho Ng  
Department of Biochemistry, Hong Kong University of Science and Technology, Hong Kong, China

Toshio Ohshima  
Laboratory for Molecular Brain Science, Department of Life Science and Medical Bio-Science, Science and Engineering, Waseda University, Tokyo 169-8555, Japan, Phone: -81-3-5286-3358, Fax: -81-3-5286-3382, e-mail: ohshima@waseda.jp

Harish C. Pant  
Laboratory of Neurochemistry, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20814, USA, Phone: 301-402 2124, Fax: 301-496 1339, e-mail: panth@ninds.nih.gov

Tej K. Pareek  
Rainbow Babies and Children Hospital, Department of Pediatrics, Case Western Reserve University, Cleveland, OH, e-mail: tkp5@case.edu

Florian Plattner  
Institute of Neurology, University College London, London, UK, Phone: 44-0-207-837 8370, Fax: 44-0-207-278 4993, e-mail: f.plattner@ucl.ac.uk

Robert Z. Qi  
Department of Biochemistry, Hong Kong University of Science and Technology, Hong Kong, China, Phone: 852-2358 7273, Fax: 852-2358 1552, e-mail: qizr@ust.hk

Parvathi Rudrabhatla  
Laboratory of Neurochemistry, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20814, USA

Marjan Rupnik  
Institute of Physiology, Faculty of Medicine, University of Maribor, Maribor, Slovenia

Yukio Sasaki  
Department of Molecular Pharmacology & Neurobiology, Yokohama City University Graduate School of Medicine, 236-0004, Japan
Kazuhito Tomizawa
Department of Physiology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8558, Japan, Phone: 81-86-2357107, Fax: 81-86-235-7111, e-mail: tomikt@md.okayama-u.ac.jp

Li-Huei Tsai
Picower Institute for Learning and Memory, Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Howard Hughes Medical Institute, RIKEN-MIT center for Neuroscience research, Stanley Center for Psychiatric Research, Cambridge, MA, USA, Phone: 617.324.1660, Fax: 617.324.1657, e-mail: lhtsai@mit.edu

Yutaka Uchida
Department of Molecular Pharmacology & Neurobiology, Yokohama City University Graduate School of Medicine, 236-0004, Japan

Li Wang
Department Genetics, Case Western Reserve University, School of Medicine, 10900 Euclid Ave., Cleveland, OH 44016

Fan-Yan Wei
Department of Physiology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8558, Japan

Jochen H. Weishaupt
University of Göttingen, Department of Neurology, Robert-Koch-Str. 40, 37075 Göttingen, Germany, Phone: 49-551-39-14343, Mobile: 49-177-414-6790, e-mail: jweisha@gwdg.de

Yi Wen
Department of Pathology, Taub Institute for Alzheimer’s Disease research, Columbia University/NYS Psychiatric Institute, BB-513, Black Building, 650 W 168th St., New York 10032, USA

Naoya Yamashita
Department of Molecular Pharmacology & Neurobiology, Yokohama City University Graduate School of Medicine, 236-0004, Japan

Qian Yang
Departments of Pharmacology and Neurology, Center for Neurodegenerative Disease, Emory University School of Medicine, 615 Michael St., Atlanta, GA 30322 USA

Haung Yu
Department of Pathology, Taub Institute for Alzheimer’s Disease research, Columbia University/NYS Psychiatric Institute, BB-513, Black Building, 650 W 168th St., New York 10032, USA
Jie Zhang
Department Cell Biology and Neuroscience, Nelson Biological Laboratories, Rutgers University, 604 Allison Road, Piscataway, NJ 08854, USA

Ya-Li Zheng
Laboratory of Neurochemistry, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20814, USA
About the Editors

Prof. Nancy Ip was born in Hong Kong, and spent the early years of her scientific career in the United States. After obtaining her PhD in pharmacology at Harvard Medical School, she continued her scientific training as a post-doctoral fellow at Harvard and Sloan-Kettering Institute. Thereafter, she left academia to work in the biopharmaceutical industry as the Laboratory Head at Lifecodes Corporation and then at Regeneron Pharmaceuticals Inc, both based in New York. In 1993, Nancy Ip returned to an academic career at the newly established Hong Kong University of Science and Technology. She currently serves as Chair Professor and Head of the Department of Biochemistry, as well as Director of the Biotechnology Research Institute and the Molecular Neuroscience Center.

Nancy Ip is well known for her seminal discoveries on the biology of neurotrophic factors, proteins that promote the survival, development and maintenance of neurons in the nervous system. Her research has identified neurotrophic factors as potential pharmaceutical agents for the treatment of neurodegenerative diseases such as Alzheimer’s disease. She is also internationally recognized for her work on elucidating the signaling mechanisms of neuronal plasticity, such as establishing the pivotal involvement of cyclin-dependent kinase 5 in the regulation of synapse development and maintenance. Given the association of synaptic dysfunction with the pathophysiology of neurodegenerative diseases, her findings provide important clues in the development of therapeutics for these disorders.

As a highly accomplished researcher, Ip has published over 170 scientific papers with more than 12,000 SCI citations, and holds 18 patents. She is the Editor-in-Chief of NeuroSignals and is on the editorial board of journals such as J. Biol. Chem., J. Neurosci., and Dev. Neurobiol. She is also an Academician of the Chinese Academy of Sciences, a fellow of the Academy of Sciences for the Developing World, and a founding member of the Asia-Pacific International Molecular Biology Network. Additionally, she has received numerous awards including the National Natural Science Award and the L’OREAL-UNESCO for Women in Science Award.

Dr. Li-Huei Tsai was born in Taipei, Taiwan. In 1986, she started her Ph.D. at the University of Texas Southwestern. Under the direction of Bradford
Ozanne, she graduated in 1990 and joined Ed Harlow’s laboratory at Cold Spring Harbor Laboratory and Massachusetts General Hospital for post-doctoral training. During her time in the Harlow lab, she isolated two proteins prominently expressed in the nervous system: cyclin-dependent kinase 5 (Cdk5) and its regulatory activator p35. She was appointed Assistant Professor of Pathology at Harvard Medical School in 1994, elected Investigator of Howard Hughes Medical Institute in 1997, and promoted to Professor of Pathology in 2002. In 2006, she relocated her lab to MIT and became the Picower Professor of Neuroscience in the Picower Institute for Learning and Memory. She began directing the Neurobiology Program at the Stanley Center for Psychiatric Research in 2007.

A major research interest of the Tsai lab is to understand neurodegenerative diseases associated with cognitive decline such as Alzheimer’s disease. Her findings have led to the hypothesis that deregulation of Cdk5, through conversion of p35 to p25, plays an important role in the pathogenesis of Alzheimer’s disease. Recently, she found that chromatin remodeling via increased histone acetylation is beneficial for learning impairment and memory loss caused by severe neurodegeneration in the inducible p25 mouse model.

Li-Huei Tsai is on the editorial boards of the journal *Neuron, Journal of Neuroscience and NeuroSignals*, and has been awarded the Young Investigator Award from Metropolitan Life Foundation and the Outstanding Contributor Award from the Alzheimer Research Forum. She sits on the scientific advisory boards and committees for NINDS, Gruber Foundation, Alzheimer Research Forum, Hotchkiss Brain Institute at the University of Calgary, among other organizations.
Cdk5/p35 Regulates Neuronal Migration

Toshio Ohshima

Abstract Neurons migrate from proliferative zone to their final position during brain development. Cyclin-dependent kinase 5 (Cdk5) plays an important role in neuronal migration to establish a proper structure of the brain.

Analyses of Cdk5/p35-deficient mice have provided the knowledge about the role of Cdk5/p35 in neuronal migration. Over the past years, migration-related substrates of Cdk5 have been identified. Imaging analyses of neuronal migration of Cdk5/p35-deficient neurons have begun to elucidate how proper phosphorylations of these proteins by Cdk5/p35 are required for the regulation of cytoskeletal dynamics and cellular adhesion during neuronal migration.

Expression of Cdk5, p35, and p39 During Brain Development

Cyclin-dependent kinase 5 (Cdk5), a proline-directed serine (Ser)/threonine (Thr) kinase, had been identified as a member of the CDK family because of its close sequence homology to human CDC2 (Meyerson et al., 1992; Hellmich et al., 1992; Lew et al., 1992). Since the activity of Cdk5 is regulated by binding it with one of its neuron-specific regulatory subunits, either p35 (Lew et al., 1994; Tsai et al., 1994) or its isoform p39 (Tang et al., 1995), its activity is correlated with the level of expression of p35 and p39. Cdk5 expression is basically ubiquitous, and it is abundant in neuronal cells (Tsai et al., 1993). Expression of p35 and p39 overlaps throughout the central nervous system (CNS) during brain development, except for their expression in the cerebral cortex in the early stage, where only p35 is expressed till around E16 (Ohshima et al., 2001). High Cdk5 activity during neuronal differentiation and brain development reflects high-level expression of p35 and p39 (Tsai et al., 1993).

T. Ohshima
Laboratory for Molecular Brain Science, Department of Life Science and Medical Bio-Science, Science and Engineering, Waseda University, Tokyo 169-8555, Japan
e-mail: ohshima@waseda.jp

Cdk5/p35 Deficiency Causes Neuronal Migration Defects in CNS

Studies of the phenotypes of knockout (KO) mice have shown that Cdk5 and p35 are critical for migration of neurons to their final positions in the developing brain (Ohshima and Mikoshiba, 2002; Dhavan and Tsai, 2002). Migration defects in the cortical neurons of Cdk5 KO mice result in disruption of the laminar structures in the cerebral cortex, olfactory bulb, hippocampus, and cerebellum (Ohshima et al., 1996). p35 KO mice display a milder phenotype than Cdk5 KO mice because of the redundancy of p39 (Chae et al., 1997; Ohshima et al., 2001). p39 KO mice display no phenotype; however, p35 and p39 double KO mice display a phenotype identical to that of Cdk5 KO mice (Ko et al., 2001), confirming the redundancy of these subunits. Neuronal migration defects in Cdk5 KO mice are observed in many types of neuronal migration, but not in all types. These observations about Cdk5/p35 mutant mice indicate the occurrence of Cdk5-dependent and -independent neuronal migration. For example, radial migration of cortical neurons is Cdk5 dependent, but migration of subplate neurons seems to be Cdk5 independent in the cerebral cortex (Gilmore and Herrup, 2001). Tangential migration of GABAergic neurons from ganglionic eminence to cerebral cortex is also Cdk5 independent (Gilmore and Herrup, 2001). Migration along radial glial fibers is Cdk5 dependent in many cases including radial migration of cerebral cortical neurons and inward migration of granule cells in the cerebellum (Table 1). The list of examples of Cdk5-dependent migration (Table 1) will be expanded by further analysis of mutant mice.

### Table 1 Comparison of migration defects in neuronal types in CNS among mutant mice

<table>
<thead>
<tr>
<th>Structure or neuronal type in CNS</th>
<th>Cdk5 KO</th>
<th>p35 KO</th>
<th>Reeler/Dab1 mutant</th>
</tr>
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<tbody>
<tr>
<td>Olfactory bulb mitral cell</td>
<td>++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) Subplate neurons</td>
<td>–</td>
<td>–</td>
<td>++</td>
</tr>
<tr>
<td>(2) Cortical neurons</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>(3) GABAergic neurons</td>
<td>–</td>
<td>–</td>
<td>±</td>
</tr>
<tr>
<td>Hippocampus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) Pamidal cell layer</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>(2) Dentate gyrus</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Midbrain dopamin neurons in SN</td>
<td>++</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Cerebellum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) Purkinje cells</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>(2) Granule cells (inward)</td>
<td>n.d.</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Brain stem</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) Facial motor nucleus</td>
<td>++</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>(2) Inferior olive</td>
<td>++</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

++, severe defect; +, mild defect; n.d., could not be determined because of perinatal lethality.

This type of migration of granule cells is Cdk5 dependent (Ohshima et al., 1999). CNS, central nervous system; SN, substantia nigra.
Normal Migration of Cortical Neurons and Alteration by Cdk5/p35 Deficiency

Neurons settle on six layers in the cerebral cortex of the mammalian brain in an inside-out manner, with the earliest-generated neurons positioning themselves in the deepest layer and the later-generated neurons occupying the more superficial layers (Angevine and Sidman, 1961). The first wave of radially migrating neurons (earliest-generated neurons) splits the pre-existing subpial layer, which is called the preplate zone (PPZ), and “somal translocation” type migration is observed at this stage (Nadarajah et al., 2001). Successive waves of neurons (later-generated neurons) exhibit distinct migratory behaviors. In the subventricular zone (SVZ) and lower intermediate zone (IZ), immature neurons transiently become multipolar, and extend multiple neurites, and just before migrating to the cortical plate (CP), they change shape to become bipolar (Tabata and Nakajima, 2003; Noctor et al., 2004). In the CP, neurons migrate by “locomotion” along radial glial fibers (Nadarajah et al., 2001).

Analyses of Cdk5-null mice and p35-null mice have revealed defects in migratory behavior in Cdk5/p35 deficiency (Gupta et al., 2003; Ohshima et al., 2007b). The PPZ splits into the marginal zone and the subplate in the developing cerebral cortex of Cdk5 KO and p35 KO mice (Chae et al., 1997; Gilmore et al., 1998), in contrast to the impaired PPZ splitting that occurs in reeler as discussed below. The migration of Cdk5-null neurons destined for layer II–V stalls below the subplate (Gilmore et al., 1998), indicating that the locomotion type of radial migration is greatly affected by Cdk5/p35 deficiency. A time-lapse imaging study of p35-null brain slices revealed abnormal leading process morphology in migrating p35-null neurons (Gupta et al., 2003). In Cdk5 KO mice, the multipolar-to-bipolar transition is completely blocked, and neurons retain their multipolar morphology (Ohshima et al., 2007b). Introduction of Cdk5-dominant negative (DN) recaptures the phenotype of the migration defect of Cdk5/p35 deficiency in a cell-autonomous manner. When Cdk5-DN was introduced in high levels, the multipolar-to-bipolar transition was impaired, and radial migration was greatly disturbed. When Cdk5-DN was introduced at low levels, branched leading processes were observed, and migration was impaired to a moderate degree (Ohshima et al., 2007b). These observations suggest that the branched leading processes observed in p35-null migrating neurons represent a compensated phenotype in which there is an incomplete transition to the bipolar morphology. The results of an analysis of the migratory behavior of p35-null neurons also suggested reduced association of migrating neurons during locomotion with radial glial fibers (Gupta et al., 2003). Cdk5/p35 deficiency causes migration defects in later-generated cortical neurons and results in inverted layering of the cerebral cortex (Fig. 1).
Molecular Mechanism of the Regulatory Function of Cdk5/p35 in Neuronal Migration

How does Cdk5 regulate neuronal migration and positioning? Initially, a possible relation with Reelin signaling was discussed because of the phenotypic similarities between Cdk5 KO mice and reeler mice in which Reelin is defective. As summarized in Table 1, there is considerable overlap between neuronal populations affected in reeler and Cdk5 KO mice. An unsplit preplate is a characteristic feature (reeler phenotype) of deficiency of any component of Reelin signaling pathway (Fig. 2). The relationship between Cdk5/p35 and Reelin signaling was investigated by using double mutant mice with the defects of components of these two signaling pathways (Ohshima et al., 2001; Beffert et al., 2004). Genetic evidence has shown that Reelin and its receptors, VLDLR and ApoER2, and Dab1 belong to the same pathway called “Reelin signaling” (see review by Ohshima and Mikoshiba, 2002). Exaggerated neuronal migration defects were typically observed in the hippocampus of p35/Dab1, p35/Reelin, p35/ApoER2, and p35/VLDLR double KO mice compared with KO mice for the respective components (Ohshima et al., 2001; Beffert et al., 2004). Exaggerated defects in Purkinje cell migration in the cerebellum have also been reported in p35/Reelin and p35/Dab1 double KO mice (Ohshima et al., 2001). These findings indicate that Reelin signaling and Cdk5 function in parallel to effect proper neuronal migration and positioning in the developing brain (Ohshima and Mikoshiba, 2002; Beffert et al., 2004).

Cdk5 modulates actin-cytoskeleton dynamics through phosphorylation of Pak1 (Nikolic et al., 1998; Rashid et al., 2001) and filamin 1 (Fox et al.,...
and it modulates microtubule dynamics through phosphorylation of microtubule-associated proteins, including tau (Kobayashi et al., 1993), MAP1b (Paglini et al., 1998), doublecortin (Tanaka et al., 2004), Nudel (Sasaki et al., 2000; Niethammer et al., 2000), and CRMPs (Uchida et al., 2005). A defect in one of these substrates, filamin 1 in humans, causes periventricular heterotopia (Fox et al., 1998), and defects in two other substrates, Lis1 and doublecortin in humans, cause lissencephaly type 1 (Reiner et al., 1993; des Portes et al., 1998; Gleeson et al., 1998). These findings indicate that Cdk5 regulates cytoskeletal dynamics that determine the speed of migration, extension of the leading processes, and cell-soma propulsion in migrating neurons, in addition to proper transition from multipolar-to-bipolar morphology (Fig. 3). Impaired regulation of cytoskeletal proteins of migrating neurons may produce defective migration in Cdk5/p35 deficiency as described above. Cdk5 may also mediate cellular adhesion in neuronal–glial interactions through phosphorylation of β-catenin to regulate the interaction between β-catenin and N-cadherin (Kwon et al., 2000), and this Cdk5-mediated adhesion may also be important for neuronal migration along radial glial fibers (Fig. 3), as reduced association of p35-null migrating neurons with radial glial fiber was reported (Gupta et al., 2003). Although Cdk5 phosphorylates Dab1 at Ser491 in vivo (Keshvara et al., 2002) and at multiple sites in vitro (Ohshima et al., 2007a), the functional significance of the Cdk5-mediated Dab1 phosphorylation in Reelin signaling remains to be elucidated.
Because Cdk5 phosphorylates a number of proteins in the developing brain, Cdk5 deficiency causes multiple defects in the phosphorylation-mediated regulation of protein interactions required for proper neuronal migration. In addition to the impacts on the dynamics of cytoskeletal proteins, defective phosphorylation of substrate proteins may also alter expression of genes for neuronal differentiation and maturation. Analysis of Cdk5-deficient mouse models may reveal much more about the precise cellular and molecular mechanisms of neuronal migration in the developing brain, and recent studies have begun to demonstrate the involvement of Cdk5 in neuronal migration in the adult brain, and this field remains to be explored.

**Summary**

Because Cdk5 phosphorylates a number of proteins in the developing brain, Cdk5 deficiency causes multiple defects in the phosphorylation-mediated regulation of protein interactions required for proper neuronal migration. In addition to the impacts on the dynamics of cytoskeletal proteins, defective phosphorylation of substrate proteins may also alter expression of genes for neuronal differentiation and maturation. Analysis of Cdk5-deficient mouse models may reveal much more about the precise cellular and molecular mechanisms of neuronal migration in the developing brain, and recent studies have begun to demonstrate the involvement of Cdk5 in neuronal migration in the adult brain, and this field remains to be explored.

**References**


Cdk5/p35 Regulates Neuronal Migration


CRMP Family Protein: Novel Targets for Cdk5 That Regulates Axon Guidance, Synapse Maturation, and Cell Migration

Yoshio Goshima, Yukio Sasaki, Yutaka Uchida, Naoya Yamashita, and Fumio Nakamura

Abstract In the developing nervous system, post-mitotic neurons migrate and extend their neurites and form precise patterns of connections that emerge through the interaction between the growth cone and a myriad of environmental cues such as attractive or repulsive axon guidance molecules. Semaphorin3A (Sema3A) is the prototypical repulsive axon guidance molecule that potently induces growth cone collapse stalling neurite extension. Neuropilin-1 (NRP-1) and Plexin-As are ligand-binding and signal-transducing receptor components for Sema3A, respectively. Collapsin response mediator protein (CRMP) was identified as a signaling molecule of Sema3A. However, its molecular mechanisms have been ill-defined. CRMPs are now known to be composed of five homologous cytosolic proteins CRMP1–5; all of the family proteins are highly phosphorylated in developing brains. By screening pharmaceutical reagents and utilizing gene-deficient mice and through biochemical analysis, we found that Fyn and cyclin-dependent kinase 5 (Cdk5) mediate Sema3A-induced response in dorsal root ganglion (DRG) neurons. Cdk5 was associated with PlexA2 through the active state of Fyn. This raised the possibility that Sema3A induced growth cone collapse response through phosphorylation of CRMPs by Cdk5. The 2-D gel analysis of brain lysate from Cdk5-deficient mice revealed that CRMP2 was a substrate for Cdk5 in vivo. In vitro kinase assay revealed that Ser522 was the major site of CRMP1 and CRMP2 phosphorylation by Cdk5. Cdk5 primarily phosphorylated CRMP2 at Ser522, and GSK3β secondarily phosphorylates at Thr509. The dual-phosphorylated CRMP2 was recognized by the antibody 3F4, which is highly reactive with the neurofibrillary tangles of Alzheimer’s disease. In DRG neurons, Sema3A stimulation enhanced the levels of the phosphorylated form of CRMP2 detected by 3F4. Overexpression of CRMP2 mutant substituting either Ser522 or Thr509 with Ala attenuated Sema3A-induced growth cone collapse. Knockdown of CRMP1 and CRMP2 inhibited Sema3A-induced growth cone collapse. The phosphorylation of

Y. Goshima
Department of Molecular Pharmacology & Neurobiology, Yokohama City University Graduate School of Medicine, 236-0004, Japan
e-mail: goshima@med.yokohama-cu.ac.jp

CRMP1 and/or CRMP2 is therefore an essential step for Sema3A signaling. CRMP1 and CRMP2 were also good substrates for Fyn. The phosphorylation of CRMP1 by Cdk5 and Fyn also appears to be involved in Sema3A and Reelin signaling, contributing to spine maturation and the regulation of cell migration during the development of the cerebral cortex.

Introduction

Cyclin-dependent kinase 5 (Cdk5), a member of the serine/threonine kinase Cdk family, has enzymatic activity only in post-mitotic neurons due to a neuron-specific expression of the regulatory subunit p35 (Lew and Wang, 1995). Cdk5 has been implicated in various aspects of neural development, such as neurite outgrowth, axonal path finding, dendritic branching, and neural plasticity. Knockout mice studies reveal that Cdk5 and p35 play critical roles in laminar formation of the cerebral cortex by regulating the migration of neurons (Ohshima et al., 1996; Chae et al., 1997). However, the molecular mechanisms by which Cdk5 regulates the processes of neural development are unclear.

Neurons form precise patterns of connections that emerge through the interaction between the growth cone and extracellular signals in the developing nervous system. Neuronal extension from somas and navigating extensive length to their crucial targets are controlled by four types of axon guidance cues, membrane-bound or soluble attractive and repulsive molecules (Tessier-Lavigne and Goodman, 1996). Morphological changes and motility of neuronal growth cones are closely related to reorganization of actin, tubulin, and other cytoskeletal proteins, and interplay between actin and microtubule cytoskeletons has been shown to be critical for growth cone navigation (Tanaka and Sabry, 1995). Semaphorins, a large family of guidance cues for axonal/dendritic projections, are comprised of both secreting and membrane-bound proteins sharing a Sema domain (Kolodkin, 1998; Raper, 2000). The class 3 subfamily of the eight semaphorin subfamilies, composed of at least seven known members including Sema3A, has been the best-characterized subfamily. Sema3A promotes a variety of cellular responses, including cytoskeletal reorganization, endocytosis, and facilitation of axonal transport (Goshima et al., 1997; Fournier et al., 2000; Raper, 2000; Li et al., 2004). The semaphorin receptor mediating class 3 semaphorin signals has been identified as a complex of neuropilins and plexins (Takahashi et al., 1999; Tamagnone et al., 1999). Neuropilin (NRP)-1 and -2 bind to class 3 semaphorins with high affinities and are necessary for Sema3A-mediated repulsive guidance events. The mammalian plexin (Plex)-A subfamily does not bind directly to class 3 semaphorins. NRPs and Plex-As are ligand-binding and signal-transducing subunits of class 3 semaphorin receptor complexes, respectively (Takahashi et al., 1999; Tamagnone et al., 1999). Recent studies have shown that several intracellular molecules, including the small GTPase Rac1, R-Ras, the collapsin (Sema3A) response mediator protein (CRMP), LIM kinase, and FARP2, are implicated...
as mediators of Sema3A signaling (Puschel, 2007). However, the whole picture of the mechanisms involved in transducing Sema3A to actin and microtubule cytoskeleton for neuronal guidance remains obscure.

The chicken collapsin (formerly called semaphorin) response mediator protein (CRMP-62) molecule (also known as CRMP2) was originally identified as signaling molecule of Sema3A (Goshima et al., 1995). CRMP2, which has also been independently identified, is one of the five isoforms (CRMP1–5). The interactions among CRMP isomers favor heterophilic oligomerization over homophilic oligomerization (Wang and Strittmatter, 1997). CRMPs are extensively phosphorylated during neuronal development (Byk et al., 1996). This raised the possibility that CRMPs act as downstream components of semaphorin–PlexA signal transduction pathway through their phosphorylation to regulate cytoskeletal reorganization.

This chapter will discuss the role of CRMPs as substrate of Cdk5 that regulate axon guidance, spine maturation, and cell migration in developing nervous system.

### Fyn and Cdk5 Are Involved in Sema3A Signaling

Sema3A is a common guidance cue for nerve projection in the CNS and PNS. The collapse assay system using dorsal root ganglion (DRG) is the most useful method to characterize Sema3A responses. To search protein kinases involved in Sema3A signaling, we first screened various pharmacological reagents against Sema3A-induced growth cone collapse of DRG neurons. Among the more than 20 compounds we tested, lavendustin A (10 μM), a tyrosine kinase inhibitor, significantly suppressed Sema3A-induced growth cone collapse. We previously reported that olomoucine, a Cdk inhibitor, blocks growth cone collapse induced by chick caudal tectal membrane containing ephrins (Nakayama et al., 1999). Olomoucine (10 μM) also blocks Sema3A-induced growth cone collapse of chick DRG neurons. These data suggest that tyrosine kinase(s) and Cdk(s) are involved in Sema3A-induced growth cone collapse.

Because lavendustin A is thought to inhibit a variety of tyrosine kinases, it was difficult to identify tyrosine kinase(s) responsible for Sema3A signaling by using pharmacological methods. Among nonreceptor tyrosine kinases, Fyn is one of the plausible candidates for Sema3A signaling because Fyn is enriched in growth cones (Helmke and Pfenninger, 1995), and fyn-deficient mice show neural phenotypes (Yagi et al., 1993). In embryonic homogygous null mutant mice lacking fyn gene, the trajectory of the olfactory nerve displays defasciculation (Morse et al., 1998). Recently, Fyn has been appreciated as a mediator for axon guidance, since phosphorylation of deleted colorectal cancer receptor by Fyn mediates Netrin-1 signaling in growth cone (Meriane et al., 2004), and Netrin requires focal adhesion kinase and Src family kinases for axon outgrowth and attraction (Liu et al., 2004).
To examine whether Fyn is involved in Sema3A signaling, we analyzed Sema3A response in fyn-deficient DRG neurons. The rate of growth cone collapse induced by Sema3A in fyn\(^{-/-}\) DRG neurons at E17 showed a significant decrease compared with that in fyn\(^{+/+}\) neurons at lower concentrations (Sasaki et al., 2002). Olomoucine is reported to inhibit kinase activities of several kinds of Cdk/cyclin complexes as well as Cdk5/p35. Within the Cdk family, Cdk5 is the most plausible candidate for Sema3A signaling because Cdk5 activity is detected only in neurons, and cdk5-deficient mice show neural phenotypes (Ohshima et al., 1996). To assess whether Cdk5 participates in Sema3A-induced growth cone collapse, we used a DRG explant culture of E12 cdk5-deficient mice. The collapse rate induced by Sema3A in cdk5\(^{-/-}\) mice decreased to 30–40\%, whereas the rate reached 80\% in cdk5\(^{+/+}\) mice at 0.3 nM Sema3A (Sasaki et al., 2002). These findings demonstrate that Fyn and Cdk5 are involved in Sema3A-induced growth cone collapse.

After discovery of CRMP-62, we investigated whether Sema3A had some effects on axonal transport, because CRMPs are related to UNC-33: unc-33 mutant shows multiple axon guidance defects, and abnormalities in the form and number of microtubules (Li et al., 1992). UNC-33 has been therefore implicated in the regulation of tubulin dynamics. We found that Sema3A facilitates antero- and retrograde axonal transport (Goshima et al., 1997, 1999). To test the possibility that Fyn and Cdk5 mediate Sema3A-induced axonal transport as well, we examined the effects of lavendustin A and olomoucine on axonal transport (Li et al., 2004). Pretreatment of lavendustin A or olomoucine inhibited Sema3A-induced facilitation of antero- and retrograde axonal transport, without affecting basal levels of the antero- and retrograde axonal transport. Furthermore, Sema3A-induced facilitation of axonal transport was attenuated in fyn- and p35-deficient mouse DRG (Li et al., 2004). These findings indicate that Fyn and Cdk5 mediate Sema3A-induced axonal transport as well as growth cone collapse.

**Plex-A2 Associates with Cdk5 via Interaction with Active Fyn, and Fyn Activates Cdk5 via Tyr15 Phosphorylation**

Plex-A3 and -B1 has been reported to associate with some unknown kinase activity (Tamagnone et al., 1999). We thus hypothesized that Fyn associated with the Plex-A/NRP-1 heterodimer receptor to transduce Sema3A signaling. Coimmunoprecipitation experiments demonstrated that Plex-A2/NRP-1 complex could associate with all Fyn variants, irrespective of their kinase activities (Sasaki et al., 2002). Constitutively, active and wild-type Fyn induced tyrosine phosphorylation of Plex-A2, whereas kinase-negative and dominant-negative mutants did not promote the phosphorylation. Fyn was coimmunoprecipitated with Plex-A2 in the absence of NRP-1. The tyrosine phosphorylation level of Plex-A2 was reduced without NRP-1, suggesting that NRP-1 might have a
cooperative effect on Plex-A2 phosphorylation by Fyn. Fyn was not coimmunoprecipitated with Plex-A2 lacking most of the cytosolic region (Plex-A2Δcyto), and no tyrosine phosphorylation of Plex-A2Δcyto was detected, indicating that Fyn phosphorylated and associated with the cytosolic region of Plex-A2. We also examined whether Cdk5 was associated with Plex-A2. Cdk5 was not coimmunoprecipitated with Plex-A2 in the absence of Fyn. When constitutively active Fyn was co-expressed with Cdk5 and Plex-A2, Cdk5 was detected in the immunoprecipitate of Plex-A2. The association of Cdk5 with Plex-A2 was hardly observed in the presence of either wild-type or dominant-negative Fyn. Furthermore, Cdk5 was associated with constitutively active Fyn in the absence of Plex-A2. These data indicate that Cdk5 is associated with PlexA2 through the active state of Src family kinases.

Because Cdk5 cannot associate with Plex-A2 in the absence of Fyn, it was possible that the Src family kinases were necessary to regulate Cdk5 activity downstream of Plex-As. Thus, we examined whether Fyn promoted activation of Cdk5. Transfection of COS-7 cells with Cdk5, p35, and constitutively active Fyn enhanced kinase activity of the Cdk5/p35 complex by about 2-fold compared with Cdk5/p35 alone. Dominant-negative Fyn did not facilitate Cdk5 activity. Wild-type Fyn activated Cdk5 to a lesser extent than did the constitutively active form. It is reported that a nonreceptor tyrosine kinase, Abl, facilitates Cdk5 kinase activity via Tyr15 phosphorylation of Cdk5 (Zukerberg et al., 2000). The kinase activity of a Cdk5 mutant in which Tyr15 was converted to Ala (Cdk5Y15A) was not facilitated by constitutively active Fyn. An antiphospho-Cdc2 (Tyr15) antibody also recognizes Tyr15-phosphorylated Cdk5. Immunoblot analysis using this antibody showed that constitutively active Fyn phosphorylated Tyr15 of Cdk5. These results clearly show that Fyn facilitates Cdk5 activity mediated through Tyr15 phosphorylation of Cdk5.

Sema3A-Induced Growth Cone Collapse Is Mediated Through Cdk5 Activation via Tyr15 Phosphorylation

To examine whether Sema3A promoted Cdk5 activation via Tyr15 phosphorylation, we performed a Cdk5 kinase assay in COS cells expressing Plex-A2, NRP-1, Cdk5, and p35. Sema3A facilitated kinase activity of Cdk5/p35 within 5 min, and the activity persisted up to 20 min. We examined the effect of the Cdk5Y15A mutant on Sema3A-induced growth cone collapse because Fyn did not facilitate the activity of the Cdk5Y15A mutant. We expressed wild-type Cdk5, Cdk5Y15A, or kinase-negative mutant in DRG neurons. The expression of Cdk5Y15A and kinase-negative mutant suppressed the Sema3A-induced response. In contrast, wild-type Cdk5 in DRG neurons did not block the growth cone collapse. In culture without Sema3A, anti-phospho-Cdc2 (Tyr15) antibody stained only faintly in well-spread growth cones of DRG explants. At 0.5–2 min after Sema3A application, the anti-phospho-Cdc2 (Tyr15) antibody intensely
stained partially collapsed, but not fully extended, growth cones. The staining was visible in the central domain of growth cones and in most of the filopodial processes. The staining by the phospho-specific antibody was not detected within 5 min. These data suggest that Sema3A-induced growth cone collapse is mediated through Tyr15 phosphorylation of Cdk5.

Tau is a known substrate of Cdk5 that regulates the dynamics of tubulin (Hosoi et al., 1995). To examine whether phosphorylated tau increased in growth cones after Sema3A stimulation, we used an anti-phosphorylated tau antibody AT-8, which recognized phosphorylation sites by Cdk5. In nonstimulated culture, phosphorylated tau was localized in the axon, but not in the growth cone. At 2–5 min after Sema3A application, phosphorylated tau was mainly localized at the proximal region of the axon. Anti-tau antibody showed that total tau content in growth cones did not appear to change until complete collapse. These results indicate that Sema3A promotes activation of Cdk5 in growth cones, followed by phosphorylation of tau (Sasaki et al., 2002).

**CRMP2 Is an In Vivo Substrate of Cdk5**

These findings suggest that Sema3A regulates growth cone motility through Fyn-Cdk5 cascade. This prompted us to determine whether Sema3A signaling was mediated through phosphorylation of CRMP by Cdk5. To examine whether CRMP was a substrate for Cdk5, we performed in vitro kinase assay using purified CRMP2, Cdk5, and p25 or p35. CRMP2 was phosphorylated in the presence of both Cdk5 and p25. Cdk5/p35 enhanced phosphorylation of CRMP2 in HEK293T cells. Phosphorylation of CRMP1, 4, and 5 were also enhanced by Cdk5/p35 in HEK293T cells, a result consistent with the fact that CRMP1, 4, and 5 had the consensus sequence of phosphorylation by Cdk5 near amino acid residue 522 as follows. The consensus sequence of phosphorylation by Cdk5 was amino acid sequence (S/T)PX(K/H/R), where S or T was the phosphorylatable serine or threonine (Songyang et al., 1996). Amino acids 522–525 (SPAK) of CRMP2 matched with the consensus sequence. To test whether Ser522 was the phosphorylation site of CRMP2 by Cdk5, we produced a non-phosphorylated CRMP2 mutant, CRMP2S522A, in which Ser522 was replaced by Ala. Cdk5 phosphorylated wild-type CRMP2 (CRMP2wt), but not CRMP2S522A in vitro. We prepared a rabbit polyclonal antibody, anti-pS522-CRMP1/2 antibody, that recognized CRMP1 and CRMP2 phosphorylated at Ser522 in vitro, but not CRMP3, CRMP4, and CRMP5. Immunoblot analysis using anti-pS522-CRMP1/2 antibody revealed that the bands were at low intensity or were missing in lysate of embryonic brains from cdk5−/− when compared to wild-type mice.

During a search for in vivo substrate of Cdk5 using cdk5−/− mice and a monoclonal antibody, which recognized phospho-threonine followed by proline (pThr-Pro antibody), Ohshima and his colleagues found that the threonine...
phosphorylation was not seen with brain lysate from \textit{cdk5}^{-/-} mice. They found that three bands were at low intensity or missing in \textit{cdk5}^{-/-} brain lysate. These bands corresponded to 65 kDa, 70 kDa, and 120 kDa in SDS-PAGE electrophoresis. 2-D gel electrophoresis followed by Western blot with pThr-Pro antibody showed that five spots were at very low intensity or absent in \textit{cdk5}^{-/-} brain lysate. Spot 1 and 2 corresponded to 65 kDa, spot 3 and 4 to 70 kDa, and spot 5 to 120 kDa bands. MALDI-TOP mass spectrometry analysis and database search indicated that spot 3 fitted to CRMP2. Immunoblot analysis of 2-D gel with pThr-Pro antibody and C4G, which was phosphorylation-independent anti-CRMP2 monoclonal antibody (Gu et al., 2000), revealed that two of them matched spot 3 and 4. Phosphorylation of CRMP2 was previously reported in Alzheimer’s disease (AD) patient brains using monoclonal antibody 3F4 (Yoshida et al., 1998). 3F4 recognized three sites of phosphorylation of CRMP2 at Thr509, Ser518, and Ser522 (Gu et al., 2000). Immunoblot analysis with this monoclonal antibody confirmed that spots 3 and 4 completely matched those sites detected by 3F4 and C4G antibodies. Furthermore, immunoblotting with these antibodies revealed that CRMP2 was phosphorylated at 3F4 recognition site in \textit{cdk5}^{-/-} embryonic brain. These results indicated that CRMP2 was phosphorylated by Cdk5 in the embryonic mouse brain, and the phosphorylation site(s) was (were) Thr509 and/or Ser518 and/or Ser522. These findings suggest that CRMP2 is also phosphorylated at the threonine residue(s) \textit{in vivo}. In addition, no band shift was observed with CRMP2S522A co-expressed with Cdk5/p35.

\textbf{Cdk5-Primed GSK3\textbeta{} Phosphorylation of CRMP2}

Based on these findings, we speculated that phosphorylation of Ser522 was required for phosphorylation of other sites, and that other Ser/Thr kinase(s) might be involved in phosphorylation of CRMP2 at threonine residue. Tau is known for its Cdk5-primed phosphorylation by a Ser/Thr kinase GSK3\textbeta{} (Cho and Johnson, 2003). Indeed, when CRMP2 was co-expressed with GSK3\textbeta{}, CRMP2 was phosphorylated and intensity of upper band increased compared with CRMP2 co-expressed with Cdk5/p35 in HEK293T cells. When co-expressed with GSK3\textbeta{} and Cdk5/p35, CRMP2 was more phosphorylated and the intensity of upper band relatively increased, thereby indicating phosphorylation at additional sites. No phosphorylation and band shift were observed in the co-expression of CRMP2S522A with Cdk5/p35, GSK3\textbeta{}, or both. This result suggests that CSK3\textbeta{} as well as Cdk5 phosphorylated CRMP2 at Ser522, or Cdk5 phosphorylation of Ser522 is essential for the additional phosphorylation of CRMP2 by GSK3\textbeta{}. \textit{In vitro} kinase assay using purified protein GSK3\textbeta{} alone did not phosphorylate CRMP2. To examine whether the phosphorylation by Cdk5 enhances additional phosphorylation of CRMP2 by GSK3\textbeta{}, we prepared GST-CRMP2c (aa486 to carboxyl terminus), and then