



Evolution of mitochondrial cell death pathway: Proapoptotic role of HtrA2/Omi in *Drosophila*

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Abstract

Despite the essential role of mitochondria in a variety of mammalian cell death processes, the involvement of mitochondrial pathway in *Drosophila* cell death has remained unclear. To address this, we cloned and characterized DmHtrA2, a *Drosophila* homolog of a mitochondrial serine protease HtrA2/Omi. We show that DmHtrA2 normally resides in mitochondria and is up-regulated by UV-irradiation. Upon receipt of apoptotic stimuli, DmHtrA2 is translocated to extramitochondrial compartment; however, unlike its mammalian counterpart, the extramitochondrial DmHtrA2 does not diffuse throughout the cytosol but stays near the mitochondria. RNAi-mediated knock-down of DmHtrA2 in larvae or adult flies results in a resistance to stress stimuli. DmHtrA2 specifically cleaves *Drosophila* inhibitor-of-apoptosis protein 1 (DIAP1), a cellular caspase inhibitor, and induces cell death both *in vitro* and *in vivo* as potent as other fly cell death proteins. Our observations suggest that DmHtrA2 promotes cell death through a cleavage of DIAP1 in the vicinity of mitochondria, which may represent a prototype of mitochondrial cell death pathway in evolution.

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Keywords: Apoptosis; Cell death; *Drosophila*; HtrA2/Omi; Mitochondria

Mitochondria play crucial roles in regulating cell death in mammals [1,2]. Upon receipt of apoptotic stimuli, the cell activates caspase protease cascade to execute cell death. The activation of caspases is largely regulated by mitochondrial proteins such as cytochrome *c* (cyt *c*), Smac/DIABLO, and HtrA2/Omi, which are released to cytosol following cell death stimuli [1,2]. cyt *c* directly activates the cytosolic caspase-activating protein Apaf-1, thereby triggering a cascade of caspase activations [1,3]. On the

other hand, Smac/DIABLO and HtrA2/Omi indirectly activate caspases by antagonizing inhibitor-of-apoptosis proteins (IAPs), a family of cellular caspase inhibitors [4–7]. HtrA2/Omi, as well as cyt *c*, has also been shown to be important for cell survival, as loss of HtrA2/Omi gene results in a neurodegeneration in mice [8,9].

Despite abundant similarities in cell death mechanisms between vertebrates and flies, the involvement of mitochondria in *Drosophila* cell death machinery has remained unclear. *Drosophila* cell death is largely regulated by three killer proteins Reaper, Hid, and Grim. These proteins are thought to be functional counterparts of mammalian mitochondrial killer proteins Smac/DIABLO and HtrA2/Omi, as they bind to and antagonize IAPs through their

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conserved four-amino-acid sequences called RHG (Reaper, Hid, and Grim) motif or IBM (IAP-Binding Motif) [3,10]. Unlike mammalian counterparts, however, RHG normally reside in the cytoplasm and their activities are regulated either at transcriptional levels or by phosphorylation [11]. The presence of two *Drosophila* Bcl-2 family proteins that localize to mitochondria [12,13] indicates that mitochondrial cell death pathway may also exist in flies [14]. Here, in order to investigate the role of mitochondria in *Drosophila* cell death pathway, we cloned and characterized DmHtrA2, a *Drosophila* homolog of HtrA2/Omi. Our data suggest that DmHtrA2 promotes cell death through a cleavage of *Drosophila* IAP1 (DIAP1) in the vicinity of mitochondria, which may represent a prototype of mitochondrial cell death pathway in evolution.

Materials and methods

Stress resistance. For UV-resistance, third-instar larvae were irradiated with 5 mJ/cm² UV and allowed to develop at 25 °C. Paraquat resistance was tested essentially as described [15]. Adult flies (age 10–20 days) were starved for 6 h and transferred to vials containing two 2 cm × 2 cm filter squares wetted with 20 mM paraquat (Sigma) in 5% sucrose solution. Survival was scored at 18 or 24 h after the transfer. The ingestion rate was determined by dye intake by adding 10 mg/ml bromophenol blue instead of paraquat. Fly lysate was analyzed by monitoring the absorbance at 595 nm at 18 h.

Recombinant proteins and cleavage assay. The C-terminally His₆-tagged recombinant human HtrA2 protein (HsHtrA2ΔN133-His₆) was described previously [6]. The N-terminally GST-tagged recombinant DmHtrA2 protein (GST-DmHtrA2ΔN92) was produced in the *Escherichia coli*. The protein was purified by affinity chromatography using Glutathione Sepharose™ 4B (Amersham Bioscience). N-terminally FLAG-tagged DIAP1 or Hop was translated *in vitro* in the presence of [³⁵S]-methionine using a TNT T7 Quick Coupled Transcription/Translation System (Promega). [³⁵S]-labeled proteins were incubated with recombinant HtrA2 proteins (100 nM) in Tris buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1 mM DTT for 1 h at 37 °C. The reaction mixtures were subjected to SDS-PAGE and visualized by autoradiography.

Cell death assay. Cell death assay was performed as described previously [12]. In brief, S2 cells were transfected with pUAST-derived expression constructs with a driver plasmid pWAGAL4 (actin promoter-GAL4), together with a reporter plasmid pCaspaseR-hs-lacZ that encodes β-galactosidase under the control of the *hsp70* promoter. Twenty-four hours after transfection, the cells were heat-shocked at 37 °C and cultured for another 24 h. The cells were lysed at 48 h and assayed for β-galactosidase activity in a reaction mixture containing *o*-nitrophenyl-β-D-galactopyranoside.

For information regarding cloning, expression constructs, antibodies, cell culture, subcellular fractionation, Western blotting, and fly stocks, see Supplementary information.

Results and discussion

We cloned the DmHtrA2 cDNA (DDBJ/EMBL/GenBank, Accession No. AB112473) from the total RNA of wild-type fly embryo. DmHtrA2 encoded a protein of 422 amino acids with an N-terminal transmembrane (TM) domain, a central trypsin-like serine protease domain, and a C-terminal PDZ domain, as well as an IBM-like (ASKM) sequence that locates adjacent to the TM (Supplemental Fig. 1). RT-PCR analysis revealed that DmHtrA2

mRNA was expressed at all stages of *Drosophila* development (Supplemental Fig. 1).

To investigate the role of DmHtrA2 in cell death, we induced cell death in *Drosophila* S2 cells by UV-irradiation. Four hours after irradiation, the cells began to exhibit apoptotic morphological changes (Fig. 1A and B). We found that the protein level of DmHtrA2 was significantly up-regulated in the irradiated cells in a time-dependent

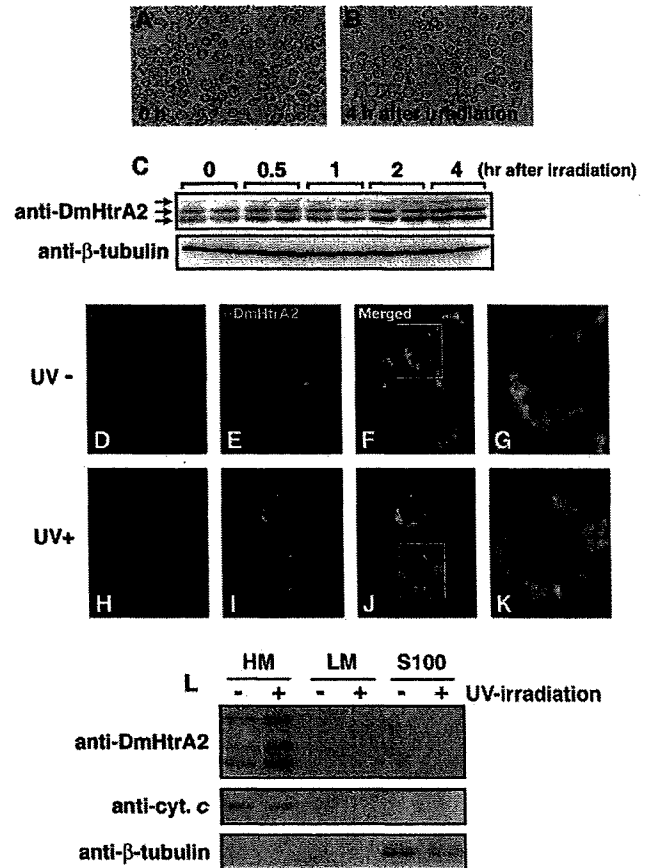


Fig. 1. Apoptotic stimulus up-regulates DmHtrA2 and induces its translocation from mitochondria to extramitochondrial compartment. (A,B) S2 cells were irradiated with UV (200 mJ/cm²) and cultured for another 4 h. (C) Endogenous DmHtrA2 levels were assessed by Western blotting using an anti-DmHtrA2 antibody before and after UV-irradiation. An anti-β-tubulin antibody was used for a loading control. The anti-DmHtrA2 antibody recognized three bands; the highest band corresponded to the size of full-length form, and the lowest band corresponded to the size of putative mitochondrial mature (ΔN) form (C, upper panel). The experiments were performed using duplicate dishes and were repeated three times. (D–K) Confocal images of S2 cells co-stained with Mitotracker (magenta) and an anti-DmHtrA2 antibody (green). Mitochondria and the endogenous DmHtrA2 protein were visualized before (D–G) or 4 h after (H–K) UV-irradiation (200 mJ/cm²). G and K show magnified images from F and J, respectively. (L) S2 cells were fractionated before (–) or 4 h after (+) UV-irradiation (200 mJ/cm²), and the cell lysate was subjected to Western blot analysis using anti-DmHtrA2, anti-cytochrome c, and anti-β-tubulin antibodies. For DmHtrA2 protein, the total amount of protein from each fraction was adjusted to 2.5 μg. For cytochrome c and β-tubulin, the mitochondrial and cytosolic markers, respectively, an equal volume (10 μl) from each fraction was used for Western analysis.

manner (Fig. 1C). This up-regulation was observed even at 2 h after irradiation, preceding the morphological changes (Fig. 1C). Immunostaining of DmHtrA2 revealed that it exclusively localized to mitochondria under the normal condition (Fig. 1D–G). Four hours after UV-irradiation, the anti-DmHtrA2 staining still showed a punctate pattern with a higher signal intensity, but it no longer merged with the Mitotracker-labeled mitochondria (Fig. 1H–K). This suggests that DmHtrA2 is translocated to extramitochondrial compartment in response to UV-irradiation. We further analyzed the subcellular localization of DmHtrA2 by fractionating S2 cell lysates before and after irradiation. DmHtrA2 protein was detected in the heavy membrane (HM) fraction, as was *cyt c*, but not in either the light membrane (LM) or cytosolic (S100) fraction (Fig. 1L). We found that UV-irradiation did not alter this distribution (Fig. 1L), suggesting that DmHtrA2 is released to the extramitochondrial compartment but stays in the vicinity of the mitochondria. Since no difference was observed in the mitochondrial membrane potential between control and UV-irradiated S2 cells (Fig. 1D and H; [16]), it is unlikely that the complementary staining of anti-DmHtrA2 and Mitotracker in irradiated cells was due to a loss of membrane potential in a subset of mitochondria.

To examine the physiological role of DmHtrA2, we generated DmHtrA2 knock-down flies using an RNAi construct of DmHtrA2 and a ubiquitous driver *da-GAL4*. In the knock-down larvae and adults, DmHtrA2 protein level was greatly reduced (Fig. 2A). The knock-down flies were viable and fertile with no detectable morphological abnormalities. We found that the DmHtrA2 knock-down larvae were more resistant to UV-induced lethality compared to control larvae (Fig. 2B). Furthermore, the DmHtrA2

knock-down adult flies were more resistant to dietary paraquat, a superoxide stress agent (Fig. 2C). The ingestion rate of the knock-down flies was not affected (data not shown). These observations suggest that DmHtrA2 is involved in stress-induced toxicity *in vivo*.

Mammalian HtrA2/Omi induces cell death by cleaving and thereby inactivating IAPs through its serine protease activity [4–7]. We therefore assumed that the stress-induced toxicity mediated by DmHtrA2 is through a cleavage of DIAP1. We performed an *in vitro* cleavage assay using recombinant DmHtrA2 and DIAP1 proteins, and found that both *Drosophila* and human HtrA2 proteins specifically cleaved DIAP1 (Fig. 3A). These HtrA2 proteins did not cleave a control protein FLAG-tagged Hop (Hsp70/Hsp90-organizing protein) (Fig. 3A). Thus, the specific serine protease activity of HtrA2/Omi is conserved in *Drosophila*.

Finally, we examined whether DmHtrA2 can kill the cell. Overexpression of DmHtrA2 in S2 cells significantly reduced their viability as potent as other *Drosophila* killer proteins such as Reaper (Fig. 3B and data not shown). This cell death could not be blocked by caspase inhibitors such as DIAP1, p35, or p49, similar to the one caused by human HtrA2/Omi [4,6]. We further examined the toxicity of DmHtrA2 *in vivo*. Overexpression of DmHtrA2 in developing *Drosophila* eye resulted in “no eye” phenotype (Fig. 3C), suggestive of an extensive cell death during development. This phenotype was also resistant to caspase inhibitors (Fig. 3C). Together, these data suggest that DmHtrA2 potentially induces cell death through a cleavage of DIAP1.

Our observations suggest that stress stimuli such as UV-irradiation cause translocation of DmHtrA2 from mito-

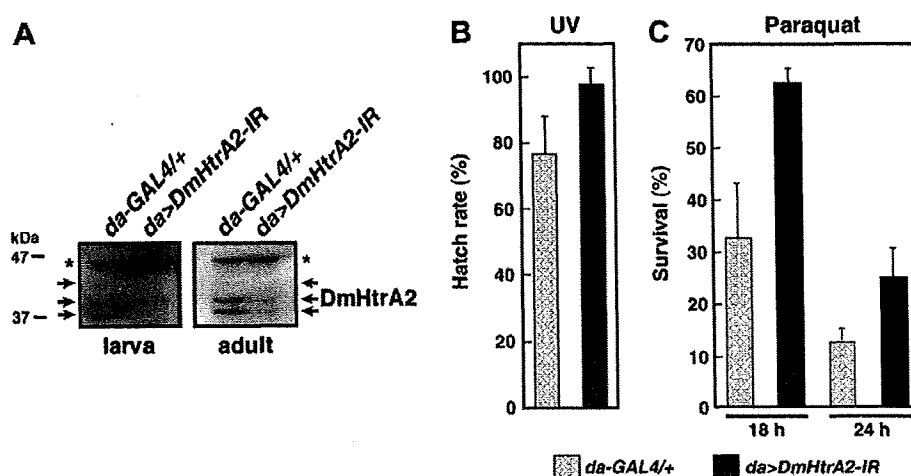


Fig. 2. DmHtrA2 mediates stress stimuli *in vivo*. (A) An RNAi construct (inverted-repeat; IR) for DmHtrA2 (UAS-DmHtrA2-IR) was driven by ubiquitous expression driver *da-GAL4* in third-instar larvae or adult flies. Eight animals were homogenized with 96 μ l of conventional SDS loading buffer and subjected to SDS-PAGE (4 μ l/lane) followed by an anti-DmHtrA2 blotting. The non-specific bands detected by the anti-DmHtrA2 antibody (asterisks) show equal loadings of the protein. (B) Control (*da-GAL4*) or knock-down (*da-GAL4*, UAS-DmHtrA2-IR) third-instar larvae were irradiated with UV (5 mJ/cm²), and the resistance was assessed by the number of adult flies that hatched. (C) Control or knock-down adult flies were starved and subjected to dietary paraquat. Survival was scored at 18 or 24 h after the beginning of ingestion.

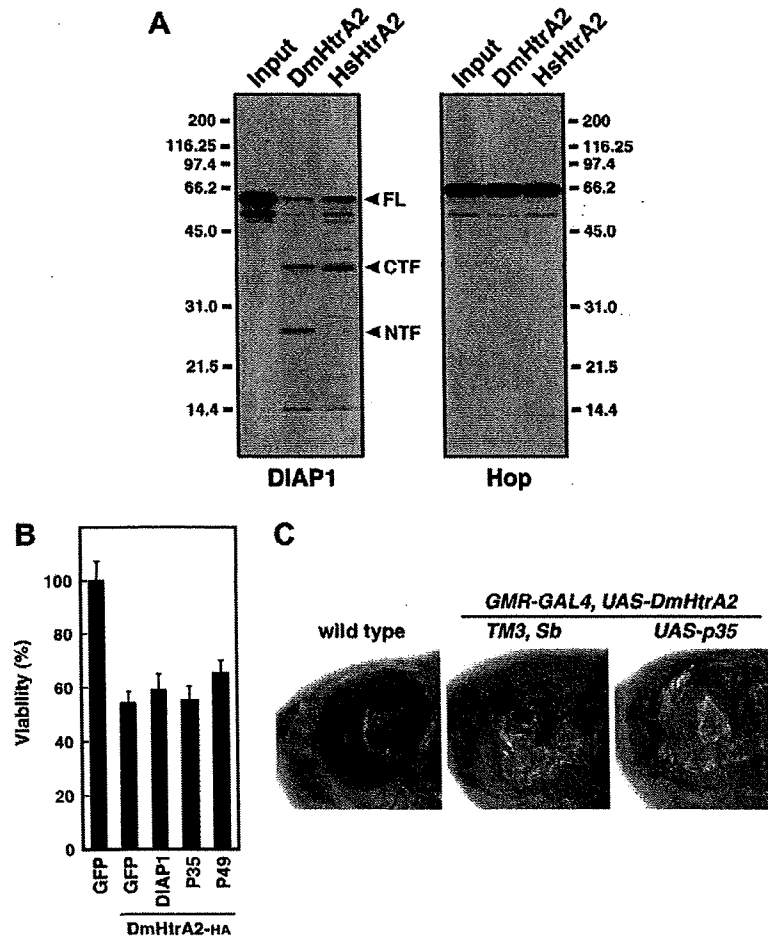


Fig. 3. DmHtrA2 is a potent cell death inducer that cleaves DIAP1. (A) [35 S]-labeled DIAP1 or Hop was incubated with *Drosophila* or human HtrA2 recombinant protein, subjected to SDS-PAGE, and visualized by autoradiography. The bands of C-terminal fragment (CTF) and N-terminal fragment (NTF) were predicted by Western blotting of S2 cell lysate co-expressing DmHtrA2 and FLAG-tagged DIAP1 (data not shown). (B) S2 cells were transfected with expression constructs for the indicated proteins and subjected to Cell Death assay. (C) DmHtrA2 was overexpressed in developing *Drosophila* eye using the GMR-GAL4 driver. The eyes at late pupal stage of wild-type, GMR-GAL4/UAS-DmHtrA2²⁻⁶, TM3, Sb/+, and GMR-GAL4/UAS-DmHtrA2²⁻⁶; UAS-p35/+ are shown.

chondria to extramitochondrial compartment, which in turn promotes cell death through inactivation of DIAP1. Indeed, a significant proportion of the *Drosophila* caspase DRONC, a fly counterpart of caspase-9, has been shown to localize near mitochondria [17]. Intriguingly, overexpression of DmHtrA2 caused caspase-independent cell death both *in vitro* and *in vivo*. This is consistent with the previous report that down-regulation of DIAP1 triggers a caspase activity-independent cell death pathway that is mediated by DRONC [18]. Our findings suggest that the mitochondrial regulation of cell death machinery could be conserved in *Drosophila*, and that the diverse roles of mitochondria in mammalian systems may have been coopted through the evolution of cell death mechanisms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.03.079.

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Rac-GAP α -Chimerin Regulates Motor-Circuit Formation as a Key Mediator of EphrinB3/EphA4 Forward Signaling

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SUMMARY

The ephrin/Eph system plays a central role in neuronal circuit formation; however, its downstream effectors are poorly understood. Here we show that α -chimerin Rac GTPase-activating protein mediates ephrinB3/EphA4 forward signaling. We discovered a spontaneous mouse mutation, *miffy* (*mfy*), which results in a rabbit-like hopping gait, impaired corticospinal axon guidance, and abnormal spinal central pattern generators. Using positional cloning, transgene rescue, and gene targeting, we demonstrated that loss of α -chimerin leads to *mfy* phenotypes similar to those of *EphA4*^{-/-} and *ephrinB3*^{-/-} mice. α -chimerin interacts with EphA4 and, in response to ephrinB3/EphA4 signaling, inactivates Rac, which is a positive regulator of process outgrowth. Moreover, downregulation of α -chimerin suppresses ephrinB3-induced growth cone collapse in cultured neurons. Our findings indicate that ephrinB3/EphA4 signaling prevents growth cone extension in motor circuit formation via α -chimerin-induced inactivation of Rac. They also highlight the role of a Rho family GTPase-activating protein as a key mediator of ephrin/Eph signaling.

INTRODUCTION

Ephrins are cell-surface-bound ligands for Eph receptors, which comprise the largest family of receptor tyrosine kinases (Eph Nomenclature Committee, 1997). The ephrin/Eph interaction induces bidirectional signaling: ephrin \rightarrow Eph forward and Eph \rightarrow ephrin reverse (Noren

and Pasquale, 2004; Palmer and Klein, 2003). Ephrin/Eph signaling, which functions in short-range cell-to-cell communication primarily through repulsive effects, is central to neuronal circuit development (Flanagan and Vanderhaeghen, 1998; Palmer and Klein, 2003; Pasquale, 2005).

Downstream signaling has been studied mostly through in vitro cell culture experiments. Particularly important players in both forward and reverse signaling are the Rho-family GTPases (Rho-GTPases), such as RhoA, Rac, and Cdc42, which are the key regulators of actin dynamics (Etienne-Manneville and Hall, 2002; Luo, 2000; Noren and Pasquale, 2004; Wahl et al., 2000). Rho-GTPases are directly activated by Rho-guanine nucleotide-exchange factors (Rho-GEFs) and are inactivated by Rho-GTPase-activating proteins (Rho-GAPs). Accumulating evidence suggests that ephrin/Eph regulates Rho-GTPases through Rho-GEFs (Cowan et al., 2005; Irie and Yamaguchi, 2002; Murai and Pasquale, 2005; Ogita et al., 2003; Penzes et al., 2003; Shamah et al., 2001; Tanaka et al., 2004).

Among the numerous Rho-GEFs involved in ephrin/Eph signaling, ephexin1 is the best characterized. EphA receptors are thought to regulate growth cone dynamics through ephexin1 in axon guidance (Sahin et al., 2005; Shamah et al., 2001). Activation of RhoA induces growth cone retraction and/or collapse, while activated Rac and Cdc42 promote its extension (Etienne-Manneville and Hall, 2002; Luo, 2000). The engagement of Ephs by ephrin leads to activation of the GEF activity of ephexin1 toward RhoA, thereby causing growth cone collapse in vitro (Shamah et al., 2001). However, as *ephexin1*-knockout (KO) mice are apparently normal (Sahin et al., 2005), the role of ephexin1 remains largely unknown. It is also noteworthy that, compared with the considerable attention given to Rho-GEFs, the possible contribution of Rho-GAPs in actin dynamics controlled by ephrin/Eph signaling has largely been neglected.

The roles of ephrin/Eph signaling *in vivo* have been studied using mouse reverse genetics. An extremely well-characterized case is ephrinB3 \rightarrow EphA4 forward signaling. *EphrinB3*^{-/-} and *EphA4*^{-/-} mice both display several neural phenotypes, including a rabbit-like hopping gait and impairment of two major motor circuits: the corticospinal tract (CST) and the spinal neuronal circuit controlling locomotion, which is also known as the central pattern generator (CPG). Similar phenotypes are also displayed by *EphA4*^{KD/KD} and *EphA4*^{FF/FF} mice, both of which have impaired kinase activity of EphA4, but not by mice expressing a truncated form of ephrinB3 lacking its cytoplasmic domain (Dottori et al., 1998; Kullander et al., 2003, 2001a, 2001b; Yokoyama et al., 2001). Thus, it is apparent that ephrinB3 \rightarrow EphA4 forward signaling, but not EphA4 \rightarrow ephrinB3 reverse signaling, is essential for the formation of these motor circuits.

CST axons controlling voluntary movements arise in the motor cortex, cross into the contralateral side of the medulla, and enter the spinal cord (Gianino et al., 1999; Liang et al., 1991). In wild-type (WT) mice, they rarely cross back to the other side in the spinal cord because ephrinB3 is anchored at the midline and generates repulsive signals through EphA4 receptors expressed on the surface of the CST axon membranes. In *ephrinB3* or *EphA4* mutant mice, due to a lack of repulsive ephrinB3/EphA4 forward signaling, many CST axons fail to stop at the midline and recross it (Kullander et al., 2001a; Yokoyama et al., 2001). Spinal CPGs are thought to generate the repetitive sequential stepping of limbs during walking (Grillner and Wallen, 1985). Locomotor-like rhythmic activity, alternating between the left and right sides, can be evoked in the isolated spinal cords of WT mice (Nishimaru and Kudo, 2000), whereas the activity of the two sides is synchronous in the spinal cords of *ephrinB3*^{-/-} or *EphA4*^{-/-} mice (Kullander et al., 2003). Aberrant midline crossing of axons from EphA4-expressing CPG interneurons is thought to be responsible for the CPG abnormality in *ephrinB3*^{-/-} and *EphA4*^{-/-} mice (Kiehn and Kullander, 2004; Kullander et al., 2003).

The current study provides both *in vivo* and *in vitro* evidence that the Rac-specific GAP α -chimerin plays a critical role in the formation of CST and CPGs as an essential downstream component of ephrinB3/EphA4 forward signaling.

RESULTS

A Novel Spontaneous Autosomal Recessive Mutation leading to a Rabbit-like Hopping Gait

We unexpectedly discovered mutant mice with a rabbit-like gait while generating mice homozygous for a ChAT-Cre#23 construct (Inoue et al., 2003). This mutation, which we designated as *miffy* (*mfy*), was autosomal recessive. We initially assumed that *mfy* was caused by gene disruption during transgene integration. However, this was not the case because the *mfy* locus segregated from the transgene, suggesting that the mutation arose spontane-

ously. *mfy/mfy* mice appeared in crosses at the expected Mendelian frequency and were healthy and fertile. Their bodies were slightly smaller than their littermate controls during the postnatal developmental period. For instance, on postnatal day 10 (P10), the average body weights \pm standard error of the mean (SEM) were 4.68 ± 0.17 g, 5.68 ± 0.17 g, and 5.80 ± 0.15 g for the *mfy/mfy* ($n = 10$), *mfy/+* ($n = 12$), and $+/+$ ($n = 5$) pups, respectively. However, these differences were not evident in adulthood.

WT and *mfy/+* mice moved with a normal alternate step gait. By contrast, all of the *mfy/mfy* mice ($n > 100$) tended to move their left and right hind limbs synchronously, resulting in a rabbit-like hopping gait (Figure 1A). The mean \pm SEM proportion of left-right synchronized gaits among 40 randomly selected gaits was 0 for *mfy/+* ($n = 13$) and $98.42\% \pm 0.61\%$ for *mfy/mfy* ($n = 19$) mice ($p < 0.0001$, unpaired *t* test). The gross morphology of the *mfy/mfy* mouse brain was indistinguishable from that of its littermate controls (WT and *mfy/+* mice; Figures S1A and S1B). By contrast, a morphological analysis of cross-sections of the spinal cord of *mfy/mfy* mice revealed that the white matter in the dorsal funiculus was reduced, most prominently at the lumbar levels (Figure S1C).

Aberrant Recrossing of CST Axons at the Midline of the Spinal Cord in *mfy/mfy* Mice

The CST axons control voluntary movements through direct or indirect contact with spinal motor neurons (Liang et al., 1991). To visualize these axons, we injected anterograde tracer into the left motor cortex and stained the projections of the CST axons in the medulla and spinal cord. CST axons arising in the left motor cortex crossed to the right side of the medulla in both *mfy/mfy* and control mice (Figure S1D). However, in the spinal cord of control mice ($n = 11$), CST axons projected only into the contralateral (right) gray matter and barely recrossed the midline into the ipsilateral gray matter, whereas we observed aberrant midline recrossing of these axons in all of the preparations of *mfy/mfy* mouse spinal cords examined ($n = 7$; Figures 1B and 1C).

To confirm the anterograde tracing results, we injected a retrograde tracer unilaterally into the lumbar spinal cord (Figure 1D). In WT mice, most of the retrograde-labeled CST neurons were located in layer V of the contralateral motor cortex (Figure 1E). However, in *mfy/mfy* mice, the ipsilateral cortex also contained many labeled neurons (Figure 1F). Cell-count analyses of the motor cortex revealed that the ipsilateral cortex of WT ($n = 4$) and *mfy/mfy* ($n = 7$) mice contained $2.02\% \pm 0.92\%$ and $34.26\% \pm 5.22\%$ times as many labeled cells as the contralateral cortex, respectively, which was statistically significant ($p < 0.002$; Figure 1G).

Abnormal CPGs in the Spinal Cord of *mfy/mfy* Mice

To examine the locomotor CPGs underlying limb movements during walking, we isolated the spinal cords from newborn (P1–P3) *mfy/mfy* and control (*mfy/+* and WT) mice and induced locomotor-like activity by applying

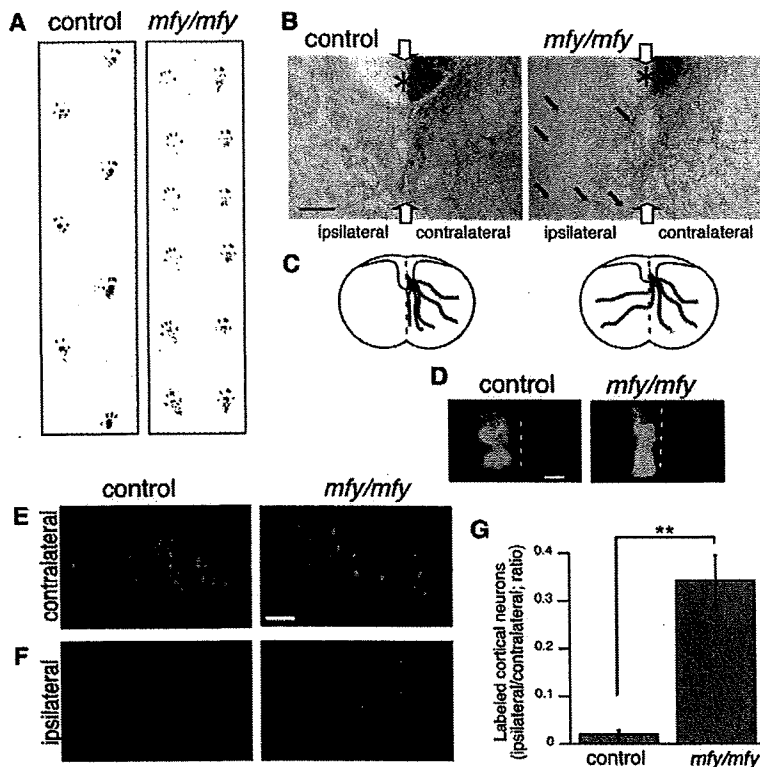


Figure 1. Abnormal Walking and CST Axon Guidance in the Novel Spontaneous Mutant *mfy*

(A) Representative hind-limb footprint patterns. Hind limbs were painted with black ink, and the mice were placed on white paper.

(B) Anterograde tracings of CST axons by biotinylated dextran amine (BDA) and sectioning at cervical levels of the spinal cord. CST axons positioned within the dorsal funiculus (asterisk) on the contralateral side to the tracer injection projected into the spinal gray matter in both control and *mfy/mfy* mice; however, in the *mfy/mfy* mice only, many of the CST axons (blue arrows) recrossed the midline (white arrows).

(C) Schematics of CST axons (red) in the spinal cord.

(D) A fluorescent retrograde tracer cholera toxin B (CTB) was unilaterally injected into the lumbar spinal cord.

(E and F) In the motor cortex layer V of control (WT) mice, most of the labeled neurons were located in the contralateral side (E). In *mfy/mfy* mice, the ipsilateral side also contained many labeled neurons (F).

(G) The ipsilateral/contralateral ratio of numbers of labeled cortical neurons in *mfy/mfy* mice ($n = 7$) was significantly higher than that of control mice ($n = 4$). Data are represented as the mean \pm SEM: Student's *t* test, $p < 0.002$. Scale bars: 100 μ m.

N-methyl-D-aspartate (NMDA) and serotonin. The ventral root (VR) activity of lumbar 2 (L2) represents flexor muscle activity during locomotion, while that of L5 represents extensor activity (Whelan et al., 2000). In control mice, we observed alternation between the left and right L2s (Figures 2A–2C), whereas the cords of *mfy/mfy* mice displayed a synchronous rhythm of the left and right L2s (Figures 2A–2C). The rhythmic activity of the flexors (L2) and extensors (L5) of each limb alternated in both control and *mfy/mfy* mice spinal cords (Figures 2A–2C). We injected a tracer unilaterally into the ventral side of the spinal cords of P4 *mfy/mfy* and control pups, and we found that more neuronal fibers, presumably derived from ipsilateral-projecting interneurons, crossed the midline in the *mfy/mfy* spinal cords than in the control spinal cords (Figure 2D; for quantitative analyses, see Figures S2A and S2B).

The *mfy* Locus Encodes the Rac-Specific GAP α -Chimerin

To locate the *mfy* locus, we employed microsatellites and single-nucleotide polymorphisms (SNPs) that distinguish between the alleles of two inbred mouse strains, DBA/2 (DBA) and C57BL/6 (B6). The *mfy* mutant was identified and maintained in a pure B6 genetic background. We therefore crossed *mfy/mfy* mice with WT DBA mice and obtained *mfy/+* mice in a B6/DBA F_1 genetic background. By backcrossing these F_1 mice to *mfy/mfy* mice and genotyping the DNA of the 299 backcross progeny, we mapped the *mfy* locus to the region between SNPs

rs13476571 and *rs13459064* on chromosome 2 (Figure S3).

In total, 30 genes are known within this 3.27 Mb interval (Table S1). Among these, 10 genes could be excluded because their KO mouse lines have been previously reported to survive to adulthood, and they do not appear to have hopping gaits (for references, see Table S1). We compared the sizes and transcript levels of the remaining 20 genes in the brains of WT and *mfy/mfy* mice at P5 using the reverse transcription-polymerase chain reaction (RT-PCR) with primer sets amplifying the complementary DNA (cDNA) between the 5' and 3' untranslated regions (UTRs) and found that the transcripts of only one gene, the α -chimerin (α -*Chn*) gene, differed in size (Figures 3A and 3B; Table S1). α -chimerin is a Rho-GAP that is specific for the positive regulator of actin polymerization, Rac (Diekmann et al., 1991; Hall et al., 1990, 1993). Recent reports using overexpression and/or small-interfering RNA (siRNA)-mediated knockdown in cultured neurons and/or tissue slices suggest that α -chimerin is involved in regulating dendritic morphology and spine density (Buttery et al., 2006; Van de Ven et al., 2005) as well as semaphorin3A-induced growth cone collapse (Brown et al., 2004). However, the role of α -chimerin in living animals has not yet been defined.

α -*Chn* has two splice isoforms: $\alpha 1$ and $\alpha 2$ (Hall et al., 1990, 1993). A comparison of the sequences of the $\alpha 1$ -*Chn* and $\alpha 2$ -*Chn* cDNAs of WT and *mfy/mfy* mice revealed that exon 9 (174 base pairs [bp]) was deleted in the

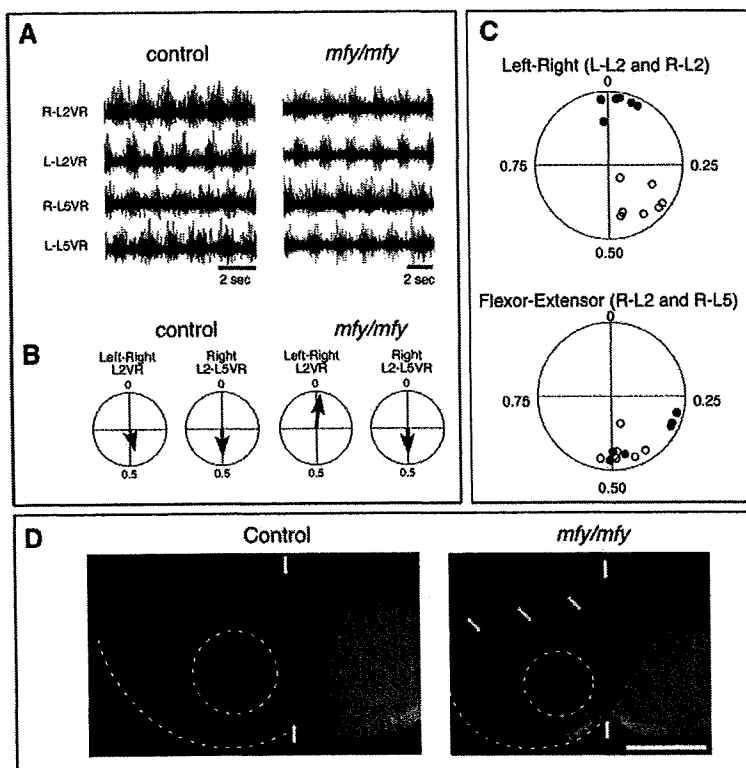


Figure 2. Synchronous Left-Right VR Activity in the Lumbar Spinal Cord of *mfy/mfy* Mice

(A) Locomotor-like motor activity recorded after bath-application of NMDA and serotonin to isolated spinal cords of control and *mfy/mfy* mice. VR activity of the second (L2) and fifth (L5) lumbar segments on the left (L-) and right (R-) sides was recorded using glass-suction electrodes.

(B) Circular phase diagrams for the locomotor-like rhythmic activity of the control and *mfy/mfy* mice shown in (A). Phase relationships between L-L2VR and R-L2VR (left panel) and flexor (R-L2VR) and extensor (R-L5VR) on one side of the lumbar cord (right panel) are indicated in each diagram derived from 30 locomotor cycles. Locomotor cycles in which the two VR activities are in complete alternation have phase values of 0.5. Those that are completely synchronous have phase values of 0 or 1. The mean phase and the *r* value, which describes the concentration of phase values around the mean, are shown by the direction and magnitude, respectively, of the vector originating from the center of the circle.

(C) Summary plots of seven control (open circles) and six *mfy/mfy* (filled circles) mice at P1–P3.

(D) Transverse spinal cord sections (100 μ m thick) at the L2 level after unilateral application of Dil on the ventral side of L4. More neuronal fibers (yellow arrows), which are likely to be derived from ipsilateral-projecting interneurons, crossed the midline (white arrows) to the contralateral (left) side in *mfy/mfy* spinal cords than in control spinal cords. The cell bodies of descending commissural interneurons, shown by dotted circles, were indistinguishable. Scale bar: 500 μ m.

$\alpha 1$ -*Chn*^{*mfy*} and $\alpha 2$ -*Chn*^{*mfy*} transcripts (Figure 3A). Exon 9 encodes 58 amino acids, three of which (EIE) are known to be essential for the GAP activity of α -chimerin that inactivates Rac (Ahmed et al., 1994); $\alpha 2$ -chimerin^{*mfy*} was indeed found to lack Rac-GAP activity in vitro (Figure S4). In addition to the $\alpha 1$ and $\alpha 2$ isoforms, we found transcripts of a putative novel isoform that we termed $\alpha 3$ (Figures 3A and 3B). In the $\alpha 3$ -*Chn*^{*mfy*} transcript, four nucleotides (GATG) of exon 9, including the putative initiation codon, were replaced with retroposon sequences, and intron 9 failed to be excised (Figure 3A). The cloning and sequencing of the genomic DNA of the *mfy* allele revealed an insertion of a retroposon into exon 9, which appeared to impair both the donor and the acceptor splicing functions (Figure 3A). Quantitative RT-PCR demonstrated strong $\alpha 2$ -*Chn* expression, weak $\alpha 1$ -*Chn* expression, and little $\alpha 3$ -*Chn* expression in the motor cortex and spinal cord of P4 WT mice (Figure 3C). We therefore focused on the $\alpha 2$ isoform in subsequent studies. We raised an $\alpha 2$ -chimerin-specific polyclonal antibody and confirmed by western blot analysis that the $\alpha 2$ -chimerin^{WT} protein could not be detected in the *mfy/mfy* brain (Figure 3D). Further-

more, even the $\alpha 2$ -chimerin^{*mfy*} protein was barely detected in the *mfy/mfy* brain and spinal cord (Figures 3D and 3E), suggesting that endogenous $\alpha 2$ -chimerin^{*mfy*} is much less stable in neurons than $\alpha 2$ -chimerin^{*mfy*} overexpressed in cultured cells (Figure S4B).

Improved Locomotor Behavior of *mfy/mfy* Mice Expressing Transgenic α -*Chn*

To confirm that α -*Chn* was the *mfy* gene, we tested whether transgenic (Tg) expression of α -*Chn* rescued the *mfy* phenotype. We modified a bacterial artificial chromosome (BAC) clone that covered the 49 kb upstream region and exons 1–7 of α -*Chn* using Red/ET homologous recombination and flip/FRT recombination in order to make a BAC α -*Chn* construct in which exon 7 was followed by a cDNA encoding exons 8–13 and a poly(A) signal (Figure 4A). We then generated two lines (#539 and #883) of BAC Tg mice by microinjecting the construct into pronuclei and mated them with *mfy/mfy* mice to obtain Tg mice in the *mfy/mfy* background (Figure 4B). There were no improvements in the gaits of the Tg#539:*mfy/mfy* mice (see Figure S5 legend), consistent with no detectable

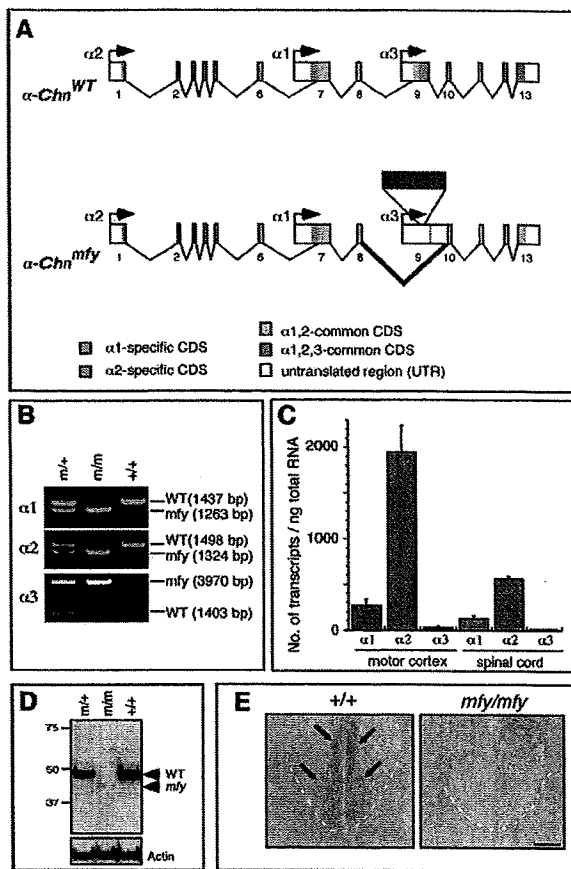


Figure 3. The *mfy* Locus Encodes α -Chimerin Rac-GAP

(A) Schematic exon-intron structures of $\alpha 1$ -*Chn*, $\alpha 2$ -*Chn*, and $\alpha 3$ -*Chn* splicing variants for the WT (α -*Chn*^{WT}) and *mfy* (α -*Chn*^{mfy}) alleles. Retroposon (Tr) insertion into exon 9 in the *mfy* mutant resulted in deletion (red lines) of exon 9 (174 bp) in the $\alpha 1$ and $\alpha 2$ transcripts, and deletion of four nucleotides (including the putative initiation codon) and failure of intron 9 splicing in the $\alpha 3$ transcript. CDS: coding sequences.

(B) RT-PCR products from between the 5' UTR and the 3' UTR of the $\alpha 1$ -*Chn*, $\alpha 2$ -*Chn*, and $\alpha 3$ -*Chn* splicing isoforms in cDNAs derived from WT (+/+), *mfy*+/+ (m/+), and *mfy*/*mfy* (m/m) brains at P5. PCR products from the WT and mutant (*mfy*) $\alpha 1$ -*Chn*, $\alpha 2$ -*Chn*, and $\alpha 3$ -*Chn* isoforms were purified and cloned, and their nucleotide sequences were determined.

(C) $\alpha 2$ -*Chn* predominates in the developing motor cortex and spinal cord. Real-time quantitative RT-PCR of $\alpha 1$ -*Chn*, $\alpha 2$ -*Chn*, and $\alpha 3$ -*Chn* in the motor cortex and spinal cord of WT mice at P4. All data are presented as the mean \pm SEM (n = 3 mice).

(D) Western blot using an $\alpha 2$ -chimerin-specific antibody revealed that $\alpha 2$ -chimerin^{WT} protein (WT) was absent from *mfy*/*mfy* mice. Unexpectedly the mutant protein (*mfy*) was barely detectable in *mfy*+/+ and *mfy*/*mfy* mice. Total lysates from P10 of the mouse telencephalon were used.

(E) Immunohistochemistry using $\alpha 2$ -chimerin-specific antibody revealed strong expression of $\alpha 2$ -chimerin in the CST (arrows) of the dorsal funiculus (dotted line) of the WT spinal cord, suggesting that $\alpha 2$ -chimerin functions in developing CST axons. α -chimerin was not detectable in *mfy*/*mfy* spinal cords. Scale bar: 50 μ m.

α -chimerin expression in these mice (Figures S5A and S5B). By contrast, considerable improvement was observed in the gaits of the Tg#883:*mfy*/*mfy* mice (Figure 4C). The average \pm SEM proportion of left-right synchronized gaits in 40 randomly selected gaits of Tg#883:*mfy*/*mfy* mice (n = 13) was 53.65% \pm 6.56%, which was substantially less than in their littermate *mfy*/*mfy* mice (n = 6, 99.58% \pm 0.42%; p < 0.0001, unpaired t test). α -chimerin protein was expressed at low levels in Tg#883:*mfy*/*mfy* mice (Figures S5A–S5C), which was consistent with the moderate rescue of gait observed in these mice. These results strongly suggest that α -*Chn* is the causal gene of the *mfy* mutation.

Generation and Characterization of α -*Chn* KO Mice

To further confirm that the *mfy* gene encoded α -*Chn*, we deleted exons 9 and 10 from the allele by a gene-targeting technique in embryonic stem (ES) cells and Cre/loxP recombination in the mouse germline and generated mice homozygous for the targeted allele (α -*Chn* KO mice; Figures 4D and S5D). These mice had a hopping gait similar to that of *mfy*/*mfy* mice (Figure 4E). They also demonstrated aberrant midline recrossing of CST axons (Figure 4F), aberrant midline crossing of spinal local circuit neurons (Figure S2C), and shorter ventral extension of the dorsal funiculus in the spinal cord (data not shown). Furthermore, they showed abnormal spinal CPGs (Figures 4G and S6). All of these phenotypes were similar to those of *mfy*/*mfy* mice. Thus, we concluded that the *mfy* phenotypes were caused by α -*Chn* disruption.

Localization of α -Chimerin Proteins in CST

The phenotypes of *mfy*/*mfy* and α -*Chn* KO mice (hopping gait, Figures 1A and 4E), abnormal spinal-cord morphology (Figure S1C), abnormal CPGs (Figures 2C and 4G), aberrant midline crossing by CPG axons (Figures 2D and S2), and aberrant midline recrossing by CST axons (Figures 1B and 4F) appeared to be identical to those reported for *ephrinB3*^{-/-} and *EphA4*^{-/-} mice (Dottori et al., 1998; Kullander et al., 2003, 2001a; Yokoyama et al., 2001) and for *EphA4*^{FFIFF} and *EphA4*^{KDIKD} mice expressing a mutant EphA4 lacking kinase activity (Kullander et al., 2001b). Nevertheless, the anterior commissure, the formation of which is known to be dependent on EphA4 reverse signaling (Kullander et al., 2001b), appeared normal in *mfy*/*mfy* mice (Figure S1B). Hence, it seemed likely that the *mfy* phenotype was caused by impairment of ephrinB3/EphA4 forward signaling.

Using immunohistochemistry, we found that $\alpha 2$ -chimerin colocalized with EphA4 in the developing CST (Figures S7A and S7C). In α -*Chn* KO CST, $\alpha 2$ -chimerin was not detected, but the levels of EphA4 expression appeared to be unaltered (Figures S7B and S7D). We also found that the $\alpha 2$ -chimerin protein was present in the growth cones of cultured neurons derived from the anterior dorsomedial to dorsal neocortex (motor cortex; Figure S8C). These results suggest that α -chimerin functions

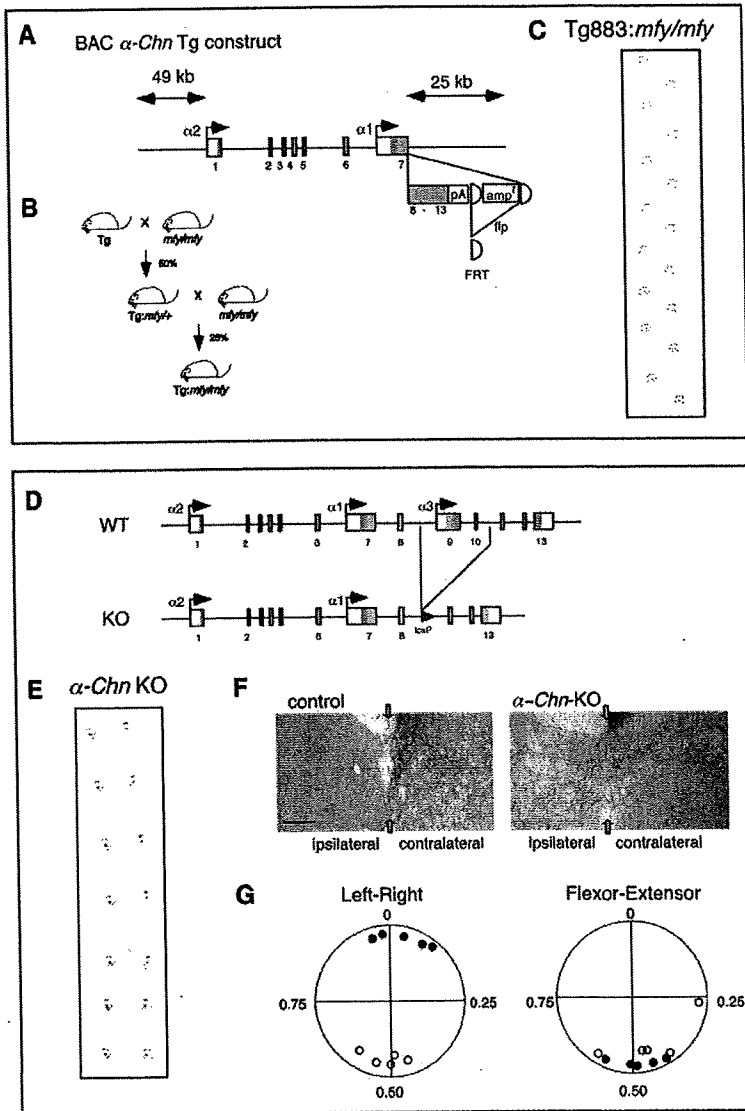


Figure 4. Disruption of α -Chn Is Responsible for the *mfy* Phenotypes

(A) Schematic of the BAC Tg construct. A cDNA fragment containing exons 8–13, a poly(A) signal, and an *amp^r* selection marker flanked by two FRT sites was inserted immediately after exon 7 of a BAC clone covering exons 1–7 of α -Chn, using Red/ET homologous recombination in bacteria. *Amp^r* was removed from the BAC construct by expressing *flp* recombinase in the bacterial clone. (B) Tg mouse lines were generated by injecting the linearized construct into fertilized mouse eggs. Two lines of Tg mice were crossed with *mfy/mfy* mice to yield Tg:*mfy*⁺ mice. These were then crossed with *mfy/mfy* mice to obtain Tg:*mfy/mfy* mice. (C) Improved walking of Tg#883:*mfy/mfy* mice. All Tg#883:*mfy/mfy* mice showed a marked improvement in the control of walking. (D) Schematic of the KO allele, in which exons 9 and 10 were deleted by homologous recombination in ES cells, and Cre/loxP recombination in the mouse germline. (E) Representative hind-limb footprint patterns of α -Chn KO mice showing a hopping gait similar to that of *mfy/mfy* mice. (F) Anterograde tracing of CST axons and sectioning at cervical levels of the spinal cord. CST axons of α -Chn KO mice aberrantly recrossed the spinal cord midline, as seen in *mfy/mfy* mice. Scale bar: 100 μ m. (G) Circular phase diagrams for the locomotor-like activity of five control (open circles) and five α -Chn KO (filled circles) mice at P1–P3. Left-right VR activity in the lumbar spinal cord of α -Chn KO mice was synchronous as that of *mfy/mfy* mice.

as a downstream mediator of ephrinB3/EphA4 forward signaling in developing CST axons.

Interaction between α -Chimerin and EphA4

To test whether α -chimerin interacts with EphA4, we co-transfected 293T cells with EphA4 and α -Chn-expression constructs and immunoprecipitated EphA4 from cell lysates with Fc region-fused ephrinA1. Both α 1-chimerin and α 2-chimerin were precipitated with EphA4^{WT} and kinase-dead EphA4^{FF} (Figure 5A), and α 2-chimerin^{*mfy*} was also precipitated with EphA4 (Figure S9). These results suggest that α -chimerin interacts with EphA4 in vitro and that this interaction does not require the kinase activity of EphA4 or the GAP activity of α -chimerin. To identify the region of α -chimerin responsible for interacting with EphA4, we performed an in vitro glutathione S-transferase (GST) fusion protein pull-down assay. EphA4 was co-

precipitated with full-length α 2-chimerin and the α 1- and α 2-common carboxy (C) terminus, but not with the α 2-specific amino (N)-terminus (Figure 5B). These results show that EphA4 associates with α 1-chimerin and α 2-chimerin at their C termini.

Next we examined whether α -chimerin interacts with EphA4 in neurons, and, if so, whether ligand stimulation enhances the interaction. EphA4 was coprecipitated with α 2-chimerin without ligand stimulation, and the amount of EphA4 precipitated with α 2-chimerin was not increased following stimulation with clustered ephrinB3 (Figure 5C). These results indicate that α -chimerin associates with EphA4 in neurons and that this interaction is independent of ephrinB3-stimulation. Finally, we prepared lysates from the developing motor cortex of WT mice and immunoprecipitated them with anti- α 2-chimerin antibody. EphA4 was again precipitated with α 2-chimerin (Figure 5D).

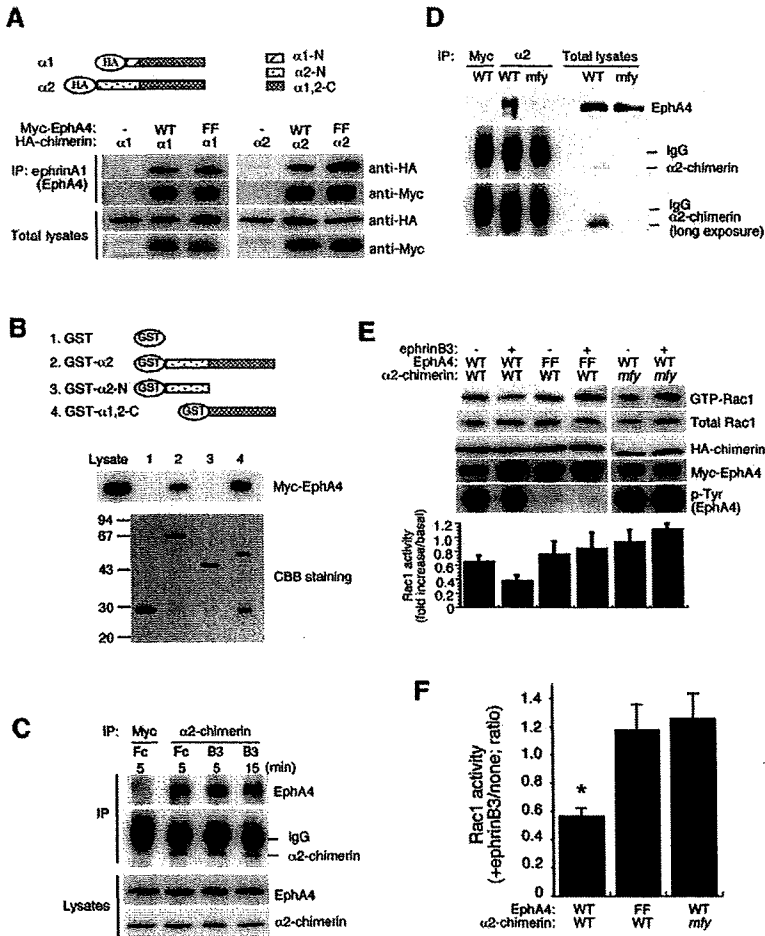


Figure 5. α -Chimerin Interacts with EphA4 and Regulates Rac1 Activity in Response to EphrinB3 \rightarrow EphA4 Forward Signaling

(A) EphA4 bound to α 1-chimerin and α 2-chimerin in vitro. Kinase-dead EphA4^{FF} also bound to both isoforms. 293T cells were transfected with the indicated expression plasmids. Total lysates were precipitated with Fc region-fused ephrinA1 and immunoblotted with anti-HA and anti-Myc antibodies to detect α -chimerin and EphA4, respectively. Total lysates were also blotted with anti-HA and anti-Myc antibodies.

(B) EphA4 interacted with the α -chimerin C terminus that is common to both α 1 and α 2 isoforms. For pulldown assays, HEK293T cells transfected with Myc-tagged EphA4 were lysed, and supernatants were incubated with glutathione-Sepharose beads. Bound proteins were analyzed by SDS-PAGE and immunoblotted with anti-Myc antibody. The lower panel shows Coomassie brilliant blue (CBB) staining of GST-fusion proteins used in this experiment.

(C) Endogenous α -chimerin and EphA4 interacted in neurons, and ephrinB3-stimulation did not enhance this interaction. Cultured cortical neurons from E18.5 rats were stimulated with preclustered ephrinB3-Fc or control Fc for 5 or 15 min. Bound and total proteins were analyzed by immunoblotting with antibodies against EphA4 and α 2-chimerin.

(D) Endogenous α -chimerin and EphA4 interacted in the mouse brain. Lysates from a P3 mouse cortex were immunoprecipitated with anti- α 2-chimerin antibody or anti-Myc antibody and total proteins were analyzed by immunoblotting

with antibodies against EphA4 and α 2-chimerin. The *mfy/mfy* cortex was used as a second negative control. Bound and total proteins were analyzed by immunoblotting

with antibodies against EphA4 and α 2-chimerin. (E) COS-7 cells transfected with the indicated plasmids were stimulated with preclustered ephrinB3-Fc (+) or Fc (-) for 10 min. GTP-bound Rac1 was identified by SDS-PAGE and immunoblotting, and its levels were measured and normalized to the corresponding total Rac1 levels. The means and SEMs of Rac1 activity (fold increase/basal) are shown at the bottom.

(F) Statistical analysis of the data shown in (E). EphrinB3-stimulation inactivated Rac1 in cells expressing both EphA4^{WT} and α -chimerin^{WT}, but not in cells expressing EphA4^{FF} and α -chimerin^{WT} or EphA4^{WT} and α -chimerin^{mfy}. *n* = 4 (EphA4^{WT} + α 2-chimerin^{WT}), 5 (EphA4^{FF} + α 2-chimerin^{WT}), or 3 (EphA4^{WT} + α 2-chimerin^{mfy}). All data are presented as the mean \pm SEM. (**p* < 0.05, ANOVA/Tukey HSD).

α -Chimerin Inactivates Rac in Response to EphrinB3/EphA4 Forward Signaling In Vitro

No previous reports have suggested the involvement of α -chimerin in ephrin/Eph signaling. To determine whether α -chimerin regulates Rac activity in response to ephrinB3/EphA4 forward signaling, we transfected COS-7 cells with EphA4 (EphA4^{WT} or kinase-dead EphA4^{FF}) and α 2-chimerin (α -chimerin^{WT} or α -chimerin^{mfy}) and measured the Rac activity in the presence or absence of ephrinB3 stimulation (Figure 5E). We found that ephrinB3 stimulation inactivated Rac in cells expressing EphA4^{WT} and α -chimerin^{WT}, but not in cells expressing either EphA4^{FF} and α -chimerin^{WT} or EphA4^{WT} and α -chimerin^{mfy} (Figure 5F). These results indicate that both EphA4 kinase activity and functional α -chimerin are required for ephrinB3 stimulation to inactivate Rac. It is noteworthy that the EphA4

proteins overexpressed in COS cells were phosphorylated, and, therefore, kinase active, but were unable to fully activate α -chimerin in the absence of ephrinB3 stimulation (Figure 5E). These results suggest that EphA4 kinase activity alone is not sufficient for activating the Rac-GAP of α -chimerin (see Discussion).

Downregulation of α -Chimerin Suppresses EphrinB3-Induced Growth Cone Collapse in Cultured Cortical Neurons

EphrinB3/EphA4 forward signaling induces growth cone collapse in cultured neurons derived from the motor cortex (Egea et al., 2005; Kullander et al., 2001a). To determine whether α -chimerin regulates growth cone dynamics downstream of ephrinB3/EphA4 forward signaling, we performed two experiments. First, we cultured neurons

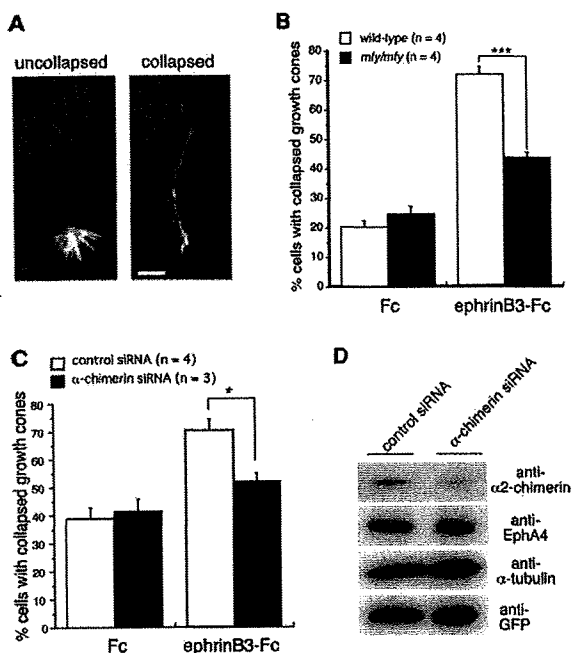


Figure 6. Downregulation of α -Chimerin Suppresses EphrinB3-Induced Growth Cone Collapse

(A) Representative examples of growth cones in phalloidin-stained cultured cortical neurons treated with preclustered Fc control (uncollapsed) or ephrinB3-Fc (collapsed). Scale bar: 10 μ m.

(B) Growth cone collapse was induced by ephrinB3 in cultured neurons derived from the WT motor cortex, but was barely seen in those derived from the *mfy/mfy* cortex. All data are presented as the mean \pm SEM of four independent experiments in each of which 100 neurons were counted (***p* < 0.001, Student's *t* test).

(C) Downregulation of α -chimerin by siRNA suppressed ephrinB3-induced growth cone collapse in cultured cortical neurons. Cultured neurons derived from E18.5 rat cortex were transfected with a plasmid expressing both siRNA and enhanced yellow fluorescent protein (EYFP). In each experiment, 34–67 neurons expressing EYFP were counted. (**p* < 0.05, Student's *t* test).

(D) α -chimerin-specific siRNA reduced the level of α -chimerin protein but not EphA4 protein. A 58.5% reduction of α -chimerin protein was observed in total lysates from cultured neurons in which 62% (not shown) of cells expressed α -chimerin-specific siRNA/EYFP, suggesting a drastic (>90%) reduction of α -chimerin.

derived from the motor cortex of WT and *mfy/mfy* mice on embryonic day 16.5 (E16.5) and then stimulated them with preclustered Fc or ephrinB3-Fc. EphrinB3 efficiently induced growth cone collapse in WT cortical neurons, as previously reported (Kullander et al., 2001a; Figures 6A and 6B); however, in the *mfy/mfy* cortical neurons, the frequency of collapse was significantly reduced (Figure 6B). Second, we expressed α -chimerin-specific siRNA by transfecting an expression vector into cultured neurons derived from the E18.5 rat motor cortex (Figure S8). The frequency of ephrinB3-induced growth cone collapse was significantly reduced in transfected neurons (Figure 6C), and western blotting with the anti- α 2-chimerin antibody demonstrated that α -chimerin protein levels were

drastically reduced (>90%) in the transfected neurons (Figure 6D). We concluded that α -chimerin Rac-GAP acts downstream of ephrinB3/Eph signaling to cause growth cone collapse of cortical neurons.

Taken together, the results of our *in vivo* and *in vitro* studies confirm that α -chimerin Rac-GAP is a key molecule linking ephrinB3-induced EphA4 activation to the inactivation of Rac—a positive regulator of process outgrowth—thereby causing growth cone retraction and, eventually, axonal repulsion at the spinal cord midline.

DISCUSSION

α -Chimerin Is a Key Mediator of EphrinB3/EphA4 Forward Signaling and Causes Repulsion of CST Axons at the Spinal Cord Midline

We have shown the following. First, α -chimerin is colocalized with EphA4 in the developing CST (Figure S7). Second, it interacts with EphA4 both *in vitro* and *in vivo* (Figures 5A–5D). Third, when EphA4 is stimulated by ephrinB3, α -chimerin inactivates Rac, which is a positive regulator of process outgrowth, *in vitro* (Figure 5F). Fourth, ephrinB3-induced growth cone collapse is suppressed in cultured cortical neurons in which α -chimerin is downregulated by RNAi or *mfy* mutation (Figure 6). Fifth and finally, repulsion of CST axons at the spinal cord midline (Figures 1B and 4F) and formation of spinal CPGs (Figures 2C and 4G) are impaired in *mfy/mfy* mice and α -*Chn* KO mice, and both of these processes depend on ephrinB3/EphA4 forward signaling (Kullander et al., 2003, 2001a, 2001b; Yokoyama et al., 2001). Our results show that the Rac-GAP α -chimerin regulates CST axon guidance and CPG formation by mediating ephrinB3/EphA4 forward signaling (Figure 7).

It has been proposed that EphA receptors regulate growth cone dynamics through Rho-GEF ephexin1 (Shamah et al., 2001). During this process, activation of RhoA induces growth cone retraction and/or collapse, while activated Rac and Cdc42 promote its extension (Etienne-Manneville and Hall, 2002; Luo, 2000). The engagement of Ephs by ephrin leads to preferential activation of the exchange activity of ephexin toward RhoA, thus leading to growth cone collapse *in vitro* (Figure 7A; Shamah et al., 2001). We showed that the ephrin/Eph interaction leads to growth cone collapse due to Rac inactivation via the GAP activity of α -chimerin (Figure 7A). As both ephexin1 and α -chimerin are enriched in the central nervous system (Hall et al., 2001, 1993; Shamah et al., 2001), it is likely that the cooperative action of ephexin1-induced RhoA activation and α -chimerin-induced Rac inactivation function to induce retraction of growth cones during axon guidance in various circuits. *Ephexin1* KO mice appear to be normal (Sahin et al., 2005). This absence of an obvious phenotype might be due to compensation by other ephexin family members. Alternatively, it might be due to the activity of α -chimerin, which could prevent growth cone extension by inactivating Rac in response to ephrin/Eph signaling, even in the absence of

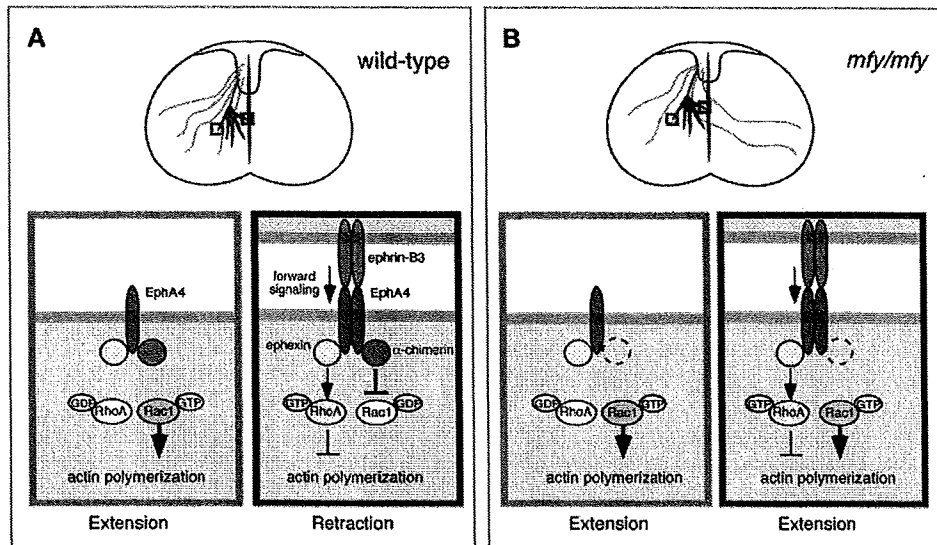


Figure 7. Model of EphrinB3/EphA4 Forward Signaling in Midline Repulsion of CST Axons

(A) In WT mice, the growth cones of CST axons extend due to basal activity of Rac, which is a positive regulator of process outgrowth, in the absence of ephrinB3 stimulation (green box). At the spinal cord midline that anchors ephrinB3, CST axons that express EphA4 receive forward signals. This ephrinB3/EphA4 forward signaling inactivates Rac through α -chimerin, leading to growth cone retraction (red box). Ephrin/EphA signaling might also activate RhoA, which is a negative regulator of process outgrowth, via ephexin (Shamah et al., 2001). The cooperative action of the RhoA-activator ephexin and the Rac-inactivator α -chimerin might induce efficient retraction of growth cones.

(B) In *mfy/mfy* mice, CST axons fail to stop at the midline due to the absence of α -chimerin-induced Rac inactivation, even in the presence of ephexin.

ephexin-induced RhoA activation. In contrast to the absence of an *Ephexin1* KO phenotype, we demonstrated that the formation of CST and CPGs is impaired in α -*Chn* mutant mice.

Critical Role of Rho-GTPase Inactivation in Ephrin/Eph Signaling

Ephrin/Eph signaling is important in a wide range of biological processes, including oocyte maturation, early morphogenesis, segmentation, guidance of migrating cells, synaptic plasticity, dendritic-spine formation, and axon guidance (Flanagan and Vanderhaeghen, 1998; Palmer and Klein, 2003; Pasquale, 2005). Recent studies of *in vitro* orientation suggest that in various biological phenomena, ephrin/Eph signaling regulates actin dynamics by activating Rho-GTPases, such as RhoA, Rac, and Cdc42, through Rho-GEFs (Cowan et al., 2005; Fu et al., 2007; Irie and Yamaguchi, 2002; Murai and Pasquale, 2005; Ogita et al., 2003; Penzes et al., 2003; Shamah et al., 2001; Tanaka et al., 2004). We suggest that a further mode of ephrin/Eph signaling exists, in which Ephs regulate actin dynamics by inactivating Rho-GTPases through Rho-GAPs.

Ephrin/Eph signaling might control actin dynamics by regulating the balance between negative regulators of actin polymerization (such as RhoA) and positive regulators (such as Rac and Cdc42; Sahin et al., 2005; Shamah et al., 2001). This theory only takes into account Rho-activation; however, we have now shown that Rho-inactivation

is another important aspect of the ephrin/Eph regulation of actin dynamics. The activity of each Rho-GTPase might be controlled by the balance between its activation by Rho-GEFs and its inactivation by Rho-GAPs. In mammalian cells, there are many diverse Rho-GEFs and Rho-GAPs with different substrate specificities that are controlled by different mechanisms (Etienne-Manneville and Hall, 2002). Thus, it is possible that ephrin/Eph signaling in different processes employs specific Rho-GEFs and/or Rho-GAPs to achieve the appropriate balance of activity of each Rho-GTPase. In short, we propose that ephrin/Eph signaling regulates actin dynamics in two ways: first, by regulating the balance between the activities of different Rho-GTPases (such as RhoA and Rac or Cdc42), and second, by regulating the balance between activation and inactivation of each individual Rho-GTPase.

Regulation and Roles of α -Chimerin in Neural Development and Function

The present study provides evidence for the involvement of α -chimerin in ephrinB3/EphA4 forward signaling. How does ephrinB3/EphA4 signaling activate α -chimerin? As the kinase-inactive EphA4^{FF} mutant protein could not activate α -chimerin (Figure 5F), it appeared that the kinase activity of EphA4 was necessary for the activation of α -chimerin. However, EphA4 kinase activity alone did not appear to be sufficient as the EphA4 proteins over-expressed in COS cells were phosphorylated and, thus,

kinase active, but were unable to fully activate α -chimerin in the absence of ephrinB3 stimulation (Figures 5E and 5F). In fact, it has been shown that the kinase activity of EphA4 alone is not sufficient for ephrinB/EphA4 forward signaling in vivo: the *EphA4^{EE/EE}* mouse, the kinase of which is constitutively activated, has a normal alternate gait, normal CST axon midline guidance, normal CPG midline guidance, and normal morphology of the spinal cord (Egea et al., 2005). Cultured cortical neurons derived from *EphA4^{EE/EE}* mice undergo normal growth cone collapse in response to clustered ephrinB3. Based on these observations, it has been proposed that higher-order clustering of EphA4 by stimulation of ephrinB is an essential component of ephrinB/EphA4 forward signaling (Egea et al., 2005). Our results are consistent with this idea.

Both the $\alpha 1$ and $\alpha 2$ isoforms of α -chimerin have a single copy of the C1 domain, which is a cysteine-rich motif, and can be activated by the binding of phorbol esters or diacylglycerol (DAG) to the C1 domain (Hall et al., 1990, 1993). Recently, it was reported that cyclin-dependent kinase 5 (Cdk5) regulates EphA4-mediated dendritic-spine retraction (Fu et al., 2007). Cdk5 is known to interact with $\alpha 2$ -chimerin (Qi et al., 2004), so it is possible that a protein complex formed by clustering of EphA4 recruits some additional molecules required for linking activated EphA4 to α -chimerin activation. Such additional factors could include Cdk5/p35 and DAG-producing enzymes, such as phospholipase C γ . It should be noted that these (or equivalent) factors do not appear to be specific to neurons, but rather are ubiquitous, as the expression of EphA4 and $\alpha 2$ isoforms in COS cells was sufficient to inactivate Rac upon stimulation by clustered-ephrinB3 (Figures 5E and 5F).

The *α -Chn* gene is widely expressed in the central nervous system both during development and in adulthood (Hall et al., 2001, 1990, 1993), and α -chimerin is found not only in axons but also in neuronal dendrites (Hall et al., 2001). It would be interesting to test whether α -chimerin plays a role in dendrite development in the brain. Recent studies using overexpression and/or siRNA-induced knockdown of $\alpha 1$ -chimerin in cultured hippocampal neurons and cerebellar slices suggest that it regulates dendritic morphology and dendritic-spine density (Buttery et al., 2006; Van de Ven et al., 2005). α -chimerin might also be involved in NMDA receptor-dependent developmental plasticity, such as maturation of the barrel cortex (Iwasato et al., 2000, 1997), as well as in learning and memory, as it has been shown to interact with NMDA receptors in vitro (Van de Ven et al., 2005). The *mfy/mfy* mouse is thus a promising experimental model for elucidating the roles of α -chimerin Rac-GAP in the development and function of the central nervous system.

EXPERIMENTAL PROCEDURES

Animals

A Tg construct was made by modifying the RP23-413N9 BAC clone derived from B6 mouse genomic DNA (Roswell Park Cancer Institute,

NY, USA). The Tg founder mice were generated by microinjection of linearized constructs into fertilized eggs. *α -Chn* KO mice were generated using MS12 ES cells derived from the B6 strain (for details see the Supplemental Experimental Procedures). All of the mice were maintained according to the institutional guidelines of the animal facilities of the RIKEN-BSI.

VR Recordings

P1–P3 mice were anesthetized with isoflurane, and their spinal cords were removed as described elsewhere (Nishimaru et al., 2006). Electrical recordings of the VRs were made with glass-suction electrodes, and locomotor-like rhythmic activity was evoked by the concomitant bath application of NMDA (4–7 μ M) and serotonin (5HT; 4–7 μ M). Details of the recording procedure and data analyses are provided in the Supplemental Data.

Linkage Analyses

PCR primers for the microsatellite markers and SNPs were designed using the Mouse Genome Informatics (<http://www.informatics.jax.org/>) and National Centre for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/SNP/>) databases, respectively, and were verified by PCR using the genomic DNAs of inbred B6 and DBA/2 mice and B6/DBA F₁ hybrids (for detailed methods, see Figure S3 and Supplemental Data).

Generation of Antibody

KLH-coupled synthetic peptides (MALTLFDTDEYRPPVWKC) corresponding to the N terminus of $\alpha 2$ -chimerin (Figure S4A) were used to raise a rabbit polyclonal antibody (BSI Research Resources Center). Sera were affinity purified on the same peptides.

Measurement of Rac1 Activity

Transfected COS-7 cells were stimulated with 2 μ g/ml preclustered ephrinB3-Fc (R&D Systems) in serum-free medium for 10 min. The cells were lysed with ice-cold cell lysis buffer containing 4 μ g of GST-CRIB of α Pak (amino acids 70–150). After centrifugation, the supernatant was incubated with glutathione-Sepharose beads, and bound proteins were analyzed by SDS-PAGE and immunoblotting (for detailed methods, see Supplemental Data).

Growth Cone Collapse Assay

Primary neurons were dissociated from the anterior dorsomedial to dorsal neocortex of E16.5 mice or E18.5 rats as described elsewhere (Ishikawa et al., 2003; Kullander et al., 2001a). The neurons were plated onto poly-D-lysine and laminin-coated coverslips and cultured in Neurobasal medium supplemented with 2% B27 (Invitrogen). They were then stimulated with 5 μ g/ml preclustered ephrinB3-Fc for 30 min, fixed with 4% paraformaldehyde, and stained with rhodamine-conjugated phalloidin (Invitrogen; for detailed methods, see Supplemental Data).

Supplemental Data

Supplemental Data include Experimental Procedures, References, nine figures, and one table and can be found with this article online at <http://www.cell.com/cgi/content/full/130/4/742/DC1/>.

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Accession Numbers

The DNA sequences have been deposited in the DNA Data Bank of Japan (DDBJ) under the following accession numbers: AB264771 ($\alpha1$ -*Chn*^{my} cDNA), AB264772 ($\alpha2$ -*Chn*^{my} cDNA), AB264773 ($\alpha3$ -*Chn*^{my} cDNA), AB264774 ($\alpha3$ -*Chn*^{WT} cDNA), and AB264775 (retroposon).



Pael receptor is involved in dopamine metabolism in the nigrostriatal system

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Abstract

Pael receptor (Pael-R) has been identified as one of the substrates of Parkin, a ubiquitin ligase responsible for autosomal recessive juvenile Parkinsonism (AR-JP). When Parkin is inactivated, unfolded Pael-R accumulates in the endoplasmic reticulum and results in neuronal death by unfolded protein stress, suggesting that Pael-R has an important role in the pathogenesis of AR-JP. Here we report the analyses on Pael-R-deficient (KO) and Pael-R-transgenic (Tg) mice. The striatal dopamine (DA) level of Pael-R KO mice was only 60% of that in normal mice, while in Pael-R Tg mice, striatal 3,4-dihydroxyphenylacetic acid (DOPAC) as well as vesicular DA content increased. Moreover, the nigrostriatal dopaminergic neurons of Pael-R Tg mice are more vulnerable to Parkinson's disease-related neurotoxins while those of Pael-R KO mice are less. These results strongly suggest that the Pael-R signal regulates the amount of DA in the dopaminergic neurons and that excessive Pael-R expression renders dopaminergic neurons susceptible to chronic DA toxicity.

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Keywords: Parkinson's disease; Parkin; Striatum; Substantia nigra; AR-JP; GPR37; MPTP; 6-OHDA

1. Introduction

DA is one of the important neurotransmitters of the central nervous system (CNS). Animal studies reveal that increasing synaptic DA concentration by inhibiting DA transporters or blocking DA autoreceptors stimulates locomotion and exploratory behavior. Conversely, blocking DA receptors attenuates the effects of food rewards, intracranial self-stimulation, and psychomotor stimulants. DA-deficient animals show severe adipsia, aphagia, hypoactivity, and impaired complicated motor behaviors (Zhou and Palmiter, 1995). About 75% of the dopaminergic neurons in the brain have their cell bodies in the substantia nigra (SN) of the basal ganglia and project to

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the striatum. Progressive loss of this population underlies Parkinson's disease (PD) in humans (Betarbet et al., 2002). Most cases of PD are sporadic and the etiology of common PD remains unknown. However, recent identification of gene mutations in familial cases of PD has advanced our understanding of the molecular mechanisms behind the neurodegeneration associated with this disease.

A major cause of juvenile PD, autosomal recessive juvenile Parkinsonism (AR-JP), results from mutations in the Parkin gene (Kitada et al., 1998). Parkin protein is a RING-finger type ubiquitin ligase (E3) and AR-JP-linked Parkin mutants are defective in E3 activity (Imai et al., 2000; Shimura et al., 2000; Zhang et al., 2000). The accumulation of unfolded Pael-R, a substrate for Parkin recently identified in our laboratory, causes unfolded protein stress, which results in cell death *in vivo* and *in vitro* (Imai et al., 2001; Yang et al., 2003). Parkin eliminates unfolded Pael-R in cooperation with the molecular chaperone Hsp70 and a U box protein known as CHIP (Imai et al., 2002). Pael-R, which is abundantly expressed in dopaminergic neurons of the SN, other regions (such as the hippocampus), and some nuclei of the brainstem and in oligodendrocytes, is a putative G protein coupled receptor whose ligand is unknown (Donohue et al., 1998; Imai et al., 2001) and whose physiological function in the brain remains unclear.

Here we provide genetic evidence in mice that Pael-R is involved in the regulation of nigrostriatal dopamine metabolism and propose a hypothesis of DA neuron-specific pathological mechanism by Pael-R expression.

2. Materials and methods

2.1. Targeted disruption of the mouse Pael-R gene

Pael-R KO mouse was generated by standard technique of gene targeting (Gomi et al., 1995). A targeting vector was constructed using 17.5-kb genomic DNA fragments containing exon 1 of Pael-R gene (Fig. 1C). Floxed pgk-neo cassette and loxP sequence were inserted at the sites of the intron 1 and untranslated region of exon 1, respectively. DT-Apa cassette was flanked at 5' end of homologous arm for negative selection (Yanagawa et al., 1999). The linearized targeting vector was transfected into MS12 (C57BL/6J [B6]) ES cells. Positive clones were selected by Southern blots analysis, and then injected into Balb/c blastocysts. Offsprings harboring targeted allele were generated from the crossing of chimera mice with B6 mice and confirmed by Southern blots analysis. To generate a null allele, B6 eggs *in vitro* fertilized by F1 sperm were microinjected with circular plasmid DNA containing the Cre recombinase gene as described previously (Sunaga et al., 1997). After the confirmation of null allele by Southern blots analysis, heterozygous mice harboring null allele were intercrossed for obtaining the homozygous mice as Pael-R KO mice.

2.2. Generation of Pael-R Tg mice

Human platelet-derived growth factor β 2 promoter-driven human Pael-R transgenic (PDGF β -Tg) mice, and mouse prion protein promoter-driven Pael-R Tg (PrP-Tg) mice were generated using the human PDGF β 2 vector (Chui et al., 1999) (kindly provided by Drs. Tanahashi H. and Tabira T., National Institute of Neuroscience, Japan), and MoPrP vector (Borchelt et al., 1996) (kindly provided by Drs. Borchelt D.R. and Fromholt D., Johns Hopkins University). The NotI fragments from the PDGF β -Pael-R and the PrP-Pael-R plasmids were microinjected into B6 and C3H/B6 mouse eggs, respectively. Potential founders were identified using Southern blot analysis and PCR. Stable Tg lines were established by breeding founders to B6 mice (for PDGF β -Tg) and C3H/B6 mice (for PrP-Tg).

2.3. Antibodies

Anti-Pael-R (#202) and anti-LP-2 (#23) monoclonal antibodies (mAbs) were raised in mice against the C-terminal 61 amino acids of human Pael-R and the C-terminal 62 amino acids of human ET_BR-LP2 (LP-2), respectively, expressed in bacteria. Anti-TH (MAB318) and anti-actin (MAB1501R) Abs were purchased from Chemicon. Anti-transferrin receptor (H68.4) and anti-NSE (BBS/NC/VI-H14) Abs were obtained from Zymed and Dako, respectively.

2.4. Western blots and immunohistochemistry

A crude homogenate was prepared by adding 10 μ l of ice-cold buffer A (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 5 mM EDTA containing Complete Protease Inhibitors [Roche Diagnostics])/mg of brain tissue and using a Dounce homogenizer with a tight pestle (40 strokes). The suspension was fractionated by centrifugation at 1000 \times g for 5 min and then by ultracentrifugation at 165,000 \times g for 60 min. The pellet fraction was washed three times with buffer A, and then extracted by shaking in 5 μ l of buffer B (buffer A containing 1% Triton X-100 and 1% SDS)/mg of brain tissue for 60 min at RT. After centrifugation at 18,500 \times g for 30 min, the pooled supernatants were used as the total membrane fraction. Each fraction was Western blotted using ECL detection reagents (GE Healthcare). Protein was quantified using the Coomassie protein assay reagent (Pierce).

2.5. Animal treatment, drugs, and behavior

Three-month-old male littermates of heterozygous interbreedings were used in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) experiments. Mice received either two intraperitoneal injections of MPTP (Sigma; 10 h apart; 30 mg/kg each), or vehicle control (saline). The animal experiments described were approved by Animal Experiments Committee of RIKEN Brain Science Institute. All procedures were performed according to guidelines of RIKEN Brain Science Institute. Treatment with 6-hydroxydopamine (6-OHDA) was performed as described previously (Kaneko et al., 2000). In short, the male Tg or KO mice at 2–3 months of age and their littermates were deeply anesthetized with sodium pentobarbital (50 mg/kg i.p.). A needle was introduced into the left hemisphere using stereotaxic techniques, and phosphate-buffered saline (PBS, 0.5 μ l) containing 6-OHDA hydrobromide (4 mg/ml) and 0.016% ascorbic acid was injected into two sites in the left striatum over 5 min. After 1 week (for the MPTP experiments) and 3 weeks (for the 6-OHDA experiments), mice were subjected to neurochemical analysis or counting of the number of tyrosine hydroxylase (TH)-positive neurons as follows. The brain perfused with cold PBS followed by 4% paraformaldehyde in PBS, was removed, embedded in paraffin, sectioned (12 μ m), deparaffinized using standard protocols, and stained with anti-TH mAb (Chemicon, 1:100). The total number of TH-stained cells with clearly visible nuclear borders in the substantia nigra pars compacta was estimated in every fifth section, using a previously described unbiased stereological method (Nelson et al., 1996; West, 1999). A complete reduction of TH immunosignal in the striatum was also used to confirm that the injection sites of each animal were optimally located. For spontaneous locomotion activities, mice were placed in white chambers (50 cm \times 50 cm). After introduction of mice without acclimation, horizontal migration up to 30 min was recorded by overhead CCD cameras and analyzed by Image OF4, a program based on the NIH image program (O'Hara & Co. Ltd., Tokyo, Japan).

2.6. Neurochemical analysis

Striatal tissue was homogenized in solution H (0.4 M HClO₄ containing 4 mM Na₂S₂O₅, 4 mM diethylenetriaminepentaacetic acid and 5 mM 1,4-dithiothreitol). The supernatant by centrifugation at 18,500 \times g for 10 min was used for measurement of free catechols. The alumina extraction of free catechols was basically performed as described previously (Ito et al., 1988).

2.7. *In vivo* microdialysis

Mice were anesthetized with sodium pentobarbital and placed in a stereotaxic frame. Dialysis probes (membrane length, 2.0 mm; outer diameter,

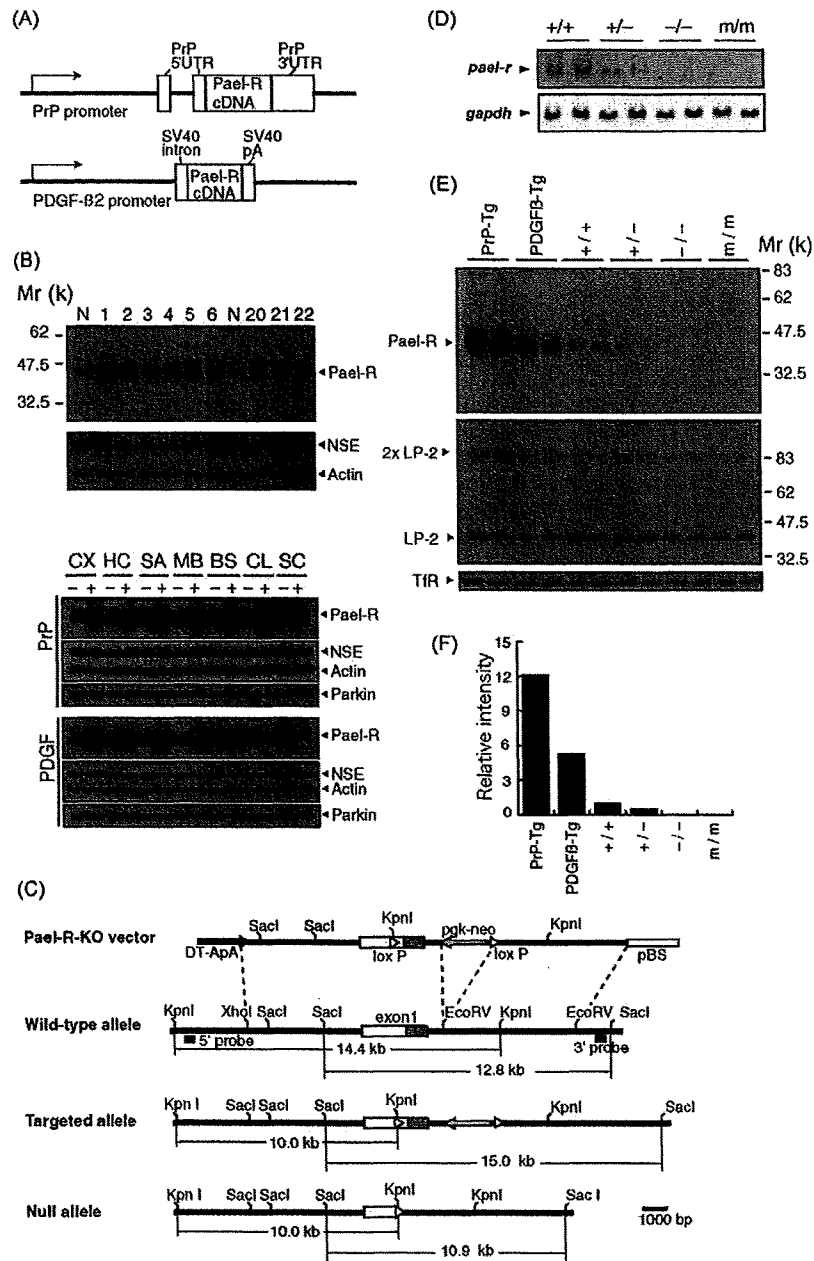


Fig. 1. Generation of Pael-R Tg and Pael-R KO mice. (A) Expression of the Pael-R gene was under the control of the PrP and PDGF β2 promoter. pA, polyadenylation sequence. (B) Expression levels of Pael-R in the whole brain of each PrP-Tg line (1–6), PDGFβ-Tg (20–22) and non-Tg mouse (N) are shown (upper). Expression levels of Pael-R and Parkin in each region of the brain of PrP-Tg line 1 and PDGFβ-Tg line 20 mice and their non-Tg littermates (lower). CX, cerebral cortex; HC, hippocampus; SA, striatum; MB, midbrain; BS, pons and medulla; CL, cerebellum, and SC, spinal cord. The amounts of neuron-specific enolase (NSE) and actin in each lane were shown as loading controls. (C) The murine Pael-R gene and targeting vector. A region of the Pael-R gene that includes the proximal exon 1 with a start codon is shown. Two loxP sites were inserted on the both sides of the Pael-R coding region within exon 1 (5'-UTR, white box; CDS1, gray box; a loxP site in the exon 1, 461 bp upstream from the translation initiation site). A neo cassette was inserted after exon 1. Locations of probes for southern blot analysis are indicated. DT-ApA, the diphtheria toxin gene with poly(A) sequence for negative selection; pBS, pBluescript (Stratagene). (D) Northern blot analysis of Pael-R transcripts (*pael-r*) in representative brain samples. '+', '-', and 'm' indicates the WT, null and targeted allele in (C), respectively. The same blot was hybridized with a *gapdh* probe as a loading control (*gapdh*). (E) Western blot analysis with the membrane fractions from the whole brain of 2.5-month-old mice. LP-2, ET_BR-LP2; 2× LP-2, a putative SDS-resistant dimer form of LP2; TfR, transferrin receptor. (F) Quantitative analysis of expression levels of Pael-R protein normalized against each TfR level in (E). The mean amounts of Pael-R in WT (+/+) are defined as one.

0.22 mm; D-I-6-02, Eicom, Kyoto, Japan) were implanted in the left striatum. The stereotaxic coordinates for implantation of microdialysis probes were anterior–posterior, 0.26 mm; dorsal–ventral, 3.5 mm; and lateral, 2.0 mm relative to the bregma (Goldberg et al., 2003). Placement of the probe was

verified by histological examination subsequent to the experiments. Following surgery, animals were returned to their home cages and given free access to food and water. Twenty hours later, to avoid transient changes, if any, caused by surgical damage, the dialysis probe was connected to a syringe pump and