Fbxw7-dependent degradation of Notch is required for control of stemness and neuronal-glial differentiation in neural stem cells

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Running title: Role of Fbxw7 in neural stem cells

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Control of the growth and differentiation of neural stem cells is fundamental to brain development and is largely dependent on the Notch signaling pathway. The mechanism by which the activity of Notch is regulated during brain development has remained unclear, however. Fbxw7 (also known as Fbw7, SEL-10, hCde4, or hAgo) is the F-box protein subunit of an SCF-type ubiquitin ligase complex that plays a central role in the degradation of Notch family members. We now show that mice with brain-specific deletion of Fbxw7 (Nestin-Cre/Fbxw7<sup>F/F</sup> mice) die shortly after birth with morphological abnormalities of the brain and the absence of suckling behavior. The maintenance of neural stem cells was sustained in association with the accumulation of Notch1 and Notch3 as well as up-regulation of Notch target genes in the mutant mice. Astrogenesis was also enhanced in the mutant mice in vivo, and the differentiation of neural progenitor cells was skewed toward astrocytes rather than neurons in vitro, with this latter effect being reversed by treatment of the cells with a pharmacological inhibitor of the Notch signaling pathway. Our results thus implicate Fbxw7 as a key regulator of the maintenance and differentiation of neural stem cells in the brain.

The Notch signaling pathway plays pivotal roles in cell fate decisions in a wide variety of cell types (1-3). During brain development, Notch activity maintains neural progenitor cells and inhibits neuronal differentiation (4). In addition, Notch signaling is thought to regulate glial versus neuronal identity (5-8).

Notch regulates the expression of a cascade of transcription factors belonging to the basic helix-loop-helix (bHLH) family (1,9,10). Notch signaling is regulated by a complex proteolytic process (11,12). Multiple cleavages of the extracellular domain of the receptor are followed by ligand-induced proteolysis of the intracellular juxtamembrane domain by a γ-secretase that may include members of the presenilin family (12,13). The cleaved Notch intracellular domain (NICD) activates proteins of the Hes family present in a complex that includes RBP-J [also known as CSL, CBF1, Su(H), and LAG-1]. The Hes proteins subsequently suppress the expression of proneural bHLH transcription factors, which play key roles in the induction of neurogenesis and regulation of neuronal differentiation (14-18).

The regulation of Notch signaling is largely dependent on ubiquitylation events. Notch ligands undergo monoubiquitylation mediated by neutralized or mind bomb 1 (mib1), which triggers ligand endocytosis and consequent Notch activation (19,20). Conditional inactivation of mib1 in mouse brain resulted in
the complete loss of Notch activation associated with the depletion of neural stem cells and the promotion of neural differentiation (21). Notch stability is also controlled by ubiquitin ligases. Drosophila Su(dx), a member of the HECT family of ubiquitin ligases, has been characterized as a negative regulator of Notch signaling, acting at the level of the postendocytic sorting of Notch. The mammalian ortholog of Su(dx), Itch (AIP4), has been shown to have multiple substrates, including Notch. Itch promotes polyubiquitylation of the membrane-anchored form of Notch, resulting in its lysosomal degradation (22). Furthermore, the released NICD is targeted for degradation by Fbxw7 (F box– and WD repeat domain–containing 7; also known as Fbw7, SEL-10, hCdc4, and hAgo), a member of the F-box protein family that determines the substrate specificity of the SCF-type ubiquitin ligase complex (23,24). However, the precise role of Fbxw7-dependent regulation of Notch stability in control of neural stem cells has remained unknown.

We have now examined the consequences of Fbxw7 deficiency in the brain. The major phenotypes associated with such deficiency were found to be neonatal death accompanied by morphological abnormalities in the brain and the apparent accumulation of Notch. The growth of neural stem cells was also enhanced, and the differentiation of these cells was skewed toward astrocytes. We thus propose that Fbxw7 plays a pivotal role in regulation of the abundance of Notch proteins by ubiquitin-dependent degradation in neural stem cells and thereby contributes to generation of the proper number and ratio of neurons and glial cells, a process that is essential for normal brain development.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Antibodies to Notch3 (M-20), to c-Myc (N-262), to N-Myc (C-19), or to cyclin E (M-20) were obtained from Santa Cruz Biotechnology. Antibodies to cleaved Notch1 (Val1744), to c-Jun, to pH3, or to mTOR (7C10) were from Cell Signaling Technology. Antibodies to HSP70 or to HSP90 were from BD Biosciences. Antibodies to Tbr2 or to MBP were from Abcam. Antibodies to βIII-tubulin, to GFAP, to BrdU, or to Ki67 were from Sigma, DakoCytomation, BD PharMingen, or Novocastra, respectively.

**Generation of Fbxw7 conditional knockout mice**—Mice homozygous for a “floxed” Fbxw7 allele (Fbxw7<sup>F/F</sup>) (25) were crossed with Nestin-Cre transgenic mice (26). Deletion of exon 5 of the floxed Fbxw7 allele was confirmed by PCR analysis of genomic DNA as previously described (25). Examination of embryonic and neonatal phenotypes was performed with mice of the C57BL/6J × 129/Ola background. All mouse experiments were approved by the animal ethics committee of Kyushu University.

**Quantitation of mRNA by RT-PCR**—Total RNA was extracted by the guanidinium thiocyanate–phenol–chloroform method, purified, and subjected (1 µg) to RT with random hexanucleotide primers (QuantiTect RT kit, Qiagen). The resulting cDNA was then subjected to real-time PCR analysis with 1× SYBR Green PCR master mix (Takara) and 200 nM gene-specific primers. Assays were performed in triplicate with a StepOnePlus Real-Time PCR System (Applied Biosystems). The amplification protocol comprised initial incubation at 60°C for 30 s and 95°C for 3 s followed by 40 cycles. The sequences of the various primers (sense and antisense, respectively) were 5′-GGACCCGAGAGGACTT-3′ and 5′-GCACATCCTCAAGATAATTTCAATGG-3′ for acidic ribosomal phosphoprotein (ARBP), 5′-TGCAAGTGTCGATTATACCC-3′ and 5′-ACTTCTCTGTCCGCTCCAGC-3′ for Fbxw7 (exon 5), 5′-TATCATGGAGAAGGGCGAAGG-3′ and 5′-TTCTCTAGGCTGGATGCGG-3′ for Hes1, 5′-TCAACGCAGCATAGAGCACGT-3′ and 5′-GGCAGGGCTTTCTGTGTTTT-3′ for Hes5, 5′-AAAATGCTGCACACTGCAGG-3′ and 5′-CGGTGAATTGGACCCGAGAAGCC-3′ for Hey1, 5′-AACAGACCCCTCCAGAAAGCGA-3′ and 5′-CGGTGAATTGGACCCGAGAAGCGA-3′ for Hey2, and 5′-AAAGTGATCGGCAGACACAAT-3′ and 5′-CCAACCGAACCACAGATTACACT-3′ for BLBP. Reactions for ARBP mRNA were performed concurrently on the same plate as those for the test mRNAs, and results were normalized by the corresponding amount of ARBP mRNA.

**Immunoblot analysis**—Total protein extracts were prepared from tissue or cells with the use of radioimmunoprecipitation assay buffer and were subjected to immunoblot analysis as previously described (27). Where indicated, protein extracts were diluted in alkaline phosphatase buffer and incubated with
calf intestinal alkaline phosphatase (40 U/30 µl, Takara) for 1 h at 37°C before immunoblot analysis.

**Histological and immunohistofluorescence analysis**—Tissue was fixed with 4% paraformaldehyde in PBS. For H&E staining, the tissue was embedded in paraffin and cut into serial sections with a thickness of 4 µm. For immunofluorescence analysis, tissue was embedded in OCT compound (Tissue Tek) and sectioned at a thickness of 10 µm with a cryostat. Immunohistofluorescence analysis was performed as described previously (28). Immune complexes were detected with Alexa Fluor 488– or Alexa Fluor 546–conjugated goat secondary antibodies (Molecular Probes). The sections were mounted in Vectashield medium (Vector) and examined with a laser-scanning confocal microscope (LSM510, Carl Zeiss). For BrdU labeling, BrdU (50 µg per gram of body weight, BD PharMingen) was injected intraperitoneally in pregnant females.

**In situ hybridization**—Tissue was fixed with 4% paraformaldehyde in PBS, embedded in paraffin, and cut into serial sections with a thickness of 5 µm. A riboprobe specific for Hes5 mRNA was synthesized with the use of a DIG RNA labeling kit (Roche). In situ hybridization was performed with the Ventana HX system.

**Primary culture**—Neurons were isolated from the cerebral cortex at embryonic day (E) 18.5 or postnatal day (P) 0.5 and were cultured with a Nerve-Cell Culture System (Sumitomo) in dishes coated with poly-L-lysine (Sigma). For inhibition of Notch signaling, the cells were cultured with the γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT, Calbiochem). After culture for 7 days (unless indicated otherwise), the cells were fixed with 4% paraformaldehyde and then incubated with antibodies to βIII-tubulin, to GFAP, or to MBP. Immune complexes were detected with Alexa Fluor 488– or Alexa Fluor 546–conjugated goat secondary antibodies (Molecular Probes). Nuclei were stained blue with Hoechst 33258. About 1000 cells per sample were examined for quantitation of marker-positive cells.

**Neurosphere assay**—For primary neurosphere formation, mechanically dissociated telencephalic neurons derived from E14.5 embryos were plated at a density of 1 × 10³ cells per 2 ml of neurobasal medium (Invitrogen) supplemented with recombinant mouse EGF (20 ng/ml, Sigma) and recombinant mouse FGF2 (10 ng/ml, R & D Systems) in each well of a six-well plate (Corning). For secondary and tertiary sphere formation, primary or secondary spheres, respectively, were collected and digested with TrypLE Select (Invitrogen) for 5 min at 37°C. The cells were then subjected to gentle trituration, isolated by centrifugation at 130 × g for 5 min, resuspended in proliferation medium with or without DAPT, and plated at a density of 500 cells per 200 µl in each well of a 96-well ultra-low cluster plate (Corning). The numbers of secondary or tertiary neurospheres were counted after culture for 7 days.

**Statistical analysis**—Quantitative data are presented as means ± SD and were analyzed by Student’s t test. A P value of <0.05 was considered statistically significant.

**RESULTS**

**Conditional ablation of Fbxw7 in the brain**—We generated mice harboring floxed Fbxw7 alleles in which exon 5 (which encodes the F-box domain) is flanked by loxP sites (25). To ablate Fbxw7 in the brain, we crossed these Fbxw7²/F² mice with mice harboring a Cre transgene under the control of the Nestin gene promoter (Nestin-Cre mice), which is active throughout the nervous system including in neural stem cells. We confirmed that almost all floxed alleles were deleted by Cre recombinase and that the corresponding mRNA was depleted in the brain of Nestin-Cre/Fbxw7²/F² mice (Fig. 1, A and B). Newborn Nestin-Cre/Fbxw7²/F² mice were found to die within 24 h after birth and were readily distinguished from control animals by their smaller body and absence of milk in the stomach (Fig. 1, C and D), suggesting that their death resulted from a defect in suckling behavior. Given that pulmonary alveoli were normally inflated in the mutant neonates (Fig. 1E), respiratory distress appeared unlikely to be responsible for their death. Nestin-Cre/Fbxw7²/F² embryos were recovered at the expected Mendelian ratio until E17.5 to E18.5, suggesting that the homozygous mutation is not embryonic lethal.

Histopathological examination revealed the presence of substantial changes in brain structure in Nestin-Cre/Fbxw7²/F² mice. At E16.5, the third ventricle of the mutant mice was dilated and distorted with a characteristic horizontal sulcus that is not present in the normal brain (Fig. 2A). The abnormal sulcus was also observed in the E18.5 brain. Furthermore, the pons appeared hypoplastic and the
cerebellum appeared defective in the mutant embryos (Fig. 2B). The hypoplasia of the brain stem was likely responsible for the defect in suckling behavior, although the precise cause of the defective behavior remains to be elucidated. In addition, the cellularity of the cortex was markedly reduced in the Fbxw7-deficient brain (Fig. 2C).

**Accumulation of Notch in the Fbxw7-deficient brain**—Given that neurogenesis is regulated by the Notch signaling pathway, we examined the expression of Notch proteins and their downstream targets in the brain of the mutant animals. Immunoblot analysis revealed that the abundance of Notch1 and Notch3 was increased in Nestin-Cre/Fbxw7<sup>F/F</sup> mice, and that this difference with control embryos was maximal at E14.5 to E16.5 (Fig. 3A). The intensity of the more slowly migrating of the two Notch1 bands, which likely corresponded to the phosphorylated form of Notch1 and was virtually undetectable in control embryos, was especially increased in the mutant mice, consistent with the previous observation that the phosphorylated form of Notch is targeted by Fbxw7, as is generally the case for Fbxw7 substrates (25,29). The slower band disappeared after treatment of samples with calf intestinal alkaline phosphatase (Fig. 3B), supporting the notion that it indeed corresponded to phosphorylated Notch1. Fbxw7 also targets for degradation various mammalian proteins that control cell cycle progression (23,24), including cyclin E (30-32), Myc (33,34), c-Jun (35,36) and mammalian target of rapamycin (mTOR) (37). The amounts of c-Jun, N-Myc, cyclin E, and mTOR (total or phosphorylated forms) were unaffected by deletion of Fbxw7 in the brain (Fig. 3A). The abundance of c-Myc in the brain was not increased in the mutant during embryogenesis, but it was increased in mutant neonates.

Consistent with the observed Notch accumulation, RT and real-time PCR analysis revealed that the amounts of mRNAs for downstream targets of Notch, including those for Hes5, Hey1, Hey2, and brain lipid-binding protein (BLBP), were increased in the brain of Fbxw7-deficient embryos (Fig. 3C). The abundance of Hes1 mRNA was not affected by the deletion of Fbxw7, consistent with previous observations showing that the expression of Hes1 is not necessarily dependent on Notch–RBP-J signaling, especially in the central nervous system at this stage (38,39). In situ hybridization analysis also showed that the intensity of the signal for Hes5 mRNA was increased in the brain of Nestin-Cre/Fbxw7<sup>F/F</sup> mice at E14.5, although ectopic expression of Hes5 was not observed (Fig. 3D). Collectively, these observations suggested that the abnormal morphology of the brain in Nestin-Cre/Fbxw7<sup>F/F</sup> mice was likely attributable to the accumulation of Notch proteins resulting from Fbxw7 ablation and was responsible for the neonatal death of these animals as a result of defective suckling behavior.

**Skew of differentiation toward astrocytes in the Fbxw7-deficient brain**—Notch plays a key role in cell fate decisions associated with the differentiation of neurons and glia. We examined the differentiation orientation of primary cultures of cells derived from the neonatal mouse cerebrum at P0.5. The percentage of neurons, as revealed by immunostaining with antibodies to βIII-tubulin, for cells derived from Nestin-Cre/Fbxw7<sup>F/F</sup> mice was reduced to about half of that for those derived from control mice (Fig. 4A and B). In contrast, the percentage of astrocytes, as revealed by staining with antibodies to glial fibrillary acidic protein (GFAP), was markedly increased for Nestin-Cre/Fbxw7<sup>F/F</sup> mice compared with that for control animals. The percentage of oligodendrocytes, revealed by staining with antibodies to myelin basic protein (MBP), did not differ significantly between the two genotypes. These results thus indicated that Fbxw7 is required for generation of neurons and astrocytes in the proper ratio.

Neurogenesis occurs predominantly between E12.5 and E16.5, which corresponds to the period when Fbxw7 contributes to the degradation of Notch1 and Notch3 (Fig. 3A). To examine whether Fbxw7 promotes neurogenesis at this stage, we monitored the number of postmitotic cells generated as a result of asymmetrical division at E13 to E14. Neurogenesis was found to be defective in Nestin-Cre/Fbxw7<sup>F/F</sup> mice. Thirty hours after labeling with a single pulse of BrdU at E13.25, the number of BrdU-positive cells that had migrated into the Ki67-negative differentiated mantle zone was greater for control mice than for Nestin-Cre/Fbxw7<sup>F/F</sup> mice (Fig. 4A and D). In contrast, astrogenesis, which is initiated at the late embryonic stage, was enhanced in Nestin-Cre/Fbxw7<sup>F/F</sup> mice at P0.5 compared with that in control animals (Fig. 4E). These in vitro and in vivo results thus indicated that
differentiation was skewed toward astrocytes rather than neurons in the mutant mice, consistent with the observed accumulation of Notch proteins, which inhibit neurogenesis and promote astroglial differentiation.

*Increased “stemness” of Fbxw7-deficient neural stem cells*—Given that Notch and its downstream target genes contribute to the maintenance of neural stem cells (17,40), the accumulation of Notch and transcriptional activation of its downstream targets apparent in Nestin-Cre/Fbxw7^Cre/ mice might have been expected to result in an increase in the number of neural stem cells. We therefore measured the proliferative potential of neural stem or progenitor cells in the ventricular zone by staining of mouse brain sections with antibodies to phosphorylated histone H3 (pHH3). Whereas the number of pHH3-positive cells did not differ significantly between Nestin-Cre/Fbxw7^Cre/ and control mice at E14.5, it was substantially greater in the mutant mice at E16.5 (Fig. 5, A and B). In contrast to this increased proliferation of neural stem cells in the mutant mice, the number of pHH3-positive progenitor cells in the subventricular and intermediate zones was reduced in Nestin-Cre/Fbxw7^Cre/ mice at E14.5 (Fig. 5B). The number of intermediate neural progenitor cells, which are stained with antibodies to Tbr2, was decreased in Nestin-Cre/Fbxw7^Cre/ mice at both E14.5 and E16.5 compared with controls (Fig. 5, C and D). The frequency of apoptosis in the brain did not differ between control and mutant mice during the late embryogenesis (E14.5 to P0.5) (data not shown). These data suggested that the abnormal accumulation of Notch in Nestin-Cre/Fbxw7^Cre/ mice inhibits the differentiation of neural stem cells, resulting in an increase in the number of these cells and a decrease in the number of mature neurons.

We next examined the effect of Fbxw7 deletion on the ability of neural stem cells to proliferate and maintain stemness in vitro with the use of a colony formation (neurosphere) assay. We generated neurospheres from single neural stem cells of the E14.5 mouse brain and examined the expression of genes for proteins in the Notch signaling pathway. Single primary neurosphere colonies were capable of producing secondary neurosphere colonies after 7 days in vitro. The number of descendant neurospheres is thought to provide an estimate of the extent to which the initial neural stem cell underwent symmetrical (expansionary) divisions (41). Sequential culture experiments revealed that the numbers of secondary and tertiary neurospheres were significantly greater for cells derived from Fbxw7-deficient mice than for those derived from control mice (Fig. 6, A and B). As was evident in the brain, the accumulation of phosphorylated Notch1 was apparent in the tertiary neurospheres derived from Fbxw7-deficient cells (Fig. 6C). In contrast, the abundance of Notch3 was not increased in the Fbxw7-deficient neurospheres, suggesting that the accumulation of Notch1 may be primarily responsible or sufficient for the proliferative nature of the Fbxw7-deficient spheres (Fig. 6D). The abundance of other substrates of Fbxw7 did not differ between the two genotypes (Fig. 6D). These results suggested that neural stemness is excessively maintained in Fbxw7-deficient mice.

We also measured the expression of downstream target genes of Notch including those for Hes1, Hes5, Hey1, Hey2, and BLBP, most of which contribute to the maintenance of neural stem cells. RT and real-time PCR analysis revealed that the expression of Notch target genes was greater in the tertiary neurospheres derived from Nestin-Cre/Fbxw7^Cre/ mice than in those from control mice (Fig. 6E). These results suggested that Fbxw7 controls the maintenance of neural stem cells by regulating the level of Notch and the activation of its downstream target genes.

Reversal by a Notch inhibitor of the increased stemness and skewed differentiation of Fbxw7-deficient neural stem cells—To examine whether the increased stemness and skewed neuronal-glial differentiation of neural stem cells induced by Fbxw7 loss were attributable to excessive Notch activation, we exposed primary neuronal cultures or neurospheres to DAPT, an inhibitor of the γ-secretase activity that is necessary for Notch activation. DAPT increased the number of βIII-tubulin–positive neurons and reduced the number of GFAP-positive astrocytes in primary cultures prepared from the cerebrum of Nestin-Cre/Fbxw7^Cre/ mice at E18.5 (Fig. 7, A and B). It also attenuated the increases in the amounts of Hes5 and BLBP mRNAs associated with Fbxw7 deficiency (Fig. 7C). Furthermore, the overproliferation of Fbxw7-deficient neurospheres (Fig. 7D) and the associated transcriptional activation of Notch target genes (Fig. 7E) were reversed by treatment of the cells with DAPT. Together, these results suggested that the up-regulation of Notch abundance is responsible for the increased stemness of neural
stem cells and the increased astrogenesis in the Fbxw7-deficient brain.

**DISCUSSION**

The Notch signaling pathway is thought to play a pivotal role in neuronal and glial cell fate decisions, acting via a process known as lateral inhibition. The products of the proneural genes Mash1 and Ngn2 activate the transcription of Dll1, which encodes a ligand of Notch (Delta-like 1), and the signaling triggered by the Dll1-Notch interaction suppresses the expression of the proneural genes via activation of Hes1 expression and thereby prevents neuronal differentiation (42). We have now provided genetic evidence implicating Fbxw7-mediated control of Notch protein stability in this process, in particular in regulation of the maintenance and differentiation orientation of neural stem cells. Loss of Fbxw7 resulted in Notch accumulation as well as in abnormal activation of Notch target genes, leading to excessive proliferation of neural stem cells and skewed differentiation toward the astrocyte lineage (Fig. 8).

The development of the brain initially requires expansion of the progenitor pool by symmetrical division, which is then followed by generation of differentiated cells such as neurons, astrocytes, and oligodendrocytes through asymmetrical division. Notch inactivation promotes premature neurogenesis, resulting in exhaustion of the progenitor pool (38,43). The final number of mature neurons in mice lacking Notch1 in the brain is thus decreased compared with that in control animals. Mice deficient in presenilin 1 (PS1), in which Notch is not cleaved on interaction with ligands such as Dll1 and the Notch signaling pathway is therefore disabled, show a similar phenotype (44,45). In contrast, Fbxw7 inactivation in the brain resulted in the accumulation of Notch proteins, leading to expansion of the progenitor pool and a delay in the generation of mature neurons. The formation of neospheres was thus impaired in cells prepared from mice deficient in Notch1, RBP-J, or PS1 (39), whereas it was promoted in those from Fbxw7-deficient mice. The effects of inactivation of Notch and those of Fbxw7 inactivation therefore appear opposite, consistent with the notion that Fbxw7 is a negative regulator of the Notch signaling pathway.

The HECT-type ubiquitin ligase Itch (AIP4) also contributes to the control of Notch stability (22). Numb, a negative regulator of Notch, recruits Itch to Notch and thereby promotes Notch ubiquitylation (46). A mouse strain harboring a mutation in Itch (Itchy mouse) develops severe autoimmune disease (47) but does not manifest a neural defect at the embryonic stage similar to that apparent in Fbxw7-deficient mice. Although the role of Itch in neural development remains unknown, we conclude that Fbxw7-mediated degradation is primarily responsible for the regulation of Notch stability during neural development.

In mice lacking Fbxw7 specifically in hematopoietic lineage, the pool of hematopoietic stem cells becomes exhausted as a result of premature proliferation followed by induction of p53-dependent apoptosis in response to c-Myc accumulation (48). Inhibition of the cell cycle as a result of maintenance of c-Myc at a low level by Fbxw7-dependent proteolysis is thought to be required for the maintenance of stemness in HSCs. In contrast, neural stem cells have not been fully characterized and their properties remain unclear. The neosphere assay, one of the most widely applied procedures to estimate neural stemness, evaluate the growth of neural stem cells as well as their ability to differentiate into cells of multiple lineages, but the requirement for cell cycle inhibition in neural stem cells remains largely unknown. In this assay, the loss of Fbxw7 promoted cell proliferation in association with the accumulation of Notch1. In contrast to its effects in HSCs, deletion of Fbxw7 in the embryonic brain elicited neither the accumulation of c-Myc nor apoptosis as a result of p53 induction. However, the final number of generated mature neurons was greatly reduced and that of astrocytes was increased at birth as a result of Fbxw7 deficiency, suggesting that the excessive and persistent Notch signaling skews the orientation of differentiation of neural stem cells from neurons to astrocytes.

During preparation of the present manuscript, Hoeck et al. described the generation and characterization of mice that lack Fbxw7 in the brain (49). Although the results of the two independent studies overlap in part, there are differences with regard to astrogenesis, neosphere formation, and apoptosis between the brain-specific Fbxw7-deficient mice in the previous study and those in our present study (see Table S1). First, Hoeck et al. found no change in gliogenesis in the brain of their mutant mice at E18.5, whereas we detected a marked increase in astrogenesis in the brain of our
Fbxw7-deficient mice at P0.5. Given that gliogenesis begins to increase at the late stage of embryogenesis, a change in this process might be more evident at P0.5 than at E18.5. Second, Hoeck et al. analyzed primary neurospheres, finding that their number and size were decreased for the mutant mice, whereas we studied secondary and tertiary neurospheres and found that the efficiency of sphere formation was significantly increased as a result of Fbxw7 loss. Hoeck et al. also showed that the relative proportion of immature neurons in the Fbxw7-deficient neurospheres was increased, suggesting that this increase might underlie the enhanced formation of secondary spheres observed in our study. The apparently opposite results of the two studies are therefore likely attributable to the difference in the stage of analysis (primary versus secondary and tertiary neurospheres). Third, Hoeck et al. showed that the frequency of apoptosis was increased in neurons of the Fbxw7-deficient brain, and they concluded that the accumulation of c-Jun may be responsible for this phenotype. However, we observed neither an increased frequency of apoptosis (data not shown) nor an increased abundance of c-Jun (Fig. 3A and Fig. 6D) in the mutant mice. The reason for this discrepancy remains unclear, but it might be attributable to a difference in genetic background of the mouse strains studied. We also demonstrated a defect in suckling behavior of Fbxw7-deficient neonates as well as anatomic anomalies in the mutant brain. Overall, many results of the two studies are not overlapping but rather complementary, and both provide a better understanding of Fbxw7 function in the brain.

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ACKNOWLEDGMENTS

We thank D. R. Littman for pL2-Neo and pMC-Cre; A. Niihara, C. Mitai, Y. Yamada, K. Takeda, and M. Tanaka for technical assistance; and members of our laboratories for comments on the manuscript. This work was supported in part by a grant from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.
FOOTNOTES

1Nonstandard abbreviations: bHLH, basic helix-loop-helix; NICD, Notch intracellular domain; ARBP, acidic ribosomal phosphoprotein; E, embryonic day; P, postnatal day; DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; mTOR, mammalian target of rapamycin; BLBP, brain lipid-binding protein; GFAP, glial fibrillary acidic protein; MBP, myelin basic protein; pHH3, phosphorylated histone H3; HSC, hematopoietic stem cell.

FIGURE LEGENDS

FIGURE 1. Generation of conditional knockout mice lacking Fbxw7 specifically in the nervous system. A, PCR analysis of genomic DNA from the brain of mice of the indicated genotypes at P0.5. The positions of amplified fragments corresponding to wild-type (WT), floxed, and ΔE5 (exon 5-deleted) alleles are indicated. B, RT and real-time PCR analysis of Fbxw7 mRNA in the brain of Fbxw7<sup>−/−</sup>, Nestin-Cre/Fbxw7<sup>−/−</sup>, and Nestin-Cre/Fbxw7<sup>+/−</sup> mice at E14.5. Normalized data are expressed relative to the value for Fbxw7<sup>−/−</sup> mice and are means ± SD from three independent experiments. C, Gross appearance of newborns and the neonatal stomach of Nestin-Cre/Fbxw7<sup>+/−</sup> (Fbxw7 CKO) and control mice. Arrows indicate the position of the stomach. Scale bars, 5 mm. D, Summary of suckling behavior of Nestin-Cre/Fbxw7<sup>+/−</sup> (control) and Fbxw7 CKO mice at P0.5. E, H&E staining of lung tissue from Nestin-Cre/Fbxw7<sup>+/−</sup> (control) and Fbxw7 CKO mice at P0.5. Scale bars, 100 µm.

FIGURE 2. Morphological abnormalities of the brain of Nestin-Cre/Fbxw7<sup>+/−</sup> mice. A and B, H&E staining of coronal sections (A) and sagittal sections (B) of the brain of Nestin-Cre/Fbxw7<sup>+/−</sup> (control) and Nestin-Cre/Fbxw7<sup>+/−</sup> (Fbxw7 CKO) mice at E16.5 and E18.5 (A) or P0.5 (B). Arrows in (A) indicate a horizontal sulcus characteristic of the mutant mice, which is shown at higher magnification in the inset. The arrow and arrowhead in (B) indicate the pons and cerebellum, respectively. Scale bars, 500 µm (A) and 1 mm (B). C, H&E staining of coronal sections of the cortex of Nestin-Cre/Fbxw7<sup>+/−</sup> (control) and Fbxw7 CKO mice at E18.5. CP, cortical plate; IZ, intermediate zone; VZ, ventricular zone; SVZ, subventricular zone. Scale bars, 100 µm.

FIGURE 3. Notch accumulation in the Fbxw7-deficient brain. A, Immunoblot (IB) analysis of Fbxw7 substrates and HSP70 (loading control) in brain extracts from Nestin-Cre/Fbxw7<sup>+/−</sup> (control) and Nestin-Cre/Fbxw7<sup>+/−</sup> (Fbxw7 CKO) mice at E12.5 to P0.5. The amounts of the NICD of Notch1 or Notch3 (both phosphorylated and nonphosphorylated forms) in the mutant brain relative to those in the control brain at each developmental stage were determined by densitometry and are shown below each lane. B, Protein extracts from the brain of Nestin-Cre/Fbxw7<sup>+/−</sup> (control) and Fbxw7 CKO mice at E14.5 were incubated with or without calf intestinal alkaline phosphatase (CIAP) for 1 h before immunoblot analysis of Notch1. C, RT and real-time PCR analysis of Notch target gene expression in the brain of Nestin-Cre/Fbxw7<sup>+/−</sup> (control) and Fbxw7 CKO mice at E14.5. Normalized data are expressed relative to the corresponding value for control mice and are means ± SD from three independent experiments. *P < 0.05, **P < 0.01; N.S., not significant. D, In situ hybridization analysis of Hes5 mRNA in the brain of Nestin-Cre/Fbxw7<sup>+/−</sup> (control) and Fbxw7 CKO mice at E14.5. Scale bars, 500 µm.

FIGURE 4. Impairment of neuronal differentiation and enhanced astroglial differentiation in the Fbxw7-deficient brain. A, Immunofluorescence analysis of βIII-tubulin (green) and GFAP (red) in primary cultured cells from the cerebrum of Nestin-Cre/Fbxw7<sup>+/−</sup> (control) and Nestin-Cre/Fbxw7<sup>+/−</sup> (Fbxw7 CKO) mice at P0.5. Nuclei were stained blue with Hoechst 33258. Scale bars, 40 µm. B, Quantitative analysis of cells positive for βIII-tubulin, GFAP, or MBP in experiments similar to that in A. Data are means ± SD from three independent experiments. **P < 0.01, ***P < 0.005. C, Immunofluorescence analysis of BrdU (green) and Ki67 (red) in the brain of Nestin-Cre/Fbxw7<sup>+/−</sup> (control) and Fbxw7 CKO mice at 30 h after labeling with a single pulse of BrdU at E13.25. More
BrdU-positive cells had migrated into the Ki67-negative differentiated mantle zone (MZ) of control mice than into that of Fbxw7 CKO mice. Scale bars, 100 µm (left panels) and 50 µm (right panels). D, Quantitative analysis of cells positive for BrdU in the mantle zone in experiments similar to that in C. Data are means ± SD from three independent experiments. ***P < 0.005. E, Immunofluorescence analysis of GFAP in the posterior midbrain of Nestin-Cre/Fbxw7^{+/F} (control) and Fbxw7 CKO mice at P0.5. Scale bars, 100 µm.

FIGURE 5. Promotion of neural stem cell proliferation by loss of Fbxw7. A, Immunofluorescence analysis of pHH3 (green) in the brain of Nestin-Cre/Fbxw7^{+/F} (control) and Nestin-Cre/Fbxw7^{+/F} (Fbxw7 CKO) mice at E14.5 and E16.5. Nuclei were stained blue with Hoechst 33258. Scale bars, 50 µm. B, Quantitative analysis of cells positive for pHH3 in the ventricular zone (VZ) or in the subventricular zone (SVZ) and intermediate zone (IZ) per image area in experiments similar to that in A. Data are means ± SD from four independent experiments. ***P < 0.005. C, Immunofluorescence analysis of Tbr2 (green) in the brain of Nestin-Cre/Fbxw7^{+/F} (control) and Fbxw7 CKO mice at E14.5 and E16.5. Nuclei were stained blue with Hoechst 33258. Scale bars, 50 µm. D, Quantitative analysis of cells positive for Tbr2 per image area in experiments similar to that in C. Data are means ± SD from four independent experiments. ***P < 0.005.

FIGURE 6. Increased efficiency of neurosphere formation associated with Notch1 accumulation resulting from the loss of Fbxw7. A, Light microscopy of neurospheres (tertiary) derived from the telencephalon of E14.5 Nestin-Cre/Fbxw7^{+/F} (control) and Nestin-Cre/Fbxw7^{+/F} (Fbxw7 CKO) mice. Scale bars, 200 µm. B, Numbers of secondary and tertiary neurospheres formed by 4000 dissociated cells from Nestin-Cre/Fbxw7^{+/F} (control) and Fbxw7 CKO mice. Data are means ± SD from four and three independent experiments for secondary and tertiary neurospheres, respectively. *P < 0.05. C, Immunoblot analysis of Notch1 and HSP90 (loading control) in neurospheres (tertiary, 7 days after replating) and brain extracts (E14.5) derived from Nestin-Cre/Fbxw7^{+/F} (control) and Fbxw7 CKO mice. The amounts of the NICD of Notch1 (both phosphorylated and nonphosphorylated forms) for the mutant genotype relative to those for the control genotype were determined by densitometry and are shown below each lane. D, Immunoblot analysis of other Fbxw7 substrates in neurospheres (tertiary) from Nestin-Cre/Fbxw7^{+/F} (control) and Fbxw7 CKO mice at 7 days after replating. E, RT and real-time PCR analysis of Notch target gene expression in tertiary neurospheres from Nestin-Cre/Fbxw7^{+/F} (control) and Fbxw7 CKO mice at 3 days after replating. Normalized data are expressed relative to the corresponding value for control mice and are means ± SD from three independent experiments. *P < 0.05, ***P < 0.005.

FIGURE 7. Attenuation of the abnormalities in the differentiation and maintenance of neural stem cells of Fbxw7-deficient mice by inhibition of Notch signaling. A, Immunofluorescence analysis of βIII-tubulin (green) and GFAP (red) in primary cultured cells derived from the cerebrum of Nestin-Cre/Fbxw7^{+/F} (control) and Nestin-Cre/Fbxw7^{+/F} (Fbxw7 CKO) mice at E18.5. The cells were cultured with 0.5 µM DAPT or 0.05% DMSO vehicle for 7 days. Nuclei were stained blue with Hoechst 33258. Scale bars, 40 µm. B, Quantitative analysis of cells positive for βIII-tubulin or GFAP in experiments similar to that in A. Data are means ± SD from three independent experiments. *P < 0.05, ***P < 0.005. C, RT and real-time PCR analysis of Notch target gene expression in primary cultured cells from Nestin-Cre/Fbxw7^{+/F} (control) and Fbxw7 CKO mice. The cells were cultured with or without 0.5 µM DAPT for 2 days. Normalized data are expressed relative to the corresponding value for control cells treated with DMSO and are means ± SD from three independent experiments. ***P < 0.005. D, Number of tertiary neurospheres formed by 6000 dissociated cells derived from Nestin-Cre/Fbxw7^{+/F} (control) or Fbxw7 CKO mice and cultured with or without 0.5 µM DAPT. Data are means ± SD from three independent experiments. *P < 0.05, ***P < 0.005. E, RT and real-time PCR analysis of Notch target gene expression in tertiary neurospheres derived from Nestin-Cre/Fbxw7^{+/F} (control) or Fbxw7 CKO mice. Dissociated cells were cultured with or without 0.5 µM DAPT for 3 days. Normalized data are expressed relative to the corresponding value for control cells treated with DMSO and are means ± SD from three independent experiments. ***P < 0.005.
FIGURE 8. Model for Fbxw7-mediated regulation of Notch signaling and neural development. Fbxw7 regulates the abundance of Notch by mediating its phosphorylation-dependent degradation. Loss of Fbxw7 results in the accumulation of Notch, an increase in the number of neural stem cells, and skewed differentiation of these cells into astrocytes, leading to aberrant brain development.
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Matsumoto et al. Figure 2
Matsumoto et al. Figure 3

A

| Age  | Control | Fbxw7 CKO |
|------|---------|-----------|
| E12.5| IB: Notch1 (NICD) | 1.00 |
| E14.5| IB: Notch3 (NICD)  | 1.00 |
| E16.5| IB: c-Jun        | 1.00 |
| E18.5| IB: c-Myc        | 1.00 |
| P0.5 | IB: N-Myc        | 1.00 |
|      | IB: Cyclin E    | 1.00 |
|      | IB: mTOR        | 1.00 |
|      | IB: HSP70       | 1.00 |

B

Control | Fbxw7 CKO
---|---
CIAP: - | -
IB: Notch1 (NICD) | - +
IB: HSP70 | - +

C

| Protein | Control | Fbxw7 CKO |
|---------|---------|-----------|
| Hes1    | 1.00    | 1.53      |
| Hes5    | 1.17    | 1.58      |
| Hey1    | 1.00    | 1.53      |
| Hey2    | 1.00    | 1.53      |
| BLBP    | 1.00    | 1.53      |

D

Control | Fbxw7 CKO
---|---

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Matsumoto et al. Figure 6
Matsumoto et al. Figure 7
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Fbxw7-dependent degradation of Notch is required for control of stemness and neuronal-glial differentiation in neural stem cells
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J. Biol. Chem. published online February 24, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M110.194936

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