Mouse Fbw7/Sel-10/Cdc4 Is Required for Notch Degradation during Vascular Development*

Received for publication, December 11, 2003
Published, JBC Papers in Press, December 12, 2003, DOI 10.1074/jbc.M312337200

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Mammalian Fbw7 (also known as Sel-10, hCdc4, or hAgo) is the F-box protein component of an SCF (Skp1-Cul1-F-box protein-Rbx1)-type ubiquitin ligase, and the mouse Fbw7 is expressed prominently in the endothelial cell lineage of embryos. We generated mice deficient in Fbw7 and found that the embryos died in utero at embryonic day 10.5–11.5, manifesting marked abnormalities in vascular development. Vascular remodeling was impaired in the brain and yolk sac, and the major trunk veins were not formed. In vitro para-aortic splanchnopleural explant cultures from Fbw7−/− embryos also manifested an impairment of vascular network formation. Notch4, which is the product of the proto-oncogene Int3 and an endothelial cell-specific mammalian isoform of Notch, accumulated in Fbw7−/− embryos, resulting in an increased expression of Hey1, which encodes a transcriptional repressor that acts downstream of Notch signaling and is implicated in vascular development. Expression of Notch1, -2, or -3 or of cyclin E was unaffected in Fbw7−/− embryos. Mammalian Fbw7 thus appears to play an indispensable role in negative regulation of the Notch4-Hey1 pathway and is required for vascular development.

The Notch signaling pathway is evolutionarily conserved and essential both for pattern formation during development and for execution of a wide variety of cell fate decisions. In Caenorhabditis elegans, the Notch homolog LIN-12 ensures that only one of two undifferentiated gonadal cells develops into an anchor cell, whereas the other cell becomes a ventral uterine precursor cell (1, 2). Given that relatively small differences in the activity of the Notch signaling pathway result in marked differences in cell behavior, this pathway is strictly regulated by various mechanisms (3). The pathway is activated by interaction of the receptor Notch with Delta-like ligands presented on the surface of adjacent cells, which results in proteolytic cleavage of Notch and the consequent release of its intracellular domain (ICD) from the plasma membrane into the cytosol. The Notch ICD then migrates to the nucleus, forms a complex with the transcriptional suppressor CBF1 (also known as Su(H) or RBP-Jk), and acts as a transcriptional activator of Notch target genes.

Several recent studies have highlighted the importance of Notch signaling in vascular development. Endothelial cell precursors differentiate and coalesce into a network of uniformly sized primitive blood vessels, a process known as vasculogenesis, both in the embryo and in extraembryonic tissues such as the yolk sac during the early stages of mammalian vascular development. This primary vascular plexus is then remodeled by angiogenesis, a process that includes the sprouting, branching, splitting, and differential growth of vessels and that results in the formation of the large and small vessels of the mature vascular system (4, 5). In mice, several Notch isoforms are expressed in endothelial cells of the embryonic vasculature (6), and both Notch1 loss-of-function mutant embryos and Notch1-Notch4 double mutant embryos manifest severe defects in angiogenic vascular remodeling (7). Expression of an activated form of Notch4 also results in abnormal vessel structure and patterning (8). Furthermore, Notch signaling has been shown to control an arterial versus venous cell fate decision (9–11). A role for the Notch pathway in vascular homeostasis has also been suggested by the observation that the human degenerative vascular disease CADASIL is caused by missense mutations in the Notch3 gene (12).

The activity of the Notch pathway is regulated by the ubiquitin-proteasome system of protein degradation (13). Protein ubiquitylation is mediated by several enzymes that act in concert (14, 15). A ubiquitin-activating enzyme, with ATP as a substrate, catalyzes the formation of a thioester bond between itself and ubiquitin, and it then transfers the activated ubiquitin to a ubiquitin-conjugating enzyme. Certain ubiquitin-conjugating enzymes transfer ubiquitin directly to the protein substrate, whereas others require the participation of a third component, termed a ubiquitin ligase, to achieve this effect. Mammalian Fbw7 (also known as Sel-10, hCdc4, or hAgo) (16–18) is the F-box protein component of an SCF-type ubiquitin ligase complex (19–22) and a homolog of C. elegans SEL-10, which was identified as a negative regulator of Notch (LIN-12) (2, 23). Biochemical evidence has suggested that the ICDs of both Notch1 (24–26) and Notch4 (2, 26) as well as presenilin

* This work was supported in part by a grant from the Ministry of Education, Science, Sports, and Culture of Japan and by a research grant from the Human Frontier Science Program. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡‡ The abbreviations used are: ICD, intracellular domain; PBS, phosphate-buffered saline; HA, hemagglutinin; ES, embryonic stem; E, embryonic day; P-Sp, para-aortic splanchnopleural; CDK, cyclin-dependent kinase.
(1) and cyclin E (16–18) are targets for ubiquitination mediated by mammalian Fbw7. The human FBW7 gene has also been shown to be mutated in breast and ovarian cancer cell lines (19, 25). However, the physiological substrates and functions of mammalian Fbw7 have been unclear. We now present genetic evidence that mouse Fbw7 is essential for normal vascular development as a result of its regulation of the abundance both of Notch4 and of the downstream effector Hey1.

**EXPERIMENTAL PROCEDURES**

**Construction of an Fbw7 Targeting Vector and Generation of Fbw7−/− Mice**—Cloned DNA corresponding to the Fbw7 locus was isolated from a 129/Sv mouse genomic library (Stratagene). The targeting vector was constructed by replacing a 15-kb Swal-HpaII fragment of genomic DNA containing exons 1–5 of Fbw7 with a PGK-lox-neo-poly(A) cassette. The vector thus created included 1.2- and 7.3-kb regions of homology located 5′ and 3′, respectively, relative to the neomycin resistance gene (neo). The PGK-k-lox-poly(A) cassette was ligated at the 3′ end of the targeting construct. The maintenance, transfection, and selection of ES cells were performed as described (27). The recombination event was confirmed by Southern blot analysis with a 0.5-kb SpeI fragment of genomic DNA that flanked the 5′ homology region (Fig. 1A). The expected 8-kb hybridizing fragment was observed in all ES clones. A small size variation of 2 kb was observed among clones, consistent with our previous results (27). The cassette was excised by homologous recombination in ES cells, and the clones were then re transfected with the same targeting vector, selected with G418 and puromycin (1 μg/ml) for 2 days, and the tissue was fixed with 4% paraformaldehyde in PBS for 10 min at 4°C, washed twice with PBS, and incubated for 30 min at room temperature with 0.3% H2O2 in PBS to block endogenous peroxidase activity. The cultures were then incubated for 30 min at room temperature with 1% normal goat serum and 0.2% bovine serum albumin in PBS containing 0.1% Triton X-100 to block nonspecific sites. After incubation overnight at 4°C with monoclonal anti-PECAM-1, the tissue was washed three times with PBS containing 0.1% Triton X-100, and immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies and diaminobenzidine. The cultures were finally examined with a dissection microscope.

**RESULTS**

**Generation of Fbw7−/− Mice**—We generated mice deficient in Fbw7. The Fbw7 gene was disrupted in mouse embryonic stem (ES) cells by replacement of the first five exons (33) with a neo cassette (Fig. 1A). Heterozygous mice appeared normal, but no homozygous mutants were detected among 299 newborn animals generated from heterozygote crosses (Fig. 1B) even though the ratio of wild-type to heterozygous offspring was normal. The mutation thus appeared to be lethal in the homozygous state. In situ hybridization analysis revealed that Fbw7 mRNA was localized predominantly to cells of the endothelial lineage, including immature mesenchymal cells of lateral plate mesoderm and endothelial cells of the atrium and major trunk arteries but was not apparent in the large veins of wild-type embryos at embryonic day (E) 9.5 (Fig. 1, C and D). The Fbw7 gene was not expressed in the neural tube of wild-type embryos at E9.5, even though it is highly expressed in the adult brain (16, 33). No hybridization signal was detected in Fbw7−/− embryos (Fig. 1E).

**Impaired Vascular Development in Fbw7−/− Mice**—To determine the time at which the Fbw7 mutation becomes lethal, we examined embryos from Fbw7−/− intercrosses at various developmental stages. Most Fbw7−/− embryos had been resorbed by E11.5, but they were still recoverable at E10.5 (Fig. 2). Growth retardation, especially in the head region, was evident in all homozygous mutant embryos (n = 181) (Fig. 2, A–E). They were pale and anemic in appearance, and often exhibited pericardial edema and impaired closure of the neural tube. Somite formation appeared normal, with the exception that the gap between adjacent somites was slightly narrower, and their pericardial edema and impaired closure of the neural tube. Somite formation appeared normal, with the exception that the gap between adjacent somites was slightly narrower, and their arrangement was slightly irregular in comparison with wild-type embryos (see Fig. 5, K and L). Both Notch1−/− (34, 35) and Dll1 (Delta-like 1)−/− (36) embryos exhibit severe abnormalities of somite patterning.

The vascular networks of Fbw7−/− embryos were generally less complex than were those of wild-type embryos. We immunostained the vessels of Fbw7−/− and Fbw7+/− embryos with antibodies to (anti-) PECAM-1, a marker of endothelial cells. The tree-like vascular networks apparent in the brain of Fbw7−/− embryos (Fig. 2D) failed to form in Fbw7−/− embryos.
A

Targeting vector

Fbw7 locus

B S SS W X XB H X Xh

Probes

5.2 kb

Mutant allele

B S SS W X XB H X Xh

Probes

5.8 kb

2 kb

(B) Targeting strategy for Fbw7. A 15-kb targeting vector, and the mutant allele were generated by homologous recombination. A 15-kb genomic fragment including exons 1-5 of Fbw7, which encode the NH2-terminal and F-box domains of the Fbw7 protein, was replaced by a loxP-neo cassette. Exons and the probe used for hybridization are denoted by open and filled boxes, respectively. Restriction sites are as follows: B, BglII; H, HpaII; S, SpeI; W, Swal; X, XhoI. Southern blot analysis was performed with the probe shown in A of tail genomic DNA from the offspring of heterozygote crosses after its digestion with BglII and XhoI. The 5.2- and 5.8-kb bands corresponding to the wild-type and mutant alleles, respectively, are indicated. C-E, in situ hybridization analysis of Fbw7+/− (C and D) and Fbw7−/− (E) embryos at E9.5 with an Fbw7 riboprobe. Hybridization signals were apparent in mesenchymal cells of lateral plate mesoderm (arrowheads in C) and in endothelial cells of the atrium and dorsal aorta (arrows in C and D) but not in the umbilical vein in wild-type embryos. Abbreviations used are as follows: nt, neural tube; acv, anterior cardinal vein; da, dorsal aorta; at, atrium; hg, hindgut. Scale bar, 250 μm.

Abnormal Vascular Development in Fbw7−/− Mice

(Fig. 2E). Rather, the homozygous mutants exhibited plexus-like vascular networks, and vessels in the brain periphery either did not develop or degenerated. Many Fbw7−/− embryos also manifested intracranial hemorrhage (Fig. 2, B and C), presumably as a result of the compromised integrity of the endothelial wall of the abnormal vessels. The deficit in vascular remodeling was most evident in the yolk sac vasculature. Extensive remodeling, resulting in the formation of large and small vessels, takes place in the yolk sac of wild-type embryos (Fig. 2F). However, although the honeycomb-like vascular plexus appeared to form normally at E8.5 in Fbw7−/− embryos, it remained largely unchanged across most of the surface of the yolk sac at E10.5 (Fig. 2G), suggesting that Fbw7 is essential for the remodeling of these vessels. Furthermore, formation of the major vessels, including the anterior cardinal vein, was severely impaired in Fbw7−/− embryos (Fig. 2, H and I).

To confirm that the vascular phenotype of Fbw7−/− embryos was not a secondary effect of a general failure in embryonic development, we studied in vitro para-aortic splanchnopleural (P-Sp) explant cultures, which support the growth of endothelial cells (31, 32). P-Sp explants from E9.5 Fbw7+/− and Fbw7−/− embryos were co-cultured with OP9 stromal cells and then immunostained with anti-PECAM-1. The formation of an extensive PECAM-1-positive vascular network apparent in the Fbw7+/− P-Sp explants was almost completely inhibited in the Fbw7−/− P-Sp explants (Fig. 3), suggesting that the failure of normal vascular development in Fbw7−/− embryos is attributable to a defect in endothelial cells or supporting tissues, not to a general retardation of embryonic growth.

Accumulation of Notch1 in Fbw7−/− Mice—Given that the phenotype of Fbw7−/− mice is similar to those of mutant zebrfish or mice in which Notch signaling is impaired, we hypothesized that accumulation of the Notch ICD, a potential target for Fbw7-mediated ubiquitylation, is responsible for the abnormal vascular development in Fbw7−/− embryos. We therefore examined the expression of Notch1−4, all of which are thought to be important for normal vascular development (7, 8), in Fbw7−/− embryos. The abundance of the ICDs of Notch1−3 did not appear to be affected by the Fbw7 mutation, whereas that of Notch4-ICD was markedly increased in Fbw7−/− embryos (Fig. 4A). The amount of Notch4-ICD in Fbw7−/− embryos appeared to be intermediate between that in Fbw7+/− and that in Fbw7+/− embryos.

To evaluate the turnover rate of Notch4-ICD, we generated ES cells in which both Fbw7 alleles were disrupted and then transfected both these cells and wild-type cells with expression vectors for Notch1-ICD or Notch4-ICD. The rate of degradation of Notch1-ICD was similar in Fbw7+/− and Fbw7−/− ES cells, whereas the turnover of Notch4-ICD was markedly slower in Fbw7−/− cells than in Fbw7+/− cells (Fig. 4B). These data thus
suggest that Fbw7 is required for proteolysis of Notch4-ICD but not for that of Notch1-ICD.

Expression of an Fbw7 mutant (Fbw7WD) that lacks the NH₂-terminal and F-box domains was shown previously (26) to result in accumulation of both Notch1-ICD and Notch4-ICD in Bosc23 cells. We performed similar experiments with HEK293T cells, and we found that the expression of Fbw7WD induced the accumulation of Notch4-ICD but not that of Notch1-ICD (Fig. 4C). Although the reason for this discrepancy with regard to Notch1-ICD is unclear, the difference in the cell lines studied might be a contributing factor. We also found that the abundance of presenilin did not differ between Fbw7+/+ and Fbw7−/− embryos (data not shown).

Enhanced Expression of Hey1 in Fbw7−/− Mice—We next examined the expression of members of the Hes and Hey families, downstream components of the Notch signaling pathway, by whole mount in situ hybridization (Fig. 5). Whereas the abundance of Hes5, Hes7, Hey2, HeyL, and Uncx4.1 mRNAs in Fbw7−/− embryos was almost identical to that in Fbw7+/+ embryos, the amount of Hey1 mRNA was markedly increased in the vessels of Fbw7−/− embryos compared with that in the vessels of Fbw7+/+ embryos. The accumulation of Notch4-ICD in Fbw7−/− embryos may thus lead to increased expression of Hey1 and a consequent effect on vascular development.

Normal Expression of Cyclin E in Fbw7−/− Mice—Given that biochemical and genetic evidence in Drosophila suggests that cyclin E is a target for Fbw7-mediated ubiquitylation and subsequent proteolysis (16–18), we also examined the expression of cyclin E in Fbw7+/+ embryos. Neither immunoblot nor immunohistochemical analysis with specific antibodies detected accumulation of cyclin E in Fbw7−/− embryos (Fig. 6, A and C). Immunoblot analysis also revealed a similar abundance of cyclin-dependent kinase 2 (CDK2) and p27Kip1 in Fbw7+/+ and Fbw7−/− embryos (Fig. 6A). Furthermore, the kinase activity associated with CDK2, cyclin A, or cyclin E was not increased in Fbw7−/− embryos (Fig. 6B). In contrast, the abundance of cyclin E and p27Kip1 was greatly increased in Skp2−/− embryos (Fig. 6, A and C), consistent with our previous data (37). Cyclin E may thus be subject to dual control by two F-box proteins, Fbw7 and Skp2, with Fbw7 being dispensable for the regulation of cyclin E abundance at least until the mid-stage of embryonic development.

**DISCUSSION**

Notch signaling is implicated in vascular remodeling during embryonic development. Several isoforms of Notch are expressed in the developing vasculature (38), with Notch4 expression being restricted to cells of the endothelial lineage (39, 40). Vascular remodeling deficits similar to those evident in Fbw7−/− embryos have been observed in mice lacking Notch1, both Notch1 and Notch4 (7), Tie2 (41), or angiopoietin-1 (42) but not in Notch4−/− embryos (7), suggesting that Notch1 compensates for the loss of function of Notch4. In contrast, increased expression of Notch4 impairs vascular development, as is evident in mice harboring a transgene for an activated form of Notch4 (8). The similarity

**Fig. 2. Phenotype of Fbw7 heterozygous mutant embryos.** A–C, gross appearance of Fbw7+/+ (A) and Fbw7−/− (B and C) embryos at E10.5. Arrows in B and C indicate hemorrhage in the head region. D–G, whole mount immunohistochemical analysis with anti-PECAM-1 of the head region (D and E) and yolk sac (F and G) of E10.5 embryos. Fbw7−/− embryos (E and G) exhibited a disorganized vasculature compared with the fine vascular network of Fbw7+/+ embryos (D and F). Arrows and arrowheads in D indicate head veins and the internal carotid artery, respectively. H and I, immunohistochemical staining with anti-P-Sp of Fbw7−/− (H) and Fbw7+/+ (I) embryos at E9.5. Arrowheads in I indicate the absence of anterior cardinal veins in Fbw7−/− embryos. Abbreviations are as in Fig. 1 legend. Scale bars: 500 μm (A–C, F, and G), 250 μm (D and E), or 100 μm (H and I).

**Fig. 3. Impaired vascular network formation by Fbw7−/− P-Sp explants.** P-Sp explants from E9.5 Fbw7−/− (A) or Fbw7+/+ (B) embryos were cultured on OP9 stromal cells and then immunostained with anti-PECAM-1. P-Sp explants from Fbw7−/− embryos, but not those from Fbw7+/− embryos, formed an extensive PECAM-1-positive vascular network. Abbreviations used are as follows: vb, vascular bed; vn, vascular network. Scale bar, 1 mm.
between Fbw7−/− mice and Notch4 transgenic mice suggests that Fbw7 and Notch4 are genetically related. In addition to its apparent role in vascular remodeling, Notch signaling is also implicated in arterial versus venous cell fate determination during embryonic vascular development (9, 11). Loss of Notch signaling thus results both in the loss of arterial markers and in the ectopic expression of venous markers in the dorsal aorta. Conversely, activation of Notch signaling results in repression of venous cell fate determination. The impaired formation of major veins, including the anterior cardinal vein, in Fbw7−/− embryos suggests that the accumulation in these embryos of Notch4-ICD, which is a potential target for Fbw7-mediated ubiquitylation, may result in the repression of venous cell fate determination. Members of the Hey (also referred to as HRT or HESR) family of proteins function as transcriptional repressors downstream of Notch signaling (43) and play an important role in vascular network formation and determination of arterial-venous cell fate. Our data indicate that Fbw7 is indispensable for normal vascular development and that the inactivation of Fbw7 in mice affects the expression of Notch4 and Hey1 but not that of Notch1, Notch2, Notch3, Hes5, Hes7, Hey2, or HeyL. The molecular mechanism underlying the specificity of the effect of Fbw7 on Notch4-Hey1 signaling remains to be determined.

In zebrafish, the gridlock protein, which is a homolog of mammalian Hey2 and contributes to the arterial-venous cell fate decision (9, 10, 44), is a candidate downstream target of venous cell fate determination. FIG. 4. Accumulation of Notch4-ICD in Fbw7−/− embryos. A, immunoblot analysis of Fbw7+/+, Fbw7−/−, and Fbw7−/− embryos at E9.5 for the abundance of the ICDs of Notch1–4. The expression of α-tubulin was examined as a loading control. B, turnover rate of Notch4-ICD and Notch1-ICD in Fbw7+/+ and Fbw7−/− ES cells. Cells expressing Notch1-ICD-HA (upper panels) or Notch4-ICD-mycHis (lower panels) were lysed at the indicated times after exposure to cycloheximide and then subjected to immunoblot analysis with anti-HA or anti-Myc, respectively, and with anti-α-tubulin. C, specific stabilization of Notch-ICD by Fbw7WD. HEK293T cells were transfected both with an expression vector for Notch1-ICD-HA (upper panels) or Notch4-ICD-mycHis (lower panels) and with the indicated amounts of a vector for FLAG epitope-tagged Fbw7WD. The transfected cells were subsequently subjected to immunoblot analysis with anti-HA or anti-Myc and with anti-α-tubulin as a control.

FIG. 5. Accumulation of Hey1 mRNA in Fbw7−/− embryos. Both Fbw7+/+ (A, C, E, G, I, and K) and Fbw7−/− (B, D, F, H, J, and L) embryos at E9.5 were subjected to whole-mount in situ hybridization with probes specific for Hes5 (A and B), Hes7 (C and D), Hey1 (E and F), Hey2 (G and H), HeyL (I and J), or UnCx4.1 (K and L) mRNAs. The amount of Hey1 mRNA was increased in Fbw7−/− embryos, especially along the dorsal aorta (arrowheads in F) compared with that in Fbw7+/+ embryos. Scale bar, 500 μm.
The secondary effect of p27

Notch signaling. A mutation in gridlock results in a localized defect of the aorta that resembles aortic co-arctation in humans. However, inactivation of Hey2 in mice does not appear to affect aortic development but rather results in cardiac malformations including ventricular septal defects, tetralogy of Fallot, and tricuspid atresia (45, 46). The dissimilarity in these mutant phenotypes between zebrafish and mice suggests that gridlock and Hey2 perform different functions. On the other hand, overexpression of Hey1 in human endothelial cells was shown to block vascular network formation (47). Our present results also suggest that an increased abundance of Hey1 that results from inactivation of Fbw7 affects vascular remodeling as well as arteriovenous differentiation. The similarity in phenotypes between zebrafish gridlock and mouse Hey1 mutants suggests that mouse Hey1 is the functional homolog of gridlock, even though molecular phylogenetic analysis indicates that mouse Hey2 is the ortholog of zebrafish gridlock (45).

Although many F-box proteins have been identified, their physiological targets and functions in most instances remain unclear. We previously identified the F-box protein Skp2 as an important regulator of cyclin E and p27Kip1. Mice lacking Skp2 manifest cellular accumulation of cyclin E and p27Kip1 as well as overreplication of chromosomes and centrosomes (37). The increased abundance of cyclin E in Skp2−/− embryos is not a secondary effect of p27Kip1 accumulation, given that it was also apparent in Skp2−/− p27−/− double mutant mice and that cyclin E ubiquitylation was markedly increased by recombiant Skp2 in vitro (37). In addition, CDK9 and p130, other potential targets of Skp2-mediated ubiquitylation, also accumulate in Skp2−/− mice. In contrast, E2F-1, another Skp2

target, does not accumulate in Skp2−/− mice, suggesting the existence of another redundant pathway for the control of E2F-1 abundance. Potential targets of mammalian Fbw7 have been thought to include Notch1, Notch4, cyclin E, and prese

ilin. However, the inactivation of Fbw7 affected the abundance of Notch4 but not that of Notch1, cyclin E, or prese

nilin, suggesting that the amount of the latter three proteins is also controlled by other mechanisms. Four ubiquitin ligases have been shown to contribute to the control of Notch1 signaling (13). These enzymes include the following: Suppressor of deltex (Itch) and Fbw7, both of which regulate Notch1; Neutralized, which regulates the Notch ligand Delta; and LNX, which regulates the Notch antagonist Numb. Although it has not been clear whether Notch4 is controlled by the same mechanisms, our data suggest that Notch1 and Notch4 may be regulated by different mechanisms.

Truncated Notch proteins exhibit transforming activity both in vitro (48–50) and in animal models (51–53). Deregulated expression of wild-type Notch or of Notch ligands or downstream targets has been detected in cervical, lung, colon, head and neck, and renal carcinomas (54–57), acute myeloid leukemia (58), and Hodgkin’s and large cell lymphomas (59). Notch1 was recently shown to be a downstream effector of oncogenic Ras, and down-regulation of Notch1 in Ras-transformed human cells was sufficient to abolish key elements of the neoplastic phenotype in vitro and in vivo (60). Notch4 was also originally identified as Int8, a proto-oncogene that is a frequent target for integration of mouse mammary tumor virus in mammary carcinomas (39, 40, 61). Mutation of human FBW7 and an associated accumulation of cyclin E has been detected in various cancer cell lines (17, 18). Our present findings suggest that mutation of human FBW7 may result in impairment of Notch4 degradation and consequent Notch4 accumulation, which, together with the accumulation of cyclin E, might then contribute to carcinogenesis.

Acknowledgments—We thank N. Osumi, T. Takeuchi, M. Shibuya, and S. Nishikawa for helpful discussions; Y. Yamada, K. Shimoharada, S. Matsuhashi, N. Nishimura, R. Yasukochi, and other laboratory members for technical assistance; and C. Sugita for help in preparation of the manuscript.

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K. Nakayama and K. I. Nakayama, manuscript in preparation.
