**Functional Regulation of FEZ1 by the U-box-type Ubiquitin Ligase E4B Contributes to Neuritogenesis***

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E4B (also known as UFD2a) is a mammalian homolog of *Saccharomyces cerevisiae* Ufd2, which was originally described as a ubiquitin chain assembly factor (E4). E4B is a U-box-type ubiquitin-protein isopeptide ligase (E3) and likely functions as either an E3 or an E4. With a yeast two-hybrid screen, we have now identified FEZ1 (fasciulation and elongation protein zeta 1) as a protein that interacts with E4B. FEZ1 is implicated in neuritogenesis when phosphorylated by protein kinase Cζ (PKCζ). Interaction between E4B and FEZ1 in mammalian cells was enhanced by coexpression of constitutively active PKCζ. E4B mediated the polyubiquitylation of FEZ1 but did not affect its intracellular stability, suggesting that such modification of FEZ1 is not a signal for its proteolysis. Polyubiquitylation of FEZ1 by E4B required Lys27 of ubiquitin. Expression of a dominant-negative mutant of E4B in rat pheochromocytoma PC12 cells resulted in inhibition of neurite extension induced either by nerve growth factor or by coexpression of FEZ1 and constitutively active PKCζ. These findings indicate that E4B serves as a ubiquitin ligase for FEZ1 and thereby regulates its function but not its degradation.

The steady-state concentrations of cellular proteins are maintained by the balance between their synthesis and degradation, with the regulation of this balance underlying the control of many cellular functions. The ubiquitin proteolytic pathway plays an important role in the elimination of short-lived regulatory proteins (1), including those that contribute to the cell cycle, cellular signaling in response to environmental stress or extracellular ligands, morphogenesis, secretion, DNA repair, and organelle biogenesis (2, 3). This pathway includes two key steps: covalent attachment of multiple ubiquitin molecules to the protein substrate and degradation of the ubiquitylated protein by the 26 S proteasome complex. The system responsible for the attachment of ubiquitin to the target protein consists of several components that act in concert (4, 5),

including a ubiquitin-activating enzyme (E1), a ubiquitin carrier protein (E2), and a ubiquitin-protein isopeptide ligase (E3). E3 is thought to be the component of the ubiquitin conjugation system that is most directly responsible for substrate recognition (5, 6).

On the basis of structural similarity, E3 enzymes have been classified into two families: the HECT (homologous to E6-AP COOH terminus) family (7) and the RING finger-containing protein family (8–10). Recently, however, we (11) and others (12–16) identified a third family of E3 enzymes known as the U-box proteins. The prototype U-box protein, *Saccharomyces cerevisiae* Ufd2, was originally described as a ubiquitin chain assembly factor (also designated E4) that promotes the polyubiquitylation of artificial ubiquitin fusion proteins in conjunction with E1 (Uba1), E2 (Ubc4), and E3 (Ufd4, a HECT-type E3) enzymes (17, 18). Ufd2 and its homologs in other eukaryotes share a conserved domain of ~70 amino acids termed the U-box. E4 is required for further elongation of an oligoubiquitin chain on certain types of substrates, and the resulting polyubiquitylated proteins are then recognized by the 26 S proteasome for degradation. Aravind and Koonin (19) showed, by means of sequence profile analysis, that the U-box is a derived version of the RING finger domain. However, most of the signature cysteines, the hallmark metal-chelating residues of the RING finger, are not conserved in the U-box. Nevertheless, the predicted three-dimensional structure of the U-box is similar to that of the RING finger domain (19, 20). This observation suggested the possibility that U-box proteins in general are able to function as E3 enzymes. Indeed, Patterson and co-workers (12) showed that CHIP (COOH terminus of Hsc70-interacting protein) functions as an E3. We also showed that all six mammalian U-box proteins tested mediate polyubiquitylation in the presence of E1 and E2 enzymes independent of the presence of other E3 enzymes (11). Thus, U-box proteins do possess E3 activity, and their E4 activity likely represents a specialized type of E3 activity apparent with oligoubiquitylated artificial fusion proteins as substrates.

In yeast, Ufd2 is implicated in cell survival under stressful conditions and is associated with Cdc48 (18), which belongs to the large family of AAA-type ATPases that are thought to possess chaperone activity (21, 22). We have shown previously that mouse E4B (UFD2a), a homolog of yeast Ufd2, is abundant in neurons (23). E4B interacts with VCP, a mammalian or-

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The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; E4, ubiquitin chain assembly factor; PKCζ, protein kinase Cζ; caPKCζ, constitutively active PKCζ; HA, hemagglutinin; CHAPS, 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; NGF, nerve growth factor; shRNA, short hairpin RNA; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.
olog of yeast Cdc48, suggesting that the association of AAA-type ATPases with Ufd2-like proteins has been conserved through evolution and thus may be functionally important (23).

We also found that E4B interacts with, and thereby mediates the polyubiquitylation of, ataxin-3, the protein in which the abnormal expansion of a polyglutamine tract is responsible for spinocerebellar ataxia type 3 (24).

In this study, we isolated FEZ1 (fasciculation and elongation protein zeta 1), a protein implicated in neurite extension, as a binding partner of E4B. FEZ1 is a mammalian homolog of Caenorhabditis elegans UNC-76, which is required for axonal bundling and elongation in the nematode (25), suggesting that FEZ1 also participates in axonal outgrowth and fasciculation in mammals. FEZ1 is phosphorylated by protein kinase Cζ (PKCζ), and this phosphorylation reaction appears to be required for neurite extension in rat pheochromocytoma PC12 cells (26, 27). The possible role of E4B in the functional regulation of FEZ1 as well as in neuritogenesis was investigated.

**EXPERIMENTAL PROCEDURES**

Antibodies—Immunoblot analysis was performed with antibodies to Myc (1 μg/ml; clone 9E10, Covance), to FLAG (1 μg/ml; clone M5, Sigma), to the hemagglutinin epitope (1 μg/ml; clone HA.11/16B12, Babco), to Hsp70 (1 μg/ml; clone 7, Transduction Laboratories), to E4B (0.25 μg/ml; clone 7, Transduction Laboratories), to ubiquitin (1 μg/ml; clone 1B3, Medical Biological Laboratories), and to hexahistidine (0.2 μg/ml; clone H15, Santa Cruz Biotechnology). Rabbit polyclonal antibodies to human FEZ1 (used at 1 μg/ml) were generated by standard procedures with the use of a recombinant protein expressed in Sf21 insect cells.

**Yeast Two-hybrid System**—Yeast strain L40 (MATα LYS2-LexA-HIS3 URA3::LexA-lucZ trp1 leu2 his3) (Invitrogen) was transformed both with the plasmid pGBKLexA encoding the U-box domain (amino acids 919–1173) of mouse E4B (GenBank™/EBI accession number NP_071305) and with a human brain cDNA library in the pACT2 vector (Clontech). The cells were then streaked on plates of medium lacking histidine to detect interaction-dependent activation of HIS3 according to the assay protocol (Matchmaker, Toyobo).

**Cloning of cDNAs**—The cDNAs for human FEZ1 (GenBank™/EBI...
accession number U48249) and human PKC\mu/H9256 (accession number XM_049769) were amplified from a brain cDNA library (BD Biosciences) by PCR with Taq polymerase (Takara). The amplified fragments were subcloned into pBluescript II SK\mu/H11001 (Stratagene) and sequenced. The cDNA for constitutively active PKC\mu/H9256 (caPKC\mu/H9256) was prepared by deleting the nucleotide sequence corresponding to the pseudo-substrate region (Arg116–Trp122) from the full-length cDNA (28). These various cDNAs were subcloned into pcDNA3 (Invitrogen), pCGN (kindly provided by A. Nagafuchi), pCR (Invitrogen), pFASTBAC (Invitrogen), or pGBKT7 (BD Biosciences). The plasmid pT7-7-UbcH5c (Novagen) was described previously (11), and pMT107 encoding His6-ubiquitin was kindly provided by D. Bohmann. The plasmids pcDNA3-FLAG-E4B, pGEX-6P-HA-ubiquitin, and pBacPAC9-GST-FLAG-E4B were described previously (11). A cDNA for E4B tagged at its N terminus with the hemagglutinin (HA) epitope was generated by PCR, sequenced, and cloned into pcDNA3. A cDNA for \beta-galactosidase was subcloned into pcDNA3.1-Myc-His (Invitrogen).

Transfection, Immunoprecipitation, and Immunoblot Analysis—Transfection, immunoprecipitation, and immunoblot analysis were performed as described previously (29). Urea (8 M) was added to the cell lysis buffer.
for the purification of Hist-ubiquitin-conjugated proteins by chromatography on ProBond resin (Invitrogen); proteins were eluted from the resin with a solution containing 50 mM sodium phosphate buffer (pH 8.0), 100 mM KCl, 20% glycerol, 0.2% Nonidet P-40, and 200 mM imidazole. For detection of the interaction between endogenous E4B and Myc-FEZ1 in PC12 cells, the cells were lysed in a solution containing 50 mM Tris-HCl (pH 7.4), 120 mM NaCl, 0.2% CHAPS, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 0.4 mM NaF, 0.4 mM EDTA, 10 mM NaF, and 10 mM sodium pyrophosphate.

Phosphatase Treatment—α-Protein phosphatase (New England BioLabs Inc.) was used to dephosphorylate immunoprecipitated HA-FEZ1 expressed in HEK293T cells. The reaction was performed according to the manufacturer’s instructions. In brief, the immunoprecipitates were incubated with or without 800 units of enzyme in 100 μl of α-phosphatase reaction buffer at 30 °C for 2 h. After washing three times with in vitro ubiquitination buffer, the immune complex was used as substrate in an in vitro ubiquitination assay.

In Vitro Ubiquitination Assay—Recombinant glutathione S-transferase-HA-ubiquitin, glutathione S-transferase-FLAG-E4B, His6-UbcH5c, and His6-Myc-FEZ1 were expressed and purified as described previously (11). The in vitro ubiquitination assay was performed as described previously (8) with some modifications. In brief, reaction mixtures (10 μl) containing 15 ng of FLAG-E4B, 0.1 μg of recombinant rabbit E1 (Boston Biomedica), 0.5 μg of purified His6-UbcH5c, 3.5 units of phosphocreatine kinase, 0.6 units of inorganic pyrophosphatase, 0.3 μg of HA-tagged wild-type or mutant ubiquitin, 40 mM Tris-HCl (pH 7.4), 50 mM KCl, 2 mM ATP, 5 mM MgCl2, 1 mM dithiothreitol, 10% glycerol, and 10 mM creatine phosphate were incubated for 2 h at 30 °C with 50 ng of Hist6-Myc-FEZ1 as substrate. The reaction was terminated by adding SDS sample buffer containing 4% β-mercaptoethanol and heating at 95 °C for 5 min.

Pulse-Chase Analysis—Pulse-chase analysis was performed as described previously (30) with slight modifications. In brief, HEK293T cells grown in 15-cm dishes to 50% confluency were transiently transfected with 45 μg of pCGN-HA-FEZ1 and 45 μg of either pcDNA3-FLAG-E4B or pcDNA3-FLAG-E4B(P1140A). After 36 h, the cells were metabolically labeled by incubation for 1 h with [35S]methionine, and subjected (30) with slight modifications. In brief, HEK293T cells transfected with the indicated combinations of vectors for HA-FEZ1, Myc-caPKCζ, FLAG-E4B, and FLAG-CHIP were pulse-labeled with [35S]methionine and then incubated in the absence of isotope with or without MG132 for the indicated chase periods.

B, a portion of the cell lysate corresponding to 10% of the input for pulse-chase analysis was subjected to immunoblot (IB) analysis with anti-E4B, anti-FLAG, anti-CHIP, anti-HA, anti-Myc, or anti-Hsp70 (loading control) antibody. The intensity of the immunoreactive bands obtained with anti-E4B antibody was determined relative to that obtained for cells not expressing exogenous U-box protein.

C, cell lysates were subjected to immunoprecipitation with anti-HA antibody, and the resulting precipitates were analyzed by SDS-PAGE, autoradiography, and scanning densitometry. The amount of 35S-labeled HA-FEZ1 remaining is expressed as a percentage of that present at the beginning of the chase period.

D, the amount of 35S-labeled HA-FEZ1 remaining is expressed as a percentage of that present at the beginning of the chase period.
Assay of Nerve Growth Factor (NGF)-induced Neuritogenesis in PC12 Cells—PC12 cells were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum, 10% horse serum, penicillin, and streptomycin. They were subsequently seeded on poly-L-lysine-coated 2-cm² dishes at a density of 5\times10^3 cells/cm². After incubation overnight in the same culture medium, the cells were exposed for 3 days to NGF (50 ng/ml; Alomone Labs) in Dulbecco's modified Eagle's medium supplemented with 1% horse serum, penicillin, and streptomycin. The cells were then examined by phase-contrast microscopy, and photographs were taken at 40 magnification. About 100 cells were scored for the presence of neurites with a length greater than the diameter of the soma.

Retroviral Expression System—cDNAs encoding mouse wild-type E4B or E4B(P1140A) containing an N-terminal FLAG tag and a cDNA for human FEZ1 containing an N-terminal Myc epitope tag were subcloned into pMX-puro (kindly provided by T. Kitamura), and the resulting vectors were used to transfect Plat E cells and thereby to generate recombinant retroviruses. PC12 cells were infected with the recombinant retroviruses and subjected to selection in medium containing puromycin (5\mu g/ml) for 14 days. Cells expressing the recombinant proteins were pooled for experiments.

RNA Interference—The pMX-puro II vector was constructed by deletion of the U3 portion of the 3'long terminal repeat of pMX-puro. The mouse U6 gene promoter followed by a DNA sequence encoding a short hairpin RNA (shRNA) was subcloned into the NotI and XhoI sites of pMX-puro II, yielding pMX-puro II-U6/shRNA. The DNA sequence for the shRNA comprised two 21-nucleotide complementary tracts specific for the mRNA target separated by a loop sequence (-TTCAAGAGA-) and followed by a string of five T nucleotides to terminate transcription. The hairpin sequences specific for mouse E4B and enhanced green fluorescent protein (Clontech) mRNAs corresponded to nucleotides 2466–2486 and nucleotides 126–146 of the respective coding regions. Recombinant retroviruses were generated and used to infect PC12 cells as described above. After selection in medium containing puromycin (5 \mu g/ml), cells stably expressing the shRNAs were pooled for experiments.

Electroporation and X-Gal Staining—PC12 cells (2.0 \times 10^7) were...
suspended in 0.4 ml of RPMI 1640 medium supplemented with 5% fetal bovine serum and 10% horse serum and containing 33 μg of pCGN-HA-PKCζ, 38 μg of pcDNA3-FLAG-E4B, and 4 μg of pcDNA3.1-LacZ-Myc-His6 as a reporter plasmid (10:10:1 molar ratio). After incubation on ice for 10 min, the cells were subjected to electroporation using a Gene-Pulser II (Bio-Rad) at 1.25 kV/cm and 975 microfarads. The cells were then incubated on ice for an additional 10 min before plating in the same culture medium on 10-cm Petri dishes coated with poly-L-lysine. After culture for 72 h, the cells were fixed and stained for β-galactosidase activity with X-gal as described previously (26). About 100 β-galactosidase-positive (blue) cells were scored by phase-contrast microscopy for the presence of neurites with a length greater than the diameter of the soma.

RESULTS

FEZ1 Interacts with E4B—We screened a human brain cDNA library with the yeast two-hybrid assay and the U-box domain of mouse E4B (amino acids 919–1173) as bait. From 1 × 10⁶ transformants that were able to grow on Leu- and Trp-deficient medium, eight positive clones were isolated after two rounds of growth in the absence of His and screening for β-galactosidase activity with X-gal as described previously (26). About 100 β-galactosidase-positive (blue) cells were scored by phase-contrast microscopy for the presence of neurites with a length greater than the diameter of the soma.

mammalian cells, we stably transfected PC12 cells with an expression plasmid encoding Myc epitope-tagged FEZ1. Lysates of the transfected cells were then subjected to immunoprecipitation with anti-Myc antibody, and the resulting precipitates were analyzed by SDS-PAGE, autoradiography, and scanning densitometry. C, the amount of ³⁵S-labeled HA-FEZ1 remaining is expressed as a percentage of that present at the beginning of the chase period.

![Image of a graph showing FEZ1 interactions with E4B](image-url)

**Fig. 5.** A dominant-negative mutant of E4B does not inhibit the degradation of FEZ1. HEK293T cells were transfected with the indicated combinations of vectors for HA-FEZ1, Myc epitope-tagged wild-type (WT) or constitutively active (CA) PKCζ, and FLAG-tagged wild-type E4B or E4B(P1140A). The transfected cells were pulse-labeled with [³⁵S]methionine and then incubated for the indicated chase periods in the absence of isotope with or without the proteasome inhibitor MG132. A, a portion of the cell lysate corresponding to 3% of the input for pulse-chase analysis was subjected to immunoblot (IB) analysis with anti-FLAG, anti-E4B, anti-HA, anti-Myc, or anti-Hsp70 antibody. The numbers shown below the E4B blot represent the relative abundance of total E4B. B, cell lysates were subjected to immunoprecipitation (IP) with anti-HA antibody or normal mouse IgG (negative control), and the resulting precipitates were analyzed by SDS-PAGE, autoradiography, and scanning densitometry. C, the amount of ³⁵S-labeled HA-FEZ1 remaining is expressed as a percentage of that present at the beginning of the chase period.
amined them for the ability to interact with FEZ1 (Fig. 1D).
Although we identified FEZ1 as a protein that interacts with the U-box domain of E4B, E4BΔC, which lacks the C-terminal region containing the U-box domain, interacted with FEZ1 as well as did wild-type E4B. E4BΔNM, which lacks the N-terminal and middle regions, and E4BΔNC, which lacks the N- and C-terminal regions, weakly associated with FEZ1, whereas E4BΔMC, which lacks the middle and C-terminal regions, did not bind to FEZ1. These findings suggest that multiple sites of E4B contribute to the interaction with FEZ1.

Polyubiquitylation of FEZ1 Mediated by E4B in Vivo and in Vitro—We next investigated whether the interaction of FEZ1 with the U-box-type ubiquitin ligase E4B results in the polyubiquitylation of the former by the latter. Coexpression of FLAG-E4B with HA-FEZ1 in HEK293T cells resulted in a marked increase in the extent of polyubiquitylation of HA-FEZ1 (Fig. 2A). To rule out the possibility that a protein bound to FEZ1 was polyubiquitylated by E4B, we transfected HEK293T cells with a vector for His6-ubiquitin as well as with vectors for HA-FEZ1 and FLAG-E4B and then purified the FLAG-E4B or FLAG-E4B(P1140A) or those infected with the corresponding empty retrovirus were incubated with cycloheximide (50 μg/ml) for the indicated times, after which cell lysates were subjected to immunoblot (IB) analysis with the indicated antibodies. The arrow indicates FEZ1, and the asterisks indicate nonspecific bands. B, the amount of FEZ1 remaining after the various times of incubation of cells with cycloheximide was determined by scanning densitometry of the immunoblots. Data are expressed as a percentage of the amount of FEZ1 present at time 0 and are the means ± S.D. from three independent experiments.

**Fig. 6. Exogenous E4B or E4B(P1140A) does not affect the degradation of endogenous FEZ1 in PC12 cells.** A, cells stably expressing FLAG-E4B or FLAG-E4B(P1140A) or those infected with the corresponding empty retrovirus were incubated with cycloheximide (50 μg/ml) for the indicated times, after which cell lysates were subjected to immunoblot (IB) analysis with the indicated antibodies. The arrow indicates FEZ1, and the asterisks indicate nonspecific bands. B, the amount of FEZ1 remaining after the various times of incubation of cells with cycloheximide was determined by scanning densitometry of the immunoblots. Data are expressed as a percentage of the amount of FEZ1 present at time 0 and are the means ± S.D. from three independent experiments.

**Polyubiquitylation of FEZ1 mediated by E4B in Vivo and in Vitro**—We next investigated whether the interaction of FEZ1 with the U-box-type ubiquitin ligase E4B results in the polyubiquitylation of the former by the latter. Coexpression of FLAG-E4B with HA-FEZ1 in HEK293T cells resulted in a marked increase in the extent of polyubiquitylation of HA-FEZ1 (Fig. 2A). To rule out the possibility that a protein bound to FEZ1 was polyubiquitylated by E4B, we transfected HEK293T cells with a vector for His6-ubiquitin as well as with vectors for HA-FEZ1 and FLAG-E4B and then purified the His6-ubiquitin-labeled proteins under denaturing conditions (Fig. 2B). This approach again showed that overexpression of E4B increased the ubiquitylation of FEZ1. Given that expression of caPKCζ enhanced the interaction between E4B and FEZ1 (Fig. 1B), we examined the effect of caPKCζ on the polyubiquitylation of FEZ1 mediated by E4B. Coexpression of caPKCζ with E4B further increased the polyubiquitylation of FEZ1 compared with overexpression of E4B alone or E4B and wild-type PKCζ (Fig. 2B). These results thus indicate that E4B catalyzes the polyubiquitylation of FEZ1 in mammalian cells. We also performed an in vitro ubiquitylation assay with His6-Myc-tagged FEZ1 that was expressed in and purified from Sf21 insect cells (Fig. 2C). FEZ1 was polyubiquitylated by recombinant E4B that was also expressed in and purified from Sf21 cells (Fig. 2C, compare fifth and eighth lanes), and this reaction was dependent on the presence of ATP, ubiquitin, E1, and E2 (UbcH5c).

To investigate the relationship between phosphorylation and polyubiquitylation of FEZ1, experiments with phosphatase treatment of FEZ1 were performed. HA-FEZ1 was coexpressed with caPKCζ in HEK293T cells, and the lysates of the cells were immunoprecipitated with anti-HA antibodies. Treatment of the immunoprecipitates with λ-protein phosphatase resulted in the disappearance of the lower mobility form of FEZ1 (Fig. 1F, compare third and fourth lanes). These results thus suggest that phosphorylation is responsible for the observed shift in the electrophoretic mobility of FEZ1. Furthermore, phosphatase treatment significantly decreased the polyubiquitylation of FEZ1 (Fig. 2D), suggesting that phosphorylation of this protein is critical for its polyubiquitylation.

**E4B-mediated Ubiquitylation Does Not Induce the Degradation of FEZ1—**Pulse-chase analysis revealed that the proteasome inhibitors MG132 and clasto-lactacystin β-lactone inhibited the degradation of HA-FEZ1 expressed in HEK293T cells (Fig. 3A), indicating that FEZ1 is degraded by the ubiquitin-proteasome system. We therefore examined the possible effect of overexpression of E4B or CHIP, another member of the U-box family of ubiquitin ligases, on the degradation of FEZ1. However, overexpression of neither E4B nor CHIP affected the stability of FEZ1, whereas MG132 markedly inhibited FEZ1 degradation in the same cells (Fig. 3, B–D). These results suggest that E4B-mediated ubiquitylation does not promote the degradation of FEZ1 by the proteasome.

**Inhibition of E4B Function by a Dominant-negative Mutant—**We have shown that Pro1140 in the U-box domain of E4B is highly conserved in all U-box-type E3 enzymes and is essential for E3 activity (11). A mutant form of E4B in which Pro1140 is replaced with alanine (P1140A) might thus be expected to function in a dominant-negative manner. As with the interaction between wild-type E4B and FEZ1, that between E4B(P1140A) and FEZ1 was increased by expression of caPKCζ (Fig. 4A), although the extent of the increase was less pronounced than that observed with wild-type E4B. To examine the possible dominant-negative effect of E4B(P1140A), we performed in vitro ubiquitylation assays with wild-type E4B and FEZ1 in the presence of various amounts of E4B(P1140A) (Fig. 4B). E4B(P1140A) markedly inhibited the polyubiquitylation of FEZ1 mediated by wild-type E4B in a concentration-dependent manner. Furthermore, expression of E4B(P1140A) in HEK293T cells also inhibited the polyubiquitylation of FEZ1...
induced by wild-type E4B (Fig. 4C). These in vitro and in vivo findings thus suggest that E4B(P1140A) acts in a dominant-negative manner.

Pulse-chase analysis revealed that the turnover rate of FEZ1 in HEK293T cells was not affected by overexpression of wild-type E4B or E4B(P1140A), even in the presence of CaPKCζ (Fig. 5). The degradation of FEZ1 was again inhibited by the proteasome inhibitor MG132, indicating that FEZ1 is degraded by the proteasome under these conditions. These data thus provide further support for the notion that the polyubiquitylation of FEZ1 mediated by E4B does not result in FEZ1 degradation.

E4B Does Not Promote the Degradation of FEZ1 in PC12 Cells—To examine whether E4B promotes the degradation of endogenous FEZ1 in PC12 cells, we infected the cells with recombinant retroviruses encoding either FLAG-E4B or FLAG-E4B(P1140A) or with the corresponding empty virus. Cell clones stably expressing the exogenous proteins were established and examined for FEZ1 stability by treatment with the protein synthesis inhibitor cycloheximide. Immunoblot analysis of cell lysates with anti-FEZ1 antibody revealed that the turnover rate of FEZ1 was not affected by expression of either wild-type E4B or the dominant-negative E4B(P1140A) mutant (Fig. 6).

E4B Mediates Lys27-linked Polyubiquitylation of FEZ1—Given that yeast Ufd4 (E3) and Ufd2 (E4) require Lys27 of the ubiquitin moiety of ubiquitin fusion proteins to mediate their polyubiquitylation (18), we examined which lysine residues of ubiquitin are required for the formation of a polyubiquitin chain on FEZ1 catalyzed by E4B. We therefore performed the in vitro ubiquitylation assay with wild-type ubiquitin or with a series of ubiquitin mutants that possess only one lysine residue, the other six lysines having been replaced with arginine (Fig. 7A). Performance of the assay with a ubiquitin mutant containing no lysine residues (K0) resulted in the generation of oligoubiquitylated FEZ1 (Fig. 7B), suggesting that FEZ1 is monoubiquitylated on its several lysine residues by E4B. A level of FEZ1 polyubiquitylation similar to that observed in the presence of wild-type ubiquitin was apparent only with the ubiquitin mutant containing Lys27 (K27); indeed, the extent of FEZ1 polyubiquitylation was greater with this mutant than with wild-type ubiquitin (Fig. 7B). To confirm that Lys27 of ubiquitin contributed to polyubiquitin chain formation, we performed the assay with a ubiquitin mutant in which only this lysine residue was replaced with arginine (K27R) (Fig. 7C). A polyubiquitin chain was not formed on FEZ1 in the presence of K27R mutant ubiquitin (Fig. 7D). These data thus suggest that Lys27 of ubiquitin is the preferred residue for polyubiquitylation of FEZ1 by E4B. We also investigated the autoubiquitylation of E4B in this assay (Fig. 7E). Our results indicate that the Lys27 linkage is also preferred by E4B for its autoubiquitylation. The Lys48 linkage, which is functionally linked to proteasomal degradation of modified proteins, was not prominent for either FEZ1 ubiquitylation or E4B autoubiquitylation. Given that substrate recognition by the proteasome requires at least four ubiquitin moieties linked via Lys48, the polyubiquitin chain formed on FEZ1 by E4B is likely not recognized as a signal for proteasome-mediated degradation.

E4B Is Required for Neuritogenesis in PC12 Cells—Phosphorylation of FEZ1 by PKCζ is thought to promote neurite extension. To assess the possible role of E4B in neuritogenesis, we exposed PC12 cells stably expressing wild-type E4B or E4B(P1140A) to NGF to induce this process. Whereas expression of wild-type E4B did not affect the percentage of cells bearing neurites in the presence of NGF, that of E4B(P1140A) resulted in a marked decrease in this parameter (Fig. 8, A and B), suggesting that the ubiquitin ligase activity of E4B is required for neuritogenesis.

Fig. 7. E4B prefers Lys27 of ubiquitin for the formation of a polyubiquitin chain on FEZ1. A, immunoblot (IB) analysis with anti-HA antibody of purified HA-tagged wild-type (WT) or mutant ubiquitin used for the in vitro assay of FEZ1 ubiquitylation. B, formation of a Lys27-linked polyubiquitin chain on FEZ1 mediated by E4B. The in vitro ubiquitylation assay was performed with FLAG-E4B, His6-Myc-FEZ1, and equal amounts of HA-tagged wild-type or mutant ubiquitin (HA-Ub) as indicated. The reaction mixtures were subjected to immunoblot analysis with anti-Myc antibody. The asterisk denotes a nonspecific band that possibly corresponds to a dimeric form of His6-Myc-FEZ1. C, immunoblot analysis with anti-HA antibody of purified HA-tagged wild-type or K27R mutant ubiquitin used for the in vitro assay. D, the in vitro ubiquitylation assay performed as described for B with FLAG-E4B and equal amounts of HA-tagged wild-type or mutant ubiquitin, e, the in vitro ubiquitylation assay performed as described for B with FLAG-E4B and equal amounts of HA-tagged wild-type or mutant ubiquitin, but in the absence of His6-Myc-FEZ1, as indicated. The reaction mixtures were subjected to immunoblot analysis with anti-FLAG antibody.
required for NGF-induced neuritogenesis in PC12 cells. We also applied RNA interference to determine the effect of depletion of endogenous E4B on neuritogenesis induced by NGF in PC12 cells. Cells infected with a retroviral vector encoding an shRNA specific for E4B mRNA exhibited a marked decrease in the abundance of E4B compared with cells infected with a control vector (Fig. 8C). The neurite extension induced by NGF was significantly inhibited by expression of the E4B shRNA (Fig. 8, D and E).

Cotransfection of PC12 cells with expression vectors for FEZ1 and caPKC\(\zeta\) was shown previously to induce neuritogenesis in the absence of NGF stimulation (26). We thus examined the effect of E4B on FEZ1-dependent neuritogenesis stimulated by PKC\(\zeta\). PC12 cells were infected with a recombinant retroviral vector encoding Myc-FEZ1; selected in the presence of puromycin for 14 days (Fig. 9A); and then subjected (together with control PC12 cells) to electroporation with vectors for wild-type PKC\(\zeta\) or caPKC\(\zeta\), for wild-type E4B or E4B(P1140A), and for \(\beta\)-galactosidase as a marker gene (31). As described previously (26), expression of caPKC\(\zeta\) alone did not increase the percentage of cells bearing neuritis, whereas the combined expression of FEZ1 and caPKC\(\zeta\) markedly increased neurite formation (Fig. 9, B and C). Coexpression of E4B with FEZ1 and caPKC\(\zeta\) resulted in a further increase in the proportion of cells with neurites. In contrast, coexpression of E4B(P1140A) greatly inhibited neurite formation in cells expressing FEZ1 and caPKC\(\zeta\) (Fig. 9, B and C). These data suggest that neuritogenesis in PC12 cells induced either by NGF or by coexpression of PKC\(\zeta\) and FEZ1 requires the ubiquitin ligase activity of E4B.

**DISCUSSION**

E3 enzymes have been thought to mark their substrates for proteolysis by the 26 S proteasome through the attachment of Lys\(^{48}\)-linked polyubiquitin chains (32). However, the existence of polyubiquitin chains linked through other lysine residues of ubiquitin has been demonstrated. Although the physiological functions of these latter chains have been largely unclear, Lys\(^{11}\)-linked polyubiquitin chains have been implicated in proteasomal degradation (33); Lys\(^{63}\)-linked chains in DNA repair (34, 35), activation of I\(\kappa\)B kinase (36), the cellular response to stress (37), inheritance of mitochondrial DNA (38), endocytosis (39), and ribosome activity (40); and Lys\(^{29}\)-linked chains in proteasomal degradation mediated by Ufd4/Ufd2 (18, 41). Ufd2
is a ubiquitin chain assembly factor (E4) that attaches ubiquitin to oligoubiquitylated artificial substrates (18), but its physiological role remains unclear.

We have now identified FEZ1 as a protein that interacts with E4B. FEZ1, a mammalian homolog of C. elegans UNC-76, is thought to participate in axonal outgrowth and fasciculation. Kinesin and UNC-76 were shown recently to form a stable complex that contributes to the transport of synaptic vesicle precursors (42). We could not formally prove that E4B directly binds to FEZ1. However, given that this interaction was identified in budding yeast, it is likely that E4B directly binds to FEZ1. The protein DISC1 (disrupted in schizophrenia 1), which is encoded by a gene that is disrupted by a (1;11)(q42.1;q14.3) chromosomal translocation that segregates with schizophrenia in a Scottish family, also interacts with FEZ1 and contributes with it to neurite outgrowth (43). Given that both E4B and FEZ1 are predominantly expressed in the brain (23, 26), they may together play an important role in neuronal development.

PKCζ phosphorylates FEZ1, and this phosphorylation reaction appears to be required for neurite extension in the cells (26, 27). We have demonstrated here that expression of caPKCζ in PC12 cells (26, 27). We have demonstrated here that expression of caPKCζ in HEK293T cells enhanced the interaction between E4B and FEZ1, suggesting that E4B preferentially binds to phosphorylated FEZ1. Although it was technically difficult to express caPKCζ in PC12 cells to the level that is detected by immunoblotting (data not shown), indirect evidence suggests that phosphorylation and ubiquitylation of FEZ1 may contribute to neurite extension in the cells (Fig. 9). The phosphorylation site(s) of FEZ1 targeted by PKCζ remains to be determined.

Our present results show that, although E4B activity appears to be required for neurite extension induced either by NGF or by PKCζ and FEZ1 in PC12 cells, the turnover of FEZ1 does not seem to be regulated by E4B. Thus, modification of FEZ1 by E4B is unlikely to be a signal for proteasomal degradation. This conclusion is consistent with our observation that the polyubiquitin chain added to FEZ1 by E4B appears to be linked via Lys27, not Lys48. The U-box-type E3 CHIP was shown recently to mediate the attachment of a Lys27-linked polyubiquitin chain to BAG-1, a ubiquitin-like protein that links the molecular chaperones Hsc70 and Hsp70 to the proteasome (44). This Lys27-linked polyubiquitin chain does not stabilize or induce the proteasomal degradation of BAG-1, but rather promotes the association of BAG-1 with the proteasome. The polyubiquitin chain attached by E4B to FEZ1 might therefore facilitate the interaction of FEZ1 with an unidentified target that functions in neuritogenesis.

Treatment of PC12 cells with NGF induces the accumulation of high molecular mass (40–1000 kDa) ubiquitin-protein conjugates, an effect that is inhibited by staurosporine, a protein kinase inhibitor that also blocks NGF-induced neurite outgrowth (45). Furthermore, proteasome inhibitors induce neurite elongation in PC12 cells (46). In addition, lysates of NGF-
treated PC12 cells exhibit an increased rate of formation of ubiquitin-E1 and ubiquitin-E2 thiol esters (47). These various observations suggest that the ubiquitin-proteasome pathway and ubiquitylation system may play a role in neurite elongation in PC12 cells.

Given that the proteasome inhibitors MG132 and elostostacystin β-lactone inhibit the degradation of FEZ1, FEZ1 is likely degraded by the 26 S proteasome in proliferating cells, although the ubiquitin ligase responsible for this degradation remains to be identified. Under differentiation conditions, FEZ1 in PC12 cells may be polyubiquitylated by E4B, with the ubiquitin chain being linked through Lys27 of ubiquitin, and the modified FEZ1 then appears to contribute to neuritogenesis. Two different ubiquitin ligases may therefore mediate the ubiquitylation of FEZ1 and thereby either target it for degradation or promote its action in neurite elongation.

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