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Structural basis for the selection of glycosylated substrates by SCF^{Fbs1} ubiquitin ligase

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The ubiquitin ligase complex SCF^{Fbs1}, which contributes to the ubiquitination of glycoproteins, is involved in the endoplasmic reticulum-associated degradation pathway. In SCF ubiquitin ligases, a diverse array of F-box proteins confers substrate specificity. Fbs1/Fbx2, a member of the F-box protein family, recognizes high-mannose oligosaccharides. To elucidate the structural basis of SCF^{Fbs1} function, we determined the crystal structures of the Skp1–Fbs1 complex and the sugar-binding domain (SBD) of the Fbs1–glycoprotein complex. The mechanistic model indicated by the structures appears to be well conserved among the SCF ubiquitin ligases. The structure of the SBD–glycoprotein complex indicates that the SBD primarily recognizes Man₃GlcNAc₂, thereby explaining the broad activity of the enzyme against various glycoproteins. Comparison of two crystal structures of the Skp1–Fbs1 complex revealed the relative motion of a linker segment between the F-box and the SBD domains, which might underlie the ability of the complex to recognize different acceptor lysine residues for ubiquitination.

glycoprotein | tertiary structure | ubiquitin system

Ubiquitin-mediated proteolysis plays a regulatory role in a number of diverse cellular processes and involves the selective destruction of short-lived functional proteins (1). The ubiquitin–proteasome system also is responsible for the disposal of misfolded and unfolded cellular proteins, the aberrant accumulation of which usually causes cell death, which can lead to neurodegenerative diseases (2). Protein ubiquitination is catalyzed by a sophisticated cascade system consisting of the ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin-ligating (E3) enzymes (3). Among these enzymes, the E3 enzymes are responsible for the selection of target proteins. E3 enzymes are a diverse family of proteins and protein complexes. One of the best characterized groups of E3 enzymes is the SCF complex [composed of Skp1, Cul1, Rbx1 (also called Roc1), and an F-box protein], which regulates the degradation of a broad range of cellular proteins (4). F-box proteins consist of an N-terminal ≈40-aa F-box domain that binds to Skp1 and various C-terminal substrate-recognition regions and are subcategorized into three classes according to their substrate-binding domains. The Fbw (or FBXW) and Fbl (or FBXL) families possess WD40 repeats and leucine-rich repeats in their binding domains, respectively (5). The third class of F-box proteins is the Fbx (or FBXO) family, which does not contain any presumptive functional domains. However, we recently identified a subfamily within the Fbx family that consists of at least five homologous F-box proteins that recognize N-glycan; we named the sugar-binding domain (SBD)-containing proteins of this subfamily the Fbs (F-box protein that recognizes sugar chains; known previously as FBG) proteins (6).

In the SCF complex, Cul1 functions as a molecular scaffold that simultaneously interacts through its N and C termini with the crucial adaptor subunits Skp1 and Rbx1 together with a specific E2 enzyme, respectively. Skp1 is an adaptor protein that

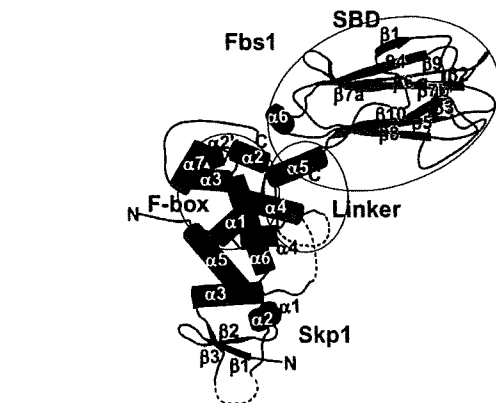


Fig. 1. Structure of the Skp1–Fbs1 complex. Skp1 and Fbs1 are colored blue and red, respectively. The secondary structure elements for Skp1 and Fbs1 are labeled. Dotted lines represent disordered regions. The F-box, linker, and SBD domains of Fbs1 are circled.

links Cul1 and one of the F-box proteins (6). Fbw and Fbl proteins usually recognize the phosphorylation status of the substrate, and structural models of the SCF complexes of some of these proteins, such as Fbw1/β-TrCP1 (7), Fbw7/Cdc4 (8), and Fbl1/Skp2 (9–11), have been reported. Structural information, however, is available only for the recognition of phosphorylated protein substrates.

N-linked glycosylation of proteins takes place in the endoplasmic reticulum and plays a key role in protein quality control (12). Misfolded proteins and unassembled protein complexes that fail to assume their functional states in the endoplasmic reticulum are subjected to endoplasmic reticulum-associated degradation, which involves retrotranslocation into the cytosol and degradation by the ubiquitin–proteasome system. SCF^{Fbs1} is an N-linked glycoprotein-specific ubiquitin ligase complex that contains the neuron-specific F-box protein Fbs1/Fbx2/NFB42 (13–16). We previously reported x-ray crystal structures of the Fbs1 SBD alone and in complex with di-N-acetylchitobiose (chitobiose, GlcNAc–GlcNAc, or GlcNAc₂), which revealed that the binding site is formed by a small hydrophobic pocket located at the top of a β-sandwich (17). The molecular mechanism

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Abbreviations: RNaseB, ribonuclease B; SBD, sugar-binding domain.

Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 2E31, 2E32, and 2E33).

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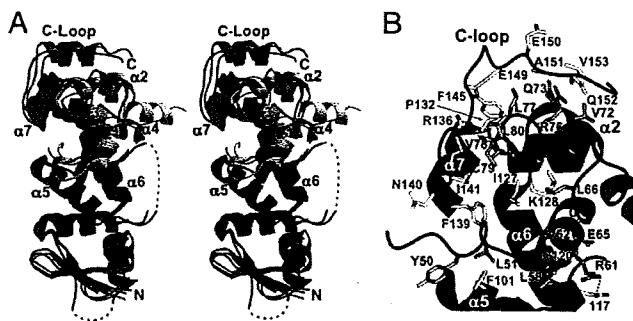


Fig. 2. Structure of the Skp1-Fbs1 interface. (A) Stereo views of the Fbs1 F-box domain (red) and Skp1 (blue) are compared with the structures of Skp1-Skp2 and Skp1- β -TrCP1. Skp1 is purple, Skp2 is green, Skp1 is orange, and β -TrCP1 is yellow. The secondary structure elements for Skp1 and Fbs1 are labeled with black and red letters, respectively. (B) Close-up view of the interface between Skp1 and Fbs1 showing intermolecular contacts. Fbs1 is red and Skp1 is blue.

underlying the ubiquitination of N-glycoproteins by the SCF^{Fbs1} ubiquitin ligase, however, is unknown at present. To understand the mechanistic details of the SCF^{Fbs1}-mediated ubiquitination reaction, we determined the crystal structures of the SBD-glycoprotein and Skp1-Fbs1 complexes.

Results

Overall Structure of the Skp1-Fbs1 Complex. The Skp1-Fbs1 complex has an overall L-shaped structure with Skp1 and the Fbs1 subunits oriented $\approx 90^\circ$ to each other (Fig. 1). Skp1 and the chitobiose-binding site (17) are located at the opposite ends of

Fbs1. Fbs1 consists of four distinct domains: the PEST domain (residues 1-54), the F-box domain (residues 55-95), a linker domain (residues 96-124), and the SBD (residues 125-297). The electron densities of the N-terminal PEST domain (residues 1-47) and part of the linker domain (residues 104-108) are not visible, suggesting that these regions are flexible. Although the Skp1-Fbs1 complex was crystallized in the presence of 30 mM chitobiose, the chitobiose molecule is not visible in this structure. The F-box domain comprises four α helices, which is the same structural motif observed in the Skp1-binding domains of Skp2 and β -TrCP1 (Fig. 2A). The SBD in the Skp1-Fbs1 complex is composed of a 10-stranded antiparallel β -sandwich, and it can be superposed on the previously reported structure of SBD alone with an average rms deviation of 0.39 Å for the C α atoms, indicating that the structures are very similar. On the other hand, the C-terminal linker helix $\alpha 5$ assumes slightly different positions in the two structures because of crystal packing and the flexibility of the linker domain, which consists of $\alpha 5$ and a loop structure.

The Skp1 in the Skp1-Fbs1 complex adopts the same BTB/POZ fold (18) observed in previously reported structures of Skp1 (7-9). Interestingly, the C-terminal α -helix $\alpha 8$ of previously reported structures of Skp1 complexed with other F-box proteins is replaced with an extended structure (C loop: residues 146-155) (Fig. 2A). The differences in the secondary structure of Skp1 may reflect different roles of the protein. With the exception of the C-terminal loop, the overall structure of Skp1 is almost identical to those of the protein in the Skp1-Skp2 and Skp1- β -TrCP1 complexes (rms deviations for the C α atoms: 1.8 and 2.1 Å, respectively).

Skp1-F-Box Interface. Whereas the F-box domain of Fbs1 contains the same four-helix ($\alpha 1$ - $\alpha 4$) structure seen in the domains of

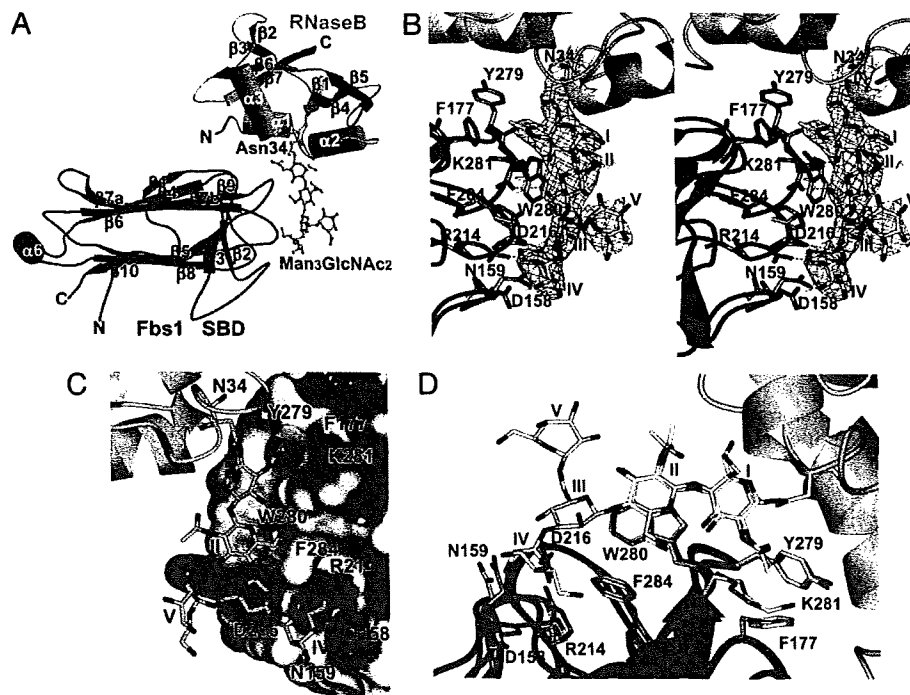


Fig. 3. Structure of the SBD-RNaseB complex. (A) The SBD is red and RNaseB is cyan. The secondary structure elements of the SBD and RNaseB are labeled. (B) Stereo view of the interface between the substrate-binding pocket of the SBD and the sugar of RNaseB. The Man₃GlcNAc₂ was modeled with the electron density map ($2F_o - F_c$ omit map of Man₃GlcNAc₂) contoured at 1.1 rms deviation. Fbs1 is red and RNaseB is cyan. (C) Surface representation of the substrate-binding pocket of the SBD bound to Man₃GlcNAc₂. The surface is colored according to the electrostatic potential of the residues (blue, positive; red, negative). (D) Comparison between the substrate-binding sites of the Fbs1 SBD (green) bound to chitobiose (yellow) and the Fbs1 SBD (red) bound to glycosylated RNaseB (cyan).

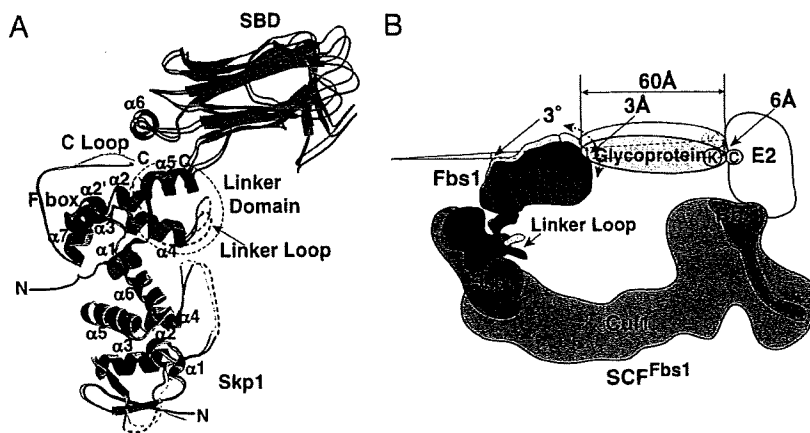


Fig. 4. Regulatory mechanism of SCF^{Fbs1} glycoprotein ubiquitination. (A) Comparison of the two crystal structures of the Skp1-Fbs1 complex. Skp1 (form 1), Fbs1 (form 1), Skp1 (form 2), and Fbs1 (form 2) are blue, red, yellow, and green, respectively. (B) Schematic representation of the model for ubiquitination on SCF^{Fbs1}. The E2 active-site cysteine and acceptor lysine residues are depicted with circled letters.

Skp2 (1.5-Å rms deviation for 27 C α atoms) and β -TrCP1 (1.7-Å rms deviation for 28 C α atoms), there are several differences. Helix $\alpha 2$ in Fbs1 is composed of two segments ($\alpha 2$ and $\alpha 2'$) separated by a turn, which causes it to bulge into the C loop of Skp1. The orientations of helices $\alpha 3$ and $\alpha 4$ of Fbs1 are similar to those of Skp2 but not to those of β -TrCP1 (Fig. 2A). Moreover, whereas the C-terminal region of the F-box domain of Skp2 is a loop structure, those of Fbs1 and β -TrCP1 form α -helices ($\alpha 4$).

The binding mode between Skp1 and the F-box domain of Fbs1 is almost identical to those between Skp1 and Skp2 (1.0-Å rms deviation for 120 C α atoms) and between Skp1 and β -TrCP1 (1.1-Å rms deviation for 117 C α atoms), except that the C loop of Skp1 interacts with the F-box domain through van der Waals contacts (Phe-145, Glu-149, Glu-150, Ala-151, Gln-152, and Val-153 of Skp1 interact with Val-72, Gln-73, Arg-76, Leu-77, and Leu-80 of Fbs1) (Fig. 2B). The positions of the Fbs1 F-box domain and $\alpha 7$ in Skp1 are shifted away from the N-terminal domain of Skp1 by ≈ 4.0 Å (Skp1-Skp2) or ≈ 2.5 Å (Skp1- β -TrCP1). These differences in the distances from the F-box domains to $\alpha 7$ of Skp1 and the C-terminal structures of Skp1 are likely due to the F-box structure.

Structure of the Glycoprotein Complex of the SBD. Ribonuclease B (RNaseB) is a glycoprotein that has a single high-mannose

oligosaccharide (Man₆₋₈GlcNAc₂) attached at Asn-34 (19). RNaseB binds to the edge of the β -sandwich of the SBD (Fig. 3A). Clear electron density demonstrates the presence of Man₃GlcNAc₂ bound to the Fbs1 monomer, but the outer branches of the carbohydrate are disordered and not visible in the electron density map. The structure of the Fbs1-bound RNaseB, which consists of three α -helices and seven β -strands, is essentially identical to the previously reported structures of apo-RNaseB (20, 21); these structures have an average 0.59-Å rms deviation for the C α positions. Similarly, the SBDs in the structures of the Skp1-Fbs1 and SBD-RNaseB complexes can be superposed with an average 0.48-Å rms deviation for the C α positions, indicating that RNaseB binding does not cause any significant structural changes in the SBD.

Glycoprotein Recognition by the SBD in the SBD-RNaseB Complex.

The sugar-binding surface consists of the four loops between $\beta 2$ and $\beta 3$, $\beta 3$ and $\beta 4$, $\beta 5$ and $\beta 6$, and $\beta 9$ and $\beta 10$. Man₃GlcNAc₂ interacts with nine Fbs1 residues (Asp-158, Asn-159, Phe-177, Arg-214, Asp-216, Tyr-279, Trp-280, Lys-281, and Phe-284) through hydrogen bonds and/or van der Waals contacts (Fig. 3B and C). The molecular recognition mechanism between the chitobiose moiety and the amino acid residues in Fbs1 is similar to that reported previously for the SBD-chitobiose complex (17). The methyl group of the *N*-acetyl moiety from the GlcNAc (I) residue is inserted into a small hydrophobic pocket surrounded by the side chains of Phe-177, Tyr-279, and Lys-281; and the O3 atom forms a hydrogen bond with the main chain N atom of Lys-281. The GlcNAc (II) residue is stacked on the aromatic ring of Trp-280 and the O6 atom forms a hydrogen bond with the carbonyl oxygen atom of Lys-281. The two GlcNAc residues form an intramolecular hydrogen bond between the O3 atom of GlcNAc (I) and the O6 atom of GlcNAc (II). Formation of these hydrogen bonds fixes the orientation of the $\beta(1\rightarrow4)$ -linked GlcNAc residues. Comparison of the SBD-RNaseB complex and the previously reported SBD-chitobiose complex reveals that the substrate binding pockets (Phe-177, Tyr-279, Trp-280, and Lys-281) and the chitobiose structures can be superposed with an average rms deviation of 0.69 Å for all of the atoms (Fig. 3D). Only the side chain of Lys-281 has a different conformation, and it has been shown that Lys-281 is not essential for the binding to chitobiose (17). The outer mannose-binding residues of the SBD also do not exhibit significant conformational changes. We have reported that Fbs1 recognizes not only chitobiose but also the outer mannose branches (17). The O4 atom of Man(III)

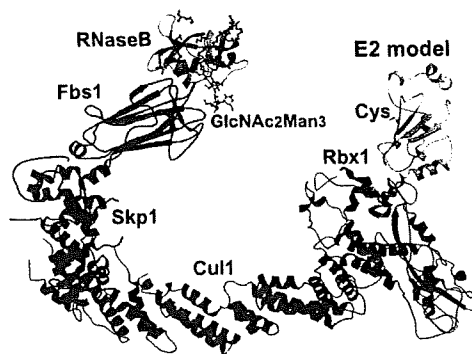


Fig. 5. Model of the SCF^{Fbs1}-RNaseB complex bound to E2. Cul1, Rbx1, Skp1, Fbs1, RNaseB, and E2 are green, orange, blue, red, cyan, and yellow, respectively. Lysine residues on the RNaseB surface are presented in ball-and-stick format and are coral.

Table 1. Data collection, phasing, and refinement statistics

	Skp1-Fbs1	Skp1-Fbs1/Thimerosal	Skp1-Fbs1	SBD-RNaseB
Data collection				
Space group	$P3_221$	$P3_221$	$P2_12_12_1$	$P432$
Cell parameters, Å				
<i>a</i>	106.7	106.6	66.2	148.6
<i>b</i>	106.7	106.6	111.1	148.6
<i>c</i>	110.2	113.7	153.3	148.6
Unique reflections	27,964	17,765	12,269	15,839
Resolution range, Å	2.40 (2.49–2.40)	2.80 (2.90–2.80)	3.50 (3.63–3.50)	2.70 (2.80–2.70)
R_{merge}	0.045 (0.327)	0.058 (0.328)	0.103 (0.225)	0.078 (0.325)
$I/\sigma I$	18.1 (2.7)	16.0 (2.2)	7.0 (4.1)	13.9 (5.2)
Completeness, %	97.7 (93.0)	95.7 (79.6)	85.2 (79.0)	99.4 (97.9)
Redundancy	5.4 (2.9)	6.2 (2.3)	2.6 (2.1)	11.8 (6.4)
Refinement				
No. of reflections	26,521		12,279	15,049
$R_{\text{work}}/R_{\text{free}}$	0.233/0.291		0.223/0.299	0.216/0.288
rms deviations				
Bond lengths, Å	0.010		0.022	0.026
Bond angles, °	1.33		2.08	2.51

Values in parentheses represent the highest-resolution shell.

forms a hydrogen bond with the sidechain O^δ atom of the Asp-216 sidechain; this hydrogen bond stabilizes the complex with the Man β (1→4)GlcNAc₂ moiety. Clear electron density is observed between the β (1→4)-linked Man residue and Asp-216 in Fbs1. Furthermore, Asp-216 is conserved in other F-box proteins that contain an F-box-associated domain, suggesting that it plays a role in the recognition of oligosaccharides. Man(IV) forms hydrogen bonds with the sidechain N^δ atom of Asn-159 through the O2 atom and with the sidechain N^γ atom of Arg-214 through the O5 atom. On the other hand, Man(V) protrudes from the binding site and does not interact with Fbs1. These results indicate that the GlcNAc₂ core, β (1→4)-linked Man(III), and α (1→3)-linked Man(IV) play significant roles in the binding to Fbs1. Whereas Man₈GlcNAc₂ is thought to be the major N-glycan among unfolded glycoproteins that are translocated into the cytosol for endoplasmic reticulum-associated degradation, our results indicate that Man₃GlcNAc₂ can be sufficiently recognized by the SCF^{Fbs1} complex. Indeed, various synthetic oligosaccharides were used to show that Man₃GlcNAc₂ and Man₈GlcNAc₂ have similar affinities for Fbs1 (22). On the other hand, the binding affinities of Man₈GlcNAc₁ and chitobiose for Fbs1 are several orders of magnitude lower than those of Man₃GlcNAc₂ and Man₈GlcNAc₂, indicating that Man₃GlcNAc₂ is required for efficient binding to Fbs1 (15, 22). Moreover, the binding site provides substrate selectivity and specificity based on its shape and hydrogen-bonding network.

There are limited contacts between the SBD and RNaseB; the interface involves only 514 Å² of surface-accessible area. The surface areas occupied by Man₃GlcNAc₂ and the protein portion of the substrate are 349 and 165 Å², respectively. In addition to the smaller contact area, the protein portion of RNaseB does not form a hydrogen bond with Fbs1, suggesting that Man₃GlcNAc₂, but not the protein in RNaseB, defines the interaction with Fbs1.

Linker Flexibility Might Accommodate a Range of Substrates. Two crystal forms were identified for Skp1-Fbs1 ($P3_221$ and $P2_12_12_1$ define form 1 and form 2, respectively). These two forms have essentially the same overall structure (rms deviation of 1.1 Å for the C^α atoms) (Fig. 4A). Whereas the Skp1 proteins are well aligned with each other, the SBD of Fbs1 in form 2 is tilted farther away from Skp1 by $\approx 3^\circ$. The 3° tilt angle of the SBD creates a 3-Å gap at the substrate-binding site and a 6-Å shift at the E2 active site. This flexibility seems to be due in part to the

linker-domain structure of Fbs1 (Fig. 4A). Residues 100–103 and 113–115 are shifted significantly compared with form 1. Although the linkage between the F-box and WD40 domains does not seem to be exceedingly rigid in the yeast Cdc4 structure, deletion of helix $\alpha 5$ or the lengthening of helix $\alpha 6$ due to an insertion of amino acid residues disrupts Cdc4 function *in vivo*, suggesting that the orientation and rigidity of the linkage between the F-box and the substrate-binding domains is important for SCF function (8). In the structure of Fbs1, the interaction between $\alpha 5$ and the linker loop through van der Waals contacts (His-113 of the linker loop to Gln-115, Phe-119, and Arg-123 of $\alpha 5$) is not rigid, and residues 104–108 of the linker domain are not visible in the electron density map. This structure indicates that the linkage between the F-box domain and the SBD of Fbs1 is somewhat flexible. This feature might allow the protein to accommodate a range of substrates (Fig. 4B).

Discussion

In this study, we determined the crystal structures of two crystal forms of the Skp1-Fbs1 complex and the SBD-RNaseB complex at 2.4-, 3.5-, and 2.7-Å resolutions, respectively. The structure of the Skp1-Fbs1 complex illustrates a different class of F-box proteins within the SCF ubiquitin ligase model. A model of the SCF^{Fbs1}-RNaseB-E2 complex was generated simply by superposition of the Skp1 subunits from the Skp1-Fbs1 and Skp1-Cull1-Rbx1 structures (PDB ID code 1LDK), the RING-finger domains derived from Rbx1 and from the c-Cbl subunit of the c-Cbl-UbcH7 structure (PDB ID code 1FBV) (23), the E2 subunits of the c-Cbl-UbcH7 structure, and the SBD-RNaseB structure. In this SCF^{Fbs1}-RNaseB model, RNaseB points toward the E2-binding site on Rbx1 (Fig. 5). The distance between the E2 active-site cysteine and the substrate-binding site is ≈ 60 Å, which is similar to the value that was reported previously (7, 8, 10). Despite differences in the sizes of the substrates and the positions of ubiquitinated lysine residues, the distance between the E2 active-site cysteine and the substrate-binding site is conserved among the SCF complexes. The same mechanism that allows the E2 active-site cysteine to reach the ubiquitinated lysine residues of the substrates is used independently of the type of F-box protein.

In the case of RNaseB, the distances between the E2 active-site cysteine and the lysine residues in RNaseB are 58.6–88.4 Å in the model of the SCF^{Fbs1}-RNaseB-E2 complex, whereas the

lysine residues in RNaseB are between 5.3 and 36.9 Å away from Asn-34. RNaseB is smaller than the minimum distance required to reach E2. Actually, SCF^{Fbs1} was not able to ubiquitinate RNaseB *in vitro*. This could be because RNaseB is too small, the lysine residues are at the wrong positions, or RNaseB is fixed because of contacts between Man₃GlcNAc₂ and Fbs1.

One of the most important properties for ubiquitination is the rigidity of the SCF-ubiquitin-ligase complex structure, because it serves to correctly position the target protein and E2. The two crystal structures of the Skp1-Fbs1 complex described in the present study, however, show differences in the orientation of the SBD. We propose that SCF^{Fbs1} has the ability to nonspecifically ubiquitinate glycoproteins targeted to the endoplasmic reticulum-associated degradation pathway. The protein portion of a target glycoprotein bound to Fbs1 may rotate at the linkage site between the innermost GlcNAc moiety and the asparagine residue, and the acceptor lysine residue can be located at a variety of positions. In SCF^{Fbs1}, the relative motion of the linker domain between the F-box domain and the SBD might be necessary to accommodate the different positions of the acceptor lysine residues in the various endoplasmic reticulum-associated degradation substrates.

SCF^{Fbs1} is a functionally unique molecule that recognizes the innermost Man₃GlcNAc₂ in N-glycans as a marker of denatured proteins. Our results provide a mechanistic basis for the recognition and ubiquitination of various glycoproteins by SCF^{Fbs1}.

Materials and Methods

Protein Expression and Purification. The Skp1-Fbs1 complex was coexpressed from the pET28b plasmid (Novagen, Madison, WI) in BL21 (DE3) cells. Full-length Skp1 was expressed as a 6× His-tagged protein, and full-length Fbs1 was expressed as an untagged protein. The complex was purified stepwise by Ni affinity, anion exchange, and gel-filtration chromatography. The Skp1-Fbs1 complex was then concentrated to ≈10 mg/ml by ultrafiltration in 25 mM Tris-HCl (pH 7.5) and 1 mM DTT.

For the SBD-RNaseB complex (with SBD residues 105–297 of Fbs1), the SBD and RNaseB (Sigma, St. Louis, MO) were combined in a 1:1 molar ratio and purified by gel-filtration chromatography. Fractions containing the SBD-RNaseB complex were then concentrated to ≈10 mg/ml and used for crystallization.

Crystallization and Data Collection. Crystals of Skp1-Fbs1 and SBD-RNaseB were obtained at 20°C by using the sitting-drop

vapor diffusion method. Skp1-Fbs1 crystals were grown from 2.0 M ammonium sulfate, 0.1 M sodium citrate (pH 5.7), and 30 mM chitobiose, which produced two crystal forms. The SBD-RNaseB crystals were prepared by using 2.0% (vol/vol) PEG 400, 0.1 M Hepes (pH 7.5), and 2.1 M ammonium sulfate.

Diffraction data sets for Skp1-Fbs1 and SBD-RNaseB were collected at beamline BL44XU (SPring-8, Hyogo, Japan). Data processing and reduction were carried out with the HKL program suite (24). The two crystal forms of Skp1-Fbs1 and the SBD-RNaseB crystals belong to the *P*3₂21 (Skp1-Fbs1 form 1), *P*2₁2₁2₁ (Skp1-Fbs1 form 2), and *P*432 (SBD-RNaseB) space groups. Heavy-atom soaks of the Skp1-Fbs1 crystals (form 1) were performed in crystallization buffer with 1 mM thimerosal for 5 min. Data collection, phasing, and refinement statistics are summarized in Table 1.

Structure Determination and Refinement. The structure of Skp1-Fbs1 was determined by a combination of molecular replacement, single isomorphous replacement, and anomalous scattering with an Hg derivative. The initial single isomorphous replacement and anomalous scattering phases were calculated with SHARP (25) and then improved by density modification with DM (26). Molecular replacement with the program MOLREP (27) was used to locate the Skp1 and SBD portions of the complex with search models consisting of Skp1 from SCF (PDB ID code 1LDK) and the SBD of Fbs1 (PDB ID code 1UMH). The model was further built with the program COOT (28) and then was improved by several cycles of manual rebuilding and refinement with the program REFMAC5 (29). The structure of crystal form 2 was solved by molecular replacement using MOLREP with the refined model of form 1.

The structure of SBD-RNaseB was determined by using the molecular replacement technique, MOLREP, and the structures of the SBD and RNaseB. The refined model contains residues 123–297 of the SBD and residues 1–124 of RNaseB. Phasing and refinement statistics are summarized in Table 1. There are no residues in disallowed regions of the Ramachandran plot. Structure figures were generated by using MOLSCRIPT (30), RASTER3D (31), and CCP4MG (32).

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Regulation of CD8⁺ T Cell Development by Thymus-Specific Proteasomes

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Proteasomes are responsible for generating peptides presented by the class I major histocompatibility complex (MHC) molecules of the immune system. Here, we report the identification of a previously unrecognized catalytic subunit called $\beta 5t$. $\beta 5t$ is expressed exclusively in cortical thymic epithelial cells, which are responsible for the positive selection of developing thymocytes. Although the chymotrypsin-like activity of proteasomes is considered to be important for the production of peptides with high affinities for MHC class I clefts, incorporation of $\beta 5t$ into proteasomes in place of $\beta 5$ or $\beta 5i$ selectively reduces this activity. We also found that $\beta 5t$ -deficient mice displayed defective development of CD8⁺ T cells in the thymus. Our results suggest a key role for $\beta 5t$ in generating the MHC class I-restricted CD8⁺ T cell repertoire during thymic selection.

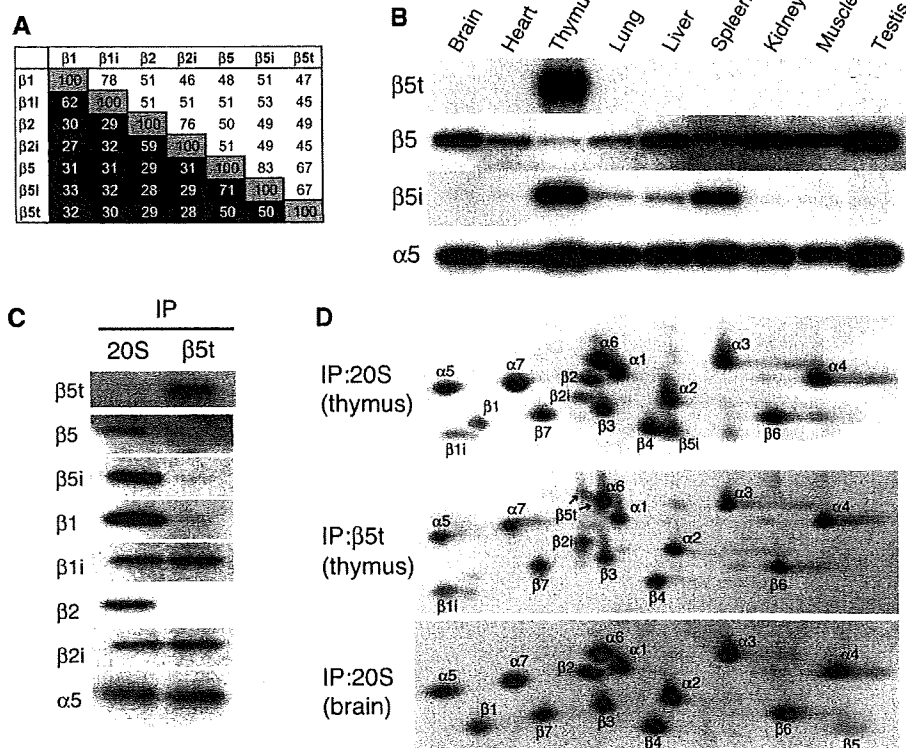
Proteasomes are multicatalytic proteinase complexes that are responsible for regulated proteolysis in eukaryotic cells and essential for the generation of antigenic peptides presented by major histocompatibility complex (MHC) class I molecules of the immune system in jawed vertebrates (*J*). Proteolysis is conducted by 20S proteasomes, which are large complexes composed of 28 subunits arranged as a cylinder in four heteroheptameric rings: α_{1-7} , β_{1-7} , β_{1-7} , and α_{1-7} . Among these, the three subunits called $\beta 1$, $\beta 2$, and $\beta 5$ perform the catalytic function. In vertebrates, three additional $\beta 1i$, $\beta 2i$, and $\beta 5i$ subunits are induced by interferon (IFN)- γ and are preferentially incorporated into proteasomes. These immunoproteasomes produce antigenic peptides more efficiently than do constitutive proteasomes, and they play an important role in the elimination of virus-infected and tumor cells by CD8⁺ T cells (2).

During a search for proteasome-related genes in a genome database (www.ensembl.org), we found a previously unrecognized gene product with high homology to β subunits of 20S proteasomes. The gene encoding it is located adjacent to the gene for $\beta 5$, and the gene product is encoded by a single exon in both human and mouse genomes (fig. S1A). Orthologous genes are found specifically in vertebrates, implying that this gene emerged synchronously with genes involved in adaptive immunity in evolution, as did the immunoproteasomes (2). The expression

of this gene was biased toward the thymus in an expressed sequence tag database (UniGene Mm.32009). Multiple sequence alignments showed that its putative active-site threonine residue was preceded by a propeptide that ends with a glycine residue, a hallmark of active β subunits (3–6) (fig. S1B). The dendrogram and homologies among catalytic β subunits indicated its close relation with $\beta 5$ and $\beta 5i$ (Fig. 1A and fig. S1C),

and we therefore named this gene $\beta 5t$, as a thymus-specific $\beta 5$ family member. Northern blot analysis confirmed the exclusive transcription of $\beta 5t$ in the thymus (fig. S1D). Immunoblot analysis of various mouse organs, with the use of an antibody specific to $\beta 5t$ (anti- $\beta 5t$), further confirmed that the expression was thymus-specific (Fig. 1B). In comparison, $\beta 5i$ was predominantly expressed in a broad range of immune tissues (including the thymus and spleen and to a lesser extent in lung and liver), and it further differed from the wide distribution of $\beta 5$ (Fig. 1B).

We next examined how $\beta 5t$ is incorporated into proteasomes. Thymic extracts were immunoprecipitated with an antibody to the 20S proteasome (anti-20S), (cross-specific for all 20S proteasomes) and with anti- $\beta 5t$. These antibodies immunodepleted 20S proteasomes and $\beta 5t$, respectively (fig. S2A). Quantitative immunoblotting of $\alpha 5$, an invariable subunit of 20S proteasomes, revealed that ~20% of the 20S proteasomes in the thymus contain $\beta 5t$ (fig. S2B). $\beta 5$ and $\beta 5i$ were barely detected in the $\beta 5t$ -containing proteasomes, indicating that $\beta 5t$ was incorporated into 20S proteasomes, in place of $\beta 5$ or $\beta 5i$ (Fig. 1C). Moreover, the IFN- γ -inducible subunits $\beta 1i$ and $\beta 2i$ were preferentially incorporated into the $\beta 5t$ -containing proteasomes, in



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Fig. 1. $\beta 5t$ is a catalytic proteasome subunit in the thymus. (A) Percentages of amino acid sequence identities (boxed in black) and similarities (white box) among primary structures of mouse catalytic subunits obtained with the BLAST2 program (28). (B) Immunoblot analysis of various tissues of 3-week-old mice with the indicated antibodies. (C) Extracts of mouse thymus were immunoprecipitated with anti-20S or anti- $\beta 5t$, followed by immunoblotting with the indicated antibodies. IP, immunoprecipitation. (D) 2D-PAGE analysis of samples in (C) with Coomassie staining. 20S proteasomes of brain extracts represent constitutive proteasomes. All spots were identified by tandem mass spectrometry. One of the $\beta 5t$ spots (represented by arrows) overlaps with that of $\alpha 6$.

place of constitutive subunits $\beta 1$ and $\beta 2$ (Fig. 1C). The above findings were further confirmed by analysis of the precipitated products by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (Fig. 1D). Spots for $\beta 1$, $\beta 2$, $\beta 5$, and $\beta 5i$ were faintly detected in the $\beta 5t$ -containing proteasomes, whereas spots for $\beta 5t$ emerged together with those for $\beta 1i$ and $\beta 2i$ (Fig. 1D, middle). We therefore propose to call this distinct subtype of proteasomes "thymoproteasomes."

Proteasomes are responsible for the production of MHC class I-binding peptides and the sole enzymes that determine the C termini of the peptides (7, 8). Previous crystal structural studies of MHC class I complexes have revealed that the hydrophobic C-terminal anchor residues of the peptides are essential for high-affinity

peptide binding into the clefts of MHC class I complexes (9). Some types of MHC molecules prefer basic C termini (1). The 20S proteasomes have at least three types of peptidase activities (chymotrypsin-like, trypsin-like, and caspase-like activities), which cleave peptide bonds after hydrophobic, basic, and acidic amino acids, respectively (10). The specificity is determined by the nature of the amino acids that compose the so-called S1 pocket (11, 12). For the production of high-affinity MHC class I ligands, the chymotrypsin-like activity carried by $\beta 5$ and $\beta 5i$, whose S1 pockets are composed mostly of hydrophobic amino acids, is important (13). A comparison of the S1 pockets of the three $\beta 5$ families revealed that the pocket of $\beta 5t$ is mainly composed of hydrophilic residues, in marked contrast to those of $\beta 5$ and $\beta 5i$ (Fig. 2A), suggesting that

thymoproteasomes have weaker chymotrypsin-like activity. To test this idea, we analyzed the peptidase activities of $\beta 5t$ -overexpressing cells, where ~90% of $\beta 5$ was replaced by $\beta 5t$ (Fig. 2, B and C). We observed that the precursor forms of $\beta 5t$ were processed into mature forms after incorporation into 20S and 26S proteasomes (Fig. 2B, bottom left). In $\beta 5t$ -expressing cells, the chymotrypsin-like activity was exclusively reduced by 60 to 70%, without affecting the other two activities in both 20S and 26S fractions (Fig. 2B). Kinetic analysis of the chymotrypsin-like activity revealed that $\beta 5t$ reduced both the maximum velocity and the Michaelis constant values for the activity (Fig. 2D), which is opposite to the effect that $\beta 5i$ has on these quantities (14). However, these cells still possessed normal protein-degrading activity, as assessed by the

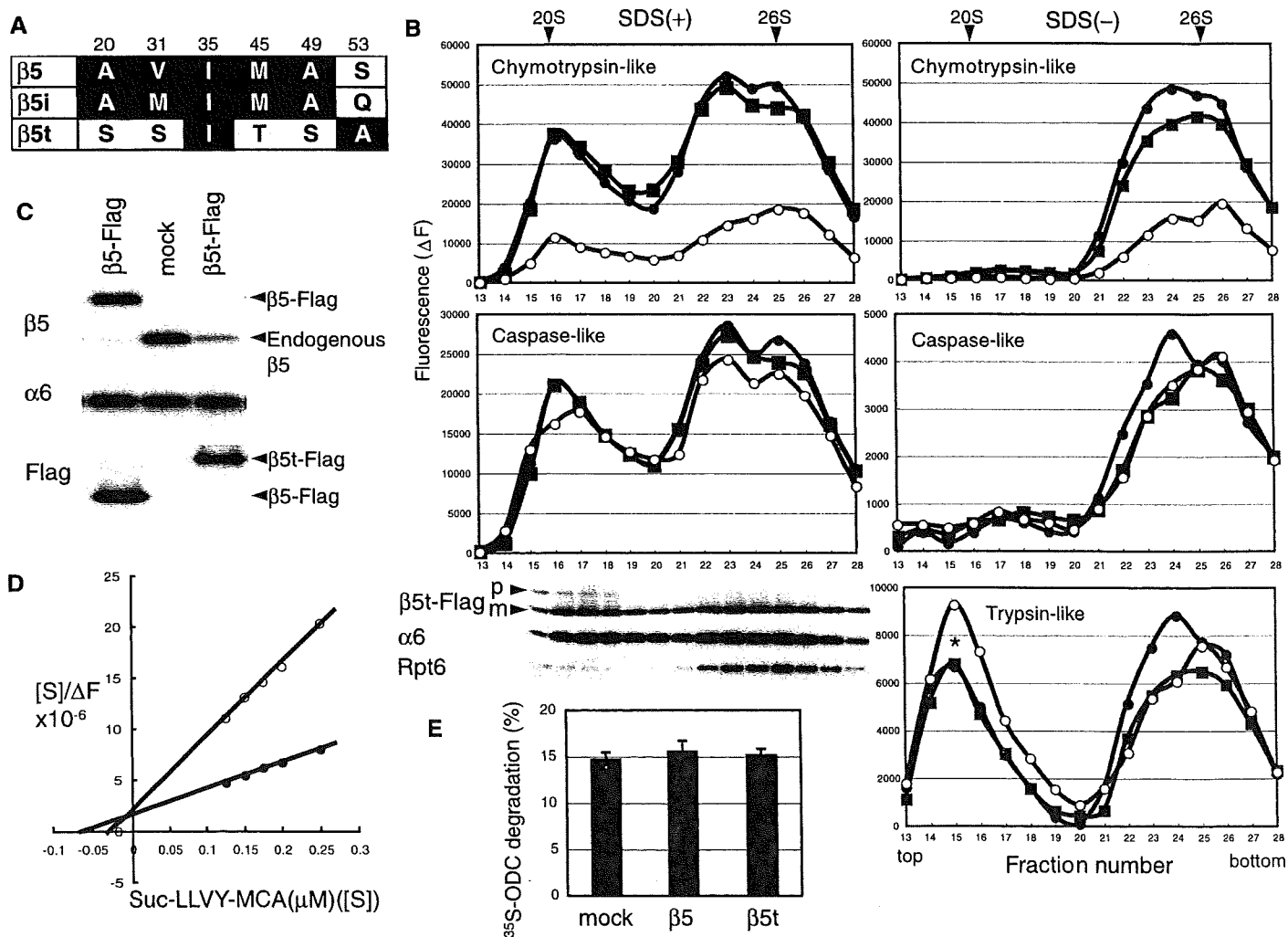


Fig. 2. $\beta 5t$ selectively reduces the chymotrypsin-like activity of proteasomes. (A) Alignment of amino acid residues organizing the S1 pockets of $\beta 5$ families. The hydrophobic and hydrophilic amino acid residues are boxed in black and white, respectively. The numbers represent the position from the active-site threonine. (B) Peptidase activities of human embryonic kidney-293 T cells expressing human $\beta 5t$ -Flag (open circles), $\beta 5$ -Flag (black squares), or mock cells (black circles) in the presence (left) and absence (right) of 0.025% SDS. Arrowheads indicate the peak locations of 20S proteasomes and 26S proteasomes, proven by

immunoblot analysis of each fraction (bottom left). p, precursor; m, mature; asterisk, nonproteasomal activity. (C) Fraction numbers 25 in (B) were immunoblotted for $\beta 5$, $\alpha 6$, and Flag. (D) Hanes-Woolf plots for chymotrypsin-like activity of 26S proteasomes (pools of fraction numbers 24 to 26) from mock cells (black circles) and $\beta 5t$ cells (open circles). Suc-LLVY-MCA, succinyl-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide; [S], substrate concentration. (E) Cell extracts from (B) were assayed for adenosine triphosphate-dependent protein degradation activity using ^{35}S -labeled ODC. Mean \pm SD; $n = 3$ experiments.

degradation of ornithine decarboxylase (ODC) (Fig. 2E) (15).

The thymus is responsible for generating a T cell repertoire that specifically recognizes self-peptide-MHC (self-pMHC) complexes and tolerates self-antigens. Thymic stromal cells represent a heterogeneous mixture of cell types and provide a proper microenvironment for developing thymocytes (16, 17). To identify a cell type that expresses $\beta 5t$, we immunostained sections of mouse thymus for $\beta 5t$ and markers for thymic stromal cells (Fig. 3, A and B). $\beta 5t$ was exclusively expressed in the thymic cortex, which is quite similar to the expression of Ly51, a marker for cortical thymic epithelial cells (cTECs). The distribution of $\beta 5t$ was distinct from that of cells that bind to *Ulex europaeus* agglutinin I (UEA-I), a marker for medullary TECs; CD11c, a marker for dendritic cells; and MTS15, a marker for fibroblasts (Fig. 3A) (18–20). A magnified image of the cortex revealed that the reticular staining pattern of $\beta 5t$ is nearly identical to that of Ly51, indicating that $\beta 5t$ is exclusively expressed in cTECs (Fig. 3B).

To confirm this conclusion, different cell populations of the thymus were isolated. Immunoblot analysis demonstrated that $\beta 5t$ is specifically expressed in Ly51-positive cells and is not detected in any other stromal cells or in CD45⁺ cells that are mostly composed of thymocytes (Fig. 3C). A comparison of Ly51-positive cells with murine embryonic fibroblasts that were treated with or without IFN- γ [in which almost all the proteasomes are immuno- and constitutive pro-

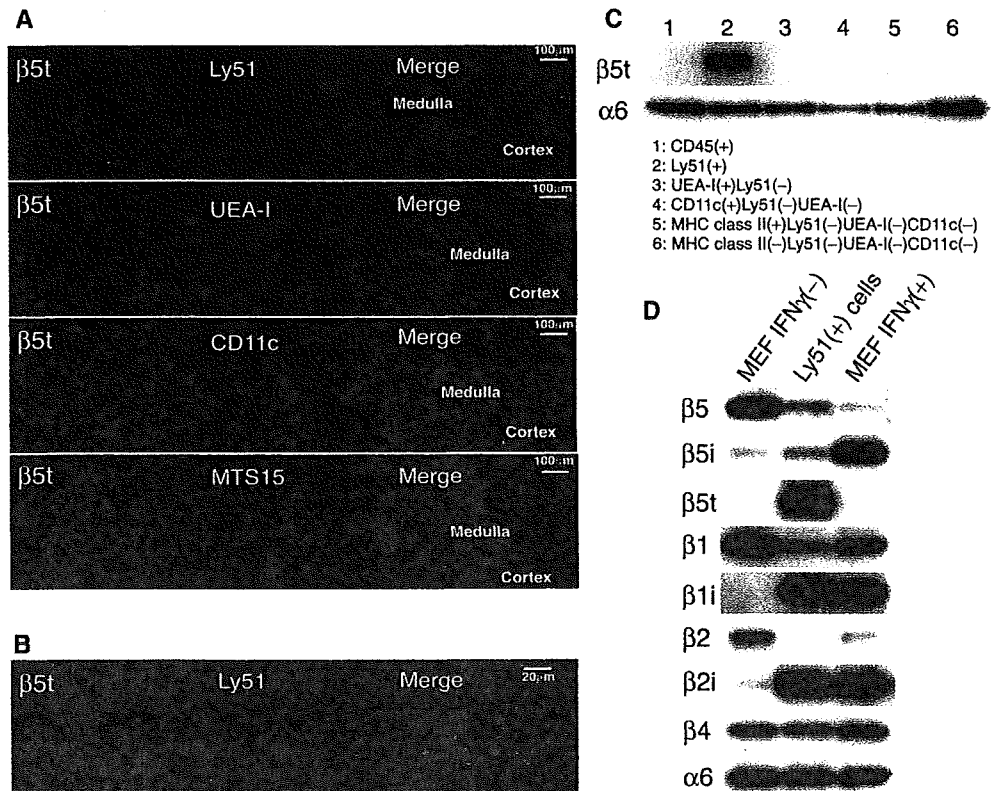
teasomes, respectively (21)] revealed that the majority of proteasomes in cTECs were thymo-proteasomes containing $\beta 5t$, together with $\beta 1i$ and $\beta 2i$. Also, the expression levels of $\beta 5$ and $\beta 5i$ were markedly down-regulated in cTECs (Fig. 3D).

To clarify the physiological role of $\beta 5t$, we generated $\beta 5t$ -deficient mice. The $\beta 5t$ -coding sequence was substituted for cDNA encoding the protein Venus to identify $\beta 5t$ -expressing cells (Fig. 4A). The loss of $\beta 5t$ proteins in the $\beta 5t^{-/-}$ thymus and the expression of Venus in the $\beta 5t^{-/-}$ and $\beta 5t^{+/-}$ thymuses were confirmed by immunoblotting (Fig. 4B). An inspection of $\beta 5t^{+/-}$ and $\beta 5t^{-/-}$ mice with fluorescence demonstrated that the expression of Venus (i.e., $\beta 5t$) was limited to the cortex of the thymus with a reticular pattern similar to that of cTECs (fig. S3, A to C). Cortical and medullary architectures, as well as the size of the thymuses of $\beta 5t^{-/-}$ mice, were indistinguishable from those of $\beta 5t^{+/-}$ mice (fig. S3, B and C). When thymic stromal cells from $\beta 5t^{+/-}$ mice were analyzed by flow cytometry, all Venus-expressing cells were MHC class II I-A^b-positive (Fig. 4C). Furthermore, we examined the relationship between Venus and Ly51 expression in the I-A^b-positive population (Fig. 4D). We identified three distinct populations. Among them, the Venus⁺Ly51⁺ population was the largest, constituting 50% of I-A^b-positive cells. There was a smaller population of Venus⁺Ly51⁻ that constituted 11% of I-A^b-positive cells. These results demonstrate that all $\beta 5t$ -expressing cells are I-A^bLy51⁺

cTECs and that this population makes up ~80% of cTECs.

It has been suggested that cTECs are mainly responsible for positive selection by presenting ligands on MHC molecules to CD4⁺CD8⁺ double-positive (DP) thymocytes (22–25). We therefore examined how $\beta 5t$ is involved in the function of cTECs. Flow cytometry analysis of thymocytes revealed that $\beta 5t$ deficiency was associated with a markedly low percentage of CD8 single-positive (SP) thymocytes but not with the percentage of CD4 SP thymocytes (Fig. 4E). A large proportion (80.7%) of wild-type (WT) CD8 SP thymocytes expressed high levels of T cell receptor β (TCR β), resembling positively selected cells (Fig. 4F, left) (26). In contrast, a much smaller proportion (50.2%) of CD8 SP thymocytes expressed high levels of TCR β in $\beta 5t^{-/-}$ mice (Fig. 4F, right), suggesting that many of the CD8 SP thymocytes of $\beta 5t^{-/-}$ mice are immature thymocytes that are transitory intermediates between double-negative (DN) and DP thymocytes (27). Consequently, the ratio of CD4 SP cells to CD8 SP cells in $\beta 5t^{-/-}$ mice was markedly higher than that in WT mice (Fig. 4G). The ratio was also significantly elevated in $\beta 5t^{+/-}$ mice, although the difference was much smaller (Fig. 4G). Total numbers of DN, DP, and CD4 SP thymocytes in $\beta 5t^{-/-}$ mice were nearly identical to those of $\beta 5t^{+/-}$ and WT mice. However, a significantly low total number of CD8 SP thymocytes was observed in $\beta 5t^{-/-}$ mice (Fig. 4H). Selective reduction of CD8 SP T cells was also observed in TCR β (+) splenocytes of $\beta 5t^{-/-}$ mice

Fig. 3. $\beta 5t$ is specifically expressed in cTECs. (A and B) Cryosections of mouse thymus were immunostained for $\beta 5t$, together with Ly51, UEA-I, CD11c, or MTS15. (C) Various populations of cells from the thymus were immunoblotted for $\beta 5t$ and $\alpha 6$ (a loading control for proteasomes). (D) Contents of thymo-proteasomes in Ly51-positive cells were analyzed by immunoblotting. MEF, murine embryonic fibroblast.



(Fig. 4, I and J). These results demonstrate that $\beta 5t$ is required for the development of $CD8^+$ T cells in the thymus and suggest the possibility that $\beta 5t$ deficiency is associated with defective positive selection of $CD8^+$ T cells.

During positive selection, DP cells that interact with self-pMHC complexes expressed on cTECs with sufficiently low affinity or avidity are rescued from intrathymic death and induced to differentiate into CD4 or CD8 SP thymocytes. The recognition of MHC class I molecules results

in commitment to the CD8 lineage (22–26). In contrast, DP cells that interact with high affinity or avidity with self-pMHC complexes are eliminated by the induction of apoptosis (22–24). To date, however, information about whether and how cTECs can offer specialized signals that are suitable for positive selection has been elusive. The present work demonstrates that $\beta 5t$, which is specifically expressed in cTECs, plays a pivotal role in the development of $CD8^+$ T cells. What, then, is the mechanism for $CD8^+$ T cell

development regulated by $\beta 5t$? The thymic architecture was apparently normal in $\beta 5t^{-/-}$ mice (fig. S3, B and C), suggesting that $\beta 5t$ is not essential for the differentiation and proliferation of cTECs. Normal development of CD4 SP thymocytes observed in $\beta 5t^{-/-}$ mice supports this argument. Surface expression levels of MHC class I on $\beta 5t^{-/-}$ cTECs were also comparable to those of $\beta 5t^{+/+}$ cTECs (fig. S4). It is assumed that other $\beta 5$ family members (e.g., $\beta 5$ and $\beta 5i$ subunits) are incorporated in place of $\beta 5t$ in cTECs of

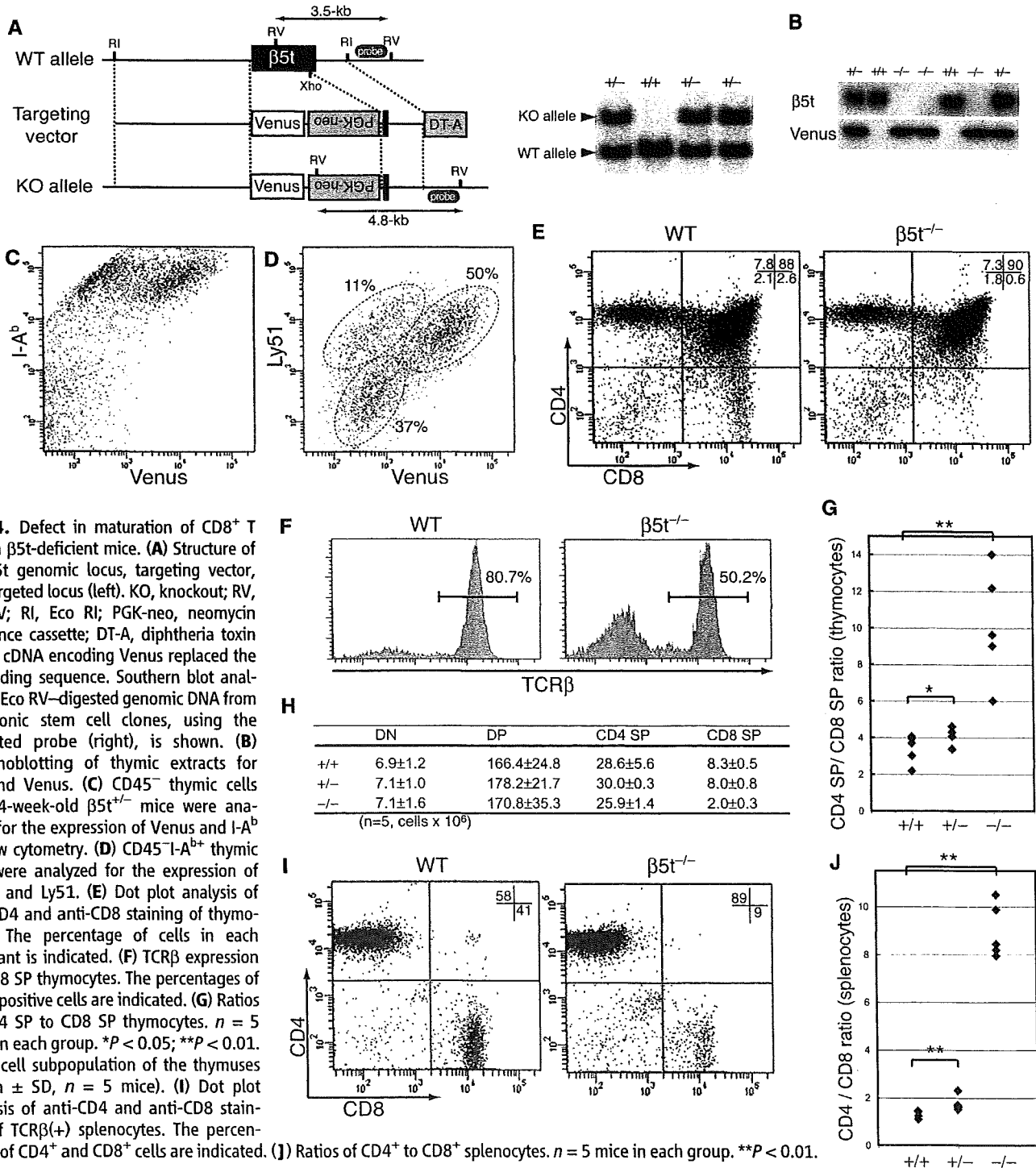


Fig. 4. Defect in maturation of $CD8^+$ T cells in $\beta 5t$ -deficient mice. (A) Structure of the $\beta 5t$ genomic locus, targeting vector, and targeted locus (left). KO, knockout; RV, Eco RV; RI, Eco RI; PGK-neo, neomycin resistance cassette; DT-A, diphtheria toxin A. The cDNA encoding Venus replaced the $\beta 5t$ coding sequence. Southern blot analysis of Eco RV-digested genomic DNA from embryonic stem cell clones, using the indicated probe (right), is shown. (B) Immunoblotting of thymic extracts for $\beta 5t$ and Venus. (C) $CD45^-$ thymic cells from 4-week-old $\beta 5t^{+/+}$ mice were analyzed for the expression of Venus and I-A^b by flow cytometry. (D) $CD45^-$ I-A^{b+} thymic cells were analyzed for the expression of Venus and Ly5.1. (E) Dot plot analysis of anti-CD4 and anti-CD8 staining of thymocytes. The percentage of cells in each quadrant is indicated. (F) TCR β expression on CD8 SP thymocytes. The percentages of TCR β -positive cells are indicated. (G) Ratios of CD4 SP to CD8 SP thymocytes. $n = 5$ mice in each group. * $P < 0.05$; ** $P < 0.01$. (H) T cell subpopulation of the thymuses (mean \pm SD, $n = 5$ mice). (I) Dot plot analysis of anti-CD4 and anti-CD8 staining of TCR β (+) splenocytes. The percentages of CD4⁺ and CD8⁺ cells are indicated. (J) Ratios of CD4⁺ to CD8⁺ splenocytes. $n = 5$ mice in each group. ** $P < 0.01$.

$\beta 5t^{-/-}$ mice. Considering that proteasomes are essential for the production of MHC class I ligands and that $\beta 5t$ specifically attenuates the peptidase activities that cleave peptide bonds after hydrophobic amino acid residues, it is possible that thymoproteasomes predominantly produce low-affinity MHC class I ligands rather than high-affinity ligands in cTECs, as compared with constitutive- and immunoproteasomes, thereby supporting positive selection.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/316/5829/1349/DC1

Materials and Methods

Figs. S1 to S4

References

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Essential role for autophagy protein Atg7 in the maintenance of axonal homeostasis and the prevention of axonal degeneration

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Autophagy is a regulated lysosomal degradation process that involves autophagosome formation and transport. Although recent evidence indicates that basal levels of autophagy protect against neurodegeneration, the exact mechanism whereby this occurs is not known. By using conditional knockout mutant mice, we report that neuronal autophagy is particularly important for the maintenance of local homeostasis of axon terminals and protection against axonal degeneration. We show that specific ablation of an essential autophagy gene, *Atg7*, in Purkinje cells initially causes cell-autonomous, progressive dystrophy (manifested by axonal swellings) and degeneration of the axon terminals. Consistent with suppression of autophagy, no autophagosomes are observed in these dystrophic swellings, which is in contrast to accumulation of autophagosomes in the axonal dystrophic swellings under pathological conditions. Axonal dystrophy of mutant Purkinje cells proceeds with little sign of dendritic or spine atrophy, indicating that axon terminals are much more vulnerable to autophagy impairment than dendrites. This early pathological event in the axons is followed by cell-autonomous Purkinje cell death and mouse behavioral deficits. Furthermore, ultrastructural analyses of mutant Purkinje cells reveal an accumulation of aberrant membrane structures in the axonal dystrophic swellings. Finally, we observe double-membrane vacuole-like structures in wild-type Purkinje cell axons, whereas these structures are abolished in mutant Purkinje cell axons. Thus, we conclude that the autophagy protein *Atg7* is required for membrane trafficking and turnover in the axons. Our study implicates impairment of axonal autophagy as a possible mechanism for axonopathy associated with neurodegeneration.

axon | axonopathy | neurodegeneration | autophagosome | Purkinje cell

Macroautophagy is characterized by dynamic membrane rearrangements, involving the formation, trafficking, and degradation of double-membrane autophagic vacuoles (autophagosomes) in the cytoplasm. Macroautophagy (hereafter referred to as autophagy) is a highly regulated process, which can be induced by nutrient starvation, trophic factors, and stress (1). Despite recent advances in characterizing autophagy in several model systems, autophagic processes in the nervous system remain poorly understood. On one hand, nutrient deprivation has not been observed to induce autophagy in the mammalian brain (2), thus suggesting a specific regulatory system for autophagy that is not typically activated by starvation. On the other hand, a variety of conditions that cause neuronal stress or degeneration can lead to the accumulation of autophagosomes in neurons, thus implicating autophagy in these neuropathogenic processes (3, 4).

The axon is a highly specialized neuronal compartment that performs many functions independently from the cell body. After axotomy or excitotoxicity, double-membrane vacuoles

resembling autophagosomes were originally observed to accumulate in dilated axon terminals that result from the injury (5, 6), a local phenomenon that is not observed in undisturbed axons. Autophagosome-like vacuoles have also been shown to be present in the dysfunctional or degenerating axons associated with a range of chronic neurodegenerative conditions, including Alzheimer's (7, 8), Parkinson's (9), Huntington's (10), and Creutzfeldt–Jakob (11) diseases and their animal models (12–14). These observations suggest a link between locally altered autophagy and axonopathy, which is one of the underlying mechanisms in neurodegeneration (15).

Although the biological significance of these autophagosome-like vacuoles in degenerating axons is unclear, recent studies have shown that genetic inactivation of autophagy in the mouse CNS causes neurodegeneration accompanied by axonal dystrophy and the formation of intracellular ubiquitin-associated inclusions (16, 17). These studies suggest a role for basal levels of autophagy in neuronal protection and in protein quality control. However, the connection between the inactivation of autophagy and the observed axonal dystrophy and neurodegeneration remains to be determined. In addition, because autophagy was suppressed in all cell types in the CNS (including neurons and nonneuronal cells) (16, 17), it is not known whether the observed axonal dystrophy and neurodegeneration is cell-autonomous.

Here, we seek to further elucidate the physiological function of neuronal autophagy by generating conditional knockout mice with Purkinje cell-specific deletion of *Atg7*, an autophagy gene encoding E1-like enzyme in the two ubiquitin-like conjugation systems that are essential for the autophagosome biogenesis (18). We show that ablation of *Atg7* leads to abnormal swellings and dystrophy of Purkinje cell axon terminals in the deep cerebellar nuclei (DCN). Subsequently, these *Atg7*-deletion mice develop cell-autonomous neurodegeneration of Purkinje cells, dendritic atrophy, and behavioral deficits. Moreover, double-membrane vacuole-like structures are formed in the distal ends of wild-type Purkinje cell axons, whereas they are absent in *Atg7*-deletion Purkinje cell axons.

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Abbreviations: DCN, deep cerebellar nucleus; Pn, postnatal day n; LC3, light chain 3; mGluR, metabotropic glutamate receptor.

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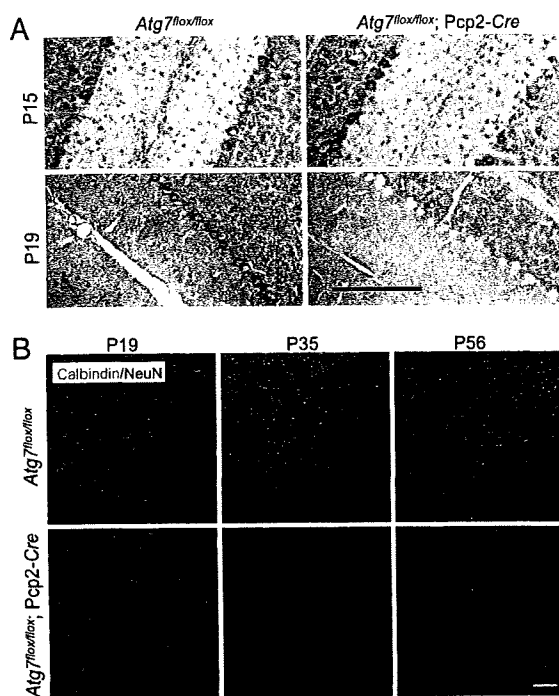


Fig. 1. Deletion of *Atg7* specifically in Purkinje cells caused progressive dystrophic swelling of axon terminals. (A) Immunohistochemistry of *Atg7* protein expression in Purkinje cells of *Atg7^{lox/lox}* and *Atg7^{lox/lox};Pcp2-Cre* mice at P15 and P19. The endogenous *Atg7* protein was present at P15 but absent at P19 in the *Atg7^{lox/lox};Pcp2-Cre* Purkinje cells. (Scale bar: 100 μ m.) (B) Progression of the abnormal Purkinje cell axon terminal swellings in the DCN of *Atg7^{lox/lox};Pcp2-Cre* mice (anti-calbindin immunofluorescent staining in green with anti-NeuN counterstained in red) at P19, P35, and P56. *Atg7^{lox/lox}* was used as control. (Scale bar: 20 μ m.) $n = 3-5$.

Instead, the mutant Purkinje cell axon terminal swellings accumulate aberrant membranous structures. Our results suggest that autophagy is required for normal axon terminal membrane trafficking and turnover, and indicate an essential role of local autophagy in the maintenance of axonal homeostasis and prevention of axonal degeneration.

Results

Specific Depletion of *Atg7* in Purkinje Cells Caused Cell-Autonomous Dystrophy and Degeneration of Axon Terminals. To generate Purkinje cell-specific deletion of *Atg7* in mice, we crossed mice harboring the floxed *Atg7* alleles (19) with transgenic mice expressing *Cre* recombinase under control of the *Pcp2* (L7) promoter (20) to establish the mouse line *Atg7^{lox/lox};Pcp2-Cre*. To determine when loss of the endogenous *Atg7* protein occurred, we examined *Atg7* expression in *Atg7^{lox/lox};Pcp2-Cre* mice at postnatal day 15 (P15) and P19. At P15, *Atg7* was expressed at similar levels in Purkinje cells of both mutant *Atg7^{lox/lox};Pcp2-Cre* and the control *Atg7^{lox/lox}* mice, whereas at P19, despite residual expression in a small number of Purkinje cells (<12%), *Atg7* immunostaining was largely diminished in *Atg7^{lox/lox};Pcp2-Cre* Purkinje cells (>88%), but unchanged in the *Atg7^{lox/lox}* Purkinje cells (Fig. 1A). At P35, >95% Purkinje cells in *Atg7^{lox/lox};Pcp2-Cre* showed no detectable *Atg7* expression (data not shown). In addition, *Atg7* deficiency in *Atg7^{lox/lox};Pcp2-Cre* mice was specific for Purkinje cells because *Atg7* is clearly present in the other cell types (Fig. 1A). Thus, the specific loss of *Atg7* in Purkinje cells occurred largely between P15 and P19 in *Atg7^{lox/lox};Pcp2-Cre* mice.

Next, we examined Purkinje cell axons in the DCN of

Atg7^{lox/lox};Pcp2-Cre mice by immunofluorescent staining using an antibody against calbindin, a Purkinje cell marker. At P15, no morphological alteration was observed in the axons of *Atg7^{lox/lox};Pcp2-Cre* Purkinje cells compared with those of *Atg7^{lox/lox}* Purkinje cells [supporting information (SI) Fig. 7], consistent with the presence of normal levels of *Atg7* in *Atg7^{lox/lox};Pcp2-Cre* Purkinje cells at this stage (Fig. 1A). However, at P19, *Atg7^{lox/lox};Pcp2-Cre* Purkinje cell axons were abnormally dilated, as visualized by green fluorescence-labeled "endbulbs" (Fig. 1B). In addition, these Purkinje cell axonal swellings were labeled with the antibody raised against synaptophysin, the presynaptic terminal marker (SI Fig. 8), suggesting that they were terminals of Purkinje cell axons. The number and size of the swollen axon terminals in the DCN of the *Atg7^{lox/lox};Pcp2-Cre* were markedly increased at P35 in comparison with those at P19 (Fig. 1B). At P56, the number of such axonal dystrophic swellings of the mutant Purkinje cells was noticeably decreased in comparison with that at P35, suggesting that many of these swollen axons had degenerated by this age (Fig. 1B). These data demonstrated that deletion of *Atg7* caused cell-autonomous axonal dystrophy and degeneration in Purkinje cells.

Axonal Dystrophic Swellings of *Atg7^{lox/lox};Pcp2-Cre* Purkinje Cells Were Devoid of GFP-Light Chain 3 (LC3)-Labeled Puncta and Exhibited Increased Levels of p62/SQSTM1.

Transgenic mice producing GFP fused with microtubule-associated protein 1 light chain 3 (LC3), a specific marker for autophagosomes (21), were previously generated to monitor autophagosomes *in vivo* (2). By expressing GFP-LC3 in *Lurcher* mice (*GFP-LC3/Lurcher*), we showed that a large number of autophagosomes were formed in the dystrophic axon terminals of *Lurcher* Purkinje cells (Fig. 2Ad), providing *in vivo* evidence for the induction of autophagy in response to *Lurcher*-induced excitotoxicity (22). To assess autophagic activity in the dystrophic axons of *Atg7^{lox/lox};Pcp2-Cre* Purkinje cells, we crossed transgenic GFP-LC3 with *Atg7^{lox/lox};Pcp2-Cre* mice (*Atg7^{lox/lox};Pcp2-Cre/GFP-LC3*). Despite intense GFP-LC3 accumulation in the axonal dystrophic swellings of Purkinje cells in *Atg7^{lox/lox};Pcp2-Cre/GFP-LC3* mice at P35, no GFP-LC3 fluorescent puncta characteristic of autophagosomes were observed in these swellings (Fig. 2A b and c). We contrasted this finding to our observation of GFP-LC3 puncta in Purkinje cell axons GFP-LC3/*Lurcher* mice (Fig. 2Ad) (22). In addition, no GFP-LC3 puncta were observed in the somata or dendrites of *Atg7^{lox/lox};Pcp2-Cre/GFP-LC3* Purkinje cells (Fig. 2A f and g), again in contrast to the observation of GFP-LC3 puncta in the somata and dendrites of GFP-LC3/*Lurcher* Purkinje cells (Fig. 2Ah) (22).

It has been shown that inhibition of autophagy is correlated with increased levels of the polyubiquitin binding protein p62/SQSTM1 (22, 23). We thus examined the levels of p62/SQSTM1 in *Atg7^{lox/lox};Pcp2-Cre* Purkinje cells. As detected with anti-p62/SQSTM1 immunofluorescent staining, p62/SQSTM1 was markedly accumulated in the axonal dystrophic swellings (Fig. 2B, arrows) and somata (SI Fig. 9) of *Atg7^{lox/lox};Pcp2-Cre* Purkinje cells in comparison with *Atg7^{lox/lox}* Purkinje cells. It is also noteworthy that the dystrophic axonal swellings in *Lurcher* Purkinje cells did not have detectable p62/SQSTM1 immunofluorescent staining (SI Fig. 10). These results provided molecular evidence for impaired autophagic activity in the dystrophic axons of *Atg7^{lox/lox};Pcp2-Cre* Purkinje cells, but not in the dystrophic axons of *Lurcher* Purkinje cells.

Atg7^{lox/lox};Pcp2-Cre Purkinje Cells Exhibited Normal Dendritic Tree and Spine Morphology at P56.

Despite the remarkable dystrophy and degeneration of Purkinje cell axon terminals in the DCN of *Atg7^{lox/lox};Pcp2-Cre* mice at P35 and P56, the cerebellar cortex displayed little change in its overall size and organization (Fig. 3A). For example, at P35 and P56, *Atg7^{lox/lox};Pcp2-Cre* mice and

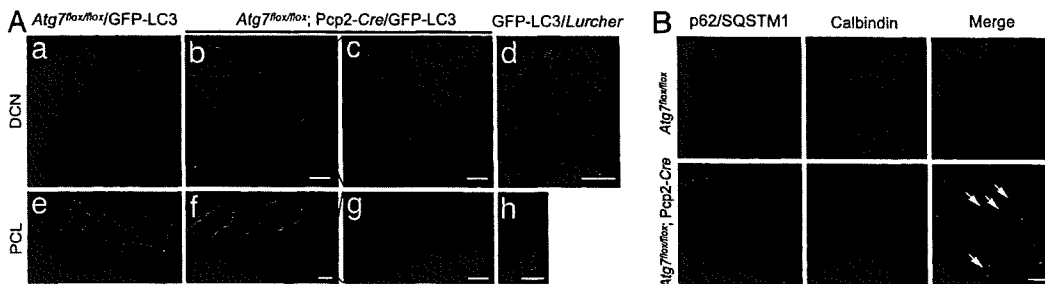


Fig. 2. The axonal dystrophic swellings of the *Atg7*-deficient Purkinje cells contained no GFP-LC3 labeled autophagosomes but accumulated p62/SQSTM1. (A) The absence of GFP-LC3 puncta in Purkinje cell axonal dystrophic swellings (b and c) and somata (f and g) of *Atg7^{lox/lox};Pcp2-Cre*/GFP-LC3 mice (P35). GFP-LC3 puncta were found in GFP-LC3/*Lurcher* Purkinje cell axonal dystrophic swellings (d) and somata (h) (P12). DCN (a) and Purkinje cell layer (PCL) (e) of control mice *Atg7^{lox/lox}*/GFP-LC3 are shown. (Scale bars: a, b, e, and f, 20 μ m; c, d, g, and h, 10 μ m.) (B) Anti-p62/SQSTM1 immunofluorescent staining (in green) showed accumulation of p62/SQSTM1 in Purkinje cell axonal dystrophic swellings (calbindin labeling in red) (white arrows) in the DCN of *Atg7^{lox/lox};Pcp2-Cre* mice at P56. *Atg7^{lox/lox}* was used as control. (Scale bar: 10 μ m.)

Atg7^{lox/lox} mice did not exhibit significant difference in their cerebellar molecular layer thickness (Fig. 3A and B). To evaluate changes in dendritic tree and spine morphology, we examined the expression pattern of metabotropic glutamate receptor 1 α (mGluR1 α) protein, a marker for parallel fiber–Purkinje cell synapses. No difference in localization and intensity of the anti-mGluR1 α immunofluorescent staining was observed between the *Atg7^{lox/lox};Pcp2-Cre* and *Atg7^{lox/lox}* cerebellar molecular layers at either P35 (data not shown) or P56 (Fig. 3C). Thus, *Atg7* deletion had little effect on Purkinje cell dendritic tree and spine morphology up to at least P56. We conclude that *Atg7*

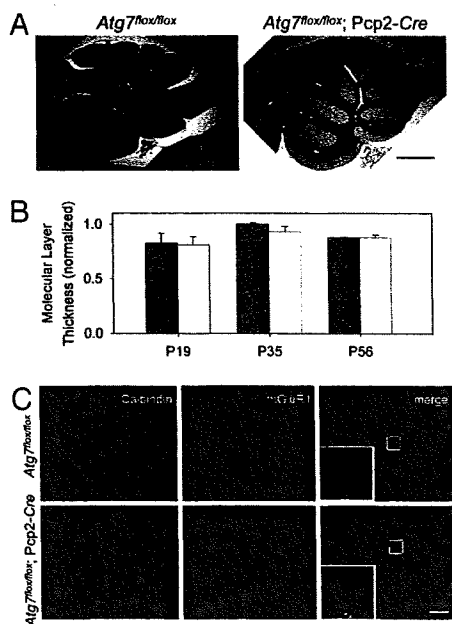


Fig. 3. Deletion of *Atg7* in Purkinje cells had little effect on the morphology of cerebellar cortex, Purkinje cell dendritic tree and spines in *Atg7^{lox/lox};Pcp2-Cre* mice at P56. (A) H&E-stained images of mid-sagittal sections from *Atg7^{lox/lox}* and *Atg7^{lox/lox};Pcp2-Cre* cerebella at P56. (Scale bar: 0.5 mm.) $n = 3-5$. (B) Quantification of the molecular layer thickness (as the distance between lobules V and VI of the Purkinje cell layer divided by 2) from the cerebellar mid-sagittal sections of *Atg7^{lox/lox}* and *Atg7^{lox/lox};Pcp2-Cre* mice at P19, P35, and P56. $n = 3-5$. (C) Immunofluorescent staining of cerebellar mid-sagittal sections shows normal localization and appearance of mGluR1 α (in red) in *Atg7^{lox/lox};Pcp2-Cre* mice compared with *Atg7^{lox/lox}* mice at P56. Green indicates anti-calbindin. (Scale bar: 10 μ m.) $n = 3$.

deletion in Purkinje cells elicit differential effects on the dendritic and axonal compartments, suggesting that the axon terminals are particularly vulnerable to autophagy deficiency.

Axonal Dystrophy Preceded Cell-Autonomous Degeneration of Purkinje Cells and Behavioral Deficits in *Atg7^{lox/lox};Pcp2-Cre* Mice. To further evaluate the effects of *Atg7* deletion, we assayed for Purkinje cell degeneration and mouse behavioral deficits. Whereas the Purkinje cell axonal dystrophic swellings in the DCN of *Atg7^{lox/lox};Pcp2-Cre* mice first became apparent at P19 and grew severe at P35 (Fig. 2A), no significant difference in Purkinje cell numbers was observed between the *Atg7^{lox/lox};Pcp2-Cre* and *Atg7^{lox/lox}* mice at both ages (Fig. 4A and B). However, at P56, the number of Purkinje cells in *Atg7^{lox/lox};Pcp2-Cre* mice was reduced by 28.4% ($P < 0.0005$) compared with *Atg7^{lox/lox}* mice (Fig. 4A and B). Thus, loss of Purkinje cells in *Atg7^{lox/lox};Pcp2-Cre* mice occurred between P35 and P56. In comparison, the onset of axonal dystrophy began as early as P19. In addition, levels of GluR δ 2 (a Purkinje cell-specific glutamate receptor subtype) in *Atg7^{lox/lox};Pcp2-Cre* cerebellar extract were not reduced until P56 (SI Fig. 11), further supporting that the onset of axon dystrophy was earlier than Purkinje cell degeneration in *Atg7^{lox/lox};Pcp2-Cre* mice.

Next, we assessed the locomotive behaviors in *Atg7^{lox/lox};Pcp2-Cre* mice by limb-clasping, rotarod, and gait analyses at different postnatal ages. At P19 and P35, *Atg7^{lox/lox};Pcp2-Cre* mice appeared normal and did not show any difference in performance compared with their control *Atg7^{lox/lox}* littermates (data not shown). At P56, *Atg7^{lox/lox};Pcp2-Cre* and *Atg7^{lox/lox}* mice performed equally well on the rotarod and gait analyses (Fig. 4C and D). However, 5 of 13 *Atg7^{lox/lox};Pcp2-Cre* mice (38.5%) displayed limb-clasping reflexes on tail suspension, in comparison with 0 of 10 of their control littermates ($P < 0.01$). Thus, at P56, despite the 28.4% loss of Purkinje cells (Fig. 4B), *Atg7^{lox/lox};Pcp2-Cre* mice displayed only mild behavioral impairment. In contrast, at 1 year, these mice demonstrated severe behavioral disorders in locomotion and motor coordination when evaluated in all three behavioral tests (Fig. 4C and D; data not shown).

We summarize the temporal relationship of the morphological alterations, differential pathology in different compartments of Purkinje cells, and behavioral changes in *Atg7^{lox/lox};Pcp2-Cre* mice in SI Table 1. These results demonstrate that axonal dystrophic swelling is an early pathogenic event and is likely to be a direct result of impairment of local autophagy in axon terminals.

Aberrant Membrane Structures Accumulated in the Dystrophic Axon Terminals of *Atg7^{lox/lox};Pcp2-Cre* Purkinje Cells. To further assess the effect of impairment of autophagy on axon terminals, we characterized the axonal dystrophic swellings of *Atg7^{lox/lox};Pcp2-*

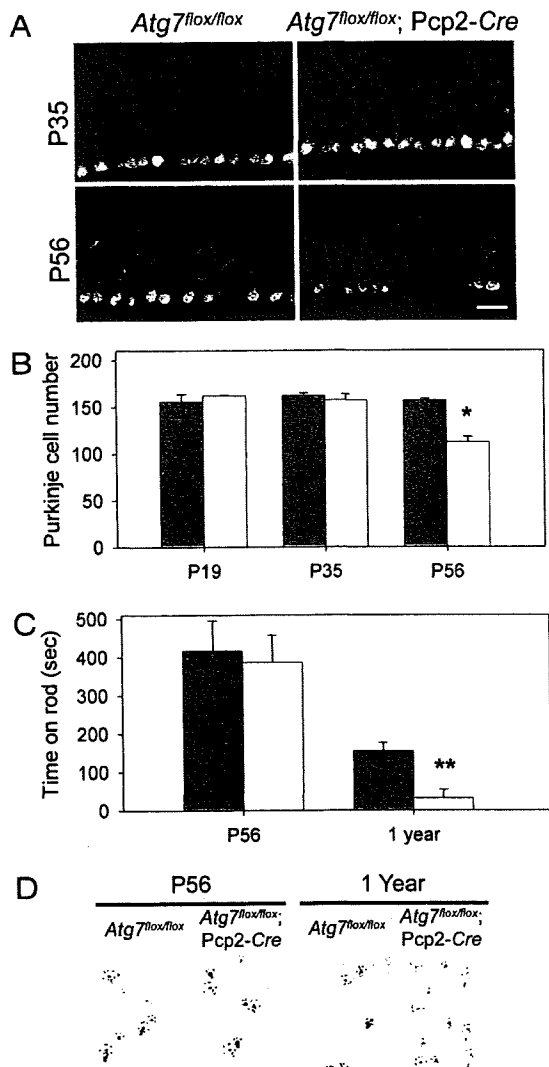


Fig. 4. Time course of Purkinje cell degeneration and locomotive behavioral deficits in *Atg7^{flox/flox};Pcp2-Cre* mice. (A) Anti-calbindin immunofluorescent staining of the cerebellar mid-sagittal sections of *Atg7^{flox/flox}* and *Atg7^{flox/flox};Pcp2-Cre* mice at P35 and P56. (B) Quantitation of Purkinje cells at lobules IV–V of the mid-sagittal sections of *Atg7^{flox/flox}* and *Atg7^{flox/flox};Pcp2-Cre* mice at P19, P35, and P56 based on H&E-stained images. $n = 3, 3,$ and 3 for *Atg7^{flox/flox}* at P19, P35, and P56, respectively. $n = 2, 3,$ and 5 for *Atg7^{flox/flox};Pcp2-Cre* at P19, P35, and P56, respectively (*, $P < 0.0005$). (C) At P56, *Atg7^{flox/flox}* and *Atg7^{flox/flox};Pcp2-Cre* mice showed no significant difference in the time spent on the rod in rotarod assay. At 1 year, *Atg7^{flox/flox}* mice spent much longer time on the rod than *Atg7^{flox/flox};Pcp2-Cre* mice (**, $P < 0.05$). (D) In gait analyses at P56, *Atg7^{flox/flox}* and *Atg7^{flox/flox};Pcp2-Cre* mice showed similar step width and overlapping of forefeet and hindfeet (forefeet, red; hindfeet, black). At 1 year old, *Atg7^{flox/flox};Pcp2-Cre* mice showed shorter step width than *Atg7^{flox/flox}* mice as well as nonoverlapping forefeet and hindfeet (left feet, black; right feet, red). For both C and D, $n = 5$ at P56; $n = 3$ and 4 at 1 year.

Cre Purkinje cells by transmission electron microscopy. The cross-sections of the Purkinje cell axon terminals in the DCN of the *Atg7^{flox/flox}* mice were normally $0.5\text{--}2\ \mu\text{m}$ in diameter (Fig. 5A, white arrows). Remarkably, the swollen Purkinje cell axon terminals in the DCN of the *Atg7^{flox/flox};Pcp2-Cre* mice often spanned $1\text{--}6\ \mu\text{m}$ in diameter (Fig. 5B–F, black arrows) and differed profoundly in their morphology from the axonal dystrophic swellings observed in *Lurcher* Purkinje cells (Fig. 5G,

black arrows). The Purkinje cell axonal dystrophic swellings of *Lurcher* mice contained a large number of autophagosomes/autolysosomes (Fig. 5G), whereas those of the *Atg7^{flox/flox};Pcp2-Cre* mice were devoid of autophagosomes (Fig. 2 and 6A). However, these autophagosome-free swellings of the *Atg7^{flox/flox};Pcp2-Cre* Purkinje cell axons often contained abnormal organelles or membrane structures (Fig. 5B–F, white arrows), including stacks of cisternal membranes that formed lamellar bodies (Fig. 5B and E, white arrows) (24), large and elaborate cisternal arrays and filaments (Fig. 5F, white arrow), and highly convoluted double-membrane whorls that occupied $1.5\text{--}2\ \mu\text{m}$ of the swollen terminal (Fig. 5C and D, white arrows). The exact nature of these aberrant structures was not clear; they were rarely seen in the somata of *Atg7^{flox/flox};Pcp2-Cre* Purkinje cells (data not shown) or in the *Atg7^{flox/flox}* Purkinje cells axons (Fig. 5A). However, it is noteworthy that the formation of convoluted membrane whorls was previously described in hepatocytes with *Atg7* deletion and was attributed to a failure in autophagic degradation (19). Our observations suggest a conserved function for autophagy in the clearance of cellular membranes and/or lipids in both axon terminals and hepatocytes.

Vacuole-Like Structures with Double Membranes Were Formed in Normal Purkinje Cell Axons but Were Absent in the Dystrophic Axon Terminals of *Atg7^{flox/flox};Pcp2-Cre* Purkinje Cells. Interestingly, through further ultrastructural analysis, we observed vacuole-like structures with double membranes in the myelinated Purkinje cell axons in the DCN of control *Atg7^{flox/flox}* mice (Fig. 6Aa and SI Fig. 12). These structures typically appeared to be closed and had diameters of $0.1\text{--}0.5\ \mu\text{m}$ (Fig. 6Aa). We occasionally observed these double-membrane vacuole-like structures in the process of formation (Fig. 6A b–e and SI Fig. 12A). Many of these developing double-membrane vacuole-like structures appeared to be formed through invagination of the axolemma along the myelinated layers (Fig. 6A b and c, SI Fig. 12A), reminiscent of invasion by oligodendrocytic processes (25, 26). Some of them appear to be continuous with the axonal plasma membrane (Fig. 6A d and e), which are enwrapping portions of axoplasm (27). The average number of these distinct double-membrane vacuole-like structures (both closed and in the process of formation) in Purkinje cell axons of *Atg7^{flox/flox}* mice was $\approx 0.9/50\ \mu\text{m}^2$ (Fig. 6B). In contrast, these structures were virtually absent in the *Atg7^{flox/flox};Pcp2-Cre* mice (Fig. 6Af, B, and C). Although we have yet to determine the exact nature of the vacuole-like structures with double membranes and their relationship with autophagosomes, our results suggested that *Atg7* was required for the formation of these distinct structures in axon terminals of normal Purkinje cells.

Discussion

Axonal dystrophic swelling is a hallmark of CNS axonopathy, which can be triggered by neuronal injuries, excitotoxicity, and various neurodegenerative conditions. Despite the prevalence of this pathology, the molecular mechanisms underlying axonopathy as well as the connection between axonopathy and neurodegeneration remain poorly understood (28). A critical question is whether axonal dystrophy and degeneration precede neuronal cell death or are secondary to neurodegeneration. Here, we analyzed the time course of pathological events after the deletion of the autophagy gene *Atg7* in cerebellar Purkinje cells. We showed that axonal dystrophy and degeneration caused by ablation of *Atg7* occurred much earlier than the onset of neuronal death, indicating that axonal dystrophy was not secondary to neurodegeneration. In addition, axonal dystrophy and degeneration was a cell-autonomous event, which precluded the action of glia as the primary cause of the axonal dystrophy in *Atg7^{flox/flox};Pcp2-Cre* mice. Although this study was limited to Purkinje cells for the purpose of cell type-specific study of autophagy, axonal dystrophies occurred widely in many

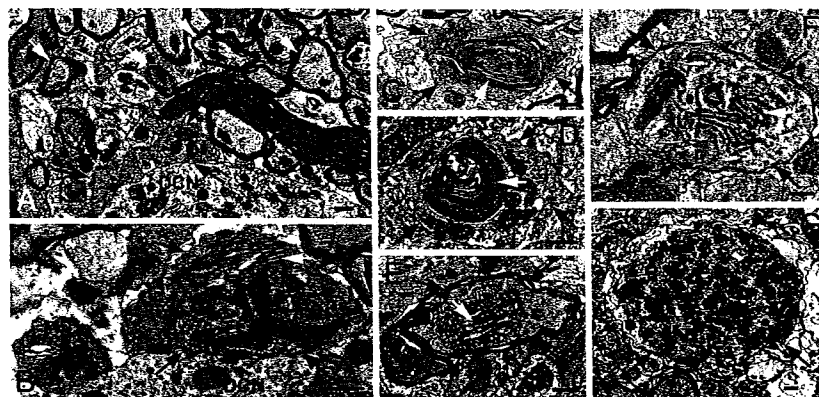


Fig. 5. Deletion of *Atg7* in Purkinje cells led to aberrant membrane structures in the axonal dystrophic swellings. Ultrastructural image of normal myelinated Purkinje cell axons (white arrows) and axon terminals (black arrows) in the DCN of *Atg7^{flox/flox}* mice (A) and Purkinje cell axonal dystrophic swellings (black arrows) in the DCN of *Atg7^{flox/flox};Pcp2-Cre* mice (B–F). (B and E) Stacks of cisternal membranes (white arrows). (C and D) Convoluted double-membrane whorls (white arrows). (F) The arrays of abnormal filaments (white arrows). (G) A dystrophic axon (black arrows) of *Lurcher* Purkinje cells containing numerous autophagosomes. (Scale bars: 500 nm.)

regions of the mutant mouse brain with *Atg7* or *Atg5* deletion (16, 17). We speculate that the axonal dystrophy associated with various types of neurons in these mutant mice are also cell-autonomous events caused by the absence of neuronal autophagy. Importantly, our results implied that interference in local autophagy would have a deleterious effect on axons, a potential mechanism of axonopathies involving impaired autophagy. Furthermore, we showed that deletion of *Atg7* had little effect on the overall morphology of the Purkinje cell dendritic arbors at a stage when axon terminals displayed massive dystrophy and degeneration. These differential effects of *Atg7* deletion suggested that basal levels of autophagy played a particularly important role in housekeeping functions in axon terminals and in protection against axonal degeneration.

We showed that *Atg7* was indispensable for the formation of the distinctive vacuole-like structures with double membranes, which were normally present within wild-type Purkinje cell axons. These structures have not been described previously in the wild-type Purkinje cell axons. Although the majority of these structures were likely derived from invagination of neighboring oligodendrocytes (Fig. 6*A b* and *c* and SI Fig. 12*A*) (25, 26), some of them appeared

to originate from axonal subsurface cisternae (27) or smooth endoplasmic reticulum (29) (Fig. 6*A d* and *e*). Although we have yet to determine the nature of these structures, we cannot exclude the possibility that some of these structures are autophagosome-related vacuoles. A previous study has shown the presence and transport of autophagosomes in the axons of cultured sympathetic neurons, despite that the physiological function of autophagy in the axons is unknown (30). Regardless of the nature of these vacuole-like structures, *Atg7* deletion abolished their formation, and caused axonal swelling and accumulation of aberrant membrane structures in these swellings. These results established an important role of *Atg7* in regulating local membrane trafficking and turnover. However, an important question arising from these results is whether the requirement of *Atg7* for the formation of the double-membrane vacuole-like structures is somehow connected to autophagy or associated with a specific *Atg7* function independent of autophagy. Current evidence suggests that *Atg7* function is exclusively associated with autophagy. Consistent with this idea, the abolishment of the double-membrane vacuole-like structures caused by *Atg7* deletion can be explained by the failure of autophagy, which would

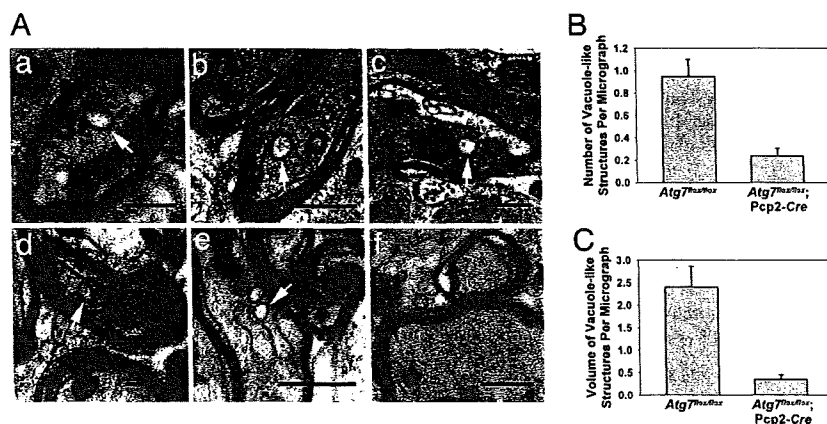


Fig. 6. Deletion of *Atg7* in Purkinje cells abolished double-membrane vacuole-like structures in their axon terminals in the DCN. (A) Ultrastructural images show the presence of vacuole-like structures with double membranes in Purkinje cell preterminal axons of *Atg7^{flox/flox}* mice (a–e, white arrows) and the abolishment of these structures in *Atg7^{flox/flox};Pcp2-Cre* mice (f) at P35. (Scale bars: 0.5 μ m.) (B) Comparison of the numbers of these vacuole-like structures per transmission electron microscopy micrograph (50 μ m²) in the DCN of *Atg7^{flox/flox};Pcp2-Cre* versus *Atg7^{flox/flox}* mice (ratio, 4.0; $P = 0.00003$). (C) Comparison of the volume fraction of double-membrane vacuole-like structures by point counting of transmission electron microscopy micrographs in the DCN of *Atg7^{flox/flox};Pcp2-Cre* versus *Atg7^{flox/flox}* mice (ratio, 6.9; $P = 0.00002$).

normally participate in the formation of these structures. Thus, we hypothesize that, in addition to the role in protein quality control (16, 17), neuronal autophagy regulates membrane homeostasis in the axon terminals. Furthermore, we showed that deletion of *Atg7* caused the axonal terminal swelling, which was a reminiscence of hepatic cell swelling in *Atg7*-deleted mouse liver (19). It is conceivable that axon terminals and hepatocytes may share a similar mechanism for cell (or cellular compartment) size control, which requires autophagy (31).

Previous morphological studies have consistently shown the presence of large numbers of autophagosome-like vacuoles in axonal dystrophic swellings of injured neurons (4–13). We have previously demonstrated that induction of autophagy in *Lurcher* Purkinje cells involved accumulation of autophagosomes in the dystrophic axons (22). In contrast, our present study shows that the axonal swellings of Purkinje cells in *Atg7^{flox/flox};Pcp2-Cre* mice were devoid of vacuoles that resembled the autophagosomes observed in *Lurcher* mice. Although our present study demonstrates a role for basal levels of autophagy in axonal protection and indicates that altered autophagy could serve as an adaptive response for remodeling the axon terminals for regeneration (3, 22), we cannot exclude the possibility that up-regulation of autophagy in dystrophic axons is actually destructive, causing overdegradation of axonal structures. This possibility can be tested, in principle, by genetic crossing of the autophagy-deficient mice with diseased mice containing the autophagosome-like vacuoles in dystrophic axons or cell bodies.

In summary, our study provides genetic and molecular evidence for the indispensable role of neuronal autophagy in the maintenance of axonal homeostasis, particularly in local membrane trafficking and turnover. Perturbation of local autophagy in the axons leads to axonopathy. We believe that it is important to study the connection between axonal autophagy impairment and human neuropathological conditions associated with axonal dystrophy.

Materials and Methods

Antibodies. Antibodies used were mouse monoclonal anti-calbindin D-28K (Swant, Bellinzona, Switzerland), anti-calbindin (Sigma, St. Louis, MO), anti-GluR δ 2 (BD Transduction Laboratories, San Diego, CA), anti-p62 (American Research Products, Belmont, MA), Cy3-conjugated anti-mouse and anti-rabbit IgG (Upstate Biotechnology, Lake Placid, NY), and NeuN, mGluR1 α , and actin antibodies (Chemicon International, Temecula, CA). Anti-*Atg7* is described in ref. 19.

Animals. *Pcp2-Cre* transgenic mice (20) (The Jackson Laboratory, Bar Harbor, ME) and *Atg7^{flox/flox}* mice (19) were crossed to produce *Atg7^{flox/flox};Pcp2-Cre* mice. *Atg7^{flox/flox};Pcp2-Cre* mice and GFP-LC3 transgenic mice (2) were crossed to produce *Atg7^{flox/flox};Pcp2-Cre*/GFP-LC3 mice.

Histological Examination. Mice were fixed by cardiac perfusion with 0.1 M phosphate buffer containing 4% paraformaldehyde. The color images of the Meyer's H&E-stained midsagittal cryosections (10 μ m) of cerebella were acquired with a 20 \times objective lens and a color CCD camera, and later assembled to full images in Photoshop for the quantification of Purkinje cells. Immunofluorescent stained cerebellar samples were prepared as described in ref. 22 and examined by using confocal microscopy.

Behavioral Analyses. Motor function was assessed by the limb-clasping test, rotarod assay, and gait analysis (*SI Materials and Methods*).

Electron Microscopy. Tissue samples were obtained from three *Atg7^{flox/flox}* mice and three *Atg7^{flox/flox};Pcp2-Cre* mice. Details of the experiment are described in *SI Materials and Methods*. In brief, thin sections (70 nm) of the lateral cerebellar nucleus and cerebellar cortex were prepared and examined by transmission electron microscopy (H7500; Hitachi, Tokyo, Japan). A double-blind point-counting method was used to quantify double-membrane vacuole-like structures in 55 micrographs of either *Atg7^{flox/flox}* or *Atg7^{flox/flox};Pcp2-Cre* mice. The total number of these structures overlying at least one intersection of a Photoshop-generated grid was counted to be 52 and 13 for *Atg7^{flox/flox}* and *Atg7^{flox/flox};Pcp2-Cre* samples, respectively. The total number of intersections within these structures was 132 and 19 for *Atg7^{flox/flox}* and *Atg7^{flox/flox};Pcp2-Cre* samples, respectively.

Statistical Analyses. The equality of the variance was first tested by using the *F* test. Pair-wise comparisons were calculated by using one-tailed Student's *t* test. The standard error was calculated for each sample.

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Rpn10-Mediated Degradation of Ubiquitinated Proteins Is Essential for Mouse Development[∇]

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Rpn10 is a subunit of the 26S proteasome that recognizes polyubiquitinated proteins. The importance of Rpn10 in ubiquitin-mediated proteolysis is debatable, since a deficiency of Rpn10 causes different phenotypes in different organisms. To date, the role of mammalian Rpn10 has not been examined genetically. Moreover, vertebrates have five splice variants of Rpn10 whose expressions are developmentally regulated, but their biological significance is not understood. To address these issues, we generated three kinds of Rpn10 mutant mice. Rpn10 knockout resulted in early-embryonic lethality, demonstrating the essential role of Rpn10 in mouse development. Rpn10a knock-in mice, which exclusively expressed the constitutive type of Rpn10 and did not express vertebrate-specific variants, grew normally, indicating that Rpn10 diversity is not essential for conventional development. Mice expressing the N-terminal portion of Rpn10, which contained a von Willebrand factor A (VWA) domain but lacked ubiquitin-interacting motifs (Rpn10ΔUIM), also exhibited embryonic lethality, suggesting the important contribution of UIM domains to viability, but survived longer than Rpn10-null mice, consistent with a “facilitator” function of the VWA domain. Biochemical analysis of the Rpn10ΔUIM liver showed specific impairment of degradation of ubiquitinated proteins. Our results demonstrate that Rpn10-mediated degradation of ubiquitinated proteins, catalyzed by UIMs, is indispensable for mammalian life.

The ubiquitin-proteasome system is the main nonlysosomal apparatus for intracellular protein degradation that is conserved in all eukaryotes from *Saccharomyces cerevisiae* to mammals (10, 15). Short-lived proteins as well as abnormal proteins are mostly recognized by the ubiquitin system and are tagged with ubiquitin chains as degradation signals. The polyubiquitinated proteins are then targeted for degradation by 26S proteasomes.

The 26S proteasome is composed of one proteolytically active 20S proteasome (also called the core particle) and two 19S regulatory particles (RP), each attached to one end of the 20S proteasome (1). The 19S RP plays an essential role in the degradation of ubiquitinated proteins. The 19S RP can be divided into two subcomplexes, known as the “base” and the “lid” (11). Structurally, the base subcomplex is made up of six different ATPases (Rpt1 to Rpt6) and two large subunits called Rpn1 and Rpn2, which act as scaffolds for molecules that modulate proteasome functions, such as Rpn13, Ubp6 (an USP14 orthologue), and Rad23 (an mHR23A/B orthologue) (4, 5, 13, 17, 24, 29, 35, 50). The base binds to the α -ring of the 20S proteasome and opens its narrow gate in an ATP-dependent manner (39). In addition, the ATPase subunits supply energy for unfolding target proteins, so that they can be trans-

located into the interior cavities of 20S proteasomes, where the active sites are located. The lid subcomplex consists of multiple non-ATPase subunits (Rpn3, Rpn5 to -9, Rpn11, Rpn12, and Rpn15). The role of the lid complex is less well understood, but it is reported to be essential for the degradation of ubiquitinated proteins, at least through the function of Rpn11, which deubiquitinates ubiquitin chains of proteasome substrates prior to degradation (44, 49).

In the ubiquitin-proteasome pathway, the process through which the polyubiquitin chains are recognized by the proteasome remains elusive. To date, several proteins have been identified as receptors that bind ubiquitinated proteins to ferry them to proteasomes for degradation. The UBL-UBA proteins, which contain ubiquitin-like (UBL) and ubiquitin-associated (UBA) domains, can interact with the proteasome through their N-terminal UBL domains as well as with polyubiquitin chains through their C-terminal UBA domains and are thought to shuttle ubiquitinated substrates to the proteasome and to facilitate their degradation (2, 8, 34). There are three UBL-UBA proteins in budding yeast called Rad23, Ddi1, and Dsk2 (2, 8, 34). Furthermore, several UBL-UBA proteins are also found in mammals, and some of them act in a manner similar to that of their yeast counterparts (12, 21).

Polyubiquitinated proteins are also recognized directly by the 19S proteasome subunit Rpn10 (3). Rpn10 is composed of one N-terminal von Willebrand factor A (VWA) domain and one or two C-terminal ubiquitin-interacting motifs (UIM). Rpn10 was the first protein recognized to bind to polyubiquitin chains through UIM domains. Surprisingly, genetic studies

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TABLE 1. Genotyping PCR primers^a

Primer name	Sequence (5'-3')
a	CAAGTAGTGCCTCTGGCTGCAAGA
b	TCCTGTCATCTCACCTTGCTCTG
c	CTTGGGAGGCAGAGACAGATGGAT
d	AGGACAAGAACAGCCCATGTCTGATTC
e	GATGCAATGCGGGCGCTGCATACGCTTG
f	ATGGCCGCTCACAACTGTCTGCAACTCC
g	CCTGTGTAGGATACCACAGCATCGACT
h	ATTGCTGGGAGTATGAACCACCATGCTG

^a Primers a, b, and c were used for genotyping of Rpn10 knockout mice. Primers d, e, and f were used for Rpn10a knock-in mouse genotyping. Primers f, g, and h were used for Rpn10ΔUIM mouse genotyping.

with yeast showed that deletion of the gene resulted in little loss of viability, in contrast to most other proteasome genes, which are essential for life (6, 43). The degradation defect of the *rpn10Δ* strain is modest, and the defect becomes evident when the strain is crossed with strains that lack UBL-UBA proteins such as Rad23 and Dsk2 (23, 31). In vitro analysis of the degradation of polyubiquitinated Sic1 showed that while its degradation was defective in *rpn10Δ* proteasomes and *rad23Δ* proteasomes, simultaneous addition of Rad23 protein and the VWA domain of Rpn10 restored the degradation of ubiquitinated Sic1, suggesting a "facilitator" activity within the VWA domain of Rpn10 (45). Finally, a yeast strain that has mutations in the UIM domain of Rpn10 showed a clear deficiency in ubiquitin chain recognition, and this *rpn10-uim* mutation confers synthetic sensitivity to canavanine when combined with either a *rad23Δ* or a *dsk2Δ* mutation, indicating that the UIM domain of Rpn10 and UBL-UBA proteins work redundantly (4). It is now suggested that UBL-UBA proteins and Rpn10 define a layer of substrate selectivity, which might depend on the length of polyubiquitin chains (6, 20, 30, 45).

Although Rpn10 is not essential for life or for overall ubiquitin-mediated protein turnover in yeast and worm (6, 36, 41, 43), Rpn10-deficient mutants of *Physcomitrella patens*, *Arabidopsis thaliana*, and *Drosophila melanogaster* showed more-severe phenotypes such as developmental arrest and lethality (9, 38, 40). These results suggest that higher eukaryotes depend on Rpn10-mediated degradation of polyubiquitinated proteins for their development.

Vertebrates have acquired a diversity of proteasomes by creating new subunits. For example, the gamma interferon-inducible subunits β1i, β2i, and β5i evolved to produce immunoproteasomes, which enable viral proteins to be presented on major histocompatibility complex class I molecules more efficiently than constitutive proteasomes (42). Vertebrates have also acquired a diversified Rpn10 subunit, which was accomplished by developmentally regulated alternative splicing, resulting in the generation of five isoforms named Rpn10a to Rpn10e (see Fig. 1A) (18). It is reported that Rpn10c in *Xenopus laevis* associates with Scythe/BAG-6 and regulates apoptosis (19). However, the significance and distinct functions of these isoforms in mammals are not understood at all. In addition, the importance of the UIM and VWA domains of Rpn10 for mammalian development has not been examined.

In the present study, we generated and analyzed three types of Rpn10 mutant mice in order to understand the role(s) of

TABLE 2. PCR primers for Rpn10 splicing variants

Primer name	Sequence (5'-3')
Rpn10a,c,d,e forward	CAAAGGCAAGATCACCTTCTGCACTGGCA
Rpn10b forward	TACACCTGGGACTGAAGGTGAAGA
Rpn10a,b reverse	GTTCTCTAGGACGCTCTG
Rpn10c reverse	AAGTGTTTCCCTCTGTGCTCCAAG
Rpn10d reverse	GGCCCTGCCACCAAGCCATGGCCAC
Rpn10e reverse	TCGTGAAATGGCTAGCAC

Rpn10 in mammals. Our results provide genetic evidence for the in vivo significance of Rpn10 in mammals.

MATERIALS AND METHODS

Gene targeting of Rpn10. A targeting vector for Rpn10-null mice was constructed by replacing exons 2 to 8 with a neomycin resistance gene (*neo*) cassette. For Rpn10a knock-in mice (with, at the same time, conditional deletion of UIM domains), exons 7 to 10 were replaced with the corresponding cDNA of Rpn10a, with a polyadenylation signal attached at its 3' end. A *neo* cassette was inserted at the 3' end of the cDNA. LoxP sequences were inserted at the 5' end of the cDNA and the 3' end of the *neo* cassette. TT2 embryonic stem cells were screened as described previously (27). For Southern blot analysis, genomic DNA was digested with EcoRI for Rpn10 knockout or with EcoRV for Rpn10a knock-in and was hybridized with the probes shown in Fig. 1A and 2A, respectively. EIIa-Cre and Alb-Cre were purchased from The Jackson Laboratory. PCR primers used for mouse genotyping are listed in Table 1. Mice were housed in pathogen-free facilities, and the experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the Tokyo Metropolitan Institute of Medical Science.

RNA isolation, reverse transcription, and real-time PCR. Expression levels of Rpn10 variants were determined by reverse transcription-PCR (RT-PCR) as described previously (18). Specific primers for each variant are listed in Table 2. For real-time PCR analysis, total RNAs were isolated from the livers of 5-week-old mice by using an RNAspin minikit (GE Healthcare), reverse transcribed to cDNA using a Transcriptor first-strand cDNA synthesis kit (Roche), and subjected to real-time PCR using the LightCycler 480 system (Roche). PCR primers and universal probes (Roche), which are listed in Table 3, were designed according to the Universal Probe Assay Design Center (<http://www.roche-applied-science.com/sis/rtPCR/upl/adc.jsp>). Glucuronidase beta (GUSβ) was used for normalization. Real-time PCR data were analyzed by the ΔΔC_T method.

Immunological analysis. Mouse livers were homogenized and subjected to immunoblotting and immunoprecipitation as described previously (13). The antibodies against Rpn1, Rpn3, Rpt6, the VWA domain of Rpn10 [Rpn10(N)], USP14, polyubiquitin, and actin have been described previously (13). Polyclonal antibodies against a6, a7, Rpn6, Rpn7, Rpt3, Rpt5, the UIM domain of Rpn10 [Rpn10(C)], and mHR23B (all sequences were derived from mice) were raised in rabbits by using recombinant proteins expressed in and purified from strain BL21RIL (Novagen) as His₆ fusion proteins of a6 (residues 152 to 263), a7 (residues 157 to 255), Rpn6 (residues 1 to 162), Rpn7 (residues 1 to 137), Rpt3 (residues 1 to 100), and Rpt5 (residues 1 to 131) and as glutathione S-transferase fusion proteins of Rpn10 (residues 255 to 376) and mHR23B (full length), respectively. For immunoprecipitation, a liver homogenate from an Rpn10^{neo} or an Rpn10^{neo:Alb} mouse (the two mice had equal Suc-LLVY hydrolyzing activities) was immunoprecipitated with an anti-Rpt6 antibody.

Glycerol gradient analysis. Mouse liver homogenates were clarified by centrifugation at 20,000 × g and subjected to 10 to 40% (vol/vol) linear glycerol gradient centrifugation (22 h, 83,000 × g) as described previously (16).

Assay of proteasome activity. The assays of proteasome chymotryptic peptidase activity, degradation of recombinant ³⁵S-labeled ornithine decarboxylase (ODC), and degradation of polyubiquitinated ³⁵S-labeled cIAP1 protein have been described previously (13, 16).

Histological examination. Embryos in utero were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned. Sections were stained with Mayer's hematoxylin, followed by eosin staining.

Culture of blastocysts. Blastocysts were flushed out from pregnant female uteri at embryonic day 3.5 (E3.5) and were cultured in M16 medium (Sigma-Aldrich) at 37°C under 5% CO₂ on a gelatin-coated chambered coverglass (Nalgene).