<arttitle> E3 ubiquitin-ligase that recognises sugar chains

<aug> Yukiko Yoshida[*], Tomoki Chiba[†‡], Fuminori Tokunaga[§], Hiroshi Kawasaki[‖], Kazuhiro Iwai[§], Toshiaki Suzuki[†‡], Yukishige Ito[¶], Koji Matsuoka[#], Minoru Yoshida[‡**], Keiji Tanaka[†‡] & Tadashi Tai[*]

<aff> Departments of [*]Tumor Immunology and [†]Molecular Oncology, Tokyo Metropolitan Institute of Medical Science, Bunkyo-ku, Tokyo 113-8613, Japan,
[‡]CREST, Japan Science and Technology Corporation (JST), Saitama 332-0012, Japan,
[§]Department of Molecular Cell Biology, Graduate School of Medicine, Osaka City University, Osaka 545-8585, Japan,
[‖]Kihara Institute for Biological Research, Graduate School of Integrated Science, Yokohama City University, Yokohama, Kanagawa 244-0813, Japan,
[¶]Synthetic Cellular Chemistry Laboratory, and [**]Chemical Genetics Laboratory, RIKEN, Wako, Saitama 351-0198, Japan,
[#]Department of Functional Materials Science, Saitama University, Saitama, 338-8570, Japan,

<footnote> Corresponding author: Tadashi Tai

Phone: +81-3-3823-2105

Fax: +81-3-3823-2965

E-mail: tai@rinshoken.or.jp

Keiji Tanaka

Phone: +81-4463-7592

Fax: +81-3-3823-2237

E-mail: tanakak@rinshoken.or.jp

<abs> *N*-glycosylation of proteins in the endoplasmic reticulum (ER) plays a key role in the protein quality control[1-3]. Here we report that *N*-glycan serves as a degradation signal by the novel SCF^{Fbx2} ubiquitin-ligase complex. Fbx2[4], an F-box protein, binds specifically to proteins attached with *N*-linked high-mannose type oligosaccharides, and subsequently contributes to ubiquitylation of *N*-glycosylated proteins. We identified pre-integrin β1 as one of the Fbx2 targets. This interaction was detected in the cytosol when the proteasome was inhibited. Furthermore, expression of Fbx2ΔF lacking the F-box domain essential for the formation of SCF complex appreciably blocked degradation of typical substrates of ER-associated degradation (ERAD)[5,6]. These results indicate that SCF^{Fbx2} ubiquitylates *N*-glycosylated proteins, which are translocated from the ER to the cytosol by the quality control mechanism.

<p-ni> In an attempt to find novel biological functions of *N*-glycans, we screened mouse brain extracts for proteins bound to various sugar probes. When we used GlcNAc-terminated fetuin[7] (GTF)-immobilised beads, we found two proteins, which were specifically eluted with *N, N'*-diacetylchitobiose (chitobiose) but not with EDTA/EGTA, unlike C-type lectins[8] (Fig. 1a). Protein sequencing analysis revealed that they were Skp1 and Fbx2/NFB42 (neural F-box 42 kDa)[4]. The interaction of Skp1 and Fbx2 to GTF was further examined by expressing these proteins in 293T cells. Fbx2, but not Skp1, interacted with GTF, indicating that the interaction between Skp1 and GTF is mediated by Fbx2. The GTF-binding domain of Fbx2 was mapped on the entire C-terminal domain (95-296 a.a.) (Fig. 1b and Supplementary Information Fig. 1). Fbx2, as well as other F-box proteins, formed a complex with Skp1, Cul1 and Roc1 (Fig. 1c and Supplementary Information Fig.2). We further devised an *in vitro* ubiquitylation assay of GTF by using the recombinant SCF^{Fbx2} complex[9] (Fig. 1c). Ubiquitylation of GTF was detected in a time-dependent manner by immunoblotting using an anti-fetuin antibody, as judged by shifting the GTF migration to higher molecular mass positions with ubiquitin (Ub) or GST-Ub (Fig. 1d, lanes 2-5 and 8-9). On the other hand, ubiquitylation of GTF was not detected without SCF^{Fbx2} or with $SCF^{\beta TrCP1}$ (lanes 6-7). The ubiquitylation of GTF was inhibited by chitobiose (Fig. 1e), or when GTF was deglycosylated with peptide: *N*-glycanase F (PNGase) (Fig. 1f). These results show that SCF^{Fbx2} is an E3 ubiquitin-ligase that recognises target proteins in an *N*-glycosylation dependent manner.

We next examined the ability of Fbx2 to bind endogenous proteins using overlay assay (Fig. 2a). Several proteins were detected by full-length Fbx2 and ΔN-2 probes mainly in the membrane fractions of mouse brains and neuroblastoma cells. The most prominent one was a 120-kDa protein (p120) detected in Neuro2a cells. In the adult mouse brain, we detected a broad 75-80 kDa protein band(s) (p75) instead of p120. These interactions were specifically inhibited by chitobiose, but not by other

monosaccharides or disaccharides tested (Fig. 2a and not shown). Removal of *N*-glycans by PNGase completely inhibited Fbx2 binding. Moreover, treatment with endo-β-*N*-acetylglucosaminidase H (endo H) that cleaves the high-mannose type oligosaccharides (High-Man) also interfered with the binding. To test whether the interaction of Fbx2 with glycoproteins also occurs *in vivo*, we analysed the proteins co-immunoprecipitated with ΔN-2 by lectin blotting (Fig. 2b). Several proteins including p120 were detected with Con A, a lectin that binds to High-Man, but not with WGA, specific for terminal GlcNAc or sialic acids. These proteins were not bound to βTrCP1.

We further characterised the binding specificity of Fbx2 by pull-down assay using different *N*-glycans on fetuin and ribonuclease B that had been treated with various glycosidases (Fig. 2c). Fbx2 could bind to mannose-terminated fetuin (MTF) more efficiently than to asialofetuin and GTF, but not to DGF or intact fetuin. Moreover, Fbx2 bound to intact ribonuclease, but its binding was reduced when ribonuclease was treated with α-mannosidase and endo H. When we examined the efficiency of elution of ΔN-2 bound to ribonuclease by oligosaccharides, $Man_9GlcNAc_2$ was found to elute ΔN-2 at much lower concentrations than those of chitobiose (Fig. 2d). These data indicate that *N*-glycans containing diacetylchitobiose structure with mannose residues are required for the efficient Fbx2 binding and that further modification of mannose residues by other sugars reduces the Fbx2 binding.

We next investigated whether Fbx2 is involved in proteolysis *in vivo*. The full-length Fbx2 but not ΔN-2 was associated with ubiquitylated proteins in Neuro2a cells under inhibition of proteasome activity, but this binding was decreased in the presence of chitobiose during the immunoprecipitation (Supplementary Information Fig.2), suggesting that the formation of SCF^{Fbx2} is necessary for *N*-glycoprotein ubiquitylation. We next examined the amounts of Fbx2 substrates bound to each constructs by overlay assay (Fig. 3a). Despite the presence of a larger amount of the substrates in the

membrane fraction (input), only the cytoplasmic substrates were co-immunoprecipitated with ΔN-2. The full-length Fbx2 apparently failed to interact with endogenous substrates, probably due to proteasomal degradation of substrates. Indeed, the interaction became detectable in the cytoplasm by MG132 treatment. We next purified p120 and determined the amino acid sequences. The peptide-sequence LPDGVTINYK was identical to part of mouse integrin β1 (389-398 a.a.). The integrin β1 proteins with different oligosaccharide chains were shown to run separately as two bands on SDS-PAGE[10,11]. As shown in Fig. 3b, ΔN-2 was co-immunoprecipitated with the precursor (pre-integrin β1) but not the mature form. Fbx2 failed to interact with deglycosylated integrin β1 in the cells treated with tunicamycin, whereas it did bind to pre-integrin β1, indicating a specific interaction of Fbx2 with pre-integrin β1containing High-Man (Fig. 3c). As ubiquitylation reaction occurs in the cytosolic compartment, we sought to determine where the interaction of Fbx2 with pre-integrin β1 occurs in cells. To this end, we fractionated Neuro2a cells prior to immunoprecipitation. While the cytosol fraction contained only an almost undetectable amount of pre-integrin β1, pre-integrin β1 bound to ΔN-2 was detected in the cytosol fraction (Fig. 3d, lanes 2, 5, 13). Although the interaction between the full-length Fbx2 and pre-integrin β1 was hardly detected in the cytosol, it was restored by MG132 treatment (lanes 1, 4). The interaction was also detected in the microsomal fraction, suggesting that a part of pre-integrin β1 is complexed with Fbx2 upon translocation to the cytosol. These results suggest that Fbx2 is responsible for the proteasomal elimination of proteins containing High-Man in the cytosol.

Cystic fibrosis transmembrane conductance regulator (CFTR) is rapidly degraded in the cytosol when it fails to be properly folded in the ER[12,13]. This degradation is dependent on the ERAD pathway and especially enhanced when CFTR has mutations such as ΔF508[14]. The degradation of misfolded CFTR requires the proteasomal activity, and when cells are treated with a proteasome inhibitor, CFTR

accumulates in intracellular inclusions termed aggresomes[14]. We therefore assessed the role of Fbx2 in the degradation of CFTRΔF508. Immunoprecipitation of CFTRΔF508-GFP (CFTR-GFP) followed by immunoblotting showed that full-length Fbx2, ΔN-2 and ΔF but not ΔC-1 could interact with CFTR-GFP, and this interaction appeared to be mediated by its glycosylation, because tunicamycin markedly reduced the interaction (Fig. 4a). Since ΔF could bind to CFTR-GFP more efficiently than ΔN-2, we subsequently tested whether the expression of ΔF affects the ERAD pathway. Pulse-chase experiments revealed that whereas CFTR-GFP was rapidly degraded in control cells (17.1% at 2 h), its degradation was significantly suppressed by ΔF overexpression (31.5 % at 2 h) (Fig. 4b). In the case of another ERAD substrate T cell receptor α subunit (TCRα)[15], the decay was more efficiently suppressed by ΔF (vector; 17.7%, ΔF; 70.6% at 2 h). These results show the involvement of Fbx2 in the ERAD pathway *in vivo*. COS7 cells transfected with CFTR-GFP formed a large aggregate adjacent to the nucleus when treated with MG132, as described[14] (Fig. 4c). This aggresome formation was significantly suppressed when Fbx2 was co-expressed, whereas ΔN-2, ΔC-1, ΔF or full-length βTrCP1 did not cause any change in aggresome formation (Fig. 4d and not shown). These results suggest that SCF^{Fbx2} plays a role in the elimination of misfolded glycoproteins that are translocated from the ER to the cytosol.

E3 ubiquitin-ligase constitutes a large protein family including SCF[16,17]. F-box proteins are also diverse[18,19] and play a pivotal role in the selection of target proteins for ubiquitylation[16]. Accumulating evidence suggests that modification of target proteins is a prerequisite for the recognition by a certain SCF-type E3 and subsequent destruction by the 26S proteasome[16-18]. These include phosphorylation and proline hydroxylation[20,21]. In the present study, we show that *N*-glycosylation acts as a novel targeting signal to eliminate intracellular glycoproteins by Fbx2-dependent ubiquitylation and proteasomal degradation. Fbx2 recognises High-Man on the

substrates. Since High-Man are present in the ER, it is conceivable that SCF^{Fbx2} plays an important role in the quality control coupled to the ER. Indeed, we identified pre-integrin β1 as one of the Fbx2 targets. Integrin β1 is expressed in excess over α-chains[10,22], and the excess of non-complexed β1 is either immediately degraded probably by the ERAD pathway or remains in the ER by calnexin ready to associate with freshly synthesised α-chains[10,23]. Moreover, we showed direct evidence for the role of Fbx2 in the ERAD pathway *in vivo* using typical ERAD substrates (Fig. 4b). Thus we propose that SCF^{Fbx2} is an E3 specific for *N*-linked glycoproteins in the ERAD pathway. It is noteworthy that Fbx2 is expressed mainly in neuronal cells in the adult brain (ref. 3 and our unpublished data). Accordingly, it is likely that the SCF^{Fbx2}-mediated ERAD pathway contributes to the rapid elimination of glycoproteins present in neurons. Further studies should be designed to identify the physiological substrates for SCF^{Fbx2} in neuronal cells and their carbohydrate structure, which may help to understand the *in vivo* function of Fbx2 in the brain.

<meth1ttl> Methods

<meth1hd> Isolation and identification of GlcNAc-binding proteins

GlcNAc-terminated fetuin (GTF) was prepared by incubating asialofetuin type II (Sigma) with β-galactosidase (*Streptococcus* 6646K, Seikagaku-kogyo) and passed through RCA120 lectin agarose. Brains of ICR mice (8-weeks old) were homogenized in 10 volumes of 20 mM Tris-HCl (pH 7.5) / 150 mM NaCl (TBS) containing 5 mM $CaCl_2$ and protease inhibitor cocktail. After centrifugation of the homogenate at 30,000 x *g* for 30 min, the supernatant was incubated with GTF-immobilised beads (Affi-gel 15, Bio-Rad) for 18 h. The beads were washed with TBS containing 0.1% TritonX-100 (TBS-T). The proteins adsorbed were successively eluted by 0.5 M NaCl, 20 mM EDTA/EGTA, 0.2 M GlcNAc or 0.1 M chitobiose in TBS-T. Eluted proteins were separated by SDS-PAGE, excised, digested with lysylendopeptidase, and their sequences were determined by a protein sequencer.

Transfection, immunoprecipitation and immunoblotting

The 293T, Neuro2a, and COS-7 cell lines were transfected with various plasmids by lipofection (Lipofectamine Plus, Gibco). For inhibition of cellular proteasome activity, cells were treated with 50 μM of MG132 (Peptide Institute) for 4 h unless otherwise noted. For inhibition of biosynthesis of *N*-glycans, cells were treated with 5 μg/ml of tunicamycin for 24 h. The whole cell extracts (WCE) were prepared with TBS containing 0.5% NP-40. The supernatant by centrifugation at 24,000 x *g* for 20 min of freezing-and-thawing cell extracts in TBS was used as the cytoplasmic fraction (abbreviated simply C), while the precipitate solubilised with 1% Triton X-100 was used as the membranous fraction (abbreviated M). Cytosol and microsome fractions were obtained by centrifugation of the above cytoplasmic fraction at 100,000 x *g* for 60 min. The microsomal proteins were solubilised with Triton X-100. We performed

immunoprecipitation using mouse monoclonal antibodies, anti-FLAG (M2, Sigma), anti-Myc (9E10, Santa Cruz), anti-HA (HA.11, Babco), and anti-GFP (clones 7.1 and 13.1, Roche). Mouse monoclonal antibodies, anti-FLAG, anti-HA, and anti-V5 (Invitrogen), rat monoclonal anti-integrin β1 (Mab1997, Chemicon) and rabbit polyclonal antibodies, anti-Myc (A-14, Santa Cruz) and anti-fetuin (Chemicon), were used for immunoblotting.

***In vitro* ubiquitylation assay**

SCF^{Fbx2} were produced in insect cells by co-infection of baculovirus encoding His-Fbx2, FALG-Skp1, Cul1-HA, T7-Roc1, then affinity purified by a HiTrap HP column (Amersham Pharmacia). Other recombinant proteins for *in vitro* ubiquitylation assay were described [9]. Deglycosylated fetuin (DGF) were prepared by incubating asialofetuin with *Flavobacterium meningosepticum* recombinant PNGase F (Roche). Each 1 μg of fetuin, GTF or DGF was incubated in 50 μl of the reaction mixture containing ATP regenerating system[9], 0.5 μg E1, 1 μg Ubc4 (E2), 2 μg SCF^{Fbx2} and 6 μg bovine ubiquitin (Sigma) or 6.5 μg recombinant GST-ubiquitin at 30˚C.

Overlay assay

The cytoplasm (C) and membranous (M) fractions from mouse and cultured cells were separated with SDS-PAGE, and blotted onto a membrane (immobilon, Millipore). To prepare the [^{35}S]-Fbx2 probes, we constructed Fbx2-Metx3 (full and ΔN-2) plasmids for Flag-tagged (at N-termini) full-length Fbx2 and ΔN-2 with additional three methionines at C-termini by subcloning them into pTracer-EF (Invitrogen). The TNT Coupled Reticulocyte Lysate System (Promega) was used for generating [^{35}S]-Fbx2 and its derivatives. Membranes were blocked by 5% skim milk in TBS, and incubated with probes in 1% skim milk at 4˚C for 18 h. Membranes were washed with TBS containing 0.05% Tween20 and analysed by autoradiography. In endo-H or PNGase-

treatment experiments, the blotted membranes were blocked with 3% BSA in PBS. Each membrane was treated with 1 unit of recombinant endo H (Seikagaku-kogyo) in citrate-phosphate buffer, pH 5.5, or 10 units of PNGase F in phosphate buffer, pH 8.2, at 37˚C for 18 h. The enzyme-treated membranes were washed with TBS, and incubated with 5% skim milk, before the overlay.

Pull-down assay

Two mg of asialofetuin or ribonuclease B was treated with various glycosidases (β-galactosidase, Jack Bean β-*N*-acetylhexosaminidase, PNGase F, Jack Bean α-mannosidase, and endo H), and was immobilised to 0.5 ml of Affi-gel 10 or 15. WCE of ΔN-2-expressing cells (25 μg) was incubated with 15 μl of various glycoprotein-immobilised beads, and bound proteins were eluted by boiling with SDS sample buffer or incubation with 15 μl of various concentrations of chitobiose or $Man_9GlcNAc_2$ at room temperature for 10 min. The eluates were separated by spin filtration.

Immunofluorescence microscopy

COS-7 cells that were transfected with CFTRΔF508 (CFTR-GFP) and cultured onto glass-bottomed 35-mm dishes were fixed in 4% paraformaldehyde, and permeabilised by 0.5% NP-40/PBS for 30 min. The cells were washed with PBS and blocked with 2% FCS/PBS for 2 h. The fixed cells were incubated with the primary antibody and washed with PBS, and then exposed to a 1,000-fold diluted Cy3-conjugated secondary antibody. Immunofluorescence was examined using an Olympus IX70 microscope equipped with a digital camera (C4732-5 Hamamatsu) operated by QED Camera Plug-in software (QED Imaging).

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<corr> Correspondence and requests for materials should be addressed to T.T. (e-mail: tai@rinshoken.or.jp) or K.T. (e-mail: tanakak@rinshoken.or.jp).

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Figure 1 SCF^{Fbx2} complex ubiquitylates the *N*-glycosylated substrate *in vitro*. **a,** Identification of Fbx2 and Skp1 as GlcNAc-binding proteins (silver-staining). The GlcNAc-terminated fetuin (GTF)-bound proteins were successively eluted with NaCl, EDTA / EGTA, GlcNAc, or chitobiose. Following amino acid sequences were obtained; ^{83}ELVDGAPL and ^{182}AQVIDLQAExY for Fbx2, and ^{81}RTDDIP and ^{138}TFNIK for Skp1. **b,** Interaction between GTF and various mutants of Fbx2. FBA represents F-box-associated domain[19]. The abilities of Fbx2 mutants to bind GTF were summerised with schematic representation of Fbx2 and its deletion mutants (+ or -) (see Supplementary Information Fig. 1 for details). **c,** Electrophoretic pattern of the recombinant SCF^{Fbx2} complex produced by the baculovirus system. **d,** *In vitro* ubiquitylation of GTF by the SCF^{Fbx2} ubiquitin-ligase system. **e,** Effect of saccharides on the *in vitro* ubiquitylation of GTF. *In vitro* reactions were performed in the presence of 0.1 M galactose (Gal), 0.1 M GlcNAc, or 0.1 M chitobiose for 2 h. **f,** *N*-linked oligosaccharides are required for ubiquitylation. *In vitro* ubiquitylation was performed with GTF and deglycosylated fetuin (DGF).

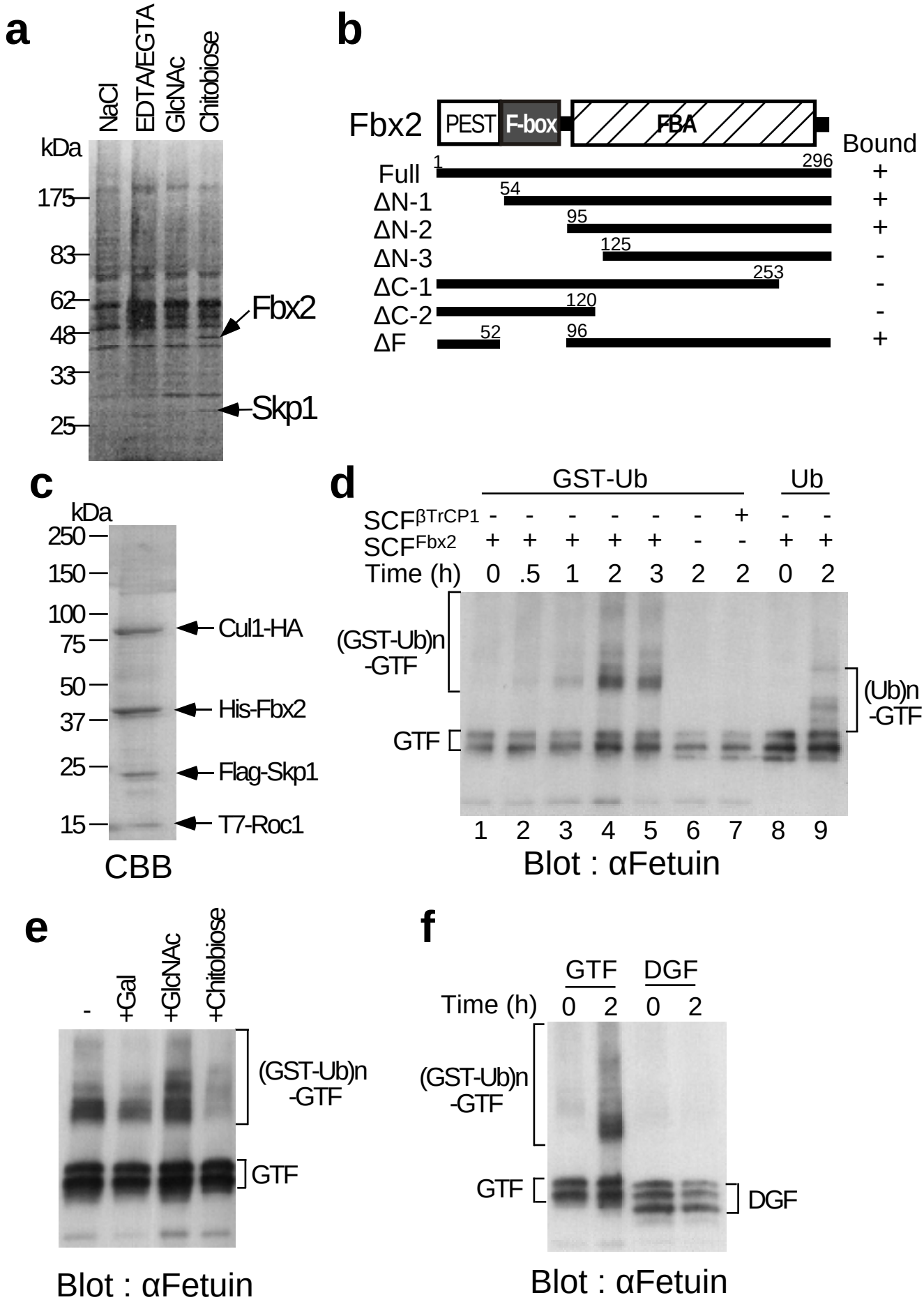
Figure 2 Interaction of Fbx2 with endogenous glycoproteins containing High-Man. **a,** Detection of Fbx2-binding proteins by overlay assay using [^{35}S]-labelled-Fbx2. Cytoplasmic proteins (C) and membranous fractions (M) in brains of newborn (NB) and adult (A) mice and Neuro2a (N2a) cells were separated with SDS-PAGE, and then transferred to membranes. 0.1 M chitobiose was added with the probe for competition. Each blot was untreated or treated with PNGase or endo H before the overlay assay. Arrows indicate p120 and p75. **b,** *In vivo* interaction of Fbx2 with endogenous glycoproteins. Neuro2a cells were transfected with Flag-tagged Fbx2 (ΔN-2) or βTrCP1 lacking its F-box domain. After immunoprecipitation from WCE, the Fbx2-binding proteins were analysed by immunoblotting or lectin-blotting. Arrows denote similar signals detected with overlay assay in **a**. **c,** Pull-down analysis of the interaction of Fbx2 with *N*-glycoproteins. Bound proteins were analysed by immunoblotting (upper) and their binding efficiencies were expressed in the order of -, ±, +, ++ (lower). **d,** Elution of Fbx2 bound to ribonuclease-B immobilised beads with oligosaccharides. The ribonuclease-bound ΔN-2 was eluted by boiling with sample buffer (B) or by incubation with chitobiose or $Man_9GlcNAc_2$.

Figure 3 SCF^{Fbx2} promotes proteasomal degradation of glycoproteins in the cytosol. **a,** *In vivo* detection of Fbx2-binding proteins in the cytoplasm. Neuro2a cells transfected with Flag-tagged Fbx2 were treated with (+) or without (-) MG132. After fractionation, Fbx2 was immunoprecipitated from cytoplasmic (C) and membranous (M) fractions. Lysate (Input) and the immunoprecipitates were analysed by overlay assay. Arrows denote similar signals observed in Figure 2**a**. **b,** Identification of p120 as integrin β1. Neuro2a cells were transfected with or without Flag-tagged Fbx2 (ΔN-2) or βTrCP1 lacking its F-box domain. Each F-box protein was immunoprecitiated from WCE and blotted with

integrin β1 antibody. Cytoplasmic (C) and membranous (M) lysates were loaded to compare the sizes of integrin β1 proteins. **c,** *In vivo* interaction of Fbx2 with integrin β1 containing High-Man. Neuro2a cells transfected with Flag-tagged ΔN-2 were treated with (+) or without (-) tunicamycin (TM). After ΔN-2 had been immunoprecipitated from WCE, the integrin β1 proteins were analysed by immunoblotting. **d,** Detection of the Fbx2-integrin β1 complex in the cytosol. Cytosolic (Cs) and microsomal (Mi) fractions were prepared from the cells transfected with or without Fbx2 constructs. The amounts of integrin β1 associated with Fbx2 were determined by immunoblotting.

Figure 4 Involvement of Fbx2 on the ERAD pathway. **a,** Interaction between CFTRΔF508-GFP and Fbx2. 293T cells transfected with CFTR-GFP and various Fbx2 constructs were treated with (+) or without (-) tunicamycin (TM). After immunoprecipitation of CFTR-GFP from WCE, the bound Fbx2 was analysed using anti-Flag antibody. **b,** Effect of ΔF on the stability of CFTR and TCRα. The [^{35}S]-pulse-labelled (30 min) CFTR-GFP and HA-TCRα were chased at indicated periods in the absence or presence of ΔF. Pulse-chase experiments were performed as described[23]. **c,** CFTR-GFP aggresome formation by treatment with a proteasome inhibitor. COS-7 cells were transiently transfected with CFTR-GFP. Forty-eight h after transfection, cells were treated with (+) or without (-) 20 μM MG132 in the presence of 10 μg ml^{-1} cycloheximide for 24 h. Treatment of cells with MG132 leads to the aggregation of the misfolded protein termed aggresomes (white arrowheads). Bar = 10 μm. **d,** Suppression of aggresome formation by Fbx2. COS-7 cells were transfected with CFTR-GFP and various Flag-tagged Fbx2 constructs. Note the lack of aggresomes (yellow arrowhead) in cells expressing full-length Fbx2. Bar = 10 μm. The right panel shows the rates of aggresome formation

in the presence of various Fbx2 constructs. N indicates the number to experiments. 50 ~ 100 cells were counted in each experiment. Results are expressed as mean ± SEM.



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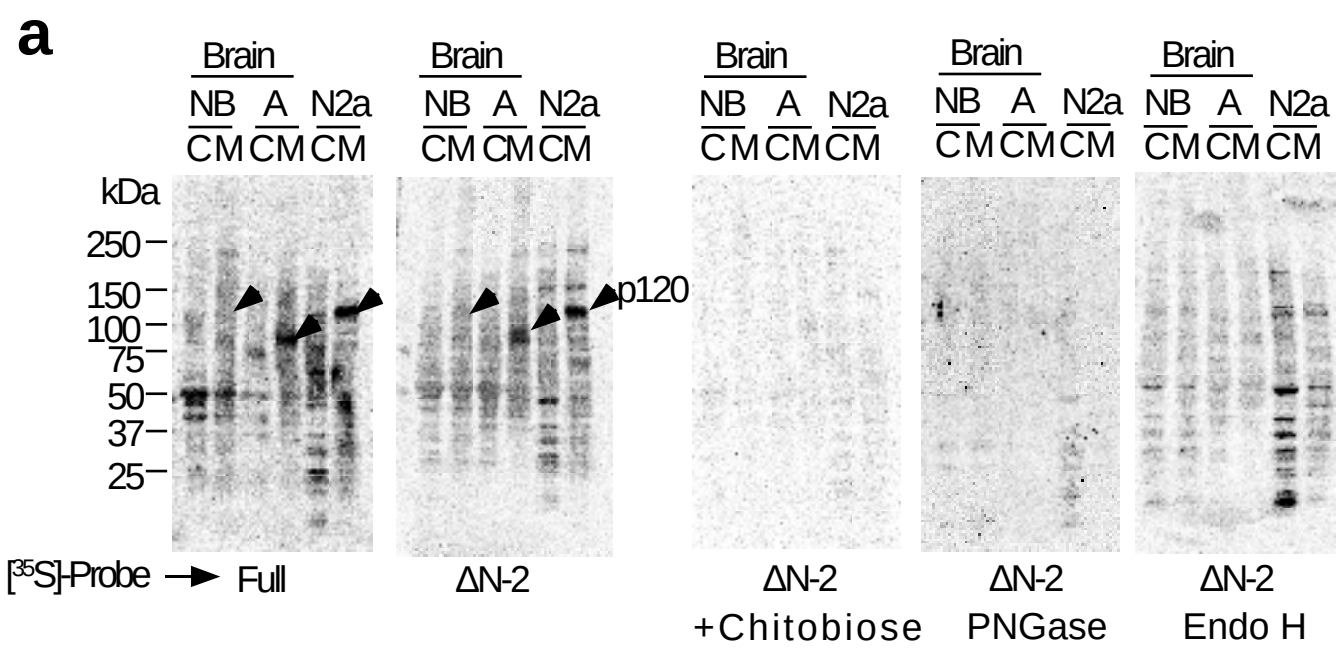
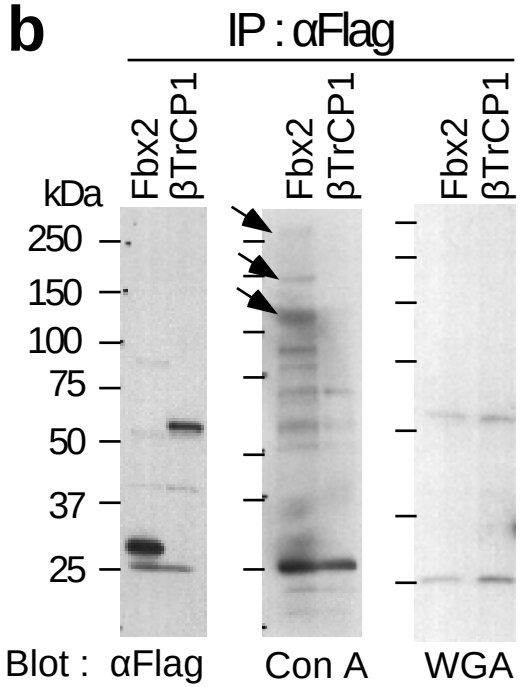
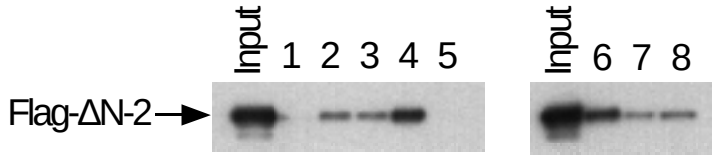






	Proteins (N-Glycan)	Bound
1	Fetuin (Tri-antennary Complex)	±
2	Asialo Fetuin (Gal-terminal Tri-antennary)	+
3	GTF (GlcNAc-terminal Tri-antennary)	+
4	MTF ($Man_3GlcNAc_2$)	++
5	DGF (None)	-
6	Ribonuclease B ($Man_{5-7}GlcNAc_2$)	++
7	α-Mannosidase-treated Ribonuclease B ($ManGlcNAc_2$)	+
8	Endo-H-treated Ribonuclease B (GlcNAc)	+

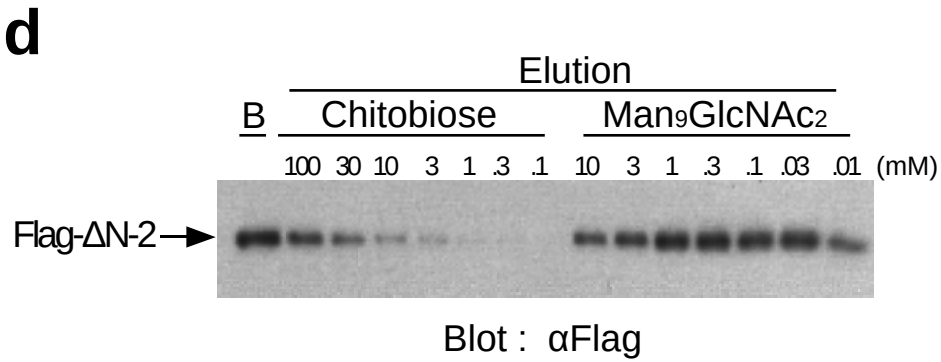


Figure 2 Tai et al.

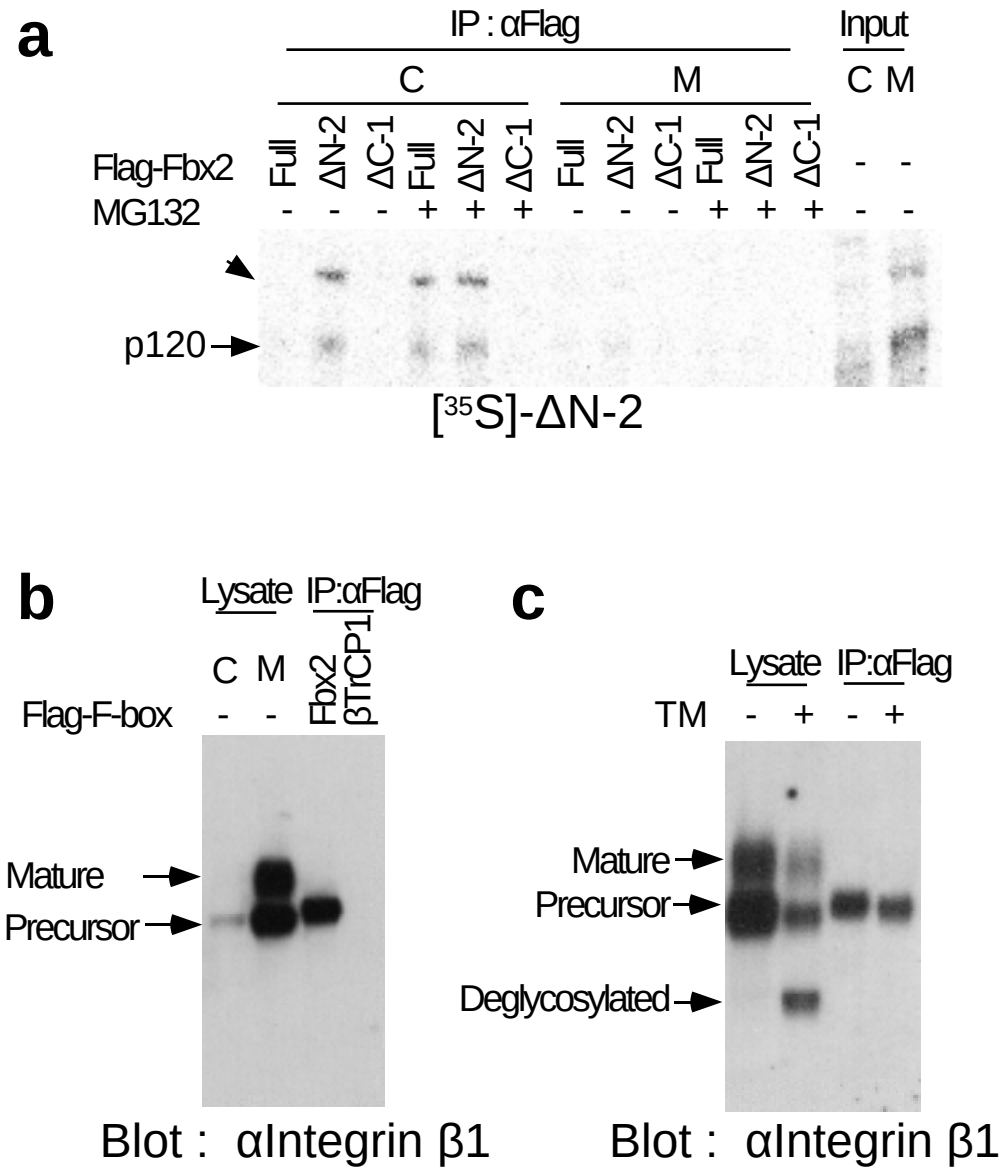
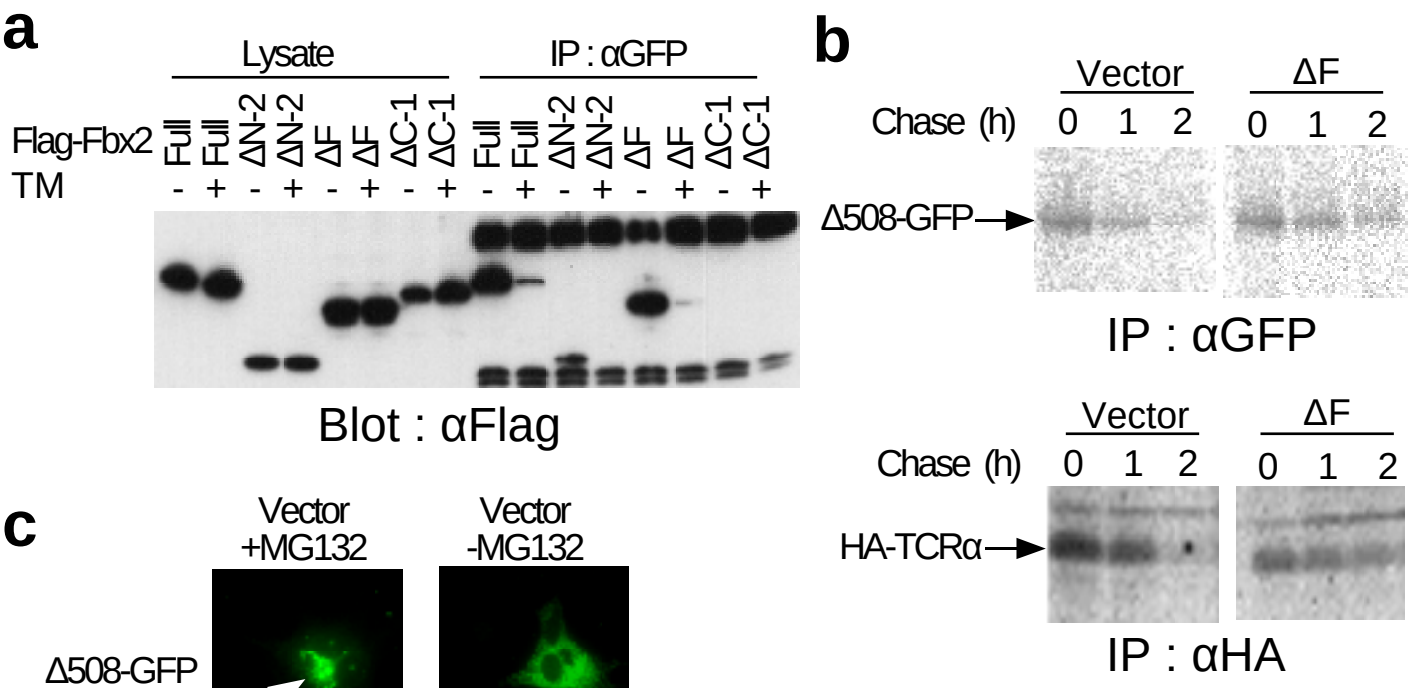


Figure 3 Tai et al.



		Rate of Aggresome Formation (%)		
Full	+ MG132	27.4	±11.1	(n=4)
ΔN-2	+ MG132	66.2	± 6.7	(n=3)
ΔC-1	+ MG132	58.0		(n=2)
ΔF	+ MG132	59.8	± 5.4	(n=5)

Figure 4 Tai et al.

Supplementary Information

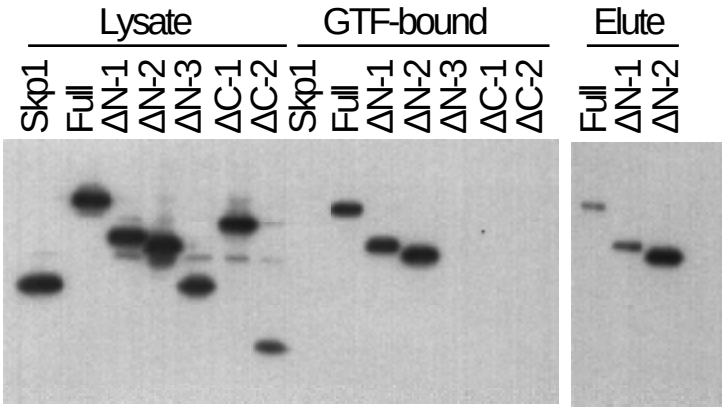


Figure 1. Interaction between GTF and various mutants of Fbx2. Schematic representation of Fbx2 and its deletion mutants are shown in Figure 1b. 293T cells were transfected with Flag-tagged Skp1 or various Flag-tagged Fbx2 mutants. The 0.5% NP40-solubilised whole cell extracts (WCE) were incubated with GTF-immobilised beads. The beads were washed and then halves of the beads were boiled with sample buffer (GTF-bound) and the other halves were eluted with *N, N*'-diacetylchitobiose (Elute). Lysates (15 μg), bound proteins, and eluates were analysed by immunoblotting with an anti-Flag antibody.

Supplementary Information

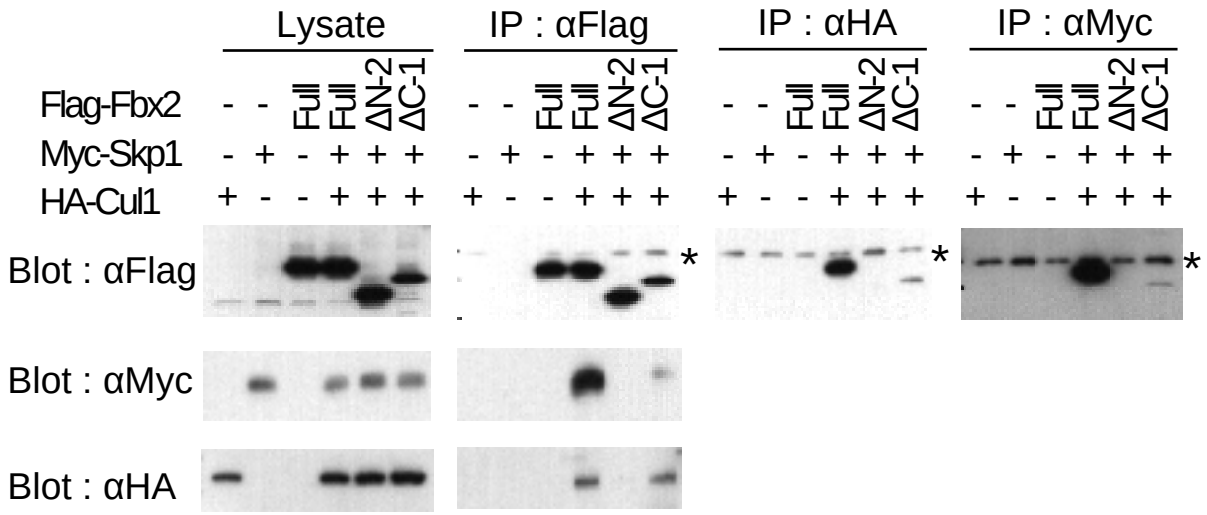


Figure 2. Formation of the SCF complex. 293T cells were co-transfected with the indicated combinations of pTracer-EF-Flag-fbx2 (Full, ΔN-2, ΔC-1), pcDNA3.1(+)Myc-skp1, and pcDNA3.1(+)HA-cul1 (4 μg each). 46 h after transfection, cells were treated with 50 mM MG132 for 90 min. Fbx2, Cul1, and Skp1 were immunoprecipitated with the indicated antibodies. These proteins in WCE (10 μg each) and the immunoprecipitates were analysed by immunoblotting using the indicated antibodies. Asterisks indicate the heavy chain of IgG.

Supplementary Information

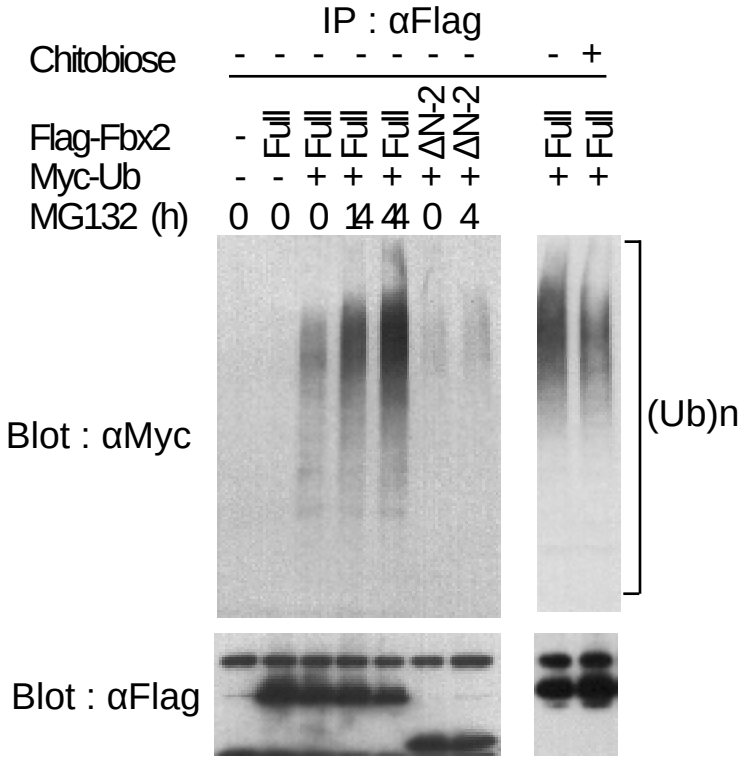


Figure 3. Association of ubiquitylated cellular proteins with Fbx2 after treatment with MG132. Neuro2a cells were co-transfected with pTracer-EF-Flag-fbx2 (Full, ΔN-2) and pcDNA3.1(+)Myc-ubiquitin (5 μg each). Cells were treated with 50 mM MG132 for the indicated time periods before harvesting at 48 h after transfection. Ubiquitylated proteins [(Ub)n] in the immunoprecipitates in the absence or presence of 0.1 M *N, N*'-diacetylchitobiose were analysed by immunoblotting using anti-Myc (top) and anti-Flag (bottom) antibodies.