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Supplementary Information for

Loss of peptide:*N*-glycanase causes proteasome dysfunction mediated by a sugar-recognizing ubiquitin ligase.

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Other supplementary materials for this manuscript include the following:

Supplementary Information Text
Supplementary Materials and Methods

Mice
C57BL/6J mice were obtained from Charles River Laboratories Japan. All animal care procedures and experiments conformed to the Association for Assessment and Accreditation of Laboratory Animal Care guidelines and were approved by the Experimental Animal Care and Use Committee of Takeda Pharmaceutical Company Limited and Tokyo Metropolitan Institute of Medical Science. All mice were housed in individual cages in a room with controlled temperature (23 °C), humidity (55%), and lighting (lights on from 7:00 am to 7:00 pm), and were fed with a normal chow diet (CE-2; CLEA Japan) with free access to water. Mice were sacrificed by exsanguination. Organs were eviscerated, and some were weighed.

Cell lines
HeLa cells were authenticated by the JCRB cell bank (KBN0573); HCT116 cells were obtained from Dr. Richard Youle (NIH); HEK293T (CRL-3216) cells were obtained from ATCC. HeLa, and HEK293T cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin/glutamine, and HCT116 cells were cultured in McCoy's 5A supplemented with 10% FBS, 2 mM GlutaMAX (Thermo Fisher Scientific) and nonessential amino acids. Cells were cultured at 37 °C in a 5% CO_2 incubator.

Generation of *Fbs2*-KO mice
Genomic DNA containing the mouse *MAD2L2-Fbs1* gene (Fig. S1*A*) was isolated from a BAC (ATCC clone No. RP23-139J21), and a targeting vector was constructed by replacing a part of exon 1 and exons 2–4 of the *Fbs2* gene with a *loxP* site and MC1-*neo* resistance gene. Another *loxP* site was also inserted into 3' of the *Fbs1* gene. The vector was transfected into ES cells (TT2) (1), and G418-resistant colonies were selected. A homologous recombinant ES clone containing the appropriately targeted allele was microinjected into 8-cell embryos of ICR mice. The resultant chimeric mice were backcrossed with C57BL/6 mice more than 12 times. Genotyping of *Fbs2*$^{-/-}$ mice was performed by PCR with the following primer pairs: *Fbs2* KO forward primer, 5'-GAACAAGATGGATTGCACGC-3' in NeoR; reverse primer, 5'-AGCTGTACCCGGAGTTGATAGGTGCA-3' in *Fbs2* exon 5. *Fbs2* WT forward primer, 5'-AGCTGTGGCACAAGCTTTCC-3' in *Fbs2* exon 3; reverse primer, 5'-CCGAAAGGTGTCCATCAGC-3' in *Fbs2* exon 4.

mRNA expression analysis in mice
Total RNA derived from C57BL embryos was purchased from GenoStaff (Japan). Reverse transcription was carried out with the SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific) using Oligo (dT) primer. Expression levels of mouse *Ngly1*, *Fbxo6*, and *Actb* mRNA were measured by quantitative PCR (qPCR) analysis with TaqMan Gene Expression Assays (Mm00451878_m1, Mm00489422_m1, and Mm00607939_s1) (Thermo Fisher Scientific). *Actb* mRNA was used as an endogenous normalization control.

Phenotypic analyses of *Fbs2*$^{-/-}$; *Ngly1*$^{-/-}$ mice
Hindlimb clasping tests: Mice were individually lifted by grasping their tails near the base, and their hindlimb positions were observed for 10 sec. If both hindlimbs were partially or entirely retracted toward the abdomen for more than 5 sec, we defined them as having an abnormal hindlimb clasping reflex.
Rotarod test: motor performance and coordination behaviors were tested using an accelerating rotarod (MK610; Muromachi Kikai) at different ages. Each mouse underwent two days of training sessions and a one-day test session. Each session consisted of four separates 5-min trials with a 15-min interval between each trial. The speed of the rotarod was accelerated from 4.5 to 45 rpm over the course of 4 min and maintained at 45 rpm for another 1 min. The time taken for the mice to fall from the rod was measured. If the mice stayed on the rod until the end of the 5-min trial, a time of 300 sec was recorded.

Histological analysis: For light microscopy analysis, tissues from mice at 25 weeks of age were dissected and fixed in 10% neutral-buffered formalin. Brains were trimmed coronally at 4 levels (Level 2, 3, 5, and 6) based on the STP position paper(2). The brains were paraffin-embedded, sectioned at 4–6 μm thickness, and mounted on slides. Sections were stained with H&E.

DNA constructs

Plasmids expressing FLAG-tagged CUL1, FBS2, and FBS2 Y241A/W242A (YW/AA) were described previously (3, 4). Mutations including FBS2 L16A/P17A (LP/AA) were generated by site-directed mutagenesis using the QuikChange Lightning Kit (Agilent Technologies) using primers 5'-GACAGCATTAACGAGGCGGCCGAGAACATCCTGC-3' and 5'-GCAGGATGTTCTCGGCCGCCTCGTTAATGCTGTC-3'. Plasmid expressing C-terminally HA-tagged Ngly1 was subcloned from the mouse PNG1 (Ngly1) clone (5) using the primer pair PNG-Eco-ATG (AGCAGAGAATTCCGCGGCGCAGCCATGGCGTCGGCCACACTG) and PNG-Xho-3 (TGGGTACTCGAGGTCATTGAACGTTATAATTATTTCC) into pcDNA3/C-terminal HA vector using the *Eco*RI and *Xho*I sites. Human NRF1 cDNA was synthesized from total RNA extracted from PANC-1 cells (Yoshida et al., 2017) (primers: Forward, 5'-TTCTGGTCCTTCAGCAATGCTTTC-3'; Reverse: 5'-CGGTCTTGGTGGGCTTCAAAC-3', and the amplified fragments were re-amplified using internal primer sets that added restriction enzyme sites (NRF1-Eco-ATG: 5'-TCTGGTGAATTCGCAATGCTTTCTCTGAAGAAATAC-3', and NRF1-Not-3: 5'-CTTCCCCGCGGCCGCTTTCTCCGGTCC-3'). The fragment was subcloned into the *Eco*RI/*Not*I sites of the pcDNA3/C-terminal HA vector. NRF18N/D-HA was constructed by inserting synthetic DNA encoding NRF1 (from 326M to 373F, replacing 329N, 348N, 360N, 400N, 405N, 412N, 423N, and 427N with D) into the *Nco*I/*Apo*I sites of NRF1 cDNA.

For the Fluoppi assay, *FBS2* (WT, YW/AA, and LP/AA) and *NRF1* genes were amplified (primers: FBS2-AG-Eco, 5'-AGCAGAGAATTCGGCCATGGATGCTCCCCACTCCAAAGC-3'; FBS2-AG-HIII, 5'-TGATCCAAGCTTGAAAATCTGGACAACAGCTCGG-3'; NRF1-Ash-Eco, 5'-AGCAGAGAATTCGGCAATGCTTTCTCTGAAGAAATAC-3'; NRF1-Ash-HIII, 5'-TGATCCAAGCTTCTTTCTCCGGTCCTTTGGCTTCC-3') and subcloned into the *Eco*RI/*Hin*dIII sites of phAG-MCL and pAsh-MNL (MBL International).

Retrovirus plasmids pMXs-puro-FLAG-FBS2, pMXs-puro-FLAG-FBS2 (YW/AA), pMXs-puro-FLAG-FBS2 (LP/AA), pMXs-puro-Ngly1-HA, pMXs-puro-NRF1-HA, and pMXs-puro-NRF1 8N/D-HA were generated by cloning amplified fragments (primers: pMXs-pcDNA-fw, 5'-GGTGGTACGGGAATTAGACCCAAGCTTGGTACCGAGCTC-3'; pMXs-pcDNA-rv, 5'-CGCTCAGGCTGGAATTGGCAACTAGAAGGCACAGTCG-3') from FLAG-FBS2, FLAG-FBS2 (YW/AA), FLAG-FBS2 (LP/AA), FLAG-FBS1 (Yoshida et al., 2002), FLAG-FBS3, FBS3-FLAG (Yoshida et al., 2017), Ngly1-HA, NRF1-HA, and NRF1 8N/D-HA into the *Eco*RI site of retroviral vector pMXs-puro (Cell Biolabs) using the In-Fusion HD cloning kit (Clontech). pMXs-puro-NRF1 9N/D-HA was generated by replacing Asn574 of pMXs-puro-NRF1 8N/D-HA with Asp using the QuikChange Lightning Kit (Agilent Technologies) with primers 5'-AGCACGTGGGCCACGACCACACATACAAC-3' and 5'-GTTGTATGTGTGGTCGTGGCCCACGTGCT-3'. pMXs-puro-GFP was EGFP fragment amplified from pEGFP-C1 (Clontech) using primers pMXs-GFP-fw (5'-GGTGGTACGGGAATTGCCACCATGGTGAGCAAGGGCGAGGAGCTG-3') and pMXs-GFP-rv: (5'-CGCTCAGCTGGAATTAAAACCTCTACAAATGTGGTATGGCTG-3') and cloned into *Eco*RI site of pMXs-puro. Ub-G76V-GFP (Addgene plasmid #11941) fragment amplified with primers pMXs-GFP-fw (5'-GGTGGTACGGGAATTCGTCAGATCCGCTAGCGCTACCGGACTC-3') and pMXs-GFP-rv (5'-CGCTCAGCTGGAATTAAAACCTCTACAAATGTGGTATGGCTG-3') was subcloned into the *Eco*RI site of retroviral vector pMXs-Neo (Cell Biolabs) using In-Fusion HD to yield pMXs-Neo-UbG76V-GFP. All constructs were confirmed by sequencing.

Establishment of *NGLY1*-KO, *FBS2;NGLY1*-dKO, *NRF1*-KO, *NGLY;NRF1*-dKO, and *NGLY1*-KO;*NRF1*-low cell lines

To generate knockout cell lines, CRISPR target sites were designed using CHOPCHOP (https://chopchop.cbu.uib.no/) and cloned into a pX330-U6-Chimeric_BB-Cbh-hSpCas9 (Addgene plasmid #42230). The following single guide RNA (sgRNA) sequences were used:

FBS2 exon 6, 5'-AACAGCTCGGTAGGGCGATTGGG-3'; NGLY1 exon 6, 5'-CGCTATGTTTGGATTACACAGG-3'; NGLY1 exon 11, 5'-GTATATTTGGCCCGAAAGGAAGG-3'; NRF1 exon 6, 5'-GTATGCGCTCCAGTACGCCGGGG-3'; and NRF1 exon 2, 5'-TTTCTCGCACCCCGTTGTCTGGG-3'. To generate *FBS2;NGLY1*-dKO HCT116 cells, cells were co-transfected with gRNA plasmids (for NGLY1 exon 11 and FBS2 exon 6) and pMXs-puro using FuGENE 6 (Promega). To generate *NGLY1*-KO HeLa cells, cells were co-transfected with an NGLY1-gRNA plasmid (exon 6 or 11) and pMXs-puro using Lipofectamine 2000 (Thermo Fisher Scientific). *NRF1*-KO and *NGLY1;NRF1*-dKO HeLa cells were generated by co-transfection with NRF1-gRNA plasmids (for exons 2 and 6) and pMXs-puro with or without NGLY1-gRNA plasmid (exon 11), using Lipofectamine 2000. After 30 h of transfection, cells were cultured for 3 days in medium supplemented with 5 μg/mL puromycin. Each puromycin-resistant cell was plated into 96-well plates for single-colony isolation. Genomic DNA was isolated from single colonies. PCR amplification of the target site was carried out using the following primer pairs, and the PCR products were cloned using the TOPO TA Cloning kit for sequencing (Thermo Fisher Scientific). NGLY1 (exon 6), 5'-CCCTGAGAAACTTTTGGAAACA-3'and 5'-GCTCAGAATGTAAACCCAAACC-3'; NGLY1 (exon 11), 5'-TAAAAGATGCCATGGGAAAAAT-3' and 5'- ACTGACCCACACTCAAACTTCC-3'; FBS2 (exon 6), 5'-AGTCACCAACAGCAGCATTG-3' and 5'-GTTACCACAAGCTGGTAGGGTT-3'; NRF1 (exon 2), 5'-GACCTGGACAATTACTTCACTGC-3' and 5'- GGTTAAGTCTCCACTGACTGGG-3'; and NRF1 (exon 6). 5'-AGATGAAGCAGAAGGTCCAGAG-3' and 5'- TTTAAGTCCCCGCTGTAGGTC-3'. Gene knockout clones were confirmed by immunoblotting. For the *NRF1*-KO construct, the NRF1-gRNA for exon 2 spans an exon–intron junction sequence, and we were able to obtain KO cells more efficiently than for exon 6. We also used mixed gRNA plasmids and cells containing frame-shift mutations in exon 6, and the cells obtained expressed low levels of NRF1 (*NRF1*-low cells). Because the expression of NRF1 seems to be critical for cell growth in HeLa cells, these *NGLY1*-KO;*NRF1*-low cells were also used for further analyses.

Transfections and generation of stable cell lines

For transient transfection of plasmids, Lipofectamine 2000 (Thermo Fisher Scientific) was used for HeLa cells and FuGENE6 transfection reagent (Promega) was used for HCT116 cells. Stable cell lines were established by recombinant retrovirus infection as follows. Virus particles were produced in HEK293T cells by co-transfection with a retrovirus plasmid (expressing in pMXs-puro vector), Gag-Pol (Addgene plasmid #14887), and VSV-G (Addgene plasmid #8454) by Lipofectamine LTX with Plus reagent (Thermo Fisher). Twenty hours after transfection, the medium was changed to fresh medium and cells were further cultured for 24 h. The filtrated viral supernatants were then used to infect HeLa cells or HCT116 cells with 8 μg/mL Polybrene (Sigma). Stable transfectants were selected in medium containing 1 μg/mL puromycin. For monitoring proteasome activity, stable cells expressing UbG76-GFP were selected in medium containing 500 μg/mL G418 after infection of virus generated by co-transfection using pMXs-neo-UbG76-GFP. The selected clones expressing UbG76-GFP were expanded and used for further experiments.

Immunoprecipitation, ConA-pulldown, immunoblotting, and lectin blotting

Harvested cells were washed with PBS, solubilized with TBS-N buffer (10 mM Tris-HCl (pH7.5), 150 mM NaCl, 0.5% Nonidet P-40) containing a protease inhibitor mixture (Roche) on ice, and cell lysates were cleared by centrifugation at 20,000 x *g* for 20 min at 4˚C. The heads and torsos of murine embryos were suspended in 400 μl of TBS-N buffer containing a protease inhibitor mixture, and homogenized on ice using a BioMasher II (Nippi). The homogenate was centrifuged twice at 20,000 × *g* for 20 min at 4°C. For immunoprecipitation, supernatants (whole cell lysates; WCLs) were immunoprecipitated with anti-FLAG antibody–conjugated agarose (anti-DDDDK, FLA-2 clone; MBL International) for 1.5 h at 4°C. The beads were subsequently washed five times with 1-mL portions of TBS-N, and immune complexes were eluted by heating in SDS/PAGE sample buffer. For ConA-pulldown, 30 μl of ConA-agarose (FujiFilm) was mixed with WCL (equivalent of 800 μg of protein), in which glycosylated NRF1 in *NGLY1*-KO cells was not detected in the flow-through fraction. ConA-agarose was reacted with WCL for 2 h at 4°C and

washed five times with TBS-N, and glycoproteins were recovered from the resin by heating in SDS/PAGE sample buffer.

Appropriate amounts of immunoprecipitates, ConA-bound glycoproteins, and WCL were applied and separated on 5–20% SDS-PAGE. After transfer, PVDF membranes were blocked with 5% skim milk/PBS, and incubated with primary antibodies. The following antibodies were used for western blotting: mouse anti-FLAG (1:2000 DDDDK-tag mAb-HRP DirecT; MBL International), rabbit anti-NRF1 (1:1000, D5B10; Cell Signaling Technology), rabbit anti-NGLY1 (1:200; Sigma-Aldrich), rabbit anti-Cullin1 (1:1000; Thermo Fisher), rabbit anti-Skp1(1:1000, Abcam), rabbit anti–human FBS2 (1:1000, ABclonal), mouse anti-actin (1:1000, C4; Millipore), rabbit anti-LDH (1:5000; Abcam), rabbit anti-calnexin (1:2000; Abcam), rabbit anti-SP1(1:1000, D4C3; Cell Signaling Technology), rabbit anti–Histone H3 (1:2000, D11H2; Cell Signaling Technology), mouse anti-ubiquitin (1:500, P4D1; Santa Cruz Biotechnology), rabbit anti-ubiquitin, Lys48-specific (1:1000, clone Apu2; Sigma-Aldrich), rabbit anti-ubiquitin, linkage-specific K63 (1:1000, Abcam), mouse anti-GFP (1:500, clone7.1 and 13.1, Merck), rabbit anti–c-Jun (1:1000, 60A8, Cell Signaling Technology), rabbit anti-p53 (1:1000, 7F5; Cell Signaling Technology), rabbit anti-p27 (1:1000, D69C12; Cell Signaling Technology), and mouse anti-HA (1:1000, clone 16B12; BioLegend). Proteins were detected with peroxidase-linked secondary antibodies (1:10000; Jackson Immuno Research). For the ConA lectin blots, PVDF membranes were blocked with 3% bovine serum albumin (BSA)/PBS for 2 h, and incubated with 1:1000 ConA-HRP (J-Oil Mills) in 1% BSA/PBS. Western Lightning Plus-ECL (Perkin Elmer) was used to develop the blots, and all images were acquired using a LAS 4000 biomolecular imager (GE Healthcare). ImageQuant TL version 8.1(GE Healthcare) was used for quantification of the images.

Immunofluorescence and confocal image analysis

In the Fluoppi Assay, 1×10^5 HeLa cells were cultured on glass-bottomed 35-mm dishes for 24 h, and then transfected with plasmids encoding hAG-FBS proteins or FBS2 YW/AA mutant in combination with NRF1-Ash. Transfections were performed using Lipofectamine 2000 (Thermo Fisher Scientific). After transfection for 24 h, cells were fixed with 4% paraformaldehyde (PFA) in PBS and stained with DAPI.

For immunostaining of NRF1-HA, 2×10^5 stable HeLa cells were cultured on glass-bottomed 35-mm dishes for 24 h, and then treated with 20 nM Btz for 5 h. The cells were fixed with 4% PFA in PBS for 15 min and permeabilized with 0.2% Triton X-100 in PBS for 15 min. After blocking with 0.1% gelatin (Difco), the fixed cells were incubated with mouse anti-HA (1:1000, clone 16B12; BioLegend) and then with goat anti–mouse IgG Alexa Fluor 488 (1:200 dilution; Life Technologies). For quantification of cells with nuclear translocation of NRF1 and their biological replicates, over 110 cells were counted in each of three replicate dishes (Figures 2F and S4D). Samples were mounted using Slow Fade Gold (Thermo Fisher Scientific) and observed with a laser-scanning confocal microscope (LSM710; Carl Zeiss) with a Plan-Apochromat 63X NA 1.4 oil differential interference contrast objective lens. In the proteasome activity assay using UbG76V-GFP, samples were observed with Plan-Apochromat 10X NA 0.45 (for HeLa cells) and 20X NA 0.8 (for HCT116 cells) differential interference contrast objective lens. The ZEN microscope software (Carl Zeiss) and Photoshop CC (Adobe) were used for image analyses.

Quantitative RT-PCR

Cells were harvested 16 h after 20 nM bortezomib treatment. Total RNA was isolated using TRIzol Reagent (Thermo Fisher Scientific) and reverse-transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Quantitative RT-PCR was performed with LightCycler 480 SYBR Green I Master (Nippon Genetics) on a LightCycler 480 (Roche). Primer sequences were as follows: *PSMA1*, 5'-TGTATTCGATAGACCACTGCC-3' and 5'-GCAATAAGGAGACCAACACCATA-3'; *PSMB1*, 5'-ACCAGCTCGGTTTCCACA-3' and 5'-CCCGGTATCGGTAACACATC-3'; *PSMC4*, 5'-GGTGCAGGAGGAATACATCAAA-3' and 5'-CTCCAGAAATTGTCCGATGACC-3'; *GAPDH*, 5'-AGCCACATCGCTCAGACAC-3' and 5'-GCCCAATACGACCAAATCC-3'.

Proteasome peptidase activity measurement

Because *NGLY1*-KO cells stably expressing FBS2 began to die 7–8 days after retrovirus infection, we measured proteasome activity 6 days after infection. Harvested cells were washed with PBS, solubilized with 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, 5 mM ATP, and 1 mM DTT by brief sonication on ice, and cell lysates were cleared by centrifugation at 20,000 × *g* for 20 min at 4°C. Peptidase activity of the 26S proteasome in cell lysates was measured as described previously (6). The peptidase activity assay was set up in a 50-μl reaction (384-well format) containing 0.5 μg lysate protein, 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 50 μM Suc-LLVY-MCA. Fluorescence of AMC was measured in an EnSpire Multimode Plate Reader (PerkinElmer) with an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Fluorescence was read every 30 sec for a total duration of 1 h, and the rate of hydrolysis was calculated based on the slope of fluorescence over time between 20 and 50 min (linear phase of the reaction).

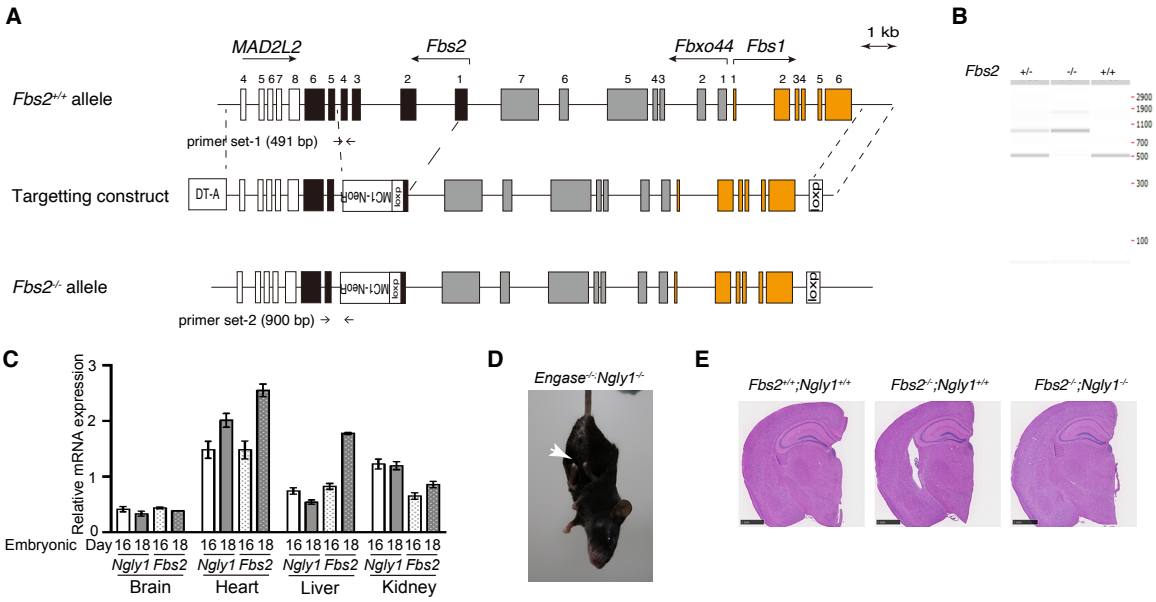

Fig. S1. *Fbs2;Ngly1* dKO mice are viable and have normal motor function. (Related to Fig. 1)

(*A*) Diagram of the targeting construct used to generate *Fbs2*-KO mice. In the *Fbs1*$^{-/-}$ allele, a region spanning from part of exon 1 to exon 4 of the *Fbs2* gene was replaced with *loxP* and a MC1-neo resistance gene. Another *loxP* site was also inserted into the 3' of the *Fbs1* gene. Primer sites for genotyping are shown. Primer set-1: wild-type (*Fbs2*$^{+/+}$) allele; primer set-2: *Fbs2*$^{-/-}$ allele. (*B*) Representative results from genotyping of Fbs2$^{+/-}$, $^{-/-}$, and $^{+/+}$ mice. Wild-type and *Fbs2*$^{-/-}$ alleles could be distinguished by PCR and electrophoresis. (*C*) Relative mRNA expression of Ngly1 and Fbs2 in wild-type mice at E16.5 (16) and E18.5 (18) was quantified by qPCR. mRNA levels of target genes were normalized against the corresponding levels of *Actb* mRNA. Error bars show means ± SD of three replicates. (*D*) Hindlimb clasping of 79-wk-old *Engase;Ngly1* dKO mouse. White arrow indicates clasped hindlimbs. (*D*) H&E-stained sections of the brain from female *Fbs2;Ngly1* dKO mice at 25 wk of age. Black scale bar, 1 mm.

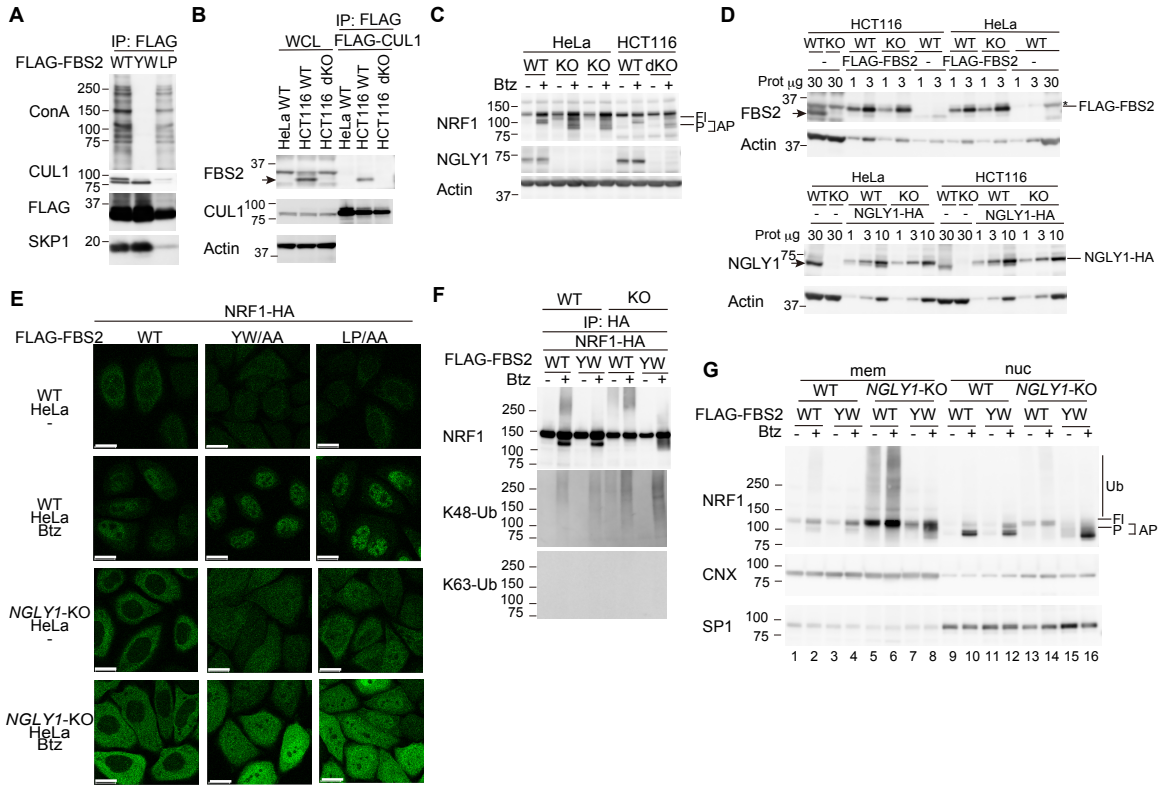

Fig. S2. Ubiquitinated NRF1 accumulates in the cytosol in FBS2-expressing *NGLY1*-KO cells. (Related to Fig. 2)

(*A*) Glycoprotein-binding activity and SCF complex formation in FBS2 mutants. Lysates prepared from HeLa cells transiently expressing a FLAG-FBS2 (WT) or its mutants (YW/AA and LP/AA) were immunoprecipitated with FLAG antibody, and the immunoprecipitates were analyzed by concanavalin A (ConA) lectin blot or immunoblots. The results are summarized in Fig. 2*A*. (*B*) FBS2 was expressed in HCT116 cells. Whole-cell lysates (WCLs) and immunoprecipitates prepared from wild-type HeLa, HCT116, and *NGLY1;FBS2* double KO (dKO) HCT116 cells transiently expressing FLAG-CUL1 were analyzed by immunoblotting. Arrow indicates the position of FBS2. (*C*) Differences in NRF1 processing between wild-type (WT) and *NGLY1*-KO (KO) cells in the presence of proteasome inhibitor. Cells were treated with or without 20 nM bortezomib (Btz) for 5 h before harvest. WCLs prepared from WT, *NGLY1*-KO (KO), and *NGLY1;FBS2* double-KO (dKO) cells were analyzed by immunoblotting. Fl, unprocessed full-length NRF1; P, processed NRF1 detected in WT cells; AP, abnormally processed NRF1. (*D*) Overexpression of FBS2 and NGLY1 expressed from pMXs-puro retrovirus vector in HCT116 and HeLa cells. The position of endogenous FBS2 or NGLY1 is shown by arrow. Asterisk indicates a non-specific band that is cross-reactive with FBS2 antibody. FBS2 was overexpressed ~20-fold relative to its endogenous level in HCT116. On the other hand, NGLY1 was overexpressed by ~3 fold and ~6-fold in HeLa and HCT116, respectively. (*E*) Immunofluorescence micrographs of WT and *NGLY1*-KO HeLa cells overexpressing NRF1-HA in combination with FLAG-FBS2 (WT or mutants) with or without 20 nM bortezomib (Btz) for 5 h. Cells were subjected to immunostaining with anti-HA antibody. Scale bars, 20 μm. Quantifications are shown in Fig. 2*C*. (*F*) K48-linked ubiquitination of NRF1 by SCF^{FBS2}. HeLa cells overexpressing NRF1-HA in combination with FLAG-FBS2 (WT or YW/AA) were treated with or without 20 nM Btz for 5 h, and then immunoprecipitated with anti-HA antibody. Immunoprecipitates were analyzed by immunoblotting.

(*G*) Subcellular distribution of endogenous NRF1 in WT and *NGLY1*-KO cells overexpressing FBS2 or its lectin mutant (YW). Fl, unprocessed full-length NRF1; P, processed NRF1; AP, abnormally processed NRF1; vertical line (Ub), ubiquitinated NRF1; mem, membrane fraction; nuc, nuclear soluble fraction. CNX, calnexin (ER marker); SP1 (nuclear soluble protein marker).

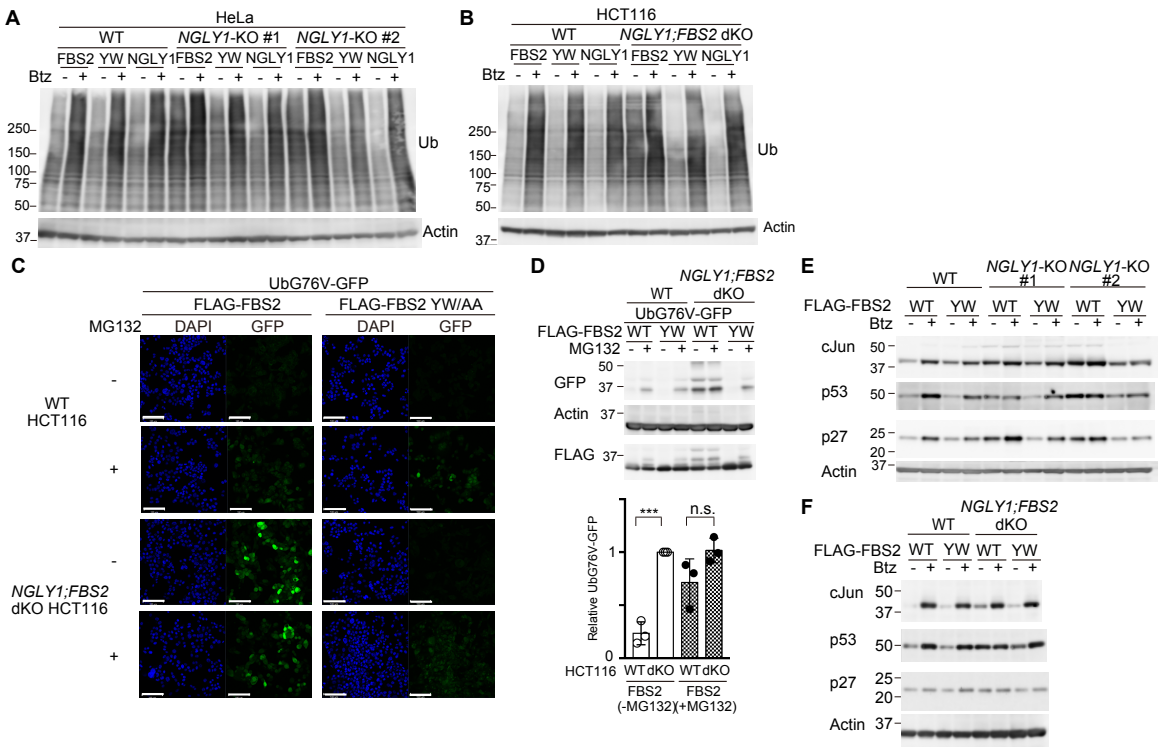

Fig.S3. FBS2 overexpression inhibits proteasome activity in *NGLY1*-KO cells. (Related to Fig. 3)

(*A* and *B*) Accumulation of ubiquitinated proteins in *NGLY1*-KO cells overexpressing FBS2. In A, wild-type (WT) and *NGLY1*-KO HeLa cells (#1, exon 6 mutant; #2, exon 11 mutant) B, WT and *NGLY1*;*FBS2*-dKO HCT116 cells were used. Cells overexpressing FBS2, YW/AA mutant, or NGLY1 were treated with or without 20 nM bortezomib (Btz) for 5 h prior to harvest. Cell lysates were analyzed by immunoblotting with anti-ubiquitin (Ub) antibody. Quantifications are shown in Fig. 3*A*. (*C*) Low-magnification fluorescence micrographs of WT and *NGLY1*;*FBS2*-dKO HCT116 cells overexpressing both UbG76V-GFP and FBS2 (WT or YW/AA mutant) cultured for 5 h in the absence or presence of 50 μM MG132. Scale bars, 100 μm. (*D*) Detection of UbG76V-GFP accumulation by immunoblotting. *(upper)* Cell lysates in (*C*) were analyzed by immunoblotting with anti-GFP antibody. *(lower)* Relative levels of UbG76V-GFP in FBS2-expressing WT and *NGLY1;FBS2*-dKO (dKO) cells were quantified; the intensity of the band from FBS2-overexpressing *NGLY1*;*FBS2*-dKO cells without MG132 treatment was defined as 1. Error bars show means ± SD of three biological replicates. Statistical significance was determined using two-tailed Student's *t*-tests. ***P<0.0002; n.s., not significant. (*E* and F) Accumulation of short-lived proteins without *N*-glycans in *NGLY1*-KO (*E*, HeLa, *F*, HCT116) cells overexpressing FLAG-FBS2 or FLAG-FBS2YW/AA mutant.

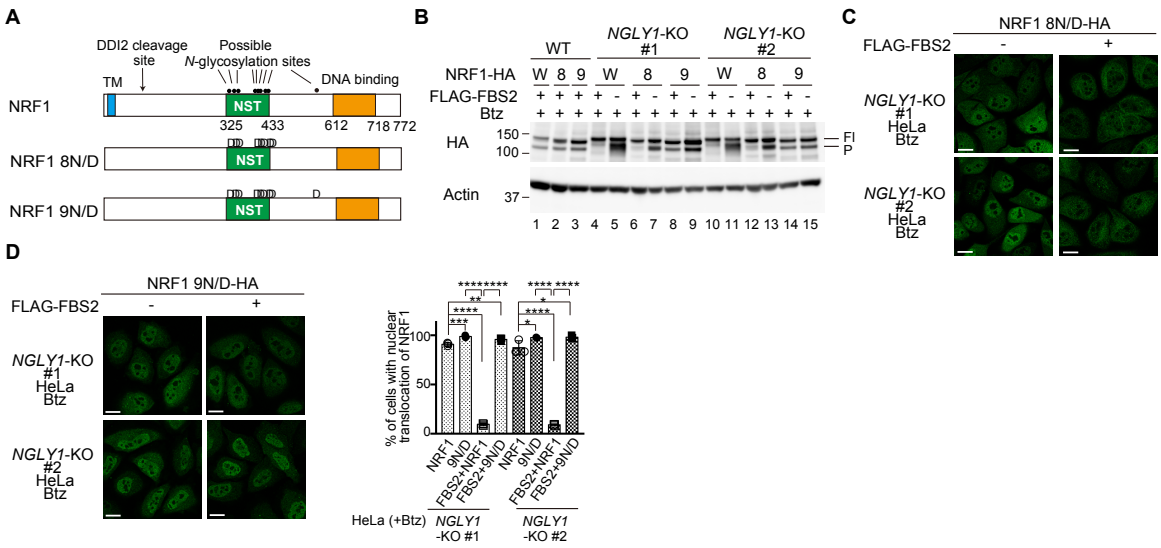

Fig. S4. *N*-Glycosylation occurs at all nine potential sites in NRF1. (Related to Fig. 4) (*A*) Schematic representation of NRF1 and its 8N/D and 9N/D mutants. TM, transmembrane domain; NST, Asn/Ser/Thr-rich region. (*B*) Effect of FBS2 overexpression on processing of wild-type NRF1 (W), NRF1 8N/D (8), or NRF1 9N/D (9) in *NGLY1*-KO cells. Wild-type or *NGLY1*-KO HeLa cells stably expressing NRF1, NRF1 8N/D-HA, or NRF1 9N/D-HA with or without FLAG-FBS2 were treated with or without 20 nM Btz for 5 h. Cell lysates were analyzed by immunoblotting. Fl, unprocessed full-length NRF1-HA; P, processed NRF1-HA. (*C*) Immunofluorescence microscopy of *NGLY1*-KO HeLa cells overexpressing NRF1 8N/D-HA with FBS2 in the presence of 20 nM bortezomib (Btz) for 5 h. Cells were subjected to immunostaining with anti-HA antibody. Scale bars, 20 μm. (*D*) (left) Immunofluorescence microscopy of *NGLY1*-KO HeLa cells overexpressing NRF1 9N/D-HA with FBS2 in the presence of 20 nM bortezomib (Btz) for 5 h. Cells were subjected to immunostaining with anti-HA antibody. Scale bars, 20 μm. Graphs at right show quantification of immunofluorescence analysis of *NGLY1*-KO HeLa cells (#1, exon 6 mutant; #2, exon 11 mutant) overexpressing NRF1-HA or NRF1 N/D-HA with or without FLAG-FBS2 in the presence of 20 nM bortezomib (Btz) for 5 h. Error bars show means ± SD of three biological replicates. Statistical significance was determined by one-way ANOVA with Tukey's post-test. ****P<0.0001; ***P<0.0002; **P<0.002; *P<0.003.

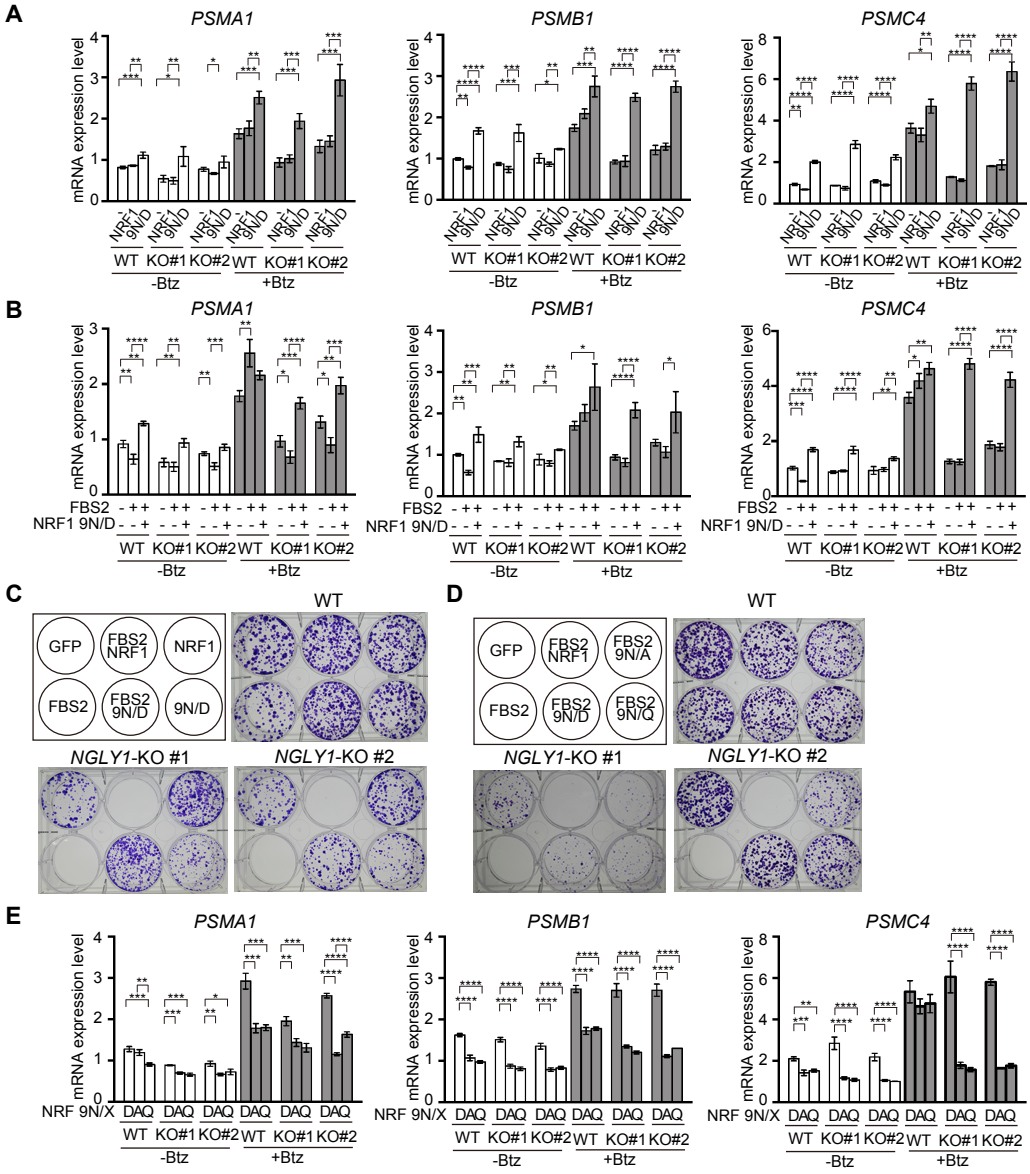

Fig. S5. Cytotoxicity caused by FBS2 in *NGLY1*-KO cells is restored by "glycan-less" NRF1 mutants. (Related to Fig. 4)
(*A*, *B*, and *E*) Relative mRNA expression of the proteasome genes *PSMA1*, *PSMB1*, and *PSMC4* in wild-type and *NGLY1*-KO HeLa cells overexpressing the proteins indicated. Cells were treated with 20 nM Btz for 16 h. mRNA levels of target genes were normalized against the corresponding levels of *GAPDH* mRNA. Error bars show means ± SD of three biological replicates. Statistical significance was determined by one-way ANOVA with Tukey's post-test ****P<0.0001; ***P<0.0002; **P<0.002; *P<0.03. (*C* and *D*) Colony formation assays in wild-type (WT) and *NGLY1*-KO HeLa cells (#1, exon 6 mutant; #2, exon11 mutant) overexpressing the indicated proteins. WT and *NGLY1*-KO HeLa cells were infected with recombinant retrovirus for expression of the indicated proteins produced using pMXs-puro. After infection for 36 h, 500 cells were plated in a 6-well dish in the presence of 1 μg/ml puromycin. After culture for 11–14 days, the colonies that developed were stained with crystal violet and counted. Counts are shown in Figs. 4*B* and 4*C*.

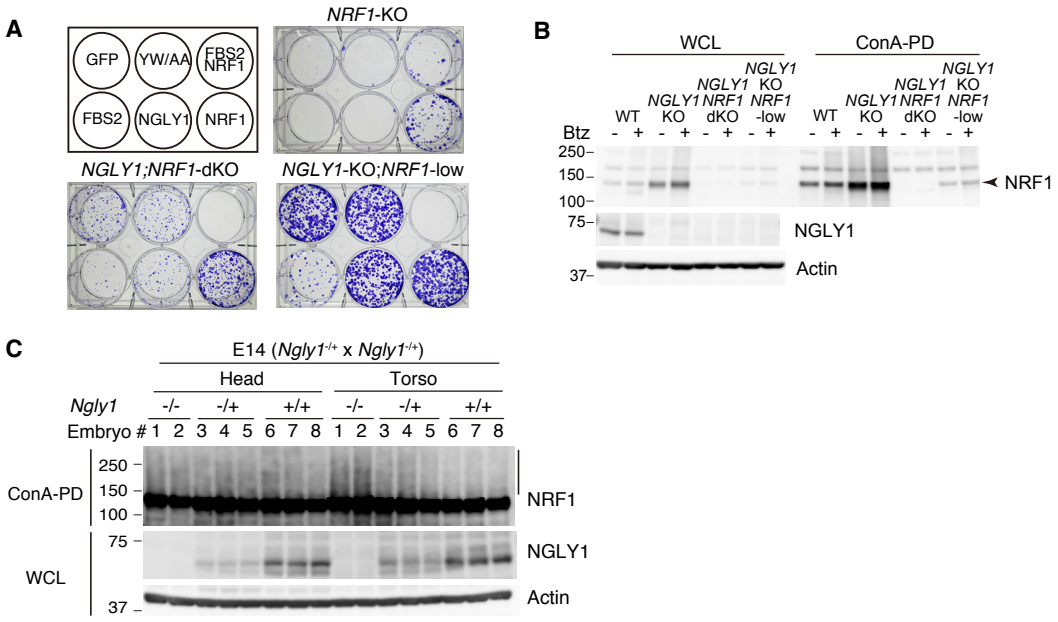

Fig. S6. NRF1 is primarily responsible for the induction of cytotoxicity in *NGLY1*-KO cells. (Related to Fig. 5)
(*A*) Colony formation assays in *NRF1*-KO, *NGLY1*;*NRF1*-dKO, and *NGLY1*-KO;*NRF1*-low HeLa cells overexpressing the indicated proteins. After infection, *NRF1*-KO, *NGLY1*;*NRF1*-dKO, and *NGLY1*-KO;*NRF1*-low HeLa cells were plated in 6-well dishes at 1000 cells/well, respectively. In *NRF1*-KO cells, only NRF1-restored cells formed colonies. Quantifications for *NGLY1*;*NRF1*-dKO and *NGLY1*-KO;*NRF1*-low HeLa cells are shown in Fig. 5*A*. (*B*) Expression level of NRF1 in WT, NGLY1-KO, *NGLY1*;*NRF1*-dKO, and *NGLY1*-KO;*NRF1*-low HeLa cells. (*C*) Ubiquitination of NRF1 in *Ngly1*-KO day 14 embryos. ConA-bound glycoproteins from whole-cell lysates (WCLs) were subjected to immunoblotting with anti-NRF1 antibody. Vertical line, ubiquitinated NRF1. In lanes 1 and 2, *Ngly1*$^{-/-}$ embryos are shown.

Table S1. Genotypes of pups obtained by crossing of *Fbs2*$^{-/+}$; *Ngly1*$^{-/+}$ mice, counted at P1 and P28. Related to Fig. 1*D*

Genotype	Number of pups (%)					
	P1			P28		(Expected%)
Fbs2$^{+/+}$; *Ngly1*$^{+/+}$	15	(6.5)		12	(6.2)	(6.3)
Fbs2$^{+/+}$; *Ngly1*$^{-/+}$	34	(14.8)		33	(17.0)	(12.5)
Fbs2$^{+/+}$; *Ngly1*$^{-/-}$	2	(0.9)	**	0	(0.0)	** (6.3)
Fbs2$^{-/+}$; *Ngly1*$^{+/+}$	37	(16.1)		34	(17.5)	* (12.5)
Fbs2$^{-/+}$; *Ngly1*$^{-/+}$	76	(33.0)	*	70	(36.1)	** (25.0)
Fbs2$^{-/+}$; *Ngly1*$^{-/-}$	10	(4.3)	**	2	(1.0)	** (12.5)
Fbs2$^{-/-}$; *Ngly1*$^{+/+}$	15	(6.5)		13	(6.7)	(6.3)
Fbs2$^{-/-}$; *Ngly1*$^{-/+}$	30	(13.0)		26	(13.4)	(12.5)
Fbs2$^{-/-}$; *Ngly1*$^{-/-}$	11	(4.8)		4	(2.1)	* (6.3)

Significance of the difference vs. expected value was evaluated by Chi-squared test.

*P<0.05, **P<0.01

P: Postnatal day

Movie S1 (separate file). Behavior of a 22-month-old *Fbs2*$^{-/-}$;*Ngly1*$^{-/-}$-dKO mouse (right)

Movie S1 (separate file). A *Fbs2;Ngly1*-dKO mouse (23-month-old male) exhibited no sign of abnormal hindlimb clasping.

SI References

1. T. Yagi *et al.*, A novel ES cell line, TT2, with high germline-differentiating potency. *Anal Biochem* **214**, 70-76 (1993).
2. B. Bolon *et al.*, STP position paper: Recommended practices for sampling and processing the nervous system (brain, spinal cord, nerve, and eye) during nonclinical general toxicity studies. *Toxicol Pathol* **41**, 1028-1048 (2013).
3. Y. Yoshida *et al.*, Ubiquitination of exposed glycoproteins by SCF(FBXO27) directs damaged lysosomes for autophagy. *Proc Natl Acad Sci U S A* **114**, 8574-8579 (2017).
4. Y. Yoshida *et al.*, E3 ubiquitin ligase that recognizes sugar chains. *Nature* **418**, 438-442 (2002).
5. T. Suzuki, H. Park, N. M. Hollingsworth, R. Sternglanz, W. J. Lennarz, PNG1, a yeast gene encoding a highly conserved peptide:N-glycanase. *J Cell Biol* **149**, 1039-1052 (2000).
6. Z. Sha, A. L. Goldberg, Proteasome-mediated processing of Nrf1 is essential for coordinate induction of all proteasome subunits and p97. *Curr Biol* **24**, 1573-1583 (2014).