Supporting Information

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SI Materials and Methods

Factor VIII Activity and Antigen Analysis. FVIII activity was measured by (*i*) a one-stage activated partial thromboplastin time clotting assay using an MLA Electra 750 fibrinometer (Medical Laboratory Automation) by reconstitution of human FVIII–deficient plasma, or (ii) a two-stage chromogenic assay using the COAMATIC assay kit according to the manufacturer's instructions. The FVIII plasma standard was FACT (normal human pooled plasma) from George King Biomedical for the one-stage activated partial thromboplastin time clotting assay. The calibration standard included with the COAMATIC chromogenic assay was assayed according to the Fourth International WHO standard. FVIII antigen was quantified by an anti-human FVIII light-chain sandwich ELISA using commercial kits from Affinity Biologicals.

Analysis of Oxidative Markers

Lipid peroxidation. Lipids were extracted from liver tissue using a modified Dole procedure (1). HODEs were quantified by reverse-phase hydrolysis C-18 HPLC analysis of triphenylphosphine-reduced lipid extracts after base hydrolysis. The protein content of tissue pellets was determined by modified Bradford assay using BSA as standard.

Protein Carbonyls. The quantity of protein carbonyls in liver extracts were determined by derivatizing with dinitrophenylhydrazine (DNP) and measuring bound DNP by ELISA using biotinylated anti-DNP antibody followed by streptavidin-linked horseradish peroxidase. Absorbances were related to a standard curve prepared for serum albumin containing increasing proportions of hypochlorous acid-oxidized protein that had been calibrated colorimetrically.

Glutathione. Reduced GSH and total glutathione (GSH plus oxidized glutathione) contents were measured by an enzymatic assay using glutathione reductase and 5,5′-dithiobis(2-nitrobenzoic acid). Cell pellets were homogenized in 200 μl of 50 mM phosphate buffer (pH 7.4). The homogenates were centrifuged at 6,000 $\times$ *g* for 15 min. The glutathione assays were performed using glutathione assay kit from Cayman Chemicals (2).

Malondialdehyde. Lipid peroxidation was measured using an assay kit (Calbiochem) that detects malondialdehyde generated by peroxidation of polyunsaturated fatty acids and related esters. Malondialdehyde levels were quantified as an index of lipid peroxidation according to the supplier's instructions (3).

Analysis of Liver Injury and Oxidative Stress. Livers were fixed in 4% formaldehyde, dehydrated, embedded in paraffin, and sectioned. For immunostaining, liver sections were embedded in optimal cutting temperature compound (Sakura Finetek) and then cryosectioned at 10-μM thickness. TUNEL staining was performed using *in situ* cell death detection kit and fluorescein from Roche Diagnostics. Apoptotic cells were visualized under an Olympus IX81disc spinning unit fluorescence microscope.

Fluorescence Analysis of ROS Production. CHO cells (clone H9) engineered for NaB-inducible FVIII expression were propagated as previously described (4). Cells were plated on 35-mm dishes, treated with 5 mM NaB for 24 h, and then loaded with CM-H_2DCFDA (Molecular Probes). After 30 min, cells were collected and analyzed using a flow cytometer (FACS Caliber) and the CellQuest computer program (5).

Immunoblotting. Cultured cells or liver tissues were solubilized in 50 mM Tris·HCl (pH 7.4), 250 mM NaCl, 1% Triton-X 100, 250 μM Na orthovanadate, 1 mM PMSF, 1 μg/ml aprotinin, 10 μg/ml pepstatin and 10 μg/ml leupeptin. Lysates were centrifuged at 16,000 $\times$ *g* for 20 min. The protein concentrations in the supernatants were measured using the Bradford assay (Bio-Rad). Equal amounts of total protein were separated by SDS/PAGE and transferred to nitrocellulose membranes (Amersham) and probed with the antibodies CHOP (Santa Cruz Biotechnology), BiP (Stressgen), *p*-eIF2α (Biosource), or α-actin (Sigma). After incubation with secondary antibodies conjugated with horseradish peroxidase (Pierce) for 1 h at room temperature, the signals were detected using enhanced chemiluminescence (Pierce). Densitometric analysis was performed using Quantity One software from Bio-Rad. FVIII antigen levels in the lysates were measured by ELISA (Affinity Biologicals). Western blotting for FVIII was performed using anti-FVIII antibody (Green Mountain).

RNA Extraction and Quantitative Real-Time RT-PCR Analysis. Total RNA was extracted from liver tissue using TRIzol reagent (Invitrogen). RNA was reverse-transcribed into cDNA in a 20-μl reaction using iScript cDNA synthesis kit (Bio-Rad). RT reactions were incubated for 5 min at 25°C. An iQTM SYBR Green Supermix kit (Bio-Rad) was used for quantitative real-time PCR (20 μl) using the iCycler iQ real-time PCR detection system (Bio-Rad). The relative amounts of mRNA were calculated from the comparative threshold cycle (Ct) values relative to 18S rRNA. The fold induction of mRNA levels for each gene was then expressed relative to vector-injected mice. Real-time primer sequences were designed by Primer Express (Applied Biosystems) and are shown in Table S1.

Preparation of BHA Diet. Standard rodent chow (Formulab Diet 5008) was crushed into water and mixed with BHA in 100% ethanol (ETOH) to a final concentration of 0.7% (18). Small pieces were dried completely overnight to exclude ETOH. Mice were fed for 4 days with the BHA-supplemented chow or standard chow before tail vein injection of DNA.

Statistical Analysis. All data are presented as mean ± SD. Statistical significance of differences between groups was evaluated using Student *t* test or ANOVA one-way test (Tukey). (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

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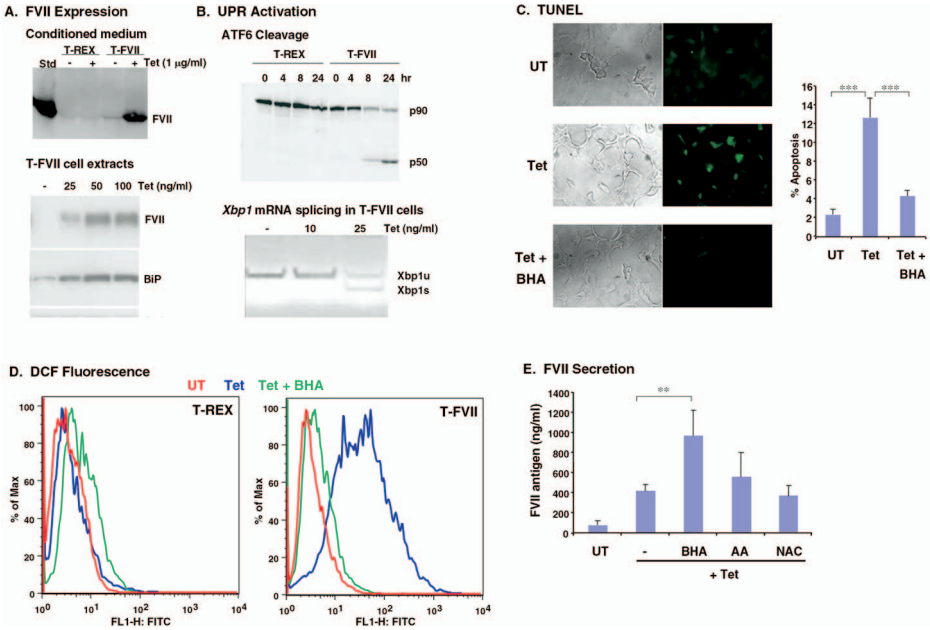

Fig. S1. Induction of FVII causes ROS in CHO cells. A. tetracycline (Tet) induces FVII expression in T-FVII cells. CHO cells that express the tetracycline *trans*-activator (T-REX) were stably transfected with a tetracycline-responsive plasmid for expression of FVII to obtain T-FVII cells. Cells were treated with the indicated concentrations of tetracycline. After 24 h, conditioned media was harvested and cell lysates were prepared for Western blot analysis of FVII, BiP, and α-actin. FVII was not detected in control cells (T-REX) stably transfected with empty vector. B. FVII expression induces ATF6α cleavage and *Xbp1* mRNA splicing. *Top*, T-REX and T-FVII cells were treated for increasing periods of time with 1 μg/ml tetracycline. Cell extracts were prepared for Western blot analysis of ATF6α cleavage. The intact (p90) and cleaved (p50) forms of ATF6α are indicated. Tetracycline-induced FVII expression causes ATF6α cleavage after 8 h. *Bottom*, T-FVII cells were treated with 0, 10, or 25 ng/ml tetracycline. After 24 h, RNA was prepared for analysis of *Xbp1* mRNA splicing by RT-PCR and gel electrophoresis. Note that 10 ng/ml tetracycline is insufficient to elicit *Xbp1* mRNA splicing, whereas 25 ng/ml tetracycline induces *Xbp1* splicing. (*C*) Tetracycline induction of FVII causes apoptosis. T-FVII cells were untreated (UT) or treated with 1 μg/ml tetracycline in the absence (Tet) or presence (Tet + BHA) of 10 μM butylated hydroxyanisole (BHA). Apoptosis was analyzed using TUNEL assay as described in *Methods*. TUNEL-positive cells from three independent experiments were analyzed for quantification. (*D*) Control T-REX cells and T-FVII cells were stained with dichlorofluorescein at 24 h after induction under the indicated conditions (tetracycline 1 μg/ml, BHA 10 μM). Tetracycline-induced FVII expression causes oxidative stress that is prevented by BHA. E. T-FVII cells were treated with tetracycline (1 μg/ml) in the presence of BHA (10 μM), ascorbic acid (AA; 500 μM), or N-acetylcysteine (NAC; 500 μM) for 24 h and samples of conditioned medium harvested for analysis of FVII by ELISA. We gratefully thank Drs. Jens Jacob Hansen, D. Thomas Rutkowski, and Kazutoshi Mori for analysis of FVII and UPR induction in the T-FVII cells (*A* and *B*).

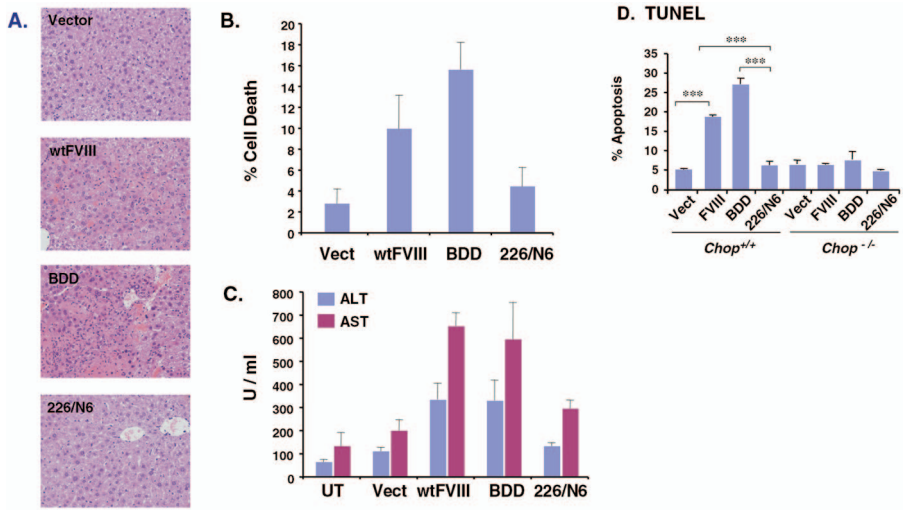

Fig. S2. wtFVIII and BDD expression in liver induce cell death. (*A*) Hematoxylin and eosin staining of liver sections from mice at 24 h after injection with empty vector, wtFVIII, BDD, and 226/N6. (*B*) Quantitation of cell death from four different animals at 24 h after injection with empty vector, wtFVIII, BDD, and 226/N6. Lighter areas represent in cell death were measured by analysis of 5 fields per mouse. Data represent mean ± SD. (*C*) Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) assays were performed to assess the liver damage at 24 h after DNA injection into the tail vein. The serum levels of ALT and AST were measured using an autoanalyzer (Idexx Laboratories). Values between 28 and 132 (ALT) and between 59 and 247 (AST) are considered within the normal range. (*D*) Sections from four different mice were analyzed by TUNEL as in Fig. 3*A* and quantified.

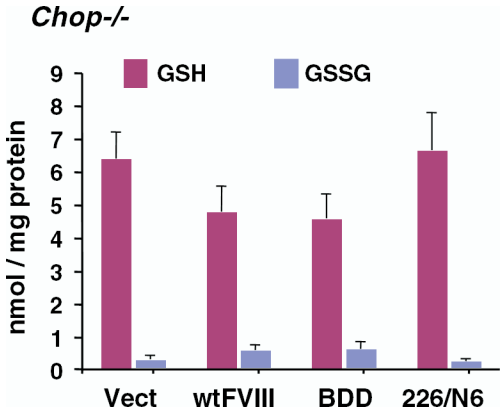

Fig. S3. Analysis of GSH levels in *Chop*−/− mice after DNA injection. *Chop*−/− mice were injected with the indicated plasmid DNAs and liver tissues were harvested after 24 h for analysis of GSH and GSH + GSSG (oxidized glutathione) levels as described in *Methods*. ($n = 3$).

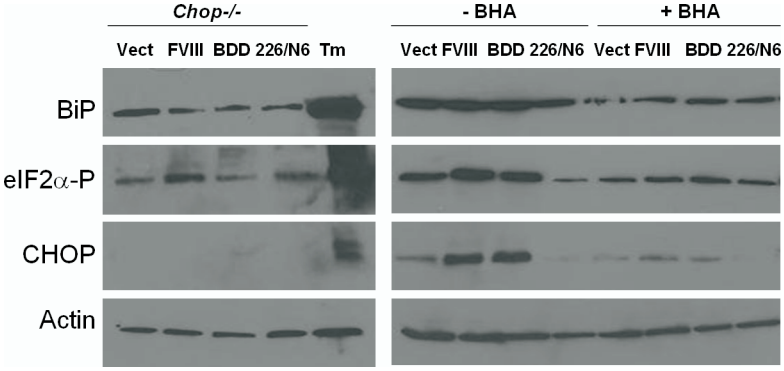

Fig. S4. WT were fed with normal chow or chow supplemented with BHA for 4 days and then DNA expression vectors were injected into the tail vein. WT and *Chop*−/− mice (6) were injected with the indicated plasmid DNAs. For positive control, WT mice were injected with tunicamycin 1 mg/kg body weight as described (7). After 24 h, plasma and liver samples were harvested. Western blot analysis of liver tissue was performed for detection of BiP, phospho-eIF2α, and CHOP.

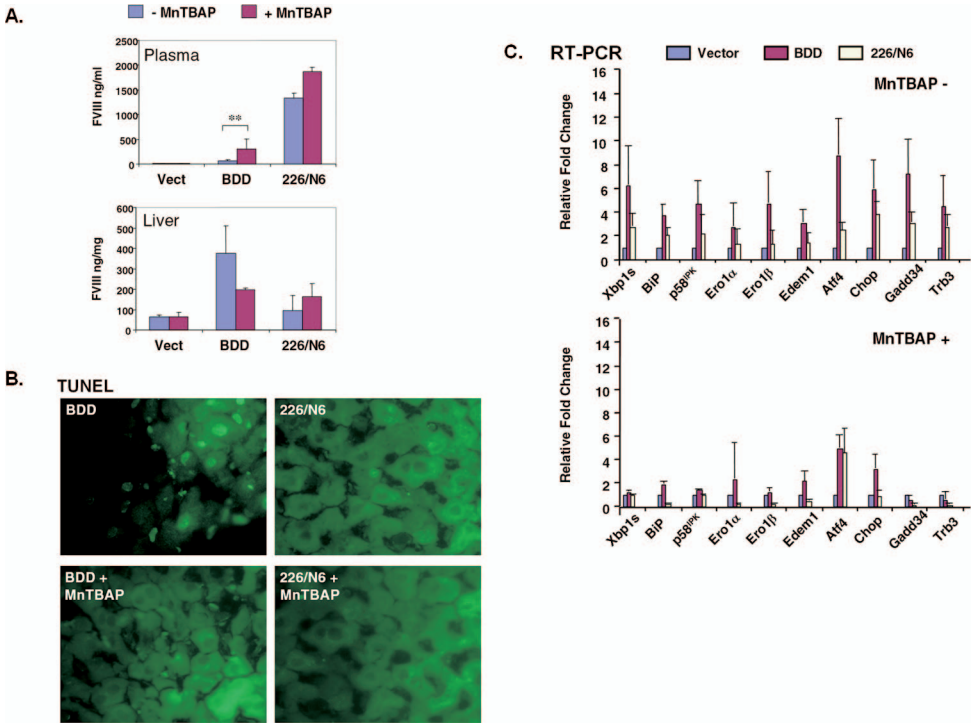

Fig. S5. MnTBAP improves secretion of BDD *in vivo*. C57BL/6 mice were treated with MnTBAP (10 mg kg^{-1}) by daily s.c. injection for 4 days (8). DNA vectors encoding BDD or 226/N6 and empty vector control were injected into the tail vein. After 24 h, plasma and liver samples were isolated. (*A*) FVIII antigen in liver extracts and plasma samples was measured by ELISA. (*B*) Liver samples were harvested for TUNEL analysis by fluorescence microscopy. (*C*) Real-time RT-PCR. Expression values were normalized to 18S rRNA and the fold induction is expressed relative to empty vector. Experiments were conducted in three independent mice.

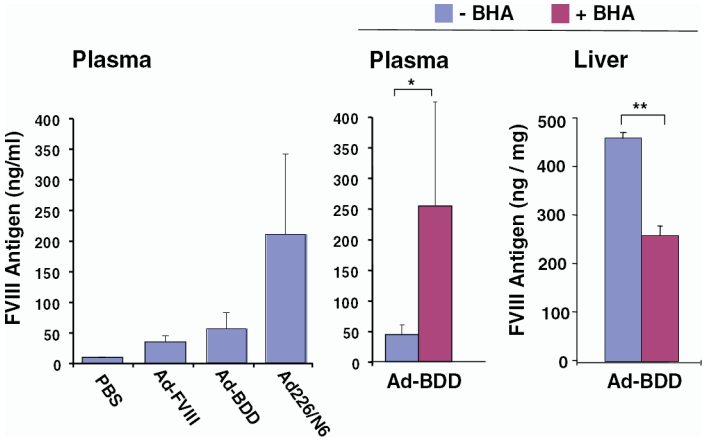

Fig. S6. BHA improves secretion of BDD delivered by adenovirus. Adenoviral constructs encoding wtFVIII, BDD, or 226/N6 (9) were injected into the tail vein (5 × 10e12 VP/kg). Adenovirus preparations were prepared and kindly provided by Brendan Lee (Houston, TX). For BHA feeding, mice were fed BHA-supplemented chow for 4 days before adenovirus delivery and were maintained on BHA-supplemented chow after adenovirus delivery. At 48 h after infection, plasma and liver samples were harvested for analysis of FVIII antigen.

Table S1. Real-time RT-PCR primer sequences

Gene name	5′ Oligonucleotide	3′ Oligonucleotide
18S rRNA	CGCTTCCTTACCTGGTTGAT	GAGCGACCAAAGGAACCATA
β-Actin	GATCTGGCACCACACACCTTCT	GGGGTGTTGAAGGTCTCAAA
Atf4	ATGGCCGGCTATGGATGAT	CGAAGTCAAACTCTTTCAGATCCATT
Bip/Grp78	GGTGCAGCAGGACATCAAGTT	CCCACCTCCAATATCAACTTGA
Chop	CTGCCTTTCACCTTGGAGAC	CGTTTCCTGGGGATGAGATA
Edem1	GCAATGAAGGAGAAGGAGACCC	TAGAAGGCGTGTAGGCAGATGG
Ero1α	GCATTGAAGAAGGTGAGCAA	ATCATGCTTGGTCCACTGAA
Ero1β	GGGCCAAGTCATTAAAGGAA	TTTATCGCACCCAACACAGT
Gadd34	CCCGAGATTCCTCTAAAAGC	CCAGACAGCAAGGAAATGG
Gpx1	ACAGTCCACCGTGTATGCCTTC	CTCTTCATTCTTGCCATTCTCCTG
Ho1	CCACACAGCACTATGTAAAGCGTC	GTTCGGGAAGGTAAAAAAAGCC
p58^{IPK}	TCCTGGTGGACCTGCAGTACG	CTGCGAGTAATTTCTTCCCC
Sod1	GGCCCGGCGGATGA	CGTCCTTTCCAGCAGTCACA
Sod2	GGGTTGGCTTGGTTTCAATAAGGAA	AGGTAGTAAGCGTGCTCCCACACAT
Trb3	TCTCCTCCGCAAGGAACCT	TCTCAACCAGGGATGCAAGAG
Ucp2	TACCAGAGCACTGTCGAAGCC	AGTCCCTTTCCAGAGGCCC
Xbp1 s	GAGTCCGCAGCAGGTG	GTGTCAGAGTCCATGGGA