**Supporting Information**

**Fig. S1.** Schematic representation of amino acid substitutions in human XBP1u to disrupt two NLS sequences.

**Fig. S2.** Supporting information for Figure 2.

(A) Preparation of PK-treated microsomes. CMM was treated with the indicated concentration of proteinase K (PK). The efficiency of surface protein digestion was evaluated by the removal of a short cytosolic tail of calnexin. The membrane topology of calnexin is shown on the right. The full-length and protected fragment of calnexin were detected using the antibody against the ER-luminal portion of calnexin. (B) XBP1u translation was not affected by PK treatment in the presence of a PK inhibitor. XBP1u was synthesized via the *in vitro* translation system with mock-treated CMM or PK-treated CMM with PMSF (2 mM) and benzamidine (2 mM). (C) Endogenous Sec61β and/or HA-tagged Sec61β were co-immunoprecipitated with FH-XBP1u and FH-XBP1u-tRNA using anti-FLAG antibodies. In contrast, FH-XBP1u and/or FH-XBP1u-tRNA were co-immunoprecipitated with Sec61β and/or Sec61β-HA from the cell lysate derived from HEK293T cells transiently expressing FH-XBP1u and/or Sec61β-HA. Anti-FLAG or anti-HA antibody was used for immunoprecipitation. (D) Co-immunoprecipitation of endogenous Sec61β with FH-XBP1u from the cell lysate derived from HEK293T cells transiently expressing FH-XBP1u. Anti-Sec61β antibody was used for immunoprecipitation.

**Fig. S3.** Supporting information for Fig. 5.

(A) EDTA and high-salt treatment of microsomes in HEK293T cells transiently expressing wild-type (WT) or 3L mutant (3L) FH-XBP1u were analyzed by western blotting. Calnexin (CNX) and GAPDH were used as control membrane and cytosolic proteins, respectively. (B) Kyte and Doolittle hydrophobicity plots for HR2 of wild-type and 3L mutant XBP1u (window size: 9) are visualized using a ProtoScale analysis implemented in ExPASy (http://web.expasy.org/protscale/). (C) Variants of FH-XBP1u transiently expressed in HeLa cells were co-stained with Sec61β. (D) FH-XBP1u variants translated with RRL were co-immunoprecipitated with SRP54. XBP1u was detected by autoradiography, whereas SRP54 was detected by immunoblotting. (E) Post-translational ER-targeting assay of FH-XBP1u[W256A] and FH-XBP1u[W256A/3L]. Total protein before fractionation is shown as the input. Floated and bottom fractions are shown. The presence and absence of the ER membrane
(EKRM) are indicated by “-” and “+”, respectively. See Materials and methods for additional information.

**Fig. S4.** Splicing efficiency and membrane-localization efficiency of XBP1u mRNA. (A) Relationship between splicing efficiency and membrane-localization efficiency of XBP1u mRNA according to Figure 2C and Figure 5D and E are shown. Bar: SD. (B) Primary structure of variants of XBP1u and C-terminal extended XBP1u, XBP1u+s. PS, HR2, and WA indicate Pausing Sequence, Hydrophobic Region 2, and W256A, respectively. (C) The membrane-localization efficiencies of XBP1u variants mRNA transiently expressed in HEK293T cells were quantified as described in Figure 2C. Bars, SD; *P < 0.05 (n=4) using analysis of variance (ANOVA).

**Table S1.** Identification of proteins that interact with XBP1u by tandem mass spectrometry. The results obtained from FLAG-tag and His-tag pulldown assays are shown in FLAG-tag and His-tag, respectively. Columns colored in yellow indicate the components of Sec61 or SRP.