Supporting Information 1 of

SARS-CoV-2 ORF8 protein induces endoplasmic reticulum stress-like responses and facilitates virus replication by triggering Calnexin: an unbiased study

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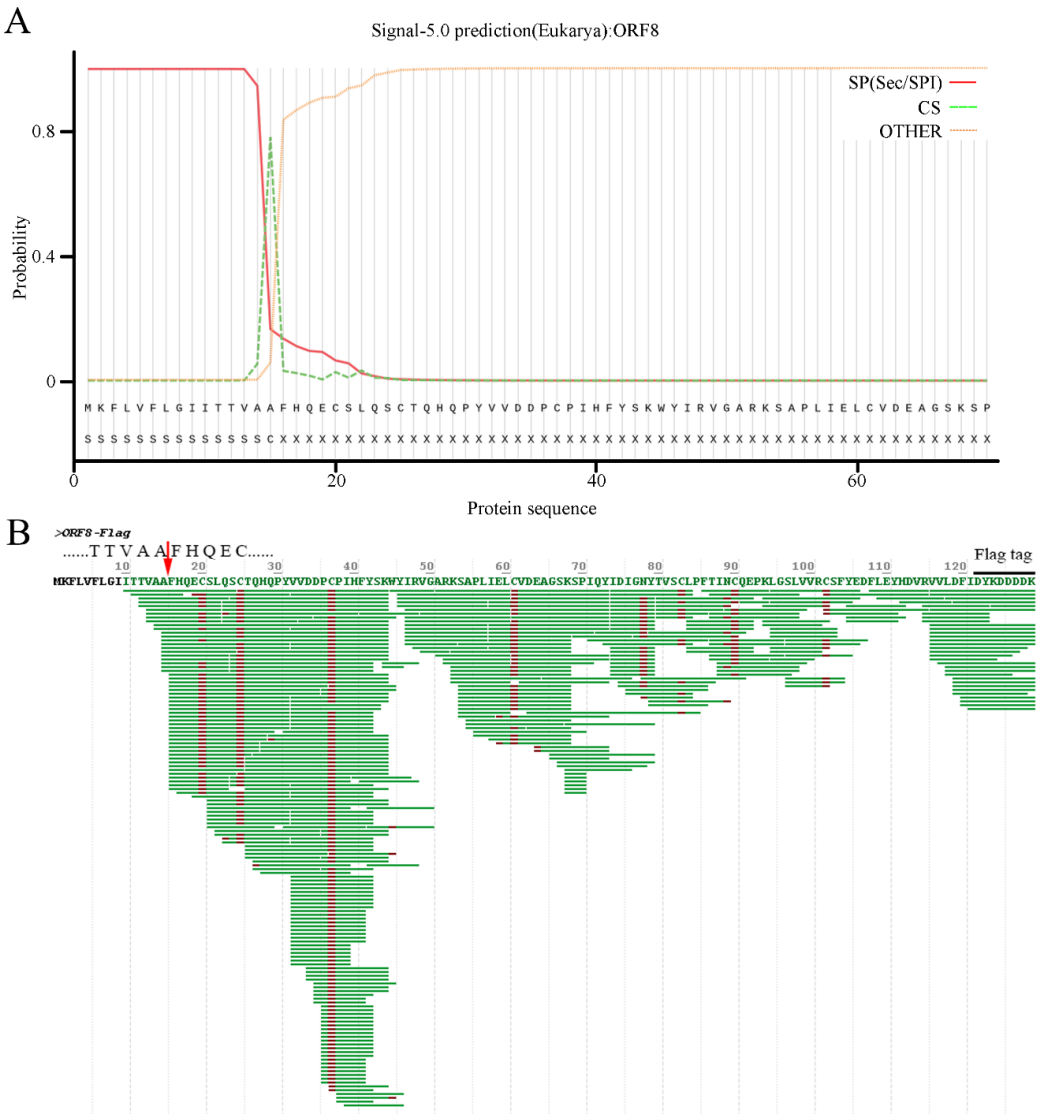

Fig S1. ORF8 signal peptide includes its N-terminal 1-15 amino acids. (A) Prediction of ORF8 signal peptide using Signal 5.0. (B) Identified peptides of ORF8-Flag by mass spectrometry. Immunoprecipitation products of ORF8-Flag (protein sequence and the C-terminal Flag as indicated) overexpressing 293T cells were analyzed by mass spectrometry. Each identified peptide of ORF8 is demonstrated by a green line segment with inside modifications (if exist) in red. The recognized signal peptidase cleavage site between the indicated amino acids (indicated with enlarged black texts) is demonstrated by a red arrow.

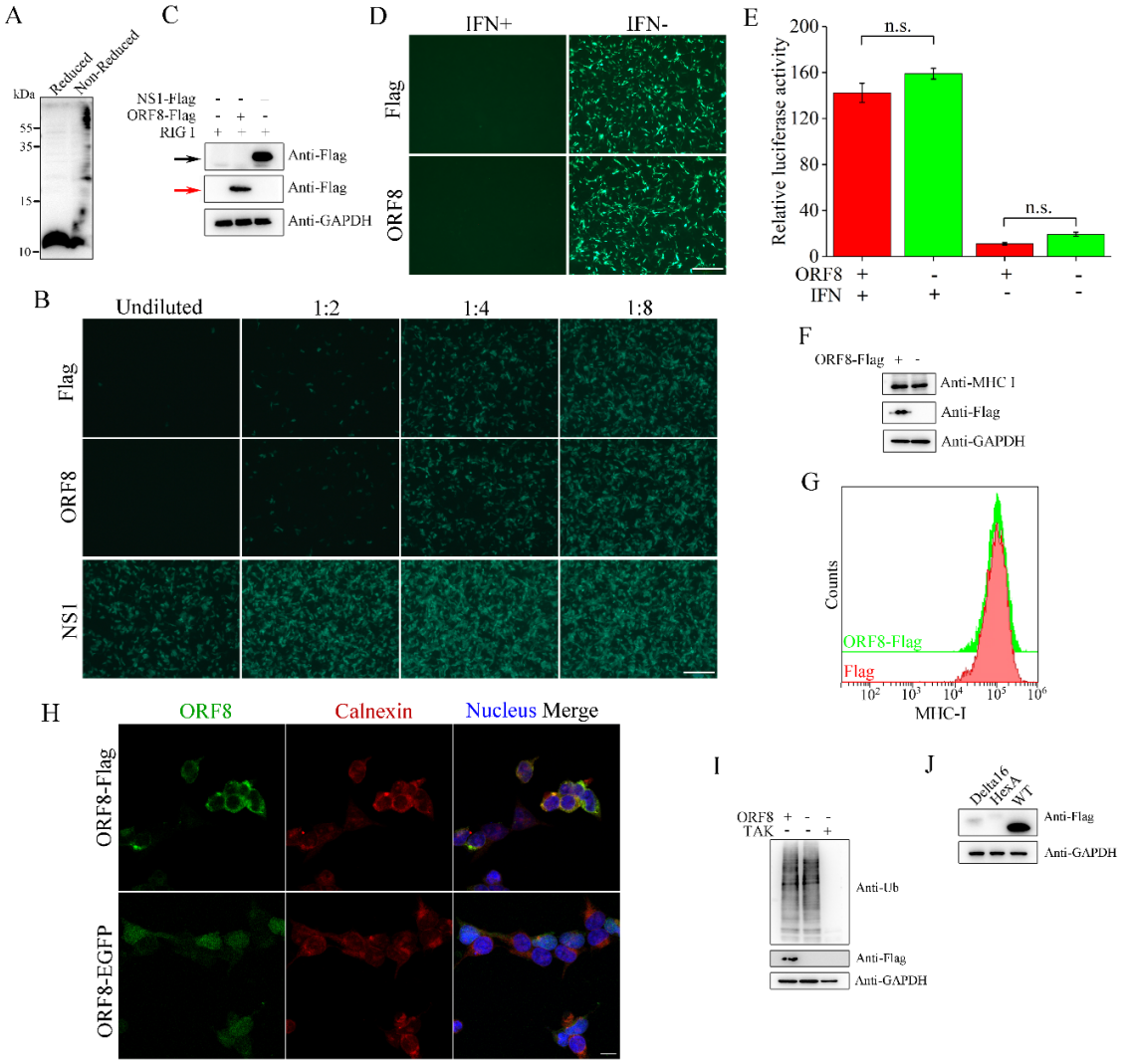

Fig S2. ORF8 fails to function as an immune antagonist. (A) ORF8 from mammalian cells fails to form a stable disulfide-linked homodimer. Lysates of ORF8-Flag overexpressing 293T cells were analyzed by anti-Flag WB after tricine gel electrophoresis in either reduced or non-reduced condition. (B-C) ORF8 fails to suppress IFN production, demonstrated by NDV-GFP assay (B) although both NS1 (in C, black arrow) and ORF8 (in C, red arrow) are well-expressed. Scale bars in (B) and (D): 300 μm. (D-E) ORF8 fails to suppress IFN signaling, demonstrated by NDV-GFP assays (D) and ISRE responses (E). (F-G) ORF8 fails to inhibit MHC-I expression. (F) ORF8 was overexpressed in 293T for 60 hours cells, and lysates were analyzed by WB. (G) Cells in (F) were analyzed by flow cytometry. (H) ORF8-EGFP fails to co-localize with Calnexin. ORF8-Flag and

ORF8-EGFP overexpressing 293T cells were analyzed by multi-immunofluorescence. Left row: ORF8 signals in green; middle row: Calnexin signals in green; right row: merge signals of left and middle with nucleus in blue. Scale bar: 15 μm. (I) ORF8 fails to inhibit ubiquitination. Lysates of ORF8 overexpressing or TAK treated 293T were analyzed by WB. (J) ORF8 mutants Delat16 and HexA were expressed in low levels. Lysates of ORF8 WT and mutant overexpressing 293T cells were analyzed by WB after tricine gel electrophoresis.

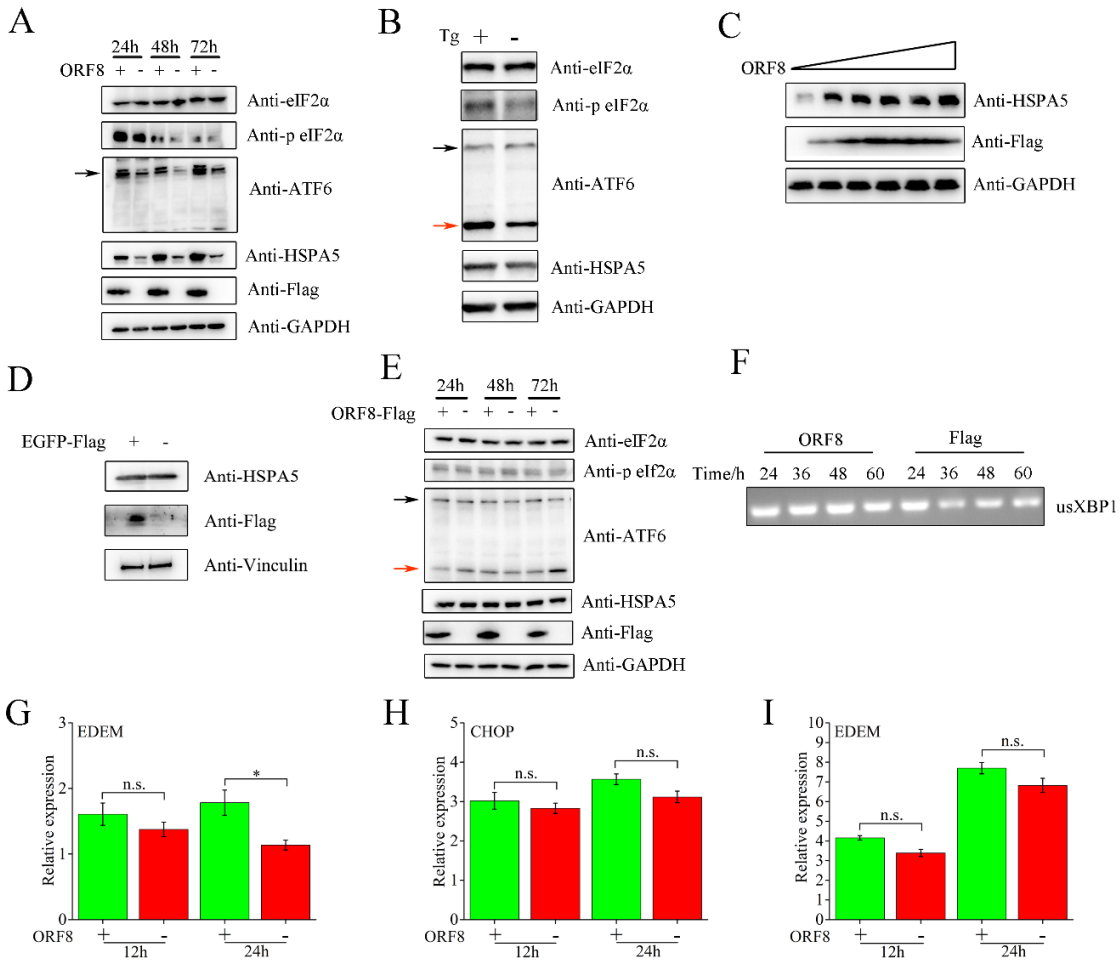

Fig S3. ORF8 leads to stress-like responses in human but not in mouse cells. (A) ORF8 induces HSPA5 upregulation via neither PERK nor ATF6 branch. 293T cells were transfected with ORF8 and lysates were obtained at the indicated time post transfection and analyzed by WB. In anti-ATF6

WB results (A), (B) and (D), full-length ATF6 is marked by black arrows and ATF6(N) is marked by red arrows. (B) Tg induces ER stress of three branches. As is in (A) but using Hela cells treated by Tg. (C) A tiny amount of ORF8 is able to induce stress-like response. 293T cells in 6-well plates were transfected with 0, 100, 200, 1000, 2500 and 5000 ng ORF8 (from left to right lanes), respectively, and lysates were obtained at 60 hours post transfection and analyzed by WB. (D) EGFP overexpression fails to induce HSPA5 upregulation in 293T cells. (E) ORF8 fails to activate PERK or ATF6 branch of ER stress in MEF cells. As is in (A) but using MEF cells. (F) ORF8 fails to activate IRE1 branch of ER stress in MEF cells. As is in Figure 5B but using MEF cells. (G-I) Influence of ORF8 overexpression in 293T cells (G), or MEF cells (H-I). As is in Figure 5D. Not significant: n.s..

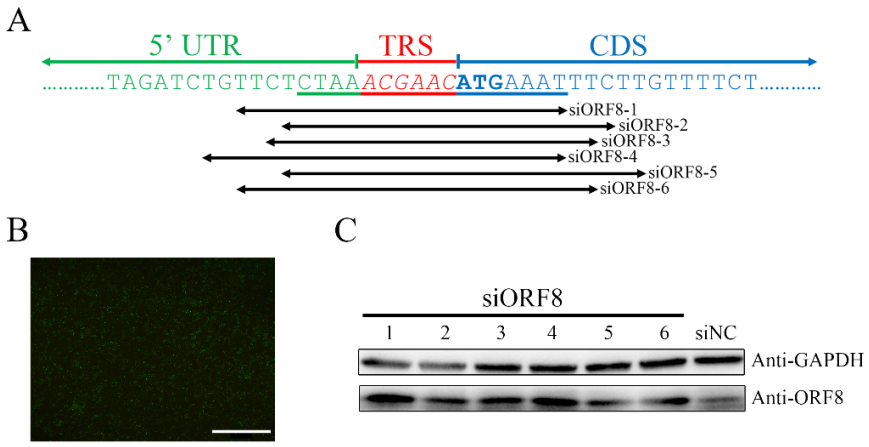

Fig S4. ORF8 siRNAs fail to knockdown ORF8. (A) Designs of 6 ORF8 specific siRNAs according to the sequence of ORF8 sgmRNA. (B) Successful transfection of siORF8. Scale bar: 400 μm. (C) ORF8 siRNAs fail to knockdown ORF8. Hela^{ACE2+} cells were transfected using each siORF8/siNC for 48 hours, and infected by SARS-CoV-2 at an MOI of 1. WB analyses were performed at 24 hpi.

Table S1. Details for ORF8-Calnexin docking

Name	Distance	Category	Types	From	From Chemistry	To	To Chemistry
A:LYS90:HZ1 - B:ASP107:OD2	2.26938	Hydrogen Bond; Electrostatic	Salt Bridge; Attractive Charge	A: LYS90:HZ1	H-Donor; Positive	B:ASP107:OD2	H-Acceptor; Negative
A:ARG221:NH2 - B:GLU19:OE2	4.93241	Electrostatic	Attractive Charge	A: ARG221:NH2	Positive	B:GLU19:OE2	Negative
A:GLY195:H - B:ILE39:O	2.89138	Hydrogen Bond	Conventional Hydrogen Bond	A: GLY195:H	H-Donor	B:ILE39:O	H-Acceptor
A:HIS201:HE2 - B:GLU110:OE1	2.25439	Hydrogen Bond	Conventional Hydrogen Bond	A:HIS201:HE 2	H-Donor	B:GLU110:OE1	H-Acceptor
B:HIS40:HE2 - A:GLU159:OE2	2.94436	Hydrogen Bond	Conventional Hydrogen Bond	B:HIS40:HE2	H-Donor	A:GLU159:OE2	H-Acceptor
B:HIS40:HE1 - A:GLU159:OE2	3.09197	Hydrogen Bond	Carbon Hydrogen Bond	B:HIS40:HE1	H-Donor	A:GLU159:OE2	H-Acceptor
A:ARG221:NH2 - B:HIS112	4.26075	Electrostatic	Pi-Cation	A: ARG221:NH2	Positive	B:HIS112	Pi-Orbitals
A:ASP192:OD1 - B:PHE104	4.24739	Electrostatic	Pi-Anion	A: ASP192:OD1	Negative	B:PHE104	Pi-Orbitals
A:ASP197:OD2 - B:HIS112	3.36826	Electrostatic	Pi-Anion	A: ASP197:OD2	Negative	B:HIS112	Pi-Orbitals
B:GLU110:OE2 - A:TYR164	4.20693	Electrostatic	Pi-Anion	B: GLU110:OE2	Negative	A:TYR164	Pi-Orbitals
B:GLU110:OE2 - A:HIS201	3.24915	Electrostatic	Pi-Anion	B: GLU110:OE2	Negative	A:HIS201	Pi-Orbitals

A:CYS160:SG - B:PHE104	4.36697	Other	Pi-Sulfur	A:CYS160:SG	Sulfur	B:PHE104	Pi-Orbitals
A:CYS194:SG - B:PHE104	5.21006	Other	Pi-Sulfur	A:CYS194:SG	Sulfur	B:PHE104	Pi-Orbitals
B:HIS40 - A:CYS160	4.66622	Hydrophobic	Pi-Alkyl	B:HIS40	Pi-Orbitals	A:CYS160	Alkyl
B:HIS40 - A:MET429	4.82283	Hydrophobic	Pi-Alkyl	B:HIS40	Pi-Orbitals	A:MET429	Alkyl
B:PHE41 - A:CYS194	4.39346	Hydrophobic	Pi-Alkyl	B:PHE41	Pi-Orbitals	A:CYS194	Alkyl

Table S2. Primer applications and sequences

Primer applications	Primer sequences (5' to 3')
RT-PCR for Human XBP-1 splicing	CCTTGTAGTTGAGAACCAGG
	GGGGCTTGGTATATATGTGG
qRT-PCR for Human CHOP	ACCTCCTGGAAATGAAGAGGAAG
	GCTCTGACTGGAATCTGGAGAG
qRT-PCR for Human EDEM	TATCTCCTCTACCAGGCAACCA
	GGACTTGTCAATGACGTGATGC
qRT-PCR for Human HSPA5	CAACGCCAAGCAACCAAAGA
	GTTCTTCTCCCCCTCCCTCT
qRT-PCR for Human PDIA4	AGCAGGGCTCCACCCA
	CCTGGTGTAGCGCTTAGCAT
RT-PCR for Mouse XBP-1 splicing	GAACCAGGAGTTAAGAACACG
	AGGCAACAGTGTCAGAGTCC
qRT-PCR for Mouse CHOP	CCCAGGAAACGAAGAGGAAGAA
	CTCTGACTGGAATCTGGAGAGC
qRT-PCR for Mouse EDEM	TATCTCCTCTACCAGGCAACCA
	TCTATGACGTGATGCAGCGTAG
site-directed mutagenesis to construct pCAGGS-Flag-Stop	ACGACGATAATATTCGAGCTCATC
	CATCCTTGTAATCCATGG
PCR to linearize pCAGGS-Flag-Stop	GATTACAAGGATGACGACGATAAG
	GATGAGACAGCACAACAACCAG

RT-PCR to construct ORF8-Flag	TTGTGCTGTCTCATCATGAAATTTCTTGTTTTCTTAGG
	GTCATCCTTGTAATCGATGAAATCTAAAACAACACGAAC
subcloned PCR to construct ORF8-EGFP	GGCGCTAGCATGAAATTTCTTGTTTTCTTAGGAATCATC
	CTTGGATCCCGGATGAAATCTAAAACAACACGAACG
site-directed mutagenesis to construct ORF8-T12R	TGTTTTCTTAGGAATCATCACAAGAGTAGCTGCATTTCACCAAGAATG
	CATTCTTGGTGAAATGCAGCTACTCTTGTGATGATTCCTAAGAAAACA
site-directed mutagenesis to construct ORF8-V13A	ATTCTTGGTGAAATGCAGCTGCAGTTGTGATGATTCCTAAG
	CTTAGGAATCATCACAACTGCAGCTGCATTTCACCAAGAAT
site-directed mutagenesis to construct ORF8-A15R	CTGTAAACTACATTCTTGGTGAAATCTAGCTACAGTTGTGATGATTCCTAAG
	CTTAGGAATCATCACAACTGTAGCTAGATTTCACCAAGAATGTAGTTTACAG
site-directed mutagenesis to construct ORF8-V13R	CTTGGTGAAATGCAGCTCTAGTTGTGATGATTCCTAAGAAAACAAGAAATTT
	AAATTTCTTGTTTTCTTAGGAATCATCACAACTAGAGCTGCATTTCACCAAG
site-directed mutagenesis to construct ORF8-△SR	ACGACGATAATATTCGAGCTCATC
	CATCCTTGTAATCCATGG
site-directed mutagenesis to construct ORF8-SR (1st round)	TATATTAGAGTAGGAGCTAG
	TTCTTGGTGAAATGCAGC
site-directed mutagenesis to construct ORF8-SR (2nd round)	GATTACAAGGATGACGAC
	AGGTAAACAGGAAACTGTATAATTAC
site-directed mutagenesis to construct ORF8-N78Q	CGATATCGGTCAATATACAGTTTCCTG
	ATGTACTGAATGGGTGATTTAG
RT-PCR to construct HSPA5-HA	TAAAAGCTTATTATGAAGCTCTCCCTGGTGGC
	TCTCGAGCAACTCATCTTTTTCTGCTGTATCC
RT-PCR to construct Calnexin-HA	ATTGGATCCATTATGGAAGGGAAGTGGTTGCTGT

	TCTCGAGCTCTCTTCGTGGCTTTCTGTTTCTT
RT-PCR to construct TUBB-HA	ATCAAGCTTATTATGGACTCTGTTCGCTCAGGTCCTT
	TCTCGAGGGCCTCCTCTTCGGCCTCCTC
RT-PCR to construct TUBA1B-HA	ATTGGATCCATTATGCGTGAGTGCATCTCCATCC
	TCTCGAGGTATTCCTCTCCTTCTTCCTCACCC
RT-PCR to construct PDIA6-HA	TTCAAGCTTATTATGATCTTAGGTCTGGTGAGCTGTA
	TCTCGAGCAACTCATCTTTCCCTAAGTCATCA
RT-PCR to construct EEF1A1-HA	ATTGGATCCATTATGGGAAAGGAAAAGACTCATATCA
	TCTCGAGTTTAGCCTTCTGAGCTTTCTGGG
site-directed mutagenesis to construct Calnexin-ΔGlobular (1st round)	CTGCTCAATGACATGACTC
	GAGCTTTGTAAGTAACCTTG
site-directed mutagenesis to construct Calnexin-ΔGlobular (2nd round)	AAGAAAGCTGCTGATGGG
	AGGTTCCAGATCTTCAAAG
site-directed mutagenesis to construct Calnexin-ΔArm	TTCAGAATGACTCCTTTTAG
	ATTTCCACTATTCACCAC
site-directed mutagenesis to construct Calnexin-ΔCtem	CTCGAGTCTAGAGGGCCC
	CAGGCCCCATCCATCATTG
site-directed mutagenesis to construct Calnexin-Arm (1st round)	ATAGAATTCGCCACCATGGAAGGGAAGTG
	GTGAAGGATTTACAGGATCAATCACATCATCATCATGTCCATCATG
site-directed mutagenesis to construct Calnexin-Arm (2nd round)	TGATGATGTGATTGATCCTGTAAATCCTTCACGTGAAATTGAGG
	AGGTTCCAGATCTTCAAAGAAATCTGGATTTGCTCGAGATA
homologous recombination to construct Calnexin-Ctem (N-terminal part)	AATGAATTCGCCACCATGGAAGGGAAGTGGTTGCTG
	TCTCGAGATCAATCACATCATCATCATGTCCA

homologous recombination to construct Calnexin-Ctem (C-terminal part)	ATGATGATGATGTGATTGATAAGAAAGCTGCTGATGGGGC
	TAGGGCCCTCTAGACTCGAGCTCTCTTCGTGGCTTTCTGT
homologous recombination to construct Calnexin-Globular (N-terminal part)	AATGAATTCGCCACCATGGAAGGGAAGTGGTTGCTG
	TCTCGAGATTTCCACTATTCACCACAGATTGG
homologous recombination to construct Calnexin-Globular (C-terminal part)	CTGTGGTGAATAGTGGAAATTTCAGAATGACTCCTTTTAG
	TAGGGCCCTCTAGACTCGAGCAGGCCCCATCCATCATTGG
site-directed mutagenesis to construct HSPA5-ΔNBD	CAAGATACAGGTGACCTG
	CTTCTTGTCCTCCTCCTC
site-directed mutagenesis to construct HSPA5-ΔSBD	AAACTCTATGGAAGTGCAGGCCCTC
	TACATCAAGCAGTACCAGGTCACCTG
site-directed mutagenesis to construct HSPA5-ΔN	AAACCATACATTCAAGTTGATATTGGAGGTGGGCAAACAAAGACATTTGCTCC
	CTCCTCGGCCCGCGCCGC
site-directed mutagenesis to construct HSPA5-Δcore NBD	GACAATAGAGCTGTGCAGAAACTCCGG
	AGTTTTCTTTTCAACCACCTTGAACGGC
site-directed mutagenesis to construct HSPA5-Δcore SBD	TTTGAGATAGATGTGAATGG
	TACATCAAGCAGTACCAG

Table S3. Antibodies information

Antibody Name	Vendor	Application
Anti-HA	Biolegend	WB
Anti-Flag	Sigma	WB
Anti-eIf2α	Cell Signaling	WB
Anti-p eIf2α	Cell Signaling	WB
Anti-Calneixn	Cell Signaling	WB
Anti-Calnexin	Abcam	IP/IF
Anti-HSPA5	Proteintech	WB/IP/IF
Anti-beta tubulin	Cell Signaling	WB/IF
Anti-GAPDH	Cell Signaling	WB
Anti-GM130	Abcam	IF
Anti-ATF6	Novus	WB
Anti-MHC I	Abcam	WB
Anti-CD63	Santa Cruz	WB
Anti-Ubiquitin	Cell Signaling	WB

Table S4. siRNA information

Name	Sequences (5' to 3')	
siORF8-1	sense	UUCUCUAAACGAACAUGAAAUdTdT
	antisense	AUUUCAUGUUCGUUUAGAGAAdTdT
siORF8-2	sense	UCUAAACGAACAUGAAAUUUCdTdT
	antisense	GAAAUUUCAUGUUCGUUUAGAdTdT
siORF8-3	sense	CUCUAAACGAACAUGAAAUUUdTdT
	antisense	AAAUUUCAUGUUCGUUUAGAGdTdT
siORF8-4	sense	UGUUCUCUAAACGAACAUGAAAUdTdT
	antisense	AUUUCAUGUUCGUUUAGAGAACAdTdT
siORF8-5	sense	UCUAAACGAACAUGAAAUUUCUUdTdT
	antisense	AAGAAAUUUCAUGUUCGUUUAGAdTdT
siORF8-6	sense	UUCUCUAAACGAACAUGAAAUUUdTdT
	antisense	AAAUUUCAUGUUCGUUUAGAGAAdTdT
siCalnexin-1	sense	CACCAGAACUCAACCUGGAUCAGUU
	antisense	AACUGAUCCAGGUUGAGUUCUGGUG
siCalnexin-2	sense	UGUGGUGGUGCCUAUGUGATT
	antisense	UCACAUAGGCACCACCACAUU
siNC	Sense	UUCUCCGAACGUGUCACGUTT
	antisense	ACGUGACACGUUCGGAGAATT