Supplementary Figures for

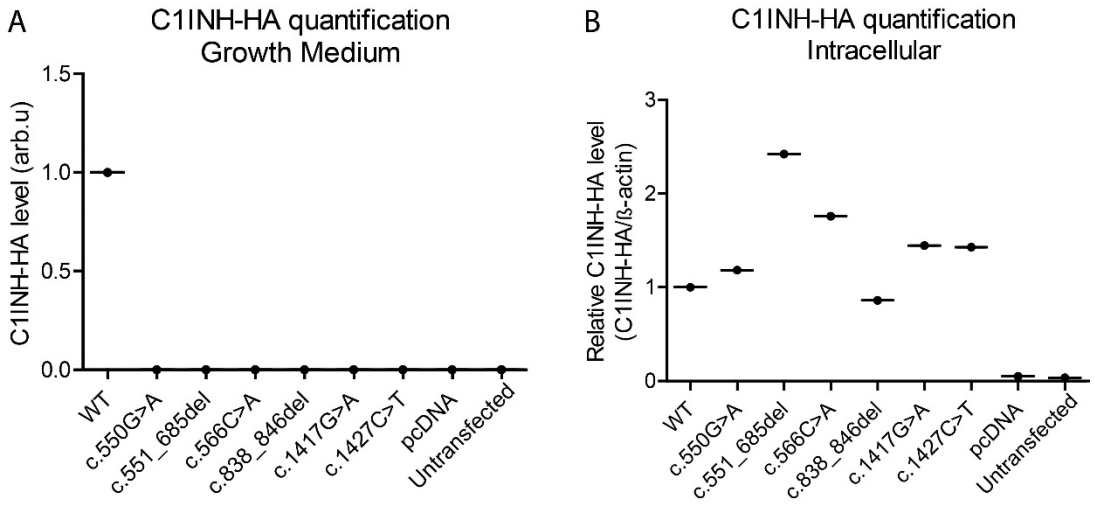
Dominant negative *SERPING1* variants cause intracellular retention of C1-inhibitor in hereditary angioedema

Didde Haslund, Laura Barrett Ryø, Sara Seidelin Majidi, Iben Kløvgaard Rose, Kristian Alsbjerg Skipper, Tue Fryland, Anja Bille Bohn, Claus Koch, Martin K. Thomsen, Yaseelan Palarasah, Thomas J. Corydon, Anette Bygum, Lene N. Nejsum and Jacob Giehm Mikkelsen

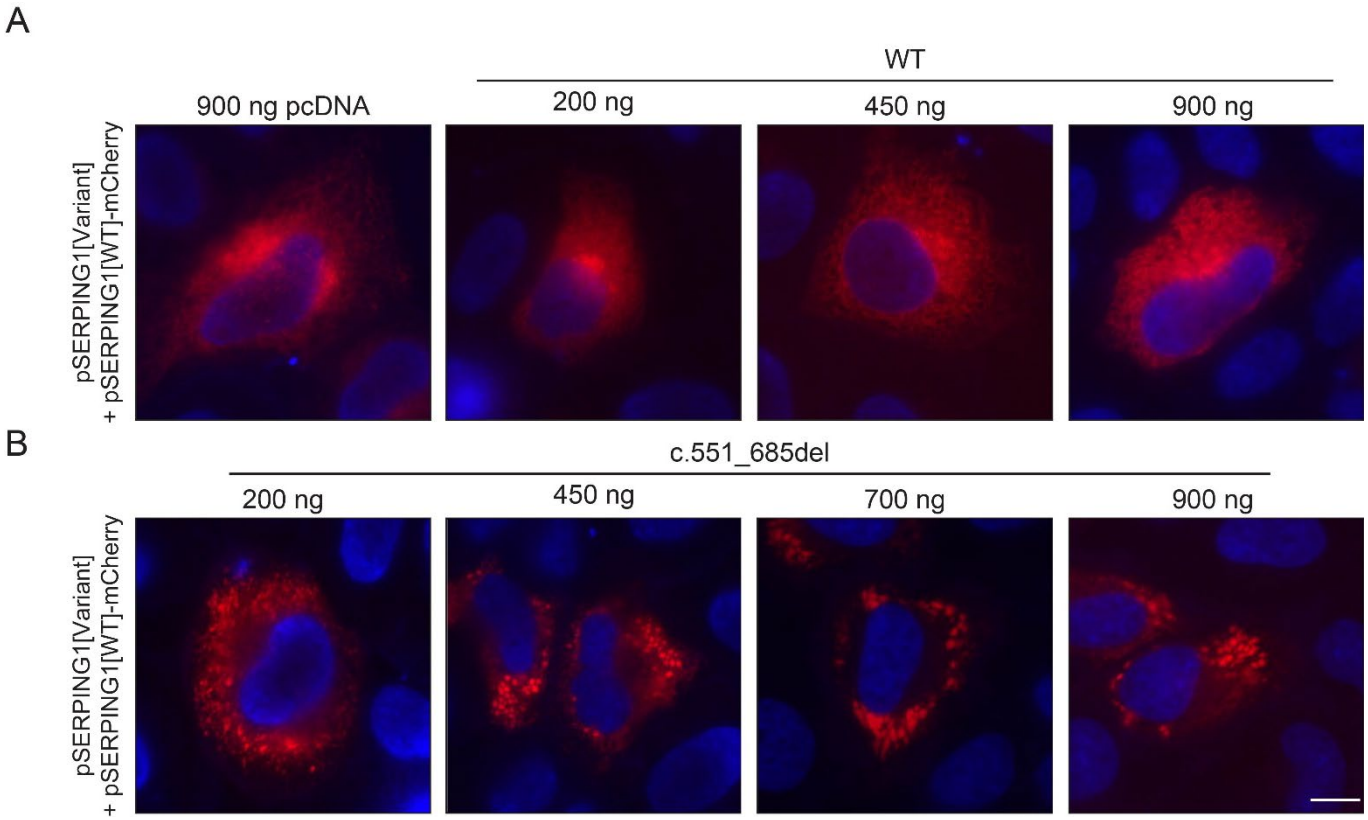
This PDF file includes:

Supplementary Figures S1-S13

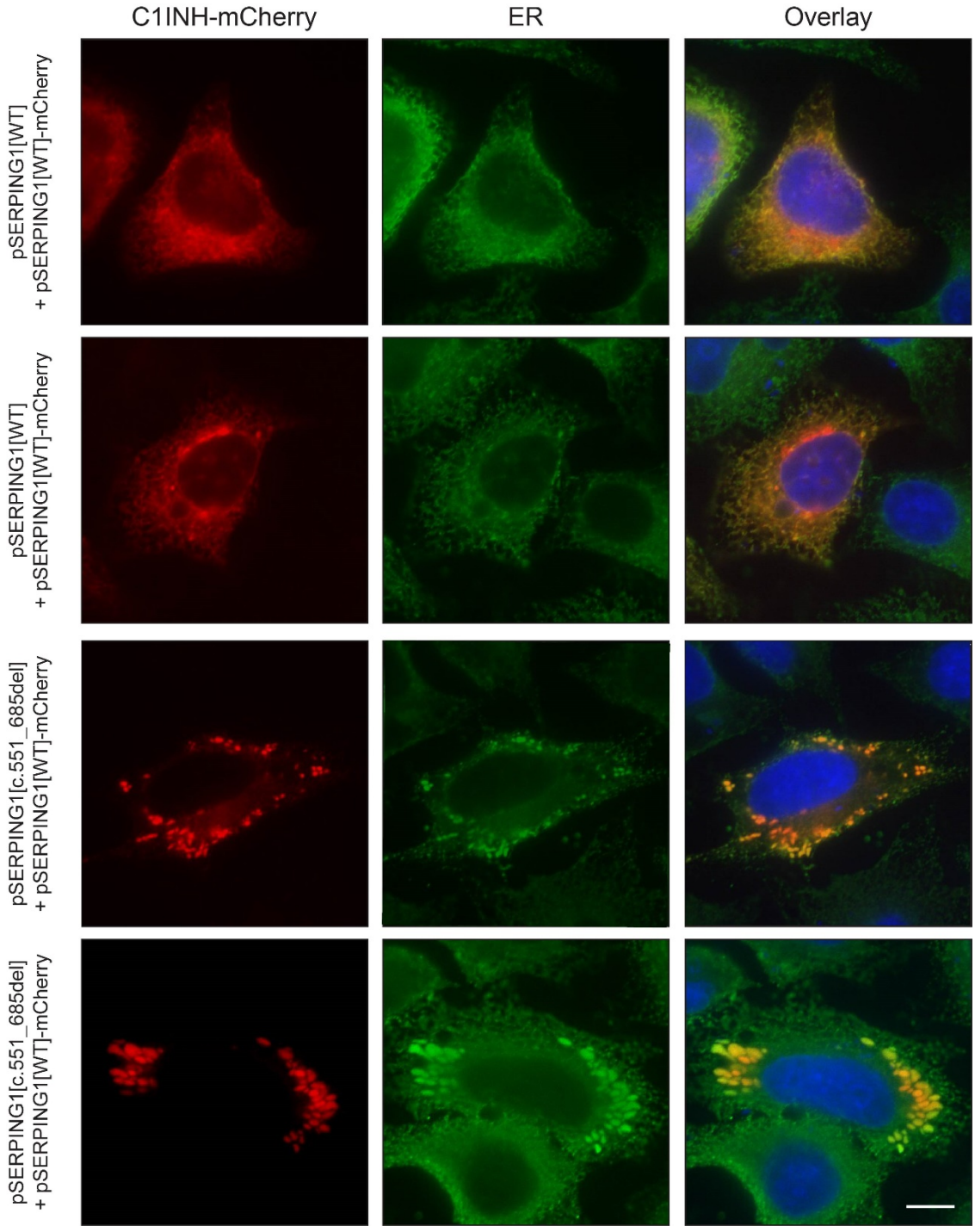
Supplementary Tables S1-S2



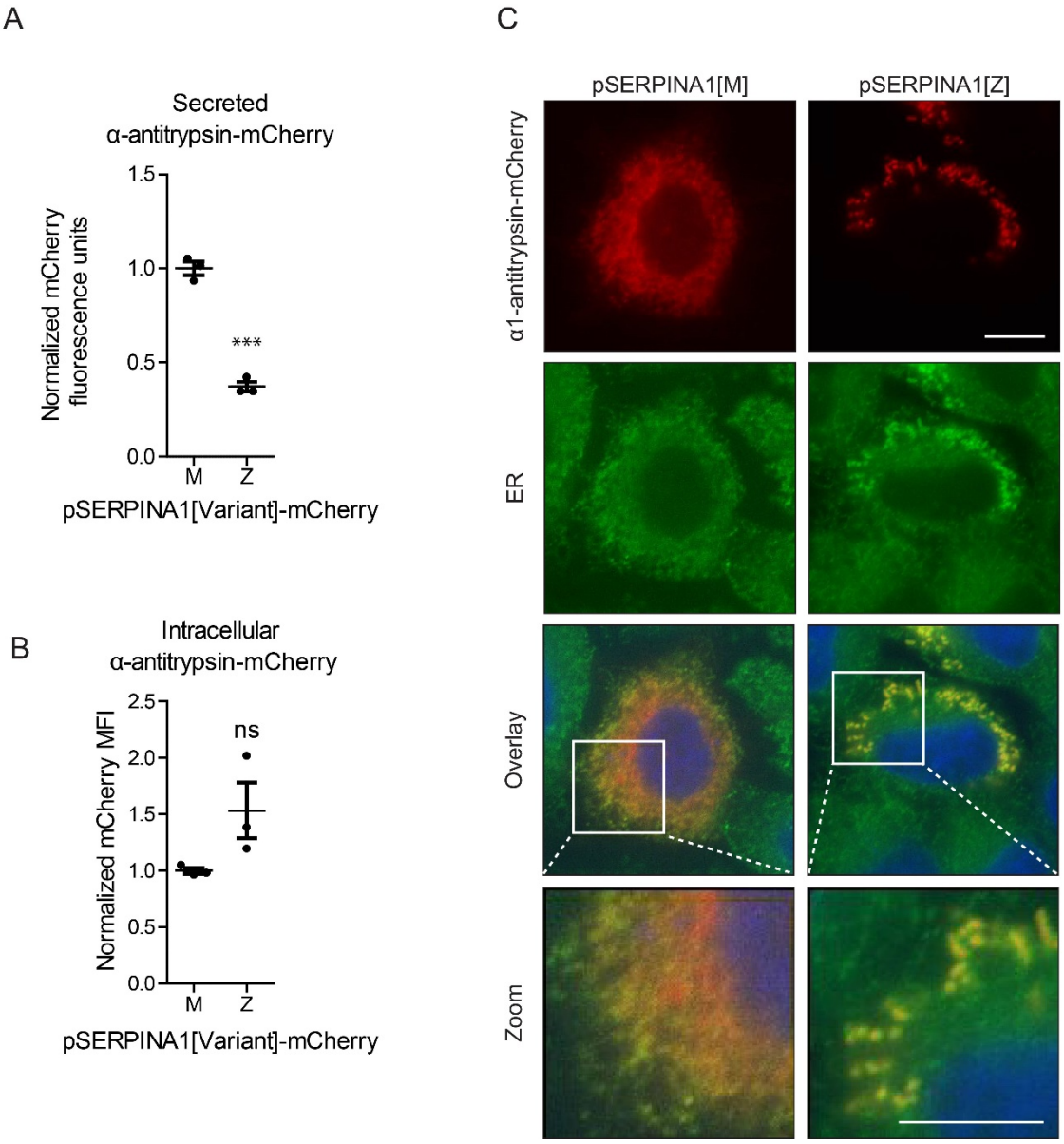
Supplementary Figure S1. Reduced C1INH secretion and increased intracellular C1INH levels in HeLa cells transiently transfected with a HAE-causing SERPING1 variant. Western blot quantification of the signal intensity of C1INH[Variant]-HA protein in Figure 2C using Image J.



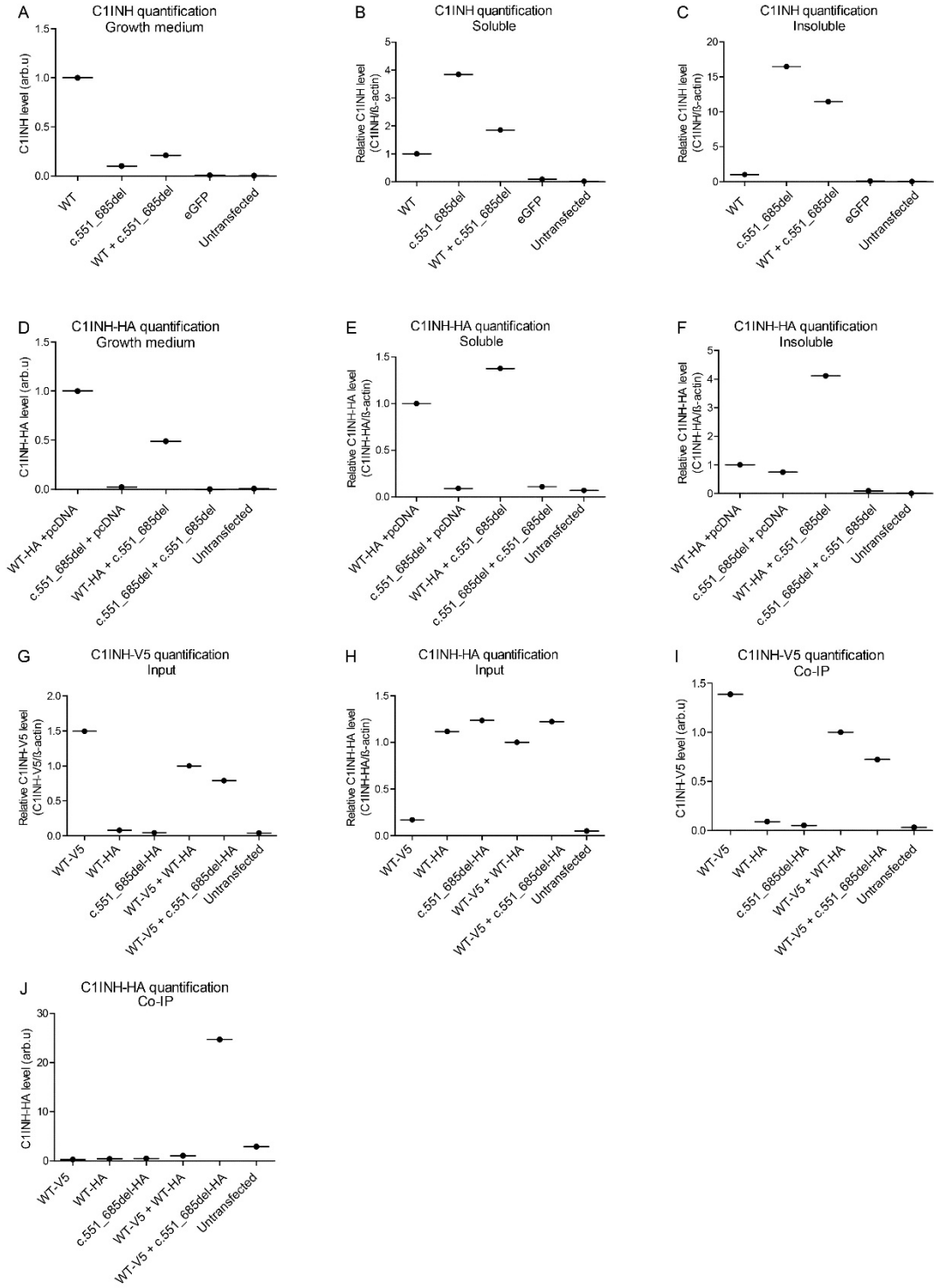
Supplementary Figure S2. Comparable levels of C1INH-mCherry aggregate formation with low and high amounts of pSERPING1[c.551_685del]. Live widefield microscopy of HeLa cells cultured for 48 hours after co-transfection with 450 ng pSERPING1[WT]-mCherry and 200, 450, 700 or 900 ng of either **(A)** pSERPING1[WT] or **(B)** pSERPING1[c.551_685del]. Empty pcDNA was included in all transfections to ensure transfection with equal amounts of plasmid DNA. Cells were incubated with Hoechst to visualize nuclei (blue). Scale bars: 10 µm. Data are representative of findings from more than three biological replicates.



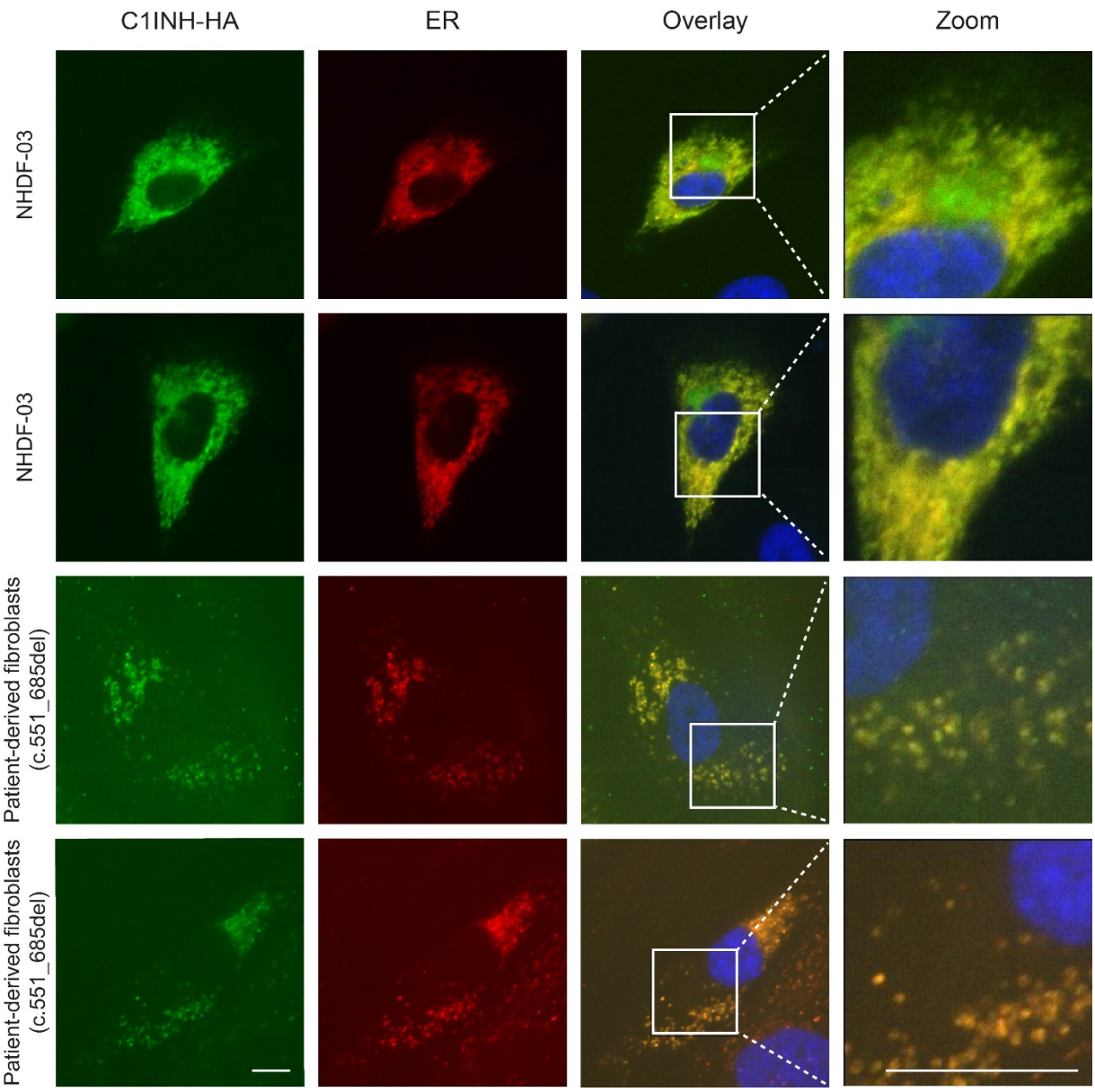
Supplementary Figure S3. Intracellular accumulation of normal C1INH in ER. Widefield microscopy of HeLa cells cultured for 48 hours after co-transfection with 450 ng of pSERPING1[WT]-mCherry and 450 ng pSERPING1[WT] or pSERPING1[c.551_685del]. ER was visualized with an antibody recognizing KDEL (green). Scale bars: 10 µm. Data are representative of findings from more than three biological replicates.



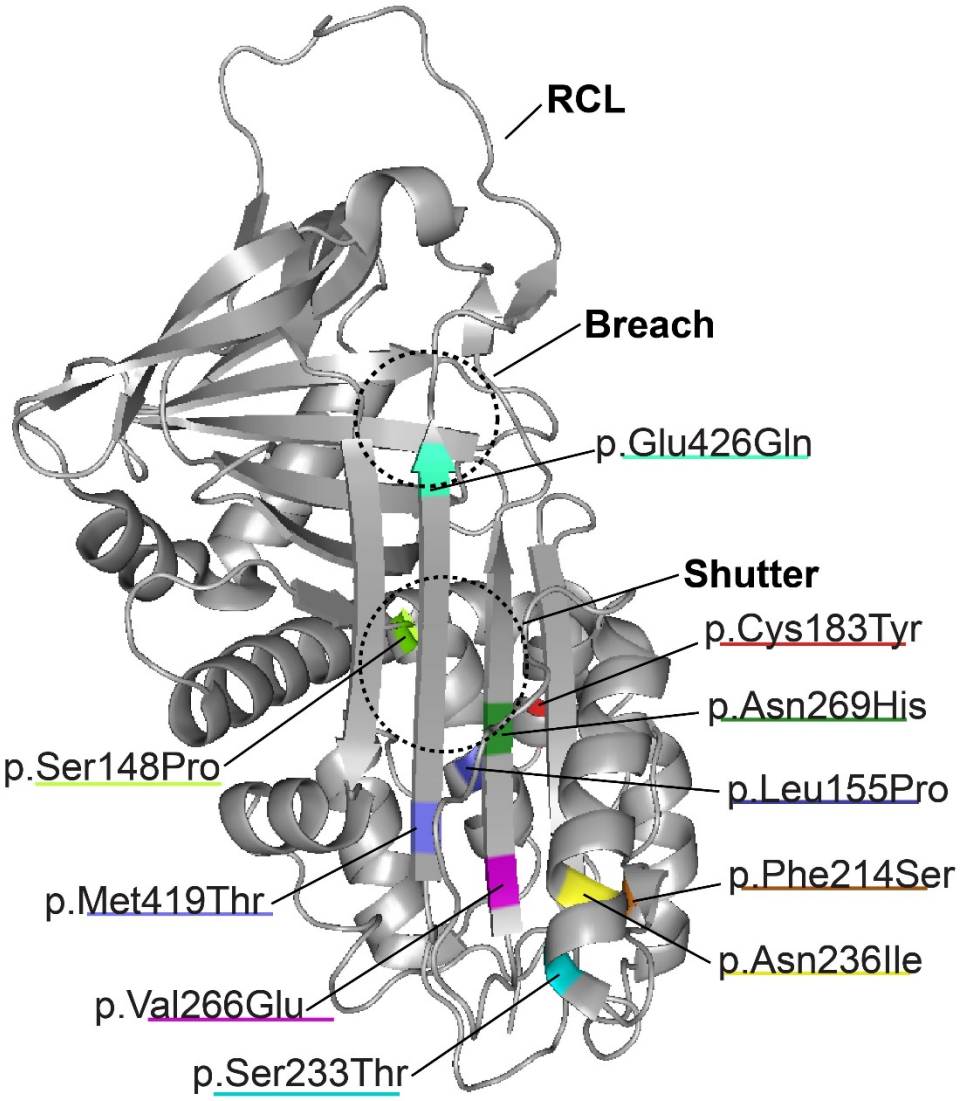
Supplementary Figure S4. Intracellular retention of α1-antitrypsin in the ER. Reduced secretion and ER accumulation of the Z variant of α1-antitrypsin-mCherry. HeLa cells were transfected with 900 ng of pα1-antitrypsin[Variant]-mCherry and the levels of (**A)** secreted and **(B)** intracellular α1-antitrypsin-mCherry were determined, as described elsewhere. **(C)** Intracellular localization of α1-antitrypsin-mCherry. HeLa cells were transfected with pα1-antitrypsin[M]-mCherry or pα1-antitrypsin[Z]-mCherry. 48 hours after transfection, the cells were fixated and incubated with a KDEL antibody and Hoechst to visualize ER (green) and the nuclei (blue), respectively. **(A-B)** Transfections were carried out in triplicates (*n* = 3). * P < 0.05, **P < 0.01, ***P < 0.001, compared with M. Statistical analyses were performed by 2-tailed, paired Student's t test. **(C)** Scale bars: 10 µm. Data are representative of findings from more than three biological replicates. Similar results were seen in at least three independent experiments.



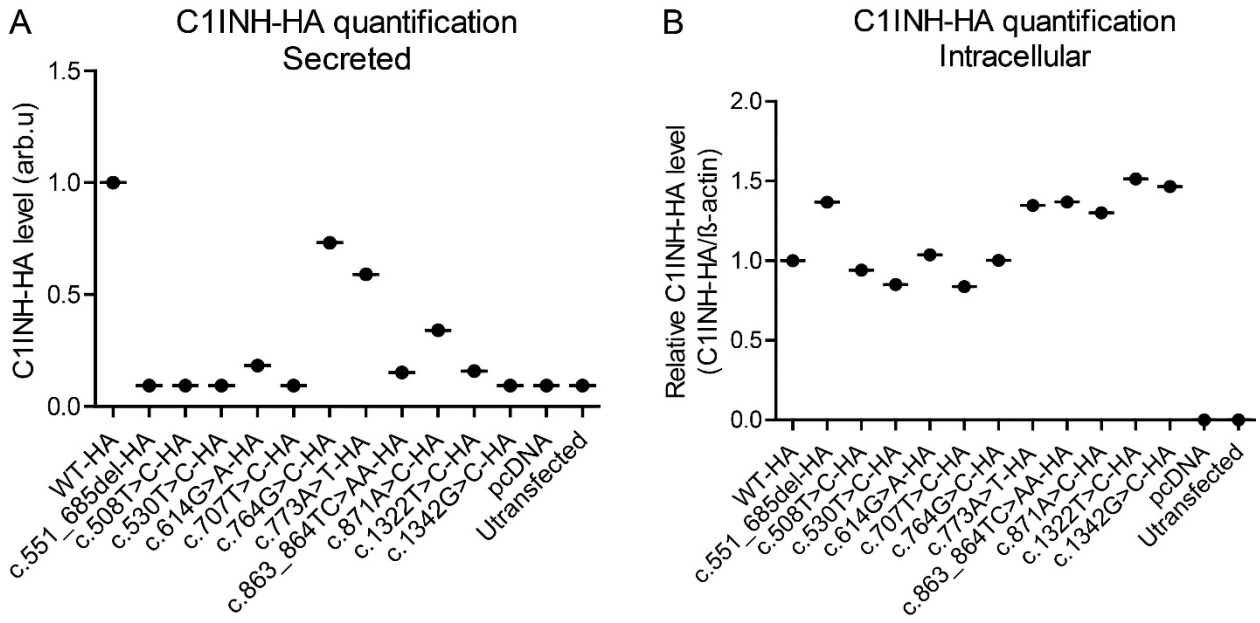
Supplementary Figure S5. Western blot quantification of the signal intensity of C1INH. (A-C) Quantification of signal intensity of C1INH[Variant] protein detected in medium **(A)** as well as in soluble **(B)** and insoluble **(C)** fractions shown in Figure 6B. Quantification of signal intensity of C1INH[Variant]-HA protein detected in medium **(D)** as well as in soluble **(E)** and insoluble **(F)** fractions shown in Figure 6C. Quantification of the signal intensity of normal C1INH-V5 and C1INH[Variant]-HA in the input **(G-H)** and co-IP **(I-J)** shown in Figure 6D. The C1INH signal was normalized to the β-actin signal (panels B, C, E, F, G, H). The Western blot quantification was done using Image J.



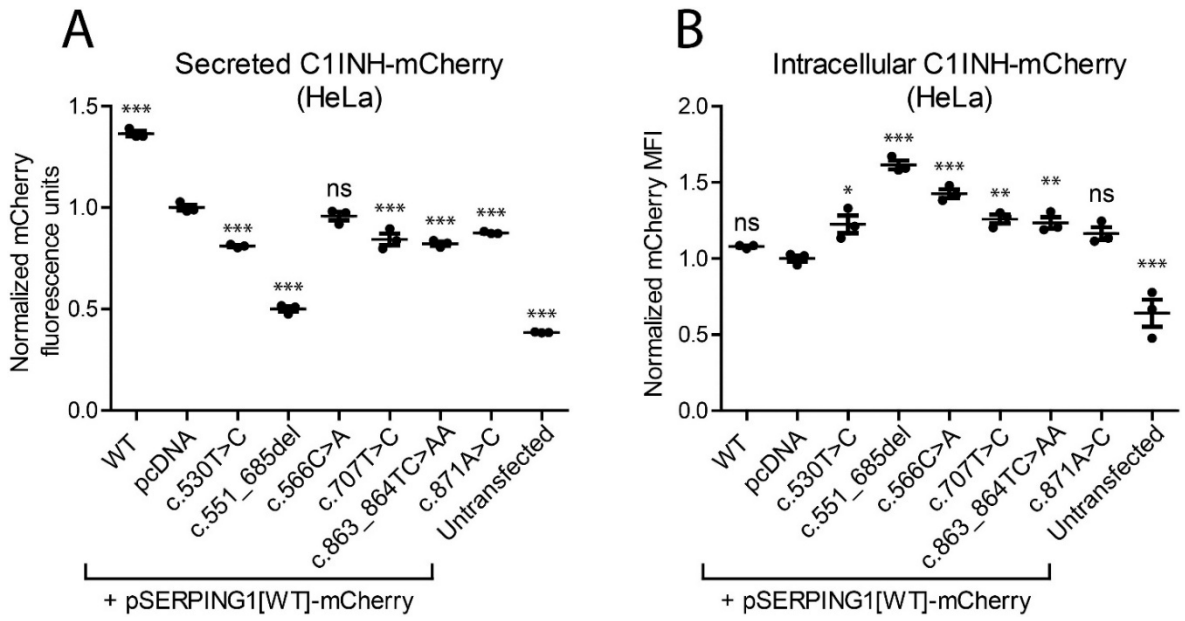
Supplementary Figure S6. Transiently expressed C1INH-HA colocalizes with endogenously expressed C1INH in patient-derived fibroblasts. NHDF-03 control fibroblasts and fibroblasts derived from the HAE patient carrying the c.551_685del SERPING1 gene variant were co-transfected with 450 ng pSERPING1[WT]-HA, and Tomato-Calreticulin to visualize ER. 48 hours after the co-transfection, the fibroblasts were fixated and incubated with a HA antibody and Hoechst to visualize C1INH-HA (green) and nuclei (blue), respectively. Scale bars: 10 µm. Data are representative of findings from more than three biological replicates.



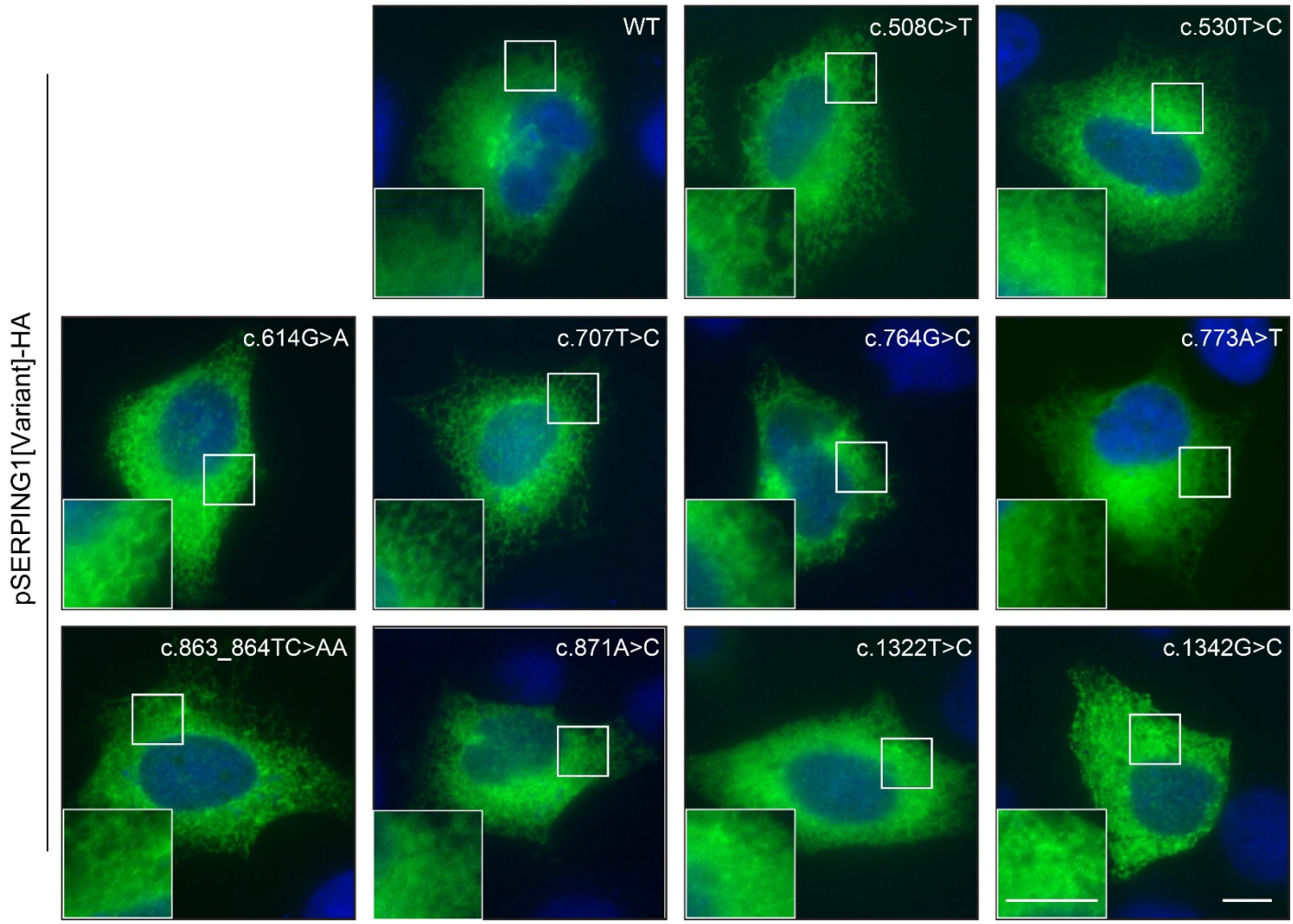
Supplementary Figure S7. The structure of active C1INH protein (PDB entry 5DU3). The location of the ten studied HAE-causing SERPING1 variations are marked as follows: p.Ser148Pro (light green), p.Leu155Pro (dark blue), p.Cys183Tyr (red), p.Phe214Ser(brown), p.Ser233Thr (cyan), p.Asn236Ile (yellow), p.Val266Glu (magenta), p.Asn269His (dark green), p.Met419Thr (purple), p.Glu426Gln (turquoise).



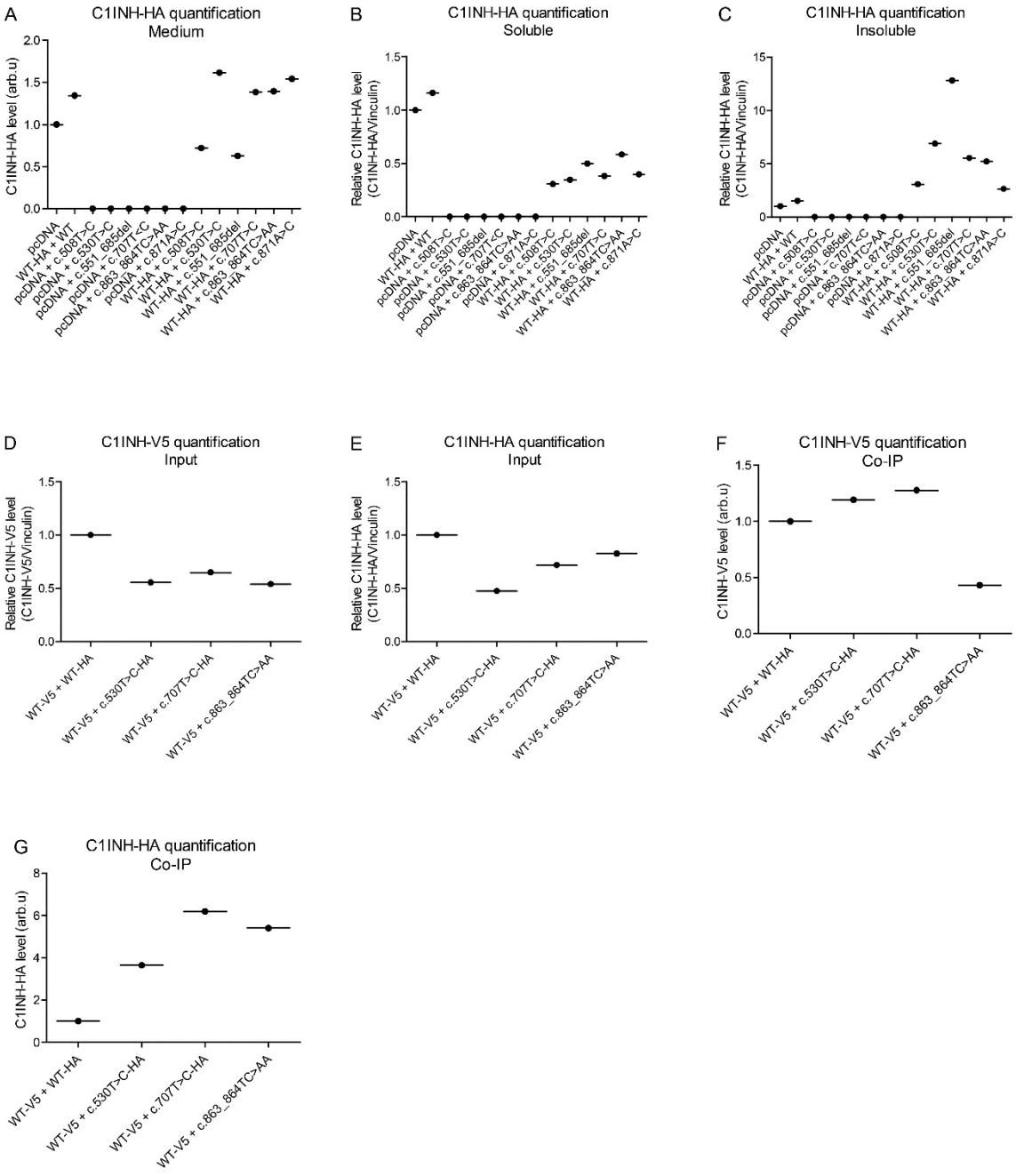
Supplementary Figure S8. Reduced secretion and increased intracellular levels of C1INH[Variant]-HA among analysed variants. Western blot quantification of the signal intensity of secreted **(A)** and intracellular **(B)** C1INH[Variant]-HA shown in Figure 8B using Image J.



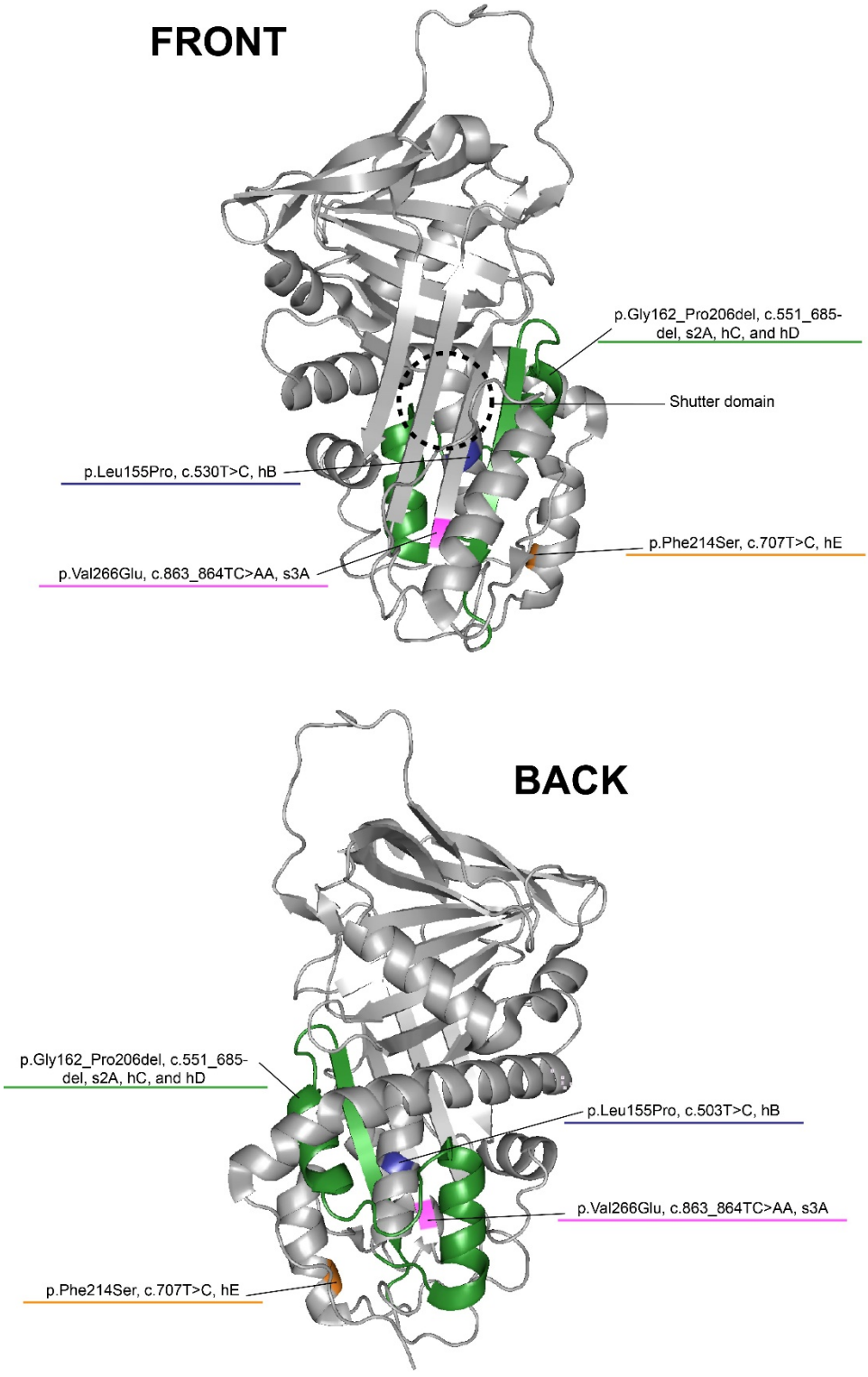
Supplementary Figure S9. Negative impact on secretion and intracellur C1INH levels by HAE-causing variants is prominent 48 hours after transfection. (A,B) Levels of secreted and intracellular normal C1INH-mCherry in HeLa cells co-transfected with 450 ng of pSERPING1[Variant] and 450 ng of pSERPING1[WT]-mCherry. Secretion of normal C1INH-mCherry was measured by fluorescence scanning **(A)** and the intracellular level by flow cytometry **(B)**. Transfections were carried out in triplicates (*n* = 3), and data are depicted as mean ± SEM. * P < 0.05, **P < 0.01, ***P < 0.001, compared with pcDNA. Statistical analyses were performed by 1-way ANOVA with Dunnett's multiple comparison test. MFI, Median Fluorescence Intensity



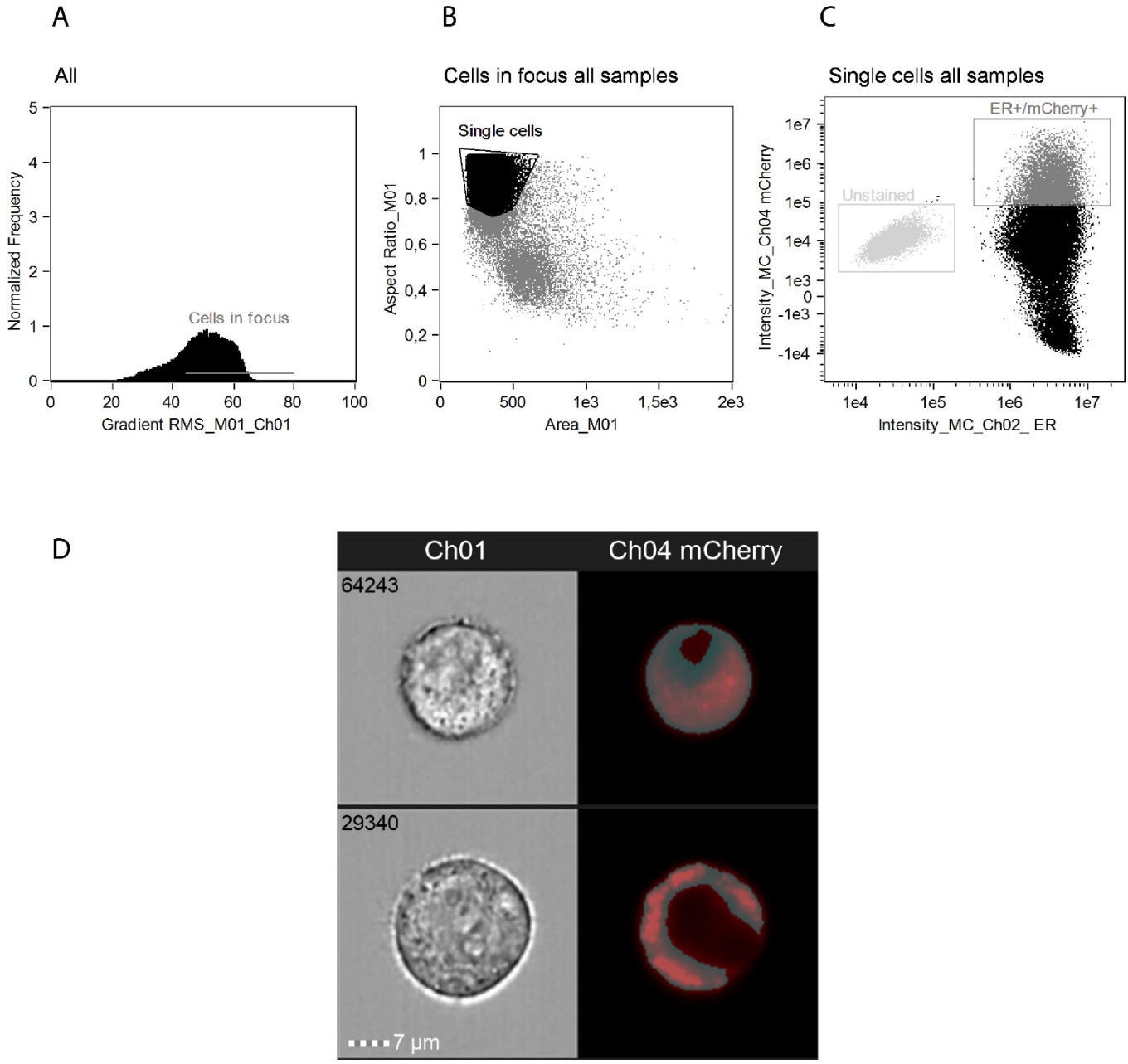
Supplementary Figure S10. Lack of detectable C1INH aggregates in HeLa cells expressing HAE-causing *SERPING1* gene variants separately. Intracellular localization of normal and mutated C1INH-HA. Widefield microscopy of HeLa cells cultured for 48 hours after transfection with 900 ng pSERPING1[Variant]-HA. C1INH-HA was visualized with a HA-specific antibody (green).



Supplementary Figure S11. Direct protein-protein interaction between normal and three different C1INH variants. Western blot quantification of the signal intensity of normal C1INH-HA in the medium**(A)**, and in the soluble **(B)** and insoluble **(C)** fractions of total cellular protein in Figure 9B. Western blot quantification of the signal intensity of normal C1INH-V5 and C1INH[Variant]-HA in the input **(D-E)** and co-IP **(F-G)** in Figure 9C. The Western blot quantifications were done using Image J.



Supplementary Figure S12. Dominant negative HAE-causing SERPING1 variants are in close proximity to the shutter domain. The structure of active C1INH protein (PDB entry 5DU3). The location of the four dominant negative HAE-causing SERPING1 variations are marked as follow: p.Leu155Pro (dark blue), p.Gly162_Pro206del (green), p.Phe214Ser(orange), p.Val266Glu (magenta).



Supplementary Figure S13. Gating strategy and mask design for Imagestream analysis. The gating strategy demonstrates **(A)** selection of cells in focus, **(B)** single cells, and **(C)** ER and mCherry double-positive cells. **(D)** Column one shows the brightfield image of cells without a mask, and column two illustrates cells with a threshold mask. The lower of the two cells is identical to the cell shown at the top of the second column in Figure 4C.

Table S1| ***SERPING1* variants found in Danish Type I patients**

SERPING1 Variant	Localization	Protein effect	Type of variation	Structural localization	C1INH protein length	C1INH plasma level (g/L)
WT	-	-	-	-	478 aa	0.21-0.39
c.550G>A	Exon 3	p.Gly162Arg	Missense	In turn between hC and hB	478 aa	0.07
c.551_685del	Exon 4	p.Gly162_Pro206del	Deletion	s2A, hD and hC	433 aa	0.05-0.13
c.566C>A	Exon 4	p.Thr167Asn	Missense	hC	478 aa	0.2
c.838_846del	Exon 5	p.Ser258_Pro260del	Deletion	In loop between s3A and hF	475 aa	0.06
c.1417G>A	Exon 8	p.Val451Met	Missense	In loop between s1C and s4B	478 aa	0.05-0.12
c.1427C>T	Exon	p.Pro454Leu	Missense	In loop between s1C and s4B	478 aa	0.06

Table S2| **Second set of *SERPING1* variants chosen for analysis of dominant negative effects**

SERPING1 Variant	Localization	Protein effect	Type of variation	Structural localization	C1INH protein length	Reference
WT	-	-	-	-	478 aa	
c.508T>C	Exon 3	p.Ser148Pro	Missense	hB	478 aa	Pappalardo et al., J Allergy Clin Immunol 2000;106:1147-54
c.530T>C	Exon 3	p.Leu155Pro	Missense	hB	478 aa	Bissler JJ et al (Proc Assoc Am Physicians. 1997 Mar;109(2):164-73.)
c.614G>A	Exon 4	p.Cys183Tyr	Missense	hD	478 aa	Zuraw BL et al (J Allergy Clin Immunol. 2000 Mar;105(3):541-6.)
c.707T>C	Exon 5	p.Phe214Ser	Missense	hE	478 aa	Verpy et al. (Am J Hum Genet 1996 Aug;59(2):308-19)
c.764G>C	Exon 5	p.Ser233Thr	Missense	hF	478 aa	Gößwein et al. (Cytogenet Genome Res 2008; 121(3-4):181-8)
c.773A>T	Exon 5	p.Asn236Ile	Missense	hF	478 aa	Gößwein et al. (Cytogenet Genome Res 2008; 121(3-4):181-8)
c.863_864TC>AA	Exon 5	p.Val266Glu	Missense	s3A	478 aa	Roche O et al. (Human Mutation 2005 26(2), 135-144)
c.871A>C	Exon 5	p.Asn269His	Missense	s3A	478 aa	Gößwein et al. (Cytogenet Genome Res 2008; 121(3-4):181-8)
c.1322T>C	Exon 8	Met419Thr	Missense	s5A	478 aa	Gößwein et al. (Cytogenet Genome Res 2008; 121(3-4):181-8)
c.1342G>C	Exon 8	p.Glu426Gln	Missense	s5A	478 aa	Gößwein et al. (Cytogenet Genome Res 2008; 121(3-4):181-8)

Supplementary Methods for

Dominant negative *SERPING1* variants cause intracellular retention of C1-inhibitor in hereditary angioedema

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Materials and Methods

Selection of HAE-causing SERPING1 gene variants

Six HAE type I-causing SERPING1 variants (c.550G>A, c.551_685del, c.566C>A, c.838_846del, c.1417G>A, and c.1427C>T) were selected among variants found in the Danish HAE type I cohort. These variants were chosen based on the following criteria: (i) intact reading frame, (ii) premature stop codons not present, (iii) encode full-length or near full-length C1INH protein. Variants matching these criteria were considered most likely to possess dominant negative effects. To identify additional SERPING1 gene variants with a dominant negative effect, as seen for the two variants c.551_685del and c.566C>A, we focused on variations within the shutter domain, as this domain is afflicted in the above-mentioned C1INH variants displaying dominant negative effects. Using the software PyMOL, amino acids located in the β-sheets and α-helices in close proximity to the shutter domain were identified. By searching the 'C1 inhibitor gene mutation database' (1) a total of ten HAE-causing SERPING1 variants (c.508T>C, c.530T>C, c.614G>A, c.707T>C,. c.764G>C, c.773A>T, c.863_864TC>AA, c.871A>C, c.1322T>C and c.1342G>C) located in the selected β-sheets and α-helices were selected for further analysis.

Vector construction

The expression plasmid pSERPING1[WT] encoding the human SERPING1 gene was generated by replacing the eGFP gene in pT2/CMV-eGFP(s).SV40-neo (2) with a NotI-digested PCR amplicon encompassing the human SERPING1 cDNA sequence. The different HAE-causing mutations were introduced into the wildtype SERPING1 sequence by overlap PCR or by using the NEBuilder® HiFi DNA Assembly Cloning Kit (New England Biolabs). To generate pSERPING1[Variant]-mCherry encoding mCherry-tagged SERPING1 we first generated the expression plasmid pT2/CMV-mCherry.SV40-neo by replacing the eGFP gene in pT2/CMV-eGFP(s).SV40-neo (2) with a PCR amplicon carrying the mCherry cDNA sequence. Thereafter, PCR amplicons encompassing the different SERPING1 sequences were inserted upstream from the mCherry gene in pT2/CMV-

mCherry.SV40-neo using the NEBuilder® HiFi DNA Assembly Cloning Kit (New England Biolabs) to generate pSERPING1[Variant]-mCherry. Plasmids expressing SERPINA1 (referred to as pSERPINA1[Variant]) were generated by replacing the eGFP gene in pT2/CMV-eGFP(s).SV40-neo with an amplicon encompassing the human SERPINA1 cDNA sequence. The Z mutation in the SERPINA1 gene was introduced by using the NEBuilder® HiFi DNA Assembly Cloning Kit (New England Biolabs). pSERPINA1[Variant]-mCherry was created by insertion of the SERPINA1 gene variants upstream from the mCherry gene in pT2/CMV-mCherry.SV40-neo. pSERPING1[Variant]-HA carrying the SERPING1 gene linked to an HA tag was generated by PCR-amplifying the SERPING1 gene from pSERPING1[Variant], using a matching forward primer and a reverse primer carrying the HA tag sequence. The SERPING1[Variant]-HA amplicon was inserted into Not-digested pT2/CMV-eGFP.SV40-neo. pSERPING1[WT]-V5 carrying the SERPING1 gene linked to the sequence for the V5 tag was generated by PCR-amplifying the SERPING1 gene from pSERPING1[WT]. The SERPING1 PCR amplicon was digested with XhoI and ligated into XhoI-digested pcDNA™6/V5-His A (Thermo Fischer Scientific™, V22020). To produce lentiviral vectors carrying SERPING1[Variant] cDNA, the eGFP gene in pCCL/PGK-eGFP(3) was replaced with a SERPING1[Variant] cDNA amplicon amplified from pSERPING1[Variant]. The SERPING1[Variant] PCR amplicon was digested with XhoI and BclI, and ligated into BamHI/XhoI-digested pCCL/PGK-eGFP. The expression plasmid encoding the ER-resident calreticulin-Tomato fusion protein, the tdTomato-ER-3 vector, was a gift from Dr. Matteo Beretta, King´s College London (Addgene #58097).

Cell culture

Skin biopsies were donated from three Danish type I HAE patients. The biopsies were obtained by a dermatologist under local anesthetic and preserved in AmnioChrome (Lonza). The skin biopsies were divided into small pieces and cultured in AminioMax C-100 medium (Gibco®, Life Technologies). When the growth was well established, the patient-derived dermal fibroblasts as well as NHDF-3 (Promocell, Lot#4090701.2), NHDF-5 (ATCC, ATCC# CRL-2211) and NHDF-15

(Cellsystems, Lot# 03410) cells were all maintained in Roswell Park Memorial Institute medium (RPMI) (lonza) supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 µg/ml streptomycin and 5 mL glutamine (2.92 g/100mL). The cells were cultured at 37°C in 5% (v/v) CO_2. HepG2 (liver hepatocellular cells) and HeLa (Human cervix epithelioid carcinoma cells), originally acquired through ATCC, were maintained in Dulbecco´s Modified Eagle´s Medium (Lonza) supplemented with 5% fetal calf serum, 100 U/mL penicillin and 100 µg/ml streptomycin and cultured at 37°C in 5% (v/v) CO_2.

Enzyme linked immunosorbent assay (ELISA)

To measure the amount of C1INH secreted into the medium from transfected HeLa or HepG2 cells, the cells were seeded in 6-well plates at a density of 2 x 10^5 cells/well at day one and transfected with a total amount of 900 ng plasmid DNA. For evaluating the C1INH secretion from the different SERPING1 variants, the cells were transfected with either 900 ng pSERPING1[Variant] or pSERPING1[Variant]-mCherry. For evaluating the effect of HAE-causing SERPING1 gene variants on wildtype C1INH, cells were transfected 450 ng SERPING1[WT]-mCherry and 450 ng of the vector encoding the SERPING1 variant in question. Six hours after transfection, the medium was changed. All transfections were performed using Turbofect (Thermo Fischer Scientific™,). 24 hours after the transfection, the medium was removed, and 2 ml fresh medium was added. The medium was harvested for ELISA 48 hours after the new medium was added. To measure the amount of C1INH secreted into the medium from control and patient-derived fibroblasts, the cells were seeded in 6-well plates at a density of 2 x 10^5 cells/well at day one. At day two, the medium was removed, and 1.5 ml fresh medium was added. The medium was harvested for ELISA 48 hours after the new medium was added. The total amount of C1INH secreted into the medium from cultured HepG2 and fibroblasts cells was determined by sandwich ELISA. The capture and the detection antibody are both monoclonal antibodies from mouse, designed to detect the total amount of C1INH. Briefly, 96-well polystyrene flat bottom MicroWell™ Maxisorp™ plates (Thermo Fisher Scientific™) were

coated with 3 µg/mL of anti-C1INH mAb 11-28-21 and incubated overnight at 4° in a moist environment. The following day, the plate was washed (PBS, 0.05% tween) and diluted samples were loaded onto the plate and incubated for one hour at room temperature on a tilt. After incubation, the plate was washed (PBS, 0.05% tween) and loaded with 100 µl/well biotin-conjugated detection antibody anti-C1INH mAb 11-28-1 diluted 1:1000 (PBS, 0.05% tween) and incubated for one hour at room temperature on a tilt. The plate was washed (PBS, 0.05% tween), and 100 µl/well of 0.83 µg/mL horseradish peroxidase conjugated streptavidin (Thermo Fisher Scientific™) diluted in PBS 0.05% tween was loaded onto the plate followed by half an hour incubation at room temperature on a tilt. After the incubation the plate was washed. Next OPD tablets (Kem-En-Tec-Diagnostics A/S) were dissolved in 2 M citric acid (2 mg/2.5mL) and supplemented with 1 µL 30% hydrogen peroxide (VWR International) pr. 1 mL OPD solution. 100 µL of the OPD solution was added to each well. The reaction was stopped by adding 1 M sulphuric acid. The absorbance was measured at 490 nm, and a reference measurement at 620 nm was included. Serial dilutions of Berinert® (CSL Behring) were included for generation of the standard curve.

In vitro fluorescence assays

For analysis of the effect of HAE-causing SERPING1 gene variants on secretion, intracellular level and intracellular location of normal C1INH protein, HeLa or HepG2 cells were seeded in 6-well plates at a density of 2 x 10^5 cells/well one day prior to transfection. In studies of C1INH expression from the different SERPING1 expressing vectors, cells were transfected with a total of 900 ng SERPING1-encoding plasmids. For evaluating the effect of HAE-causing SERPING1 gene variants on normal C1INH, cells were transfected with 450 ng SERPING1[WT]-mCherry and 450 ng of the plasmid encoding the SERPING1 variant in question. For analysis of dose-response relation, cells were co-transfected with 450 ng SERPING1[WT]-mCherry, and either 50, 200, 450, 700 ng of the relevant pSERPING1[Variant] and pcDNA to a total of 1350 ng plasmid DNA). To analyze if a dominant negative effect was evident already 48 hours after transfection, cells were

transfected with 450 ng pSERPING1[WT]-mCherry and 450 ng of pSERPING1[Variant]. All transfections were performed using Turbofect (Thermo Fischer Scientific™). Medium was changed six hours post-transfection. Forty-eight hours after transfection medium was changed to serum-reduced Opti-MEM medium (Gibco, Life Technologies™Opti-MEM medium was used to omit additional fluorescence from the medium during fluorescence scanning analysis. Medium and cells were harvested 72 hours post transfection. However, for the analysis performed after 48 hours, the medium was changed to Opti-MEM 24 hours after transfection, and cells and medium were harvested 48 hours post transfection. The level of C1INH-mCherry secreted into the medium was determined by measuring the mCherry fluorescence intensity in the medium. The harvested cell medium was centrifuged to remove cell debris, and 200 µl of the medium was transferred to a 96-well non-transparent plate. The fluorescence intensity was analyzed using a fluorescence scanner (Thermo Fischer Scientific™) with excitation and emission parameters set to 530 nm and 590 nm, respectively. Flow cytometry was used for analyzing the intracellular level of C1INH-mCherry. Cells were trypsinized and transferred to a transparent V-bottom 96-well plate, and after repeated washing, cells were resuspended in 200 µL PBS. Ten or hundred thousand events were obtained during flow cytometry analysis on the BD LRSFortessa flow cytometer (BD Biosciences). mCherry was excited by the 561 nm laser and detected by the 600 LP and 615/20 bandpass filter. Data was analyzed using FlowJo, and the median fluorescence intensity (MFI) for mCherry positive cells was determined.

Widefield microscopy

To study the intracellular localization of C1INH, widefield microscopy was performed on either live or fixed cells. The cells were imaged with a Nikon T*i* ECLIPSE inverted microscope equipped with a pE-300 WHITE LED illumination unit, a Perfect Focus 3 system, a 100X objective and an Andor Zyla 5.5 mPixel camera. For live imaging an Oko-Lab heating chamber maintaining 37°C and 5% CO_2 was used. Image analysis was performed using ImageJ software.

Live microscopy

HeLa cells were seeded on round collagen-coated coverslips in 6-well plates at a density of 1.2 x 10^5 cells/well one day prior transfection. To study the intracellular localization of normal and mutated C1INH-mCherry, the cells were transfected with 900 ng pSERPING1[Variant]-mCherry. For studying the intracellular localization of normal C1INH in the presence of a HAE-causing SERPING1 variant, the cells were transfected with 450 ng pSERPING1[WT]-mCherry and either 450 ng pSERPING1[Variant] or 200, 450, 700 or 900 ng of pSERPING1[WT] or pSERPING1[c.551_685del]. In the dose experiment, an empty pcDNA vector was included to ensure all cells were transfected with equal amounts of plasmid DNA. Six hours after transfection, the medium was changed. All transfections were performed using Turbofect (Thermo Fischer Scientific™). The medium was removed after 48 hours after transfection, and reduced Opti-MEM medium supplemented with Hoechst was added to visualize the nucleus.

Immunofluorescent staining and imaging of fixed cells

HeLa cells or fibroblasts were seeded on collagen-coated coverslips in a 6-well plate at a density of 1.5 x 10^5 cells/well one day prior to transfection. The cells were fixed for 10 minutes in 4% paraformaldehyde 48 hours after transfection, and then permeabilized in ice-cold 70% ethanol. Coverslips were then washed three times in PBS and were then incubated for one hour at room temperature with primary antibody. The coverslips were then washed three times in PBS before incubating with the secondary antibody. The cells were transfected as described in the Results section. Transfection of HeLa cells was performed using Turbofect (Thermo Fischer Scientific™), whereas transfection of fibroblasts was performed using X-tremeGENE 9 DNA Transfection Reagent (Sigma-Aldrich) in the presence of penicillin, streptomycin and serum free medium. To label C1INH-HA, coverslips were stained with Anti-HA monoclonal primary antibody (16B12, Abcam) diluted at 1:100. In experiments where ER was not visualized with Tomato fused to an endoplasmic reticulum targeting sequence, ER was stained with a KDEL monoclonal primary antibody (10C3, Enzo) diluted 1:100. Endogenously expressed C1INH in fibroblasts were

visualized with Anti-C1INH monoclonal primary antibody (batch 11-28-1). All primary antibodies were subsequently labeled with AlexaFluor-488-conjugated secondary antibody diluted 1:400 (Thermo Fischer Scientific™). For staining of nucleic acids, 2 µg/mL Hoechst was added to the dilution of the secondary antibody. All stains and antibodies were diluted in PBS + 3% BSA. After staining, coverslips were washed three times in PBS before mounting on microscope slides with Glycergel mounting medium (Agilent Technologies Dako).

Quantification of protein aggregation in cell populations by imaging flow cytometry analysis

HeLa cells were seeded in 6-well plates at a density of 2 x 10^5 cells/well one day prior to transfection. The cells were co-transfected with 450 ng SERPING1[WT]-mCherry and 450 ng SERPING1[WT], SERPING1[c.551_685del], or pcDNA. Untransfected cells were included as a negative control. The cells were harvested for imaging flow cytometry analysis 72 hours post transfection. Twenty thousand events were obtained during imaging flow cytometry analysis on ImageStream®X Mark II (Amnis®). Samples were acquired with X60 magnification with low flow rate/high sensitivity using INSPIRE software (version 6.2). mCherry was excited by the 561nm laser (200 mW), and was detected in channel 4 (595-642 nm). Bright field images were collected in channel 1 (420-480 nm) and a 785nm laser (0.50mW) was responsible for darkfield images collected in channel 6 (745-800 nm). The data was analyzed using IDEAS® software. Gating of cells was performed as described in Supplementary Figure S13A-C. During analysis, a threshold mask (Supplementary Figure S13D, column 2) was used to define the area of interest within the cells. The threshold mask is designed to exclude pixels from analysis based on a threshold, which in our analysis was set to 76, creating a mask covering area with a high intensity of C1INH-mCherry (Supplementary Figure S13B, column 2). Mask and feature optimization revealed the "Max Contour Position" feature combined with the threshold mask Max Contour Position_Threshold (M04, Ch04 mCherry, 76)_Ch04 mCherry (mCherry Max Contour Position) to be able to differentiate cells with condensed C1-INH protein in the cell periphery from cells with diffusely located C1-INH protein.

Western blot analysis

For all Western blots, the samples were lysed in RIPA Lysis and Extraction buffer (ThermoFisher Scientific, 89901) supplemented with 10 mM NaF and 1 x complete protease inhibitor cocktail (Roche). Thereafter the samples were added XT Sample Buffer, 4X (Biorad, 161-0791) and XT Reduction Detergent 20X (Biorad, 161-0792) and boiled for 5 minutes before being loaded on the gel. Proteins were separated by SDS-PAGE and blotted in to a polyvinylidene fluoride (PVDF) membrane. Membranes were blocked in 5% skim-milk in TBS supplemented with 0.005 % Tween-20 (Sigma-Aldrich) and incubated overnight at 4°C with the primary antibody. The membranes were then washed in TBS supplemented with 0.005 % Tween-20 (Sigma-Aldrich) and incubated with a horseradish peroxidase (HPR)-conjugated secondary antibody and visualized by chemiluminescence using a horseradish peroxidase substrate (Bio-Rad, 170-5061). If more than one protein were to be visualized on a membrane, the membrane was then washed in TBS supplemented with 0.005 % Tween-20 (Sigma-Aldrich), stripped using the Restore PLUS Western Blot Stripping Buffer (ThermoFisher Scientific, 46430), washed in TBS supplemented with 0.005 % Tween-20 (Sigma-Aldrich), blocked in 5% skim-milk in TBS supplemented with 0.005 % Tween-20 (Sigma-Aldrich) and incubated overnight at 4°C with a new primary antibody. The remaining proteins were visualized as described above. Scanned blots were analysed using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, USA).

Analysis of separately expressed SERPING1 gene variants by western blot.

HepG2 cells were seeded in 6-well plates at a density of 2 x 10^5 cells/well at day one. At day two the cells were transfected with 900 ng of pSERPING1[Variant]-HA, and all transfections were performed using Turbofect (Thermo Fischer Scientific™). The medium was changed 24 hours post transfection, and the medium was harvested after additional 48 hours and the cells lysed. Samples were sonicated (Bioruptor, Diagnode) for 5 minutes and prepared for Western blotting as described earlier. C1INH-HA was detected using a monoclonal HA antibody diluted 1:1000

(ab130275, abcam) and a HRP-conjugated anti-mouse secondary antibody (Agilent Technologies Dako, P0447) and visualized as described above.

Separation of soluble and insoluble C1INH proteins

To evaluate the polymerogenic properties of the different SERPING1 variants, HeLa cells were seeded in 6-well plates at a density of 2 x 10^5 cells/well and transfected or co-transfected as described in the results. 24 hours after transfection the medium was removed, and 1.5 mL fresh medium was added. Cells and medium were harvested 72 hours after transfection. All transfections were performed using Turbofect (Thermo Fischer Scientific™) Cells were lysed in 200µL RIPA Lysis and Extraction buffer (ThermoFisher Scientific, 89901) supplemented with 10 mM NaF and 1 x complete protease inhibitor cocktail (Roche). Samples were centrifuged at 12,000g for 20 minutes at 4°C, the supernatant moved to a fresh tube and the pellets resuspended in 100 µL buffer containing 60 nM Tris-HCL, pH 6.8, 5 % SDS and 10% glycerol as described in Marques *et al*[2]. Samples were sonicated (Bioruptor, Diagnode) for 5 minutes and prepared for Western blotting as described earlier. C1INH protein was detected using a polyclonal C1INH antibody (CKBeR-2014) diluted 1:300 and a HPR-conjugated anti-rabbit (Agilent Technologies, P0448) secondary antibody and visualized as described above. The Human influenza hemagglutinin (HA)-tag was detected using a monoclonal HA antibody diluted 1:1000 (ab130275, abcam) and a HPR-conjugated anti-mouse (Agilent Technologies, P0447) secondary antibody and visualised as described above. Beta-actin was detected using a monoclonal Beta-actin antibody (ab6276, abcam) and a HPR-conjugated anti-mouse (Agilent Technologies, P0447) secondary antibody and visualized as described above. Vinculin was detected using a monoclonal Vinculin antibody diluted 1:10000 (Sigma-Aldrich, V9131), and HPR-conjugated anti-mouse (Agilent Technologies, P0447).

Co-immunoprecipitation

Co- Immunoprecipitation was performed to study the direct interaction between normal C1INH and C1INH encoded by HAE-causing SERPING1 gene variants. HeLa cells were seeded in p15 dishes at a density of 60 x 10^5 cells/dish at day one. At day two, the medium was removed, and medium supplemented with 25 µM Chloroquine was added one hour before transfection. The cells were transfected or co-transfected with 40 µg plasmid DNA in total as described in the text. All transfections were performed using calcium phosphate. To increase the transfection efficiency the medium was removed 24 hours after the transfection and the cells were shocked by incubating the cells with 10% dimethyl sulfoxide (DMSO) for two minutes. The DMSO was removed by washing twice in PBS. The cells were lysed as described above, and 2 µl DNAse I (Sigma-Aldrich, AMPD1-1KT) was added to the cell lysate. From then on the cell lysate was kept on ice. All samples were sonicated (Bioruptor, Diagnode) in 3 x 15 minutes and centrifuged at 4,000 rpm (Eppendorf Centrifuge, 5810R) for 15 minutes at 4°C. A small amount of the cell lysate was saved (input). Monoclonal anti-V5 antibody conjugated to cyanogen bromide-activated beaded agarose (Sigma-Aldrich, A7345) was washed three times in IP buffer (20mM Trish-HCL, 100 mM NaCl, 1% (v/v) Triton X-100, 2 mM $MgCl_2$, 1% (v/v) glycerol and 1 x complete protease inhibitor cocktail (Roche)) and then four times in RIPA Lysis and Extraction buffer (ThermoFisher Scientific, 89901). Between each wash step, the beads were centrifuged at 3.5g for one minute at 4°C. The beads were blocked in 1% (v/v) Bovine Serum Albumin (BSA) dissolved in IP buffer for 15 minutes at room temperature with rotation. The BSA solution was removed after centrifugation, and the beads were washed six times in the IP buffer. The cell lysate was added to the beads and incubated with rotation at 4°C overnight. The cell lysate was removed the following day, and the beads were washed six times with the IP buffer, 6 times with an IP buffer with a higher salt concentration (150 mM NaCl instead of 100mM NaCl) and once with the normal IP buffer. To elute the proteins from the beads, elution buffer (20mM Tris-HCL, 100mM NaCl, 1% (v/v) Triton X-100, 2mM $MgCl_2$, 1% SDS and sodium dodecyl sulphate (SDS) and 1 x complete protease inhibitor cocktail (Roche)) was added, and the beads were kept at 50°C for

ten minutes. The input and co-IP (elute) fractions were prepared for Western blotting as described earlier. C1INH-HA protein was detected using a monoclonal HA antibody diluted 1:1000 (ab130275, abcam) and a HPR-conjugated anti-mouse (Agilent Technologies, P0447) secondary antibody, and visualized as described above. C1INH-V5 was detected using a monoclonal V5-Tag antibody (Cell Signaling Technology, 13202) and a HPR-conjugated anti-rabbit (Agilent Technologies, P0448) secondary antibody, and visualized as described above.

mRNA quantification by qPCR

For SERPING1 mRNA quantification, total RNA was isolated from control and patient-derived fibroblasts using TRIzol® reagent (Thermo Fisher Scientific™) according to manufacturer's protocol. The total RNA samples were treated with DNase I (Thermo Fisher Scientific™) according to manufacturer's protocol. The RNA concentration in each sample was normalized following spectrophotometric measurements using Nanodrop. First strand cDNA synthesis was performed on 500 ng RNA using the Maxima First Strand cDNA Synthesis for qPCR (Thermo Fisher Scientific™) according to manufacturer's protocol. All qPCR reactions were performed using the Maxima Probe qPCR Master Mix (2X) (Thermo Fisher Scientific™). For SERPING1 mRNA quantification the following primers and probes were used: SERPING1-forward: 5'-AACCTGTGGCCCATTTCATT-3', SERPING1-reverse: 5'-TCTGGGGTACCAGGATCAC-3', SERPING1-probe: 5'-AGCTCTCCCACAATCTGAGTT-3' (FAM-BHQ-1). For quantification of the RPLP0 reference gene, the following primers and probes were used: RPLP0-forward:5'-GGCGACCTGGAAGTCCAACT-3', RPLP0-reverse: 5'-CCATCAGCACCACAGCCTTC-3', RPLP0-probe: 5'-ATCTGCTGCATCTGCTTGGAGCCCA-3' (FAM-Tamra). The relative mRNA expression levels were calculated using the X0-method (4).

Identification of mRNA with c.551_685 deletion in patient-derived fibroblasts by gel analysis

PCR was performed on cDNA synthesized from RNA purified from either control NHDF-03 fibroblasts or fibroblasts derived from the patient carrying the c.551_685del mutation. We used

primers spanning the c.551_685 SERPING1 deletion (exon 4 deletion), by using a forward primer (5' TCTGCTCTGACTTGGAGAGTCA 3') and a reverse primer (5' TGGCGTCACTGTTGTTGCTTAG 3'), binding to exon 3 and exon 5, respectively. The PCR products were visualized on an agarose gel. PCR products amplified from the wildtype and mutated SERPING1 alleles are 387 bp and 252 bp, respectively.

Production of lentiviral vectors

Lentiviral vectors were produced by calcium phosphate transfection of 293T cells (seeded at 3 x $4x10^6$ cells/p10 dish) with the three packaging plasmids (3 µg pRSV-Rev, 3.75 µg pMD.2G and 13 µg pMDLg/pRRE) and one of the transfer plasmids (13 µg pCCL/PGK-SERPING1[Variant] or pCCL/PGK-eGFP). Twenty-four hours post transfection the medium was changed, and 72 hours post transfection the medium was harvested and filtered through a 0.45µm filter (Sarstedt) and polybrene (Sigma Aldrich) was added to a final concentration of 8µg/mL.

Transduction of fibroblasts

To evaluate the inhibitory effect of c.551_685del on endogenously expressed C1INH in control fibroblasts, NHDF-03 fibroblasts were seeded at a density of 1 x 10^5 cells/well in 6-well plates in triplicates one day prior to transduction. The cells were transduced with lentiviral vectors encoding eGFP or c.551_685del 24 hours after seeding at an estimated MOI of 1 with a final concentration of 8 µg/mL polybrene and left overnight. The following day, the medium was replaced with 1.5 mL fresh medium and harvested 72 hours later for measuring C1INH secretion by sandwich ELISA. To study the effect of delivery of a therapeutic SERPING1 gene to control and patient-derived fibroblast, control and patient-derived fibroblasts were seeded at a density of 1 x 10^5 cells/well in 6-wells plates in triplicates one day prior to transduction. The cells were transduced with lentiviral vectors encoding normal C1INH or eGFP 24 hours after seeding at an estimated MOI of 5 with a final concentration of 8 µg/mL polybrene and left overnight. The following day, the medium was replaced with 1.5 mL fresh medium and harvested 72 hours later for measuring C1INH secretion by sandwich ELISA.

HeLa population expressing normal and C1INH$_{Gly162_Pro206del}$

To create HeLa-WT and HeLa-c.551_685del cell populations, naïve HeLa cells were seeded at a density of of 2 x 10^5 cells/well in 6-wells plates at day 1. At day 2, the cells were transduced with lentiviral vector encoding eGFP, normal or C1INH$_{Gly162_Pro206del}$ at an estimated MOI of 1.25 with a final concentration of 8 µg/mL polybrene and left overnight. At day 3, the medium was replaced with 1.5 mL fresh medium, and at day 11 each of the HeLa populations (three of each) and naïve HeLa cells were reseeded in triplicates at a density of 2 x 10^5 cells/well in 6-wells plates. The following day, at day 12, the cells were either kept as a naïve population or transduced with lentiviral vectors encoding normal C1INH or eGFP at an estimated MOI of 2.5 with a final concentration of 8 µg/mL polybrene and left overnight. The following day, the medium was replaced with 1.5 mL fresh medium. 72 hours later, the medium from each population seeded in triplicates was harvested and pooled before measuring the C1INH secretion by sandwich ELISA.

Statistical analysis

All numerical data are presented as the mean ± SEM. Statistical analyses were performed with One-way ANOVA with multiple comparisons for analysis of three or more groups, whereas 2-tailed Student's t test was only used for comparison of fewer than three groups. In all cases, P less than 0.05 was considered significant.

Ethics

All subjects gave informed written consent to the skin biopsy. The study was conducted in accordance with the Helsinki II Declaration and approved by the Regional Scientific Ethics Committee for Southern Denmark (Project-id: S-20120116).

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