Creation of the first monoclonal antibody recognizing an extracellular epitope of hABCC6

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Mutations in the ABCC6 gene result in calcification diseases such as pseudoxanthoma elasticum or Generalized Arterial Calcification of Infancy. Generation of antibodies recognizing an extracellular (EC) epitope of ABCC6 has been hampered by the short EC segments of the protein. To overcome this limitation, we immunized bovine FcRn transgenic mice exhibiting an augmented humoral immune response with Human Embryonic Kidney 293 cells expressing human ABCC6 (hABCC6). We obtained a monoclonal antibody recognizing an EC epitope of hABCC6 that we named mEChC6. Limited proteolysis revealed that the epitope is within a loop in the N-terminal half of ABCC6 and probably spans amino acids 338–347. mEChC6 recognizes hABCC6 in the liver of hABCC6 transgenic mice, verifying both specificity and EC binding to intact hepatocytes.

Keywords: ABCC6; ectopic calcification; extracellular epitope; monoclonal antibody

Connective tissue calcification (also called ectopic or soft tissue calcification) is pathological mineralization (mostly Ca-phosphate precipitation) of tissues or organs. Plasma concentrations of the endogenous metabolite pyrophosphate (PPi) control ectopic calcification by inhibiting calcium hydroxyapatite crystal formation [1,2]. PPi levels are regulated by ABCC6, which facilitates nucleoside triphosphates (mostly ATP) release from hepatocytes, and Ectonucleotide Pyrophosphatase/Phosphodiesterase 1 (ENPP1), an ectonuclease that cleaves ATP promptly in the microvasculature of the liver, generating AMP and PPi. ABCC6 is expressed in the basolateral membrane of hepatocytes [3,4] and at much lower levels in the kidneys. Two inherited calcification disorders were connected to hepatic PPi generation. Mutations in the ABCC6 gene result in pseudoxanthoma elasticum (PXE), while mutations in the ENPP1 gene cause Generalized Arterial Calcification of Infancy (GACI). In some cases, GACI is due to ABCC6 mutations and the two diseases have a considerable overlap in their phenotype [5]. Both phenotypes include ectopic calcification; however, whereas PXE is

Abbreviations
bFcRn, bovine neonatal Fc receptor; DPBS, Dulbecco’s phosphate-buffered saline, with 0.9 mM CaCl2 and 0.5 mM MgCl2; EC, extracellular; ENPP1, Ectonucleotide Pyrophosphatase/Phosphodiesterase 1; FA, Freund’s adjuvant; GACI, Generalized Arterial Calcification of Infancy; hABCC6, human ATP-binding cassette subfamily C member 6; HEC, Human Embryonic Kidney 293 cells; HTVI, hydrodynamic tail vein injection; MDCKII, Madin-Darby Canine Kidney II cells; mmAb, mouse monoclonal antibody; pAb, polyclonal antibody; PPi, inorganic pyrophosphate; PXE, pseudoxanthoma elasticum; rmAb, rat monoclonal antibody; wt, wild-type.
characterized by a later onset and less severe symptoms, GACI affects newborns (calcification can be detected even \textit{in utero}) and it often leads to early death. Patients with PXE typically have a normal life span, but the disease has substantial adverse effects that have a negative impact on the quality of life. Plasma PPi levels are low in both disorders, and the same is true for the \textit{Abcc6} and \textit{Enpp1} knockout mouse strains [1,6]. However, whereas PPi levels are not detectable in GACI, PPi levels in PXE reach 30–40% of the normal range (for recent reviews see Ref. [7–9]). This difference provides the most parsimonious explanation of the phenotypic difference between the two diseases. PPi levels in PXE are maintained by nucleoside triphosphates release from extrahepatic organs or cells, because the released nucleoside triphosphates can be converted to PPi by ENPP1, which is present in many tissues. On the other hand, since in GACI patients there is no substitute for the activity of ENPP1, PPi is not produced even in the presence of nucleoside triphosphates.

\textbf{ABCC6} belongs to the C-subfamily of ABC proteins, which consists of 13 members. Apart from the lack of an N-terminal TMD0 domain in ABCC4, ABCC5, and ABCC7 (CFTR), ABCC proteins share a similar membrane topology [10] (Fig. 1A), which was verified by the cryo-EM structures of CFTR, Abcc1, and ABCC8/SUR1 [11–13]. ABCC6 is an organic anion transporter [14,15], and a central question has been for a long time to link its function as a liver transporter to the phenotype and pathomechanism of systemic calcification diseases. The discovery that ABCC6 facilitates nucleoside triphosphates (mostly ATP) release from hepatocytes into the circulation has provided important insights [2]. However, it is still not known whether ABCC6 transports nucleoside triphosphates or an another ABCC6-mediated molecular mechanism facilitates nucleoside triphosphates release.

Specific antibodies are essential tools in research; furthermore, they are widely used in diagnostics and even as therapeutic drugs. There are several well-characterized antibodies developed for ABCC6 research (Fig. 1A) [3,14,16], but they all recognize intracellular epitopes. ABCC subfamily members have characteristically short extracellular (EC) loops, which has precluded the generation of antibodies recognizing EC ABCC epitopes, whereas such antibodies have long been available for other ABC transporter subfamilies [17,18]. Here, we have generated a three-dimensional homology model of human ATP-binding cassette subfamily C member 6 (hABCC6) using the recently published cryo-EM structures of bovine Abcc1 [12,19], which shows that the peptide segments accessible from the EC space are indeed very short. We also report the first successful production of a specific mouse monoclonal antibody (mmAb) that binds to a linear EC epitope in the native hABCC6 protein.

\textbf{Materials and methods}

\textbf{Study approval and animals}

\textit{Abcc6} \textsuperscript{−/−} mice were generated on 129/Ola background and backcrossed into a C57BL/6J > 10 times [20]. CD1 mice...
were obtained from the breeding of heterozygous ttw \((E_{npp1}^{+/--})\) mice [21]. All animal protocols were approved by the Food Chain Safety and Animal Health Directorate of the Government Office of Pest County, Hungary (XIV-I-001/707-4/2012). Bovine FcRn transgenic mice carry five copies of the bovine neonatal Fc receptor (bFcRn) alpha chain on a BALB/c genetic background [22]. Experiments on bFcRn transgenic mice were carried out in strict accordance with the recommendations of the Guide of the Institutional Animal Care and Ethics Committee at ImmunoGenes Ltd, in accordance with the ethical approval PEI/001/2196-2/2013 issued by the Food Chain Safety and Animal Health Directorate of the Government Office of Pest County, Hungary. All experiments were conducted according to national guidelines. Mice were kept under routine laboratory conditions with a 12-h light-dark cycle, a constant temperature of 23 °C, and with ad libitum access to water and chow.

**Comparative modeling of hABCC6**

The sequences of bovine and human ABCC1 and hABCC6 were aligned by the Modeller v9.24 [23] ‘salign’ method. Sequence regions without appropriate template structures (altogether 193 aa) were removed from the alignment; therefore, the final structure model does not contain these regions (first 71 aa, and longer loop regions on the molecular surface of ABCC6). Next, one hundred models were generated by the ‘automodel’ method. The following bovine Abcc1 structures were utilized: PDB codes: 5uj9, 5uja, 6bhu, 6u0, [12,19]. The generated models were sorted according to the Discrete Optimized Protein Energy (DOPE) and Statistically Optimized Atomic Potential (SOAP) scores, and the best models were investigated to eliminate models with charged amino acids facing the lipid phase. Then, the coordinates of the best remaining model were transformed by the TMDET algorithm [24] in order to align the membrane plane with the XY plane, and Charmm-gui [25] was applied to generate a lipid environment around the protein. For energy minimization, GROMACS version 2018.8 (http://www.gromacs.org) [26] was utilized. The quality of the final model was checked by ProCheck [27], and only 8 out of the 1209 residues (0.7%) were out of the allowed regions in the Ramachandran plot.

**Cell culture**

Human Embryonic Kidney 293 cells (HEK) and Madin-Darby Canine Kidney II cells (MDCKII) cell lines were grown in DMEM supplemented with l-glutamine, penicillin/streptomycin, and 10% FBS. Following hypoxanthine–aminopterin–thymidine medium selection, hybridoma cells were grown under similar conditions. To maintain a high level of ABCC6 expression, HEK cells expressing rat Abcc6 and HEK cells expressing hABCC6 [1] were grown under continuous puromycin selection (2 μg·mL⁻¹). Before reaching confluence, cells were harvested by scraping in ice cold PBS, washed in 50 mL cold PBS, and kept on ice until they were mixed with the adjuvant for immunization or used for flow cytometry.

**Immunization of mice and hybridoma production**

Eight-week-old female bFcRn mice were immunized by intraperitoneal injections of \(5 \times 10^8\) HEK cells expressing high levels of hABCC6, combined with adjuvant. Complete Freund’s adjuvant (CFA) was used for the first injection, and incomplete FA was used for booster injections. Blood sampling for the testing of immune sera was done by retroorbital puncture. Mice selected for hybridoma generation were euthanized by cervical dislocation, and their spleens were harvested, and splenocytes were used for fusion with SP2/0-Ag14 mouse myeloma cells (for a detailed description of the process see Ref. [28]). mAb isotype was determined using RapidYield mmAb isotyping kit (Crystal Chem, 80601) according to manufacturer’s instructions.

**Screening by flow cytometry**

HEK cells were harvested by scraping. Cells were homogenized by gently pipetting in PBS and then filtered through a 100 μm cell strainer to avoid clumps. For each sample tested, \(2 \times 10^5\) cells of the hABCC6+ and hABCC6− cell lines were fixed in 4% formaldehyde solution for 10 min at room temperature followed by a 30-min blocking step in PBS containing 1% BSA. During the screening process, supernatant samples were added to cells at a dilution of 1 : 2 and detected by a goat anti-mouse eFluor660 secondary antibody (Invitrogen, 50-4010-82). Flow cytometry measurements were done using a BD FACSCalibur cytometer. mEChC6 was conjugated to Alexa Fluor 488 (Invitrogen, A20000) according to the manufacturer’s instructions. The conjugated antibody was added to HEK cell suspensions for 30 min, diluted 1 : 100. In the case of intracellular labeling with M6II-7, an additional permeabilization step with 0.1% Triton X for 10 min was applied before adding the primary antibody at a dilution of 1 : 50 for 30 min. Cells were incubated with goat anti-rat (Invitrogen, A11081) secondary antibody for 30 min (1 : 250), and FACs analysis was performed with an Attune NxT flow cytometer.

**Screening on MDCKII cells**

MDCKII cells were seeded on 96-well plates (Greiner Bio-One, Kremsmünster, Austria, 655180) in complete DMEM containing 10% FBS, at a density of \(1.5 \times 10^4\) cells per well. Sera or supernatant samples were tested on the plates before the cells reached confluence, using the following
protocol: Plates were washed in cold Dulbecco’s phosphate-buffered saline (DPBS; Sigma-Aldrich, St. Louis, MO, USA, 56064C) containing 0.9 mM CaCl₂ and 0.5 mM MgCl₂ and then incubated for 30 min in cold blocking solution containing 2.5% bovine serum in DPBS. Tested sera were added to wells at dilutions of 1 : 100, 1 : 500, and 1 : 1000 in blocking solution, and the plates were incubated for 60 min. For supernatant samples, the initial tested dilution was 1 : 2. After washing with DPBS, goat anti-mouse secondary antibody (Invitrogen, A11029) was added at 1 : 500 in blocking solution for 60 min; the cells were then washed in cold DPBS. Cells that lost membrane integrity during the labeling process were revealed by a DAPI staining step (1 : 500, 10 min). Throughout the process, all reagents were kept on ice, and plates were also incubated on ice to avoid potential internalization of antibodies bound on the cell surface. Plates were immediately imaged by an ImageXpress Micro XLS automated microscope, Molecular Devices, San José, CA, USA.

Detection of hABCC6 in mice by immunohistochemistry

hABCC6 expressing liver samples were obtained from ~ 12-week-old wild-type (wt) or Abcc6−/− female mice by hydrodynamic tail vein injection (HTVI) of wt hABCC6 in pLIVE plasmid as described [29].

Liver lobes harvested from mice expressing hABCC6 were snap-frozen in 2-methylbutane cooled by liquid nitrogen, and immunohistochemistry was performed as described in [29]. Primary antibodies were applied for 1 h at room temperature diluted in blocking solution, A488-labeled mEChC6 at a concentration of 2.9 µg·mL⁻¹, and rat monoclonal antibody (mAb) M6II-7 [3] (Monosan, MON9047) at a dilution of 1 : 100. To detect M6II-7, goat anti-rat secondary antibody (Invitrogen, A11081) was applied 1 : 250, 60° at room temperature. Slides were mounted with coverslips in Vectashield (Vector Laboratories, Burlingame, CA, USA, H-1200), and images were acquired using a Zeiss LSM710 inverted confocal microscope with 20× and 63× objective (Carl Zeiss, Jena, Germany). To minimize the cross-talk between imaged channels, sequential image collection was used. Cells are shown as a single confocal section. All images were processed using ZEN software (Carl Zeiss).

Immunoblotting and limited proteolysis

Two microgram protein samples of hABCC6 expressing Sf9 cell membrane fractions [14] and 10 µg of ABCC1, ABCC2, ABCC3 [30–32], and rat Abcc6 [16] expressing Sf9 cell membrane fractions were loaded on 7.5% polyacrylamide gels and transferred to polyvinylidene difluoride membranes. ABCC6 variants were detected with polyclonal antibody (pAb) HB6 (1 : 2000) [14], mAb mEChC6 (1 : 2000), and pAb K14 (1 : 2000, a kind gift of Bruno Stieger, Zurich). The mAbs MRPm6 [33], M2I-4, and M2I-II-9 [34] (kind gifts from Rik J. Schepers, Amsterdam) were used for the detection of ABCC1, ABCC2, and ABCC3, respectively. HRP-conjugated anti-rabbit (Sigma-Aldrich, 12–348, 1 : 15 000) and anti-mouse (Jackson ImmunoResearch, West Grove, PA, USA, 715–035–150, 1 : 10 000) secondary antibodies and WesternBright ECL (Advansta, San Jose, CA, USA, K-12045) were used to develop chemiluminescent signals.

Limited proteolysis of hABCC6 was performed as described in [35]. Briefly, 80 µg of isolated Sf9-wtABCC6 membranes was digested by 0.1–8 µg trypsin (type XIII; Sigma) in 200 µL of reaction buffer (50 mM MOPS, 50 mM KCl, 0.5 mM EGTA, 2 mM dithiothreitol, pH 7.0) for 10 min at 4°C. Reaction was stopped by adding excess soybean trypsin inhibitor (Sigma, type 1-S). Thereafter, membranes were washed with reaction buffer, dissolved in loading buffer and loaded on 10% polyacrylamide gels, then subsequently blotted, and probed with specific Abs as described above.

In vivo labeling of hABCC6

hABCC6 was expressed in vivo by HTVI of the pLIVE expression plasmid as described earlier [29]. Three days later, A488-conjugated mmAb mEChC6 was injected in the lateral tail vein at 75 µg/animal in 100 µL sterile PBS [36]. Twenty-four hours after mAb injection, the mice were euthanized and potential unbound antibodies remaining in their circulation were washed out by perfusion with saline. Liver samples were processed and labeled with control mAb M6II-7 as described above.

Results and Discussion

The problem in generating antibodies recognizing ABCC6 from the EC space is that this protein has only short external epitopes, which as such are difficult to target. In fact, no antibodies recognizing an external epitope of any of the ABCC family members are yet available. To better identify the EC loops of ABCC6, we generated a three-dimensional homology model. Our model is based on the coordinates of the four structures of bovine Abcc1 [12,19], a close relative of hABCC6 (sequence identity is 45.95%). The EC regions in the four Abcc1 structures are highly similar; therefore, the structure of the predicted EC segments is reliable. Fig. 1B shows the molecular surface of the EC and the membrane-bound regions of hABCC6. Membrane lipids are also shown as they are an integral part of the presentation of the structure. It is obvious that the segments potentially accessible from the EC space for an antibody should extrude from the
lipid phase. As shown in Fig. 1A,B, four of the eight EC loops satisfy this criterion (residues 338–347, 559–561, 974–983, and 1193–1197).

To overcome the challenge associated with the low immunogenicity of the short EC loops, we used bFcRn transgenic mice that exhibit an increased humoral immune response [37]. Mice were immunized with intact HEK cells expressing hABCC6 in the plasma membrane, and monoclonal antibodies were produced using the hybridoma technology. The specificity of hybridoma clones was tested on adherent MDCKII cells expressing wt hABCC6, using a microscopy-based platform, and on HEK cells, using flow cytometry. For each clone, hABCC6-positive and -negative cells were labeled with supernatant samples and appropriate secondary antibodies under identical conditions, and the resulting fluorescent signals were compared. Approximately 1500 hybridoma microcultures were tested, and 80 positive cultures underwent additional cloning steps to ensure their monoclonal origin (data not shown).

The most promising clone identified by flow cytometry-based screening was mEChC6 (IgG2a isotype). The mAb produced by this clone was analyzed on liver sections of mice transiently expressing hABCC6 in the liver. In this experimental setup, hABCC6 is transiently expressed in vivo in the liver of mice by HTVI of a hABCC6 expression plasmid [29,38,39]. Our earlier work has shown that HTVI injection results in the expression of hABCC6 in its native subcellular localization, the basolateral plasma membrane of murine hepatocytes [29,38,39]. Mouse hepatocytes not expressing the human protein serve as an inherent negative control. Since mmAb mEChC6 is of mouse origin, using it to visualize hABCC6 in mouse tissue samples is problematic because of the high background resulting from the necessary use of an anti-mouse secondary antibody. To overcome this obstacle, we directly labeled mmAb mEChC6 with a fluorophore. Immunohistochemical analyses of 7 µm sections of hABCC6-expressing CD1 mouse livers revealed that mAb mEChC6 is indeed hABCC6-specific because it reacts with its target protein with the same efficiency as the well characterized rmAb M6II-7 (Fig. 2A), without any cross-reaction with mouse proteins, including mAbcc6.

To further characterize the specificity of mEChC6, we used western blot technique to test its reactivity against human ABCC1, ABCC2, and ABCC3, as well as rat Abcc6 (all expressed in S99 cells). Expression of each ABC transporter was verified using specific antibodies (see Fig. 2B for positive controls; and Fig. 2D for the Coomassie-stained blot indicating the amounts of protein loaded on the gels). As shown in Fig. 2C, the monoclonal antibody mEChC6 specifically recognizes hABCC6 without showing cross-reactivity with the human homologues or the rat ortholog. These results also imply that in addition to the demonstration of its selectivity, this also means that mEChC6 recognizes a linear epitope of hABCC6.

To map the epitope of mEChC6, HEK cells expressing hABCC6 or rAbcc6 with mEChC6-A488 and M6II-7 (the latter recognizing an intracellular epitope) were analyzed by FACS, either in fixed and permeabilized, or fixed but not permeabilized conditions. As shown in Fig. 3A, mEChC6 and M6II-7 did not label parental HEK cells or HEK cells expressing rAbcc6. Conversely, mEChC6 was able to detect hABCC6 in nonpermeabilized hABCC6-expressing HEK cells, while mAb M6II-7 reacted only when HEK-hABCC6 cells were permeabilized. To further characterize mEChC6, we performed in vivo immunolabeling, injecting mice expressing hABCC6 with mEChC6-A488 conjugate. Immunohistochemical analyses of liver cryosections (Fig. 3B) showed that A488-labeled mmAb mEChC6 reacted in vivo with wt hABCC6 in hepatocytes, and its signal overlapped with that of the positive control mmAb M6II-7, further demonstrating both the specificity and EC binding of mmAb mEChC6.

In further experiments, hABCC6 was subjected to limited tryptic proteolysis and fragments were detected with mEChC6, or HB6 and K14 (Fig. 4A), recognizing an N-terminal and C-terminal epitope, respectively (Fig. 1A). We observed a band of ~100 kDa that appeared on blots developed by HB6 or mEChC6 (indicated by arrowhead), but was absent on the blot developed by K14. Conversely, a band of ~65 kDa was apparent on the K14 blot but was missing from the HB6 and mEChC6 blots (indicated by arrowhead). The abundance of these signals corresponded to the extent of trypsin digestion. We interpret the two bands to result from the first tryptic cleavage at the linker region, splitting ABCC6 to an N-terminal and a C-terminal half (Fig. 1A). Taken together, our results indicate that mEChC6 recognizes an external epitope in the N-terminal half of ABCC6.

The N-terminal half contains the ABCC family-specific transmembrane domain and a linker region (TMD0 and L0). To further refine the epitope, we expressed ΔABCC6, a truncated version lacking TMD0 and L0 (Fig. 1B) in S99 insect cells. The microsomal (membrane) fraction of S99 cells expressing wt ABCC6 or ΔABCC6 was subjected to western blot analysis, using mEChC6 and HB6, a rabbit pAb recognizing an epitope in the L0 domain [14]. While HB6
Fig. 2. Specificity of the new anti-hABCC6 mAb, mEChC6. (A) Immunohistochemical detection of hABCC6 expressed in mouse liver with mAb mEChC6 (green) and with mAb M6II-7 (red). Nuclei are visualized with DAPI (blue). Liver cells not expressing the human protein serve as negative control. (B) Western blots of hABCC1, hABCC2, hABCC3, hABCC6, and rat Abcc6. (C) Western blot of hABCC1, hABCC2, hABCC3, hABCC6, and rat Abcc6 probed with mAb mEChC6. All ABCC proteins were expressed in Sf9 insect cells; 25 µg total protein per lane was loaded in the gels. (D) Coomassie stain of the blot shown in panel C.
does not detect ΔABCC6, mEChC6 reacts with both protein variants, indicating that the epitope of mEChC6 is most probably located in the TMD1 region of the protein.

The length of linear epitopes recognized by Abs range between 4 and 12 amino acids, and in many cases, even a single conservative amino acid substitution can result in decreased binding [40]. In light of the specificity of mEChC6, we compared the EC loops of hABCC6 with the corresponding regions of the rat and mouse Abcc6, ABCC1, ABCC2, and ABCC3 (Fig. 4C). Each ABCC protein shows more than one conservative amino acid substitution in their EC segments corresponding to aa 338–347 of human ABCC6 (Fig. 4C). Based on the alignment, we conclude that the most probable candidate for the linear epitope recognized by mEChC6 is the EC segment of 338–347 (FIGDPKPPA).

In summary, the ABCC6 homology model generated herein demonstrates that ABCC6 possesses very short EC segments. We show here that the inherent difficulty to obtain antibody against very short EC segments of hABCC6, or possibly other multispanning membrane proteins, can be overcome by cell-based immunization of a transgenic mouse strain in which FcRn overexpression augments T-cell-dependent humoral immune response. Several PXE and GACI patients harbor missense ABCC6 mutations that result in folding and/or
trafficking defects. Most of these disease-associated mutants are transport-competent but cannot reach the basolateral membrane [29,39]. Pharmacological compounds, the so-called chemical chaperones, may improve the stability of such mutants thereby rescuing trafficking to the plasma membrane and physiological function. Preclinical studies using 4-phenylbutyrate toward an allele-specific intervention in PXE have been described [39]. The antibody described here may facilitate screening for similar compounds, and we are thus interested in developing mAbs that recognize EC epitopes of ABCC1 and ABCC7/CFTR.

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Conflict of interest

PKJ, BS, and IK are employees of ImmunoGenes using patented, genetically modified animals that over-express FcRn for the production of polyclonal and monoclonal antibodies. The remaining authors have no conflict of interest to declare.

Fig. 4. Mapping the hABCC6 region bound by mmAb mEChC6. (A) Limited proteolysis of hABCC6 expressed in Sf9 cells. Eight microgram of protein was loaded per lane, digested with the indicated amounts of trypsin for 10 min. hABCC6 fragments were detected with mEChC6 and specific pAbs with known epitopes in the N-terminal (HB6) and C-terminal (K14) positions of the protein. (B) Western blot detection of wt hABCC6 and ΔABCC6 with pAb HB6 (left) and with mAb mEChC6 (right). Both proteins were expressed in Sf9 cells, and 2 µg total protein from the membrane fraction was loaded per lane. (C) Sequence alignment of the EC segments of human, rat, and mouse Abcc6 as well as that of human ABCC1, ABCC2, and ABCC3 in the TMD1 and TMD2 regions.
Author contributions

EK contributed to immunization and screening of sera, EK, ZH and ZsM performed screening and characterisation of the candidate clones; BSz, AI and PKJ generated hybridomas; EK, PKJ and GyV performed FACS analyses; EK, DD, NT, KP and VP were involved in animal experiments and in liver histochemistry; EB and EK performed and analysed the limited proteolysis experiments GET created and interpreted the 3D homology model IK supervised the project and designed experiments AV, supervised the project, designed experiments and compiled the manuscript.

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