Interphotoreceptor matrix proteoglycans IMPG1 and IMPG2 proteolyze in the SEA domain and reveal localization mutual dependency

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Abstract

The interphotoreceptor matrix (IPM) is a specialized extracellular mesh of molecules surrounding the inner and outer segments of the photoreceptor neurons. The interphotoreceptor matrix proteoglycan 1 and 2 (IMPG1 and IMPG2) are major components of the IPM. Both proteoglycans possess SEA (Sperm protein, Enterokinase and Agrin) domains, which are described to support protein proteolysis. Interestingly, humans with mutations in the SEA domains of IMPG1 and IMPG2 are associated with retinitis pigmentosa (RP) vision disease. This work investigates the SEA-mediated proteolysis of IMPG1 and IMPG2 and its significance to IPM physiology. Western blot relative molecular mobility analysis of IMPG1 from retina confirmed protein proteolysis. Point mutations in the SEA domain of IMPG1 inhibit protein proteolysis, as shown in cell culture assays. Likewise, IMPG2 also proteolyzes at the SEA domain generating two subunits: a membrane-attached and an extracellular peptide, as seen by western blot and immunohistochemical assays. Importantly, the extracellular portion of IMPG2 traffics toward the outer segment IPM by a mechanism dependent on IMPG1. These results demonstrate that proteolysis is part of the maturation of IMPG1 and IMPG2, which is important for protein localization and linked to vision diseases. Moreover, the results indicate interdependency between IMPG1 and IMPG2, which helps understand the molecular mechanisms involved in the development of vision diseases in patients with defective IMPG molecules.

Introduction

The extracellular space between neurons and glial cells is supported by a network of macromolecules known as the extracellular matrix. In the retina, the interphotoreceptor matrix (IPM) is a specialized extracellular matrix surrounding the inner and outer segment (IS and OS) of photoreceptors, extending between the Müller cells and the retina pigment epithelium (RPE). It is thought that the IPM has a fundamental role in the transportation of nutrients and metabolites in between photoreceptors and RPE. Other implied roles of the IPM are extracellular calcium buffer and facilitate growth factor presentation and cell-cell communication (1).

The IPM is primarily composed of two retinal-specific proteoglycans, the interphotoreceptor matrix proteoglycan 1 and 2 (IMPG1 and IMPG2), also known as SPARC and SPARCAN or IPM150 and IPM200, respectively (2-6). Both proteoglycans are densely glycosylated, rich in chondroitin sulfate (ChS), and share common protein domains termed SEA (Sperm protein, Enterokinase and Agrin) (7). IMPG1 and IMPG2 have two SEA domains in their structure, SEA-1 and SEA-2 (Fig. 1a) (3,4,6,8).

The SEA domains have been associated with an extensive family of highly glycosylated proteoglycans called mucins, many of which express well-studied SEA domains (9). Depending on the amino acid sequence, some mucin SEA domains undergo intramolecular proteolysis while others do not (10-13).

IMPG1 is a secreted proteoglycan homogeneously distributed through the retina IS and OS (14). Notably, missense mutations only affecting the SEA-2 domain of IMPG1 are associated with the

development of retinitis pigmentosa (RP) in humans (15). The RP disease is characterized by rod photoreceptor progressive cell death leading to tunnel vision and night blindness (16).

IMPG2 has a transmembrane domain adjacent to the SEA-2 domain, suggesting that proteolysis would generate two molecules, a membrane-anchored and extracellular peptide. While previous work with intracellular-specific antibodies indicated strict localization of IMPG2 to the photoreceptor IS (14), the generation of free extracellular IMPG2 and the impact on photoreceptor homeostasis is unknown. Interestingly, the localization of IMPG1 depends on the presence of IMPG2, as we described in our previous work (14).

This work explores the role of IMPG1 and IMPG2 SEA domains, centering on the hypothesis that SEA domains promote protein cleavage. We demonstrate that both proteins proteolyze, mutations affecting IMPG1 proteolysis associated with visual disease, and proteolysis of IMPG2 generate an extracellular peptide that requires IMPG1 to traffic towards the OS photoreceptors.

Results

Predicted SEA domain proteolysis of IMPG1 and IMPG2

Proteolytic SEA domains contain the conserved amino acid sequence GSxxV; where x may be either V or I (11-13). This sequence is found in the established proteolytic-active SEA domain of MUC1 protein. To assess the presence of such SEA sequence in IMPG1 and IMPG2, we compared the MUC1 SEA amino acid sequence with the SEA-1 and SEA-2 sequences of IMPG1 and IMPG2 (Fig. 1A). This comparison shows that the SEA-2 domains from both IMPG proteins contain the canonical amino acid sequence necessary for intramolecular proteolysis (fig. 1B). The alignment also revealed that SEA-1 domains do not contain the required sequence for proteolysis in IMPG proteins. Therefore, IMPG1 and IMPG2 have the predicted capacity to proteolyze at their respective SEA-2 domains but not at the SEA-1 domain. The alignment of murine IMPG SEA domains revealed the same anticipated catalytic activity for the SEA-2 domain (Fig. 1B), consistent with the noted conservation across species (17).

IMPG1 undergoes intramolecular proteolysis

Both IMPG1 and IMPG2 proteoglycans are heavily glycosylated and contain chondroitin sulfate (ChS) glycosaminoglycans chains (4,18). The predicted molecular weight of IMPG1 is 89 kDa. After proteolysis at the SEA-2 domain, it would generate protein fragments of 71 kDa and 18 kDa (Fig. 2A). To determine the relative molecular mobility (Rf) of the IMPG1 protein backbone, chondroitin sulfate and N-/O-glycosylation were removed using a series of enzymatic digestions in isolated mouse retina tissue using Chondroitinase ABC (ChSabc) and deglycosylation enzyme mix (Deglyco) (Fig. 2B). After sample digestion, proteins were separated on gradient

polyacrylamide gels and immunoblotted with an antibody recognizing the 71 kDa long peptide section of IMPG1.

Our Western blot results show an IMPG1 band on wildtype (WT) samples, absent in IMPG1 knockout (1-KO) mice retinas. WT samples treated with ChSabc possess an Rf of ~100 KDa; however, WT samples treated with ChSabc and Deglyco showed a ~71 kDa band, consistent with expectations of IMPG1 proteolysis. Total protein loading controls are included in the supporting information (Fig. S1). Note that samples need to be treated with Chondroitinase ABC (ChSabc) enzyme in order to be detected by the primary antibody.

To exclude the possibility that sample deglycosylation treatment leads to proteolysis in IMPG1, we examined IMPG1 Rf throughout serial dilutions of the protein deglycosylation mix (Fig. 2C). The results show that IMPG1 molecular weight increases in a series of distinct stages, proportionally to dilutions of the enzyme and always appearing as a single protein band. In other words, the serial dilution of the deglycosylation mix revealed linear increments of glycosylation on IMPG1 and not a binary state of the protein between proteolyzed and non-proteolyzed. Our results support the premise that the deglycosylation treatment does not inadvertently cause IMPG1 proteolysis.

In addition, ChSabc treatment was scrutinized as a possible cause of IMPG1 proteolysis. The molecular weight of IMPG1 was assessed in the retinas of a mouse model constitutively lacking ChS, which is known as "*small with kinky tail*" SKT mice (Fig. 2D). These ChS-free SKT retinas showed an IMPG1 profile similar to the WT retina shown in figure 2B, verifying that IMPG1

proteolysis is not caused by ChSabc treatment. Moreover, these findings demonstrate that IMPG1 proteolysis is independent of ChS attachments to the molecule.

In our previous work (14), we demonstrated that the localization of IMPG1 depends on the presence of IMPG2. To study the influence of IMPG2 on IMPG1 proteolysis, we analyzed the molecular weight of IMPG1 in the retinas of IMPG2 KO mice (fig. 2E). The data revealed similar bands and weight distribution as those found in WT, indicating that IMPG1 proteolysis is independent of IMPG2 presence.

Finally, we expressed recombinant IMPG1 with a C-terminal DDK epitope tag in HEK293 cells. Cell samples were studied by immunoblotting, stained with a DDK-specific antibody and the above described IMPG1 antibody (Fig. 2F). These results showed two proteolyzed IMPG1 bands at ~75kDa (anti-IMPG1 antibody) and ~24kDa (anti-DDK antibody), indicating proteolysis of the expressed IMPG1 transgene. Moreover, non-proteolyzed ~100kDa bands were detected in IMPG1 and DDK staining.

Overall, these results indicate that IMPG1 undergoes proteolysis as a part of its normal maturation process in the retina and tissue culture cells. Moreover, this process occurs independently of the presence of IMPG2 and ChS.

IMPG1 proteolysis and vision disease

Proteolytic SEA domains have a characteristic globular shape constituted by four alpha helix and four beta sheets, in which proteolysis occurs between the second and the third beta sheet (Fig. 3A, B) (11). In humans, mutations affecting the SEA-2 domain of IMPG1 are linked to the development of RP disease (Fig. 3A) (15). To determine if these mutations affect the proteolysis of the SEA-2 domain, we expressed in HEK293 cells, two IMPG1 transgenes replicating mutations in the SEA-2 domain associated with RP in humans.

The Western blot analysis shows that cells expressing IMPG1-WT plasmid present 72% proteolyzed and 28% non-proteolyzed protein, whereas cells expressing IMPG1 L583P have 92%, and L630F 58% non-proteolyzed IMPG1 (Fig. 3C, D). These results suggest that RP-associated SEA-2 domain mutations linked to vision loss are likely due to a protein proteolysis deficit.

Proteolysis and translocation of IMPG2

The transmembrane proteoglycan IMPG2 has a theoretical molecular weight of 138 kDa, where SEA-2-mediated proteolysis is expected to liberate a 106 kDa portion of the extracellular region from a membrane-retained 32 kDa fragment (Fig. 4A). Western blot analysis comparing WT and IMPG2 KO retinas using an antibody against the theoretical membrane portion of IMPG2 (IMPG2m) revealed a single band at 32 kDa with no additional bands at higher molecular weight in WT retinas (Fig. 4B). This result supports the hypothesized proteolysis of IMPG2. Furthermore, proteolysis of IMPG2 does not appear to depend on the presence of IMPG1 or ChS, as tested in retinas lysates from mice lacking IMPG1 or ChS. The antibody targeting the 106 KDa extracellular

portion of IMPG2 could not detect IMPG2 by western blot, despite sample treatments with Deglyco and ChSabc enzymes (not shown). Loading controls in supporting information (Fig. S3). We previously described the localization of IMPG2 at the photoreceptor IS using a primary antibody recognizing the membrane portion of IMPG2 (IMPG2m) (14). In light of the ability of IMPG2 to proteolyze, the extracellular portion of IMPG2 may not co-localize with the IMPG2-membrane portion previously described. Immunohistochemistry analysis on WT retinas, using antibodies targeting the extracellular portion of IMPG2 (IMPG2ec), revealed that IMPG2ec localizes around the IS and the OS of photoreceptors (Fig 4 C, D). These key findings demonstrate that IMPG2 undergoes intramolecular proteolysis and generates an extracellular portion that travels from the IS to the OS extracellular matrix.

Past work indicated that IMPG2 KO in retinas induces aggregation and mislocalization of IMPG1. However, the lack of IMPG1 does not affect the localization of the intracellular portion of IMPG2 (14). To determine the role of IMPG1 in the localization of IMPG2ec, we examined the localization of IMPG2ec in IMPG1 KO retinas. The immunohistochemistry analysis reveals mislocalization of IMPG2ec in mice lacking IMPG1. IMPG2ec mislocalizes at the intersection between IS and OS, leaving the rest of the OS depleted of IMPG2ec (Fig 4 G, H). These results indicate that the localization of the IMPG2ec peptide depends on the presence of IMPG1, similar to the localization dependence of IMPG1 on IMPG2 expression (14). Together, these results imply a reciprocal dependency for proper photoreceptor localization between IMPG1 and IMPG2.

Discussion

This work investigates the intramolecular proteolysis of IMPG1 and IMPG2. Our results indicate that both IMPG molecules undergo intramolecular proteolysis on the SEA-2 domain as part of the protein maturation process. This work shows that IMPG1 SEA-2 mutations which have been linked to vision disease in humans, affect IMPG1 proteolysis. Moreover, this work demonstrates that the extracellular portion of IMPG2 migrates from the IPM around the IS to the OS in an IMPG1-dependent manner.

SEA domains are associated with secreted and glycosylated membrane proteins, where they are classified as either proteolytic or non-proteolytic (9,10). IMPG1 and IMPG2 possess two SEA domains in their molecule structure: SEA-1, which is a non-proteolytic domain and SEA-2, a proteolytic domain (Fig. 1A) (6,8,19).

The role of non-proteolytic SEA domains remains unclear; however, similar non-proteolytic SEA molecules in the mature ectodomain of the ICA512 receptor generate homodimer through β strand hydrogen bonds (20,21). This dimerization likely occurs in the endoplasmic reticulum (ER), which is required for extracellular secretion (20,21). In the case of IMPG2, cells expressing mutated forms of the non-proteolytic SEA-1 domain do not reach the cell membrane, being retained and accumulated in the ER (22).

SEA-1 domains in IMPG1 and IMPG2 have amino acid sequences that do not match with proteolysis (Fig. 1B). Our data support this prediction, given that the hypothetical proteolysis in IMPG1 at SEA-1 would generate a peptide with a lower molecular weight than observed in our

assays (Fig. 2). In the case of IMPG2, we were not able to test the non-proteolytic prediction in the SEA-1 domain since we could not analyze the extracellular IMPG2 peptide in western blot analysis. The function of SEA-1 domains in IMPG molecules remains to be tested in future studies.

Proteolytic SEA domains possess the conserved amino acid sequence, GSxxV, where x represents either V or I; this motif is found in the SEA-2 domains of both IMPG proteins (Fig. 1B) (11,12,17). Cell culture assays expressing MUC3 proteoglycan containing a SEA domain demonstrate that proteolytic cleavage occurs in the endoplasmic reticulum (23). Interestingly, after SEA proteolysis, the generated subunits recognize each other to form a heterodimeric complex associated by a non-covalent SDS-sensitive interaction (11,13,23,24). Consequently, it has been proposed that apical cells expressing membrane-associated proteoglycans with self-cleaving SEA domains have evolved to dissociate due to mechanical stress, protecting epithelial cells from rupture. In this sense, it is thought that the dissociation of the non-covalent interactions can subsequently generate an intracellular signaling cascade responding to the mechanically shear from the mucosal surface (12).

Our results indicate that IMPG1 proteolyze independently of the presence of IMPG2 or the existence of ChS in the molecule (Fig. 2). In humans, IMPG1 point-mutations within the SEA-2 domain generate photoreceptor degeneration linked to RP, whereas mutations outside the SEA-2 lead to vitelliform macular dystrophy (15,25,26). This well-defined demarcation between mutations linked to RP or vitelliform macular dystrophy in IMPG1 is not mirrored in IMPG2, where mutations within and outside the SEA domains can generate either pathology (22,27-30). We show that two

mutations in IMPG1 linked to RP obstruct IMPG1 proteolysis *in-vitro* (Fig. 3). These results suggest that deficits in IMPG1 proteolysis are involved in the pathophysiology of the RP-IMPG1 linked disease.

The potential for IMPG2 to undergo SEA-mediated proteolysis has been previously overlooked. Therefore, we described IMPG2 as a membrane-attached proteoglycan localized only at the photoreceptors IS (14). We now show by western blot analysis that IMPG2 undergoes cleavage at the SEA-2 domain generate extracellular and membrane-bound forms (Fig. 4A, B). Localization studies indicate that the extracellular portion of IMPG2 is released from the IS to distribute evenly between the IS and OS regions (Fig. 4C, G). These studies aid in refining our understanding of the parameters governing IMPG localization (Fig. 5). In this model, both IMPG molecules are synthesized at the photoreceptor IS and secreted to the IPM adjacent to the IS (IS-IPM). Next, IMPG1 and the extracellular portion of IMPG2 traffic to the IPM around the OS (OS-IPM) by an undetermined mechanism. Our study shows that the traffic mechanism of the extracellular portion of IMPG2 depends on the presence of IMPG1 (Fig 4C, F). Likewise, we previously demonstrated that the proper localization of IMPG1 relies on the presence of IMPG2 (14). Together these results indicate that both IMPG molecules depend on each other for their final localization around the OS.

As mentioned, the proteolysis of IMPG molecules generates SEA “sticky” ends with the potential to associate by non-covalent interactions (9,24). This speculative scenario lets us hypothesize about the generation of homo and heterodimers between IMPG1 and IMPG2 subunits as part of a hypothetical IMPG1-IMPG2-complex assembly. Specifically, we hypothesize that non-covalent interactions between the IMPG2 membrane and extracellular subunits occur at the photoreceptor

IS. Still, they rapidly detach after being exposed to the extracellular space containing IMPG1 and extracellular molecules (Fig. 5 red rectangle).

In summary, these results imply that proteolysis is a critical maturation step for both IMPG molecules. These results identified that mutations linked to vision disease affect proteolysis of IMPG1. Importantly, these results indicate a mutual localization dependency between IMPG1 and IMPG2 to traffic from the IPM around the IS to the OS.

Materials and methods

Animals

The IMPG1 KO and IMPG2 KO animal models were generated at the West Virginia University transgenic core with the use of Crispr-Cas9, as described in Salido *et al.* (2020) (14). SKT (small with kinky tail) mice, also known as CHSY1 KO mice, were purchased from Jackson Lab (Stock No: 001433). All mouse populations were backcrossed with C57BL/6J (The Jackson Laboratory) and consistently genotyped to discover possible RD1 and RD10 mutations. The animals were maintained under 12 h light/dark cycles with food and water *ad libitum*. Both sexes of mice were equally used throughout our experiments. All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of West Virginia University.

Western Blot

Mice were euthanized using CO2, followed by cervical dislocation as a secondary method. Subject eyes were enucleated, corneas and lenses surgically removed, and retinas carefully extracted from the eyecup and rapidly frozen on dry ice.

Frozen retinal samples were sonicated in T-PER buffer (Thermo Fisher, Cat. 78510) with HALT protease and phosphatase inhibitor/EDTA (Thermo Fisher, Cat. 78430) as recommended. Protein concentrations were measured using a standard BCA protein assay (Pierce, Cat. 23225). Samples treated with Chondroitinase ABC (ChSabc) (Sigma, cat. C3667) were incubated for 3 hours at 37°C with 0.1 Units per sample, with periodic vortex homogenization per hour. Likewise, the deglycosylation treatment (Dglyco) consisted of sample incubation for 3 hours at 37°C: 20 nL of Protein Deglycosylation Mix II (NEB, Cat. P6044S) per µL of the sample. Immediately following

enzyme treatments, LDS buffer (Genscript, Cat. M00676) mixed with B-mercaptoethanol was added to the samples and stored at -20°C, until used.

Equal sample concentrations (100 µg of total protein per well) were resolved in 4-20% electrophoresis gradient gel (Genscript, Cat. M00656). Then, the gels were transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-FL; Millipore). Subsequently, membranes were stained with Total Protein Staining kit (LI-CORE, Cat. 926-11010), imaged at 700nm, and immediately blocked (Thermo Fisher, Cat. 37570) for 1 hour at room temperature. Next, primary antibodies were incubated overnight at 4°C, using a bidirectional rocker. Primary antibodies IMPG1 and IMPG2m are the same as those described in our previous work (14). Following primary antibody incubation, membranes were washed in PBST (PBS with 0.1% Tween 20) 3 times for 5 min each at room temperature. Secondary antibody incubation was conducted at room temperature using goat anti-mouse Alexa Fluor 680 (Invitrogen, Cat. A28183) or donkey anti-mouse IRDye 800 (Li-Cor, Cat. 926-32212), and goat anti-rabbit Alexa Fluor 488 (Invitrogen, Cat. A11034). After 45 min antibody treatment, the membranes were washed 3 times for 5 min with PBST and scanned using a Typhoon 9410 imager (GE).

Plasmid design and protein expression

Human IMPG1 with a DDK tag in the c-terminus, under CMV promotor in an MR210410 vector, was generated by Origene company. The second set of plasmids contains three versions of mouse IMPG1: the WT and two-point mutations in L583P and L630F. The mutations was confirmed by sequencing. Full-length mouse IMPG1 variants were synthesized and inserted in a pcDNA3.1(+)-P2A-eGFP vector under a CMV promotor by a commercial company (Genscript). All

the IMPG1 proteins were expressed in HEK293 cells. Cells were collected after 48hs post-transfection and sonicated in PBS with protease inhibitor mixture (Roche). Samples were next processed and run by western blot as described above.

Immunohistochemistry

All mice were euthanized with CO2 and cervical dislocation, after which their eyes were carefully enucleated. A small hole was cut into each cornea and the enucleated eyes placed in 3% paraformaldehyde (PFA) for 3 hours at room temperature. Next, increasing serial dilutions of sucrose solution 7.5%, 15%, and 20% were interchanged over a 2-day period. The eyecups were placed in optimal cutting temperature compound (Sakura) and flash-frozen in an alcohol bath on dry ice prior to storage at -80°C.

Using a Leica CM1850 Cryostat, 16 µm cross-sections were cut and carefully placed on Superfrost Plus slides (Fisher Scientific). Immunofluorescent staining and image acquisition were concurrently performed as described in past studies (14), with the addition of the treatment described below.

In order to specifically target the extracellular region of IMPG2, two steps of enzyme digestion were required before proceeding with the standard immunostaining: First, 1 U/mL of Chondroitinase ABC (ChSabc) (Sigma, cat. C3667) enzyme was added to the slide and incubated for 1 hour at 37°C; next, 7uL of Protein Deglycosylation Mix II (NEB, Cat. P6044S) per slide accomplished overnight deglycosylation at 37°C. After both assays, the standard immunostaining technique resumed, as previously described in Salido et al. (2020) (14). This work used a primary antibody against the extracellular region of IMPG2 (Sigma, HPA015907) at 1:500 dilution.

Experimental design

All mice used in this work were post-natal day 45 at the time of euthanasia. Two retinas from the same mouse were collected in one tube for western blot experiments. All results were reproduced at least 3 times using samples from different mice. For immunohistochemical analysis, at least 4 sections were imaged per sample, and data were derived from 3 independent experimental runs. Sex differences were assessed for each outcome measured, with no significant variance observed.

This article contains supporting information.

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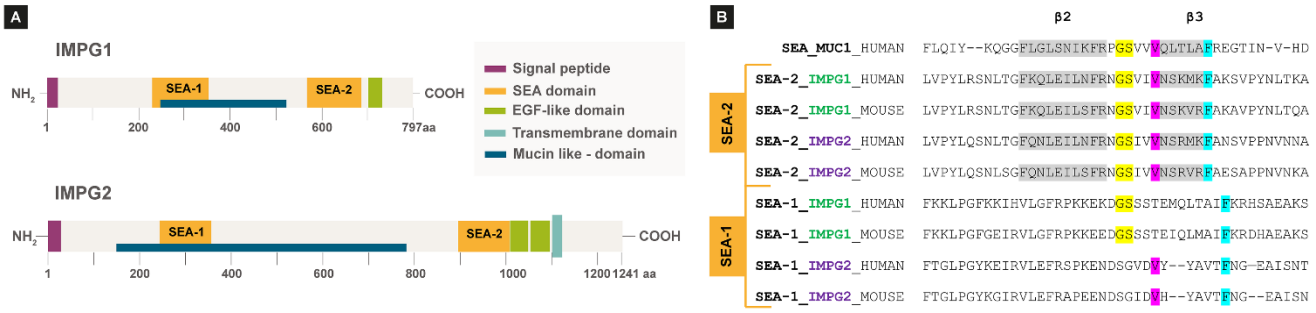

Figure 1. IMPG1 and IMPG2 SEA-2 domains have amino acid sequences compatible with proteolysis. **A**, Schematic representation of IMPG1 and IMPG2 domains. Only IMPG2 has a transmembrane domain; SEA-1 and SEA-2 are present in both proteoglycans. **B**, Amino acid alignment of known self-proteolytic SEA domain of MUC1 protein against SEA-2 and SEA-1 sequence from IMPG1 and IMPG2, in humans and mice. Established proteolytic GSxxV sequence (yellow and violet). Conserved amino acid sequences between SEA domains (grey and cyan).

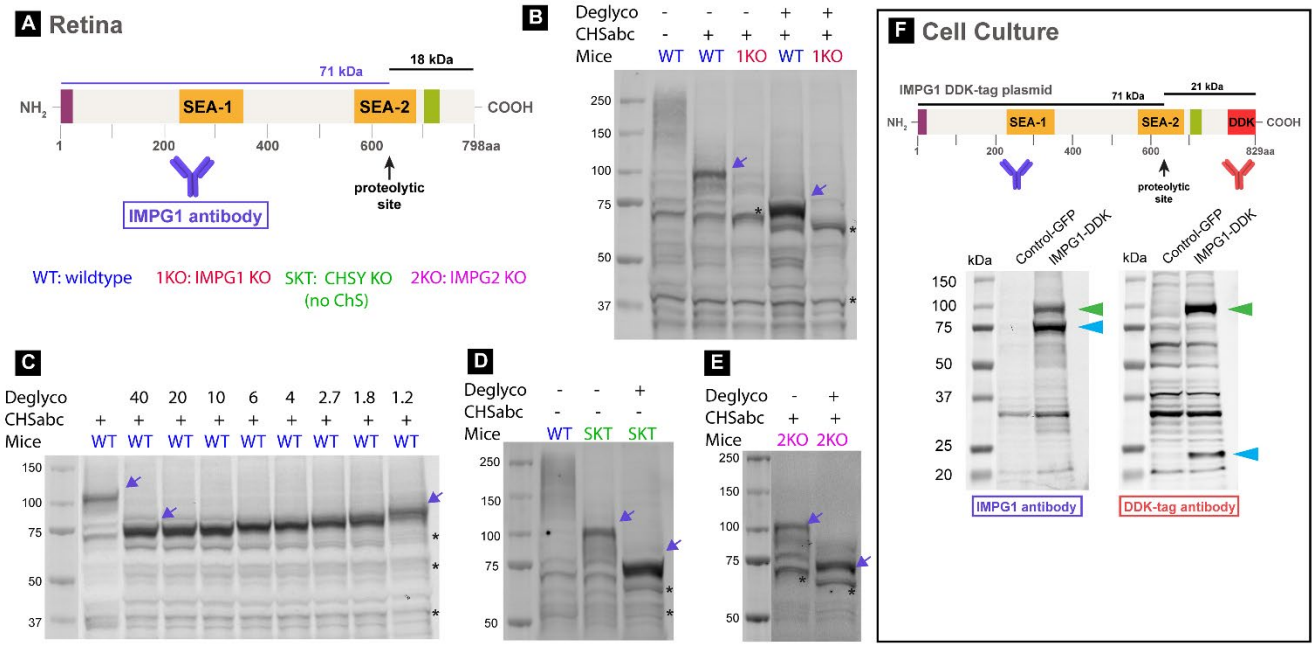

Figure 2. IMPG1 undergoes proteolysis. A, Schematic of IMPG1 structure representing the two peptides expected after proteolysis and respective relative molecular mobility (Rf). **B**, Western blot assay using retina samples from wildtype (WT) and IMPG1 KO mice (M1), treated with and without Chondroitinase abc (ChSabc), as well as with and without deglycosylation enzymes (Deglyco). Membranes stained with an antibody against the long IMPG1 predicted peptide (71 kDa) (violet arrows). The WT samples co-treat with ChSabc and Deglyco enzymes yield one single IMPG1 band with an Rf near 71 kDa. Black asterisks mark nonspecific bands. **C**, WT samples treated with ChSabc, exposed to a series of decreased amounts of Dglyco enzyme (nL/µL of sample). Only one single IMPG1 band is present in all the serial dilutions, and its molecular weight increases stepwise with less Dglyco enzyme. **D-E**, Molecular weight profile of IMPG1 in a mice model lacking chondroitin sulfate (SKT mice) (D) and in IMPG2 KO mice (M2) (E). **F**, Western blot assay of HEK293 cell samples expressing GFP as a negative control or IMPG1-DDK transgenes. Antibody against IMPG1 and DDK-tag was used in the same membrane. The light blue arrows indicate the long and the short IMPG1 peptides, while the green arrows label non-proteolyzed IMPG1. All western blots have been replicated at least three times in different samples; total protein staining was used as a loading control for all repeated experiments (Fig. S1).

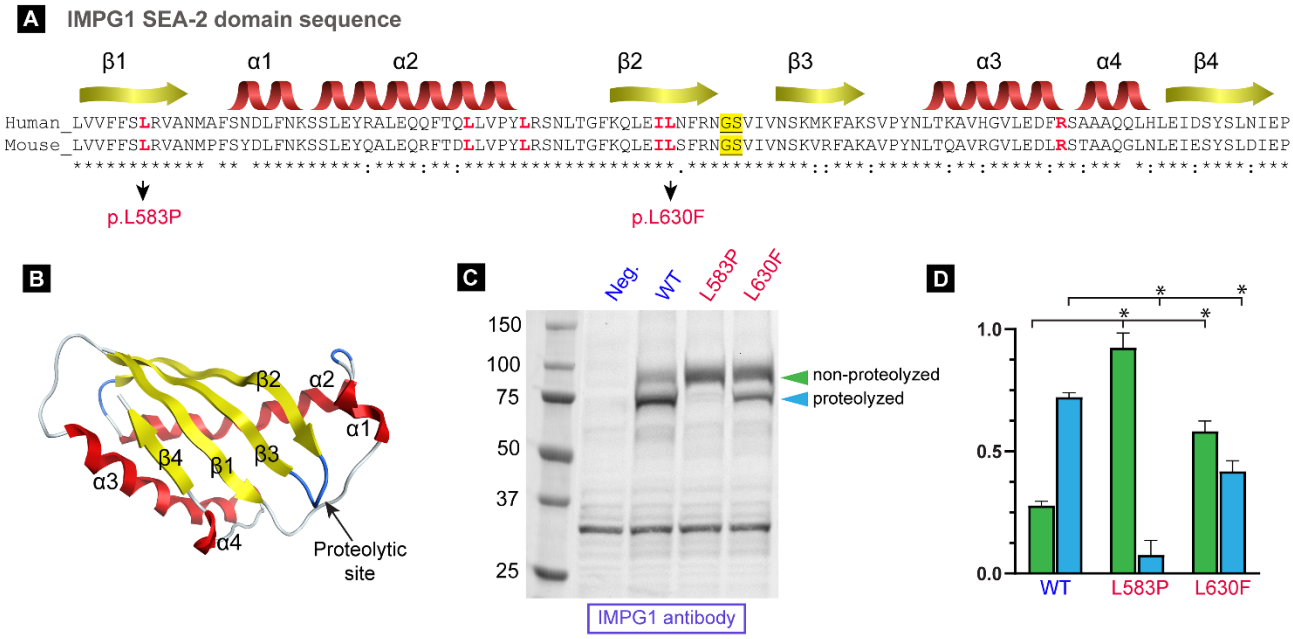

Figure 3. Mutations in the SEA-2 domain of IMPG1 prevent proteolysis. A-B, IMPG1 SEA-2 amino acid sequence and predicted tertiary structure, including described mutations linked to RP pathology in human patients (red letters). Protein structure prediction made with the AlphaFold server. **C**, Western blot assay detecting IMPG1 molecules synthesized by HEK293 cells expressing either GFP as a negative control, IMPG1-WT, IMPG1-L583P, or IMPG1-L630F transgenes. **D**, Protein expression quantification of proteolyzed (light blue) and non-proteolyzed (green) IMPG1. Results were obtained by triplicates from different expression assays and referenced with total protein stain. Bars are the mean ± SEM of N = 3 samples. Both WT groups are significantly different (*p<0.01) from each mutant, two-way T-test. All Western blot samples were deglycosylated before loading into the gel; untreated protein and total protein are in Figure S2.

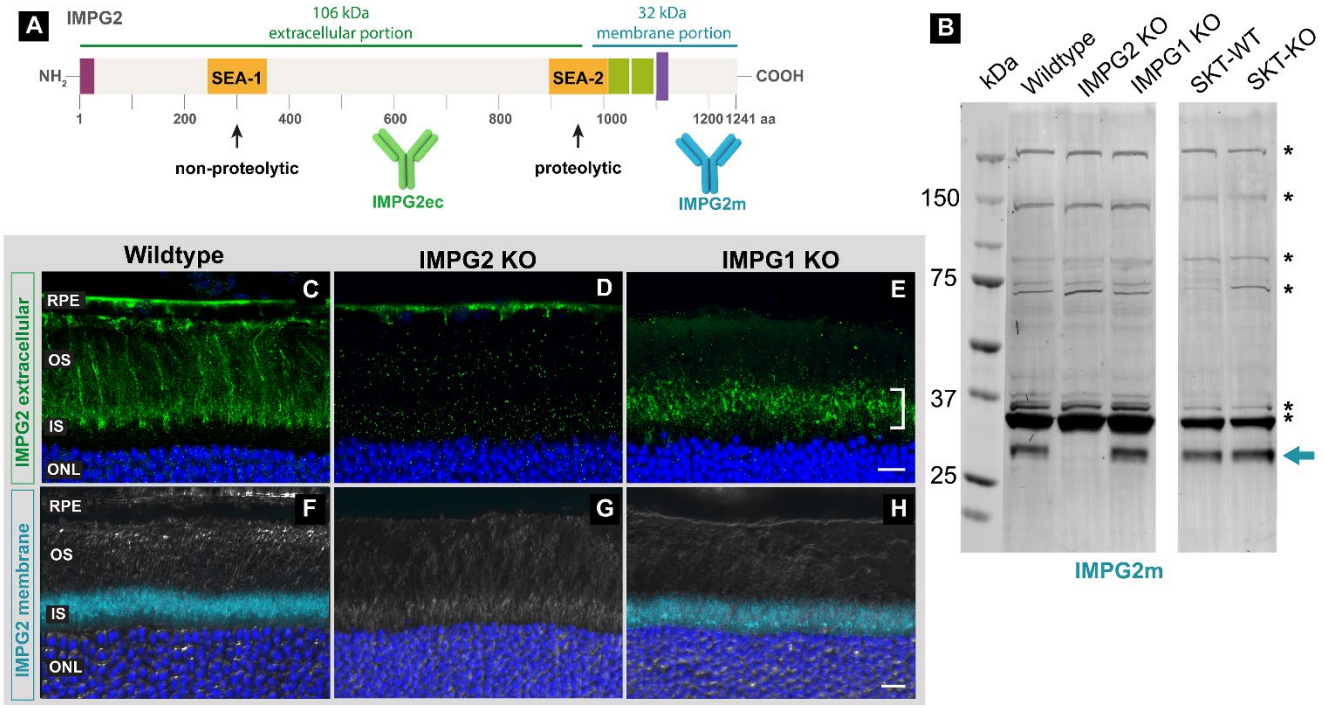

Figure 4. IMPG2 proteolyze and its extracellular portion migrates from the matrix IS to the OS in a process dependent on IMPG1. **A**, Schematic of IMPG2 shows the expected proteolytic and non-proteolytic SEA domains along with extracellular and membrane-portion antibody recognition sites. **B**, Representative western blot assay comparing retina samples from wildtype, IMPG2 KO, IMPG1 KO, and SKT mice wildtype and mutant. A single band at 32 kDa is present in wildtype, IMPG1 KO and SKT mice (cyan arrow). **C-H**, immunohistochemistry staining on retina cross-section from wildtype (C, F), IMPG2 KO (D, G), and IMPG1 KO (E, H) using a specific antibody against the extracellular portion of IMPG2 (IMPG2ec, green) and the membrane portion (IMPG2m, cyan). **C**, IMPG2ec antibody stains the outer part of the IS and the entire OS. **E**, IMPG2ec localizes at the outer IS and inner OS region (white bracket), with a substantial reduction in the rest of the OS. Outer nuclear layer (ONL) stained with DAPI (blue). Differential interference contrast (DIC) in white. Inner segment (IS), outer segment (OS), retina pigment epithelium (RPE). Scale bar equals 10 µm. N=3 mice per experiment at post-natal day 45.

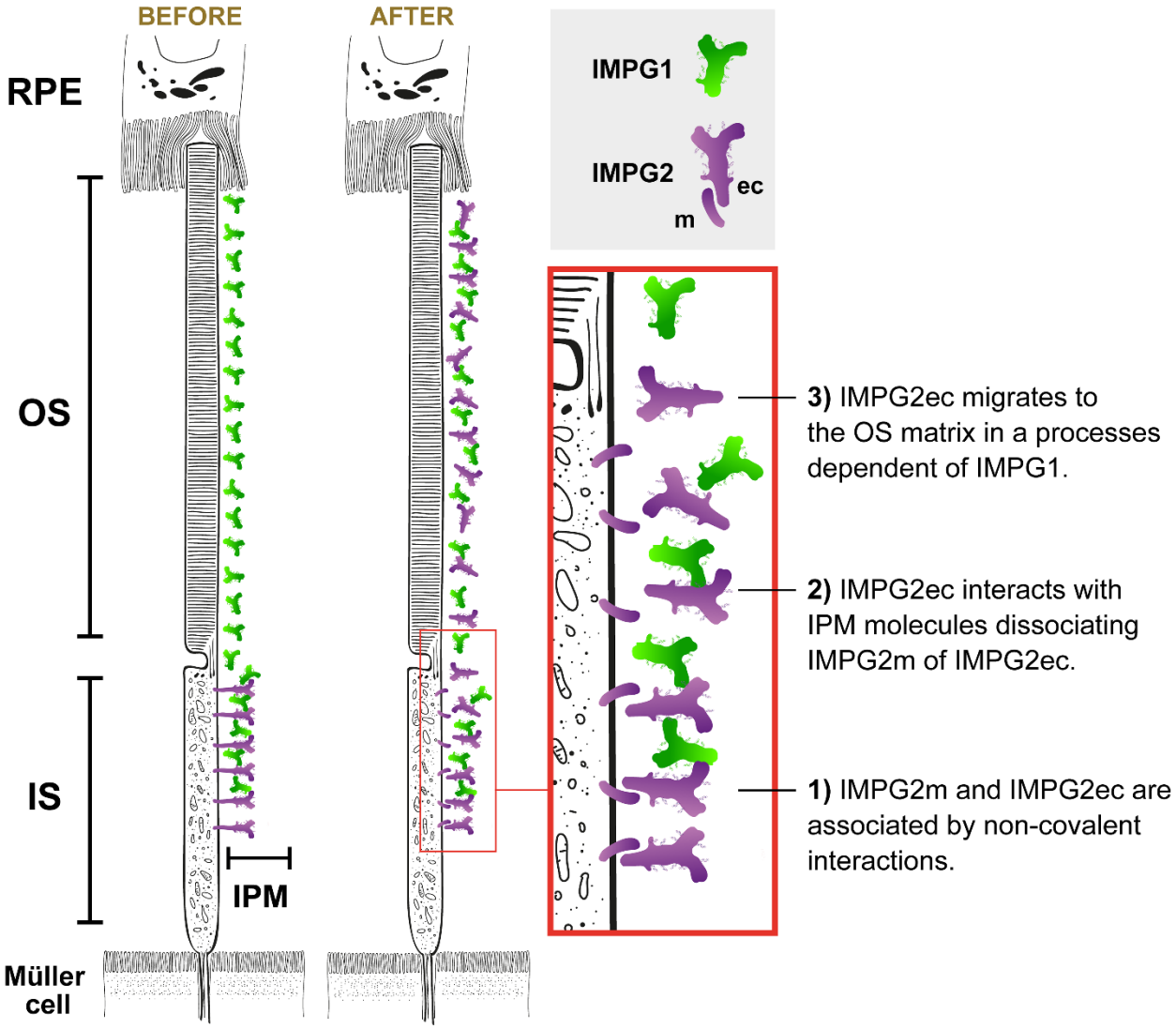

Figure 5. Schematic representation of IMPG1 and IMPG2 localization before and after integration of the findings from our study: Left panel representing IMPG2 (purple) anchored at the photoreceptor IS as a single molecule, as we conceived before this work; right panel illustrating the current understanding of IMPG2 localization where the IMPG2 membrane portion (m) anchors to the IS membrane and the extracellular portion (ec) localizes at the IS-IPM and OS-IPM. **Red box** shows a magnified view of the IS with a speculative representation of the IMPG2 dissociation and translocation of the IMPG2ec to the OS-IPM along with IMPG1 (green).