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RESEARCH ARTICLE

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GPR37 is processed in the N-terminal ectodomain by ADAM10 and furin

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Abstract

GPR37 is an orphan G protein-coupled receptor (GPCR) implicated in several neurological diseases and important physiological pathways in the brain. We previously reported that its long N-terminal ectodomain undergoes constitutive metalloprotease-mediated cleavage and shedding, which have been rarely described for class A GPCRs. Here, we demonstrate that the protease that cleaves GPR37 at Glu167↓Gln168 is a disintegrin and metalloprotease 10 (ADAM10). This was achieved by employing selective inhibition, RNAi-mediated downregulation, and genetic depletion of ADAM10 in cultured cells as well as in vitro cleavage of the purified receptor with recombinant ADAM10. In addition, the cleavage was restored in ADAM10 knockout cells by overexpression of the wild type but not the inactive mutant ADAM10. Finally, postnatal conditional depletion of ADAM10 in mouse neuronal cells was found to reduce cleavage of the endogenous receptor in the brain cortex and hippocampus, confirming the physiological relevance of ADAM10 as a GPR37 sheddase. Additionally, we discovered that the receptor is subject to another cleavage step in cultured cells. Using site-directed mutagenesis, the site (Arg54↓Asp55) was localized to a highly conserved region at the distal end of the ectodomain that contains a recognition site for the proprotein convertase furin. The cleavage by furin was confirmed by using furin-deficient human colon carcinoma LoVo cells and proprotein convertase inhibitors. GPR37 is thus the first multispanning membrane protein that

Abbreviations: ADAM, a disintegrin and metalloprotease; cKO, conditional knock out; decCMK, decanoyl-RVKR-chloromethylketone; EndoH, endoglycosidase H; GPCR, G protein-coupled receptor; GPR37, G protein-coupled receptor 37; h, human; KO, knock out; m, mouse; MEF, mouse embryonic fibroblast; MMP, matrix metalloprotease; PAR, protease-activated receptor; PC, proprotein convertase; PNGaseF, peptide-N-glycosidase F; r, recombinant; WT, wild type.

has been validated as an ADAM10 substrate and the first GPCR that is processed by both furin and ADAM10. The unconventional N-terminal processing may represent an important regulatory element for GPR37.

KEYWORDS

ADAM, GPR37, G protein-coupled receptor (GPCR), furin, shedding

1 | INTRODUCTION

The G protein-coupled receptor 37 (GPR37) is a class A (rhodopsin-type) G protein-coupled receptor (GPCR) that is highly expressed in several brain regions.[1] It is involved in dopaminergic and adenosinergic signaling pathways,[2-6] and consequently has been implicated in the pathophysiology of Parkinson's disease[7,8] along with certain other brain pathologies.[9,10] The receptor is also found in astrocytes,[11] and especially in oligodendrocytes,[12,13] with a demonstrated role in their differentiation and myelination.[13] Thus, GPR37 is a promising target for the treatment of neurological diseases. However, attempts to identify the activating ligand for GPR37 have resulted in controversial results [reviewed[14,15]], and the receptor is still considered an orphan GPCR. Moreover, the mechanisms, by which GPR37 is activated and regulated in cells, are poorly understood.

We have previously demonstrated that the long extracellular N-terminus of human GPR37 (238 amino acids) is subject to constitutive proteolytic cleavage between Glu167 and Gln168 in cultured cells and that the cleaved ectodomain is shed to the culture medium.[16] In line with this finding, the cerebrospinal fluid has been shown to contain GPR37 N-terminal peptides,[17,18] suggesting that the proteolytic processing of the receptor occurs in native brain tissue. Indeed, we reported recently that these peptides are elevated in Parkinson's disease patients.[18] While ectodomain cleavage and shedding are common mechanisms regulating single-pass membrane proteins, these mechanisms have quite rarely been described for multi-spanning membrane proteins, such as GPCRs. Important exceptions include adhesion family receptors and protease-activated receptors (PARs) that display a unique activation mechanism involving N-terminal proteolytic processing.[19,20] Whereas the former receptors are characteristically cleaved in the endoplasmic reticulum in an autoproteolytic manner, PARs are cleaved by extracellular proteases at the cell surface. For both GPCR subgroups, the limited proteolysis reveals a new N-terminus that acts as a tethered peptide agonist and activates the receptor. Examples of other GPCRs that are cleaved at their N-terminal ectodomain have remained scarce and in most cases, the cleavage mechanism and its functional consequences are poorly known. Intriguingly, among these few GPCRs are two receptors closely related to GPR37, the endothelin B receptor, and GPR37L1.[21,22] Whereas GPR37L1, an orphan receptor-like GPR37, is cleaved constitutively, cleavage of the endothelin B receptor occurs in an agonist-dependent manner and has been found to alter receptor function in vascular smooth muscle cells.[23]

Proteolytic processing of membrane proteins involves several proteases, many of which belong to proprotein convertase (PC) and metalloprotease families. PCs comprise a family of nine secretory serine proteases that activate, or in some cases inactivate, secreted or membrane-bound proteins by cleaving them at specific sites.[24] Their substrates range from hormones and cytokines to growth factors, receptors, and other proteases [reviewed[24]], highlighting the role of PCs in many vital biological processes. MMPs (matrix metalloproteases) and ADAMs (a disintegrin and metalloproteases) constitute the main metalloprotease families involved in limited proteolysis and shedding,[25,26] Among these, ADAM10, along with ADAM17, is the most extensively characterized proteolytically active ADAM family member.[26,27] ADAM10 is involved in the cleavage and shedding of several type I and II or glycosylphosphatidylinositol-anchored membrane proteins, including Notch, amyloid precursor protein, N-cadherin, ephrins 2 and 5, and cellular prion protein [reviewed[26,27]]. In comparison, the possible role of ADAM10 in the cleavage of multi-spanning membrane proteins has remained elusive, although a few ADAM10 candidate substrates have emerged in proteomic studies.[28]

In our previous study, we showed that metalloproteases are involved in GPR37 processing as the cleavage is significantly reduced with broad-spectrum metalloprotease inhibitors at the Glu167↓Gln168 site.[16] However, the actual enzymes involved in GPR37 processing and their mechanisms of action have remained unknown. As GPR37 is found at the plasma membrane predominantly in its N-terminally truncated form,[16] a better understanding of receptor cleavage mechanisms and identification of the proteases involved will benefit studies aiming to assess GPR37 activation and signaling mechanisms. In the present study, we set out to identify the specific enzymes involved in GPR37 processing. We demonstrate that ADAM10 cleaves human and mouse GPR37 (hGPR37 and mGPR37, respectively) in cultured cells, and furthermore participates in receptor processing in the mouse brain. In addition, GPR37 is processed by the PC furin at a highly conserved site in the distal end of the N-terminal

domain (Arg^{54}↓$Asp^{55}$of hGPR37). Thus, this study provides evidence for diverse multistep proteolytic processing of a class A orphan GPCR involving proteases belonging to two distinct families, PCs and ADAMs.

2 | MATERIALS AND METHODS

2.1 | Chemicals and antibodies

Protease inhibitors were obtained from Tocris Bioscience [decanoyl-RVKR-chloromethylketone (decCMK), GI254023X, marimastat; Abingdon, UK], Enzo Life Sciences (aprotinin, GM6001, leupeptin; Farmingdale, NY, USA), Calbiochem (hexa-D-arginine; San Diego, CA, USA), Merck Millipore (inactive GM6001, TAPI-1; Burlington, MA, USA) or Sigma-Aldrich (E-64d, pepstatin A, phosphoramidon; St. Louis, MO, USA). The recombinant (r) human ADAM10 was obtained from R&D Systems (936-AD; Abingdon, UK). The following commercially available primary polyclonal and monoclonal antibodies (pAb and mAb, respectively) were used for Western blotting: cMyc [rabbit pAb A14, sc-789, 1:1000; SantaCruz Biotechnology (Dallas, Tx, USA) and mouse mAb 9E10, 626802, 1:1000; BioLegend (San Diego, CA, USA)], FLAG [HRP-conjugated mouse mAb M2, A8592, 1:50 000, Sigma-Aldrich], translocon-associated protein α-subunit (TRAPα, rabbit pAb 10583-1-AP, 1:2000; Proteintech, Manchester, UK), ADAM10 [rabbit pAb ab1997, 1:1000, Figure 3 and rabbit mAb (EPR5622) ab124695, 1:2000, Figure 9; Abcam, Cambridge, UK], ADAM17 (rabbit pAb ADI-905-249, 1:2000; Enzo Life Sciences) and α-actinin (mouse mAb B-12, sc-166524, 1:1000; Santa-Cruz Biotechnology). The rabbit pAbs directed against hGPR37 and mGPR37 N-terminal domain and GPR37 C-terminal domain (all used at 0.5-2 μg/mL) have been described previously.[2,5,29] Mouse and rabbit HRP-conjugated secondary antibodies were obtained from Sigma-Aldrich or Thermo Fisher Scientific (GENA9310 and GENA9340, 1:40 000; 31460 and 31430, 1:10 000, respectively). The antibodies for confocal microscopy were: cMyc (rabbit pAb C3956, 2 μg/mL; Sigma-Aldrich), α-1 Na^+/K^+ ATPase [mouse mAb (464.6) ab7671, 2 μg/mL; Abcam] and Alexa Fluor 488 and 568 goat anti-rabbit IgG (A11008 and A11004, respectively, 8 μg/mL; Invitrogen, Carlsbad, CA, USA). All other reagents were purchased from Sigma-Aldrich or Thermo Fisher Scientific unless indicated otherwise.

2.2 | Animals

Eight-week-old C57BL/6J wild type (WT, RRID: MGI:5657312) and GPR37 knock out (KO, stock no: 005806) male mice (B6.129P2-*GPR37*^{tm1Dgen}/J; Jackson Laboratory, Bar Harbor, ME, USA) were used for analyzing GPR37 species in various brain regions. The University of Barcelona Committee on Animal Use and Care approved the protocol (no: 7085). Animals were housed and tested in compliance with the European Union directives (2010/63/EU), FELASA, and ARRIVE guidelines. The animals were euthanized by cervical dislocation and the fresh brain regions (cortex, striatum, hippocampus, and cerebellum) were used for membrane preparation immediately after harvesting. Samples from cortex, hippocampus and cerebellum of four *Adam10* conditional KO (cKO) mice (*Adam10*$^{f/f}$; *CamK*IIα-*Cre*)[30] and three Cre-negative *Adam10*$^{F/F}$ or *Adam10*$^{F/+}$ WT littermate controls harvested at postnatal day 20 were provided by Prof. Paul Saftig.

2.3 | DNA constructs

The N-terminally Myc-tagged and C-terminally FLAG-tagged hGPR37 with a cleavable HA signal peptide in pFT-SMMF and the C-terminally FLAG-tagged mGPR37 in pcDNA3 have been described previously.[16] Constructs with mutations or with short deletions were generated using the QuikChange Lightning mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) and Myc-hGPR37-FLAG as a template. The following oligonucleotides and corresponding reverse complements were used: CCCAGGCGTCCGCGCCGCGGCGCTG (R54A), GGTCCCCAGGCGTCCGCGCCGGCGGCCTGG ATCACTGTAGG (R51;52;54A), GGTCCCCAGGCGTCC TGGATCACTGTAG (ΔR51-R54), GCCCCCTGCTCCTC CAGAACGTCTCTTG (ΔR68-R73), and GGCTCCTGACC CCAGGGGCCAGG (ΔR121-R126). The hADAM10 E384A construct was prepared in a similar manner using the oligonucleotide and the corresponding reverse complement CTCACATTACTTTTGCTCACGCAGTTGGACATAACT TTGGATCC and the WT HA-tagged hADAM10 in pEAK12[31] (from Prof. Mikko Hiltunen, University of Eastern Finland, Kuopio, Finland) as a template. The hGPR37 tagged at its N-terminus with NanoLuc and HA was prepared as described previously.[18] The prepared constructs were verified by DNA sequencing.

2.4 | Cell culture and transfections

The stably transfected tetracycline-inducible $HEK293_i$ cell line expressing Myc-hGPR37-FLAG was prepared using Invitrogen's T-REx System, as described.[16] Briefly, the receptor construct and pOG44 plasmid were co-transfected into the Tet repressor expressing $HEK293_i$ cells under blasticidin S (4 μg/mL, InvivoGen, Toulouse, France) and hygromycin (400 μg/mL, InvivoGen) selection. The furin-deficient human colon carcinoma LoVo cells[32] were obtained from Sigma-Aldrich. ADAM10KO, ADAM17KO,

and the parental HEK293T cells were a kind gift from Prof. Christoph Garbers (Otto-von-Guericke-University Magdeburg, Magdeburg, Germany),[33] and SH-SY5Y cells from Prof. Mikko Hiltunen (University of Eastern Finland, Kuopio, Finland). The ADAM10KO and the parental WT mouse embryonic fibroblast (MEF) cells have been described previously.[34]

All cell lines were maintained at 37 ºC in a humidified atmosphere of 5% CO_2. HEK293, MEF and SH-SY5Y cells were cultured in DMEM and LoVo cells in Ham's F-12 containing 10% FBS. The media were supplemented with 100 units/mL penicillin and 0.1 mg/mL streptomycin as well as with selection antibiotics in the case of the stably transfected $HEK293_i$ cells. Receptor expression was induced in $HEK293_i$ cells by adding 0.5 μg/mL tetracycline to the fresh culture medium in the absence of selection antibiotics. For transient transfections, cells were transfected at 60-80% confluency with linear 25-kDa polyethyleneimine (Polysciences, Warrington, PA, USA), Lipofectamine 3000 (Invitrogen), or jetOPTIMUS (Polyplus-transfection, Illkirch, France). All transfection reagents were used at 1:3 or 1:1 DNA-to-reagent ratio. The cells were transfected with 0.75-2 μg (6-cm plate) or 2-5 μg (10-cm plate) of receptor DNA and harvested after 24-48 hours. For co-transfections, GPR37 and ADAM10 plasmids were used at 1:1 ratio. Protease inhibitors, when used, were added to the culture medium 4 hours or 1 hour after starting the transfection or induction.

2.5 | Small interfering RNA-mediated gene silencing

For siRNA-mediated knock-down of ADAM10 and ADAM17, stable $HEK293_i$ cells at 20% confluency were transfected with Lipofectamine RNAiMAX (Invitrogen) using 20 nM siGENOME SMART pool against ADAM10 or ADAM17 and siGENOME non-targeting siRNA pool for control cells (Dharmacon, Lafayette, CO, USA) in Opti-MEM medium (Invitrogen) containing 4% FBS. Medium was changed to DMEM containing FBS, penicillin, and streptomycin 24 hours after transfection, and after 42 hours the cells were induced to express the receptor for 6 hours.

2.6 | Metabolic pulse-chase labeling and cell surface biotinylation

Stably transfected $HEK293_i$ cells in 25-cm^2 flasks were induced to express GPR37 for 6-16 hours and subjected to metabolic labeling with 70-100 μCi/mL [^{35}S]-methionine/cysteine (PerkinElmer, Waltham, MA, USA) as described previously.[35] GI254023X and decCMK were added to the depletion medium and were maintained thereafter. At the end of the chase, cell surface proteins were biotinylated with 0.5 mg/mL EZ-Link™ sulfo-NHS-Biotin (Thermo Fisher Scientific), as described.[36]

2.7 | Preparation of cellular membranes and whole-cell extracts

Total cellular lysates and cellular membranes from cultured cells were prepared as described.[37] The membrane homogenization buffer (25 mM Tris-HCl, pH 7.4, 20 mM N-ethylmaleimide) contained a protease inhibitor mix: EDTA (2 mM), 1,10-phenanthroline (2 mM), phenylmethylsulfonyl fluoride (0.5 mM), leupeptin (5 μg/mL), soybean trypsin inhibitor (5 μg/mL) and benzamidine (10 μg/mL). For membrane solubilization and preparation of cellular lysates, the homogenization buffer was supplemented with 0.5% n-dodecyl-β-D-maltoside (Enzo Life Sciences) or Triton X-100 and 140 mM NaCl. N-ethylmaleimide was omitted from the membrane solubilization buffer. Membrane preparation from mouse brain tissue samples was performed by suspending the samples in 50 mM Tris-HCl, pH 7.4 containing the protease inhibitor mix and homogenizing with the VDI12 homogenizer (VWR International, Darmstadt, Germany) three times for 10 seconds at 800 rpm on ice. Tissue homogenates were centrifuged at 12 000× g for 30 minutes and the membrane pellets were resuspended in the homogenization buffer before adding SDS-sample buffer. Protein concentrations were determined using either Bio-Rad (Hercules, CA, USA) DC or Pierce (Thermo Fisher Scientific) BCA protein assays with BSA as a standard.

2.8 | Receptor purification

Receptors were purified with FLAG M2 or cMyc antibody resins, and ADAM10 with the HA antibody resin (all from Sigma-Aldrich). One-step receptor immunoprecipitation was performed before Western blotting and two-step purification before fluorography of [^{35}S]-methionine/cysteine-labeled receptors following previously described protocols.[36] For the in vitro ADAM10 cleavage assay, protease inhibitors were omitted during the second immunoprecipitation step, excluding leupeptin. Biotinylated receptors were first purified with streptavidin agarose (Thermo Fisher Scientific) and then immunoprecipitated with FLAG M2 antibody as described.[36] The purified receptors were eluted from the resins with SDS-sample buffer for direct analysis by SDS-PAGE or with 1% SDS, 50 mM Na-phosphate, pH 5.5 before enzymatic deglycosylation.

2.9 | Deglycosylation

The purified receptors were deglycosylated with 50 mU/mL endoglycosidase H (Endo H) and 20-30 U/mL peptide-N-glycosidase F (PNGase F, Sigma-Aldrich), as described.[35] Membranes prepared from the mouse striatum were deglycosylated with Endo H and PNGase F using reagents from New England Biolabs (Ipswich, MA, USA) according to the protocol provided by the manufacturer. Briefly, 50 µg of protein was treated with denaturing buffer at 100°C for 10 minutes. After adding the glycobuffer and enzymes (15 U/mL), the samples were incubated at 37°C for 1 hour.

2.10 | In vitro cleavage assay with recombinant ADAM10

The purified hGPR37 was cleaved with rADAM10 while still attached to the FLAG antibody resin. Before the assay, the resin was washed with the incubation buffer (0.1% Triton X-100, 25 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM $CaCl_2$, 2.5 mM $ZnCl_2$) and the samples were incubated with rADAM10 (10 µg/mL) as specified in Figure 4. The reaction was stopped by adding 10 mM EDTA in 0.1% Triton X-100, 25 mM Tris-HCl, pH 7.4, 140 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride, 2 mM 1,10-phenantroline, 5 µg/mL leupeptin, 5 µg/mL soybean trypsin inhibitor and 10 µg/mL benzamidine. After washing the resin, the receptor was eluted with an SDS-sample buffer.

2.11 | Purification of the shed N-terminal receptor fragment

For Figures 1 and 3, the N-terminal receptor fragment was immunoprecipitated and analyzed using Tris-Tricine gel electrophoresis as described previously.[16] For Figure 7, the cells were transiently transfected with NanoLuc-HA-hGPR37 on 6-cm plates and Myc-hGPR37-FLAG on 10-cm plates for 16 hours and treated with decCMK and GI254023X for 12 hours in serum-free medium. The conditioned medium was then collected and concentrated to 500 µL using the Amicon Ultra-15 10K centrifugal filter devices (Merck Millipore). Aliquots (25 µL) of the concentrated samples were diluted 1:1 with SDS sample buffer and separated on 12% SDS-PAGE gels.

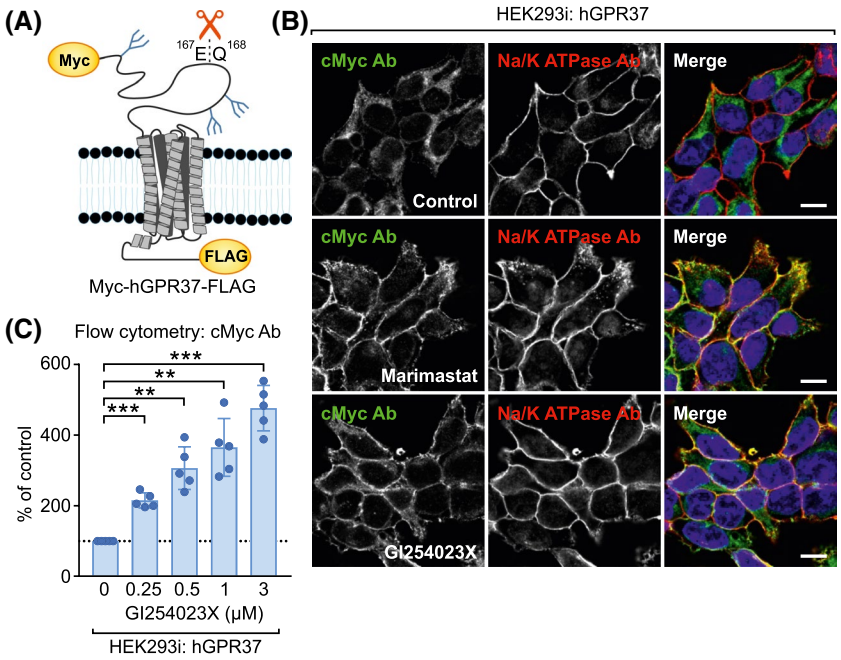

FIGURE 1 Nonselective and ADAM10-selective metalloprotease inhibitors increase the amount of full-length hGPR37 at the cell surface. A, Schematic model of hGPR37 with the metalloprotease cleavage site at Glu167↓Gln168[16] and with three N-glycosylation sites[16] shown as blue glycan structures. The added Myc and FLAG epitope tags are also indicated. B, HEK293$_i$ cells stably transfected with Myc-hGPR37-FLAG were induced to express the receptor for 24 hours and treated for the last 23 hours with the indicated metalloprotease inhibitors (20 µM marimastat, 2 µM GI254023X) or vehicle. Cells were fixed, permeabilized, and stained with the indicated antibodies followed by Alexa-Fluor-488- and -568-conjugated secondary antibodies. The nuclei were stained with TO-PRO-3 iodine. The cellular localization of cMyc antibody-labeled full-length receptors was analyzed by confocal microscopy. Scale bars: 10 µm. C, Induced HEK293$_i$ cells were treated with the indicated concentrations of GI254023X for 23 hours and analyzed by flow cytometry after labeling cell surface receptors with cMyc antibody and the phycoerythrin-conjugated secondary antibody. The results represent five independent experiments performed with triplicate samples. The fluorescence intensity values were normalized to the mean value obtained from cells treated with vehicle only. The results were analyzed before normalization using repeated measures one-way ANOVA followed by Dunnett's multiple comparison test. ***$P < .001$; **$P < .01$. Ab, antibody

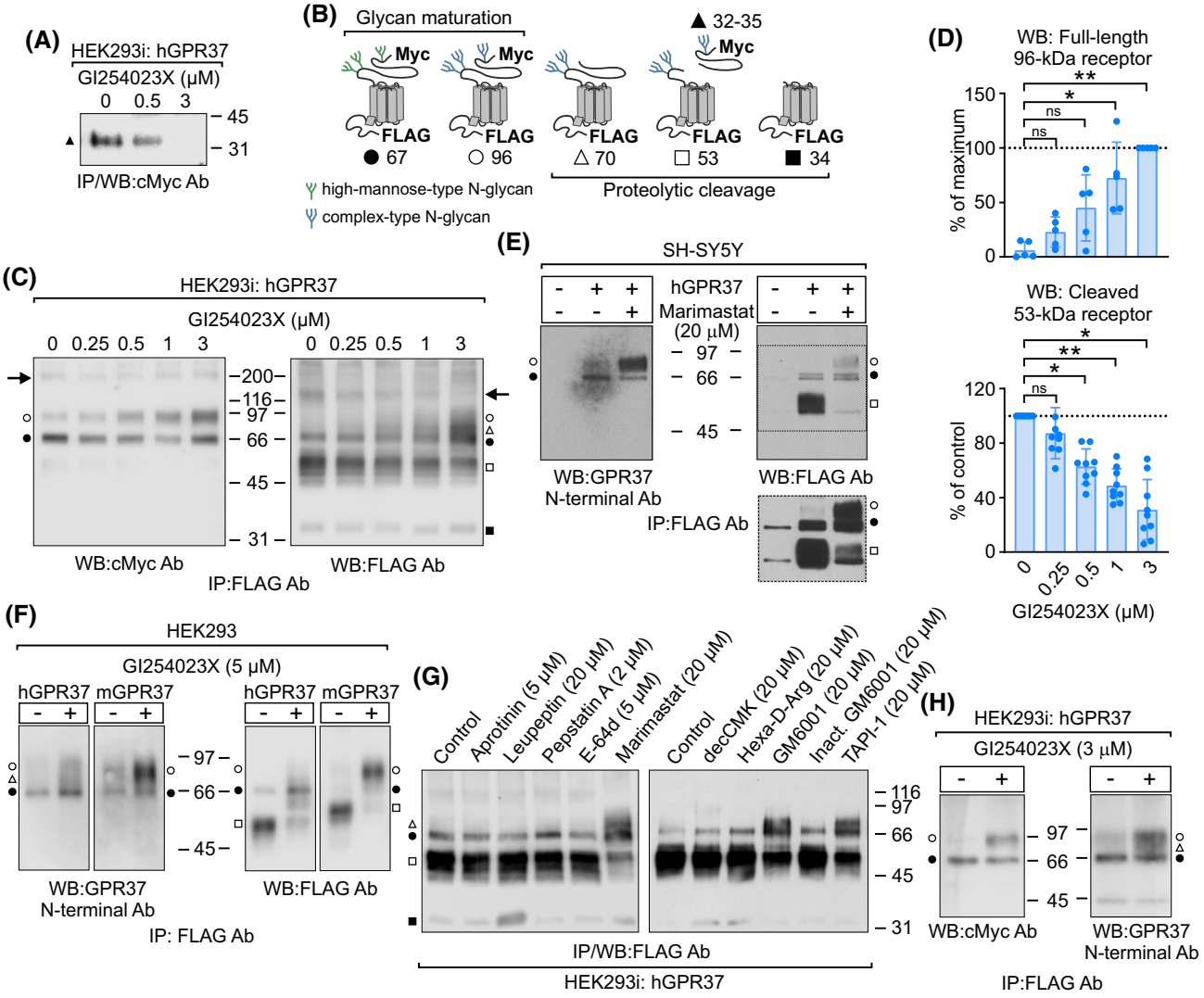

FIGURE 2 ADAM10 selective inhibitor GI254023X inhibits N-terminal cleavage of human and mouse GPR37. Stably transfected HEK293$_i$ cells were induced to express Myc-hGPR37-FLAG (A, C, G, H) or, alternatively, mGPR37-FLAG and Myc-hGPR37-FLAG were transiently expressed in either SH-SY5Y (E) or HEK293 (F) cells for 24 hours. The cells were treated with the indicated concentrations of protease inhibitors or vehicle for 20-23 hours. The shed N-terminal receptor fragment from the conditioned culture medium (A) and membrane-bound receptors from cellular lysates (C, E-H) were immunoprecipitated and analyzed by SDS-PAGE and Western blotting. The outlined area in panel E is shown with a longer exposure time. The identified hGPR37 species are depicted in panel B. The 67-kDa precursor and the 96-kDa full-length mature receptor are indicated with closed and open circles, respectively. The shed 32- 35-kDa N-terminal receptor fragment is indicated with a closed triangle and the 53- and 34-kDa cleaved C-terminal fragments with open and closed squares, respectively. The 70-kDa receptor species are indicated with an open triangle. Arrows in panel C indicate receptor oligomers and higher molecular mass aggregates. Panel D shows relative changes in the intensity of the 96-kDa full-length and 53-kDa cleaved receptors seen in panel C (left and right panels, respectively). The values were normalized to the 67-kDa precursor and are shown as means ± SD from four to nine independent experiments. The repeated-measures one-way ANOVA followed by Dunnett's multiple comparison test was used for the statistical comparison. $^{**}P < .01$; $^{*}P < .05$; ns, nonsignificant. Ab, antibody, decCMK, Decanoyl-RVKR-CMK; Hexa-D-Arg, hexa-D-arginine; IP, immunoprecipitation; WB, Western blotting

2.12 | SDS-PAGE, Western blotting, and fluorography

Protein samples were reduced with 50 mM DTT for 2-5 minutes at 95°C and separated by SDS-PAGE using reagents and equipment from the Bio-Rad Laboratories. Proteins were transferred to 0.45 μm polyvinylidene difluoride membranes (Immobilon-P, Merck Millipore) using the Mini Trans-Blot cell apparatus or a semidry transfer system (Trans-Blot SD or Trans-Blot Turbo). The membranes were blocked with 5% nonfat dry milk in TBS-T (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) or PBS-T (2 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.2, 0.05% Tween-20; Figures 8-9 and S5-S6) and probed with the appropriate antibodies in

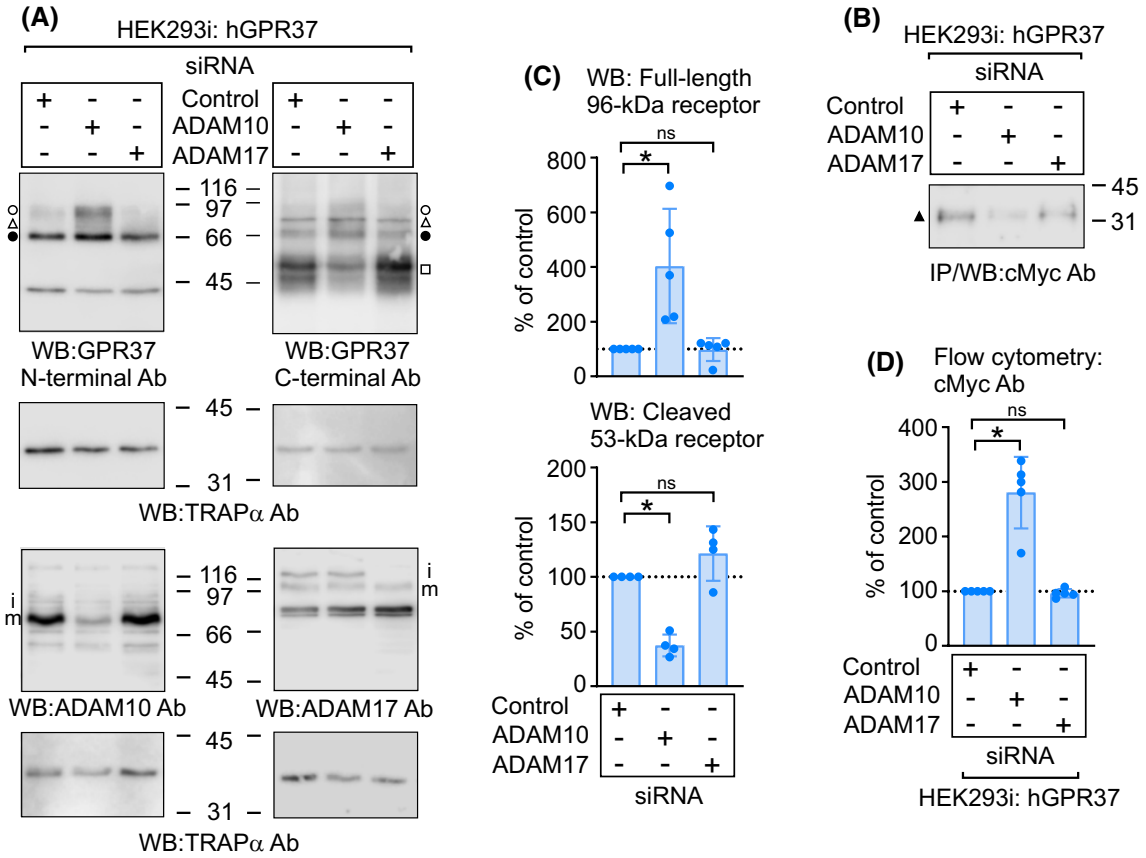

FIGURE 3 ADAM10 downregulation in HEK293 cells inhibits hGPR37 ectodomain cleavage and shedding. Inducible HEK293$_i$ cells were treated with siRNA pools targeting ADAM10 or ADAM17 or with the control siRNA pool for 72 hours and were induced to express hGPR37 for 8 hours at the end of the siRNA transfection. A, Receptors from solubilized cellular membranes were analyzed by SDS-PAGE and Western blotting. The knock-down of ADAM10 and ADAM17 was confirmed with the corresponding selective antibodies. The translocon-associated protein α-subunit (TRAPα) was used as a loading control. B, N-terminal receptor fragments in the cell culture medium were immunoprecipitated and analyzed by Western blotting with cMyc antibody. D, Cell surface cMyc antibody labeled receptors from five independent experiments were analyzed in triplicate by flow cytometry. Relative changes in the 96-kDa full-length and 53-kDa cleaved receptors seen in panel A are shown in panel C as means ± SD from 4-5 independent experiments. The values were normalized to the loading control before statistical analysis. The data were analyzed by repeated measures one-way ANOVA and Dunnett's multiple comparison test. *$P < .05$; ns, nonsignificant. The abbreviations and symbols describing GPR37 species are explained in the legend for Figure 2. i, immature ADAM17; m, mature ADAM10/17

TBS/0.1% BSA or PBS-T/5% nonfat dry milk. The Luminata Crescendo Western HRP substrate (Merck Millipore) was used to reveal the specific protein bands on the membranes that were analyzed by digital imaging with the Odyssey FC imager (LI-COR Biosciences, Lincoln, NE, USA) or Amersham Imager 600 (GE Healthcare). Alternatively, the membranes were exposed on UltraCruz™ autoradiography film (SantaCruz Biotechnology). Gels containing radiolabeled samples were treated for fluorography as described[36] and exposed on Kodak Biomax MR film (Sigma-Aldrich). The relative intensities of bands on films were analyzed by densitometric scanning with the Umax PowerLook 1120 color scanner (Umax Technologies, Dallas, TX, USA) and the Image Master 2D Platinum 6.0 software (GE Healthcare, Chicago, IL, USA). The results were quantified using the LI-COR Image studio v. 5.2, subtracting the local background from each lane.

2.13 | Flow cytometry

The total pool of full-length cell surface receptors was analyzed using mouse mAb cMyc (9E10, 3 μg/mL, 626802; BioLegend) and phycoerythrin-conjugated rat anti-mouse IgG1 (2 μg/mL, 550 083; BD Biosciences, Franklin Lakes, NJ, USA) as described.[38] Induced HEK293$_i$ cells were used in parallel for flow cytometry and Western blot analyses for Figures 1C and 2B, and for Figures 3A,D, respectively. For Figure 7C, HEK293 cells were transiently transfected with 5 μg of Myc-hGPR37-FLAG or Myc-hGPR37(Δ51-54)-FLAG on 10-cm plates. Cells were reseeded onto 6-well plates 4 hours after transfection and treated with decCMK and GI254023X for 19 hours. A total of 10 000 cells from each sample were analyzed with the FACSCalibur flow cytometer and the CellQuestPro 6.0 software (BD Biosciences) (Figures 1 and 3). Alternatively, the ACCURI C6 flow cytometer and FlowJo

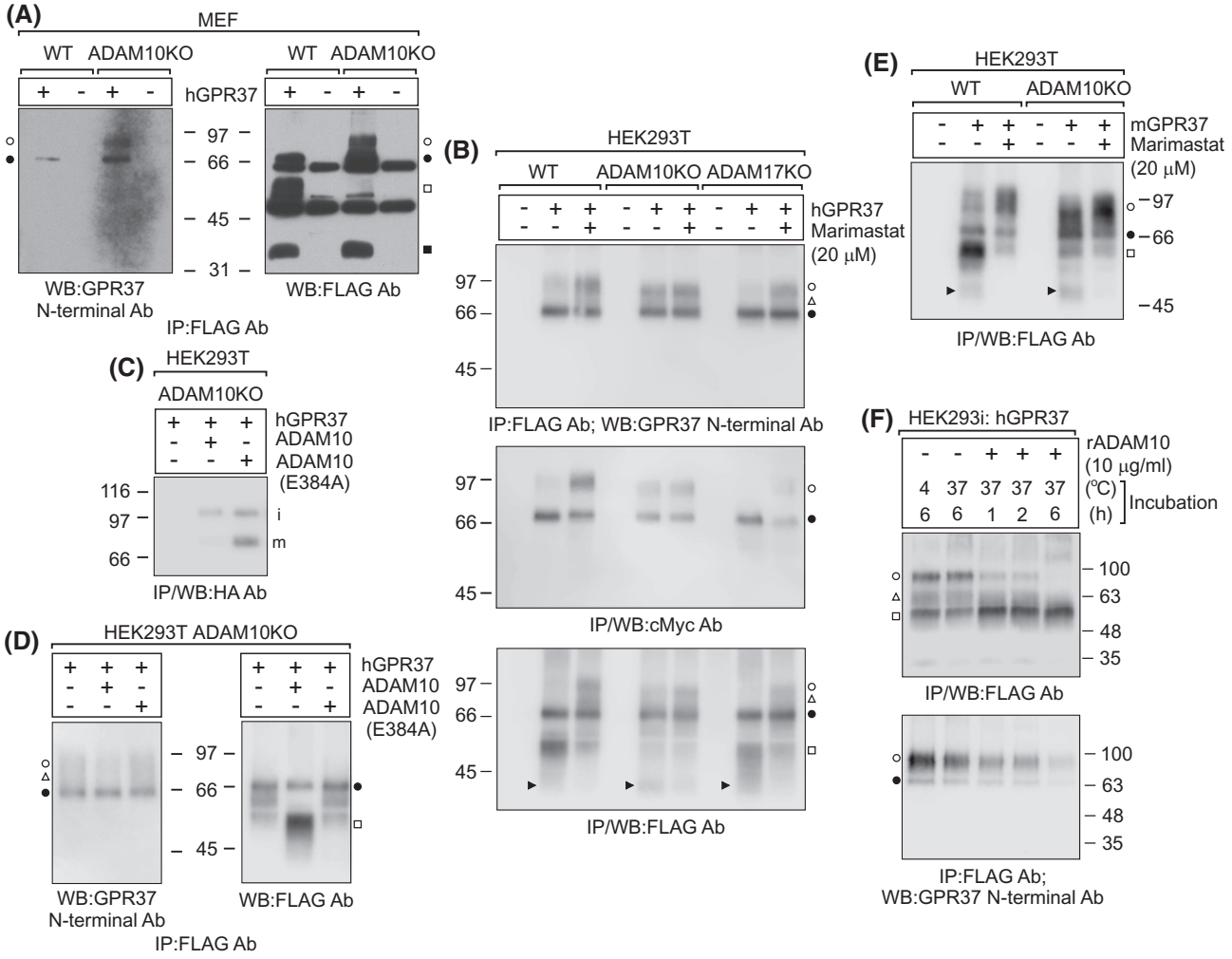

FIGURE 4 N-terminal cleavage of GPR37 is impaired in MEF and HEK293T cells lacking ADAM10 and is induced in vitro with rADAM10. The Myc- and FLAG-tagged hGPR37 was transiently expressed for 24 hours in WT and ADAM10KO MEF cells (A) or in WT, ADAM10KO, and ADAM17KO HEK293T cells that were treated or not with marimastat for 20 hours (B). For C-D, WT and ADAM10KO HEK293T cells were transiently transfected with Myc-hGPR37-FLAG together with the HA-tagged WT hADAM10 or its inactive E384A mutant (1:1 DNA ratio) for 48 hours. For E, mGPR37-FLAG was transiently expressed for 24 hours in WT and ADAM10KO HEK293T cells treated or not with marimastat for 20 hours, and for F, hGPR37 was expressed for 24 hours in stably transfected HEK293$_i$ cells treated with decCMK (10 μM) and GI254023X (5 μM) for 20 hours. Receptors and ADAM10 were immunoprecipitated from cellular lysates with FLAG and HA-antibodies, respectively, before analysis by Western blotting. For F, receptors were treated before elution from the FLAG antibody resin with rADAM10 as indicated. The additional GPR37 C-terminal fragment seen in HEK293T cells is indicated with an arrowhead (B, E). The other symbols and abbreviations are explained in the legends for Figures 2 and 3

V10 software (BD Biosciences) were used for measurements and data analysis (Figure 7). The background fluorescence of live cells stained only with the secondary antibody was subtracted from the mean fluorescence of live cells stained with the cMyc and secondary antibodies.

2.14 | Confocal microscopy

HEK293$_i$ cells expressing Myc-hGPR37-FLAG were cultured, fixed, and permeabilized as described elsewhere,[16] and stained with the appropriate antibodies.[39] The nuclei were stained with TO-PRO-3 iodine (2 μM, Invitrogen) for 10 minutes and the fluorescence was detected with the Zeiss LSM780 laser scanning confocal microscope (Carl Zeiss Microscopy, Jena, Germany), using the Plan-Apochromat 63x1.4 numerical aperture oil-immersion objective. The confocal images were processed with the Zen lite 2012 and Zen 2012 black edition software (Carl Zeiss Microscopy).

2.15 | Sequence alignment

The sequence logo of the GPR37 N-terminus corresponding to hGPR37 amino acids 27-178 was generated using WebLogo[40] (http://weblogo.threeplusone.com/). The respective sequences

of fourteen placental mammals (Table S1) were aligned using CLUSTAL Omega (1.2.4) and submitted to Weblogo for the generation of graphic representation. CLUSTAL Omega multiple sequence alignment was also performed for the first 300 and 278 amino acids of hGPR37 and mGPR37, respectively.

2.16 | Data analysis

The data were analyzed with the GraphPad Prism 8.3 software (GraphPad Software, La Jolla, CA, USA). Statistical analyses were performed using unpaired *t*-test or repeated measures one-way or two-way ANOVA followed by Dunnett's or Tukey's multiple comparison test. The limit of significance was set at $P < .05$.

3 | RESULTS

3.1 | N-terminal cleavage of GPR37 is inhibited by the ADAM10-selective metalloprotease inhibitor GI254023X

To initiate identification of the protease(s) involved in GPR37 processing, we first tested the ability of broad-spectrum and selective metalloprotease inhibitors to inhibit cleavage of the Myc- and FLAG-epitope tagged receptor (Figure 1A) in stably transfected inducible HEK293$_i$ cells. The subcellular localization of cMyc antibody-stained full-length receptors was analyzed by confocal microscopy after 24-hour induction of receptor expression. Treatment with the broad-spectrum inhibitor marimastat increased the number of full-length receptors at the cell surface observed as enhanced co-localization with the plasma membrane marker Na^+/K^+-ATPase (Figure 1B). Similar results were obtained with GI254023X (Figure 1B), an inhibitor selective for ADAM10.[41] Likewise, the amount of cMyc-stained full-length receptors detected at the plasma membrane by flow cytometry was elevated (Figure 1C). This effect was dependent on the inhibitor concentration used, with a significant increase detected already at 0.25 μM GI254023X.

We then investigated the effect of GI254023X on receptor cleavage by analyzing membrane-bound and shed receptor fragments by Western blotting. Under steady-state conditions, an N-terminal fragment of about 32-35 kDa was purified with cMyc antibody from the conditioned culture medium of induced HEK293$_i$ cells (Figure 2A, first lane; Figure 2B). The same antibody also detected two specific full-length receptor species of about 67 and 96 kDa from cellular lysates following immunoprecipitation with FLAG antibody (Figure 2C, left panel, first lane, Figure S1). As demonstrated earlier,[16] these species represent intracellular receptor precursors and mature plasma membrane receptors, respectively (Figure 2B). Immunoblotting of these samples with FLAG antibody revealed a prominent C-terminal receptor fragment of about 53 kDa and a minor 34-kDa one in addition to the 67-kDa precursor (Figure 2C, right panel, first lane, Figure S1). While the 53-kDa form represents GPR37 that is cleaved between Glu167 and Gln168 at the receptor N-terminal ectodomain, the 34-kDa species is most likely cleaved adjacent to the first transmembrane domain[16] (Figure 2B). The mature 96-kDa receptor, however, was poorly recognized with FLAG antibody and was typically not seen (Figure 2C, right panel, first lane; Figures S1 and S2).

As expected, treatment with GI254023X resulted in a significant concentration-dependent increase in the 96-kDa full-length receptor and a corresponding decrease in the 53-kDa cleaved form (Figure 2C,D) and the shed 32-35-kDa fragment (Figure 2A). No changes were seen for the 34-kDa cleaved form (Figure 2C, right panel). Similar findings were obtained when hGPR37 and mGPR37 were transiently expressed in metalloprotease inhibitor-treated human neuroblastoma SH-SY5Y cells and in HEK293 cells, respectively (Figure 2E,F). These results suggest that the metalloprotease mediated GPR37 ectodomain cleavage is neither a cell line nor species-specific event. Interestingly, the major C-terminal receptor fragment identified for mGPR37 migrated slower in SDS-PAGE than the corresponding human receptor species (Figure 2F). The difference remained after removing N-glycans with PNGase F (Figure S3), suggesting nonidentical metalloprotease cleavage sites for the two receptors.

Intriguingly, treatment of stably transfected HEK293$_i$ cells with GI254023X resulted in the appearance of an additional hGPR37 form on Western blots that was not normally seen. This species that appeared either as a distinct species of about 70 kDa or as an ill-defined smear above the 67-kDa receptor precursor (hereafter called the 70-kDa receptor species) was detected with FLAG antibody, but not with cMyc antibody (Figure 2C, right panel; B). This receptor form was likewise detected when cells were treated with broad-spectrum metalloprotease inhibitors, such as GM6001, TAPI-1, or marimastat, but not with inactive GM6001 or inhibitors against other protease families (Figure 2G). In addition to the FLAG antibody, a GPR37 N-terminal antibody raised against amino acids 27-265 of hGPR37,[29] was also able to detect the 70-kDa form (Figure 2H). Thus, the ectodomain of hGPR37 must be proteolytically processed at yet another site that is located upstream of the identified metalloprotease cleavage site.

3.2 | The N-terminal ectodomain of GPR37 is cleaved by ADAM10

The ability of GI254023X to inhibit hGPR37 N-terminal cleavage and shedding at low concentrations suggests that cleavage

at the identified Glu167↓Gln168 site is mediated by ADAM10. To investigate this possibility further, we utilized siRNA-mediated knock-down of ADAM10 and ADAM17, another metalloprotease commonly involved in ectodomain shedding,[26] in HEK293$_i$ cells stably expressing hGPR37. The knock-down of the desired enzymes was verified by subjecting detergent-solubilized membrane fractions to Western blotting with selective antibodies against ADAM10 and ADAM17 (Figure 3A). Analysis of the solubilized receptors with the hGPR37 N-terminal antibody revealed that knock-down of ADAM10, but not ADAM17, increased the amount of the full-length receptor significantly (Figure 3A,C). Correspondingly, the amount of the cleaved receptor species, detected with an antibody against the GPR37 C-terminus,[5] was decreased (Figure 3A,C). Likewise, the amount of the shed N-terminal fragment in the culture medium was reduced when the cells were treated with the ADAM10 siRNA pool (Figure 3B). These results were further supported by flow cytometry as knock-down of ADAM10, but not ADAM17, significantly increased the number of full-length receptors at the cell surface (Figure 3D).

Further evidence for the role of ADAM10 in GPR37 cleavage was acquired by expressing the receptor in stable cell lines that lack ADAM10. As expected, transient expression of hGPR37 in ADAM10 deficient MEF cells[34] showed that the full-length mature 96-kDa species was substantially increased and the 53-kDa cleaved form decreased compared with the corresponding WT cells (Figure 4A). These results were reproduced by employing ADAM10 deficient HEK293T cells generated using CRISPR/Cas9-mediated genome editing, but not by using the corresponding ADAM17 deficient cells[33] (Figure 4B). The full-length receptor in the ADAM10KO and ADAM17KO cells was slightly smaller in size compared to the WT cells (Figure 4B), most likely due to somewhat altered receptor glycosylation in the KO cells (Figure S4).

Next, to test whether we could rescue the enzymatic activity of ADAM10, we co-transfected hGPR37 either with the HA-tagged WT or an inactive Glu384Ala mutant ADAM10[42] in ADAM10KO HEK293T cells. Both WT and mutant ADAM10 were expressed as two forms, the larger immature and the smaller mature species[42] (Figure 4C). The abundance of the full-length mature receptor was decreased when cells were co-transfected with the WT ADAM10 (Figure 4D). This was accompanied by a corresponding dramatic increase in the cleaved 53-kDa receptor (Figure 4D). In contrast, overexpression of the inactive mutant ADAM10 had no effect (Figure 4D). We also transfected the WT and ADAM10KO HEK293T cells with mGPR37. As expected, cleavage of the receptor was reduced in KO cells and this inhibitory effect was further enhanced when cells were treated with marimastat (Figure 4E). Finally, we subjected the purified hGPR37 to rADAM10-mediated cleavage in vitro, and as anticipated, the enzyme cleaved the receptor in a time-dependent manner at 37°C (Figure 4F). Taken together, these results imply that the major enzyme responsible for cleaving GPR37 is indeed ADAM10. Nevertheless, we cannot exclude the possibility that other metalloproteases are also involved in GPR37 processing, especially in cells lacking ADAM10. This was suggested by the finding that an additional smaller-sized C-terminal receptor fragment seen in HEK293T cells was responsive to marimastat treatment (Figure 4B,E).

3.3 | The GPR37 ectodomain is cleaved at an additional N-terminal site

The 70-kDa hGPR37 species detected in metalloprotease inhibitor-treated cells indicates that the receptor ectodomain is cleaved at an additional site upstream of the identified ADAM10 site. Furthermore, this additional cleavage appears to be mediated by a protease not belonging to the metalloprotease family as it was detected in the presence of broad-spectrum metalloprotease inhibitors (see Figure 2G). The appearance and identity of the 70-kDa receptor species were first studied by metabolic pulse-chase labeling and cell surface biotinylation. Under control conditions, the 67-kDa receptor precursor was rapidly converted to the 96-kDa mature full-length receptor that was then cleaved to the 53-kDa proteolytically processed form and to the less abundant 34-kDa species (Figure 5A, upper panel). When cells were treated with GI254023X during depletion, pulse, and chase, the appearance of the cleaved 53-kDa form was inhibited (Figure 5A). This was, however, less evident than was normally seen in Western blot experiments, most likely due to the shorter treatment time with the inhibitor. The 70-kDa receptor form became apparent during the chase and was clearly detectable after 3 hours (Figure 5A). Importantly, this receptor species was detected at the cell surface together with the 96-kDa full-length and the 53-kDa cleaved receptor (Figure 5A, lower panel). Enzymatic deglycosylation of the 2-hour chase samples from cells treated or not with GI254023X revealed that the 70-kDa species was insensitive to Endo H (Figure 5B) that digests N-glycans typical to endoplasmic reticulum-localized proteins. However, upon treatment with PNGase F, which releases also fully processed N-glycans, it was deglycosylated to a slightly smaller size than the deglycosylated receptor precursor (Figure 5B). Thus, the 70-kDa receptor form represents a mature cell surface receptor that has been cleaved at a site close to the extreme end of the N-terminus but downstream of the N-glycosylation site at Asn36 (see Figure 2B).

To determine the protease responsible for the conversion of hGPR37 to the 70-kDa species, we first tested the ability of various protease inhibitors to inhibit this cleavage step in HEK293$_i$ cells treated simultaneously with marimastat (Figure 5C). No differences in the amount of the 70-kDa species or the full-length 96-kDa form were detected with protease

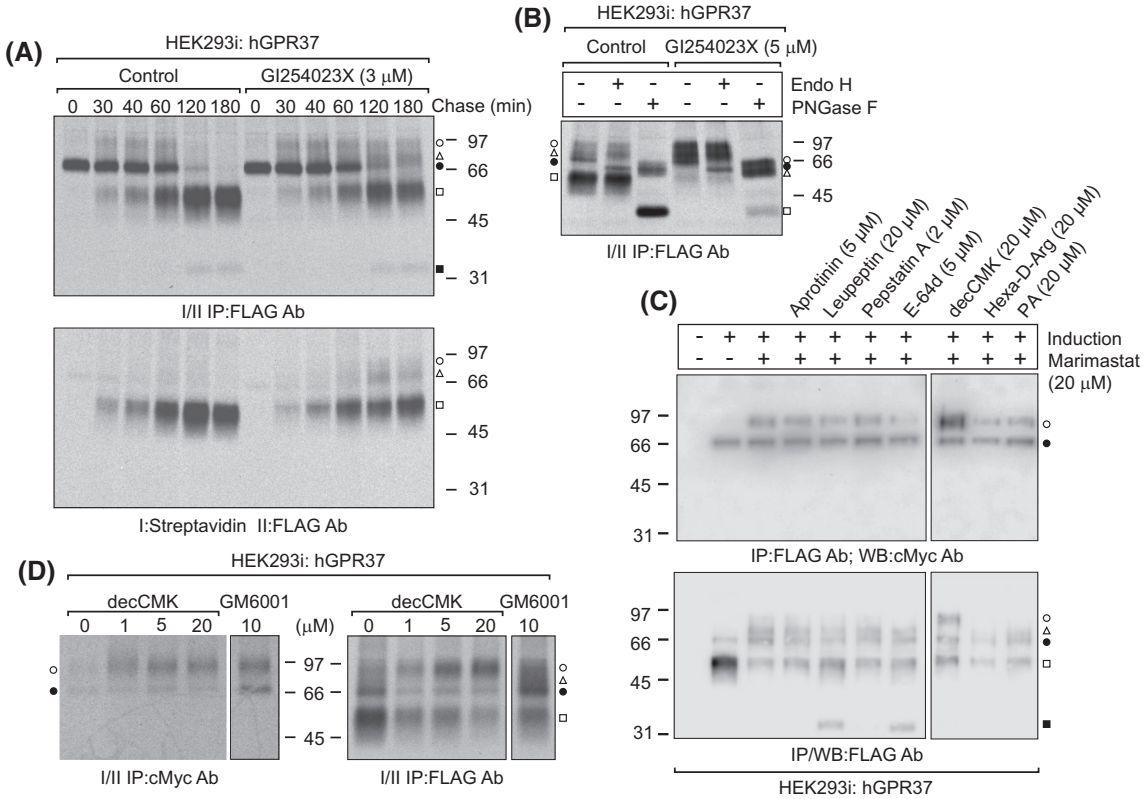

FIGURE 5 GPR37 is cleaved in its N-terminal domain by a non-metalloprotease. A, B and D, HEK293$_i$ cells were induced to express hGPR37 for 6 hours (A, B) or 16 hours (D), labeled with [^{35}S]methionine/cysteine for 30 minutes and chased for various time periods before cell surface proteins were biotinylated (A) or not (B, D) with sulfo-NHS-biotin. GI254023X, decCMK, or vehicle were added to the medium during depletion, labeling, and chase. Receptors were subjected to two-step immunoprecipitation with FLAG antibody (A, upper panel, B, D) or were first purified with streptavidin agarose and then with FLAG antibody (A, lower panel) before analysis by SDS-PAGE and fluorography. For panel B, immunoprecipitated receptors from 120-minute chase samples were deglycosylated with Endo H (50 mU/mL) or PNGase F (20 U/mL) before SDS-PAGE. C, HEK293$_i$ cells were induced for 24 hours and treated with the indicated protease inhibitors or vehicle for 20 hours. Immunoprecipitated receptors were analyzed by SDS-PAGE and Western blotting. Note that GI254023X inhibits receptor cleavage more efficiently at 5 μM (B) than at 3 μM (A). PA, phosphoramidon. Other abbreviations and symbols are as described in the legends for Figures 2 and 3

inhibitors belonging to serine-, cysteine-, trypsin-, aspartyl- or metallo-endopeptidase families (Figure 5C). In contrast, treatment with the PC inhibitor decCMK resulted in a clear increase in the full-length GPR37 while the 70-kDa species was no longer apparent (Figure 5C). These changes, however, were not observed with the corresponding membrane-impermeable inhibitor hexa-D-arginine (Figure 5C). Metabolically labeled cells chased for 2 hours and treated with decCMK without the metalloprotease inhibitor showed a similar increase in the full-length GPR37, but not in the 70-kDa species (Figure 5D). Treatment with the metalloprotease inhibitor GM6001, in contrast, led to an increased abundance of both the 70-kDa form and the full-length receptor (Figure 5D). Since decCMK is selective for furin and furin-like PCs, these data imply that GPR37 is cleaved by member(s) of this protease family. Furthermore, this cleavage step appears to take place intracellularly before the receptor reaches the cell surface or, alternatively, after endocytosis, as only the membrane-permeable inhibitor was able to reduce the cleavage. Interestingly, the two tested cysteine protease inhibitors, leupeptin and E-64d, increased the amount of the 34-kDa C-terminal receptor fragment that was not responsive to metalloproteinase inhibitors (Figure 5C, see also Figure 2C,G). Thus, this species most likely represents a receptor degradation intermediate in the endosomal pathway.

3.4 | GPR37 is cleaved by furin at a conserved Arg^{54}↓Asp^{55} site in the N-terminus

Furin, together with other basic amino acid-specific PCs, possesses a strong preference for the cleavage motif (Arg/Lys)-2nX-Arg↓ (where n = 0-3 aa).[24] The N-terminal domain of hGPR37 contains four potential sites upstream of the Glu167↓Gln168 cleavage site that fit these criteria. These potential sites are well conserved according to a sequence logo generated from N-terminal GPR37 sequences of 14 placental mammals (Figure 6).

To test if any of these sites are used, we created constructs with short deletions at the putative sites and transiently expressed them in HEK293 cells. The site closest to the

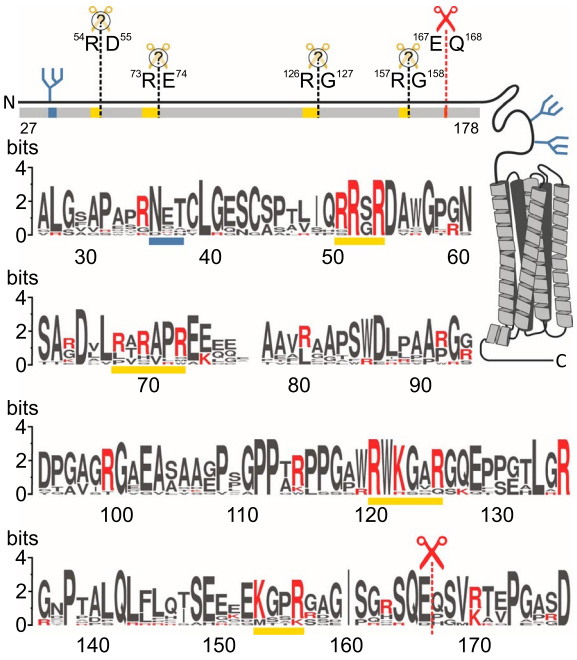

FIGURE 6 Conservation of GPR37 N-terminal tail. WebLogo[40] was used to generate a graphical representation of conservation between fourteen placental mammalian species (see Table S1). The generated sequence logo corresponding to residues 27-178 of hGPR37 (shown as a gray bar below the schematic model of the N-terminal domain) displays the conservation of each residue, indicated by the overall height of the letter. The relative height of each letter in the stack indicates the frequency of the corresponding amino acid at the site. The positively charged amino acids Arg and Lys are shown in red. The consensus sequence for N-glycosylation and putative PC cleavage sites are indicated with blue and yellow bars, respectively, below the sequence. The metalloprotease cleavage site (Glu167↓Gln168) identified for hGPR37[16] is indicated with the red scissors. The corresponding sites for hGPR37 are also shown in the schematic model

Glu167↓Gln168 cleavage site was omitted from the analysis as it is located only about 10 amino acids upstream of the metalloprotease cleavage site. Deletion of the most distal site at the receptor N-terminus (Δ51-54) increased the abundance of the full-length receptor and abolished the cleavage step that produces the 70-kDa receptor species in GI254023X-treated WT receptor-expressing cells (Figure 7A). No clear effect was observed when the two other putative cleavage sites (Δ68-73 and Δ121-126) were deleted (Figure 7A). Similar results were obtained when the three arginine residues at positions 51, 52, and 54 or only the arginine residue 54 preceding the expected cleavage site were mutated to alanine (Figure 7B). These results indicate that PC(s) cleave the GPR37 ectodomain at a highly conserved site at Arg54↓Asp55. Further evidence for this finding was provided by flow cytometric analysis of cells transiently transfected with the WT and Δ51-54 mutant GPR37 (Figure 7C). The number of full-length WT, but not mutant receptors, was increased significantly at the cell surface when cells were treated simultaneously with decCMK and GI254023X compared to cells treated only with the metalloprotease inhibitor (Figure 7C).

To test if inhibition of the PC-mediated cleavage also affects the shed N-terminal receptor fragment, we treated transiently transfected HEK293 cells with decCMK, GI254023X, or both inhibitors simultaneously, and analyzed the shed fragments in the concentrated conditioned medium by Western blotting. As expected, the amount of the receptor fragment detected with the N-terminal antibody was decreased with GI254023X (Figure 7D). In contrast, its abundance was increased with decCMK but only when the cells were not treated simultaneously with GI254023 (Figure 7D). This observation, together with the fact that the fragment could also be detected with cMyc antibody that recognizes the N-terminal Myc tag (see Figures 2A and 3B), implies that it originates from receptors that are cleaved by ADAM10 but not by PC(s). Similar results were obtained when the cells were transfected with hGPR37 carrying N-terminal NanoLuc and HA tags (Figure 7E), indicating that the added tags do no alter proteolytic processing and shedding of the receptor ectodomain. Altogether, these results imply that the ADAM10-mediated receptor cleavage occurs efficiently, whereas the cleavage by PC(s) is inefficient.

Finally, to further identify the enzyme that cleaves hGPR37 at the Arg54↓Asp55 site, we expressed the receptor in furin-deficient human colon carcinoma LoVo cells[32] (Figure 7F). Both marimastat and GI254023X were able to inhibit receptor cleavage at the Glu167↓Gln168 site in these cells (Figure 7F). However, the 70-kDa species was not detected (Figure 7F), although it was clearly visible in HEK293 cells tested in parallel (data not shown). These results are in line with the notion that the major PC involved in GPR37 processing at the Arg54↓Asp55 site is furin.

3.5 | Processing of GPR37 in the mouse brain occurs in an ADAM10-dependent manner

The finding that both human and mouse GPR37 undergo ADAM10-mediated cleavage in cultured cells prompted us to investigate the processing of the receptor in a more physiologically relevant environment. To this end, membrane fractions were prepared from various brain regions of GPR37KO mice and corresponding WT animals and expressed GPR37 species were studied by Western blotting using antibodies against the receptor N- and C-terminal domains. As reported earlier,[5] a specific receptor species of about 62 kDa was detected with the N-terminal antibody from the cerebellum, cortex, hippocampus, and striatum of GPR37WT, but not from KO animals (Figure 8). This species was sensitive to digestion with PNGase F but not with Endo H (Figure S5), thus representing a mature receptor with fully processed N-glycans.

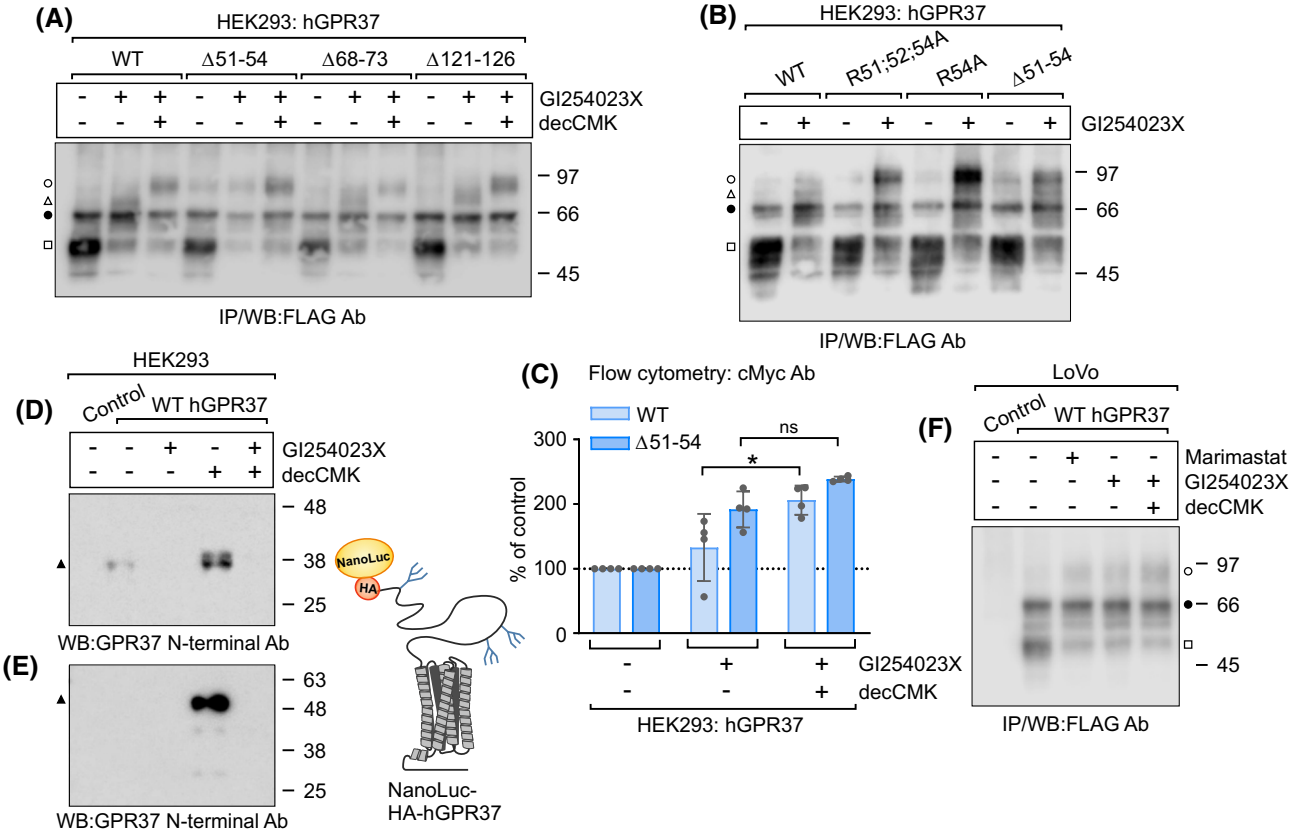

FIGURE 7 GPR37 is cleaved at the conserved Arg^{54}↓Asp^{55} site in the N-terminus by furin. HEK293 (A-E) and LoVo (F) cells were transiently transfected with the indicated Myc-hGPR37-FLAG constructs (A-D, F), or alternatively, with the NanoLuc- and HA-tagged hGPR37 (E) for 16-24 hours and treated or not with GI254023X (5 μM), decCMK (20 μM) or marimastat (20 μM) for 20 (A, B, F), 19 (C) or 12 (D, E) h. Immunoprecipitated receptors from cellular lysates (A, B, F) or aliquots of the concentrated conditioned culture medium without immunoprecipitation (D, E) were analyzed by SDS-PAGE and Western blotting. For panel C, cell surface cMyc antibody labeled receptors were analyzed by flow cytometry. Triplicate or quadruplicate samples from four independent experiments were analyzed by repeated-measures two-way ANOVA and Tukey's multiple comparison test before normalization to the respective controls without the inhibitor-treatment. *$P < .05$; ns, nonsignificant. Abbreviations and symbols are as in the legend for Figure 2

In comparison, the C-terminal antibody detected a distinct smaller receptor form with an apparent molecular weight of about 42 kDa in the same samples. For an unknown reason it was not able to detect the 62 kDa species (Figure 8). Likewise, it detected the full-length hGPR37 and mGPR37 in cultured cells poorly (see Figure 3A, Figure S2). These results suggest that GPR37 undergoes proteolytic processing also in native brain tissue. The smaller size of receptor species in the brain compared to those detected in cultured cells might suggest more extensive proteolytic processing of the receptor in the native tissue or otherwise different post-translational processing.

Finally, to assess the role of ADAM10 in GPR37 processing in the brain, we analyzed samples of various brain regions of *Adam10*cKO mice[30] at postnatal day 20. In this mouse model, the *CamK*IIa-*Cre* promoter was used to obtain deletion of ADAM10 after birth, especially in forebrain neurons. The levels in the cerebellum, however, remain unaltered. Consistent with previous results,[28,30] a significant decrease in ADAM10 levels was detected in solubilized membrane samples from cortex and hippocampus, but not from cerebellum, of the *Adam10*cKO mice compared with the WT littermate controls (Figure 9B,F, Figure S6B). The amount of mature ADAM10 was decreased to 28 ± 4% and 39 ± 6% in the cortex and hippocampus, respectively and the immature form was practically undetectable in the KO mouse samples. This change in the cortex and hippocampus was accompanied by a highly significant increase in the 62-kDa mGPR37 detected with the N-terminal antibody (2200 ± 420% and 480 ± 445%, respectively) (Figure 9). No changes were detected in the cerebellum (Figure S6). Thus, we can conclude that ADAM10 is the major protease involved in GPR37 processing in the mouse brain.

4 | DISCUSSION

GPR37 is an orphan GPCR that is abundantly expressed in the brain and has been linked to several important physiological functions in health and disease. However, the mechanisms of receptor regulation are poorly understood. The

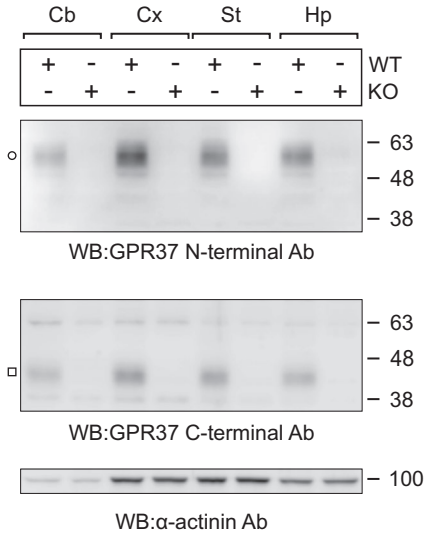

FIGURE 8 Distinct GPR37 species are detected in the mouse brain with antibodies directed against N- and C-terminal domains of the receptor. Cellular membranes were isolated from the cerebellum (Cb), cortex (Cx), striatum (St), and hippocampus (Hp) of WT and GPR37KO mice, and expressed receptors were analyzed by SDS-PAGE and Western blotting. The α-actinin was used as a loading control. Abbreviations and symbols are as in the legend for Figure 2

present study was prompted by our previous finding that the receptor undergoes constitutive metalloprotease-mediated N-terminal cleavage and is thus present at the cell surface mainly in the truncated form.[16] Here, we present evidence that the processing of GPR37 occurs in two steps involving proteases from two enzyme families. The cleavage at the previously identified Glu167↓Gln168 site is mediated by the well-characterized metalloprotease and sheddase ADAM10. Moreover, GPR37 is also cleaved by the PC furin at the conserved Arg54↓Asp55 site.

Several lines of evidence support the notion that ADAM10 is involved in the cleavage and ectodomain shedding of human and mouse GPR37. The cleavage was inhibited by the ADAM10-selective inhibitor GI254023X at low concentrations, and knock-down and knock-out of ADAM10 in HEK293 and MEF cells reduced the cleavage. In addition, the cleavage could be rescued in the HEK293TKO cells by over-expression of WT ADAM10, but not by the inactive mutant ADAM10. Furthermore, the cleavage was induced in vitro by rADAM10. Finally, the conditional KO of *Adam10* in mouse neuronal cells was found to reduce GPR37 cleavage in the cortex and hippocampus in vivo, confirming the physiological relevance of ADAM10 as a GPR37 cleaving enzyme. Although several membrane-anchored and single-spanning membrane proteins have been shown to be processed by ADAM10, GPR37, to our knowledge, is the first multispanning membrane protein that has been validated as an ADAM10 substrate.

While we demonstrate the ADAM10-mediated cleavage for both human and mouse GPR37, the results indicate that there are some species-specific differences in receptor processing. In cultured cells, the human receptor appears to be cleaved more efficiently than the mouse receptor at steady state conditions and the cleaved C-terminal fragment is smaller in size. Moreover, the mouse receptor species are smaller in the brain than in cultured cells. These observations, together with the fact that the N-terminal domain of the mouse receptor is shorter in length and sequences around the Glu167↓Gln168 cleavage site are not identical (Figure S7), indicates a distinct ADAM10 cleavage site for the two receptors and possibly differential regulation. However, we cannot exclude the possibility that the primary ADAM10 cleavage site of hGPR37 is located upstream of the identified site and the receptor is prone to additional processing in a similar manner as has been shown for the thyrotropin receptor N-terminal domain.[43] It can also be suggested that other ADAM or MMP family members may compensate ADAM10 function under certain conditions. For example, they might cleave GPR37 under cellular stress conditions, while ADAM10 cleaves GPR37 in a constitutive manner. In contrast to species-specific differences, no clear differences in receptor processing were detected in the tested mouse brain regions. The specific bands detected with the receptor antibodies were heterogenous and wide, but no apparent differences were detected in receptor size or number of species detected.

Although metalloproteases have been shown to cleave GPCRs, only a few studies have identified the responsible enzymes. The limited proteolysis and shedding of the thyrotropin receptor ectodomain have been extensively studied and metalloproteases, including ADAMs have been implicated in the process. However, the corresponding enzyme has remained under dispute [reviewed[44]]. The N-terminus of the adhesion GPCR B1 (ADGRB1, also known as BAI1) is cleaved by MMP14,[45] and several MMPs have been suggested to be involved in PAR1 cleavage.[46,47] Intriguingly, cleavage of PAR1 in a noncanonical site by MMP2 distinct from the thrombin cleavage site results in biased signaling,[46] suggesting that the metalloprotease-mediated cleavage has a role in the functional regulation of the receptor. Metalloproteases have also been associated with the proteolytic processing of a few other class A GPCRs but the responsible enzymes have remained unknown. These receptors include the β_1-adrenergic receptor,[35,48] V2 vasopressin receptor,[49] C5a receptor,[50] sphingosine 1-phosphate receptor,[51] parathyroid hormone 1 receptor[52] as well as the endothelin B receptor[21] and GPR37LI.[22] This prevalent role of metalloproteases in the cleavage of GPCRs suggests that these enzymes, including ADAMs, might be more generally involved in the processing and functional regulation of GPCRs.

The mechanisms that determine ADAM10 substrate specificity and govern substrate recognition are not fully

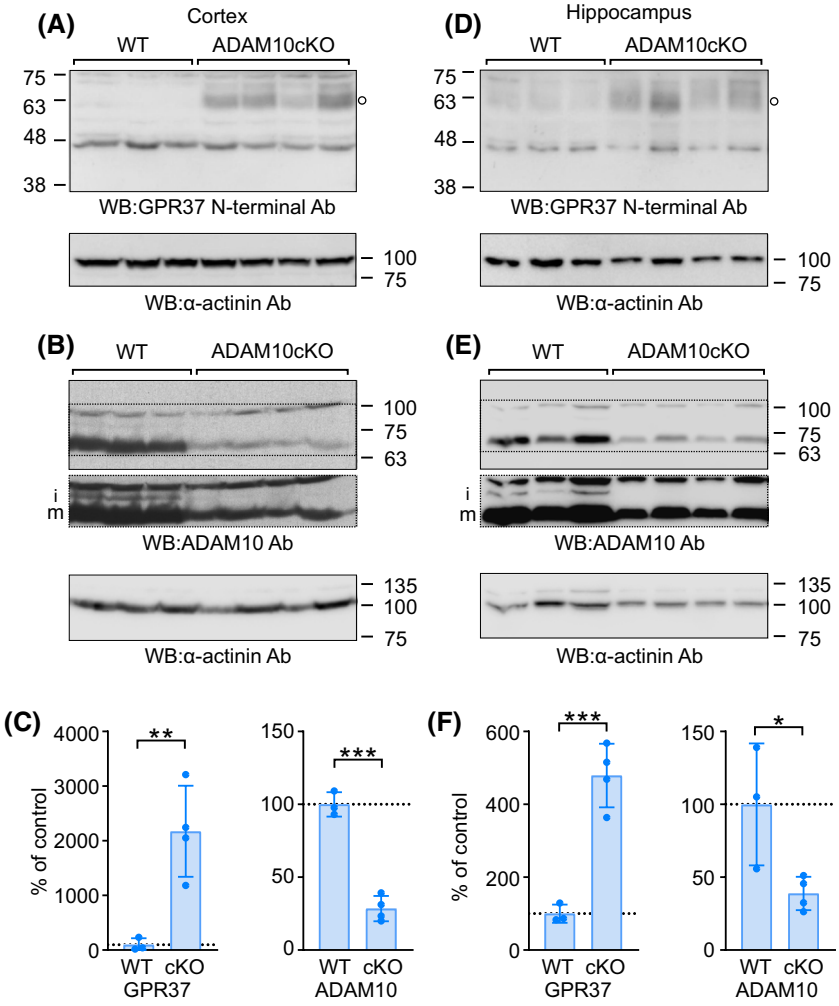

FIGURE 9 GPR37 is cleaved by ADAM10 in the mouse brain. Cellular membranes were isolated from the cortex (A-C) and hippocampus (D-F) of 20-day-old ADAM10cKO mice and the corresponding WT littermate controls. The receptor (A, D) and ADAM10 (B, E) were analyzed by SDS-PAGE and Western blotting. The α-actinin was used as a loading control. The outlined areas in panels B and E are shown with enhanced exposure time. In panels C and F, the normalized intensity values for the full-length receptor and mature ADAM10 are shown as means ± SD from three WT and four cKO animals. The unpaired *t*-test was used for the statistical comparison. ***$P < .001$; **$P < .01$; *$P < .05$; ns, nonsignificant. Abbreviations and symbols are as in the legends for Figures 2 and 3

understood. Although the protease has been shown to favor certain amino acids surrounding the substrate cleavage site,[53,54] no distinct recognition motif has been identified. The distance of the cleavage site from the cell membrane and protein conformation appear to be important determinants.[33] Furthermore, the noncatalytic domains of the enzyme play a role in defining substrate selectivity. For instance, the disintegrin- and cysteine-rich domains of ADAM10 may facilitate substrate binding at a distinct site from the metalloprotease cleavage site, thus promoting accessibility to the cleavage site.[55] In line with these notions, we have previously reported that mutations at the identified Glu167↓Gln168 cleavage site are not sufficient to abolish GPR37 cleavage.[16] Surprisingly, the distance between this cleavage site in the GPR37 ectodomain and the first transmembrane domain is almost 100 amino acids. As ADAM10 typically cleaves its substrates close to the plasma membrane, this raises a question as to how ADAM10 can access the GPR37 cleavage site. One possibility is that the favorable proximity between the ADAM10 metalloprotease domain and GPR37 cleavage site is attained by conformational rearrangements of the GPR37 ectodomain. Indeed, it has been shown that the conformation of ADAM substrates that enables cleavage is typically tightly regulated.[56] Additionally, the conformation of ADAM10 itself can vary depending on its association with different members of the TspanC8 subgroup of tetraspanins,[57] and this could play a role in positioning the metalloprotease domain in a favorable orientation to cleave GPR37. It can also be speculated that GPR37 and the cleaving enzyme might not be in the same cell. Most ADAM10 substrates are cleaved *in cis* within the same cell but the unusual mechanism occurring *in trans* has been demonstrated for ephrin A5.[55] In line with this, ADAM10 in neurons might cleave the receptor in the adjacent glial cells, in which it is abundantly expressed.[12,13]

In addition to ADAMs, PCs are another family of enzymes typically involved in limited proteolysis.[24] Like for ADAMs, their potential involvement in the processing of GPCRs is poorly understood. The PC-mediated cleavage has been demonstrated for the adhesion GPCRs G6 (ADGRG6), F5 (ADGRF5), and B2 (ADGRB2), also known as GPR126, GPR116, and BAI2, respectively.[58-60] In addition, GPR107, a proposed GPCR for neuronostatin, is processed by furin.[61] More recently, PCs have been shown to be involved in the processing of PAR1 and PAR2.[62,63] Thus, PARs provide

another example of GPCRs in addition to GPR37 that can be cleaved by members of both PC and metalloprotease family members at distinct sites in their N-termini.

The N-terminal domain of GPR37 contains several potential cleavage sites for PC-mediated processing, but only the highly conserved $Arg^{54}\downarrow Asp^{55}$ site was found to be used. This was supported by the finding that deletions spanning this site, but not the other putative sites, blocks the cleavage. Mutation of Arg54 to alanine was sufficient for this effect. The cleavage was inhibited by the membrane-permeable PC inhibitor decCMK, but not by the membrane-impermeable hexa-D-arginine, and furthermore, it was abolished in furin-deficient LoVo cells, indicating that the cleaving enzyme is the *trans*-Golgi network localized furin. In line with this, the full-length receptor is found in abundant amounts in the *trans*-Golgi network.[16] The high conservation of the GPR37 furin cleavage site in mammals suggests that the PC-mediated processing of the receptor has functional relevance. The distal N-terminus of GPR37 removed by furin may act as a prodomain in a similar manner as has been described for several other membrane proteins. Such prodomains have been shown, for example, to inhibit the functional activity of the protein or to promote protein folding in the early secretory pathway.[64]

As the limited proteolytic processing of the N-terminal domain of PARs and adhesion family GPCRs is intimately involved in receptor functional activation,[19,20] it is tempting to speculate a similar role for GPR37 ectodomain processing. It can be hypothesized that the new N-terminus revealed by limited proteolysis might act as a tethered peptide agonist that binds and activates the receptor. Alternatively, the shed N-terminal fragment(s) may act as ligands for the receptor itself or for other proteins/receptors in adjacent cells, or even as a scavenger for the natural yet unidentified soluble ligand in the extracellular space. It is also possible that limited proteolysis merely represents a way to inactivate the receptor, in which case only the full-length receptor is expected to be functionally active. Future research will reveal whether any of these intriguing possibilities turn out to be accurate.

In summary, we provide evidence that ADAM10 cleaves GPR37 in various cell lines of diverse tissue origin as well as in a more physiological context in the mouse brain. Furthermore, GPR37 ectodomain is subject to additional processing mediated by furin. These findings together support the notion that the two enzyme families ADAMs and PCs could be more commonly involved in the processing and functional regulation of GPCRs.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

S.O. Mattila and U.E. Petäjä-Repo designed research and wrote the manuscript. S.O. Mattila, H.E. Tuhkanen, J.J. Lackman, A. Konzack, X. Morató, J. Argerich, and U.E. Petäjä-Repo performed experiments. S.O. Mattila, J.J. Lackman, and U.E. Petäjä-Repo analyzed data. P. Saftig provided cell lines and tissue samples and F. Ciruela antibodies and tissue samples. All authors contributed to the editing of the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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