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RESEARCH ARTICLE

# The Parkinson's-disease-associated receptor GPR37 undergoes metalloproteinase-mediated N-terminal cleavage and ectodomain shedding

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## ABSTRACT

The G-protein-coupled receptor 37 ( GPR37) has been implicated in the juvenile form of Parkinson's disease, in dopamine signalling and in the survival of dopaminergic cells in animal models. The structure and function of the receptor, however, have remained enigmatic. Here, we demonstrate that although GPR37 matures and is exported from the endoplasmic reticulum in a normal manner upon heterologous expression in HEK293 and SH-SY5Y cells, its long extracellular N-terminus is subject to metalloproteinase-mediated limited proteolysis between E167 and Q168. The proteolytic processing is a rapid and efficient process that occurs constitutively. Moreover, the GPR37 ectodomain is released from cells by shedding, a phenomenon rarely described for GPCRs. Immunofluorescence microscopy further established that although full-length receptors are present in the secretory pathway until the trans-Golgi network, GPR37 is expressed at the cell surface predominantly in the N-terminally truncated form. This notion was verified by flow cytometry and cell surface biotinylation assays. These new findings on the GPR37 N-terminal limited proteolysis may help us to understand the role of this GPCR in the pathophysiology of Parkinson's disease and in neuronal function in general.


**KEY WORDS: GPR37, Ectodomain shedding, Metalloproteinase, N-terminal cleavage, Pael receptor, Parkinson's disease**

## INTRODUCTION

The G-protein-coupled receptor 37 (GPR37) is a class A (rhodopsin-type) G-protein-coupled receptor (GPCR) sharing highest sequence similarity with endothelin and bombesin receptors (Marazziti et al., 1997; Zeng et al., 1997; Donohue et al., 1998; Imai et al., 2001). It is abundantly expressed in glial and neuronal cells in several brain regions, including dopaminergic cells of the substantia nigra, and has been reported to have a role in dopaminergic signalling (Marazziti et al., 2004, 2007; Imai et al., 2007). Incidentally, GPR37 has been implicated in certain brain disorders (Imai et al., 2001; Fujita-Jimbo et al., 2012; Tomita et al., 2013), the most widely acknowledged of which is the autosomal recessive juvenile parkinsonism (AR-JP), an early onset familial Parkinson's disease. The receptor was originally identified as a substrate of parkin, an E3 ubiquitin ligase encoded by the *PARK2* gene, and is therefore also known as the Parkin-associated endothelin-receptor-like receptor [Pael receptor (Imai et al., 2001)]. Mutations in the *PARK2* gene leading to the loss of the ubiquitin ligase activity of parkin are the most common causes of AR-JP (Kitada et al., 1998). An insoluble form of GPR37 has been reported to accumulate in the brains of AR-JP patients (Imai et al., 2001) and also in the core of Lewy bodies of Parkinson's disease patients in general (Murakami et al., 2004). Thus, the intracellular aggregation and impaired ubiquitylation of unfolded GPR37 by parkin have been proposed to be linked with the death of dopaminergic neurons characteristic of Parkinson's disease (Imai et al., 2001; Kitao et al., 2007). Based on these early findings, the research on GPR37 has mainly focused on strategies to improve its plasma membrane trafficking and to reduce the receptor-induced cell toxicity (Dunham et al., 2009; Gandía et al., 2013; Lundius et al., 2013; Dutta et al., 2014). Recently, GPR37, together with its closest homolog GPR37-like 1 (GPR37L1; Valdenaire et al., 1998), has been reported to act as a receptor for prosaposin and a prosaposin-derived peptide prosaptide (Meyer et al., 2013). These peptides, which possess neuroprotective and glioprotective effects (reviewed in Meyer et al., 2013), have been found to induce receptor internalization and to stimulate GPR37-mediated signalling in HEK293 cells and primary cortical astrocytes (Meyer et al., 2013). Prosaposin has also been reported to elevate plasma membrane GPR37 levels and to promote receptor interaction with lipid rafts enriched in GM1 gangliosides (Lundius et al., 2014). These findings raise the possibility that GPR37 has a more general and far-reaching functional significance in the nervous system than was originally anticipated. However, the basic structural and functional characteristics of the receptor and the mechanisms of its regulation at the molecular level have remained poorly understood.

Limited proteolysis is a post-translational process involved in the regulation of several membrane-anchored and single-pass plasma membrane proteins leading to a range of functional consequences. In some cases, the proteolytic cut adjacent to the plasma membrane is followed by a release of the extracellular ectodomain in a process called shedding (Overall and Blobel, 2007; Weber and Saftig, 2012). The cleaved fragments can act as ligands in a paracrine or autocrine fashion, or alternatively as decoy receptors. The limited proteolysis can also lead to regulated intramembrane proteolysis and signal-transducing intracellular domain fragments, or it can be involved in the regulation of protein turnover. In comparison to single-pass membrane proteins, very little is known about the limited ectodomain proteolysis of multi-pass membrane proteins, including GPCRs. An exceptional group among GPCRs are adhesion receptors, which contain a conserved autoproteolytic site in their large N-termini (reviewed in Paavola and Hall, 2012). After the cleavage of the GPCR autoproteolysis-inducing (GAIN) domain, the N-terminus of these receptors remains noncovalently attached to the transmembrane

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region forming a two-subunit structure. Recent reports suggest that the dissociation of the receptor ectodomain is directly related to the activation mechanism of adhesion receptors in a manner in which the GAIN domain stalk region acts as a tethered agonist for the receptor (Liebscher et al., 2014; Stoveken et al., 2015). In contrast to the adhesion receptors, only a few other GPCRs have been reported to undergo N-terminal proteolysis. One of them is the endothelin B receptor ($ET_BR$, also known as EDNRB; Kozuka et al., 1991; Grantcharova et al., 2002), a close relative of GPR37. Another more widely studied example is the thyroid-stimulating hormone receptor (TSHR). The cleavage of this GPCR leads to the formation of a two-subunit receptor structure that is held together by disulphide bonds, the reduction of which can lead to the shedding of the receptor ectodomain (Couet et al., 1996). In addition to TSHR, the angiotensin II type 1 receptor and the protease-activated receptor-1 are the only other class A GPCRs, for which ectodomain shedding has been demonstrated (Ludeman et al., 2004; Cook et al., 2007).

To gain new insights to GPR37 regulatory mechanisms, we set out to investigate its biosynthesis and processing using an inducible stable expression system. We provide evidence that, unlike in transiently transfected cells, GPR37 does not aggregate and accumulate intracellularly in stably transfected cells. Instead, the unusually long extracellular N-terminus (261 amino acids) of the receptor is subject to constitutive proteolytic cleavage and the N-terminal fragment is released from the cells by shedding. The full-length and cleaved receptors are differentially localised, with the cleaved form being the predominant species at the cell surface, suggesting a potential role for the limited N-terminal cleavage in the functional regulation of GPR37.

## RESULTS

### GPR37 undergoes proteolytic cleavage in its large extracellular N-terminus

We modified the human GPR37 to contain Myc and FLAG epitope tags at the receptor N- and C-termini, respectively, and replaced the endogenous signal peptide with a hemagglutinin (HA) signal peptide (Fig. 1A). This construct was stably transfected into a tetracycline-inducible $HEK293_i$ cell line (Apaja et al., 2006), allowing receptor expression in a controlled manner. Western blot analysis of immunoprecipitated receptors from induced, but not from non-induced, cells with the anti-Myc antibody revealed two specific bands with an $M_r$ of ~67,000 and ~96,000 (Fig. 1B), the sizes of which are close to the expected size of the receptor polypeptide. In addition, the anti-Myc antibody detected a third band with a higher $M_r$ of ~200,000 (Fig. 1B), probably representing oligomeric receptor forms. The same three bands were recognised with the anti-FLAG antibody (Fig. 1B), indicating that they correspond to full-length receptor forms. Importantly, the anti-FLAG antibody, but not the anti-Myc antibody, also detected a very intense and heterogeneous band with

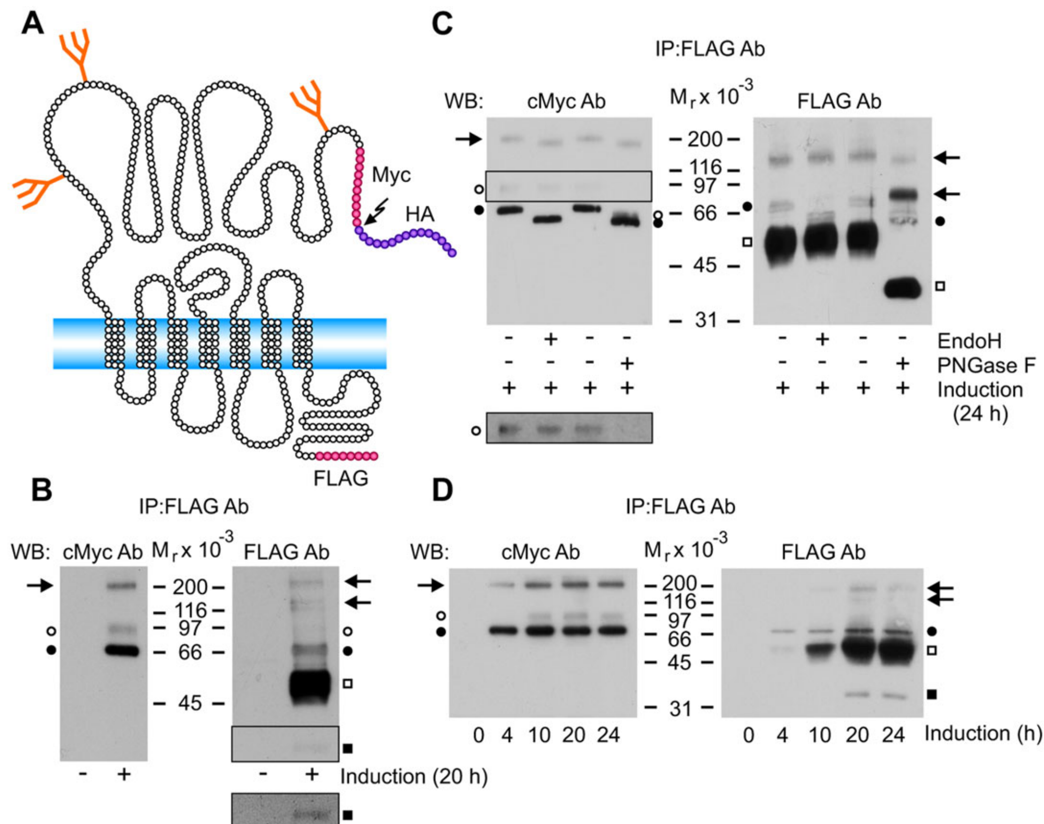


**Fig. 1. GPR37 N-terminus is subject to proteolytic cleavage.** (A) Topography of the human GPR37. The added Myc and FLAG epitope tags are shown in pink, the cleavable hemagglutinin (HA) signal peptide in purple, and the signal peptide cleavage site with an arrow. The three putative N-glycans at the receptor N-terminus are also indicated. (B–D) $HEK293_i$ cells stably transfected with the Myc–GPR37–FLAG construct were induced or not to express the receptor as indicated. Receptors were immunoprecipitated from cellular lysates and analysed by western blotting. For C, the purified receptors were deglycosylated with Endo H (100 mU/ml) or PNGase F (50 U/ml), or were left untreated before SDS-PAGE. The outlined areas are shown with enhanced contrast. The precursor and full-length mature receptor forms are indicated with closed and open circles, respectively. The two cleaved C-terminal fragments are indicated with open and closed squares, and the higher molecular mass species with arrows. All experiments were repeated independently at least five times. IP, immunoprecipitation; WB, western blotting.

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an $M_r$ of ∼53,000 and a much less abundant one with an $M_r$ of ∼34,000 (Fig. 1B). These two receptor species are likely to represent proteolytically processed forms of GPR37 missing part of the extracellular N-terminal domain.

The N-terminus of GPR37 contains three potential N-glycosylation sites (see Fig. 1A), and, therefore, to identify the expressed receptor forms more specifically, enzymatic digestions with peptide-N-glycosidase F (PNGase F) and endo-β-N-acetylglucosaminidase H (Endo H) were performed. PNGase F is capable of releasing all types of N-linked oligosaccharides from glycoproteins modified in the endoplasmic reticulum (ER) or the Golgi complex, whereas Endo H can cleave hybrid and high-mannose type oligosaccharides, but does not remove Golgi-modified complex glycans. As seen in Fig. 1C, the $M_r$ 67,000 species was sensitive to both Endo H and PNGase F, indicating that it represents a receptor glycoform that is typical for an ER-localised precursor. The shift in its electrophoretic mobility reflects a decrease in the molecular mass of ∼7000 Da, suggesting that the receptor carries two or three N-glycans. In contrast, the $M_r$ 53,000 and the $M_r$ 96,000 receptor forms were insensitive to Endo H, and were digested only with PNGase F (Fig. 1C). Thus, they correspond to mature receptor forms carrying N-glycans that have been processed in the Golgi. The calculated change in the electrophoretic mobility was about 15,000 Da for the PNGase-F-digested cleaved receptor form and 33,000 Da for the full-length mature receptor. Therefore, the major cleavage site is located in the receptor N-terminal domain somewhere between the sites of N-glycosylation. Furthermore, the $M_r$ 96,000 species is likely to carry other post-translational modifications given that its $M_r$ value remained slightly above that of the precursor after full de-glycosylation of N-glycans (Fig. 1C, see also Fig. S1). Given that the GPR37 N-terminus contains several serine and threonine residues, this species is most probably further processed by O-glycosylation in the Golgi.

To determine the timecourse of the appearance of the detected receptor forms, the stably transfected HEK293$_i$ cells were treated with tetracycline for increasing periods of time. The full-length receptor forms were detectable 4 h after the addition of tetracycline, with no significant changes in their relative abundance with longer induction times (Fig. 1D). The proteolytically cleaved $M_r$ 53,000 species was also apparent after the 4-h induction, but unlike the full-length mature form, the abundance of this species increased substantially in a time-dependent manner (Fig. 1D). In addition, the $M_r$ 34,000 species became more abundant with time. These data indicate that the cleavage of GPR37 occurs *in vivo* in cultured cells and is not a mere *in vitro* artefact.

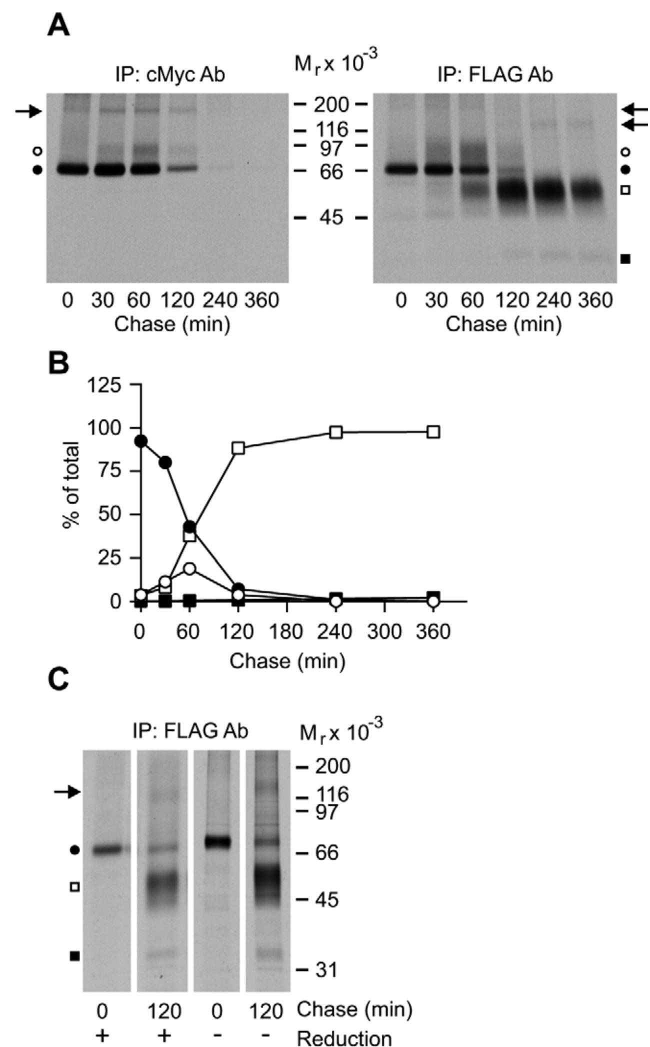


**Fig. 2. GPR37 matures efficiently and is quickly converted into the cleaved form.** Receptor expression in HEK293$_i$ cells was induced for 16 h and cells were labelled with [$^{35}$S]methionine and [$^{35}$S]cysteine for 30 min and chased for various times before harvesting. Cellular membranes were prepared and subjected to immunoprecipitation. The labelled receptors were analysed by SDS-PAGE and fluorography. For B, the intensities of the labelled receptor species immunoprecipitated with the anti-FLAG antibody and shown in A were quantified by densitometric scanning. The values were calculated as percentages of total receptor in each lane. For C, receptors were denatured under reducing or non-reducing conditions before SDS-PAGE. Results are representative of three (A) and one (C) independent experiments. Symbols and abbreviations are as explained in Fig. 1.

## GPR37 is converted into the cleaved form rapidly after maturation

To further investigate the proteolytic processing of GPR37 and to assess receptor maturation, the stably transfected HEK293$_i$ cells were subjected to metabolic pulse-chase labelling with [$^{35}$S]methionine and [$^{35}$S]cysteine (Fig. 2A,B). As expected, the $M_r$ 67,000 species, representing the receptor precursor, was clearly detectable at the end of the pulse. This species disappeared after 4 h. The mature $M_r$ 96,000 species was visible after the 30-min chase, but had already started to disappear after 60 min, at the same time as the cleaved $M_r$ 53,000 species became apparent. This data is consistent with the notion that the full-length receptor is efficiently cleaved shortly after the processing of receptor N-glycans to the mature form has been completed. Furthermore, the maturation efficiency of GPR37 appears to be high, as essentially all synthesised precursors were ultimately converted to the $M_r$ 53,000 cleaved form. Given that the GPR37 N-terminus contains two cysteine residues (C39 and C44), we further tested the possibility that their bonding with the conserved cysteines in the third transmembrane domain and the second extracellular loop (C334 and C419, respectively) might keep the full-length receptor intact in spite of the cleavage. This, however, was not the case, as no changes in the relative abundance of the different receptor forms were detected when receptors were analysed by non-reducing SDS-PAGE (Fig. 2C).

## GPR37 is cleaved in transiently transfected Flp-In-293 and SH-SY5Y cells

The Myc–GPR37–FLAG construct was then transiently transfected into Flp-In-293 and SH-SY5Y human neuroblastoma cells to rule out the possibility that the observed receptor cleavage occurs only in

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the stably transfected $HEK293_i$ cells. Both HEK293 and SH-SY5Y cells are known to express GPR37 endogenously (Imai et al., 2001; Steentoft et al., 2013), thus providing a natural cellular environment for the transfected receptor. Results from the western blot analysis show that GPR37 is subjected to N-terminal cleavage in both cell lines (Fig. 3A). However, some differences between the transfection systems were observed. The transient expression resulted in the accumulation of higher molecular mass receptor species at the top of the gel in addition to the receptor oligomers seen upon stable expression (compare Figs 3A and 1B). This accumulation is unlikely to be an *in vitro* artefact, given that alkylation of receptor cysteine residues with iodoacetamide to prevent disulphide bond formation during SDS-PAGE had no impact on the higher molecular mass species (Fig. 3B). These results imply that a proportion of receptors have a tendency to aggregate in transient expression systems, consistent with previously published observations (Imai et al., 2001; Rezgaoui et al., 2006). As the relative abundance of the cleaved $M_r$ 53,000 receptor species in transiently transfected cells appeared to be less than in cells stably expressing GPR37 (compare Figs 3A and 1B), it can be hypothesised that the aggregation in the former cells involves receptor precursors, which might then lead to their impaired export from the ER and subsequent processing to the cleaved form. In spite of the apparent difference between the two transfection systems, the results allow the conclusion that GPR37 is subjected to N-terminal cleavage in both transiently and stably transfected cells, and is not a cell-type-specific phenomenon. Furthermore, as the mouse GPR37 was found to be cleaved in transiently transfected Flp-In-293 cells (Fig. S2) in a similar manner to the human receptor, the cleavage is not a species-specific event.

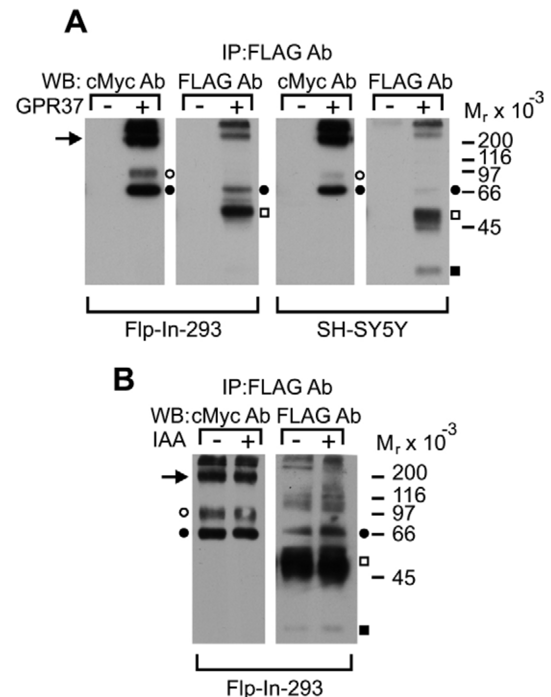


**Fig. 3. GPR37 cleavage occurs in transiently transfected Flp-In-293 and SH-SY5Y cells.** Cells were transiently transfected with Myc–GPR37–FLAG or the plain vector for 24 h. Receptors were immunoprecipitated from cellular lysates and analysed by western blotting. For B, the immunoprecipitated samples were reduced and then alkylated or not with iodoacetamide (IAA) before SDS-PAGE. Results are representative of five (A) and one (B) independent experiments. Receptor accumulation at the top of the gel is marked with an asterisk. Other symbols and abbreviations are as explained in Fig. 1.

### The full-length GPR37 is predominantly localised in intracellular compartments

To examine the cellular localisation of GPR37, we stained the Myc- and FLAG-tagged receptors in $HEK293_i$ cells with anti-Myc and -FLAG antibodies, and analysed the cells by confocal microscopy. Very little cell surface receptor staining was detected with the anti-Myc antibody (Fig. 4A). Instead, most of the staining appeared to localise around the perinuclear region, forming a web-like structure that resembles ER staining, and showed some accumulation close to the nucleus. The receptors stained with the anti-FLAG antibody, in contrast, were mostly localised at the cell surface with only a small fraction seen intracellularly (Fig. 4A). Similar results were obtained using transiently transfected Flp-In-293 cells (Fig. 4B). Given that the western blotting data suggests that the anti-FLAG antibody predominantly recognises the N-terminally processed receptor forms (see Fig. 1B), most of the receptors detected at the cell surface are likely to represent cleaved receptor species. This was confirmed by a cell surface biotinylation assay (Fig. S3). Further analysis of the full-length receptors stained by the anti-Myc antibody revealed some colocalisation with the plasma membrane marker Na/K-ATPase (Fig. 4C). The most intense anti-Myc antibody staining was, however, observed in intracellular rosette-like structures typically localising slightly above the nucleus (Fig. 4D).

To identify the organelles, in which the full-length GPR37 is mainly localised, we immunostained GPR37 expressing $HEK293_i$ cells with either the monoclonal or polyclonal anti-Myc antibody in combination with antibodies recognising marker proteins for various subcellular compartments. The full-length GPR37 colocalised to some degree with the ER markers Sec61β and calreticulin, the ER-Golgi intermediate compartment (ERGIC) 53 (also known as LMAN1) and a cis-Golgi marker GM130 (also known as GOLGA2) (Fig. 5A–D). However, the most profound co-staining was observed with the trans-Golgi network protein TGN46 (also known as TGOLN2) in the rosette-like structures (Fig. 5E), indicating that most of the intracellular receptors seen with the anti-Myc antibody localise in this compartment. These results indicate that GPR37 remains in its full-length form in the secretory pathway from the ER to the trans-Golgi network and is cleaved only after it leaves the latter compartment, either at the cell surface or possibly in recycling vesicles.

### GPR37 cleavage is mediated by a metalloproteinase

To characterise the enzyme(s) responsible for GPR37 processing, we tested the ability of various protease inhibitors to prevent or reduce receptor cleavage. No substantial change in the abundance of the $M_r$ 96,000 full-length mature receptor was detected with inhibitors against serine, cysteine or aspartyl proteases, but treatment with the broad-range metalloproteinase inhibitors GM6001 and marimastat resulted in a clear increase in the amount of this species (Fig. 6A). Similar results were obtained with another metalloproteinase inhibitor TAPI-1 (data not shown). Consistent with the western blot data, the metalloproteinase inhibitors increased the amount of full-length receptors detected at the plasma membrane by flow cytometry, whereas inactive GM6001 had no effect (Fig. 6B). The effect mediated by GM6001 was concentration dependent (Fig. 6C). A clear increase in the amount of full-length receptors was also seen in the cell surface biotinylation assay following protease inhibitor treatment (Fig. S3). Further evidence that the cleavage of the GPR37 N-terminus is mediated by

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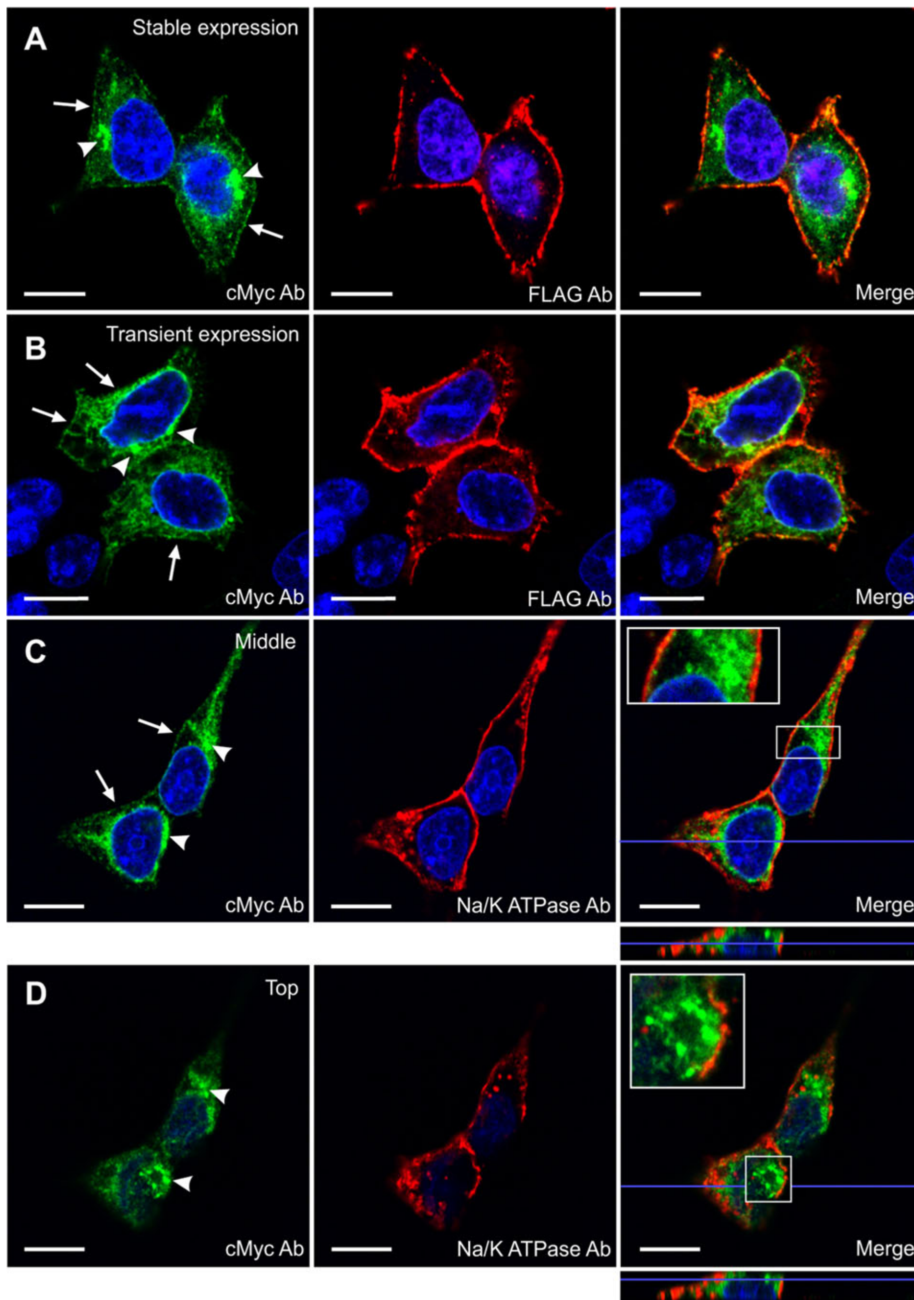


**Fig. 4. Cellular localisation of GPR37.** The Myc–GPR37–FLAG was expressed stably in HEK293$_i$ cells (A,C,D) or transiently in Flp-In-293 cells (B) for 24 h. Cells were fixed and permeabilised, and receptors were stained with the indicated antibodies, followed by Alexa-Fluor-488- and -568-conjugated secondary antibodies. The nuclei were stained with TO-PRO-3 iodine. Cells were analysed by confocal microscopy. C and D represent the same image viewed from the plane of the nucleus and slightly above the nucleus, respectively. Orthogonal projections are presented below the merged images in panels C and D, and the outlined areas are shown magnified at the top left corner of the respective image. The arrowheads in the anti-Myc antibody panels indicate the intense GPR37 perinuclear staining, and receptors at the cell surface are designated with arrows. The experiment was repeated five (A), three (B) or two (C-D) times, using triplicate samples for each antibody. Scale bars: 10 µm.

metalloproteinases was obtained by confocal microscopy, showing that the cell surface anti-Myc antibody staining increased upon marimastat treatment (Fig. 6D).

Interestingly, the cleavage of GPR37 was also inhibited with the furin inhibitor decanoyl-RVKR-chloromethylketone (Fig. 6A,B), which is able to inhibit furin as well as some other proprotein convertases. Several metalloproteinases are known to require removal of their pro-domain by proprotein convertases in order to attain their proteolytic activity (Weber and Saftig, 2012). Taking this into account, together with the identified receptor cleavage site (see below), which is not a typical furin recognition site, it is reasonable to assume that the effect observed with the furin inhibitor was indirect and resulted from the blocked activation of the actual cleaving enzyme. This was also supported by the fact that the membrane-impermeable furin inhibitor (FI II), unlike the membrane-permeable one (FI I), had no effect on receptor cleavage (Fig. 6B). Thus, we conclude that GPR37 is cleaved by a metalloproteinase, which requires proprotein-convertase-mediated activation.

### GPR37 is cleaved between E167 and Q168, leading to the release of receptor ectodomain by shedding

To test whether the cleaved ectodomain of GPR37 is extracted to the cell culture medium, we subjected the conditioned medium from 24-h induced stably transfected HEK293$_i$ cells to immunoprecipitation and western blot analysis with the anti-Myc antibody. As anticipated, the antibody was able to detect a species with $M_r$ of ∼32,000 from the medium collected from induced but not from uninduced or marimastat-treated cells (Fig. 7A).

We then tested whether the observed receptor cleavage and ectodomain shedding might be influenced by the added epitope tags. GPR37 carries a PSD-95, Discs-large, ZO-1-homology (PDZ)-domain-binding motif at its C-terminus that is known to



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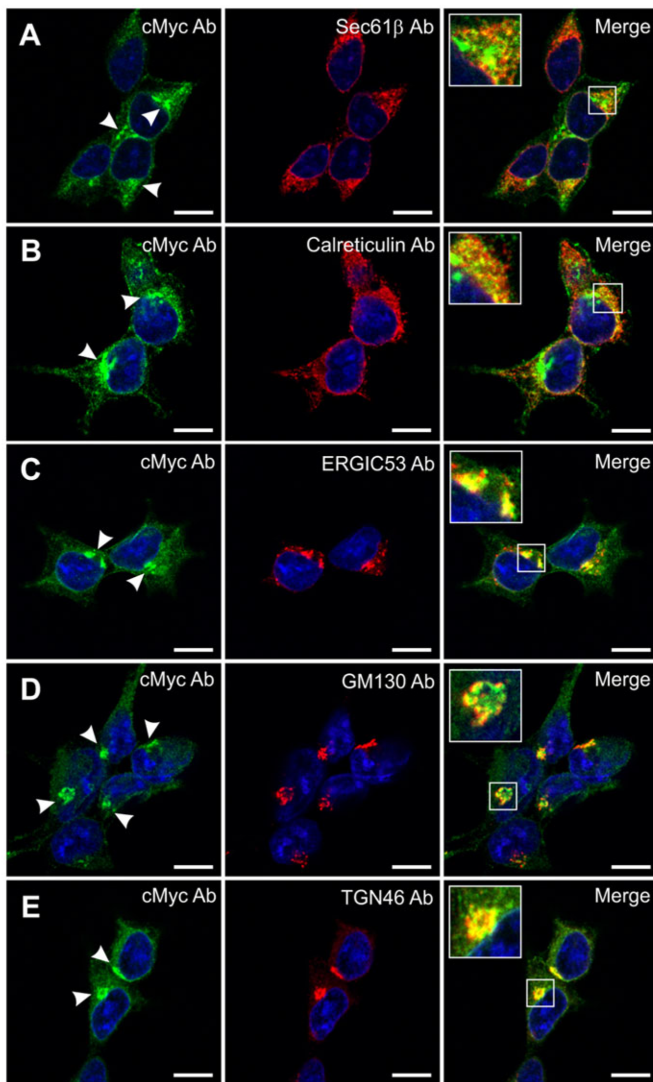


**Fig. 5. Subcellular localisation of the full-length GPR37 in the secretory pathway.** Stably transfected $HEK293_i$ cells were induced for 24 h, fixed, permeabilised, stained with the indicated antibodies and analysed as described in Fig. 4. Intense perinuclear receptor staining is indicated with arrowheads and the outlined areas are magnified in the insets at the top left corner of each image. The experiment was replicated four (B-E) times or once (A), by using triplicate samples for each antibody. Scale bars: 10 µm.

mediate interaction with a few PDZ-domain-containing proteins (Dunham et al., 2009; Dutta et al., 2014). Such interactions are known to alter receptor function and trafficking (Romero et al., 2011), and thus could modify the susceptibility to cleavage. Towards this end, we created two GPR37 constructs, one missing the N-terminal Myc epitope and carrying the endogenous signal peptide, and another without the C-terminal FLAG epitope tag (Fig. 7B). Western blot analysis of these receptors transiently expressed in Flp-In-293 cells showed that the GPR37–FLAG construct was cleaved in a similar manner as the receptor carrying both epitope tags (Fig. 7C; see also Fig. S2). As expected, no cleaved receptor species were detected for the Myc–GPR37 construct with the anti-FLAG antibody, owing to the missing FLAG epitope, but the N-terminal ectodomain could be purified from the culture medium, indicating that this construct is also cleaved (Fig. 7D). The shedding of this construct was abolished by inhibiting the cleavage with marimastat (Fig. 7D). Subsequently, we tested whether the observed intracellular localisation of the Myc- and FLAG-tagged GPR37 was affected by the FLAG epitope. No apparent difference in cellular localisation was detected between cells transiently expressing the Myc–GPR37–FLAG or the Myc–GPR37 constructs (Fig. 7E).

To identify the location of the major GPR37 cleavage site within the receptor N-terminus in relation to the added N-glycans, the $M_r$ 53,000 C-terminal fragment and the $M_r$ 32,000 N-terminal fragment were subjected to digestion with PNGase F. Sequential deglycosylation of the [$^{35}$S]methionine- and [$^{35}$S]cysteine-labelled $M_r$ 53,000 species showed two shifts in the electrophoretic mobility, indicating that this receptor form carries two N-glycans (Fig. 8A). The N-terminal $M_r$ 32,000 species purified from the conditioned medium was also sensitive to PNGase F (Fig. 8B). Thus, these data indicate that all three putative N-glycosylation sites of the receptor are used, and furthermore, imply that the main cleavage site is located upstream of N222 and N239. In comparison, the PNGase F treatment had no effect on the electrophoretic mobility of the smaller $M_r$ 34,000 C-terminal fragment (data not shown), suggesting that the secondary cleavage site locates downstream from N239.

Finally, the cleaved $M_r$ 53,000 receptor species was subjected to N-terminal sequencing by Edman degradation in order to more accurately identify the main cleavage site. The analysis of the five first amino acids gave a sequence QSVXT, which is found in the GPR37 N-terminus ($Q^{168}SVKT^{172}$, Fig. 8C) upstream of the two N-glycosylation sites at N222 and N239. Interestingly, we were unable to prevent receptor cleavage with point mutations or short deletions of two to four amino acids at the identified cleavage site (Fig. 8D). This finding is not surprising as the metalloproteinase-mediated cleavage of several substrate proteins is known to depend on protein conformation and on the distance of the site from the transmembrane region rather than a specific recognition motif (Overall and Blobel, 2007). Similar results have been obtained with other integral membrane proteins cleaved by metalloproteinases, including $ET_BR$ (Grantcharova et al., 2002).

## DISCUSSION

Limited proteolysis is a common way to modify membrane-anchored and single-pass integral membrane proteins. Although much less frequent, this mechanism has also been reported for some multi-pass proteins at the plasma membrane, including GPCRs. Here, we identified GPR37 as a novel substrate for such post-translational processing. The GPR37 N-terminal ectodomain was found to be susceptible to metalloproteinase-mediated cleavage, which occurs constitutively without any external stimulation and leads to the shedding of the receptor N-terminus. Thus, although receptor precursors are efficiently converted into the mature form, by far the majority of cell surface receptors are in the cleaved form under steady-state conditions, revealing a putative mechanism to regulate GPR37 at the post-translational level.

Several lines of evidence presented in this study support the notion that the N-terminal proteolytic processing of GPR37 is an *in vivo* event. First, a pulse-chase labelling analysis of receptor maturation revealed that the cleavage occurred only after the receptor precursor was converted into the full-length mature form, which then started to disappear concomitantly with the appearance of the cleaved receptor form. Second, in western blot assays, the cleaved receptor species accumulated in a time-dependent manner after the initiation of receptor expression. Third, receptor cleavage was inhibited by the addition of metalloproteinase inhibitors to the cell culture medium and, fourth, the cleaved N-terminal ectodomain

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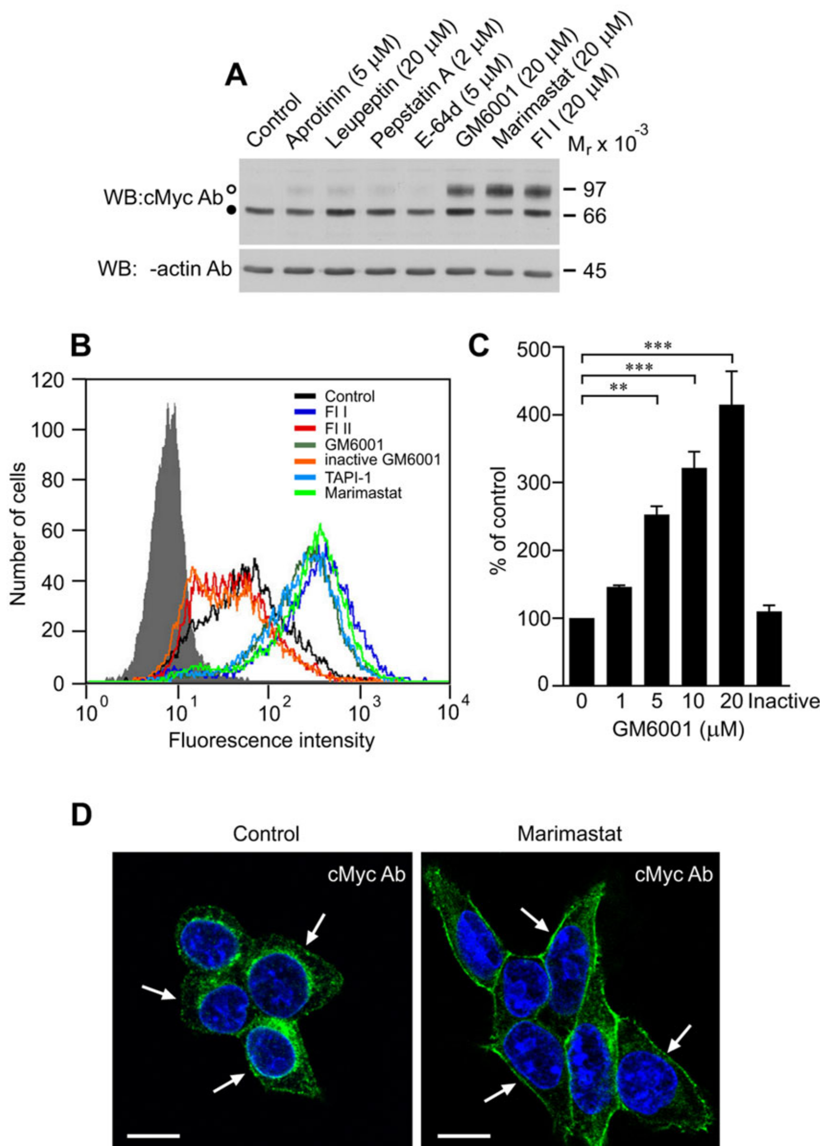


**Fig. 6. GPR37 N-terminal cleavage is attenuated with metalloproteinase inhibitors.** (A) The Myc–GPR37–FLAG expression in $HEK293_i$ cells was induced for 16 h and cells were treated with the indicated protease inhibitors or vehicle. Cellular lysates were analysed by western blotting. The anti-β-actin antibody was used as a control for protein loading. FI I, furin inhibitor I. (B) Cells were induced for 24 h and proteinase inhibitors were added to the culture medium to the final concentration of 20 µM. Intact cells were treated with anti-Myc antibody and phycoerythrin-conjugated secondary antibody before analysis by flow cytometry. The shaded curve represents the background signal obtained in the absence of the primary antibody. FI II, membrane-impermeable furin inhibitor II. (C) Cells were treated with GM6001 at various concentrations or with 20 µM inactive GM6001 during the 24-h induction and analysed by flow cytometry. The values are shown as means±s.e.m. of six independent experiments performed in duplicate. The fluorescence intensity values were normalised to those obtained from cells treated with vehicle only, and the controls were set at 100%. ***P*<0.01; ****P*<0.001 (analysed before normalisation using repeated measures one-way ANOVA followed by the Bonferroni's multiple comparison test). (D) Cells were induced for 24 h and either treated with 20 µM marimastat or vehicle during the induction. The cellular localisation of the anti-Myc-antibody-labelled full-length receptors was analysed by confocal microscopy. The cell surface staining is indicated with arrows. Scale bars: 10 µm. Results are representative of six (A), three (B) and two (D) independent experiments. Symbols and abbreviations are as described in Fig. 1.

was recovered from the conditioned medium in the absence, but not in the presence, of metalloproteinase inhibitors. Finally, flow cytometry, confocal microscopy and cell surface biotinylation analyses demonstrated that the level of full-length plasma membrane receptors increased upon addition of metalloproteinase inhibitors to the culture medium. The cleavage and shedding of the GPR37 ectodomain were found to occur in a similar manner whether the receptor C-terminus was modified with a FLAG epitope tag or remained intact, indicating that the processing can take place even when putative interactions with PDZ-domain-containing proteins are attenuated. Moreover, the cleavage was shown to occur in two different cells lines endogenously expressing the receptor, was not dependent on the transfection system used and was demonstrated for both human and mouse receptors, implying that the processing event is an intrinsic property of the receptor protein.

Although the possibility that GPR37 undergoes proteolytic cleavage has not been taken into consideration before, the findings presented here are well in line with already existing data. Although the predicted molecular mass of the full-length human GPR37 polypeptide is ∼67,000 Da (Marazziti et al., 1997; Donohue et al., 1998), the main receptor species detected in various heterologous expression systems and in natural tissues using antibodies against the receptor C-terminus or C-terminally placed epitope tags (Imai et al., 2001; Rezgaoui et al., 2006; Omura et al., 2008) has been generally smaller with a comparable size to the identified C-terminal fragment ($M_r$ ∼53,000). Furthermore, the expression pattern observed for the mouse GPR37 in previously published studies (e.g. Imai et al., 2001) is very similar to that of the human receptor, in line with the present observations. This implies that the cleavage of GPR37 is evolutionarily conserved in a similar manner as has been demonstrated for $ET_BR$ (Kozuka et al., 1991; Grantcharova et al., 2002) and the $\beta_1$ adrenergic receptor (Hakalahti et al., 2013). Interestingly, one of the studies aiming to enhance GPR37 plasma membrane trafficking demonstrated that the receptor cell surface expression could be increased significantly by N-terminal truncations (Dunham et al., 2009). In this study, an N-terminal FLAG epitope tag was used to detect the full-length and truncated receptors. Therefore, one possible explanation for these findings is that the FLAG epitope was lost when placed N-terminally to the cleavage site, whereas larger deletions enabled receptor detection given that the epitope tag was placed C-terminally to the cleavage site.

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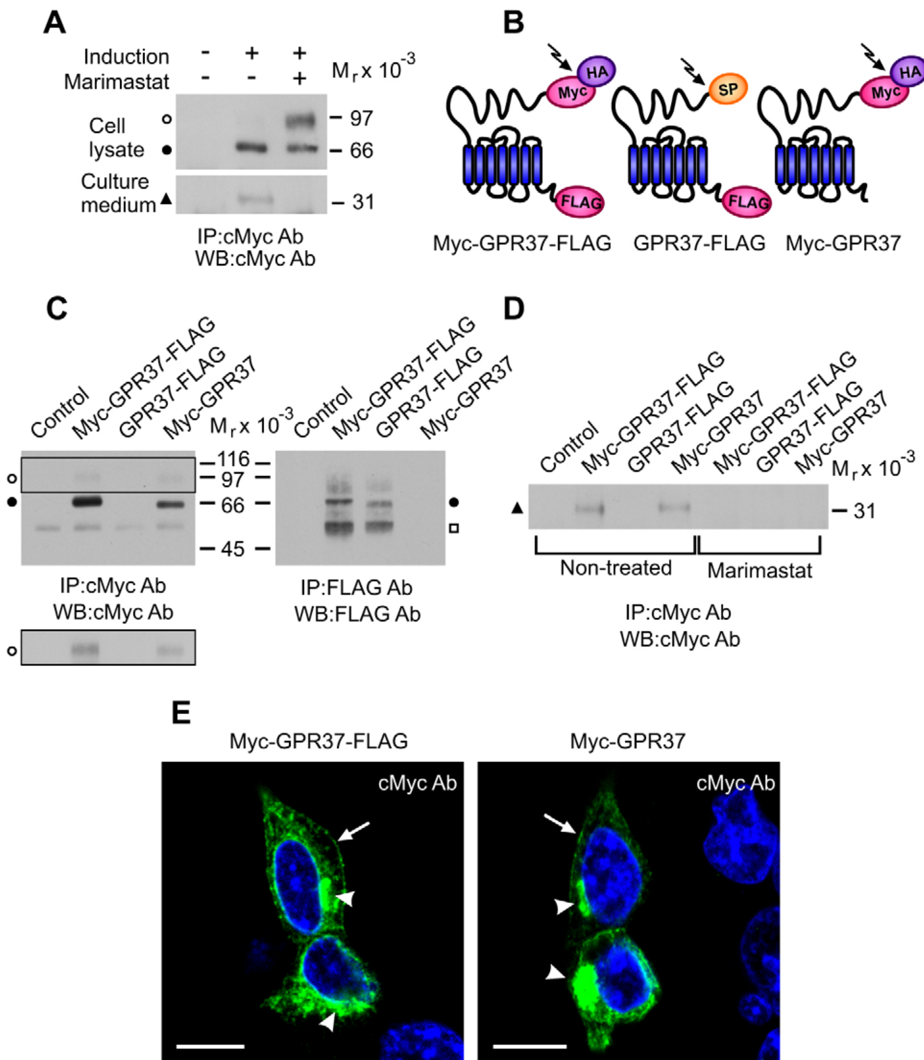


**Fig. 7. GPR37 N-terminal cleavage leads to ectodomain shedding and is not dependent on epitope tags or the HA signal peptide added to the receptor.** (A) Stably transfected HEK293$_i$ cells were induced or not for 24 h and were simultaneously treated or not with marimastat (20 µM). The conditioned medium was collected and cellular lysates were prepared from the harvested cells. The medium and lysates were subjected to immunoprecipitation with the anti-Myc antibody, and the purified receptor species were analysed by western blotting. The N-terminal receptor fragment is indicated with a triangle. Other symbols are as explained in Fig. 1. (B) Schematic of the differently epitope-tagged receptor constructs. Myc and FLAG epitopes are shown in pink, the HA signal peptide in purple and the endogenous signal peptide (SP) in yellow. The signal peptide cleavage site is indicated with an arrow. (C) The receptor constructs shown in B or vector only (control) were transiently transfected into Flp-In-293 cells for 24 h. Receptors were immunoprecipitated from cellular lysates and analysed by western blotting. The outlined area is shown with a longer exposure time. (D) Flp-In-293 cells were transiently transfected with the various GPR37 constructs and treated or not with marimastat. The shed receptor N-terminal fragment was immunoprecipitated from the conditioned medium and analysed by western blotting. Symbols and abbreviations are as described in Fig. 1. (E) Flp-In-293 cells were transiently transfected with the indicated GPR37 constructs for 24 h, and the anti-Myc-antibody-labelled full-length receptors were analysed by confocal microscopy as described in Fig. 4. The intracellular intense receptor staining is indicated with arrowheads and the cell surface staining with arrows. Scale bars: 10 µm. Results are representative of four (A) and two (C–E) replicates.

Our finding that the GPR37 N-terminus is subject to proteolytic processing is further supported by a recent report in which glycoproteomic tools were used to identify O-glycosylated peptides. An O-glycosylated N-terminal GPR37 peptide (TVPGASDLFYWPR) was found in the secretome of HEK293 cells using SimpleCell technology (Steentoft et al., 2013). It is noteworthy, that, in addition to two frizzled receptors and a couple of adhesion receptors, the latter of which are known to undergo N-terminal proteolysis (Paavola and Hall, 2012), GPR37 was the only GPCR for which O-glycopeptides were isolated. Altogether, secretomes of 12 different human cell lines were analysed. This study also confirms that, in addition to the three N-glycans, GPR37 is modified by O-glycosylation, as was suspected in the present study on the basis of the molecular mass difference seen between the precursor and the full-length mature receptor after full deglycosylation of N-glycans (see Fig. 1C, Fig. S1). Likewise, two glycosylated peptides corresponding to the N-terminal region of the mouse GPR37 have been found in a screen for O-glycosylated peptides in a murine synaptosome (Trinidad et al., 2013). It is notable that, in some cases, O-glycans have been found to modulate proteolytic cleavage in a manner that is subject to regulation (Boskovski et al., 2013; Goth et al., 2015). Therefore, the potential for this kind of functional interplay between GPR37 O-glycosylation and proteolytic processing will provide an interesting topic for future research.

Interestingly, GPR37L1 is another GPCR that has shown up in recent glycoproteomic studies. Several O-glycopeptides representing the GPR37L1 N-terminal domain have been identified from human cerebrospinal fluid (Halim et al., 2013), implying that this close relative of GPR37 might also be susceptible to proteolytic processing. Similarly, $ET_BR$, another receptor with significant sequence similarity to GPR37, has been reported to undergo N-terminal cleavage in several studies and in various species (Kozuka et al., 1991; Grantcharova et al., 2002). Therefore, N-terminal processing might represent a common mechanism to modify receptors closely related to GPR37. However, it should be pointed out that the N-termini of GPR37L1 and $ET_BR$ are significantly shorter than that of GPR37, suggesting that the mechanism and functional role of the limited cleavage are likely to be dissimilar. The $ET_BR$ is cleaved at the plasma membrane by a mechanism that depends on agonist binding (Grantcharova et al., 2002), whereas the proteolytic processing of GPR37 occurs constitutively, leading to a high level of cleaved receptors at the cell surface.

Although limited proteolysis has been demonstrated for a few GPCRs, most commonly occurring at their N-termini, the physiological significance of this modification is still largely

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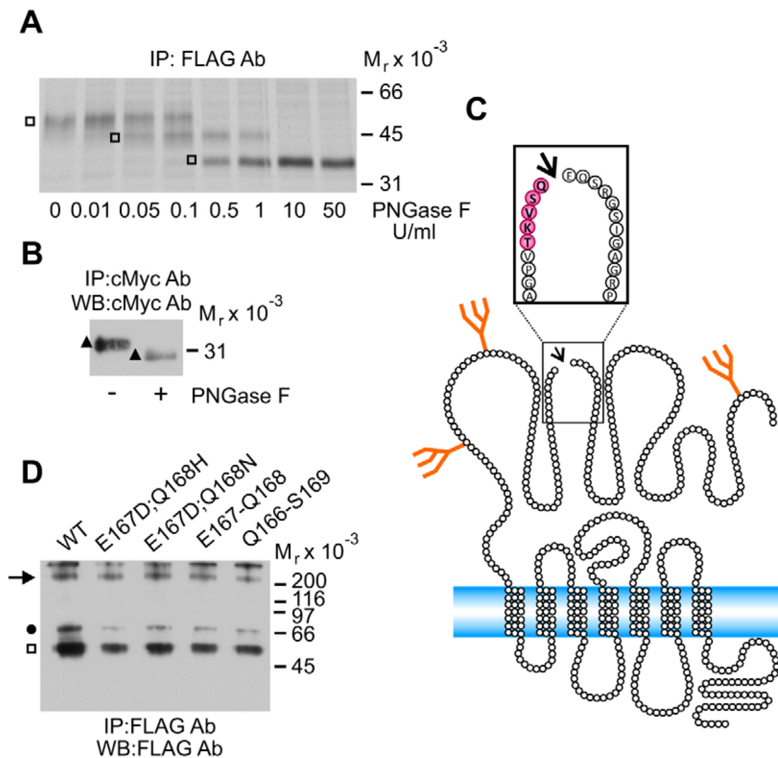


**Fig. 8. Identification of the major GPR37 cleavage site.** (A) Stably transfected HEK293$_i$ cells were induced for 16 h, labelled with [$^{35}$S]methionine and [$^{35}$S]cysteine for 30 min and chased for 4 h. Cellular membranes were prepared and immunoprecipitated receptors were subjected to digestion with an increasing concentration of PNGase F. Receptors were detected by SDS-PAGE and fluorography. (B) The N-terminal receptor fragment was immunoprecipitated from the conditioned medium and subjected to deglycosylation with 50 U/ml PNGase F and analysed by western blotting. (C) Topography of GPR37 showing the cleavage site between E167 and Q168 identified by Edman degradation of the major cleaved C-terminal receptor fragment. The five amino acids corresponding to the identified QSVXT sequence are highlighted in pink. (D) The wild-type (WT) GPR37 and the indicated cleavage site mutant constructs were transiently transfected into Flp-In-293 cells for 24 h. Receptors were immunoprecipitated from cellular lysates and analysed by western blotting. The results shown are representative of two (A, B) and three (D) independent experiments. The N-terminal sequencing was performed twice. Symbols and abbreviations are as described in Fig. 1.

unknown. In addition to $ET_BR$, the cleavage of $\beta_1$ adrenergic and $V_2$ vasopressin receptors has been reported to occur in an activation-dependent manner following ligand binding (Kojro and Fahrenholz, 1995; Hakalahti et al., 2010, 2013). A similar mechanism has been implicated for TSHR (Latif et al., 2004; Kaczur et al., 2007), although conflicting information exists (Chazenbalk et al., 2004). In the case of the parathyroid hormone receptor, the cleavage leads to reduced protein stability and possible degradation of the receptor protein (Klenk et al., 2010), whereas the exposed new N-termini of the cleaved protease-activated receptors and adhesion family receptors act as tethered agonists for the cognate receptors (Soh et al., 2010; Stoveken et al., 2015). In the case of GPR37, the question about the functional significance of the proteolytic processing is further complicated by the fact that very little is known about the natural physiological function of the receptor. The receptor was only very recently suggested to respond to prosaposin and prosaptide (Meyer et al., 2013), indicating that GPR37 might actually function in pathways providing neuronal protection in the brain. Given that only a very small fraction of full-length receptors were detected at the cell surface under steady-state conditions, it is plausible to hypothesise that the cleavage is related to the functional activity of the receptor. Whether this event has a role in ligand binding and receptor activation, or is related to downregulation and recycling remains to be elucidated in the future. It also remains to be investigated whether the shed ectodomain has any functional role. Neither can we fully exclude the possibility that the N-terminal processing has a role in a functional interplay of GPR37 with other receptors, a phenomenon that has been suggested to occur between GPR37 and adenosine A2A and dopamine D2 receptors (Gandía et al., 2015; Lopes et al., 2015), as well as the dopamine transporter (Marazziti et al., 2007). In addition, GPR37 has been recently identified in at least two screens for novel interacting partners for GPCRs, one identifying partners for the $\beta_2$ adrenergic receptor (Kittanakom et al., 2014) and the other for the glucagon-like peptide 1 receptor (Huang et al., 2013). The potential functional relationship between these proteins and the specific role of receptor cleavage in this process remains to be investigated.

The link between GPR37 and the inheritable early onset Parkinson's disease was first established when GPR37 was identified as a substrate for the parkin ubiquitin ligase and the receptor was reported to aggregate in cells upon transient overexpression leading to cell toxicity (Imai et al., 2001). Concurrently, the tendency for aggregation was suggested to be the cause for the observed low-level cell surface expression of GPR37, although later on receptor accumulation was found to be significantly reduced upon stable expression (Rezgaoui et al., 2006). Similar to this latter observation, we found no signs of GPR37 aggregation under stable expression conditions. In comparison, the transient expression led to the appearance of high-molecular-mass aggregates at the top of SDS-PAGE gels. Based on our observations using the stable transfection system, we can claim that GPR37 is a GPCR that folds and matures efficiently. However, it can be speculated that the receptor might be sensitive to perturbations upon cellular stress especially when the parkin-mediated receptor degradation is impaired. Such conditions might exist specifically in dopaminergic cells as it is known that dopamine is easily converted into toxic oxidising derivatives (Miyazaki and Asanuma, 2008). However, our observations argue against the notion that low cell surface GPR37 levels result from the tendency of the receptor to form intracellular aggregates. Rather, we suggest that the N-terminally cleaved receptors that appear to be the predominant receptor forms at the cell surface have simply not been detected in previous studies due to the use of antibodies that are directed against the receptor N-terminus or N-terminally added epitope tags.

In conclusion, we provide evidence that GPR37 is subject to N-terminal proteolytic cleavage and ectodomain shedding. Considering the functional importance of limited proteolysis in the regulation of membrane proteins together with our finding that GPR37 is expressed at the plasma membrane predominantly in the

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proteolytically cleaved form, we propose that this process is related to the natural physiological role of the receptor. Taking into account that the cleavage of GPR37 is extremely efficient, leading to the loss of the receptor N-terminus, the findings presented here are also of importance for future research aiming to assess GPR37 function and role in Parkinson's disease and other nervous system disorders.

## MATERIALS AND METHODS

### DNA constructs

The human and mouse GPR37 cDNAs in the pcDNA3 vector were generous gifts from Ryosuke Takahashi (Kyoto University, Japan). The human δ-opioid receptor construct has been described previously (Leskelä et al., 2007). Two C-terminally FLAG epitope (EQKLISEEDL)-tagged human GPR37 constructs were prepared: Myc–GPR37–FLAG and GPR37–FLAG. The former contains a Myc epitope tag (EQKLISEEDL) and a cleavable influenza HA signal peptide (KTIIALSYIFCLVFA) in the N-terminus, whereas the latter has the endogenous signal peptide. The constructs were generated using the GPR37-pcDNA3 plasmid as a template, and PCR amplification was carried out using the oligonucleotides listed in Table S1. The PCR products were digested with NheI and AvrII (Myc–GPR–FLAG) or HindIII and AvrII (GPR37–FLAG) (New England Biolabs), ligated into the pFT-SMMF vector [modified from the pcDNA5/FRT/TO vector (Invitrogen) as described previously (Pietilä et al., 2005)] and transformed into *Escherichia coli* JM109. Constructs containing mutations or short deletions at the identified cleavage site were created using the QuikChange Lightning mutagenesis kit (Agilent Technologies). A construct missing the C-terminal FLAG epitope tag was generated by introducing a Stop codon before the tag sequence. The oligonucleotide primers used for mutagenesis are indicated in Table S1. The generated modified constructs and desired mutations were confirmed by DNA sequencing.

### Cell culture and transfections

A stable inducible cell line expressing the Myc- and FLAG-tagged human GPR37 was created by co-transfecting the receptor construct and pOG44 plasmid (Invitrogen) into $HEK293_i$ cells (Apaja et al., 2006) with the Lipofectamine 2000 transfection reagent (Invitrogen) under blasticidin S (4 µg/ml, InvivoGen) and hygromycin (400 µg/ml, InvivoGen) selection. The selected clone was sensitive to zeocin (Invitrogen), lacked β-galactosidase activity and showed very low basal but highly inducible GPR37 expression (see Fig. 1B). All cell lines were cultured in a humidified atmosphere at 37°C with 5% $CO_2$ in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, 100 units/ml penicillin and 0.1 mg/ml streptomycin (complete DMEM). The culture medium was supplemented with selection antibiotics: 100 µg/ml hygromycin and 4 µg/ml blasticidin S for stable $HEK293_i$ cells, and 100 µg/ml zeocin for Flp-In-293 cells (Invitrogen). For experiments, cells were seeded into 25-cm$^2$ flasks ($1.5\times10^6$ cells/flask) or 10-cm plates ($4.5\times10^6$ cells/plate) and cultured for 2 days to 80–90% confluency. Receptor expression in the stably transfected $HEK293_i$ cells was induced by adding 0.5 µg/ml tetracycline (Invitrogen) to the fresh culture medium (complete DMEM without antibiotics). Proteinase inhibitors (see Table S2) or vehicle were added to the culture medium 60 min after starting the induction. For transient transfections, Flp-In-293 and SH-SY5Y cells (a kind gift of Mikko Hiltunen, University of Eastern Finland, Finland) were seeded onto 10-cm plates ($3\times10^6$ cells/plate) and cultured for 24 h to 50–70% confluency. Cells were transfected with 5 µg of receptor constructs for 24 h using Lipofectamine 2000 according to the manufacturer's instructions, or alternatively, linear 25-kDa polyethyleneimine (PEI; Polysciences) as described previously (Breton et al., 2010). Both reagents were used at 1:3 DNA-to-reagent ratio. Protease inhibitors (Table S2), when used, were added 4 h after starting the transfection.

### Cell surface biotinylation

Cell surface proteins were biotinylated with 0.5 mg/ml sulfo-NHS-biotin (Thermo Fisher Scientific) and purified by immobilised streptavidin as described previously (Petäjä-Repo et al., 2000).

### Metabolic pulse-chase labelling with [$^{35}$S]methionine and [$^{35}$S]cysteine

Stably transfected $HEK293_i$ cells were grown in 25-cm$^2$ flasks and treated with tetracycline for 16 h. Metabolic labelling was performed as described previously (Hakalahti et al., 2010). Briefly, cells were starved in methionine and cysteine-free DMEM for 60 min, labelled in fresh medium containing 100 µCi/ml [$^{35}$S]methionine and [$^{35}$S]cysteine (PerkinElmer) for 30 min, and chased in complete DMEM supplemented with 5 mM methionine for the time periods indicated in the figures.

### Preparation and solubilisation of cellular membranes and whole-cell extracts

Cellular membranes were prepared and solubilised, and total cellular lysates were prepared as described previously (Leskelä et al., 2007).

### Immunoprecipitation of solubilised receptors

Solubilised receptors were purified by one- or two-step immunoprecipitation using immobilised monoclonal anti-FLAG M2 or polyclonal anti-Myc antibody resins (Sigma-Aldrich) as described previously (Petäjä-Repo et al., 2000, 2001). The purified receptors were eluted with SDS-sample buffer.

### Deglycosylation of immunoprecipitated receptors

For deglycosylation, samples were eluted from the antibody affinity resin with 1% SDS in 50 mM sodium phosphate, pH 5.5, and diluted eluates were digested with PNGase F and Endo H (Roche Applied Science) as described previously (Apaja et al., 2006). For Fig. 8B, receptors were reduced with 50 mM dithiothreitol prior to PNGase F digestion.

### SDS-PAGE and western blotting

SDS-PAGE and western blotting were carried out as described (Apaja et al., 2006). To alkylate cysteine residues (Fig. 3B), reduced immunoprecipitated samples were treated with 0.05 M iodoacetamide at 37°C for 30 min before loading to the gel. Antibodies used for western blotting are listed in Table S3. Gels containing radioactively labelled receptors were treated for fluorography as explained elsewhere (Pietilä et al., 2005).

### Purification of the N-terminal receptor fragment from the conditioned medium

Cells were grown on 10-cm plates for 2 days. The medium was then changed to 4 ml of fresh complete DMEM supplemented with tetracycline for 24 h. The conditioned medium was collected and centrifuged at 1000 ***g*** for 30 min to remove cellular debris. A total of 3 ml of each medium sample was supplemented with 25 mM Tris-HCl, pH 7.4, 0.5% n-dodecyl-β-D-maltoside, 20 mM *N*-ethylmaleimide, 2 mM EDTA, 2 µg/ml aprotinin, 0.5 mM phenylmethylsulphonyl fluoride, 2 mM 1,10-phenantroline, 5 µg/ml leupeptin, 5 µg/ml soybean trypsin inhibitor and 10 µg/ml bentzamidine, and subjected to immunoprecipitation with the anti-Myc antibody resin. The immunoprecipitated receptors were separated on 10–20% Mini-PROTEAN Tris-tricine precast gels (Bio-Rad) for 90 min.

### Flow cytometry

Cell surface receptors were stained for flow cytometry with the anti-Myc (9E10) antibody (1:350, Covance) and the phycoerythrin-conjugated rat anti-mouse-$IgG_1$ secondary antibody (2 µg/ml, BD Biosciences) as described previously (Petäjä-Repo et al., 2006). A total of 10,000 cells from each sample were analysed using the FACSCalibur™ flow cytometer and the CellQuest™Pro 6.0 software (BD Biosciences).

### Immunofluorescence microscopy

Cells (15,000 cells/well on 12-well plates) were cultured on poly-L-lysine (100 µg/ml; Sigma-Aldrich)-coated 12-mm glass coverslips for 2 days and receptor expression was induced with tetracycline (stably transfected $HEK293_i$ cells) for 24 h. Alternatively, Flp-In-293 cells were transiently transfected with GPR37 for 24 h. Cells were fixed with 100% methanol at −20°C for 15 min and permeabilised with 0.1% Triton X-100 and 0.5% bovine serum albumin in phosphate-buffered saline for 45 min. Antibody incubations were performed as described previously (Leskelä et al., 2009).



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Antibodies used to label receptors and marker proteins are listed in Table S4. The nuclei were stained with TO-PRO-3 iodine (1:500, Invitrogen) for 10 min and coverslips were mounted on glass microscope slides with Immumount (Thermo Fisher Scientific). Fluorescence was detected with a Zeiss LSM780 laser scanning confocal microscope (Carl Zeiss Microscopy) using a Plan-Apochromat 63×1.4 numerical aperture oil-immersion objective. The figures were processed using the Zen lite 2012 and Zen 2012 black edition software (Carl Zeiss Microscopy).

### N-terminal sequencing of cleaved receptors

Samples were prepared according to instructions provided by the Biocenter Oulu proteomics and protein analysis core facility. Briefly, cells from 20 10-cm plates were collected after 24 h of receptor expression, and receptors from solubilised membranes were purified by two-step immunoprecipitation and eluted with 200 µg/ml of FLAG peptide (Sigma-Aldrich). Purified receptors were blotted on ProBlot membranes (Thermo Fisher Scientific) and stained with the Serva Blue staining solution (0.1% Serva Blue, 1% acetic acid, 40% ethanol). Protein bands representing the cleaved receptor were excised from the membrane and analysed by automated Edman degradation. Five cycles were performed on the protein sequencer Procise™ 492 (Thermo Fisher Scientific).

### Data analysis

The data were analysed with the GraphPad Prism 6.01 software (GraphPad Software). The statistical analysis was carried out using one-way ANOVA followed by Bonferroni's multiple comparison test. The limit of significance was set at $P<0.05$. The data are presented as mean±s.e.m.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

The original observation that GPR37 is cleaved was made by J.T.T., who also prepared the Myc–GPR37–FLAG construct and the stably transfected $HEK293_i$ cell line. S.O.M. and U.E.P.-R. planned, and S.O.M. and J.T.T. (Fig. 2A) performed the experiments and analysed the data. S.O.M. and U.E.P.-R. wrote the manuscript with input from J.T.T.

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#### Supplementary information

Supplementary information available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.176115/-/DC1

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