

1 **The epilepsy gene *LGII* encodes a secreted glycoprotein that binds to the cell surface.**

2

3 M^a Salomé. Sirerol-Piquer^{1, †}, Ana Ayerdi-Izquierdo^{1, †}, José Manuel Morante-Redolat¹, Vicente
4 Herranz-Pérez¹, Kristy Favell², Philip A. Barker², Jordi Pérez-Tur^{1, *}

5

6 1. Unitat de Genètica Molecular. Departament de Genòmica i Proteòmica. Institut de
7 Biomedicina de València-CSIC, València (Spain).

8 2. Montreal Neurological Institute, McGill University, Montreal, Quebec (Canada)

9

10 †: The authors wish it to be known that, in their opinion, the first two authors should be
11 regarded as joint First Authors

12

13 *: To whom correspondence should be addressed at

14 Institut de Biomedicina de València-CSIC

15 Unitat de Genètica Molecular

16 C/ Jaume Roig, 11

17 E46010 València (Spain)

18 Tel. +34 96 339 1755

19 Fax: +34 96 339 3774

20 e-mail: jpereztur@ibv.csic.es

21

22

1 ABSTRACT.

2 Autosomal dominant lateral temporal epilepsy (ADTLE) is a partial epilepsy caused by mutations
3 in *LGII*, a multidomain protein of unknown function. To begin to understand the biological
4 function of LGI1, we have determined its pattern of glycosylation, subcellular expression and
5 capacity for secretion. LGI1 is expressed as two different isoforms in the brain and we show that
6 the long isoform is a secreted protein whereas the short isoform is retained in an intracellular
7 pool. ADLTE-related mutants of the long form are defective for secretion and are retained in the
8 endoplasmic reticulum and Golgi complex. Finally, we show that normal secreted LGI1
9 specifically binds to the cell surface of differentiated PC12 cells. We propose that LGI1 is a
10 secreted factor important for neuronal development and that ADTLE is a disease that results from
11 the loss of regulation in the protein available either extracellular or intracellularly.

12

1 INTRODUCTION

2 Autosomal dominant lateral temporal epilepsy (ADLTE, OMIM #600512; also known by
3 some authors as autosomal dominant epilepsy with auditory features or ADPEAF) is a
4 neurological disorder characterized by auditory auras and focal seizures affecting the lateral
5 temporal lobe of the brain. The disease was mapped to a 3cM locus on human chromosome 10 by
6 linkage analysis (1, 2). In 2002, our group (3) and others (4) demonstrated that mutations in
7 *LGII* (Leucine-rich Glioma-Inactivated 1) cause ADLTE. Numerous mutations have been found
8 throughout the protein coding regions of this gene but their effect on protein function is unknown.
9 (3-11).

10 *LGII* was originally localized to the breakpoint of a balanced translocation between
11 chromosomes 10 and 19 ($t\{10;19\}\{q24;13\}$) present in the glioblastoma cell line T98G (12).
12 Because *LGII* showed low expression levels in human high grade glial tumor samples and
13 glioblastoma cell lines compared to normal brain samples, it was proposed to act as a tumor
14 suppressor (12). However, more recent studies have challenged this view and concluded that
15 *LGII* loss of function is unlikely to play a role in glial tumor progression (13). The protein
16 encoded by *LGII* consists of a signal peptide and 3 leucine-rich repeats flanked by two cysteine-
17 rich regions in the N-terminal part of the protein. Additionally, a novel domain comprised of
18 seven tandem arrays of 50 amino acids, termed EPTP repeats, was found in the C-terminal part of
19 the protein. These EPTP repeats likely form a seven-bladed beta-propeller structure (14). The
20 EPTP repeat is also found in three paralogues of *LGII* (*LGI2*, *LGI3* and *LGI4*) as well as in two
21 otherwise unrelated genes (*TNEPI* and *VLGRI*) and together, these constitute the EPTP
22 superfamily (14, 15).

23 The function of *LGII* remains unclear. Recent studies from Senechal and coworkers have
24 shown that showed that *LGII* is a secreted protein (16), consistent with our earlier prediction (3)

1 and some studies have suggested that LGI1 is involved in regulation of cell movement, growth
2 and survival (17, 18). Interestingly, LGI4 has also been shown to be a secreted protein that may
3 play a role in peripheral nerve myelination (19) whereas Schulte and colleagues have provided
4 evidence that LGI1 is a membrane-associated protein that regulates the activity of the Kv1.1
5 voltage-dependent potassium channel subunit (20).

6 In this work we confirm that LGI1 is a secreted glycoprotein **even in neuronal-like cells** and
7 show that a truncated naturally occurring spliced isoform is retained within cells. We show that
8 the secretion of the full-length isoform depends on the integrity of each of its domains and that
9 disease-causing ADLTE mutations block LGI1 secretion. Moreover, we provide evidence for an
10 LGI1 receptor on cell surfaces. Together, our data indicate that manifestation of ADLTE is
11 related to defects in LGI1 secretion.

12

13

1 RESULTS

2 *The LGI1 isoforms are glycoproteins with different cellular fates.*

3 Human LGI1-Flag^{LONG} and LGI1-Flag^{SHORT}, shown schematically in Figure 1A, were
4 transiently transfected in HEK293T cells, the extracellular media was concentrated and the cell
5 extracts were immunoprecipitated with anti-Flag monoclonal antibodies. LGI1-Flag^{LONG} was
6 clearly present in both lysates and media whereas LGI1-Flag^{SHORT} was only detected in cell
7 lysates, suggesting that only LGI1-Flag^{LONG} was capable of being secreted (Figure 1B). Similar
8 experiments were performed on T98G cells, a glioblastoma-derived cell line lacking LGI1
9 expression (Figure 1B) and on COS7 and CHO cells. In all cases, LGI1-Flag^{LONG} accumulated in
10 media and LGI1-Flag^{SHORT} did not, indicating that the secretion of LGI1-Flag^{LONG} does not show
11 cell type specificity. In some instances, a minor band is observed in cell lysates over-expressing
12 the LGI1-Flag^{LONG} or the LGI1-Flag^{SHORT} isoforms, the identity of such band has not been further
13 investigated, therefore we cannot exclude the possibility that LGI1 is being post-transcriptionally
14 processed at a position close to the C-terminus end of the protein which is common to both
15 isoforms. Finally, we tested the ability of LGI1 to be secreted on differentiated PC12 cells, a
16 neuronal-like cell type. As is shown on figure 1B, NGF-differentiated PC12 cells are also capable
17 of secrete LGI1-Flag^{LONG} whereas retain LGI1-Flag^{SHORT} as well as an ADLTE-causing mutation
18 (LGI1-Flag^{758delC}).

19 In addition to verify that the secretion was cell-line independent, we also tested the influence
20 that the different tags used had on this process and found that the secretion of LGI1 was not
21 altered by attaching a larger tag such as GFP, at its C-terminus (data not shown). To confirm that
22 accumulation of LGI1 in media reflects active secretion from living cells, pulse-chase analyses
23 were performed. Figure 1C shows that HEK 293T cells transfected with LGI1-Flag^{LONG} and, 24

1 hours later, labelled with ^{35}S -Cys/Met showed accumulation of radioactive LGI1 in media within
2 30 minutes. This time course suggests that LGI1 is actively secreted.

3 If LGI1 enters the secretory pathway, it is likely to undergo N-linked glycosylation. To test
4 this, we performed PNGase F treatment of LGI1-Flag^{LONG} and LGI1-Flag^{SHORT}. Figure 2B shows
5 that both isoforms undergo a substantial molecular weight shift when treated with PNGase F,
6 suggesting that both become N-glycosylated in the endoplasmic reticulum. We introduced point
7 mutations at three predicted N-glycosylation sites in the full length isoform (LGI1-Flag^{LONG-}
8 ^{N192Q/N277Q/N422Q}) and found that the protein produced no longer underwent a molecular weight
9 shift when treated with PNGase F, indicating that some or all of these sites are required for N-
10 glycosylation of the protein. We also examined truncated forms of LGI1-Flag in this assay and
11 found that a truncated protein lacking the LRR domain was sensitive to PNGase F treatment
12 whereas a corresponding construct in which the glycosylation sites had been mutated to glutamic
13 acid (LGI1-Flag ^{Δ LRR-N277Q/N422Q}) was not (Figure 2C). If glycosylation sites in this construct were
14 mutated individually (ie. LGI1-Flag ^{Δ LRR-N277Q} and LGI1-Flag ^{Δ LRR-N422Q}), the resulting proteins
15 were sensitive to PNGase F treatment. We also examined a construct in which the EPTP domain
16 was deleted and, using a similar approach, found that a single potential N-linked glycosylation
17 residue in the resulting product (LGI1-Flag ^{Δ EPTP-N192Q}) conferred sensitivity to PNGase F
18 treatment. Together, these data indicate that N197Q, N277Q, and N422Q are sites of N-linked
19 glycosylation in LGI1.

20 We next assessed whether N-glycosylation of LGI1 was required for its secretion and evaluated
21 the effect of the three potential N-glycosylation sites on the secretion of LGI1. For this, we
22 compared secretion of the normal protein to the triple mutant and to each of the single
23 glycosylation mutants (LGI1-Flag^{N197Q}, LGI1-Flag^{N277Q} and LGI1-Flag^{N422Q}). Figure 2D shows

1 that triple mutant (LGI1-Flag LGI1-Flag^{LONG-N197Q/N277Q/N422Q}) was not secreted and that
2 secretion of the N197Q mutant was severely attenuated.

3 ***Role of LGI1 domains in the secretion of the protein***

4 To determine whether the LRR and EPTP domains were required for secretion, two artificial
5 constructs, LGI1-Flag^{ΔEPTP} and LGI1-Flag^{ΔLRR} were transfected in HEK 293T cells and their
6 accumulation in media was assessed. Figure 3B shows that LGI1 lacking its LRR domain is
7 efficiently secreted, although this is in contrast with Senechal *et al* results, the constructs used in
8 each work were not comparable. Whereas in this work, our construct deleted the full LRR-
9 domain, including the flanking Cys-rich domains, in Senechal *et al* the construct used in this
10 same experiments maintained the Cys-rich domains thus it is possible that this construct behaves
11 as a pathogenic mutation rather than as a wild-type protein. On the other hand, LGI1 lacking its
12 EPTP domain is retained in the transfected HEK293T cells. We attempted to rescue LGI1
13 secretion in the latter mutant by stepwise addition of each of the EPTP domains from one to six,
14 using the scheme described in (15). Only the intact wild-type protein that contains all seven
15 EPTP domains was secreted from cells.

16 ***Secretion of LGI1, but not N-glycosylation, is blocked by ADLTE-causing mutations.***

17 We next assessed whether naturally occurring mutations in LGI1 that cause ADLTE alter the
18 secretion or glycosylation of the protein. HEK293T cells were transfected with normal LGI1-
19 Flag^{LONG} or with LGI1-Flag^{758delC}, LGI1-Flag^{R474X}, LGI1-Flag^{C46R}, LGI1-Flag^{S145R}, LGI1-
20 Flag^{C200R}, LGI1-Flag^{F318C} and LGI1-Flag^{E383A}. All of these constructs resulted in robust LGI1
21 expression but only LGI1-Flag^{LONG} accumulated in media, indicating that the naturally occurring
22 mutations in LGI1 that cause disease do not exit the cell, regardless of whether they were
23 truncating or missense mutations (Figure 4B). We also examined the sensitivity of these proteins

1 to PNGase F treatment and found, despite these trafficking defects, all the LGI1 mutants tested
2 were N-glycosylated (Figure 4C).

3 ***Subcellular localization of LGI1***

4 To determine the subcellular localization of LGI1-GFP^{LONG}, LGI1-GFP^{SHORT} and ADLTE-
5 related mutants LGI1-GFP^{758delC}, LGI1-GFP^{R474X}, LGI1-GFP^{F318C} and LGI1-GFP^{E383A}, COS7
6 cells were transiently transfected with each construct and transfected cells were immunostained
7 with an antibody recognising calnexin, an endoplasmic reticulum resident protein, or labelled
8 with the fluorescent Golgi marker Bodipy TR ceramide. Confocal analysis revealed that LGI1-
9 GFP^{LONG} is enriched in the Golgi apparatus, whereas LGI1-GFP^{SHORT} and the mutants analyzed,
10 excluding LGI1-GFP^{R474X}, accumulate in the ER chaperone. LGI1-GFP^{R474X} is present in both
11 ER and Golgi (Figure 5). Colocalization of LGI1 and the ER was also demonstrated by co-
12 immunoprecipitating the wild-type and the mutant constructs with an antibody that recognizes
13 calnexin (data not shown).

14

15 ***LGI1 binds to the surface of PC12 cells.***

16 Because LGI1 is secreted to the extracellular medium, we assessed whether LGI1 could
17 specifically interact with cell surface proteins. For this, we prepared conditioned media
18 containing either placental alkaline phosphatase alone or placental alkaline phosphatase fused to
19 LGI1^{LONG} (AP-LGI^{LONG}) and compared these for their ability to bind NGF-differentiated PC12
20 cells as well as to the surface of non differentiated PC12 and COS7 cells (data not shown).
21 Figure 6 shows highly specific, dose dependent and saturable binding of AP-LGI^{LONG}, with half-
22 maximal binding observed at a concentration of 4 nM, suggesting that specific LGI1 receptor
23 complex is present on these cells.

24 ***Binding of AP-LGI1 to the cell surface results in reduction of activated ERK1/2***

1 To determine the effect that AP-LGI1 binding had on the cells, we assessed the activation state of
2 ERK1/2 as it has been shown that over-expression of LGI1 caused a decrease in their activated
3 levels in T98G cells (Kunapuli *et al.*, 2004). Figure 7 shows that incubating NGF-differentiated
4 PC12 cells with AP-LGI1^{LONG} results in a significant reduction in the level of activated ERK1/2
5 compared to AP treated cells.

6

1 DISCUSSION

2 We demonstrate here that LGI1 is a glycoprotein secreted to the extracellular media in several *in*
3 *vitro* models including a neurona-like cell type. The shorter and much less prevalent spliced
4 isoform is retained within the cell. This is true even though the short isoform is expressed at
5 higher levels in our *in vitro* system. In some instances, usage of alternative splice sites within
6 coding exons has been show to produce isoforms with different, even opposite, functions (24, 25)
7 thus, the existence of two LGI1 isoforms with different cellular locations suggest that these
8 proteins may have different functions.

9 *LGII* is the gene responsible for ADLTE and distinct mutations have been described in several
10 families. Disease-causing *LGII* mutations have been identified throughout the LGI1 open reading
11 frame (3, 4, 6-11, 21) and in this study, we have tested the hypothesis that disease-causing
12 mutations alter LGI1 secretion. We show here that each of the disease-causing forms of LGI1 are
13 defective in secretion from transfected cells. We did not detect changes in the glycosylation of
14 the mutant proteins but instead demonstrate that the mutant proteins are largely retained in the
15 endoplasmic reticulum. The only exception was LGI1-Flag^{R474X}, which was present in both in the
16 endoplasmic reticulum and the Golgi apparatus. This mutation produces a truncated protein
17 lacking the C-terminal 83 amino acids and it is possible that it retains structural elements that
18 allow it to partially mimic the normal protein and reach the Golgi apparatus. Using artificial
19 mutations, we also show that the LRR domain is not required for secretion but that an intact
20 EPTP domain with all seven tandem repeats is required for the protein to exit the cell and find
21 that N-linked glycosylation of LGI1 is necessary for its secretion but individual mutation of any
22 the three putative N-linked residues does not block its exit from the cell. We conclude that the
23 disease-related missense mutations are exerting their effect by altering the tertiary structure of the
24 protein, causing their retention and degradation through protein quality control mechanisms.

1 The secretion of the full-length isoform together with the retention of the spliced isoform raises
2 also interesting questions regarding the function of LGI1. Kunapuli and coworkers showed that
3 when T98G cells were forced to re-express the long isoform, the malignancy of this glioma cell
4 line was severely reduced and this correlated with reduced ERK1/2 phosphorylation and reduced
5 metalloproteinase production (26). Given that we have demonstrated that LGI1 binds in a dose-
6 dependent manner to differentiated PC12 cells and that this binding has a functional effect in the
7 cells, it is possible that forced overexpression of LGI1 in T98G cells may have activated a ligand-
8 receptor autocrine loop in these cells that altered cellular signalling pathways.

9 Schulte and colleagues (2006) have recently shown that LGI1 is part of a Kv1 channel complex
10 and provided evidence that LGI1 acts to prevent the inhibitory effect of the Kv β 1 channel
11 subunit. They report that mutant forms of LGI1 assemble into Kv1 channel complexes yet are
12 incapable of altering Kv β 1 channel inhibitor activity. They propose that the C-terminal domain of
13 LGI1 lost in truncated mutants of the protein is required for Kv β 1 channel antagonizing activity.

14 We show here that a major consequence of LGI1 mutation is ER retention and a failure to enter
15 Golgi and the secretory pathway. Therefore, an equally plausible alternative explanation for the
16 findings of Schulte *et al.* (2006) is that the mutant LGI1 assembles into Kv1 complexes in the ER
17 and the resulting assembly remains trapped in this compartment. In this scenario, only Kv1
18 complexes lacking LGI1 would actually reach the cell surface.

19 Taken together, our data suggest a hypothesis for the molecular pathology of ADLTE based on
20 the retention of LGI1 in intracellular compartments. This retention could have a gain of function
21 with cell autonomous effects, such as on Kv1 channels, or could result in a lost of function from
22 non-autonomous effects that include loss of a critical cell communication between cells that
23 secrete LGI1 and cells that respond to it.

1 METHODS

2 *Constructs.*

3 Several constructs were prepared for this work as fusion proteins. Proteins fused to GFP were
4 cloned into pEGFP-N1 (Clontech), proteins fused to AP were cloned into pc3.1AP6 whereas
5 fusions to the Flag epitope were cloned into pSalo-Flag, a modified version of pEGFP-N1 with
6 the GFP protein removed and substituted by a Flag epitope. For simplicity each construct is
7 named by placing the name of the tag showing its position with respect to the cloned LGI1.

8 Both LGI1 isoforms, the full length (long, amino acids 1-557) and the splicing variant (short,
9 amino acids 1-259), were cloned from a human cDNA library (Invitrogen). For this study we
10 have selected mutations producing truncated proteins (c.758delC and p.R474X; (3)) and some
11 missense mutations affecting different domains of the protein: the N-terminal cysteine rich
12 domain (p.C46R;(21)), the third leucine rich repeat (p.S145R; (10)), the C-terminal cysteine rich
13 domain (p.C200R; (8)) and the third and fourth EPTP repeats (p.F318C; (6) and p.E383A; (4)
14 respectively). In addition to these, we have also introduced the mutations affecting the three
15 potential N-glycosylation sites predicted by NetNGlyc
16 (<http://www.cbs.dtu.dk/services/NetNGlyc/>), p.N192Q, p.N277Q and p.N422Q. Finally, we have
17 created two deletion constructs; one lacks the EPTP repeat region (Δ EPTP, lacking amino acids
18 224-556) and one lacks the LRR region (Δ LRR) and contains the signal peptide (amino acids 1-
19 34) fused to the EPTP-repeat region (amino acids 224-556). In addition to those, we have
20 produced several chimeric forms lacking part or all of a particular domain as shown in figures 1
21 to 3. For the serial deletions of the EPTP repeats, different primers were used in order to obtain 7
22 constructs containing from 0 to 6 EPTP repeats tagged with FLAG; 0EPTPs (ending at amino
23 acid 223), 1EPTP (ending at 267), 2EPTPs (ending at 314), 3EPTPs (ending at 364), 4EPTPs
24 (ending at 415), 5EPTPs (ending at 462), 6EPTPs (ending at 506). To obtain the AP-LGI1 vector,

1 the cDNA encoding amino acids 35-557 was ligated into the pc3.1AP6 vector in frame with
2 signal sequence and placental AP coding region. All mutagenesis were done with the Quick
3 Change Mutagenesis kit (Stratagene) and all constructs generated by PCR were fully sequenced
4 to ensure integrity of the cloned ORFs. All primers used as well as PCR conditions are available
5 from authors on request.

6 ***Cell culture, transient transfection and media collection***

7 CHO, COS7 and HEK 293T cells were grown in DMEM (Cambrex), supplemented with 10%
8 (v/v) heat inactivated foetal bovine serum (FBS, Gibco) 2mM Glutamine (Gibco) and 2% (v/v)
9 Penicillin/Streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin, Gibco) on 10 cm
10 plates and maintained at 37°C and 5% CO₂. T98G cells, a glioblastoma derived cell line that do
11 not express LGI1 (12), were grown in EMEM (Cambrex) 10% (v/v) FBS, 2 mM Glutamine, 1%
12 Non Essential amino Acids (Gibco) and 1% Sodium Piruvate (Gibco). PC12 cells were grown on
13 poly-D-lysine coated plates in DMEM supplemented with 2mM Glutamine, 2% (v/v)
14 Penicillin/Streptomycin and 0.1% BSA. When required PC12 cells were differentiated with
15 50ng/mL NGF in complete medium for 48 hours.

16 T98G, COS7 and CHO cells were transiently transfected with liposomes (Fugene, Roche), while
17 HEK 293T were transfected by calcium phosphate method. PC12 cells were transfected with
18 lipofectamine and differentiated with NGF twenty-four hours post-transfection. For media
19 production, HEK 293T cells were grown to approximately 60-70% confluence and were
20 transiently transfected with 5µg DNA. After 16-20 hr of transfection the media were replaced
21 with a reduced serum media (Optimem I, Gibco), and incubated for an additional 24 hr. Then,
22 media were collected, supplemented with a protein inhibitor cocktail (Complete, Roche), filtrated
23 through a 0,2 µm filter and concentrated 10-15 times to 300 µl using an Amicon Ultra15 device
24 (MWCO: 30,000 KDa; Millipore) and analyzed by SDS-PAGE.

1 For binding experiments, after two days on serum-free DMEM, secreted AP-LGI1 and AP were
2 first concentrated as before and then the amount of protein was estimated according to (22, 23)
3 measuring the AP activity using p-nitro phenyl phosphate as substrate (Sigma). For the binding
4 experiments, the concentrated media was diluted with PBS 1x medium to 5 - 40nM.

5 ***SDS-PAGE and Western-blotting***

6 Equivalent volumes of concentrated media and immunoprecipitates were loaded onto 10%
7 SDS-PAGE gels to resolve proteins. Following transfer, nitrocellulose membranes (Amersham)
8 were blocked at room temperature for 1 hr with non-fat dry milk powder in Tris-HCl 20 mM pH
9 7.6, NaCl 140 mM, Tween-20 0.1% (v/v) (TBST) to reduce non specific binding. The Flag
10 epitope was detected by incubation with either mouse anti-Flag antibody (1:500, in blocking
11 solution; Sigma) or rabbit anti-Flag antibody (1:1000, in 3% BSA; Sigma) at 4°C overnight. The
12 membranes were washed in TBST prior to incubation with the HRP-conjugated anti-mouse IgG
13 antibody or HRP-conjugated anti-rabbit IgG antibody (1:5000, in blocking solution, Jackson).
14 The blots were further washed in TBST and subsequently incubated in ECL or ECL plus
15 (Amersham).

16 ***Immunoprecipitation and pulse-chase analyses***

17 HEK 293T cells were lysed and scraped in 1 ml NP40-containing lysis buffer (10 mM Tris
18 HCl pH8, 150mM NaCl, 1% Nonidet-P40, 10% Glycerol, 1% Triton-X100 and protease
19 inhibitors) for 30 min at 4°C. Cell lysates were clarified by centrifugation at 13,000 rpm at 4°C
20 for 10 min and then the supernatants were incubated with protein G sepharose beads (Amersham)
21 conjugated with M2 mouse anti-Flag antibody (Sigma) at 4°C for 2 hr. The beads were washed
22 three times with lysis buffer and then resuspended in 60µl 2x Laemmli sample buffer for SDS-
23 PAGE. For pulse chase analyses, cells were transfected with LGI1 or with parental vector and 24
24 hours later, pre-incubated in cysteine- and methionine-free media for 30 minutes followed by

1 incubation for 30 minutes in labeling media consisting of 50 $\mu\text{Ci/ml}$ ^{35}S -Trans-label (ICN) and 3
2 mg/L unlabelled cysteine and methionine, respectively, for 30 minutes. Cells were then switched
3 to normal media (30 mg/L cysteine and 30 mg/L methionine) for 10, 30 or 60 minutes. After
4 lysis, immunoprecipitations were performed using monoclonal anti-Flag antibodies.
5 Immunoprecipitates were separated by SDS-PAGE, gels were impregnated with Enhance (NEB)
6 and dried, using the protocol provided by the manufacturer.

7 ***PNGase F digestion***

8 Cell lysates and culture medium obtained as described from transiently transfected HEK 293T
9 cells were immunoprecipitated as above and resuspended and denatured in 25 μl of denaturing
10 buffer at 100°C for 10 min and cooled to room temperature. The denatured samples were
11 subjected to digestion with 4 mU of PNGase F (New England Biolabs) at 37°C for 90 min. The
12 samples were then analyzed by SDS-PAGE.

13 ***Subcellular localization of wild type and mutant LGII***

14 Twenty-four hours after transfection with the LGI1-GFP constructs using FuGene (Roche),
15 COS7 cells grown on glass cover slips were fixed with 4% (w/v) paraformaldehyde in PBS a
16 37°C for 30 minutes. Slides were blocked at room temperature for two hours with 5% BSA in
17 PBS and then immunostained with the ER marker anti-Calnexin (1:500, Calbiochem) at 4°C
18 overnight. Slides were washed and incubated with Texas Red-conjugated anti-rabbit antibody
19 (1:5000, Molecular Probes) at room temperature for 2 hr. Alternatively, COS7 cells were
20 incubated with the Golgi marker Bodipy TR Ceramide (Molecular Probes) at a 3 μM
21 concentration in culture media, for 30 min at 37°C. Images were collected on a Leica TCS SL
22 spectral confocal microscope coupled to an inverted microscope (Leica DMIRE2; Leica
23 Microsystems) at room temperature with a 63x immersion objective (HCX PLAPO CS, 63x/1.40-

1 0.60 1.40-0.60. Images were captured on a computer system by the Leica Confocal Software v2.5
2 (Build 1347, Leica Microsystems).

3 ***Cell binding assays of AP fusion proteins***

4 AP-tagged fusion proteins were produced by transient transfection of HEK 293T cells with
5 either AP-LGI1 or empty vector producing secreted AP. PC-12 cells, 5×10^5 cells/well, were
6 plated in 6 well plates coated with poly-D-lysine and differentiated with NGF (50ng/mL) for 48
7 hours. Afterwards, plates were washed twice with PBS 1x and then cells were incubated with
8 dilutions of either AP or AP-LGI1 for 90 min at 37°C. Cultures were then washed three times
9 with ice-cold Hanks balanced Salt solution containing 20mM sodium HEPES, 0.1% sodium azide
10 and 0.5mg/mL BSA. Plates where the binding was quantified were processed with lysis buffer
11 (20mM Tris-HCl pH 8.0, 0.1% Triton-X100 and protease inhibitors). Endogenous AP activity
12 was heat inactivated at 65° for 15 minutes and bound AP activity was assessed using p-nitro
13 phenyl phosphate as substrate measuring absorbance at 405nm. In order to normalize the results,
14 total protein present in the lysates was measured by DC protein assay (Bio-Rad). Plates where
15 pictures were taken were incubated at 65° for 90 minutes to heat inactivate the endogenous AP
16 activity and the AP-LGI1 binding was revealed in the presence of NBT (nitro blue tetrazolium)
17 and BCIP (5-Bromo-4-chloro-3-indolyl phosphate) (Sigma).

18 ***Determination of ERK1/2 levels.***

19 Conditioned media from HEK 293T cells expressing either AP-LGI1^{LONG} or the AP alone, were
20 incubated with NGF-differentiated PC12 cells for the times shown in figure 7 at 37°C
21 normalising for the AP activity at 15nM. As the process of differentiation of the PC12 cells by
22 NGF requires activation of the ERK1/2 pathway, and the incubation with conditioned media
23 could affect the state of activation of ERK1/2 by itself, we pre-treated all cultures with an
24 equivalent amount of conditioned media coming from mock-transfected HEK 293T for 2h prior

1 to AP-LGII^{LONG} or AP treatment. After treatment, the cells were washed with PBS and harvested
2 in RIPA buffer (1.5 M NaCl, 1% Nonidet-P40, 0.5% deoxycholate, 1 % SDS, 50 mM Tris-HCl,
3 pH 8.0) with a cocktail of protease inhibitors (Complete, Roche) supplemented with 1 mM
4 sodium orthovanadate. Cell lysates were incubated on ice for 30 min prior to clarification at 4°C
5 and 16,000 g for 15 min. Fifteen micrograms of protein from each condition were assayed by
6 Western blotting using an antibody that recognizes phosphorylated ERK1/2. After stripping of
7 the blot, it was incubated with a phosphorylation-independent antibody that recognized total
8 ERK1/2. In both cases, the Western was developed and an image was captured and the intensity
9 of each band was measured with the help of the MultiGauge v2.1 software (Fujifilm). After this,
10 we estimated the relative amount of phospho-ERK1/2 over total ERK1/2.
11

1 ACKNOWLEDGEMENTS

2 The authors thank two anonymous reviewers for their helpful criticism of the work presented
3 herein. The authors are indebted to Silvia Aparicio-Domingo, Raquel Rodríguez-de Pablos and
4 Benito Alarcón, for their technical support as well as to the Unidad de Genética y Medicina
5 Molecular and Unidad de Biología Vascular of the Instituto de Biomedicina de Valencia-CSIC
6 for technical assistance. JP-T is part of the Grupos de Excelencia of the Generalitat Valenciana
7 (Grupos 03/015) and PAB is a CIHR Scientist. This work was supported by grants from the
8 Ministerio de Educación y Ciencia (SAF2002-00060 and SAF2005-00136) to JP-T and from the
9 Canadian Institute of Health Research (PPP147918) to PAB. SS-P is funded by a fellowship of the
10 Generalitat Valenciana (CTBPRB/2002/35), JMM-R is funded by an FPU and a Bancaixa
11 fellowship. Support from the Ministerio de Educación y Ciencia (BES-2003-0243, to AA-I) and
12 from the Ministerio de Sanidad y Consumo (BF03/00182, to VH-P) is also acknowledged. KF is
13 funded by a Canadian NSERC award.

14

1 **CONFLICTS OF INTEREST**

2 The authors do not have any conflict of interest regarding the work reported in this manuscript.

3

1 REFERENCES

- 2 1. Ottman, R., Risch, N., Hauser, W.A., Pedley, T.A., Lee, J.H., Barker-Cummings, C.,
3 Lustenberger, A., Nagle, K.J., Lee, K.S., Scheuer, M.L. *et al.* (1995) Localization of a
4 gene for partial epilepsy to chromosome 10q. *Nat. Genet.*, **10**, 56-60.
- 5 2. Poza, J.J., Saenz, A., Martinez-Gil, A., Cheron, N., Cobo, A.M., Urtasun, M., Marti-
6 Masso, J.F., Grid, D., Beckmann, J.S., Prud'homme, J.F. *et al.* (1999) Autosomal
7 dominant lateral temporal epilepsy: clinical and genetic study of a large Basque pedigree
8 linked to chromosome 10q. *Ann. Neurol.*, **45**, 182-188.
- 9 3. Morante-Redolat, J.M., Gorostidi-Pagola, A., Piquer-Sirerol, S., Saenz, A., Poza, J.J.,
10 Galan, J., Gesk, S., Sarafidou, T., Mautner, V.F., Binelli, S. *et al.* (2002) Mutations in the
11 LGI1 gene on 10q24 cause autosomal dominant lateral temporal epilepsy. *Hum. Mol.*
12 *Genet.*, **11**, 1119-1128.
- 13 4. Kalachikov, S., Evgrafov, O., Ross, B., Winawer, M., Barker-Cummings, C., Martinelli
14 Boneschi, F., Choi, C., Morozov, P., Das, K., Teplitskaya, E. *et al.* (2002) Mutations in
15 LGI1 cause autosomal-dominant partial epilepsy with auditory features. *Nat. Genet.*, **30**,
16 335-341.
- 17 5. Gu, W., Brodtkorb, E. and Steinlein, O.K. (2002) LGI1 is mutated in familial temporal
18 lobe epilepsy characterized by aphasic seizures. *Ann. Neurol.*, **52**, 364-367.
- 19 6. Fertig, E., Lincoln, A., Martinuzzi, A., Mattson, R.H. and Hisama, F.M. (2003) Novel
20 LGI1 mutation in a family with autosomal dominant partial epilepsy with auditory
21 features. *Neurology*, **60**, 1687-1690.
- 22 7. Kobayashi, E., Santos, N.F., Torres, F.R., Secolin, R., Sardinha, L.A., Lopez-Cendes, I.
23 and Cendes, F. (2003) Magnetic resonance imaging abnormalities in familial temporal
24 lobe epilepsy with auditory auras. *Arch. Neurol.*, **60**, 1546-1551.

- 1 8. Michelucci, R., Poza, J.J., Sofia, V., de Feo, M.R., Binelli, S., Bisulli, F., Scudellaro, E.,
2 Simionati, B., Zimbello, R., D'Orsi, G. *et al.* (2003) Autosomal dominant lateral temporal
3 epilepsy: clinical spectrum, new epitempin mutations, and genetic heterogeneity in seven
4 European families. *Epilepsia*, **44**, 1289-1297.
- 5 9. Berkovic, S.F., Izzillo, P., McMahon, J.M., Harkin, L.A., McIntosh, A.M., Phillips, H.A.,
6 Briellmann, R.S., Wallace, R.H., Mazarib, A., Neufeld, M.Y. *et al.* (2004) LGI1
7 mutations in temporal lobe epilepsies. *Neurology*, **62**, 1115-1119.
- 8 10. Hedera, P., Abou-Khalil, B., Crunk, A.E., Taylor, K.A., Haines, J.L. and Sutcliffe, J.S.
9 (2004) Autosomal dominant lateral temporal epilepsy: two families with novel mutations
10 in the LGI1 gene. *Epilepsia*, **45**, 218-222.
- 11 11. Ottman, R., Winawer, M.R., Kalachikov, S., Barker-Cummings, C., Gilliam, T.C., Pedley,
12 T.A. and Hauser, W.A. (2004) LGI1 mutations in autosomal dominant partial epilepsy
13 with auditory features. *Neurology*, **62**, 1120-1126.
- 14 12. Chernova, O.B., Somerville, R.P. and Cowell, J.K. (1998) A novel gene, LGI1, from
15 10q24 is rearranged and downregulated in malignant brain tumors. *Oncogene*, **17**, 2873-
16 2881.
- 17 13. Piepoli, T., Jakupoglu, C., Gu, W., Lualdi, E., Suarez-Merino, B., Poliani, P.L., Cattaneo,
18 M.G., Ortino, B., Goplen, D., Wang, J. *et al.* (2006) Expression studies in gliomas and
19 glial cells do not support a tumor suppressor role for LGI1. *Neuro-oncol.*, **8**, 96-108.
- 20 14. Staub, E., Perez-Tur, J., Siebert, R., Nobile, C., Moschonas, N.K., Deloukas, P. and
21 Hinzmann, B. (2002) The novel EPTP repeat defines a superfamily of proteins implicated
22 in epileptic disorders. *Trends Biochem. Sci.*, **27**, 441-444.
- 23 15. Scheel, H., Tomiuk, S. and Hofmann, K. (2002) A common protein interaction domain
24 links two recently identified epilepsy genes. *Hum. Mol. Genet.*, **11**, 1757-1762.

- 1 16. Senechal, K.R., Thaller, C. and Noebels, J.L. (2005) ADPEAF mutations reduce levels of
2 secreted LGI1, a putative tumor suppressor protein linked to epilepsy. *Hum. Mol. Genet.*,
3 **14**, 1613-1620.
- 4 17. Kunapuli, P., Chitta, K.S. and Cowell, J.K. (2003) Suppression of the cell proliferation
5 and invasion phenotypes in glioma cells by the LGI1 gene. *Oncogene*, **22**, 3985-3991.
- 6 18. Gabellini, N., Masola, V., Quartesan, S., Oselladore, B., Nobile, C., Michelucci, R.,
7 Curtarello, M., Parolin, C. and Palu, G. (2006) Increased expression of LGI1 gene triggers
8 growth inhibition and apoptosis of neuroblastoma cells. *J. Cell. Physiol.*, **207**, 711-721.
- 9 19. Bermingham, J.R., Shearin, H., Pennington, J., O'Moore, J., Jaegle, M., Driegen, S., van
10 Zon, A., Darbas, A., Ozkaynak, E., Ryu, E.J. *et al.* (2006) The claw paw mutation reveals
11 a role for Lgi4 in peripheral nerve development. *Nat. Neurosci.*, **9**, 76-84.
- 12 20. Schulte, U., Thumfart, J.O., Klocker, N., Sailer, C.A., Bildl, W., Biniiossek, M., Dehn, D.,
13 Deller, T., Eble, S., Abbass, K. *et al.* (2006) The epilepsy-linked lgi1 protein assembles
14 into presynaptic kv1 channels and inhibits inactivation by kvbeta1. *Neuron*, **49**, 697-706.
- 15 21. Gu, W., Wevers, A., Schroder, H., Grzeschik, K.H., Derst, C., Brodtkorb, E., de Vos, R.
16 and Steinlein, O.K. (2002) The LGI1 gene involved in lateral temporal lobe epilepsy
17 belongs to a new subfamily of leucine-rich repeat proteins. *FEBS Lett.*, **519**, 71-76.
- 18 22. Flanagan, J.G. and Cheng, H.J. (2000) Alkaline phosphatase fusion proteins for molecular
19 characterization and cloning of receptors and their ligands. *Methods Enzymol.*, **327**, 198-
20 210.
- 21 23. Flanagan, J.G., Cheng, H.J., Feldheim, D.A., Hattori, M., Lu, Q. and Vanderhaeghen, P.
22 (2000) Alkaline phosphatase fusions of ligands or receptors as in situ probes for staining
23 of cells, tissues, and embryos. *Methods Enzymol.*, **327**, 19-35.

-
- 1 24. Boise, L.H., Gonzalez-Garcia, M., Postema, C.E., Ding, L., Lindsten, T., Turka, L.A.,
2 Mao, X., Nunez, G. and Thompson, C.B. (1993) *bcl-x*, a *bcl-2*-related gene that functions
3 as a dominant regulator of apoptotic cell death. *Cell*, **74**, 597-608.
- 4 25. Roos, K.L. and Simmons, D.L. (2005) Cyclooxygenase variants: the role of alternative
5 splicing. *Biochem. Biophys. Res. Commun.*, **338**, 62-69.
- 6 26. Kunapuli, P., Kasyapa, C.S., Hawthorn, L. and Cowell, J.K. (2004) *LGI1*, a putative
7 tumor metastasis suppressor gene, controls in vitro invasiveness and expression of matrix
8 metalloproteinases in glioma cells through the ERK1/2 pathway. *J. Biol. Chem.*, **279**,
9 23151-23157.
- 10
11

1 FIGURE LEGENDS

2 **Figure 1. LGI1-Flag^{LONG} is secreted from HEK293T cells.** (A) Schematic representation of the
3 LGI1-Flag constructs with the discrete domains used The white box represents de signal peptide,
4 ovals represent the N-term and C-term Cys-rich domains flanking the LRR domains (light grey
5 rectangles) Dark grey rectangles represent the EPTP repeats. The pentagon indicates the position
6 of the FLAG epitope. (B) Analysis of the secretion of LGI1-Flag^{LONG} and LGI1-Flag^{SHORT} in
7 NGF-differentiated PC12 (left), HEK293T (middle) and T98G (right) cells. Cells were
8 transfected with each construct and the lysates and the concentrated media were analyzed by
9 Western blot using an anti-Flag antibody as detailed in the Methods section. **Note the presence of**
10 **an unspecific band close to the size of the LGI1-Flag^{SHORT} isoform in T98G cells.** (C) HEK293
11 cells transfected with plasmid encoding LGI1-FLAG or control vector were incubated with ³⁵S-
12 Translabel (ICN) for 30 minutes and then chased with media containing an excess of unlabelled
13 cysteine and methionine for 10, 30 and 60 minutes, as indicated. LGI1-FLAG was
14 immunoprecipitated using an anti-FLAG antibody and analyzed by SDS-PAGE/fluorography.
15 LONG refers to the LGI1-Flag^{LONG} construct, SHORT refers to LGI1-Flag^{SHORT}.
16

17 **Figure 2. LGI1-Flag is N-glycosylated at N192, N277 and N422.** (A) Constructs used in these
18 experiments showing the position of the predicted N-glycosylation sites. See figure 1 for details
19 on the domains of the protein. (B) Both LGI1-Flag^{LONG} (LONG) and LGI1-Flag^{SHORT} (SHORT)
20 were transfected in HEK293T cells and extracts treated in the absence (-) or presence (+) of
21 PNGFase and subjected to SDS-PAGE followed by immunodetection with an anti-Flag antibody.
22 (C) Analysis of the sites of glycosylation in the constructs. Each of the LGI1-Flag constructs
23 shown with the N-glycosylation sites mutated was subjected to treatment without (-) or with (+)
24 PNGFase. Whereas the LGI1-Flag^{LONG} isoform is glycosylated, the equivalent construct with all

1 three predicted glycosylation sites removed shows lack of glycosylation. Mutation of the N192,
2 N277 or N422 residues clearly show a modification in the pattern of PNGFase-sensitive
3 glycosylation. (2x: construct with both predicted sites mutated simultaneously; 3x: construct with
4 all three predicted N-glycosylation sites mutated). Note the presence of bands resulting from
5 incomplete digestion with PNGFase in some lanes. (D) Effect of abolishing the sites of N-
6 glycosylation on the secretion of LGI1-Flag^{LONG}. Removing any individual N-glycosylation site
7 does not block the secretion of LGI1-Flag^{LONG}; on the contrary, LGI1-Flag^{N192Q/1277Q/N422Q} (first
8 lane on the right) is not secreted (right panel) and gets retained within the cell (left panel).

9
10 **Figure 3. LGI1 domains required for protein secretion.** (A) Representation of the constructs
11 employed in this experiment. See legend of figure 1 for details on the protein domains. (B)
12 Analysis of the effect on the secretion of the two regions of the protein. As detailed in the
13 Material and Methods section, HEK293T cells were transfected with the indicated constructs and,
14 48h post-transfection, cell lysates and concentrated culture media were analyzed by Western
15 blotting with an anti-Flag antibody. (C) The requirement for a complete EPTP-repeat region was
16 tested by transfecting HEK293T cells with constructs containing increasing numbers of EPTP
17 repeats and analyzing both the cell extracts and concentrated culture media as before.

18
19 **Figure 4. LGI1 mutations that cause ADLTE block secretion, but not the glycosylation, of**
20 **LGI1-Flag.** (A) Constructs employed in these experiments. See legend for figure 1 for an
21 explanation of the different domains of the protein. (B). HEK293T cells were transfected with
22 each construct and cell lysates (left panel) and concentrated culture media (right panel) were
23 subjected to SDS-PAGE followed by immunoblotting with an anti-Flag antibody. (C) PNGFase
24 treatment of ADLTE-causing mutations. HEK293T cells were transfected with each construct

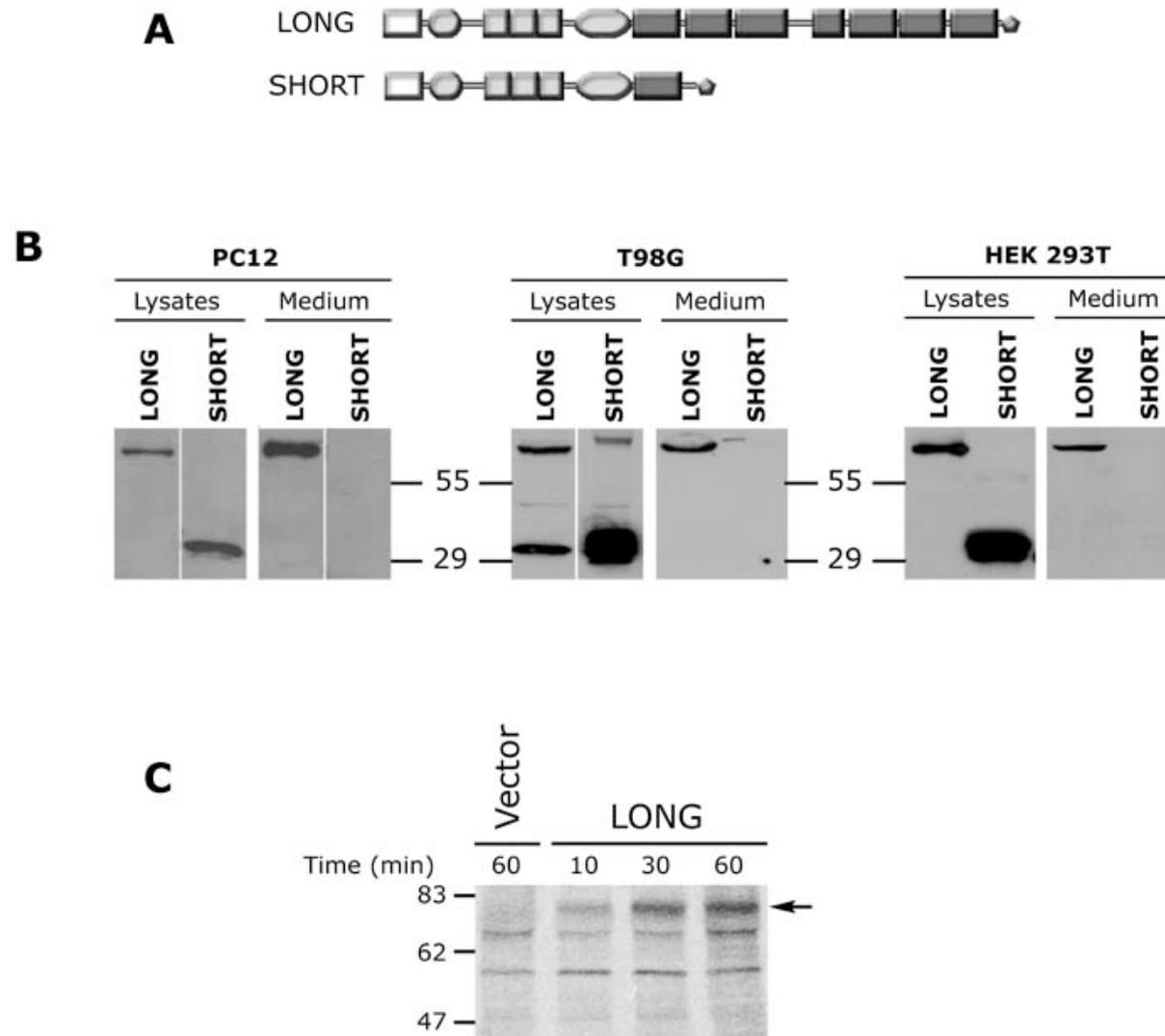
1 and, 48h post-transfection, cell extracts were subjected to treatment without (-) or with (+)
2 PNGFase prior to Western blotting.

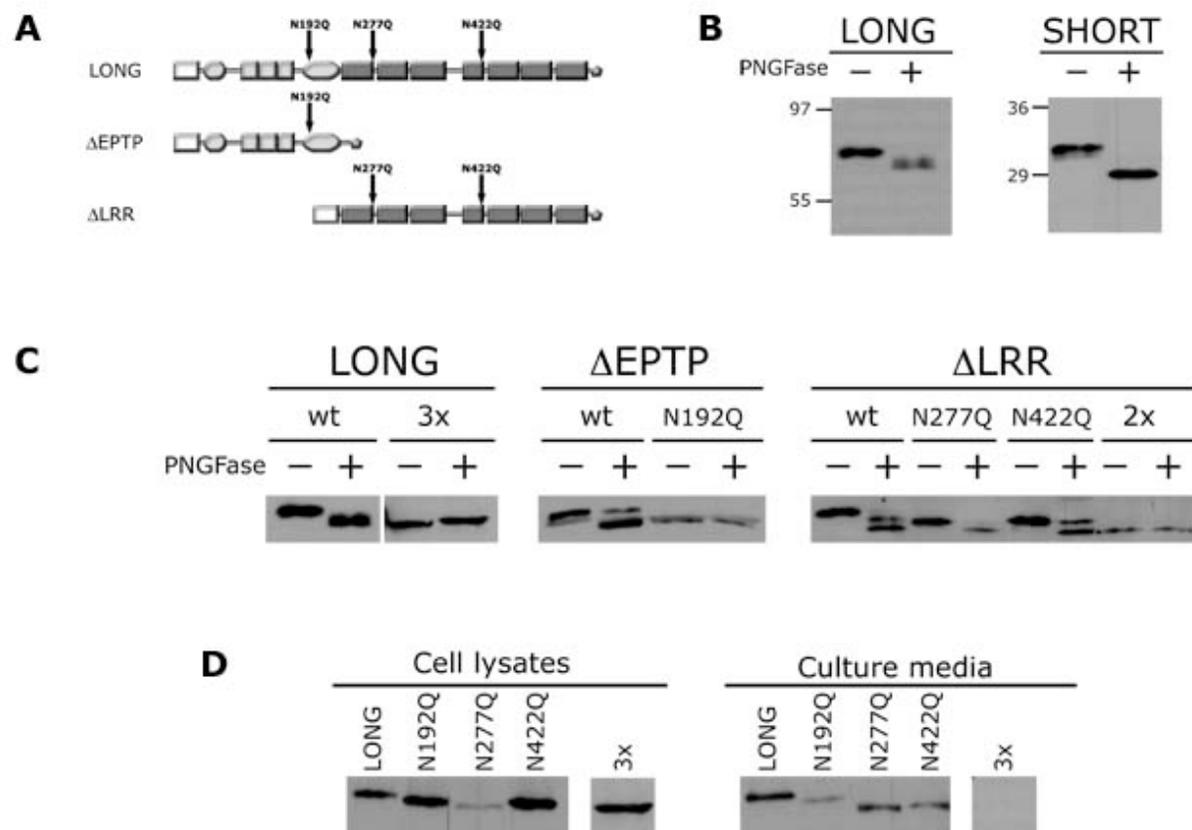
3
4 **Figure 5. Subcellular localization of disease-causing mutations of LGI1.** COS7 cells were
5 transfected with each GFP-fused protein as indicated and treated with either an anti-calnexin
6 antibody to identify the endoplasmic reticulum or the Bodipy probe to detect the Golgi apparatus.
7 Confocal images for each construct are shown together with the merged image. Bar: 8 μ m.

8
9 **Figure 6. LGI1 binds to the cell surface of differentiated PC12 cells.** AP-LGI1^{LONG} was
10 obtained from the concentrated culture media of transfected HEK293T and incubated with NGF-
11 differentiated PC12 cells. After 1h incubation, the reagents were removed and the cells washed
12 and treated to (A) detect membrane-bound AP-LGI1^{LONG} (left) or membrane-bound AP as a
13 control (right) or subjected to a quantitative analysis as detailed in the Methods section (B).
14 Magnification of pictures: 200x

15
16 **Figure 7. Binding of LGI1 to the surface of NGF-differentiated PC12 cells reduces the**
17 **activation of ERK1/2.** AP-LGI1^{LONG} was incubated with NGF-differentiated PC12 cells as in
18 figure 6. After the times indicated, cells were harvested and analyzed to determine the level of
19 phospho-ERK1/2 and total ERK1/2. (A) Western blot showing the reduction in phosphoERK1/2
20 at 5 and 15 minutes after AP-LGI1^{LONG} treatment compared to AP treatment. (B) Quantification
21 of the relative levels of phosphoERK1/2 over total ERK1/2 expressed as percentage over the
22 levels at t=0. * p=0.02 using a Student's t. Values are given as means \pm SEM for three independent
23 experiments.

24

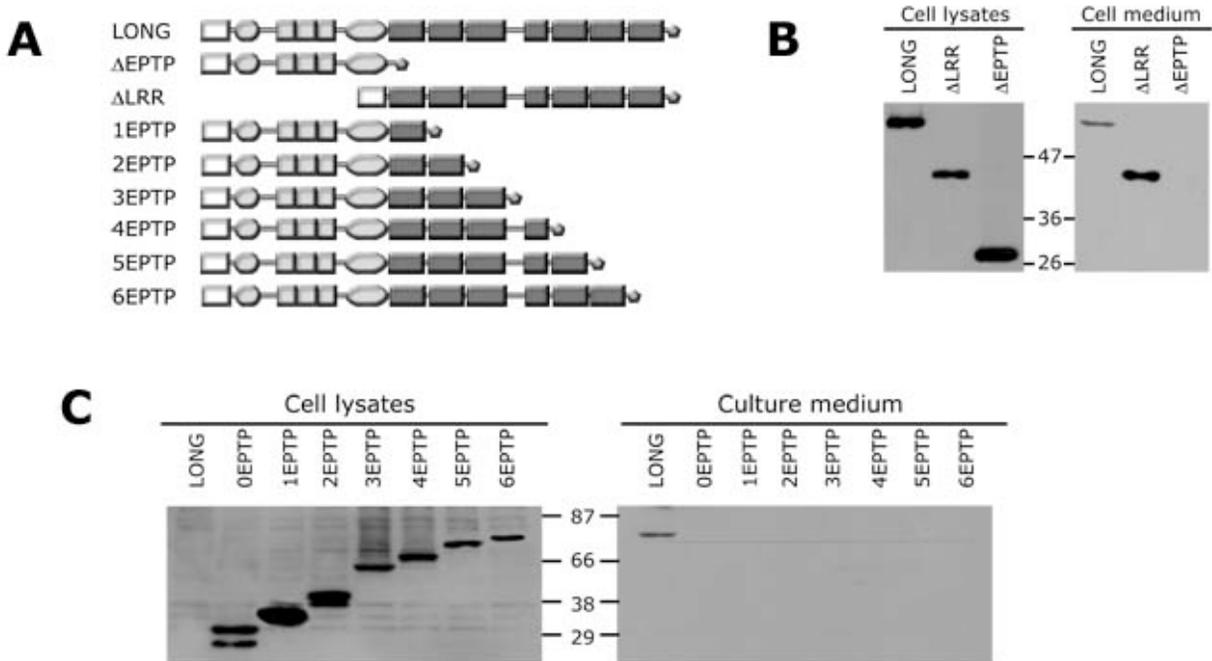
1 **Figure 1**

1 **Figure 2**

2

3

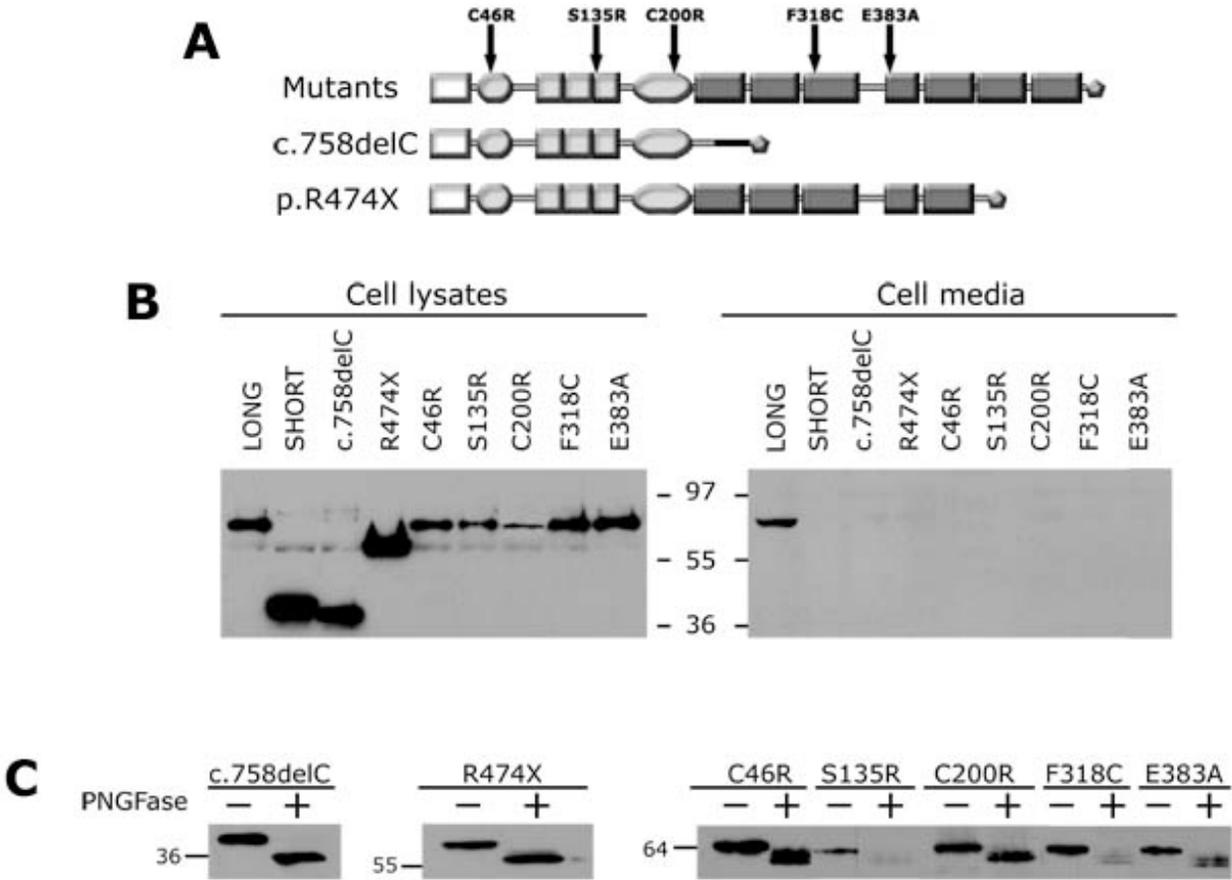
1 **Figure 3**

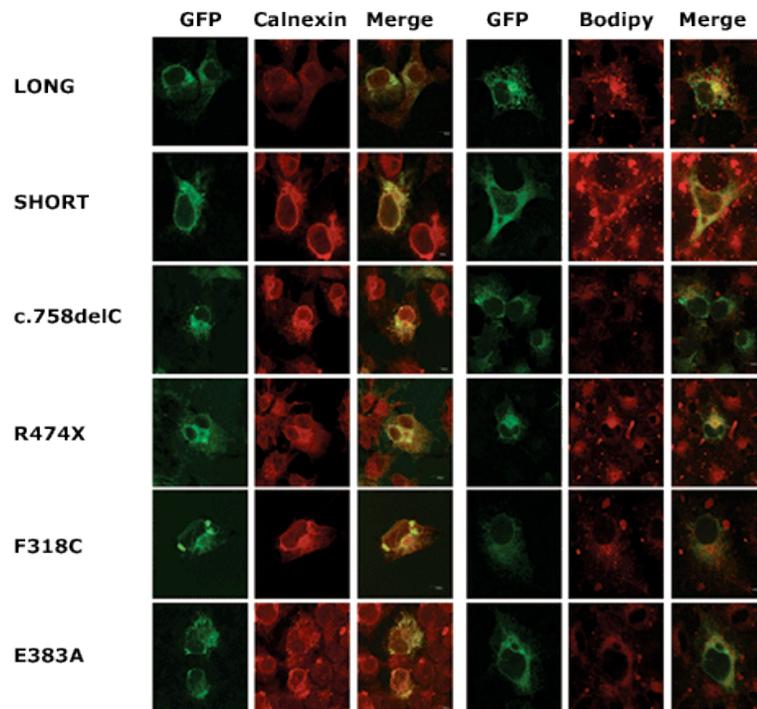


2

3

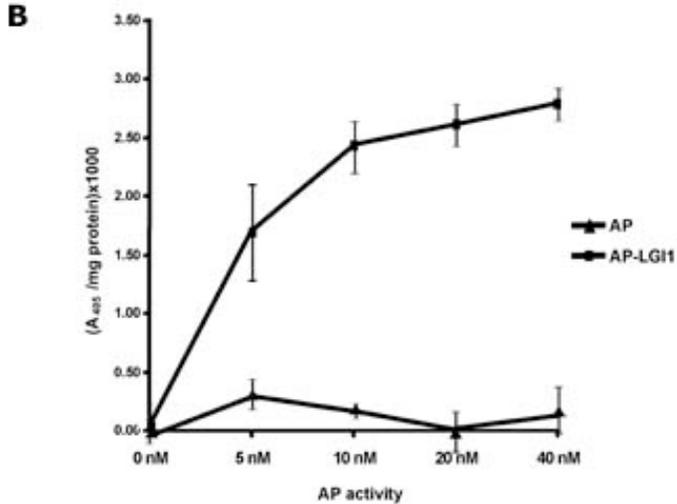
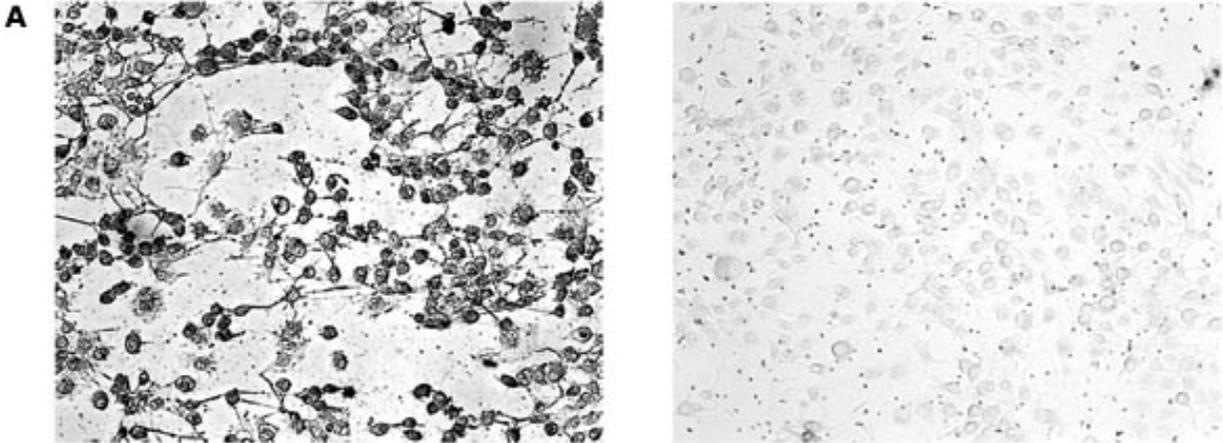
1 **Figure 4**



1 **Figure 5**

2

1 **Figure 6**



2

3

1 **Figure 7**