The kinase PERK represses translation of the G-protein–coupled receptor LGR5 and receptor tyrosine kinase ERBB3 during ER stress in cancer cells

Yuka Okamoto, Takuya Saito, Yuri Tani, Tamami Toki, Akiko Hasebe, Masaru Koido, and Akihiro Tomida

From Genome Research, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, 3-8-31 Ariake, Koto-ku, Tokyo 135-8550, Japan

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As a branch of the unfolded protein response, protein kinase R-like endoplasmic reticulum kinase (PERK) represses global translation in response to endoplasmic reticulum (ER) stress. This pathophysiological condition is associated with the tumor microenvironment in cancer. Previous findings in our lab have suggested that PERK selectively represses translation of some mRNAs, but this possibility awaits additional investigation. In this study, we show that a stem-cell marker protein, leucine-rich repeat-containing G-protein–coupled receptor 5 (LGR5), is rapidly depleted in colon cancer cells during ER stress, an effect that depended on the PERK-mediated translational repression. Indeed, the PERK inhibition led to the accumulation of premature, underglycosylated forms of LGR5, which were produced only at low levels during proper PERK activation. Unlike the mature LGR5 form, which is constitutively degraded regardless of PERK activation, the underglycosylated LGR5 exhibited a prolonged half-life and accumulated inside the cells without being expressed on the cell surface. We also found that Erb-B2 receptor tyrosine kinase 3 (ERBB3) is subjected to a similarly-regulated depletion by PERK, whereas the epidermal growth factor receptor (EGFR), stress-inducible heat-shock protein family A (Hsp70) member 5 (HSPA5), and anterior gradient 2 protein-disulfide isomerase family member (AGR2) were relatively insensitive to the PERK-mediated repression of translation. These results indicate that LGR5 and ERBB3 are targets for PERK-mediated translational repression during ER stress.

Cancer cells in tumor tissues are often exposed to conditions of nutrient deprivation and/or low levels of oxygen, which are associated with inadequate vasculatization. These stress conditions may lead to the accumulation of misfolded proteins, resulting in endoplasmic reticulum (ER) stress. In response to ER stress, cells activate survival responses, such as the UPR. The UPR comprises a complex network of processes, including both transcriptional reprogramming (mediated by multiple transcription factors) and translational reprogramming (1, 2). This signaling pathway is initiated through the activation of ER-localized transmembrane signal transducers, including protein kinase R-like ER kinase (PERK), inositol-requiring 1 (IRE1), and activating transcription factor (ATF) 6 (1–3). Activated PERK phosphorylates eukaryotic translation initiation factor 2α (eIF2α), leading to global translational repression. Conversely, it promotes the translation of specific mRNAs with an upstream ORF (uORF), including ATF4 (4–7). Meanwhile, IRE1 mediates the splicing of X-box–binding protein 1 (XBP1) mRNA to produce transcriptionally-active XBP1-s (8), whereas ATF6 is translocated to the Golgi apparatus and converted to its active form by proteolytic cleavage (9, 10). As downstream transcription factors, ATF4, XBP1-s, and ATF6 coordinately induce numerous UPR-associated genes, which restore ER homeostasis by enhancing ER function and ER-associated degradation (1–3, 11–13).

Leucine-rich repeat-containing G-protein–coupled receptor 5 (LGR5) is a G-protein–coupled receptor that is an established stem cell marker for the adult small intestine, colon, hair follicles, eyes, stomach, and mammary glands (14–18). Notably, several studies have shown a rapid turnover of the LGR5 protein in colorectal cancer cell lines (19, 20). As a membrane glycoprotein, nascent LGR5 protein is subjected to maturation, including glycosylation at multiple sites (19) in the ER and is subsequently trafficked to the plasma membrane. After delivery to the cell surface, mature LGR5 is constitutively internalized by clathrin-mediated endocytosis, followed by degradation via the endosome–lysosome pathway (20).

It was previously reported that expression of LGR5 was reduced under conditions of ER stress (e.g. glucose starvation) (19). However, the mechanisms involved in this reduction are

1 To whom correspondence should be addressed: Genome Research, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, 3-8-31, Ariake, Koto-ku, Tokyo 135-8550, Japan. Tel.: 81-3-3570-0514; Fax: 81-3-3570-0484; E-mail: akihiro.tomida@jfcr.or.jp.

2 The abbreviations used are: ER, endoplasmic reticulum; 2DG, 2-deoxy-D-glucose; TM, tunicamycin; TG, thapsigargin; GSK, GSK2656157; SN, soluble nuclear; Cyt, cytoplasm; MM, microsomal membrane; PARP, poly(ADP-ribbose); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; UPR, unfolded protein response; uORF, upstream open reading frame; PERK, protein kinase R-like endoplasmic reticulum kinase; PNGase F, peptide–N-glycosidase F; Endo H, endoglycosidase H; EGFR, epidermal growth factor receptor; qRT-PCR, quantitative RT-PCR.
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Results

Expression of LGR5 during ER stress

We determined whether the expression of LGR5 was reduced under ER stress conditions in human colorectal cancer cell lines by exposing the cells to the chemical ER stressors hypoglycemia-mimicking reagent 2-deoxy-D-glucose (2DG), N-glycosylation inhibitor tunicamycin (TM), or the sarco/ER Ca\(^{2+}\)-ATPase inhibitor thapsigargin (TG) for 8 h. These chemical stressors successfully activated the UPR in HT29 cells, as assessed by the phosphorylation of PERK (band shift), and the downstream induction of ATF4 (Fig. 1A). The expression levels of the LGR5 protein (~100 kDa) were decreased significantly under these stress conditions, whereas a small amount of LGR5 protein with lower molecular mass (~80 kDa) was generated under 2DG or TM treatment (Fig. 1A). Similarly, the levels of LGR5 protein decreased in KM12 and LoVo cells treated with chemical ER stressors. However, unlike in HT29 cells, the levels of LGR5 with a lower molecular weight in TM-treated LoVo cells were comparable with those of the mature form under normal conditions (Fig. 1, B and C). We further explored the glycosylation status of LGR5 under normal and stress conditions using two endoglycosidases, namely peptide–N–glycosidase F (PNGase F), which removes all forms of N-linked glycosylation, and endoglycosidase H (Endo H), which removes only high-mannose and hybrid types of N-glycans on premature sugar chains (see supporting methods). Under normal conditions, LGR5 protein existed as a mixture of Endo H–resistant and -sensitive proteins, whereas only Endo H–sensitive protein corresponded to the protein generated under TM treatment (Fig. S1). These results indicated that the glycosylation status of LGR5 varied among normal conditions, TM treatment, and 2DG or 2DG treatment. Overall, ER stress was involved in the disappearance of the mature form of LGR5 and the appearance of relatively low levels of underglycosylated LGR5 compared with the levels of the mature form under normal conditions.

We examined the extent of the degradation of mature and underglycosylated LGR5 protein during ER stress. HT29 cells were treated with proteasome inhibitors (MG132 or bortezomib) and lysosome inhibitors (hydroxychloroquine or bafilomycin A) in the presence or absence of 10 μg/ml TM for 4 h. Immunoblotting analysis with the LGR5 antibody is shown. RPS3 was used as an internal control.
whereas unglycosylated LGR5 (~80 kDa) was predominant under TM-stress conditions (Fig. 1D). The expression levels of mature LGR5 were drastically increased by lysosome inhibitors, regardless of TM treatment. In contrast, the levels of unglycosylated LGR5 were hardly affected by the lysosome inhibitors. Meanwhile, the levels of both the mature and unglycosylated forms of LGR5 remained unchanged by proteasome inhibitors, indicating that the proteasome-dependent degradation pathway was less important than the lysosome pathway in the reduction of LGR5 protein expression during ER stress. These results suggested that the mature LGR5 protein was more vulnerable to lysosome-dependent degradation than the underglycosylated form of LGR5.

**Role of PERK in LGR5 down-regulation during ER stress**

We determined whether the activation of UPR signal initiators IRE1, PERK, and ATF6 was directly involved in the LGR5 protein reduction during ER stress by knockdown of these molecules. HT29 cells were transfected with specific siRNAs. After 48 h, the cells were treated with TG for 8 h. The knockdown of PERK specifically increased the levels of the underglycosylated forms of LGR5 remaining unchanged by proteasome inhibitors, indicating that the proteasome-dependent degradation pathway was less important than the lysosome pathway in the reduction of LGR5 protein expression during ER stress. These results suggested that the mature LGR5 protein was more vulnerable to lysosome-dependent degradation than the underglycosylated form of LGR5.

**Figure 2. Accumulation of underglycosylated LGR5 by knockdown of PERK.** Immunoblotting analyses under indicated experimental conditions. A and B, HT29 (A) or LoVo (B) cells were transfected with ON-TARGETplus SMART pool siRNAs for nontargeting control, IRE1, PERK, or ATF6. 48 h after transfection, cells were treated with 300 nM TG for 8 h. C, indicated samples from A and B were re-analyzed with lower concentration gel (7.5%) to distinguish the molecular weight of LGR5 between normal condition and TG treatment. D, siRNA-transfected HT29 cells as in A were treated with 10 μg/ml TM for 8 h. E and F, HT29 (E) or LoVo (F) cells were transfected with ON-TARGETplus SMART pool siRNAs for nontargeting control, PERK, GCN2, or ATF4. 48 h after transfection, cells were treated with 300 nM TG for 8 h. RPS3 was used as an internal control in A–D, and RPL7 was used as an internal control in E and F.

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both HT29 and LoVo cells under stress conditions (Fig. 3, A and B). We further examined whether the activation and inactivation of PERK also affected the mRNA levels of LGR5. Similar to the protein expression, the mRNA expression levels of LGR5 tended to decline following treatment with a stressor, and this effect was attenuated by GSK2656157 (Fig. 3, C and D). However, changes in the mRNA expression levels of LGR5 differed among stressors and cell lines. These results suggested that PERK may also be involved in the regulation of LGR5 mRNA expression. Furthermore, we determined whether the PERK-mediated down-regulation of LGR5 occurred in cells that did not naturally express LGR5, by exogenously expressing LGR5 in human sarcoma HT1080 cells and human embryonic kidney (HEK293) cells. GSK2656157 enhanced the levels of underglycosylated and exogenously-expressed LGR5 during treatment with TG in both cell lines (Fig. 3, E and F). These results indicated that PERK is responsible for regulating the protein expression of LGR5 in both intrinsically LGR5-positive and -negative cell lines.

inverse correlation between the accumulation of underglycosylated LGR5 and PERK-mediated repression of translation

We examined the relationship between the decreased expression of LGR5 protein and the PERK-mediated repression of translation. To this end, we assessed the global translational activity by the cellular incorporation of puromycin in HT29 cells treated with TG in the presence or absence of GSK2656157 for 8 h. In accordance with the expression levels of the underglycosylated LGR5 protein, global translational activity was reduced by treatment with TG alone and was recovered by co-treatment with GSK2656157 (Fig. 4A, B).
Quantitatively, global translational activity was reduced to ∼30% of the basal levels by treatment with TG and was recovered to ∼50% by co-treatment with GSK2656157 (Fig. 4A, right bottom). The levels of underglycosylated LGR5 under treatment with TG were reduced to ∼10% of the levels of mature LGR5 under normal conditions and increased to ∼200% following co-treatment with GSK2656157 (Fig. 4A, right top). Similarly, global translation activity was reduced by treatment with TG and recovered by co-treatment with GSK2656157 in LoVo cells (Fig. 4B). The accumulation of underglycosylated LGR5 was thus inversely correlated with the PERK-dependent repression of translation. Unexpectedly, both the extent of reduction in the expression of the LGR5 protein by treatment with TG alone

Figure 4. Inverse correlation between the accumulation of underglycosylated LGR5 and PERK-dependent repression of translation. Incorporation of puromycin into HT29 (A) or LoVo (B) cells treated with 300 nM TG in the presence or absence of 300 nM GSK2656157 and the expression of LGR5 protein are shown. Immunoblots are shown (left). RPS3 was used as an internal control. The relative expressions of the LGR5 protein (right top) and relative incorporation of puromycin (right bottom) were determined through the quantification of immunoblots using densitometry, with levels under normal conditions set as 100%. The mean and S.D. of three biological replicates are shown.
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and the recovery by co-treatment with GSK2656157 were greater than those observed in global translation activity.

Intracellular localization and prolonged half-life of underglycosylated LGR5 protein

We examined the expression of LGR5 on the cell surface of LoVo cells to further elucidate the regulation of LGR5 protein expression under ER stress conditions. Treatment with TG for 8 h reduced the expression of LGR5 on the cell surface to almost the limit of detection (isotype control). These levels remained undetectable after co-treatment with GSK2656157 (Fig. 5A), indicating that underglycosylated LGR5 was not expressed on the cell surface. Subcellular fractionation under the same experimental conditions revealed that LGR5 was recovered mainly from the microsomal membrane fraction, where PERK and a heat-shock protein family A (Hsp70) member 5 (HSPA5) were also recovered. The observed patterns of LGR5 expression in the microsomal membrane fraction were very similar to those noted in the total lysates (Fig. 5B). Similarly, underglycosylated LGR5 was also recovered from the microsomal membrane fraction in HT29 cells treated with TG and GSK2656157 (Fig. 5C) and in LoVo cells treated with TM and GSK2656157 (Fig. S3A).

We evaluated the stability of underglycosylated LGR5 during ER stress by measuring its half-life in the presence of cycloheximide. To this end, we pre-treated HT29 cells with TG or TM in the presence of GSK2656157 for 10 h prior to the addition of cycloheximide. The half-life of the mature form of LGR5 under normal conditions was ~1 h, as it is known to be degraded rapidly by constitutive internalization after maturation (20). However, the half-life of the underglycosylated forms of LGR5 produced under co-treatment with GSK2656157 and TM or TG was prolonged to ~3 h (Fig. 5D and Fig. S3B). We pre-treated HT29 cells with TM in the presence or absence of GSK2656157 for 4 h to investigate the effect of ER stress alone on the half-life of underglycosylated LGR5. The half-life of mature LGR5 was ~1 h, regardless of GSK2656157. The half-life of the underglycosylated LGR5 was ~2 h under treatment with TM alone and ~3 h under co-treatment with TM and GSK2656157 (Fig. 5E and Fig. S3C). The observed difference in the half-life of underglycosylated LGR5 may be attributed to co-treatment with TM and GSK2656157, which strongly suppressed the generation of mature LGR5 that was weakly synthesized during treatment with TM alone. These data suggested that underglycosylated forms of LGR5 synthesized under conditions of ER stress were not expressed on the cell surface and had a longer half-life than the mature form. Failure in expression on the cell surface due to inadequate glycosylation could have led to circumvention from the degradation pathway to which the mature LGR5 is subjected.

Various effects of PERK-dependent repression of translation on the expression of membrane proteins

HT29 and LoVo cells were treated with TM in the presence or absence of GSK2656157 for 8 h to determine whether the PERK-dependent repression of translation uniformly suppressed the expression of membrane proteins. LGR5 expression and global translation activity declined by TM treatment and recovered by co-treatment with GSK2656157, although the changes in the translation activity seemed to be moderate compared with those observed in TG-treated cells (Fig. 6, A and B, and Fig. S5). The expression levels of the well-established short-lived protein MYC proto-oncogene (Myc) were measured to determine whether the expression pattern of the LGR5 protein was due to its short half-life. Similar to the expression of LGR5, the expression levels of Myc were reduced by treatment with TM alone; however, they were not significantly recovered by co-treatment with GSK2656157. The expression patterns of ERBB3 and CD44 variant 9 (CD44v9) proteins under PERK activation and inactivation were similar to that of the LGR5 protein. In particular, the expression levels of the unglycosylated forms of these proteins were low under treatment with TM alone and increased by co-treatment with GSK2656157. Notably, the expression of CD44v9 was undetectable in LoVo cells under these conditions. In contrast, the expression pattern of another ERBB family protein, epidermal growth factor receptor (EGFR), was distinct from that of LGR5. The amount of underglycosylated EGFR protein under treatment with TM alone was not changed by co-treatment with GSK2656157, indicating that EGFR was constitutively synthesized regardless of PERK activation under ER stress conditions. In addition, there were membrane proteins whose expression levels were increased by TM treatment. The expression levels of the cystine–glutamate antiporter solute carrier family member 7A11 (SLC7A11), HSPA5, and anterior gradient 2 protein-disulfide isomerase family member (AGR2) were increased by TM treatment, and these increases were attenuated by co-treatment with GSK2656157.

ER stress-specific involvement of PERK in the down-regulation of LGR5

Given the involvement of the PERK–eIF2α signaling pathway in the ER stress-induced down-regulation of LGR5, we determined whether LGR5 was also down-regulated under different types of stress conditions that may lead to the activation of another eIF2α kinase, GCN2 (24, 25). We subjected HT29 and LoVo cells to conditions mimicking amino acid starvation using histidinol (a competitive inhibitor of histidinyl-tRNA synthase) or halofuginone (an inhibitor of glutamyl-prolyl-tRNA synthase) (26, 27). The mature form of LGR5 was also depleted under these experimental conditions, together with the activation (phosphorylation) of GCN2 and eIF2α, the subsequent repression of global translation, and the induction of ATF4; however, there was no activation of PERK (Fig. 7, A and B). Furthermore, the stem cell-targeted inhibitor PTC-209 (28) also reduced the expression of the mature LGR5 protein with the activation of GCN2, but not PERK (Fig. 7, A and B). These decreases in the expression of the LGR5 protein were not pre-
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**Figure 5. Intracellular localization and prolonged half-life of underglycosylated LGR5 protein during ER stress.** A, LoVo cells were treated with 300 nM TG for 8 h in the presence or absence of 300 nM GSK2656157 (GSK). The expression of LGR5 on the cell surface was determined using a phycoerythrin-conjugated rat anti-LGR5 mAb. The specific signal was determined using an appropriately-matched isotype control. B, LoVo cells (B) or HT29 cells (C) were treated with 300 nM TG for 8 h in the presence or absence of 300 nM GSK. The cells were subsequently divided into cytoplasm (Cyt), microsomal membrane (MM), and soluble nuclear (SN) fractions. Amounts of lysate equivalent to 1 × 10⁵ cells for the total lysate, Cyt, and MM fractions and 2 × 10⁴ cells for the SN fractions were subjected to immunoblotting analysis. β-Tubulin, calnexin, and PARP were used as representative markers of the Cyt, MM, and SN fractions, respectively. D, HT29 cells were treated with 300 nM TG or 10 μg/ml TM in the presence or absence of 300 nM GSK for 10 h. Cycloheximide was subsequently added to the medium, and cell lysates were collected at the indicated time. The expression of LGR5 under each condition over the time course was quantified by setting the levels in each sample at 0 min as 100%. Means of three biological replicates and S.D. are shown. E, HT29 cells were treated with 10 μg/ml TM for 4 h in the presence or absence of GSK2656157. Cycloheximide was subsequently added to the medium, and cell lysates were collected at the indicated time. The quantification of LGR5 expression was performed as in D. Means of three biological replicates and S.D. are shown. See also Fig. S3.

**Discussion**

We demonstrated that the PERK–eIF2α branch of the UPR played a crucial role in the down-regulation of LGR5 during ER stress. The activation of PERK prevented the accumulation of underglycosylated LGR5 during ER stress, possibly through translation repression. This mechanism, together with the constitutive internalization and degradation of mature LGR5 present on the cell surface, led to the rapid depletion of LGR5. Following the inhibition of PERK during ER stress, the generation of underglycosylated LGR5 increased in accordance with the recovery of global translational activity. In this case, the nascent proteins were retained in the ER rather than being delivered to the cell surface, possibly because the maturation (including glycosylation) of the LGR5 protein was incomplete (Fig. 8).

It seemed that the production of underglycosylated LGR5 was tightly regulated by PERK activation. Indeed, although changes in global translation were modest during TM treatment, the expression levels of underglycosylated LGR5 were very low and drastically increased by the inhibition of PERK. The expression of c-Myc, which is known to be a very short-lived protein, was decreased by TM treatment and remained decreased following the inactivation of PERK. Based on these observations, the pattern of LGR5 expression could not be entirely attributed to its short half-life. We were curious whether this sensitivity to PERK activation also occurred in other membrane proteins, because most membrane proteins are delivered in the ER for post-translational modifications, such as glycosylation. The expression patterns of membrane proteins under PERK activation and inactivation during stress varied depending on the properties of the individual protein. In fact, among the membrane proteins tested, only ERBB3 was highly sensitive to PERK activation.

The underlying mechanisms of constant or preferential synthesis under ER stress conditions depended on the individual proteins. During eIF2α phosphorylation, which inhibits the initiation of translation by inhibiting ternary complex formation, the noncanonical translation of mRNAs utilizing cis-elements within their 5′-UTR (e.g., uORFs or internal ribosome entry sites) preferentially occurs. The observed increase in the expression of HSPA5 is related to this noncanonical translation (29, 30) under the phosphorylation of eIF2α.

Furthermore, the increase in HSPA5 protein expression was also attributable to an increase in mRNA levels, because HSPA5 transcription is drastically activated during ER stress. The increase in the expression of the SLC7A11 protein (a transcriptional target of ATF4) (31) and the AGR2 protein may also reflect an increase in mRNA expression. These mechanisms were enabled because eIF2α-mediated translational repression was alleviated through de-phosphorylation of eIF2α by phosphatases, such as growth arrest and DNA damage-inducible enzymes.
protein 34, which occurred after the phosphorylation of eIF2α during ER stress. Therefore, the strong suppression of the expression of the LGR5 protein at this time point raised the possibility that the synthesis of specific proteins, such as LGR5 and ERBB3, was selectively restricted even under the alleviation of the phosphorylation of eIF2α. Indeed, a recent report provided evidence that the PERK-dependent repressive gene expression program selectively targets proteins undergoing ER-dependent post-translational modification during early and mid-response to ER stress conditions (32). Therefore, it is highly possible that the PERK-mediated repression of translation can be selectively applied to some proteins (e.g., LGR5) that are prone to accumulate in their premature forms during PERK inhibition. The underlying mechanism of this selective repression of translation remains to be determined. However, it is possible that signal sequence-dependent mRNA release from the ER is involved in this process (33).

Cancer cells are often exposed to pathophysiological conditions, including nutrient deprivation and ER stress, as a consequence of inefficient vascularization. The phosphorylation of eIF2α was performed by PERK during ER stress and by GCN2 under conditions of amino acid deprivation (34, 35). The expression of LGR5 also declined quickly under GCN2 activation. Considering the vulnerability of LGR5 expression presented in this study and a previous study (19) under stress conditions, we are reluctant to determine the stemness of tumor cells based on its levels on the cell surface alone. Furthermore, the biological roles of LGR5 in the regulation of tumor cell stemness remain debatable. Several studies have claimed that LGR5 did not contribute to the proliferation of tumor cells in

**Figure 7. ER stress-specific involvement of PERK in the down-regulation of LGR5.** A and B, HT29 (A) or LoVo (B) cells were treated with L-histidinol (His; 2 mM), halofuginone (Hal; 30 nM), or PTC-209 (1 μM) for 6 h. Immunoblots against the indicated antibodies are shown. RPS3 was used as an internal control. C, HT29 cells were treated with L-histidinol (2 mM), halofuginone (30 nM), PTC-209 (PTC; 1 μM), or TG (300 nM) in the presence or absence of the PERK inhibitor GSK (300 nM) for 6 h. Cell lysates were subjected to immunoblotting analysis with indicated antibodies. RPS3 was used as an internal control. D, HT29 cells were deprived of glucose or glutamine for 24 h in the presence of the indicated dose of GSK. Immunoblots against indicated antibodies are shown.
two-dimensional cultures or to the tumorigenicity in vivo (36, 37). Consistent with these reports, in our preliminary examination, the knockdown of LGR5 had no significant effects on the growth in two-dimensional (monolayer) and three-dimensional (sphere) culture of HT29 and LoVo cells (data not shown). In contrast, the ER stress-associated regulation of LGR5 expression may play a significant role in vivo. It has been shown that LGR5 at the plasma membrane binds its R-spondin ligands and modulates Wnt signaling in both tumor and normal tissues (37–42). Hence, the loss of cell-surface expression of LGR5 under stress conditions may modulate the cellular response via the negative regulation of R-spondin signaling mediated by LGR5. Indeed, several recent studies showed that LGR5 expression is negatively regulated in ER-stressed cells in intestinal stem cells, leading to subsequent loss of stemness (43, 44). Thus, the PERK-mediated mechanisms of rapid response of cell-surface LGR5 to ER stress reported in this study may play important roles in the maintenance of normal and malignant tissues by sensing stress conditions and transmitting the response signals.

**Experimental procedures**

**Cell culture**

The human colorectal cancer cell line KM12 was generously provided by the National Cancer Institute (Frederick, MD). The

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![Diagram](image-url)

Figure 8. Schematic depicting the biosynthesis of LGR5 under normal and ER stress conditions. Under normal conditions, LGR5 is glycosylated and folded in the ER after translation and is trafficked to the plasma membrane. Subsequently, the mature LGR5 is constitutively internalized, followed by endosome–lysosome-dependent degradation (top). Under ER stress conditions, PERK-mediated translational repression leads to the inhibition of LGR5 translation. This mechanism, together with the constitutive internalization and degradation of the mature LGR5 pre-existing on the cell surface, leads to the rapid depletion of LGR5 (bottom left). Following the inhibition of PERK under ER stress conditions, the generation of nascent LGR5 polypeptides is restored in concordance with the recovery of global translation activity. However, the maturation steps (including glycosylation) continue to be perturbed independently of PERK, resulting in the accumulation of underglycosylated LGR5 in the ER without expression on the cell surface (bottom right).
human colorectal cancer cell lines HT29 and LoVo, human embryonic kidney cell line HEK293, and human fibrosarcoma cell line HT1080 were purchased from the American Type Culture Collection (Manassas, VA). All cell lines were maintained in RPMI 1640 medium (Wako, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Nichirei Biosciences, Tokyo, Japan) and 100 µg/ml kanamycin (Meiji Seika Pharma Co., Ltd., Tokyo, Japan) at 37 °C in a humidified atmosphere containing 5% carbon dioxide.

**Chemicals and treatments**

Thapsigargin (Wako), tunicamycin (Nacalai Tesque, Tokyo, Japan), bafilomycin A1 (Sigma-Aldrich, Tokyo, Japan), bortezomib (Millennium Pharmaceuticals, Cambridge, MA), GSK2656157 (Selleckchem, Houston, TX), PTC-209 (Selleckchem, Tokyo, Japan), halofuginone (Sigma-Aldrich), and cycloheximide (Sigma-Aldrich) were dissolved in DMSO (Wako). MG132 (Pepchem), bafilomycin A1 (Sigma-Aldrich, Tokyo, Japan), bortezomib (Millennium Pharmaceuticals, Cambridge, MA), and 2-deoxy-D-glucose (Wako) were dissolved in distilled sterile water (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan). These compounds were added to the culture medium with the solvent comprising <0.5% of the total volume of the medium for DMSO or <1% for sterilized water. Cells were seeded onto six-well plates at a density of 5 × 10^5 cells/well for transient transfection using Lipofectamine RNAiMAX (Invitrogen) at a final concentration of 20 nM. For PERK-dependent depletion of LGR5, the following primers were used: 5'-TGAAGGTCGGAGTCAA-3' and 3'-TCAAGATTGTGGCTG-5'. The mRNA expression levels of LGR5 were normalized to those of GAPDH in HT29 and LoVo cells, respectively. Relative expression levels of LGR5 were calculated by setting the expression levels under normal conditions as 100%.

**qRT-PCR**

Total RNA was isolated from cells using the RNeasy mini kit (Qiagen, Hilden, Germany). qRT-PCR was performed on a Chromo4 real-time detection system (Bio-Rad) using a SuperScript® III Platinum® SYBR® Green One-Step qRT-PCR kit (Invitrogen), according to the manufacturer’s instructions. The following primers were used: LGR5-forward, 5'-CTGTTGGCCT-GATGACAATGC-3', and LGR5-reverse, 5'-CAGTGATAGAGTGCTCCTCCTC-3', and ACTNB-forward, 5'-ATTCATACCAAGCTGGA-3', and ACTNB-reverse, 5'-GATGTAGACTCTGATGGA-3'. 

**Knockdown with siRNA**

ON-TARGETplus SMART pool siRNAs against PERK (L-004883-00), GCN2 (L-005314-00), ATF4 (L-005125-00), IRE1 (L-004951-02), and ATF6 (L-009917-00) were purchased from Dharmacon (Lafayette, CO). Silencer® Select siRNAs against PERK (s-18101, s-18102, and s-18103) were purchased from Dharmacon (Lafayette, CO). Silencer SMART pool siRNAs against PERK plus LGR5 were purchased from Dharmacon (Lafayette, CO). Each siRNA was introduced to cells by reverse transfection using Lipofectamine RNAiMAX (Invitrogen) at a final concentration of 20 nM for ON-TARGETplus SMART pool siRNAs or 5 nM for Silencer® Select siRNAs.

**Immunoblotting analysis**

Immunoblotting analysis was performed as described previously (45). Briefly, cells were lysed in 1× SDS sample buffer. Equal amounts of proteins were resolved on a 12% polyacrylamide gel (Cosmo Bio Ltd., Tokyo, Japan) with a 4–20% gradient (unless otherwise described) and transferred to a nitrocellulose membrane (GE Healthcare). The membranes were probed with antibodies, and their specific signals were detected using an enhanced chemiluminescence detection system (PerkinElmer Life Sciences). The following antibodies were purchased from Abcam: rabbit anti-LGR5 (clone EPR3065Y, ab75850), rabbit anti-PERK (ab65142), rabbit anti-GCN2 (phospho-T899, clone EPR2320Y, ab75836), and mouse anti-EIF2S1 (ab5369). The following antibodies were purchased from Cell Signaling Technology (Danvers, MA): rabbit anti-GCN2 (catalog no. 3302); rabbit anti-ribosomal protein L7 (catalog no. 2403, note that this product is discontinued); rabbit anti-ribosomal protein S3 (clone D50G7, catalog no. 9538); rabbit anti-β-tubulin (clone D2N5G, catalog no. 15115); rabbit anti-ATF4 (clone D48B, catalog no. 11815BC); rabbit anti-SLC7A11 (clone D2M7A, catalog no. 12691); rabbit anti-elf2α (phospho-S51, clone D9G8, catalog no. 3398); rabbit anti-Myc (clone D84C12, catalog no. 5605); rabbit anti-ERBB2 (clone 29D8, catalog no. 2165); rabbit anti-EGFR (clone D38B1, catalog no. 4267); rabbit anti-calnexin (clone C5C9, catalog no. 2679); rabbit anti-H2A histone family member X (H2AX) (clone D17A3, catalog no. 7631); and rabbit anti-poly(ADP-ribose) polymerase 1 (PARP) (clone 46D11, catalog no. 9532). The following antibodies were also used: rabbit anti-ATF6 (24169-1-AP; Proteintech Group Inc., Rosemont, IL); mouse anti-puromycin (clone 3RH11, EQ0001, Kerafast Inc., Boston, MA); rat anti-CD44v9 (clone RV3, LKG-M003, Cosmobio); Bafilomycin A1 (Millennium Pharmaceuticals, Cambridge, MA), and 2-deoxy-D-glucose (Wako) were dissolved in distilled sterile water (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan). These compounds were added to the culture medium with the solvent comprising <0.5% of the total volume of the medium for DMSO or <1% for sterilized water. Cells were seeded onto six-well plates at a density of 5 × 10^5 cells/well for transient transfection using Lipofectamine RNAiMAX (Invitrogen) at a final concentration of 20 nM. For PERK-dependent depletion of LGR5, the following primers were used: 5'-TGAAGGTCGGAGTCAA-3' and 3'-TCAAGATTGTGGCTG-5'. The mRNA expression levels of LGR5 were normalized to those of β-actin (ACTNB) and GAPDH in HT29 and LoVo cells, respectively. Relative expression levels of LGR5 were calculated by setting the expression levels under normal conditions as 100%.

**Overexpression of LGR5**

The ORF of LGR5 was amplified from the cDNA product in HT29 cells using KOD-Plus Neo (Toyobo, Osaka, Japan) and cloned into pcDNA3. The following primers were used: 5'-GGTACCGAACACCTCCCCCGCTGTTGTTG-3' and 5'-CCCAAGTTAGGTTTTTTT-3'; and GAPDH-forward, 5'-AAGGGTGAAAGTGCTGATGCA-3', and GAPDH-reverse, 5'-AATGAAGGGGTCATTGAGG-3'. The mRNA expression levels of LGR5 were normalized to those of β-actin (ACTNB) and GAPDH in HT29 and LoVo cells, respectively. Relative expression levels of LGR5 were calculated by setting the expression levels under normal conditions as 100%.

**PERK-dependent depletion of LGR5**

The ORF of LGR5 was amplified from the cDNA product in HT29 cells using KOD-Plus Neo (Toyobo, Osaka, Japan) and cloned into pcDNA. The following primers were used: 5'-GGTACCGAACACCTCCCCCGCTGTTGTTG-3' and 5'-CCCAAGTTAGGTTTTTTT-3'; and GAPDH-forward, 5'-AAGGGTGAAAGTGCTGATGCA-3', and GAPDH-reverse, 5'-AATGAAGGGGTCATTGAGG-3'. The mRNA expression levels of LGR5 were normalized to those of β-actin (ACTNB) and GAPDH in HT29 and LoVo cells, respectively. Relative expression levels of LGR5 were calculated by setting the expression levels under normal conditions as 100%.
PERK-dependent depletion of LGR5

Monitoring translational activity

The cellular translational activity was monitored using a nonradioactive method, as described previously (46, 47). Briefly, after treatment of HT29 or LoVo cells with 300 nM TG in the presence or absence of 300 nM GSK2656157 for 8 h, the cells were further cultured for 30 min in the presence of 1 μg/ml puromycin to allow incorporation. Puromycin incorporation was determined by immunoblotting with an anti-puromycin antibody and densitometric quantification using the ImageJ software (48).

Flow cytometry

The expression of LGR5 on the cell surface was detected through immunolabeling with a phycoerythrin-conjugated rat anti-LGR5 mAb (clone DA03-22H2.8, 130-100 – 849; Miltenyi Biotec, Bergisch-Gladbach, Germany) or the appropriately matched isotype control antibody (555848; Pharamingen), followed by staining with 7-aminomycin D (559925, Pharmingen) to assess cell viability. Multiparameter flow cytometric measurements were performed using a FACSCantoTM flow cytometer (BD Biosciences) in conjunction with the FACSuiteTM software (BD Biosciences). The acquired data were analyzed using the FlowJo software version 10 (Tree Star Inc., Ashland, OR).

Subcellular fractionation

HT29 or LoVo cells were seeded onto 60-mm culture dishes at a density of 1 × 10⁶ cells/well (HT29 cells) or 1.6 × 10⁶ cells/well (LoVo cells) and treated the following day with 300 nM TG or 10 μg/ml TM in the presence or absence of 300 nM GSK2656157 for 8 h. The collected cells were divided into cytoplasm, microsomal membrane, and soluble nuclear fractions using a subcellular protein fractionation kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s instructions.

Half-life analysis

After treatment of HT29 cells with 300 nM TG or 10 μg/ml TM in the presence of 300 nM GSK2656157 for 10 h, cycloheximide was added to the culture medium at a final concentration of 10 μg/ml. The levels of LGR5 protein were measured in the cell lysates collected sequentially at 0 and 30 min and at 1, 2, and 4 h after the addition of cycloheximide. The immunoblots were quantified by densitometry using the ImageJ software (48).

Data reproducibility

The reproducibility of the data presented in the figures was confirmed in at least three independent experiments except for those in Fig. 5, which were performed twice.

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