Inhibiting clathrin-mediated endocytosis of the leucine-rich G protein-coupled receptor-5 diminishes cell fitness

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Joshua C. Snyder‡§1, Lauren K. Rochelle‡, Caroline Ray‡, Thomas F. Pack‡, Cheryl B. Bock‖, Veronica Lubkov‖, H. Kim Lyerly‡, Alan S. Waggone‡∗2, Larry S. Barak†, and Marc G. Caron‡3

From the Departments of Cell Biology, Surgery, and Pharmacology and Cancer Biology and Duke Cancer Institute Transgenic Core, Duke University Medical Center, Durham, North Carolina 27712 and **Department of Biological Sciences and Molecular Biosensor and Imaging Center, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213

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The leucine-rich G protein-coupled receptor-5 (LGR5) is expressed in adult tissue stem cells of many epithelia, and its overexpression is negatively correlated with cancer prognosis. LGR5 potentiates WNT/β-catenin signaling through its unique constitutive internalization property that clears negative regulators of the WNT-receptor complex from the membrane. However, both the mechanism and physiological relevance of LGR5 internalization are unclear. Therefore, a natural product library was screened to discover LGR5 internalization inhibitors and gain mechanistic insight into LGR5 internalization. The plant lignan justicidin B blocked the constitutive internalization of LGR5. Justicidin B is structurally similar to more potent vacuolar-type H+-ATPase inhibitors, which all inhibited LGR5 internalization by blocking clathrin-mediated endocytosis. We then tested the physiological relevance of LGR5 internalization blockade in vivo. A LGR5-rainbow (LBOW) mouse line was engineered to express three different LGR5 isoforms along with unique fluorescent protein lineage reporters in the same mouse. In this manner, the effects of each isoform on cell fate can be simultaneously assessed through simple fluorescent imaging for each lineage reporter. LBOW mice express three different forms of LGR5, a wild-type form that constitutively internalizes and two mutant forms whose internalization properties have been compromised by genetic perturbations within the carboxyl-terminal tail. LBOW was activated in the intestinal epithelium, and a year-long lineage-tracing course revealed that genetic blockade of LGR5 internalization diminished cell fitness. Together these data provide proof-of-concept genetic evidence that blocking the clathrin-mediated endocytosis of LGR5 could be used to pharmacologically control cell behavior.

The dynamic and multimodal signaling properties of GPCRs transduce cell signaling and are essential for a diversity of physiological functions (1, 2). Among the rhodopsin-like family of GPCRs are the glycoprotein hormone receptors. These receptors diverge from other GPCRs due to their large amino-terminal ectodomain, which comprises leucine-rich repeats (3). LGR5 is the founding member of a subfamily of glycoprotein hormone receptors that also includes LGR4 and LGR6 (4, 5). Remarkably, the expression of LGR5 can be used to identify epithelial stem cells of both the small and large intestine. These LGR5-positive stem cells reside at the base of the intestinal crypt, rapidly proliferate, and are capable of producing the entire lineage of differentiated epithelial progeny (6). LGR5-positive stem cells have also been described in a myriad of epithelial tissues (7). In subsequent work, LGR5-positive cells were also shown to function as cancer stem cells in mouse models of intestinal cancer (8, 9). Clinical research findings also support the notion that LGR5 can be used as a prognostic indicator in colon cancer (10). Therefore, LGR5 is a tantalizing drug target for cancer therapy and regenerative medicine.

Several groups have reported that Rspondins stimulate LGR5 to potentiate WNT/β-catenin signaling (11–13). In addition, the extracellular matrix-associated protein Norrin was also described as another endogenous high-affinity ligand that could potentiate WNT/β-catenin signaling (14, 15). However, none of these ligands were able to stimulate canonical GPCR signaling. This includes both G protein coupling and recruitment of the GPCR adaptor protein β-arrestin (16). This is surprising given the fact that LGR5, in addition to its prototypical seven-transmembrane domain, possesses both the discrete

4 The abbreviations used are: GPCR, G protein-coupled receptor; BafA1, bafilomycin A1; LGR, leucine-rich G protein-coupled receptor; IRFAF, infrared fluorochrome-activating protein; HTS, high-throughput screen; NPL, natural product library; vATPase, vacuolar-type H+-ATPase; LBOW, LGR5-rainbow; D2R, dopamine D2 receptor; EGFP, enhanced GFP; bBRET, bystander bioluminescence energy transfer; RLuc, Renilla luciferase; V2R, vasopressin receptor 2; EYFP, enhanced YFP; CAS, Chemical Abstracts Service; PFA, paraformaldehyde; MEM, minimum Eagle’s medium; PGK-Neo, phosphoglycerate kinase-neomycin resistance gene; RFP, red fluorescent protein; ANOVA, analysis of variance; Vil, villin.
“DRY” motif in the second intracellular loop necessary for G protein signaling and a serine cluster in the carboxyl-terminal tail (amino acids 873–875) necessary for β-arrestin recruitment. In fact, LGR5 can recruit β-arrestin when G protein-coupled receptor kinases are overexpressed. As would be expected, this “SSS” cluster at amino acids 873–875 is necessary for this response (17). Therefore, although Rspontins and Norrin can serve as endogenous ligands for LGR4–6, the molecular details of canonical GPCR signaling are likely still incomplete.

Despite the inability to describe canonical GPCR signaling modes for LGR5, it is becoming clear that the trafficking of LGR5 might be one critical feature underlying its signaling (18–20). At steady state, most rhodopsin-like GPCRs (e.g. dopamine D2 receptor (D2R)) are expressed at the plasma membrane and internalized upon ligand binding. Once activated, the receptor is desensitized following phosphorylation by G protein-coupled receptor kinases, recruitment of β-arrestin, and clathrin-mediated endocytosis (16, 21, 22). In contrast, LGR5 is constitutively internalized from the plasma membrane in the absence of a ligand and in a dynamin-dependent manner (23).

Previously, we have shown that in the absence of exogenous ligand, LGR5 constitutively internalizes from the plasma membrane and that this process is likely regulated by phosphorylation at Ser861/Ser864 in the carboxyl-terminal tail (23). We demonstrated that LGR5 constitutively internalizes from the plasma membrane and internalized upon ligand binding. Once activated, the receptor is desensitized following phosphorylation by G protein-coupled receptor kinases, recruitment of β-arrestin, and clathrin-mediated endocytosis (16, 21, 22). In contrast, LGR5 is constitutively internalized from the plasma membrane in the absence of a ligand and in a dynamin-dependent manner (23).

The previously described glucocorticoid derivatives, which modestly increase LGR5 cell surface expression, were among these hits and thereby validate this screen and confirm previous findings (Fig. 1B, yellow) (24). Two plant products were also found to induce a significantly higher increase in LGR5 surface expression compared with all other high true-actives. The aryl-naphthelene lignan 9-(1,3-benzodioxol-5-yl)-6,7-dimethoxy-naphtho[2,3-c]furan-1(3H)-one (justicidin B) isolated from the Justicia genus (28) increased LGR5 surface expression ~7-fold above vehicle (Fig. 1C, green). The alkaloid berbamine (red) isolated from the Berberis genus (29, 30) increased LGR5 surface ~10-fold above vehicle but was later found to be due to a library contaminant. Therefore, we focused on justicidin B.

**Justicidin B treatment increases the cell surface expression of LGR5**

LGR5 trafficking was visualized in living cells to validate IRFAP-HTS and characterize the kinetics of the justicidin B response. Confocal imaging of LGR5-EGFP was performed by acquiring images every 30 min for 15.5 h. We previously demonstrated that LGR5 constitutively internalizes from the plasma membrane and accumulates in the trans-Golgi network (23). Therefore, as expected, LGR5 was found primarily in intracellular vesicles and in a perinuclear distribution immediately after stimulus (Fig. 2A). In contrast, approximately 2 h after justicidin B treatment, appreciable surface expression of LGR5 could be observed (Fig. 2B). The expression surface of LGR5 continued to increase throughout the time course and reached a peak 15.5 h post-treatment (Fig. 2C–E). IRFAP detection was used to quantify this time-course analysis of membrane redistribution in a dose-dependent manner (Fig. 2F). The EC_{50} for justicidin B was ~1–2 μM for each time course (2 h, 0.8 μM; 4 h, 0.6 μM; 6 h, 2.4 μM; and 24 h, 1.7 μM).
These data confirm the findings of the IRFAP-HTS assay and indicate that the effects of justicidin B can be observed as early as 2 h after treatment.

**Justicidin B blocks the constitutive internalization of LGR5**

Justicidin B could act by either increasing the rate of receptor recycling or by directly inhibiting internalization of the receptor. Our live cell imaging for LGR5-EGFP trafficking (Fig. 2) suggests that justicidin B inhibits the internalization of LGR5. To test this hypothesis, we utilized the Mars1-LGR5-EGFP cell line and pulse-labeled the cell-surface pool of LGR5 with the membrane-impermeant SCi1 probe after overnight incubation with justicidin B. Vehicle-treated cells possessed weak SCi1 staining of membrane-localized LGR5 that accumulated in intracellular vesicles (Fig. 3A). As expected, cells treated with either 2 μM justicidin B or 20 μM justicidin B possessed greater SCi1 staining of LGR5 compared with vehicle (Fig. 3B and C). LGR5 internalization was slowed by 2 μM justicidin B treatment and almost completely inhibited by 20 μM justicidin B.

We have shown previously that internalized LGR5 transits through early endosomes in route to its final destination in the trans-Golgi network (23). Thus, to further support our live cell trafficking findings, we next quantified the transit of LGR5 into early endosomes. We adapted a "bystander" bioluminescence energy transfer (bBRET) assay to quantify receptor transit into early endosomes (31). This assay was first tested on two GPCRs whose ligand-dependent internalization properties are well known. The RLuc domain was fused to the carboxyl-terminal tail for the human vasopressin receptor 2 (V2R) or the mouse D2R. Cells were transfected with the donor and the 2xFYVE early endosome sensor and then stimulated with receptor-specific agonists. A dose-response increase in net BRET can be observed for V2R stimulated with arginine vasopressin (Fig. 3D, dashed line) and for D2R stimulated with quinpirole (Fig. 3E, dashed line). Justicidin B partially inhibited V2R and D2R transit to early endosomes (Fig. 3, D and E). As expected, justicidin B also inhibited LGR5 transit into the early endosomes (Fig. 3F). Collectively these data demonstrate that justicidin B inhibits the constitutive internalization of LGR5 and its transit into early endosomes. Justicidin B also inhibits ligand-dependent localization of other GPCRs into early endosomes. These data suggest that justicidin B blocks an early event in receptor internalization or endosomal sorting.
Blockade of vacuolar-type H⁺-ATPases inhibits clathrin-mediated internalization of LGR5

A PubChem search for structural analogues of justicidin B identified diphyllin (justicidin A) (Fig. 4A). Previous reports have demonstrated that diphyllin is a selective vacuolar-type H⁺-ATPases (vATPase) inhibitor and can prevent the normal acidification of endosomes (32, 33). A recognized specific inhibitor of vATPase is bafilomycin A1 (BafA1) (34). In addition to its defined roles for inhibiting endosomal acidification, a recent report demonstrated that prolonged BafA1 treatment can inhibit receptor internalization (35).

Therefore, we hypothesized that, like justicidin B, diphyllin or BafA1 treatment would inhibit the constitutive internalization of LGR5. The results demonstrate that each compound increased the expression of LGR5 at the cell surface (Fig. 4, B and C). BafA1 is the most potent (EC₅₀ = 2.6 nM) and is well below the inhibitory con-
centrations of F$_0$/F$_1$-ATPases (10–100 µM) that are toxic in cells (Fig. 4B, *asterisk*) (34). Diphyllin and justicidin B are far less potent (EC$_{50}$ = 0.2 and 3.0 µM, respectively). Similar to justicidin B, IRFAP live cell trafficking revealed that both BafA1 and diphyllin inhibit LGR5 internalization and result in accumulation of fluorescent punctae at the cell surface (Fig. 4, D and E).

Prolonged BafA1 vATPase inhibition prevents cholesterol recycling and inhibits clathrin-mediated endocytosis (35). We
hypothesized that direct inhibition of clathrin would similarly inhibit constitutive internalization of LGR5. The internalization of LGR5 was blocked by dominant-negative inhibitors of dynamin (36) or clathrin (37–39), indicating that LGR5 internalization is both dynamin- and clathrin-dependent (Fig. 5, A and B). We can reverse the effects of vATPase inhibition on LGR5 internalization by restoring plasma membrane cholesterol levels through simple co-incubation with the cholesterol donor cholesterol-methyl-β-cyclodextrin (Fig. 5, C and D) (35, 40). These data unequivocally demonstrate that the constitutive internalization of LGR5 is dynamin- and clathrin-dependent.

**Genetic blockade of LGR5 internalization in vivo impedes cell fitness**

The physiological relevance of LGR5 internalization is still unclear. Although vATPase inhibitors can be used in vitro as tool compounds to block LGR5 internalization, their pleiotropic effects are not advantageous for specifically studying LGR5 in vivo. For instance, vATPase inhibitors block WNT secretion and thereby inhibit WNT signaling (41). Therefore, an alternative model was needed to more precisely investigate roles for LGR5 internalization in vivo. To accomplish this, we adapted and improved upon a Brainbow expression approach (42, 43) to express multiple forms of LGR5 and simultaneously compare their effects on the stem cell lineage. The proposed LGR5-rainbow (LBOW) construct drives constitutive expression of a membrane orange fluorescent protein in all cells (Fig. 6 A). The cell type-specific expression of a Cre recombinase results in recombination and expression of one of three distinct genetic outcomes (Fig. 6A) including 1) nuclear EYFP and wild-type (WT) LGR5, which constitutively internalizes and traffics to the trans-Golgi network; 2) cytosolic mCherry and 834del LGR5, which has a truncated carboxyl-terminal tail and is unable to internalize; and 3) E2-Crimson and LGR5/V2R-tail in which the carboxyl-terminal tail of LGR5 was exchanged for the V2R carboxyl-terminal tail and is unable to internalize but can

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**Figure 4. Identification of vATPase inhibitors as inhibitors of LGR5 internalization.** A, a PubChem search for structural analogues of justicidin B identified diphyllin (justicidin A). B and C, Mars1-LGR5-EGFP-expressing U2OS cells were stimulated overnight with an eight-point dose response of vATPase inhibitors and justicidin B (B, plate image; C, quantification (an average of three independent experiments)). Mars1-LGR5-EGFP-expressing cells were stimulated overnight with 2 μM BafA1 (D) or 20 μM diphyllin (E). The membrane-impermeant fluorogen SCi1 was added to cells for 1 min to stain the cell surface fraction of LGR5. Cells were tile-imaged for SCi1 (excitation, 633 nm; magenta) and EGFP (excitation, 488 nm; see insets) every 2 min, and a subset of images is shown here (0, 8, 16, and 24 min as annotated). Arrows in B and C depict strong membrane staining at time 0 that only modestly internalized. Imaging was reproduced across multiple independent experiments. Scale bars represent 20 μm. Error bars depict S.E.
recruit βarrestin-2 (17, 23). Each spectrally resolvable fluorescent protein is co-expressed with an LGR5 isoform and can be used as a proxy to visualize their effects on cell behavior.

The bicistronic expression cassettes were first tested in vitro. We chose to compartmentalize the fluorescent proteins to maximally discriminate their signal in the event that their fluorescence was suboptimal. We also utilized a P2A self-cleaving peptide to encode both a fluorescent protein and LGR5 isoform from the same mRNA (44) (Fig. 6B). Importantly, each compartmentalized fluorescent protein and its cognate LGR5 isoform were expressed according to the predicted subcellular localization (Fig. 6, C–E). It was also clear by Western blotting that efficient P2A-mediated self-cleavage had occurred (Fig. 6, C–E).

The LBOW transgene was targeted to the ROSA locus for uniform expression in the mouse (45) (Fig. 7). Germ line founder mice harboring LBOW in the ROSA locus expressed the membrane orange fluorescent protein ubiquitously in all tissues grossly examined (Fig. 8A). In particular, the intestinal epithelium clearly expressed LBOW ubiquitously throughout the epithelium (Fig. 8, A–C). LBOW mice were crossed to mice expressing Cre recombinase under the control of the villin promoter (Vil-Cre) to test Cre recombinase-directed activation of LBOW in the intestinal epithelium and verify that each resultant fate could be observed. Vil-Cre mice initiate recombination uniformly throughout the intestinal epithelium beginning at 12.5 days postcoitus (46).

Fluorescent protein clones for each fate could be observed (Fig. 8, D–F), including mKO1-positive, non-recombined, and non-epithelial cells (pseudocolored blue) and recombined cells that expressed i) nuclear EYFP (Fig. 8, E and F, arrows, pseudocolored yellow), ii) cytosolic mCherry (Fig. 8, E and F, arrowheads, pseudocolored red), and iii) non-compartmentalized E2-Crimson (Fig. 8, E and F, asterisks, pseudocolored red). Antibody staining was required for the best visualization of each lineage reporter. The antibody to mCherry cross-reacts with E2-Crimson; however, the engineered fail-safe that expresses each fluorescent protein reporter in distinct subcellular compartments allowed for discrimination of each protein. Clearly two distinct subcellular localizations of each fluorescent protein can be observed (Fig. 8F, mCherry cytosolic signal with dark nuclei denoted by arrowheads versus E2-Crimson signal with uniform expression throughout the cell denoted by asterisks). Serendipitously, their staining intensity also markedly differed, which enabled discrimination of both lineages by compartmentalization and signal intensity thresholding.

Previous in vitro reports demonstrated that blocking LGR5 internalization potentiates WNT/βcatenin signaling (19). We hypothesized that in vivo this would translate into a competitive advantage for clones expressing forms of LGR5 that are...
unable to internalize. To test this hypothesis, LBOW mice were crossed with ROSA-CreER mice (47) so that sparse recombination could be initiated in the adult intestinal epithelium. In this manner, recombined cells and their clones can then be chased and competed with one another over time. This competition experiment enables the deterministic evaluation for each recombined clone’s fitness, that is the ability to confer a competitive advantage or disadvantage to the stem cell and/or its progeny long term (48). 14 days after tamoxifen activation, small patches of yellow, red, and magenta clones were present throughout the villus and crypt epithelium (Fig. 9, d14, small arrows). 28 days later, short-lived progenitors and their differentiated progeny are lost, resulting in survival of clones formed from recombined stem cells within the crypt. This corresponds to a decrease in small clones at day 28 and an increase in full-length clones from the crypt base to the tip of the villus (Fig. 9, d28, large arrows). These long-lived clones are present 56, 112, 224, and 365 days later (supplemental Movie 1).

Figure 6. An LGR5-rainbow expression system for the simultaneous comparison of multiple LGR5 isoforms. A, schematic of the LGR5-rainbow expression system. The proposed construct promotes ubiquitous membrane mKO1 expression in all cells. Cre recombinase drives elimination of mKO1 and the stochastic expression of one of three fates per cell including 1) a nuclear EYFP (3xNLS, nuclear localization signal; yellow, EYFP) and 3xHA epitope-tagged WT LGR5, 2) cytosolic mCherry (NES, nuclear export signal; red, mCherry) and Myc-tagged truncated LGR5 (834del), and 3) ubiquitous E2-Crimson (violet) and a V5 epitope-tagged chimeric LGR5/V2R. Therefore, in a single tissue from the same mouse, the effects of each receptor on cell fate can be compared by imaging for each spectrally resolvable XFP. A P2A peptide was inserted between the fluorescent protein and LGR5 to enable stoichiometric expression of both the fluorescent protein and LGR5 (44). B, HEK cells were transfected with plasmids for independently testing the cassettes activated in fates 1, 2, and 3. Antibody staining for HA, Myc, and V5 antibody in transfected HEK cells verified that each form of LGR5 is expressed, traffics to the plasma membrane, and is co-expressed with a compartmentalized XFP: HA-WT LGR5 (green) and nuclear EYFP (yellow) (C), Myc-834del LGR5 and cytosolic mCherry (red) (D), V5-LGR5/V2R-tail and ubiquitous E2-Crimson (violet) (E). C–E, right, Western blotting analysis of HEK cells overexpressing each vector demonstrates the correct molecular mass for each form of LGR5 (asterisk; probed for with epitope-specific antibodies) and XFP (arrowhead; probed for with a P2A antibody that recognizes the P2A fragment remaining on the XFP following cleavage).
contract. In this way, one would expect three different outcomes: i) competitive advantage with relative fitness $>1$, ii) no advantage with relative fitness $= 1$, and iii) competitive disadvantage with relative fitness $< 1$. Relative fitness indices demonstrated that EYFP clones (WT LGR5-positive) have a competitive advantage and increase their relative contribution to the total pool of intestinal epithelial cells over time. In contrast, both the mCherry (834del LGR5-positive) and E2-Crimson (LGR5/V2R-positive) clones are at a competitive disadvantage, resulting in a decreased fractional contribution over time (Fig. 10C). Together these data demonstrate that the clathrin-mediated internalization of LGR5 is critical to regulating cell behavior in vivo.

**Discussion**

LGR5 is a tantalizing target for pharmacological control of stem cell behavior. Recent observations suggest that LGR5 and Rspondsins act together to tune WNT/β-catenin signaling by controlling the plasma membrane expression of the WNT receptor negative regulators RNF43/ZNRF3 (49, 50). However, the mechanistic underpinnings of these various interactions still remain to be explored in vivo. This is due in part to LGR5’s unique trafficking program, which remains unclear. The results of the present study clarify the early events in LGR5 internalization and provide in vivo physiological evidence for the importance of this trafficking program.

Using genetic and chemical inhibitors of clathrin, we conclusively demonstrate that the constitutive internalization of LGR5 is both clathrin- and dynamin-dependent. This provides necessary clarity over the early events driving LGR5 internalization. Our data also highlight an important and underappreciated property of vATPase inhibitors, namely that the long-term inhibition of vATPase results in a potent blockade of clathrin-mediated endocytosis (33). LGR5 internalization can be inhibited with a 2-h treatment of justicidin B, but the maximal effect is attained with prolonged treatment. This effect can be reversed by resupplying the plasma membrane with cholesterol as in previous studies (33). Therefore, these data confirm the observations that vATPase activity is essential for proper plasma membrane fluidity and clathrin-mediated endocytosis. These findings also suggest potential new avenues to pursue in LGR5 biology and more broadly all plasma membrane receptors. vATPases can be targeted to many different compartments within the cell, including intracellular vesicles, and the plasma membrane of specialized epithelial cell types (51, 52). The stoichiometry and localization of vATPase appear to be important in cancer (53). Experimental evidence demonstrates that plasma membrane-localized vATPase requires the incorporation of the α3 subunit and that this form is overexpressed in breast cancer cells (54, 55). Therefore, it is possible that aberrant vATPase activity and its subcellular distribution could influence important receptor signaling cascades, including the...
Pharmacological and genetic blockade of LGR5 internalization

Epithelial homeostasis in the intestine is driven by the stochastic and neutral competition of approximately 14 stem cells resident to the crypt base. This mechanism is modeled by “neutral-drift” dynamics in which each stem cell is of similar fitness and thereby has an equal probability of renewal or differentiation. In this manner, intestinal crypts become clonal though an unbiased process (56, 57). Therefore, seemingly small perturbations to cell behavior may bias this process and could drive tumorigenesis (58) or field cancerization (59). Importantly, only a subtle change to any number of processes, like apoptosis and proliferation, or cell signaling itself can provide a selective advantage for one population of cells over another. This “cell-fitness” mechanism is thought to drive tissue development and underlie cancer by allowing for selection of the most fit cells and their progeny (48). The LGR5-rainbow model that we developed allowed us to empirically test the relative “fitness” for three different clones simultaneously. We found that clones expressing isoforms of LGR5 that are unable to internalize (834del or LGR5/V2R) are at a competitive disadvantage and less fit relative to WT-expressing clones. Future work will determine whether changes to proliferation index, differentiation potential, or apoptosis underlie these observations.

Our LGR5-rainbow system provides the means to characterize multiple receptor mutants at the same time, in vivo, and with single cell resolution. This technological advance provides a paradigm for visualizing the confluence of biochemistry and physiology at a previously unattainable level. Previously, in vitro experiments demonstrated that inhibiting LGR5 internalization potentiated WNT/βcatenin signaling (19). Therefore, we were surprised that blocking LGR5 internalization in vivo disadvantaged cell fitness. One reason for this discrepancy may lie in the inherent limitations of the in vitro model systems, which are unable to recapitulate the complexity of cell signaling in vivo. For example, these systems do not take into account the concentrations of effectors (e.g. ZNRF3/RNF43, WNT receptors, and downstream effectors), nor do they account for the stem cell and its microenvironment. Although our LGR5-rainbow model is currently limited to overexpression of LGR5 in traffick of LGR5 and its subsequent signaling in vivo and control of epithelial homeostasis.

Figure 8. LGR5-rainbow mouse: in vivo validation. A, gross fluorescence imaging of transgenic (Tg +) tissues shows fluorescent orange signal in the entire gastrointestinal tract (G.I.), kidney (Ki), lung (Lu), pancreas (Pa), heart (Ht), liver (Li), skin (Sk), and brain (Br). Relative to the brain, the fluorescence in skin, lung, liver, and heart is weaker. Inset, higher power confocal imaging of lung tissue demonstrates that the conducting airways and alveolar compartment are fluorescent. B, 14 mm of small intestinal lumen was imaged for mKO1 at 34X (shown at 3.4X magnification) using a fluorescence dissecting microscope. C, inset 1 from B is shown at 100% (34X magnification) with orange fluorescent intestinal villi. D, LGR5-rainbow mice were crossed to mice expressing Cre recombinase under the control of the villin promoter. Adult mice were sacrificed (>6 weeks of age), imaged, and tiled at 40X for EYFP, mCherry, and E2-Crimson. Small intestine was sectioned with a Vibratome, stained with antibodies for EYFP and mCherry/E2-Crimson, and then tile-imaged by confocal microscopy (mKO1, blue; EYFP, yellow; mCherry, red; and E2-Crimson, red) (scale bar, 400 μm). E, higher magnification view of box in D (scale bar, 40 μm). F, higher magnification view of box in E (scale bar, 40 μm) (arrows, EYFP; arrowheads, mCherry; asterisks, E2-Crimson; #, mKO1).
stem cells and ectopic expression in their progeny, it is able to better model these other complexities. However, we also cannot exclude the possibility that truncating the carboxyl-terminal tail eliminates a key motif for directing its engagement of other unknown signaling effectors independently of its effects on trafficking. In subsequent evolutions of this technology, we can address these shortcomings by incorporating more precise DNA-editing tools and epitope tagging of the endogenous receptor. These experiments will be necessary to definitively characterize LGR5 trafficking in vivo and its role in stem cell signaling.

Our results provide new tool compounds that inhibit LGR5 function and unequivocally confirm that its internalization is both clathrin- and dynamin-dependent. Our proof-of-concept in vivo studies demonstrate that LGR5 internalization is important for regulating epithelial homeostasis. Therefore, small molecule control of LGR5 trafficking may provide a new avenue for driving cell fate. This work provides the rationale for continued high-throughput screening to identify small molecules that could be used to control stem cell fate for regenerative medicine or cancer therapy.

**Experimental procedures**

**Plasmids and chemicals**

Mammalian expression plasmids encoding rat dynamin I K44A (36) and Mars1-LGR5-EGFP (24) were used. The carboxyl-terminal fragment of AP180 was a gift from Dr. Harvey McMahon and was described previously (37, 39). The 2xFYVE-mVenus expression vector was cloned by codon optimizing for expression in mammalian cells using the following primary amino acid sequence from hepatic growth factor-regulated tyrosine kinase substrate (60): SDAMFAAERPADVDAEECHRCRVQFGVMTRKHHCRAGQIFCGKCSSKYST-
IPKFGIEKERVCEPCYEQLNKRKE. A gene block encoding a 2xFYVE domain was synthesized (Integrated DNA Technologies, Inc.) and cloned into pcDNA3.1. mVenus cDNA was cloned in-frame and 3’ to the 2xFYVE domain. bBRET donor plasmids were made by cloning cDNA constructs encoding human V2R and mouse D2R fused in-frame with a carboxy-terminal RlucII or human LGR5 fused in-frame with a carboxy-terminal RlucI. Justicidin B (TimTec, catalogue number ST077116, CAS number 17951-19-8) was dissolved in DMSO to a final concentration of 10 mM. [Arg8]Vasopressin acetate salt (Sigma-Aldrich, catalogue number V9879, CAS number 113-79-1) was dissolved in 0.1% BSA, H2O to a final concentration of 10 mM. Bafilomycin A1 (Santa Cruz Biotechnology, catalogue number sc-201550, CAS number 88899-55-2) was dissolved in DMSO to a final concentration of 1 mM. Dipyillin (Hirt2Lead, catalogue number 5468327, CAS number 22055-22-7) was dissolved in DMSO to a final concentration of 10 mM. Quinpirole hydrochloride (Tocris, catalogue number 1061, CAS number 85798-08-9) was dissolved in H2O to a final concentration of 10 mM.

**IRFAP-HTS**

The IRFAP-HTS was performed on a 384-well plate using a U2OS cell line stably expressing Mars1-LGR5-EGFP as described previously (24). Extensive materials and methods, data analysis procedures, and data mining are provided in supplemental File 1. Briefly, 384-well tissue culture plates were seeded with 5550 Mars1-LGR5-EGFP cells and cultured in 40 μl of cell culture medium. Compounds from the TimTec 720 compound NP were added the next day to a final concentration of 20 μM with 0.4% DMSO and incubated overnight. Cells were fixed with 4% PFA (Sigma, catalogue number P6148) for 8 min at room temperature and then washed one time with PBS. SCi1 was incubated with fixed cells for 15 min, and then unbound SCi1 was diluted with 30 μl of PBS. Plates were scanned on a LI-COR Biosciences Odyssey imaging system (Lincoln, NE), and intensity measurements were exported into Excel. Hits were designated, as presented in supplemental File 1, based on >1.3-fold change over DMSO-treated wells and >3 S.D. away from untreated mean. A proportional Venn diagram was made using the online resource BioVenn (61). For time-course and dose-response analyses, IRFAP-HTS was modified for 96-well or 24-well formats as indicated in the text. Briefly, for 96-well dose-response or time-course analysis, the Mars1-LGR5-EGFP cell line was plated at 15,000 cells/well of a tissue culture treated 96-well plate (Denville Scientific, catalogue number T1096). Drugs were diluted in DMEM, 10% FBS, 10 mM HEPES (Gibco, catalogue number 15630080) at 10× and then added to each well as indicated. For time-course analysis, the cells were fixed in 4% PFA for 5 min, washed with PBS, and stained with SCi1 in PBS (1:5000) for 10 min. Cells were washed one time with PBS and then scanned at 700 nm on the Odyssey imaging system. For dose-response analysis, cells were stained live with SCi1 diluted 1:5000 in MEM without phenol red, 1× GlutaMAX (Gibco, catalogue number 35050061), 10 mM HEPES (complete MEM) for 5 min on ice and then scanned on the Odyssey imaging system at 700 nm. For 24-well experiments, 600,000 U2OS Mars1-LGR5-EGFP cells were plated on a 6-well plate and transfected overnight with 4 μg of DNA with 10 μl of Lipofectamine2000 protocol (ThermoFisher, catalogue number 11668027). The next morning 300,000 transfected cells were plated on tissue culture treated 24-well plates (Corning Costar, catalogue number 3524) and 8 h later exposed to the indicated vATPase inhibitor. The next day cells were stained live with SCi1 diluted 1:5000 in complete MEM for 5 min on ice and then scanned on the Odyssey imaging system at 700 nm.

**Live cell confocal imaging**

Confocal microscopy was performed using a Zeiss LSM 780 microscope (Carl Zeiss Microscopy, Germany) equipped with a motorized live cell incubation stage. Experiments were performed at 5% CO2 and 37 °C in cell culture medium (1× DMEM (Mediatech/Cellgro, catalogue number 10-013-CV), 10% FBS (Sigma, catalogue number F2442), 1× antibiotic-antimycotic (Invitrogen, catalogue number 15240-062)). 200,000 U2OS Mars1-LGR5-EGFP cells were plated on 35-mm glass-bottom dishes (MatTek Corp., Ashland, MA, catalogue number P35G-0-10C) that were coated with 75 μg/ml fibronectin (Millipore, catalogue number FC010-5MG). The next day cells were exposed to the indicated vATPase inhibitor overnight, then stained with SCi1, and imaged. For transient expression experiments, 4 × 10⁶ HEKT cells (ATCC, catalogue number CRL-11268) grown on a 100-mm dish were calcium phosphate-transfected with 10 μg of a Mars1-LGR5-EGFP expression construct as described previously (24). The next day 400,000 cells were plated on fibronectin-coated 35-mm glass-bottom dishes. Cells were exposed to the indicated vATPase inhibitor overnight. SCi1 was diluted to 0.2 μM in cell culture medium, and then 200 μl was added to cells for live confocal imaging. After ~1 min of incubation, time-lapse imaging was performed every 2 min. Imaging was performed using a 40×/1.4-numerical-aperture oil Plan-Apochromat differential interference contrast UV-visible-IR objective (Carl Zeiss Microscopy, catalogue number 420762-9900) and the Definite Focus module. Tiled images (5 × 5) were acquired and stitched together for each time point. Images were imported into IMARIS® V8.0 (Bitplane AG, Switzerland) for analysis and conversion to QuickTime movie format.

**bBRET**

bBRET was performed similarly as described (31). On day 1, 800,000 HEKT cells/well were plated in a 6-well plate, and the next day cells were transfected by calcium phosphate (0.4 μg of bBRET donor, 5.0 μg of bBRET acceptor, and 5.0 μg of empty vector pcDNA3.1). On day 2, cells were trypsinized and plated on a poly-d-lysine (100 μg/ml) (Sigma, catalogue number P0899)-coated clear-bottomed 96-well BRET plate (Corning, catalogue number 3903). On day 3, medium was aspirated, and cells were incubated with vATPase inhibitor or vehicle for 2 h in Hanks’ balanced salt solution (Gibco, catalogue number 14025) at 37 °C and 10% CO2. After 2 h, the indicated ligands were diluted in Hanks’ balanced salt solution and then added to the cells for 30 min. Coelenterazine h (Promega, catalogue number S2011) was added to each well, and the plate was immediately read on a Mithras LB 940 luminometer (Berthold Technologies). Rluc was read for 1 s with an F485 emission filter, and...
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EYFP was read for 1.0 s with an F530 emission filter. Measurements were taken at 0, 5, 10, and 15 min after the addition of coelenterazine. The 15-min reading was used to calculate BRET ratios (Venus/RLuc), and net BRET was determined by normalizing to the −12 dose for each transfection group.

LGR5-rainbow cloning, testing, and targeting

A Gateway-compatible ROSA-targeting construct (supplemental File 2) was generated by PCR amplification of the gateway DEST cassette and then overlap-exchanged into ROSA26 mT/mG (Addgene, catalogue number 177787), thereby replacing the Tomato and GFP transgenes (62). Constructs from Fig. 8 were cloned using a standard molecular biology technique and by PCR or gene block synthesis (Integrated DNA Technologies, Inc.) for EYFP, mCherry (Clontech), and E2-Crimson sequences (Clontech) (63). Each test vector was transfected into HEKT cells on MatTek dishes either for live staining and confoocal imaging or for protein isolation and Western blotting. Primary antibodies used were mouse anti-HA (in-house preparation from hybridoma library (23)), rabbit anti-Myc (Abcam, catalogue number ab9106-100), mouse anti-V5 (Thermo-Fisher, catalogue number R960-25), and rabbit anti-P2A (Abcam, catalogue number ab85607). Live cell imaging was performed as described previously (23). Western blotting was performed, and blots were imaged on an Odyssey imaging system.

An empty LGR5-rainbow vector was synthesized (Thermo-Fisher) that included the plasma membrane mKO1 (MBL International Corp.) and downstream restriction digestion sites for incorporation of each in vitro validated transgenic cassette. Each transgenic cassette was iteratively cloned into the empty LGR5-rainbow vector by restriction cloning. Flanking L1/L2 sites were added to enable Gateway cloning into the ROSA-DEST vector. A final map of the targeted construct is published in supplemental File 2, which includes annotations for signal sequences, epitope tags, and localization signals.

All mouse experiments were performed according to a Duke Institutional Animal Care and Use Committee-approved protocol. The Duke Transgenic Mouse facility performed mouse targeting according to established protocols. The G4 ES cell line (129S6/SvEvTac × C57BL/6Ncr) was used for targeting (64). 1.5 × 10⁷ G4 ES cells (passage 13) were electroporated (Bio-Rad Gene Pulser II; 4-mm gap cuvette, 0.8 ml of cells in electroporation buffer (Specialty Media, catalogue number ES-003-D) with 35 µg of LBOW linearized DNA, 0.250 kV, 0.5 microfarad × 1000, pulsed two times) with 35 µg of LBOW linearized DNA and then plated onto to 10-cm plates with feeder cells. Cells were selected in 250 µg/ml G418 (Gibco, catalogue number 11811-031), and medium was changed every day for 8 days. Clones were picked, trypsinized, and either replated in a 96-well feeder plate well or lysed for PCR analysis of transgene targeting (see Fig. 9 for primer strategy and supplemental File 2 for primer sequences). 18 PCR positive clones were expanded and reconfirmed by PCR. A minimum of two clones were injected into ICR (Envigo) morulae (10–12 ES cells per injection) and cultured overnight. 24 injected embryos were transplanted into the uteruses of pseudopregnant ICR surrogate females. Resultant pups were assessed for transgene integration using PCR analysis described above. Germ line transmission and removal of PGK-Neo occurred by breeding to ROSA26::FLPe mice (129S4/SvJaeSorGt(ROSA)26Sortm1(FLP1)Dym/J; The Jackson Laboratory, stock number 003946) and were assessed by PCR genotyping.

Lineage tracing

LBOW mice were crossed with either Vil-Cre mice (B6.Cg-Tg(Vil1-cre)997Gum/J; The Jackson Laboratory, stock number 004586) or ROSA-CreERT2 mice (B6.129-Gt(ROSA)26Sortm1(cre/ERT2)Tyj/J; The Jackson Laboratory, stock number 008463). 200 mg/kg tamoxifen (Sigma, catalogue number T5648) dissolved in corn oil (Sigma, catalogue number C8267) was given via intraperitoneal injection every other day for three injections total. At sacrifice, a surgical plane was induced via intraperitoneal injection of ketamine/xylazine (100 and 10 mg/kg, respectively, in saline). Mice were perfusion-fixed with freshly made 4% PFA. The entire intestine was removed and fixed overnight in 4% PFA at 4°C and then stored in PBS.

Fixed tissue was embedded in 4% agar and then sectioned in 100–150-µm slices with a Vibratome. Intestinal slices were fixed and permeabilized in FISHX comprising fish gelatin extract (Rockland, catalogue number MB-067-0100) and 0.2% Triton-X-100 for 30 min at room temperature. Primary antibodies to EYFP (chicken anti-EGFP, ThermoFisher, catalogue number A-11122) and mCherry/E2-Crimson (rabbit anti-RFP; Rockland, catalogue number 600-401-379) were incubated overnight in FISHX overnight at 4°C. Sections were washed in PBS and reblocked in FISHX + 5% normal goat serum for 30 min. The following secondary antibodies were incubated for 1.5 h in FISHX + 5% normal goat serum: goat anti-chicken 488 (ThermoFisher, catalogue number A11039) and goat anti-rabbit 594 (ThermoFisher, catalogue number A11005). Sections were mounted on glass slides (VWR, 48311-703) with glass coverslips (VWR, catalogue number 48393-194) and Vectashield (Vector Laboratories, catalogue number H-1000). Tiled images at 40× were acquired on a Zeiss LSM 780 microscope using 488 and 568 nm laser lines and 34-channel spectral array detectors. Images were stitched and then exported into IMARIS. Before surface-rendering, the background was eliminated from each recombined channel. This was accomplished by iterative subtraction of the non-recombined control channel (orange) and the channels for the other recombined cell fates. Channels were surface-rendered using the surface function in IMARIS. Clear demarcation of the cytosolic mCherry signal intensity and ubiquitous E2-Crimson signal intensity was observed despite staining with the same antibody. Therefore, a simple threshold of high-intensity clones and low-intensity RFP clones was used to demarcate mCherry clones from E2-Crimson clones and was validated by each reporter’s unique subcellular distribution. The surface area for each clone was exported into Excel as described in the text.

Software

Statistical analysis was performed in Prism® version 6.0h (GraphPad Software Inc., La Jolla, CA) as indicated in the text.
The chemical structures for justicidin B and diphyllin were drawn in ChemDraw Professional (PerkinElmer Life Sciences). Tiled images were acquired using Zen Black (Carl Zeiss Microscopy), exported as a ZVI file, and imported into IMARIS V8.1.2x64 for surface rendering and data analysis.

Author contributions—J. C. S. designed and performed experiments, analyzed data, and wrote the manuscript. L. K. R. designed and performed experiments and analyzed data. T. F. P. developed the bRET donor vectors and performed experiments. C. B. B. generated targeted ES cell clones and chimeric mice. A. S. W. synthesized bBRET donor vectors and performed experiments. C. R. provided SCi1 fluorogen and provided technical support. H. K. L. provided collaborative discussions and experimental insight. L. S. B. and M. G. C. designed experiments, provided equipment, and edited the manuscript.

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