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Growth hormone receptor targeting to lipid rafts requires extracellular subdomain 2

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ABSTRACT

GH receptor (GHR) is a single membrane-spanning glycoprotein dimer that binds GH in its extracellular domain (ECD). GH activates the GHR intracellular domain (ICD)-associated tyrosine kinase, JAK2, which causes intracellular signaling. We previously found that plasma membrane (PM)-associated GHR was dramatically enriched in the lipid raft (LR) component of the membrane and that localization of GHR within PM regions may regulate GH signaling by influencing the profile of pathway activation. In this study, we examined determinants of LR localization of the GHR using a reconstitution system which lacks endogenous JAK2 and GHR. By non-detergent extraction and multistep fractionation, we found that GHR was highly enriched in the LR fraction independent of JAK2 expression. Various GHR mutants were examined in transfectants harboring JAK2. LR concentration was observed for a GHR in which the native transmembrane domain (TMD) is replaced by that of the unrelated LDL receptor and for a GHR that lacks its ICD. Thus, LR association requires neither the TMD nor the ICD. Similarly, a GHR that lacks the ECD, except for the membrane-proximal ECD stem region, was only minimally LR-concentrated. Mutants with internal stem deletions in the context of the full-length receptor were LR-concentrated similar to the wild-type. A GHR lacking ECD subdomain 1 reached the PM and was LR-concentrated, while one lacking ECD subdomain 2, also reached the PM, but was not LR-concentrated. These data suggest LR targeting resides in ECD subdomain 2, a region relatively uninvolved in GH binding.

Introduction

The receptor for GH (GHR) is a glycoprotein displayed on the surface of many cell types. Plasma membrane (PM) GHR possesses a large (~245 residue), heavily glycosylated extracellular domain (ECD), a single transmembrane domain (TMD), and an intracellular domain (ICD) of roughly 350 residues [1]. An initial step in GH signaling is thought to be activation of the cytoplasmic tyrosine kinase, JAK2, which associates noncovalently with the cell surface GHR via Box 1, a short proline-rich segment in GHR's proximal ICD [2–4]. In most systems, GH induces tyrosine phosphorylation and nuclear translocation of signal transducer and activator of transcription 5 (STAT5) to regulate important GH target genes [5,6]. GH-induced STAT5 activation requires GHR–JAK2 association and the presence of tyrosine residues distributed in the GHR ICD [7–9]. GH can also activate the ERK 1/2 pathway in many cell types [10,11]. In contrast to STAT5 signaling, neither distal ICD regions nor tyrosine phosphorylation of GHR are required for GH-induced ERK activity.

Our studies suggest GH's capacity to activate ERKs relative to STAT5 relates to whether GHR/JAK2 activation emanates from lipid raft (LR) microdomains within the PM [12]. LR are PM microdomains enriched in glycosphingolipids and cholesterol and characterized biochemically as relatively detergent-resistant and with low buoyant density compared to bulk PM. LR have multiple functions, including endocytosis, transcytosis, cholesterol transport, and signal transduction [13]. Various signaling proteins are concentrated within these liquid-ordered microdomains, including glycosylphosphatidylinositol (GPI)-anchored and transmembrane receptors, enzymes associated with the inner plasma membrane leaflet, and cytosolic scaffolding proteins [14]. We estimated that in mouse 3T3-F442A preadipocytes roughly one-third of

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PM-associated GHR resides within the LR fraction; however, as LR membrane protein accounts for only a small fraction of total PM protein, GHR is highly LR-concentrated [12]. GH-induced ERK signaling is nearly exclusively from LR and dramatically reduced by cholesterol depletion; in contrast, GH-induced STAT5 signaling is mainly in the non-membraneous fraction and unaffected by cholesterol depletion [12].

Little is known about the mechanisms and determinants that govern LR segregation of PM-associated receptors. Herein, we assess GHR regions required for preferential LR association. Our analysis indicates that the GHR's ECD subdomain 2, a region relatively uninvolved in GH binding, is required for LR association.

Materials and methods

Materials. Routine reagents were purchased from Sigma–Aldrich Corp. (St. Louis, MO) unless otherwise noted. Zeocin was purchased from Invitrogen Life Technologies, Inc. (Carlsbad, CA). G418 and hygromycin B were from Mediatech (Herndon, VA). Fetal bovine serum, gentamicin sulfate, penicillin, and streptomycin were purchased from BioFluids (Rockville, MD). Optiprep™ was purchased from Accurate Chemical and Scientific Corp. (Westbury, NY). Percoll was purchased from M.P. Biomedical, Inc.

Antibodies. The rabbit polyclonal antisera, anti-$GHR_{cytAL-47}$ against the GHR ICD, and anti-$JAK2_{AL33}$, have been described [15,16]. Monoclonal anti-Myc (9E10) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The 3F10 anti-HA rat monoclonal antibody was from Roche. Polyclonal anti-caveolin and monoclonal anti-clathrin heavy chain antibodies were from BD Transduction Laboratories and were used for blotting as in [12]. Horseradish peroxidase-conjugated anti-rabbit (1:15,000) or anti-mouse (1:15,000) were from Pierce Chemical Co. (Rockford, IL).

Plasmid construction. The rabbit GHR cDNA was a gift of Dr. W. Wood, Genentech, Inc. The human LDLR expression vector was a gift of Dr. Alan Attie, Univeristy of Wisconsin. Construction of the pcDNA-$GHR_{1-274\text{-Myc-His}}$ has been described [17], as has the construction of pcDNA-GHR_{LDLR} [18], pcDNA-$GHR_{239-620\text{-HA}}$ [19], pcDNA-$GHR_{\Delta 297-406\text{-His}}$ [2,17], and pcDNA-$GHR_{\Delta 240-242\ \Delta 297-406\text{-His}}$ and pcDNA-$GHR_{\Delta 242-244\ \Delta 297-406\text{-His}}$ [17]. pcDNA-$GHR_{\Delta 237-243\ \Delta 297-406\text{-His}}$ was constructed using the ExSite (Stratagene) PCR-based site-directed mutagenesis method using the pcDNA-$GHR_{\Delta 297-406\text{-His}}$ as the template. Similarly, pcDNA-$GHR_{1-274\text{-Myc-His-LDLR}}$ was constructed using ExSite to replace the GHR TMD with that of human LDLR by PCR-based site-directed mutagenesis using pcDNA-$GHR_{1-274\text{-Myc-His}}$ as the template. For construction of pcDNA-$GHR_{124-620}$ and pcDNA-$GHR_{\Delta 128-238}$, PCR mutagenesis was performed using pcDNA-GHR as a template. Sequences for the mutagenic oligonucleotides are available upon request. The entire protein coding sequence of each selected mutant cDNA was subjected to dideoxy DNA sequencing (UAB Genetics Core Facility), which verified the desired mutations and the absence of unwanted mutations.

Cells, cell culture, and transfection. γ2A is a JAK2-deficient human fibrosarcoma cell line kindly provided by Dr. G. Stark (Cleveland Clinic Foundation, Cleveland, OH) [20]. A stable γ2A cell line expressing rabbit GHR (γ2A-GHR) and its cell culture conditions have been described [15]. A stable γ2A cell line expressing rabbit GHR and mouse JAK2 (γ2A-GHR–JAK2; C14) has been described [4]. Stable cell lines expressing mutant GHRs in the context of JAK2 were prepared by cotransfecting the plasmids above that encode GHR_{LDLR}, $GHR_{1-274\text{-Myc-His}}$, $GHR_{239-620\text{-HA}}$, $GHR_{\Delta 297-406}$, $GHR_{\Delta 240-242\ \Delta 297-406\text{-His}}$, $GHR_{\Delta 242-244\ \Delta 297-406\text{-His}}$, $GHR_{\Delta 237-243\ \Delta 297-406\text{-His}}$, $GHR_{124-620}$, or $GHR_{\Delta 128-238}$, along with an empty vector carrying the hygromycin resistance marker and selecting in G418, Zeocin and hygromycin, as previously described [15]. Expression of mutant GHRs was screened for by blotting with anti-$GHR_{cytAL-47}$, anti-HA, or anti-Myc, as indicated. Both transient and stable transfections were performed using Lipofectamine Plus (Invitrogen Life Technologies, Inc.) according to the manufacturer's instructions.

Protein extraction, fractionation, immunoprecipitation, electrophoresis, immunoblotting, cell surface biotinylation, and enzymatic deglycosylation. Serum starvation of all cell lines was accomplished by substitution of 0.25% (wt/vol) BSA (fraction V; Roche) for serum in their respective culture media for 16 h before experiments. Prior to harvesting, cells were washed twice with ice-cold PBS in the presence of 0.4 mM sodium orthovanadate (PBS–vanadate). Isolation of subcellular fractions was performed according to the method of Smart et al. [21] and as reported in detail previously [12]. For immunoprecipitation, cells were solubilized in Triton X-100-containing lysis buffer and previously published procedures were followed [15]. Fractionated proteins or eluates from immunoprecipitation were resolved by SDS–PAGE and immunoblotted as previously described [12]. Immunoblotting detection reagents (SuperSignal West Pico chemiluminescent substrate) are from Pierce Chemical Co. Stripping and reprobing of blots was accomplished according to the manufacturer's suggestions. All blots shown are representative of at least two independent experiments. Surface biotinylation was achieved using Sulpho-NHS-LC-biotin, according to the manufacturer's protocol (Pierce) and our previous methods [22]. Enzymatic deglycosylation was achieved as reported previously [23].

Densitometric analysis. Densitometric quantitation of immunoblots was performed using a high resolution scanner and the ImageJ 1.30 program (developed by W.S. Rasband, Research Services Branch, National Institute of Mental Health, National Institutes of Health, Bethesda, MD).

Results and discussion

JAK2 is not required for GHR raft localization

We previously used non-detergent extraction and a multistep fractionation scheme [21] that exploits low density characteristics of the cholesterol-rich LR (and/or caveolae) membrane to allow their isolation (Fig. 1 of Ref. [12]). Using this method, we demonstrated that GHR endogenously expressed in mouse 3T3-F442A preadipocytes and in human IM-9 lymphoblasts exhibits dramatic concentration in the LR fraction of the PM (~7-fold enriched in LR in 3T3-F442A). Notably, although to a lesser degree than GHR, JAK2

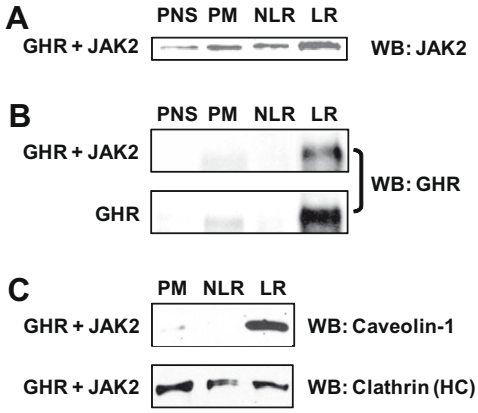

Fig. 1. JAK2 is not required for GHR raft localization. (A–C) Serum-starved γ2A-GHR–JAK2 (GHR + JAK2; A–C) or γ2A-GHR (GHR; B) cells were subjected to subcellular fractionation, as in Methods. Equal amounts of protein in fraction (PNS, PM, NLR, LR) were separated by SDS–PAGE and immunoblotted with anti-JAK2 (A), anti-$GHR_{cyt\text{-}AL47}$ (B), anti-caveolin-1 (C), or anti-clathrin heavy chain (C).

is also enriched in the LR fraction of cells in which it is endogenously expressed (~2.5-fold in 3T3-F442A cells, for example) [12].

Using our human γ2A fibrosarcoma cell reconstitution system [4,22], we studied the effects of JAK2 and GHR mutations on GHR LR association. γ2A cells lack JAK2 and can be reconstituted with GHR or mutants thereof either in the presence or absence of JAK2. Equal amounts of protein from the PM, NLR, and LR fractions were resolved by SDS–PAGE and immunoblotted. γ2A cells expressing JAK2 manifested LR concentration of JAK2 (Fig. 1A), although the concentration of exogenously-expressed JAK2 in rafts appears less than that observed in endogenous JAK2-expressing cells. We next examined GHR distribution in γ2A-GHR cells compared to γ2A-GHR–JAK2 cells (Fig. 1B, "GHR" and "GHR + JAK2", respectively). GHR expressed in γ2A cells was highly enriched (~9-fold) in LR vs. PM, independent of JAK2 coexpression. As expected [12], caveolin-1 (a known raft component) was dramatically enriched in the LR fraction while clathrin heavy chain was not enriched. Thus, GHR is similarly LR-concentrated in reconstituted human fibrosarcoma cells as in murine preadipocytes and human lymphoblasts and JAK2 expression is not required for GHR concentration in the LR fraction.

The GHR ICD is dispensable for the receptor's concentration in LR

Parameters dictating LR association are largely unknown. Few mapping studies have been reported. Influenza neuraminidase's association with LR localizes to its TMD and nearby ICD residues [24] and the TMDs of several other surface proteins have also been implicated. These include the T-cell receptor ζ chain [25], CD40 [26], CD44 [27], and latent membrane protein-1 (LMP-1) [28]. For the transmembrane tyrosine kinase receptor, epidermal growth factor receptor (EGFR), a 60-residue juxtamembrane ECD region is sufficient for LR localization [29]. Similarly, an ECD region in the adhesion protein CD2 has been implicated in LR association [30]. ICD regions have also been implicated, as with the T-cell coreceptor CD4 [31] and the AC5 adenylyl cyclase isoform [32]. Although other cytokine receptors in addition to GHR are LR-associated [33–36], regions within these receptors that mediate LR disposition have yet to be mapped.

As JAK2 is not required for GHR's LR localization, we next asked which receptor region – the ECD, TMD, or ICD – most contributes to this localization (Fig. 2). γ2A-JAK2 cells expressing wild-type GHR or mutant receptors were isolated and subjected to fractionation. In GHR_{LDLR} [18], the native GHR TMD is replaced by that of the human LDL receptor. Replacement with the LDLR TMD did not alter LR enrichment (8.9-fold for GHR_{LDLR}). $GHR_{1-274\text{-}Myc\text{-}His}$ has an intact ECD and TMD, but lacks all but four ICD residues [17]. As this mutant is not detectable by our anti-GHR ICD serum, we used anti-Myc for immunoblotting. Despite lacking the ICD, $GHR_{1-274\text{-}Myc\text{-}His}$ achieved substantial (8.6-fold) LR enrichment, suggesting the ICD is not required for LR localization.

$GHR_{1-274\text{-}Myc\text{-}His\text{-}LDLR}$ is equivalent to $GHR_{1-274\text{-}Myc\text{-}His}$, but has the LDLR TMD. Again, this truncated chimera localized to the LR (8.1-fold). These data strongly suggest that the GHR ECD is required for raft association. To test this, we expressed a GHR mutant lacking the majority of the ECD. $GHR_{239-620}$ contains eight ECD juxtamembraneous stem region residues, the TMD, and the ICD, and corresponds to the GHR remnant that results upon inducible metalloprotease-mediated GHR proteolysis [17]. Notably, $GHR_{239-620}$ present in the PM fraction was only minimally LR-concentrated (2.6-fold). While these data do not rule out a role for the ICD (perhaps particularly in the absence of the ECD), deletion of nearly the entire ECD has substantially greater detrimental impact on GHR LR accumulation than does deletion of the ICD.

An intact GHR ECD stem is not required for LR accumulation

The GHR ECD (residues 1–246) is comprised of three main regions. Subdomains 1 and 2 (residues 1–123 and 128–238, respectively) are "β sandwiches", each incorporating seven β strands in two antiparallel β sheets [37]. These subdomains are connected by a 4 residue hinge region. In contrast to subdomains 1 and 2 and the hinge, little is known about the stem region (residues 238–246) of the ECD that links subdomain 2 to the TMD; the stem was not included in crystallographic studies [37]. $GHR_{239-620}$, which retains the stem region, was only slightly enriched in LR. To determine whether the stem is required for LR localization in the context of an otherwise normal ECD, we examined GHR mutants with internal deletions of stem residues (Fig. 3). These were evaluated in the setting of a GHR with internal deletion of residues 297–406 within the ICD (termed $GHR_{\Delta 297-406}$), which we previously characterized [2,15,17,38]. $GHR_{\Delta 297-406}$ is highly expressed at the cell surface, binds GH normally, and allows GH-induced JAK2 activation. When expressed in γ2A-JAK2 cells, $GHR_{\Delta 297-406}$ was highly LR-concentrated. $GHR_{\Delta 240-242\ \Delta 297-406}$, $GHR_{\Delta 242-244\ \Delta 297-406}$, and $GHR_{\Delta 237-243\ \Delta 297-406}$, lack 3, 3, and 7 residues, respectively, from within the stem region. Despite this, each appears at the cell surface and is engaged by GH ([17,38] and data not shown). These deletion mutants were evaluated by fractionation in the same reconstitution system. Each stem mutant concentrated in LR to a very similar degree to that seen for

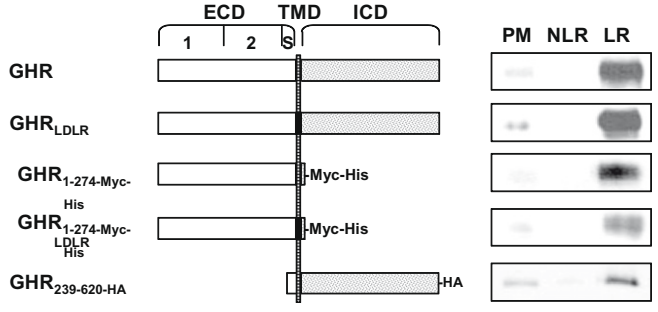

Fig. 2. GHR ICD is dispensable for raft GHR concentration. γ2A-JAK2 cells stably expressing wild-type GHR, GHR_{LDLR}, $GHR_{1-274\text{-}Myc\text{-}His}$, or $GHR_{239-620\text{-}HA}$ or transiently transfected with $GHR_{1-274\text{-}Myc\text{-}His\text{-}LDLR}$ were serum-starved and subjected to fractionation, SDS–PAGE, and blotting with anti-$GHR_{cyt\text{-}AL47}$ (GHR and GHR_{LDLR}), anti-Myc ($GHR_{1-274\text{-}Myc\text{-}His}$ and $GHR_{1-274\text{-}Myc\text{-}His\text{-}LDLR}$), or anti-HA ($GHR_{239-620\text{-}HA}$).

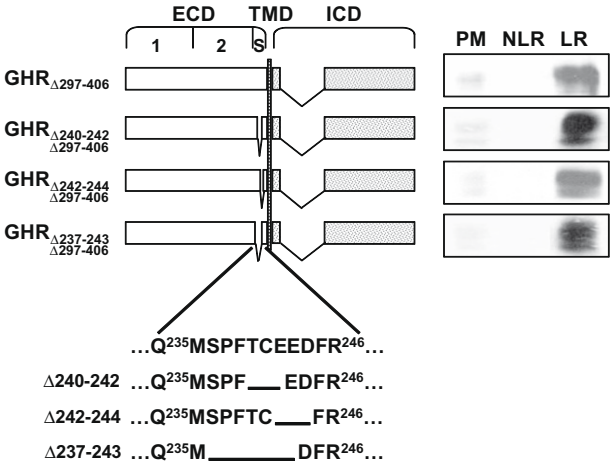

Fig. 3. An intact GHR ECD stem is not required for raft accumulation. γ2A-JAK2 cells stably expressing $GHR_{\Delta 297-406}$, $GHR_{\Delta 240-242\ \Delta 297-406}$, $GHR_{\Delta 242-244\ \Delta 297-406}$, or $GHR_{\Delta 237-243\ \Delta 297-406}$ were serum-starved and subjected to fractionation, SDS–PAGE, and blotting with anti-$GHR_{cyt\text{-}AL47}$.

$GHR_{\Delta 297-406}$. We conclude that the juxtamembraneous ECD stem region is not required for efficient targeting of GHR to the LR fraction of the PM.

Deletion of GHR ECD subdomain 2, but not subdomain 1, markedly impairs LR concentration

As neither association with JAK2 nor the presence of the receptor's ICD, TMD, or ECD stem region is required, we infer that the determinant(s) for LR localization resides in subdomain 1 and/or 2 of the ECD. Subdomain 1 and the hinge contain nearly all GHR residues critical for GH binding [37] and one of the three ECD *N*-glycosylation sites [39]. Unlike subdomain 1, subdomain 2 (with the exception of W-169) is largely unassociated with GH binding. Rather, subdomain 2 harbors the "dimerization interface" that mediates GHR dimerization and the F′–G′ loop that undergoes ligand-induced conformational change [18,40,41]. We prepared two mutants, $GHR_{124-620}$ and $GHR_{\Delta 128-238}$, in which subdomains 1 and 2, respectively, are deleted. To address potential disruptions in receptor structure, maturation, and surface presentation, we first analyzed whether each mutant, expressed in γ2A-JAK2 cells, was detected by GHR antibodies and exhibited expected glycosylation (Fig. 4A). Receptors were immunoprecipitated with a monoclonal antibody to the GHR ICD and precipitates were treated with endoglycosidase-H. Endo-H-resistant (mature) GHR does not change migration in SDS–PAGE, whereas endo-H-sensitive (precursor) GHR's mobility is enhanced [23]. For control, we treated with a combination of *N*-glycosidase F and Neuraminidase (F/N) to remove all carbohydrate chains [23]. A substantial fraction of $GHR_{124-620}$ (which retains two *N*-glycosylation sites) achieved maturity (endo-H-resistance) and thus likely resided on the cell surface when expressed in γ2A-JAK2 cells. $GHR_{\Delta 128-238}$, albeit to a lesser extent, also achieved endo-H-resistance. Surface biotinylation of cells expressing $GHR_{\Delta 128-238}$ vs. WT GHR (Fig. 4B) verified that the mutant reached the PM and comigrated with the endo-H-resistant form. When cells expressing either $GHR_{124-620}$ or $GHR_{\Delta 128-238}$ were subjected to fractionation (Fig. 4C), the mature forms of each were detected in the PM fraction. $GHR_{124-620}$ exhibited LR accumulation similar to that of WT GHR. In contrast, $GHR_{\Delta 128-238}$ was not concentrated in the LR. Thus, despite undergoing maturation and appearing at the cell surface, mature $GHR_{\Delta 128-238}$ present in the PM was quite deficient in localizing to LR. These data suggest that a LR-targeting determinant(s) resides in subdomain 2 and not subdomain 1.

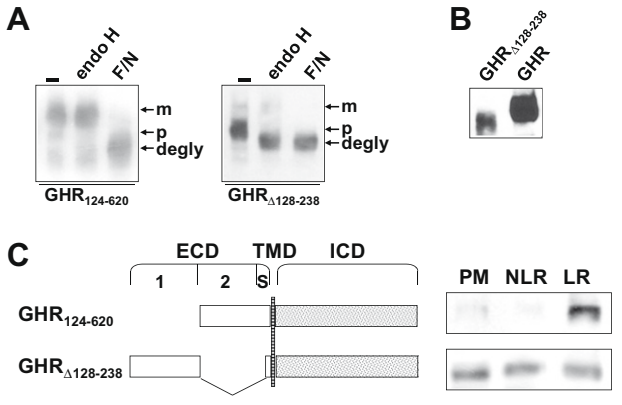

Fig. 4. Deletion of GHR ECD subdomain 2, but not subdomain 1, markedly impairs GHR raft concentration. (A, B) Characterization of $GHR_{124-620}$ and $GHR_{\Delta 128-238}$. (A) γ2A-JAK2 cells stably expressing each mutant were serum-starved. Detergent extracts were immunoprecipitated with anti-$GHR_{cyt\text{-}mAb}$. Eluates were treated with endoH and F/N, as in Methods and then resolved by SDS–PAGE and blotted with anti-$GHR_{cyt\text{-}AL47}$. (B) γ2A-JAK2 cells stably expressing WT GHR or $GHR_{\Delta 128-238}$ were surface biotinylated, anti-$GHR_{cyt\text{-}AL47}$ immunoprecipitated, and blotted with streptavidin-HRP. (C) γ2A-JAK2 cells stably expressing $GHR_{124-620}$ or $GHR_{\Delta 128-238}$ were serum-starved and subjected to fractionation, SDS–PAGE, and blotting with anti-$GHR_{cyt\text{-}AL47}$.

Conclusions

The data in this report indicate that LR association of GHR and JAK2 are observed in a reconstitution system in a pattern similar to that seen in cells endogenously expressing these two proteins. Association of GHR with rafts does not depend on coexpression with JAK2. By mutagenesis and reconstitution, we found that removal of the GHR ICD did not impair LR localization; nor did replacement of the native GHR TMD with that of the LDLR, either in the context of the full-length GHR or an ICD-truncated version. In contrast, removal of most of the ECD substantially reduced LR association and ECD mutagenesis implicated subdomain 2, but not subdomain 1 or the stem region, as harboring a determinant(s) for raft localization.

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