

A role for JAK2 in mediating cell surface GHR-PRLR interaction

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eLife Assessment

This is an **important** study that characterizes a surprising interaction between two different cytokine/hormone receptors using nanoscale resolution (dSTORM) microscopy. The study provides **solid** evidence that the interaction is ligand-dependent, and is mediated by the receptor-associated intracellular signalling molecule JAK2. While at present limited to growth hormone and prolactin receptors in a limited number of cell lines, there are potentially broad implications for cytokine signalling, as such JAK2-mediated interactions could occur between a range of different cytokines. Moreover, the specific hormone interactions shown in the manuscript may have significant implications for understanding how these hormones can have differential effects in breast cancer, under different conditions.

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Abstract

Growth hormone (GH) receptor (GHR) and (full-length) prolactin (PRL) receptor (PRLR) are transmembrane class I cytokine receptors that co-exist in various normal and cancerous cells. Both receptors respond to their associated ligands predominantly by activating the Janus Kinase 2 (JAK2)-signal transducer and activator of transcription (STAT) signaling pathways, and both are also known to initiate receptor-specific JAK2-independent signaling. Together with their cognate ligands, these receptors have been associated with pro-tumorigenic effects in various cancers, including breast cancer (BC). Human GH is known to bind GHR and PRLR, while PRL can only bind PRLR. A growing body of work suggests that GHR and PRLR can form heteromers in BC cells, modulating GH signal transduction. However, the dynamics of PRLR and GHR on the plasma membrane and how these could affect their respective signaling still need to be understood.

To this end, we set out to unravel the spatiotemporal dynamics of GHR and PRLR on the surface of human T47D breast cancer cells and γ 2A-JAK2 cells. We applied direct stochastic optical reconstruction microscopy (dSTORM) and quantified the colocalization and availability of both receptors on the plasma membrane at the nanometer scale at different time points following treatment with GH and PRL. In cells co-expressing GHR and PRLR, we surprisingly observed that not only GH but also PRL treatment induces a significant loss of surface GHR. In cells lacking PRLR or expressing a mutant PRLR deficient in JAK2 binding, we observed that GH induces downregulation of cell surface membrane-bound GHR, but PRL no longer induces loss of surface GHR. Colocalizations of GHR and PRLR were confirmed by proximity ligation (PL) assay.

Our results suggest that PRLR-GHR interaction, direct or indirect, is indispensable for PRL- but not GH- induced loss of surface GHR and for both GH-induced and PRL-induced increase of surface PRLR, with potential consequences for downstream signaling. Furthermore, our results suggest that JAK2 binding via the receptor intracellular domain's Box1 element is crucial for the observed regulation of one class I cytokine receptor's cell surface availability via ligand-induced activation of another class I cytokine receptor. Our findings shed new light on the reciprocal and collective role that PRLR and GHR play in regulating cell signaling.

Introduction

Growth hormone (GH) and prolactin (PRL) are hormones emanating mainly from the anterior pituitary. The primary function of GH is regulating anabolism and metabolism [1, 2], while PRL has important roles in breast development and lactation [3]. There is mounting evidence pointing at both hormones and their receptors playing roles in various types of cancer [4–11], including breast cancer (BC) [12–17], where GHR is frequently present and PRLR is often found overexpressed [18–25]. While they have been mostly associated with pro-tumorigenic effects, PRL has also been reported to show anti-tumor effects and, like PRLR, has been associated with good prognosis in certain BC subtypes [26–29]. However, a humanized neutralizing monoclonal antibody directed against the extracellular domain of PRLR showed no anti-tumor effect when administered in patients with PRLR-positive metastatic BC [30]. This suggests that PRLRs' pro-tumorigenic function may not be as relevant as previously thought or depends on other circumstances such as the presence or absence of other hormone receptors, with which they may interact.

Both GH receptor (GHR) and PRL receptor (PRLR) are structurally similar transmembrane glycoproteins and belong to the class I cytokine receptor superfamily [31, 32]. As is characteristic of the majority of their superfamily members, both receptors contain a proline-rich Box1 motif in the membrane-proximal region of their intracellular domains (ICDs), which is followed by a short inter-box region, and a less conserved Box2 motif that is rich in acidic and aromatic residues [33–35]. For both receptors different isoforms have been identified: GHR has three additional isoforms two of which are created through alternative splicing events and lack the Box1-Box2 motif [36] and the third is the exon 3 deleted GHR (d3-GHR). Aside from the PRLR long (or full length) isoform (LF), other membrane-bound isoforms have been identified, including a long form lacking domain D1 (Δ S1), an intermediate form (IF), and the short forms S1a, S1b, and S1c [3, 37]. All isoforms except for S1b and S1c contain the Box1-Box2 motif, but S1b and S1c still contain the Box1 motif. If not specifically stated otherwise, we will refer here by default to the receptors' full-length (or long) isoforms.

GH can bind and introduce a conformational change to both GHR and PRLR, allowing receptor activation and downstream signaling [38–42], but, unlike GH, PRL can only bind to PRLR [41, 43–45]. Both GHR and PRLR lack intrinsic kinase activity. Following ligand binding,

downstream signal transduction involves predominantly activating the associated cytoplasmic tyrosine kinase, Janus kinase 2 (JAK2), bound to the receptors' Box1 elements. This is followed by phosphorylation of the signal transducer and activator of transcription 5 (STAT5a and STAT5b) [46, 47], although other receptor-specific JAK2-independent signal transduction pathways may also be activated. Of note, JAK2 is not only activating the canonical JAK2- STAT5 pathway, but it can also signal via STAT1 and STAT3 [48–51] and activate non-canonical kinase pathways such as Ras/Raf/MAPK/ERK [52–54] and PI3K/Akt/mTOR [55–58], as well as influence gene expression through histone modification [59, 60]. Moreover, activation of PRLR downstream pathways is isoform-specific, with the short isoforms activating the MAPK and PI3K pathways [61–63]. For comprehensive reviews of JAK2-independent signaling pathways, we refer the reader to [64, 65].

Increasing evidence indicates that GHR and PRLR interact. Two decades ago, it was shown that ovine GHR (oGHR) and PRLR (oPRLR) can tightly associate with each other following stimulation with placental lactogen [66]. These studies utilized chimeric receptors consisting of the extracellular domain (ECD) of human granulocyte and macrophage colony-stimulating factor (hGM-CSF) receptor (hGM-CSFR) along with a part of either oGHR ICD or oPRLR ICD. After hGM-CSF treatment of cells co-expressing oGHR chimera and oPRLR chimera, JAK2 was effectively activated, and protein-protein interaction of both chimeric receptors was detected via co-immunoprecipitation [66, 67]. Additionally, our previous work revealed a specific ligand-independent human GHR (hGHR) - human PRLR (hPRLR) association in human T47D breast cancer cells, which endogenously express both receptors [68]. Further, the use of split luciferase complementation assays has suggested that hGHR homodimers and hPRLR homodimers form hGHR/hPRLR multimers [69] and extracellular subdomain 2 of the hGHR or hPRLR determines the dimerization partner [70]. Although biochemical studies and luciferase complementation assays strongly support the notion that an interplay between hGHR and hPRLR exists, the observed outcomes are ascribed to total receptors within cells, irrespective of subcellular localization.

In the present study, we directly visualized the cell surface interaction of hGHR and hPRLR and how it changes upon ligand treatment. Specifically, we used direct stochastic optical reconstruction microscopy (dSTORM) [71] to visualize single receptor clusters of hGHR and hPRLR on cell surfaces. In dSTORM, a super-resolution microscopy technique, individual fluorophores cycle through reversible transitions between a dark and a fluorescent state [72]. Thus, a fluorophore emits photons multiple times before permanently being photobleached. These blinking events and their localizations are recorded and, although the exact number of proteins in clusters is difficult to determine, the number of localizations is strongly correlated with the number of receptors [73, 74]. Thus, the dSTORM approach can deliver high-resolution images to reveal the localization or arrangement of individual membrane receptor systems providing valuable insight into their interactions with other proteins at the cell surface. Given that receptors are highly trafficked to and from the cell surface and to avoid signal detection from cytosolic receptors, we used monoclonal antibodies to distinctly label the extracellular S2 domain of GHR and PRLR on non-permeabilized cells. Descriptive spatial analysis using Ripley's K- and L-function [75] indicates that both hGHR and hPRLR are organized in nanometer-scale clusters on the T47D cell surface. To further gain quantitative information about GHR and PRLR nanoclusters, we applied DBSCAN (density-based spatial clustering of applications with noise) [76]. Subsequently, individual cluster contours were delineated, individual clusters were assigned a cluster ID, and receptor abundance was analyzed separately for each cluster and receptor. By doing so, we detected and calculated homomeric and heteromeric hGHR and hPRLR clusters on cell surfaces. Colocalizations of GHR and PRLR were also confirmed by proximity ligation (PL) assay. Lastly, we explored which receptor domains determine the interaction of hGHR and hPRLR by creating different truncated or modified hPRLR variants. Our findings indicate that the intracellular Box1

region is an essential determinant of hGHR and hPRLR association, suggesting JAK2 may play an important role in the observed ligand-induced and PRLR-mediated downregulation of cell surface GHR – which could also elicit or add to PRLR's observed anti-tumor effect.

Results

Ligands induce an increase in PRLR localizations and a decrease in GHR localizations at the surface of T47D cells

To investigate the dynamic spatial distribution of endogenous hGHR and hPRLR on the surface of breast cancer cells, we used dSTORM under the total internal reflection fluorescence (TIRF) illumination mode [77]. The dSTORM images show that hGHR and hPRLR form nanometer-scale clusters and are broadly distributed on the surface of T47D cells (Fig. 1). To assess the ligands' effects on the spatial distribution of hGHR and hPRLR on the cell surface, we conducted time-course experiments using T47D cells with human GH or human PRL (500 ng/ml each). Such stimulation is known to induce rapid and substantial STAT5 phosphorylation [68]. We first analyzed the cell surface localization density (number of localizations per μm^2) of hGHR or hPRLR in both resting and ligand-stimulated conditions. The abundance of surface-hPRLR was rapidly increased, reaching its maximum after 3 min of GH or PRL treatment with a ~ 5.6 -fold and ~ 4.5 -fold increase compared to the basal value, respectively (Fig. 2A and 2B). This increase was followed by a rapid decline: after GH treatment for 5 min, the localization density dropped from 200.6 ± 14.7 per μm^2 to 57.2 ± 3.6 per μm^2 ; after PRL treatment for 5 min, the density fell from 159.4 ± 16.2 per μm^2 to 94.8 ± 16.0 per μm^2 . In contrast to hPRLR, the density of hGHR significantly decreased from 43.3 ± 5.5 per μm^2 basally to 25.8 ± 2.7 per μm^2 after 3 min of GH stimulation. After 5 min and 10 min of GH stimulation, the density of hGHR remained relatively low at 26.6 ± 2.3 per μm^2 and 19.3 ± 1.5 per μm^2 , respectively (Fig. 2C). Surprisingly, PRL, which does not bind to hGHR, also induced a loss of surface hGHR on T47D cells. After 1 min of PRL treatment, only 36% of hGHR remained on the cell surface compared to the basal state, and hGHR density remained low for at least 10 min (Fig. 2D). A schematic illustration of GH- and PRL- induced hGHR and hPRLR density changes is shown in Fig. 2E. We previously observed that hGHR and hPRLR specifically co-immunoprecipitate in the absence of added ligands in T47D cells [68]; thus, we postulate that the propensity of hGHR and hPRLR to physically interact underlies our observed loss of hGHR in response to PRL stimulation.

GH or PRL stimulation induces a redistribution of hGHR and hPRLR clusters

We analyzed the dSTORM images using the DBSCAN algorithm to identify different clusters and determine the number of receptor localizations within a cluster (termed 'cluster size') (Fig. 3A). We then performed a localization distribution analysis and plotted a histogram of the relative frequency of localizations (termed 'distribution plot' in this study). Representative dSTORM images of hGHR as well as associated distribution plots are shown in Fig. 3B.

To gain a better understanding of the changes in GHR-PRLR colocalizations, we obtained and analyzed the corresponding bivariate cluster size distributions. Their median values are summarized in Fig. 3C and 3D. As can be seen, hGHR responds quickly and transiently to GH stimulation by forming larger clusters. After GH treatment for 1 min, the median number of hGHR blinking events in a cluster reaches its peak and amounts to 85.6 ± 20.9 localizations in comparison with 33.3 ± 1.7 localizations per cluster in the basal state (P value = 0.0035). After 5 min, this number is reduced approximately to its pre-stimulation value (29.1 ± 2.2) but appears to continue to fluctuate for at least another 25 minutes (Fig. 3C). In turn, hPRLR responds to GH in a less

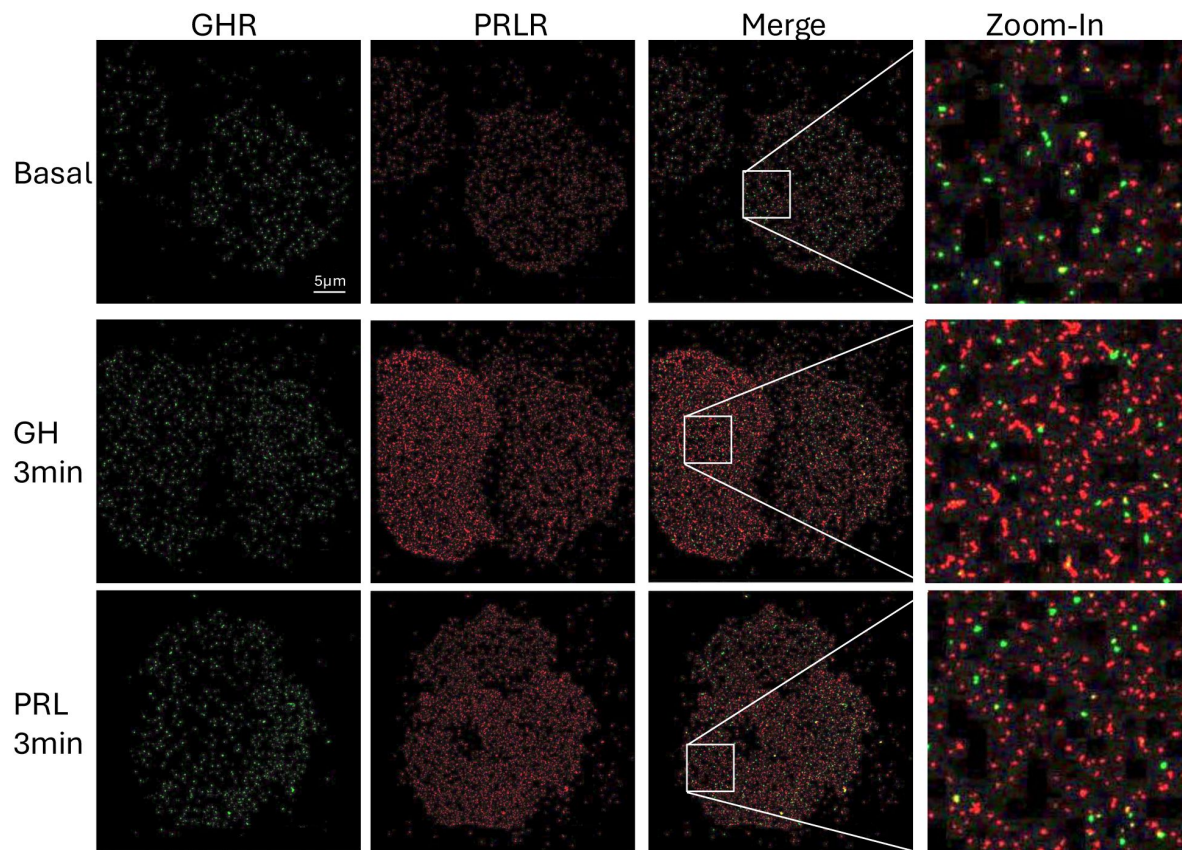


Fig. 1.

Ligand-induced increase of PRLR localizations and decrease of GHR localizations at the surface of T47D cells. A representative set of reconstructed dSTORM images, showing Gaussian-fitted localizations obtained from fluorophore blinking events. GHR is labeled with Alexa 568-conjugated antibody (green, channel 1), and PRLR is labeled with Alexa 647-conjugated antibody (red, channel 2). The last column of images shows the merging of these two channels. T47D cells were either left untreated (upper row), exposed to 500ng/mL GH for 3 min (middle row), or exposed to 500ng/mL PRL for 3 min (lower row). Brightness was increased by 40% and Contrast was reduced by 40% to increase visibility. The images in the first three columns (GHR, PRLR, Merge) have the same scale. The scale bar (5µm) is shown in the upper left image. The images in the right most column are zoomed-in sections from the corresponding merged images.

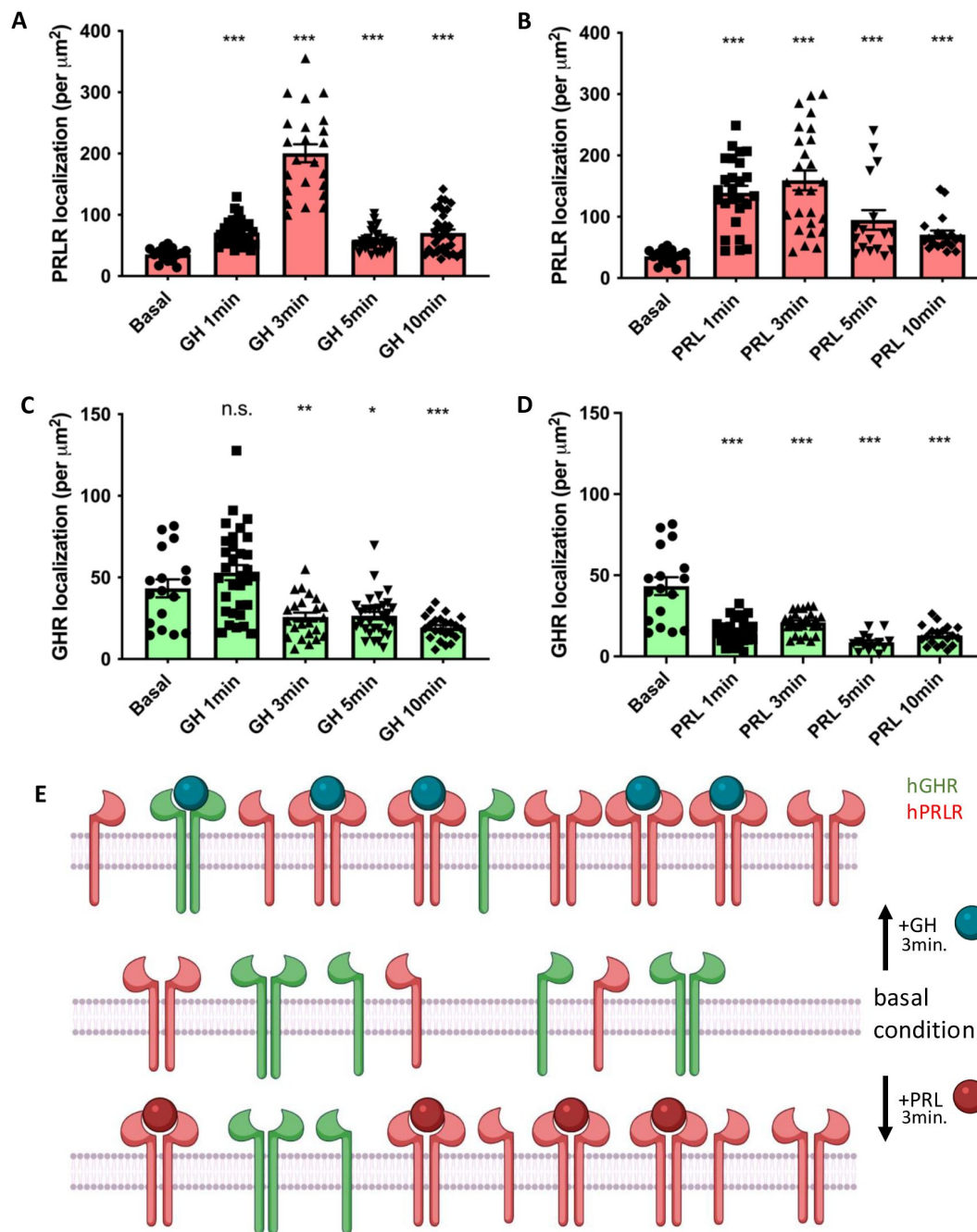


Fig. 2.

(A,B) Quantification of GH-induced (500 ng/ml) (A) and PRL-induced (500 ng/ml) (B) changes of PRLR localizations on the cell surface. **(C, D)** Quantification of GH-induced (C) and PRL-induced (D) changes of GHR localizations on the cell surface. Each data point represents the density of receptors in a $6.25 \mu\text{m} \times 6.25 \mu\text{m}$ area. Data are collected from at least 6 cells from each group and displayed as mean $\pm$ SE. * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$) indicate the statistical significance in comparison with Basal and are calculated by two-tailed t-tests assuming unequal variances. **(E)** Model depicting hPRLR and hGHR densities and activation schemes in T47D cells. The middle of the three states depicts the basal condition.

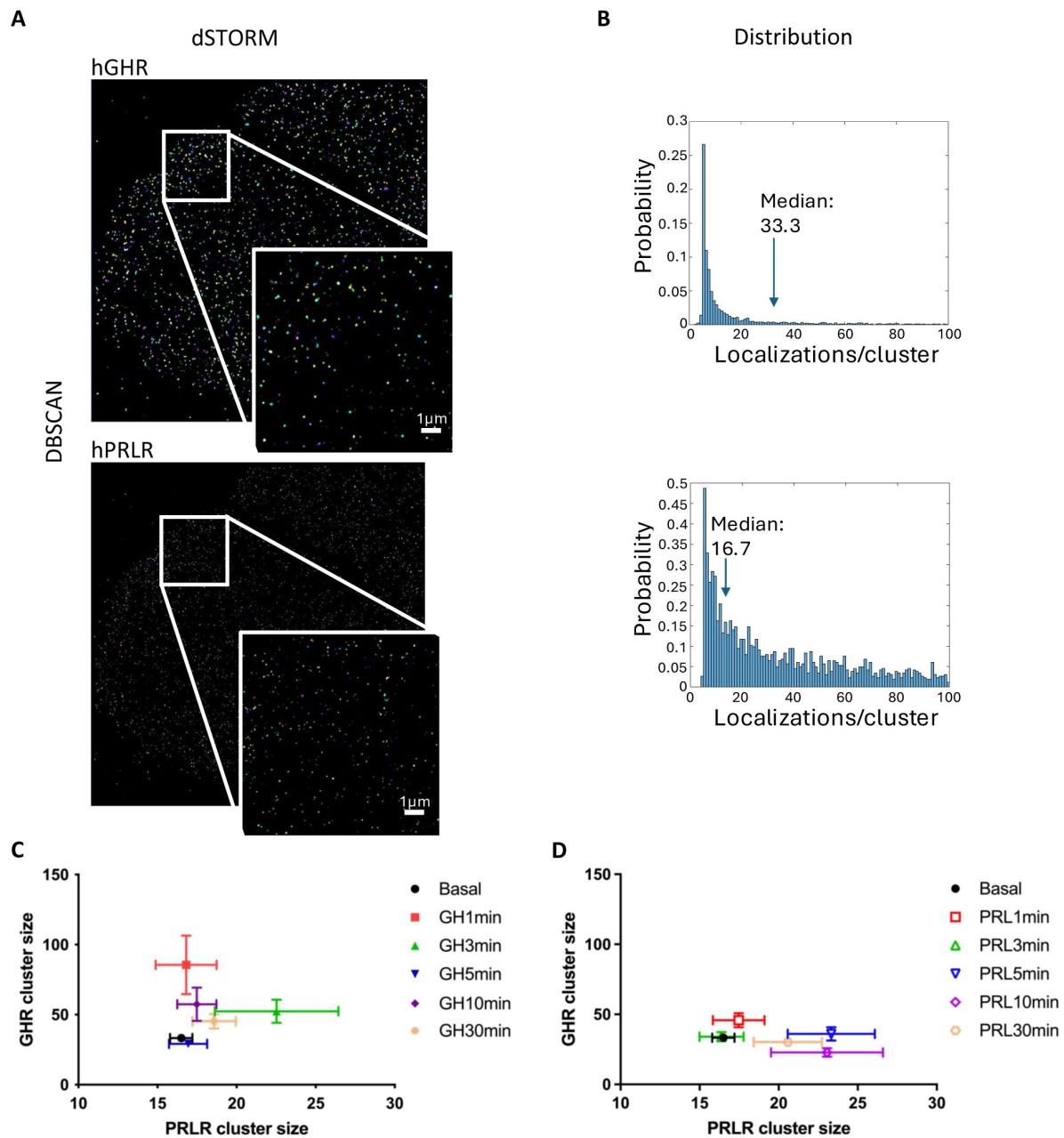


Fig. 3.

GH/PRL stimulation induces a redistribution of hGHR and hPRLR clusters. **(A)** Sample dSTORM images and application of DBSCAN to identify and measure clusters (scale bar, 1 μ m). To distinguish different clusters, each cluster has a different color assigned. **(B)** For similar images corresponding histograms of the relative frequencies of localizations are shown. Median cluster sizes are indicated. **(C, D)** The medians of hGHR and hPRLR cluster sizes following GH (C) and PRL (D) treatment are summarized in these plots. The x-axis and y-axis represent hPRLR and hGHR cluster sizes (counts), respectively. Data are collected from at least 6 cells from each group and displayed as median $\pm$ SE.

prominent manner: The median number of hPRLR blinking events in a cluster in the basal state is 16.5 ± 0.7 . After 3 min of GH stimulation, the median of PRLR cluster size reaches its maximum, which is 22.5 ± 3.9 (P value = 0.1496), and afterward declines to its basal level (**Fig. 3C** [↗](#)).

Like the response to GH, hGHR reacts also quickly to PRL treatment. The median number of hGHR blinking events in a cluster culminates at 1 min of PRL treatment with 45.8 ± 5.0 (compared with basal level, P value = 0.0098). In distinction, hPRLR response to PRL is slower. The median number of hPRLR blinking events in a cluster reaches its peak at 5 min of PRL treatment with a median number of 23.3 ± 2.8 (compared with basal level, P value = 0.0233), and after 10 min the median is only slightly less than that maximum (**Fig. 3D** [↗](#)). Together, these results indicate that upon ligand stimulation hGHR cluster sizes increase transiently and significantly, while changes of hPRLR cluster sizes occur slowly and more subtly.

Spatial proximity of hGHR and hPRLR upon ligand stimulation

Nanoscale interactions of hGHR and hPRLR on the cell surface have yet to be well established. To evaluate the extent of hGHR and hPRLR surface colocalization on T47D cells, we utilized proximity ligation assays (PLAs). In PLAs, a positive signal appears only when two target proteins are in proximity (<40 nm). Notably, individual treatment with GH or PRL for 5 min decreased the PLA signal observed in untreated cells by 34.4% and 28.1%, respectively (**Fig. 4A** [↗](#)), suggesting either ligand caused a reduction in the total number of colocalized hGHR and hPRLR clusters. To further validate these observations, we calculated the ratio of colocalized clusters in dSTORM images. Treatment with GH or PRL for 1 min reduced the proportion of colocalized clusters by nearly 50% (**Fig. 4B** [↗](#) and **4C** [↗](#)). To analyze their compositions, we plotted 3D distributions for the colocalized clusters after ligand treatments (**Fig. S1A** and **S1B**). The probability of observing colocalized clusters with numbers of hGHR and hPRLR that fall into certain bins is identified by color and by the height of the bar on the z-axis. Following GH treatment for 1 min and 3 min, the number of smallest clusters decreased, and the number of medium-sized clusters increased, suggesting a shift of the bivariate distribution of co-localized cluster sizes toward medium-sized clusters (**Fig. S1A**). The distribution of colocalized clusters after 5 min of GH treatment is similar to that at the basal state. In contrast, after treatment with PRL for 3 min or more, the number of medium to large-sized clusters, majoritarily containing either GHR or PRLR, decreased, while the number of smallest clusters increased (**Fig. S1B**). Together, these results demonstrate that hGHR and hPRLR are spatially accessible to each other and form receptor complexes on the T47D cell surface and that the nature of these complexes changes differentially depending on the stimulating ligand.

Reduction of hGHR induced by PRL on the cell surface is dependent on the presence of PRLR

PRL strongly binds PRLR but not GHR. Yet, PRL induces a decrease of hGHR on the surface of cells expressing both hGHR and hPRLR. Thus, we sought to investigate the effect of PRL on hGHR in the absence of hPRLR. We utilized CRISPR/Cas9 technology to generate hPRLR knockout T47D cells (termed T47D_{ΔPRLR}). In addition, to evaluate isolated hPRLR responses to ligands, we also generated hGHR knockout T47D cells (termed T47D_{ΔGHR}). Immunoblot analysis with specific GHR and PRLR antibodies confirmed the absence of hPRLR in T47D_{ΔPRLR} cells and hGHR in T47D_{ΔGHR} cells (**Fig 5A** [↗](#)). Similar to our results with parental T47D cells, GH treatment of T47D_{ΔPRLR} cells rapidly reduces the density of surface hGHR, suggesting that hPRLR need not be present to allow this GH-induced effect (**Fig 5B** [↗](#)). However, contrary to findings in parental T47D cells, PRL treatment of T47D_{ΔPRLR} cells fails to modulate hGHR surface density (**Fig 5C** [↗](#)). Like parental T47D cells, treatment of T47D_{ΔGHR} cells with GH (**Fig 5D** [↗](#)) or PRL (**Fig 5E** [↗](#)) yielded increased hPRLR surface localizations. Thus, we conclude that the PRL-induced decrease of hGHR in T47D cells is dependent on the presence of hPRLR, but the ability of both GH and PRL to increase surface hPRLR in T47D cells is independent of hGHR's presence.

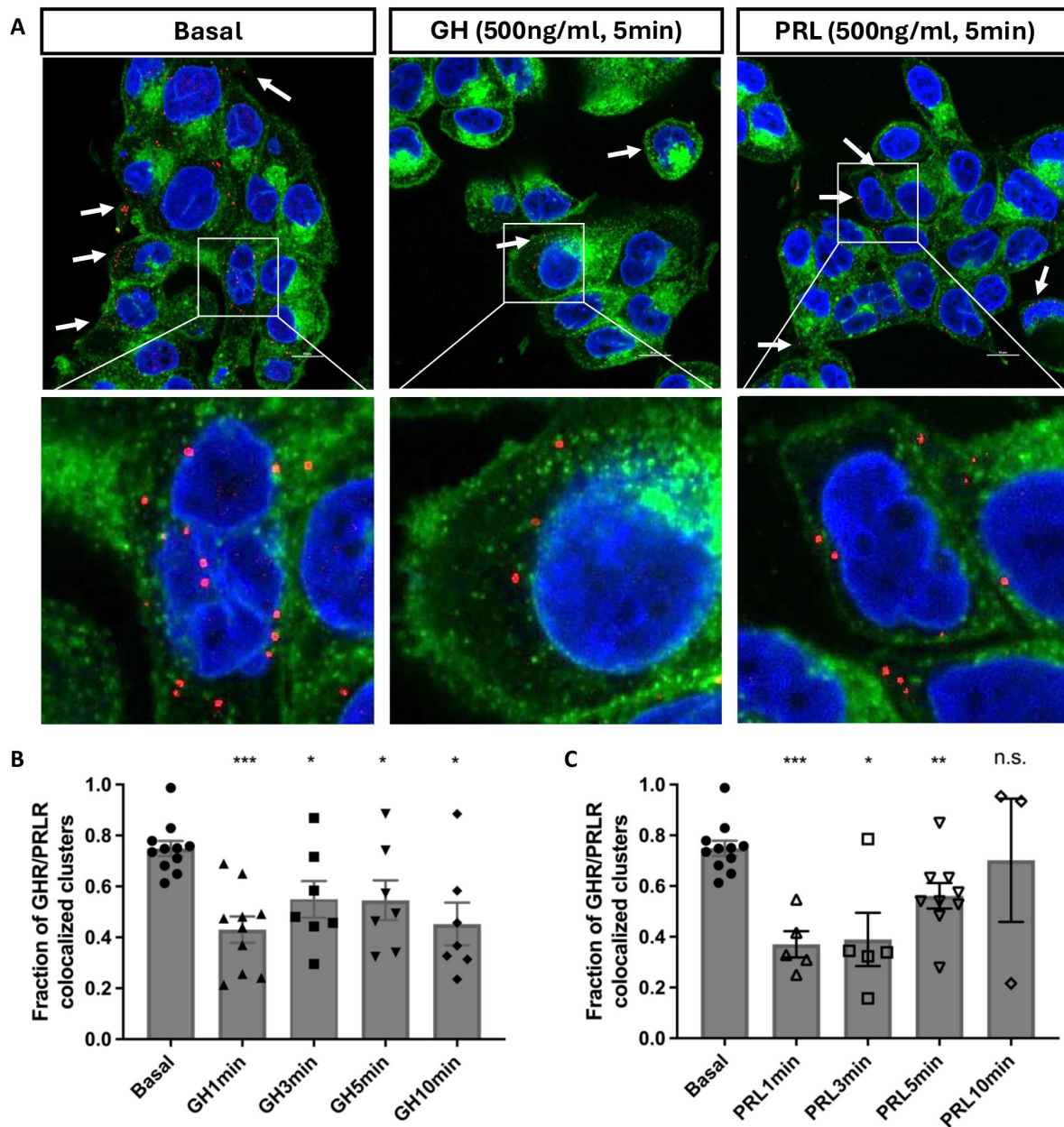


Fig. 4.

Spatial proximity of hGHR and hPRLR upon ligand stimulation. **(A)** Proximity ligation assays were performed in T47D cells (scale bar, 10 μ m). Here we show representative confocal microscopy images of T47D cells at resting and ligand stimulation conditions. The PLA signal (red dots) corresponds to hGHR and hPRLR complexes. Wheat germ agglutinin staining (green) was used to label glycoproteins for cell membrane imaging. DAPI (blue staining) corresponds to cell nuclei. Zoom-in views are provided in the second row. **(B, C)** hGHR and hPRLR colocalization changes in T47D cell surface following 500 ng/ml GH (B) or PRL (C) treatment obtained from dSTORM images using DBSCAN. Each data point represents a ratio of the number of clusters containing both hGHR and hPRLR, over the total number of clusters on the cell surface. Data are displayed as mean $\pm$ SE. * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$) indicate the statistical significance in comparison with Basal and are calculated by a two-tailed t-test assuming unequal variance.

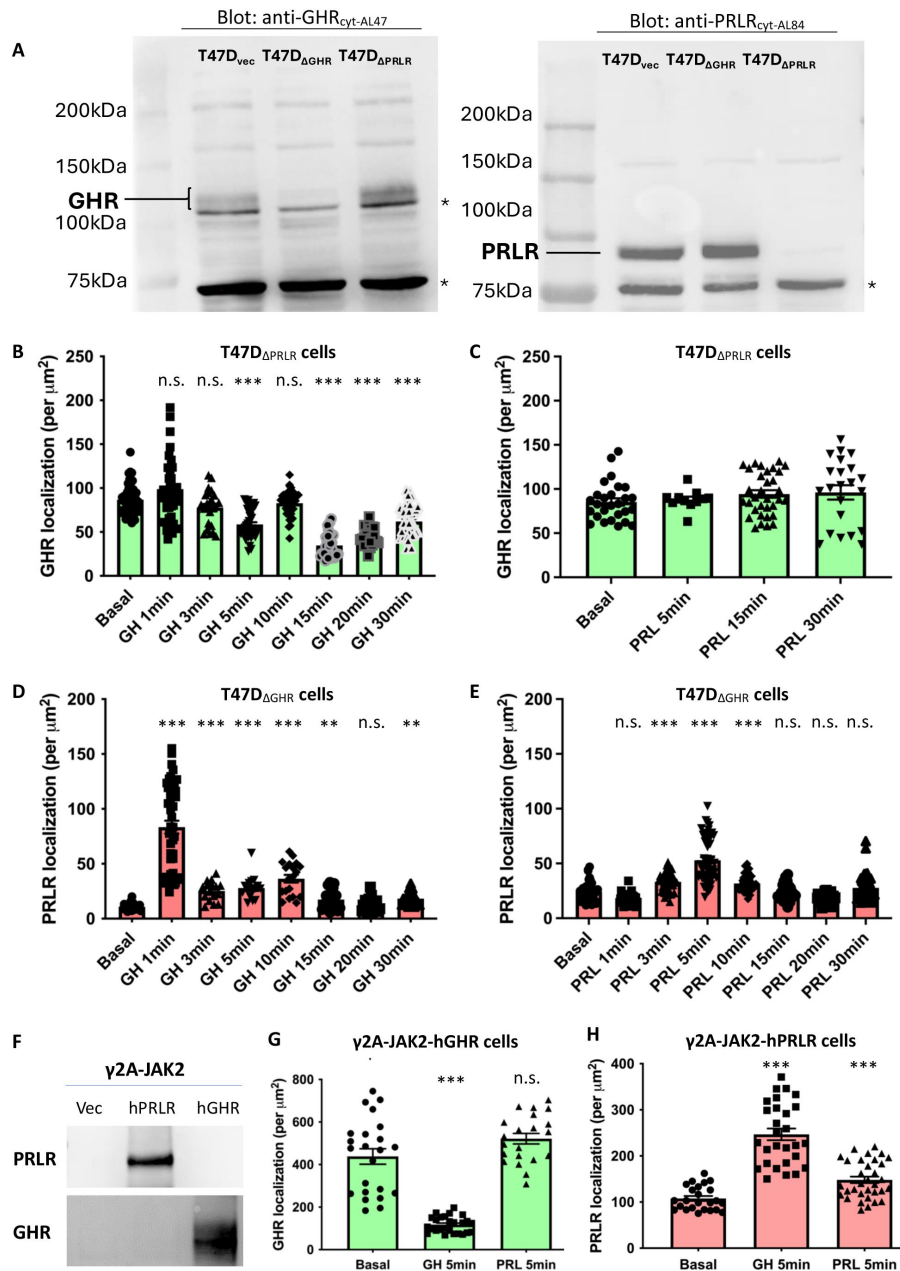


Fig. 5.

PRL-induced reduction of hGHR on the cell surface depends on the presence of PRLR. **(A)** Detergent cell extracts of T47D_{vec}, T47D_{ΔGHR}, and T47D_{ΔPRLR} were analyzed by immunoblotting with anti-GHR_{Cyt-AL47} and anti-PRLR_{Cyt-AL84} antibodies. The bands corresponding to GHR and PRLR are annotated. Asterisks indicate migration of strong non-specific bands detected in the Western Blots, which serves as a loading control for protein. **(B)** In T47D_{ΔPRLR} cells, GH (500 ng/ml) induces downregulation of hGHR localizations on the cell surface, while **(C)** PRL does not change hGHR localizations. **(D, E)** In T47D_{ΔGHR} cells, GH **(D)** and PRL **(E)** incite an increase of hPRLR on the cell surface. Data are displayed as mean ± SE. * (P<0.05), ** (P<0.01), and *** (P<0.001) indicate the statistical significance in comparison with Basal and are calculated by a two-tailed t-test assuming unequal variance. **(F)** y2A-JAK2 cells with stable expression of hGHR or hPRLR. Detergent extracts were resolved by SDS-PAGE and immunoblotted with anti-GHR_{Cyt-AL47} and anti-PRLR_{Cyt-AL84} antibodies. Vec corresponds to stable transfection with empty vector. **(G, H)** Serum-starved y2A-JAK2-hGHR cells **(G)** or y2A-JAK2-hPRLR cells **(H)** were treated with GH (500 ng/ml) or PRL (500 ng/ml) for 5 min. Localizations of hGHR in y2A-JAK2-hGHR cells were decreased after GH treatment while remaining the same after PRL treatment compared to the basal state. Localizations of hPRLR in y2A-JAK2-hPRLR cells were increased by GH or PRL treatment. Data are displayed as mean ± SE. * (P<0.05), ** (P<0.01), and *** (P<0.001) denote the statistical significance in comparison with Basal and are calculated by a two-tailed t-test assuming unequal variance.

To extend our observations, we next examined the GH and PRL responses in a cellular reconstitution system: γ 2A-JAK2 [78–80] is a human JAK2-deficient fibrosarcoma cell line reconstituted with JAK2 that stably expresses JAK2 but lacks hGHR and hPRLR. To independently study the role of each receptor in this setting, we used our previously generated stable transfectants of γ 2A-JAK2 cells that harbor either hGHR or hPRLR [81] and verified the presence of the indicated receptor by immunoblotting (Fig. 5F). Consistent with the observation in T47D_{ΔPRLR} cells, γ 2A-JAK2-hGHR cells responded with a loss of surface hGHR density to GH stimulation but not to PRL stimulation (Fig. 5G). Interestingly, as in T47D_{ΔGHR} cells, treatment of γ 2A-JAK2-hPRLR cells with either GH or PRL (Fig. 5H) promotes increased surface hPRLR. Thus, our findings in both cell systems suggest that hPRLR-hGHR interaction, direct or indirect, is indispensable for PRL-induced but not for GH-induced loss of surface hGHR.

Box 1 region in hPRLR contributes to PRL-induced cell surface GHR downregulation

The hPRLR-dependent PRL-induced downregulation of cell surface hGHR indicates that hPRLR can modulate the density of hGHR on the cell surface in response to PRL. To investigate this interaction, we generated a set of truncation or deletion mutants of hPRLR: (1) hPRLR-tr292, which truncates the intracellular domain of hPRLR distal to the membrane-proximal intracellular domain box 1 element; (2) hPRLR-tr238, which contains only 4 amino acids (aa) of the proximal intracellular domain and does not include box 1; and (3) hPRLR-Δbox1, in which the box 1 region (243aa–251aa) is internally deleted (shown in Fig. 6A). Expression of hPRLR-tr292, hPRLR-tr238, and hPRLR-Δbox1, as well as wild-type hPRLR, was detected by immunoblotting using monoclonal antibodies targeting the ECD of PRLR (mAb_{ext-1.48}). Since hPRLR-tr238 contains only 4 aa in its ICD, no immunoblot signal was detected using a polyclonal antibody (Ab_{AL-84}) targeting the hPRLR ICD. In contrast, the expression of hPRLR-tr292 was easily detected by Ab_{AL-84} (Fig. 6B). We then transiently transfected each hPRLR construct into the γ 2A-JAK2-hGHR cells, which stably express hGHR, and analyzed the changes of cell surface hGHR localizations. In cells expressing wild-type hPRLR (hPRLR-WT), the localizations of hGHR significantly decreased 3 min post-exposure to GH and PRL (Fig. 6C). Similarly, under the same treatment, in cells expressing hPRLR-tr292, the localization of hGHR on the cell membrane was reduced in response to each ligand (Fig. 6D). However, in hPRLR-tr238-expressing cells, the localization of hGHR was diminished by GH stimulation but slightly increased by PRL stimulation (Fig. 6E). To further investigate the role of the box1 region for hGHR and hPRLR functional interaction, we studied hPRLR-Δbox1 expressing cells and found that upon PRL treatment, hGHR localizations did not decrease on the cell surface (Fig. 6F). As a negative control, we transfected the cells with vector (pcDNA3.1). hGHR localization decreased upon GH stimulation but remained at basal levels upon PRL stimulation (Fig. 6G). In addition, in hPRLR-ΔBox1 and hGHR expressing cells, the JAK2 and STAT5 tyrosine phosphorylation levels were assessed in response to GH and PRL stimulations. Treatment of 500ng/ml GH induces a dramatic increase in both JAK2 and STAT5 phosphorylation. In contrast, 500ng/ml PRL treatment does not cause JAK2 or STAT5 phosphorylation (Fig. 6H), consistent with PRLR Box1 being required for effective coupling of PRL occupancy of PRLR to activation of JAK2 and phosphorylation of STAT5 and the inability of PRL to signal via GHR.

Next, we analyzed colocalization in each group. In the resting state, the ratios of GHR-PRLR-colocalization clusters are relatively higher in hPRLR-WT- and hPRLR-tr292-expressing cells in comparison with hPRLR-tr238- and hPRLR-ΔBox1-expressing cells (Fig. 6I). This suggests that the box 1 region in hPRLR plays a critical role in stabilizing the hGHR-hPRLR complexes in the basal state.

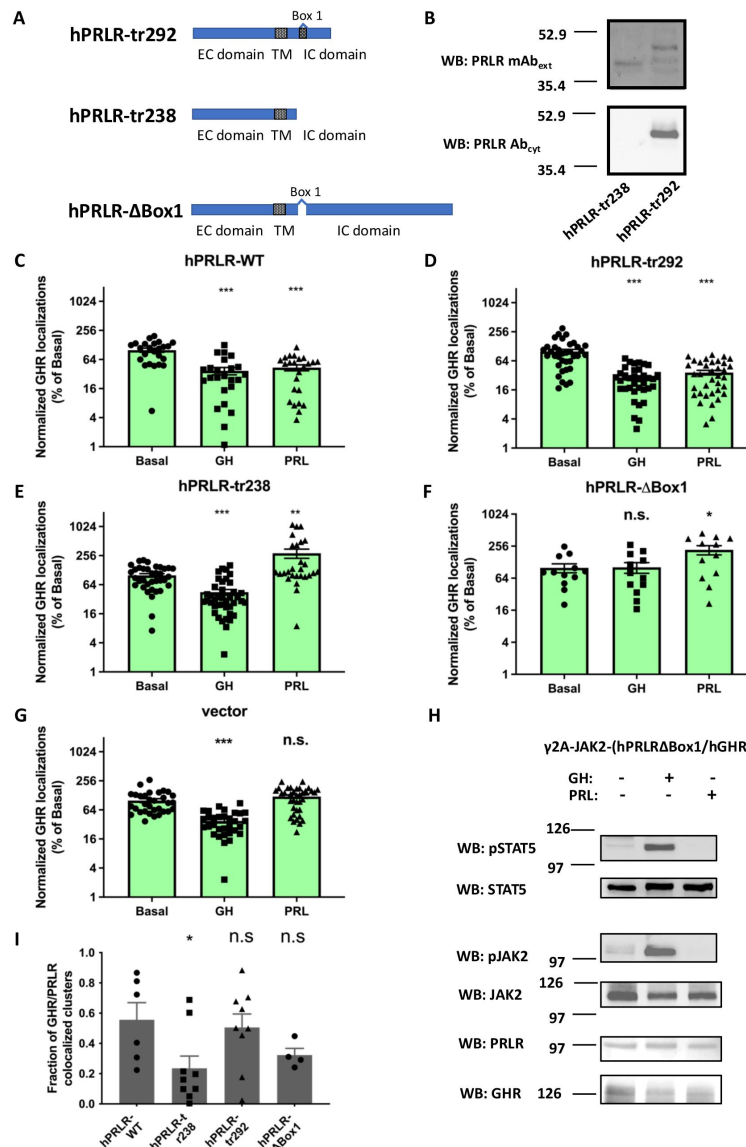


Fig. 6.

Box 1 region in hPRLR contributes to PRLR-induced downregulation of cell surface GHR. **(A)** Diagrams of employed mutant hPRLR variants with truncations or deletion in the intracellular domain. ECD, extracellular domain; TMD, transmembrane domain; ICD, intracellular domain. **(B)** γ2A-JAK2-hGHR cells were transiently transfected with hPRLR-tr292 and hPRLR-tr238. Cell extracts were resolved by SDS- PAGE and blotted with anti-PRLR mAb_{ext}-1.48 and anti-PRLR_{cyt}-AL84. **(C-G)** γ2A-JAK2-hGHR cells were transfected with (C) wild-type hPRLR (hPRLR-WT), (D) hPRLR truncated at 292 aa (hPRLR-tr292), (E) hPRLR truncated at 238 aa (hPRLR-tr238), (F) hPRLR with box1 motif deleted (hPRLR-ΔBox1) and (G) vector pcDNA3.1 (vector). Transfected cells were imaged by dSTORM microscopy and analyzed using the DBSCAN algorithm. The density of hGHR localizations on the cell surface was calculated. Each data point represents the density of hGHR in a cell surface area of size 6.25 μm X 6.25 μm. Data are collected from at least 6 cells (4 ROIs per cell) from each group, and values are displayed as mean ± SE (from three independent experiments). Data are normalized such that the basal is 100%. * (P<0.05), ** (P<0.01), and *** (P<0.001) denote the statistical significance in comparison with Basal and are calculated by a two-tailed t-test assuming unequal variance. **(H)** Detergent cell extracts of hPRLR-ΔBox1 and hGHR-expressing cells were analyzed by immunoblotting. After 5 hrs. starvation, cells were treated with GH (500 ng/ml) or PRL (500 ng/ml) for 10 min. In each experiment, the average Basal value is considered 100%. **(I)** Fraction of GHR/PRLR colocalized clusters. In the resting state, the colocalization ratio is significantly lower for cells expressing hPRLR-tr238 than for those expressing hPRLR-WT. Each data point represents the ratio of the number of clusters, which contain both hGHR and hPRLR, to the total number of clusters on the cell surface. Data are displayed as mean ± SE. * (P<0.05) indicates statistical significance in comparison with WT and is calculated by a two-tailed t-test assuming unequal variance (all bars without an asterisk are not significant).

Box 1 region in hGHR plays an essential role in regulating PRLR and GHR interaction

From our observations (Fig. 6), we concluded that the JAK2 binding site, i.e., the box 1 region, in hPRLR is required for PRL-induced hGHR down-regulation from the cell surface. To further assess whether the JAK2 binding site on hGHR is also essential, we generated hGHR-ΔBox1, in which the box 1 region (297aa – 305aa) was deleted (Fig. 7A). In cells expressing both hGHR-ΔBox1 and hPRLR we observed that GH does not alter the hGHR-ΔBox1 localizations on the cell surface, while PRL slightly increases hGHR-ΔBox1 localizations (Fig. 7B). Together, these results re-affirm that binding of JAK2 to hGHR is also required for hPRLR-mediated regulation of hGHR availability on the cell surface.

Materials and Methods

Materials

Common reagents were purchased from Sigma Aldrich Corp. (St. Louis, MO) unless otherwise noted. Fetal bovine serum was purchased from Atlanta Biologicals (Lawrenceville, GA). Cell culture medium, penicillin/streptomycin and trypsin were purchased from Corning (Corning, NY). Recombinant hGH was kindly provided by Eli Lilly & Co. (Indianapolis, IN). Recombinant human PRL was obtained from the National Hormone and Pituitary Program.

Antibodies

Polyclonal anti-GHR_{cyt-AL47} (1:1000) against the intracellular domain of GHR and polyclonal anti-PRLR_{cyt-AL84} against the human PRLR intracellular domain described previously in [82] and [83], respectively, were used as the primary antibodies for western blot analysis. Monoclonal anti-GHR_{ext-mAb} 74.3 (1:1000) against the extracellular S2 domain of GHR [40, 68, 70, 79, 81, 84–93] and monoclonal anti-PRLR_{ext-mAb} 1.48 against the extracellular S2 domain of human PRLR [70] were used for microscopy. Specifically, we let hybridoma cells secrete these antibodies into the cell medium to avoid the purification step. A detailed description of the antibodies and their previous use is provided in the references. Specificity of anti-GHR_{ext-mAb} and anti-PRLR_{ext-mAb} 1.48 was confirmed using confocal microscopy (see also Supp. Fig. S2–S4). The following secondary antibodies were used: Alexa Fluor 568 goat anti-mouse IgG1 (Invitrogen #A-21124) (1:1000), Alexa Fluor 647 goat anti-mouse IgG2b (Invitrogen #A-21242) (1:1000), Alexa Fluor 647 goat anti-mouse IgG1 (Invitrogen #A-21240) (1:1000) and Alexa Fluor 568 goat anti-mouse IgG2b (Invitrogen #A-21144) (1:1000). In the absence of primary antibodies, secondary antibodies did not show any fluorescence signals (see also Supp. Fig. S3).

Cloning and constructs

The human GHR cDNA in pcDNA1 was a generous gift from R. Ross (University of Sheffield, Sheffield, UK). The human PRLR cDNA in pEF/V5/HIS was generously provided by C. Clevenger (Virginia Commonwealth University, Richmond, VA). hPRLR-tr238 and hPRLR-tr292 were generated by amplifying with external primers containing EcoRI site and a stop codon with XhoI site after the sequence of 238 or 292 amino acid, respectively. hPRLR-ΔBox1, and hGHR-Δbox1 were generated by overlap extension polymerase chain reaction (PCR) with associated primers and cloned into pcDNA3.1(+) vector.

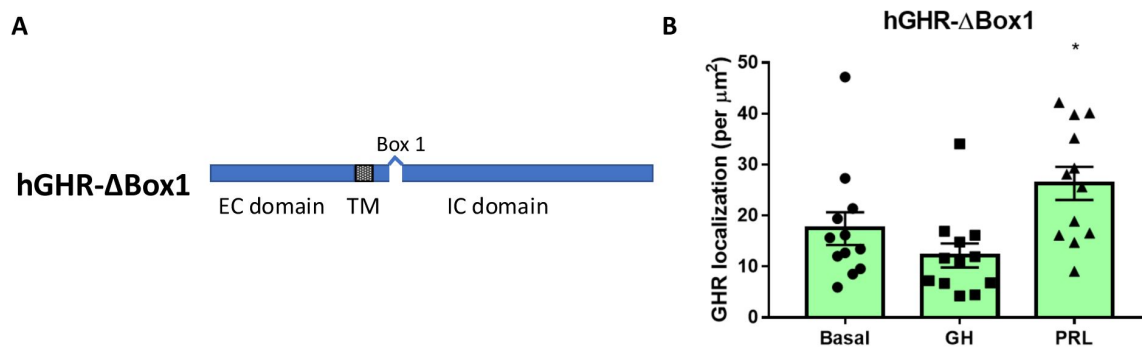


Fig. 7.

Box 1 region in hGHR also plays an important role in PRLR and GHR interaction. **(A)** Diagram of hGHR-ΔBox1 showing the deleted box 1 region in the intracellular domain of hGHR. **(B)** γ 2A-JAK2-hPRLR cells were transfected with hGHR-ΔBox1. Transfected cells were imaged by dSTORM microscopy and analyzed using DBSCAN. The density of hGHR localizations on the cell surface was calculated. Each data point represents the density of hGHR in a cell surface area of size $6.25 \mu\text{m} \times 6.25 \mu\text{m}$. Data are collected from at least 6 cells for each group and displayed as mean $\pm$ SE. * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$) indicate the statistical significance in comparison with Basal and are calculated by a two-tailed t-test assuming unequal variance.

Cell culture and transfections

Human T47D breast cancer cells were purchased from American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C.

γ2A-JAK2 cells were generated by transfection of γ2A cells [78] with pcDNA3.1(+)/zeo-JAK2 and maintained in culture, as described previously [79, 80]. The generation of γ2A-JAK2-GHR and γ2A-JAK2- PRLR cells has been previously described [81]. Transient expression of receptors was achieved by using Lipofectamine LTX Plus (Invitrogen), transfecting 0.3 pmol plasmid DNA per 6-cm² dish.

Western blot

Cells were serum starved for 5 hours and treated with 500 ng/ml GH or 500 ng/ml PRL at 37 °C for 10 min. Stimulations were terminated by washing the cells with ice-cold phosphate-buffered saline supplemented with 0.4 mM sodium orthovanadate. Cells were lysed in lysis buffer for 30 min at 4°C. Then cell lysates were centrifuged at 15,000 g for 10 min at 4°C. The protein extracts (supernatant) along with SDS sample buffer were resolved by SDS-PAGE.

Sample preparation for imaging

Cells were seeded into either eight well #1.5 coverslip bottom dishes (Ibidi) or 25 mm #1.5 coverslips (Electron Microscopy Sciences). Cells were serum starved for 5 hours, then treated with GH (500 ng/ml), PRL (500 ng/ml) at 37 °C for the indicated time. Then the cells were washed with Dulbecco's Phosphate-Buffered Saline (DPBS) three times and fixed with 4% PFA (paraformaldehyde) in 1X DPBS with calcium and magnesium (Cat # 21-030-CV) for 10 min. After multiple washes with DPBS, cells were blocked with 5% normal horse serum, 5% normal goat serum, 5% BSA for 30 min. Lastly, cells were incubated in primary antibody for 3h and secondary antibody for 70 min at 37°C.

dSTORM imaging and data analysis

dSTORM experiments were conducted on an inverted Nikon Ti2 N-STORM microscope equipped with a 100 × 1.49 NA oil immersion objective (Nikon, Japan), 488 and 647 nm lasers, and an iXon DU-897 Ultra EMCCD camera (Andor, Oxford Instruments). The dSTORM imaging buffer consists of two buffers, A and B, and a GLOX mixture. Buffer A is made of 10 mM Tris pH 8 and 50 mM NaCl, while buffer B is made of

50 mM Tris pH 8 and 10 mM NaCl. The GLOX mixture consists of 14 mg glucose oxidase (Sigma, St Louis, Missouri), 50 µl catalase (17 mg/ml) (Roche, Penzberg, Germany), and 200 µl of Buffer A. The dSTORM buffer is made by combining (on ice) 7 µl GLOX with 7 µl 2-mercaptoethanol (Sigma, St Louis, Missouri) and 690 µl Buffer B, using only the supernatant from the GLOX mixture. For a detailed description of the buffer, we refer the reader to [94].

The cell membrane was focused using TIRF excitation, which selectively images within 100-150 nm of the cell membrane, making it an excellent method to study surface distribution of membrane proteins. Of note, TIRFM exploits the unique properties of an induced evanescent field in a limited specimen region immediately adjacent to the interface between two media having different refractive indices. It thereby dramatically reduces background by rejecting fluorescence from out-of-focus areas in the detection path and illuminating only the area right near the surface.

Moreover, we reduced auto-fluorescence by ensuring that (a) cells are grown in phenol-red-free media, (b) imaging is performed in STORM buffer which reduces autofluorescence, and (c) our immunostaining protocol includes a quenching step aside from using blocking buffer with different serum, in addition to BSA (5%). Moreover, we employed extensive washing steps following antibody incubations to eliminate non-specifically bound antibodies. Ensuring that the TIRF illumination field is uniform helps reduce scatter. Additionally, an extended bleach step prior to the acquisition of frames to determine localizations helped further reduce the probability of non-bleached fluorescent molecules.

For dual color STORM imaging, the sample was illuminated with alternating 561 nm or 647 nm lasers and 40,000 frames of images were acquired per channel (9ms/frame). The chromatic offset associated with the different acquisition wavelengths was corrected using 0.1 μ m microspheres [95, 96]. Imaging captured the fluorophores' blinking events. Localizations were counted as true localizations, when at least 5 consecutive blinking events had been observed. Moreover, coordinates were filtered using a threshold radius of 25nm. dSTORM images were reconstructed using the built-in STORM module in NIS-Elements (Nikon). Nikon software was used for Gaussian fitting, and each dot represents the peak of a Gaussian. The localizations list was exported and further analyzed in *Matlab*. In particular, for the DBSCAN cluster analysis, we employed Clus-DoC directly on the list of localizations, utilizing the parameters of a minimum of 4 neighbors within a cluster radius of 20nm [97].

Proximity Ligation Assay

Cells were serum starved for 5 hours, then treated with GH (500 ng/ml) or PRL (500 ng/ml) at 37 °C for the indicated time. Then the cells were washed with DPBS three times and fixed with 4% PFA (paraformaldehyde) for 10 min. After multiple washes with DPBS, cells were blocked with 5% normal horse serum, 5% normal goat serum, 1% BSA for 30 min. Lastly, cells were incubated in primary antibody for 3h and secondary antibody for 70 min at 37°C. After secondary antibody incubation, ligation and amplification were performed according to the manufacturer's protocol (Duolink® PLA; Sigma-Aldrich). Briefly, ligation solution containing ligase was added to the samples, followed by incubation for 30 minutes at 37°C. Samples were then washed and incubated with the amplification solution, which includes polymerase, at 37°C for 100 minutes to amplify the PLA signal.

Generating hGHR knockout and hPRLR knockout T47D cells

We utilized CRISPR/Cas9 to generate T47D_{ΔPRLR} and T47D_{ΔGHR}. Two single-guide RNAs (sgRNAs) and Cas9 nickase (Cas9n) were designed to create sticky-end double-strand breaks in DNA at the targeted locus, triggering DNA repair via the Non-Homologous End Joining (NHEJ) pathway, which introduces insertions, deletions or frameshift mutations [98]. Each sgRNA was expressed using plasmid #62987 pSpCas9n(BB)- 2A-Puro(PX462) V2.0 (purchased from Addgene), which also expressed Cas9n when transfected into cells. Two vectors, built on pSpCas9n(BB)-2A-Puro(PX462) V2.0 backbone, containing a pair of sgRNAs targeting either the hGHR or hPRLR gene for knockout, as well as a built-in Cas9n gene, were transfected into newly purchased T47D cells. The transfected cells were plated at serial dilutions and selected with puromycin. Single clones were picked and expanded. The vector backbone pSpCas9n(BB)-2A-Puro(PX462) V2.0 was also used for transfection into T47D cells as a control cell line.

Genomic DNA was extracted from each clone. Primers were designed 200-400 bp upstream and downstream of the targeted locus of the hGHR or hPRLR gene to amplify the genomic DNA via PCR, which was subsequently cloned into the pUC19 vector for sequencing. Clones with mutations on both alleles were identified, and the western blot was used to confirm the absence of expression of the targeted genes.

Design of sgRNAs for hGHR or hPRLR knockout

A pair of 20 bp sequences located 10-25 bp apart at the target locus was chosen using the Feng Zhang lab's Target Finder [99]. This tool identified the best pair of guide RNA sequences with theoretically zero off-target effects. One sequence targeted the top strand, and the other targeted the bottom strand, enabling the introduction of a sticky-end double-strand DNA break. Each guide RNA sequence was synthesized as double-stranded oligonucleotides with a CACC at 5' overhang of the 20-nt guide sequence and an AAAC at the 5' overhang of the complementary strand, allowing insertion into the backbone plasmid pSpCas9n(BB)-2A-Puro(PX462) V2.0 using the BbsI restriction enzyme. An extra nucleotide 'G' was added to the 5' end of the 20-nt guide if the sequence did not begin with 'G', which is the preferred first base following the U6 RNA Polymerase III promoter.

The target locus for introducing mutations in the hGHR gene was located at exon 4. Exon 4 was chosen because many naturally occurring pathological mutations in Laron Syndrome are located in this region. Additionally, deletion of the 3' end of exon 4 and its adjacent intron has been shown to successfully generate GHR knockout mice [100]. The hPRLR gene has seven isoforms; exon 5 is included in six of these isoforms, except for the Δ S1 PRLR isoform, in which both exons 4 and 5 are skipped by alternative splicing. Therefore, we selected the end of exon 5 and the adjacent intron as the target locus for introducing mutations. The goal was to knock out all six isoforms containing exon 5, as well as the Δ S1 PRLR isoform, by interrupting alternative splicing. This was achieved by deleting the initial GU dinucleotide sequence at the 5' end of the intron, which is required for the binding of the U1 protein of the spliceosome.

Statistical analysis

Imaging and biochemical experiments were carried out at least three times to ensure reproducibility. Data were analyzed using unpaired, two-tailed t-tests. *Prism* software was used for statistical analysis (GraphPad Inc, USA). Data are presented as means $\pm$ s.e.m.

To quantify changes in localization and colocalization we randomly selected 4 ROIs (regions of interest) per cell to calculate fractions and then calculated the average of three different cells from independently repeated experiments.

Discussion

The non-ligand-bound states of hGHR and hPRLR have been extensively investigated. Pre-homodimerization of hGHR and hPRLR has been reported using structural and biochemical methods [40, 101–104]. At the same time, a recent study supports the notion that GHR, at physiological densities, exists as monomers on the cell surface and becomes activated by a ligand-induced dimerization [105]. The current study uses a highly precise and sensitive single-molecule localization microscopy approach to study the formation of hGHR and hPRLR nanoclusters and the cell surface receptor availability. Our super-resolution images reveal that in human T47D breast cancer cells and the γ 2A-JAK2 cell exogenous expression system, the cluster size of hGHR and hPRLR in the basal state range from a few localizations per cluster up to a thousand localizations per cluster.

With GH or PRL treatment, the number of hGHR on both T47D and γ 2A-JAK2 cell surfaces is decreased, indicating the removal of surface hGHR. Moreover, the distribution curve of hGHR shifts toward larger cluster sizes (Fig. 3), suggesting a ligand-induced aggregation of receptors. In turn, hPRLR numbers dramatically increase on the cell surface in response to ligand

stimulation. The newly presented hPRLR clusters may impact the distribution of cluster sizes, which may explain why the median of hPRLR cluster sizes does not change much with ligand treatment.

Our PL assay data indicate that distances between hGHR and hPRLR are small (less than 40nm). This implies that hGHR and hPRLR form co-localized clusters in unstimulated states, suggesting hGHR and hPRLR are physically accessible to one other. With GH stimulation, the fraction of co-localized receptors is decreased. Given that GH treatment enhances the coimmunoprecipitation of total cellular hGHR and hPRLR [68], the decrease in co-localized surface receptors can likely be attributed to removing co-localized receptors from the cell surface.

It is well documented that hPRLR is engaged by both PRL and GH, while hGHR only responds to GH [41, 43, 44]. Unexpectedly, we found that PRL induces a down-regulation of cell surface hGHR in cells that co-express hGHR and hPRLR (**Fig. 2**), indicating that hPRLR directly or indirectly interacts with hGHR. Interestingly, PRL was unable to induce hGHR down-regulation in cells either lacking hPRLR or expressing hPRLR without its Box1 region (hPRLR-tr238 or hPRLR-ΔBox1), suggesting that JAK2 and PRLR association is required for hGHR-hPRLR interaction that in turn allows PRL-induced downregulation of cell surface hGHR. Notably, previous work showed that GHR and JAK2 association is necessary for JAK2 to stabilize cell surface GHR and inhibit constitutive GHR down-regulation [80]. Furthermore, single-particle tracking studies showed that, in the presence of JAK2, a higher level of ligand-induced dimerization of GHR was observed [105]. Moreover, JAK2 with intact kinase activity is required for GH-induced GHR down-regulation [80]. Similarly, the box1 region of PRLR also associates with JAK2, and deletion or modulation of the last proline residue of box1 abrogates PRLR function [106]. Collectively, these findings suggest that the box1 regions in hGHR and hPRLR play a crucial role in the transduction of the individual signaling cascades and hGHR-hPRLR association.

Here we found that, in the resting state, the degree of hGHR-hPRLR colocalization is higher in cells expressing hPRLR with the box1 motif. This suggests JAK2 may not only stabilize GHR but also support the formation of hGHR-hPRLR-containing clusters. Previously, it has been suggested that such clusters are comprised of hGHR homodimers and hPRLR homodimers that together form (hGHR-hGHR) – (hPRLR-hPRLR) hetero-multimers or higher order oligomers [69]. Indeed, because the intracellular domains (ICD) of both hGHR and hPRLR are highly disordered, the flexibility of their ICDs may provide room for recruiting JAK2 and stabilizing the hGHR-hPRLR association [107, 108]. Moreover, a study of the crystal structure of erythropoietin receptor (EPOR) and leptin receptor (LEPR), which also belong to the class I cytokine receptor family, revealed recently that JAK2/EPOR and JAK2/LEPR complexes contained four JAK2 and four EPOR or LEPR molecules, respectively [109]. Hence, it is possible that hGHR-hGHR homodimers and hPRLR-hPRLR homodimers form complexes in a similar fashion (**Fig. 8**). We propose that this JAK2/Box1-mediated interaction of receptors is not limited to hGHR and hPRLR but may generalize to other receptors of the class I cytokine receptor family. We note, however, that determinants within particular receptors (perhaps residing in their extracellular and/or transmembrane domains) and their intracellular JAK2 association domains may facilitate, to varying degrees, their propensity to form multimeric aggregates. GHR and PRLR, for example, may tend to do so more avidly with each other than either one does with other cytokine receptors. Additional studies are required to determine and understand the modes and functions of the hGHR-hPRLR association.

An obvious question is to what extent our observed regulation is biologically relevant. While our work does not provide an answer to this question, one could argue that, generally speaking, such a mechanism could provide a means for crosstalk and feedback between the two cytokine receptors and allow for a more fine-tuned signaling response. In particular high levels of PRL could reduce GH signaling thereby prioritizing PRL's function over GHR-mediated growth processes. PRLR's ability to modulate GHR cell surface availability might also contribute to tissue-specific responses to hormonal signals. For instance, in situations with high PRL levels in circulation such as during

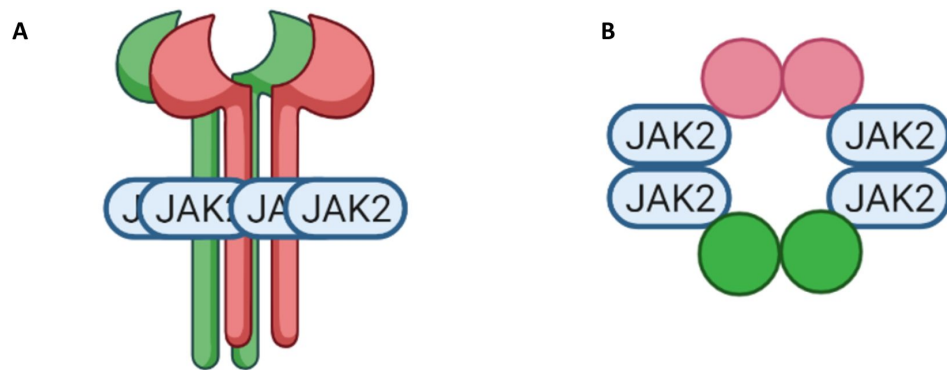


Fig. 8.

Model of JAK2 stabilizing GHR-GHR and PRLR-PRLR heteromers. **(A)** Schematic representation of GHRs homodimer and PRLRs homodimer form a heteromer, which contains two PRLRs (red), two GHRs (green), and four JAK2 (blue). **(B)** A top view of the JAK2/PRLR/GHR multimer.

lactation [110], downregulation of mammary gland cell surface GHRs could prevent GH from counteracting PRL's effects. Also, PRL and GH have important roles in metabolism, often with opposing effects [2, 111–113]. PRL promotes lipogenesis and energy storage, while GH promotes lipolysis and energy mobilization. By downregulating GHRs, PRL could shift the metabolic balance in favor of energy storage. Moreover, this regulation may also have disease implications: hPRL/hPRLR-mediated downregulation of cell surface hGHR could be a relevant factor in the observed anti-tumor effect of PRLR signaling, especially in triple-negative breast cancer (TNBC) expressing PRLR but lacking other interfering hormone receptors. As previously shown, the TNBC cell lines MDA-MB-231 and MDA-MB-453 show significantly increased GHR [15] while expressing no or only endogenous levels of PRLR, respectively [28]. PRL treatment significantly decreased cell viability and invasive capacity of MDA-MB-231 cells following the restoration of PRLR expression. In MDA-MB-453 cells, PRL caused a significant reduction in cell viability. A xenograft model with inoculated MDA-MB-453 cells confirmed the growth inhibitory effect of PRL treatment *in vivo*, suggesting PRLR expression as an indicator of a favorable prognosis. However, several studies have reported the contrary, namely that PRL promotes breast cancer [12], presenting a conundrum that so far has not been resolved. Among the observations supporting a role of PRL in promoting breast cancer are: (1) frequent PRLR overexpression in BC tissues, (2) association of elevated (serum) PRL with increased BC risk – in particular for estrogen receptor (ER) positive BC – and increased risk for metastatic progression from epidemiologic data, and (3) PRL stimulation modulating the cytoskeleton and inducing motility of human breast cancer cells (including T47D) *in vitro*, which has been associated with mammary carcinoma progression *in vivo* [12]. Of note, our hypothesis of PRL-mediated down-regulation of GHR presence on the cell surface being protective does not mean that other effects cannot also be involved or cancel this positive effect out. Moreover, timing could be a decisive element: while PRL might have a protective effect (depending on other factors) at the time of tumorigenesis, it may actually facilitate BC metastasis. But given the lack of experimental data indicating a clear protective effect through Jak2-mediated receptor interactions in PRLR+ GHR+ BC at any BC stage, our hypothesis remains speculative.

Besides, observations in MCF-7, a human breast cancer cell line, where binding of PRL causes internalization and downregulation of PRLR expression seem to contradict our observations in T47D cells [114]. Recently, a competition between two kinases (JAK2 and LYN) for binding hGHR at the box1-box2 region was reported [115], indicating that hGHR nanocluster formation at the cell membrane is regulated also by kinases. It is conceivable that similar kinase-dependent competition mechanisms could regulate also PRLR cell surface availability. Thus, differences in cells' expression of such kinases could then play a role in the perceived inconsistency. Moreover, [114] focuses on the downregulation of the long PRLR isoform in response to PRL, while all other isoforms were undetectable in MCF-7 cells. So, this and other differences between the cell lines may be the reason for the perceived contradiction.

We note that a limitation of our study is the focus on T47D and γ 2A-JAK2 cells. It remains to be seen, if we can observe and replicate the same effect also in other cell lines, for instance mammary epithelial cells or other hGHR+ PRLR+ cancer cell lines. Also, the roles of the different membrane PRLR isoforms have not been fully addressed and our study lacks isoform specific data [37]. The anti-PRLR antibody that we used does not only recognize the long form PRLR but also the intermediate form and the short forms, all of which have ECDs. All these forms contain the box1 motif. Importantly, form S1b is almost identical to hPRLR-tr292, but behaves similarly to LF (i.e. our hPRLR WT) in terms of normalized GHR cell surface localization and PRLR co-localization (Fig. 6). So, while we do not have experimental confirmation, we would expect that these other isoforms behave somewhat similarly to LF with respect to GHR surface membrane interactions – while still inducing different downstream effects as well as playing different roles in breast cancer [37, 116–118].

As part of future research, we plan to study the observed effect in cell lines other than T47D. We would also like to investigate if the mechanism is PRLR-GHR specific, or if there are other cytokine receptors that can interact in a similar asymmetric, ligand-dependent manner, such that one regulates the other receptor's surface availability. Importantly, we would like to elucidate any biological relevance of the observed effect, analyze roles and consequences of PRLR isoforms, and investigate our hypothesis that PRLR-mediated downregulation of cell surface GHR contributed to the anti-tumoral effect of PRL treatment in PRLR-positive TNBC.

Additional files

Supplementary Material [↗](#)

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Reviewer #3 (Public review):

Summary:

The authors are interested in the relative importance of PRL versus GH and their interactive signaling in breast cancer. After examining GHR-PRLR interactions in response to ligands, they suggest that a reduction in cell surface GHR in response to PRL may be a mechanism whereby PRL can sometimes be protective against breast cancer.

Strengths:

The strengths of the study include the interesting question being addressed and the application of multiple complementary techniques, including dSTORM, which is technically very challenging, especially when using double labeling. Thus, dSTORM is used to analyze co-clustering of GHR and PRLR, and, in response to PRL, rapid internalization of GHR and increased cell surface PRLR. Conclusions from Proximity ligation assays are that some GHR and PRLR are within 40 nm ($\approx$ 4 plasma membranes) of each other and that upon ligand stimulation, they move apart. Intact receptor knockin and knockout approaches and receptor constructs without the Jak2 binding domain demonstrate a) a requirement for the PRLR for there to be PRL- driven internalization of GHR, and b) that Jak2-PRLR interactions are necessary for stability of the GHR-PRLR colocalizations.

Weaknesses:

Although improved over the first version, the manuscript still suffers from a lack of detail, which in places makes it difficult to evaluate the data and would make it very difficult for the results to be replicated by others.

Comments on revised version:

Points for improvement of the manuscript:

(1) There is still insufficient detail about the proximity ligation assay. For example, PLAs that use reagents from Sigma (as now reported) require primary antibodies from two different species and yet both the anti-PRLR and anti-GHR used for dSTORM were mouse monoclonals. On line 356 it says that the ECD antibodies were used for microscopy and the PLA is microscopy. Were instead the ICD antibodies used for the PLA? If so, how do we know that one or more of the proteins in the very strong "non-specific" bands seen on Figure 5A are not what is being localized? Could you do a Western blot of just cell membrane proteins? There needs to be further clarity/explanation.

(2) Although the manuscript now shows a Western blot using the antibodies against intracellular regions of the receptor, a full Western blot is not provided for the antibodies against the S2 extracellular domain used for the dSTORM. While I haven't checked the papers showing characterization of the anti-GHR, I did re-check reference 70, which the authors say shows full characterization of the PRLR antibody, and this does not show a full Western (only portions of gels). How do we know that this antibody is not recognizing some other cell surface molecule, the surface expression of which increases upon stimulation of the cells with PRL? Is there only one band when blotting whole cell extracts with either the GHR or PRLR ECD antibodies so we can be sure of specificity? Figure S2 helps some, but these are different cells and the relative expression of the PRLR versus some other potential cell surface protein in these engineered cells may well be completely different.

Author response:

The following is the authors' response to the original reviews.

Reviewer #1 (Public Review):

(1) The questions after reading this manuscript are what novel insights have been gained that significantly go beyond what was already known about the interaction of these receptors and, more importantly, what are the physiological implications of these findings? The proposed significance of the results in the last paragraph of the Discussion section is speculative since none of the receptor interactions have been investigated in TNBC cell lines. Moreover, no physiological experiments were conducted using the PRLR and GH knockout T47D cells to provide biological relevance for the receptor heteromers. The proposed role of JAK2 in the cell surface distribution and association of both receptors as stated in the title was only derived from the analysis of box 1 domain receptor mutants. A knockout of JAK2 was not conducted to assess heteromers formation.

We thank the reviewer for these comments. The novel insight is that two different cytokine receptors can interact in an asymmetric, ligand-dependent manner, such that one receptor regulates the other receptor's surface availability, mediated by JAK2. To our knowledge this has not been reported before. Beyond our observations, there is the question if this could be a much more common regulatory mechanism and if it has therapeutic relevance. However, answering these questions is beyond the scope of this work.

Along the same line, the question regarding the biological relevance of our receptor heteromers and JAK2's role in cell surface distribution is undoubtedly very important. Studying GHR-PRLR cell surface distributions in JAK2 knockout cells and certain TNBC cell lines as proposed by the reviewer could perhaps be insightful. However, most TNBCs down-regulate PRLR [1], so we would first have to identify TNBC cell lines that actually express PRLR at sufficiently high levels. Moreover, knocking out JAK2 is known to significantly reduce GHR surface availability [2,3], such that the proposed experiment would probably provide only limited insights.

Unfortunately, our team is currently not in the position to perform any experiments (due to lack of funding and shortage of personnel). However, to address the reviewer's comment as much as possible, we have revised the respective paragraph of the discussion section to emphasize the speculative nature of our statement and have added another paragraph discussing shortcoming and future experiments (see revised manuscript, pages 23-24).

(1) López-Ozuna, V., Hachim, I., Hachim, M. *et al.* Prolactin Pro-Differentiation Pathway in Triple Negative Breast Cancer: Impact on Prognosis and Potential Therapy. *Sci Rep* 6, 30934 (2016). <https://www.nature.com/articles/srep30934>

(2) He, K., Wang, X., Jiang, J., Guan, R., Bernstein, K.E., Sayeski, P.P., Frank, S.J. Janus kinase 2 determinants for growth hormone receptor association, surface assembly, and signaling. *Mol Endocrinol.* 2003;17(11):2211-27. doi: 10.1210/me.2003-0256. PMID: 12920237.

(3) He, K., Loesch, K., Cowan, J.W., Li, X., Deng, L., Wang, X., Jiang, J., Frank, S.J. Janus Kinase 2 Enhances the Stability of the Mature Growth Hormone Receptor, *Endocrinology*, Volume 146, Issue 11, 2005, Pages 4755–4765, <https://doi.org/10.1210/en.2005-0514>

(2) Except for some investigation of γ 2A-JAK2 cells, most of the experiments in this study were conducted on a single breast cancer cell line. In terms of rigor and reproducibility,

this is somewhat borderline. The CRISPR/Cas9 mutant T47D cells were not used for rescue experiments with the corresponding full-length receptors and the box1 mutants. A missed opportunity is the lack of an investigation correlating the number of receptors with physiological changes upon ligand stimulation (e.g., cellular clustering, proliferation, downstream signaling strength).

We appreciate the reviewer's comments. While we are confident in the reproducibility of our findings, including those obtained in the T47D cell line, we acknowledge that testing in additional cell lines would have strengthened the generalizability of our results. We also recognize that performing a rescue experiment using our T47D hPRLR or hGHR KO cells would have been valuable. Furthermore, examining physiological changes, such as proliferation rates and downstream signaling responses, would have provided additional insights. Unfortunately, these experiments were not conducted at the time, and we currently lack the resources to carry them out.

(3) An obvious shortcoming of the study that was not discussed seems to be that the main methodology used in this study (super-resolution microscopy) does not distinguish the presence of various isoforms of the PRLR on the cell surface. Is it possible that the ligand stimulation changes the ratio between different isoforms? Which isoforms besides the long form may be involved in heteromers formation, presumably all that can bind JAK2?

This is a very good point. We fully agree with the reviewer that a discussion of the results in the light of different PRLR isoforms is appropriate. We have added information on PRLR isoforms to the Introduction (see revised manuscript, page 2) and Discussion sections (see revised manuscript, pages 23-24).

(4) Changes in the ligand-inducible activation of JAK2 and STAT5 were not investigated in the T47D knockout models for the PRL and GHR. It is also a missed opportunity to use super-resolution microscopy as a validation tool for the knockouts on the single cell level and how it might affect the distribution of the corresponding other receptor that is still expressed.

We thank the reviewer for his comment. We fully agree that such additional experiments could be very valuable. We are sorry but, as already mentioned above, this is not something we are able to address at this stage due to lack of personnel and funding. However, we do hope to address these and other proposed experiments in the future.

(5) Why does the binding of PRL not cause a similar decrease (internalization and downregulation) of the PRLR, and instead, an increase in cell surface localization? This seems to be contrary to previous observations in MCF-7 cells (J Biol Chem. 2005 October 7; 280(40): 33909-33916).

It has been recently reported for GHR that not only JAK2 but also LYN binds to the box1-box2 region, creating competition that results in divergent signaling cascades and affects GHR nanoclustering [1]. So, it is reasonable to assume that similar mechanisms may be at work that regulate PRLR cell surface availability. Differences in cells' expression of such kinases could perhaps play a role in the perceived inconsistency. Also, Lu et al. [2] studied the downregulation of the long PRLR isoform in response to PRL. All other PRLR isoforms were not detectable in MCF-7 cells. So, differences between MCF-7 and T47D may lead to this perceived contradiction.

At this stage, we can only speculate about the actual reasons for these seemingly contradictory results. However, for full transparency, we are now mentioning this apparent

contradiction in the Discussion section (see page 23) and have added the references below.

(1) Chhabra, Y., Seiffert, P., Gormal, R.S., *et al.* Tyrosine kinases compete for growth hormone receptor binding and regulate receptor mobility and degradation. *Cell Rep.* 2023;42(5):112490. doi: 10.1016/j.celrep.2023.112490. PMID: 37163374.

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(2) Lu, J.C., Piazza, T.M., Schuler, L.A. Proteasomes mediate prolactin-induced receptor down-regulation and fragment generation in breast cancer cells. *J Biol Chem.* 2005 Oct 7;280(40):33909-16. doi: 10.1074/jbc.M508118200. PMID: 16103113; PMCID: PMC1976473.

(6) Some figures and illustrations are of poor quality and were put together without paying attention to detail. For example, in Fig 5A, the GHR was cut off, possibly to omit other nonspecific bands, the WB images look 'washed out'. 5B, 5D: the labels are not in one line over the bars, and what is the point of showing all individual data points when the bar graphs with all annotations and SD lines are disappearing? As done for the y2A cells, the illustrations in 5B-5E should indicate what cell lines were used. No loading controls in Fig 5F, is there any protein in the first lane? No loading controls in Fig 6B and 6H.

We thank the reviewer for pointing this out. We have amended Fig. 5A to now show larger crops of the two GHR and PRLR Western Blot images and thus a greater range of proteins present in the extracts. Please note that the bands in the WBs other than what is identified as GHR and PRLR are non-specific and reflect roughly equivalent loading of protein in each lane.

We also made some changes to Figures 5B-5E.

(7) The proximity ligation method was not described in the M&M section of the manuscript.

We thank the reviewer for pointing this out. We have added a description of the PL method to the Methods section.

Reviewer #1 (Recommendations for the Authors):

A final suggestion for future investigations: Instead of focusing on the heteromer formation of the GHR/PRLR which both signal all through the same downstream effectors (JAK2, STAT5), it would have been more cancer-relevant, and perhaps even more interesting, to look for heteromers between the PRLR and receptors of the IL-6 family since it had been shown that PRL can stimulate STAT3, which is a unique feature of cancer cells. If that is the case, this would require a different modality of the interaction between different JAK kinases.

We highly appreciate the reviewer's recommendation and hope to follow up on it in the near future.

Reviewer #2 (Public Review):

(1) I could not fully evaluate some of the data, mainly because several details on acquisition and analysis are lacking. It would be useful to know what the background signal was in dSTORM and how the authors distinguished the specific signal from unspecific background fluorescence, which can be quite prominent in these experiments. Typically, one would evaluate the signal coming from antibodies randomly bound to a substrate around the cells to determine the switching properties of the dyes in their

buffer and the average number of localisations representing one antibody. This would help evaluate if GHR or PRLR appeared as monomers or multimers in the plasma membrane before stimulation, which is currently a matter of debate. It would also provide better support for the model proposed in Figure 8.

We are grateful for the reviewer's comment. In our experience, the background signal is more relevant in dSTORM when imaging proteins that are located at deeper depths ($> 3 \mu\text{m}$) above the coverslip surface. In our experiments, cells are attached to the coverslip surface and the proteins being imaged are on the cell membrane. In addition, we employed dSTORM's TIRF (total internal reflection fluorescence) microscopy mode to image membrane receptor proteins. TIRFM exploits the unique properties of an induced evanescent field in a limited specimen region immediately adjacent to the interface between two media having different refractive indices. It thereby dramatically reduces background by rejecting fluorescence from out-of-focus areas in the detection path and illuminating only the area right near the surface.

Having said that, a few other sources such as auto-fluorescence, scattering, and non-bleached fluorescent molecules close to and distant from the focal plane can contribute to the background signal. We tried to reduce auto-fluorescence by ensuring that cells are grown in phenol-red-free media, imaging is performed in STORM buffer which reduces autofluorescence, and our immunostaining protocol includes a quenching step aside from using blocking buffer with different serum, in addition to BSA. Moreover, we employed extensive washing steps following antibody incubations to eliminate non-specifically bound antibodies. Ensuring that the TIRF illumination field is uniform helps reduce scatter. Additionally, an extended bleach step prior to the acquisition of frames to determine localizations helped further reduce the probability of non-bleached fluorescent molecules.

In short, due to the experimental design we do not expect much background. However, in the future, we will address this concern and estimate background in a subtype dependent manner. To this end we will distinguish two types of background noise: (A) background with a small change between subsequent frames, which mainly consists of auto-fluorescence and non-bleached out-of-focus fluorescent molecules; and (B) background that changes every imaging frame, which is mainly from non-bleached fluorescent molecules near the focal plane. For type (A) background, temporal filters must be used for background estimation [1]; for type (B) background, low-pass filters (e.g., wavelet transform) should be used for background estimation [2].

(1) Hoogendoorn, Crosby, Leyton-Puig, Breedijk, Jalink, Gadella, and Postma (2014). The fidelity of stochastic single-molecule super-resolution reconstructions critically depends upon robust background estimation. Scientific reports, 4, 3854. <https://doi.org/10.1038/srep03854>

(2) Patel, Williamson, Owen, and Cohen (2021). Blinking statistics and molecular counting in direct stochastic reconstruction microscopy (dSTORM). Bioinformatics, Volume 37, Issue 17, September 2021, Pages 2730–2737, <https://doi.org/10.1093/bioinformatics/btab136>

(2) Since many of the findings in this work come from the evaluation of localisation clusters, an image showing actual localisations would help support the main conclusions. I believe that the dSTORM images in Figures 1 and 2 are density maps, although this was not explicitly stated. Alexa 568 and Alexa 647 typically give a very different number of localisations, and this is also dependent on the concentration of BME. Did the authors take that into account when interpreting the results and creating the model in Figures 2 and 8?

I believe that including this information is important as findings in this paper heavily rely on the number of localisations detected under different conditions.

Including information on proximity labelling and CRISPR/Cas9 in the methods section would help with the reproducibility of these findings by other groups.

Figures 1 and 2 show Gaussian interpolations of actual localizations, not density maps. Imaging captured the fluorophores' blinking events and localizations were counted as true localizations, when at least 5 consecutive blinking events had been observed. Nikon software was used for Gaussian fitting. In other words, we show *reconstructed* images based on identifying true localizations using gaussian fitting and some strict parameters to identify true fluorophore blinking. This allowed us to identify true localizations with high confidence and generate a high-resolution image for membrane receptors.

Indeed, Alexa 568 and 647 give different numbers of localization. This is dependent on the intrinsic photo-physics of the fluorophores. Specifically, each fluorophore has a different duty cycle, switching cycle, and survival fraction. However, we note that we focused on capturing the relative changes in receptor numbers over time, before and after stimulation by ligands, not the absolute numbers of surface GHR and PRLR. We are not comparing the absolute numbers of localizations or drawing comparisons for localization numbers between 568 and 647. For all these different conditions/times, the photo-physics for a particular fluorophore remains the same. This allows us to make relative comparisons.

As far as the effect of BME is concerned, the concentration of mercaptoethanol needs to be carefully optimized, as too high a concentration can potentially quench the fluorescence or affect the overall stability of the sample. However, we are using an optimized concentration which has been previously validated across multiple STORM experiments. This makes the concerns relating to the concentration of BME irrelevant to the current experimental design. Besides, the concentration of BME is maintained across all experimental conditions.

We have added information regarding PL and CRISPR/Cas9 for generating hGHR KO and hPRLR KO cells in two new subsections to the Methods section.

Reviewer #2 (Recommendations for the authors):

In the methods please include:

(1) A section with details on proximity ligation assays.

We have added a description of the PL method to the Methods section.

(2) A section on CRISPR/Cas9 technology.

We have added two new sections on “Generating hGHR knockout and hPRLR knockout T47D cells” and “Design of sgRNAs for hGHR or hPRLR knockout” to the Methods section.

(3) List the precise composition of the buffer or cite the paper that you followed.

We used the buffer recipe described in this protocol [1] and have added the components with concentrations as well as the following reference to the manuscript.

(1) Beggs, R.R., Dean, W.F., Mattheyses, A.L. (2020). dSTORM Imaging and Analysis of Desmosome Architecture. In: Turksen, K. (eds) Permeability Barrier. Methods in Molecular Biology, vol 2367. Humana, New York, NY. https://doi.org/10.1007/978107160325_325

(4) Exposure time used for image acquisition to put 40 000 frames in the context of total imaging time and clarify why you decided to take 40 000 images per channel.

Our Nikon Ti2 N-STORM microscope is equipped with an iXon DU-897 Ultra EMCCD camera from Andor (Oxford Instruments). According to the camera's manufacturer, this camera platform uses a back-illuminated 512 x 512 frame transfer sensor and overlocks readout to 17 MHz, pushing speed performance to 56 fps (in full frame mode). We note that we always tried to acquire STORM images at the maximal frame rate. As for the exposure time, according to the manufacturer it can be as short as 17.8 ms. We would like to emphasize that we did not specify/alter the exposure time.

See also: <https://andor.oxinst.com/assets/uploads/products/andor/documents/andor-ixon-ultra-emccd-specifications.pdf>

The decision to take 40,000 images per frame was based on our intention to identify the true population of the molecules of interest that are localized and accurately represented in the final reconstruction image. The total number of frames depends on the sample complexity, density of sample labeling and desired resolution. We tested a range of frames between 20,000 and 60,000 and found for our experimental design and output requirements that 40,000 frames provided the best balance between achieving maximal resolution and desired localizations to make consistent and accurate localization estimates across different stimulation conditions compared to basal controls.

(5) The lasers used to switch Alexa 568 and Alexa 647. Were you alternating between the lasers for switching and imaging of dyes? Intermittent and continuous illumination will produce very different unspecific background fluorescence.

Yes, we used an alternating approach for the lasers exciting Alexa 647 and Alexa 568, for both switching and imaging of the dyes.

(6) A paragraph with a detailed description of methods used to differentiate the background fluorescence from the signal.

We have addressed the background fluorescence under Point 1 (Public Review). We have added a paragraph in the Methods section on this issue.

(7) Minor corrections to the text:

It appears as though there is a large difference in the expression level of GHR and PRLR in basal conditions in Figure 1. This can be due to the switching properties of the dyes, which is related to the amount of BME in the buffer, or it can be because there is indeed more PRL. Would the authors be able to comment on this?

We thank the reviewer for this suggestions. According to expression data available online there is indeed more PRLR than GHR in T47D cells. According to CellMiner [1], T47D cells have an RNA-Seq gene expression level $\log_2(\text{FPKM} + 1)$ of 6.814 for PRLR, and 3.587 for GHR, strongly suggesting that there is more PRLR than GHR in basal conditions, matching the reviewer's interpretation of our images in Fig. 1 (basal). However, we would advise against using STORM images for direct comparisons of receptor expression. First, with TIRF images, we are only looking at the membrane fraction (~150 nm close to the coverslip membrane interface) that is attached to the coverslip. Secondly, as discussed above, our data represent relative cell surface receptor levels that allow for comparison of different conditions (basal vs. stimulation) and does not represent absolute quantifications. Everything is relative and in comparison to controls.

Also, BME is not going to change the level of expression. The differences in growth factor expression as estimated by relative comparison can be attributed to the actual changes in growth factors and is not an artifact of the amount of BME in the buffer or the properties of

dyes. These factors are maintained across all experimental conditions and do not influence the final outcome.

(1) <https://discover.nci.nih.gov/cellminer/>

(8) I would encourage the authors to use unspecific binding to characterize the signal coming from single antibodies bound to the substrate. This would provide a mean number of localizations that a single antibody generates. With this information, one can evaluate how many receptors there are per cluster, which would strengthen the findings and potentially provide additional support for the model presented in Figure 8. It would also explain why the distributions of localisations per cluster in Fig. 3B look very different for hGHR and hPRLR. As the authors point out in the discussion, the results on predimerization of these receptors in basal conditions are conflicting and therefore it is important to shed more light on this topic.

We thank the reviewer for this suggestions. While we are unable to perform this experiment at this stage, we will keep it in mind for future experiments.

(9) Minor corrections to the figures:

Figure 1:

In the legend, please say what representation was used. Are these density maps or another representation? Please provide examples of actual localisations (either as dots or crosses representing the peaks of the Gaussians). Most findings of this work rely on the characterisation of the clusters of localisations and therefore it is of essence to show what the clusters look like. This could potentially go to the supplemental info to minimise additional work. It's very hard to see the puncta in this figure.

If the authors created zoomed regions in each of the images (as in Figure 3), it would be much easier to evaluate the expression level and the extent of colocalisation. Halfway through GHR 3 min green pixels become grey, but this may be the issue with the document that was created. Please check. Either increase the font on the scale bars in this figure or delete it.

As described above, Figure 1 does not show density maps. Imaging captured the fluorophores' blinking events and localizations were counted as true localizations, when at least 5 consecutive blinking events had been observed. Nikon software was used for Gaussian fitting and smoothing.

We have generated zoomed regions. In our files (original as well as pdf) we do not see pixels become grey. We increased the font size above one of the scale bars and removed all others.

Figure 3:

In A, the GHR clusters are colour coded but PRLR are not. Are both DBSCAN images? Explain the meaning of colour coding or show it as black and white. Was brightness also increased in the PRLR image? The font on the scale bars is too small. In B, right panels, the font on the axes is too small. In the figure legend explain the meaning of 33.3 and 16.7

In our document, both GHR and PRLR are color coded but the hGHR clusters are certainly bigger and therefore appear brighter than the hPRLR clusters. Both are DBSCAN images. The color coding allows to distinguish different clusters (there is no other meaning). We have kept the color-coding but have added a sentence to the caption addressing this. Brightness was increased in both images of Panel B equally. 33.3 and 16.7 are the median cluster sizes. We

have added a sentence to the caption explaining this. We have increased the font on the axes in B (right panels).

Figure 4:

I struggled to see any colocalization in the 2nd and the 3rd image. Please show zoomed-in sections. In the panels B and C, the data are presented as fractions. Is this per cell? My interpretation is that ~80% of PRL clusters also contain GHR.

Is this in agreement with Figures 1 and 2? In Figure 1, PRL 3 min, Merge, colocalization seems much smaller. Could the authors give the total numbers of GHR and PRLR from which the fractions were calculated at least in basal conditions?

We have provided zoom-in views. As for panels B and C, fractions are number of clusters containing both receptors divided by the total number of clusters. We used the same strategy that we had used for calculating the localization changes: We randomly selected 4 ROIs (regions of interest) per cell to calculate fractions and then calculated the average of three different cells from independently repeated experiments. We did not calculate total numbers of GHR/PRLR. The numbers are fractions of cluster numbers.

Moreover, the reviewer interprets results in panels B and C that ~80% of PRLR clusters also contain GHR. We assume the reviewer refers to Basal state. Now, the reviewer's interpretation is not correct for the following reason: ~80% of clusters have both receptors. How many of the remaining (~20%) clusters have only PRLR or only GHR is not revealed in the panels. Only if 100% of clusters have PRLR, we can conclude that 80% of PRLR clusters also contain GHR.

Also, while Figures 1 and 2 show localization based on dSTORM images, Figure 3 indicates and quantifies co-localization based on proximity ligation assays following DBSCAN analysis using Clus-DoC. We do not think that the results are directly comparable.

Reviewer #3 (Public Review):

(1) The manuscript suffers from a lack of detail, which in places makes it difficult to evaluate the data and would make it very difficult for the results to be replicated by others. In addition, the manuscript would very much benefit from a full discussion of the limitations of the study. For example, the manuscript is written as if there is only one form of the PRLR while the anti-PRLR antibody used for dSTORM would also recognize the intermediate form and short forms 1a and 1b on the T47D cells. Given the very different roles of these other PRLR forms in breast cancer (Dufau, Vonderhaar, Clevenger, Walker and other labs), this limitation should at the very least be discussed. Similarly, the manuscript is written as if Jak2 essentially only signals through STAT5 but Jak2 is involved in multiple other signaling pathways from the multiple PRLRs, including the long form. Also, while there are papers suggesting that PRL can be protective in breast cancer, the majority of publications in this area find that PRL promotes breast cancer. How then would the authors interpret the effect of PRL on GHR in light of all those non-protective results? [Check papers by Hallgeir Rui]

We thank the reviewer for such thoughtful comments. We have added a paragraph in the Discussion section on the limitations of our study, including sole focus on T47D and γ 2A-JAK2 cells and lack of PRLR isoform-specific data. Also, we are now mentioning that these isoforms play different roles in breast cancer, citing papers by Dufau, Vonderhaar, Clevenger, and Walker labs.

We did not mean to imply that JAK2 signals only via STAT5 or by only binding the long form. We have made this point clear in the Introduction as well as in our revised Discussion

section. Moreover, we have added information and references on JAK2 signaling and PRLR isoform specific signaling.

In our Discussions section we are also mentioning the findings that PRL is promoting breast cancer. We would like to point out that it is well perceivable that PRL is protective in BC by reducing surface hGHR availability but that this effect may depend on JAK2 levels as well as on expression levels of other kinases that competitively bind Box1 and/or Box2 [1]. Besides, could it not be that PRL's effect is BC stage dependent? In any case, we have emphasized the speculative nature of our statement.

(1) Chhabra, Y., Seiffert, P., Gormal, R.S., *et al.* Tyrosine kinases compete for growth hormone receptor binding and regulate receptor mobility and degradation. *Cell Rep.* 2023;42(5):112490. doi: 10.1016/j.celrep.2023.112490. PMID: 37163374.

Reviewer #3 (Recommendations for the authors):

Points for improvement of the manuscript:

(1) Method details -

a) "we utilized CRISPR/Cas9 to generate hPRLR knockout T47D cells" Exactly how? Nothing is said under methods. Can we be sure that you knocked out the whole gene?

We have addressed this point by adding two new sections on “Generating hGHR knockout and hPRLR knockout T47D cells” and “Design of sgRNAs for hGHR or hPRLR knockout” to the Methods section.

b) Some of the Western blots are missing mol wt markers. How specific are the various antibodies used for Westerns? For example, the previous publications are quoted as providing characterization of the antibodies also seem to use just band cutouts and do not show the full molecular weight range of whole cell extracts blotted. Anti-PRLR antibodies are notoriously bad and so this is important.

There is an antibody referred to in Figure 5 that is not listed under "antibodies" in the methods.

We have modified Figure 5a, showing the entire gel as well as molecular weight markers. As for specificity of our antibodies, we used monoclonal antibodies Anti-GHR-ext-mAB 74.3 and Anti-PRLR-ext-mAB 1.48, which have been previously tested and used. In addition, we did our own control experiments to ensure specificity. We have added some of our many control results as Supplementary Figures S2 and S3.

We thank the reviewer for noticing the missing antibody in the Methods section. We have now added information about this antibody.

c) There is no description of the proximity ligation assay.

We have addressed this by adding a paragraph on PLA in the Methods section.

d) What is the level of expression of GHR, PRLR, and Jak2 in the gamma2A-JAK2 cells compared to the T47D cells? Artifacts of overexpression are always a worry.

γ 2A-JAK2 cell series are over-expressing the receptors. That's the reason we did not only rely on the observation in γ 2A-JAK2 cell lines but also did the experiment in T47D cell lines.

e) There are no concentrations given for components of the dSTORM imaging buffer. On line 380, I think the authors mean alternating lasers not alternatively.

Thank you. Indeed, we meant alternating lasers. We are referring to [1] (the protocol we followed) for information on the imaging buffer.

(1) Beggs, R.R., Dean, W.F., Mattheyses, A.L. (2020). dSTORM Imaging and Analysis of Desmosome Architecture. In: Turksen, K. (eds) Permeability Barrier. Methods in Molecular Biology, vol 2367. Humana, New York, NY. https://doi.org/10.1007/7651_2020_325

f) In general, a read-through to determine whether there is enough detail for others to replicate is required. 4% PFA in what? Do you mean PBS or should it be Dulbecco's PBS etc., etc.?

We prepared a 4% PFA in PBS solution. We mean Dulbecco's PBS.

(2) There are no controls shown or described for the dSTORM. For example, non-specific primary antibody and second antibodies alone for non-specific sticking. Do the second antibodies cross-react with the other primary antibody? Is there only one band when blotting whole cell extracts with the GHR antibody so we can be sure of specificity?

We used monoclonal antibodies Anti-GHR-ext-mAB 74.3 and Anti-PRLR-ext-mAB 1.48 (but also tested several other antibodies). While these antibodies have been previously tested and used, we performed additional control experiments to ensure specificity of our primary antibodies and absence of non-specific binding of our secondary antibodies. We have added some of our many control results as Supplementary Figures S2 and S3.

(3) Writing/figures-

a) As discussed in the public review regarding different forms of the PRLR and the presence of other Jak2-dependent signaling

We have added paragraphs on PRLR isoforms and other JAK2-dependent signaling pathways to the Introduction. Also, we have added a paragraph on PRLR isoforms (in the context of our findings) to the Discussion section.

b) What are the units for figure 3c and d?

The figures show numbers of localizations (obtained from fluorophore blinking events). In the figure caption to 3C and 3D, we have specified the unit (i.e. counts).

c) The wheat germ agglutinin stains more than the plasma membrane and so this sentence needs some adjustment.

We thank the reviewer for this comment. We have rephrased this sentence (see caption to Fig. 4).

d) It might be better not to use the term "downregulation" since this is usually associated with expression and not internalization.

While we understand the reviewer's discomfort with the use of the word "downregulation", we still think that it best describes the observed effect. Moreover, we would like to note that in the field of receptorology "downregulation" is a specific term for trafficking of cell surface receptors in response to ligands. That said, to address the reviewer's comment, we are now

using the terms “cell surface downregulation” or “downregulation of cell surface [..] receptor” throughout the manuscript in order to explicitly distinguish it from gene downregulation.

e) Line 420 talks about "previous work", a term that usually indicates work from the same lab. My apologies if I am wrong, but the reference doesn't seem to be associated with the authors.

At the end of the sentence containing the phrase “previous work”, we are referring to reference [57], which has Dr. Stuart Frank as senior and corresponding author. Dr. Frank is also a co-corresponding author on this manuscript. While in our opinion, “previous work” does not imply some sort of ownership, we are happy to confirm that one of us was responsible for the work we are referencing.

Reviewing Editor's recommendations:

The reviewers have all provided a very constructive assessment of the work and offered many useful suggestions to improve the manuscript. I'd advise thinking carefully about how many of these can be reasonably addressed. Most will not require further experiments. I consider it essential to improve the methods to ensure others could repeat the work. This includes adding methods for the PLA and including detail about the controls for the dSTORM. The reviewers have offered suggestions about types of controls to include if these have not already been done.

We thank the editor for their recommendations. We have revised the methods section, which now includes a paragraph on PLA as well as on CRISPR/Cas9-based generation of mutant cell lines. We have also added information on the dSTORM buffer to the manuscript. Data of controls indicating antibody specificity (using confocal microscopy) have been added to the manuscript's supplementary material (see Fig. S2 and S3).

I agree with the reviewers that the different isoforms of the prolactin receptor need to be considered. I think this could be done as an acknowledgment and point of discussion.

We have revised the discussions section and have added a paragraph on the different PRLR isoforms, among others.

For Figure 2E, make it clear in the figure (or at least in legend) that the middle line is the basal condition.

We thank the editor for their comment. We have made changes to Fig 2E and have added a sentence to the legend making it clear that the middle depicts the basal condition.

My biggest concern overall was the fact that this is all largely conducted in a single cell line. This was echoed by at least one of the reviewers. I wonder if you have replicated this in other breast cancer cell lines or mammary epithelial cells? I don't think this is necessary for the current manuscript but would increase confidence if available.

We thank the editor for their comment and fully agree with their assessment. Unfortunately, we have not replicated these experiments in other BC cell lines nor mammary epithelial cells but would certainly want to do so in the near future.

<https://doi.org/10.7554/eLife.89890.2.sa0>