Tyrosine kinases compete for growth hormone receptor binding and regulate receptor mobility and degradation

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Highlights
- LYN and JAK2 compete for binding to the Box1-Box2 region of the growth hormone receptor (GHR)
- Competition results in divergent signaling cascades
- LYN/ERK1/2 pathway activation promotes cytokine receptor degradation
- Nanoclustering of GHRs at the membrane is regulated by kinases

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In brief
Chhabra et al. establish a functionally relevant competition between two tyrosine kinases (JAK2/LYN) for the growth hormone receptor at overlapping sites within its intracellular domain. Their findings show that differential kinase binding leads to differential signaling cascades, receptor stabilities, and nanoclustering at the plasma membrane.
Tyrosine kinases compete for growth hormone receptor binding and regulate receptor mobility and degradation

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SUMMARY

Growth hormone (GH) acts via JAK2 and LYN to regulate growth, metabolism, and neural function. However, the relationship between these tyrosine kinases remains enigmatic. Through an interdisciplinary approach combining cell biology, structural biology, computation, and single-particle tracking on live cells, we find overlapping LYN and JAK2 Box1-Box2-binding regions in GH receptor (GHR). Our data implicate direct competition between JAK2 and LYN for GHR binding and imply divergent signaling profiles. We show that GHR exhibits distinct mobility states within the cell membrane and that activation of LYN by GH mediates GHR immobilization, thereby initiating its nanoclustering in the membrane. Importantly, we observe that LYN mediates cytokine receptor degradation, thereby controlling receptor turnover and activity, and this applies to related cytokine receptors. Our study offers insight into the molecular interactions of LYN with GHR and highlights important functions for LYN in regulating GHR nanoclustering, signaling, and degradation, traits broadly relevant to many cytokine receptors.

INTRODUCTION

The growth hormone (GH) receptor (GHR) is a paradigmatic member of the class I family of cytokine receptors, which lack intrinsic kinase activity and therefore depend on associated kinases for signaling.1,2 Ligand binding to cytokine receptors activates associated Janus kinases (JAKs), and the GHR-mediated JAK2 activation has been studied in explicit detail.3–5 However, JAK2 is not the sole tyrosine kinase that can be activated by GHR, as GH-mediated SRC family kinase (SFK) activation is JAK2 independent, as shown both in vitro and in vivo.6,7 Moreover, both kinases are present in many GHR-expressing cell types.8 Ligand-driven SFK activation has been shown for the closely related thrombopoietin receptor (TPOR), erythropoietin receptor (EPOR), and prolactin receptor (PRLR),9–12 as well as for the more distantly related class I cytokine receptors, including IL-2R, IL-6R, and GM-CSFR,13–15 implicating a common relevance of SFK activation. However, how SFKs interact with cytokine receptors and how the interplay between JAK2 and SFK activation affects receptor function remain enigmatic.

In mice with a targeted knockin mutation in the GHR Box1 motif, essential for JAK2 binding, a complete abrogation of GH-mediated JAK2 activation is evident, whereas GH-mediated hepatic SFK and subsequent ERK1/2 activation is maintained.7 Additionally, an engineered mutation in the extracellular domain (ECD) of GHR, designed to restrict the ligand induced conformational changes, shows impaired LYN (an SFK) and downstream ERK1/2 activation, while JAK2-STAT5 signaling is unaffected.6 Similarly, a GHR isoform (d3-GHR) with a deletion of 22 residues in the N terminus shows altered ERK1/2 but unaltered STAT5 signaling and, importantly,
homzygous δ3-GHR individuals have a lifespan increased by ~10 years.\textsuperscript{15} In addition, some human GH (hGH) analogues can weakly activate ERK1/2 compared with STAT5, as reported for the hGH\textsubscript{I179M} variant associated with deficient growth.\textsuperscript{17} Structural changes in the ECD resulting in an altered ratio of GHR and other related cytokine receptors. These findings resolve the enigmatic role of LYN in GHR signaling by uncovering the molecular mechanisms of LYN in GH-mediated signal transduction, a result with direct relevance to the entire cytokine class I receptors.

RESULTS

In the absence of JAK2 interaction, GHR\textsubscript{ΔBox1} shows no P-STAT5 but still activates P-ERK1/2

To investigate GH-mediated SFK activation, we utilized a GHR-ΔBox1 construct where the four prolines in the GHR-wild-type (WT) Box1 motif (PPVPVP) were mutated to alanine (AAVAVA). This construct is incapable of binding to or activating JAK2 and LYN, thus does not initiate STAT5 signaling.\textsuperscript{6,7} Initially, signaling from GHR-WT compared with GHR\textsubscript{ΔBox1} was investigated in the IL-3-dependent cell line, FDC-P1, which lacks endogenous GHR expression but can be made GH dependent upon exogenous GHR expression.\textsuperscript{9} FDC-P1 cells were transfected to express GHR-WT or GHR\textsubscript{ΔBox1} with or without hGH stimulation. Immunoblot analysis of proteins from whole-cell lysate (WCL) also shown. Data representative of three independent experiments.
cells, while P-ERK1/2 was more strongly activated in GHR-WT cells (Figure 1C). No STAT5 activation was evident in GHRΔBox1 cells, confirming that P-SFK activation by GH is JAK2 independent. Our data are congruent with previous findings that the proteasomal degradation of P-SFK and P-ERK1/2 is more rapid than for P-STAT5.24,25

Notably, compared with GHR-WT, expression of GHRΔBox1 showed reduced levels of mature GHR (cell surface levels) but increased levels of precursor GHR (Golgi) in both FDC-P1 and HEK293 cell lines, confirmed by flow cytometry in HEK293 cells (Figure S1). This would be expected due to the known ability of JAK2 to promote GHR trafficking, stability, and half-life on the cell surface,16,27 similarly to the TPOR and EPOR.28–30

Collectively, these data indicate that loss of JAK2 interaction via the GHR Box1 mutation results in loss of GH-mediated JAK2 and STAT5 activation, while GH-mediated SFK and ERK1/2 activation is maintained. In addition, mutation of GHR Box1 leads to a reduced amount of mature GHR, which can be rescued by the proteasomal inhibitor MG132.

GH-activated LYN does not phosphorylate GHR

A key role of JAK2 in GHR signal transduction is the trans-phosphorylation of tyrosines in the receptor intracellular domain (ICD) serving as docking sites for STAT5 and other signaling proteins. To evaluate if LYN can phosphorylate GHR, we used three approaches, first by overexpressing LYN in GHR-WT cells to achieve higher LYN levels than JAK2, second by incorporating GHRΔBox1 in cells to abrogate any JAK2 binding, and third by performing in vitro phosphorylation on recombinantly expressed full-length ICD (GHR-ICDFL) using an active LYN kinase. We performed immunoprecipitation experiments in cells subjected to hGH or mock treatment using an antibody against an N-terminal hemagglutinin (HA)-tag on GHR and probed with an anti-phosphotyrosine antibody. No phosphorylated GHR was detected in hGH-treated GHRΔBox1 cells even when LYN was overexpressed (Figure 1D), nor did we observe phosphorylation-induced changes around receptor tyrosines in the NMR spectrum31 when GHR-ICDFL was incubated with active LYN (Figures S2A and S2B), a kinase that readily phosphorylated a control peptide (Figures S2C and S2D). These results demonstrate that GHR is not a bona fide LYN substrate. Together, these data show that GH-mediated activation of SFK does not contribute to tyrosine phosphorylation of GHR or STAT5 but results in activation of ERK1/2.

LYN binds GHR using at least two motifs

To determine the region of GHR that interacts with LYN, a series of truncations along the GHR-ICD were generated carrying an N-terminal HA-tag (Figure 2A) used for coIP analysis with LYN. Full-length (FL) GHR-WT and GHRΔBox1 were used as positive controls based on a previous study.9 Our coIP analysis confirmed that LYN binding to GHR was constitutive and was not significantly altered by hGH stimulation (Figure 2A). The coIP analysis of GHR-ICD truncations clearly indicated that LYN maintains interactions with the membrane proximal 42 residues of the ICD (312tr), which include the Box1 motif. However, mutation of the proline residues to alanine in the Box1 motif showed these residues not to be essential for LYN interaction with the FL GHR. LYN binding to FL GHRΔBox1 was more prominent than to GHR-WT in the absence of hGH (Figures 2A and 2C), indicating that, with compromised JAK2 binding, LYN binding is increased. In addition, LYN did not co-precipitate with GHR when truncated immediately after Box1 (289tr), and, as expected, no LYN interaction with GHR was detected when the entire GHR-ICD was deleted (270tr) (Figure 2A).

We complemented the coIP analysis with NMR analysis of an 15N-labeled recombinant GHR-ICD variant encompassing the Box1 and Box2 motifs (GHR-ICD270–370). For LYN, we took advantage of SFKs sharing a common structural organization of an N-terminal lipidated SRC homology (SH) domain 4 (SH4), a unique domain, an SH3 and SH2 domain, a catalytic (SH1) domain, and a C-terminal negative regulatory tail.32,33 The SH3 domain binds proline-rich sequences with a minimal consensus motif PxxP,34 whereas the SH2 domain recognizes phosphorylated tyrosines preferentially binding to the negatively charged and hydrophobic pYEEI motif.33 Thus, for the NMR analyses, we used a double domain variant of LYN constituting only the interacting SH3 and SH2 domains (LYN32). By NMR, protein interactions will lead to different measurable effects in the spectra, depending on the timescale of the interaction. This includes chemical shift perturbations, changes in NMR peak intensity, and disappearance of signals.35 Quantification of combined chemical shift changes (ΔδNH) and peak intensity changes (lBound/lFree) of 15N-GHR-ICD270–370 upon titration with LYN32 showed evidence of an interaction. The largest chemical shift perturbations and intensity changes were seen near the Box1 and Box2 motifs, while disappearing signals were observed just N-terminal to Box1 and in the Box2 motif, prohibiting quantification of affinity. Together, this shows that the GHR-ICD

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Figure 2. LYN binds GHR exploiting at least two motifs

(A) LYN-binding region in GHR mapped by co-immunoprecipitation in HEK293T transfected with LYN and WT GHR, GHRΔBox1, or GHR truncations (tr). Numbers denote last residue before the stop codon. Serum-starved cells were subjected to hGH treatment (100 ng/mL) for 10 min prior to harvest. Data representative of three independent experiments.

(B) Schematic (left) of GHR highlighting the three domains (ECD, TMD, and ICD), the tyrosine residues, and the engineered truncation sites. Schematic (right) illustrates the GHR dimer and intrinsically disordered ICD.

(C) Densitometry quantification of LYN interaction with WT GHR or GHRΔBox1 represented as mean ± SEM.

(D) Chemical shift perturbations (ΔδNH) and peak intensity changes (lBound/lFree) of 15N-GHR-ICD270–370 by addition of LYN32. Disappearing and overlapping signals are indicated by black and gray stars, respectively. Box1 and Box2 motifs are in green and the main LYN32-binding sites in yellow. On top, the main LYN32-binding sites (dark gray boxes), additional contact points (light gray box), lipid interaction domain, and transient α-helices (TH1–3) within GHR-ICD270–370 are indicated. Insert: binding curve of GHR-ICD270–370 titrated with LYN32 obtained from microscale thermophoresis. The experiment was performed in triplicate (N = 3) and reported as the mean ± SD.

(E) Sequence alignment of LYN-binding sites in GHR. Box1 and Box2 highlighted in green and main LYN32-binding sites in yellow. Identical amino acid residues denoted by asterisks.
Figure 3. Characterization of LYN-binding sites in GHR

(A) Chemical shift perturbations (ΔδNH) and peak intensity changes (Ibound/IFree) of 15N-GHR-ICD270–370 titrated with LYN3. Box1 and Box2 are highlighted in green and the main binding site in yellow. On top, the lipid interaction domain and transient α-helices (TH1–3) within GHR-ICD 270–370 are indicated.

(B) Chemical shift perturbations (ΔδNH) and peak intensity changes (Ibound/IFree) of 15N-GHR-ICD270–370 titrated with LYN2. Disappearing and overlapping signals are indicated by black and gray stars, respectively. Box1 and Box2 are highlighted in green and the main binding site in yellow. On top, the lipid interaction domain and transient α-helices (TH1–3) within GHR-ICD 270–370 are indicated.

(C) Chemical shift perturbations (ΔδNH) and peak intensity changes (Ibound/IFree) of 15N-LYN3 titrated with GHR-ICD 270–370. Disappearing signals are indicated by black stars. Main binding sites highlighted in gray. Sequence and secondary structures of LYN3 indicated on top.

(legend continued on next page)
interacts with LYN32 at two sites within and proximal to the Box1 and Box2 motifs (Figure 2D), supporting the coIP analysis (Figure 2A). By microscale thermophoresis, the dissociation constant \( K_D \) of the complex was determined to be 14 ± 4 \( \mu \)M (Figure 2D), further supported by isothermal titration calorimetry (ITC) (Figure S3A). This region of GHR is also essential for JAK2 binding, which exploits Box1, Box2, and the interbox region, suggesting again that JAK2 and LYN compete for GHR binding. The GHR Box1-Box2 region also comprises a lipid interaction domain,\(^a\) a degron,\(^b\) and is highly conserved across species (Figures 2B and 2E). As SH3 domains bind a consensus PxxP motif, the presence of this motif within the GHR Box1 suggested an SH3:Box1 interaction.\(^c\),\(^d\) As the GHRΔBox1 mutant still co-precipitates with LYN and maintains activation of LYN and ERK1/2, the second binding site (Box2 and proximal residues C-terminal of it) is suggested to be responsible for maintaining the interaction, just as contacts to the remaining LYN domains may also be relevant. There is no consensus SH2-binding site within the GHR-389tr Box1-Box2 region, and mutating the sole tyrosine residue (Y314A/389tr) within that region had no effect on LYN binding to GHR (Figures 2A and 2E).

To determine if the LYN:LYN interaction was dependent on the combined presence of the two domains, we investigated interactions of GHR-ICD270–370 with the individual SH3 (LYN3) and SH2 (LYN2) domains of LYN by NMR. The affinities for the individual SH3 and SH2 domains were obtained from fitting changes in chemical shifts as a function of concentration (Figure 3D). Although prolines are not observed in \( ^1H,^15N \) NMR spectra, \( \Delta \delta_{4NH} \) and \( \delta_{bound}/\delta_{free} \) of \( ^15N \)-GHR-ICD270–370 titrated with LYN3 only (Figure 3A) showed interactions in, and surrounding, Box1, with the most perturbed residues extending N-terminal of Box1. In contrast, interaction with the isolated LYN2 showed no interaction at, or in proximity to, the Box1 motif, whereas an interaction was observed in Box2 residues and extending C-terminal of it (Figure 3B), albeit with a very low affinity (Figure 3D). The reverse titration showed the most perturbed regions of LYN3 to be the RT loop, nSrc loop, and the small 3 helix, forming the SH3 canonical binding pockets and with an affinity within the range of other SH3 ligands (Figures 3C–3E). Thus, since the combined affinity of the SH3:SH2 domain interaction is higher (14 ± 4 \( \mu \)M) than the individual SH3 (33 ± 7 \( \mu \)M (Figure 3D), further supported by microscale thermophoresis (MST), Figure S3B) and SH2 (252 ± 49 \( \mu \)M) affinities, LYN SH3 and SH2 domains interact with GHR-ICD via Box1 and Box2 motifs with a slight avidity effect. Mutating Box1 prolines to alanines (GHR-ICD270–370ΔBox1) essentially abolished the interaction with LYN3 (Figures S3A and S3B). This effect was likely promoted by an induced helicity (Figure S3C), further demonstrating Box1 residues as well as extended structure to be critical for LYN3 binding. These results are supported by coIP showing interaction with LYN in GHR truncated at 312tr (GHR-312(WT)tr), which was ablated in GHR-312(ΔBox1)tr (Figure S4). Although binding between LYN32 and GHRΔBox1 was not investigated by NMR, cellular experiments showed that LYN maintains interaction with the GHRABox1 and activation by hGH, likely through the interactions with LYN2 or additional contacts (e.g., via the unique domain).\(^e\) As the proline residues essential for JAK2–FERM domain binding exist in a composite motif of PxxPxxP, with those important for SH3 binding,\(^f\) LYN and JAK2 may compete for this region.

Taken together, these data show that LYN binds to the GHR within the first 42 residues of the ICD and exploits residues in both Box1 and Box2 for interactions with its SH3 and SH2 domains, sites critical for JAK2 binding. The interbox region remains largely unaffected.

### The GHR-ICD interbox region remains dynamic in complex with LYN

The NMR chemical shift perturbation data from the titration series supplemented with structural knowledge from known SFK-SH2-binding peptides was used as input to generate an ensemble model of the complex between GHR-ICD270–370 and LYN32. Here, we observed that GHR-ICD270–370 interacted mainly with LYN3 around the regions restrained during the coarse-grained (CG) simulation, while GHR-ICD270–370 interacted mainly with LYN2 in only some of the restrained regions (179–185, 195–200) but also making contacts to residues 135–138, 158–166, and 186–194 of LYN2, positioned on the same side of the protein (Figures 3F–3H). Furthermore, the total number of contacts formed between LYN32 and Box1 and Box2 of GHR-ICD270–370 was high compared with the rest of the GHR-ICD270–370 (Figure 3H), implying these to be prime interaction sites with an average number of contacts of 16 and 15 for the two domains, respectively. In the model, negatively charged residues of LYN3 (E94, E95, and E98) interacted with I275 and K276 in the N-terminal flanking region of Box1, whereas the PxxP motif docked into the hydrophobic proline-binding groove in a canonical way (Figure 3G). In contrast, LYN2 interacted less strongly with Box2 and in a non-canonical fashion, where the conserved Glu residues in Box2 mainly interacted with the positively charged patch (R136, K137, K162, K181, H182; K184, R186) of LYN2. This positive patch is part of the additional contact regions of LYN2 (Figure 3H).

### Notes

- Global fits of chemical shift perturbations of the fast exchanging and most affected residues of \( ^15N \)-LYN3 titrated with GHR-ICD270–370 (top) and \( ^15N \)-GHR-ICD270–370 titrated with LYN2 (bottom) with \( K_D \) indicated.
- Most affected binding residues (\( \Delta AVG \gamma ) \) of \( ^15N \)-LYN3 shown on the surface of LYN3 (PDB: 1WA7) and colored dark blue. Disappearing residues are light blue, prolines are white.
- Model structure of LYN32 (residues 68–228) with regions restrained during the CG simulation colored gray.
- Structure showing the interface of LYN3:Box1 (left), and LYN2:Box2 (right).
- Number of contacts formed between the CG backbone beads of LYN3 and GHR-Box1 (top), LYN2 and GHR-Box2 (middle), and LYN32 and GHR-ICD270–370 (bottom). Restricted region (in CG simulations) marked in gray.
- Structures collected every 40 ns aligned with respect to (I) LYN3, (J) LYN2, and (K) LYN32. The structures of LYN32 collected over the simulation time are colored from gray to red. The interbox regions are highlighted in gray lines.
- Representative structure from clustering of the trajectory. The LYN3:GHR-ICD270–370 interface is colored in shades of pink, the LYN2-GHR-ICD270–370 interface in shades of blue.
Analyses of the all-atom structures generated from the simulations revealed that the GHR-ICD270–370:LYN32 complex was conformationally heterogeneous and dynamic. In particular, the relative orientation of the folded SH2 and the SH3 domains varied due to the flexibility of the interdomain linker (Figures 3I and 3J). Furthermore, from superimposition of individual domains, it was apparent that the relatively long interbox region of GHR-ICD (C24 residues) allowed Box1 to stay bound to the SH3 domain, while Box2 was making fewer contacts and vice versa, explaining the small avidity effect seen from the affinity measurements (Figures 3K and 3L).

In conclusion, the ensemble model of the complex generated by integrating the NMR experiments and simulations shows Box1 and Box2 to be the predominant interaction sites for LYN SH3 and SH2 domains, respectively. Additionally, it revealed high flexibility in the GHR interbox region imposing dynamic binding at the two sites.

LYN promotes cytokine receptor degradation
JAK2 binding to GHR and related receptors such as EPOR and TPOR increases receptor stability in the membrane.26,30 To determine if LYN regulates GHR stability, HEK293 cells were transfected with GHR and LYN or GFP as control. Cells were also subjected to acute hGH treatment and signal activation analyzed. In contrast to what has been reported for JAK2, LYN reduced the total GHR levels (Figure 4A), independently of hGH treatment. This reduction occurred at the post-translational level as no significant difference in RNA levels was detectable following LYN transfection compared with GFP (Figure 4B). Exogenous expression of LYN had a dose-dependent effect on GHR levels, with increasing amount of LYN showing a concomitant decrease in GHR levels with no change in endogenous JAK2 levels (Figure 4C). This LYN-mediated decrease in GHR levels was reduced when transfected cells were treated with the proteasomal inhibitor MG132 or with chloroquine or bafilomycin A1 (Figures 4D and S5).

Importantly, we found LYN-mediated degradation was not unique to GHR as similar effects were observed for the related cytokine receptors GP130, PRLR, and TPOR (Figure S6), highlighting the general implications of our findings.

To investigate the effect of LYN on mature GHR cell surface levels, we transfected LYN in both HEK293 and SYF (SFK-deficient) cell lines. We compared the effects of transfected LYN-WT, LYN-DN (dominant negative; Y397F), and LYN-constitutively active (CA) (Y508F) on receptor levels compared with GFP (Figure 4E). We found that LYN kinase activity reduced
GHR protein levels as overexpression of either LYN-WT or LYN-CA showed low GHR levels compared with GFP and LYN-DN. Interestingly, LYN-WT and LYN-CA resulted in a sharp decline in total GHR levels in HEK293 cells but selectively reduced mature GHR levels in SYF, compared with GFP (Figure 4E). Our data would support that the increase in the ratio of mature (m) GHR to premature (p) GHR observed in SYF cells compared with HEK293 cells when transfected in the absence of LYN would be due to the lack of any SFK interaction with GHR and the absence of competition for JAK2 binding in SYF cells (Figure 4E). SFK inhibition (PP2) showed an increase in mature GHR surface levels (Figure 4F), supporting that LYN kinase activity facilitates GHR degradation. Co-transfection of varying relative levels of JAK2 and LYN had contrasting effects on GHR protein levels, with JAK2 favoring increased mature GHR surface levels while LYN expression decreased mature GHR levels (Figure 4G). As expected, GHR stabilization by JAK2 transfection was lost when co-transfected with GHRΔBox1, while GHRΔBox1 was still amenable to LYN-mediated receptor degradation (Figure 4G). Thus, taken together, these data show that, in contrast to the stabilizing effect of JAK2 on GHR levels in the membrane, LYN promotes GHR loss and its kinase activity has a role in this.

**LYN and JAK2 compete for binding to GHR and dictate surface GHR levels**

Both LYN-WT and LYN-CA overexpression promoted reduction in GHR levels, including a considerable decline in mature levels, compared with LYN-DN, particularly in the SFK-deficient SYF cells (Figure 5A), supporting that kinase activity of LYN promotes, but is not essential for, mediating GHR degradation. To investigate if LYN can associate with JAK2-bound GHR or free (non-JAK2-bound) GHR, we performed coIP by precipitating JAK2 and determining interacting proteins. JAK2 coIP was unable to show interaction with LYN, although it confirmed the interaction with GHR-WT but not GHRΔBox1 (Figure 5B).

Further, by JAK2 coIP, we were able to show that GHR binding to JAK2 was reduced with increasing LYN expression levels relative to JAK2 (Figure 5C). We altered the relative levels of JAK2 and LYN to investigate the relative contribution of each in modulating the amount of binding of JAK2 to GHR. This suggests that the relative expression levels of these kinases in a cell, or localized concentration within a cell, determine JAK2 or LYN binding to the receptor. Importantly, our data support that the relative level of GHR on the cell surface is dictated by kinase discrimination consequent to either stabilization (JAK2) or degradation (LYN) and hence the signaling outcome from each kinase.

**Kinase binding and hGH-mediated activation differentially modulate cell surface mobility of GHR**

The lateral trapping of receptors in nanoclusters in the plasma membrane has been shown to have important implications for their function and kinetics of downstream signaling cascades. Since GHR interactions with JAK2 and LYN have contrasting effects on receptor stabilization in the membrane and their interactions potentially are dictated by their local concentrations within cell membrane domains, we evaluated whether binding to JAK2 or LYN altered GHR mobility by measuring this on the cell surface using single-particle tracking photoactivated localization microscopy (sptPALM) on live cells. This was performed using GHR expression constructs tagged at the C terminus with a photo-switchable monomeric EOS2 (mEOS2) fluorescent protein.

We first expressed GHR-FL-mEOS2 in HEK293 cells exhibiting plasma membrane localization by low-resolution green-emitting total internal reflection fluorescence (TIRF) illumination (Figure 6A). We performed sptPALM and generated high-resolution maps of intensity in control cells (Figures 6B–6D) and cells treated with hGH (Figures 6E–6H). The maps revealed highly heterogeneous spatial localizations of photoconverted molecules with small domains displaying a greater detection density. Analysis of GHR-WT(FL)-mEOS2 exhibited very low diffusion...
Figure 6. Ligand-mediated activation of LYN modulates the mobility of cell surface GHR

(A–L) Representative images of HEK293 cell expressing GHR-WT(FL)-mEOS2 imaged at 50 Hz, showing the low-resolution TIRF image (scale bar, 5 μm), high-resolution intensity, diffusion coefficient (warmer colors represent lower mobility), and trajectory maps (scale bar 1 μm) (warmer tracks appear later in the acquisition) from an ROI (white box) from (A–D) GHR-WT(FL)-mEOS2 or (E–H) immediately following hGH (100 ng/mL) addition. (I) Mean square displacement (MSD) as a function of time of the trajectories, (J) area under the MSD curve (AUC), (K) frequency distribution of the diffusion coefficient, (L) mobile-to-immobile ratios. N = 14 (-hGH), 13 (+hGH) (cells), from four independent experiments.

(M–X) Representative images for GHR-ΔBox1(FL)-mEOS2 (M–P) or immediately following hGH (100 ng/mL) addition (Q–T) are depicted (color codes as above). (U–X) Analysis of single-particle tracking expressed as the MSD as a function of time, (V) AUC, (W) frequency distribution of the diffusion coefficient, (X) mobile-to-immobile ratios. N = 15 (-hGH), 14 (+hGH) (cells), from four independent experiments. Each hGH treatment was imaged for 5 min upon hGH addition. Statistics of the AUC, mobile-to-immobile ratio, and state occupancy were performed using an unpaired Student’s t test. p < 0.05 was considered significant. Data represented as mean ± SEM.
Figure 7. Kinase binding modulates the mobility of cell surface GHR

(A–L) Representative images of HEK293 cell expressing GHR-WT(322)-mEOS2 imaged at 50 Hz, showing the low-resolution TIRF image (scale bar, 5 μm), high resolution intensity, diffusion coefficient (warmer colors represent lower mobility), and trajectory maps (scale bar, 1 μm) (warmer tracks appear later in the acquisition) from an ROI (white box) from (A–D) GHR-WT(322)-mEOS2 or (E–H) GHR-ΔBox1(322)-mEOS2.

(I) MSD as a function of time of the trajectories, (J) AUC, (K) frequency distribution of the diffusion coefficient, (L) mobile-to-immobile ratios.

(M) Hidden Markov modeling (HMM) analysis showing a three-state model (state 1 = immobile, magenta; state 2 = intermediate, green; state 3 = fast mobile, blue) and transition probabilities.

(N and O) Comparison of apparent diffusion coefficients (N) and state occupancies (O). Inferred from GHR-WT(322)-mEOS2 trajectories or GHR-ΔBox1(322)-mEOS2 trajectories (N = 14 WT(322), 14 ΔBox1(322) sets of pooled trajectories from 20 and 14 cells respectively, derived from five independent experiments. To
coefficients in small discrete areas of the plasma membrane (Figure 6C). The individual trajectories for this region of interest (ROI) (Figure 6D) highlighted receptors with high and low mobility intermixing on the plasma membrane. We quantified the mobility of the GHR-WT(FL)-mEOS2 by analyzing the mean squared displacement (MSD) (Figures 6I and 6J) and the frequency distribution of the diffusion coefficient (Figures 6K and 6L). The frequency distribution of GHR-WT(FL)-mEOS2 exhibited a bimodal mobility distribution, suggesting that the receptor can either be in an immobile or a more mobile state (Figure 6K). Addition of hGH did not alter the mobile-to-immobile ratio (Figure 6L).

To investigate the contribution of LYN binding and activation to receptor mobility, we introduced the same proline to alanine substitutions within the Box1 motif (ΔBox1) as used above (Figures 6M–6X). Under basal conditions, the distribution of the GRHΔBox1(FL)-mEOS2 receptors was similar to that of GHR-WT(FL)-mEOS2 with respect to the mobile and immobile ratio (Figures 6K and 6W). However, upon addition of hGH, the mobility of the GRHΔBox1(FL)-mEOS2 was significantly reduced (Figures 6U and 6V), suggesting that hGH-induced LYN activation mediates the reduction in GHR mobility. Since deletion of Box1 removes JAK2 binding to the receptor, leading the LYN/ERK pathway to become dominant (Figures 1 and 2), our data show that GRHΔBox1(FL)-mEOS2 mobility is significantly decreased following hGH addition and in sharp contrast with the lack of mobility change observed following hGH addition to the GHR-WT(FL)-mEOS2, which preferentially binds JAK2. In addition, colIP analysis on mEOS2-tagged FL constructs showed interaction with LYN (Figure S7) and GH-mediated activation of SFK-ERK1/2 for both constructs and STAT5 activation for GHR-WT(FL)-mEOS2 (Figure S8), indicating no significant interference from the mEOS2 tag.

To further dissect the effect of hGH addition and kinase binding to the mobility states of the GHR-WT(FL)-mEOS2, we analyzed the trajectory dataset using hidden Markov models (HMMs). HMM is here used to infer hidden diffusive states, occupancies, and transitions between states from super-resolution data. We found that the GHR switches between at least three distinct diffusive states: an immobile (S1), an intermediate (S2), and a fast mobile state (S3) (Figure S9). Box1 mutations resulted in a significant reduction in the time spent in the intermediate state without affecting the time spent in the other two states or the apparent diffusion coefficient of each of the three states. This suggests that LYN binding affects the transition from free mobile receptors into nanoclusters. Addition of hGH did not significantly change the time spent in each state (Figure S9). To assess the propensity of GHR-WT(FL)-mEOS2 to form nanoclusters, we performed density-based spatial clustering of applications with noise (DBSCAN). In agreement with the lack of a hGH effect on the mobile-to-immobile ratio (Figure 6), hGH addition did not significantly alter nanoclustering of either GHR-WT(FL)-mEOS2 or GRHΔBox1(FL)-mEOS2 (Figure S10).

To investigate the effect of JAK2 and LYN binding on clustering in the absence of any tyrosine phosphorylation required for STAT5 activation, we expressed GHR truncated prior to Box2, which maintains JAK2 and LYN binding. In the absence of hGH, this truncated receptor (GHR-WT(322)-mEOS2) had similar mobility to the WT FL receptor (Figure 7). Generation of the Box1 mutant in this background resulted in loss of JAK2 binding and in almost complete abrogation of LYN binding due to the lack of both Box1 and Box2 (Figures 3 and S3). GRHΔBox1(322)-mEOS2 displayed dramatically reduced mobility in the plasma membrane compared with GHR-WT(322)-mEOS2, as indicated in the MSD and frequency distribution of diffusion coefficients (Figures 7A–7L). We next tested the response to hGH addition on the GHR-WT(322)-mEOS2 (Figure S11). Unlike the FL receptor, the mobility of GHR-WT(322)-mEOS2 decreased significantly in response to hGH (Figure S11). Since JAK2 and LYN binding is retained in both GHR-WT(322)-mEOS2 and GHR-WT(FL)-mEOS2 receptors, the reduction in mobility induced by hGH addition in the truncated receptor is likely mediated by the Box1 and the lack of phosphorylated ICD.

We used HMM to assess which specific mobility states were affected by kinase binding to GHR and their activation by GH (Figures 7M–7O). The 322-truncated receptor lacking Box1, and hence both JAK and LYN binding, had reduced mobility compared with WT truncated receptor and promoted the recruitment of freely diffusible GHR into both intermediate and immobile states (Figures S11 and S12). Nanoclustering analysis (Figure 7P) revealed that Box1 mutation in the truncated background significantly increased the number of molecules within these clusters (Figure 7Q) without affecting their size or density (Figures 7R and 7S). Most importantly, the Box1 mutation dramatically increased the percentages of tracked molecules in the clusters (Figure 7T). Addition of hGH to GHR-WT(322)-mEOS2 promoted recruitment from the freely diffusible pool of receptors to the intermediate state (Figure S12). Together, this suggests that freely diffusible receptors first transition into the intermediate state upon hGH-binding-induced kinase activation, prior to entering nanoclusters and promoting efficient downstream signaling. Accordingly, addition of hGH increased the percentage of molecules detected in clusters without affecting their size (Figure S13). Overall, our data suggest that the binding and activity of kinases directly modulate the nanoscale organization of GHRs.

**DISCUSSION**

We recently revealed an important role of GHR signaling in liver regeneration, exclusive to the SFK-ERK1/2 pathway. In the present study, we have interrogated the interaction of LYN with...
GHR and uncovered important attributes of this interaction. We have defined the minimal interacting region as located within residues 289–312 of the GHR-ICD, and NMR analyses further identified two main binding motifs located within Box1 and Box2 (Figure 2). The LYN SH3 domain interacted canonically with the PPVP motif of Box1 (residues 280–287) and non-canonically with its SH2 domain via a conserved Glu in Box2 (residues 323–331). Truncation of the GHR Box2 motif (in 312tr) retained LYN interaction by coIP, indicating the SH3 domain as sufficient to maintain interaction, while mutation of Box1 in FL GHR also co-precipitated with LYN, indicating that interaction with the SH2 domain and potentially other LYN domains, could maintain the association. Accordingly, when GHR was truncated and mutated as in GHR-312, lacking both SH2 and SH3-binding motifs, LYN binding was completely abrogated. The weak interactions within the complex and the dynamics can be reasoned to originate from a long, flexible interbox region (~35 residues) located between the two motifs in GHR-ICD. The region is not involved in LYN binding but facilitates binding to both motifs, likely via local concentration effects. It is possible that altered helicity of this region consequent to substituting Box1 prolines with alanine residues also contributes to loss of LYN association. Together, our study shows that JAK2 and LYN share overlapping binding interfaces of GHR-ICD via composite motifs (Figure 2B). This, in turn, leads to competitive GHR binding of the two tyrosine kinases (Figure 5C). Taken together, these data suggest that the binding and activity of kinases directly modulate the nanoscale organization of GHRs.

The JAK SH2-like domains specifically interact with the closely related EPOR Box2 motif and with the Box2 of distantly related class II cytokine receptors,50–52 all lacking a phosphorylated tyrosine. Similarly, we found that GHR-Box2 interacts with the SH2 domain of LYN, also in a non-phosphorylated state, but using a highly conserved glutamic acid (E326), located at the −1 position of the FIELD motif. Thus, phosphorytrosine-independent SH2:Box2 interaction may be common for class I and class II cytokine receptors and expands to more kinases than JAKs. From the NMR analyses, we modeled the complex between GHR and the SH3-SH2 domains, which revealed a highly dynamic interbox region in GHR. Thus, once LYN binds GHR, the dynamic interbox region remains accessible to JAK2 interactions, in turn increasing access at either Box1- or Box2-binding sites, allowing JAK2 to hijack GHR from LYN. Similar binding mechanisms have been seen for other intrinsically disordered proteins (IDPs), where binding partners with comparable affinities can be outcompeted due to allosteric and dynamic effects or through competitive substitution.53–55 The ability of JAK2 to outcompete LYN is likely due to additional contacts with the interbox region of GHR, as shown in the structures of EPOR with the FERM-SH2 domain of JAK2. However, we cannot rule out additional interaction between GHR and either kinase. Our model suggests that JAK2 is the primary kinase associating with GHR either via a higher affinity and/or via induced allosteric effects.

Several studies evaluating LYN and other SFK signaling cascades utilized MG132 to minimize the rapid proteasome-mediated SFK degradation, which underpins its transient nature.56,57 Using this approach, we were able to demonstrate that GHR also mediates GH-mediated SFK and ERK1/2 activation (Figures 1A and 1C), that latter of which was previously thought to be activated independently of SFK via JAK2.58 Since we have found that LYN mediates GHR degradation (Figures 4A and 4B), the detection of SFK activation is not straightforward. However, activation is distinctly detected once JAK2 binding is abrogated as in GHRΔBox1, providing an avenue by which to characterize and understand this signaling event. Our study also highlighted that unlike JAK2, GH-mediated LYN activation did not promote any detectable tyrosine phosphorylation of GHR using GHRΔBox1 (Figure 1D), which we have shown can bind and activate the LYN cascade. An earlier study showed that SRC can bind and phosphorylate GHR in F-36P cells using an SFK inhibitor, PP2 at a concentration of 100 μM.59 However, the PP2 inhibitor has been shown to inhibit JAK2 kinase activity at concentrations greater than 50 μM.60 A previous study confirmed a lack of involvement of SRC in PRL-induced tyrosine phosphorylation of WT and ΔBox1 mutant PRLRs during activation of SRC by both constructs,61 fully in concordance with our findings with LYN and GHR.

GH-mediated LYN activation has been demonstrated to promote phospholipase C-γ-driven activation of RAS guanine nucleotide exchange factor, RASGRP1, which then promotes RAS activation and initiates the ERK1/2 cascade.6 Further evidence of JAK2-independent SFK-mediated ERK1/2 activation also comes from the observation that inhibition of SFK kinase activity at low concentrations of PP2 inhibitor reduced endogenous phosphorylated ERK1/2 levels and ablated any GH-mediated increase without affecting STAT5 induction (Figure 4F). RAS nanoclusters are well known to be essential for efficient signal transduction and the number and size of these were shown to control the response to epidermal growth factor (EGF) stimulation.62 The fact that GHR is likewise organized in nanoclusters suggests these to be vis-a-vis signaling effectors such as RAS. Our study reports the nanoscale organization of GHR in clusters, and whether it is the main determinant for activation of the RAS pathway will need further study.

Another recent study used single-molecule imaging to specifically image monomer and dimer assembly of GHR in the plasma membrane, demonstrating that JAK2 plays an important role in GHR dimerization.63 It is important to note that this study excluded nanoclusters from their analysis to enable pinpointing of co-movement of GHR and effectors. As molecular crowding induced by nanoclustering may not only favor specific kinase binding but also help the formation of active dimers, the relationship between receptor dimerization and nanoclustering needs further attention. Indeed, the probability of binding (whether to hGH or intracellular kinases) increases with the square of the binding density,65 making nanoclusters perfect nanoswitches for efficient cellular signaling. As the GHR was suggested to predominantly exist as dimers1 and hGH binding to the dimer initiates signaling, it is therefore likely that nanoclusters contain dimerized GHR. However, whether nanoclustering favors dimerization or dimerization is a prerequisite for clustering remains unknown. Further, several major trapping mechanisms have been described, including fences and pickets64 and molecular interaction65; however, so far, the mechanism underpinning lateral trapping of GHR remains to be established. Future work
to directly visualize the co-occupancy of LYN/GHR within clusters may be feasible by employing alternative methods, such as using scanning-fluorescence cross-correlation spectroscopy (FCCS). 66

SFKs also activate the endocytic machinery, leading to internalization of several receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs). 67 Our data demonstrate that LYN binding to GHR resulted in receptor degradation in a proteasome-dependent manner (Figures 4A and 4D). This LYN-promoted degradation was enhanced by an increase in kinase activity as evident from LYN-CA and LYN-WT transfection compared with GFP control (Figure 4E). This clearly suggests that LYN binding to GHR, as in the case of LYN-DN, is sufficient for degradation but is further enhanced by LYN activation, all in a ligand-independent manner. In addition, we observed cell line differences in GHR degradation with a decrease in both mature and precursor levels in HEK293 and only a significant decrease in mature levels in the SYF cell line (Figure 4E). This is likely due to the lack of endogenous LYN (and other SFK members) in SYF cells, unlike HEK293 cells. Therefore, in SYF cells, GHR is expected to be bound solely to JAK2, resulting in higher levels of mature GHR compared with precursor levels.

We have shown that GH stimulation of LYN causes an increase in the population of less mobile receptors at the cell surface. This may represent receptors that increase their association with cholesterol rich membrane nanodomains known as lipid rafts, analogous to a single-molecule tracking study that indicated that unliganded epidermal growth factor receptors (EGFRs) reside outside lipid rafts and move into these upon activation that unliganded epidermal growth factor receptors (EGFRs) reside outside lipid rafts and move into these upon activation (Figures 4A and 4D). This LYN-promoted degradation was enhanced by an increase in kinase activity as evident from LYN-CA and LYN-WT transfection compared with GFP control (Figure 4E). This clearly suggests that LYN binding to GHR, as in the case of LYN-DN, is sufficient for degradation but is further enhanced by LYN activation, all in a ligand-independent manner. In addition, we observed cell line differences in GHR degradation with a decrease in both mature and precursor levels in HEK293 and only a significant decrease in mature levels in the SYF cell line (Figure 4E). This is likely due to the lack of endogenous LYN (and other SFK members) in SYF cells, unlike HEK293 cells. Therefore, in SYF cells, GHR is expected to be bound solely to JAK2, resulting in higher levels of mature GHR compared with precursor levels.

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In conclusion, our study finds that GHR-mediated SFK activation is regulated by dynamic, spatiotemporal processes 71,72 as well as cell-type-specific effects that govern the relative levels of JAK2 and LYN at the cell surface membrane.

Limitations of the study
Our work reveals that JAK2 and LYN compete for binding GHR and highlights the importance of the Box domains of GHR in mediating the intrinsic competition between two kinase activation pathways and regulation of receptor degradation. However, so far, only soluble JAK1 has been successfully produced and only in the presence of stabilizing receptor fragments, and thus the affinity for JAK2 could not be quantified in vitro. Our study used model cell lines, and the endogenous expression of JAK and LYN will vary in different cell types. In addition, the relative expression of intermediates in the ERK1/2 signaling pathway, such as RAF and MEK, may significantly differ between different cell lines. However, it is important to note that the expression of LYN is not limited to hematopoietic cells 73 and that LYN and GHR are both expressed in diverse cell types. 74 Although we show that the proteasome inhibitor MG132 is able restore the levels of GHRΔBox1 mature protein to similar levels of GHR-WT treated in the same way, we cannot exclude some effect on receptor biosynthesis for the GHRΔBox1. One of the limitations of single-particle tracking data analysis, including MSD and diffusion coefficient analysis, is that it does not provide information on changes of mobility during a trajectory’s lifetime. To mitigate this, we used HMM to assess the different mobility states and transition probabilities of individually tracked molecules. Nevertheless, our multidisciplinary approach has led us to establish the important role of kinases in the regulation surface GHR membrane mobility and degradation pathways. Our study was confined to GHR and LYN. The generality of the mechanism could be widened to include other class I cytokine receptors and other SFK members.

STAR METHODS
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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2023.112490.
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AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS

The authors declare no competing interests.

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**STAR METHODS**

**KEY RESOURCES TABLE**

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**Oligonucleotides**

For primers used for cloning and qPCR, see Table S1

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**Software and algorithms**

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**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Associate Professor Andrew J. Brooks (a.brooks@uq.edu.au).

**Materials availability**
All plasmids and stable cell lines generated in this study are available without restrictions from the lead contact and/or through Addgene.

**Data and code availability**
- All data is available in the main text or the supplementary materials. The chemical shifts of GHR-ICD (residues G270-S370) and LYN3 have been deposited in the BioMagResBank under the accession code 50478 and 50475, respectively. Simulations are available on request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Cell lines and culture**
HEK293 (human, female)77 HEK derived 293T (human, female)80 PLAT-E (human, female)79 and Src/Yes/Fyn/Lyn deficient SYF (murine, sex unspecified)78 cell lines were maintained in DMEM (Life Technologies) supplemented with 10% fetal bovine serum (FBS). FDC-P1 (murine, sex unspecified) cells 76 were maintained in RPMI 1640 (Life Technologies) supplemented with 10% FBS, 4 mM L-glutamine (Life Technologies) and 1x interleukin-3 (IL-3).3,75 All cells were maintained at 37°C in 5% CO2. Cell culture supernatants were routinely tested for mycoplasma by PCR.

**METHOD DETAILS**

**Cell treatments**
For signaling analysis, SYF, HEK293, and HEK293 stable transduced cells were seeded at 1 × 10^5 cells per well in a 6-well dish and transfected using lipofectamine 2000 as per manufacturer’s conditions, where indicated. The cells were starved overnight in serum reduced media (DMEM containing 0.5% FBS) and subjected to 100 ng/mL recombinant human GH (hGH)74 or PBS for 10 min at 37°C, where indicated. FDC-P1 stable transduced cells (1-3 × 10^5 cells/ml) were starved in RPMI-1640 supplemented with 4 mM L-glutamine, but devoid of serum and IL-3 for 8 h. Cells were then stimulated with 50 ng/mL recombinant hGH or PBS for 10 min at 37°C prior to harvesting. Proteasomal treatment with MG132 was performed for 2 h at 20 μM with DMSO as control/vehicle where indicated. Treatment with SFK inhibitor PP2 (10 μM or 50 μM) was performed for 2 h with DMSO as control/vehicle.

**Construction of cytokine receptor expression constructs**
Full-length N-terminal HA tagged human GHR76 was used as a template for the construction of GHR truncations and Box1 mutants in pQCXP and pMX destination vectors. GHR truncations were made using specific primers with an introduced stop codon for early termination of receptor cDNA. The primers were designed to include 5’ flanking attB sequence (Table S1). PCRs were carried out using Phusion DNA polymerase (Thermo Scientific). For generating Box1ΔGHR expression construct in which all four prolines (PPVPVP within residues 280–287) were mutated to alanine (AAAVAVA) using PCR-mediated overlap cloning.90 Full-length N-terminal HA tagged human PRLR and C-terminal HA-tagged GP130 were created by Gateway Cloning. The resulting clones were analyzed by restriction digestion and DNA sequencing.

**Construction of mEOS2-tagged GHR**
The mEOS2 tagged receptor expression constructs were generated by using a modified CPEC method.3 First by amplification of four DNA fragments that were joined in a CPEC reaction to generate pHA-GHR-WT(322)-mEOS2 and pHA-GHR-WT(FL)-mEOS2 expression constructs. This was used as template to generate Box1Δ (322 truncated and FL) mEOS2 tagged constructs (Table S1). Correct clones were confirmed by DNA sequencing.

**Retroviral transduction**
FDCP-1 cell lines were created by retroviral transduction. PLAT-E cells were seeded at 1.7 × 10^5 cells/well in a 6-well cell culture plate. The following day cells were transfected with 3 μg of each GHR-WT or Box1ΔGHR or GFP construct cloned into...
pMX-GW-PGK-GFP-PuroR and incubated overnight. Culture media was removed and replaced with fresh media and cells were incubated for a further 48 h. Supernatant from each well was filtered with a 0.45 µM PDVF filter (Merck) and incubated with 3 x 10^5 FDC-P1 cells for 48 h, then selected with 0.75 µg/mL puromycin (Life Technologies).

HEK293 cells were generated by retroviral transduction. HEK293T cells were seeded in a 6-well cell culture plate and cultured overnight until 40–50%. Cells were then co-transfected with plasmids containing 4 µg of retroviral vector (pQCXP expressing GHR-WT or Box1ΔGHR), 3 µg of pVPack-GP (Stratagene) and 1.5 µg of pVPack-VSV-G (Stratagene), then incubated for 16 h. Culture media was replaced fresh media and incubated for 48 h. Supernatant from each well was filtered with a 0.45 µM PDVF filter (Merck). HEK293 cells seeded at 1 x 10^5 cells per well of 6-well culture plate were subjected to viral supernatant for 48 h before selection on 1 µg/mL puromycin (Life Technologies).

**Western blot analysis**

Treated cells were washed in cold PBS and lysed in ice-cold radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100) containing 2 mM sodium orthovanadate (Sigma), 30 mM sodium fluoride (Sigma), 10 mM sodium pyrophosphate (Sigma), and 1 x complete protease inhibitor cocktail (Roche Applied Science). Protein concentration was determined using a Pierce Bicinchoninic (BCA) Protein Assay Kit. Supernatant was mixed with 3x SDS sample buffer (containing 0.1M DTT) and heated to 98°C for 5 min and subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Following gel electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad) using the Trans-Blot Turbo Transfer System (Bio-Rad) as per manufacturer’s protocol. The immunoblots were blocked in blocking buffer (5% BSA in TBST) followed by addition of primary antibodies overnight in blocking buffer with constant agitation at 4°C. The following day, blots were washed thrice for 10 min in TBST and incubated with appropriate HRP-conjugated secondary antibody at 1:10,000 dilution in blocking buffer for 1–2 h at room temperature. The blots were washed thrice for 10 min in TBST and developed using Bio-Rad Chemi-Doc following incubation with Immobilon Western Chemiluminescent HRP substrate (Merck).

**Immunoprecipitation**

HEK293T cells were plated in T-75 flask at 1 x 10^6 cells/cm² seeding density and transfected next day at 70% confluency using lipofectamine 2000 (Life Technologies) as per manufacturer’s conditions. For competition assay, varied amounts of LYN, JAK2 and GFP expression plasmids were transfected with 5 µg of GHR constructs as indicated. Transfected cells were starved overnight in serum reduced media (DMEM supplemented with 0.5% FBS). The subsequent day, cells were stimulated with 10 ng/mL hGH for 10 min at 37°C. Cells were were washed in cold PBS and harvested in cold lysis buffer comprising 150 mM NaCl, 50 mM Tris pH 7.5, 5 mM EDTA, 0.5% Triton-X-100 and supplemented with 1 x complete EDTA-free protease inhibitor cocktail tablets (Sigma), 10 mM sodium orthovanadate (Na3VO4), 30 mM sodium fluoride (NaF), and 10 mM sodium orthophosphate (Na2HPO4). Protein lysates were centrifuged at 12,000 x g for 10 min at 4°C and quantified using BCA Protein Assay kit (ThermoFisher Scientific). Equal amounts of protein lysates (3 µg) were pre-cleared using Protein G Sepharose beads (GE Healthcare) for 30 min at 4°C. The supernatant was removed from the beads by spinning at 13000 x g and subjected to immunoprecipitation.

For each immunoprecipitation, antibodies (anti-JAK2 1:100 dilution, anti-LYN 1:50 dilution, anti-MYC-tag 1:100 dilution, or anti-HA-Tag 1:150) were added to pre-cleared protein lysate and mixed gently on a rotating wheel at 4°C. After 2 h, Protein G Sepharose beads were added in the antibody-lysate tube and mixed gently on a rotating wheel at 4°C for 2 h. Beads were washed in lysis buffer (lacking Triton) twice spun at 3000 x g, then heated to 98°C in SDS sample buffer with 0.1 M DTT for 5 min and subjected to SDS-PAGE.

**Cell proliferation assay**

Retroviral transduced and selected FDC-P1 GHR-WT and Box1ΔGHR cells were seeded (500 cells per well) into a 384-well plate in RPMI-1640 medium devoid of IL-3 and phenol red (with 10% FBS, 4 mM Glutamine, 50 µg/mL penicillin-streptomycin). Increasing concentrations of hGH were added to the respective wells and live cells were counted from day 0 to day 6 after seeding using the IN Cell Analyzer 2200 (GE Healthcare Life Sciences) determined by automated counting GFP positive cells using the FITC channel.

**Quantitative real-time PCR**

RNA was extracted using Trizol (ThermoFisher Scientific) as previously described (Chhabra et al., 2019). cDNA was prepared using iScriptRT supermix synthesis kit (Bio-Rad). GHR gene expression was quantified using SYBR green method of qPCR on an ABI Viia7 detection system. Samples were normalized against the β-2microglobulin gene (key resources table). Expression was calculated using the standard curve method according to the manufacturer’s protocol.

**Flow cytometry analysis**

Retroviral transduced HEK293 GHRWT and Box1ΔGHR cells were analyzed for cell surface expression of GHR by flow cytometry. Approximately 1 x 10^6 cells were resuspended in 80 µL PBS with 2% FBS containing anti-HA primary antibody (1:100 dilution) and incubated for 2 h at 4°C. Cells were then washed twice and resuspended in 80 µL PBS (2% FBS) containing Alexa Fluor 647 secondary...
antibody, goat anti-mouse (1:500 dilution), and incubated for 40 min (in the dark) at 4°C. Cells were washed twice and resuspended in 500 μL PBS (2% FBS), and analyzed by flow cytometry (BD LSRFortessa X20, Becton Dickinson). Ten thousand gated events were collected for each sample and analyzed using FlowJo software.

In vitro phosphorylation of GHR-ICD

In vitro phosphorylation of 15N-GHR-ICDFL produced as in, and an LYN peptide substrate (EDPIYEFLPAKKK, Enzo Life Sciences) was carried out by an active LYN A kinase (SIGMA-ALDRICH) in 20 mM Na2HPO4/NaH2PO4, 25 mM NaCl, 5 mM MgCl2, 1 mM DTT, 1 mM PMSF, 0.01% NaN3, at pH 7.3 (15N-GHR-ICDFL) or pH 7.0 (LYN peptide substrate). Active LYN kinase was added in ratios of 1:5000 (15N-GHR-ICDFL) and 1:1500 (LYN peptide substrate) with 0.5 mM ATP, and the samples incubated at 37°C for 72-110h. Phosphorylation of the control peptide was followed by Maldi-TOF mass spectrometry (Bruker autoflex) and 1H 1D NMR spectra before and after phosphorylation. For the 15N-GHR-ICDFL, 1H,15N-HSQC NMR spectra were recorded before and after incubation with LYN kinase and ATP. For NMR, the samples were added 10% (v/v) D2O and 1 mM DSS as reference. The 1D 1H NMR spectra of the peptide substrate were recorded on a Bruker 750 MHz spectrometer at 25°C, whereas those of 15N-GHR-ICDFL were recorded on a Bruker 600 MHz spectrometer at 5°C, both with cryo-probes.

Expression and purification of GHR-ICD270-370 and GHR-ICD270-370 ΔBox1

Unlabeled and 15N-labeled GST-tagged GHR-ICD270-370 and GHR-ICD270-370 ΔBox1 were expressed as described. Cell pellet from 1 L of culture was resuspended in 20 mL lysis buffer (1 × PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4), pH 7.4, 0.1% Triton X-100 and 1 tablet EDTA-free protease inhibitor cocktail (Roche Diagnostics, GmbH)) and lysed using a cell disruptor Multi Shot (Constant Systems Ltd) at 20,000 psi. The lysate was cleared by centrifugation at 20,000 × g for 20 min at 4°C and to the supernatant 0.5 M GuHCl and 5 mM DTT was added and incubated for 2 h with 3 mL Gluthathione Sepharose fast flow column material (GE Healthcare) equilibrated in 1 × PBS, pH 7.4. After 2 h, the flow through was collected and unbound protein eluted by washing with 50 mL 1 × PBS, 7.4. Bound protein was eluted in 20 mL 50 mM Tris-HCl, 10 mM reduced glutathione, pH 7.4. The elution was re-incubated on the column and eluted as described. The elution was dialyzed against 2 L 20 mM Tris-HCl, 150 mM NaCl, pH 7.4 overnight at 4°C. The GST-tag was cleaved by incubating the protein with 2.5 units of thrombin per mg of protein for 3 h. The sample was added 0.1% (v/v) trifluoroacetic acid (TFA) and applied to a Zorbax 300SB-C18 column (Agilent technologies) equilibrated in 0.1% (v/v) TFA in MO to remove the GST-tag and additional degradation products. Protein was eluted over three steps of linear gradients from 0% elution buffer (70% acetonitrile, 0.1% TFA) over four column volumes (CVs), 50–70% elution buffer over three CVs and 70–100% elution buffer over three CVs. Elution fractions were analyzed by SDS-PAGE and the ones containing pure GHR-ICD270-370 or ΔBox1 GHR-ICD270-370 were pooled and freeze-dried.

Expression of LYN32, LYN3, and LYN2

A total of three variants of the LYN Src kinase were used in this study. cDNA spanning the SH3 and SH2 domains (residues 63–228) (LYN32) was cloned into a pET22b(+) vector encoding an N-terminal His-tag. cDNAs covering the SH3 domain (residues 63–127) and SH2 domain (residues 63–228) (LYN3 and LYN2) were expressed as described. Cell pellet from 1 L of vector for 30 min on ice, heated at 42°C for 90 s and incubated on ice for 5 min. The cells were added 1 mL LB media and incubated at 37°C for 45 min. The cells were then plated on LB agar plates with 100 μg/mL ampicillin and incubated at 37°C overnight. Aliquots of 10 mL LB containing 100 μg/mL ampicillin were inoculated with one colony of freshly transformed BL21(DE3) cells and sonicated on ice for 5 min. The cell pellet was re-incubated on the column and eluted as described. The elution was dialyzed against 2 L 20 mM Tris-HCl, 150 mM NaCl, pH 7.4 overnight at 4°C. The GST-tag was cleaved by incubating the protein with 2.5 units of thrombin per mg of protein for 3 h. The sample was added 0.1% (v/v) trifluoroacetic acid (TFA) and applied to a Zorbax 300SB-C18 column (Agilent technologies) equilibrated in 0.1% (v/v) TFA in MO to remove the GST-tag and additional degradation products. Protein was eluted over three steps of linear gradients from 0% elution buffer (70% acetonitrile, 0.1% TFA) over four column volumes (CVs), 50–70% elution buffer over three CVs and 70–100% elution buffer over three CVs. Elution fractions were analyzed by SDS-PAGE and the ones containing pure GHR-ICD270-370 or ΔBox1 GHR-ICD270-370 were pooled and freeze-dried.

Purification of LYN32

The cleared lysate was incubated for 1 h with 2.5 mL Ni-NTA gravity flow column material (GE Healthcare) equilibrated at room temperature in 50 mM NaH2PO4/Na2HPO4, 300 mM NaCl, 10 mM jME, pH 8. The column was washed in 10 CVs 50 mM NaH2PO4/Na2HPO4, 300 mM NaCl, 10 mM imidazole, 10 mM jME, pH 8 and protein eluted in 3 CVs 50 mM NaH2PO4/Na2HPO4, 300 mM NaCl, 250 mM imidazole, 10 mM jME, pH 8. The elution was buffer changed to 50 mM NaH2PO4/Na2HPO4, 300 mM NaCl, 10 mM jME, pH 8 using a PD10 column (GE Healthcare). The elution was applied to a HiLoad 16/600 Superdex 75 prep grade column (GE Healthcare) equilibrated in 50 mM NaH2PO4/Na2HPO4, 300 mM NaCl, 10 mM jME, pH 8. Fractions were analyzed by SDS-PAGE and fractions containing pure LYN32 were pooled.
Purification of LYN3
The cleared lysate was applied to a 5 mL HiTrap Q FF column (GE Healthcare) equilibrated in 20 mM Tris-HCl, pH 7. The column was washed in 20 mM Tris-HCl, pH 7 until a stable 280 nm absorbance was reached before eluting protein by a linear gradient from 0 to 100% elution buffer (20 mM Tris-HCl, 1 M NaCl, pH 7). Fractions containing LYN3 were pooled and applied to a HiLoad 26/600 Superdex 75 prep grade column (GE Healthcare) equilibrated in 20 mM Tris-HCl, pH 7. Fractions were analyzed by SDS-PAGE and the ones containing pure LYN3 were pooled.

Purification of LYN2
The cleared lysate was applied to a 5 mL HiTrap heparin HP column (GE Healthcare) equilibrated in 10 mM NaH2PO4/Na2HPO4, 10 mM βME, pH 6.5. The column was washed in 10 mM NaH2PO4/Na2HPO4, 10 mM βME, pH 6.5 until a stable 280 nm absorbance was reached before eluting protein by a linear gradient from 0 to 100% elution buffer (10 mM NaH2PO4/Na2HPO4, 2 M NaCl, 10 mM βME, pH 6.5). Fractions containing LYN2 were pooled and applied to a HiLoad 16/600 Superdex 75 prep grade column (GE Healthcare) equilibrated in 50 mM NaH2PO4/Na2HPO4, 300 mM NaCl, 10 mM βME, pH 6.5. Fractions were analyzed by SDS-PAGE and the ones containing pure LYN2 were pooled.

NMR spectroscopy
All NMR experiments were acquired on a 600 or 750 MHz (1H) Bruker Avance III instruments equipped with a cryogenic probe. All experiments were recorded using non-linear sampling. Fourier induction decays were transformed and processed in qMDD,92 phased in NMRDraw95 and spectra analyzed in CcpNMRAutoAnalysis 2.3.1.95 DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) was used for 1H chemical shift referencing. Assignments of GHR-ICD270-370 was obtained from,37 adjusted to the current condition, and deposited in the BioMagResBank (BMRB: 50478). Backbone resonances of LYN3 was obtained from HNCACB, CBCA(CO)NH triple resonance experiments and deposited in BioMagResBank (BMRB: 50475). Titration of LYN variants and GHR-ICD270-370 was followed by 1H-15N-HSQC experiments at each titration point. Combined chemical shift changes ($\Delta \delta_{NH}$) were calculated using the equation: $\Delta \delta_{NH} = \sqrt{(\Delta \delta_H)^2 + (0.154 \cdot \Delta \delta_N)^2}$, where 0.154 is a scaling factor compensating for the difference in gyromagnetic ratio between the 1H and 15N nuclei.38

Intensity changes were quantified by calculating the peak height ratio between the free and the bound state. For affinity determination a global fit of the chemical shift perturbations of the most affected residues using the equation: $\Delta \delta_{NH} = \Delta \delta_{\text{max}} \left( \frac{[P]_0 + [L]_0 + K_d}{[P]_0} \right) - \sqrt{\left( \frac{[P]_0 + [L]_0 + K_d}{[P]_0} \right)^2 - 4 \frac{[P]_0}{[P]_0}}$, where $\Delta \delta_{NH}$ is the chemical shift change, $\Delta \delta_{\text{max}}$ is the maximum chemical shift change and $[P]_0$ and $[L]_0$ is the concentrations of the 15N labelled protein and ligand respectively.39

Protein labeling and microscale thermophoresis
GHR-ICD270-370 and GHR-ICD270-370Box1 were fluorescently labelled with an NT-647 NHS ester fluorophore using the Monolith NT Protein Labeling kit RED-NHS (NanoTemper). GHR-ICD270-370 and GHR-ICD270-370Box1 were labelled for 30 min at room temperature in the dark in 3x molar excess of dye in in labeling buffer following the manufacturer’s protocol. The resulting protein-dye conjugates were purified using a gravity flow column provided in the Monolith NT Protein Labeling kit RED-NHS (NanoTemper). Dilution series of the following interaction partners were prepared in 20 mM NaH2PO4/Na2HPO4, 150 mM NaCl, 2 mM DTT, 0.05% Tween 20, pH 7.3, and loaded into Monolith NT.115 premium capillaries: 100 nM labelled GHR-ICD270-370 up to 208 μM LYN32, 200 nM GHR-ICD270-370Box1 + up to to 1.7 mM LYN32, 100 nM labelled GHR-ICD270-370+ up to 198 μM LYN3. To support feasibility of MST for affinity determination in this protein system, a negative control using 100 nM labelled GHR-ICD270-370 + up to 323 μM of the globular domain of nuclear protein human histone linker H1.0 (H1-GD) (purified as in40) was performed (Figure S2C) showing only non-specific binding at high protein concentration. Thermophoresis was measured on a Monolith NT.115 instrument at 20% excitation power at 25 °C. The change in thermophoresis ($\Delta F_{\text{norm}}$) upon binding was measured in triplicates, except for GHR-ICD270-370 + H1-GD which was measured twice, and reported as “fraction bound”. $K_D$ was obtained from the fit to the equation:

\[
\text{fraction bound} = \frac{([P]_0 + [L]_0 + K_0) - \sqrt{([P]_0 + [L]_0 + K_0)^2 - 4[P]_0[L]_0}}{2[P]_0}
\]

CD spectroscopy
Far-UV CD spectra were recorded on GHR-ICD270-370 and GHR-ICD270-370Box1 in 10 mM NaH2PO4, pH 7.3 adjusted with NaOH in 1 mm Quartz cuvettes. The spectra were recorded on a Jasco J-810 Spectropolarimeter purged with 8 L/min N2 at 25 °C. Acquisition parameters were as follows: 0.1 nm data pitch, 1 nm bandwidth, 2 s response time and 10 nm/min scanning speed. A total of 10 scans were accumulated for each experiment. CD was recorded in the units of ellipticity (θ in millidegrees (mdeg)) from 260 to 190 nm and converted to mean residue ellipticity (θ) in mdeg*cm²dmol⁻¹ by the following equation: $\theta = \frac{\theta}{10}$, where C is the molar concentration, n is the number of peptide bonds and I is the cuvette path length in centimetre.
Isothermal titration calorimetry

Samples of 1.2 mM GHR-ICD$_{270-370}$ and 120 μM LYN32 were dialyzed against 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mM NaCl, 1 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP), pH 7.3 at 4°C. Isothermal titration calorimetry (ITC) was performed in a MicroCal ITC200 microcalorimeter at 10°C. GHR-ICD$_{270-370}$ was loaded in the syringe and LYN32 in the cell. A total of 38 injections of 1 μL GHR-ICD$_{270-370}$ were injected into the cell with 180 s spacing in between. The baseline was corrected for heat of dilution by subtracting the average of the heat generated from the last five injections. The data was processed and analyzed using the Origin software package (MicroCal) and fitted to a one-site binding model. The experiment was performed several times, but due to low solubility combined with stirring, aggregation issues were prominent inhibiting replication.

Modeling of the GHR:LYN complex

Modeller v. 9.19 was used to construct an initial model of the SH3-SH2 domains (LYN32) (residues 63–228) of LYN using PDB IDs: 1WA7,96 and 1AOT97 for the LYN3 (residues 1–60) and LYN2 (residues 62–166), respectively. For the GHR-ICD$_{270-370}$, an extended structure was built using Modeller. HADDOCK2.2 was used to generate an initial model of the complex with LYN3-GHR-ICD$_{270-370}$ using the interfacial residues obtained from NMR shift perturbations (LYN3) and from examining the LYN2 homologs (PDB IDs: 1AOU, 1AOT) bound to various peptides in the PDB database97 as active residues for docking (Table S2). The most representative complex structure in terms of favored docking score was considered for coarse-grained (CG) simulation. A certain number of contacts (the “coordination number”) were added as restraints during CG simulation using a PLUMED plugin in Gromacs v. 5.1.4 in order to restrict the phase space of the system.

A CG model was built using the martiniize tool, adding harmonic restraints within the LYN2 and LYN3 domains, but not between the domains or to the GHR-ICD$_{270-370}$, and solvated in a cubic water box with a 10Å padding in all directions using insane script, resulting in a system with ~14500 non-polarizable water beads and 11 sodium ions. MARTINI v. martinize v. 2019.4 was used with particle mesh Ewald (PME) for non-bonded electrostatic energies with a real-space cutoff of 11Å. The system was minimized for 1000 steps using the steepest descent method. Previously, studies have shown that excessive protein-protein interaction in the martini model results in aggregation. Thus, it is essential to appropriately scale protein-water interactions to modulate hydrophobic effects. In this study, after minimization, a new topology file was generated where the Lennard-Jones term, ε, between the protein-water, was scaled up by 1.05 percent in line with previous analyses of multidomain proteins. Following this, the system was relaxed for 1ns with coordinates saved every 5 ps at constant temperature of 300K and 1 bar pressure with the compressibility of 3 × 10$^{-4}$ bar and a time constant of 12ps. The temperature of the system was controlled using the stochastic velocity rescaling thermostat and the pressure using Parrinello-Rahman barostat. For the ring systems, stiff bond constraints were defined using LinCs. Finally, isotropic NPT simulations were performed in four steps for a total of 2 μs, and with coordinates saved every 20 ps. First, the system was simulated for 100 ns with single coordination restraint between LYN3 and GHR-ICD$_{270-370}$ backbone interface beads to optimize the system (Table S2). A cutoff of 9Å was used to calculate contacts between the two groups, and a force constant of 100 kJmol$^{-1}$nm$^{-2}$ was applied when the count went below three. Second, the simulation was performed for 400 ns with an additional coordination restraint between LYN2 and GHR-ICD$_{270-370}$ interface backbone beads (Table S2). As earlier, a force constant of 100 kJmol$^{-1}$nm$^{-2}$ was applied if the count went below five for each of the restraints. Here the system was weakly restrained to have fewer contacts. Consequently, the simulations were performed for 1.5 μs wherein the coordination was increased from 5 to 10 with increasing force constants, which is from 100 to 400 kJmol$^{-1}$nm$^{-2}$ to allow the system to converge gradually to an optimal state. Lastly, the backmapping protocol was used to reverse CG the structures to obtain atomistic models, which were minimized for 500 steps and simulated for 20 fs.

Single particle tracking photoactivated localization microscopy (sptPALM)

HEK293 cells were plated on 0.1 mg/mL poly-D-lysine (Sigma) coated 35mm glass bottom dishes (Cellvis) at a seeding density of 2.5 × 10$^4$ cells. Cells were transfected the following day with 100 ng of GHR-WT (FL or 322 truncated) or 200 ng of GHRΔBox1 (FL or 322 truncated) C-terminal tagged mEOS2 constructs where indicated using lipofectamine 2000 (Life Technologies). Prior to imaging, transfected cells were washed twice and imaged in Buffer A (145 mM NaCl, 5 mM KCl, 1.2 mM Na$_2$HPO$_4$, 10 mM D-glucose, 20 mM HEPES, pH 7.4). Single particle tracking Photoactivated Localization Microscopy (sptPALM) experiments were conducted by tracking the expression of GHR-WT(322)-mEOS2, GHRΔBox1(322)-mEOS2, GHR-WT(FL)-mEOS2 and GHR-ΔBox1(FL)-mEOS2 in the presence or absence of 100 ng/ml hGH at the plasma membrane of transfected HEK293 cells. Cells were visualized on a Roper Scientific TIRF microscope, for live-cell TIRF imaging, equipped with an iLas$^2$ double laser illuminator (Roper Scientific), a Nikon CFI Apo TIRF 100×/1.49 NA oil-immersion objective and an Evolve 512 Delta EMCCD camera (Photometrics). Time-lapse TIRF image tracking was performed using MetaMorph software (version 7.10.2.240, Molecular Devices) and was captured at 50Hz (20 ms exposure per frame) at 37°C. For sptPALM, a Stradus 405 nm laser (Vortan Laser Technology) was used to photo-activate the cells expressing mEOS2-tagged constructs, and a Jive 561 nm laser (Cobolt Lasers) was used for excitation of the resulting photo-converted single-molecule fluorescence signal. The sample was illuminated simultaneously with both the lasers. To isolate the mEOS2 signal from autofluorescence and background signals, we used a double beam splitter (LF488/561-A-000, Semrock) and a double band emitter (FF01-523/610-25, Semrock). To spatially distinguish and temporally separate the stochastically activated molecules during acquisition, the power of the lasers was adjusted, such that the 405 nm laser used 1–2% of the initial laser power, and the 561 nm laser used 80% of the full laser power. Using a custom program PALM-Tracer software (Version 2.1.0.28228).
on the Metamorph platform\textsuperscript{89} we quantified the precise localization and performed single-particle tracking, from 16,000 frame TIRF movies. All trajectories were tracked, and the diffusion coefficient ($D$) distribution was calculated (range: $\log_{10} D$ \textasciitilde 5 to 1). This was further divided into two groups based on a calculated threshold division in the diffusion coefficient, as previously described.\textsuperscript{108} Super-resolution images were colour-coded (using Fiji), to represent when they first appeared in time (16 color trajectory images, warmer colors appear latest) and average intensity maps were generated, with each pixel indicative of an individual molecule. Diffusion co-efficient map color code (16 color code), with warmer colors exhibiting lower mobility.

**Hidden Markov modeling**

In order to extract the discrete diffusive states from the single molecule trajectories and analyze their spatiotemporal characteristics, we used a suitable software based on a variational Bayesian treatment of HMMs (vbSPT).\textsuperscript{42} This analysis relies on all steps “tracklets” (distance covered in 20 ms) from all trajectories to establish a model in which molecules are allocated to specific mobility states and assigned a probability of staying in or transitioning from one state to another. We used 25 iterations and full bootstrapping (100) to infer motion parameters from HEK293 cells expressing mEOS2 tagged GHR constructs in the presence or absence of 100 ng/mL hGH after performing sptPALM. We also set a default range of initial diffusion coefficient guesses to between 0.01 and 5 $\mu m^2/s$ for the analysis. Custom-written MATLAB codes were used to generate output files formats from PALMtracer suitable for vbSPT analysis and subsequent handing of data. As HMM is a probability-based analysis method which is reliant on relatively large sample size, we pooled the trajectories from cells that contained fewer than 2000 trajectories. The average number of trajectories per GHR-FL dataset for the following conditions was 8220 for WT no GH, 7507 for WT plus GH, 7207 for $\Delta$Box1 no GH and 6839 for $\Delta$Box1 plus GH respectively. The average number of trajectories per GHR-322 dataset for the following conditions was 4480 for WT no GH, 5735 for WT plus GH, 5256 for $\Delta$Box1 no GH and 5643 for $\Delta$Box1 plus GH. We initially analyzed all data sets with a maximum of 5 hidden state models. The three-state model was most chosen to compare across all groups as this was the best fit for our control group on which we would make statistical comparisons. Furthermore, three-state model had statistically distinct average diffusion co-efficient for S1, S2 and S3. Lastly, the average diffusion coefficient were consistent across experimental groups, providing an ideal platform on which to statistically compare the change in state occupancy across each group and experimental condition. We recognize a three-state HMM could be a simplification of GHR behavior, as it is possible that multiple intermediate states (of the 4 and 5 state model) could be lumped into the same intermediate states (represented within the 3-state model).

**Clustering analysis**

We used cross-correlation drift correction of the sptPALM data using the SharpViSu tool.\textsuperscript{88} Trajectory clustering analysis was performed on this drift corrected data, using density-based spatial clustering of applications with noise \textsuperscript{109} as implemented in the SciKit Learn (\texttt{sklearn.org}) module of Python (\texttt{python.org}). Selected spatial centroids for trajectories with a length of 8 or more steps were analyzed by DBSCAN such that a centroid was considered clustered if there were a minimum of 2 other centroids within a radius ($\epsilon$) of 40 nm. A convex hull of all the detections associated with a given cluster was used to determine its area and radius assuming circularity. The average cluster radius ($\mu m$), average membership (trajectories per cluster), average density (clustered trajectories per $\mu m^2$) and the percentage of trajectories in clusters were established for each imaged cell and used for quantitative comparisons. An unpaired Student t test was used to establish the significance of difference in these metrics.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Each experiment was performed independently ($n > 3$). Quantification and data analysis of immunoblots were performed in ImageJ and GraphPad Prism8. For studies where two groups were compared, an unpaired Student t test was used. ANOVA or Holm-Sidak’s adjusted $p$ values was used for multiple comparisons. Single particle tracking data (AUC and M/IM ratio) was analyzed by a Student’s $t$-test (normally distributed). To test the three-state model apparent diffusion co-efficient a one-way ANOVA with Tukey multiple comparisons was used. Data was represented as mean $\pm$ SEM.