Controlled dimerization of insulin-like growth factor-1 and insulin receptors reveal shared and distinct activities of holo and hybrid receptors

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Abstract

Breast cancer development and progression is influenced by insulin-like growth factor 1 (IGF1R) and insulin receptor (InsR) signaling that drive cancer phenotypes such as cell growth, proliferation, and migration. IGF1R and InsR form IGF1R/InsR hybrid receptors (HybR) consisting of one molecule of IGF1R and one molecule of InsR. The specific signaling and functions of HybR are largely unknown as HybR is activated by both IGF1 and insulin, and no cellular system expresses HybR in the absence of holo-IGF1R or holo-InsR. Here we studied the role of HybR by constructing inducible chimeric receptors, and compared HybR signaling with that of holo-IGF1R and holo-InsR. We cloned chemically-inducible chimeric IGF1R and InsR constructs consisting of the extracellular domains of the p75 nerve growth factor receptor fused to the intracellular β subunit of IGF1R or InsR, and a dimerization domain. Dimerization with the drugs AP20187 or AP21967 allowed specific and independent activation of holo-IGF1R, holo-InsR, or HybR, resulting in activation of the PI3K pathway. Holo-IGF1R and HybR both promoted cell proliferation and glucose uptake, whereas holo-InsR only promoted glucose uptake, and only holo-IGF1R showed anti-apoptotic effects. We also found that the three receptors differentially regulated gene expression: holo-IGF1R and HybR upregulated EGR3; holo-InsR specifically downregulated JUN and BCL2L1; holo-InsR downregulated, but HybR upregulated HK2; and HybR specifically upregulated FHL2, ITGA6, and PCK2. Our findings suggest that when expressed and activated in mammary epithelial cells, HybR acts in a manner similar to IGF1R, and supports further investigation of the role of HybR in breast cancer.

Introduction

Breast cancer is the most common cancer among women worldwide, excluding skin cancer, and it remains a significant cause of morbidity and mortality (1). The insulin-like growth factor/insulin (IGF/insulin) system, consisting of three ligands, IGF-1, IGF-2, and insulin; six ligand-binding proteins IGFBP1-6; and five receptors, insulin-like growth factor 1 receptor (IGF1R), insulin-like growth factor 2 receptor (IGF2R), insulin receptor-A and B (InsR-A and InsR-B), and IGF-1/insulin hybrid receptor (HybR) (2-4) play a crucial role in normal mammary gland development and physiology (5).

IGF1R and InsR are both tetrameric proteins composed of two extracellular α subunits covalently linked to two intracellular β subunits, which contain the tyrosine kinase domains (6,7). The binding of ligands to IGF1R and InsR leads to conformational changes in structure and transphosphorylation, which triggers the activation of two main cascades: the phosphatidylinositol 3-kinase/AKT kinase (PI3K/AKT) pathway and the RAF kinase/mitogen activated protein kinase (RAF/MAPK) pathway (4). Although IGF1R and InsR share common downstream pathways, their biological roles are not completely identical: IGF1R mainly mediates proliferation, migration, transforming, and anti-apoptotic events, whereas InsR mainly controls the metabolism of glucose (8). Nevertheless, there is crosstalk between IGF1R and InsR: IGF-1 can also bind InsR and insulin can bind IGF1R, but with a lower affinity compared to their own targeted receptors (9).

Here we focus on the role of three receptors: holo-IGF1R, holo-InsR, and HybR. Hybrid receptors (HybR) consisting of one α/β subunit of IGF1R and one α/β subunit of InsR have been reported (10-12). Assays for determination of HybR activity have shown that it responds with much greater potency to IGF1 than insulin (13), and has different binding affinity compared to holo-receptors (14). However, as most cells express a combination of IGF1R, InsR and HybR, and few methods exist to specially examine activated HybR in living cells (15), attributing specific effects to HybR has been challenging. HybR content exceeds IGF1R content in >75% breast cancer specimens (16).

To directly study the function of HybR, we used a method which has been used to demonstrate the function of ErbB1/ErbB2 heterodimers (17). We cloned chimeric holo-IGF1R, holo-InsR, and HybR, which have low affinity to growth factors and took advantage of chemically induced system to activate them respectively, and compare their different biological roles in a breast cancer cell line. Although, we understand that the chimeric
system is artificial and highly engineered, it is a powerful tool to systematically isolate the signaling cascades of each receptor individually, and provides novel insight into the potential biological effects of the holo and hybrid chimeric receptors. Additionally, while we cannot directly compare the signaling and biological effects of the chimeric receptors to the native receptors in this study due to factors such as differing potencies of the dimerizing agent and endogenous ligand(s), inability of chimeric receptor to cross-talk with endogenous receptor or other RTK family members, and altered molecular conformation of the chimeric receptor (further detail outlined in Discussion), we have included endogenous receptor action to observe if the directional trend in response is similar.

Results

Cloning and expression of chimeric dimerizable holo-IGF1R, holo-InsR, and HybR.

To construct chimeric IGF1R and InsR and allow for chemically induced dimerization, 4 parts were cloned into the pBabe retroviral vector: 1) Extracellular domain of the low-affinity nerve growth factor receptor (p75), 2) β subunit of IGF1R or InsR, 3) a binding site for a dimerizing agent (one FRB or two FKBP domains), and 4) an epitope tag (HA or Glu-Glu) (Fig 1A). The role of p75 is to replace the ligand binding α subunit of IGF1R or InsR, and prevent chimeric receptors from being activated by IGF-1 or insulin. p75 has a low affinity for nerve growth factor (which is not expressed by breast cancer cells) and thus has no ligand and/or activity. AP20187 was used to dimerize FKBP domains together to activate holo-IGF1R or holo-InsR, while AP21967 was used to dimerize an FRB and an FKBP domain to activate HybR (Fig 1A). After transient transfection and viral infection, stable cell lines were generated and immunoblotting performed. As shown in Fig 1B, compared with wild type (WT) MCF7, holo-IGF1R, holo-InsR, and HybR transfected cells expressed endogenous proteins, and chimeric receptors were also detected with a larger molecular weight (Fig S1). All three transfected cell lines expressed HA tags as expected; however, different expression levels were noted with holo-IGF1R expressing higher levels. For IGF1R, all cells expressed endogenous IGF1R, but chimeric IGF1R was only detected in the holo-IGF1R and HybR cells as expected. For InsR, endogenous levels were low, but were increased in the holo-InsR and HybR cells as expected. However, the chimeric holo-InsR had a slightly higher molecular weight. The detection of the Glu-Glu tag was challenging due to lack of sensitivity of the antibody, however, was repeatedly detected in the HybR cells. Of note, we observed slight increases in native receptor expression with the addition of chimeric receptor expression potentially due to changes in stability of the native receptor, however we did not test this in this study. In summary, we generated stable cell lines expressing vectors that allow chemical activation of holo-IGF1R, holo-InsR and HybR. We attempted to obtain clones with equal expression of the respective components, but this was not possible.

Cells were serum starved by placing in serum-free media, to maintain a low level of activation of endogenous IGF1R, InsR, and HybR, and then treated with increasing concentrations of dimerizing agent (0 to 2.5 μM) and activation of PI3K and Erk1/2 pathway detected by immunoblotting (Fig 2A-C, Fig S1-2). In all three cell model systems (holo-IGF1R (Fig. 2A), holo-InsR (Fig 2B), and HybR (Fig 2C)), as little as 10nM of dimerizer resulted in an increase in p-Akt with a dose-response increase up to 2.5μM. IGF1 and insulin were used as positive controls for signaling activation, however, insulin had only minor effects reflecting the low levels of endogenous InsR in these cells. Phosphorylation of Erk1/2 was minimally increased by dimerizer compounds, but was not as robust as Akt activation (Fig 2A-C, Fig S2). Additionally, we did a time course analysis (30m – 8hr) in holo-IGF1R and HybR cells treated with 500 nM dimerizing agent (Fig S3). As expected the signaling (p-Akt and p-Erk1/2) declines over time, however the holo-IGF1R signaling appears more potent and sustained than the HybR signaling. As a control, stimulating untransfected wild type MCF7 with dimerizing agents did not have any effect upon p-IGF1R or p-Akt levels (Fig S4) and also did not alter cell proliferation (Fig S5)

Activated chimeric holo-IGF1R does not transphosphorylate endogenous IGF1R, but
activated endogenous IGF1R can transphosphorylate chimeric holo-IGF1R.

To ensure that any results obtained with chimeric dimerizable receptors are due to specific activation of chimeric receptors, and not any interference or cross-talk with endogenous receptors, we examined whether chimeric holo-IGF1R is able to cross-phosphorylate endogenous IGF1R and vice-versa. We treated cells expressing holo-IGF1R with IGF-1 or dimerizer. We then used an antibody to the α-subunit of IGF1R to specifically isolate endogenous IGF1R by immunoprecipitation because this domain is not expressed in the chimeric receptors. We observed that immunoprecipitated endogenous IGF1R was only phosphorylated by IGF-1 treatment and not by the dimerizing compound (Fig. 3A). Conversely, when we specifically immunoprecipitated the holo-IGF1R using the HA-tag antibody, we found that both dimerizer compound and IGF-1 induced phosphorylation of chimeric IGF1R (Fig. 3B). This indicates that endogenous IGF1R can cross-phosphorylate chimeric holo-IGF1R, but that chimeric holo-IGF1R stimulated with AP20187 does not induce phosphorylation of endogenous IGF1R. Therefore, we postulate that any results we obtain using the dimerizer compound can be attributed directly to activation of the chimeric IGF1R without any interference with the endogenous IGF1R.

Effect of holo-IGF1R, holo-InsR and HybR on cellular phenotypes.

Holo-IGF1R, but not holo-InsR, regulates cell proliferation.

We investigated the role of holo-IGF1R, holo-InsR, and HybR in cell proliferation. Treatment with 100 nM homodimer AP20187 promoted holo-IGF1R-mediated proliferation (Fig. 4A), but proliferation was not affected by activation of holo-InsR (Fig. 4B). Interestingly, activation of HybR significantly promoted cell proliferation (Fig. 4C). As expected as a positive control, 100 ng/ml IGF-1 (13.3 nM) or 75 ng/ml insulin (13.3 nM) both exhibited strong effects on cell proliferation. As a control, stimulation of WT MCF7 cells with dimerizer compounds had no effect upon cell proliferation (Fig S5).

Holo-IGF1R and holo-InsR cause increased glucose uptake.

As an indirect measure of glucose uptake, we collected media and measured the decrease in concentration of glucose every two days. The amount of glucose reduction in media was normalized to cell number (see materials and methods). Activation of endogenous IGF1R and InsR both reduced glucose in media (Fig. S6). For the chimeric system, holo-IGF1R (Fig. 5A), holo-InsR (Fig. 5B), and HybR (Fig. 5C) all induced glucose uptake, but the uptake was greatest with the holo-IGF1R, likely to due to high expression of the chimeric receptor in this cell line. Similar to proliferation, dimerizer compounds did not affect glucose uptake in WT MCF7 cells indicating endogenous receptors are not activated by the dimerizer compound (Fig S6).

Holo-IGF1R, but not holo-InsR or HybR, causes anti-apoptotic effects.

Previous reports demonstrated that IGF1R protects cells from a variety of apoptotic injuries (18), mainly via the PI3K pathway. To examine this, we first induced apoptosis in MCF7 cells by treatment with 0.1 μM staurosporine (STS), which induced a 2.50-fold increase in apoptosis in holo-IGF1R, 1.40-fold in holo-InsR, and 1.53-fold in HybR (Fig 6A). Next, we induced apoptosis with 0.1 μM STS, and treated cells with ethanol, 100 nM dimerizer, 100 ng/ml IGF or 75 ng/ml insulin and measured apoptosis. While both IGF1 and insulin were able to inhibit apoptosis, only holo-IGF1R (not holo-InsR or HybR) was also able to inhibit apoptosis (Fig. 6B-D).

Holo-IGF1R, holo-InsR, and HybR show differential gene regulation.

Since holo-IGF1R, holo-InsR and HybR have different effects on proliferation, glucose uptake, and apoptosis, we investigated whether they exhibit unique gene regulation. We examined genes that were previously shown to be differentially regulated by IGF-1 (EGR3, FHL2, and ITGA6) (19) and insulin (JUN, BCL2L1, HK2, and PCK2). qRT-PCR was used to measure mRNA levels. Cells were treated with ethanol, 100 nM dimerizer (Fig. 7), 100 ng/ml IGF-1, or 75 ng/ml insulin (Fig. S7). As shown in Fig. 7, EGR3 (Fig. 7A) was upregulated by both holo-IGF1R
(p=0.019) and HybR (p=0.026), but not holo-InsR. In contrast, JUN (Fig. 7B) and BCL2L1 (Fig. 7C) were specifically down regulated by holo-InsR (p=0.036 and p=0.028) and not by holo-IGF1R or HybR. Interestingly, HK2 (Fig. 7D), which encodes the enzyme that catalyzes the first committed step of glycolysis, was downregulated by holo-InsR (p=0.041), but upregulated by HybR (p=0.015). FHL2 (Fig. 7E) and PCK2 (Fig. 7F) were induced by HybR (p=0.048, p=0.049, and p=0.025) but not by holo-IGF1R or HybR. Therefore, holo-IGF1R, holo-InsR and HybR show differential gene regulation.

Discussion

To directly compare signaling by holo-IGF1R, holo-InsR, and HybR, we used a chemically-inducible system to activate holo-IGF1R, holo-InsR, and HybR individually and specifically in the absence of endogenous receptor activation. This method has been used in similar studies to study homodimer vs. heterodimer effects of ErbB1/ErbB2 (17,20) and fibroblast growth factor receptor (FGFR) 1 and 2 (21,22). We found that holo-IGF1R activated the PI3K pathway, induced proliferation, promoted glucose uptake, and reduced apoptosis. Holo-InsR promoted glucose uptake, but did not affect proliferation or apoptosis. HybR had effects that were more similar to holo-IGF1R than holo-InsR, by inducing proliferation and promoting glucose uptake. However, HybR did not block apoptosis. Analysis of gene expression showed genes that were differentially regulated by holo-IGF1R, InsR, and HybR.

Important to our study, we showed that the chimeric receptors act independently of the endogenous receptor, with dimerizer compounds having no effect on control cells that do not express chimeric receptors, and activation of chimeric IGF1R not affecting activation of endogenous IGF1R. Interestingly, however, we found that endogenous IGF1R transphosphorylated chimeric IGF1R. While this does not affect the validity or interpretation of any of our results - as we focus solely on the specific effects of the dimerizer compounds - it will be interesting to determine how endogenous IGF1R confers this transphosphorylation as this may have broader implications. For example, this may explain why activation of endogenous IGF1R is able to transphosphorylate the single chain ErbB1 and 2, whereas activation of ErbB1/2 is incapable of transphosphorylating endogenous IGF1R.

To study the mechanism underlying the biological differences between holo-IGF1R, holo-InsR, and HybR, we examined how these receptors affected gene expression. Since there was no previous research about genes specifically activated or repressed by HybR, we started with genes that were previously shown to exhibit regulation by IGF1 or insulin (19). We found genes that were specifically increased by holo-IGF1R and HybR (EGR3) and specifically repressed by holo-InsR (JUN, BCL21, HK2). HybR specifically induced HK2, FHL2, and PCK2. These results indicate that although these three receptors share common pathways, their downstream targets are not completely similar.

We noted in most experiments that the endogenous system of ligands and receptors appeared to activate signaling and downstream phenotypes greater than the chimeric system. There are several potential explanations for this result. First, the chimeric nature of the receptors puts them in a molecular conformation that is distinct from endogenous receptors. This new conformation may inhibit interaction with potential substrate molecules such as IRS1 and 2, which bind close to the transmembrane domain, or other proteins that simply require the heterotetrameric conformation. Future studies will address this by assessing the ability of activated chimeric receptor to induce phosphorylation of IRS1 and 2. Second, the difference in signaling might be an inability of chimeric receptor to cross-talk with other endogenous receptors as discussed above. Thus, endogenous IGF1R may cross-phosphorylate ErbB1/2 and the chimeric receptor may not be able to do this. Third, the potency of endogenous IGF-1 and insulin ligand may be greater than the dimerizer agent in ability to activate the target receptors. Despite these pitfalls, it is important to note that we only compare the results between different chimeric receptors (holo-InsR, holo-IGF1R and HybR) thus eliminating any confounding results between the endogenous and chimeric receptors.
When making the stably transfected MCF-7 cell lines we attempted to isolate cells with similar expression of chimeric receptor. It is evident, though, that the holo-InsR is expressed at a much lower expression level than the holo-IGF1R. We estimate that holo-IGF1R may be expressed approximately twice that of holo-InsR. However, it seems that even generally correcting for expression level, holo-IGF1R is a more potent inducer (more than 2x) of proliferation, glucose uptake, and survival compared to holo-InsR and that the two receptors have distinct gene expression profiles in the panel we tested. In summary, based on the data we do not think that the reduction in chimeric InsR expression compared to chimeric IGF1R would change the overall result(s) of the study we have completed.

A weakness of our study is that we could not differentiate between InsR A and B isoforms, as the intracellular β subunit we cloned is downstream of the skipped exon of InsR A. A previous study indicated differences between HybR-A and -B (14) and therefore further studies are required to study this difference using the chimeric receptor system.

In conclusion, the data presented here shows similarities and differences between holo-IGF1R, holo-InsR, and HybR. The similarity between HybR and holo-IGF1R is consistent with previous studies (16). Given the overexpression of HybR in breast cancer cell lines and tumors, strategies to inhibit IGF1 and insulin action in cancer need to consider blocking this form of the receptor.

**Experimental Procedures**

**DNA constructs.** To create chemically inducible receptors, we used a system based upon rapamycin-induced dimerization of FK506 Binding Protein 12 with the FKBP Rapamycin Binding (FRB) domain of mammalian target of rapamycin, and small peptide tags for identification consisting of HA and Glu-Glu (17). Three chimeric receptor expression vectors were constructed as follows: pBabe puro (p75-FRB-Glu-Glu), pBabe G418 (p75-IGF1R-FKBP-FKBP-HA), and pBabe G418 (p75-InsR-FKBP-FKBP-HA). Chimeric InsR with Glu-Glu tag was constructed by inserting the β-subunit InsR into pBabe puro (p75-FRB-Glu-Glu). InsR was amplified by primers 5’ AATTACTAGTAAAGAGGCAGCCA 3 and 5’ AATTACTAGTGGAGATGACC 3’ from pcDNA3.1-InsR and engineered to have SpeI sites on both ends. Then it was cloned into pCRT™4-TOPO® Vector. After digestion with SpeI enzyme, InsR with cohesive ends was separated and purified by gel extraction. InsR was cloned into pBabe puro (p75-FRB-Glu-Glu) by ligation. Chimeric InsR with HA tag was constructed by replacing p75-FRB-Glu-Glu with p75-InsR-FKBP-FKBP-HA: p75-InsR-FKBP-FKBP-HA was amplified by primers 5’ TAATAGGATCCGGGGCCATGG 3’ and 5’ CTGCAGGATCTCCTCATTTCT 3’, purified by gel extraction, digested with BamHI, and ligated to pBabe puro backbone which was also digested with BamHI and purified. SpeI and BamHI were bought from New England Biolabs Inc. MinElute Gel Extraction Kit (Qiagen, 28604) was used for gel extraction.

**Cell culture and generating stable cell lines.** PT67 retro-packaging cells were cultured in DMEM (Life Technologies, 11965-118) supplemented with 10% fetal bovine serum and 1mM Sodium Pyruvate (HyClone, SH30239.01). MCF7 breast cancer cells were cultured in DMEM supplemented with 10% fetal bovine serum. On day 0, 1.3 x 10^6 PT67 cells were seeded in 60 mm dishes. On day 1, cells were transfected with chimeric receptor plasmids using Lipofectamine LTX (Life Technologies, 15338100) according to manufacturer’s instructions. On day 2, cells were transfected with chimeric receptor plasmids using Lipofectamine LTX (Life Technologies, 15338100) according to manufacturer’s instructions. On day 2, cells were transfected with chimeric receptor plasmids using Lipofectamine LTX (Life Technologies, 15338100) according to manufacturer’s instructions. On day 2, 1.3 x 10^6 MCF7 cells were plated in 60 mm dishes. On day 3, the retrovirus-containing supernatants were harvested, centrifuged briefly (500 x g for 10 min) to remove debris, filtered through a 0.45 μm syringe filter (Fisher, 09-754-21), and added to MCF7 on day 2. On day 5, the cells were separated into 10cm dishes with series dilutions (1:5, 1:10, 1:20) and 1 mg/ml geneticin (Invitrogen, 10131-035) or 2 μg/ml puromycin (Life Technologies, A11138-03) added as selection drugs. After approximately 2 weeks, single colonies were isolated and expanded.

**Immunoblotting.** Cells were lysed in buffer containing 5% SDS, 1 x EDTA (5 mM) and 1x
Protease & Phosphatase Inhibitor Cocktail (Thermo Scientific, 78442). Protein concentration was measured with Pierce BCA Protein Assay Kit (Thermo Scientific, 23225). 30 µg protein was separated by 8% SDS-PAGE. PageRuler Plus Prestained Protein Ladder (Thermo Scientific, 26619) was used as marker. After SDS-PAGE proteins were transferred onto PVDF membrane (Fisher, IPVH00010), blocked with Odyssey Blocking Buffer (LiCor, 927-40000) for one hour, and blotted with primary antibodies overnight. This was followed by washing with PBST (0.1% Tween 20) three times for half an hour and blotting with secondary antibodies for an hour. Then the membranes were washed with PBST (0.1% Tween 20) three times for half an hour and PBS twice for 20 minutes. Proteins were detected with Odyssey infrared imaging system (version 3.0). Antibodies for immunoblots: anti-HA (1:1000, Cell Signaling, 3724), anti-IGF1R (1:1000, Cell Signaling, 9750S), anti-InsR (1:1000, Cell Signaling, 3025S), anti-pAkt (1:1000, Cell Signaling, 9271S), anti-Akt (1:1000, Cell Signaling, 9272), anti-pERK (1:1000, Cell Signaling, 4695), anti-pIGF1R/InsR (1:1000, Cell Signaling, 3021S), anti-β-actin (1:5000, Sigma, A5441), anti-Mouse IRDye 680LT (Odyssey, 1:8000), anti-Mouse IRDye 800CW (1:8000), anti-Rabbit IRDye 800CW (1:8000).

Immunoprecipitation. Holo-IGF1R MCF7 cells were cultured in full serum media to about 80% confluent in three 10 cm plates and dimerization induced as previously described. Cell lysates in each plate were extracted with 0.8 ml lysis buffer (25 mM Tris-HCl pH 8, 140 mM NaCl, 0.4% NP40, 0.5 µg/ml leupeptin, 0.5 µg/ml aprotinin, 2 mM activated sodium orthovanadate). 30 µl normal mouse IgG (Santa Cruz sc-2025) was added to 0.6 mg protein lysates. After incubation on ice for an hour, 60 µl protein G agarose beads (Invitrogen, 10-1243) was added, agitated for 0.5 hr at 4°C, centrifuged at 14000 rpm at 4°C for 10 min, and supernatant harvested. To the supernatant we added 3 µl anti-IGF1Ra (Abcam, 80548) and agitated overnight at 4°C. The next day, 70 µl protein G agarose beads were added and agitated for 3 hr. Supernatant was centrifuged at 12000 rcf for 3 min, supernatant discarded, and the pellet was washed 4 times with cell lysis buffer at 3000 rcf for 1 min and then proteins denatured in sample buffer for SDS/PAGE and analyzed by immunoblotting.

Proliferation and glucose metabolism assays. 5,000 MCF7 cells/100 µl/well were plated in four 96-well plates with 6 replicates. After serum starvation and drug-induced dimerization as previously described, one plate was harvested every other day. Media was collected for glucose measurement. The proliferation assay was performed with FluoReporter® Blue Fluorometric dsDNA Quantitation Kit (Molecular Probes, F-2962). Glucose concentration was measured with Accutrend® Plus and Roche Diagnostics Glucose Test Strips (Fisher Scientific, 22-045-871).

CellTox™ Green cytotoxicity assay. 10,000 MCF7 cells/100 µl/well were plated in 96-well plates with 6 replicates on day 0. Serum starvation was performed as previously described on day 1, with 2X CellTox™ Green Dye (Promega, G8752). 0.2 µM staurosporine was diluted in SFM and 200 nM dimerizer, 200 ng/ml IGF-1, or 150 ng/ml insulin into each well. RFU was measured immediately and 48 hours thereafter.

qRT-PCR. Total RNA was isolated from treated MCF7 cells using RNeasy Spin Mini (illustra, 25-0500-72) in a 6-well plate. iScript™ Reverse Transcription Supermix (Bio-Rad, 170-8841) was
used for reverse transcription. SsoAdvanced™
Universal SYBR® Green Supermix (Bio-rad, 172-5274) was used for qPCR and Bio-Rad CFX
Manager 3.1 was used for analyzing data. Gene
expression was normalized to the housekeeper
RPL19 and plotted as fold change for each
treatment over vehicle. Primers:

JUN (5‘ AGCCCAAACTAACCCTACG 3’; 5
TGCTCTGTTCCAGATTGTCTCT 3’),
APAF1 (5′ GGCTGTGGGAAGTCTGTATTAG
3’; 5′CAACCGTGTTGAAAGATCTCTG 3’),
ITGA6 (5‘ CCTCCCTGAGCACATATTGCG 3’; 5’
CACCTCAAATTCTCCATTCTC 3’), EGR3
(5′TCGCTAGTTCCATTACAATCGATG 3’; 5’
CTTCCCAAGTAGGTCTACGG 3’), RPL19 (5’
ATGCCAGAGAAGGTCACATG 3’; 5’
ACACATTCCCTCCACCTTC 3’),
HK2 (5′ GGGACAATGGATGCCTAGATG 3’;
5′ GTTACGGACAATCTCACCCAG 3’),
BCL2L1 (5′ GACATCCCAGCTCCACATC 3’;
5′ GTTCCCATAGATGTTCCACATC 3’),
PCK2 (5’ GAGAATACTGCCACACTGACC 3’;
5’ CCGCTGAGAAGGAGTTACAATC 3’),
CEBPA (5′ TGGACAAGAACAGCAACGAG 3’;
5′ TCATTGTCTCGTGTCAGCTC 3’), FBP1 (5’
CTACGCGAGGACTTTGAC 3’; 5’
GTAGACCAGATGCGATGAC 3’), FHL2 (5’
ACTTTGCTACTGCGACTGAC 3’; 5’
AGTGTATGCGGACTGCC 3’).

Statistical analysis. Proliferation and glucose
metabolism assay results are presented as mean ±
S.D, and statistical analyses were performed by t-
test comparing drug-treated group with EtOH-
treated group on day 4 and day 6. In CellTox™
Green cytotoxicity assay, results are presented as
mean ± SEM, with n representing the number of
replicates in each group, and statistical analyses
were performed with one-way ANOVA

(Dunnett: compare all columns vs. control
column). The qPCR results are presented as mean ± SEM, and statistical analyses were performed
with two-tailed t-test for the effect of treatment on
chimeric receptors. Two-way ANOVA was used
for analyzing the interaction between receptors.
P<0.05 was required for statistical significance.

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The authors declare that they have no conflicts of
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Author contributions
All authors provided substantial contributions to
conception and design, JC and AMN acquired
data, and all authors analyzed and interpreted data;
all authors drafted the article and/or revised it
critically for important intellectual content; final
approval of the version to be published and agree
to be accountable for all aspects of the work in
ensuring that questions related to the accuracy or
integrity of any part of the work are appropriately
investigated and resolved.
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**Figure Legends**

**Figure 1.** A) Schematic of gene modification of IGF1R and InsR to create chimeric expression system. Chimeric IGF1R was cloned by linking p75, the β subunit of endogenous IGF1R, two FKBP domains and one HA tag together. Chimeric InsR was cloned in two ways: by linking p75, the β subunit of endogenous InsR, two FKBP domains, and one HA tag; or by linking p75, β subunit of endogenous InsR, one FRB domain and one Glu-Glu tag. Dimerization of chimeric holo-IGF1R and holo-InsR are induced by homodimerizer AP20187, and HybR are induced by heterodimerizer AP21967. B) Immunoblot showing expression of chimeric receptors in MCF7 cells. Stable MCF7 cell lines were generated by viral infection and immunoblotting performed for anti-IGF1R, anti-HA, anti-InsR, anti-Glu-Glu, and anti-β-actin as a loading control (n=3).

**Figure 2.** Chemically induced dimerization of chimeric receptors activates Akt. A) holo-IGF1R, B) holo-InsR, and C) HybR MCF7 chimeric cells were plated, serum starved overnight, and treated with vehicle (EtOH) and increasing doses of AP20187 (A and B) or AP21967 (C). IGF-1 (100 ng/ml) and insulin (75 ng/ml) were used as positive controls. Immunoblot was performed to detect p-Akt and p-ERK pathways. Quantification was performed using ImageJ. All experiments were performed at 2-3 times with variable dosing included per replicate experiment.

**Figure 3.** Endogenous IGF1R transphosphorylates chimeric holo-IGF1R, but not vice-versa. Holo-IGF1R MCF7 was treated with vehicle (EtOH), AP20187 (5 μM), and IGF-1 (100 ng/ml), respectively. A) Anti-IGF1R α subunit antibody was used to immunoprecipitate endogenous IGF1R and immunoblotting performed for anti-pIGF1R and total IGF1R. B) Anti-HA was used to immunoprecipitate chimeric IGF1R and IB was performed for anti-pIGF1R and anti-HA.

**Figure 4.** Holo-IGF1R and HybR, but not holo-InsR induce cell proliferation. Effects on cell proliferation following activation of A) holo-IGF1R, B) holo-InsR, or C) HybR. Growth curves were generated with FluoReporter Blue Fluorometric dsDNA Quantification Kit, and relative fluorescent units (RFU) were measured every 2 days. Each bar represents the mean ± SD (n=6). p<0.05*, p<0.005**, p<0.001***, p<0.0001****. Two-tailed t-test was used to compare AP20187- or AP21967-treated group versus EtOH-treated group on day 4 and day 6.

**Figure 5.** Holo-IGF1R, Holo-InsR and HybR all increase glucose uptake. Effects of glucose uptake following activation of A) holo-IGF1R, B) holo-InsR, or C) HybR. Cells were plated, serum starved overnight, and treated with vehicle (EtOH), 100nM drug (AP20187 or AP21967), 100ng/ml IGF or 75ng/ml insulin. Media was collected on day 0 and day 6 and glucose concentrations
were measured with Accutrend® Plus and Roche Diagnostics Glucose Test Strips. Amount of glucose uptake was calculated and normalized to cell number (RFU measured as previously described (Fig 4)). Two-tailed t-test was used for statistical analysis. Each bar represents the mean ± SD (n=3). p<0.05*, p<0.005**, p<0.001***.

Figure 6. Holo-IGF1R but not holo-InsR or HybR, decrease cell apoptosis.
Effects on apoptosis following A) STS treatment alone in the chimeric cells, and following activation of B) holo-IGF1R, C) holo-InsR, or D) HybR. 10,000 cells were plated in 96-well on day 0 in DMEM (10% FBS), washed with PBS twice and replaced with serum-free media and 2X CellTox™ Green Dye on day 1. 0.1μM STS and dimerizer drugs were added on day 2. Fluorescence (RFU) was measured immediately after adding drugs and 48 hours later. One-way ANOVA (Dunnnet: Compare all columns vs. control column) was used for statistics analysis. Each bar, mean ± SEM (n=6). p<0.05* p<0.005**, p<0.001***, p<0.0001****.

Figure 7. Differential gene regulation by chimeric receptors.
Differential gene regulation by activation of holo-IGF1R, holo-InsR, and HybR with 100nM AP20187 or AP21967 for 5 hours. mRNA levels of EGR3 (A), JUN (B), BCL2L1 (C), HK2 (D), FHL2 (E), and PCK2 (F) were measured by qRT-PCR and were represented as fold change over vehicle control (normalized to RPL19 expression levels). Two-tailed t-test was used for statistical analysis of the effect of treatment on chimeric receptors. Each bar, mean ± SEM (n=3). p<0.05*. Two-way ANOVA was used for analyzing the interaction between receptors.
**FIGURE 1**

**A)**

- **p75**: low-affinity nerve growth factor receptor
- **HA**: Hemagglutinin tag
- **Fkbp**: FKBP domain
- **FRB**: FRB domain
- **Glu-Glu**: Glutathione S-transferase tag

**Infection into MCF7 cell lines individually**

- **Holo-IGF1R**
- **HybR**
- **Holo-InsR**

**Cytoplasm**

**B)**

| Protein     | WT | Holo-IGF1R | Holo-InsR | HybR |
|-------------|----|------------|-----------|------|
| IGF1R       |    |            |           |      |
| HA          |    |            |           |      |
| InsR        |    |            |           |      |
| Glu-Glu     |    |            |           |      |
| β-actin     |    |            |           |      |

**Western Blot Analysis**

- **130KD**: Chimeric
- **100KD**: Endogenous
- **55KD**: Endogenous
- **35KD**: Endogenous
Holo-IGF1R MCF7

A) IP: Anti-IGF1R α subunit
   IB: Anti-pIGF1R; anti-IGF1R

B) IP: Anti-HA
   IB: Anti-pIGF1R; anti-HA
FIGURE 4

A) holo-IGF1R

- EtOH
- 100 nM AP20187
- 100 ng/ml IGF
- 75 ng/ml insulin

B) holo-InsR

- EtOH
- 100 nM AP20187
- 100 ng/ml IGF
- 75 ng/ml insulin

C) HybR

- EtOH
- 100 nM AP21967
- 100 ng/ml IGF
- 75 ng/ml insulin

RFU vs. Days after treatment
Controlled dimerization of insulin-like growth factor-1 and insulin receptors reveal shared and distinct activities of holo and hybrid receptors
Jingci Chen, Alison M. Nagle, Yu-Fen Wang, David N. Boone and Adrian V. Lee

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