Supporting Information

Zheng et al. 10.1073/pnas.1118359109

SI Materials and Methods

Reagents. Monoclonal antibodies against ubiquitin (P4D1), G protein-coupled receptor kinase (GRK)2 (C-9), and GRK6 (XX-4) and polyclonal antibodies against insulin-like growth factor-1 receptor (IGF-1R) (H-60), β-arrestin1 (K-16), and GAPDH (FL-335) were from Santa Cruz Biotechnology. Polyclonal antibodies against phosphorylated (p)AKT (S473), AKT, pERK1/2, ERK1/2, pIGF-1R, and IGF-1R were from Cell Signaling Technology. Polyclonal antibody against phospho-serine and Dynabeads protein G were from Invitrogen. Polyclonal antibody against flag and anti-flag M2-agarose were from Sigma.

Cell Culture. The Human Embryonic Kidney 293T cell line (HEK293T) was obtained from ATCC (via LGC Promochem). HEK293T was grown in DMEM supplemented with 10% (vol/vol) FBS and 1% penicillin-streptomycin (PS). BE and DFB, skin melanoma cells, were cultured in RPMI medium supplemented with 10% (vol/vol) FBS and 1% PS. The mouse embryonic fibroblast (MEF), MEF cell with targeted disruption of IGF-1R gene (R-), and Δ1245 mouse cell lines (IGF-1R knockout cells, stably transfected with IGF-1R with C terminus truncated at position 1245) were a kind gift from Dr. Renato Baserga (Thomas Jefferson University, Philadelphia, PA) and are as previously described (1). These cell lines were cultured in DMEM supplemented with 10% (vol/vol) FBS and 1% PS in the presence of G-418 (Promega).

Serum Starvation and IGF1 Stimulation. Cells were washed twice with PBS and changed to serum-free media for 12 h before stimulation. Stimulation with recombinant human IGF1 (Sigma) was performed at 50 ng/mL.

siRNAs and Transfection. Chemically synthesized, double-stranded siRNAs were purchased from Dharmacon. The siRNA sequences targeting endogenous GRKs are as follows: GRK2, 5′-GGAAGAAUGUGGAGCUCAAtt-3′; GRK3, 5′-GCAGCAAGAAGUAACGGAAtt-3′; GRK5, 5′-CGUCUACCGAGAUCUGAAAtt-3′; GRK6, 5′-GAGAAAAAGCGGAUCAAGAtt-3′. The cells were transfected at 40–50% confluency in six-well plates, using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions.

Plasmids and Transfection. The plasmid encoding full-length IGF-1R–YFP was previously described (2). Various mutants were constructed from IGF-1R–YFP plasmids using the QuikChange XL II kit (Agilent Technologies). Sense and antisense primers were designed to mutate the desired codon at specific positions within the IGF-1R–YFP using the QuikChange primer design program. In the C terminus of IGF-1R, three serine residues were mutated individually to alanine residues at 1248, 1272, and 1291 (S1248A, S1272A, S1291A) to eliminate phosphorylation; or to aspartate residues (S1248D, S1272D, S1291D) to mimic phosphorylation. The β-arrestin1–CFP plasmid was obtained from Dr. Carsten Hoffmann, Wuerzburg University, Germany (3). The plasmids expressing human GRK2 and GRK6 (4), the β-arrestin1-flag plasmid, and GFP–β-arrestin1 (5) were a kind gift from Dr. Robert J. Lefkowitz (Duke University Medical Center/Howard Hughes Medical Institute, Durham, NC). Cells cultured at 90% confluency in six-well plates were transfected with plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Immunoprecipitation. Cells were cultured in six-well plates. After indicated treatments, cells were lysed with 500 μL lysis buffer [110 mM KOAc, 0.5% (vol/vol) Triton X-100, 100 mM NaCl, and buffering salts (pH 7.4)]. The protein concentration was determined by bicinchoninic acid assay (Pierce). Dynabeads protein G (10 μL) (Invitrogen) and 1 μg antibody were added to 500 μg of protein. After overnight incubation at 4 °C on a rotator platform the immunoprecipitates were collected on a magnetic holder, the supernatant discarded, and the beads were washed three times with lysis buffer and then dissolved in the sample buffer for SDS/PAGE.

In Vitro Binding Assay. β-Arrestin1-flag from transfected HEK293T cell lysates was purified by anti-flag M2 agarose with overnight incubation at 4 °C, then the beads were washed with PBS four times. The cells transfected with WT IGF-1R–YFP and mutant IGF-1R–YFP were lysed as described above. YFP fluorescence was used to control for equal loading of IGF-1R–YFP containing cell lysates, and 500 μL was added to the beads-β-arrestin1-flag complex and incubated overnight at 4 °C. The pellet was collected by centrifugation at 6,000 × *g* for 1 min and washed three times with 1× Tris-buffered saline (TBS) buffer in a spin cup column (Pierce), whereupon the pellet was dissolved in sample buffer for SDS/PAGE. Western blot (WB) analysis probing with anti–IGF-1R identified IGF-1R–YFP as migrating at ≈125 kDa, compared with endogenous IGF-1R migrating at ≈95 kDa while flag detection was used as a loading control. GAPDH and IGF-1R–YFP in the cell lysates used for the binding assay were detected to validate equal protein loading.

SDS/PAGE and WB. Protein samples were dissolved in LDS sample buffer (Invitrogen). Samples corresponding to 5–50 μg of cell protein were analyzed by SDS/PAGE with 4–12% Bis-Tris gel (Invitrogen). Molecular weight markers (Fermentas) were run simultaneously. After separation the proteins were transferred to nitrocellulose membranes for 1 h at 100 V. Membranes were then blocked for 1 h at room temperature in a solution of 5% (wt/vol) skimmed milk powder and 0.1% (vol/vol) Tween 20 in TBS, pH 7.5 (TBS-T). Incubation with appropriate primary antibody was performed overnight at 4 °C. This was followed by washes with TBS-T and incubation with either a horseradish peroxidase-labeled or a biotinylated secondary antibody (Amersham Biosciences) for 1 h. After the biotinylated secondary antibody, incubation with streptavidin-labeled horseradish peroxidase was performed. The detection was made with ECL substrate (Pierce) and exposure to X-ray film.

Quantitation of GRK siRNA Depletion and Overexpression. HEK293T, BE, and DFB cells were counted and equal numbers plated and allowed to attach and then lysed. Protein content in lysates was quantified by bicinchoninic acid assay (Pierce) and fourfold serial dilutions starting with 2 μg of protein lysates amounts separated by PAGE, transferred electrophoretically to membrane, and probed with antibody against GRK2 or -6 and GAPDH. Densitometric analysis was performed and the GRK2 or GRK6 level compared with a standard curve for protein level to allow semiquantitative Western analysis. WB comparing untransfected and GRK-overexpressed or GRK-depleted cells were quantified and the fold increase in protein level interpolated from this standard curve. This information was used throughout the study to confirm similar levels of GRK2 or -6 siRNA knockdown or overexpression.

Densitometry Analysis. Band intensity was measured by Quantity One Analysis software (Bio-Rad) and displayed relative to band intensity of the stated loading control.

Confocal Microscopy. HEK293T cells sequentially transfected with GFP–β-arrestin1 and GRK2/6 or IGF-1R–YFP were cultured in collagen-coated 35-mm glass-bottom dishes (Wilco Wells) and serum starved for 8 h before being stimulated with IGF1 (50 ng/mL). The cells were maintained in HBS buffer (150 mM NaCl, 10 mM Hepes, 10 mM glucose, 2.5 mM KCl, 4 mM $CaCl_2$, and 2 mM $MgCl_2$, pH 7.4) and placed on a stage top incubator (incubation system for microscopes, Tokai Hit) at 37 °C and 5% CO_2. Confocal experiments were performed on a Leica TCS SP5 confocal microscope (Leica Microsystems). Images were taken with a 63× objective. GFP was excited using the 488-nm line of an argon laser, and fluorescence intensities were recorded from 500 to 550 nm. Kinetics of β-arrestin1 translocation was measured with continuous agonist stimulation for 15 min. Settings for recording images were kept constant: 1024 × 1024 pixel format, line average 200 Hz. Pictures were taken at 1-min intervals.

FRET Between IGF-1R–YFP and β-Arrestin1–CFP: Validation by Acceptor Photobleaching. Validation of FRET between IGF-1R–YFP and β-arrestin1–CFP was performed by acceptor photobleaching (6) using a Leica TCS SP5 confocal microscope and FRET wizards in Leica Application Suite Advanced Fluorescence software. The method is based on the fact that in case of protein–protein interaction, the donor fluorescence is unquenched after photobleaching of the acceptor. The difference of fluorescence intensity of the donor before and after photobleaching gives a direct indication of the FRET efficiency and can be quantified using the formula:

$$\text{FRET (F)} = (\text{Dpost} - \text{Dpre})/\text{Dpost}, \qquad \textbf{[S1]}$$

where Dpost is the fluorescence intensity of the donor after photobleaching, and Dpre is the fluorescence intensity of the donor before photobleaching (6). The F is considered positive when Dpost > Dpre (7, 8).

HEK293T cells were sequentially transfected with β-arrestin1–CFP (Donor) and IGF-1R–YFP (acceptor) 24 h later. The ratio between receptor DNA and β-arrestin1–CFP DNA was 2:1. If GRK2/6 DNA was present, the GRKs plasmids were cotransfected with the IGF-1R–YFP plasmids with a 1:1 ratio between receptor and GRK2/6 DNA. 24 h after IGF-1R transfection, cells were split on collagen-coated 35-mm glass-bottom dishes and FRET experiments performed after a further 24 h, with serum starvation for the last 8 h.

After addition of IGF1 (50 ng/mL), prebleaching fluorescence intensities of donor (Dpre) and acceptor were imaged and measured in defined areas (Fig. S5 *A*–*C*, pre). CFP was excited with a 430-nm diode laser using a DCLP455 dichroic mirror. Fluorescence intensities were recorded from 480 to 510 nm. YFP was excited with the 514-nm line of the argon laser and a dual beam splitter 458/514 nm. Fluorescence intensities were recorded from 530 to 580 nm.

After acceptor (IGF-1R–YFP) photobleaching at 514 nm for 200 s in the preselected areas, the donor (Dpost) and acceptor fluorescence intensities were imaged and quantified (Fig. S5 *A*–*C* post) and FRET efficiency (F) calculated using Eq. **S1**. Negative control experiments were performed in cells expressing only β-arrestin1–CFP (Fig. S5*D*).

FRET in Cell Population. FRET between IGF-1R–YFP (or mutants) and β-arrestin1–CFP was performed in cell populations essentially as previously described (9). Cells transfected as for the FRET validation were resuspended at a density of 20,000 cells per well in 96-well black microplates (Corning) and serum starved overnight. Fluorescence was measured continuously using a Tecan Infinite M1000 monochromator-based reader (Tecan), before and after IGF1 stimulation with the following settings: CFP excitation 430 nm/10 nm bandwidth, emission 480/10, YFP excitation 510/5, emission 535/5, FRET channel (donor excitation, acceptor emission) excitation 430/10 emission 535/5.

The emission intensities for the YFP (F_{YFP}) were corrected for bleed-through of CFP into the YFP channel and direct YFP excitation using the formula:

$$FC_{YFP} = F_{YFP} - CFP_{FRET} - YFP_{FRET}, \qquad \textbf{[S2]}$$

where CFP_{FRET} is spillover of CFP into the FRET channel, and YFP_{FRET} is the direct YFP bleed-through FRET channel. CFP bleed-through was determined in unstimulated HEK293T expressing CFP only by measuring CFP and FRET intensities and was found to be linear with respect to fluorophore expression and approximated by the formula:

$$CFP_{FRET} = 3,66 * CFP - 3015. \qquad \textbf{[S3]}$$

YFP_{FRET} was determined in unstimulated HEK293T expressing YFP only by measuring CFP, YFP, and FRET intensities and was found to be linear and approximated by the formula:

$$YEP_{FRET} = 0,22 * YFP. \qquad \textbf{[S4]}$$

The spillover of YFP into the CFP channel was negligible; therefore, no correction was necessary for the CFP emission intensity (F_{CFP}).

FRET was calculated as the ratio FC_{YFP}/F_{CFP} and taking into consideration Eqs. **S1–S4**:

$$\text{FRET} = [(F_{YFP} - 3,66 * F_{CFP} + 3015 - 0,22 * YFP)]/F_{CFP}.$$

Prediction of Serine Phosphorylation. Over the past decade a series of algorithms have been developed to predict phosphorylation sites within a given amino acid sequence. These algorithms can be grouped into three categories: (*i*) prediction of whether a serine residue within a query sequence is a phosphorylation site or not, (*ii*) forecasting of the protein kinase family-specific phosphorylation sites, and (*iii*) data mining to determine whether a specific motif has been previously described in the literature. Although the second approach seems to be the most valuable, it is suitable only for those kinase families in which the number of known phosphorylation sites is large. Sequence analysis was performed using (*i*) the NetPhos program (10), (*ii*) the PredPhospho program (11), and (*iii*) the Human Protein Reference Database (12).

cDNA Production. RNA was extracted from cells using the GeneJet RNA purification kit (Fermentas) according to the manufacturer's instructions. A 1-μg aliquot of RNA was treated in 10 μL with 0.4 U DNase I (Fermentas) in DNase buffer for 30 min at 37 °C. The DNase was inactivated by 5 mM Na_2EDTA and incubation at 65 °C for 10 min. DNase-treated RNA was brought to 5 μM oligo(dT_{18}) primers, 1× RevertAid transcriptase buffer, 4 mM dNTPs, and 200 U of RevertAid transcriptase (Fermentas) in 20 μL. The mixture was incubated for 60 min at 42 °C. The reverse transcriptase was inactivated by addition of 5 mM Na_2EDTA and incubation for 10 min at 70 °C.

Quantitative RT-PCR. Primers were designed using the Primer 3 program and synthesized by Sigma. Primers used were as follows: GAPDH, 5′ GGGAAGCTTGTCATCAATGG, 3′ CTCCATGGTGGTGAAGACG; GRK3, 5′ GAAGTCAATGCTGCTGATGC 3′ CCAGCGTTCAGAGATGACC. The reaction mixture contained 2 μM of each primer, 1 μL of cDNA, and 1× Maxima SYBR Green/ROX qPCR Master Mix (Fermentas) in a total volume of 10 μL. For each primer pair, 10-fold serial dilutions of HEK293T cell cDNA up to 1:1,000 were analyzed and used to construct standard curves, and a nontemplate control was prepared. The reactions were assembled in MicroAmp 384-well optical reaction plates (Applied Biosciences), with each gene and cDNA

combination analyzed in triplicate. The plates were sealed with a lid and centrifuged for 1 min at 3,000 × *g*. The reaction plates were placed in the Applied Biosystems ABI Prism 7900HT sequence detection system and heated to 50 °C for 2 min, 95 °C for 10 min to activate the polymerase and denature the primers, followed by 40 cycles of 60 °C for 1 min annealing, extension occurring during increase to 95 °C, and 95 °C for 15 s denaturation. Fluorescence was measured and data collected at 95 °C and 60 °C during each cycle. After completion of the amplification, products were denatured by heating from 60 °C to 95 °C, during which data were collected to produce dissociation curves. Levels of GRK3 cDNA from unknown samples were interpolated from the standard curves and adjusted to interpolated GAPDH content.

Statistical Analysis. Data from a minimum of three replicates of two conditions were compared using a two-tailed, unpaired *t* test assuming equal variance. NS, not significant; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$.

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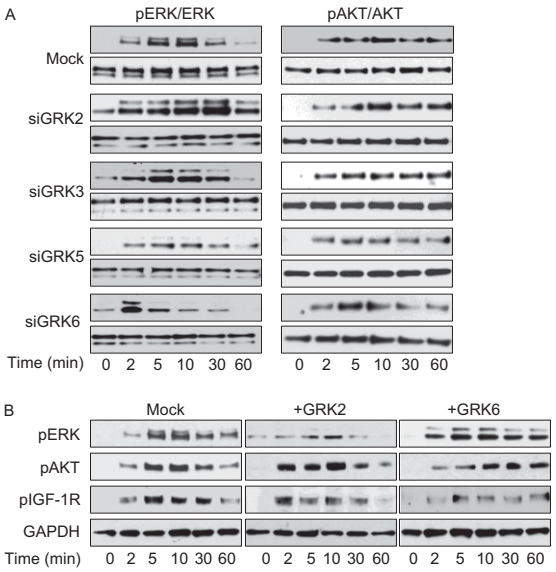


Fig. S1. Effect of GRK depletion on IGF-1R–mediated ERK/AKT signaling. (*A*) Effect of GRK depletion on IGF-1R–mediated ERK/AKT signaling. After siRNA depletion of GRK2, -3, -5, or -6, HEK293T cells were serum starved for 12 h then stimulated with IGF1 (50 ng/mL) for 0, 2, 5, 10, 30, or 60 min. The cells were lysed, and levels of phospho-ERK (pERK), phospho-AKT (pAKT), total ERK 1/2, and total AKT were analyzed by WB. (*B*) Cells were transiently transfected with either mock or indicated GRKs-encoding plasmids for 1 d. After serum starvation and IGF1 stimulation for the indicated times, the levels of phospho-IGF-1R (pIGF-1R), pERK, pAKT, and GAPDH were visualized by WB.

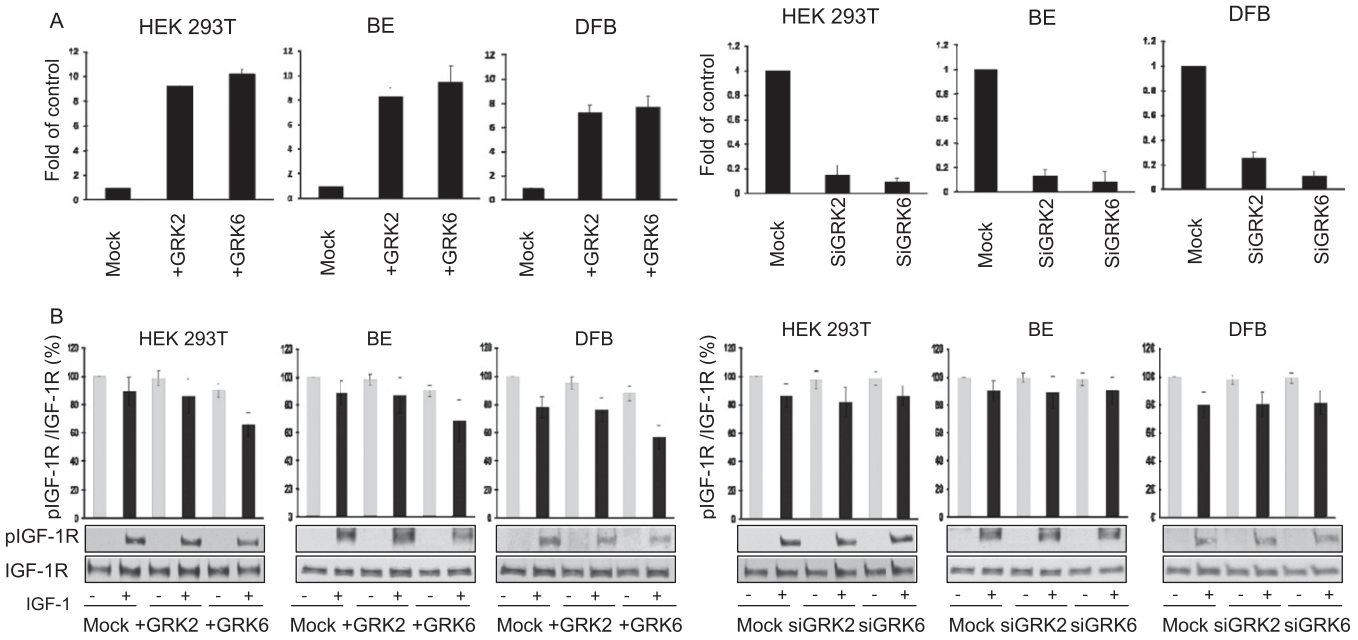


Fig. S2. Effect of GRK modulation on IGF-1R available at the cell surface. (*A*) Cell lysates were prepared from the indicated siRNA or GRKs-encoding plasmids-transfected cells. Equal amounts of proteins in each sample were used to determine expression of each GRK by WB with specific anti-GRK antibodies. Signals were quantified by densitometry and displayed as mean ± SEM relative to mock transfected cells (n = 3). (*B*) Cells were prepared as for *A* and stimulated without or with IGF1 (50 ng/mL) for 0 or 5 min and the cells lysed. Lysates were analyzed by WB for phospho-IGF-1R (pIGF-1R) and total IGF-1R levels. Western analysis was quantified using densitometry from three independent experiments and total IGF-1R (grey bars) or pIGF-1R/IGF-1R (black bars) displayed as percentage of the total IGF-1R level in mock transfected cells. Data correspond to the mean ± SEM from three independent experiments.

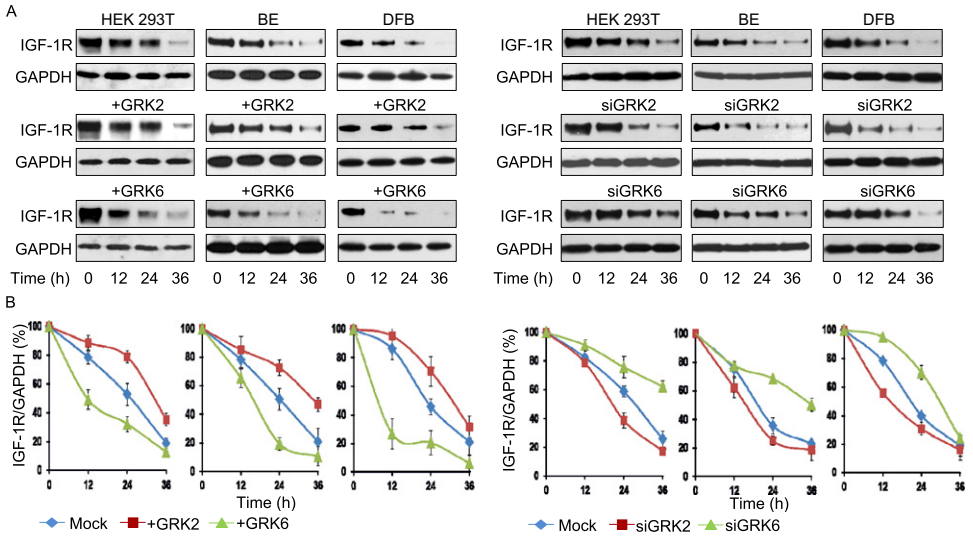


Fig. S3. Validation: effects of GRK2 and -6 on IGF-1R expression. (*A* and *B*) Cell lysates were prepared from the indicated siRNA or GRKs-encoding plasmids-transfected cells. After 12 h serum starvation, cells were stimulated with IGF1 (50 ng/mL) for the indicated times. The GAPDH and IGF-1R levels were detected by WB (*A*), quantified by densitometry from three independent experiments, and displayed as percentage mean ± SEM relative to IGF-1R in unstimulated cells (*B*).

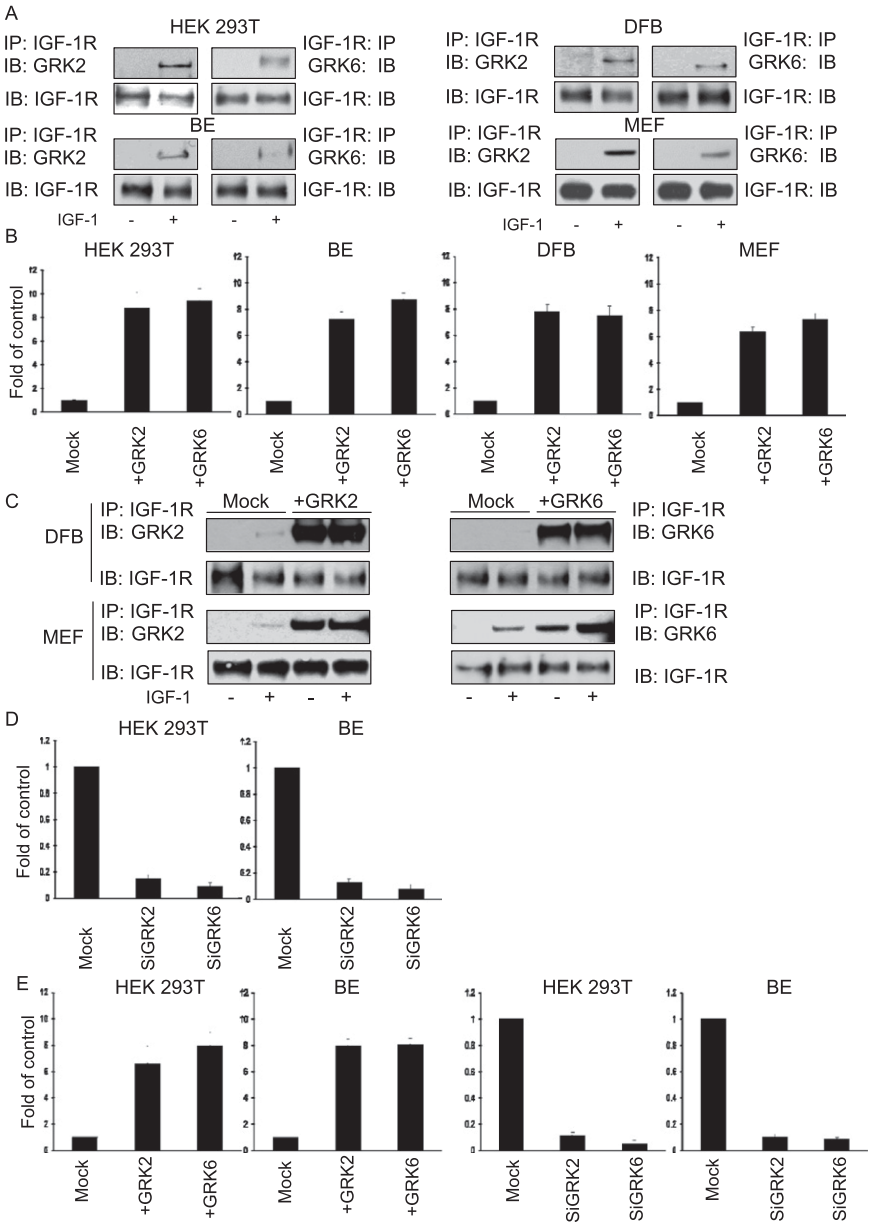
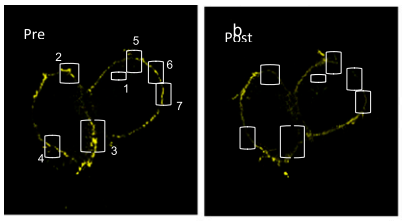
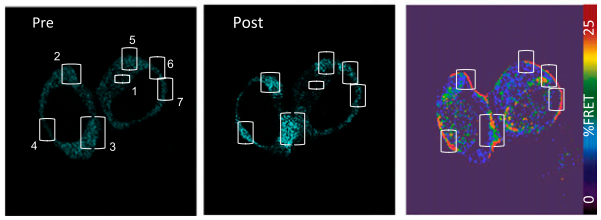


Fig. S4. Association of GRK2 and GRK6 with IGF-1R. (*A*) HEK293T, BE, DFB, and MEF cells were serum starved for 12 h and then stimulated or not with IGF1 (50 ng/mL) for 10 min and the cells lysed. IGF-1R complexes were isolated by immunoprecipitation (IP) and total IGF-1R and associated GRKs were detected by WB (IB). (*B*, *D*, and *E*) Cell lysates were prepared from the indicated siRNA or GRKs-encoding plasmids-transfected cells. Equal amounts of proteins in each sample were used to determine expression of each GRK by WB with specific anti-GRK antibodies. Signals were quantified by densitometry from at least three independent experiments and displayed as mean ± SEM relative to control transfected cells. (*B*) For Fig. 2 and Fig. S4*C*. (*D*) For Fig. 2*B*. (*E*) For Fig. 3. (*C*) MEF and DFB cells were either transfected with mock or indicated GRK-encoding plasmid, serum starved for 12 h, and stimulated or not with IGF1 (50 ng/mL) for 10 min. IGF-1R complexes were isolated by immunoprecipitation (IP) and total IGF-1R and associated GRKs were detected by WB (IB).

A



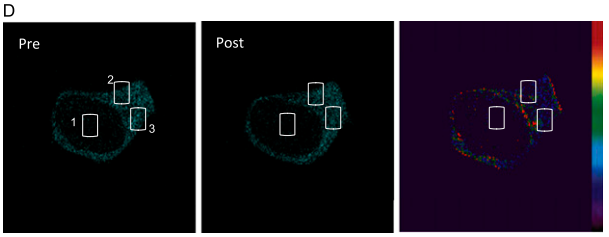
B



C

ROI	01	02	03	04	05	06	07
Donor pre	25.25	49.92	82.26	28.96	38.51	30.23	32.57
Donor post	25.25	58.66	107.43	35.87	45.31	33.88	36.60
Acceptor pre	15.37	71.75	86.01	32.87	23.75	30.26	34.56
Acceptor post	6.25	11.34	16.17	8.78	7.54	5.34	6.22
FRET	0	0.1489	0.2176	0.2379	0.1562	0.1256	0.1143

D



ROI	01	02	03
Dpre	3.05	43.4	40.1
Dpost	3.91	42.3	38.2
Apre	9.02	12.3	10.5
Apost	8.7	10.2	7.6
FRET	0	0	0

Fig. S5. IGF-1R–YFP and β-arr1–CFP FRET validation by acceptor photobleaching. (*A*–*C*) HEK293T cells were transiently transfected with IGF-1R–YFP and β-arr1–CFP. After addition of IGF1 (50 ng/mL), prebleaching fluorescence intensities were imaged for acceptor (*A*, pre) and donor (*B*, pre) and measured in regions of interest (ROI) (*C*, pre). YFP was bleached by continuous excitation at 514 nm for 200 s in the indicated areas and fluorescence of acceptor (*A*, post) and donor (*B*, post) imaged. Inside the bleached region the donor is unquenched, resulting in an intensity increase indicative of FRET. False color represents the FRET efficiency, and calculation of FRET efficiency was measured in these regions (*C*). Seven regions were selected for bleaching, ROI 01 was the negative control area without YFP expression in cytoplasm. Three independent experiments yielded similar results. (*D*) Negative control experiment was performed using cells expressing only β-arrestin1–CFP, and images and FRET efficiency are shown.

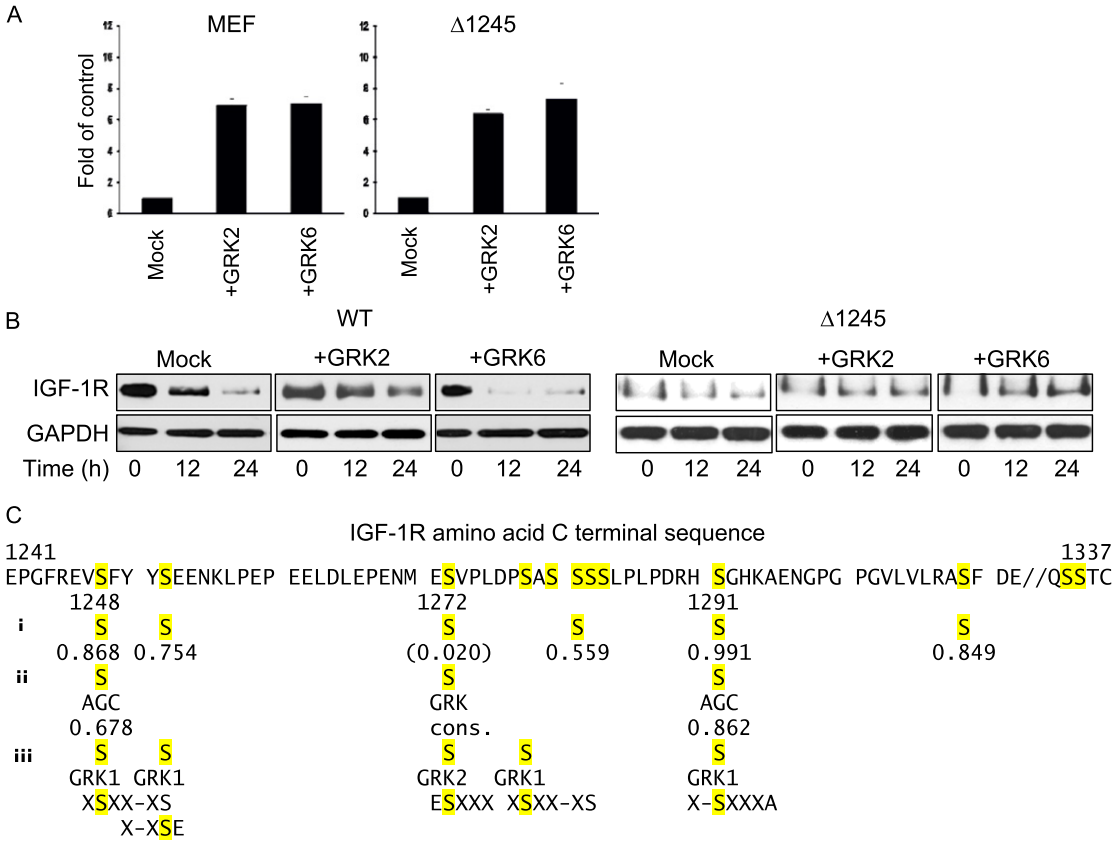


Fig. S6. Identification of serine residues involved in GRK-mediated β-arrestin binding. (*A*) MEF cells expressing WT or C terminus truncated (Δ1245) IGF-1R were transiently transfected with either mock or GRK2/6-encoding plasmids. Equal amounts of proteins in each sample were used to determine expression of each GRK by WB with specific anti-GRK antibodies. Signals were quantified by densitometry from at least three independent experiments and displayed as mean ± SEM relative to control transfected cells. (*B*) Cells prepared as described in *A* were starved, IGF1 stimulated as indicated, and IGF-1R levels were visualized by WB with GAPDH detected as a loading control. (*C*) The IGF-1R C terminus amino acid sequence was analyzed for potential serine-phosphorylation sites using (*i*) NetPhos 2.0, (*ii*) PredPhospho, and (*iii*) the Human Protein Reference Database algorithms. The prediction scores or consensus sequences are indicated below the relevant serine residues. With the NetPhos algorithm (*i*) the residues S1248 (score 0.868) and S1291 (0.991) had the highest predicted probability of being phosphorylated. With the PredPhospho program, a kinase-family prediction program, and testing for the GRKs group (AGC) phosphorylation, the highest scores were for the S1248 (score 0.678) and S1291 (score 0.862). On the basis of the consensus sequence, S1272 was also predicted to be phosphorylated by the GRKs. The motifs containing S1248 and S1291 were previously demonstrated to be substrates for GRK1, whereas the motif surrounding S1272 was previously identified as a substrate for GRK2 in a catalog of phosphorylation motifs (Human Protein Reference Database).

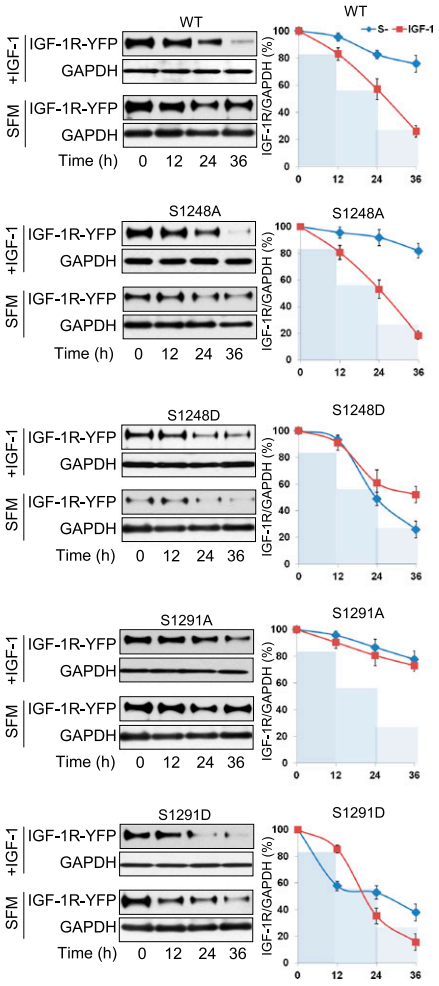


Fig. S7. Effects of serine 1248 or 1291 mutation on IGF-1R degradation. HEK293T cells were transfected with IGF-1R–YFP WT or indicated serine mutants, serum starved for 12 h, and stimulated or not with IGF1 (50 ng/mL, +IGF1) for 0, 12, 24, or 36 h and cells lysed. The GAPDH and IGF-1R–YFP levels were detected by WB (*Left*), quantified by densitometry from three independent experiments, and displayed as percentage mean ± SEM relative to IGF-1R in unstimulated cells. The levels of WT IGF-1R–YFP (IGF1 stimulated) are illustrated as a shaded area.

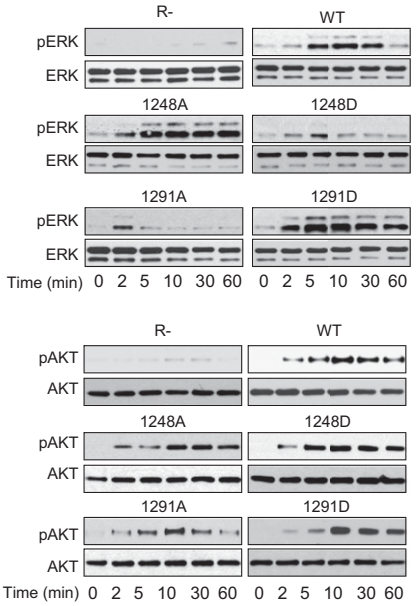


Fig. S8. Effects of serine residue 1248 or 1291 mutation on IGF1-mediated ERK and AKT signaling. Cell lysates were prepared from the indicated IGF-1R–YFP WT or serine mutants transfected IGF-1R knockout MEF cells (R-), stimulated or not with IGF1 (50 ng/mL) for the indicated times. The levels of total (ERK, AKT) and phosphorylated ERK and AKT (pERK, pAKT) were detected by WB.