SUPPLEMENTAL MATERIALS AND METHODS

Cells and epidermis

Normal human epidermal keratinocytes (NHEKs) were freshly isolated from foreskin by the Northwestern Skin Disease Research Center (SDRC) and maintained in 154 CF plus growth supplement medium (Life Technology), which contains IGF-1 (1 μg/mL) and EGF (0.2 ng/mL).

GM3 modulation

To reduce GM3 expression, NHEKs were treated for 96 h with GCSIs 1 μM C9 (Genz-123346) (Zhao et al, 2009; Zhao et al, 2007) or 200 nM GZ 161 (Genz-667161) (Cabrera-Salazar et al, 2012) (GCSIs, courtesy of Dr. Seng Cheng, Genzyme). In preliminary studies, dosing was assessed in the range of 500 nM-10 μM for C9 and 50-500 nM for GZ 161, and the final concentration for testing (1 μM for C9 and 200 nM for GZ 161) was determined based on reduction in NHEK GM3 expression (ELISA and immunofluorescence) and lack of cell toxicity. As an alternative technique to reduce GM3, NHEKs were treated with 2 nM GM3S siRNA (si) SNA (Table S1), using an oligomer sequence that recognizes a region of mRNA with 100% homologous between humans and mice (see ***SNA Synthesis and Characterization***, below). This GM3S siRNA SNA was previously shown to decrease GM3S/GM3 in the margin of diabetic mouse wounds by >80%, accelerating healing (Randeria et al, 2015). GM3 was increased by treatment of cells with: i) 5 nM bispecific M2D3 ASO SNA as previously described (Wang et al, 2002a; Wang et al, 2007b) for 48 h or 72 h; ii) high glucose medium (standard 6 mM glucose medium supplemented with 12 mM glucose Sigma, St. Louis, MO) for 96 h to simulate hyperglycemia (18 mM is equivalent to 360 mg/dl glucose); or iii) 100 μM purified GM3 (Sigma

Aldrich) for 48 h. All studies were performed with controls (vehicle for GCSIs; scrambled (Scr) siRNA SNA; sense AS SNA; and untreated).

The expression of GM3S, GM2/GD2S and GD3S in cells treated with SNAs for 48 h was quantified at least 3 times in triplicate by RT-qPCR with specific primers (Table S1). GM3S protein expression was also examined by immunoblotting and the expression of GM3 by immunofluorescence and flow cytometry with anti-GM3 antibody (Amsbio) (see below).

SNA synthesis and characterization

siRNA and DNA Synthesis

Oligonucleotides were synthesized using standard solid-phase phosphoramidite chemistry (MerMade-12, BioAutomation). All reagents were purchased from Glen Research and the oligonucleotides were purified by reverse-phase high performance liquid chromatography (HPLC Pro Star, Varian). Oligonucleotide sequences are shown in Table S1. HPLC-graded, lyophilized, thiolated ASO DNA was purchased from IDT-DNA. Lyophilized DNA was dissolved in DNase and RNase free water to 1 mM concentration.

Screening siRNA sequences to specifically target GM3S

Although not always concordant with SNA effects, knockdown of free siRNA duplexes introduced by lipofection often correlates with knockdown by the SNAs and, thus, was used as a screening tool before SNA development. As such, we initially developed GM3S siRNA duplexes and screened and synthesized potential knockdown targeting homologous sequences in mouse and human GM3S mRNA. In brief, 100 nM of each siRNA duplex for testing was mixed with 2

μL of DharmaFECT1 transfection reagent (GE Life Sciences) and incubated with NHEKs in complete 154CF media for 48 h per manufacturer's instruction. Each treatment was conducted in triplicate. Total RNA was extracted using RNeasy™ Mini Kit (Qiagen) and converted to cDNA; GM3S mRNA expression was detected using a gene specific primer for human GM3S (Table S1) and RT-qPCR (LightCycler 480-II, Roche) with the SuperScript®III One Step RT-PCR System (Life Technologies). Two siRNA sequences that maximally knocked down GM3S mRNA and protein (>90%) were chosen for further in vitro testing of knockdown and effect on migration (GM3S si #1 and GM3S si #2); one of these ("697"/ GM3S si #1) was then used for all other studies in both human and mouse keratinocytes, as well as our previously published in vivo studies (Randeria et al, 2015). Results from RT-qPCR were expressed as fold-change in means ± SD.

Preparation of spherical nucleic acid nanoparticles (SNAs)

13 ± 1 nm citrate-capped gold particles were prepared through reduction of Au(III) salt in citric buffer (Cutler et al, 2012; Cutler et al, 2011; Rosi et al, 2006). To sterilize the particles of all nucleases, 0.01% diethyl propylcarbonate (DEPC, Sigma-Aldrich) was added to the nanoparticles for 1 h and the solution was subsequently autoclaved. Upon cooling, 0.2% surfactant (Tween 20, Sigma-Aldrich) was added to the particles and the solution was vigorously mixed for 5 minutes. Next, 150 mM sodium chloride (NaCl) were added to the gold nanoparticles. All stock solutions were made using nuclease free water (RNAse Free Water, Integrated DNA Technologies). The thiolated siRNA duplex or antisense DNA was added to the solution of gold nanoparticles and shaken on a benchtop shaker. After 4 h, the final NaCl concentration in the gold nanoparticle-oligonucleotide solution was brought to 350 mM and

incubated overnight at room temperature (RT). 10 μM oligo ethylene glycol (hexaethylene glycol, Sigma-Aldrich) (OEG), was added to the particle solution for 3 h at RT to complete the SNA functionalization process. Unbound siRNA and OEG from the SNAs were removed using centrifugation. UV-visible spectroscopy measurements were performed to determine SNA concentration based on the absorbance of gold nanoparticles (Cary 5000, Agilent). The measured absorbance was related to the concentration of gold particles using Beer's Law ($A = \varepsilon bc$), where $\varepsilon = 2.77 \times 10^8$. The size distribution and polydispersity of the SNAs was determined using dynamic light scattering. A 5 nM aliquot of SNA was used to make dynamic light scattering measurements (NanoZS, Malvern).

Measurement of ganglioside synthase and ganglioside GM3 in human foot epidermis and keratinocytes

RNA and protein were analyzed for GM3S expression by RT-qPCR and Western blotting, respectively, as described (Wang et al, 2014). GM2/GD2S and GD3S expression in NHEKs was also evaluated by RT-qPCR. Human skin was obtained from the Northwestern SDRC Repository after IRB-approved written consent according to Declaration of Helsinki guidelines from diabetic patients undergoing amputation and age- and site-matched controls; in the diabetics, non-ulcerated plantar skin was taken 5-10 mm from the ulcer edge, usually proximal to the big toe. Three samples were acquired from three different patients. Control plantar skin was obtained through the Repository from healthy, age-matched volunteers. Fat was quickly removed and epidermis was separated from dermis by overnight incubation with 5 mg/mL dispase (Roche) in RNase-free solution at 4^{o}C. Total RNA was extracted in Trizol reagent (Invitrogen, CA) from either freshly treated keratinocytes or fresh human epidermis that were acquired from patients. Homogenization of samples was performed in tissue homogenizer tube using 1 mL of TRIzol

Reagent per 10 mg of tissue. Insoluble material from the homogenate was discared by centrifugation at 12,000 g for 10 minutes at 4°C. The purified RNA was dissolved in 40 ul DEPC-treated water and stored at -80°C.

cDNA was synthesized using qScript™ cDNA SuperMix (Quanta Biosciences, MD) per manufacturer's instructions. 1 ug of RNA (from the DNase treatment reaction mixture) was first incubated with 1 ul Oligo dT 0.5 ug/ul, 1 ul dNTP Mix 10 mM and 2 ul of specific primer for 18S. This mixture was heated to 65°C for10 min and incubated on ice for 1 min for primer annealing. Finally, the sample was mixed with 2 ul 10X RT Buffer (Quanta Bioscience), 1 ul of 0.1 M DTT, 1 ul RNase OUT 40 U/ul (Invitrogen), 1 uL of MultiScribe RT (50U/ul) (Quanta Bioscience). Reactions were conducted using GeneAmp PCR System 2000 (PerkinElmer) at 37ºC for 60 min, and then 85ºC for 7 min. The reactions were stopped and kept at 4ºC. Purity of the cDNA was assured by measuring the A260/A280 ratio (typically about 1.8).

PCR reactions were performed in a 7000 Sequence Detection System (ABI PRISM) using SYBR Green I (Invitrogen) in a final volume of 25 ul. The reaction mix contained 2.5 ul of 10X PCR Buffer, 0.75 ul of 50 mM $MgCl_2$, 0.5 ul of 10 mM dNTP Mix (Quanta Bioscience), 1 ul of SYBR Green I (Invitrogen), 0.1 ul of 5 U/ul Platinum® Taq DNA Polymerase (Invitrogen), 0.5 ul of 10 uM primer mix (forward and reverse primers) and 3 ul of cDNA. The PCR reactions were initiated with a 1 min incubation at 95°C, followed by 30 cycles of 95°C for 15 secs, 65°C for 15 secs and 72°C for 30 secs. All reactions were performed in triplicate. Melting curve analysis involved ramping from 55°C to 95°C in 21 mins, with fluorescence data measured continously. Gene-specific amplification was confirmed by a single band in 2% agarose gel

electrophoresis SYBR safe RNA gel stain (Invitrogen). Data was analyzed using QuantaSoft Program. Results from RT-qPCR were expressed as fold-change (Mean±S.D.).

GM3 expression in treated cells and in human skin was analyzed by immunofluorescence microscopy, flow cytometry, and ganglioside ELISA with anti-GM3 antibody (Seikagaku Biobusiness Corp.). For immunofluorescence staining (Wang et al, 2007a), treated NHEKs, either at 80% confluence on glass cover slips or as 10^6/mL of single cell suspensions, were fixed in 4% methanol-free paraformaldehyde for 10 min at 4^{o}C before incubating with 2% BSA-PBS for 1 h at RT to block non-specific binding. After incubation with anti-GM3 antibody overnight at 4^{o}C followed by washing with 1% BSA-PBS for 30 min, FITC-labeled goat anti-mouse IgM antibody was incubated with cells for 45 min and counterstained with 10 mmol L-4′,6-diamidino-2-phenylindole (DAPI). Images were captured using the UV LSM 510 Meta confocal imaging system (Zeiss, MA). GM3 expression levels in flow studies were detected in a Beckman Coulter Epics XL-MCL counter as described (Wang et al, 2001b). Background fluorescence was detected by treating with secondary antibody only and was subtracted. For ganglioside ELISA, total ganglioside was extracted and purified from foot skin using chloroform/methanol (2:1, 1:1 and 1:2) as previously described (Wang et al, 2001a). After desalting with a C18 column, total ganglioside was coated onto 96-well plates overnight, and incubated serially with anti-GM3 antibody, HRP-conjugated anti-mouse IgM antibody, and peroxidase (POD) solution (Roche). The reaction was terminated and GM3 was detected at OD 450 nm using an EL808 Ultra microplate reader linked with the KC Junior program (Bio-Tek Instrument Inc).

Migration and chemotaxis assays

To perform scratch assays, NHEKs with modulated GM3 content were treated with or without: i) transient transfection of lentiviruses with shRNAs targeting IGF1R, IR or Rac1; ii) small molecule inhibitors to IGF1R, Rac1 or ROCK (see below). Treated cells were then densely plated in complete serum-free medium, treated with mitomycin C (5 μg/mL) to prevent proliferation and, 1 h later, a 1 mm wide scratch occupying 8-10% of the monolayer area was made. Wound closure was serially imaged on an inverted light microscope (Nikon) at baseline, 6 h, 12 h, and 18 h after the scratch or monitored every 5 min for 30 min by live imaging (BioStation, Nikon). The open surface area for each well at time 0 was measured as A, the open surface area at times 6 h, 12 h and 18 h as B, and the % wound closure for that well determined as (A-B)/A x100. Gaps were measured at 10 sites along the wound and a mean was derived for each time point. Studies were performed at least 3 different times in triplicate for each condition.

For Boyden chamber assays, $2x10^4$ treated starved or unstarved NHEKs were plated onto 12-well chambers in basal 154 CF medium with or without extra glucose, and the bottom well was filled with complete medium, basal medium, or basal medium with 100 ng/mL IGF-1, all either with or without excess glucose. Cells were allowed to migrate for 18 h, and then were stained with crystal violet. After removal of cells remaining in the top chamber, cells that migrated into the bottom side of the chamber were counted. Results were shown as Mean+S.D. from triplicate chambers per trial with experiments conducted at least 3 separate times.

To evaluate the specific role of IGF1R, IR, Rac-1 and RhoA in the regulation of NHEK migration with GM3 modulation, cells with manipulated GM3 expression were transiently transfected with lentiviral shRNAs specifically targeting human IGF1R, IR, or Rac1 (Table S1)

(or their vectors as a control) per manufacturer's instructions. In brief, cells were starved overnight before incubation with polybrene (8 μg/mL, Sigma-Aldrich) for 1 h, then treated with shRNAs for 48 h (multiplicity of infection (MOI) = 3 for IGF1R; MOI=5 for IR and Rac1), and densely re-plated for scratch assay. Cells with manipulated GM3 levels (and controls) were also incubated overnight with small molecule inhibitors to IGF1R (AG538, inactivates *IGF-1R* and not IR at 50 nM, Millipore), Rac1 (NSC23766, 50 uM, Millipore), or ROCK inhibitor (Y-27632, 200 nM to inhibit RhoA, Selleckchem) before densely re-plating for scratch assays. All inhibitors and GM3 regulators were continued during the period of migration analysis. At least 3 independent trials in triplicate were conducted for each treatment.

Transduction of NHEKs with lentiviral shRNAs

To transduce NHEKs with IR shRNA , IGF1R shRNA and Rac1 shRNA, 1 mL (~10^7 virus/mL) of lentiviruses containing, IGF1R shRNA, IR shRNA or Rac1 shRNA or their respective control vectors was transduced into cells pre-treated with polybrene (8 μg/mL) for 1 h. The virus solution was then added to NHEK at 20-30% confluence in 6-well plates and incubated overnight at 37ºC; 3 days after transduction, shRNA-transfected NHEKs were treated with growth media containing 3 μg/mL puromycin for 1 wk or Western blotting (for shRNA).

Analysis of migration dynamics of single cells

Treated NHEKs were placed onto glass bottom plates (MatTek Corp., Ashland, MA, USA) and allowed to attach for 2 h. Images of single live cells were captured every 4 min for 100-140 min using BioStation (Nikon) imaging (Hamill et al, 2011) and were analyzed using FIJI software.

Velocity, persistence, and final displacement from the origin were measured for each cell using the Manual Tracking plug-in. Plane-coordinates from the Manual Tracking plug-in were used to generate vector maps using Microsoft Excel. Micrometer units were calibrated using the scale bar provided on the Biostation platform. At least 100 single KCs were analyzed per group to generate vector maps.

Cell Adhesion Assay:

Cells with manipulated GM3 expression and with or without exposure to excess glucose were plated onto 96-well plates (10,000 cells/well) pre-coated with collagen I (5 μg/cm^2) or poly-L-lysine (10 μg /cm^2) as a control and allowed to attach for 90 min before unattached cells were removed and gently washed twice with PBS (pH 7.4, 1X). Adherent cells were stained with 0.2% crystal violet and quantified at OD 560 nm using an EL808 Ultra microplate reader linked with the KC Junior program (Bio-Tek Instrument Inc) as previously described (Wang et al, 2002b). Results are presented as Mean+S.D. from at least triplicate testing in 3 different trials.

Immunoblotting

Immunoblotting was performed to detect IGF-1R, p-IGF-IR, cofilin and p-cofilin, IR, Rac-1, and, to normalize expression, GAPDH. NHEKs were treated with or without SNAs, GCSIs, or supplemental glucose in complete medium for 72 h. Cells were then starved of growth supplements and stimulated with or without IGF-1 (100 ng/mL, Prospect, East Brunswick, NJ) for 30 min. Selected times represent maximal stimulation times of IGF-1 based on preliminary studies with exposure times of 5, 15, 30, and 60 mins; similarly, concentrations of IGF-1 were chosen based on yielding maximal IGF1R phosphorylation in preliminary studies with 50, 100,

200, and 400 ng/mL for 30 min. 40 μg total protein from each treatment was separated on a precast gradient TGX^{TM} gel (4-15%, Bio-Rad), transferred to a 0.2 μm nitrocellulose membrane (Bio-Rad), and incubated with antibodies against GM3S (1:500), IGF1R (1:1000), p-IGF1R (1:500), IR (1:1000) (Santa Cruz Biotechnology), cofilin (1:1000), p-cofilin (1:500) (Cell Signaling Technology), Rac1 (1:2000, AbCam) or GAPDH antibody (1:1000, Santa Cruz Biotechnology) for 24 h at 4°C. After washing unbound primary antibody, goat anti-rabbit or anti-mouse secondary antibody was incubated with the membrane for 1 h at RT. Blots were performed at least three times with each experiment. Membranes were imaged after incubation with ECL reagents and exposed to X-ray film. Densitometric analysis was conducted using ImageJ (NIH).

G-LISA Assays:

Cells with manipulated GM3 expression at 60-75% confluence were starved overnight, then stimulated with 100 ng/mL IGF-1 for 2 min before lysis and analysis using the G-LISA Kit per manufacturer's instruction (Cytoskeleton Inc). Anti-GM3 IgM (Amsbio) antibody was used as primary antibody. Background was defined as the reading in samples treated with secondary antibody only, and was subtracted from each set of conditions. Results are presented as Mean+S.D. from 3 replicate wells per experiment, and at least 3 independent trials were run.

Statistical analysis: Paired Student's t-testing was used for all analyses. $p<0.05$ was considered significant.

Supplemental Tables

Table S1. SNAs, shRNA and primers

SNAs	
Name	**Sequence of sense and antisense strands**
GM3S-S1	5'- AAC UAC UAU AAG GAU GAC UUA - propylthiol -3'
GM3S-AS1	5'- UAA GUC AUC CUU AUA GUA GUU -3'
GM3S-S2	5'- CUA CUA UAA GGA UGA CUU A-propylthiol -3'
GM3S-AS2	5'- UAA GUC AUC CUU AUA GUA G-3'
Scr-S	5'- AUC GAA UUC CUG CAG CCC GUU – propylthiol -3'
Scr-AS	5'- AAC GGG CUG CAG GAA UUC GAU -3'
M2D3-S	5'- GAC AGG ATG TGG CTG GGC CGC A10- propylthiol -3'
	5'-AGA GGG GCC ATG GCT GTA CTG A10 -3'
M2D3-AS	5'- GCG GCC CAG CCA CAT CCT GTC A10- propylthiol -3'
	5'- CAG TAC AGC CAT GGC CCC TCT A10 -3'
shRNAs	
h- IGF1R	Santa Cruz, sc-29358-V
h- IR	Santa Cruz, sc-29370-V
h- RAC1	Open Biosystems (Themo Scientific), V2LHS-228604
Ctrl shRNA	Santa Cruz, sc-108084
Primers for PCR	
GM3S (Kang et al, 2007)	5'-CCCTGCCATTCTGGGTACGAC-3' (S); 5'-CACGATCAATGCCTCCACTGAGATC-3' (AS)
GM2/GD2S (Okada et al, 1996)	5'-AGGCCCGAGCTGCCAGATCT-3' (S); 5'-GGTCTGGTAGCTTCGGCTGC-3' (AS)
GD3S (Kang et al, 2007)	5'-TGTGGTCCAGAAAGACATTTGTGGA-3' (S); 5'-TGGAGTGAGGTATCTTCACATGGGT-3' (AS)
GAPDH	5'-TTAAAAGCAGCCCTGGTGAC-3' (S); 5'-CTCTGCTCCTCCTGTTCGAC-3' (AS)

Supplemental Figures

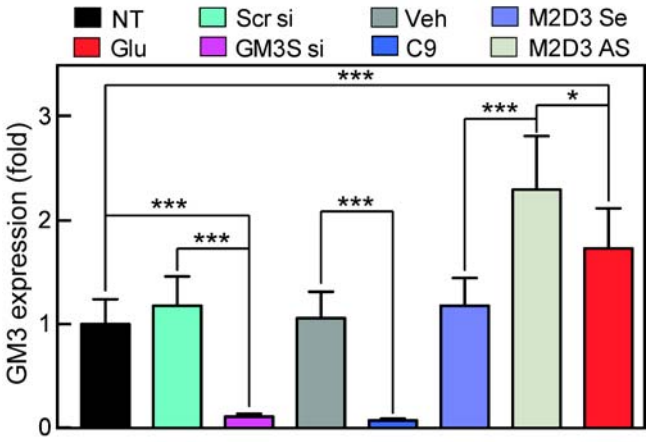

Fig. S1. Flow studies to detect GM3. GM3 content in GM3-modulated NHEKs was detected using anti-GM3 antibodies and flow cytometry. Expressed as mean+S.D. *p<0.05; ***p<0.001. NT=Untreated control.

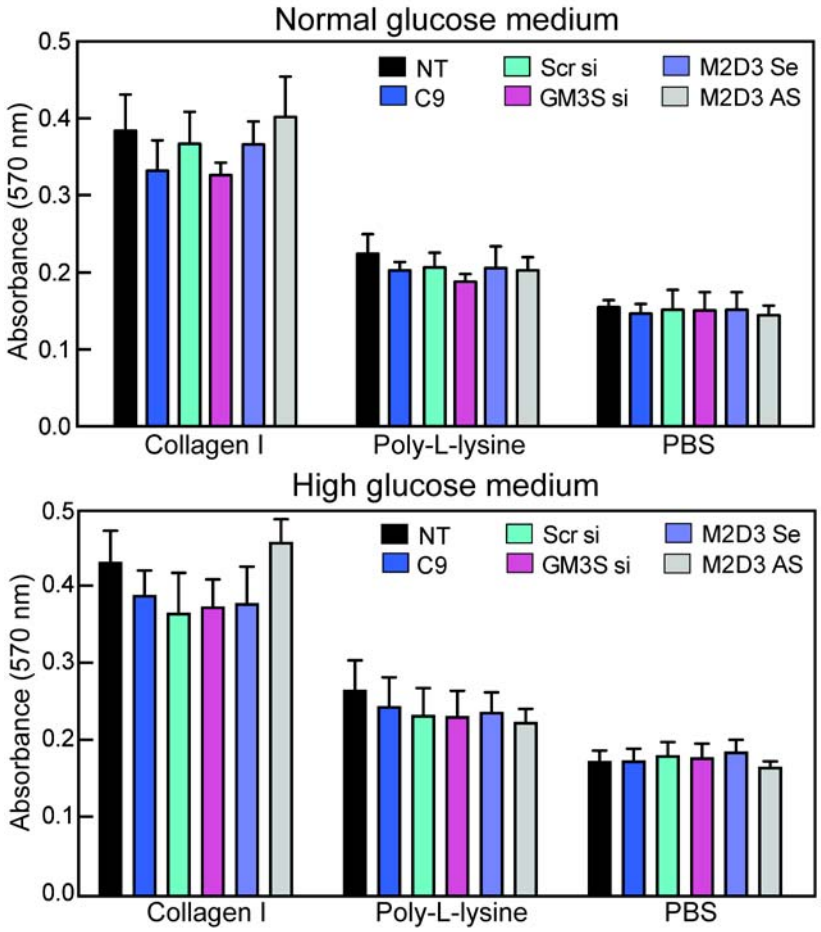

Fig. S2. Adhesion assays. GM3-modulated NHEKs were plated on 96-well plates coated with collagen I, poly-L-lysine or PBS, and adhesion was quantified by absorption at OD 560 nm after the addition of Rose Bengal dye. Data are expressed as mean+S.D.

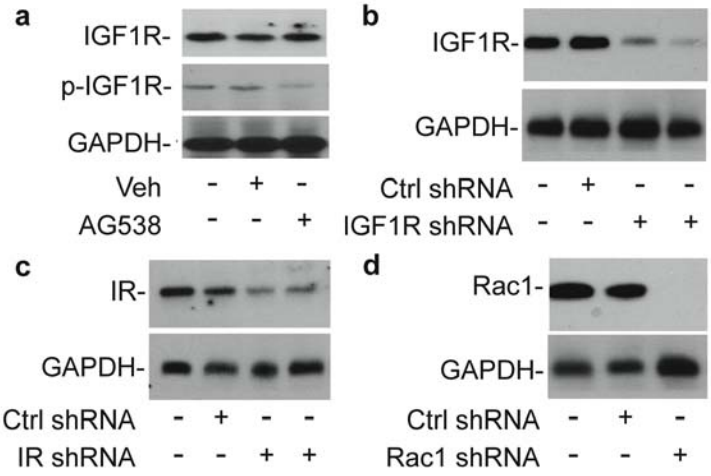

Fig. S3. shRNA and small molecule inhibition. (**a**) AG538-induced inhibition of IGF1R phosphorylation (p-IGF1R), but not of IGF1R expression. (**b**) Lentiviral shRNA-induced knockdown of IGF1R. (**c**) Lentiviral shRNA-induced knockdown of IR. (**d**) Lentiviral shRNA-induced knockdown of Rac1. Ctrl=Control; Veh=Vehicle.

References for Supplemental Materials and Methods

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