

SUPPLEMENTAL MATERIAL FOR:

GSK3 inhibition reduces ECM production and prevents age-related macular degeneration-like pathology

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The authors declare no conflicts of interest

SUPPLEMENTAL METHODS

Expanded Methods from Main Document

Cell culture. ARPE-19 cells (CRL-2302, ATCC) were cultured in DMEM/F12 (Corning) with 10% FBS (Omega Scientific) and 1% PSQ (ThermoFisher). Cells were kept at 37°C with 5% CO₂, and passages were performed every 3-4 days, or when confluent. For experiments, ARPE-19 cells were plated at confluence (unless otherwise noted), typically at a concentration of ~100,000 cells/cm², which equates to ~200,000 cells/mL. HEK-293T cells (Life Technologies) were cultured in DMEM High Glucose (4.5 g/L) supplemented with 10% FBS and 1% PSQ. Mouse NIH-3T3 fibroblasts (CRL-1658, ATCC) were maintained in DMEM high-glucose with 10% calf serum and 1% PSQ. Primary dermal fibroblasts (PCS-201-012, ATCC) were maintained in either low glucose DMEM with 10% FBS and 1% PSQ, or fibroblast growth media (Fibroblast Basal Medium [PCS-201-012, ATCC] supplemented with Low-Serum Fibroblast Growth Kit [PCS-201-041, ATCC]). Cells were periodically confirmed to be free of mycoplasma contamination using the Universal Mycoplasma Detection Kit (30-1012k, ATCC).

HiBiT F3 cell line generation. Low passage ARPE-19 cells were genomically edited to introduce a 2xFLAG-VS-HiBiT sequence immediately after the signal sequence cleavage site (Ser16) of F3 (Figure 1A). This area in F3 was specifically chosen because an extreme N-terminal tag would be cleaved off after co-translational import into the endoplasmic reticulum (ER), and because we have anecdotally noticed that C-terminal appendages (of any size) compromise the secretion efficiency of F3, in line with recent observations that a stop codon mutation in *EFEMP1* results in intracellular retention and causes a juvenile form of glaucoma (12). Introduction of the 29 amino acid 2xFLAG-VS-HiBiT tag at this position was not predicted to affect the native F3 signal sequence cleavage (Supplemental Figure 1, A and B), and FLAG tags have been used in a similar manner to force signal sequence cleavage immediately prior to the tag (85). For an in-depth

description of the editing process, please refer to a recent protocol paper (86). Briefly, a CRISPR/Cas9 ribonucleoprotein (RNP) was generated using Alt-R Sp. Cas9 Nuclease V3 (Integrated DNA Technologies, IDT) loaded with a crRNA/tracrRNA duplex (Supplemental Table 1). This RNP, combined with a single-stranded oligodeoxynucleotide (ssODN) repair template (Supplemental Table 1) were introduced into ARPE-19 cells using electroporation (1400 V, 20 ms, 2 pulses, Neon Transfection System, Life Technologies). After electroporation, cells were incubated in antibiotic-free media containing the DNA ligase IV inhibitor, SCR7 (1 μ M, Sigma) for 48 h to promote homology-directed repair (HDR). Heterogenous cultures were expanded, assayed for secreted HiBiT via a luciferase assay and HiBiT blotting, and verified genomically for insertion of the 2xFLAG-VS-HiBiT sequence (Figure 1B, primers listed in Supplemental Table 2). Full, uncropped gel images are located in Supplemental Figure 12.

High-throughput screening (HTS). Heterogenous ARPE-19 edited cells were used for all screening experiments due to their growth properties in comparison to single colony clones, which generally had much slower growth, making them subpar for large-scale HTS purposes. HiBiT F3 ARPE-19 cells were seeded at a density of \sim 5,000 cells/well (in 30 μ L with a MultiFlo, BioTek) in a white 384 well plate (781098, Greiner), and incubated at 37°C for 24 h. Media was exchanged using an EL406 microplate washer (BioTek). Compounds from the Prestwick Compound Library (1,200 compounds, Prestwick) and the National Institutes of Health clinical collection (446 compounds) were added using an Echo 655 (Beckman Coulter) at a final concentration of 5 μ M and 0.1% DMSO. As a positive control for reduction of F3 secretion/production, brefeldin A (BFA, 50 μ M) was used for the first column of each screening plate. Twenty-four hours after compound addition, plates were cooled to RT on the benchtop for 20 min followed by assaying for HiBiT signal in a whole well reading. LgBiT and lytic substrate (Promega) were added 1:100 and 1:50, respectively, into lytic buffer to generate a master mix. Fifteen microliters of the

lytic/LgBiT/substrate master mix was dispensed into each plate using a MultiFlo, shaken for 5 min, and luminescence was detected on an Envision plate reader (PerkinElmer).

HiBiT assay performance was calculated by determining the Z factor (Z') using the following equation:

$$Z' = 1 - \left(\frac{3 * (\sigma_{DMSO} + \sigma_{BFA})}{\mu_{DMSO} - \mu_{BFA}} \right)$$

Hit compounds were identified as having HiBiT F3 values less than three standard deviations from the mean of all compounds. A counter screen was used to identify toxic compounds that would be predicted to yield a false positive result (i.e., reduction in F3 signal due to cell death). Cells were plated using the same methods as the initial screen. Cherry-picked hit compounds were added the following day at the standard screening concentration (5 μ M) and an additional dose that was 0.5 log below the screening dose. The Cell Titer Glo 2.0 (Promega) viability assay was performed after 24 h of treatment. A whole-well analysis was completed by adding 10 μ L of Cell Titer Glo diluted 1:1 with PBS + 0.1% TX-100 to the plate and shaking for 5 min. Luminescence was again read on the Envision plate reader (PerkinElmer).

In addition to the cell viability counter screen, media alone was treated with the hit compounds to identify any false reductions in luminescent signal caused by NanoBiT luciferase inhibition. Briefly, conditioned media was collected from ARPE-19 HiBiT F3 cells, spun, and plated into 384-well plates. Hit compounds were added in a dose-response format and incubated for 24 h. Lytic buffer/LgBiT/substrate master mix was added to the plates, shaken for 5 min, then read on an Envision plate reader. Wells that had significantly lower amounts of HiBiT signal were noted and the corresponding compounds were removed from subsequent experiments. Remaining hit compounds were verified in a confirmatory screen using fresh compound from a new source plate.

Additional secondary metrics verified the effect of a hit compound in multiple assays and in different cell lines.

Short interfering RNA (siRNA). siRNA knockdown was used to verify the specificity of CRISPR HiBiT editing of F3 or fibulin-5 (F5). siRNAs (Silencer Select, Ambion, Supplemental Table 3) were introduced into cells containing HiBiT-tagged human F3, mouse F3, or human F5 by reverse transfection. For a 24 well plate, 2.14 μL of DharmaFECT4 (Horizon) was diluted into 250 μL OptiMEM (Thermo Fisher Scientific). siRNAs were added at a concentration of 100 nM. All samples were vortexed for 15 sec, then incubated at RT for 20 min. During this incubation period, ARPE-19 cells were trypsinized and normalized to a density of 466,000 cells/mL in full DMEM/F12 media. Two-hundred and fifty microliters of the siRNA/OptiMEM/DharmaFECT4 complex was added to a 24-well plate, after which an equivalent volume of cell suspension was added, yielding a final siRNA concentration of 50 nM. Plates were rocked to ensure cell distribution and to prevent clumping. After 24 h, the media was changed with full DMEM/F12. Seventy-two hours post knockdown, the media was changed again, and the HiBiT levels were determined the next day (96 h post knockdown) via an extracellular and intracellular HiBiT assay.

Non-HTS HiBiT assay. Cells were plated at a high density of 200,000 cells/mL for all non-HTS HiBiT assay experiments. The following day, media containing the indicted compound was added and incubated for either 72 h or 1 week. For one-week treatment wells, media was replaced after 96 h with fresh media containing compound. After treatment, extracellular and intracellular assays were performed to determine levels of HiBiT-tagged F3 using either the Nano-Glo HiBiT Extracellular Detection System or the Nano-Glo HiBiT Lytic Detection System (Promega). For the extracellular HiBiT protocol, 25 μL of the conditioned media was reacted with an equal volume of HiBiT master mix (extracellular or lytic buffer, 1:100 LgBiT, and 1:50 extracellular or lytic substrate) in a black-bottomed 96-well plate for 5 min with shaking. For intracellular HiBiT

detection, cells were washed in well with HBSS (Gibco), followed by addition of 50 μ L of lytic HiBiT master mix (described above) and 5 min shaking. Forty microliters of the lysate was transferred to a black 96-well plate for a luminescent reading on a Synergy 2 (BioTek) or a GloMax (Promega).

Western blotting. Cells for western blotting were rinsed with HBSS followed by lysis in buffer containing radioimmunoprecipitation buffer (RIPA, Santa Cruz), protease inhibitor (Pierce) and benzonase (Millipore Sigma), or the Nano-Go HiBiT Lytic Detection System. Lysis buffer was added to each well at a volume of 50 μ L (for a 24 well plate, for example) followed by shaking for 5-15 min, and lysate was collected into Eppendorf tubes on ice. The samples were spun at 4°C for 10 min at 21,000 x g, and the soluble supernatant was collected. For cells lysed in RIPA buffer, a bicinchoninic acid assay (BCA, Pierce Thermo Scientific) was used to quantify protein levels and normalize them to 20 μ g. For cells lysed in HiBiT Lytic buffer, samples were normalized by volume due to the presence of DTT, which interferes with the BCA assay. Samples were boiled in 1x reducing Laemmli buffer for 5 min, then loaded onto a 4-20% Tris-Glyc SDS-PAGE gel and run at 140 V for 80 min. Proteins were transferred to 0.2 μ m nitrocellulose membrane using a semidry apparatus (P0 protocol, iBlot2, Life Technologies). Total transferred protein was visualized with Ponceau S (Sigma-Aldrich) and incubated overnight in blocking buffer (Intercept Blocking Buffer, LI-COR). The following day, blots were incubated for 1 h at RT with primary antibody [GSK3 α (sc-5264, Santa Cruz), GSK3 β (9315S, Cell Signaling), or GAPDH (sc-47724, Santa Cruz)] diluted in 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) and 0.05% NaN₃. Blots were rinsed in TBS with 0.05% Tween (TBS-T) and incubated for 40 min at RT in an appropriate anti-mouse or anti-rabbit secondary antibody (1:10,000-15,000, LI-COR) dissolved in 5% milk in TBS-T. Blots were again washed in TBS-T, then TBS, followed by water, and imaged

on an Odyssey CLx (LI-COR), followed by analysis using the corresponding ImageStudio Software (LI-COR). Full, uncropped blot images are located in Supplemental Figure 12.

HiBiT blotting. Samples were lysed, prepared, and separated by SDS-PAGE as described above for western blotting. Normalized protein was reduced, denatured, and run through an SDS-PAGE gel, followed by transfer to a nitrocellulose membrane (P0 program, iBlot2). Once transferred, the membrane was incubated in TBS-T (10 min to 1 h, RT) to expose the HiBiT epitope. Blots were then incubated with blotting buffer containing 1:200 LgBiT for 1-2 h with rocking at RT (N2410, Promega). Next, NanoGlo substrate was added (1:500) and the blots were incubated for 5-10 min at RT. The resulting chemiluminescence signal was imaged on an Odyssey Fc (LI-COR). Full, uncropped blot images are located in Supplemental Figure 12.

Lactate dehydrogenase (LDH) release assay. Cells were treated with CHIR for either 72 h or 1 week. Media was then collected and spun at 1,000 RPM (100 x g) for 5 min to remove potential floating cells. Fifty microliters (technical triplicates) of cleared media was mixed with an equal amount of LDH reaction buffer (LDH Cytotoxicity Assay Kit, Pierce) in a clear 96-well plate for 30 min at 37°C. A DMSO-treated media sample served as a control while cells treated with cell lysis buffer (provided in the LDH Cytotoxicity Kit) served as a positive control. Fifty microliters of stopping solution was added to each well and the absorbance at 490 nm was read on a GloMax plate reader(Promega).

7xTCF-eGFP mCherry (7TGC) and 7xTCF-firefly luciferase puromycin (7TFP) lentivirus production. VSV-g-pseudotyped replication incompetent lentivirus was generated as we have described previously (88, 89). Briefly, lentiviral plasmids (Addgene plasmids #24304 and #24308, kind gifts of Roel Nusse (42)) were co-transfected with psPAX2 and VSV-g plasmids (Addgene plasmids #12260 and #12259, gifts of Didier Trono) into low passage HEK-293T cells plated at

1x10⁶ cells/well of a poly-D-lysine-coated 6-well plate using Lipofectamine 3000 (Life Technologies). The next day, media was discarded and replaced with fresh media. Media collected 24 h and 48 h later was pooled and filtered through a 0.45 µm filter. Lenti-X GoStix (Takeda) were used to confirm the production of virus. To establish 7TGC and 7TFP cell lines, HiBiT F3 ARPE-19 cells were plated at 1x10⁶ cells/well of a 6 well plate and infected with lentivirus in full media containing polybrene for 24 h. 7TGC cells were not put under selective pressure whereas 7TFP cells were then selected with 1 µg/mL of puromycin for 1-2 weeks.

Firefly luciferase (FLuc) assay. The HiBiT F3 7TFP ARPE-19 cells were treated with CHIR for 1 week (with a 72 h media change) and assayed for FLuc expression. Briefly, after a total of 1 week of treatment, media was aspirated and cells were washed with HBSS. Fifty microliters lysis buffer (Firefly Luciferase Glow Assay, Pierce) was added to each well, then the plate was rocked for 15 min. To monitor FLuc activity, 50 µL of working solution containing luciferin and Firefly Glow Assay Buffer was added to a black 96-well plate. Ten microliters of the lysate was added to respective wells, then read on a Synergy2 plate reader.

MMP2 zymography. HiBiT WT F3 or HiBiT R345W F3 ARPE-19 cells were plated at a density of ~100,000 cells per well of a 12-well, or ~30,000 cells per well of a 24-well, 0.4 µm polyester transwell plate (Corning), and media was changed to serum free media the following day and every 3-4 days thereafter. After 1 week growing on transwells, cells were treated with either DMSO (0.1%) or 1 µM CHIR in serum free media. After 2 weeks on transwells (1 week of treatment), an extracellular HiBiT assay and MMP2 zymography were performed. Briefly, for zymography, media from the apical and basal chamber was collected and combined with non-reducing SDS buffer followed by running on a 10% gelatin gel (Novex) for 90 minutes at 140 V. MMP2 was renatured in buffer (G-biosciences) for 30 min at RT. Gels were then changed to

developing buffer (G-Biosciences) for an additional 30 min at RT. After changing to fresh developing buffer, gels were gently shaken overnight at 37°C. The following day, the gels were stained in Coomassie R-250 for 1 h, then destained and imaged on an Odyssey Clx (LI-COR). Bands were quantified using Image Studio software (LI-COR). Full, uncropped gel images are located in Supplemental Figure 12.

Secreted proteome visualization and mass spectrometry. HiBiT ARPE-19 cells were treated with CHIR for 72 h in serum-free DMEM/F12 media. To visualize separated total protein, conditioned media was concentrated (Amicon Ultra 0.5 mL centrifuge unit, 3,000 MWCO, Millipore Sigma) and run on a 4-20% Tris-Gly SDS-PAGE gel for 80 min at 140 V. Protein bands were imaged by silver staining (SilverQuest Silver Staining Kit, Invitrogen). In parallel, to prepare samples for mass spectrometry, an aliquot of the same concentrated media sample was run for 10 min at 140 V on a 4-20% Tris-Gly SDS-PAGE gel, stained with Coomassie Blue, and the single band corresponding to total secreted protein was excised and submitted for mass spectrometry to the UT Southwestern Proteomics Core. Briefly, samples were digested overnight with trypsin (Pierce) following reduction and alkylation with DTT and iodoacetamide (Sigma Aldrich). The samples then underwent solid-phase extraction cleanup with an Oasis HLB plate (Waters) and 2 μ L of each sample was injected onto a QExactive HF mass spectrometer (Thermo Fisher Scientific) coupled to an Ultimate 3000 RSLC-Nano liquid chromatography system (Thermo Fisher Scientific). Samples were injected onto a 75 μ m i.d., 15-cm long EasySpray column (Thermo) and eluted with a gradient from 0-28% buffer B over 90 min with a flow rate of 250 nL/min. Buffer A contained 2% (v/v) ACN and 0.1% formic acid in water, and buffer B contained 80% (v/v) ACN, 10% (v/v) trifluoroethanol, and 0.1% formic acid in water. The mass spectrometer operated in positive ion mode with a source voltage of 2.5 kV and an ion transfer tube temperature of 275°C. MS scans were acquired at 120,000 resolution in the Orbitrap and up to 20 MS/MS spectra were obtained

for each full spectrum acquired using higher-energy collisional dissociation (HCD) for ions with charges 2-8. Dynamic exclusion was set for 20 s after an ion was selected for fragmentation. Raw MS data files were analyzed using Proteome Discoverer v. 2.4 SP1 (Thermo Fisher Scientific), with peptide identification performed using Sequest HT searching against the human protein database from UniProt (<https://www.uniprot.org/>). Fragment and precursor tolerances of 10 ppm and 0.6 Da were specified, and three missed cleavages were allowed. Carbamidomethylation of Cys was set as a fixed peptide modification, with oxidation of Met set as a peptide variable modification. The false-discovery rate (FDR) cutoff was 1% for all peptides.

Sample preparation for RNAseq. HiBiT F3 ARPE-19 cells were plated at 200,000 cells/mL in DMEM/F12 media with 10% FBS and 1% PSQ. The following day, media containing DMSO (1:1000) or 1 μ M CHIR was added to the cells. Cells were treated for 96 h before an additional media change. Seven days after beginning treatment, RNA from vehicle (DMSO)-treated and 1 μ M CHIR-treated cells was extracted using an Aurum Total RNA Kit (BioRad) and stored at -80°C. Fifteen microliters of sample was provided to Novogene. A total amount of 1 μ g RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext Ultra™ RNA Library Prep Kit for Illumina (NEB) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3' ends of DNA fragments, NEBNext Adaptor with hairpin loop structure were ligated to prepare for

hybridization. In order to select cDNA fragments of preferentially 150~200 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter). Then 3 μ L USER Enzyme (NEB) was used with size-selected, adaptor ligated cDNA at 37 °C for 15 min followed by 5 min at 95 °C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. At last, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system.

CHIR PK experiment. C57BL/6J (originally from Jackson Laboratories) were dosed via intraperitoneal injection with CHIR trihydrochloride (Tocris) dissolved in 2% DMSO with 5.7% Captisol (Ligand Pharmaceuticals) in 1x PBS at a concentration of 5 mg/mL. Mice were dosed at 25 mg/kg (average weight of 24.7 g). The dosing time points were 30, 180, 360, 960, and 1440 min, with a 0-min control group. Each timepoint included three mice, with at least one male and one female in each group. At select time points, isoflurane was used to anesthetize mice prior to tissue collection. Plasma, liver, and neural retina (both eyes pooled) were collected, and the samples were flash frozen in liquid nitrogen. All samples were then sent to the UT Southwestern Preclinical Pharmacology Core and the amount of CHIR was measured in each tissue using LC-MS/MS (see below).

LC-MS/MS analysis of CHIR: Retina tissue, liver tissue and plasma were analyzed for CHIR concentrations using an LC-MS/MS method. Retinas and livers were homogenized in PBS. Retina homogenates were made using BeadBug prefilled tubes with 3.0 mm Zirconium beads (Z763802, Sigma) and a BeadBug microtube homogenizer run for 1 min at 2800 RPM (800 x g). For standards, blank commercial plasma (Bioreclamation) or untreated liver or brain tissue homogenate was spiked with varying concentrations of compound. Standards and samples were mixed with three-fold volume of 100% acetonitrile containing 0.133% formic acid and 33.3 ng internal standard (tolbutamide, Sigma), vortexed, and then spun 5 min at 16,100 x g. Supernatant

was removed and spun again and the resulting second supernatant was put into an HPLC 96-well plate and analyzed by LC-MS/MS using a Sciex Triple Quad™ 4500 mass spectrometer coupled to a Shimadzu Prominence LC. CHIR was detected with the mass spectrometer in MRM (multiple reaction monitoring) mode by following the precursor to fragment ion transition 465 → 146.1. An Agilent Zorbax XDB-C18 column (50 x 4.6mm, 5 μm packing) was used for chromatography with the following conditions: Buffer A: dH2O + 0.1% formic acid, Buffer B: MeOH + 0.1% formic acid, 1.5mL/min flow rate, 0-1.5 min 3%B, 1.5-2.0 min gradient to 100%B, 2.0-3.5 min 100%B, 3.5-3.6 min gradient to 3%B, 3.6-4.5 3%B. Tolbutamide (transition 271.2 → 91.2) was used as an internal standard. Back-calculation of standard curve and quality control samples were accurate to within 15% for 85-100% of these samples at concentrations ranging from 0.5 ng/ml to 10000 ng/ml.

Additional Methods (appearing only in Supplemental Material)

Porcine retinal pigment epithelium (RPE) culture. To assess the long-term effects of CHIR on RPE tight junction formation and maintenance, primary porcine RPE cultures were used. Briefly, procured pig eyes were processed according to Toops et al 2014 Exp. Eye Res. with slight modification. Posterior eye cups were dissected and RPE were isolated by trypsinization followed by expansion in T25 culture dishes (5 eyes per dish) for 2 weeks in differentiation media (with 10 mM nicotinamide). Cells were then trypsinized and seeded on transwells at a density of 300,000 cells/cm² on collagen-coated (5 μg/cm²) 0.4 μm transwells (Corning). Cells were allowed to differentiate for 2 additional weeks prior to treatment with DMSO (1:1,000) or CHIR (1 μM). Cells were then washed 3x with HBSS, fixed in 4% PFA (30 min, RT), washed again with HBSS, and stained with CytoPainter phalloidin-iFluor 647 (Abcam, 1:250 in PBS, 1% BSA, 0.5% Triton-X-100, 0.5% Tween-20, 1 h, RT). Transwells were washed again, excised and mounted using Fluoromount G (ThermoFisher) on microscope slides and imaged on a Keyence BZ-X810 at 40x magnification.

HiBiT mouse F3 cell line generation. To add HiBiT to mouse F3, NIH-3T3 fibroblasts (ATCC CRL-1658) were used. Similar to the procedure described above, a 2xFLAG VS HiBiT sequence was inserted immediately after the Ser17 residue of mouse F3 (Supplemental Figure 5, A and B). Unlike in *EFEMP1*, no mutation of the PAM site was required in *Efemp1* due to significant disruption of the gRNA sequence after HiBiT insertion (Supplemental Figure 6A). RNP, ssODN, and electroporation enhancer were introduced into the NIH-3T3 cells (1400 V, 20 ms, 2 pulses, Neon Transfection System). After electroporation, cells were incubated in antibiotic-free media containing HDR Enhancer V3 (1 μ M, IDT) for 48 h to promote homology-directed repair. Heterogenous cultures were expanded and assayed for secreted HiBiT via a luciferase assay.

HiBiT fibulin-5 (F5) cell line generation. As a control cell line for HiBiT F3 cells, we appended HiBiT onto endogenous fibulin-5 (FBLN5, DANCE, or F5), a highly homologous protein to F3 (19), in a similar manner, immediately after the F5 signal sequence cleavage after residue Ala23 (Supplemental Figure 8A). No mutation of the PAM site was required in *FBLN5* due to significant disruption of the gRNA sequence after HiBiT insertion (Table S1). RNP, ssODN, and electroporation enhancer were introduced into ARPE-19 cells using electroporation (1400V, 20 ms, 2 pulses, Neon Transfection System). After electroporation, cells were incubated in antibiotic-free media containing HDR Enhancer V3 (1 μ M, IDT) for 48 h to promote homology-directed repair. Heterogenous cultures were expanded and assayed for secreted HiBiT via a luciferase assay.

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1: Introduction of the 2xFLAG-VS-HiBiT tag is not predicted to alter human F3 signal sequence cleavage. (A) SignalP5.0 predicted signal sequence cleavage of human wild-type (WT) F3 and (B) human F3 after introduction of the 2xFLAG-VS-HiBiT tag.

Supplemental Figure 2: Example data from HTS plate indicating assay uniformity and identification of potential hit compounds. (A) Example plate data from a whole-well HiBiT assay mock screen performed with DMSO and BFA (as a positive control) to identify an assay Z'-score. (B, C) Representative screening data identifying a potential F3 reducer (B) or a potential F3 enhancer (C). (D, E) Linearity of the HiBiT luminescence assay. Conditioned media from WT HiBiT F3 cells was diluted over a range of concentrations followed by a HiBiT assay. Regression analysis shows excellent linearity over 6 logs of dilution. n = technical triplicates performed in two independent experiments for D, E.

Supplemental Figure 3: Select hit compounds identified in the primary screen were confirmed in dose-response. (A) Seven hit compounds were tested at 0.2 nM – 50 μ M for 24 h. All hits reproduced and demonstrated varying levels of dose-responsiveness. (B) Table of 24 h, whole-well EC₅₀ values obtained from panel A.

Supplemental Figure 4: Verification of CHIR99021 activity toward F3 in a human non-RPE cell line and primary porcine RPE. (A) F3 in primary human dermal fibroblasts was edited with a HiBiT tag followed by treatment with CHIR99021 for 72 h. n \geq 3 independent experiments with mean of each experiment presented as a single data point in this graph. ** p < 0.01, one sample t-test vs. hypothetical mean of 1 (i.e., unchanged). (B,C) Primary porcine RPE grown on

transwells were treated with DMSO (1:1,000) or CHIR (1 μ M) for 11 weeks followed by staining for phalloidin (F-actin) to delineate RPE cell borders. Scale bar = 100 μ m.

Supplemental Figure 5: Introduction of the 2xFLAG-VS-HiBiT tag is not predicted to alter mouse F3 signal sequence cleavage. (A) SignalP5.0 predicted signal sequence cleavage of mouse (mus) wild-type (WT) F3 and (B) human F3 after introduction of the 2xFLAG-VS-HiBiT tag.

Supplemental Figure 6: HiBiT editing of mouse fibroblast NIH-3T3 cells followed by treatment with CHIR99021 demonstrates cross species activity of the compound. (A) Schematic demonstrating the 2xFLAG-VS-HiBiT F3 design for genomic insertion into the mouse genome. The upward arrow indicates the predicted signal sequence cleavage site based on SignalP5.0 prediction. (B) The origins of the HiBiT signal were confirmed to be from mouse F3 as demonstrated by siRNA knockdown experiments. $n = 3$ independent experiments, **** $p < 0.0001$, t-test vs. non-targeting siRNA. (C) Seventy-two hour treatment with CHIR99021 reduces F3 production in mouse cells. $n = 3$ independent experiments, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA with Dunnett's multiple comparison test vs. vehicle (DMSO) treated samples.

Supplemental Figure 7: Introduction of the 2xFLAG-VS-HiBiT tag is not predicted to alter human fibulin-5 (F5) signal sequence cleavage. (A) SignalP5.0 predicted signal sequence cleavage of human wild-type (WT) F5 and (B) human F5 after introduction of the 2xFLAG-VS-HiBiT tag.

Supplemental Figure 8: Production of HiBiT-tagged human F5 is also reduced by CHIR99021 treatment. (A) Schematic demonstrating the design of 2xFLAG-VS-HiBiT genomic

insertion onto the N-terminus of human F5 in ARPE-19 cells. The origins of the resulting HiBiT signal were confirmed to be F5 gene expression-dependent using siRNA. Representative data of $n = 3$ independent experiments, average \pm S.D. of technical triplicates. *** $p < 0.001$. (C) HiBiT F5-expressing ARPE-19 cells were treated with CHIR99021 for 72 h, followed by a HiBiT assay on conditioned media. $n = 3$ independent experiments performed in biological duplicates each time, * $p < 0.05$, **** $p < 0.0001$. (D) One-week CHIR99021 treatment also leads to reduced extracellular and intracellular F5 levels. $n = 3$ independent experiments performed in biological triplicate, ** $p < 0.01$, **** $p < 0.0001$. one-way ANOVA with Dunnett's multiple comparison test vs. non-targeting or vehicle (DMSO) treated samples for all panels.

Supplemental Figure 9: Low level, 1-week CHIR99021 treatment does not substantially affect the secreted proteome from ARPE-19 cells. Total concentrated secreted protein after 1 week of CHIR99021 treatment silver stained to visualize differences in overall protein abundance. Representative data of three independent experiments shown in biological duplicate.

Supplemental Figure 10: Design and validation of R345W^{+/+} ARPE-19 knockin cells and their subsequent HiBiT editing. (A) Design of the R345W knockin strategy using CRISPR/Cas9 editing and homology-directed repair. (B) Genomic DNA from single colonies was isolated and validated to be homozygous (^{+/+}) for the R345W mutation. (C) The validated R345W^{+/+} clone was then edited to include the 2xFLAG-VS-HiBiT tag.

Supplemental Figure 11: Not all TEM fields of view show BLamD. Some TEM fields were found to contain no BLamDs in both (A) vehicle (A) and CHIR99021-treated mice (B). Note the healthy basal infoldings in these images also. Scale bar = 2 μ m.

Supplemental Figure 12: Full gel and blot images for the indicated figure.

Supplemental Table 1: CRISPR reagent sequences used for guide RNAs, PAM sites, and ssODN design for insertion of the 2xFLAG-VS-HiBiT tag.

Supplemental Table 2: Primer sequences for exon 2 amplification and gDNA verification of 2xFLAG-VS-HiBiT insertion into the F3 gene.

Supplemental Table 3: siRNA sense sequences used for confirming the origins of the HiBiT signal in generated cell lines.

Supplemental Table 4: Processed data received from HTS with eight hit compounds of interest and additional information found about MOA, SMILES, HiBiT readout, etc.

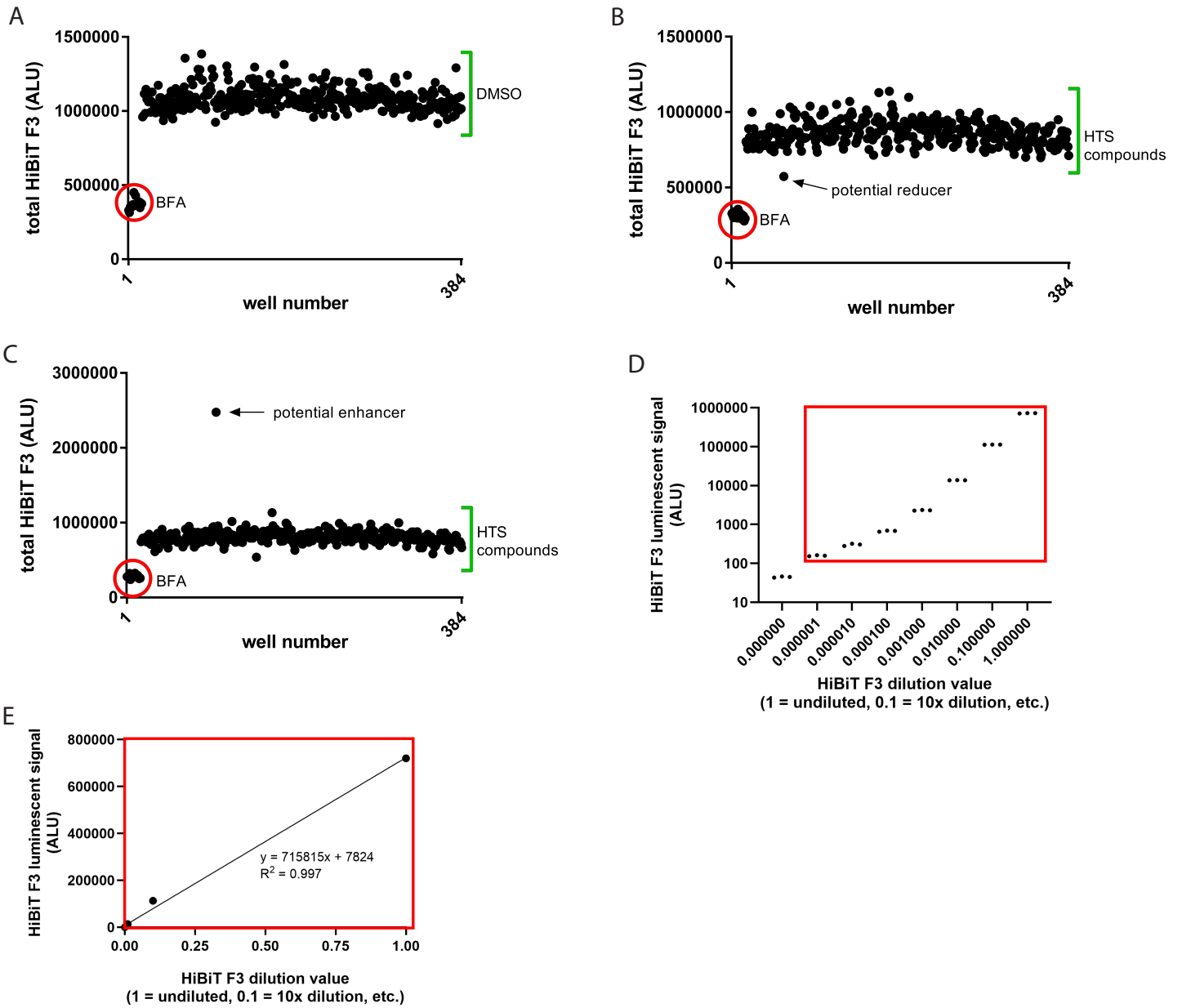
Supplemental Table 5: Top 50 increased proteins between DMSO and CHIR treated cells.

Supplemental Table 6: GO analysis of top proteins and their involvement in cellular composition.

Supplemental Table 7: RNA-seq genes identified to have ≥ 2 -fold decrease between CHIR treated and DMSO treated cells.

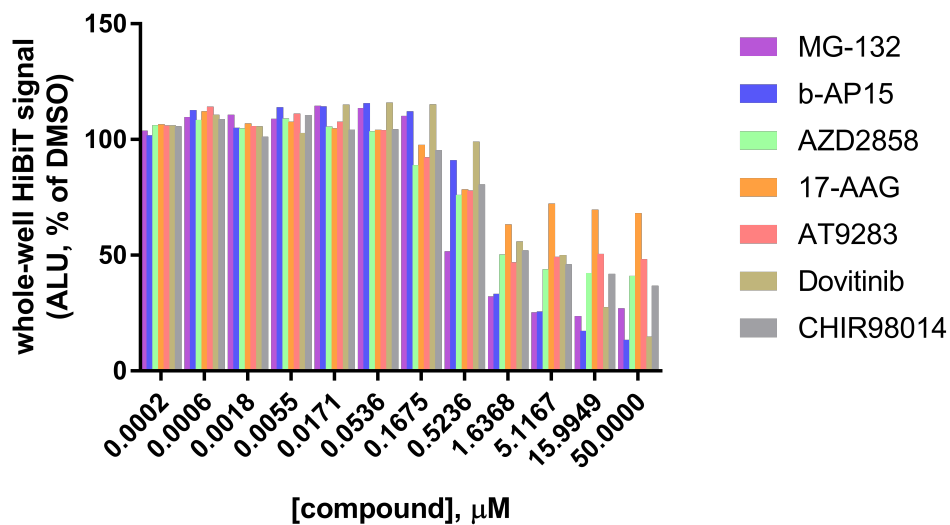
Supplemental Table 8: RNA-seq genes identified to have ≥ 2 -fold increase between CHIR treated and DMSO treated cells.

Supplemental Figure 2.



Supplemental Figure 3.

A

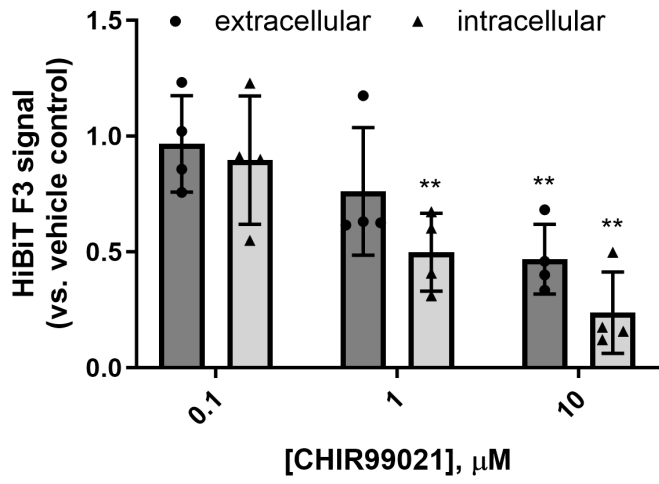


B

	whole well 24 h EC_{50}
MG-132	490.9 nM
b-AP15	1,120 nM
AZD2858	495.7 nM
17-AAG	264.6 nM
AT9283	393.7 nM
Dovitinib	2,212 nM
CHIR98014	721 nM

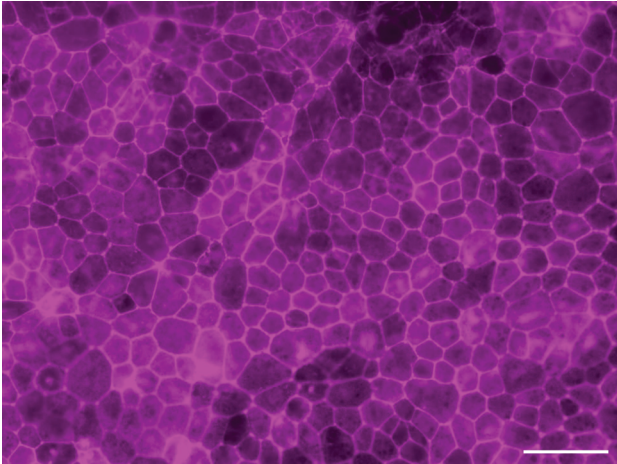
Supplemental Figure 4

A



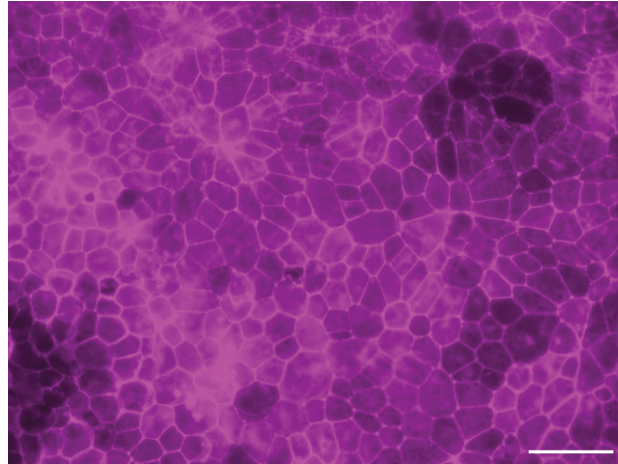
B

DMSO



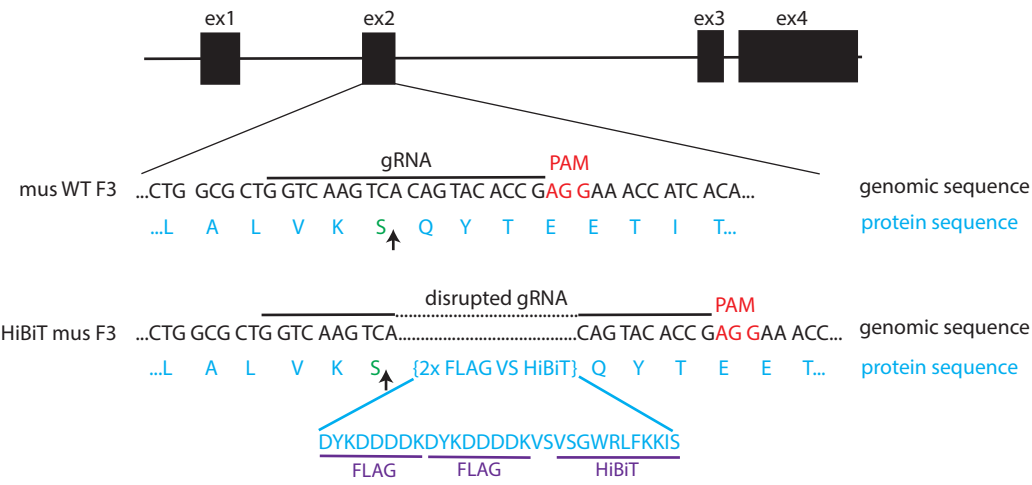
C

CHIR99021, 1 μM , 11 weeks

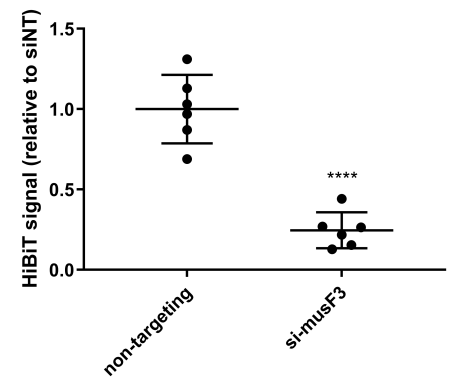


Supplemental Figure 6.

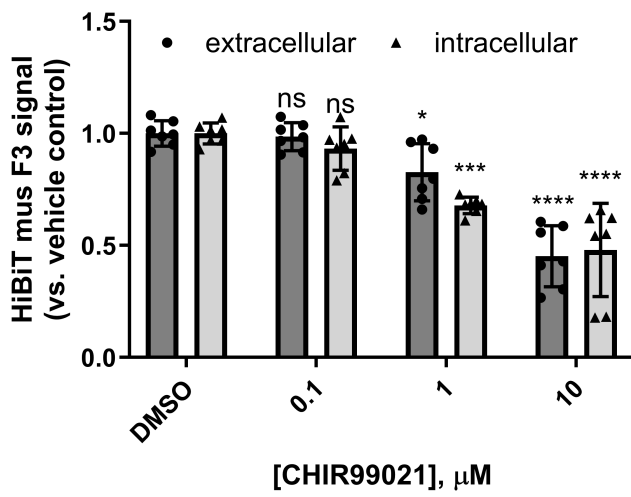
A



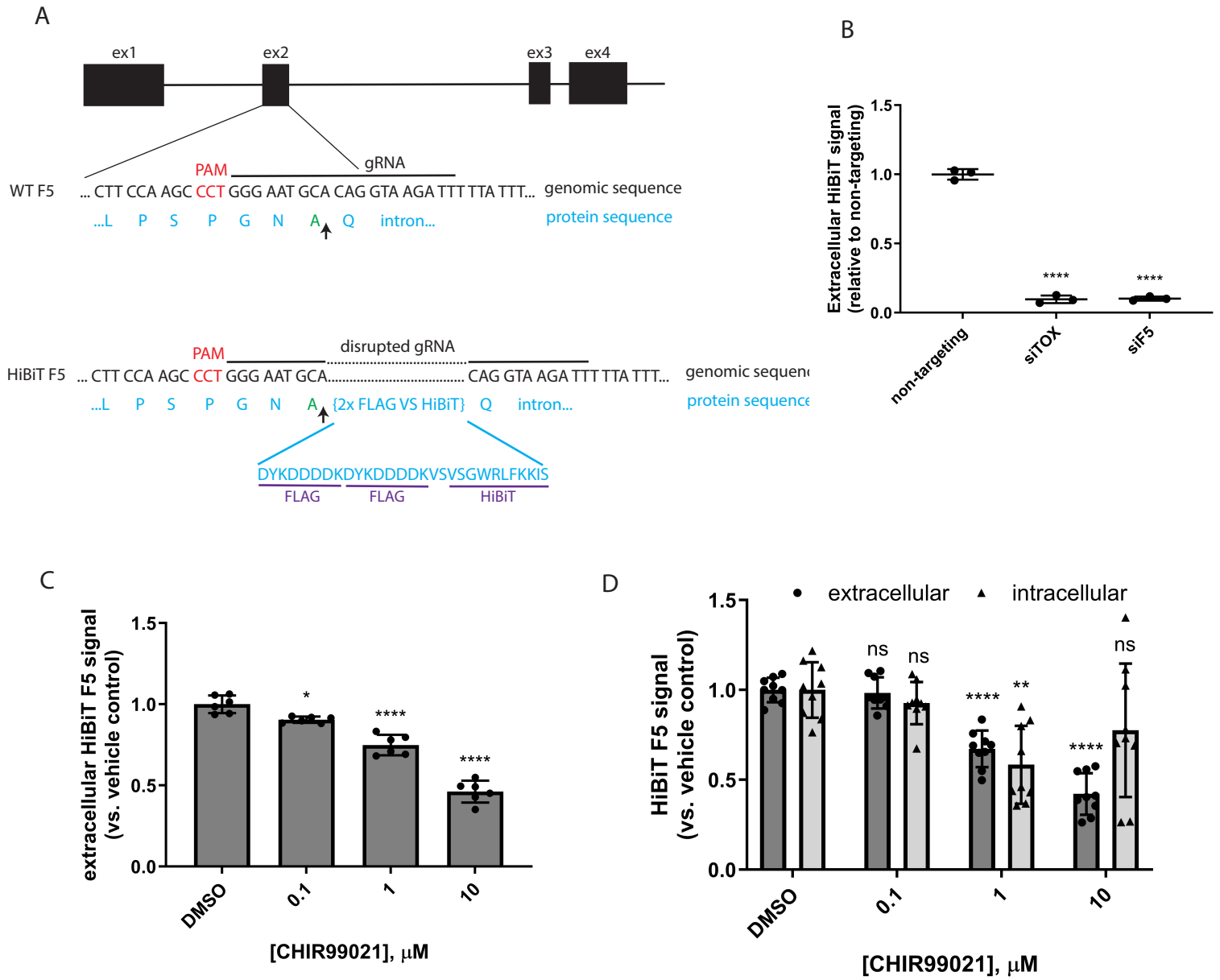
B



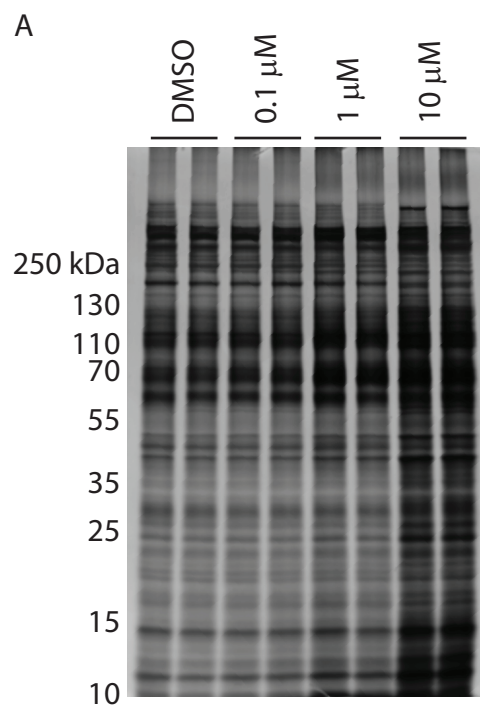
C



Supplemental Figure 8.

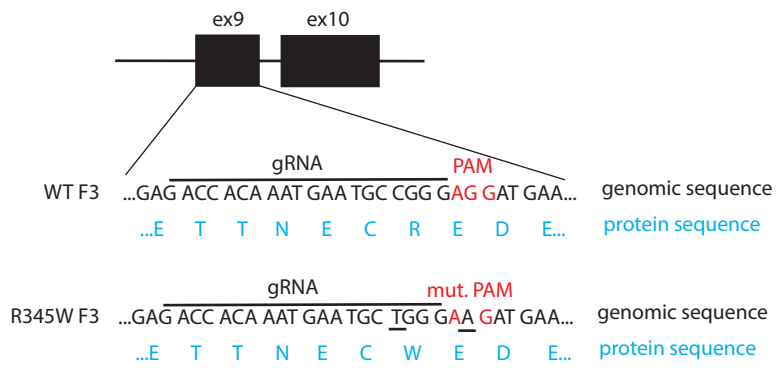


Supplemental Figure 9.

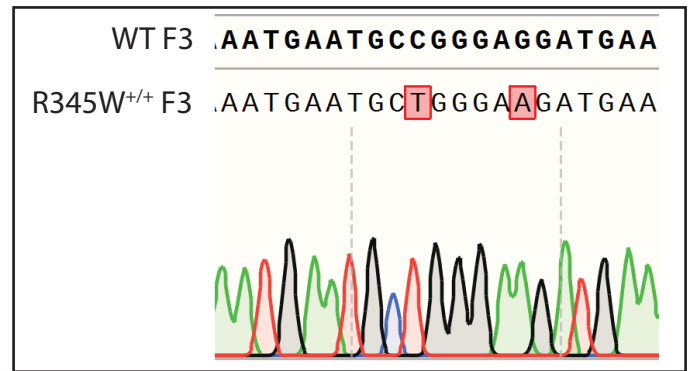


Supplemental Figure 10.

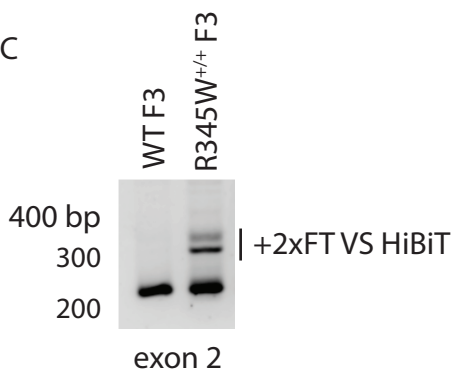
A



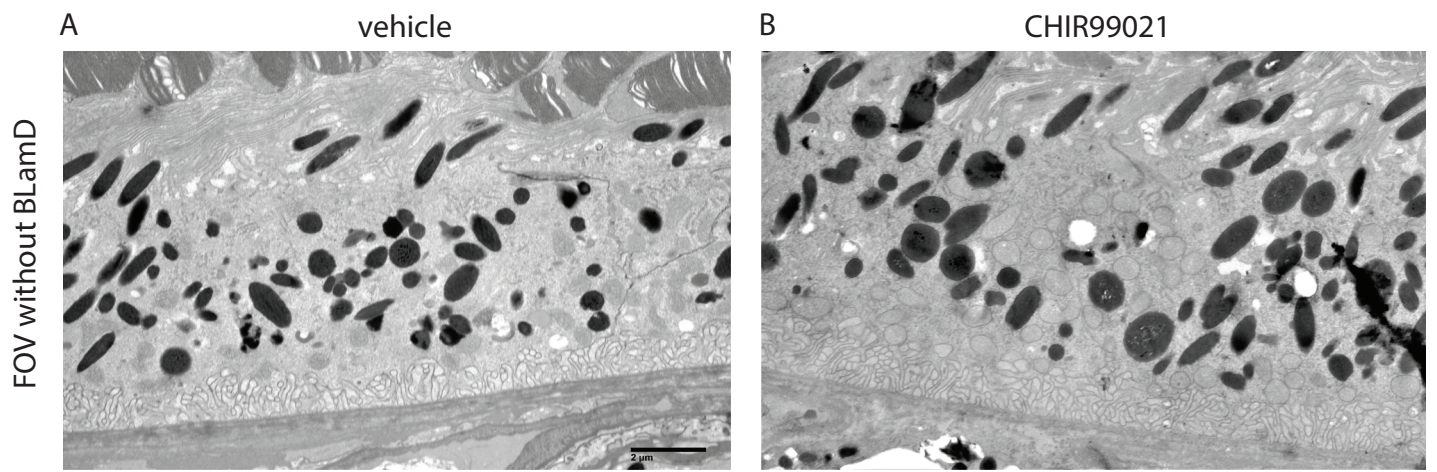
B



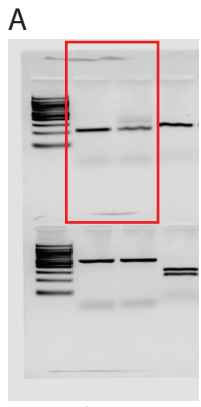
C



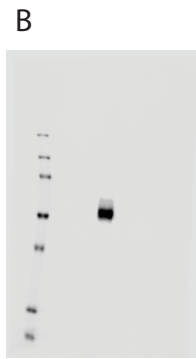
Supplemental Figure 11.



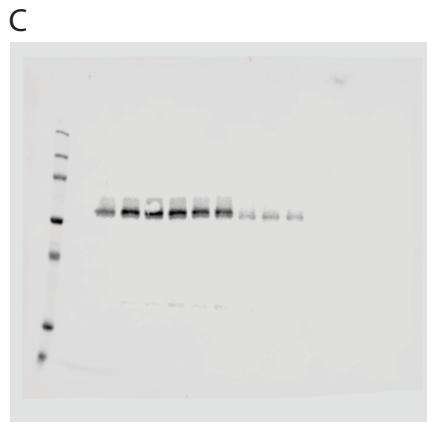
Supplemental Figure 12.



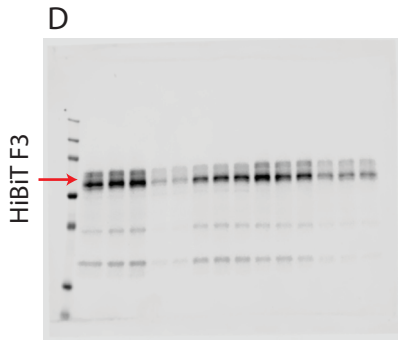
used for Fig. 1B



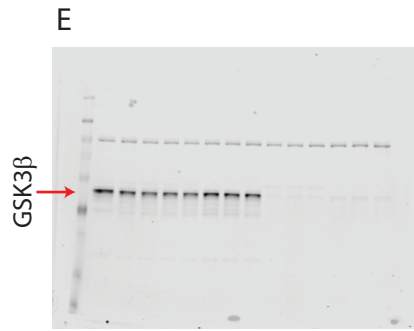
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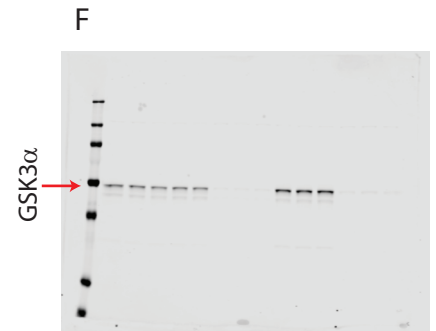
used for Fig. 2C



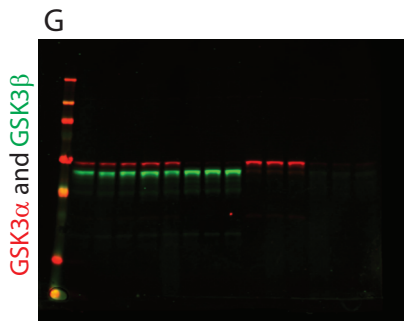
used for Fig. 3B



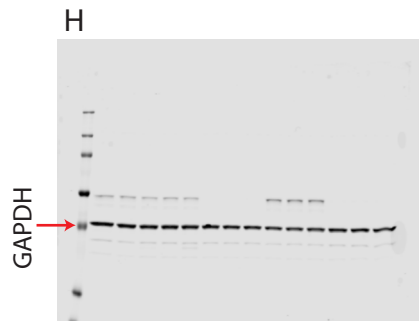
used for Fig. 3B (monochrome)



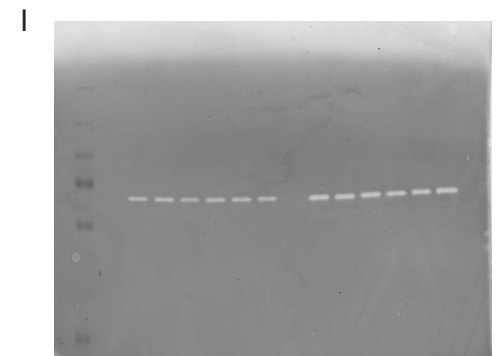
used for Fig. 3B (monochrome)



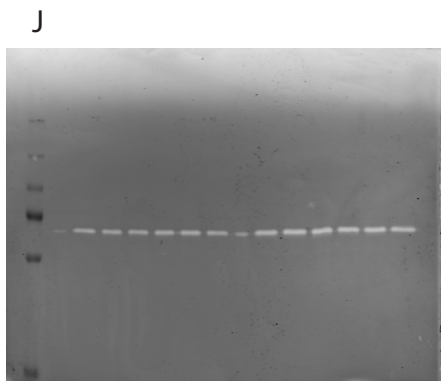
used for Fig. 3B (multiplex)



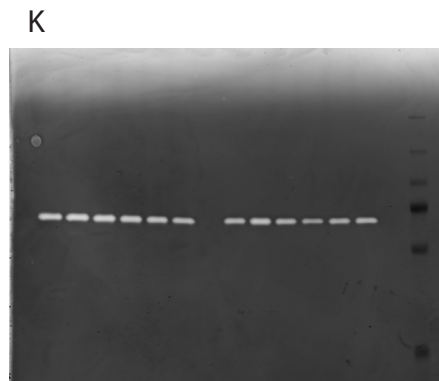
used for Fig. 3B



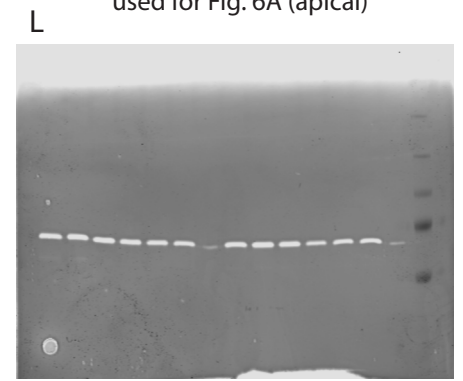
used for Fig. 6A (apical)



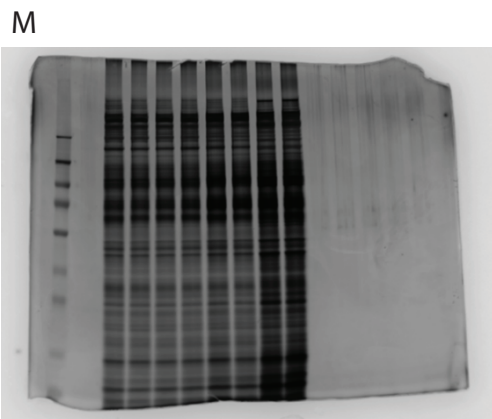
used for Fig. 6A (basal)



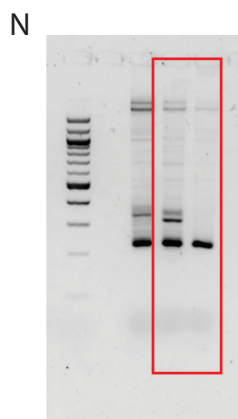
used for Fig. 6C (apical)



used for Fig. 6C (basal)



used for Supplemental Fig. 9



used for Supplemental Fig. 10C

Supplemental Table 1:

HiBiT edit	gRNA	PAM	ssODN
2xFLAG VS HiBiT F3 (human)	CGTGACGTGATGGTTTCTT	CGG	TGTTGAAAGCCCTTTTCCTAACTATGCTGACTCTGGCGCTGGTCAAGTCAG ATTACAAGGATGACGACGATAAGGATTACAAGGATGACGACGATAAGGT GAGCGTGAGCGGCTGGCGGCTGTTCAAGAAGATTAGCCAGGACACTGAA GAAACCATCACGTACACGGTAAGGGGTGATGGAATTTG
2xFLAG VS HiBiT F3 (mouse)	GGTCAAGTCACAGTACACCG	AGG	TGTTGCAAACACTTTTCCTAACTATGCTGACTCTGGCGCTGGTCAAGTCAG ATTACAAGGATGACGACGATAAGGATTACAAGGATGACGACGATAAGGT GAGCGTGAGCGGCTGGCGGCTGTTCAAGAAGATTAGCCAGTACACCGAG GAAACCATCACATACACGGTAAGGCATGATGACATTTG
2xFLAG VS HiBiT F5 (human)	AATCTTACCTGTGCATTCCC	AGG	ACAGGATACTCACTGTTACCATTCTGGCTCTCTGTCTTCCAAGCCCTGGGA ATGCAGATTACAAGGATGACGACGATAAGGATTACAAGGATGACGACGA TAAGGTGAGCGTGAGCGGCTGGCGGCTGTTCAAGAAGATTAGCCAGGTA AGATTTTTATTTTTAAAAAATATCTACAAGCGTATTTAAATCCGGGGTGA

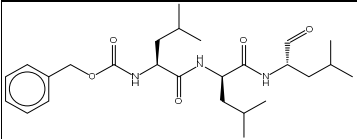
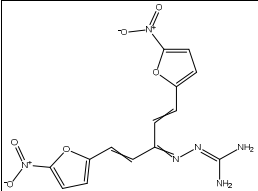
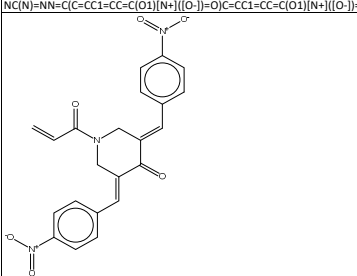
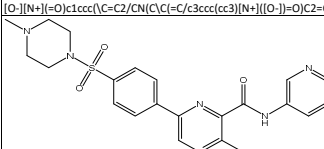
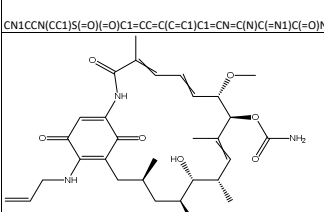
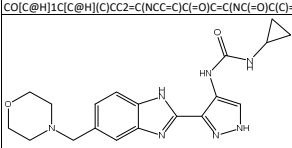
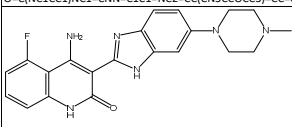
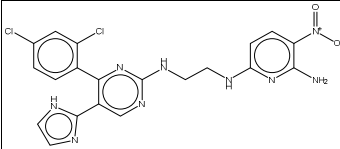
Supplemental Table 2:

HiBiT edit	exon	forward primer	reverse primer
2xFLAG VS HiBiT F3 (human)	2	TAAAGTTTTGTTACTTTCCCGCAGCAGA	GTGTGTAAGTCTTTTTTGTACAGAAATCCCG

Supplemental Table 3:

Target	sense sequence
siTOX transfection control	proprietary
non-targeting siRNA control	proprietary
siF3#1 (human)	GCAAUGCACUGACGGAUUtt
siF3#2 (human)	CAUCCGAUCUGAUAGGUCUtt
siF3 (mouse)	CAUUAUACUUUUCGGAUUtt
siF5 (human)	GCAGCUCUGUGCGAAUGUUt
siGSK3 α (human)	GAAAGACGAGCUUUACCUAtt
siGSK3 β (human)	CUCAAGAACUGUCAAGUAAtt

Supplemental Table 4 - Information on selected hit compounds based on primary, confirmatory, and counterscreening

Structure, SMILES	common name	Target	Pathway	whole well HIBIT F3 (1.66 μM)	whole well HIBIT F3 (5 μM)	media only HIBIT F3 (1.66 μM, counterscreen)	media only HIBIT F3 (5 μM, counterscreen)	CTG (1.66 μM, counterscreen, 48 h Rx)	CTG (5 μM, counterscreen, 48 h Rx)
 <chem>CC(C)[C@H](NC(=O)[C@@H](CC(C)C)NC(=O)[C@H](CC(C)C)NC(=O)OCc1ccccc1)C=O</chem>	MG132	Proteasome, Cysteine Protease	Proteases/Proteasome; Ubiquitination	-55.52	-69.49	-1.61	-1.32	-2.25	-26
 <chem>NC(N)=NN=C(C=CC1=CC=C(O1)[N+](=[O-])=O)C=CC1=CC=C(O1)[N+](=[O-])=O</chem>		unknown	unknown	-47.01	-69.62	-5.07	-7.72	7.42	-40
 <chem>[O-][N+](=O)c1ccc(C=C2/CN(C)C(=C/c3ccc(cc3)[N+](=[O-])=O)C=CC2=O)[C]=O)C=Cc1</chem>	B-AP15	DUB inhibitor	Ubiquitination	-38.36	-63.01	-5.24	-5.82	-5.54	-7
 <chem>CN1CC(NC1S(=O)(=O)C(=O)C1=CC=C(C=C1)C1=CN=C(NC(=N1)C)O)NC1=CC=C(C1)</chem>	AZD2858	GSK-3 inhibitor	PI3K/Akt/mTOR signaling; Stem Cells	-37.85	-46.41	-3.60	-6.55	9.55	16
 <chem>CO[C@H]1[C@H](C)C2=C(NCC=C(C)O)C=C(NC(=O)C)C=CC=C[C@H](OC)[C@H](OC(N)=O)C(C)=C[C@H](C)C@H1O)C2=O</chem>	Tanespimycin (17-AAG)	HSP (e.g. HSP90)	Cytoskeletal Signaling	-36.00	-34.14	-0.30	-5.02	-9.89	-12
 <chem>O=C(NC1CC1)NC1=CN=C1C1=NC2=CC(=C(C)C)C(=O)C=C2N1</chem>	AT9283	Aurora Kinase inhibitor; Bcr-Abl inhibitor; JAK inhibitor	Angiogenesis; Cell Cycle/Checkpoint; Chromatin/Epigenetic; Cytoskeletal Signaling; JAK/STAT signaling; Stem Cells	-29.84	-37.86	-0.68	-4.14	10.02	7
 <chem>CN1CC(NC1)C1=CC2=C(C=C1)N=C(N2)C1=C(N)C2=C(NC1=O)C=CC=C2F</chem>	Dovitinib (TKI-258, CHR-258)	TKI; FGFR, FLT3, PDGFR, VEGFR	Angiogenesis	-26.64	-44.03	1.94	2.43	-5.14	-21
 <chem>Nc1nc(NCCNC2ncc(-c3ncc[nH]3)c(n2)-c2ccc(Cl)cc2Cl)ccc1[N+](=[O-])=O</chem>	CHIR98014	GSK-3 inhibitor; S6 Kinase inhibitor	PI3K/Akt/mTOR signaling; Stem Cells	-26.41	-37.93	-4.02	-3.26	32.09	37

Supplemental Table 5.

accession #	gene name	description	fold change vs. DMSO
Q99470	SDF2	Stromal cell-derived factor 2	13.910
O43660	PLRG1	Pleiotropic regulator 1	9.797
Q9NRV9	HEBP1	Heme-binding protein 1	9.605
P02452	COL1A1	Collagen alpha-1(I)	8.871
P48307	TFPI2	Tissue factor pathway inhibitor 2	4.974
P36955	SERPINF1	Pigment epithelium-derived factor	4.923
P52803	EFNA5	Ephrin-A5	4.225
Q14376	GALE	UDP-glucose 4-epimerase	3.823
P81605	DCD	Dermcidin	3.809
Q5D862	FLG2	Filaggrin-2	3.777
P07311	ACYP1	Acylphosphatase-1	3.681
P61081	UBE2M	NEDD8-conjugating enzyme Ubc12	3.608
O60245	PCDH7	Protocadherin-7	3.484
P39748	FEN1	Flap endonuclease 1	3.471
P49770	EIF2B2	Translation initiation factor eIF-2B subunit beta	3.456
P62266	RPS23	40S ribosomal protein S23	3.443
P20930	FLG	Filaggrin	3.417
Q14112	NID2	Nidogen-2	3.395
O14817	TSPAN4	Tetraspanin-4	3.372
Q92520	FAM3C	Protein FAM3C	3.330
P83916	CBX1	Chromobox protein homolog 1	3.301
Q9UJH6	SHPK	Sedoheptulokinase	3.228
P61769	B2M	Beta-2-microglobulin	3.169
Q6FI81	CIAPIN1	Anamorsin	3.092
O43488	AKR7A2	Aflatoxin B1 aldehyde reductase member 2	3.060
Q9BXJ0	C1QTNF5	Complement C1q tumor necrosis factor-related protein 5	3.031
Q9NRX4	PHPT1	14 kDa phosphohistidine phosphatase	3.014
P15104	GLUL	Glutamine synthetase	2.966
P21926	CD9	CD9 antigen	2.966
Q9UJU6	DBNL	Drebrin-like protein	2.962
O75663	TIPRL	TIP41-like protein	2.873
P10909	CLU	Clusterin	2.861
P53611	RABGGTB	Geranylgeranyl transferase type-2 subunit beta	2.844
P61204	ARF3	ADP-ribosylation factor 3	2.834
P53990	IST1	IST1 homolog	2.804
P01009	SERPINA1	Alpha-1-antitrypsin	2.801
P27986	PIK3R1	Phosphatidylinositol 3-kinase regulatory subunit alpha	2.793
P52758	RIDA	2-iminobutanoate/2-iminopropanoate deaminase	2.774
P45877	PPIC	Peptidyl-prolyl cis-trans isomerase C	2.748
P33908	MAN1A1	Mannosyl-oligosaccharide 1,2-alpha-mannosidase IA	2.728
O75144	ICOSLG	ICOS ligand	2.727
P17900	GM2A	Ganglioside GM2 activator	2.710
Q13045	FLII	Protein flightless-1 homolog	2.706
Q14956	GPNMB	Transmembrane glycoprotein NMB	2.684
O15400	STX7	Syntaxin-7	2.684
Q8NBJ4	GOLM1	Golgi membrane protein 1	2.665
Q969H8	MYDGF	Myeloid-derived growth factor	2.653
Q9UII2	ATP5IF1	ATPase inhibitor, mitochondrial	2.645
P55196	AFDN	Afadin	2.638
Q92824	PCSK5	Proprotein convertase subtilisin/kexin type 5	2.622

Supplemental Table 6.

GO cellular component complete	# of genes in H. sapiens reference list	# of genes in dataset	expected frequency	fold enrichment	raw p value	FDR
keratohyalin granule (GO:0036457)	4	2	0.03	66.85	8.06E-04	2.94E-02
retromer complex (GO:0030904)	12	3	0.09	33.42	1.71E-04	9.71E-03
platelet alpha granule membrane (GO:0031092)	17	3	0.13	23.59	4.17E-04	1.74E-02
cytosolic small ribosomal subunit (GO:0022627)	45	5	0.34	14.85	3.42E-05	2.59E-03
chaperone complex (GO:0101031)	42	4	0.31	12.73	3.74E-04	1.70E-02
tertiary granule lumen (GO:1904724)	55	5	0.41	12.15	8.31E-05	5.47E-03
lysosomal lumen (GO:0043202)	98	7	0.73	9.55	1.34E-05	1.10E-03
specific granule lumen (GO:0035580)	61	4	0.46	8.77	1.39E-03	4.59E-02
small ribosomal subunit (GO:0015935)	78	5	0.58	8.57	3.85E-04	1.71E-02
platelet alpha granule (GO:0031091)	91	5	0.68	7.35	7.50E-04	2.79E-02

