

Drug-repurposing screen identifies thiostrepton as a novel regulator of the tumor suppressor DAB2IP

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Supplementary Materials and Methods

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Analysis of hDAB2IP isoforms

For assessing the expression of predicted C-terminal splice isoforms of hDAB2IP, 500 ng of total RNA was reverse transcribed with iScript Advanced cDNA Synthesis Kit (mRNA) [1725038, Biorad]. PCR reactions were done using Taq DNA Polymerase [201203, Qiagen] on a MiniAmp™ Thermal Cycler [Thermo Fisher]. PCR products were analyzed by agarose gel electrophoresis. Primer sequences are listed in Table 4.

DAB2IP-HiBiT plasmid generation

To generate a plasmid encoding the DAB2IP-HiBiT protein, a C-terminal fragment of DAB2IP in fusion with the HiBiT tag was generated by PCR and inserted in the pcDNA3-Myc-hDAB2IP plasmid (kindly provided by Sidney Yu, University of Hong Kong). Transfection of pcDNA3-hDAB2IP-HiBiT in H1299 cells was performed using Lipofectamine 2000 (Invitrogen), following manufacturer's instructions.

Retroviral Transduction

For generation of PC3 cells stably expressing hDAB2IP, low confluence (~20%) 293GP packaging cells, stably expressing retroviral gag and pol proteins, were transfected by calcium-phosphate precipitation. Briefly, cells were plated the day before the transfection in 10 cm-diameter dishes in DMEM with 10% FBS and antibiotics. Each different virus was produced by co-transfection of 10 µg of the vector of interest and 5 µg of pEnv encoding vector. After 8 hours, medium was changed and cells were incubated at 37°C. After 48 hours the virus-containing medium was filtered (0.45 µm filter) and supplemented with 10% FBS and polybrene (8 µg/ml). Target cells growing at low confluence (~ 30-40%) were treated with the appropriate viral supernatant for 24 hours, and subsequently selected with puromycin (0.5 µg/ml). Single clones with the lowest levels of ectopic DAB2IP protein were selected from the bulk of infected cells and one such clone was used to perform phenotypic assays. pLPC-Myc-DAB2IP was obtained by cloning into the pLPC vector the insert from pcDNA3-Myc-hDAB2IP.

Endogenous DAB2IP Tagging

All synthetic nucleic acids were purchased from Integrative DNA Technologies (IDT). For CRISPR-Cas9 knock-in, crRNAs were designed using multiple online tools, providing as search base an 80–100 bp genomic sequence centered around the preferred editing site.

To perform HDR-driven gene editing, a 150 bp ca. single stranded donor DNA (Ultramer[®] DNA Oligos, IDT) was synthesized containing two flanking regions (50 bp each) providing left and right homology arms, and a central sequence for the desired tag. The knock-in sequence encoded a short flexible linker (Gly-Ser) followed by the HiBiT sequence. Single stranded DNA donor was designed using an online tool (<https://horizondiscovery.com/en/dharmacon>) with the insertion site as close as possible to the predicted editing site. The sequence of the ssDNA is reported in Table 2. Cas9 was delivered as recombinant protein (120 pmol) together with crRNA (150 pmol) and tracrRNA (150 pmol) [Alt-R[®] CRISPR-Cas9 tracrRNA, IDT, 1072533] and single stranded donor DNAs (120 pmol) as previously described [1]. The mixture was prepared in 16-well Nucleocuvette StripsTM [Lonza]. Cells were electroporated using the 4D-NucleofectorTM [Lonza, AAF-1002B] using the following programs: DS-137 for PC3, CA-137 for DU145. 10 min after electroporation, 75 μ l of complete medium with NU7441 (1 μ M) were added to nucleofected wells and cells were transferred into new 12-well plate to allow for recovery. 48 hours post- nucleofection the culture medium was removed and replaced with complete medium without drug.

To obtain genetically homogenous edited clones, nucleofected cells were seeded with a density of 0.5-1 cell/well in a 96-well plate previously filled with 200 μ l/well of complete medium. Cells were incubated for at least 2 weeks before characterization. At least 10 isolated monoclonal lines per condition were characterized by immunoblotting and genomic PCR.

In order to assess the allelic status of HiBiT positive clones, the integration was verified by Sanger sequencing of PCR products of target genomic region. Primers used for genomic amplification are listed in Table 4.

References

1. Ghetti, S., Burigotto, M., Mattivi, A., Magnani, G., Casini, A., Bianchi, A., Cereseto, A. & Fava, L. L. (2021) CRISPR/Cas9 ribonucleoprotein-mediated knockin generation in hTERT-RPE1 cells, STAR Protoc. 2, 100407. [10.1016/j.xpro.2021.100407](https://doi.org/10.1016/j.xpro.2021.100407).

Supplementary figure legends

Fig. S1 Characterization of human DAB2IP isoforms and tagging site.

A-B Human DAB2IP is expressed with two C-terminal variants in multiple cell lines. **(A)** Schematic representation of predicted C-terminal variants, with position of primers used to detect the unspliced (NM_138709.2) or spliced isoforms (NM_032552.3). Lines represent introns, and boxes exons. Filled boxes represent the translated coding sequence. **(B)** Multiple normal and transformed cell lines express both C-terminal variants. Total RNA extracted from the indicated cell lines was reverse-transcribed and amplified using primers designed to distinguish alternatively spliced DAB2IP isoforms. In THP-1 monocytic leukemia cells none of the isoforms could be detected, in line with the notion that DAB2IP is not expressed in blood. Note: some electrophoresis images are cropped. **C.** Schematic representation of DAB2IP isoforms, with a blow-up of the tag insertion site. Greyed area, the C-terminal genomic sequence of human DAB2IP; exon sequence in uppercase, intron in lowercase. Underlined is the guide recognition site in the target sequence, with the PAM sequence in yellow. The vertical dotted line indicates the predicted cut site. The insertion site is arbitrarily positioned in correspondence of the cut site at junction between codons. In the lower part, codons (above) and the corresponding translation (below) of the insert are reported. Red capital letters refer to a flexible Gly/Ser linker followed by the HiBiT-tag (in blue) and two stop codons (in black). The donor DNA also included two homology arms (HA) of 50 bp (adapted from [1]). **D.** H1299 cells were transfected with pcDNA3 myc-hDAB2IP-HiBiT plasmid, encoding a fusion protein identical to the predicted endogenous tagged product. An empty pcDNA3 plasmid was transfected as a negative control. 24 hours after transfection, variable numbers of transfected cells were processed for luciferase assay. On the right, western blot confirmed expression of the transfected fusion protein, with HSP90 and actin as loading control.

Fig. S2. Validation of DAB2IP HiBiT-tagged cells for high-throughput screening.

A Workflow of CRISPR/Cas9-mediated gene knock-in. See material and methods for details. **B** Bulk HiBiT nucleofected cells are positive to luminescence. An identical number of the indicated cells were lysed and processed for luciferase assay. **C.** Bulk nucleofected cells display edited target sequence. Genomic DNA was amplified using primers designed to produce ca. 350 bp amplicons with a clear upshift in case of HiBiT integration. Parental genomic DNAs were amplified as

control. **D.** DAB2IP knockdown reduces DAB2IP-HiBiT luciferase activity. Bulk PC3-HiBiT cells were transfected with DAB2IP siRNA or with DAB2IP-targeting miR-149-3p for 48 hours before luminescence reading. Data are mean \pm SD of 3 wells per condition. On the right, western blot confirmed downregulation of DAB2IP protein. Actin was blotted as a loading control. **E.** Fluorescence intensity and luminescence intensity (DAB2IP levels) correlate with the number of cells. The indicated numbers of parental non-edited PC3 and PC3-HiBiT cells were plated in 384 well-plates. 24 hours later, cells were incubated with the CellTiterFluor (Promega) reagent and fluorescence was detected (left panel). After fluorescence detection, the same cells were incubated with NanoGlo lytic reagent (Promega) and luminescence was measured (right panel). Data are mean \pm SD of three wells per condition (****P < 0.0001; two-way ANOVA with Sidak correction). **F-G.** The Lum/Fluo ratio readily detects changes in DAB2IP protein levels. PC3-HiBiT (**F**) and DU145-HiBiT (**G**) cells were reverse transfected with DAB2IP siRNA in 384 well-plates. 72 hours after transfection, fluorescence and luminescence were read. Results are mean \pm SD of 3 wells per condition (****P < 0.0001; one-way ANOVA with Dunnett's post hoc).

Fig. S3 Validation of identified drugs in DU145 prostate cancer and MCF7 breast cancer cell lines.

A Histogram summarizes the Lum/Fluo ratio measured in DU145-HiBiT cells treated as in Fig. 1C. Data are mean \pm SD of three independent experiments (*P < 0.05, **P < 0.01, ****P < 0.0001); one-way ANOVA with Dunnett's post hoc). **B** Dose-response curves of the three best candidate drugs in DU145-HiBiT cells. The graphs summarize the effect of each drug on DAB2IP levels (Lum/Fluo ratio) and the effect on viability (Fluorescence). The half-maximal effective concentration (EC₅₀) and half-maximal inhibitory concentration (IC₅₀) are also indicated. Data are mean of *n* independent experiments as indicated in figure \pm SD. **C-D.** Effects of candidate drugs on DAB2IP in non-edited parental DU145. Cells were treated with drugs or DMSO (Ctrl) for 36h. When not indicated, drug concentration was 10uM. **(C)** Representative immunoblotting of DAB2IP, with HSP90 as loading control. **(D)** Cells were treated as in C. DAB2IP mRNA levels were measured by RT-qPCR. Values were normalized on histone H3 and compared to DMSO-treated controls. Data are median with interquartile range of three independent experiments (ns= non-significant; Kruskal-Wallis test followed by Dunn's multiple comparisons). **E-F.** Effects of candidate drugs on DAB2IP in the breast cancer cell line MCF7. **(E)** Cells were treated with the

indicated drugs at 10 μ M for 36h. Representative immunoblotting of DAB2IP, with HSP90 as loading control. **(F)** Cells were treated as in E. DAB2IP mRNA levels were measured by RT-qPCR. Values were normalized on histone H3 and compared to DMSO-treated controls. Data are mean \pm SD of three independent experiments (ns= non-significant; one-way ANOVA with Dunnett's post hoc).

Fig. S4 The identified drugs likely act on DAB2IP via different molecular mechanisms.

A. Perturbagens and Compounds significantly associated to Thio, Ube, or Sal in the Broad Institute ConnectivityMap accessed via the CLUE platform. **B.** Venn diagram summarizing the top 1500 genes affected by 24 hours treatment with 10 μ M Thio, Ube, or Sal in MCF7 cells (from ConnectivityMap). **C.** For each drug, the top 1500 genes were used for gene set enrichment analysis (KEGG pathways) using the ShinyGO platform.

Fig. S5 Hallmarks enriched in PC3 and MCF7 after drug treatment.

The top 1500 genes affected by 24 hours treatment with 10 μ M Thio, Ube, or Sal in PC3 or MCF7 cells were used for gene-set enrichment analysis (Hallmarks.MsigDB) using the ShinyGO platform.

Figure S1

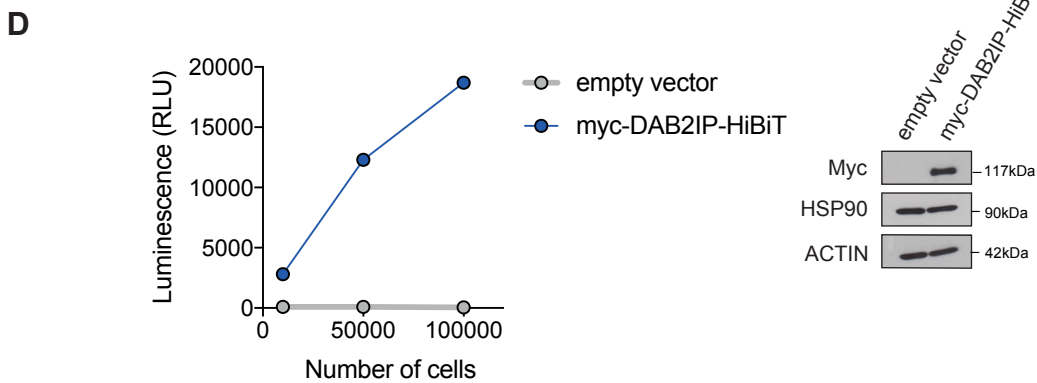
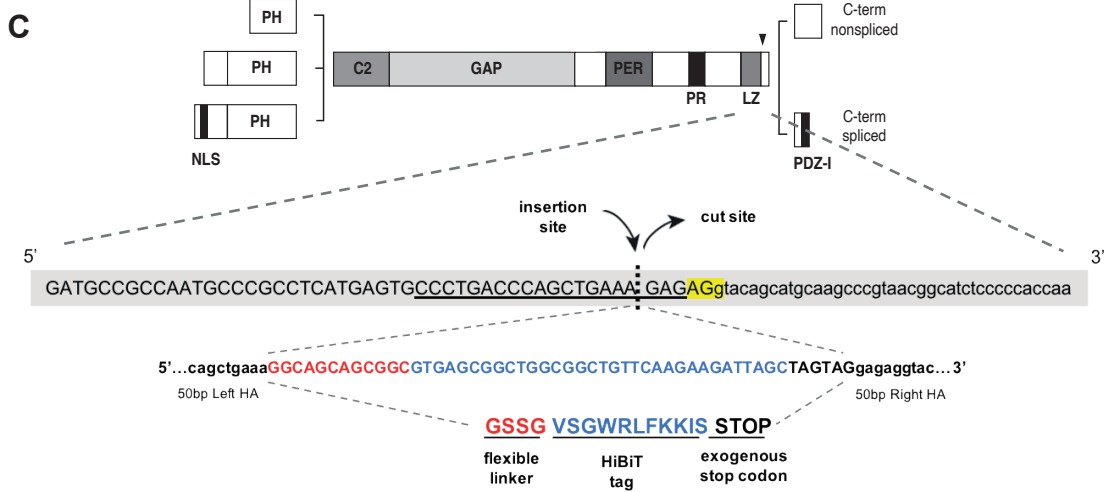
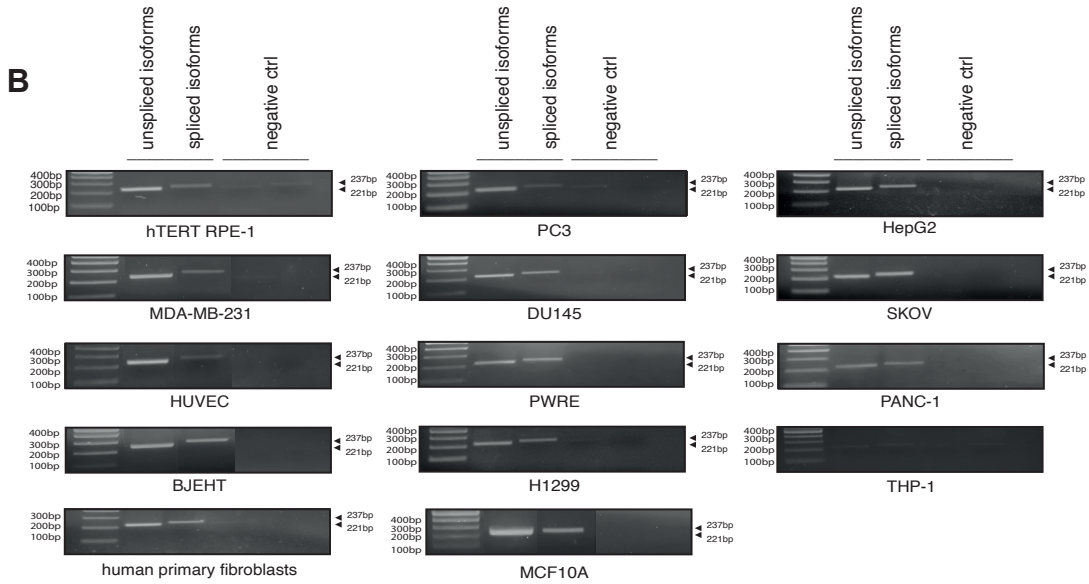
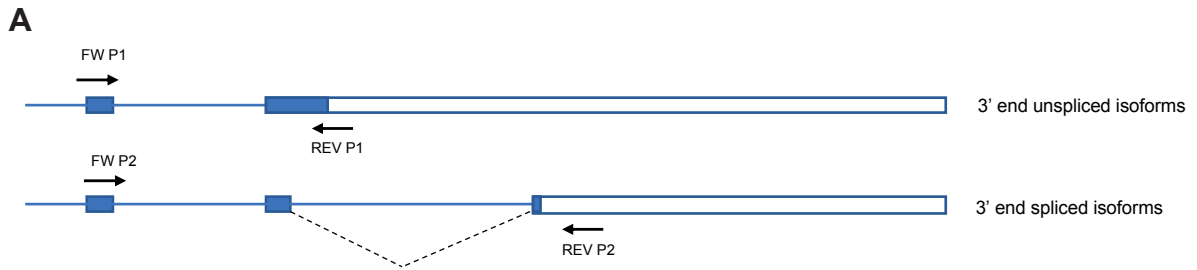


Figure S2

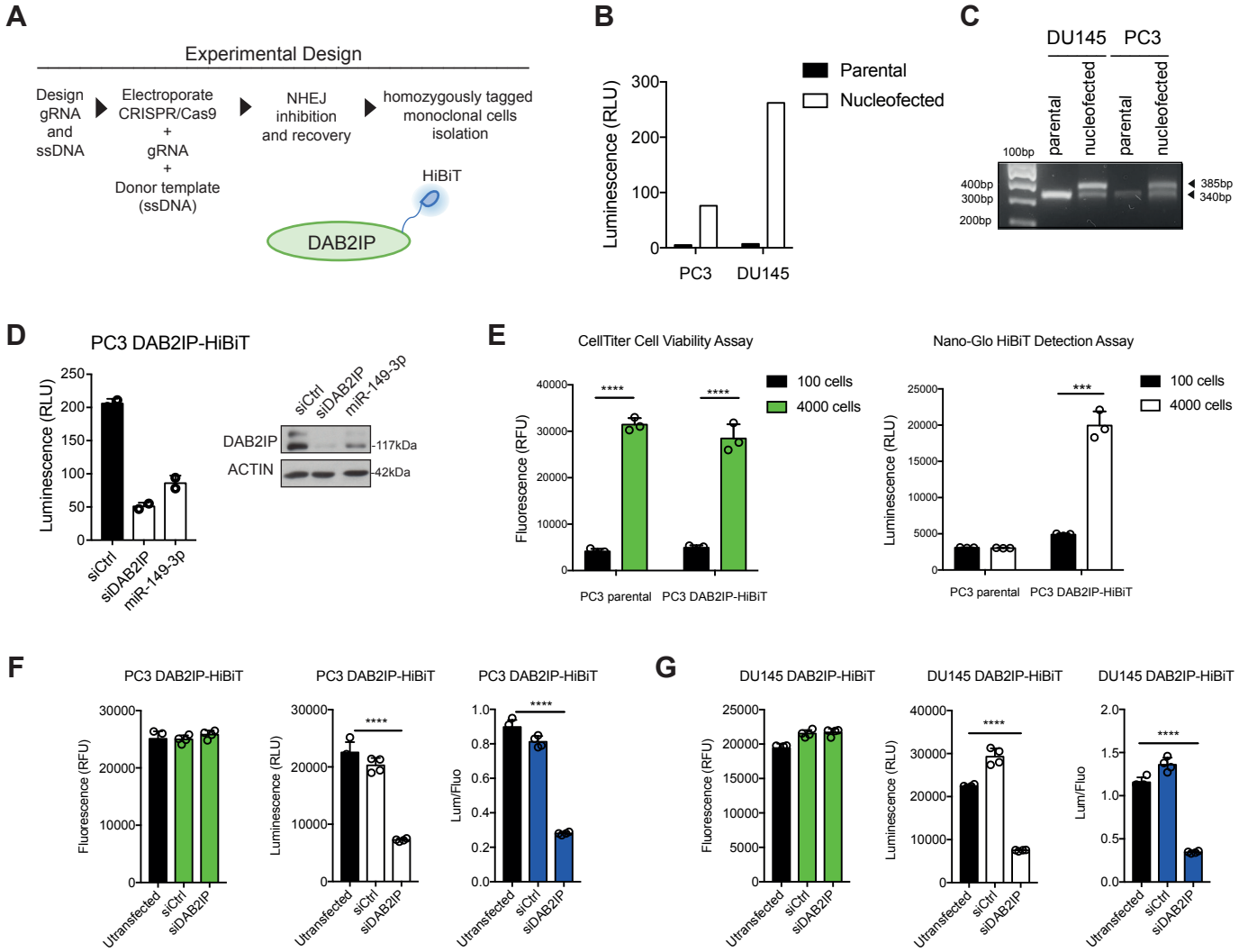


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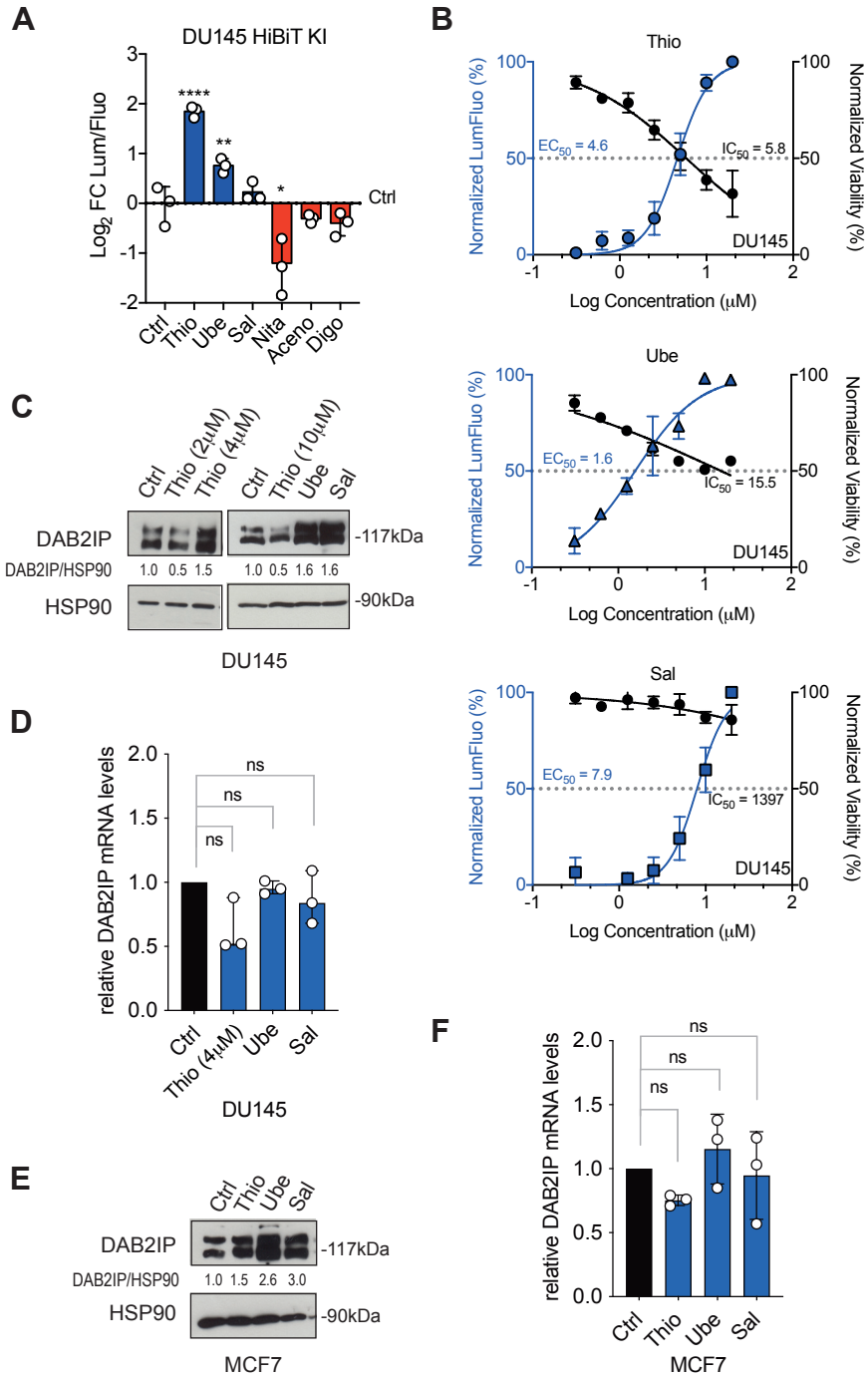
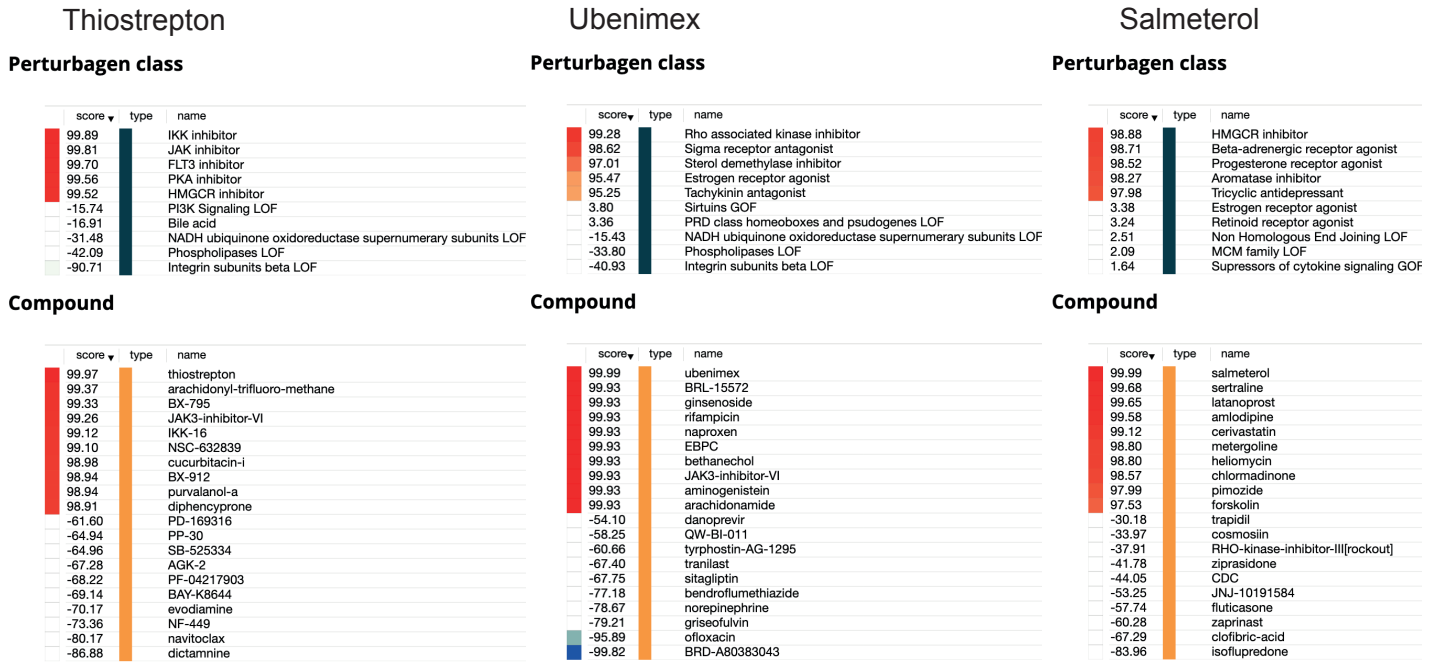
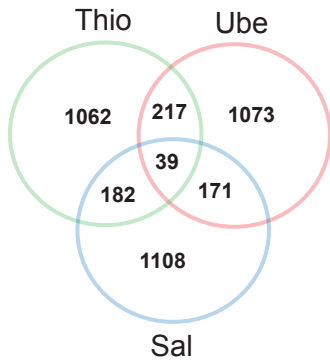


Figure S4

A



B



C

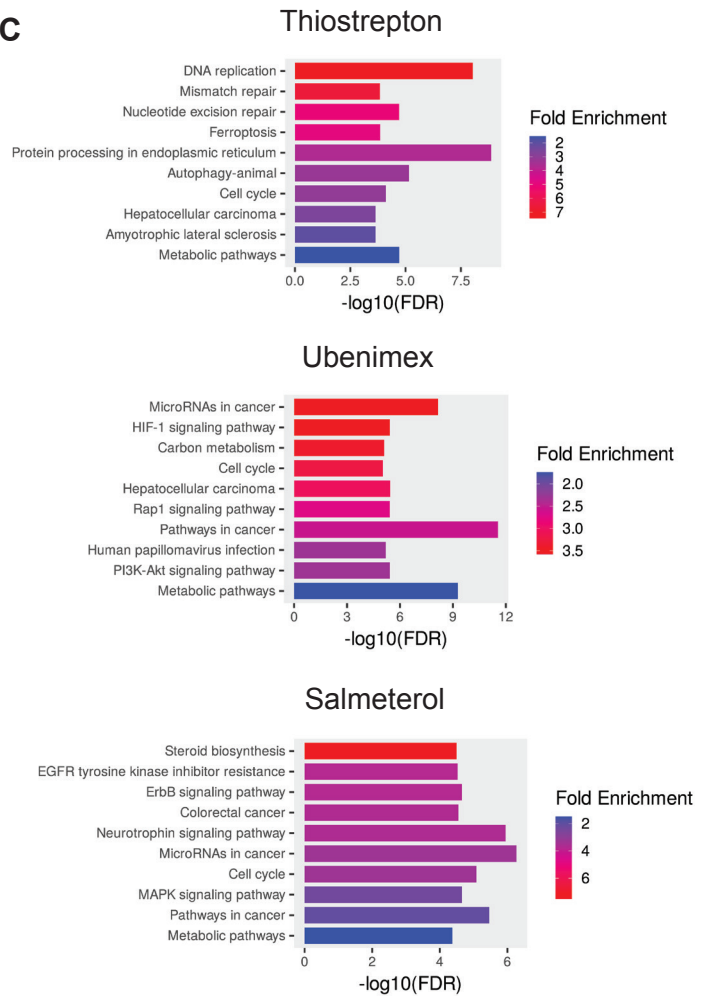
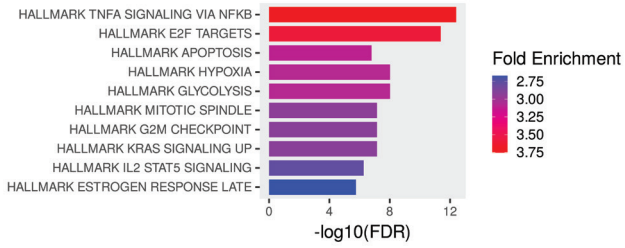


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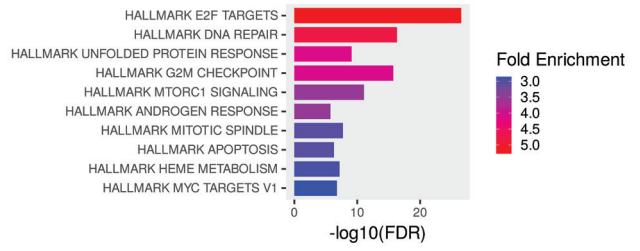
PC3

Thiostrepton

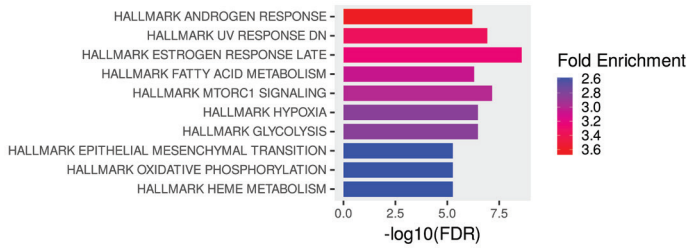


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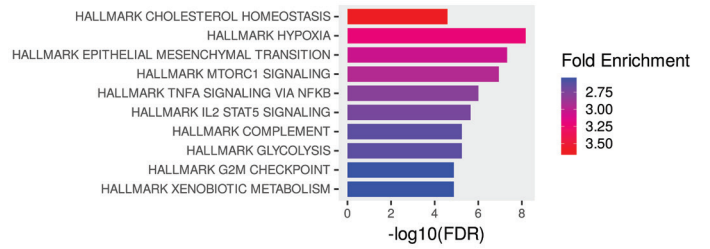
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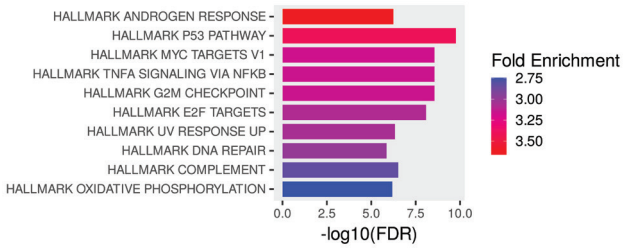
Ubenimex



Ubenimex



Salmeterol



Salmeterol

