GLI1-inducible glucuronidation targets a broad spectrum of drugs

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Supplementary Figure Legends

Supplemental Figure 1: GLI1 inducible drug glucuronidation underpins broad spectrum drug resistance

A. Western blots for GLI1 and UGT1A to show expression in FaDu-2FLAG, FaDu-GLI1 and FRII cell lines. HSP90 and Actin B are provided as loading controls for respective blots.

B. Protein quantification of GLI1 and UGT1A levels corresponding to blots in part A. Data shown for triplicate experiments as mean +/- standard error of the mean (SEM). Experiments were performed in triplicates, at least three independent times. *** *P-value*<0.001, ** *P-value*<0.01 (Student's t-test).

C. RT-qPCR analysis of *UGT1A* using a pan-UGT1A primer. mRNA levels were normalized to ubiquitin C (UBC) which is not a GLI1 target.

D. Western blots for UGT1A in FaDu-2FLAG and FaDu-GLI1 cell lines following treatment with compounds tested in **Figure 1 B**. No change in UGT1A levels were observed. Actin B is provided for loading control. Drug names as in **Figure 1**; UN: untreated.

E. Cell proliferation inhibition in untreated versus treatment with compounds tested in **Figure 1B** in the presence or absence of GDC-0449, GANT-61 or Imipramine is shown in FaDu-2FLAG, FaDu-GLI1 and FRII cells. Proliferation inhibition is measured as the mean +/- SD (error bars). 0% indicates no proliferation inhibition and is derived from the untreated control for each respective cell line. Experiments were performed in triplicates, at least three independent times. *** *P-value*<0.001, ** *P-value*<0.01 (Student's t-test). P-values for a given treatment are in comparison to the inhibition observed in FaDu-2FLAG cells for that particular treatment.

F. Western blot for GLI1 and UGT1A in FaDu-GLI1 cells following treatments with Imipramine, GDC-0449 or Gant-61. Imipramine did not change GLI1 and UGT1A levels while GDC-0449 and Gant61 did (as expected). HSP90 is used as a loading control.

G-I. Full UGT1A blots and membranes corresponding to Figures 1a, Supplemental Figure 1d and f, respectively.

J. Protein quantification of GLI1 and UGT1A levels corresponding to blots in part F. Data shown for triplicate experiments as mean +/- standard error of the mean (SEM). Experiments were performed in triplicates, at least three independent times. *** *P-value*<0.001, ** *P-value*<0.01 (Student's t-test).

Supplemental Figure 2: Mass Spectrometry Analysis of 5-Fluorouracil and Sunitinib glucuronidation

A-E MS/MS extracted ion chromatographs (EIC) are shown in FaDu-GLI1 cells for (a) 5-fluorouracil at the m/z 129.0107 Da for the parent compound and (c) the glucuronide at the mass of the parent compound + 176.0325 Da. Electrospray ionization (ESI) mass spectra showing the parent compound and (b) the glucuronide at m/z 305.0432 Da (d). EIC for the glucuronide of 5-fluorouracil at m/z (305.0432 +/- 5 ppm). No glucuronides were detected for 5-fluorouracil in FaDu-FLAG cells.

F-K. MS/MS extracted ion chromatographs (EIC) are shown in FaDu-GLI1 cells for (a) sunitinib at the m/z 399.2187 Da for the parent compound and (c) the glucuronide at the mass of the parent compound + 176.0325 Da. Electrospray ionization (ESI) mass spectra showing the parent compound and (b) the glucuronide at m/z 575.2512 Da (d). EIC for the glucuronide of sunitinib

at m/z (575.2512 +/- 5 ppm). No glucuronides were detected for 5-Fluorouracil in FaDu-FLAG cells.

Supplemental Figure 3: ATP synthesis inhibitors restore drug sensitivity in GLI1 overexpressing cells

A. Western blots for GLI1 and UGT1A in FaDu-2FLAG, FaDu-GLI1 and FRII cells following treatment with Ribavirin in the presence or absence of GDC-0449 or Oligomycin A versus untreated controls. HSP90 serves as a loading control. A full blot is shown in the bottom panel demonstrating the quality of the UGT1A antibody in FaDu cells. The major band is at 55 kDa, as expected. The origin of the upper band at 63 kDa is not known, but varies with the major 55 kDa band suggesting it could be a modified form of UGT1A.

B. Full UGT1A blots and membranes corresponding to part A.

C. Protein quantification of GLI1 and UGT1A levels corresponding to blots in part A, B. Data shown for triplicate experiments as mean +/- standard error of the mean (SEM). Experiments were performed in triplicates, at least three independent times. *** *P-value*<0.001, ** *P-value*<0.01, *ns: statistically not significant* (Student's t-test).

Supplemental Figure 4: CALR is required for GLI1-dependent inducible drug glucuronidation

A. Protein quantification of CALR levels corresponding to blots in Figure 3b. Data shown for triplicate experiments as mean +/- standard error of the mean (SEM). Experiments were

performed in triplicate, at least three independent times. *** *P-value*<0.001, ** *P-value*<0.01 (Student's t-test).

B. Western blots for UGT1A and CALR in FaDu-2FLAG, FaDu-GLI1 and FRII cells following siRNA mediated knockdown of calreticulin (siCALR) versus siLuciferase (siLuc) controls. Actin B serves as a loading control.

C. Protein quantification of CALR levels corresponding to blots part B. Data shown for triplicate experiments as mean +/- standard error of the mean (SEM). Experiments were performed in triplicate, at least three independent times. *** *P-value*<0.001, ** *P-value*<0.01 (Student's t-test).

D. Full UGT1A blots and membranes corresponding to Figures 3c and Supplemental Figure 3b. Not related refers to experiments conducted not related to this report.

Supplemental Table 1: Clients of GLI1-inducible glucuronidation

Fold change of a given compound is measured as the ratio of its IC50 observed in FaDu-2FLAG cells versus IC50 observed in FaDu-GLI1 cells. For compounds shown in purple, the IC50 was greater than the maximum tested concentration (10 μM) and as such the fold change for those compounds is measured as the difference in the % inhibition observed at 10 μM in FaDu-2FLAG versus FaDu-GLI1. Compounds showing a fold change greater than or equal to 1.5 or a difference of 20% inhibition between cell lines are considered resistant. Only compounds with statistically significant changes (*p-value*<0.05) are included in this list. Asterisk represents compounds where fold change is measured in manual cell viability validation assays.

Supplemental Table 2: Compounds reversing GLI1-inducible glucuronidation

Fold change of a given compound is measured as the ratio of its IC50 observed in FaDu-GLI1 cells versus IC50 observed in FaDu-2FLAG cells. For compounds shown in purple, the IC50 was greater than the maximum tested concentration (10 μM) and as such the fold change for those compounds is measured as the difference in the % inhibition observed at 10 μM in FaDu-GLI1 versus FaDu-2FLAG. Compounds showing a fold change greater than or equal to 1.5 or a difference of 20% inhibition between cell lines are considered reversing resistance. Only compounds with statistically significant changes (*p-value*<0.05) are included in this list. Asterisk represents compounds whose fold change is measured in manual cell viability validation assays.

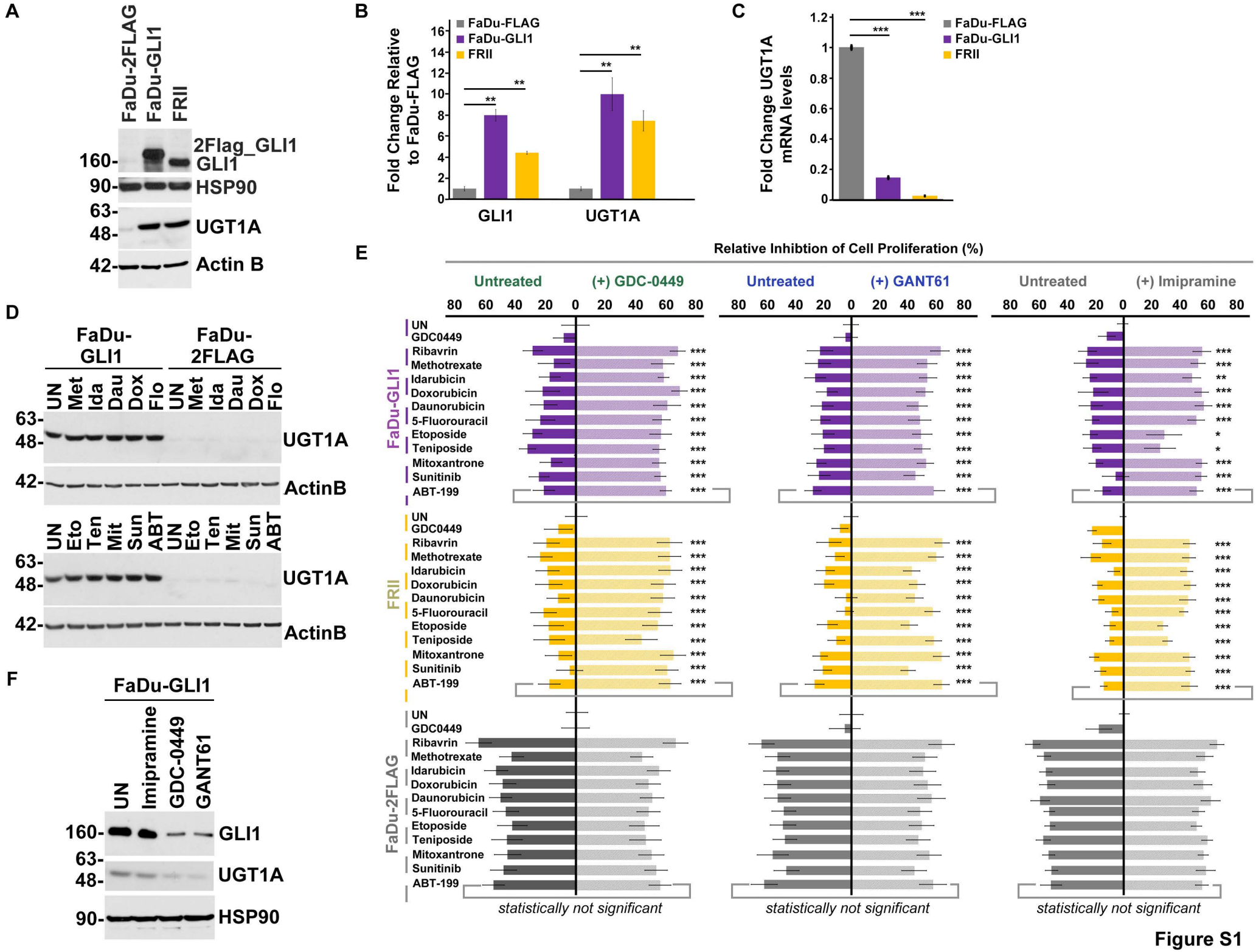
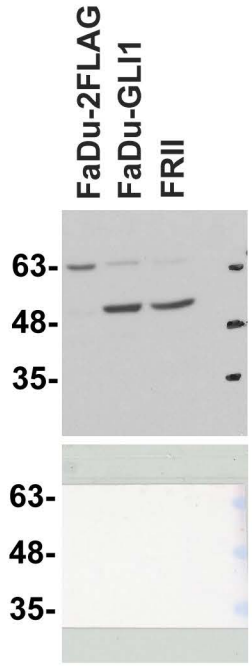

A
FaDu-2FLAG
FaDu-GLI1
FRII
160-
90-
63-
48-
42-
2Flag_GLI1
GLI1
HSP90
UGT1A
Actin B
B
FaDu-FLAG
FaDu-GLI1
FRII
Fold Change Relative to FaDu-FLAG
GLI1
UGT1A
C
Fold Change UGT1A mRNA levels
D
FaDu-GLI1
FaDu-2FLAG
UN Met Ida Dau Dox Flo
UN Eto Ten Mit Sun ABT
UGT1A
ActinB
E
Relative Inhibtion of Cell Proliferation (%)
Untreated
(+) GDC-0449
(+) GANT61
(+) Imipramine
FaDu-GLI1
FRII
FaDu-2FLAG
UN
GDC0449
Ribavrin
Methotrexate
Idarubicin
Doxorubicin
Daunorubicin
5-Fluorouracil
Etoposide
Teniposide
Mitoxantrone
Sunitinib
ABT-199
statistically not significant
F
FaDu-GLI1
UN
Imipramine
GDC-0449
GANT61
GLI1
UGT1A
HSP90

Figure S1

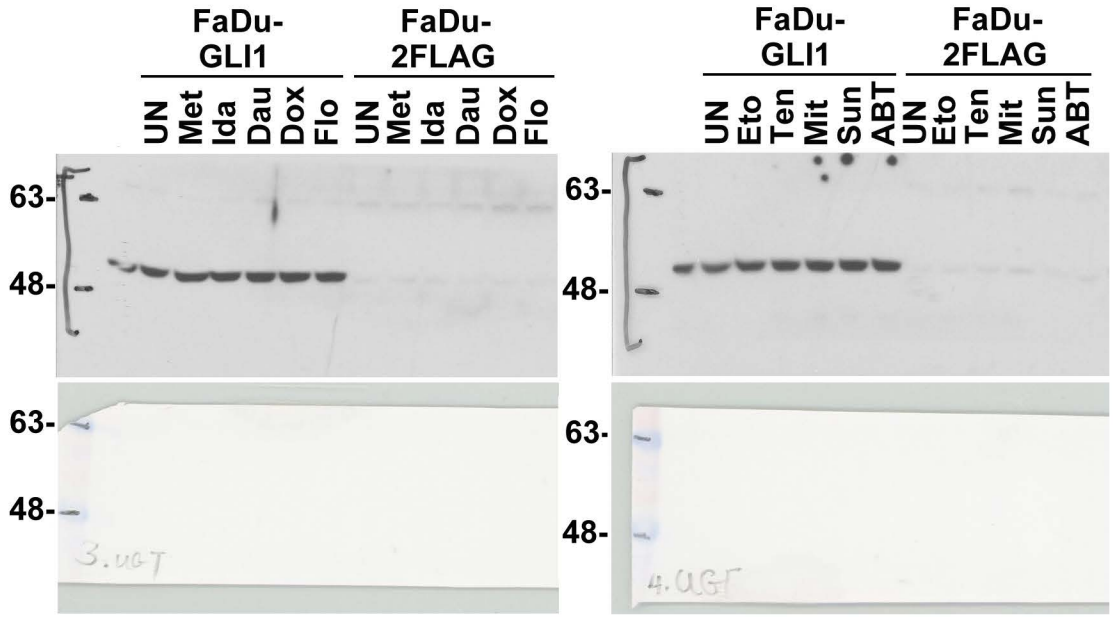
G

UGT1A WB Control Corresponding to Part a



H

UGT1A WB Control Corresponding to Part d



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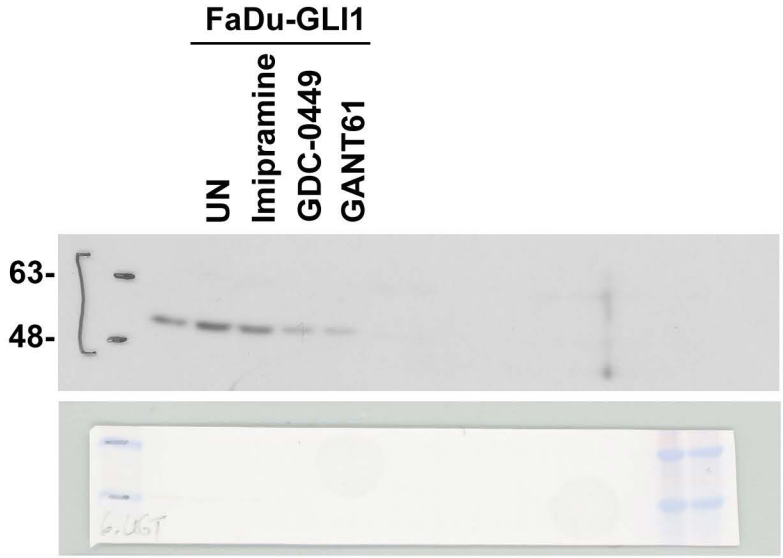
UGT1A WB Control Corresponding to Part f



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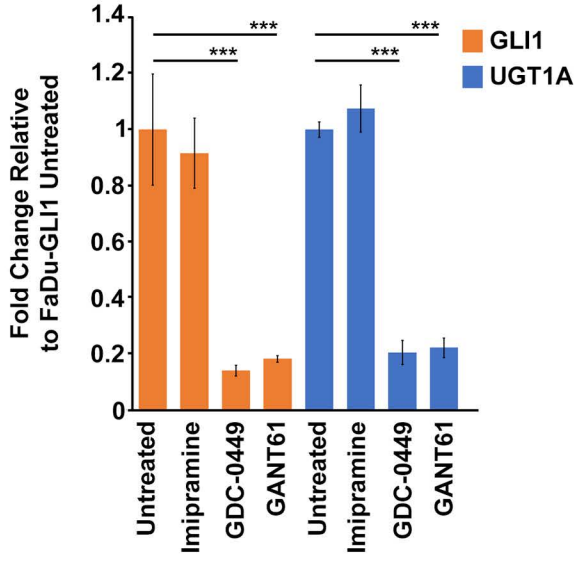


Figure S1 Cont'd

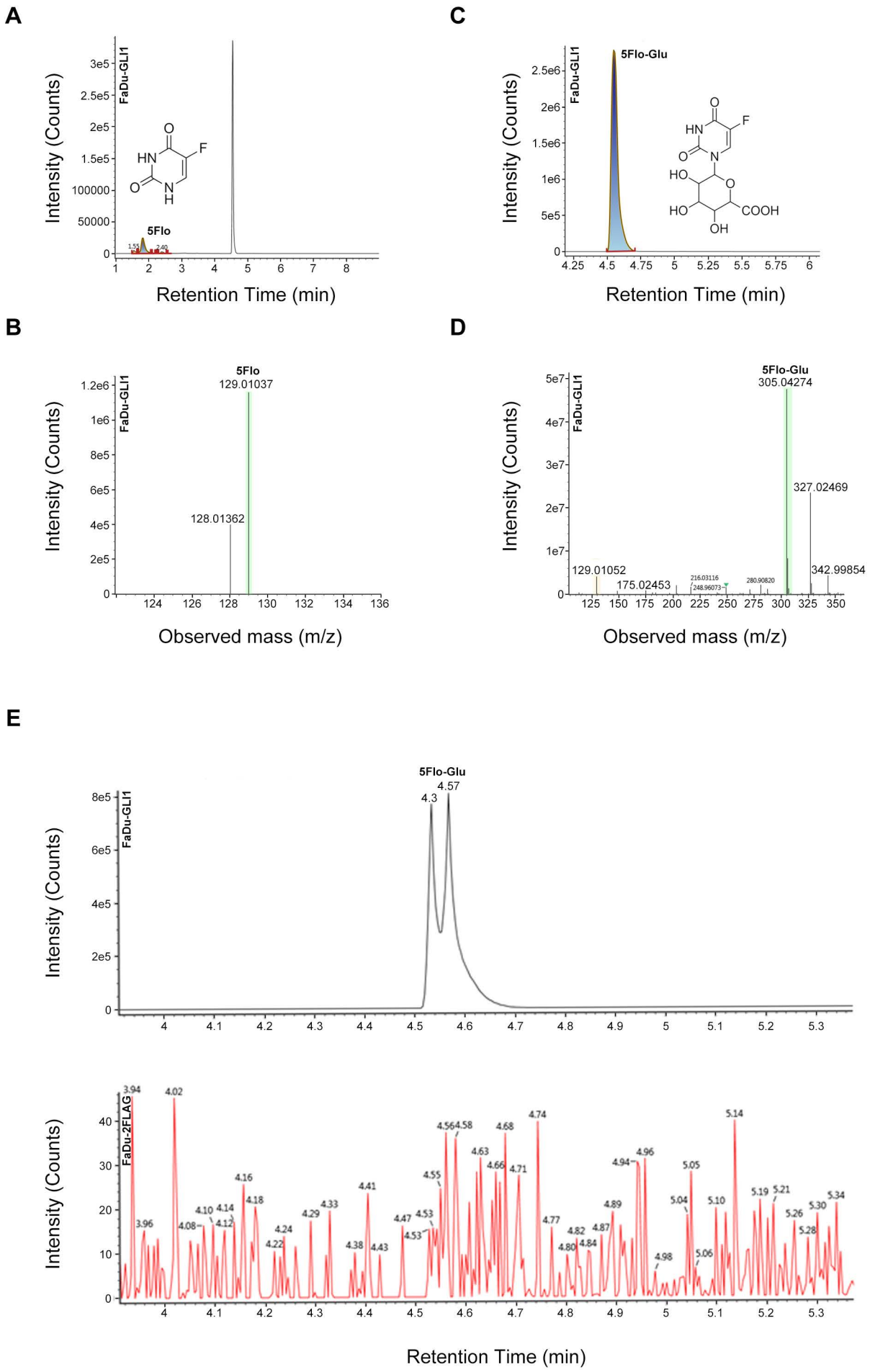

A
FaDu-GLI1
Intensity (Counts)
5Flo
Retention Time (min)
C
FaDu-GLI1
5Flo-Glu
Intensity (Counts)
Retention Time (min)
B
FaDu-GLI1
5Flo
129.01037
128.01362
Intensity (Counts)
Observed mass (m/z)
D
FaDu-GLI1
5Flo-Glu
305.04274
327.02469
342.99854
129.01052
175.02453
Intensity (Counts)
Observed mass (m/z)
E
FaDu-GLI1
5Flo-Glu
4.57
4.3
Intensity (Counts)
FaDu-2FLAG
Intensity (Counts)
Retention Time (min)

Figure S2

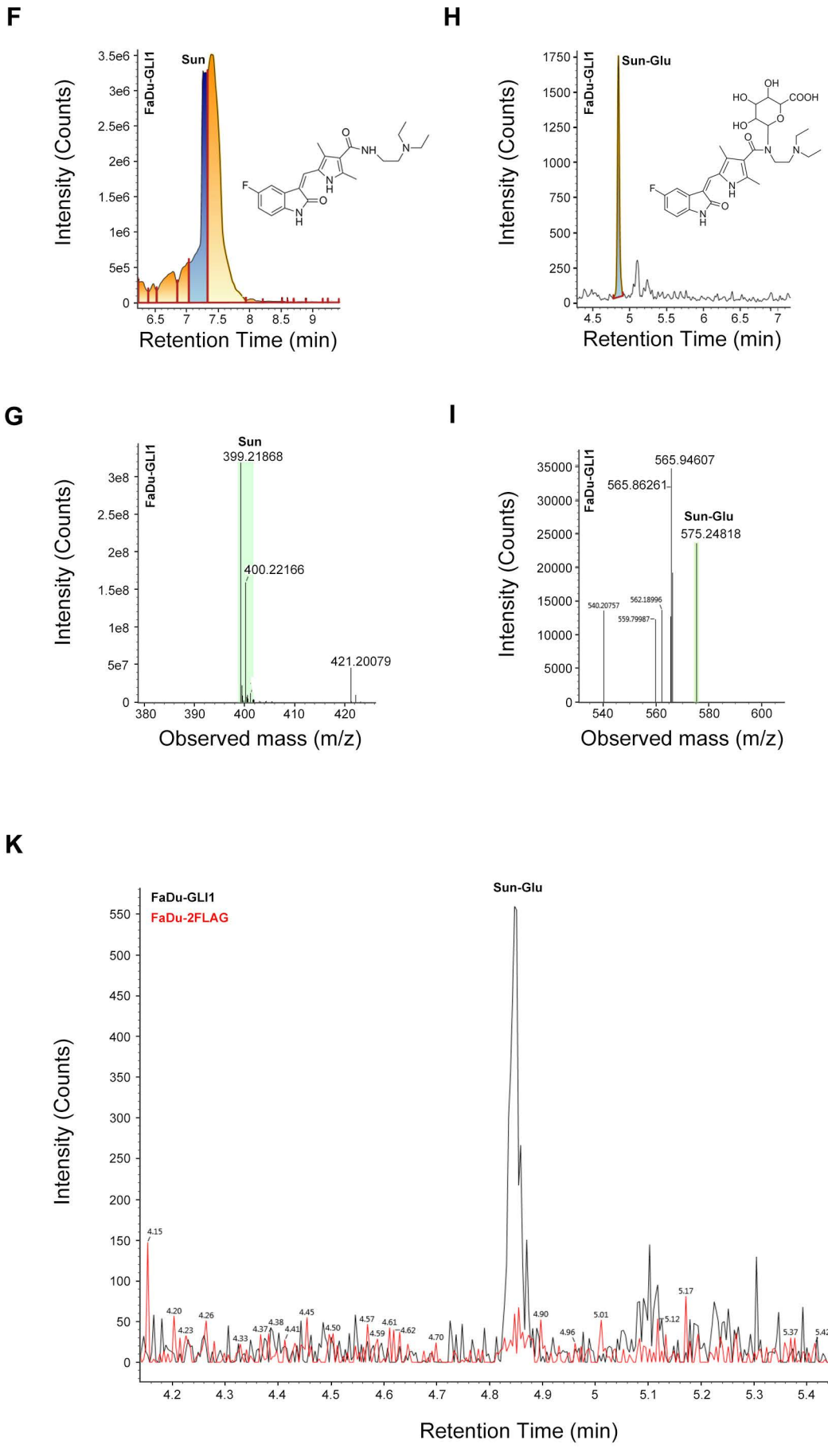


Figure S2 Cont'd

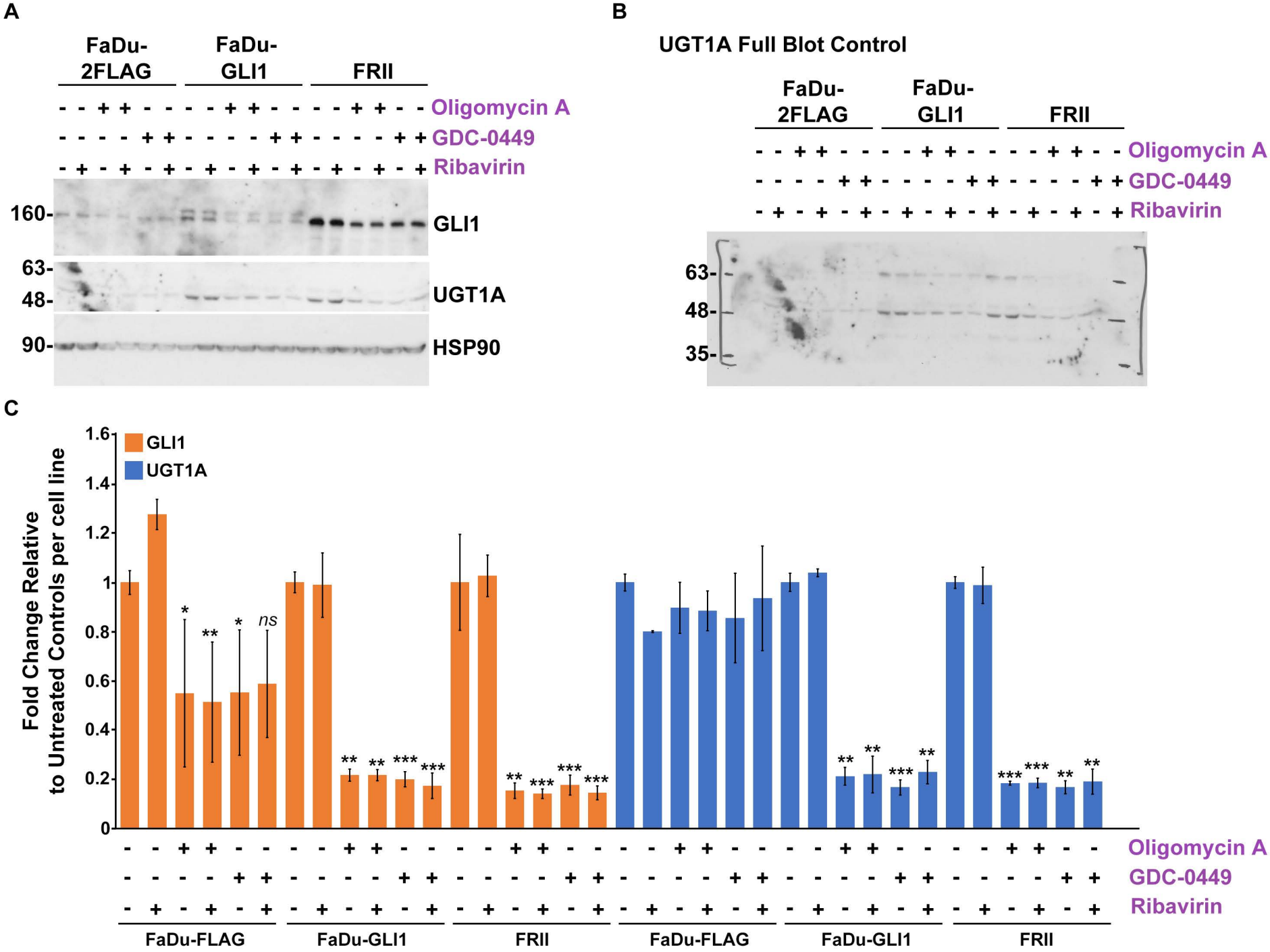
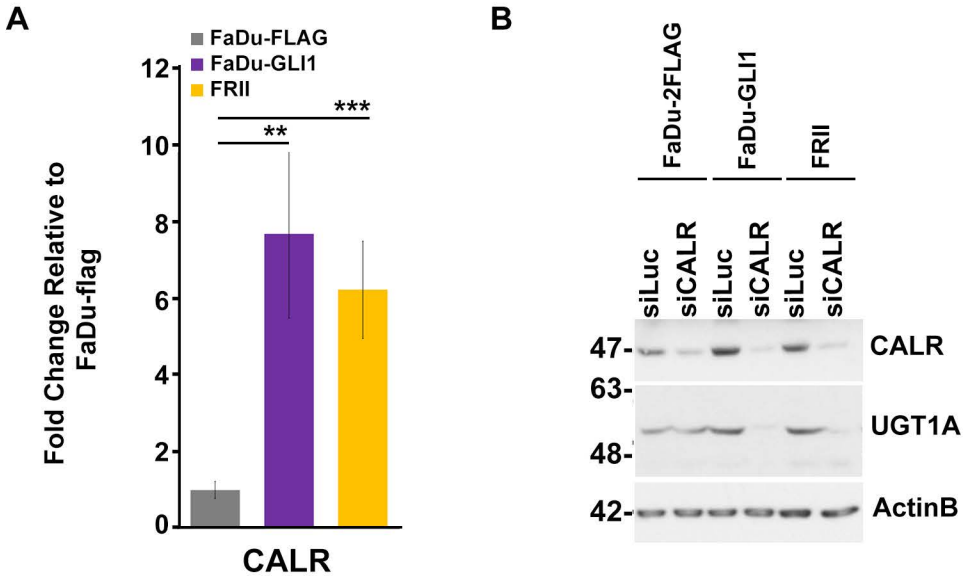

A
FaDu-2FLAG
FaDu-GLI1
FRII
Oligomycin A
GDC-0449
Ribavirin
160-
63-
48-
90-
GLI1
UGT1A
HSP90
B
UGT1A Full Blot Control
FaDu-2FLAG
FaDu-GLI1
FRII
Oligomycin A
GDC-0449
Ribavirin
63-
48-
35-
C
GLI1
UGT1A
Fold Change Relative to Untreated Controls per cell line
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
ns
Oligomycin A
GDC-0449
Ribavirin
FaDu-FLAG
FaDu-GLI1
FRII
FaDu-FLAG
FaDu-GLI1
FRII

Figure S3


A
FaDu-FLAG
FaDu-GLI1
FRII
Fold Change Relative to FaDu-flag
CALR
**
***
B
FaDu-2FLAG
FaDu-GLI1
FRII
siLuc
siCALR
47-
63-
48-
42-
CALR
UGT1A
ActinB
C
CALR
UGT1A
Fold Change Relative to FaDu-FLAG_siLuc
FaDu-FLAG_siLuc
FaDu-FLAG_siCALR
FaDu-GLI1_siLuc
FaDu-GLI1_siCALR
FRII_siLuc
FRII_siCALR

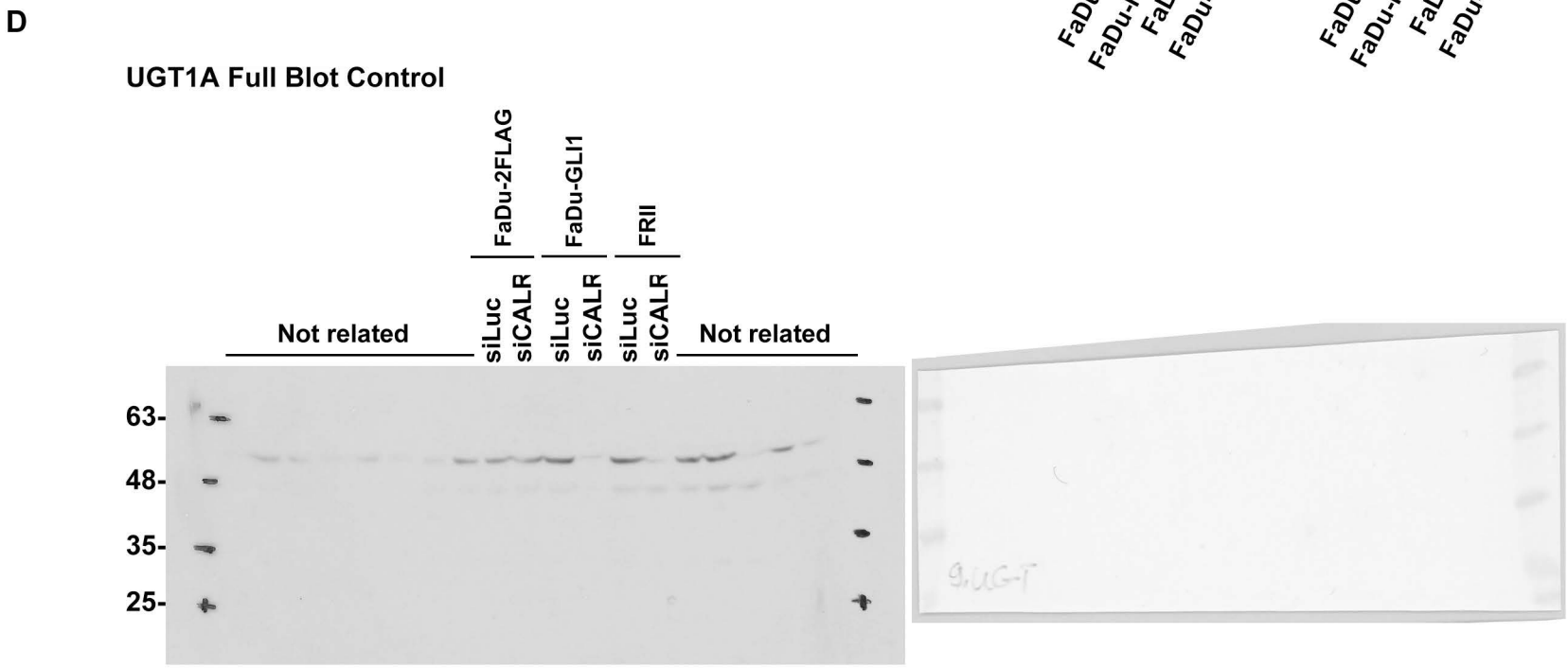

D
UGT1A Full Blot Control
FaDu-2FLAG
FaDu-GLI1
FRII
siLuc
siCALR
Not related
Not related
63-
48-
35-
25-

Figure S4

Table S1

Compound Name	Fold Change
Methotrexate	100.00 *
Doxorubicin	10.00 *
Daunorubicin	10.00 *
ABT-199	5.00 *
Azacytidine	4.00 *
Idarubicin	2.55 *
Teniposide	3.20 *
Etoposide	3.00 *
5-Fluorouracil	2.50 *
Mitxoantrone dihydrochloride	2.00 *
Sunitinib	2.00 *
Floxuridine	> 7.20
Edoxudine	> 4.30
Carmofur	3.88
Vineomycin A1	2.70
Cytochalasin E	2.63
Z-L-Phe chloromethyl ketone	2.42
Dequalinium analog, C-14 linker	2.17
Cetylpyridinium Chloride	1.98
Narasin	1.89
Actinomycin D	1.82
Tetrachloroisophthakonitrile	1.78
Vinorelbine Base	1.76
Tryptanthrin	> 1.75
Echinomycin	1.67
Helenine	1.66
Dihydrocelastryl Diacetate	1.66
Salinomycin	1.56
Cetrimonium Bromide	1.56
Atovaquone	> 1.53
Phytosphingosine	>> 1.40
Salsolinol hydrobromide	59.02
Dantrolene sodium salt	44.38
Chloroxylenol	38.94
SB 224289 hydrochloride	35.57
S-(4-Nitrobenzyl)-6-thioguanosine	30.89
5,7-Dihydroxy-4-MethylCoumarin	23.22

Table S2

Compound Name	Fold Change
Oligomycin A	1000.00 *
Gossypol	5.00 *
Quinidine sulfate	5.00 *
Aurovertin B	>>10.63
Disulfiram	2.73
GW2974	1.96
Avocadyne	1.52
Deguelin	1.50
Qunidine Gluconate	30.38
Quinine	20.08