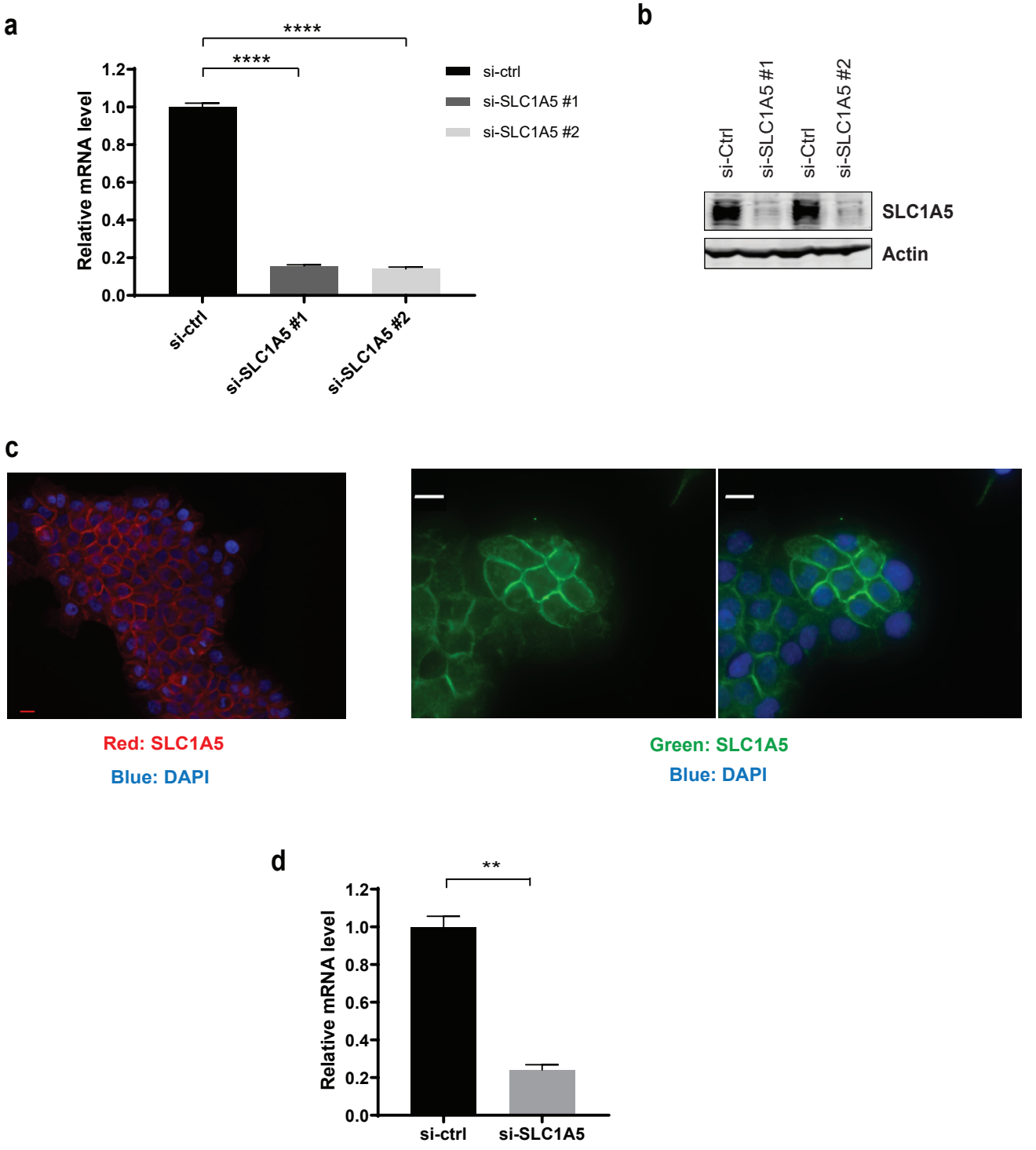
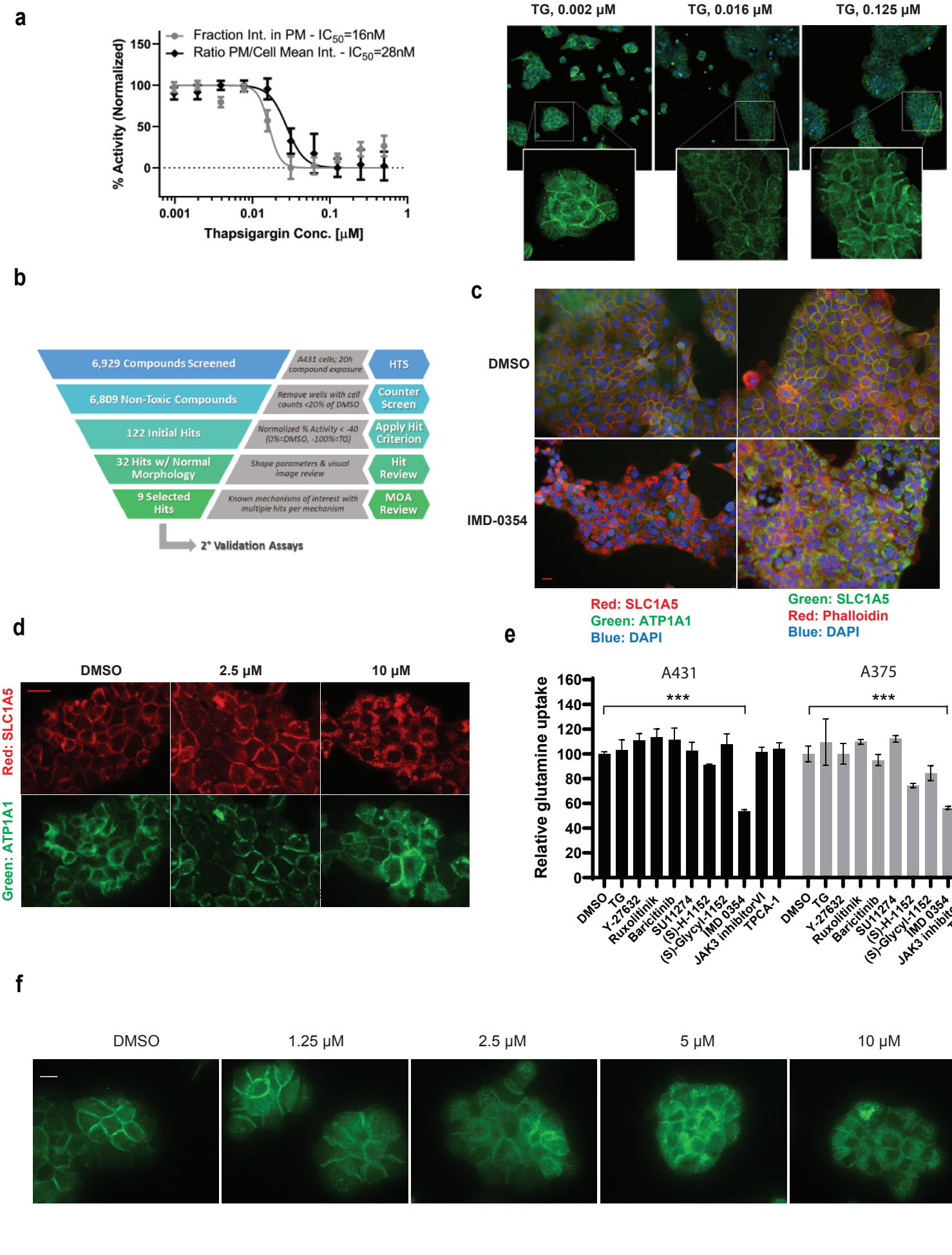


Legend Figure S1. Inhibition of SLC1A5 Expression in A431 cells.
(a) A375 cells were transfected with si-ctrl or si-SLC1A5 for 48h. RNA was extracted and subjected to qPCR analysis for SLC1A5 expression.
(b). A375 cells were transfected with si-ctrl or si-SLC1A5 for 48h. Extracted proteins were subjected to western blotting analysis (right). (c) Immunofluorescence staining of skin epidermoid carcinoma A431 cells with anti-SLC1A5 and DAPI. Scale bar, 20 µm.
(d) A431 cells were transfected with si-ctrl or si-SLC1A5 for 48h. RNA was extracted and subjected to qPCR analysis for SLC1A5 expression. Statistical analysis was performed by one-way ANOVA for the comparison of more than two groups. Unpaired t-test was used for the comparison of two groups.
Data are shown as the mean ± SD, n = 3. **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.



Feng et al., Fig S2

Legend Figure S2. Screen and Validation of Hits.

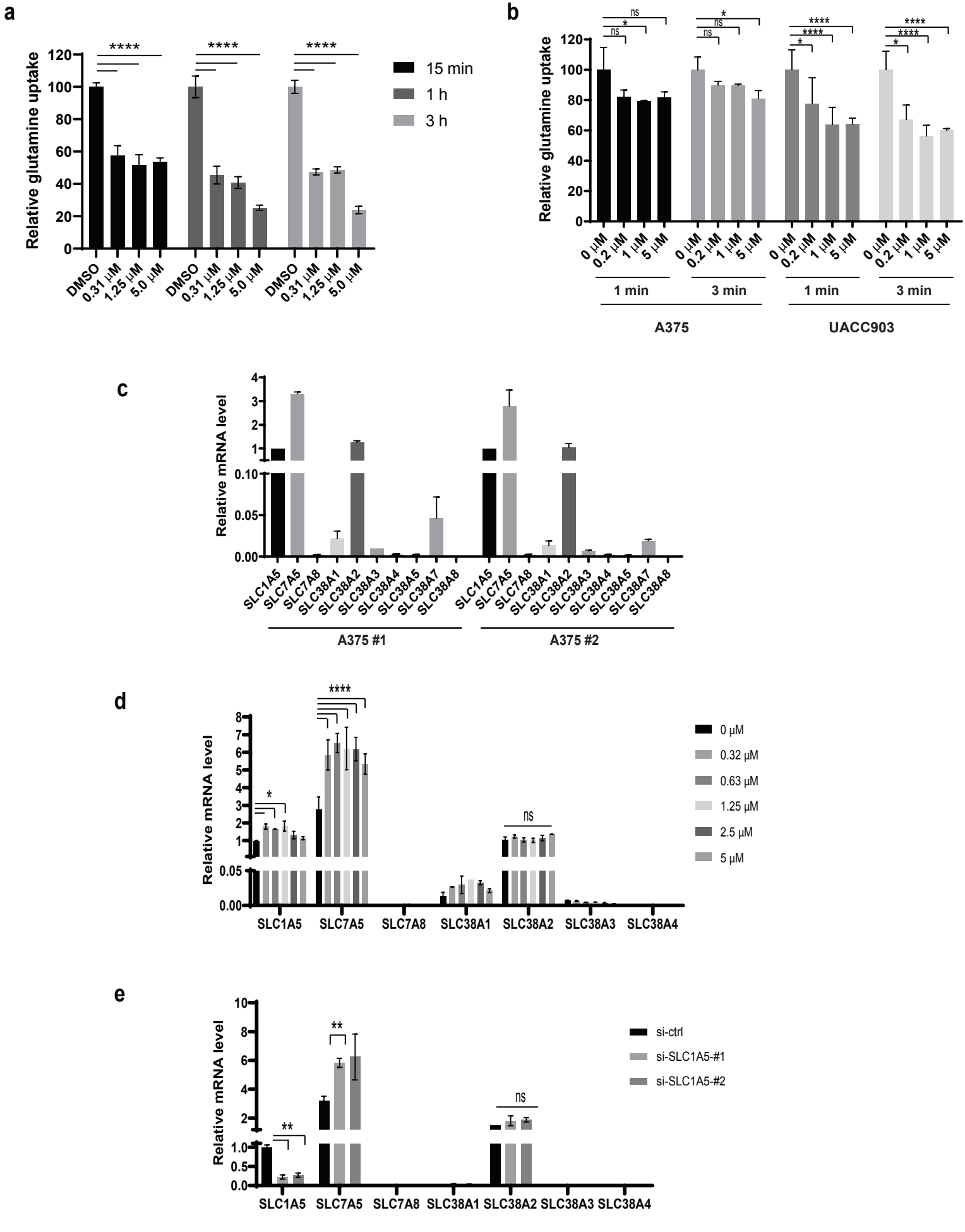
(a) Thapsigargin (as positive control) dose response for two different assay read-outs validating the quantitative HCS imaging assay in 384-well format using A431 cells (mean±SD from 4 replicate wells). Representative images for select concentrations are shown.

(b) Schema for screen and selection of hits for follow-up in secondary validation assays.

(c) Validation of translocation of SLC1A5 after IMD-0354 treatment for 24h by IF, along with the staining for membrane marker ATP1A1 and actin with Phalloidin. Scale bar, 20 µm.

(d) A431 cells were exposed to IMD-0354 (10 µM) overnight and the effect of IMD-0354 on SLC1A5 expression and translocation was examined by IF staining with anti-SLC1A5 and membrane marker protein anti-ATP1A1. Scale bar, 20 µm.

(e) A431 and A375 cells were treated with the indicated compounds (10 µM each) for 1h followed by 3H-glutamine uptake assay.


a
Relative glutamine uptake
15 min
1 h
3 h
DMSO
0.31 μM
1.25 μM
5.0 μM
b
Relative glutamine uptake
0 μM
0.2 μM
1 μM
5 μM
1 min
3 min
A375
UACC903
c
Relative mRNA level
SLC1A5
SLC7A5
SLC7A8
SLC38A1
SLC38A2
SLC38A3
SLC38A4
SLC38A5
SLC38A7
SLC38A8
A375 #1
A375 #2
d
Relative mRNA level
0 μM
0.32 μM
0.63 μM
1.25 μM
2.5 μM
5 μM
e
Relative mRNA level
si-ctrl
si-SLC1A5-#1
si-SLC1A5-#2

Legend Figure S3. IMD-0354 inhibited Glutamine Uptake.

(a) A431 cells were treated with the indicated concentrations of IMD-0354 for 15 min, 1h or 3h followed by 3H-glutamine uptake assay.

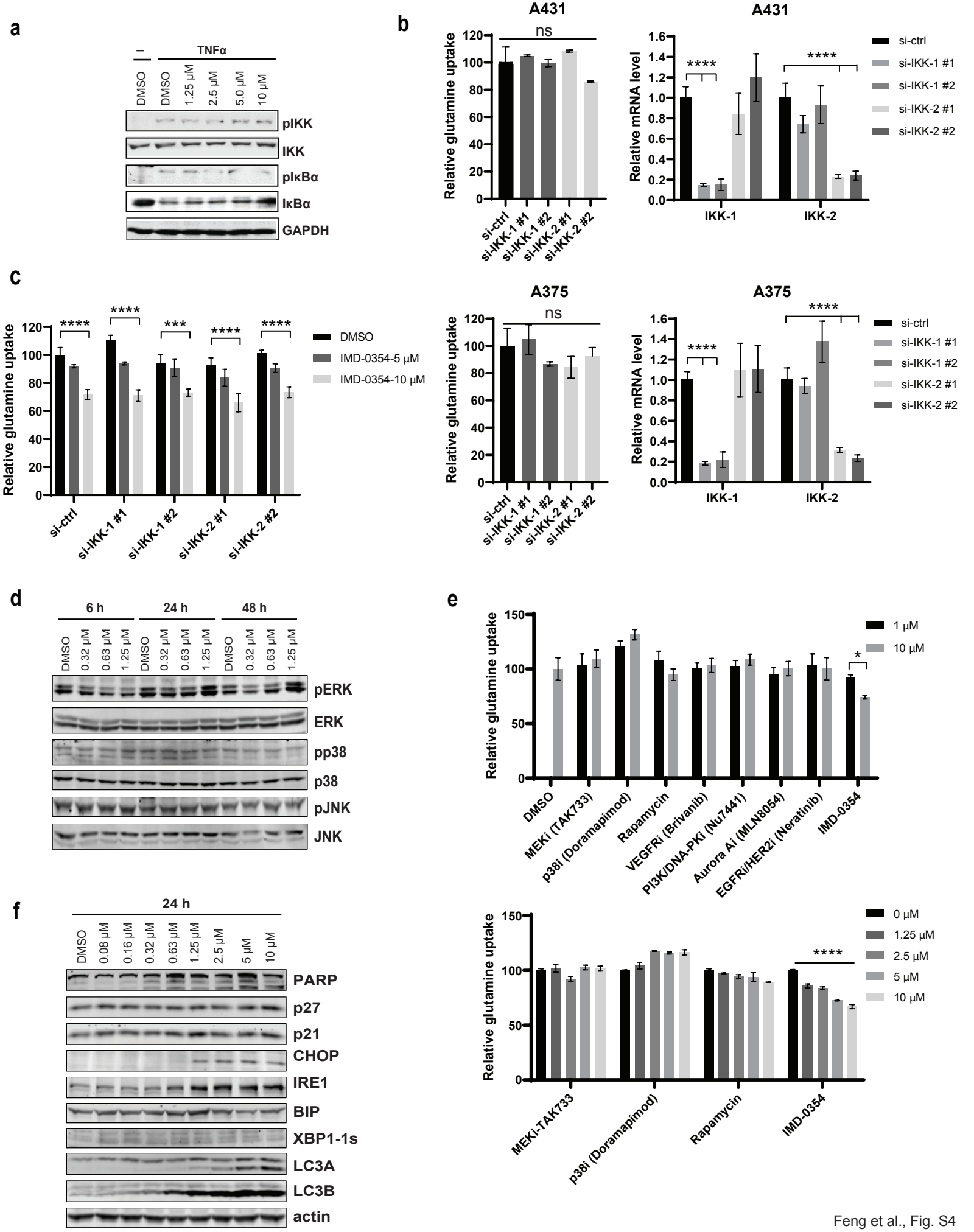
(b) A375 and UACC903 melanoma cells were treated with the indicated concentrations of IMD-0354 for 1 min or 3 min followed by 3H-glutamine uptake assay.

(c) RNA was extracted from A375 cells and subjected to qPCR analysis for expression of the indicated glutamine transporters.

(d) A375 cells were treated with indicated concentrations of IMD-0354 for 24h followed by qPCR analysis for expression of different glutamine transporters.

(e) A375 cells were transfected with si-ctrl or si-SLC1A5 for 24h. RNA was extracted and subjected to qPCR analysis for expression of different glutamine transporters.

Statistical analysis was performed by one-way ANOVA for the comparison of more than two groups. Data are shown as the mean ± SD, n = 3. ****$P \leq 0.0001$.



Feng et al., Fig. S4

Legend Figure S4.
IMD-0354 Suppresses Glutamine Uptake Independent of IKK-NF- B Signaling Suppression

(a) A431 cells were treated with the indicated concentrations of IMD-0354 for 1h and stimulated with 10 ng/ml of TNFα for 5 min. Cells were collected the levels of the indicated proteins were analyzed by Western Blotting.

(b) A431 (upper panel) and A375 (lower panel) cells were transfected with si-IKKα and si-IKKβ for 48h followed by 3H-glutamine uptake assay (left). Corresponding knockdown efficiency (right).

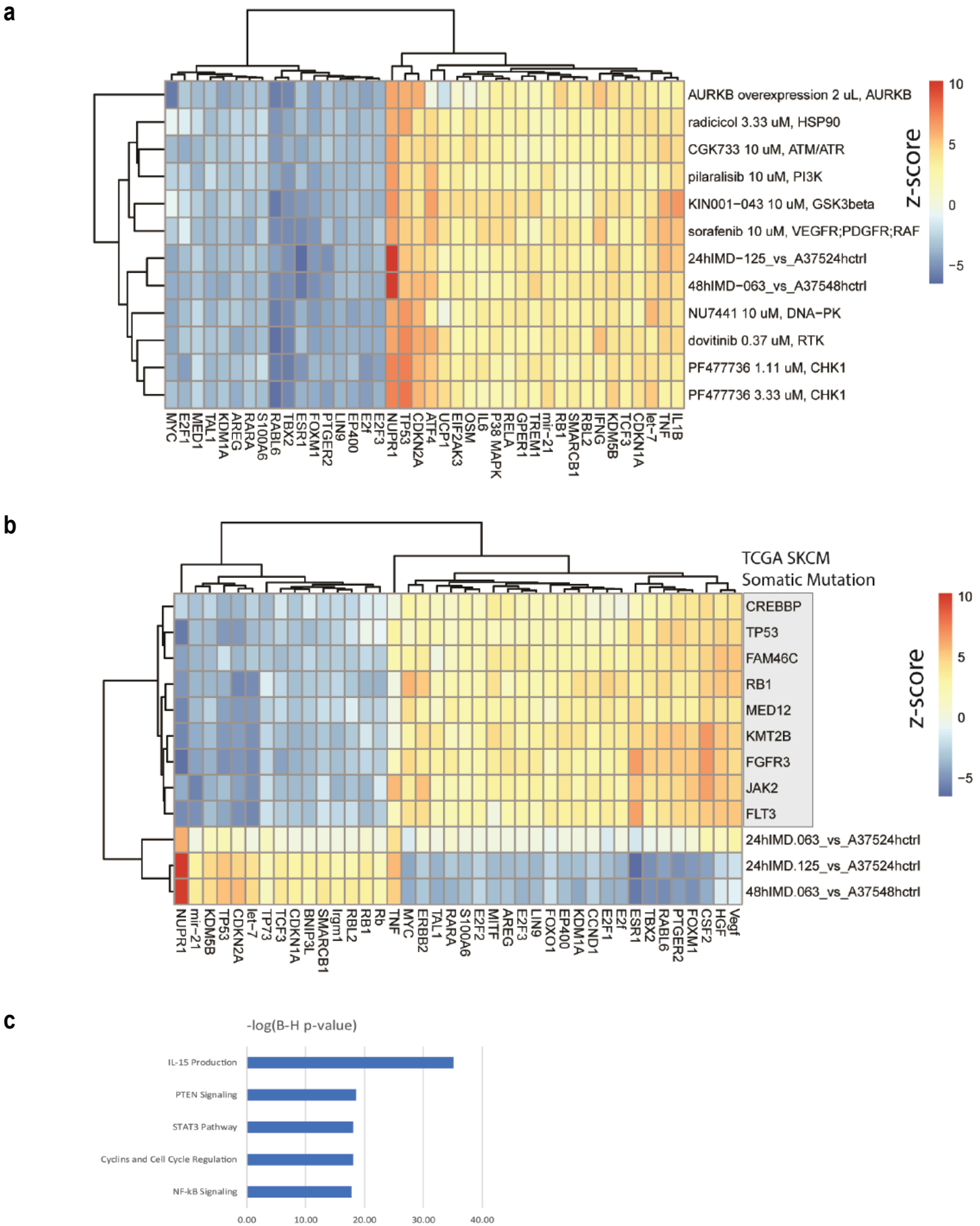
(c) A375 cells were transfected with si-IKKα and si-IKKβ for 48h followed by 3H-glutamine uptake assay in the presence of 5 μM or 10 μM of IMD-0354.

(d) Western blot analyses of indicated proteins and their phosphorylated forms prepared from melanoma cells at the indicated time points following treatment of IMD-0354.

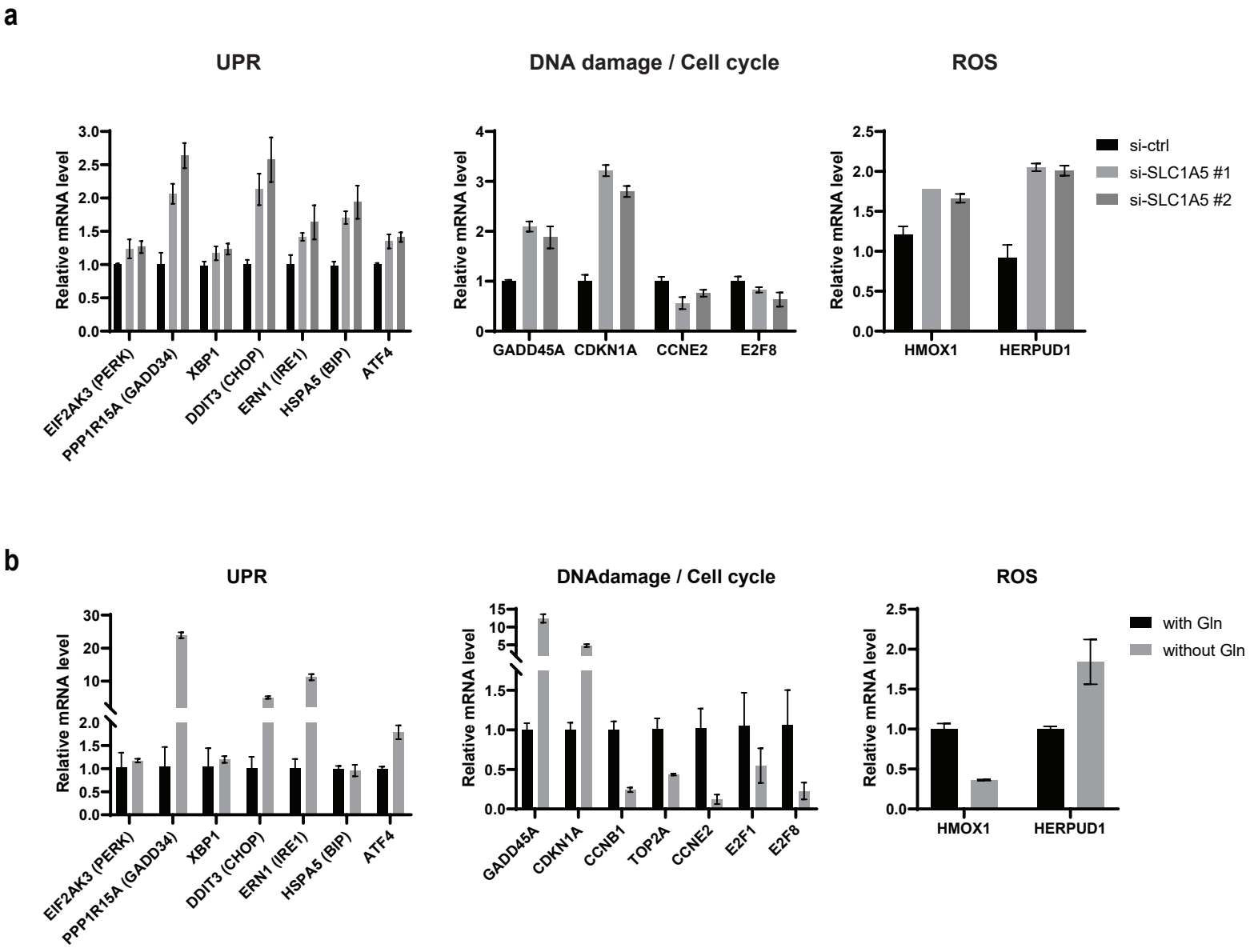
(e) A431 cells were treated with the indicated concentrations of different small molecule inhibitors for 1h followed by 3H-glutamine uptake assay.

(f) western blot analysis of indicated cell cycle, autophagy and UPR proteins prepared 24 h following treatment with the indicated IMD-0354 concentrations.

Statistical analysis was performed by one-way ANOVA for the comparison of more than two groups. Unpaired t-test was used for the comparison of two groups. Data are shown as the mean ± SD, n = 3. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.



Legend Figure S5.
Profiles of IMD-0354-Treated A375 Cells Matched with Small-Molecule Inhibitors and Melanoma Subtypes.
(a) Heatmap showing small molecule treatment with similar upstream regulators to IMD-0354.
(b) Heatmap showing melanoma subtypes by somatic mutation profiles with similar upstream regulators to IMD-0354.
(c) Significant canonical pathways for the small molecule targets and melanoma somatic mutations showing similar molecular profiles to IMD-0354.

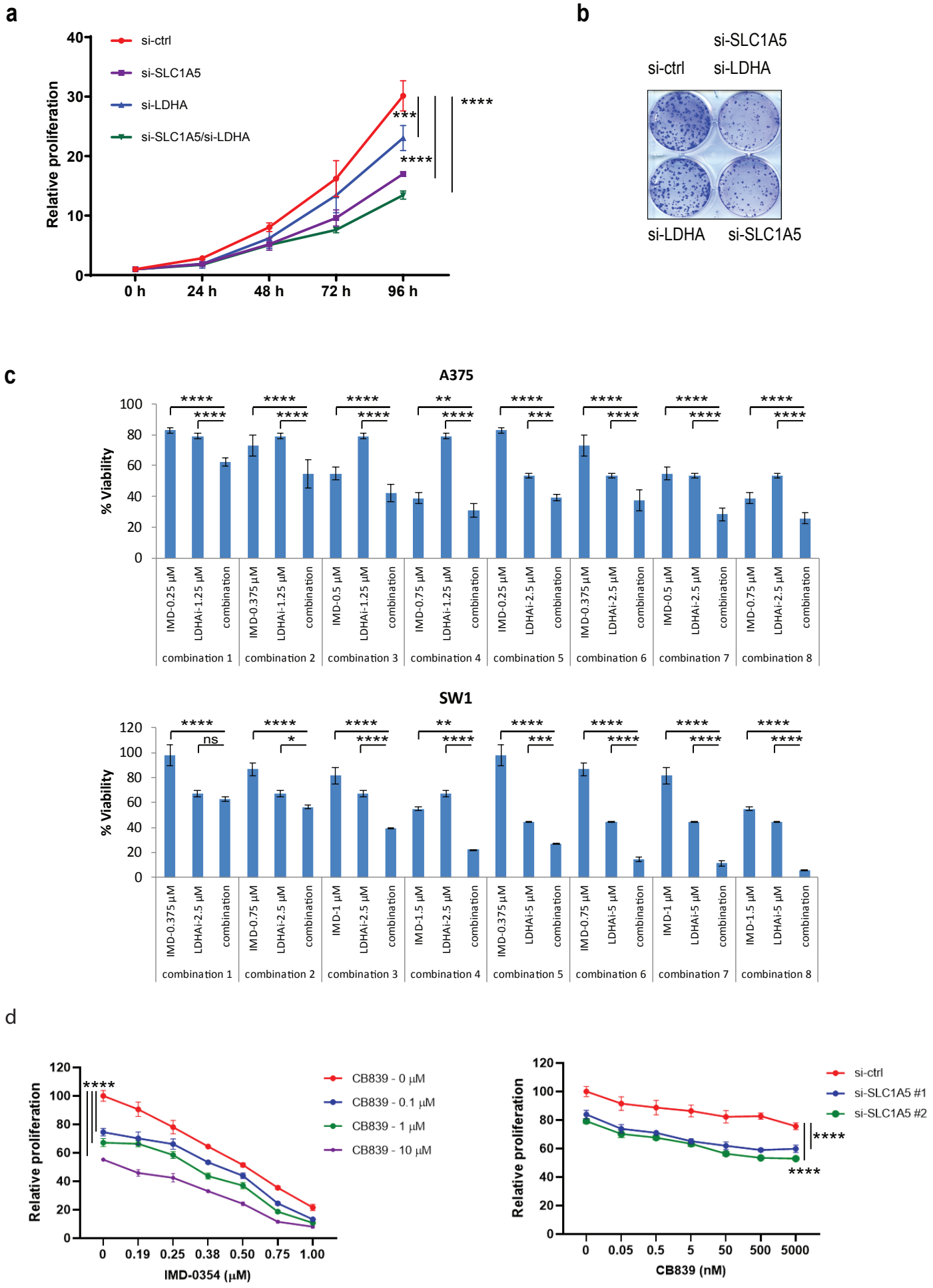


Legend Figure S6. Expression of Glutamine Deprivation-Related Genes.

(a) A375 cells were transfected with si-SLC1A5 for 48h. RNA was extracted and subjected to qPCR analysis for UPR, cell cycle/DNA damage and ROS-related genes.

(b) A375 cells were cultured in media with or without glutamine for 24h. RNA was extracted and subjected to qPCR analysis for UPR, cell cycle/DNA damage and ROS related genes.

Statistical analysis was performed by one-way ANOVA for the comparison of more than two groups.
Data are shown as the mean ± SD, n = 3. ns, not significant, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.



Feng et al., Fig. S7

Legend Figure S7.
Inhibition of Melanoma Growth with IMD-0354 in combination with LDHA or GLS1 Inhibitors.

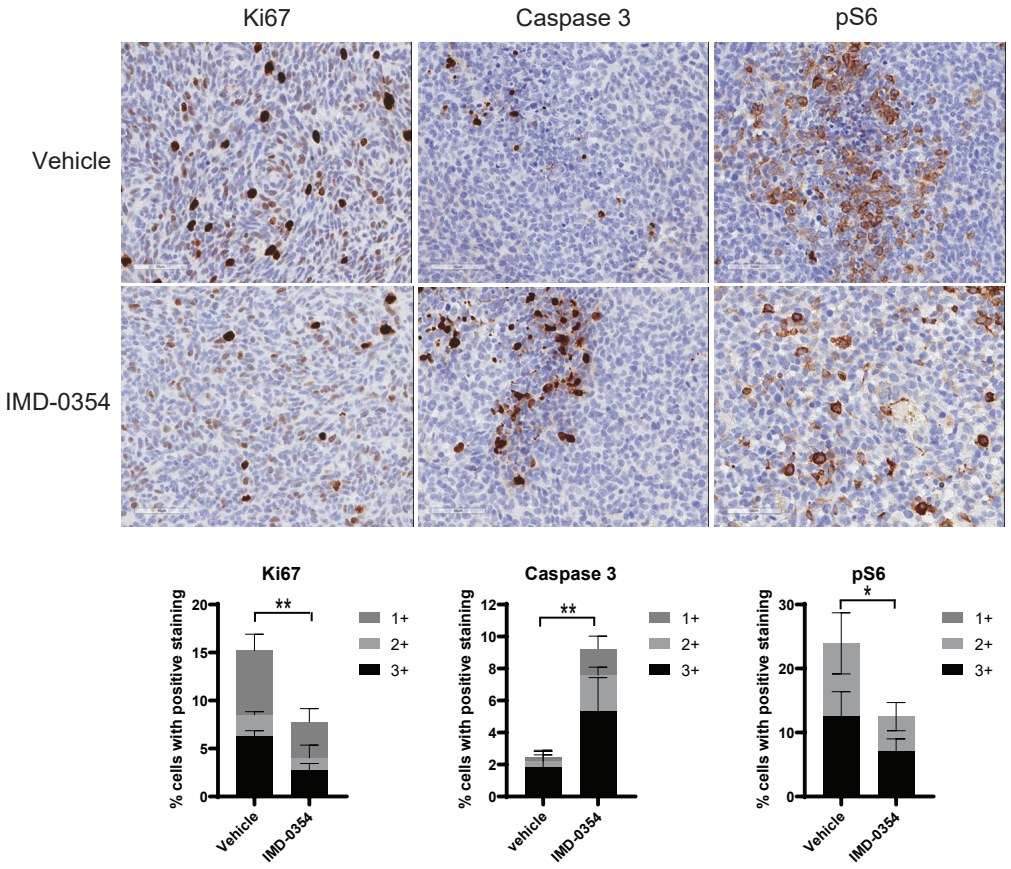
(a) A375 cells were transfected with si-ctrl, si-SLC1A5, si-LDHA, or a combination of si-SLC1A5 and si-LDHA. Cell proliferation was assessed at the indicated time points using ATPlite.

(b) Colony formation assay of A375 cells transfected with si-ctrl, si-SLC1A5, si-LDHA, or a combination of si-SLC1A5 and si-LDHA.

(c) Viability of A375 cells (upper) and SW1 cells (lower) were seeded in 96-well plates and treated with indicated concentrations of IMD-0354, LDHAi (GNE-140), or a combination of the two. Cell viability was measured by ATPlite after 72h.

(d) Left, A375 cells were treated with indicated concentrations of IMD-0354 and GLS1 inhibitor CB-839, or a combination of IMD-0354 and CB-839. Cell proliferation was assessed 72 h later by ATPlite. Right panel depicts A375 cells that were transfected with si-ctrl or si-SLC1A5 for 24h followed by treatment with indicated concentration of CB-839. Cell proliferation was assessed 72 h later by ATPlite.

Statistical analysis was performed by two-way ANOVA for viability and proliferation assay and by one-way ANOVA for the comparison of more than two groups. Data are shown as the mean ± SD, n = 3. ns, not significant, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.



Legend Figure S8. Immunohistochemistry Staining of Xenograft Tumor Samples

IHC staining of ki67, caspase 3 and pS6 in tumors obtained from control of IMD-0354-treated mice (corresponding to data shown in Fig 8d). Scale bars = 50 µm. Bottom panel depicts, quantification of ki67, caspase 3 and pS6 staining. 3+, strong staining; 2+, medium staining; 1+, weak staining.