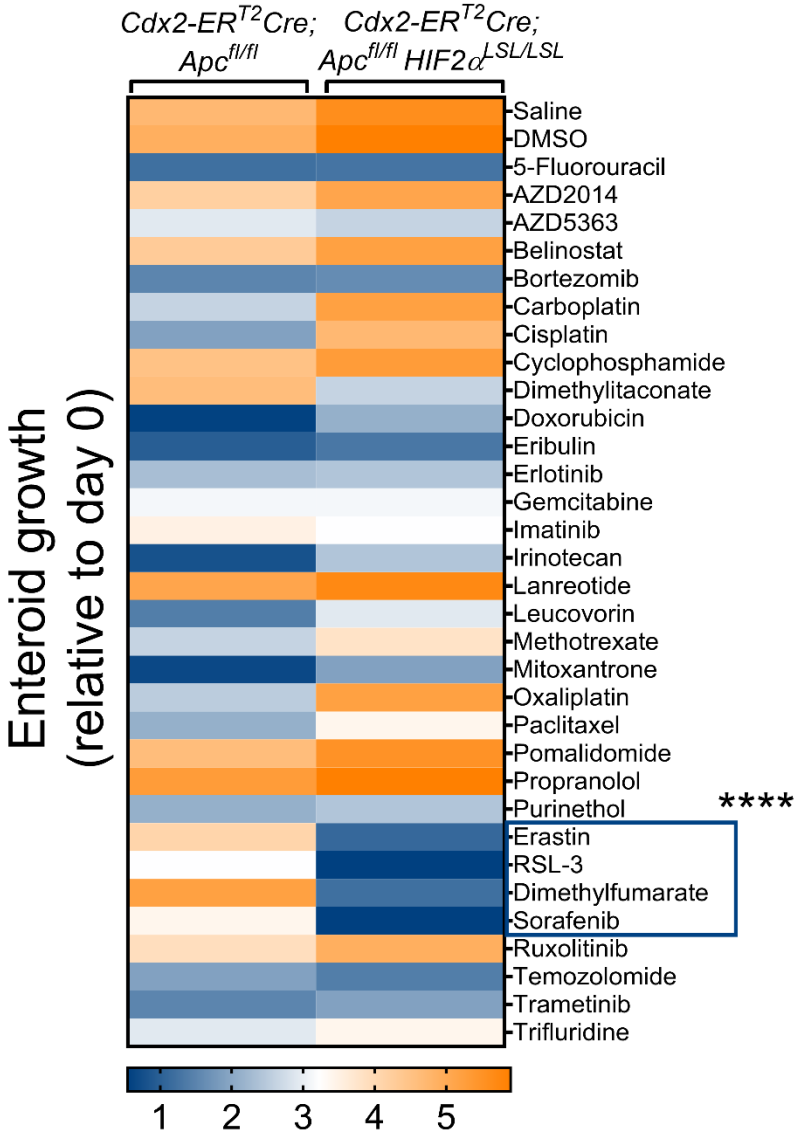
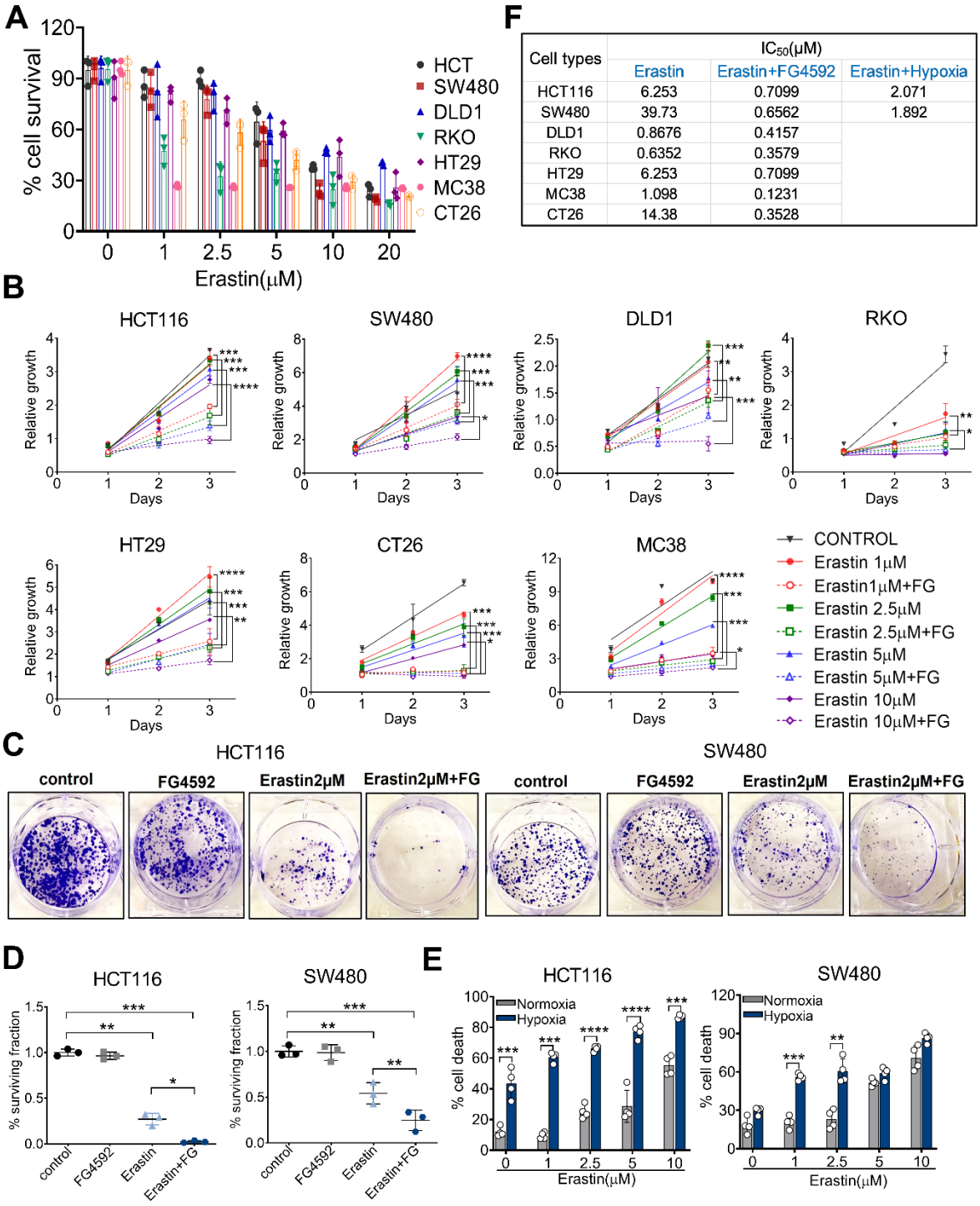
# Supplemental Figure 1




**Supplemental Figure 1. Screening of target small molecules/chemotherapeutic drugs against HIF2α overexpressing tumor enteroids.** Enteroids isolated from colon of an *Apc*$^{fl/fl}$ and *Apc*$^{fl/fl}$; *Hif2α*$^{LSL/LSL}$ mice were grown for 5 days with the screening compounds. Growth was assessed using live cell imaging. Heat map showing enteroid growth in the presence of the indicated compounds. Erastin, RSL3, dimethyl fumarate and sorafenib significantly reduced the growth of hypoxic tumors. **** P<0.0001 using unpaired t-test for the difference between Cdx2-ER$^{T2}$Cre; *Apc*$^{fl/fl}$ compared with Cdx2-ER$^{T2}$Cre; *Apc*$^{fl/fl}$*HIF2α*$^{LSL/LSL}$ mice.

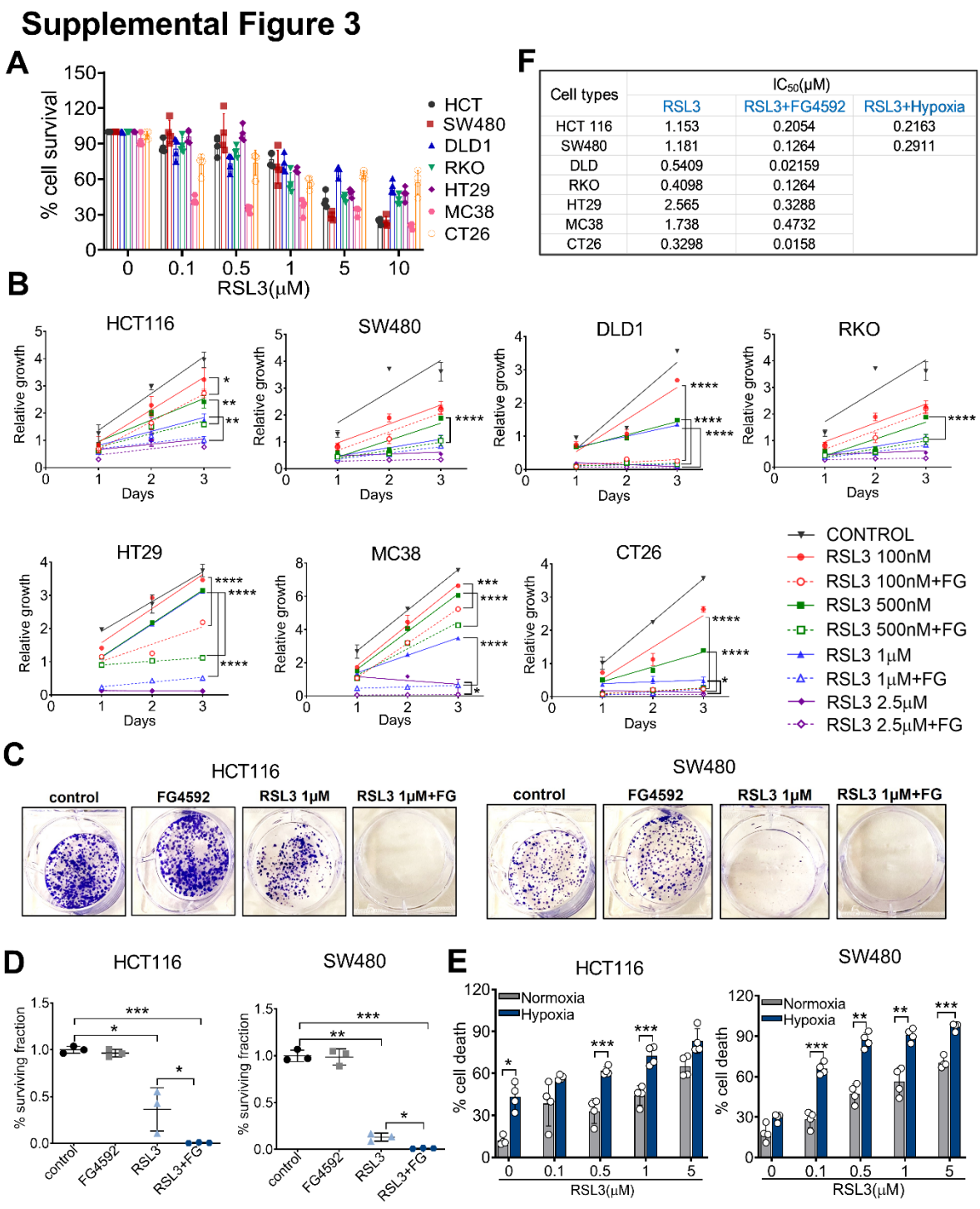
## Supplemental Figure 2




| Cell types | $IC_{50}$(μM) | | |
|---|---|---|---|
| | Erastin | Erastin+FG4592 | Erastin+Hypoxia |
| HCT116 | 6.253 | 0.7099 | 2.071 |
| SW480 | 39.73 | 0.6562 | 1.892 |
| DLD1 | 0.8676 | 0.4157 | |
| RKO | 0.6352 | 0.3579 | |
| HT29 | 6.253 | 0.7099 | |
| MC38 | 1.098 | 0.1231 | |
| CT26 | 14.38 | 0.3528 | |

**Supplemental Figure 2. HIF-activation contributes to erastin induced cell death.** HCT116, SW480, DLD1, RKO, HT29, MC38 and CT26 cells were treated with 0,1, 2.5, 5 and 10μM **(A)** erastin for 72 hours or co-treated with **(B)** FG4592 (100μM) and varying concentrations of erastin for 3 days. Cell viabilities were assessed by the MTT assay after 72 hours or after every

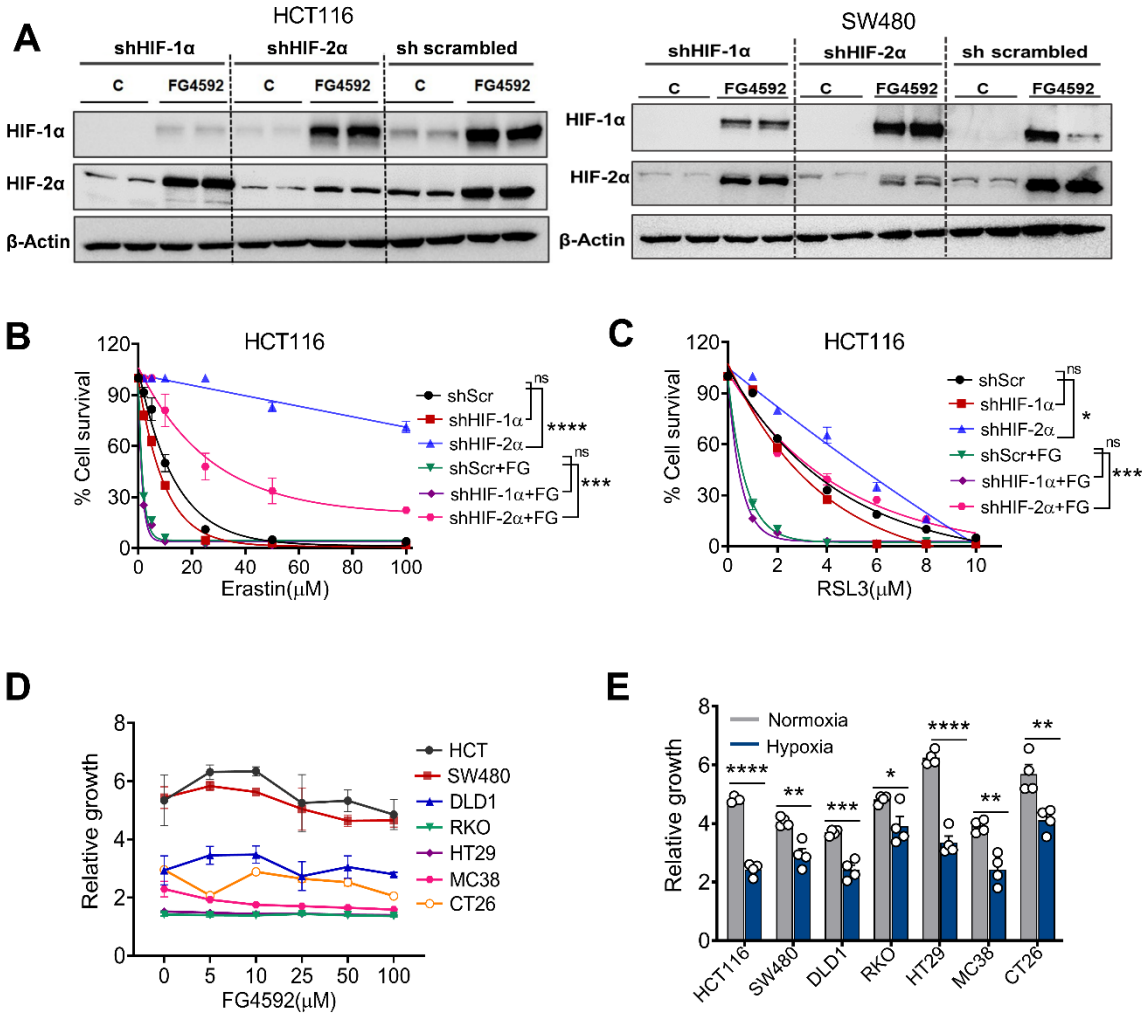
24 hours in case of FG4592 and erastin treated CRC cells. Quantitative data are presented as means ± SD from three independent experiments. Statistical significance was calculated using One-way ANOVA followed by Dunnett's multiple comparison. *P<0.05, **P<0.01, ***P<0.001, ****p<0.0001. **(C)** representative images of clonogenic assay in HCT116 and SW480 cells treated with erastin (2μM), FG4592 (100μM) or co-treated with erastin and FG4592 (**D)** quantification by blinded observers in HCT116 and SW480 cells. Quantitative data are presented as means ± SD from three independent experiments. Statistical significance was calculated using One-way ANOVA followed by Tukey's multiple comparison. *P<0.05, **P<0.01, ***P<0.001. **(E)** LDH-cell death assay in HCT116 and SW480 cells treated with erastin (1-10 μM) and cultured in normoxic and hypoxic conditions. Quantitative data are presented as means ± SD from three independent experiments. Statistical significance was calculated using unpaired t-test. **P<0.01, ***P<0.001, ****p<0.0001. **(F)** $IC_{50}$ values calculated in CRC cell lines following treatment with erastin (1-10 μM) alone or in combination with FG4592 or hypoxia.




**Supplemental Figure 3. FG4592 and hypoxia potentiates RSL3 induced ferroptosis.** HCT116, SW480, DLD1, RKO, HT29, MC38 and CT26 cells were treated with 0, 0.1, 0.5, 1, 5 and 10 μM **(A)** RSL3 for 72 hours or co-treated with **(B)** FG4592 (100 μM) with varying concentrations of RSL3 for 3 days. Cell viabilities were assessed by the MTT assay after 72

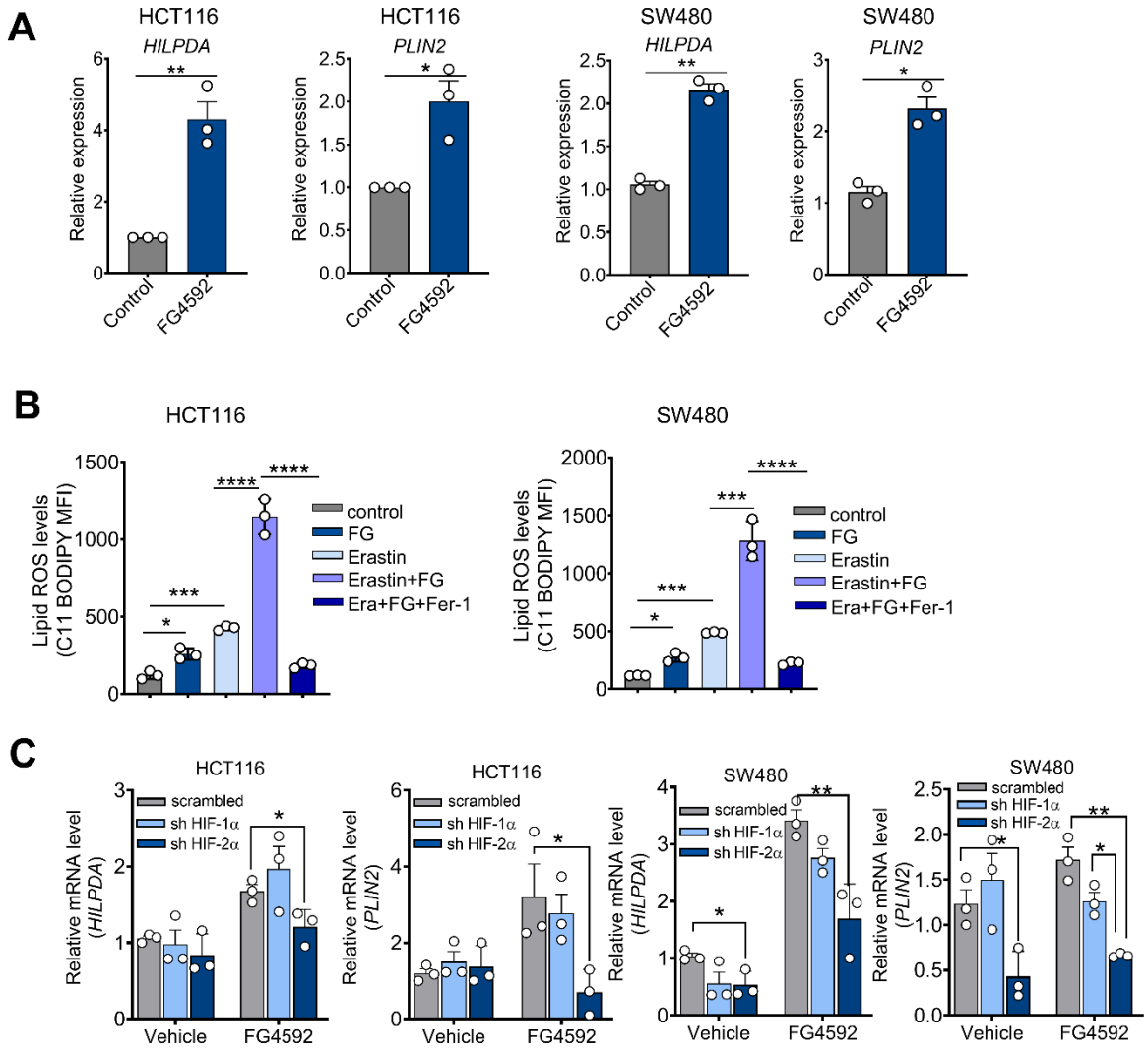
hours or after every 24-hour interval in case of FG4592 and RSL3 treated CRC cells. Quantitative data are presented as means ± SD from three independent experiments. Statistical significance was calculated using one-way ANOVA with Tukey's multiple comparison, *P<0.05, **P<0.01, ***P<0.001, ****p<0.0001. **(C)** representative images of clonogenic assay in HCT116 and SW480 cells treated with RSL3 (1 μM), FG4592 (100 μM) or co-treated with RSL3 and FG4592 (**D)** quantification of surviving colonies by blinded observers in HCT116 and SW480 cells. Quantitative data are presented as means ± SD from three independent experiments. Statistical significance was calculated using One-way ANOVA followed by Tukey's multiple comparison. *P<0.05, **P<0.01, ***P<0.001. **(E)** LDH-cell death assay in HCT116 and SW480 cells treated with RSL3 (0.1-5 μM) cultured in normoxic and hypoxic conditions. Quantitative data are presented as means ± SD from three independent experiments. Statistical significance was calculated using unpaired t-test. **P<0.01, ***P<0.001, ****p<0.0001. **(F)** $IC_{50}$ values calculated in different CRC cells after treatment with RSL3 (0.1-2.5 μM) alone or in combination with FG4592 or hypoxia.

## Supplemental Figure 4



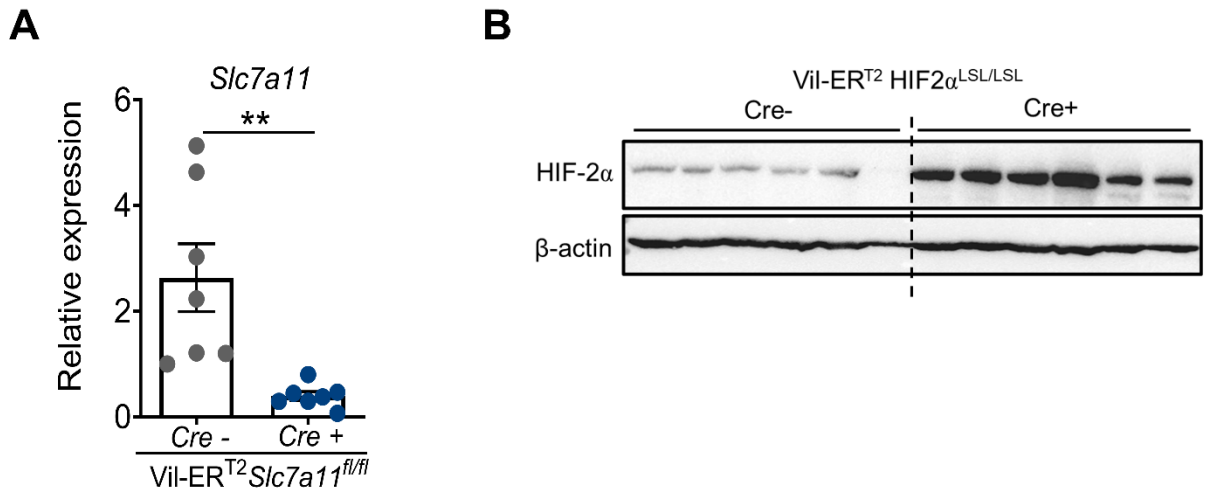

**Supplemental Figure 4. Hypoxia inhibits CRC cell growth. (A)** Protein expression in shRNA mediated HIF knockdown cells. shHIF-1α, shHIF-2α and non-target shscrambled HCT116 and SW480 cells were treated with FG4592(100 μM) for HIF activation. HIF-1α and HIF-2α expression were measured using Western blotting. Data are representative of three independent experiment. **(B)** shHIF-1α, shHIF-2α and non-target shscrambled HCT116 cells were treated with 0, 10, 20, 40, 60, 80 and 100 μM erastin alone or in combination with FG4592. These cells were also treated with **(C)** 0, 1, 2, 4, 6, 8 and 10 μM RSL3 and FG4592 (100μM). Cell survival was assessed by the MTT assay after 72 hours. Quantitative data are presented as means ± SD from three independent experiments. Statistical significance was calculated using two-way ANOVA with Tukey's multiple comparison test, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 Cell growth measured relative to day 0 of different CRC cells treated with various doses (0-100 μM) of **(D)** FG4592 or **(E)** cultured under hypoxic conditions. Statistical significance was calculated using unpaired t test, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

## Supplemental Figure 5



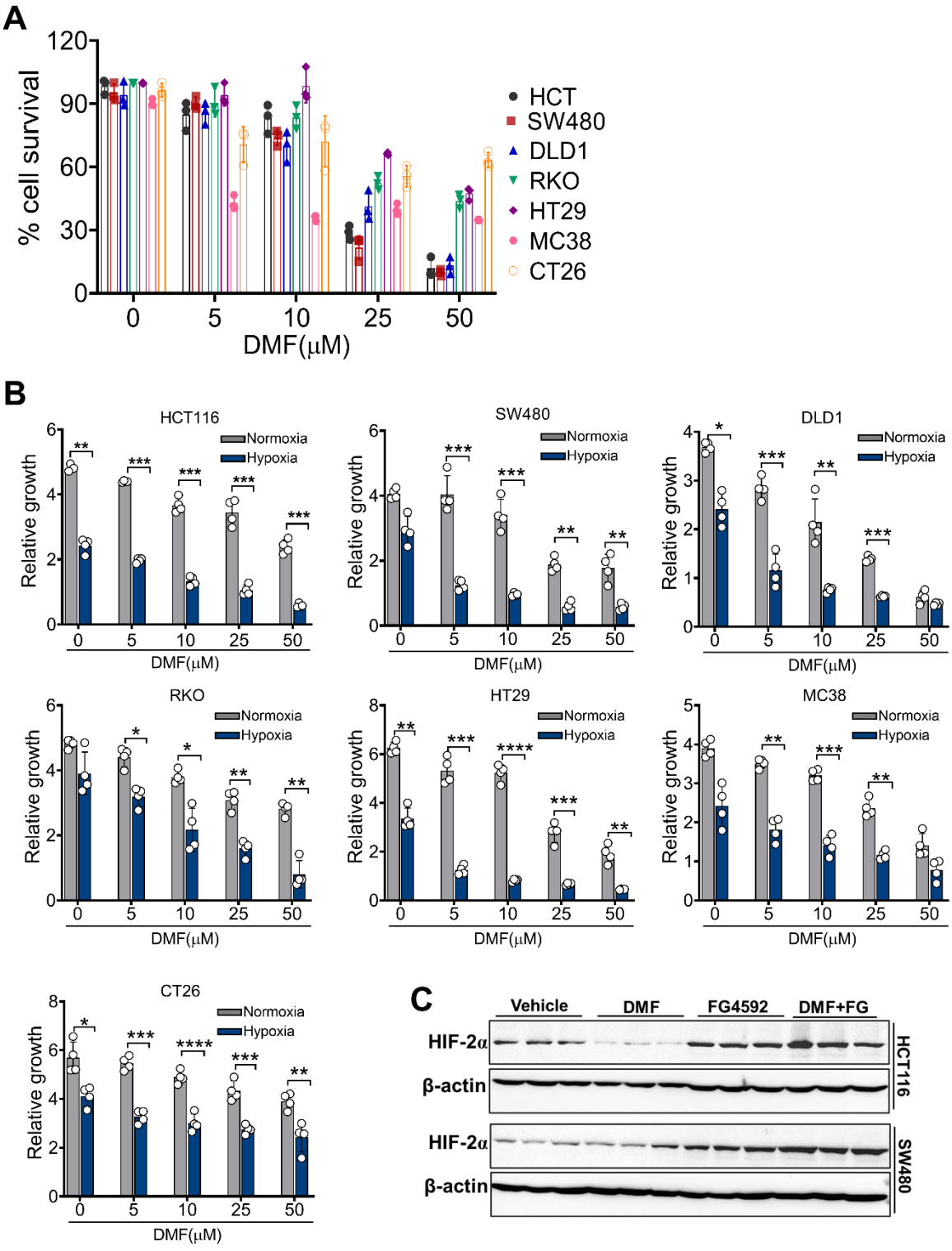

**Supplemental Figure 5. HIF2α mediates sensitivity towards erastin and RSL3 mediated ferroptosis. (A**) The mRNA levels of HILPDA and PLIN2 in HCT116 and SW480 cells treated with 100 μM FG4592 were examined by qRT-PCR. Data are means ± SD from three independent experiments. *P<0.05, **P<0.01 using unpaired t test **(B)** Lipid ROS levels were measured in HCT116 and SW480 cells through ferroptosis-dependent C11-BODIPY oxidation after 12-h incubation with FG4592(100 μM), erastin (5 μM) and ferrostatin-1 (1 μM). Data are plotted as the mean ± SD. *P* values were determined using one-way ANOVA followed by Tukey's multiple comparison test, *$P$ <0.05, **$P$ < 0.01, ***P<0.001, ****P<0.0001. **(C)** qRT-PCR analysis showing the mRNA levels of HILPDA and PLIN2 in shRNA mediated HIF-1α and HIF-2α knock down and non-target scrambled HCT116 and SW480 cells treated with vehicle or 100 μM FG4592. Quantitative data are presented as means ± SD from three independent experiments. Statistical significance was calculated using one-way ANOVA with Tukey's multiple comparison. *P<0.05, **P<0.01.

## Supplemental Figure 6




**Supplemental Figure 6. Validation of intestine specific Slc7a11 floxed and HIF-2α LSL mice. (A)** qPCR analysis confirming mRNA levels of *Slc7a11* gene in in colon tissue of Vil-ER$^{T2}$*Slc7a11*$^{fl/fl}$ cre+ (n=7) and cre-(n=7) mice. **P<0.01 using unpaired t-test for the difference between Vil-ER$^{T2}$*Slc7a11*$^{fl/fl}$ cre+ and cre- **(B)** Western blot confirming the expression of HIF-2α in colon in Vil-ER$^{T2}$ *HIF-2α*$^{LSL/LSL}$ cre+ (n=6) and cre- mice (n=6).

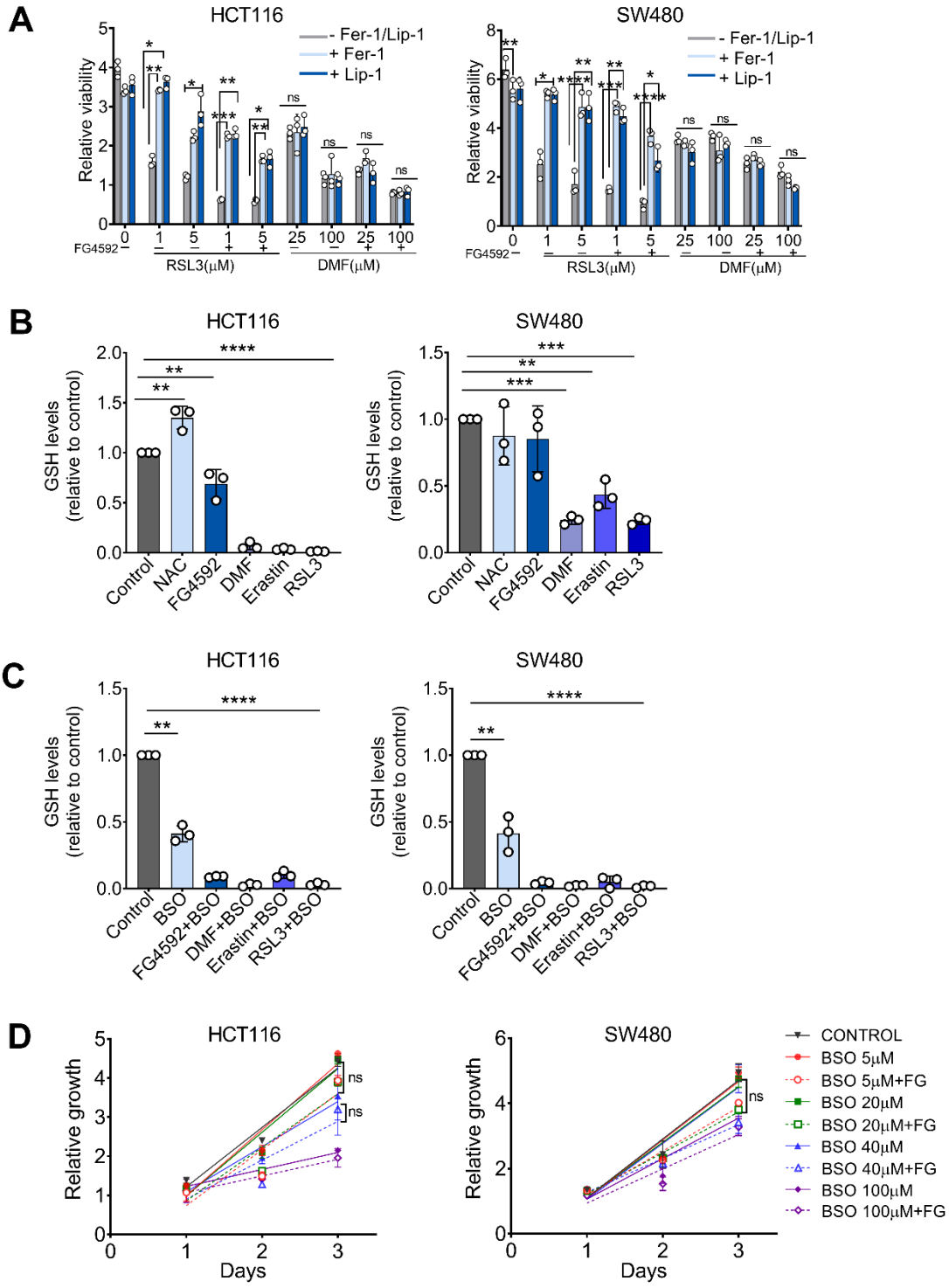
## Supplemental Figure 7




**Supplemental Figure 7. Hypoxia and DMF inhibits CRC cell growth.** Cell growth assay **(A)** following DMF dose response in different CRC cells **(B)** following DMF treatment (5-25 μM) on CRC cells cultured under normoxia and hypoxia. Quantitative data are presented as means ±

SD from three independent experiments. Statistical significance was calculated using unpaired-t test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. **(C)** Immunoblots showing expression of HIF-2α in HCT116 and SW480 cells treated with vehicle, FG4592 (100 μM), DMF (50 μM) and DMF+FG4592.

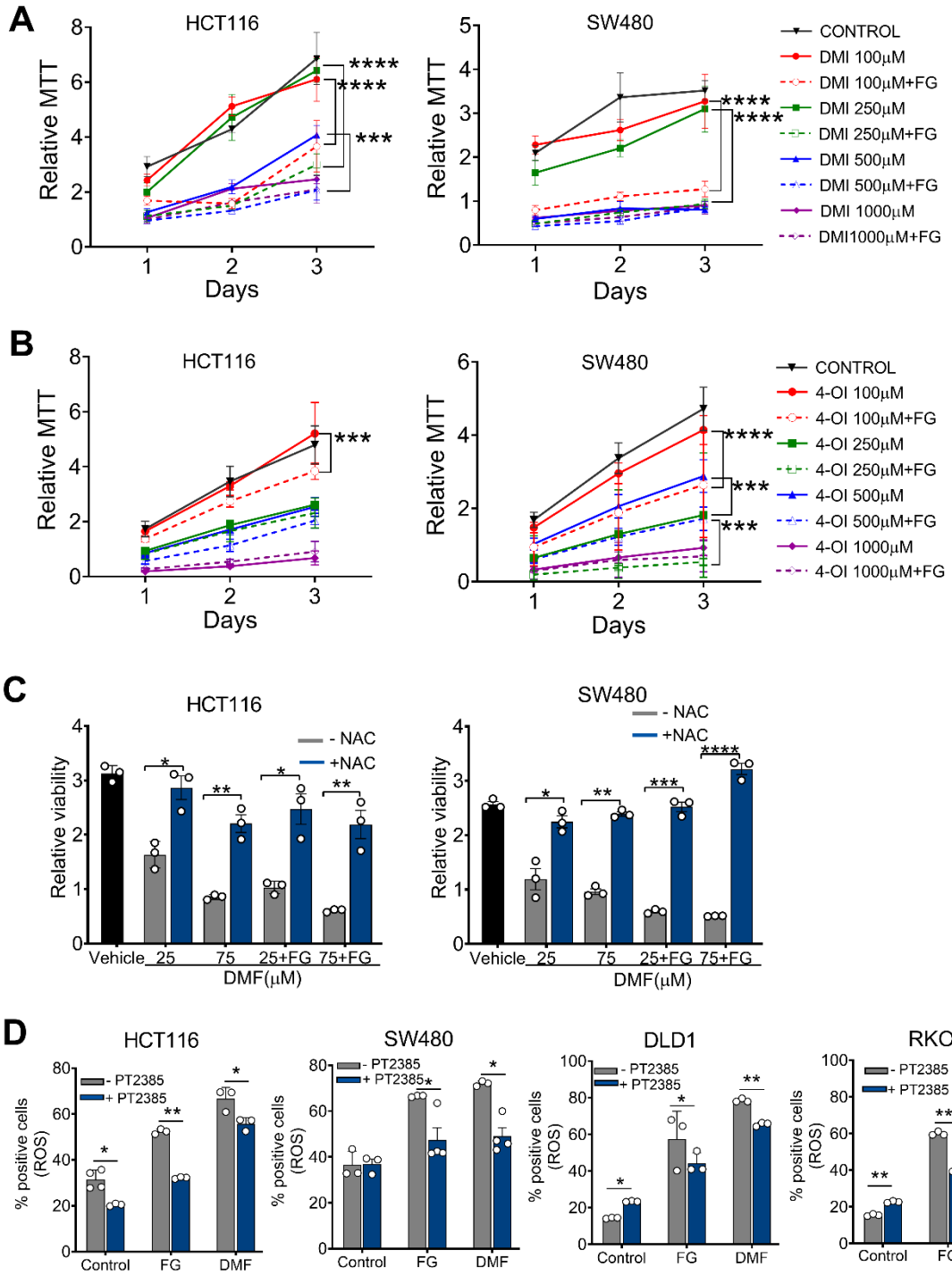
## Supplemental Figure 8




**Supplemental Figure 8. DMF induces cell death by GSH depletion. (A)** HCT116 and SW480 cells were treated with RSL3 (1 and 5 μM) or DMF (25 and 100 μM) alone or in combination with FG4592 (100μM) or without ferroptotic inhibitors- ferrostatin-1 (1μM) and liproxstatin-1 (2

μM) for 24 h and cell viability was assayed using the MTT assay. Data are mean ± SD from three independent experiments. Statistical significance was calculated using two-way ANOVA with Tukey's multiple comparison test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 ns=non-significant. **(B)** Intracellular GSH levels in HCT116 and SW480 cells treated with N-acetyl cysteine (5 mM), FG4592 (100 μM), DMF (50 μM), erastin (5 μM) and RSL3 (2.5μM) either alone or **(C)** in combination with buthionine sulfoximine (BSO) (100 μM) for 12 h. Data are plotted as mean ± SD of 3 independent experiments. Statistical significance was calculated using one-way ANOVA followed by Dunnett's multiple comparison **P <0.01, ***P < 0.001, ****P<0.0001 versus control. **(D)** HCT116 and SW480 were treated with 0, 5, 20, 40 and 100 μM of BSO either alone or in combination with FG4592 (100 μM) for 1-3 days. Cell viability was assessed by the MTT assay after every 24-h interval. Quantitative data are presented as mean ± SD of three independent experiments. Statistical significance was calculated using one-way ANOVA with Tukey's multiple comparison. ns=non-significant.

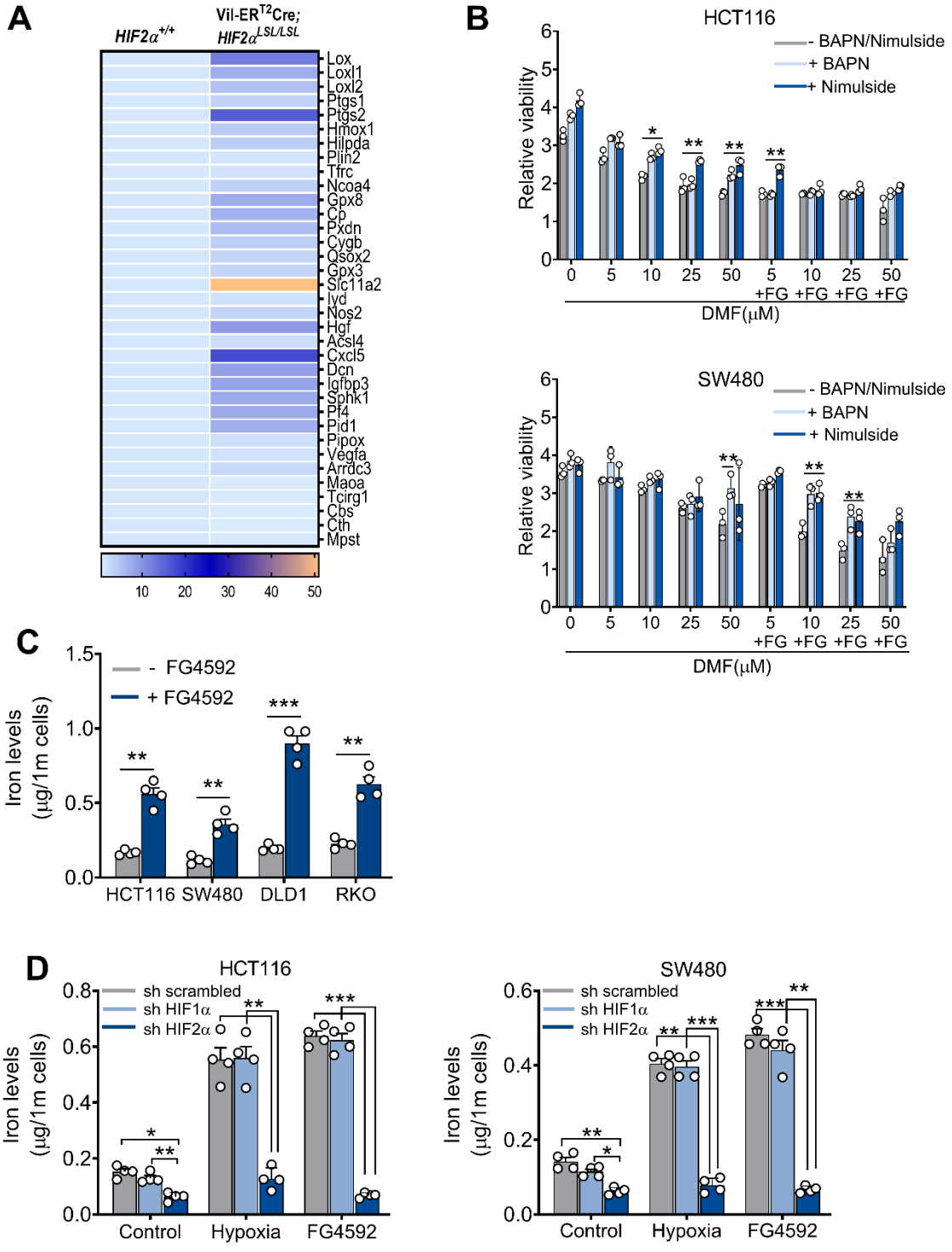
## Supplemental Figure 9




**Supplemental Figure 9. FG4592 potentiates DMI and 4-OI induced cell death.** HCT116 and SW480, cells were treated with **(A)** 100-1000 μM DMI and **(B)** 100-1000 μM 4-OI either alone or with FG4592 (100 μM) for 3 days. Cell viability was assessed by the MTT assay every 24 h.

Quantitative data are presented as mean ± SD of three independent experiments. Statistical significance was calculated using one-way ANOVA with Tukey's multiple comparison. ***P<0.001, ****p<0.0001. **(C)** Cell viability assay in HCT116 and SW480 cells treated with DMF (25 and 75 μM) or co-treated with DMF and FG4592 (100 μM) with or without N-acetyl cysteine (NAC) (5 mM). Data represent mean ± SD of three independent experiments. Statistical significance was calculated using the unpaired-t test. *p<0.05, **P<0.01, ***P <0.001, ****P < 0.0001 **(D)** ROS levels measured through flow cytometry in HCT116, SW480, DLD1 and RKO cells treated with FG4592 (100μM), DMF (50μM) in the absence or presence of PT2385 (10μM). Data represent the mean ± SEM from 3 independent experiments. Statistical significance was calculated using unpaired t-test *P<0.05, **P <0.01, ***P < 0.001.

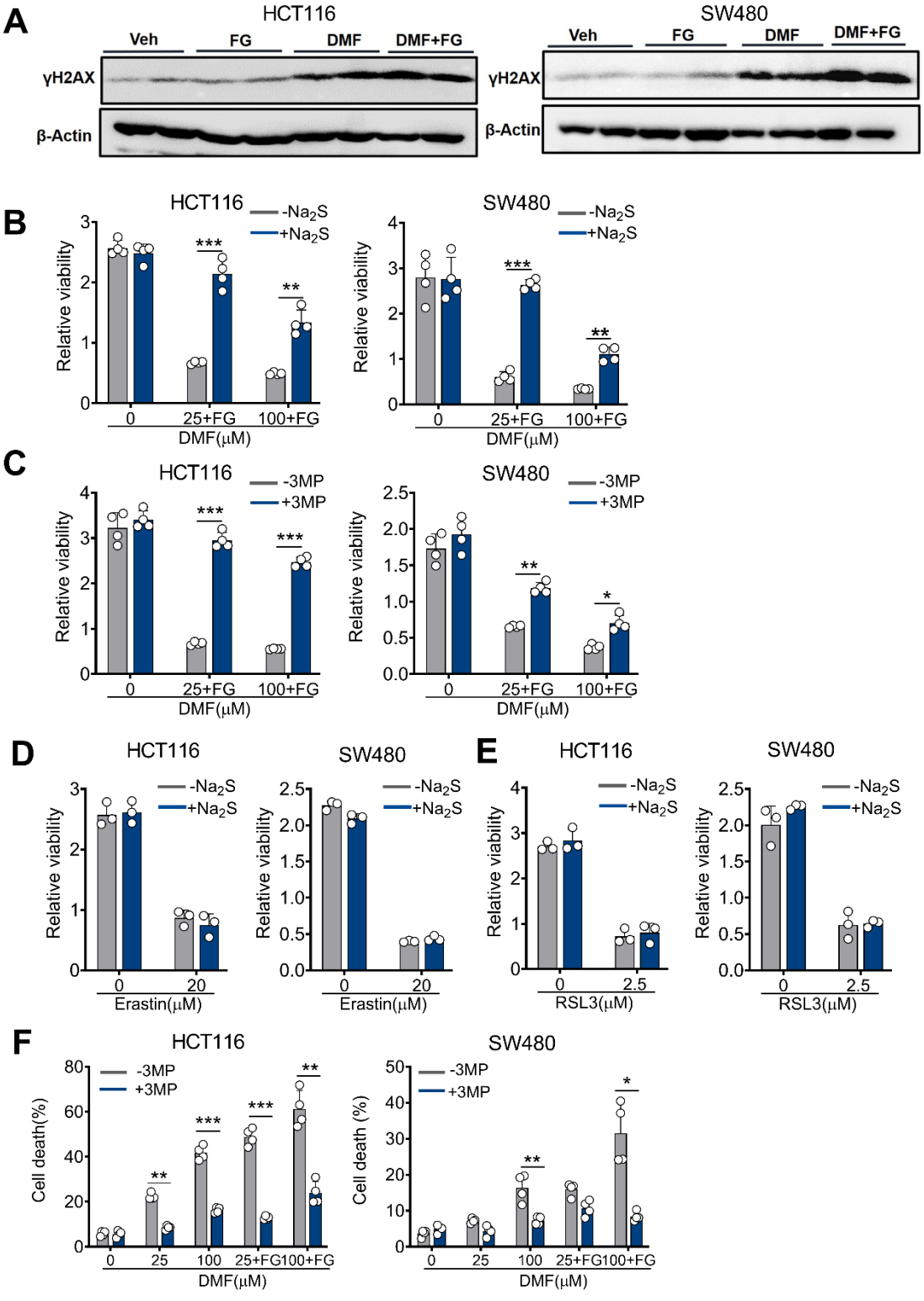
## Supplemental Figure 10




**Supplemental Figure 10. HIF-2α mediates increase in ROS and iron levels in CRC. (A)** Heat map generated from RNA-seq data on scraped colonic mucosal cells isolated from *HIF2*α$^{+/+}$ and Vil-ERT2 *HIF2*α$^{LSL/LSL}$ mice. Three mice were assessed for each group. **(B)**

HCT116 and SW480 cells were treated with DMF (0-25 μM) alone or in combination with FG4592 (100 μM) with BAPN (lox1 inhibitor) or nimulside (Cox inhibitor) and cell viability was assessed by the MTT assay. Data are plotted as the mean ± SD from 3 independent experiments. Statistical significance was calculated using one-way ANOVA with Tukey's multiple comparison, *P<0.05, **P <0.01. Iron levels were measured through ICP-MS in **(C)** HCT116, SW480, DLD1 and RKO cells treated with FG4592 (100 μM). Data represent the mean ± SD from 3 independent experiments. Statistical significance was calculated using unpaired t-test **P <0.01, ***P < 0.001, ****P<0.0001. **(D)** shRNA mediated HIF1α and HIF2α knock down and non-target scrambled HCT116 and SW480 cells cultured under hypoxia or treated with 100 μM FG4592. Statistical significance was calculated using two-way ANOVA with Tukey's multiple comparisons test, *P<0.05, **P <0.01, ***P < 0.001.

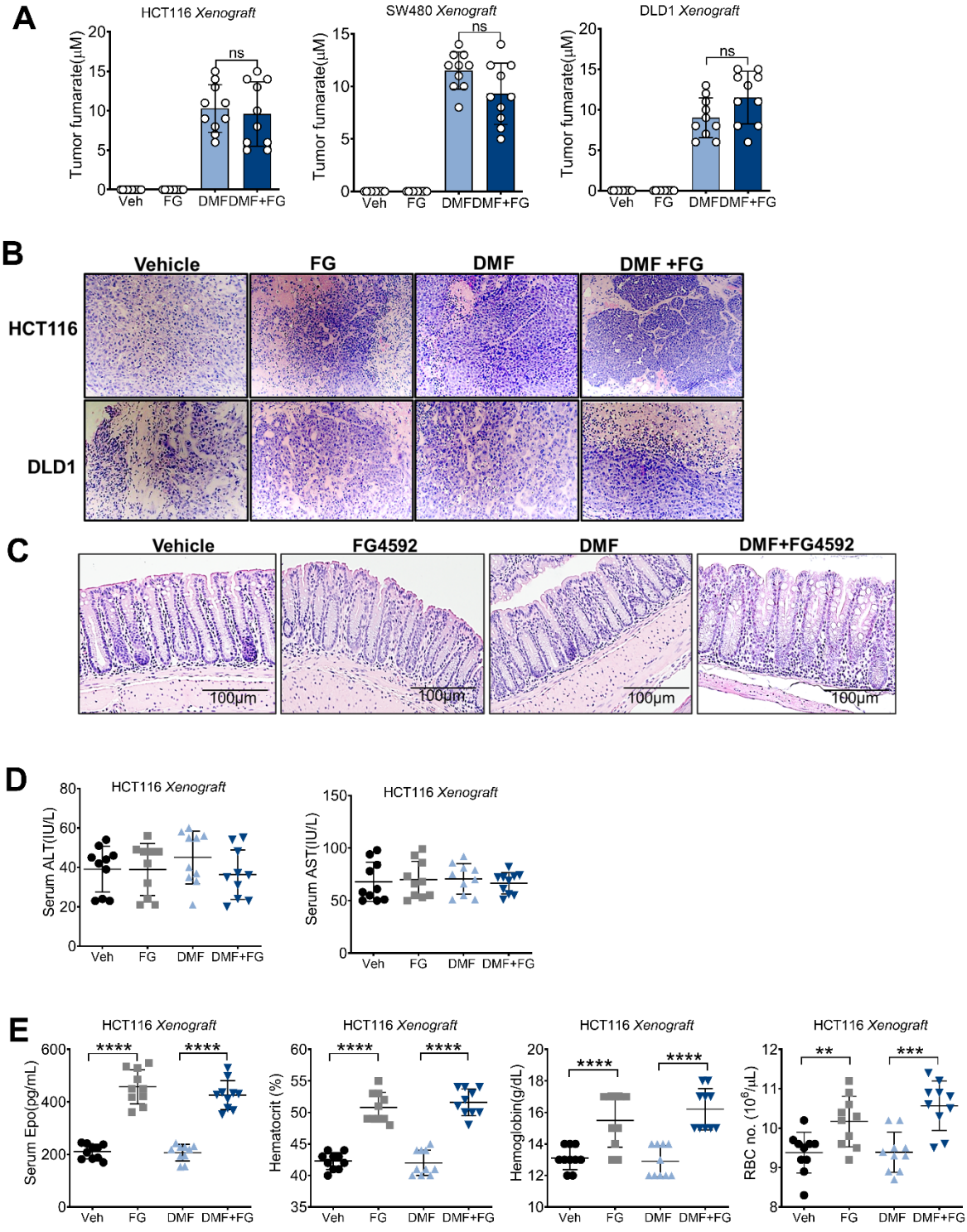
## Supplemental Figure 11




**Supplemental Figure 11. $H_2S$ protects cell death mediated by DMF and FG. (A)** Western blot analysis showing expression of γH2AX in HCT116 and SW480 cells treated with DMF (50

μM) and FG4592 (100 μM). HCT116 and SW480 cells were treated with DMF (25 and 100 μM) or co-treated with DMF and FG4592 (100 μM) with or without **(B)** 3MP (5 mM) and **(C)** $Na_2S$ (300 μM). Cell viability was analyzed after 48 h with the MTT assay. Data are plotted as mean ± SD from 3 independent experiments. Statistical significance was calculated unpaired t-test * P<0.05, **P <0.01, ***P < 0.001. HCT116 and SW480 cells were treated with **(D)** (20 μM) and **(E)** RSL3 (2.5 μM) with or without $Na_2S$**.** Cell viability was analyzed after 48 h with the MTT assay. **(F)** The cell death in HCT116 and SW480 cells treated with DMF (25 and 100μM) or co-treated with DMF and FG4592 (100μM) with or without 3MP (5mM). Data are plotted as mean ± SD from 3 independent experiments. Statistical significance was calculated using unpaired t-test * P<0.05, **P <0.01, ***P < 0.001.

## Supplemental Figure 12




**Supplemental Figure 12. DMF and FG4592 inhibits CRC growth in vivo.** HCT116, SW480 and DLD1 cells were injected subcutaneously into both flanks of C57BL/6 mice (n=10 for each group). After visible formation of tumor of day 10, the mice were subjected to DMF diet

(300mg/kg of chow) and FG4592 (10 mg/kg of mouse weight). **(A)** Fumarate levels in tumor tissue in HCT116, SW480 and DLD1 xenograft mice. Representative Hematoxylin & Eosin staining of the **(B)** tumor tissue in HCT116 and DLD1 xenografts and **(C)** normal colon tissue in HCT116 xenograft in response to different treatments **(D)** Serum AST and ALT levels in HCT116 xenograft **(E)** Serum Erythropoietin, Hematocrit, Hemoglobin and RBC number measured in HCT116 xenograft. Data represent the mean ± SD of 2 independent experiments. **P<0.01, ***P < 0.001, ****P < 0.0001 using unpaired t-test for the differences between two treatments.