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Supplemental information

Feedforward cysteine regulation maintains melanoma differentiation state and limits metastatic spread

Deyang Yu, Jiaxin Liang, Hans R. Widlund, and Pere Puigserver

Supplemental Fig.1

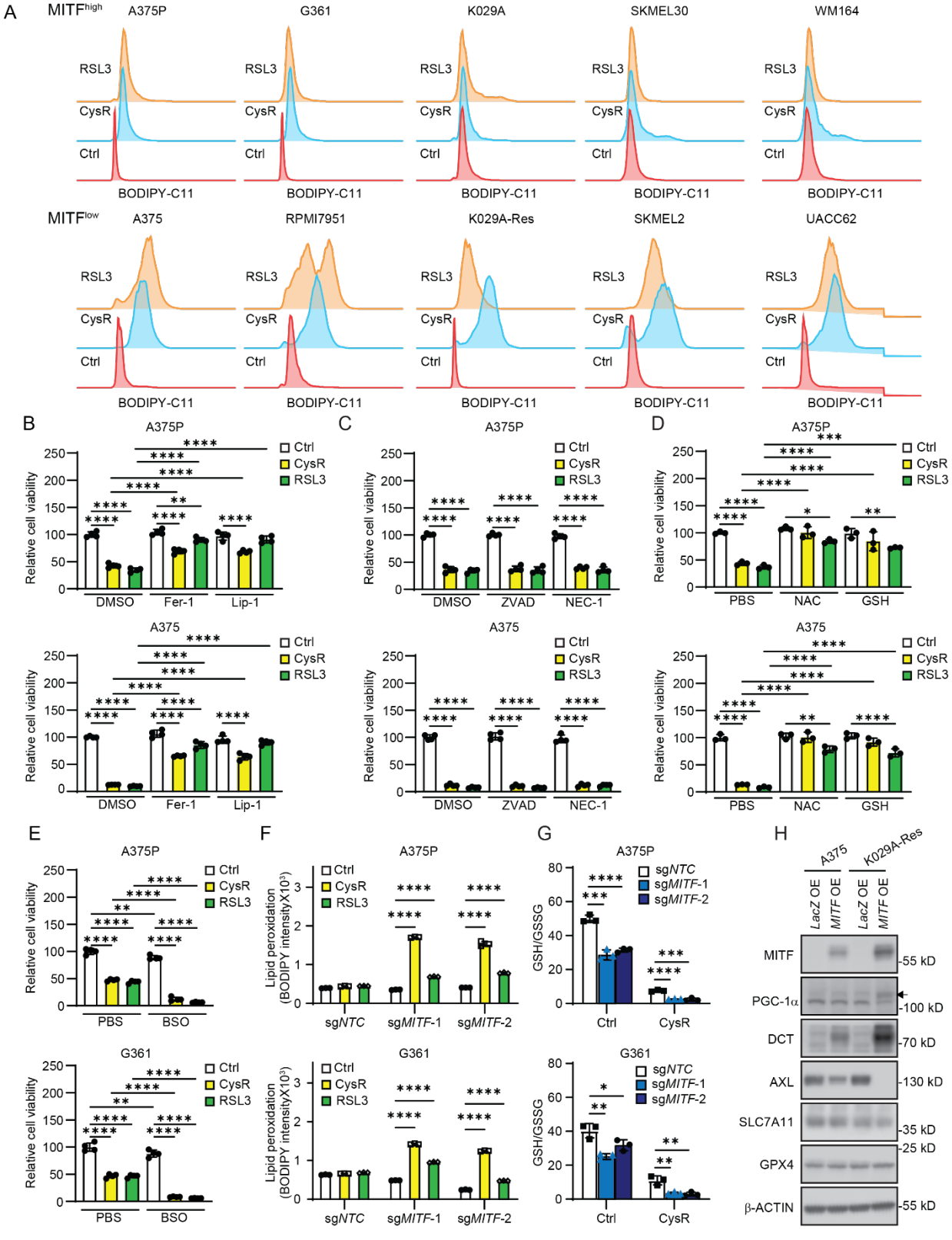

Figure S1: Loss of *MITF* sensitizes to lipid peroxidation in response to ferroptotic stress and impairs glutathione production, related to Figure 1

(A) Histogram of lipid peroxide levels of $MITF^{high}$ (top) and $MITF^{low}$ (bottom) cells in response to 16-hour cysteine restriction (CysR) and 4-hour 0.5 μM RSL3 treatment, assessed by BODIPY staining.

(B) Relative cell viability of A375P (top) and A375 (bottom) cells subjected to cysteine restriction (CysR) and 0.5 μM RSL3 treatment co-treated with 10 μM ferrostatin (Fer1) and 10 μM liproxstatin-1 (Lip-1) for 48 hours.

(C) Relative cell viability of A375P (top) and A375 (bottom) cells subjected to cysteine restriction (CysR) and 0.5 μM RSL3 treatment co-treated with 10 μM Z-VAD-FMK (Z-VAD) and 10 μM necrostatin-1 (NEC-1) for 48 hours.

(D) Relative cell viability of A375P (top) and A375 (bottom) cells subjected to cysteine restriction (CysR) and 0.5 μM RSL3 treatment co-treated with 1 mM N-acetyl cysteine (NAC) and 1 mM glutathione (GSH) for 48 hours. (E) Relative cell viability of A375P (top) and G361 (bottom) cells subjected to cysteine restriction (CysR) and 0.5 μM RSL3 treatment co-treated with 1 μM buthionine-sulfoximine (BSO) for 48 hours.

(F) Quantification of lipid peroxide levels of A375P (top) and G361 (bottom) *MITF* knockout cells in response to 16-hour cysteine restriction (CysR) and 4-hour 0.5 μM RSL3 treatment.

(G) GSH/GSSG ratio in A375P (top) and G361 (bottom) *MITF* knockout cells cultured in control media or cysteine restriction (CysR) media for 16 hours.

(H) Immunoblot analysis of the indicated proteins in A375 and K029A-Res *MITF* overexpressing cells.

Data shown as mean ± SD. Statistical analysis was performed using one-way ANOVA (B, C, D, E, F, and G). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Supplemental Fig.2

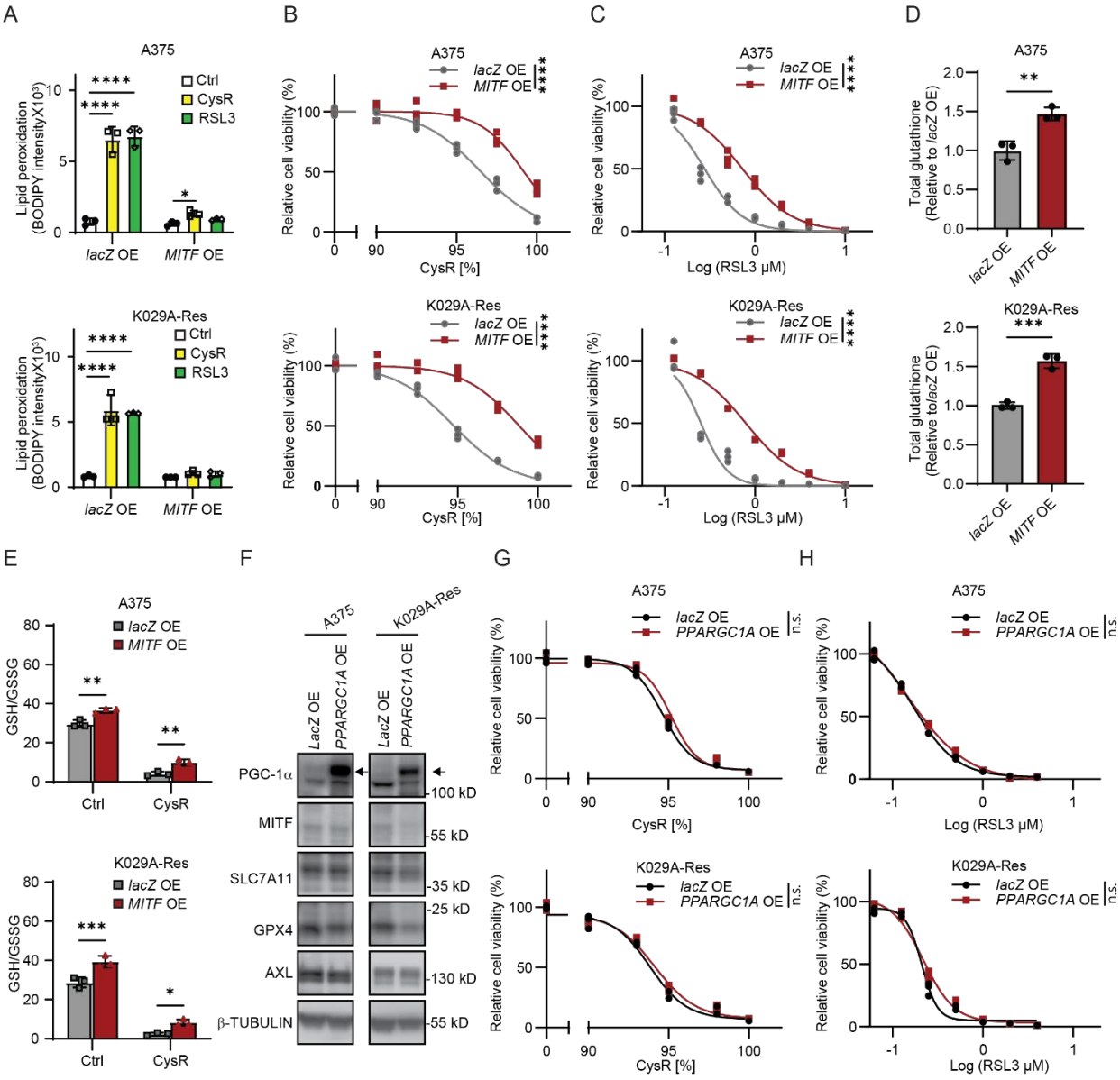

Figure S2: Overexpression of MITF, but not PGC-1α, promotes ferroptosis resistance in MITF^{low} melanoma cells, related to Figure 1

(A) Quantification of lipid peroxide levels in A375 (top) and K029A-Res (bottom) *MITF* overexpressing cells in response to 16-hour cysteine restriction (CysR) and 4-hour 0.5 μM RSL3 treatment.

(B and C) Ferroptosis sensitivity of A375 (top) and K029A-Res (bottom) *MITF* overexpressing cells to cysteine restriction (CysR) (B) and RSL3 (C).

(D) Total glutathione level in A375 (top) and K029A-Res (bottom) *MITF* overexpressing cells.

(E) GSH/GSSG ratio in A375 (top) and K029A-Res (bottom) *MITF* overexpressing cells cultured in control media or cysteine-restricted (CysR) media for 16 hours.
(F) Immunoblot analysis of the indicated proteins in A375 and K029A-Res *PPARGC1A* overexpressing cells.
(G and H) Ferroptosis sensitivity of A375 (top) and K029A-Res (bottom) *PPARGC1A* overexpressing cells to cysteine restriction (CysR) (G) and RSL3 (H).
Data shown as mean ± SD. Statistical analysis was performed using one-way ANOVA (A), two-way ANOVA (B, C, G, and H) and unpaired two-tailed t-test (D and E). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, n.s. not significant.

Supplemental Fig.3

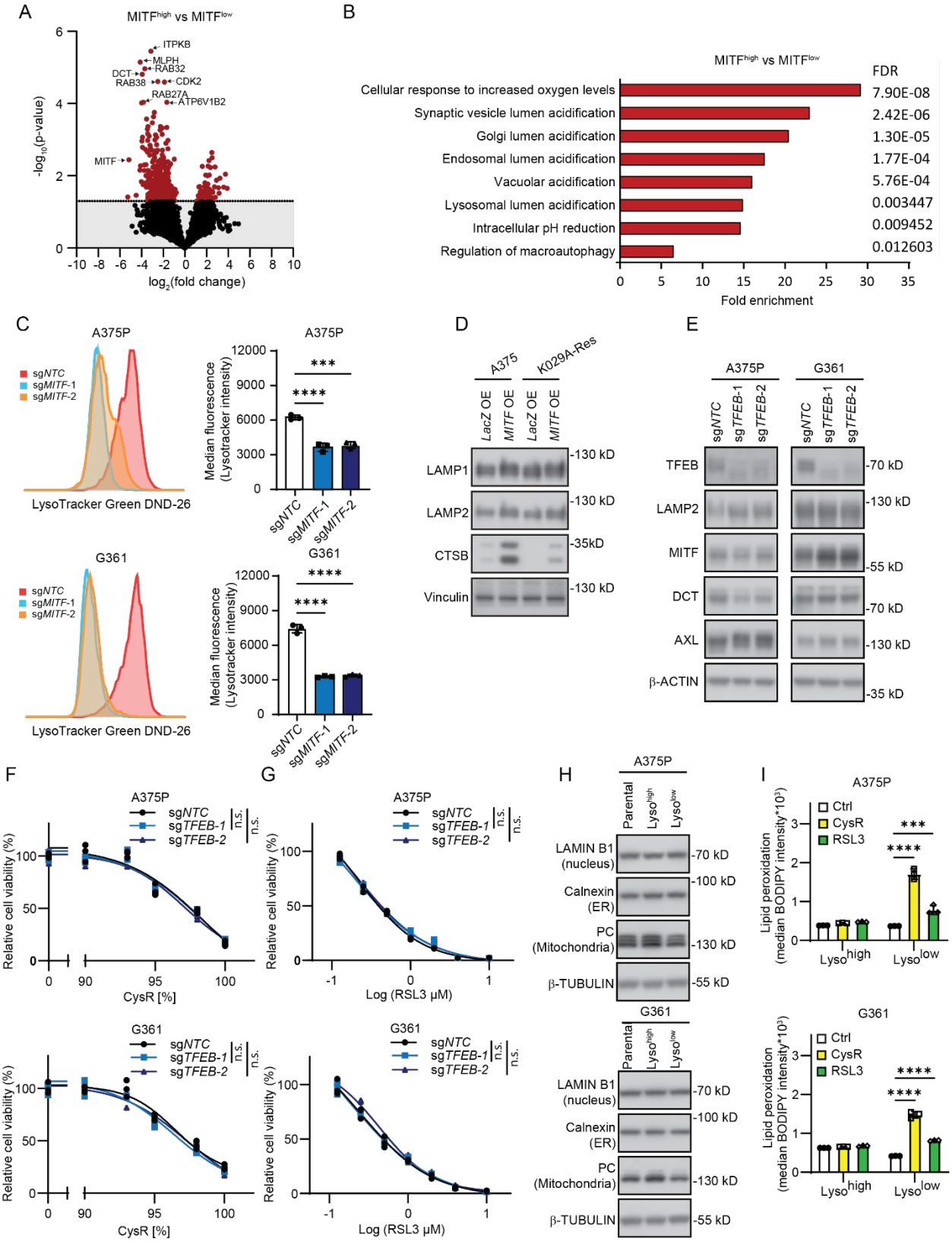

Figure S3: Decreased lysosomal abundance is associated with increased ferroptosis sensitivity, related to Figure 2

(A) Proteomic comparison between $MITF^{high}$ and $MITF^{low}$ melanoma cell lines from the CCLE melanoma dataset.

(B) Gene ontology analysis of differentially expressed genes between $MITF^{high}$ and $MITF^{low}$ melanoma cell lines.

(C) Representative histogram of LysoTracker Green staining and quantification of l LysoTracker intensity of A375P (top) and G361 (bottom) *MITF* knockout cells.

(D) Immunoblot analysis of the indicated proteins in MITF over-expressing A375 and K029A-Res.

(E) Immunoblot analysis of the indicated proteins in A375P and G361 *TFEB* knockout cells.

(F and G) Ferroptosis sensitivity of A375P (top) and G361 (bottom) *TFEB* knockout cells to cysteine restriction (CysR) (F) and RSL3 (G).

(H) Immunoblot analysis of the indicated proteins in A375P (top) and G361 (bottom) $Lyso^{high}$ and $Lyso^{low}$ cells.

(I) Quantification of BODIPY-stained lipid peroxide levels in A375P (top) and G361 (bottom) $Lyso^{high}$ and $Lyso^{low}$ cells cultured in control media or cysteine restriction (CysR) media for 16 hours.

Data shown as mean ± SD. Statistical analysis was performed using one-way ANOVA (C and I) and two-way ANOVA (F and G). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, n.s. not significant.

Supplemental Fig. 4

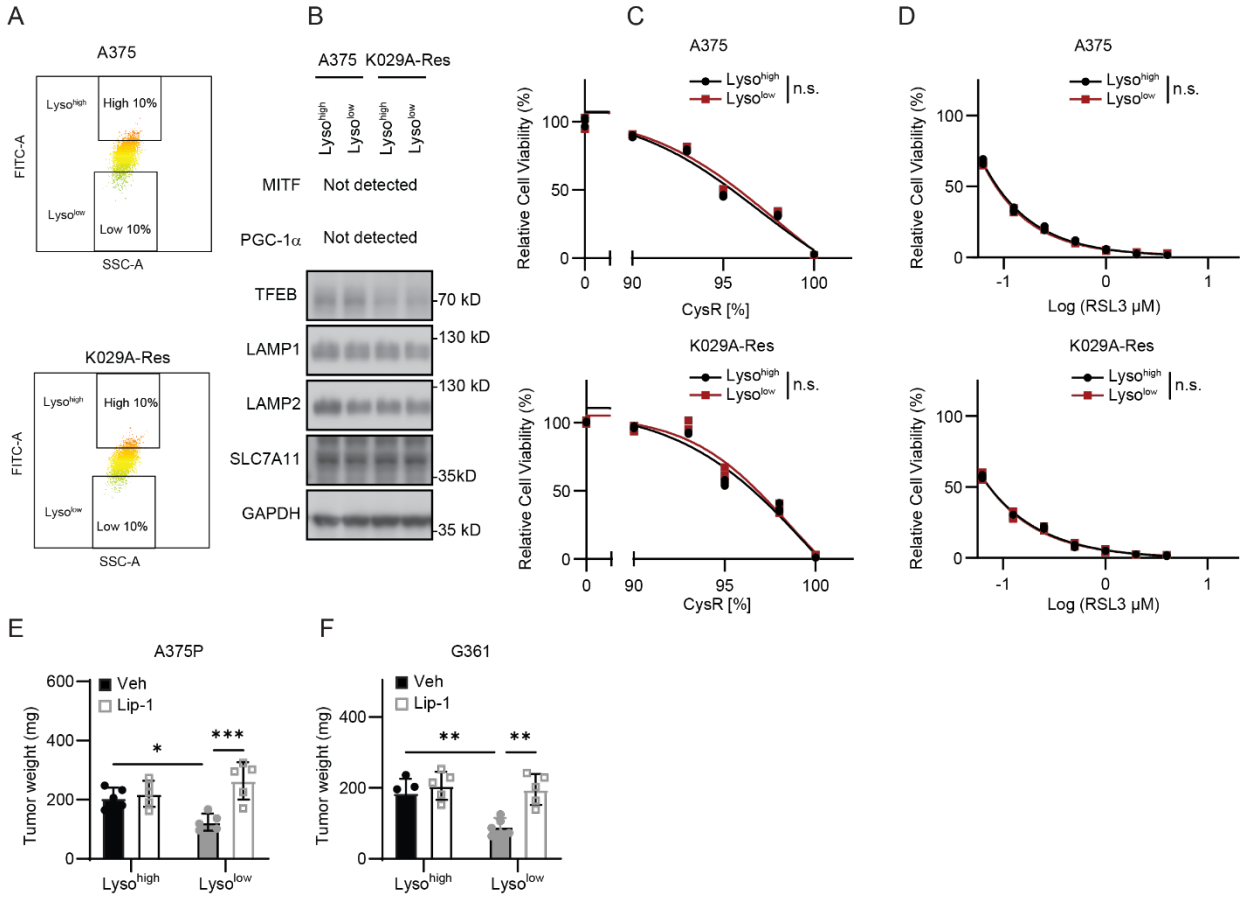

Figure S4: MITF-dependent lysosomal function is associated with increased ferroptosis sensitivity, related to Figure 2

(A) Scatter plots (SSC-A vs FITC-A) of LysoTracker-Green-stained cells by FACS sorting to separate A375 (top) and K029A-Res (bottom) cells into Lyso^{high} and Lyso^{low} populations.

(B) Immunoblot analysis of the indicated proteins in A375 and K029A-Res Lyso^{high} and Lyso^{low} cells.

(C and D) Ferroptosis sensitivity of A375 (top) and K029A-Res (bottom) Lyso^{high} and Lyso^{low} cells to cysteine restriction (CysR) (C) and RSL3 (D).

(E and F) Tumor weights derived from A375P (E) and G361 (F) Lyso^{high} and Lyso^{low} cells after 3-week treatment with vehicle (Veh, 1% DMSO in PBS) or liproxstatin1 (Lip-1, 20 mg/kg, i.p.). Data shown as mean ± SD. Statistical analysis was performed using one-way ANOVA (E and F) and two-way ANOVA (C and D). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, n.s. not significant.

Supplemental Fig.5

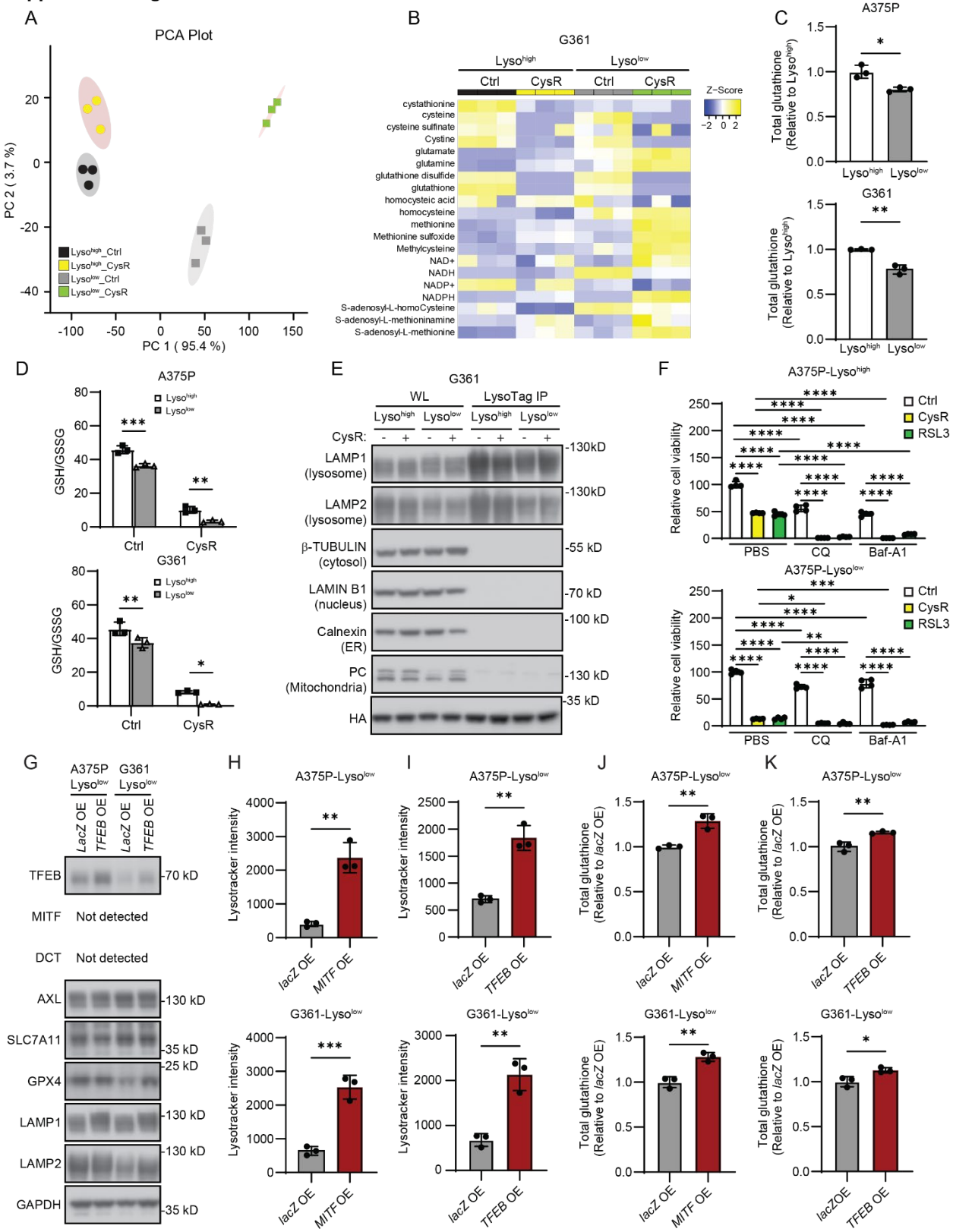

Figure S5: Overexpression of *MITF* or *TFEB* in Lyso^{low} cells promotes glutathione homeostasis, related to Figure 3

(A) Principal component analysis (PCA) plot for metabolite levels in G361 Lyso^{high} and Lyso^{low} cells in response to 16-hour cysteine restriction (CysR).

(B) Heatmap of changes in metabolites related to glutathione metabolism in G361 Lyso^{high} and Lyso^{low} cells in response to 16-hour cysteine restriction (CysR).

(C) Total glutathione in A375P (top) and G361 (bottom) Lyso^{high} and Lyso^{low} cells.

(D) GSH/GSSG ratio in A375P (top) and G361 (bottom) Lyso^{high} and Lyso^{low} cells cultured in control media or cysteine-restricted (CysR) media for 16 hours.

(E) Immunoblot analysis of the indicated proteins in G361 whole lysate (WL) and LysoTag IP in response to cysteine restriction (CysR).

(F) Relative cell viability of A375P Lyso^{high} (top) and A375P Lyso^{low} (bottom) cells subjected to cysteine restriction (CysR) and 0.5 μM RSL3 treatment co-treated with 25 μM chloroquine (CQ) and 20 nM bafilomycin A1 (Baf-A1) for 48 hours.

(G) Immunoblot analysis of the indicated proteins in A375P and G361 Lyso^{low} cells overexpressing *lacZ* or *TFEB*.

(H) LysoTracker intensity in A375P (top) and G361 (bottom) Lyso^{low} cells overexpressing *lacZ* or *MITF*.

(I) LysoTracker intensity in A375P (top) and G361 (bottom) Lyso^{low} cells overexpressing *lacZ* or *TFEB*.

(J) Total glutathione in A375P (top) and G361 (bottom) Lyso^{low} cells overexpressing *lacZ* or *MITF*.

(K) Total glutathione in A375P (top) and G361 (bottom) Lyso^{low} cells overexpressing *lacZ* or *TFEB*.

Data shown as mean ± SD. Statistical analysis was performed using unpaired two-tailed t-test (C, D, H-K) and one-way ANOVA (F). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Supplemental Fig.6

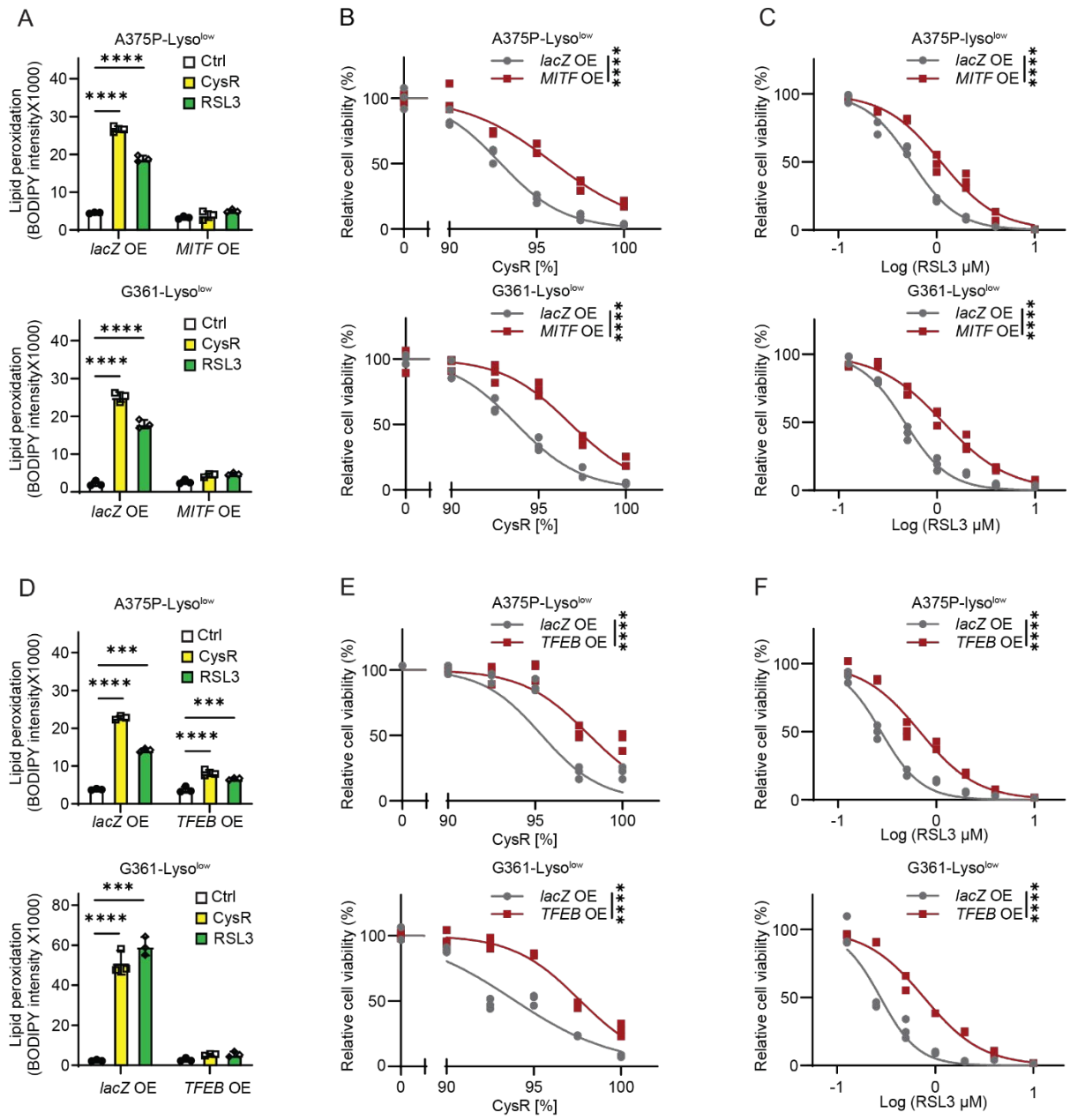

Figure S6: Overexpression of *MITF* or *TFEB* in Lyso^{low} cells promotes resistance to ferroptosis, related to Figure 3

(A) Quantification of lipid peroxide levels in A375P and G361 Lyso^{low} cells overexpressing *lacZ* or *MITF* in response to 16-hour cysteine restriction (CysR) and 4-hour 0.5 μM RSL3 treatment. (B and C) Ferroptosis sensitivity of A375P and G361 Lyso^{low} cells overexpressing *lacZ* or *MITF* to cysteine restriction (CysR) (B) and RSL3 (C).

(D) Quantification of lipid peroxide levels of A375P and G361 Lyso^{low} cells overexpressing *lacZ* or *TFEB* in response to 16-hour cysteine restriction (CysR) and 4-hour 0.5 μM RSL3 treatment. (E and F) Ferroptosis sensitivity of A375P and G361 Lyso^{low} cells overexpressing *lacZ* or *TFEB* to cysteine restriction (CysR) (E) and RSL3 (F).

Data shown as mean ± SD. Statistical analysis was performed using one-way ANOVA (A and D) and two-way ANOVA (B, C, E, and F). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Supplemental Fig.7

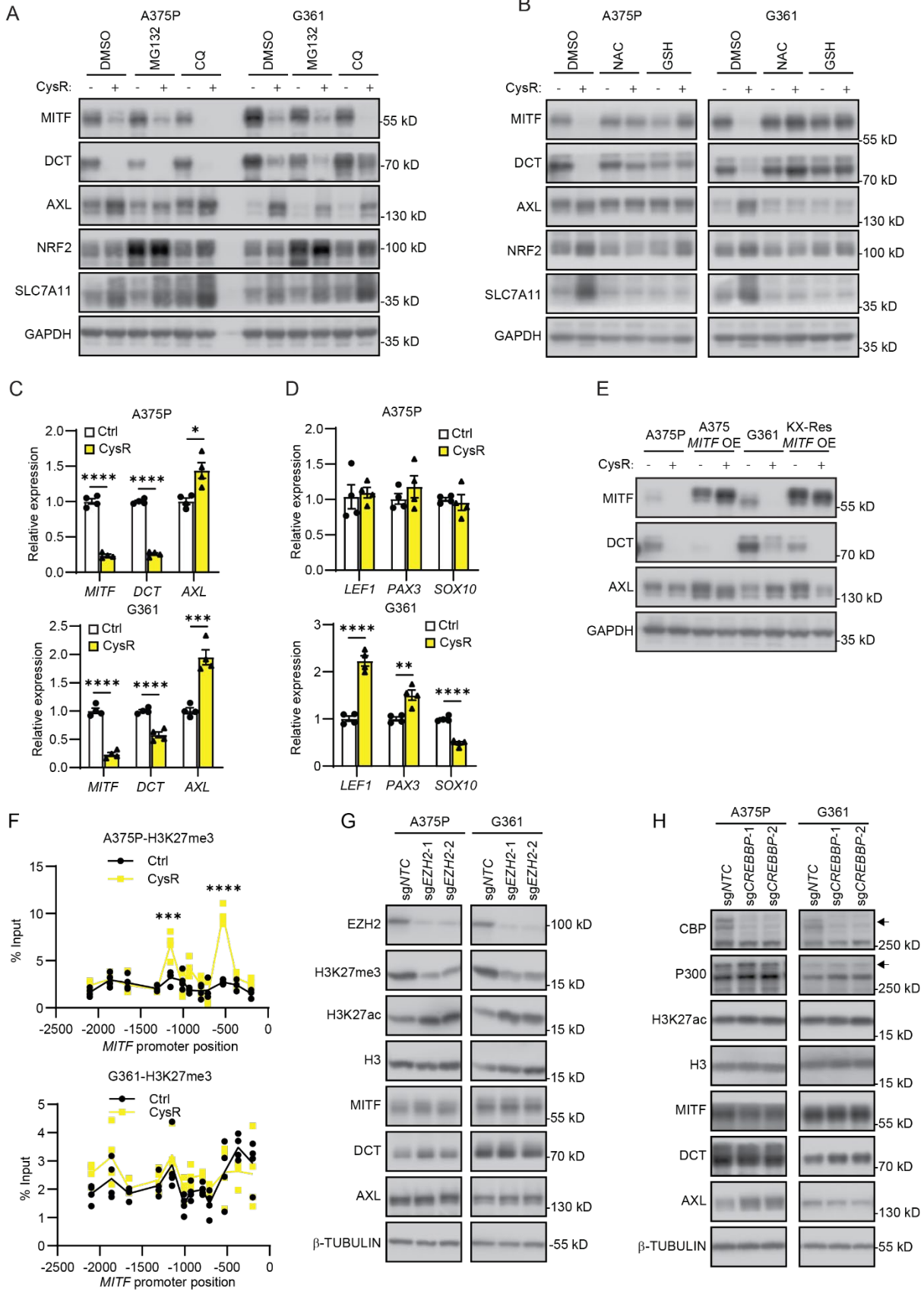

Figure S7: Neither EZH2 nor CBP regulates melanoma differentiation state in response to cysteine restriction, related to Figure 4

(A) Immunoblot analysis of the indicated proteins in A375P and G361 cells in response to 48-hour cysteine restriction (CysR),10 μM MG-132 (4h), and 100 μM chloroquine (CQ) treatment.

(B) Immunoblot analysis of the indicated proteins in A375P and G361 cells in response to 48-hour cysteine restriction (CysR),1 mM NAC, and 1 mM GSH treatment.

(C) Quantification of *MITF*, *DCT*, and *AXL* expression in A375P (top) and G361 (bottom) cells in response to 16-hour cysteine restriction (CysR) by qPCR.

(D) Quantification of *LEF1*, *PAX3*, and *SOX10* expression in A375P (top) and G361 (bottom) cells in response to 16-hour cysteine restriction (CysR) by qPCR.

(E) Immunoblot analysis of the indicated proteins in specified cells in response to 48-hour cystine free media (CysR).

(F) ChIP-PCR analysis of tri-methylated H3K27 (H3K27me3) occupancy within the *MITF* gene promoter locus in A375P (top) and G361 (bottom) cells in response to 48-hour cysteine restriction (CysR).

(G) Immunoblot analysis of the indicated proteins in A375P and G361 *EZH2* knockout cells.

(H) Immunoblot analysis of the indicated proteins in A375P and G361 *CREBBP* knockout cells.

Data shown as mean ± SD. Statistical analysis was performed using unpaired two-tailed t-test (C, D, and F). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Supplemental Fig.8

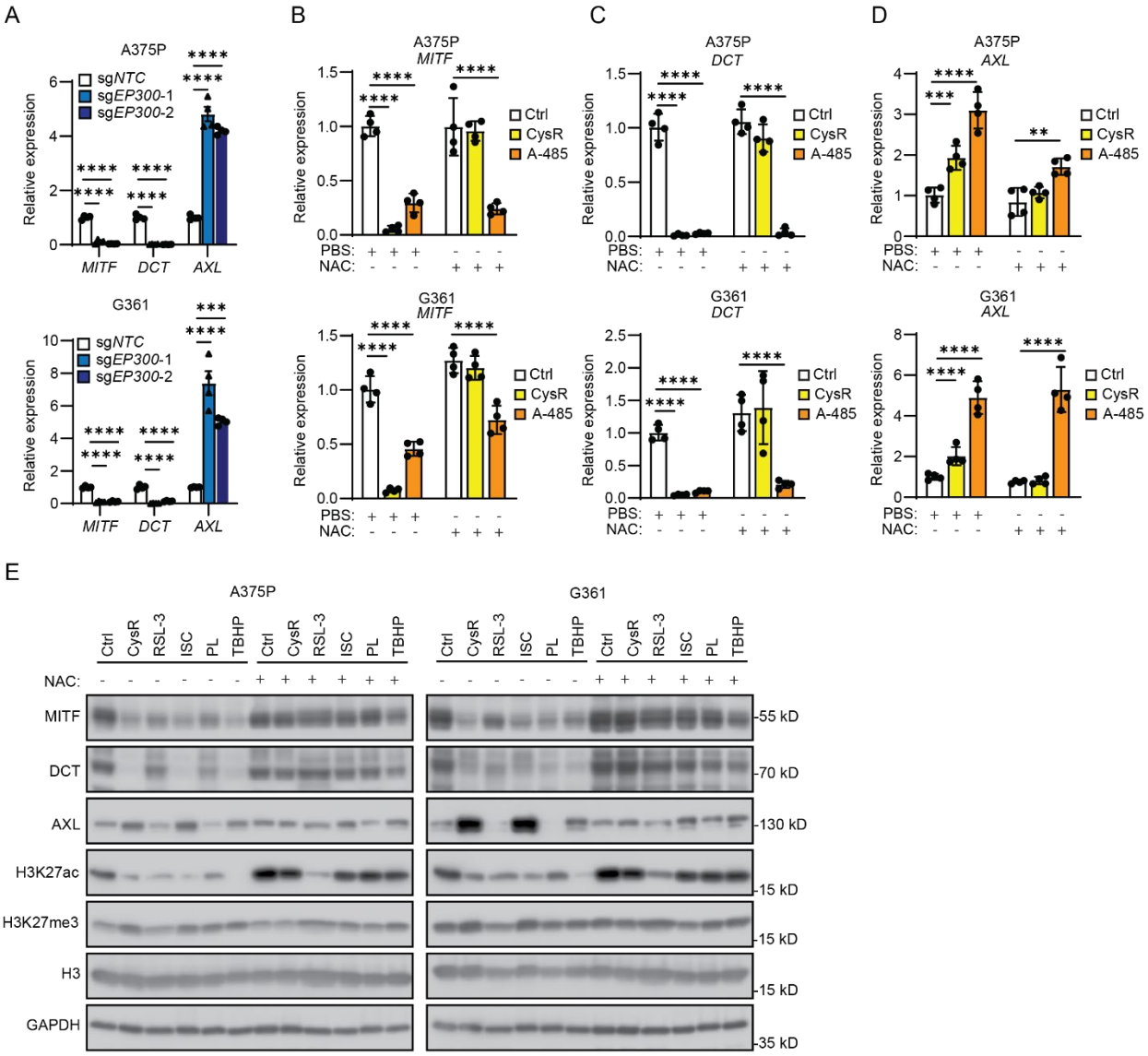

Figure S8: NAC blocks melanoma differentiation induced by reduction of H3K27ac from oxidative stress, related to Figure 4

(A) qPCR analysis of *MITF*, *DCT*, and *AXL* expression in A375P (top) and G361 (bottom) *EP300* knockout cells.

(B-D) qPCR analysis of *MITF* (B), *DCT* (C), and *AXL* (D) expression in A375P (top) and G361 (bottom) cells in response to 48-hour cysteine restriction (CysR) or 5 μM A-485 treatment in the absence or presence of 1 mM NAC.

(E) Immunoblot analysis of the indicated proteins in A375P and G361 cells in response to 48-hour cysteine restriction (CysR), 0.5 μM RSL3, 10 μM Piperlongumine (PLM), 1 μM Iron

salophene complex (ISC) RSL3, and 200 μM tert-Butyl hydroperoxide (TBHP) in the absence or presence of 1 mM NAC treatment.

Data shown as mean ± SD. Statistical analysis was performed using one-way ANOVA (A-D). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Supplemental Fig.9

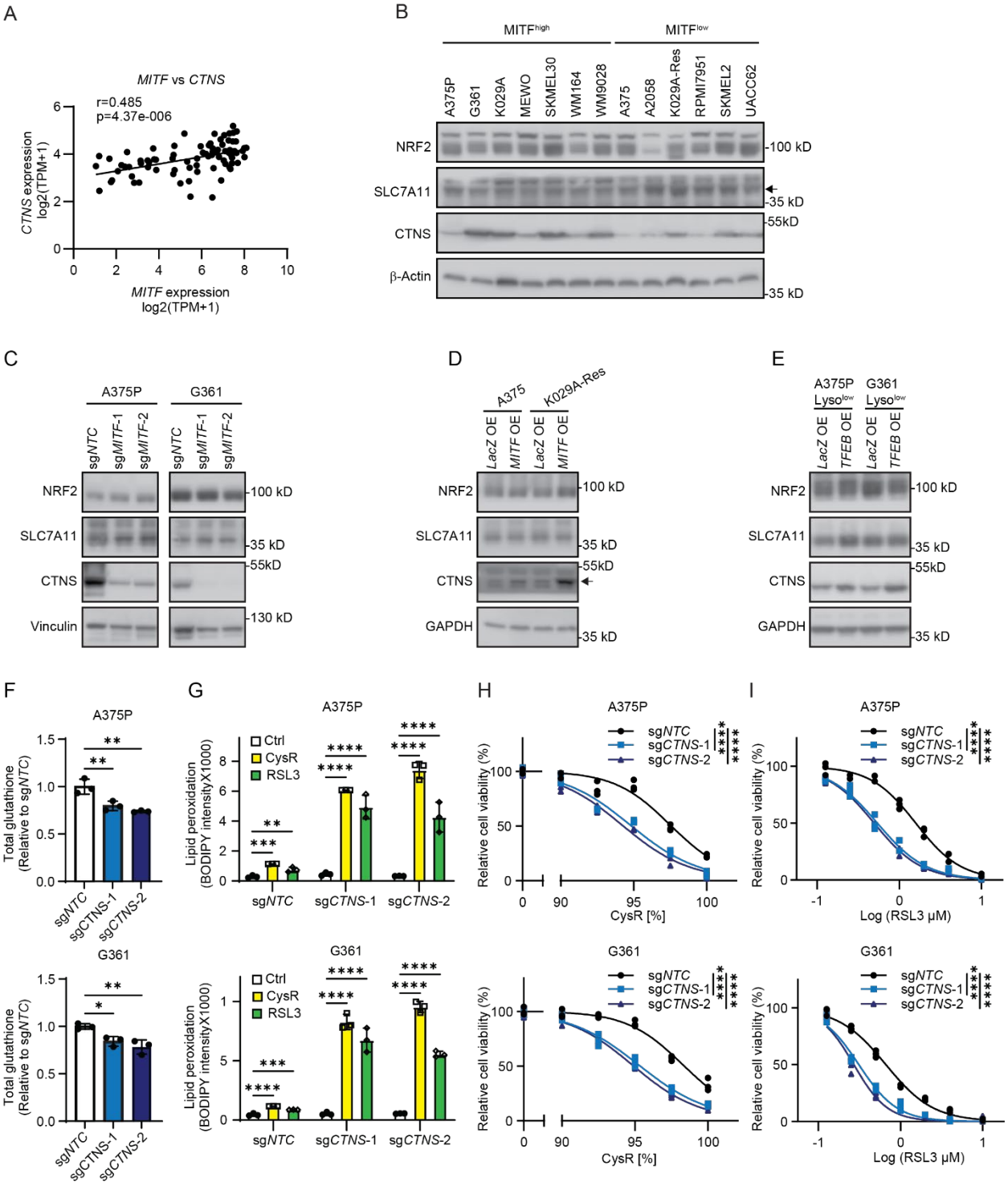

Figure S9: Deletion of *CTNS* promotes melanoma dedifferentiation and sensitizes to ferroptosis, related to Figure 5

(A) Correlation analysis of transcriptional expression between *MITF* and *CTNS* in CCLE melanoma cell lines.

(B) Immunoblot analysis of the indicated proteins in specified melanoma cell lines.
(C) Immunoblot analysis of the indicated proteins in A375P and G361 *MITF* knockout cells.
(D) Immunoblot analysis of the indicated proteins in A375 and K029A-Res cells overexpressing *lacZ* or *MITF*.
(E) Immunoblot analysis of the indicated proteins in A375P and G361 $Lyso^{low}$ cells overexpressing *lacZ* or *TFEB*.
(F) Total glutathione in A375P (top) and G361 (bottom) *CTNS* knockout cells.
(G) Lipid peroxide levels production in A375P (top) and G361 (bottom) *CTNS* knockout in response to 16-hour cysteine restriction (CysR) and 4-hour 0.5 μM RSL3 treatment.
(H and I) Ferroptosis sensitivity of A375P (top) and G361 (bottom) *CTNS* knockout cells to cysteine restriction (H) and RSL3 (I).
Data shown as mean ± SD. Statistical analysis was performed using one-way ANOVA (F and G) and two-way ANOVA (H and I). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Supplemental Fig.10

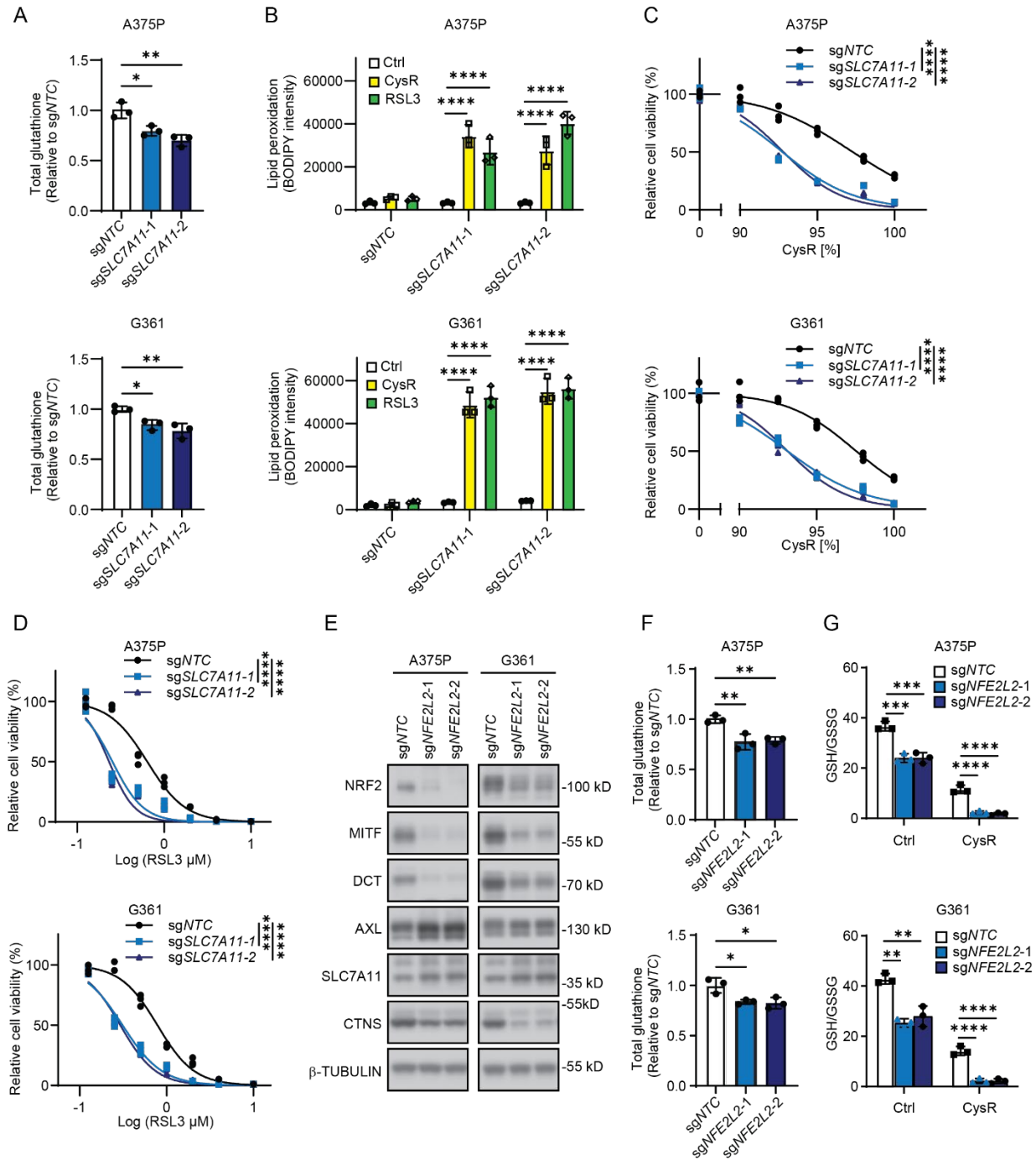

Figure S10: Deletion of *SLC7A11* or *NFE2L2* impairs glutathione capacity, related to Figure 5

(A) Total glutathione in A375P and G361 *SLC7A11* knockout cells.

(B) Lipid peroxide levels production in A375P and G361 *SLC7A11* knockout in response to 16-hour cysteine restriction (CysR) and 4-hour 0.5 μM RSL3 treatment.

(C and D) Ferroptosis sensitivity of A375P and G361 *SLC7A11* knockout cells to cysteine restriction (C) and RSL3 (D).
(E) Immunoblot analysis of the indicated proteins in A375P and G361 *NFE2L2* knockout cells.
(F) Total glutathione in A375P and G361 *NFE2L2* knockout cells.
(G) GSH/GSSG ratio in A375P and G361 *NFE2L2* knockout cells cultured in control media or cysteine restriction (CysR) media for 16 hours.
Data shown as mean ± SD. Statistical analysis was performed using one-way ANOVA (A, B, F, and G) and two-way ANOVA (C and D). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Supplemental Fig.11

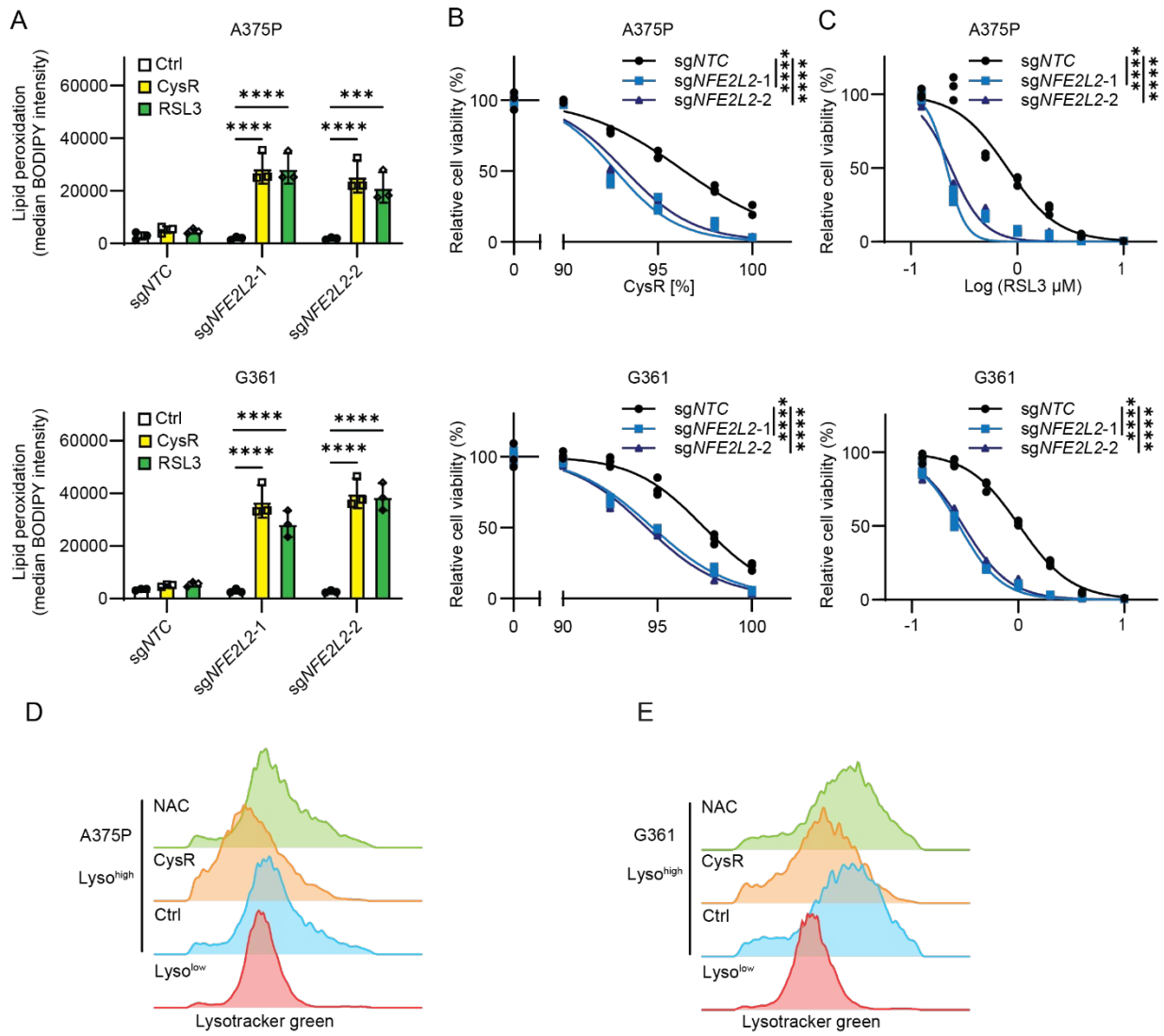

Figure S11: Deletion of *SLC7A11* or *NFE2L2* increases sensitivity to ferroptosis, related to Figure 5

(A) Lipid peroxide levels production in A375P and G361 *SLC7A11* knockout in response to 16-hour cysteine restriction (CysR) and 4-hour 0.5 μM RSL3 treatment.

(B and C) Ferroptosis sensitivity of A375P and G361 *SLC7A11* knockout cells to cysteine restriction (B) and RSL3 (C).

(D and E) Histogram of LysoTracker staining in A375P (D) and G361 (E) Lyso^{low} cells and Lyso^{high} cells cultured in DMEM with reduced cystine (10 μM cystine) or 1 mM N-acetylcysteine (NAC) for two weeks.

Data shown as mean ± SD. Statistical analysis was performed using one-way ANOVA (A) and two-way ANOVA (B and C). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Supplemental Fig.12

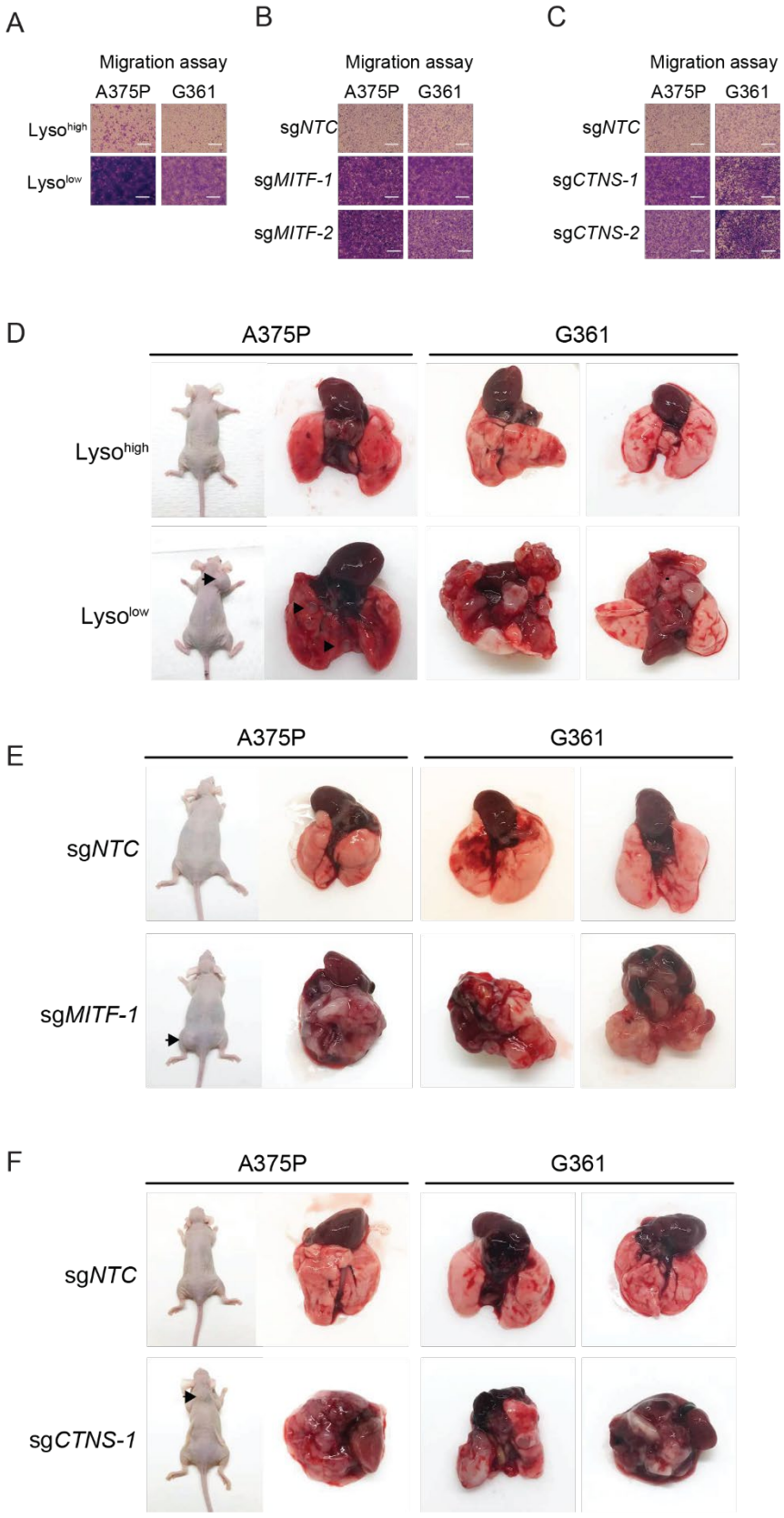

Figure S12: Cysteine restriction and decreased lysosome biogenesis are associated with increased migratory capacity and higher incidence of metastasis, related to Figure 6

(A). Representative images of transwell migration assay of A375P and G361 *MITF* knockout cells.

(B). Representative images of transwell migration assay of A375P and G361 $Lyso^{high}$ and $Lyso^{low}$ cells.

(C). Representative images of transwell migration assay of A375P and G361 *CTNS* knockout cells.

(D-F). Representative images of metastases in A375P and G361 $Lyso^{high}$ and $Lyso^{low}$ cells (D), *MITF* knockout cells (E), and *CTNS* knockout (F) cells.

Scale bar: 150 μm.