

Supplemental Information

**Transferrin Receptor Is a
Specific Ferroptosis Marker**

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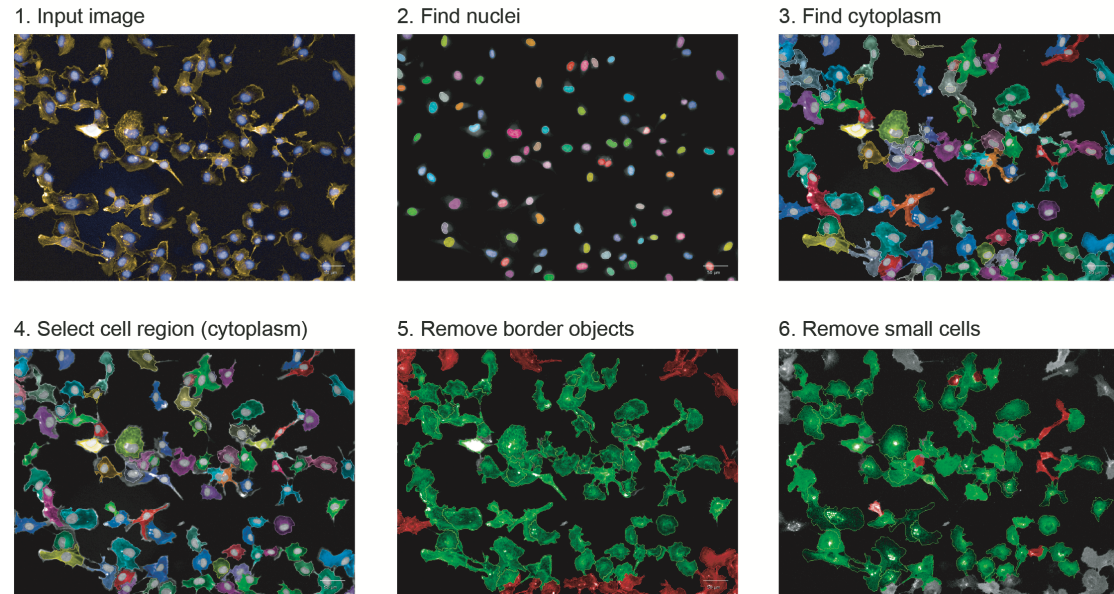
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SUPPLEMENTAL FIGURE AND LEGENDS

A. Select cell population for further analyses in B



B. Calculate properties

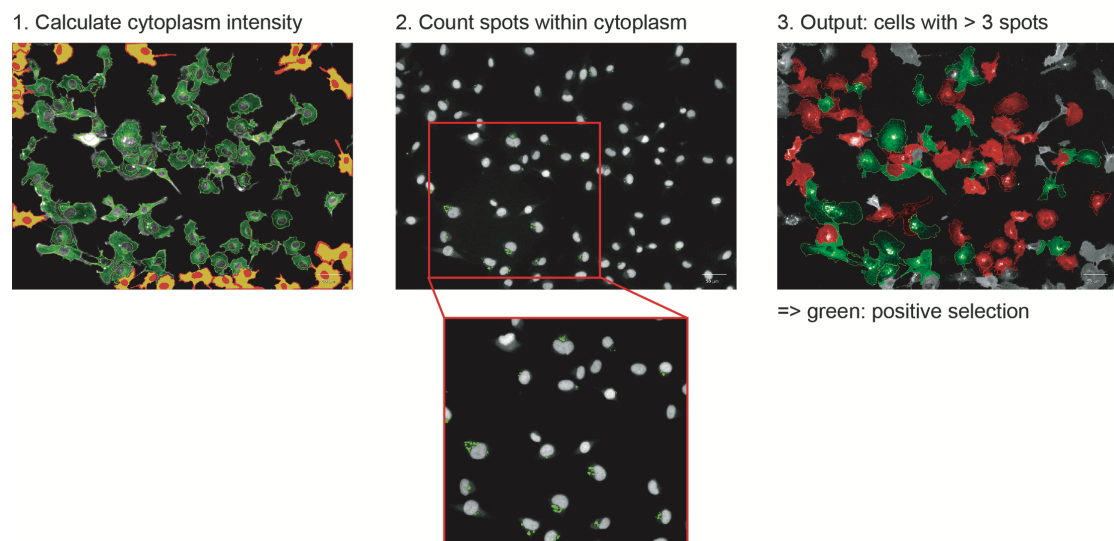


Figure 1S. High-content analysis of 3F3-FMA, Related to Figure 1.

A. HT-1080 cells were seeded in 384-well plates and treated with 0.3 μ M RSL3 for 2.5 h. Then the cells were fixed with 4% PFA, permeabilized with 0.5% Tween-20 and stained with primary antibody and secondary antibody. The cells were then stained with Hoechst 33342 (nuclei detection) and Phalloidin-TRITC (cytoplasmic region detection) prior to recording by automated Operetta® microscope. Multiparametric image analysis was performed using Columbus Software 2.8.0 (PerkinElmer). The cell

population for analysis was selected by detection and segmentation of nuclei and corresponding cytoplasm. Border objects and small cells were removed from analysis. The cells highlighted green in graph 6 were selected for further analysis.

B. Selected cells from (A) and the corresponding cell regions were used for Alexa488 intensity and spot detection. For 3F3-FMA, the number of cells with more than 3 spots in the cytoplasm (green cells in graph 3) were quantified.

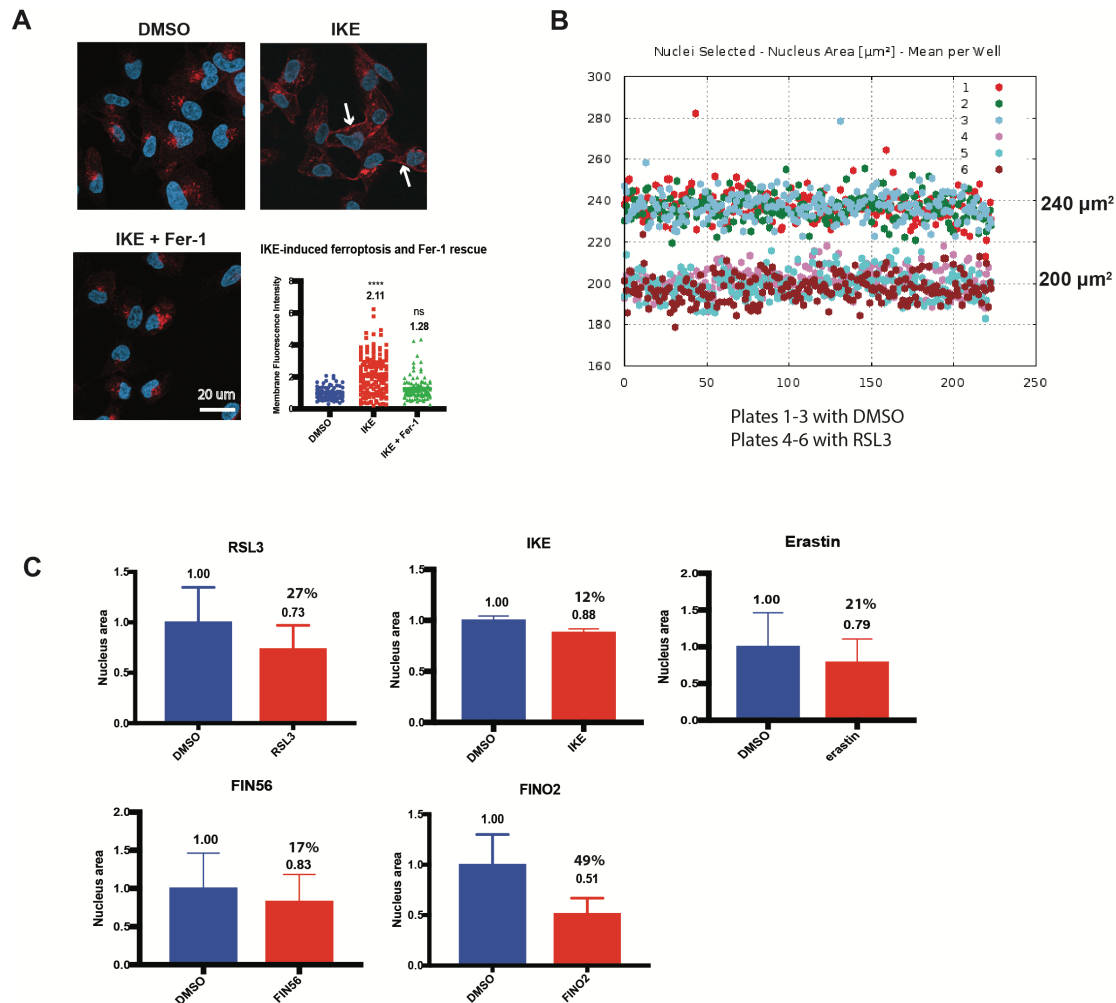


Figure S2. Nuclei are smaller in ferroptotic cells, Related to Figure 2.

A. HT-1080 cells were incubated with 10 μ M IKE +/- 5 μ M fer-1 for 8 h. Nuclei were stained with DAPI (blue) and 3F3-FMA with Alexa Fluor 594 secondary in red. 3F3-FMA showed a significantly different pattern in IKE-treated cells, but not upon Fer-1 co-treatment. White arrows indicate sample differences. Quantification of membrane intensities of 3F3-FMA is shown (DMSO, n = 68; IKE, n = 145; IKE + Fer-1, n = 98). **** indicated p value ≤ 0.0001 , ns indicates p value > 0.05 (one-way ANOVA). Data plotted are mean \pm s.e.m. Each dot represents one cell.

B. HT-1080 cells in plates 1-3 were treated with DMSO and cells in plates 4-6 were treated with RSL3. Nuclei areas were identified and measured. The average nucleus area for the DMSO group was $240 \mu\text{m}^2$ while for the RSL3 group was $200 \mu\text{m}^2$.

C. HT-1080 cells were incubated with $1 \mu\text{M}$ RSL3 for 4h, $10 \mu\text{M}$ IKE for 8 h, $15 \mu\text{M}$ erastin for 8 h, $10 \mu\text{M}$ FIN56 for 8 h and $15 \mu\text{M}$ FINO₂ for 8 h. Nuclei were stained with DAPI and identified using CellProfiler 3.1.8. Mean area of the nucleus for each cell was calculated. Quantification of nucleus area is shown (DMSO, n = 107; RSL3, n = 96; DMSO, n = 113, IKE, n = 129; DMSO, n = 68, erastin, n = 92; DMSO, n = 68, FIN56, n = 86; DMSO, n = 59, FINO₂, n = 30). Nuclei size plotted are mean \pm s.e.m. The nuclei shrank by 27% in 4 h incubation of $1 \mu\text{M}$ RSL3, 12% in 8 h incubation of $10 \mu\text{M}$ IKE, 21% in 8 h incubation of $15 \mu\text{M}$ erastin, 17% in 8 h incubation of $10 \mu\text{M}$ FIN56 and 49% in 8 h incubation of $15 \mu\text{M}$ FINO₂.

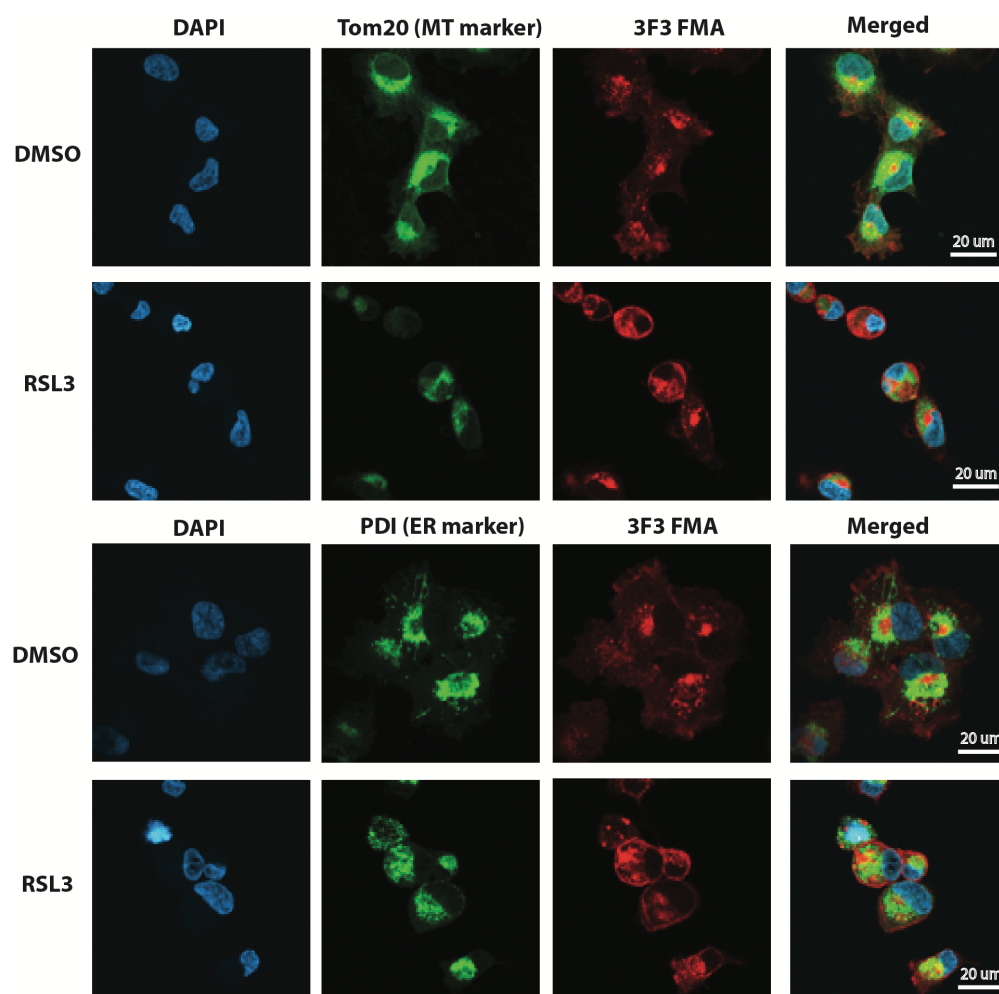


Figure S3. The target of 3F3-FMA is not in the mitochondria or in the ER, Related to Figure 3.

HT-1080 cells were incubated with 1 μ M RSL3 for 4 h, and then fixed, permeabilized and stained with DAPI (nuclei, blue), and either Tom20 (mitochondria marker, green) or PDI (ER marker, green), and also 3F3-FMA. 3F3-FMA did not co-localize with these mitochondria and ER markers.

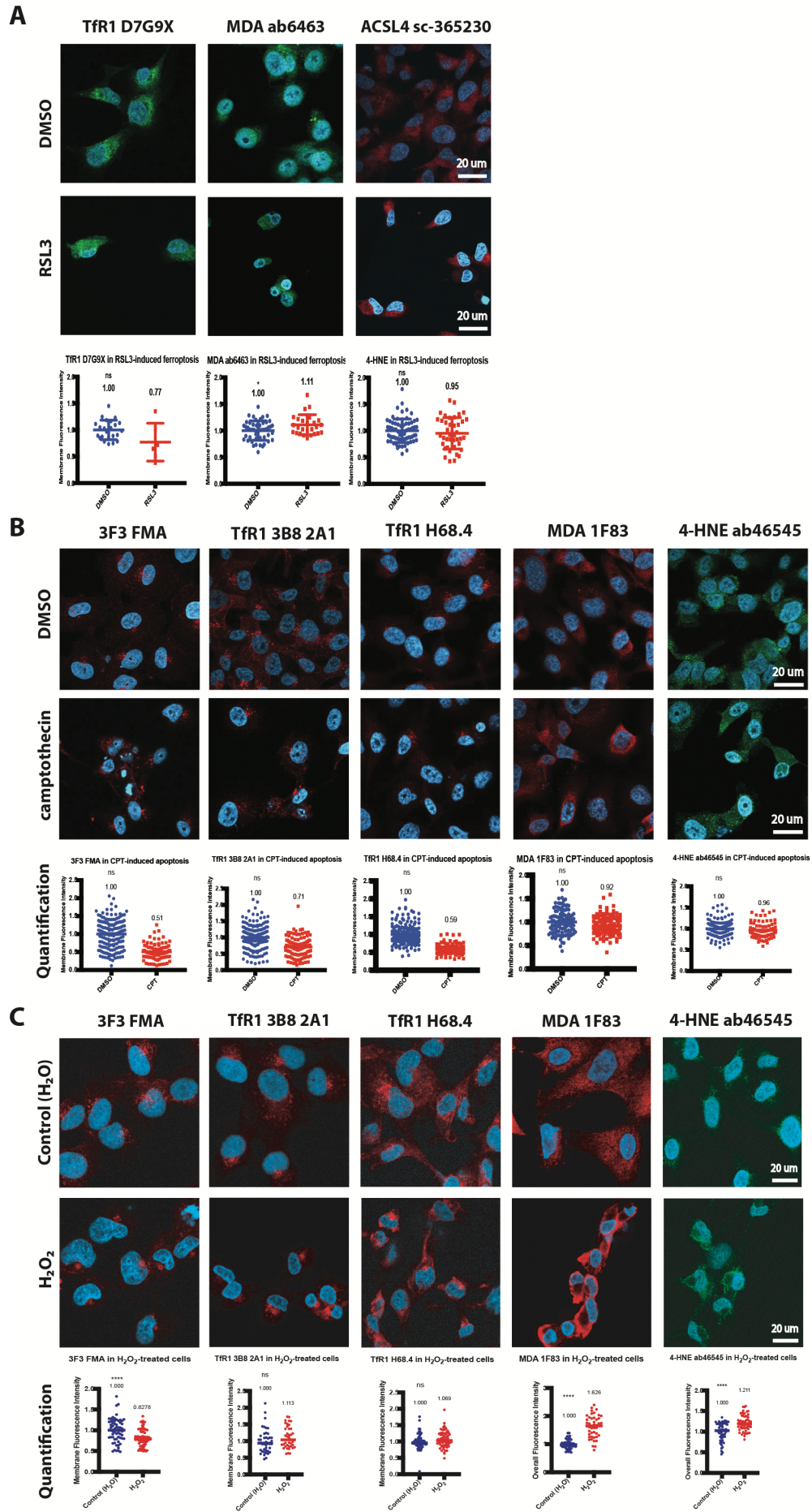


Figure S4. 3F3-FMA, anti-TfR1 3B8 2A1, anti-TfR1 H68.4, and anti-MDA 1F83 and anti-4-HNE antibodies can be used as selective ferroptosis markers using immunofluorescence, Related to Figure 4.

A. HT-1080 cells were incubated with 1 μ M RSL3 for 4 h, and then were fixed, permeabilized and stained with DAPI (nuclei, blue), anti-TfR1 D7G9X (green), anti-MDA ab6463 (green), and anti-ACSL4 sc-365230 antibodies (red). Quantification of membrane intensities of the antibodies is shown on the bottom (TfR1 D7G9X, n = 23 and 6; MDA ab6463, n = 49 and 27; ACSL4 sc-365230, n = 69 and 41. * indicated p value ≤ 0.05 and ns indicated p value > 0.05 (two-tailed *t*-test). Data plotted are mean \pm s.e.m. Each dot represents one cell.

B. HT-1080 cells were incubated with 2 μ M camptothecin for 24 h to induce apoptosis, and then were fixed, permeabilized and stained with DAPI (nuclei, blue), 3F3-FMA (red), anti-TfR1 3B8 2A1 (red), anti-TfR1 H68.4 (red), anti-MDA 1F83 (red), and anti-4-HNE ab46545 antibodies (green). Quantification of membrane intensities of the antibodies is shown on the bottom (3F3-FMA, n = 166 and 91; anti-TfR1 3B8 2A1, n = 164 and 116; anti-TfR1 H68.4, n = 186 and 112; anti-MDA 1F83, n = 109 and 119; 4-HNE ab46545, n = 122 and 74). Data plotted are mean \pm s.e.m. Each dot represents one cell.

C. HT-1080 cells were incubated with 1 mM H₂O₂ for 4 h to induce oxidative stress independent of ferroptosis, and then were fixed, permeabilized and stained with DAPI (nuclei, blue), 3F3-FMA (red), anti-TfR1 3B8 2A1 (red), anti-TfR1 H68.4 (red), anti-MDA 1F83 (red), and anti-4-HNE ab46545 antibodies (green). Quantification of membrane intensities and overall intensities of the antibodies is shown on the bottom (3F3-FMA, n = 62 and 73; anti-TfR1 3B8 2A1, n = 43 and 38; anti-TfR1 H68.4, n = 64 and 64; anti-MDA 1F83, n = 45 and 46; 4-HNE ab46545, n = 53 and 62). **** indicated p value ≤ 0.0001 , ns indicated p value > 0.05 (two-tailed *t*-test). Data plotted are mean \pm s.e.m. Each dot represents one cell.

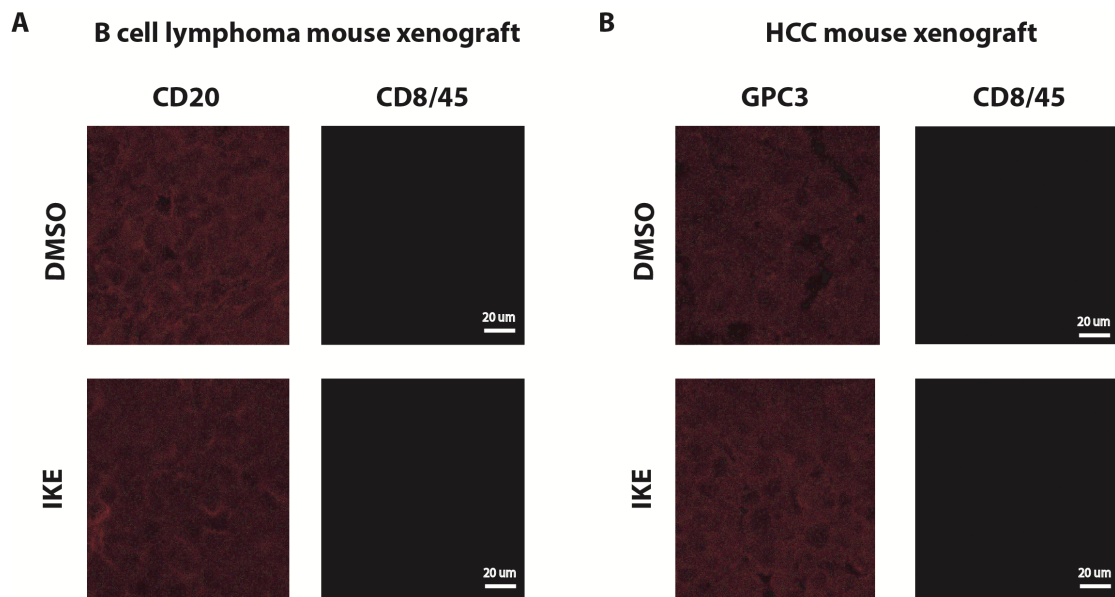


Figure S5. Diffuse large B cell lymphoma and hepatocellular carcinoma mouse xenograft tissues contain tumor cells but not immune cells, Related to Figure 6.

A. B cell lymphoma tumor tissues were fixed in 4% PFA for 24 h, perfused with 30% sucrose for 24 h, and stained with primary and secondary antibodies, as indicated. Staining of CD20, a B cell lymphoma marker, was positive, while staining of CD8 and CD45, immune cell markers, was negative, indicating that tumor cells, but not infiltrating immune cells, were present in the B cell lymphoma tissue samples. Representative images are shown.

B. Hepatocellular carcinoma (HCC) xenograft tumor tissues was fixed in 4% PFA for 24 h, perfused with 30% sucrose for 24 h, and stained with primary and secondary antibodies. Staining of GPC3, an HCC marker, was positive, while staining of CD8 and CD45 was negative, indicating that tumor cells, but not these infiltrating immune cells, were present in the HCC tissue samples. Representative images are shown.

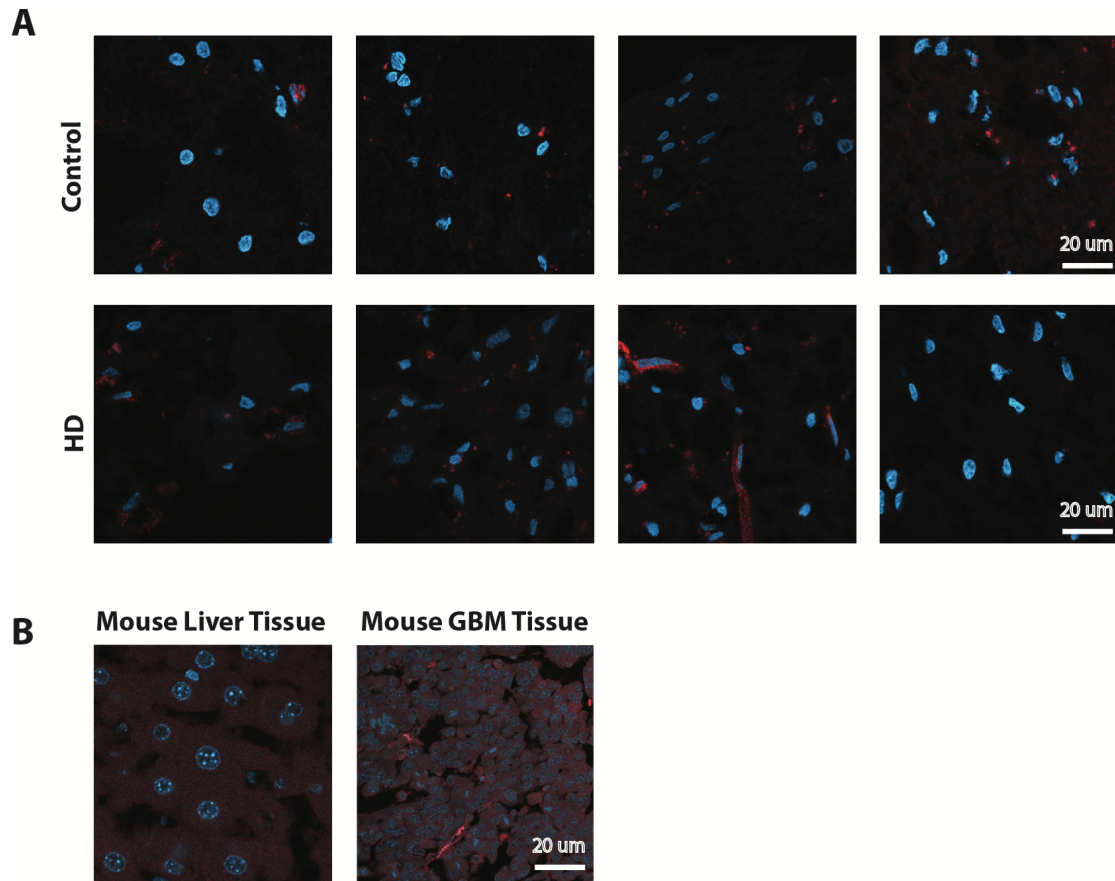


Figure S6. 3F3-FMA staining of human Huntington disease brains and mouse tissues, Related to Figure 6.

A. Five human HD and five unaffected control brain tissue samples from the NIH NeuroBioBank at the Harvard Brain Tissue Resource Center, the University of Maryland, Baltimore, MD, VA_LA, the University of Pittsburgh, and the University of Miami from the caudate nucleus of each patient were analyzed. The tissues were fixed in 4% PFA for 24 h, perfused in 30% sucrose for 24 h, then stained with DAPI (nuclei, blue) and 3F3-FMA with Alexa Fluor 594 secondary in red . The expression level of TfR1 was low in all samples, and there was no detectable difference between the HD and control samples.

B. Mouse liver tissues samples from HCC mouse xenograft model and mouse glioblastoma tissue samples from Peter Canoll's lab were fixed and stained with DAPI (nuclei, blue) and 3F3-FMA (red). 3F3-FMA was able to recognize mouse TfR1 in both types of tissue.

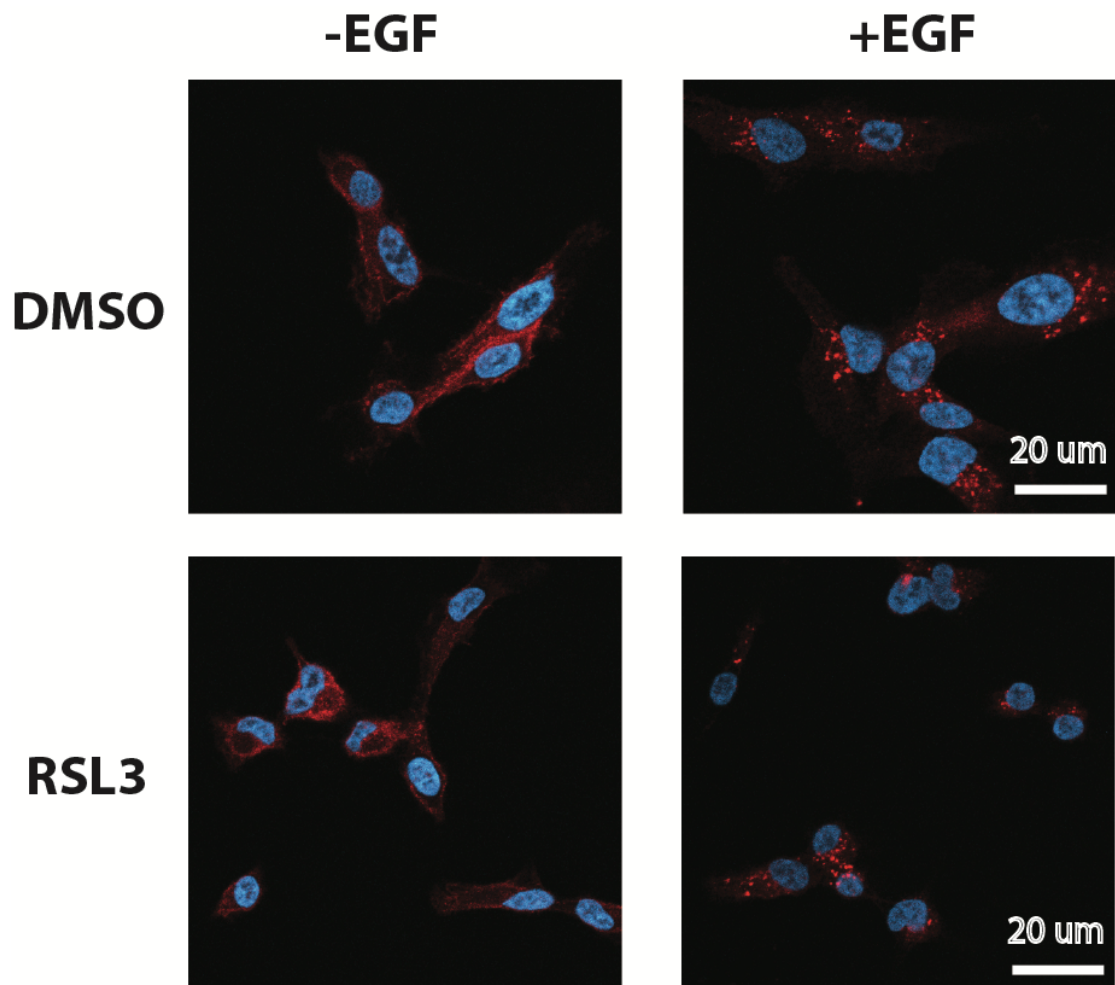


Figure S7. Internalization of EGFR is not inhibited during ferroptosis, Related to Figure 3.

HT-1080 cells were incubated with 1 μ M RSL3 in serum-free medium for 4 h (ferroptosis induction with serum starvation), and were incubated with 25 ng/mL EGF for 40 min. Cells were fixed, permeabilized and stained with DAPI (nuclei, blue) and EGFR Monoclonal Antibody (H11) (Thermo Fisher Scientific, Cat# MA513070) with Alexa Fluor 594 secondary in red. EGFR was internalized in the presence of EGF with and without RSL3 treatment, indicating that clathrin-mediated endocytosis isn't affected during ferroptosis. The shrinking nuclei indicated that ferroptosis was happening.