Determination of the subcellular localization and mechanism of action of ferrostatins in suppressing ferroptosis

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Supporting information

Supplemental figure 1. Spontaneous Raman spectrum of 10 mM ferrostatin **2** in DMSO solution, displaying a sharp diyne peak at 2262 cm^{-1}.

Supplemental figure 2. SRS imaging of protein CH_3 groups, ferrostatin **2** (10 μM), and off-resonance signal in live Panc-1 cells in the absence or absence of erastin (10 μM). Images gathered 6 h after compound addition.

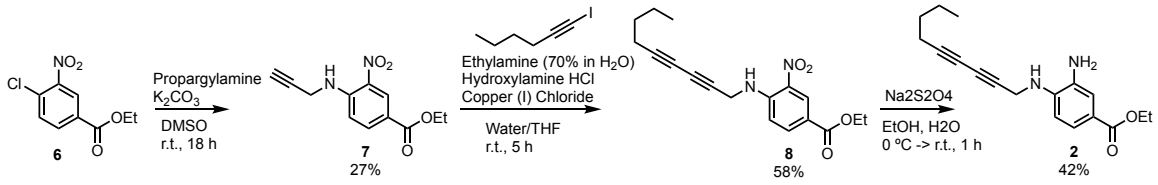
Supplemental figure 3. (A) Immunofluoresence intensity of mitochondria-specific proteins COX IV and HSP60 in wild type and YFP-parkin transfected cells in the presence and absence of CCCP (12.5 μM, 48 h). (B) Electron microscopy images of wild type and YFP-parkin transfected HT-1080 cells with

and without 48 h CCCP treatment (12.5 μM). Red arrows indicate mitochondria. (C) Dose-response curve of lethal molecules in YFP-parkin transfected HT-1080 cells that have not undergone mitophagy ("-CCCP"), that have undergone mitophagy (12.5 μM CCCP for 48 h) and are still under CCCP treatment at the time of lethal molecule addition ("+CCCP") or cells that have undergone mitophagy (12.5 μM CCCP for 48 h) but had CCCP removed at the time of lethal molecule addition ("+Mitophagy -CCCP"). Viability was measured at 24 h after lethal compound addition. (D) Abundance of GPX4 protein in wildtype and transfected HT-1080 cells treated with 12.5 μM CCCP for 48 h (E) Potency of lethal molecules in wild type HT-1080 cells. Column height represents EC_{50} value with error bars corresponding to the 95% confidence interval. (F) Dose-response curve of ferrostatin-1 rescue of erastin (10 μM) induced ferroptosis. Viability measured at 24 h. (G) Dose-dependent prevention of erastin (10 μM) induced ferroptosis by iron chelators ciclopirox olamine (CPX) and deferoxamine (DFO) with and without mitophagy.

Supplemental figure 4. Lipid peroxidation in mitochondria-depleted cells. HT-1080 cells depleted of mitochondria (+CCCP) and treated with the ferroptosis inducer IKE showed an increase in oxidative events relative to mitochondria-replete cells treated with IKE at an equivalent concentration and time.

Compound synthesis

General Procedures. All commercial reagents were used without further purification. All solvents used were reagent or HPLC grade. Chemical yields refer to isolated, spectroscopically pure compounds. ^{1}H and ^{13}C NMR spectra were recorded on a 400 or 500 MHz spectrometer (as indicated) at ambient temperature. Chemical shifts are recorded in parts per million relative to residual solvent $CDCl_3$ (^{1}H, 7.26 ppm; ^{13}C, 77.16 ppm) or $(CD_3)_2SO$ (^{1}H, 2.50 ppm; ^{13}C, 39.52 ppm). Multiplicities are reported as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, comp m = complex multiplet, dd = doublet of doublets, ddd = doublet of doublets of doublets, td = triplet of doublets. Liquid chromatography was performed on a CombiFlash Rf system (Teledyne Isco). Preparative thin layer chromatography was performed on plates coated with 1 mm silica gel. All compounds were confirmed to be at least 95% pure before use in subsequent synthetic steps or cellular assays.



Ethyl 3-nitro-4-(prop-2-yn-1-ylamino)benzoate (7). To a solution of 4-chloro-3-nitro ethylbenzoate (2.0 g, 8.71 mmol) in DMSO (20 mL) was added potassium carbonate (2.4 g, 17.42 mmol) and propargylamine (0.67 mL, 10.4 mmol). The mixture was stirred at room temperature overnight. The reaction mixture was poured into water and extracted with ethyl acetate (3 X 30 mL). The combined organic extract was dried with sodium sulfate, decanted, and concentrated under

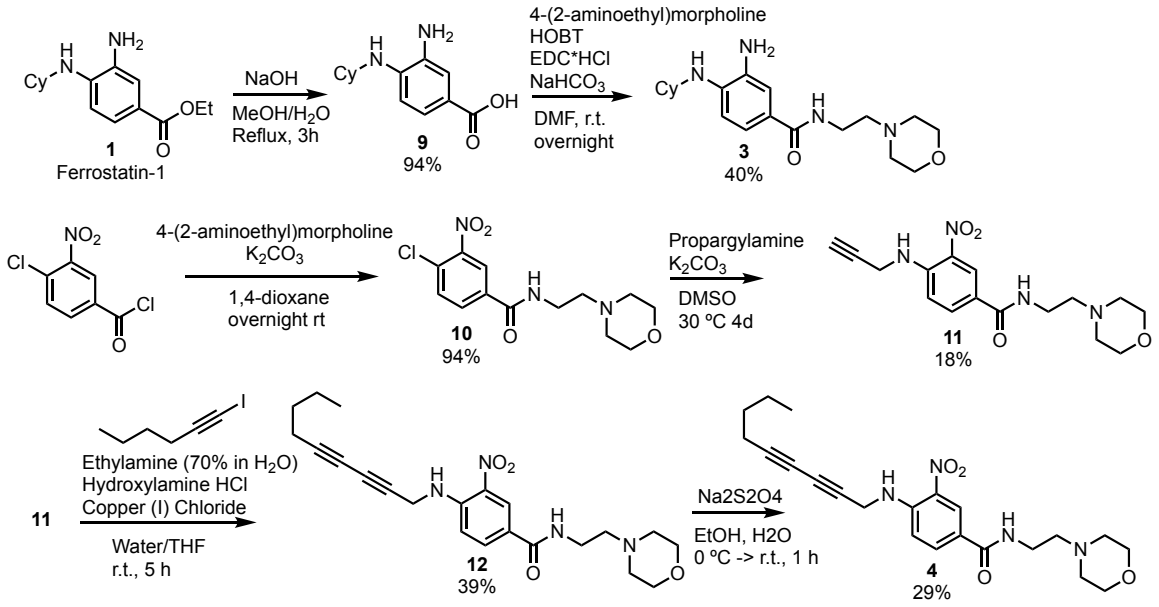
vacuum. The crude mixture was purified using a gradient of 0→30% ethyl acetate in hexanes to afford **7** (0.6 g, 27%) as a yellow solid. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.90 (d, *J* = 2.0 Hz, 1H), 8.42 (s, 1H), 8.14 (ddd, *J* = 8.9, 2.1, 0.7 Hz, 1H), 6.99 (d, *J* = 9.0 Hz, 1H), 4.38 (q, *J* = 7.1 Hz, 2H), 4.18 (dd, *J* = 5.7, 2.5 Hz, 2H), 2.33 (t, *J* = 2.5 Hz, 1H), 1.40 (t, *J* = 7.1 Hz, 3H).

1-Iodohex-1-yne. To a solution of 1-hexyne (2.0 mL, 17.4 mmol) in THF (42 mL) under a nitrogen atmosphere was added methyl lithium (1.6 M in diethyl ether, 19.5 mL, 31.33 mmol) dropwise over 10 minutes. The mixture was allowed to stir for an hour at room temperature. Separately, iodine (4.4 g, 17.3 mmol) was dissolved in THF (17 mL). This solution was added dropwise to the 1-hexyne solution portionwise over 30 minutes, allowing the brown color to dissipate fully between each addition. After complete addition of iodine, the mixture was allowed to stir for an additional 30 min. The mixture was poured over water (100 mL) and extracted with diethyl ether (3 X 25 mL). The combined ether extracts were washed with a 5% aqueous solution of sodium thiosulfate and collected, and partially concentrated under vacuum. Purification was achieved by filtration through a plug of silica gel with hexanes. The flow-through was collected and concentrated completely on vacuum to yield 1-iodohex-1-yne as a highly odorous amber liquid. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 2.36 (t, *J* = 7.0 Hz, 2H), 1.53 – 1.46 (m, 2H), 1.45 – 1.37 (m, 2H), 0.91 (t, *J* = 7.2 Hz, 3H) ^{13}C NMR (500 MHz, Chloroform-*d*) δ 94.88, 60.50, 30.67, 21.98, 20.62, 13.73.

Ethyl 3-nitro-4-(nona-2,4-diyn-1-ylamino)benzoate (8). To a solution of THF (38 mL), water (10.5 mL), and ethylamine (70% in water, 5.5 mL), was added **7** (0.6 g, 2.4 mmol) followed by hydroxylamine hydrochloride (0.119 g, 1.72 mmol) and copper (I) chloride (59 mg, 0.6 mmol). This mixture was allowed to stir at room temperature for 5 minutes. Separately, a solution of 1-iodohex-1-yne (0.25 g, 1.2 mmol) was dissolved in 10 mL THF. This solution was added dropwise and the whole mixture was allowed to stir for 5 h at room temperature. The reaction mixture was poured over 85 mL of water containing 5.2 g KCN, and extracted with diethylether (3 X 25 mL), dried over sodium sulfate and concentrated under vacuum. The crude mixture was purified using a gradient of 0→30% ethyl acetate in hexanes to afford **8** (0.23 g, 58%) as a yellow solid. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.89 (d, *J* = 2.0 Hz, 1H), 8.41 (t, *J* = 5.9 Hz, 1H), 8.14 (ddd, *J* = 9.0, 2.1, 0.7 Hz, 1H), 6.98 (d, *J* = 9.0 Hz, 1H), 4.37 (q, *J* = 7.1 Hz, 2H), 4.24 (dt, *J* = 5.8, 1.1 Hz, 2H), 2.26 (tt, *J* = 7.1, 1.1 Hz, 2H), 1.55 – 1.45 (m, 2H), 1.50 – 1.33 (m, 5H), 0.90 (t, *J* = 7.2 Hz, 3H).

Ethyl 3-amino-4-(nona-2,4-diyn-1-ylamino)benzoate (2). To a solution of ethanol (200 proof, 8 mL) was added **8** (0.23 g, 0.7 mmol). The temperature was decreased to 0 ºC by cooling with an ice bath. Sodium dithionite (0.73 g, 4.2 mmol) was added followed by water (4 mL). The ice bath was removed and the reaction was allowed to warm over the course of 90 minutes. The reaction

mixture was extracted with ethyl acetate (2 X 10 mL), dried over sodium sulfate and concentrated. The mixture was purified by preparative thin layer chromatography (2:1 hexanes:ethyl acetate) to yield **2** (20 mg, 42%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.61 (dd, *J* = 8.3, 1.9 Hz, 1H), 7.43 (d, *J* = 1.9 Hz, 1H), 6.69 (d, *J* = 8.3 Hz, 1H), 4.32 (q, *J* = 7.1 Hz, 2H), 4.14 – 4.02 (m, 3H), 3.29 (s, 2H), 2.26 (tt, *J* = 7.1, 1.0 Hz, 2H), 1.56 – 1.44 (m, 2H), 1.48 – 1.32 (m, 5H), 0.90 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (500 MHz, Chloroform-*d*) δ 167.01, 141.28, 133.23, 123.95, 121.04, 118.31, 110.66, 80.62, 77.33, 72.26, 68.92, 64.64, 60.49, 34.22, 30.28, 22.02, 19.01, 14.55, 13.61.



3-Amino-4-(cyclohexylamino)benzoic acid (9). To a solution of ferrostatin-1 (0.32 g, 1.22 mmol) in methanol (10 mL) was added 6 M sodium hydroxide (5 mL). The mixture was brought to reflux and stirred overnight. The reaction mixture was partitioned between water and dichloromethane. The organic extracts were dried over sodium sulfate, decanted, and concentrated under

vacuum to yield **9** (0.26 g, 94%) as a brown solid. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.65 (dd, *J* = 8.4, 2.0 Hz, 1H), 7.46 (d, *J* = 2.0 Hz, 1H), 6.61 (d, *J* = 8.5 Hz, 1H), 3.36 (tt, *J* = 10.1, 3.8 Hz, 1H), 2.14 – 2.03 (m, 2H), 1.79 (dt, *J* = 13.2, 3.9 Hz, 2H), 1.68 (dt, *J* = 12.7, 3.8 Hz, 1H), 1.49 – 1.33 (m, 2H), 1.33 – 1.18 (m, 3H).

3-Amino-4-(cyclohexylamino)-*N*-(2-morpholinoethyl)benzamide (3). To a solution of **9** (59 mg, 0.253 mmol) in DMF (5 mL) was added 1-hydroxybenzotriazole (102 mg, 0.76 mmol), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (96 mg, 0.506 mmol), sodium bicarbonate (63 mg, 0.76 mmol) and 4-(2-aminoethyl)morpholine (0.05 mL, 0.38 mmol). The mixture was stirred at room temperature overnight. The mixture was extracted into dichloromethane (3 X 25 mL). The combined organic layers were dried over sodium sulfate, decanted, and concentrated under vacuum. Preparative thin layer chromatography (1:9 methanol:dichloromethane) afforded **3** (35.5 mg, 40%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.25 (d, *J* = 2.1 Hz, 0H), 7.20 (dd, *J* = 8.2, 2.1 Hz, 0H), 6.60 (d, *J* = 8.4 Hz, 1H), 3.72 (t, *J* = 4.6 Hz, 2H), 3.55 – 3.48 (m, 1H), 3.30 (tt, *J* = 10.0, 3.7 Hz, 0H), 2.95 (s, 1H), 2.87 (s, 1H), 2.58 (t, *J* = 6.0 Hz, 1H), 2.50 (d, *J* = 9.4 Hz, 1H), 2.50 (s, 1H), 2.07 (dt, *J* = 12.8, 3.9 Hz, 1H), 1.78 (dt, *J* = 13.6, 3.9 Hz, 1H), 1.66 (dq, *J* = 11.5, 3.7 Hz, 0H), 1.46 – 1.33 (m, 1H), 1.31 – 1.16 (m, 2H). ^{13}C NMR (500 MHz, Chloroform-*d*) δ 167.69, 162.64,

140.66, 132.84, 123.10, 120.17, 116.61, 110.12, 77.36, 67.09, 57.28, 53.47, 51.60, 36.59, 36.02, 33.49, 31.56, 26.03, 25.08.

4-Chloro-*N*-(2-morpholinoethyl)-3-nitrobenzamide (10) To a solution of 4-chloro-3-nitrobenzoyl chloride (1 g, 4.54 mmol) in dioxane (25 mL) was added potassium carbonate (1.25 g, 9 mmol) and 4-(2-aminoethyl)-morpholine (0.65 mL, 4.99 mmol). The suspension was allowed to stir overnight at room temperature. The mixture was partitioned between dichloromethane and saturated sodium bicarbonate (50 mL each). The aqueous layer was extracted twice more with dichloromethane (20 mL). The combined organic layers were dried over sodium sulfate, decanted, and concentrated under vacuum. The crude mixture was purified using a gradient of 0→10% methanol in dichloromethane to afford **10** (1.3 g, 91%) as a white solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.27 (d, *J* = 2.1 Hz, 1H), 7.93 (dd, *J* = 8.4, 2.1 Hz, 1H), 7.63 (d, *J* = 8.4 Hz, 1H), 6.93 (s, 1H), 3.75 – 3.69 (m, 4H), 3.59 – 3.52 (m, 2H), 2.61 (t, *J* = 5.9 Hz, 2H), 2.50 (dd, *J* = 5.7, 3.7 Hz, 4H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 164.16, 147.96, 134.52, 132.42, 131.52, 130.20, 124.29, 67.03, 56.84, 53.46, 36.42.

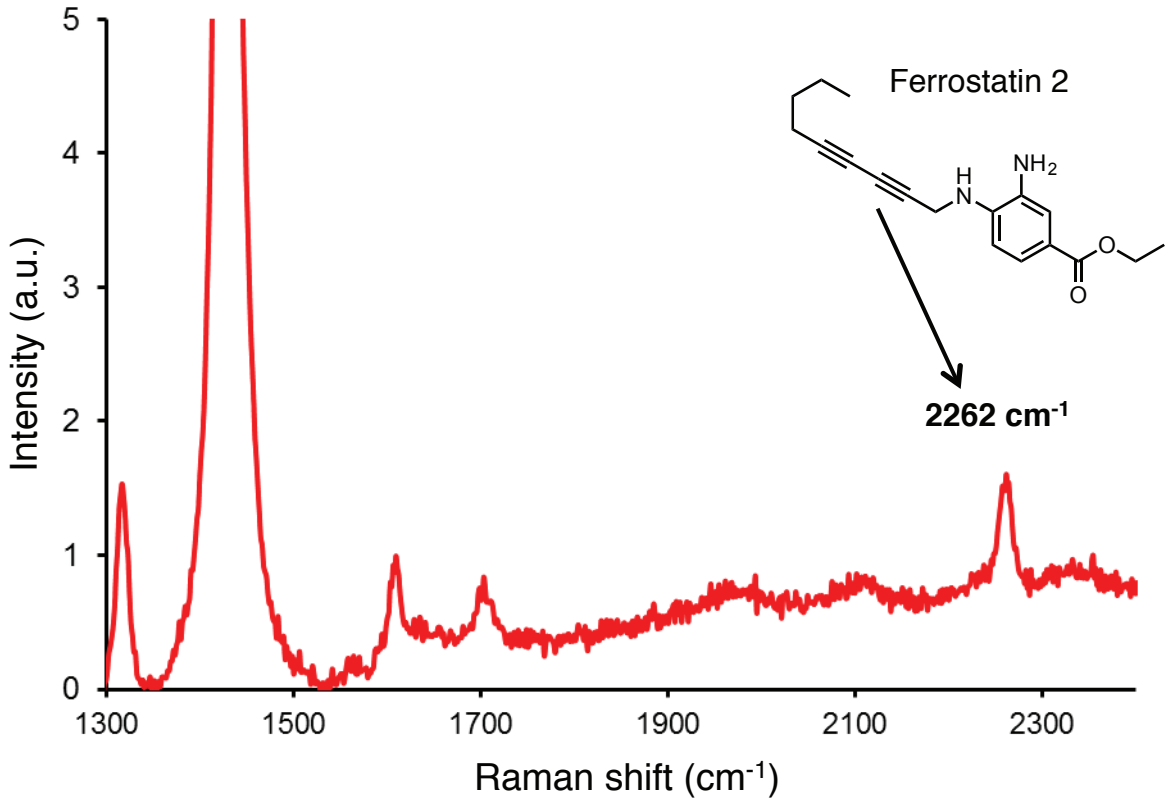
***N*-(2-morpholinoethyl)-3-nitro-4-(prop-2-yn-1-ylamino)benzamide (11)** To a solution of **10** (1 g, 3.18 mmol) in DMSO (10 mL) was added propargylamine (0.21 mL, 3.82 mmol) and potassium carbonate (0.88g, 6.34 mmol). The mixture was stirred at 30 ºC for four days. The mixture was partitioned between

dichloromethane and saturated sodium bicarbonate (25 mL each). The aqueous layer was extracted twice more with dichloromethane (10 mL). The combined organic layers were dried over sodium sulfate, decanted, and concentrated under vacuum. The crude mixture was purified preparative thin layer chromatography (9:1 dichloromethane:methanol) to yield **11** (0.18 g, 17%) as a yellow solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.61 (d, *J* = 2.2 Hz, 1H), 8.35 (s, 1H), 8.01 (dd, *J* = 8.9, 2.2 Hz, 1H), 7.04 (d, *J* = 8.9 Hz, 1H), 6.73 (s, 1H), 4.18 (dd, *J* = 5.8, 2.5 Hz, 2H), 3.74 (t, *J* = 4.7 Hz, 4H), 3.55 (m, 2H), 2.65 – 2.57 (m, 2H), 2.52 (t, *J* = 4.6 Hz, 4H), 2.33 (t, *J* = 2.4 Hz, 1H).

***N*-(2-morpholinoethyl)-3-nitro-4-(nona-2,4-diyn-1-ylamino)benzamide (12)** To a solution of THF (6 mL), water (2 mL), and ethylamine (70% in water, 1 mL), was added **11** (0.1 g, 0.3 mmol) followed by hydroxylamine hydrochloride (18 mg, 0.21 mmol) and copper (I) chloride (7 mg, 0.07 mmol). This mixture was allowed to stir at room temperature for 5 minutes. Separately, a solution of 1-iodohex-1-yne (37 mg, 0.18 mmol) was dissolved in 2 mL THF. This solution was added dropwise and the whole mixture was allowed to stir for 5 h at room temperature. A dark green color developed. The reaction mixture was poured over 13 mL of water containing 0.81 g KCN, and extracted with diethyl ether (3 X 10 mL), dried over sodium sulfate and concentrated under vacuum. The crude mixture was purified preparative thin layer chromatography (9:1 dichloromethane:methanol) to yield **12** (29 mg, 39%) as a yellow solid. ^{1}H NMR

(300 MHz, Chloroform-*d*) δ 8.60 (d, *J* = 2.2 Hz, 1H), 8.33 (t, *J* = 5.8 Hz, 1H), 8.00 (ddd, *J* = 9.0, 2.2, 0.6 Hz, 1H), 7.01 (d, *J* = 8.9 Hz, 1H), 6.75 (s, 1H), 4.23 (dt, *J* = 5.9, 1.1 Hz, 2H), 3.79 – 3.67 (m, 4H), 3.61 – 3.49 (m, 2H), 2.61 (dd, *J* = 7.1, 4.9 Hz, 2H), 2.56 – 2.45 (m, 4H), 2.26 (tt, *J* = 7.0, 1.0 Hz, 2H), 1.57 – 1.31 (m, 4H), 0.90 (t, *J* = 7.2 Hz, 3H).

3-Amino-*N*-(2-morpholinoethyl)-4-(nona-2,4-diyn-1-ylamino)benzamide (4) A solution of **12** (29 mg, 0.07 mmol) in ethanol (2 mL, 200 proof) and water (1 mL) was cooled to 0 ºC on an ice bath. Sodium dithionite (73 mg, 0.422 mmol) was added in small portions. The ice bath was removed and the reaction was allowed to stir and warm to room temperature over 4 h. The reaction mixture was extracted with ethyl acetate (2 X 10 mL), dried over sodium sulfate and concentrated under vacuum. The crude mixture was purified preparative thin layer chromatography (9:1 dichloromethane:methanol) to yield **4** (7.6 mg, 29%) as a colorless solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.04 (d, *J* = 5.8 Hz, 1H), 7.52 – 7.42 (m, 1H), 7.40 (d, *J* = 2.0 Hz, 1H), 6.72 (d, *J* = 8.4 Hz, 1H), 4.01 (d, *J* = 27.6 Hz, 7H), 3.85 (q, *J* = 5.6 Hz, 2H), 3.60 (d, *J* = 12.3 Hz, 2H), 3.34 (t, *J* = 5.5 Hz, 2H), 2.96 (d, *J* = 23.9 Hz, 2H), 2.30 – 2.22 (m, 2H), 1.53 – 1.46 (m, 2H), 1.45 – 1.36 (m, 2H), 0.90 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (500 MHz, Chloroform-*d*) δ 167.57, 139.67, 134.23, 125.62, 122.50, 119.64, 116.32, 111.19, 80.61, 72.55, 68.87, 67.19, 64.69, 57.24, 53.52, 36.11, 34.37, 30.31, 22.06, 19.05, 13.64. HRMS (ESI+) *m / z* calc'd for $C_{22}H_{30}N_4O_2$ $(M + H)^+$ 383.2447, found 383.2440.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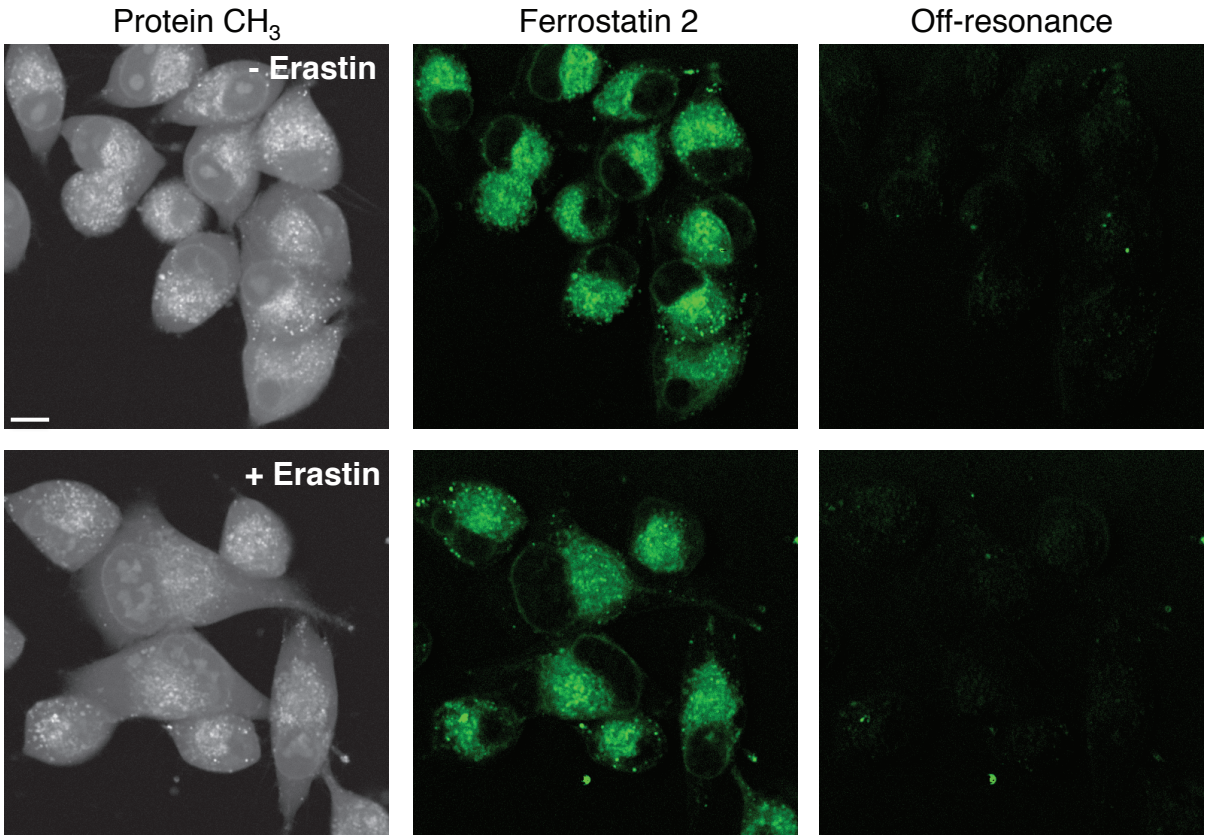

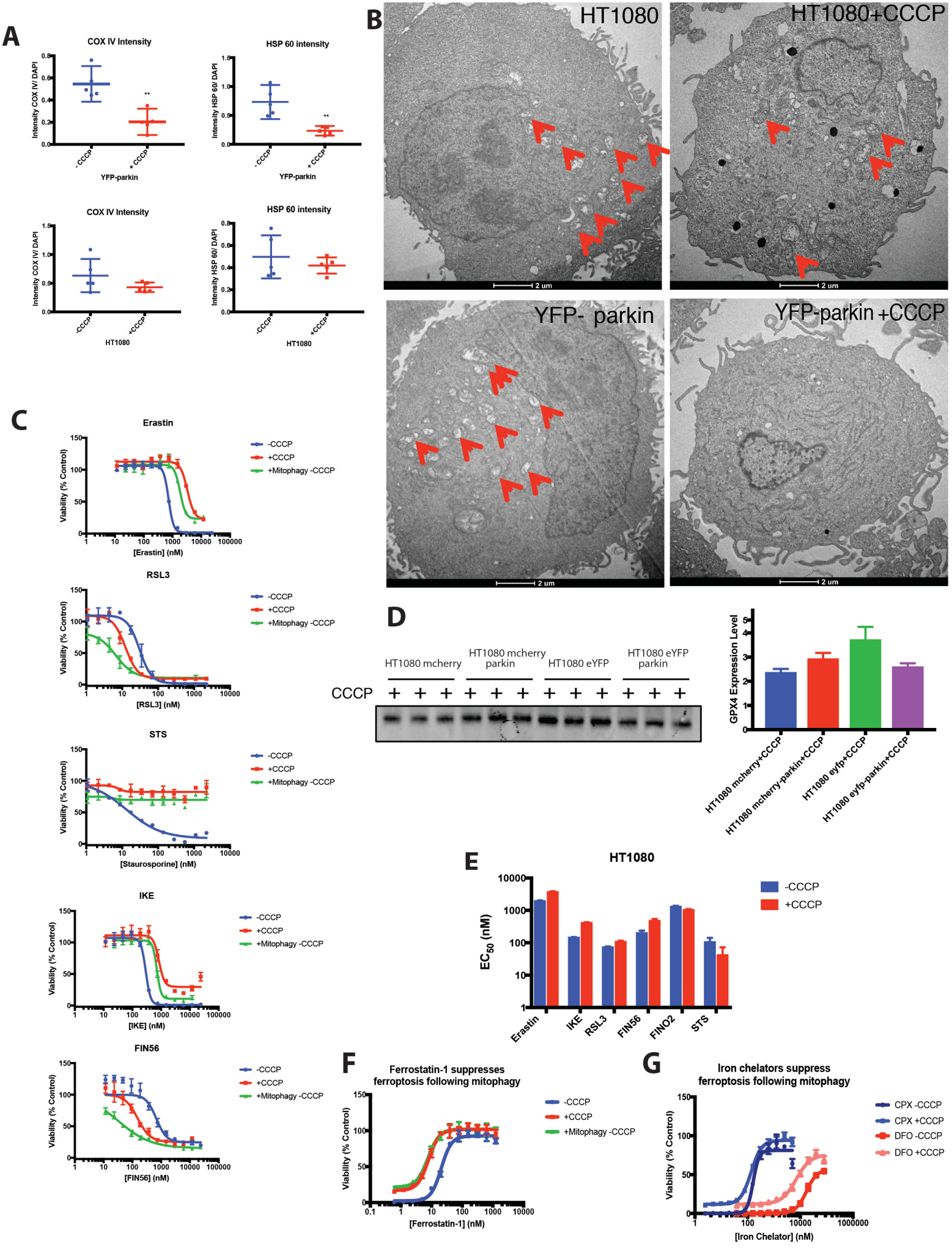
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Viability (% Control)
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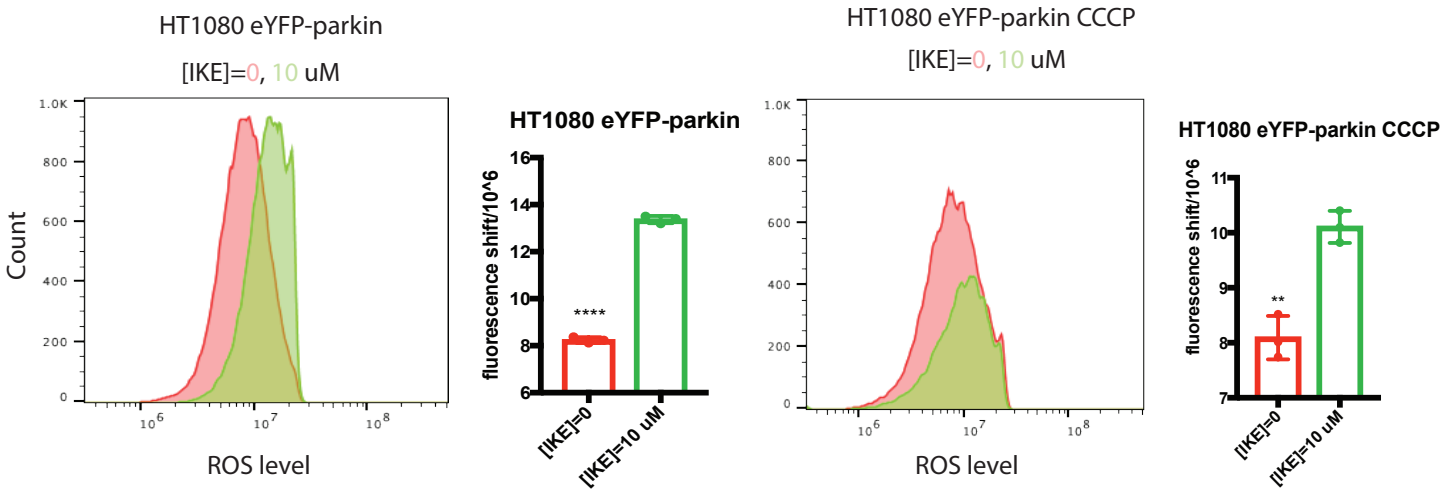
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