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Supplementary materials for

**Anomanolide C suppresses tumor progression and
metastasis by ubiquitinating GPX4-driven autophagy-
dependent ferroptosis in triple negative breast cancer**

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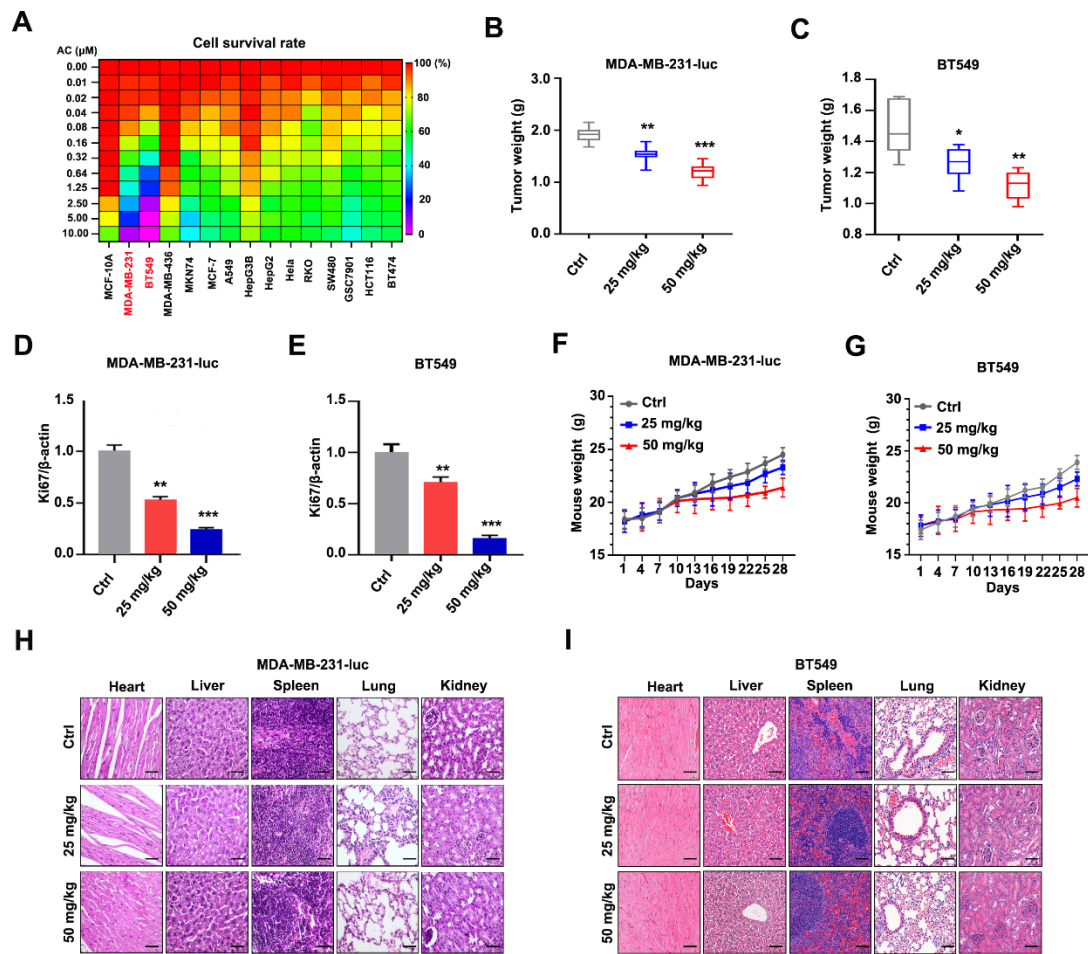
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49 **This file includes:**

50 **Supplementary figures S1 to S13.**

51 **Supplementary tables S1.**

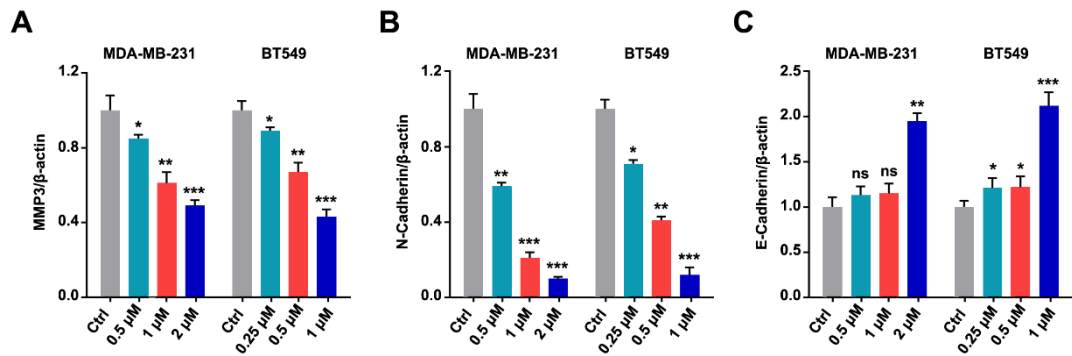
52 **Supplementary Figure 1.**



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54 **Fig. S1** Anomanolide C inhibits breast cancer cells *in vivo* and *in vitro*. (A) Heat map of
 55 mean cell survival values of AC in multiple tumor cell lines at different concentration. Cell
 56 viabilities were measured by CCK8 assay, and the cell survival values were calculated by
 57 Prism 8.0. (B-C) Quantitative analysis of the MDA-MB-231-luc (B) or BT549 (C) cells tumor
 58 weight. (D-E) The statistical diagram of Ki67 protein in MDA-MB-231-luc (D) or BT549 (E)
 59 cells tumors. (F-G) Quantitative analysis of the MDA-MB-231-luc (F) or BT549 (G) cells
 60 treated mice weight. (H-I) H&E staining of heart, liver, spleen, lung, kidney of the
 61 experimental mouse. There has been no obvious toxicity of AC-induced nude mouse. Data
 62 are presented as mean ± SEM. Data are from at least three separate experiments. ns, not
 63 significant, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$. Statistical significance was determined
 64 relative to the respective control groups.

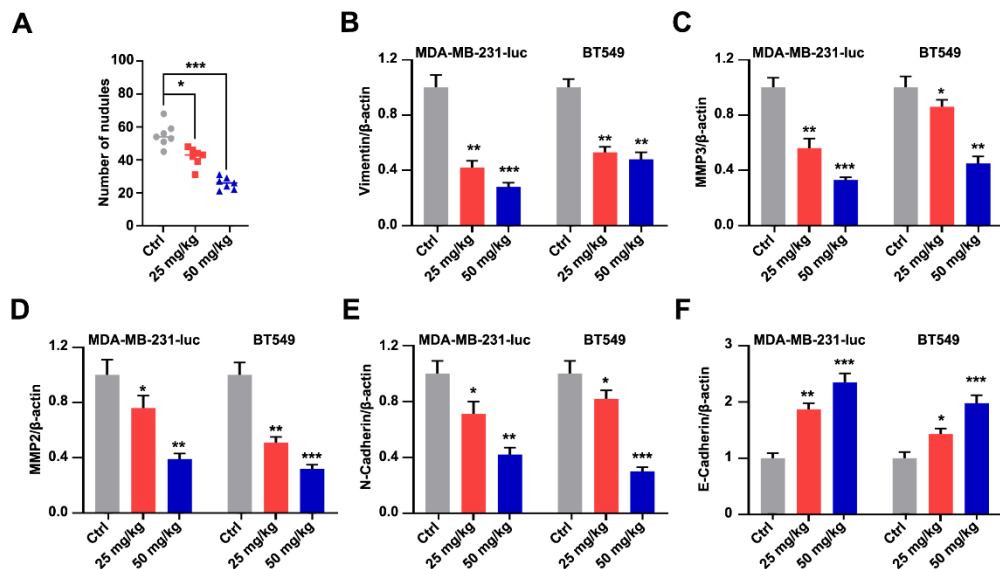
65 **Supplementary Figure 2.**



66 **Fig. S2** (A-C) Quantitative analysis of the MMP3, N-Cadherin, E-Cadherin protein in
 67 MDA-MB-231 and BT549 cells after AC treatment 24 h. Data are presented as mean ±
 68 SEM. Data are from at least three separate experiments. ns, not significant, *, $P < 0.05$, **, $P < 0.01$,
 69 $P < 0.001$. Statistical significance was determined relative to the respective
 70 control groups.

71

72 **Supplementary Figure 3.**

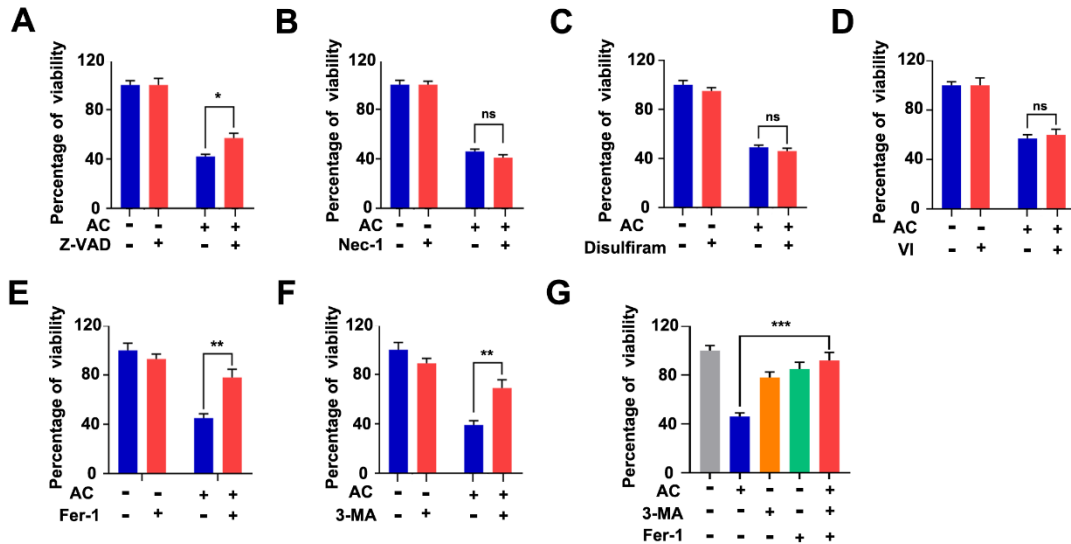


73 **Fig. S3** (A) Tumor cell metastasis was examined by counting metastatic nodules in mouse
 74 lung. (B-F) Quantitative analysis of the vimentin, MMP3, MMP2, N-Cadherin, and E-
 75 Cadherin protein in MDA-MB-231 and BT549 cells induced nude mice tumor which treated
 76 with or without AC. Data are presented as mean ± SEM. Data are from at least three

77 separate experiments. ns, not significant, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$. Statistical
 78 significance was determined relative to the respective control groups.

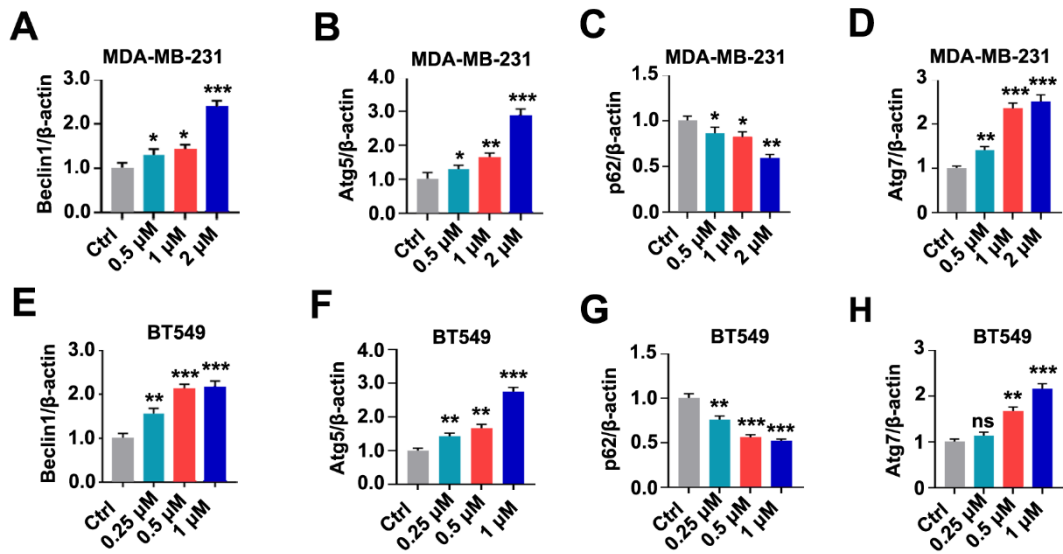
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80 **Supplementary Figure 4.**



81 **Fig. S4 (A-F)** The MDA-MB-231 cell viability of programmed cell death, containing
 82 apoptosis (apoptosis inhibitor, Z-VAD, 40 μ M), necroptosis (necroptosis inhibitor, Nec-1,
 83 30 μ M), pyroptosis (pyroptosis inhibitor, disulfiram, 10 μ M), cuproptosis (cuproptosis
 84 inhibitor, ammonium tetrathiomolybdate(VI), 5 mg/kg), ferroptosis (ferroptosis inhibitor,
 85 Fer-1, 1 μ M), autophagy (autophagy inhibitor, 3-MA, 1 mM) were analyzed after co-
 86 treatment with AC by CCK8 assay. (G) Quantitative analysis of the MDA-MB-231 cell
 87 viability after co-treatment with or without AC, Fer-1, and 3-MA. Data are presented as
 88 mean \pm SEM. Data are from at least three separate experiments. ns, not significant, *, $P <$
 89 0.05, **, $P < 0.01$, ***, $P < 0.001$. Statistical significance was determined relative to the
 90 respective control groups.

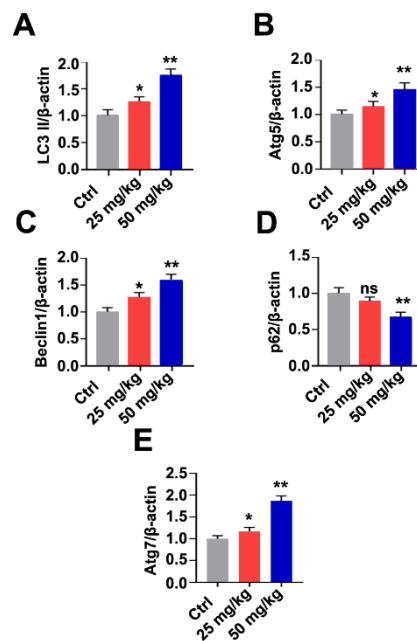
91 **Supplementary Figure 5.**



92 **Fig. S5** (A-D) The protein expression of ATG5, ATG7, Beclin1 and p62 in MDA-MB-231
 93 cells which treated with AC. (D-H) The protein expression of ATG5, ATG7, Beclin1 and p62
 94 in BT549 cells which treated with AC. Data are presented as mean ± SEM. Data are from
 95 at least three separate experiments. ns, not significant, *, $P < 0.05$, **, $P < 0.01$, ***, $P <$
 96 0.001 . Statistical significance was determined relative to the respective control groups.

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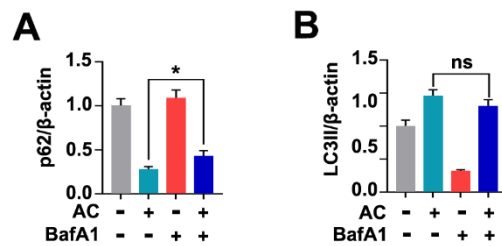
98 **Supplementary Figure 6.**



99 **Fig. S6** (A-E) Quantitative analysis of the protein expression of LC3 II, Atg5, Beclin1, p62,

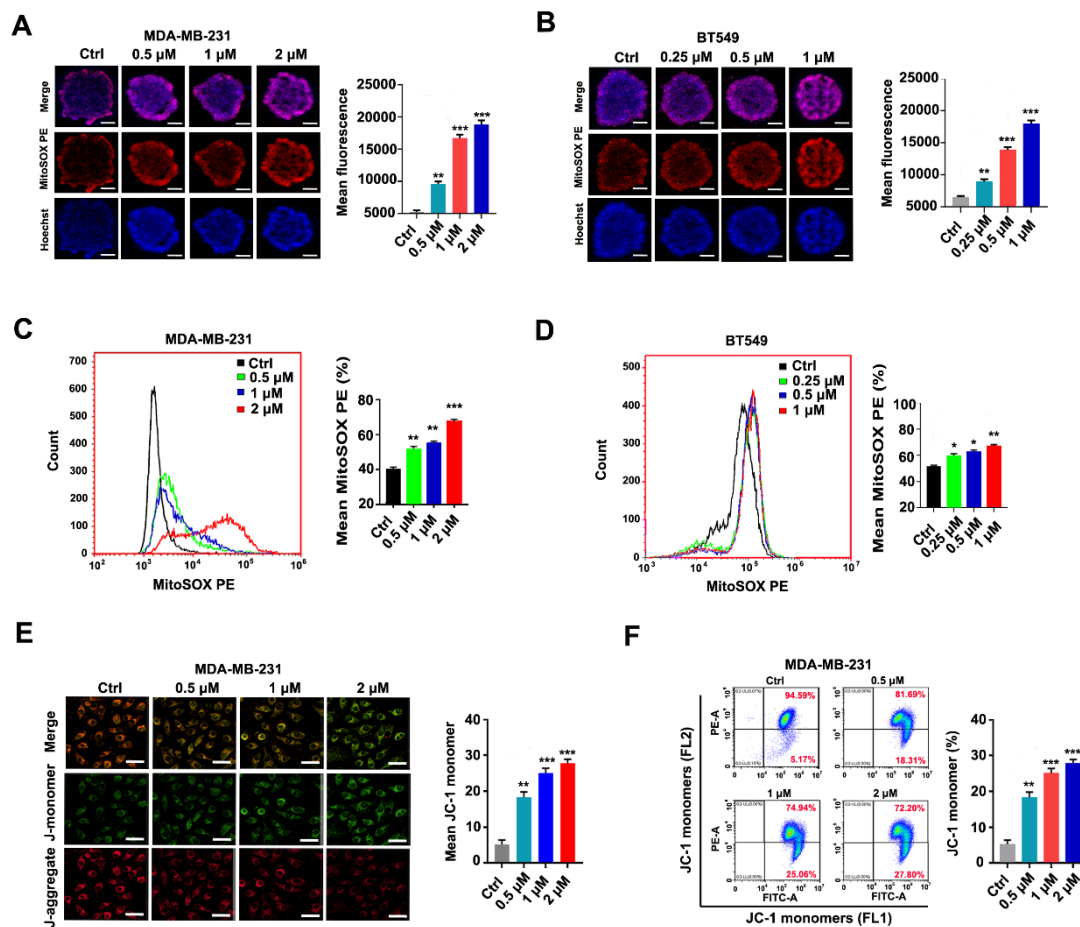
100 and Atg7 which in MDA-MB-231-luc cells induced nude mice tumor, after AC treatment.
101 Data are presented as mean \pm SEM. Data are from at least three separate experiments.
102 ns, not significant, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$. Statistical significance was
103 determined relative to the respective control groups.
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105 **Supplementary Figure 7.**



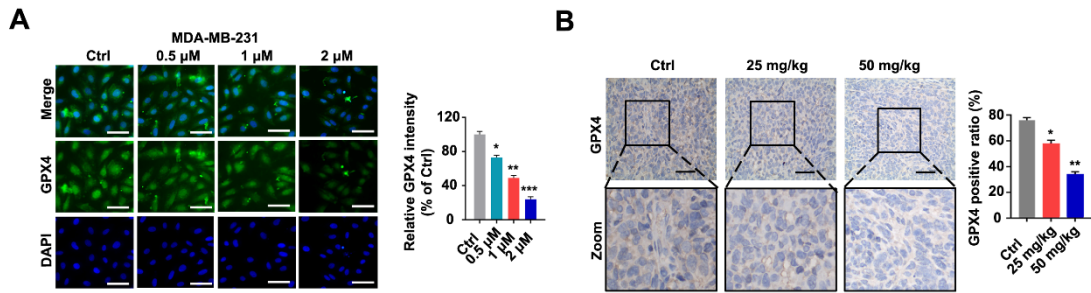
106 **Fig. S7** (A-B) Quantitative analysis of the protein expression of p62, and LC3 II after co-
107 treated with or without AC and BafA1. Data are presented as mean \pm SEM. Data are from
108 at least three separate experiments. ns, not significant, *, $P < 0.05$, **, $P < 0.01$, ***, $P <$
109 0.001. Statistical significance was determined relative to the respective control groups.

110 **Supplementary Figure 8.**



111 **Fig. S8** (A-B) Immunofluorescence analysis of mito-ROS levels in MDA-MB-231 (A) or
 112 BT549 (B) 3D spheroids treated with AC for 24 h. Quantification of immunofluorescence
 113 analysis were shown. Scale bar, 20 μ m. (C-D) The mito-ROS levels of MDA-MB-231 (C)
 114 or BT549 (D) cells were detected by flow cytometry with treated AC. Representative
 115 images and quantitative analysis of mito-ROS levels were shown. (E) Immunofluorescence
 116 analysis of Mitochondrial membrane potential (MMP) levels in MDA-MB-231 cells.
 117 Representative images and quantitative analysis of MMP levels were shown. (F) MMP
 118 levels in MDA-MB-231 cells were detected by flow cytometry using JC-1. Representative
 119 images and quantitative analysis of MMP levels were shown. Data are presented as mean
 120 \pm SEM. Data are from at least three separate experiments. ns, not significant, *, $P < 0.05$,
 121 **, $P < 0.01$, ***, $P < 0.001$. Statistical significance was determined relative to the respective
 122 control groups.

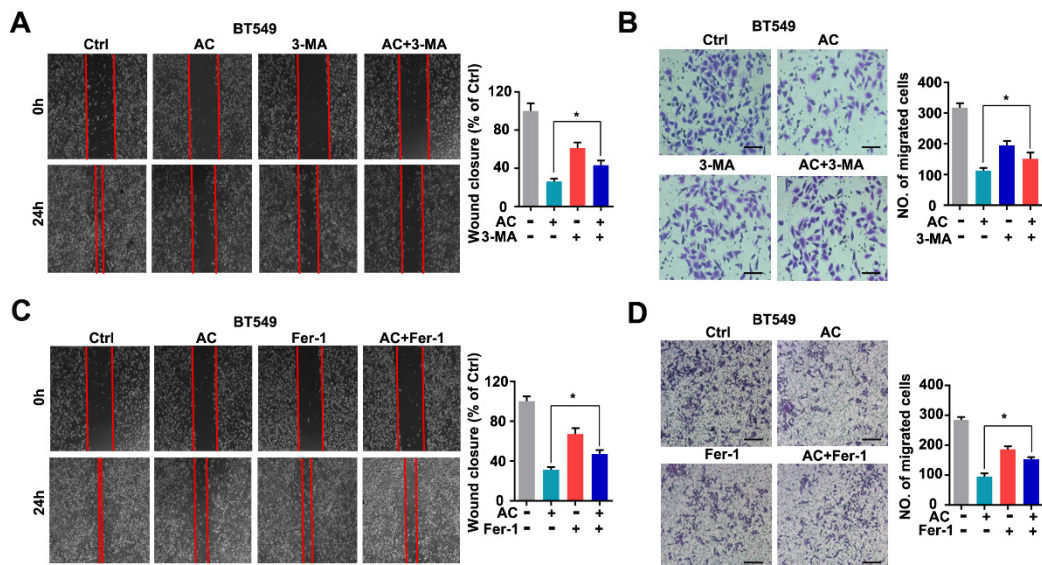
123 **Supplementary Figure 9.**



124 **Fig S9.** (A) Immunofluorescence analysis of GPX4 levels in MDA-MB-231 cells treated
 125 with or without AC for 24 h. Quantitative analysis of GPX4 fluorescence levels were shown.
 126 (B) Expression of GPX4 in tumors of naked mice from control and AC (25 and 50 mg/kg)-
 127 treated groups. The percentage of positive ratios was quantitatively analyzed and is shown
 128 in the images. Scale bar, 40 μm. Data are presented as mean ± SEM. Data are from at
 129 least three separate experiments. ns, not significant, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.
 130 Statistical significance was determined relative to the respective control groups.

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132 **Supplementary Figure 10.**

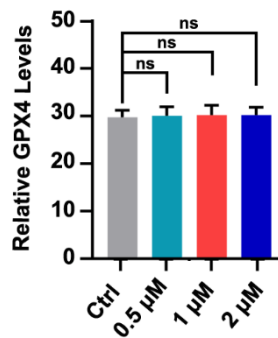


133 **Fig. S10** (A-B) BT549 cells were treated for 24 hours with AC (0.5 μM) alone or in
 134 combination with 3-MA (1 mM); 3-MA was administered 6 hours prior to the AC treatment.
 135 The cells migration' capacities were then evaluated using the scratch and transwell assays.
 136 Representative images and data are shown. Scale bar, 100 μm. (C-D) BT549 cells were

137 treated for 24 hours with AC (0.5 μ M), either alone or in combination with Fer-1 (1 μ M),
138 Fer-1 treatment was 6 6 hours prior to the AC. The ability of the cells to migrate was
139 evaluated using the scratch assay and transwell assay. Representative images and data
140 are shown. Scale bar, 100 μ m. Data are presented as mean \pm SEM. Date are from at least
141 three separate experiments. ns, not significant, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.
142 Statistical significance was determined relative to the respective control groups.

143

144 **Supplementary Figure 11.**

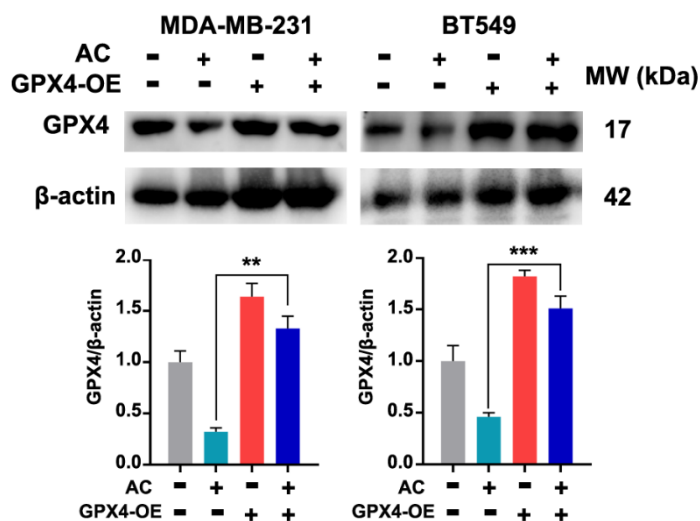


145 **Fig. S11** The expression of GPX4 was tested by qRT-PCR analysis in MDA-MB-231 cells
146 treated with or without AC (1 μ M). Data are presented as mean \pm SEM. Date are from at
147 least three separate experiments. ns, not significant, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.
148 Statistical significance was determined relative to the respective control groups.

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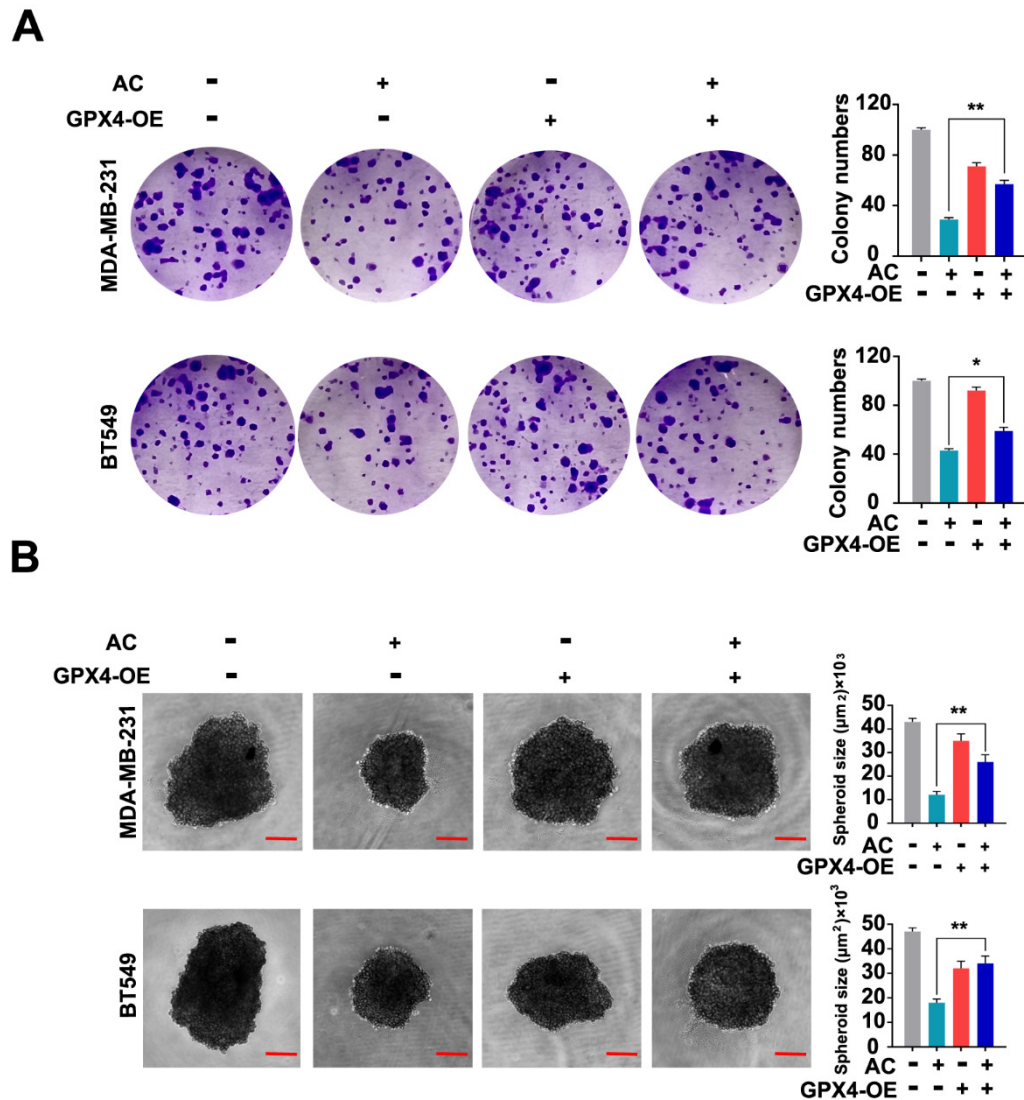
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151 **Supplementary Figure 12.**



152 **Fig. S12** MDA-MB-231 and BT549 cells were transfected with control or overexpression
 153 of GPX4. Besides, the expression levels of GPX4 were determined by immunoblotting
 154 analysis. β -Actin was measured as the loading control. Quantification analysis of GPX4
 155 expression is shown. Data are presented as mean \pm SEM. Data are from at least three
 156 separate experiments. ns, not significant, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$. Statistical
 157 significance was determined relative to the respective control groups.

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172 **Fig. S13** (A) Colony formation of MDA-MB-231 and BT549 cells were tested in the co-
 173 treated with or without AC and overexpression of GPX4 group. Quantification of colonies
 174 and representative images are shown. (C) MDA-MB-231 and BT549 3D spheroids co-
 175 treated with or without AC and overexpression of GPX4 were studied. Quantification of 3D
 176 spheroids volume and representative images are shown. Data are presented as mean \pm
 177 SEM. Data are from at least three separate experiments. ns, not significant, *, $P < 0.05$, **, $P < 0.01$,
 178 ***, $P < 0.001$. Statistical significance was determined relative to the respective
 179 control groups.

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Table S1. Primer sequences for qRT-PCR

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
Actin	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT
GPX4	AAGTTCAGTCAGAGACCTGCG	ATATCCGAGCCCTCCTCCTTC

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