Supplementary Information

Functional preservation of tumor suppressor protein p53 by formation of baicalein-induced higher-order multimers.

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Extended Methods

Protein expression and purification

The recombinant glutathione-S-transferase (GST)-tagged form of DNA-binding domain of p53 wild type (p53-DBD) (residues 94–312) was expressed by *Escherichia coli* BL21 (DE3) in LB medium containing ampicillin (50 μg/mL). *E. coli* cells were incubated at 37°C until OD_{600} ~0.4, and then ethanol was added to a final concentration of 3%. Thereafter, the cells were further incubated for 30 min at 20°C with OD_{600} ~0.6, isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 mM, followed by overnight incubation at 20°C. The cells were collected by centrifugation and the pellets were resuspended in resuspension buffer (20 mM phosphate buffer, pH 6.0, 300 mM NaCl, 1 mM phenylmethanesulfonyl fluoride) and lysed by sonication. The supernatant of lysed cells was applied to a column of DEAE-Sepharose (Cytiva, MA, USA) and affinity purified by a column of COSMOGEL® GST-Accept (Nacalai Tesque, Kyoto, Japan). After the cleavage of the GST-tag using HRV3C protease, the proteins were further purified by size exclusion chromatography with a HiLoad 26/60 Superdex 75 pg (Cytiva) equilibrated with 20 mM phosphate buffer, pH 6.0, 300 mM NaCl, 1 mM dithiothreitol (DTT). The purified proteins were concentrated and dialyzed with assay buffer (20 mM HEPES-NaOH, pH 7.4, 1 mM DTT). After dialysis, the sample was centrifuged at 15,000 ×g at 4°C for 2 min. The supernatant was used in every experiment.

NMR experiments

The samples for NMR measurements were prepared with 50 μM of ^{15}N-labeled p53-DBD solution in 20 mM phosphate buffer, 5% D_2O, pH 7.4. The final concentration of baicalein in the baicalein-added sample was 100 μM, and the final concentration of d_6-DMSO in the control sample was 0.1% (v/v) to contain the same amount of d_6-DMSO as the baicalein-added sample. The ^{1}H-^{15}N HSQC spectra were acquired at 298 K and with 128 scans using an Avance III 900 MHz NMR spectrometer (Bruker, Billerica, MA, USA) equipped with a cryogenic probe. The spectral data were analyzed using Sparky 3.115[1], and the assignments of p53-DBD peaks were referenced from the study by Rasquinha *et al.*[2]

To calculate the chemical shift changes in the ^{1}H-^{15}N HSQC spectra, we used the following eq. (1),

$$\Delta\delta = \sqrt{(\delta \mathrm{H}_1 - \delta \mathrm{H}_2)^2 + \left(\frac{\delta \mathrm{N}_1 - \delta \mathrm{N}_2}{6}\right)^2} \qquad (1)$$

where δH_1 and δH_2 are the values of the chemical shifts of ^1H in the absence and presence of baicalein, respectively, and $\delta N_1$and δN_2 are the values of the chemical shifts of ^{15}N in the absence and presence of baicalein, respectively.

Western blot

The MCF-7 cell line was seeded in 6-well plates at a density of 3.6×10^5 cells per well and treated with 50 μM baicalein for 48 h. Then the cells were lysed in RIPA buffer containing a protease inhibitor cocktail (Roche, Basel, Switzerland) and sonicated using Bioruptor (Cosmo Bio, Tokyo, Japan) for 10 min (duty cycle 50%). Protein concentration was determined by bicinchoninic acid kits (Thermo Fisher Scientific, Waltham, MA, United States). Proteins (4 μg/lane) were separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Merck, Darmstadt, Germany). Subsequently, the membrane was blocked with 5% skim milk in tris buffered saline with 0.1%-detergent (tTBS; Nacalai Tesque) for 1 h at room temperature. Then the membrane was incubated overnight at 4℃ with the primary antibodies for p53 (DO-1, mouse monoclonal, MBL Life Science, Tokyo, Japan) diluted 1:3000 in tTBS. The membrane was washed three times with tTBS and incubated at room temperature for 1 h with secondary antibodies (HRP conjugated anti-mouse IgG, Promega, Madison, WI, USA) diluted to 1:40000 in tTBS. The signals were developed with Chemi-Lumi One Super solution (Nacalai Tesque) and detected using the Lumino Graph (ATTO, Toyko, Japan). Quantification of the bands was performed using ImageJ software (version 1.54g, NIH).

Immunofluorescence imaging

The MCF-7 cell line was seeded on coverslips and treated with 50 μM baicalein for 48 h. Then the coverslips were rinsed twice with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde for 10 min at room temperature. After being rinsed with PBS, the cells were permeabilized with 0.5% Triton X-100 in PBS for 10 min and blocked with 3% BSA in PBS for 1 h at room temperature. Then the coverslips were incubated at 4℃ overnight with the primary antibodies for p53 (DO-1) diluted 1:200 in blocking buffer. The coverslips were washed three times with PBS containing 0.1% Tween 20 (PBS-T) and incubated at room temperature for 1 h with the secondary antibodies (Cy-3 conjugated goat anti-mouse, Sigma-Aldrich) diluted to 1:1000 in PBS-T. Subsequently, the coverslips were washed three times with PBS-T and mounted with VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories, Inc., Newark, CA,

United States). The fluorescence images were acquired with fluorescence microscopy (IX-71, Evident, Tokyo, Japan) equipped with a color charge-coupled device camera DP-70 (Evident) or SpinSR10 spinning disc confocal microscope (Evident). Deconvolution was performed using cellSens software on images obtained by SpinSR10. The objective lens was × 100.

Calculation of Combination Index (CI)

The MCF-7 cell line was seeded in 96-well plates at 9.6×10^3 cells per well. Following a 24 h incubation, the cells were cultured in the medium with the final concentration of baicalein at 0, 10, 30, or 50 μM and/or cisplatin at 0, 5, 15, or 25 μM for 48 h. Subsequently, the cell viability assay was performed. The CI was the values calculated on CompuSyn software [3–5] using the results of the cell viability assay with co-treatment of cisplatin and baicalein. The cell viabilities of the cells treated with baicalein only, cisplatin only, and baicalein and cisplatin in combination, obtained from the results of the WST-8 assay, were converted to *fa* (fraction affected), which is the rate of cell proliferation inhibition relative to the control, and the CI values were calculated using eq. (2),

$$\mathrm{CI} = \frac{(\mathrm{D})_1}{(\mathrm{Dx})_1} + \frac{(\mathrm{D})_2}{(\mathrm{Dx})_2} = \frac{(\mathrm{D})_1}{(\mathrm{Dm})_1\left[\frac{fa}{1-fa}\right]^{1/m_1}} + \frac{(\mathrm{D})_2}{(\mathrm{Dm})_2\left[\frac{fa}{1-fa}\right]^{1/m_2}} \quad (2)$$

where Dm and *m* are constants calculated from the concentration of each compound when treated individually and its *fa*, and $(D)_1$ and $(D)_2$ are the cisplatin and baicalein concentrations in co-treatment. $(Dx)_1$ is calculated from eq. (3),

$$(\mathrm{Dm})_1 * \left[\frac{fa}{1-fa}\right]^{1/m_1} \quad (3)$$

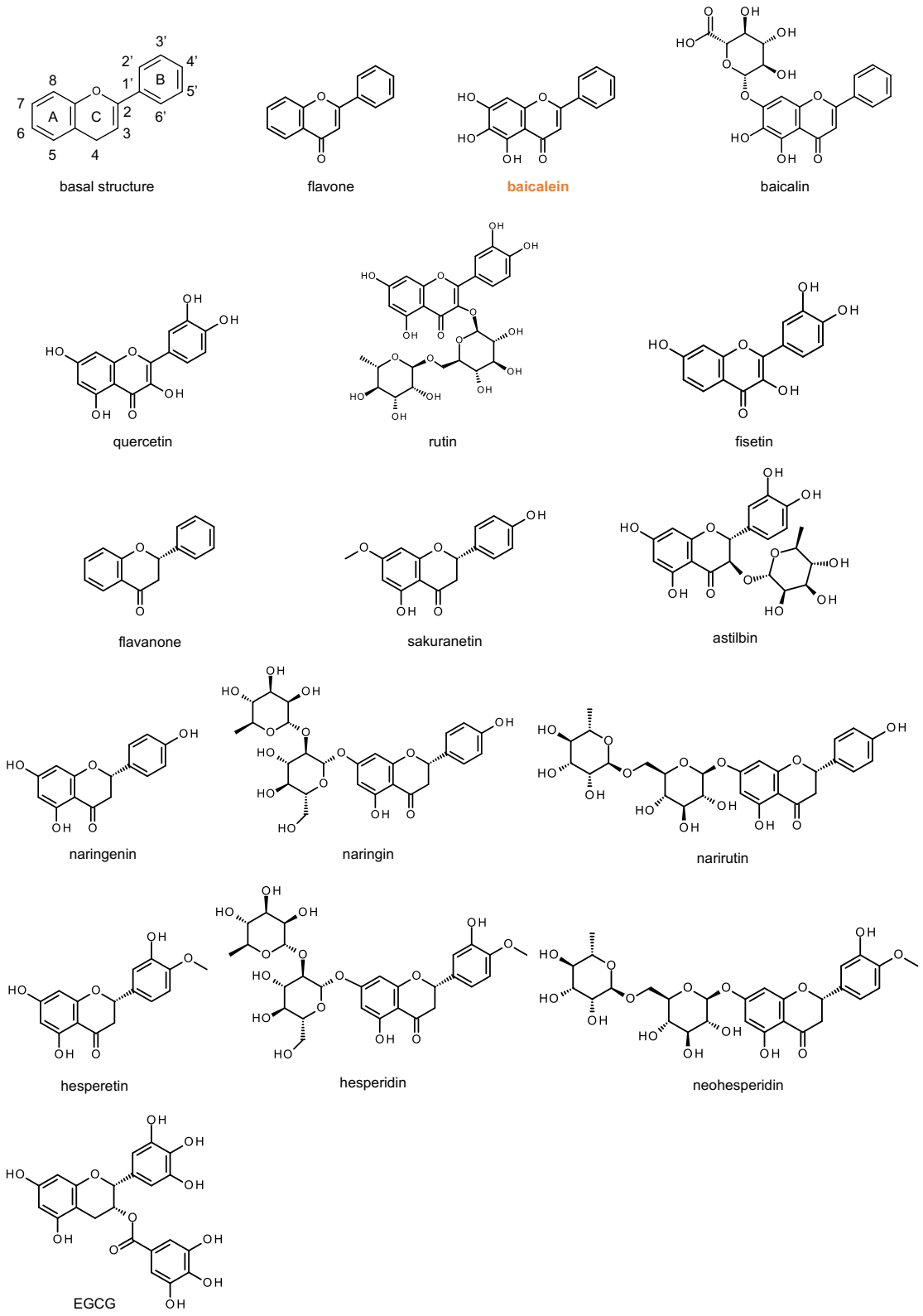
and $(Dx)_2$ is calculated from eq. (4),

$$(\mathrm{Dm})_2 * \left[\frac{fa}{1-fa}\right]^{1/m_2} \quad (4).$$

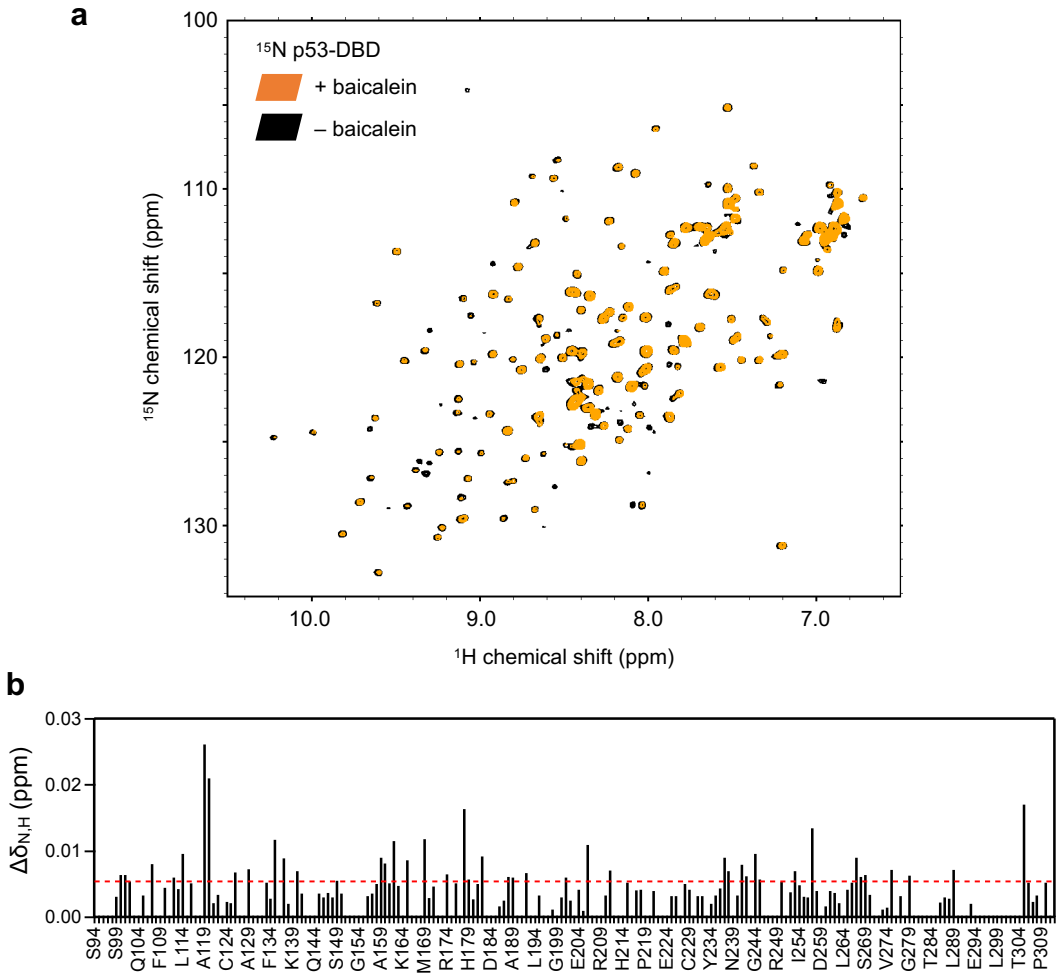
The CI values indicate synergistic (CI<0.9), additive (0.9 ≦ CI ≦ 1.1), or antagonistic effects (CI > 1.1).

RNA sequencing analysis

Total RNA was used to construct sequencing libraries with the NEBNext Poly(A) mRNA Magnetic Isolation Module and the NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, USA), following the manufacturer's protocols. The resulting libraries were assessed for quality and quantified before sequencing. Sequencing was performed on the Illumina NextSeq 550 system (Illumina, USA) using the NextSeq 500/550 High Output Kit v2.5 (75 cycles), to generate 81 bp single-end reads. The FASTQ files were analyzed using BaseSpace Sequence Hub (Illumina), and the count data were obtained. Principal component analysis and gene expression analysis were performed for the count data. The 512 genes with a fold change greater than 1 and a p-value greater than 0.01 after multiple comparison correction were listed and pathway analysis and GO analysis were performed on the Metascape (https://metascape.org)[6]. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used for pathway analysis, and biological processes were used as a group of terms for GO analysis.



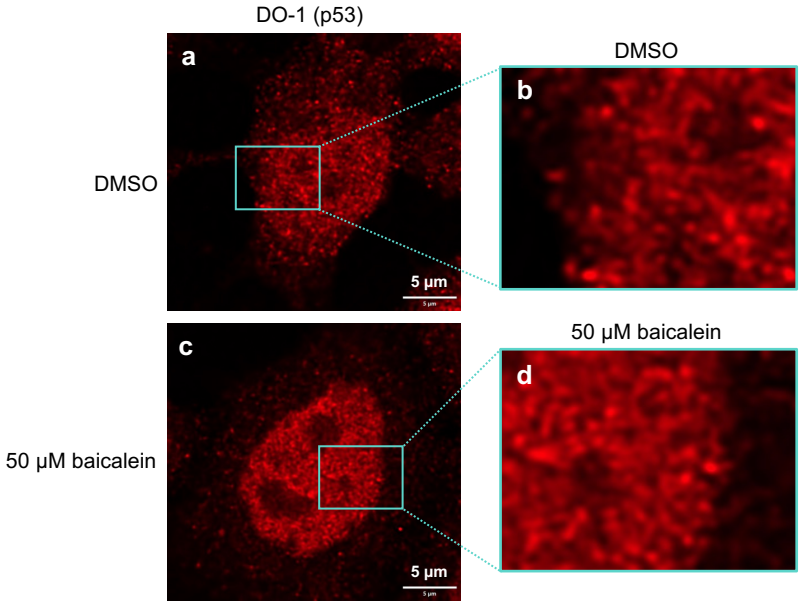
Supplementary Figure 1. The chemical structure of flavonoids used in the screening experiments.



Supplementary Figure 2. Baicalein did not change the peak positions significantly in the ^{1}H-^{15}N HSQC spectrum of ^{15}N-p53-DBD.

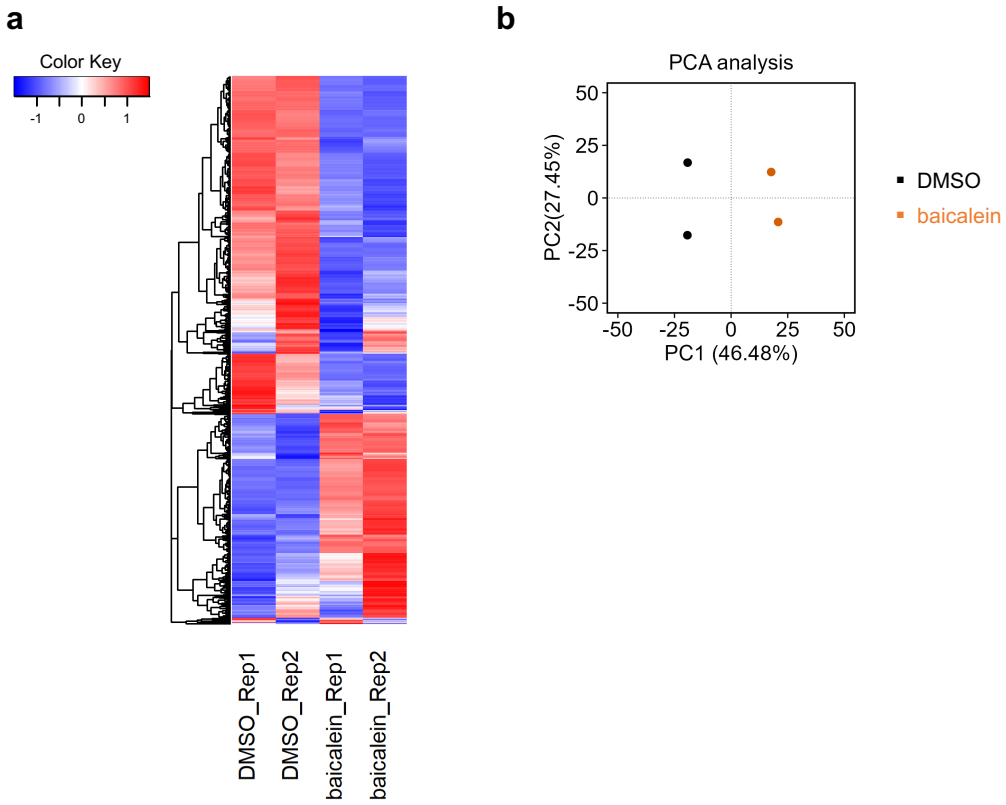
(a) ^{1}H-^{15}N HSQC spectra of ^{15}N-p53-DBD in the absence (black) and presence (orange) of baicalein.

(b) Chemical shift perturbation of the peaks of each amino acid in ^{15}N-p53-DBD with and without baicalein. The red dashed line indicates the average value.



Supplementary Figure 3. Observation of nuclear p53 granules with and without baicalein treatment.

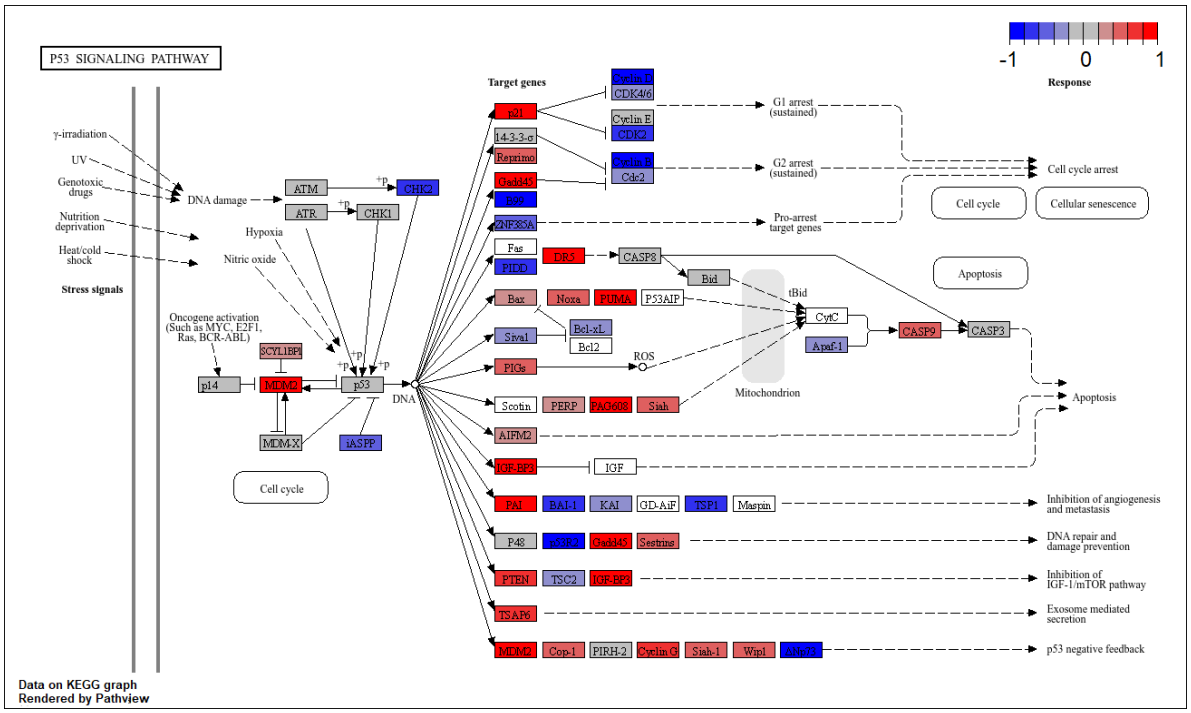
(a-d) MCF-7 cells were treated with **(a,b)** and without **(c,d)** 50 μM baicalein for 48 h, and then observed using a spinning disc confocal microscope after fluorescence immunostaining with anti-p53 antibody DO-1. **(b)** and **(d)** are enlarged images of cyan box region of **(a)** and **(c)**, respectively.



Supplementary Figure 4. Validation between replications in RNA sequencing.

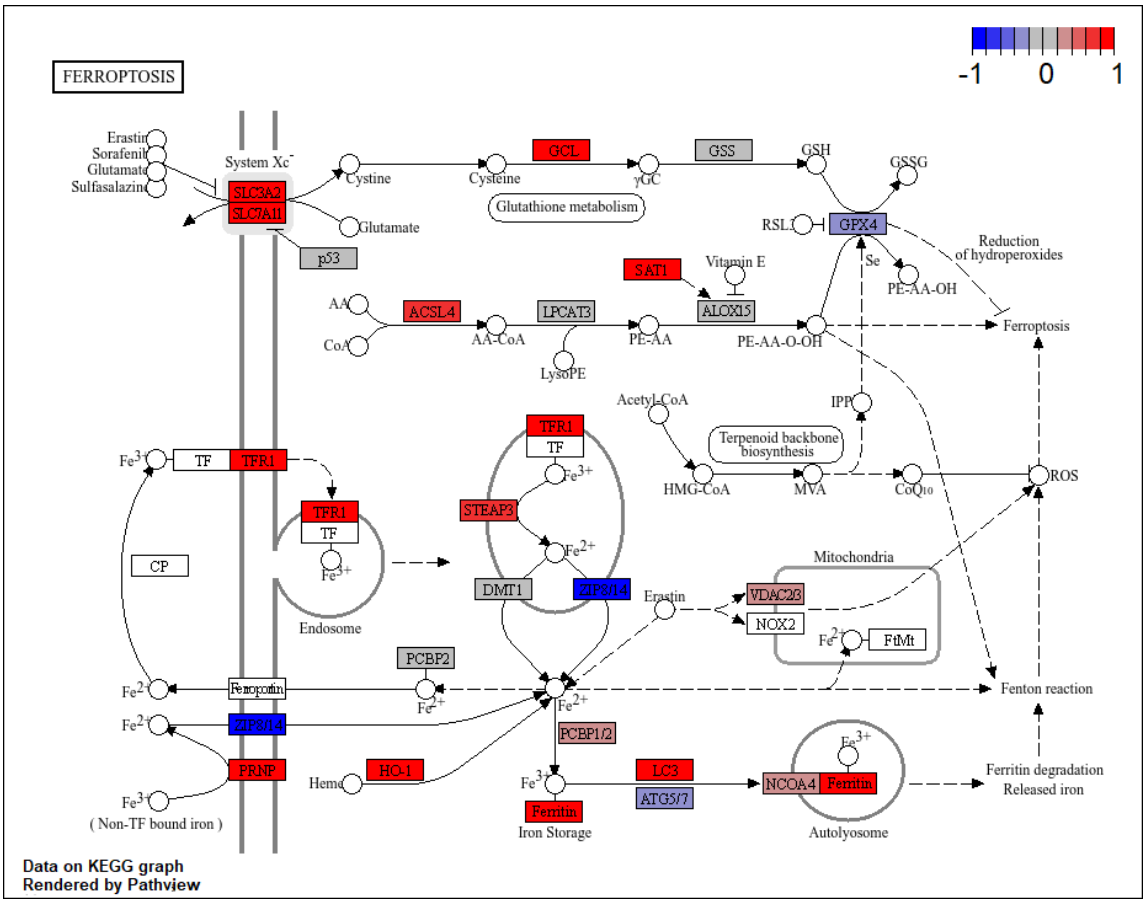
(a) A heat map of the top 2000 genes with large standard deviations in the count data between samples.

(b) PCA plot of RNA sequencing data from control and baicalein treated samples, including Rep1 and Rep2 for each condition.



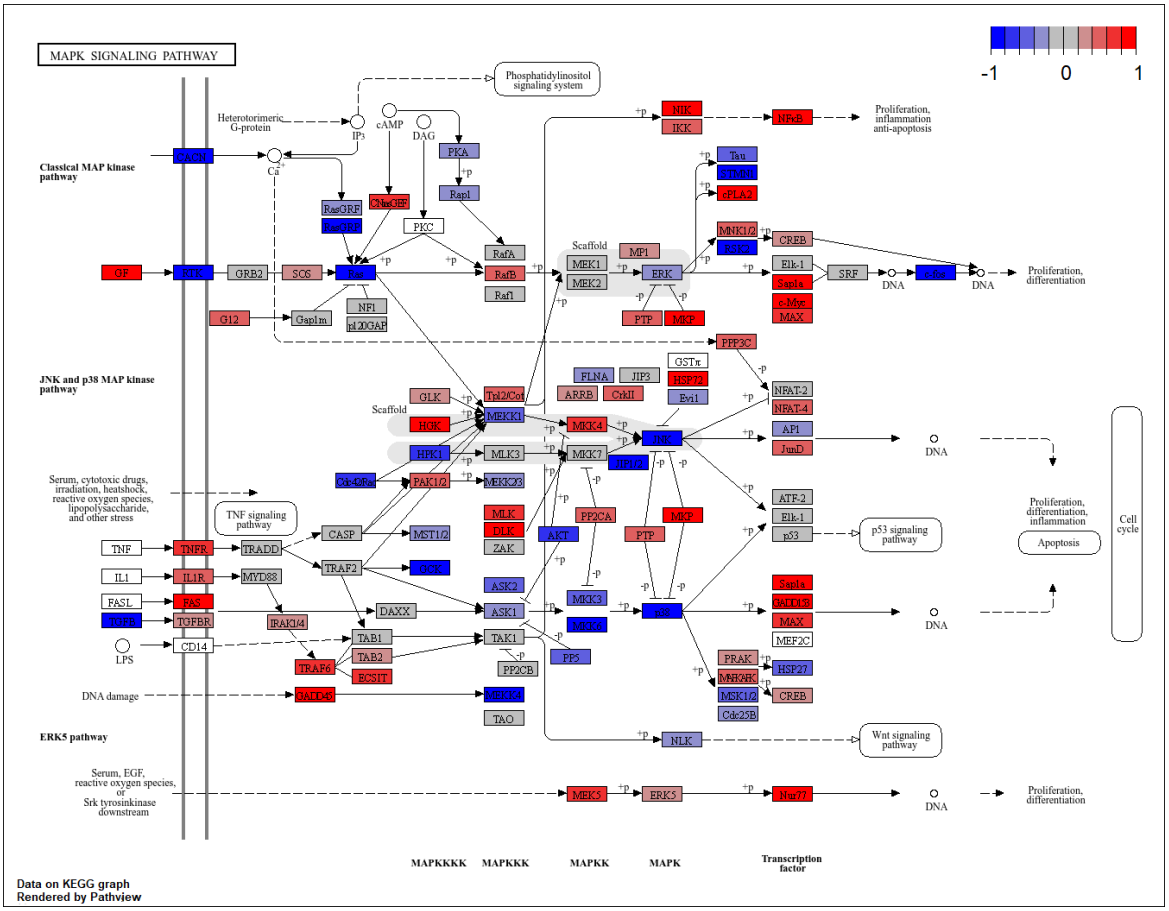
Supplementary Figure 5. Mapping of upregulated and downregulated genes in the p53 signaling pathway in MCF-7 cells treated with baicalein.

Changes in gene expression were visualized using log2 (Fold Change) values comparing baicalein-treated and control groups. The values were scaled ranging from -1 (blue, downregulated), 0 (grey, no change), to +1 (red, upregulated) and plotted onto the KEGG p53 signaling pathway.



Supplementary Figure 6. Mapping of upregulated and downregulated genes in the Ferroptosis pathway in MCF-7 cells treated with baicalein.

Changes in gene expression were visualized using log2 (Fold Change) values comparing baicalein-treated and control groups. The values were scaled ranging from -1 (blue, downregulated), 0 (grey, no change), to +1 (red, upregulated) and plotted onto the KEGG ferroptosis pathway.



Supplementary Figure 7. Mapping of upregulated and downregulated genes in the MAPK signaling pathway in MCF-7 cells treated with baicalein.

Changes in gene expression were visualized using log2 (Fold Change) values comparing baicalein-treated and control groups. The values were scaled ranging from -1 (blue, downregulated), 0 (grey, no change), to +1 (red, upregulated) and plotted onto the KEGG MAPK signaling pathway.

SI References

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