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Supplemental information

Oleanolic acid blocks the purine salvage pathway for cancer therapy by inactivating SOD1 and stimulating lysosomal proteolysis

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Supplemental information

Supplemental Materials and Methods

Apoptosis assay

A549 and MDA-MB-231 cells were seeded into 6-well plates at a density of 1.5 × 10^5/well, and then treated by 200 μM OA for 0, 24, or 48 hours. Spent medium of each well was collected individually to harvest cells by centrifuging at 1500 rpm for 5 minutes, while adherent cells of each well were digested with 0.5% trypsin and harvested. For each well, cells from spent medium and adherent cells were combined together and then washed by PBS for 2 times. Finally, cell apoptosis assay was implemented on a flow cytometer (BD FACSVerse™, New Jersey, USA) using an AnnexinV-FITC and PI Apoptosis Kit (Yuheng Biotechnology, Suzhou, China) according to the manufacturer's instructions.

Cell cycle analysis

A549 and MDA-MB-231 cells were seeded into 6-well plates at a density of 1.5 × 10^5/well and treated by 200 μM OA for 0, 24, or 48 hours. After removal of the spent medium, OA-treated cells and control cells were harvested and fixed with 70% ethanol at -20 °C overnight. Subsequently, the fixed cells were stained with propidium iodide (PI) (Sigma, St. Louis, MO, USA) at a concentration of 36 mg/mL at 37 °C for 15 minutes. Next, these cells were used for fluorescence activated cell sorting (FACS) analysis by a flow cytometer (BD FACSVerse™, New Jersey, USA). Data were analyzed with FlowJo 7.6 software.

Co-immunoprecipitation and ubiquitination assays of HGPRT

For the co-IP assay of HGPRT, A549 cells treated by vehicle or 200 μM OA for 8 hours were harvested and lysed in NP40 buffer (Beyotime, Shanghai, China) for 30 minutes on ice, and then centrifuged at 13,000 ×g for 15 minutes at 4 ℃. The lysates were incubated with anti-rabbit IgG (Cell Signaling Technology, Boston, Massachusetts, USA) or HGPRT antibody (ab109021, Abcam, Cambridge, UK) at 4 ℃ with rocking overnight. Subsequently, the lysates were incubated with protein A/G PLUS-Agarose beads (Santa Cruz, Dallas, Texas, USA) at 4 ℃ for 2 hours. The proteins binding to the beads were washed for three times by using NP40 buffer and then boiled for 10 minutes at 95 ℃, followed by SDS-PAGE, and finally analyzed by immunoblotting with primary antibodies against human hypoxanthine-guanine phosphoribosyltransferase (ab109021, Abcam, Cambridge, UK) and ubiquitin (Santa Cruz, Dallas, Texas, USA).

Table S1. Clinical characteristics of lung cancer patient cohort 1.

	Lung adenocarcinoma (n=5)
Age, years	
Median	64
Range	58-68
Gender, no. (%)	
Male	2（40）
Female	3（60）
TNM stage, no. (%)	
1	2（40）
2	2（40）
3	1（20）

Table S2. Clinical characteristics of lung cancer patient cohort 2.

	Lung adenocarcinoma (n=17)	Lung squamous cell carcinoma (n=17)
Age, years		
Median	60	60
Range	41-75	43-78
Gender, no. (%)		
Male	11（64.7）	14（82.3）
Female	6（35.3）	3（17.6）
TNM stage, no. (%)		
1	5（29.4）	
2	6（35.3）	7（41.2）
3	4（23.5）	5（29.4）
4	1（5.9）	1（5.9）
Unclassified	1（5.9）	4（23.5）

Table S3. Clinical characteristics of breast cancer patient cohort.

	Luminal-like (n=10)	Triple Negative (n=1)	HER2 positive (n=1)	Unclassified (n=8)
Age, years				
Median	52	49	59	47
Range	36-72	49	59	37-69
Gender, no. (%)				
Female	10（100）	1（100）	1（100）	8（100）
TNM stage, no. (%)				
1	3（30）	0（0）	0（0）	0（0）
2	5（50）	1（100）	1（100）	3（37.5）
3	2（20）	0（0）	0（0）	4（50.0）
4	0（0）	0（0）	0（0）	0（0）
Unclassified	0（0）	0（0）	0（0）	1（12.5）

Table S4. Clinical characteristics of lung cancer patient cohort 3.

	Lung adenocarcinoma (n=21)	Lung squamous cell carcinoma (n=13)
Age, years		
Median	60	63
Range	42-79	58-77
Gender, no. (%)		
Male	10（47.6）	11（84.6）
Female	11（52.4）	2（15.4）
TNM stage, no. (%)		
1	1（4.8）	0
2	13（61.9）	10（76.9）
3	6（28.5）	3（23.1）
4	1（4.8）	0

Table S5. Sequences of gene-specific q-PCR primers in this study.

Gene	NCBI Lous ID	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
HPRT1	NM_3251	CCTGGCGTCGTGATTAGTGAT	AGACGTTCAGTCCTGTCCATAA
NT5E	NM_4907	AAGGACTGATCGAGCCACTC	GGAAGTGTATCCAACGATTCCCA
ACTIN	NM_001101.3	CACTCTTCCAGCCTTCCTTC	GTACAGGTCTTTGCGGATGT

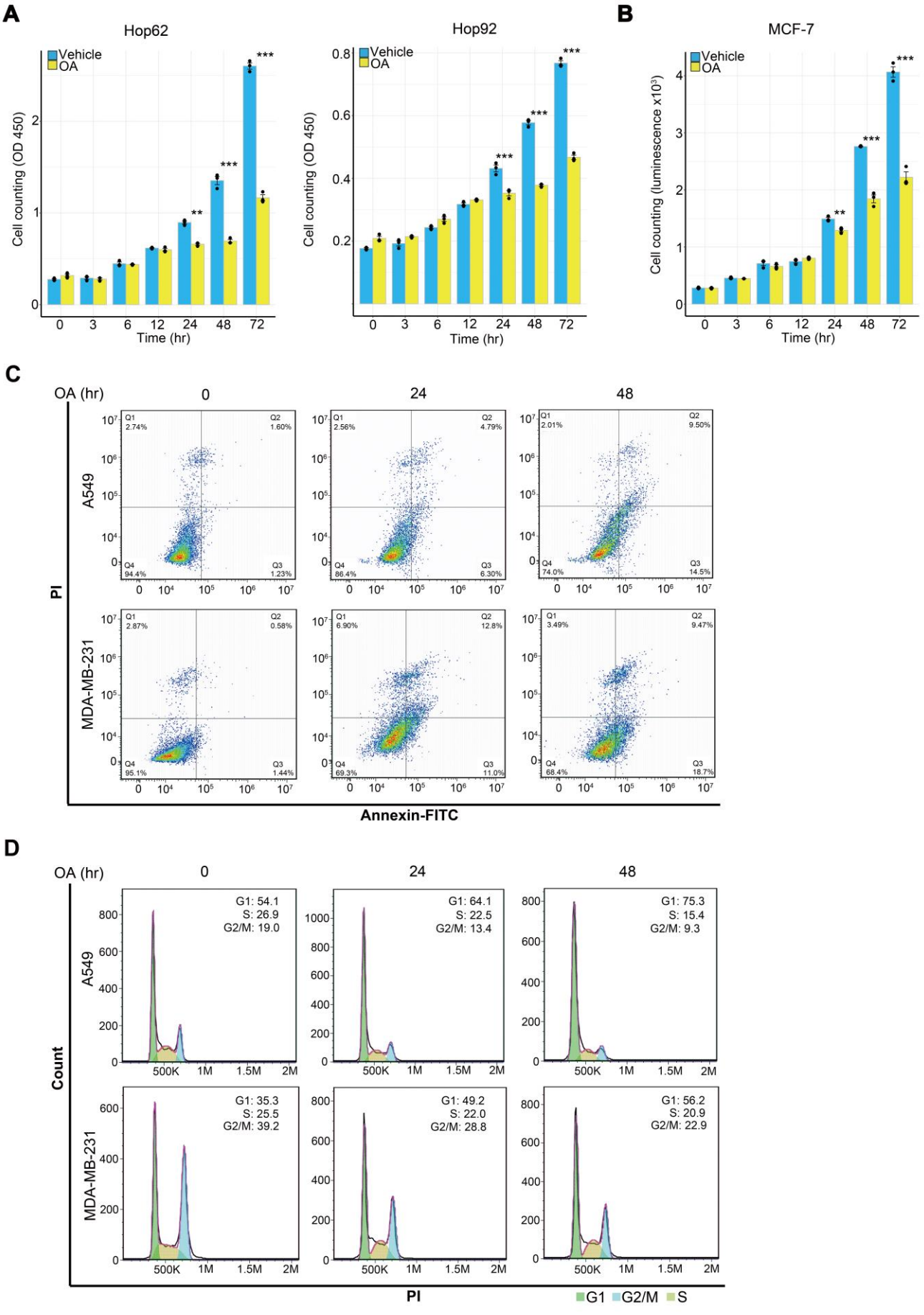
A
Hop62
Hop92
MCF-7
Vehicle
OA
Cell counting (OD 450)
Cell counting (luminescence x10³)
Time (hr)
0 3 6 12 24 48 72
**
***
B
C
OA (hr)
0
24
48
A549
MDA-MB-231
PI
Annexin-FITC
Q1
Q2
Q3
Q4
2.74%
1.60%
94.4%
1.23%
2.56%
4.79%
86.4%
6.30%
2.01%
9.50%
74.0%
14.5%
2.87%
0.58%
95.1%
1.44%
6.90%
12.8%
69.3%
11.0%
3.49%
9.47%
68.4%
18.7%
D
Count
G1: 54.1
S: 26.9
G2/M: 19.0
G1: 64.1
S: 22.5
G2/M: 13.4
G1: 75.3
S: 15.4
G2/M: 9.3
G1: 35.3
S: 25.5
G2/M: 39.2
G1: 49.2
S: 22.0
G2/M: 28.8
G1: 56.2
S: 20.9
G2/M: 22.9
500K 1M 1.5M 2M
PI
G1 G2/M S

Figure S1 Oleanolic acid impedes cancer cell growth and induces cell apoptosis and cell cycle arrest *in vitro*.

(A-B) The time-course influence of 200 μM oleanolic acid (OA) on the cell proliferation of two lung cancer cell lines Hop92 and Hop62, and one breast cancer cell line MCF-7. **(C)** The time course impact of 200 μM OA on cell apoptosis of A549 and MDA-MB-231 cells. **(D)** The time course effect of 200 μM OA on cell cycle of A549 and MDA-MB-231 cells.

Error bars represent mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, Student's t test.

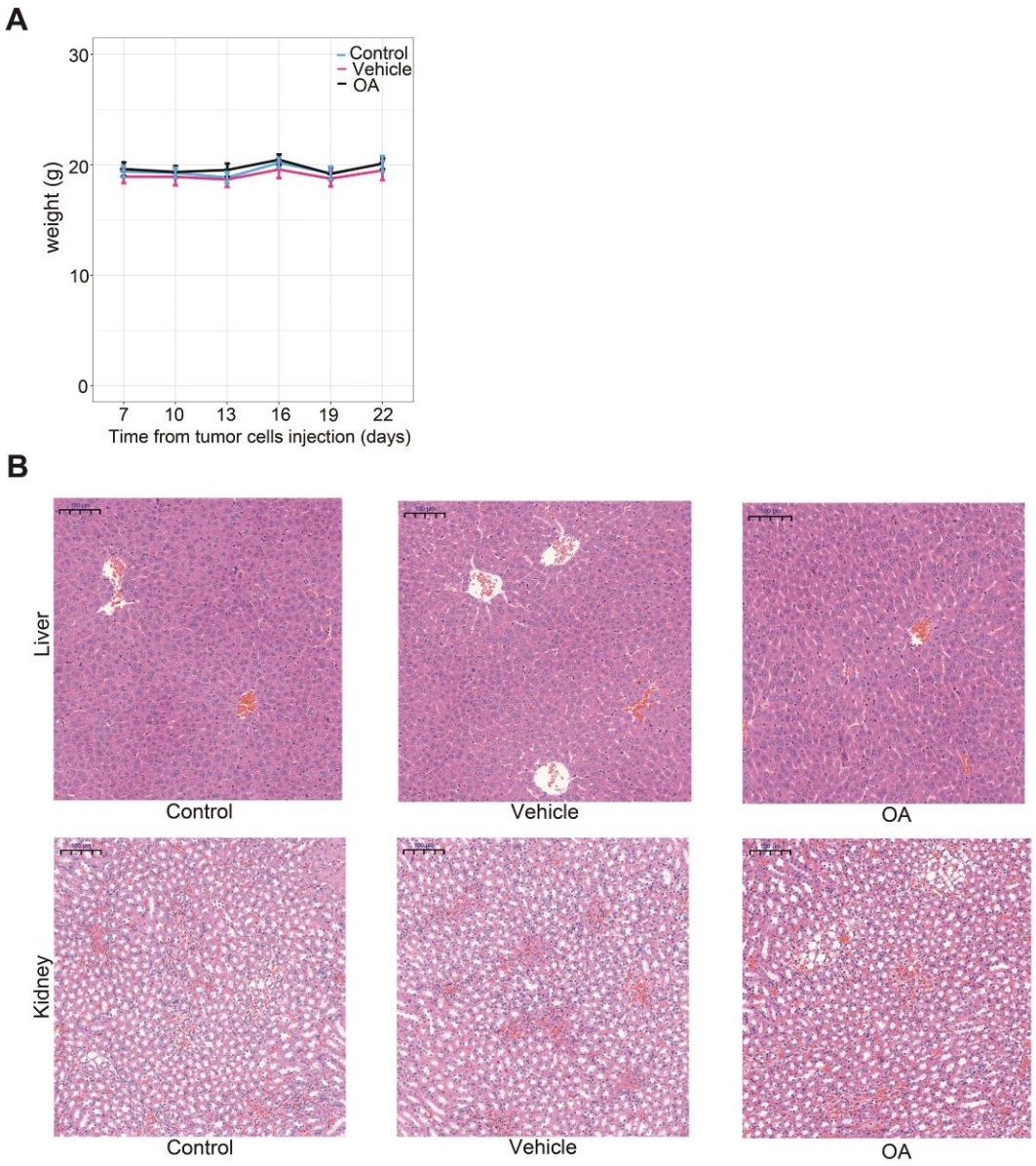

Figure S2. The influence of oleanolic acid treatment on mouse body weight and histological structure of liver and kidney.

(A) Body weight of healthy control mice, A549 tumor-bearing mice treated with vehicle, and A549 tumor-bearing mice treated with oleanolic acid (OA, 120 mg/kg/day). Error bars represent mean ± SEM. **(B)** Histological structure of liver and kidney tissues from healthy control mice, A549 tumor-bearing mice treated with vehicle, and A549 tumor-bearing mice treated with OA (120 mg/kg/day). Scale bars, 100 μm.

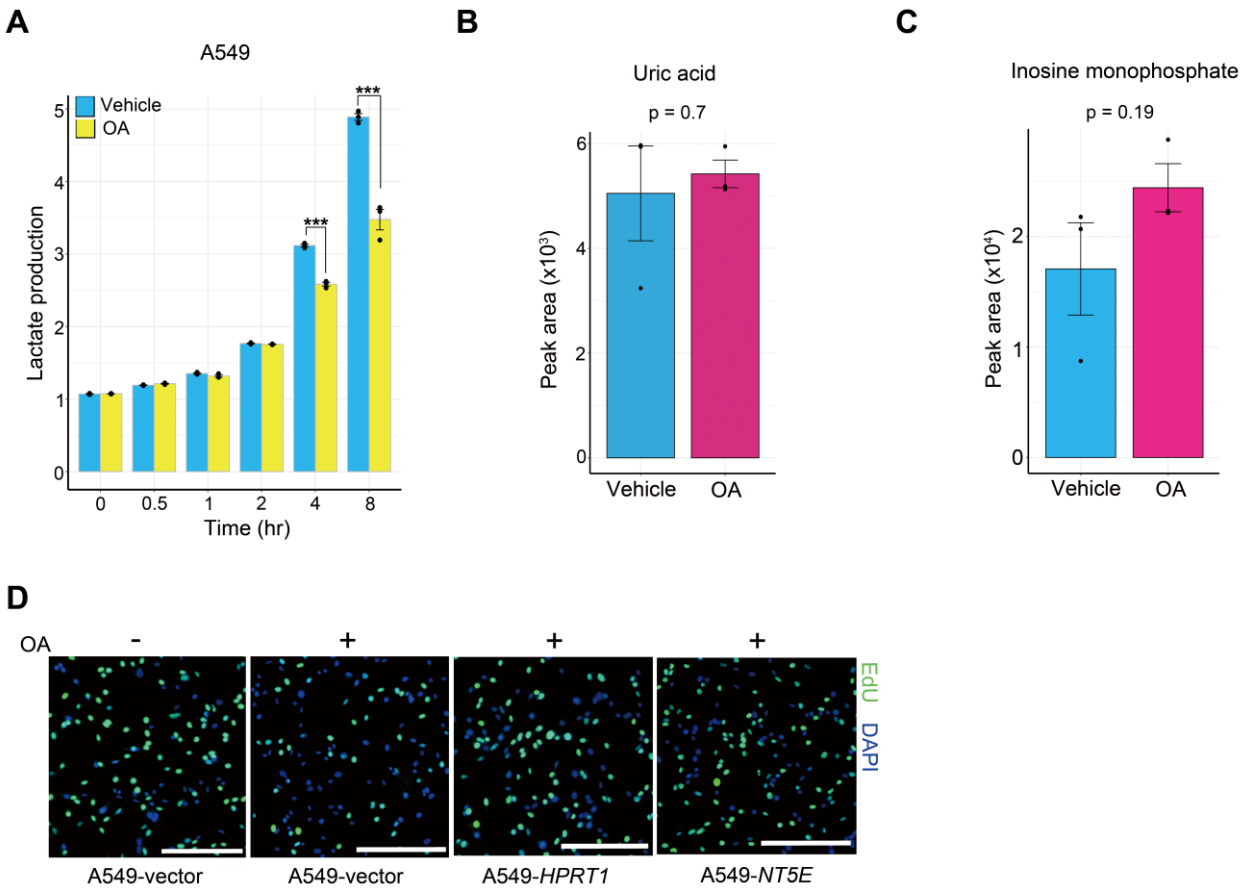

Figure S3. The impact of oleanolic acid treatment on cancer cell metabolism and the rescue effect of enforced expression of *HPRT1* or *NT5E* on DNA synthesis suppressed by oleanolic acid in A549 cells.

(A) The time course assay of lactate production of A549 cells treated by 200 μM oleanolic acid (OA). **(B)** Uric acid generation between A549 cells treated by vehicle (1/1000 DMSO) and A549 cells treated by 200 μM OA for 8 hours. **(C)** Inosine monophosphate (IMP) production between A549 cells treated by vehicle (1/1000 DMSO) and A549 cells treated by 200 μM OA for 8 hours. **(D)** DNA synthesis was remarkably refrained in A549-vector cells treated by 200 μM OA for 8 hours as compared to A549-vector cells treated by vehicle. Of note, impaired DNA synthesis elicited by OA was overtly rescued by forced expression of *HPRT1* or *NT5E*. DNA synthesis was measured by EdU incorporation (green), while cell nuclei were stained by DAPI (blue). Scale bars, 100 μm.

Error bars represent mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, Student's t test.

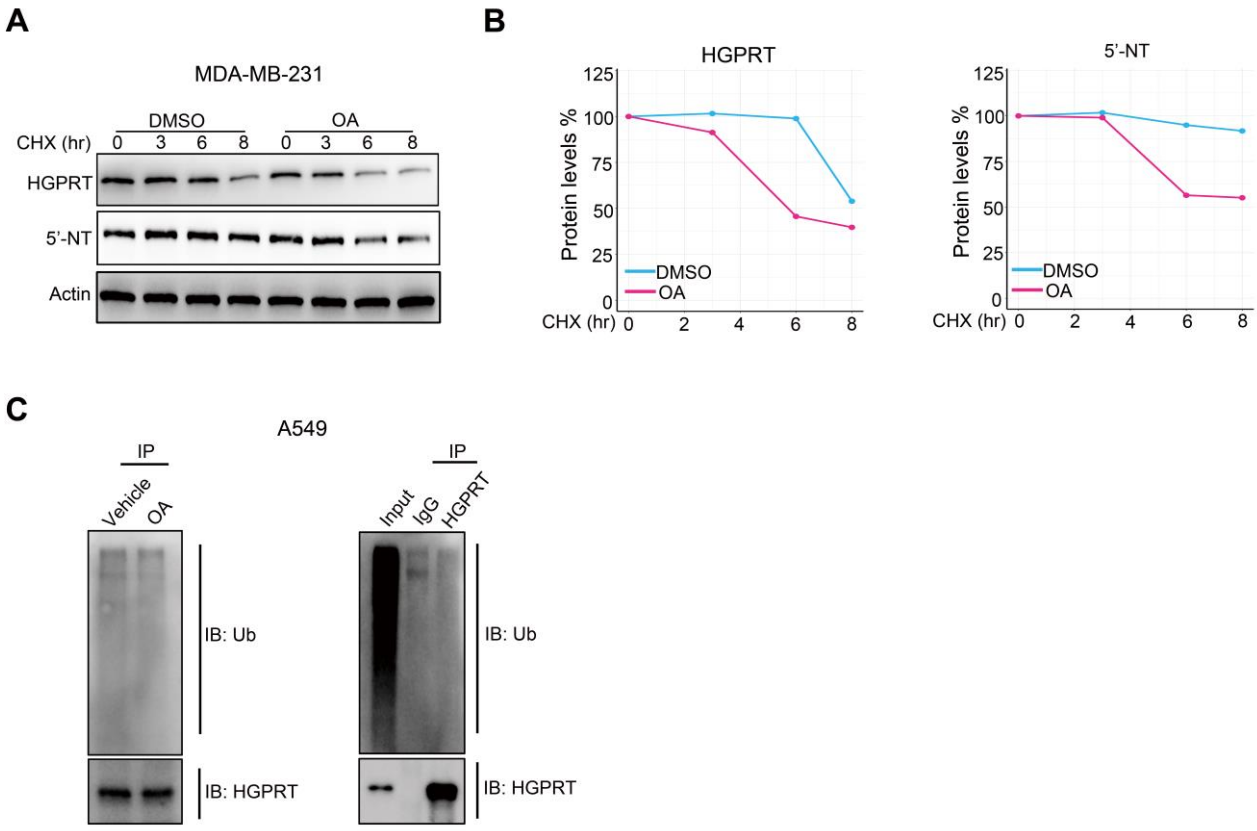

Figure S4. Oleanolic acid expedites the degradation of PSP enzymes via lysosomal pathway but not ubiquitination pathway.

(A-B) The time-course influence of 200 μM oleanolic acid (OA) treatment on HGRPT and 5'-NT degradation when using cycloheximide (CHX, 50 μg/mL) to block protein synthesis in MDA-MB-231 cells. The curves on the right side (B) displaying the quantification results of the protein levels across different time points. **(C)** The effect of 200 μM OA treatment for 8 hours on the ubiquitination status of HGPRT in A549 cells. The affinity and specificity of the anti-HGPRT antibody to cellular HGPRT protein was validated as showed by the immunoblots on the right side.

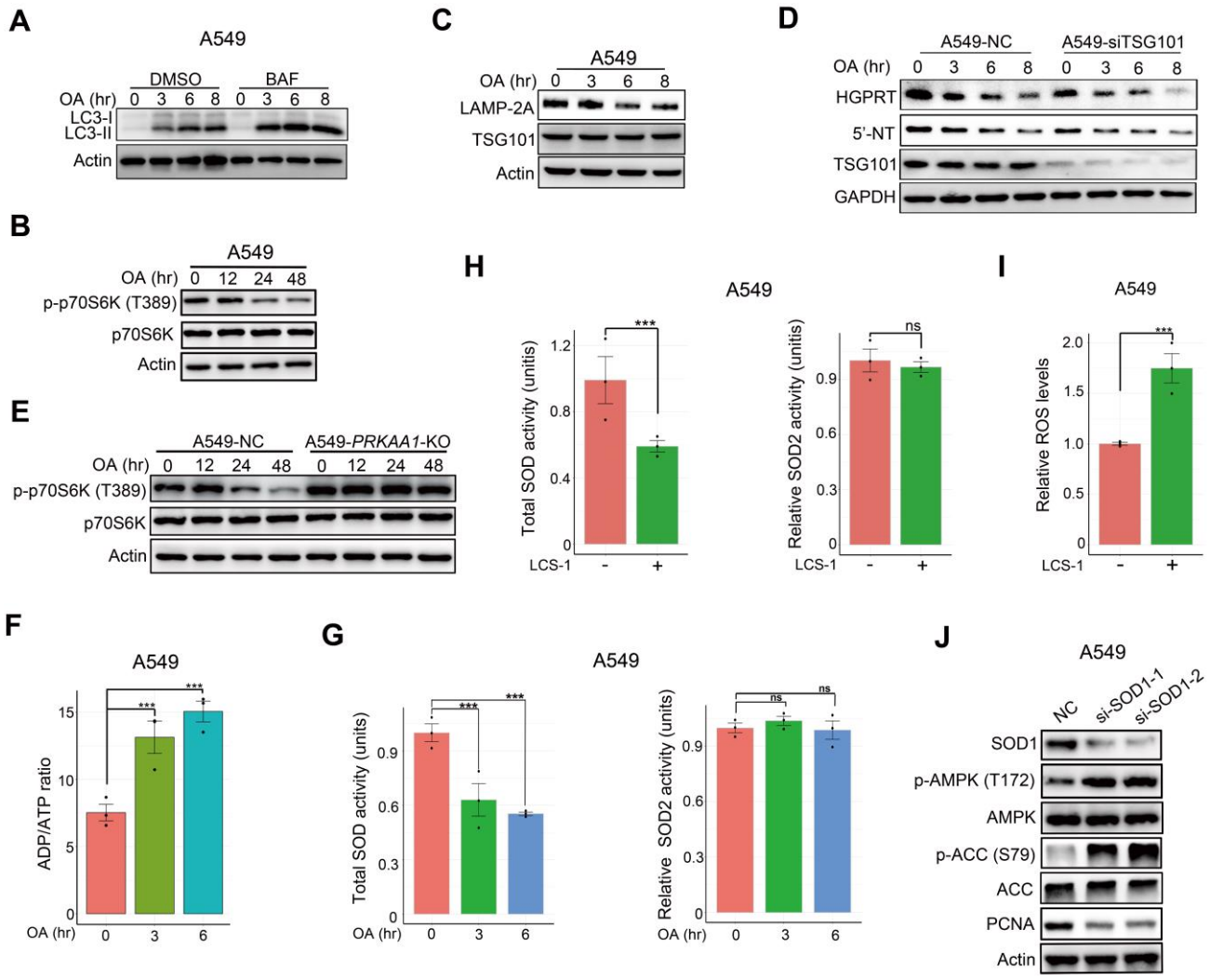

Figure S5. Stimulation of macroautophagy but not microautophagy and chaperone-mediated autophagy by oleanolic acid treatment, and the underlying mechanism of oleanolic acid-activated macroautophagy.

(A) Western blot showing the time course of a lysosome inhibitor bafilomycin A1 (BAF, 0.08 μM) treatment effect on LC3-I/II levels in A549 cells under the condition of 200 μM OA treatment. **(B)** Western blot showing the time course of the impact of 200 μM OA treatment on the expression of phospho-p70S6K (T389) and p70S6K in A549 cells. **(C)** Western blot showing the time course of the effect of 200 μM OA treatment on the expression of TSG101 and LAMP-2A in A549 cells. TSG101 is a microautophagy-related protein, while LAMP-2A is a chaperone-mediated

autophagy-related protein. **(D)** Western blot showing the time course of the effect of 200 μM OA treatment on the expression of HGPRT and 5'-NT between control A549 cells and A549 cells with TSG101 silencing. TSG101 knockdown was used to inhibit microautophagy. **(E)** Western blot showing the time course of the effect of 200 μM OA treatment on the expression of phosho-p70S6K (T389) and p70S6K in A549 cells with and without *PRKAA1*-KO. **(F)** The time course of the effect of 200 μM OA treatment on ADP/ATP ratio in A549 cells. **(G)** The time course of the influence of 200 μM OA treatment on the activities of total superoxide dismutases (SODs) and SOD2 in A549 cells. **(H)** The impact of a SOD1 inhibitor LCS-1 (5 μM**)** treatment for 6 hours on the activity of total SODs and SOD2 in A549 cells. **(I)** Measurement of ROS generation in A549 cells with or without LCS-1 (5 μM) treatment for 6 hours. **(J)** Western blot showing the impact of SOD1 silencing on the expression of SOD1, phospho-AMPK (T172), AMPK, phospho-ACC (S79), ACC and PCNA in A549 cells. The cells were treated by siRNAs for 72 hours. NC, nontarget control siRNA sequence. Error bars represent mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Student's t test. Ns, no significance.