Palmitic acid dysregulates the Hippo-YAP pathway and inhibits angiogenesis by inducing mitochondrial damage and activating the cytosolic DNA sensor cGAS-STING-IRF3 signaling

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Running Title: Cross-talk between cytosolic DNA sensor signaling and Hippo-Yap pathway in angiogenesis inhibition

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Experimental procedure

Gene silencing or overexpression. YAP siRNA, MST1 siRNA were bought from RiboBio Co. (Guangzhou, China) and GenePharma Co. (Shanghai, China). cGAS siRNA, STING siRNA, IRF3 siRNA were bought from Thermo Fisher Scientific Inc.(Shanghai, China), RiboBio Co. (Guangzhou, China) and GenePharma Co. (Shanghai, China). The best siRNA for each gene were selected and used in our study. pcDNA4/HisMaxB-YAP1-S127A (#18988, addgene) and pcDNA4/HisMaxB-YAP1 (#18978, addgene) were used in this study. HAECs were transfected with 0.5ug plasmids and then treated with PA for 24h. Cell transfection was performed with Lipofectamine 2000(12566014, Thermo Fisher Scientific Inc. Shanghai, China), and for siRNA and lipofectamine® 3000 transfection reagent(L3000015, Thermo Fisher Scientific Inc. Shanghai, China) for overexpression according to the manufacturer's instructions.

Palmitic acid preparation and treatment. Saturated palmitic acid (PA) was used in this study. PA preparation and treatment were performed as described previously (1-3). Briefly, PA was dissolved in ethanol into a 200 mM solution, and combined with 20% free fatty acid-free BSA to produce stock solutions with different concentrations from 1 to 5 mM. These stock solutions were filter-sterilized and stored at -20°C. A control solution containing ethanol and BSA was similarly prepared. For the

experiments, fresh working solutions were prepared by diluting each stock solution (1:10) in the Endothelial Cell Medium. The final BSA concentration was constant in all PA working solutions, whereas the PA-to-BSA ratio varied with the PA concentrations.

BrdU proliferation assay. 5-Bromo-2-deoxy Uridine (BrdU) was used to detect the rate of cell proliferation. HAECs with a confluence of 50%-70% on coverslips in 24 wells cell culture plates were treated with PA for 24h and 10ug/ml BrdU during the last 6h. The treated cells were washed with PBS, fixed in 4% poly formaldehyde at room temperature for 30 min, permeabilized with 0.5% Triton X 100 for 10 min. The DNA was denatured with 2mol/L HCL for 1h and the solution was neutralized with 0.1mol/L sodium borate (PH 8.3) for 20 min. The cells on the coverslips were blocked with 1% BSA at room temperature for 1h, incubated with anti-BrdU antibody at 4℃ overnight, and Alexa Fluor 488-conjugated secondary antibodies (1:500, Invitrogen) for at room temperature for 1h, and DAPI nuclear staining for 10 min. The cells were observed under Nikon eclipse Ti and UltraVIEW VOX confocal microscope. BrdU+ cells and total cells in 5 randomly selected fields per sample were counted. The ratio of BrdU+ cells/total cells (%) was compared between the groups.

Flow cytometry of BrdU proliferation assay. Flow cytometry was used to quantify 5-Bromo-2-deoxy Uridine (BrdU) proliferation assay. (4)Briefly, cells were cultured in 6-well plates and 10ug/ml BrdU was added during the last 6h. Cells were harvested by a 0.125% trypsin solution, centrifuged and resuspended in PBS and fixed by gently shaked on a shaking table in 4% poly formaldehyde for 30min at room temperature, then centrifuged at 1000rpm for 10min and washed by PBS, permeabilized with 0.5% Triton X 100 for 10 min, after being washed, The DNA was denatured with 2mol/L HCL for 1h and the solution was neutralized with 0.1mol/L sodium borate (PH 8.3) for 20 min. 1% BSA for 1h at room temperature was added for 1h at room temperature after PBS and cells were kept on gently shaking table, then stained with Anti-BrdU antibody [BU1/75 (ICR1)] (FITC) (ab74545, abcam, ShangHai, China) cells at 4°C overnight under shaking. Data was analyzed and shown by kaluza 1.5a and Flowjo 10.0.

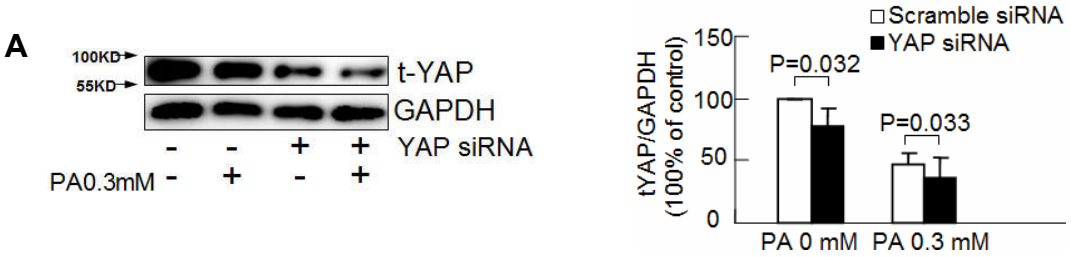
Western blot analysis. Western blot analysis was performed as described before(1-3). Protein lysates were extracted from the treated cells,and separated by SDS–polyacrylamide gels, then transferred to the polyvinylidine fluoride membranes. The membrane were blocked with antibodies were incubated at 4℃ overnight, the secondary horse radish peroxidase–labeled antibody was incubated for 1h in room temperature the second day, bands were photographed by Alpha FluorChem E system and analyzed by image J. Primary antibodies used were anti-YAP (Mouse mAb, 12395, CST); anti-MST1 antibody(ab51134,Rabbit mAb,abcam); anti-STING antibody(ab131604,mouse mAb,abcam); anti-cGAS antibody(abf124, Rabbit mAb, Millipore); anti-IRF3 antibody(ab76409,Rabbit mAb,abcam)

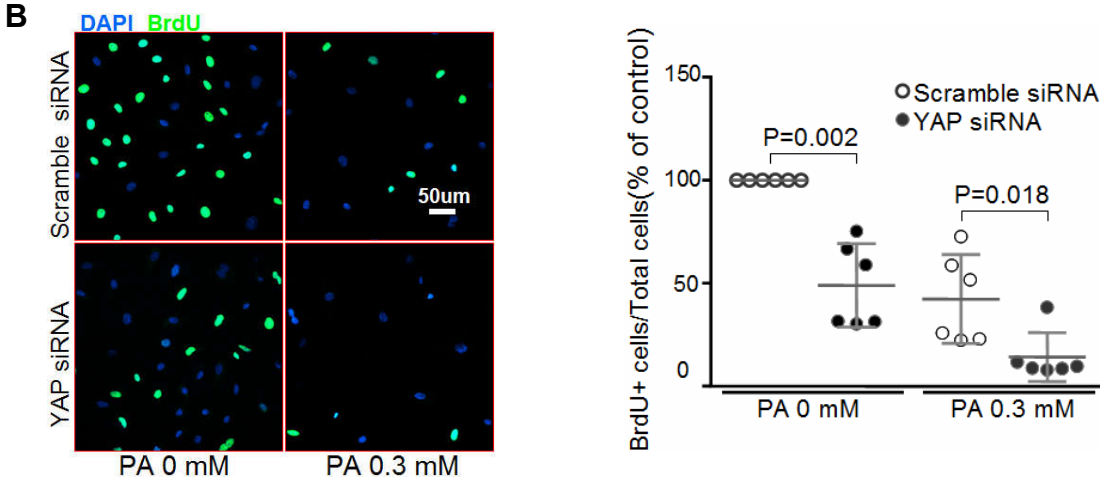
Real-time quantitative RT-PCR. We use trizol and chloroform to extract total RNA from the treated cells, PrimeScript™ RT reagent Kit (RR037A,takara) helps to reverse the RNA transcribed into cDNAs. Real-time PCR were tested and quantified by CFX Connect™ Fluorescent quantitative PCR detection system. Sequences of primers as

following Table S1:

Table S1 primers for real-time PCR

AGACCTCCAGGAGATAATCAAAGA	MST1 forword primer
AGATACAGAACCAGCCCCACA	MST1 reverse primer
TAGCCCTGCGTAGCCAGTTA	YAP1 forword primer
TCATGCTTAGTCCACTGTCTGT	YAP1 reverse primer
AGAGGCTCGTGATGGTCAAG	IRF3 forword primer
AGGTCCACAGTATTCTCCAGG	IRF3 reverse primer
CACTTGGATGCTTGCCCTC	STING forword primer
GCCACGTTGAAATTCCCTTTTT	STING reverse primer
TAACCCTGGCTTTGGAATCAAAA	cGAS forword primer
TGGGTACAAGGTAAAATGGCTTT	cGAS reverse primer
CAGGTGTTGGTTTGAGGATC	STK4 forword primer
AGAGGGTTCCACATCCACAA	STK4 reverse primer
CACCAGGGCTGCTTTTAACT	Human GAPDH forword primer
GATCTCGCTCCTGGAAGATG	Human GAPDH reverse primer





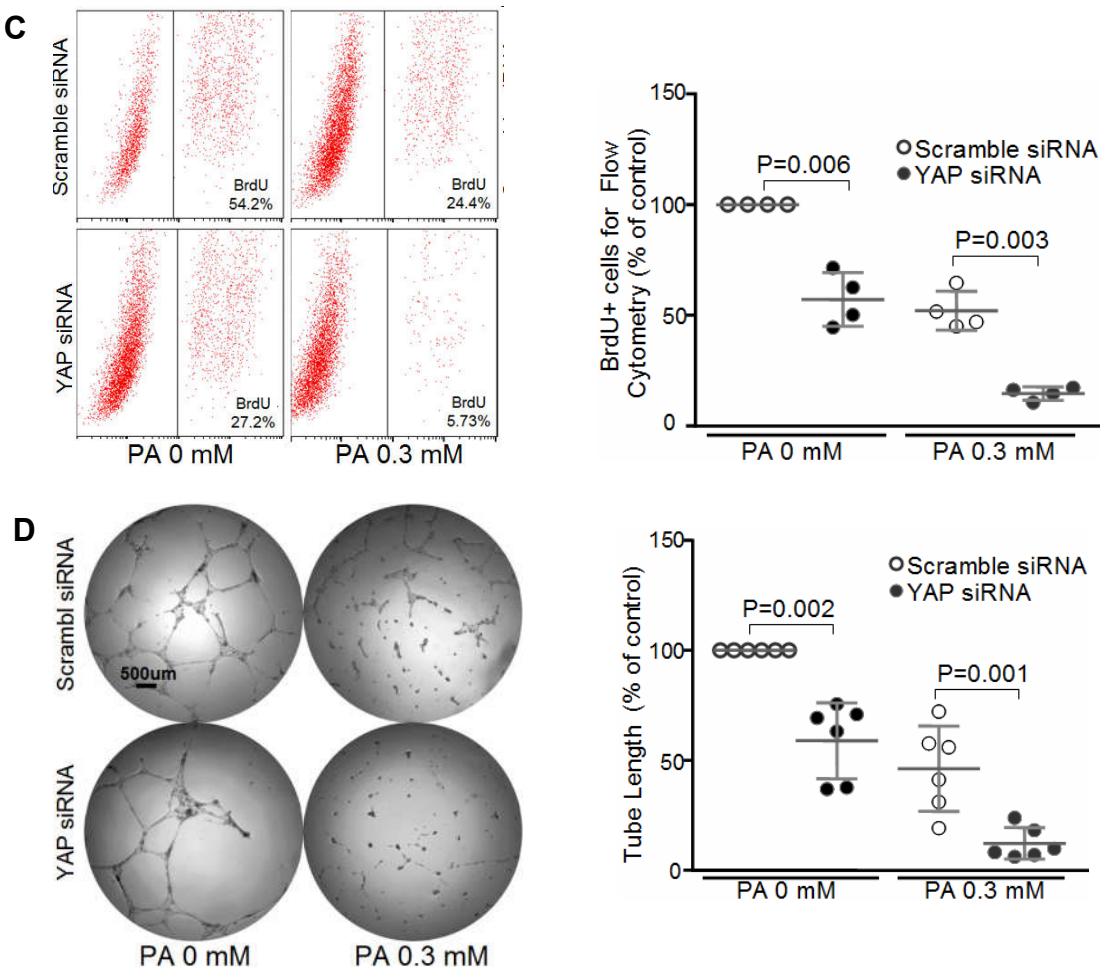


Figure S1. Silence of YAP could inhibit HAEC of proliferation and angiogenesis. HAEC were treated with scramble siRNA or YAP siRNA and then treated with PA for 24h. **A.** Western blot shows a successful knocking down of YAP(n=3 biological repeats). **B-C.** Representative images of immunostaining **(B)** (n=6 biological repeats) and flow cytometry **(C)** (n=4biological repeats) shows that knocking down of YAP could inhibit HAEC of proliferation. **D.** In vitro tube formation assay showed that knocking down YAP reduced endothelial angiogenesis (n=6 biological repeats).

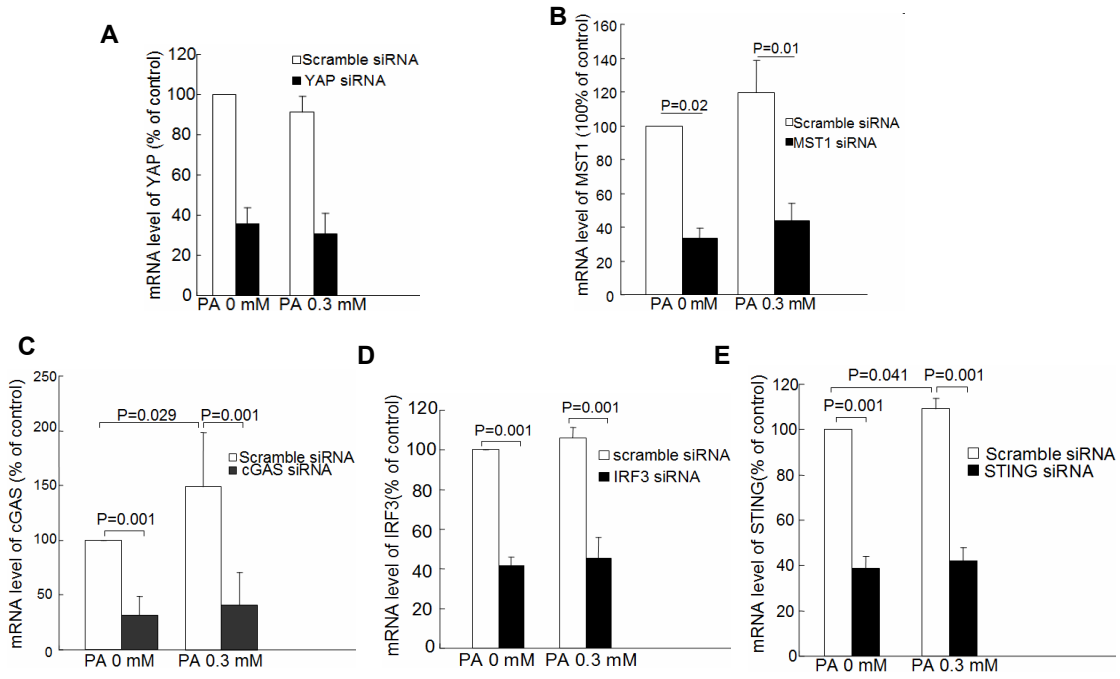


Figure S2. Real-time PCR for efficiency test of siRNAs. HAEC were treated with scramble siRNA or YAP siRNA or MST1 siRNA or IRF3 siRNA or STING siRNA and then treated with PA for 24h. **A.** mRNA level of YAP shows a successful knocking down of YAP(n=3 biological repeats). **B.** mRNA level of MST1 shows a successful knocking down of MST1(n=3 biological repeats). **C.** mRNA level of cGAS shows a successful knocking down of cGAS(n=3 biological repeats). **D.** mRNA level of IRF3 shows a successful knocking down of IRF3(n=3 biological repeats). **E.** mRNA level of STING shows a successful knocking down of STING(n=3 biological repeats).

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