Phosphorylation of 14-3-3ζ links YAP transactivation to hypoxic glycolysis for tumorigenesis

Yu Jia[1+], Hui-Yan Li[2+], Jue Wang[3], Ying Wang[4], Peng Zhang[5], Ning Ma[2], Shi-Jing Mo[2*]

Supplemental Information

Fig. S1 Hypoxia induces nuclear translocation of YAP in a HIF-1α-independent manner. a Subcellular fractionation analyses determining abundance of nuclear and cytoplasmic YAP protein in A498 RCC cells (A-4) and Huh-7 HCC cells (H-7) stimulated with hypoxia for 6h. GAPDH and Lamin B were used as internal control of cytoplasmic and nuclear extractions, respectively. Data are expressed as mean ± s.d. of three independent experiments. $*P < 0.05$, $**P < 0.01$. Two-sided Student's t test was used to calculate the *P* value. **b** Subcellular fractionation analyses comparing levels of nuclear YAP protein expression in SW-1990 PDAC cells stimulated with the indicated concentrations of $CoCl_2$ for 12h. **c** Subcellular fractionation analyses comparing accumulation of nuclear and cytoplasmic YAP protein in hypoxia-stimulated SW-1990 PDAC cells with or without HIF-1α siRNA transfection. **d** Coimmunoprecipitation assay evaluating the interaction between Myc-tagged wild-type TEAD4 and nuclear YAP in SW-1990 PDAC cells stimulated with hypoxia for 12h.

Fig. S2 Hypoxia induces nuclear translocation of YAP in a 14-3-3ζ-dependent fashion. a Subcellular fractionation analyses assessing abundance of nuclear and cytoplasmic YAP protein expression in SW-1990 PDAC cells with or without 14-3-3ζ siRNA transfection. Data are expressed as mean ± s.d. of three independent experiments. $*P < 0.05$. Two-sided Student's t test was used to calculate the *P* value. **b** Left panel: Western-blotting comparing the levels of Flag expression in SW-1990 PDAC cells with or without Flag-tagged wild-type 14-3-3ζ transfection. Right panel: Coimmunoprecipitation assay evaluating the interaction between Myc-TEAD4 and nuclear YAP in SW-1990 PDAC cells stimulated with hypoxia for 12h with or without Flag-tagged wild-type 14-3-3ζ transfection. IP, immunoprecipitation. **c** Coimmunoprecipitation assay comparing the interaction between 14-3-3ζ and YAP in SW-1990 PDAC cells treated with or without 1 mmol/L $CoCl_2$ for 12h. Data are expressed as mean ± s.d. of three independent experiments. $*P < 0.05$. Two-sided ANOVA with Bonferroni post hoc t test correction was used to calculate the *P* value. **d** Coimmunoprecipitation assay assessing the interaction between endogenous WWTR1 and YAP in SW-1990 PDAC cells stimulated with or without hypoxia for 12h.

Fig. S3 ERK1/2 is required for the hypoxia-stimulated YAP nuclear localization. a Western-blotting comparing the levels of p-IκBα and p-ERK1/2 in SW-1990 PDAC cells stimulated with hypoxia in the presence or absence of 5 μmol/L BAY 11-7085 (BAY) and 20 μmol/L U0126 pretreatment, respectively. **b** Left panel: Western-blotting testing the levels of ERK2 expression in SW-1990 PDAC cells transfected with ERK2 siRNA. Right panel: Coimmunoprecipitation assay determining the interaction between Myc-tagged wild-type TEAD4 and nuclear YAP in SW-1990 PDAC cells with or without serum stimuli (SS) in the presence or absence of ERK2 siRNA transfection. **c** Alignment of the highly conserved Ser37 residue of ERK2 phosphorylation in 14-3-3ζ from homo sapiens to drosophila.

Fig. S4 Hypoxia phosphorylates 14-3-3ζ at Ser37 via ERK2. a Coimmunoprecipitation assay detecting the levels of Flag-tagged wild-type 14-3-3ζ phosphorylation in hypoxia-stimulated SW-1990 PDAC cells in the presence or absence of 5 μmol/L BAY 11-7085 (BAY) and 20 μmol/L U0126 administration with an anti-phospho-serine antibody, respectively. **b** Sequencing analysis of Ser37 mutations in 14-3-3ζ protein. **c** *Top panel*: Co-IP assay testing the specificity of 14-3-3ζ

pS37 antibody. *Bottom panel*: Representative immunfluorescence images testing the specificity of 14-3-3ζ pS37 antibody in SW-1990 PDAC cells with Flag-tagged wild-type 14-3-3ζ (WT) or mutant 14-3-3ζ Ser37A (S37A) expression. Scale bar = 25 μm. **d** RT-qPCR analyses of YWHAZ gene expression in 14-3-3ζ shRNA (sh. 14-3-3ζ)-expressed SW-1990 PDAC cells. Experiments were performed five times, each with quantitative RT-PCR in technical duplicate and real-time values were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data are expressed as mean ± s.d. ***$P < 0.001$. Two-sided Student's t test was used to calculate the *P* value.

Fig. S5 YAP facilitates PKM2 transcription via physical interaction with HIF-1α in an 14-3-3ζ Ser37 phosphorylation-dependent manner under hypoxia. a Coimmunoprecipitation assay examining the interaction between nuclear YAP and HIF-1α in hypoxia-stimulated SW-1990 PDAC cells with or without ERK2 siRNA transfection. **b** Coimmunoprecipitation assay determining the interaction between nuclear YAP and HIF-1α in 14-3-3ζ shRNA (sh.14-3-3ζ)-expressed SW-1990 PDAC cells in the presence or absence of Flag-tagged wild-type 14-3-3ζ (WT) or mutant 14-3-3ζ S37E (S37E) reconstitution. **c** Top panel: Western blotting results depicting the expression levels of YAP with scrambled shRNA (Scr) or YAP shRNA transfection. Bottom panel: ChIP analysis for HIF-1α and HIF-2α binding to PKM2 gene HRE in SW-1990 PDAC cells stimulated with hypoxia in the presence or absence of YAP shRNA transfection using the indicated antibodies. **d** Top panel: Western blotting results depicting the expression levels of HIF-1α with control siRNA (Ctrl) or HIF-1α siRNA transfection. Bottom panel: ChIP analysis for YAP binding to PKM2 gene HRE in SW-1990 PDAC cells stimulated with hypoxia in the presence or absence of HIF-1α siRNA transfection using an anti-YAP antibody. **e** RT-qPCR analyses of PKM2 gene expression in YAP shRNA-expressed SW-1990 PDAC cells with hypoxia stimulation. Experiments were performed five times, each with quantitative RT-PCR in technical duplicate and real-time values were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data are expressed as mean ± s.d. *$P < 0.05$. Two-sided Student's t test was used to calculate the P value. **f** Western-blotting measuring levels of PKM2 protein expression in YAP shRNA-expressed SW-1990 PDAC cells with hypoxia stimulation. **g** Representative immunfluorescence images of Flag-tagged 14-3-3ζ staining in 14-3-3ζ shRNA (sh.14-3-3ζ)-expressed SW-1990 PDAC cells with Flag-tagged wild-type 14-3-3ζ (WT) or mutant 14-3-3ζ Ser37A (S37A) reconstitution. Scale bar = 100 μm. **h** Immunohistochemistry analysis of 14-3-3ζ p-S37 antibody with or without 14-3-3ζ peptide used for developing this antibody or the matched nonphosphorylated 14-3-3ζ peptide. Scale bar = 50 μm. **i** Representative cases stained by immunohistochemistry (*left panel*) and bar graph (*right panel*) showing the expression of 14-3-3ζ p-S37 in 56 primary human PDAC specimens is positively correlated with nuclear localization of YAP. Scale bar = 50 μm. The *P* value shown was calculated by Spearman order correlations.

Table S1 Clinicopathological Features in PDAC Patients (n=87)

Variable	No. of patients	%
Gender		
Male	50	57.5
Female	37	42.5
Age (years)		
<60	33	37.9
≥60	54	62.1
Tumor size		
≤40mm	56	64.4
>40mm	31	35.6
Tumor location		
Head	71	81.6
Body	9	10.3
Tail	7	8.1
Lymphatic metastasis		
Negative	49	56.3
Positive	38	43.7
TNM stage		
I	22	25.3
II	43	49.4
III	14	16.1
IV	8	9.2
Chemotherapy		
Yes	72	82.8
No	15	17.2
p-14-3-3ζ expression		
High	45	51.7
Low	42	48.3

Table S2 Correlation Between Phosphorylation of 14-3-3ζ and Clinicopathological Features in PDAC Patients

Variable	p-14-3-3ζ High (n = 45)	p-14-3-3ζ Low (n = 42)	*P* value
Gender			0.313
Male	27 (60.0%)	23 (54.8%)	
Female	18 (40.0%)	19 (45.2%)	
Age (years)			**0.015**
<60	22 (48.9%)	11 (26.2%)	
≥60	23 (51.1%)	31 (73.8%)	
Tumor size			< **0.001**
≤40mm	20 (44.4%)	36 (85.7%)	
>40mm	25 (55.6%)	6 (14.3%)	
Tumor location			0.103
Head	37 (82.2%)	34 (81.0%)	
Body	6 (13.3%)	3 (7.1%)	
Tail	2 (4.5%)	5 (11.9%)	
Lymphatic metastasis			**0.031**
Negative	21 (46.7%)	28 (66.7%)	
Positive	24 (53.3%)	14 (33.3%)	
TNM stage			0.200
I	14 (31.1%)	8 (19.0%)	
II	17 (37.8%)	26 (61.9%)	
III	8 (17.8%)	6 (14.3%)	
IV	6 (13.3%)	2 (4.8%)	
Chemotherapy			0.104
Yes	35 (77.8%)	37 (88.1%)	
No	10 (22.2%)	5 (11.9%)	