Crosstalk between hypoxia-sensing ULK1/2 and YAP-driven glycolysis fuels pancreatic ductal adenocarcinoma development

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Supplemental Figures 1-6 and Figure legends



Supplemental Figure 1. ULK1/2, but not ULK3, contributes to the hypoxic glycolysis. (A) Western-blotting examining the levels of ULK1, ULK2 and ULK3 expression in SW-1990 (S-1) cells transfected with siRNAs targeting ULK1, ULK2, ULK3 or both of each other under hypoxia, respectively. Un, Untransfected; Ctrl.siRNA, control siRNA. (B and C) Glucose consumption (B) and lactate production (C) of SW-1990 (S-1) cells transfected with siRNAs targeting ULK1, ULK2, ULK3 or both of each other under hypoxia and of HepG-2 (H-2) and MCF-7 (M-7) cells transfected with siRNAs targeting ULK1 plus ULK2 under hypoxia, respectively. Data are expressed as mean \pm s.d. of three independent experiments. *P <0.05 and **P < 0.01 versus Un. Two-sided ANOVA with Bonferroni post hoc t test correction was used to calculate the P value. (D) Western-blotting detecting the levels of ULK1 and ULK2 expression in HepG-2 (H-2) cells transfected with siRNAs targeting ULK1 plus ULK2 under hypoxia, respectively. (E and F) Glucose consumption (E) and lactate production (F) of HepG-2 (H-2) cells transfected with siRNAs targeting ULK1 plus ULK2 under hypoxia. Data are expressed as mean \pm s.d. of three independent experiments. *P < 0.05 and **P < 0.01 versus Un. Two-sided ANOVA with Bonferroni post hoc t test correction was used to calculate the P value. (G) Western-blotting evaluating the levels of ULK1 and ULK2 expression in MCF-7 (M-7) cells transfected with siRNAs targeting ULK1 plus ULK2 under hypoxia. (H and I) Glucose consumption (H) and lactate production (I) of MCF-7 (M-7) cells transfected with siRNAs targeting ULK1 plus ULK2 under hypoxia. Data are expressed as mean \pm s.d. of three independent experiments. *P < 0.05 versus Un. Two-sided ANOVA with Bonferroni post hoc t test correction was used to calculate the P value. (J) Western-blotting determining the levels of Flag-ULK1 expression in PL45 (P-45) cells transfected with Flag-tagged wild-type (WT) or kinase-inactive (KI) mutant ULK1. EV, empty vector. (K and L) Glucose consumption (K) and lactate production (L) of PL45 (P-45) cells transfected with Flag-tagged wild-type (WT) or kinase-inactive (KI) mutant ULK1 in the presence or absence of hypoxia. Data are expressed as mean \pm s.d. of three independent experiments. *P < 0.05. Two-sided Student's t test was used to calculate the *P* value.



Supplemental Figure 2. Regulatory roles of ULK1/2 in PKM2 transcription and YAP ubiquitylation. (A) Western-blotting examining the abundance of PKM2 and PKM1 expression in SW-1990 cells transfected with ULK1, ULK2, ULK1/2 and ULK3 siRNA under hypoxia, respectively. (B) RT-qPCR analyses of PKM2 and PKM1 gene expression in SW-1990 cells transfected with ULK1, ULK2, ULK1/2 and ULK3 siRNA under hypoxia, respectively. Experiments were performed three 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 \pm s.d. *P < 0.05 versus Ctrl.siRNA. Two-sided ANOVA with Bonferroni post hoc t test correction was used to calculate the P value. (C) Western-blotting comparing the levels of PKM2 and PKM1 expression in SW-1990 cells treated with 50 µmol/L SBI-0206965 (SBI) under hypoxia. (D) RT-qPCR analyses of PKM2 and PKM1 gene expression in SW-1990 cells treated with 50 µmol/L SBI-0206965 (SBI) under hypoxia. Experiments were performed three 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 \pm s.d. * $P \le 0.05$ versus D. Two-sided Student's t test was used to calculate the P value. D, DMSO. (E) Western-blotting determining the levels of PKM2 and PKM1 expression in PL45 (P-45) cells transfected with Flag-tagged wild-type (WT) or kinase-inactive (KI) mutant ULK1 under hypoxia. (F) RT-qPCR analyses of PKM2 and PKM1 gene expression in PL45 (P-45) cells transfected with Flag-tagged wild-type (WT) or kinase-inactive (KI) mutant ULK1 under hypoxia. Experiments were performed three 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 \pm s.d. *P < 0.05 versus EV. Two-sided ANOVA with Bonferroni post hoc t test correction was used to calculate the P value. EV, empty vector. (G) Luciferase-reporter PKM2 promoter activity analysis of SW-1990 cells treated with or without SBI at the indicated concentrations under hypoxia. Data are expressed as mean \pm s.d. of three independent experiments. *P < 0.05 versus 0. Two-sided ANOVA with Bonferroni post hoc t test correction was

used to calculate the P value. (H) In vitro protein binding assay with mixing nuclear extraction (NE) from SW-1990 cells expressing Flag-tagged wild-type YAP (WT) or mutant YAP Ser227A (S227A) and purified ULK1 followed by WB analyses of immunoprecipitated Flag with an anti-HIF-1a antibody in the presence or absence of CIP treatment, respectively. (I) Western-blotting detecting the levels of nuclear YAP accumulation in SW-1990 cells with or without SBI (50 µmol/L) under hypoxia. (J) Western-blotting comparing the abundance of PKM2 expression in SW-1990 cells transfected with shRNA targeting ULK1 (top panel) or ULK2 (bottom panel) in the presence or absence of YAP knockout (KO) under hypoxia. (K) Western-blotting assessing the abundance of PKM2 expression in SW-1990 cells with or without SBI (50 µmol/L) treatment in the presence or absence of YAP knockout (KO) under hypoxia. (L) RT-qPCR analyses of PKM2 gene expression in SW-1990 cells SBI (50 µmol/L) treatment in the presence or absence of YAP knockout (KO) under hypoxia, respectively. Experiments were performed three 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 \pm s.d. *P < 0.05versus D. Two-sided Student's t test was used to calculate the P value. (M) Luciferase-reporter PKM2 promoter activity analysis of SW-1990 cells with or without SBI (50 µmol/L) treatment in the presence or absence of YAP knockout (KO) under hypoxia, respectively. Data are expressed as mean \pm s.d. of three independent experiments. *P < 0.05 versus D. Two-sided Student's t test was used to calculate the P value. (N) Subcellular fractionation analyses examining abundance of nuclear and cytoplasmic YAP protein expression in SW-1990 cells transfected with ULK1/2 shRNA under hypoxia. GAPDH and Lamin B were used as internal control of cytoplasmic and nuclear extractions, respectively. NE, nuclear extraction; CE, cytoplasmic extraction; WCE, whole cell extraction. (O) Western-blotting examining the abundance of nuclear YAP in SW-1990 cells with SBI (50 µmol/L) treatment in the presence or absence of 20 µg/mL CHX for the indicated times. (P) Western-blotting testing the levels of nuclear YAP in SW-1990 cells with SBI (50 µmol/L) treatment in the presence or absence of 10 µmol/L MG132 addition.



Supplemental Figure 3. Hypoxia evokes ULK1 to interact with and phosphorylate YAP in nucleus. (A) Cellular ubiquitination assays assessing the poly-Ub levels of YAP in SW-1990 cells transfected with ULK1/2 shRNA in the presence of Flag-tagged wild-type ULK1/2 expression under hypoxia. PD, pull-down; Ni-NTA, Ni²⁺-nitrilotriacetic acid (NTA). (B) Cellular ubiquitination assays detecting the K48-linked Ub levels of YAP in SW-1990 cells transfected with ULK1/2 shRNA under hypoxia. (C) Cellular ubiquitination assays comparing the K63-linked Ub levels of YAP in SW-1990 cells transfected with ULK1/2 shRNA under hypoxia. (D) Coimmunoprecipitation assay comparing the interaction of YAP with ULK1 in SW-1990 cells with or without hypoxia stimuli. (E) His-pulldown assay for determination of ULK2-YAP interaction with mixing purified His-tagged ULK2 immobilized on Ni²⁺-nitrilotriacetic acid (NTA)-sepharose beads and nuclear lysates from hypoxia-stimulated SW-1990 cells expressing Flag-tagged wild-type YAP followed by WB analyses of proteins on beads with an anti-Flag antibody. PD, pull-down. (F) Coimmunoprecipitation assay examining the abundance of Flag-tagged wild-type YAP phosphorylation in hypoxia-stimulated SW-1990 cells in the presence or absence of 50 µmol/L SBI-0206965 (SBI) treatment with an anti-phospho-serine antibody. (G) Subcellular fractionation analyses assessing ULK1 nuclear localization in SW-1990 cells with or without hypoxia stimuli. (H) Representative immunfluorescence images of Flag-tagged wild-type (WT) or kinase inactive (KI) mutant ULK1 nuclear localization in SW-1990 cells before and after hypoxia stimuli. Scale bar = $20 \,\mu m$.



Supplemental Figure 4. ULK1/2-inducible YAP Ser227 phosphorylation upregulates PKM2 expression of PDAC cells in mice. (A and B) Glucose consumption (A) or lactate production (B) of SBI-0206965 (SBI)-treated SW-1990 cells with or without Flag-tagged wild-type YAP expression under hypoxia. Data are expressed as mean \pm s.d. of three independent experiments. *** $P \le 0.001$. Two-sided ANOVA with Bonferroni post hoc t test correction was used to calculate the P value. (C) Colony formation assays of SBI-0206965 (SBI)-treated SW-1990 cells with or without Flag-tagged wild-type YAP expression under hypoxia. Data are expressed as mean \pm s.d. of five independent experiments. *P < 0.05. Two-sided ANOVA with Bonferroni post hoc t test correction was used to calculate the P value. (D) Ki-67 staining analyses of SBI-0206965 (SBI)-treated SW-1990 cells with or without Flag-tagged wild-type YAP expression under hypoxia. Data are expressed as mean \pm s.d. of five independent experiments. $*P \le 0.05$. Two-sided ANOVA with Bonferroni post hoc t test correction was used to calculate the P value. Scale bar = $100\mu m$. (E) Representative images for H&E and PKM2 staining in mice bearing tumors from the ULK1/2 shRNA-transfected SW-1990 cells with or without Flag-tagged wild-type YAP expression at day 27 after inoculation. Scale bar = 50 μ m. (F) Representative images for H&E and PKM2 staining in mice bearing tumors from the YAP knockout (KO) SW-1990 cells with Flag-tagged wild-type YAP (WT) or mutant YAP Ser227A (S227A) reconstitution at day 27 after inoculation. Scale bar = $50 \mu m$.







ULK2		Total				
e	Positive	Iotai				Negativ
	10	30		PKM2	Negative	20
	43	65			Positive	14
	53	95		Total		34
<i>P</i> = 0.002						

		UL	Total		
		Negative	Positive	lotar	
DKMO	Negative	20	10	30	
PRIVIZ	Positive	22	43	65	
Тс	Total		53	95	
			P =	0.002	



Α

(%) **AA** 45-30-15-0-

PKM2 (%) 60 40

Ε

F

YAP

10

		ULK2		Total			
		Negative	Positive	Total			
,	Negative	25	13	38		РКМ	
	Positive	17	40	57			
Total		42	53	95			
<i>P</i> = 0.001							





Supplemental Figure 5. Correlation between ULK1/2, YAP and PKM2 in clinical PDAC samples. (A) Quantification of immunohistochemistry comparing expression of ULK2, YAP and PKM2 in pancreas tissues from the wild-type mice and PDAC tissues from the KPC (Pdx1-Cre; LSL-Kras^{G12D/+}; Trp53^{fl/+}) mice as in Figure 6A. (B) Analyses of ULK2, YAP1 and PKM2 mRNA levels in the normal pancreatic tissues and PDAC tumor samples from the publicly available GEO database (GSE15471) containing 36 normal tissues and 36 tumor tissues. (C) Bar graphs showing the frequencies of ULK1, ULK2, YAP1 and PKM genetic alterations in pancreatic tumours. Data are integrated from the cBioPortal (http://www.cbioportal.org/) using the indicated TCGA cancer datasets. (D) Kaplan-Meier curves comparing the overall survival in 177 PDAC patients with low and high expression of the combination of ULK1/2, YAP1 and PKM signature from TCGA datasets. Log-rank test was used to calculate the P value. (E) Statistic analyses for ULK2, YAP1 and PKM2 correlation from the IHC staining results in human PDAC tissues. The P value shown was calculated by Spearman order correlations. (F) Gene ontology (GO) analyses for YAP1 and PKM signature from CCLE dataset. Annotation clusters are shown according to their enrichment scores.



Supplemental Figure 6. The effects of ULK1/2 deactivation in combination with 2-DG and 3-BP on PDAC cells and mice. (A and B) Representative histograms of flow cytometry with Annexin-V/PI staining in SW-1990 cells treated with 25 mM 2-DG (A) and 100 µM 3-BP (B) with or without SBI-0206965 (SBI) administration. (C) Representative images of xenografts from the tumor-bearing mice as in Figure 7E. (D and E) Representative histograms of flow cytometry with Annexin-V/PI staining in PDC treated with 25 mM 2-DG (D) and 100 µM 3-BP (E) with or without SBI-0206965 (SBI) administration. (F) Representative images of xenografts from the tumor-bearing mice as in Figure 7L. (G-J) Body weight (G), platelets (PLT, H), alanine aminotransferase (ALT, I) and serum creatinine (Scr, J) levels from mice bearing SW-1990 xenografts in the presence of 2-DG or 3-BP in combination with or without SBI-0206965 (SBI) treatment (n=6). Data are presented as mean \pm s.d. (K-N) Body weight (K), platelets (PLT, L), alanine aminotransferase (ALT, M) and serum creatinine (Scr, N) levels from mice bearing patient-derived xenografts in the presence of 2-DG or 3-BP in combination with or without SBI-0206965 (SBI) treatment (n=6). Data are presented as mean \pm s.d.