Supplementary Information

Hydrogen Sulfide Coordinates Glucose Metabolism Switch through Destabilizing Tetrameric Pyruvate Kinase M2

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The file includes

Table S1-S4

Figure S1-S8

Other Supplementary Material for this manuscript includes the following:

Supplementary Movie 1 - Time-lapse movie of cell division in MDA-MB-231 cells expressing wild-type PKM2.

Supplementary Movie 2 - Time-lapse movie of cell division in MDA-MB-231 cells expressing $PKM2^{C326S}$.

Table S1. Overview of cysteine PTMs on PKM2 detected by LC-MS/MS.

Modification	**Formulas**	**Monoisotopic mass change**	**Detectable**	**Sites**
Beta-methylthiolation	S-SCH3 (SH modified by MMTS)	+ 45.988	Yes	Cys 31, 49, 152, 326, 358, 423, 424, and 474
SSCAM	S-C2H3NOS (SSH modified by IAM)	+ 88.994	Yes	Cys 326
Carbamidomethylation (CAM)	S-C2H3NO (SH modified by IAM)	+ 57.021	No	
S-Nitrosylation	S-NO	+28.990	No	
Glutathionylation	S-SG	+305.068	No	
Oxidation	S-OH	+15.995	No	
Dioxidation*	S-SO2H	+31.990	No	
Trioxidation*	S-SO3H	+47.985	No	

Analysis of PTMs identified from cell lysates of MDA-MB-231.

*Irreversible Modifications

Table S2. Data collection, refinement, and MolProbity statistics.

	PKM2 C326S
Data collection	
Wavelength (Å)	0.97626
Resolution range (Å)	29.69 - 3.10 (3.21 - 3.10)
Space group	C 1 2 1
Unit cell *a, b, c* (Å); α, β, γ (°)	181.51, 156.37, 121.59; 90, 114.20, 90
Total reflections	55273 (5394)
Unique reflections	15354 (1498)
Multiplicity	3.6 (3.6)
Completeness (%)	98.4 (96.5)
Mean I/sigma (I)	20.0 (2.4)
R_{meas} (%)	6.8 (60.1)
CC1/2	0.998 (0.877)
Refinement	
Reflections used in refinement	49956 (3326)
Reflections used for R-free	1999 (133)
R_{work} (%)	18.4 (29.3)
R_{free} (%)	23.8 (34.9)
Number of non-hydrogen atoms	
protein	14857
Protein residues	1938
RMS (bonds) (Å)	0.022
RMS (angles) (°)	1.86
Average *B*-factor (Å^2)	
protein	55.31
MolProbity	
All atom clashscore	11.26
Poor rotamers (%)	0.13
Ramachandran Outliers (%)	0.6
Ramachandran Favored (%)	90
MolProbity score	2.12

Statistics for the highest-resolution shell are shown in parentheses.

Table S3. Interface areas for PKM2 tetramer.

State	PDB ID	C-C interface (Å^2)	A-A interface (Å^2)
R state	4B2D	1239	2842
T state	4FXJ	1151	2469
PKM2 C326S	8HGF	1225	2379
S-sulfhydrated PKM2		877	2219

Table S4. Lists of primers and siRNA used in this study.

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Primers	
CCND1	Forward: GCGAGGAACAGAAGTG
	Reverse: GAGTTGTCGGTGTAGATGC
HIF-1α	Forward: GCCGAGGAAGAACTATGA
	Reverse: GGTTGGTTACTGTTGGTATC
LDHA	Forward: CCAGAATAAGATTACAGTTGTTG
	Reverse: GCCAAGTCCTTCATTAAGATA
GLUT1	Forward: GCAGGAGATGAAGGAAGA
	Reverse: AATAGAAGACAGCGTTGATG
GLUT12	Forward: TTGACTGTAACTGATCTTATTGG
	Reverse: ACTAATTCTTCTTGGTGATGAC
HK2	Forward: TTGACCAGGAGATTGACAT
	Reverse: GCCATCTTCACCAGGATA
GLS1	Forward: TTTGTGATTCCTGACTTTATGT
	Reverse: GCCTCTGTCCATCTACTG
siRNA sequence	
siCBS&CTH-1	siCBS: 5'-CCUAUGGUCAGAAUCAACAtt-3'
	siCTH: 5'-GGAGCUGAUAUUUCUAUCUtt-3'
siCBS&CTH-2	siCBS: 5'-CAGACCAAGUUGGCAAAGUtt-3'
	siCTH: 5'-CCUGGUGUCUGUUAAUUGUtt-3'

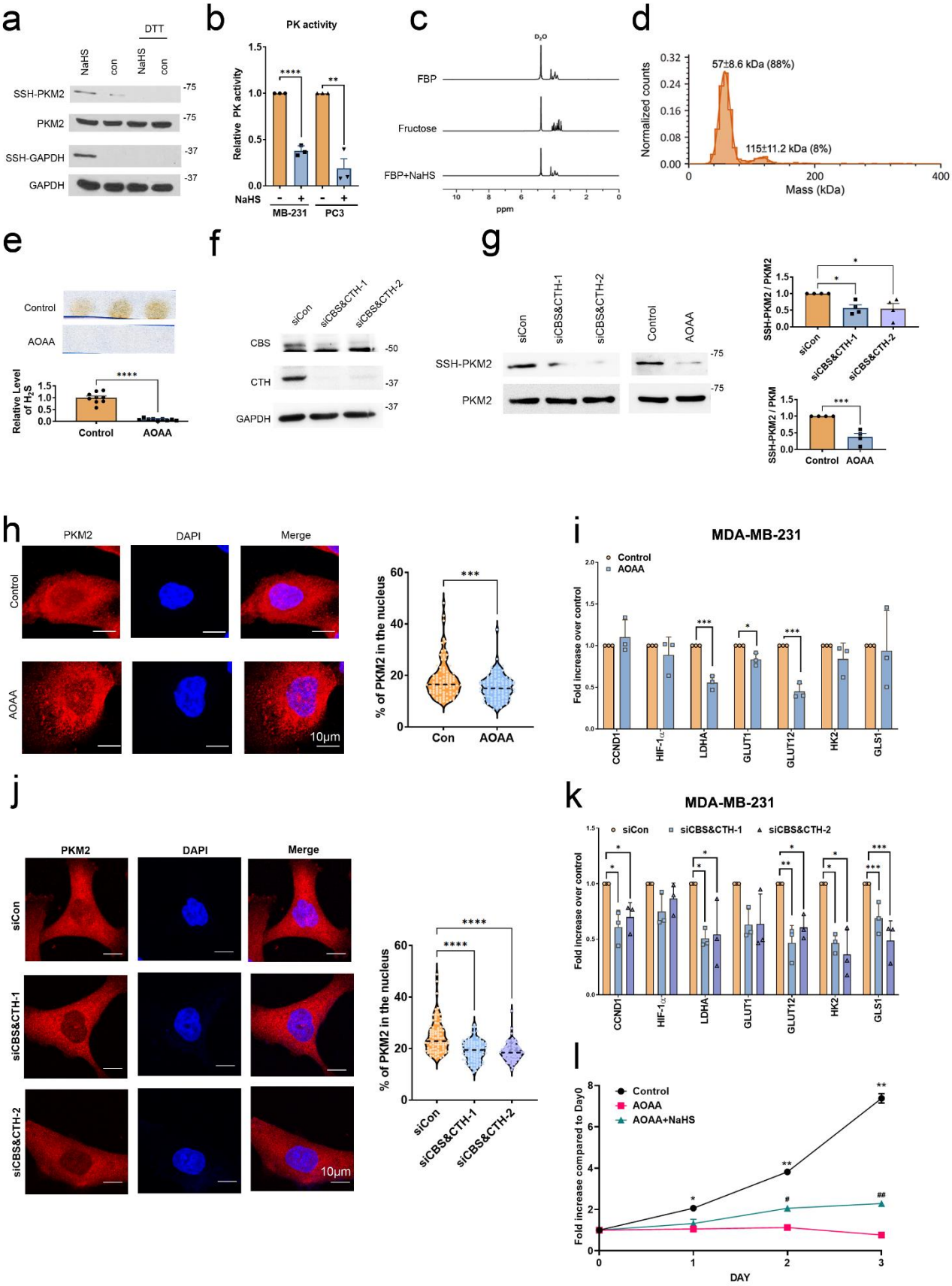
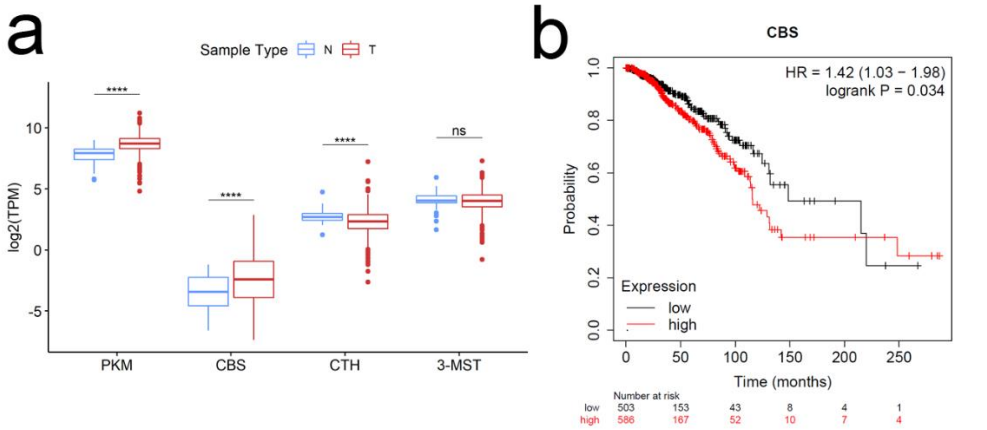

a
DTT
NaHS
con
NaHS
con
SSH-PKM2
PKM2
SSH-GAPDH
GAPDH
-75
-75
-37
-37
b
PK activity
Relative PK activity
NaHS
MB-231
PC3
c
D2O
FBP
Fructose
FBP+NaHS
ppm
d
Normalized counts
57±8.6 kDa (88%)
115±11.2 kDa (8%)
Mass (kDa)
e
Control
AOAA
Relative Level of H2S
Control
AOAA
f
siCon
siCBS&CTH-1
siCBS&CTH-2
CBS
CTH
GAPDH
g
siCon
siCBS&CTH-1
siCBS&CTH-2
Control
AOAA
SSH-PKM2
PKM2
SSH-PKM2 / PKM2
SSH-PKM2 / PKM
h
PKM2
DAPI
Merge
Control
AOAA
10μm
% of PKM2 in the nucleus
Con
AOAA
i
MDA-MB-231
Control
AOAA
Fold increase over control
CCND1
HIF-1α
LDHA
GLUT1
GLUT12
HK2
GLS1
j
PKM2
DAPI
Merge
siCon
siCBS&CTH-1
siCBS&CTH-2
10μm
% of PKM2 in the nucleus
k
MDA-MB-231
siCon
siCBS&CTH-1
siCBS&CTH-2
Fold increase over control
l
Control
AOAA
AOAA+NaHS
Fold increase compared to Day0
DAY

Figure S1. H_2S mediates PKM2 sulfhydration to inhibit PK activity. (a) PC3 cell lysates were treated with 100 μM NaHS for 30 min at 37°C and subjected with or without 1 mM DTT for 10 min. The biotin switch assay was then applied to precipitate sulfhydrated proteins. The biotin-labeled protein was analyzed by immunoblotting with anti-PKM2 or GAPDH antibody to detect sulfhydration of PKM2 and GAPDH (as positive control). Immunoblotting experiments were repeated at least 3 times with similar results. **(b)** MDA-MB-231 and PC3 cell lysates were treated with 1 μM NaHS for 30 min at 37°C and pyruvate kinase activities were then assayed by measuring the amount of pyruvate production. Data are presented as the normalized means ± SD (n=3 biological replicates). The two-tailed student's t-test was used for the statistical analysis (**p<0.01; ****p<0.0001). **(c)** ^{1}H NMR spectra of 10 mg FBP, 10 mg Fructose, or 10 mg FBP co-treated with 1 mg NaHS. **(d)** Mass measurement of individual sulfhydrated PKM2 molecules by mass photometry. Mass distributions are shown in counts normalized by the total counts. The theoretical mass of a monomer, dimer, and tetramer are 60, 120, and 240 kDa, respectively. The fitted masses are indicated. **(e)** Upper: Photo of the lead acetate embedded filter paper after a 48-h incubation at 37 °C on a 96-well plate containing MDA-MB-231 cells with or without 0.25 mM AOAA. The dark circles are due to the formation of lead sulfide on the filter paper by H_2S. Bottom: Quantification of the image using the Integrated Density (IntDen) function of ImageJ. Data are presented as the normalized means ± SD (n=9 from 3 biological replicates). The two-tailed student's t-test was used for the statistical analysis (****p<0.0001). **(f)** Western blot analysis of CBS, CTH, and GAPDH expression in MDA-MB-231 cells with siCon (40nM), siCBS&CTH-1 (20nM each), and siCBS&CTH-2 (20nM each) transfection for 48 h. The samples derive from the same experiment but different gels for CTH, and another for CBS and GAPDH were processed in parallel. GAPDH is the internal control. Immunoblotting experiments were repeated at least 3 times with similar results. **(g)** Left: MDA-MB-231 cells with siCon, siCBS&CTH-1, and siCBS&CTH-2 transfection or with 0.25mM AOAA treatment for 48h were lysed and subjected to the biotin switch assay to precipitate sulfhydrated proteins. The biotin-labeled protein was analyzed by immunoblotting with anti-PKM2 antibody to detect sulfhydration of PKM2. Right: The SSH-labeled PKM2 was normalized with the level of total PKM2. Data are presented as the normalized means ± SEM (n=3 biological replicates). One-way ANOVA followed by Tukey's multiple comparisons test (Left) or the student's t-test (Right) was used for the statistical analysis (*p<0.05; ***p<0.001). Immunoblotting experiments were repeated at least 3 times with similar results. **(h)** Left: MDA-MB-231 cells were exposed to 0.25 mM AOAA for 24h. Subcellular localization of PKM2 was detected by immunostaining. Nuclei were counterstained with DAPI. The representative images are shown. Scale bars: 10 μm. Right: The percentage of nuclear PKM2 is shown in a violin plot with individual points (n= 95-97 individual cells per group, data were combined from three independent experiments). The two-tailed student's t-test was used for the statistical analysis (***p<0.001). **(i)** MDA-MB-231 cells were treated with 0.25 mM AOAA for 24h. The relative expression of each gene was measured by qRT-PCR. Data are presented as the means ± SD (n=3 biological replicates). The two-tailed student's t-test was used for the statistical analysis (*p<0.05; ***p<0.001). **(j)** MDA-MB-231 cells with siCon, siCBS&CTH-1, and siCBS&CTH-2 transfection for 48h. Subcellular localization of PKM2 was

detected by immunostaining. Nuclei were counterstained with DAPI. The representative images are shown. Scale bars: 10 μm. Right: The percentage of nuclear PKM2 is shown in a violin plot with individual points (n= 90 individual cells per group, data were combined from three independent experiments). One-way ANOVA followed by Tukey's multiple comparisons test was used for the statistical analysis (****p<0.0001). **(k)** MDA-MB-231 cells were transfected with siCon, siCBS&CTH-1, and siCBS&CTH-2 for 48h. The relative expression of each gene was measured by qRT-PCR. Data are presented as the means ± SD (n=3 biological replicates). One-way ANOVA followed by Tukey's multiple comparisons test was used for the statistical analysis (*p<0.05; **p<0.01; ***p<0.001). **(l)** Cell proliferation assays were performed in MDA-MB-231 cells treated with 0.25mM AOAA or 0.25mM AOAA together with 1μM NaHS. Data are presented as the means ± SD (n=3 biological replicates). Two-way ANOVA followed by Tukey's multiple comparisons test was used for the statistical analysis (*p<0.05, **p<0.01, AOAA or AOAA+NaHS compared to the Control; $^{\#}p$<0.05, $^{\#\#}p$<0.01 AOAA+NaHS compared to AOAA). Source data are provided as a Source Data file.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PKM
CBS
CTH
3-MST
****
ns
b
CBS
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Probability
Expression
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high
Time (months)
Number at risk
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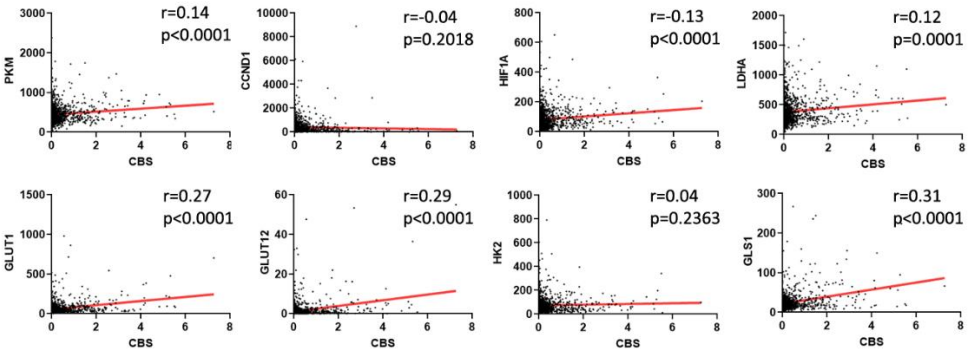
high 586 167 52 10 7 4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PKM r=0.14 p<0.0001
CCND1 r=-0.04 p=0.2018
HIF1A r=-0.13 p<0.0001
LDHA r=0.12 p=0.0001
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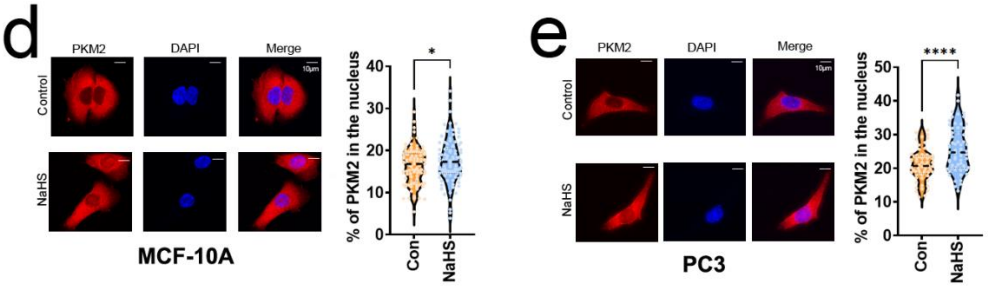
CBS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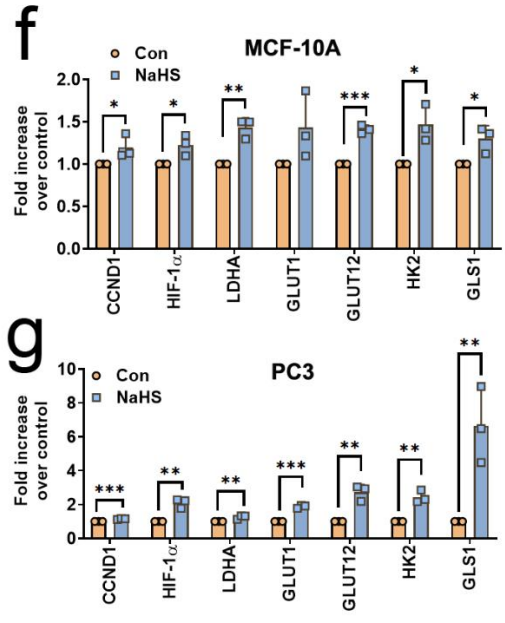
PKM2
DAPI
Merge
Control
NaHS
MCF-10A
% of PKM2 in the nucleus
Con
NaHS
e
PC3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MCF-10A
Con
NaHS
Fold increase over control
CCND1
HIF-1α
LDHA
GLUT1
GLUT12
HK2
GLS1
g
PC3

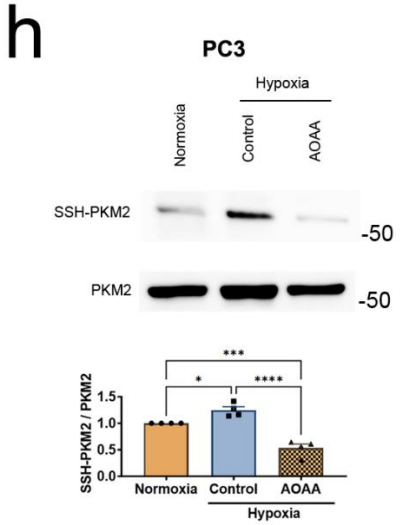

h
PC3
Hypoxia
Normoxia
Control
AOAA
SSH-PKM2
PKM2
-50
SSH-PKM2 / PKM2
Normoxia
Control
AOAA
Hypoxia

Figure S2. The expression, prognosis, and correlation of PKM and H_2S synthesizing enzymes in breast cancer patients. (a) Box plots represent gene expressions of PKM, CBS, CTH, and 3-MST between the normal breast tissues (N; n = 60) and breast tumors (T; n=1034) from the TCGA-BRCA RNA-seq dataset, measured in TPMs. The student's t-test was used for the statistical analysis (****p<0.0001; ns, not significant). **(b)** Kaplan-Meier overall survival analysis of breast cancer patients presenting high (n=586) or low (n=503) CBS expression. The RNA-seq dataset was from TCGA cohorts. The p-value was calculated using the log-rank test. **(c)** Plot of Pearson correlation of the expression level for CBS versus PKM or PKM2 response genes, including CCND1, HIF1A, LDHA, GLUT1, GLUT12, HK2, and GLS1. **(d, e)** Left: MCF-10A **(d)** and PC3 **(e)** were exposed to 1μM NaHS for 1hr. Subcellular localization of PKM2 was detected by immunocytochemistry. Nuclei were counterstained with DAPI. The representative images are shown. Scale bars: 10 μm. Right: The percentage of nuclear PKM2 is shown in a violin plot with individual points (n= 95-97 individual cells per group, data were combined from three independent experiments). One-way ANOVA followed by Tukey's multiple comparisons test was used for the statistical analysis (*p<0.05; ****p<0.0001). **(f, g)** MCF-10A **(f)** and PC3 **(g)** cells were treated with 1μM NaHS for 24 hrs. The relative expression of genes regulated by PKM2 was measured by qRT-PCR. Data are presented as the means ± SD (n=3 biological replicates). The student's t-test was used for the statistical analysis (*p<0.05; **p<0.01; ***p<0.001). **(h)** Upper: PC3 cells were under normal (20% O_2) or hypoxia incubator (1% O_2) for 48h with or without 0.25 mM AOAA. Cells were then lysed and subjected to the biotin switch assay to precipitate sulfhydrated proteins. The biotin-labeled protein was analyzed by immunoblotting with anti-PKM2 antibody to detect sulfhydration of PKM2. Bottom: The SSH-labeled PKM2 was normalized with the level of total PKM2. Data are presented as the means ± SEM (n=4 biological replicates). One-way ANOVA followed by Tukey's multiple comparisons test was used for the statistical analysis (*p<0.05; ***p<0.001; ****p<0.0001). Immunoblotting experiments were repeated at least 3 times with similar results. Source data are provided as a Source Data file.

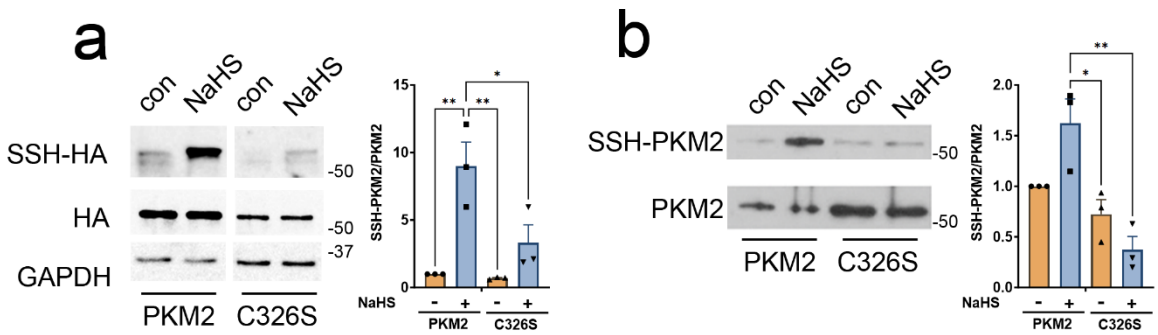
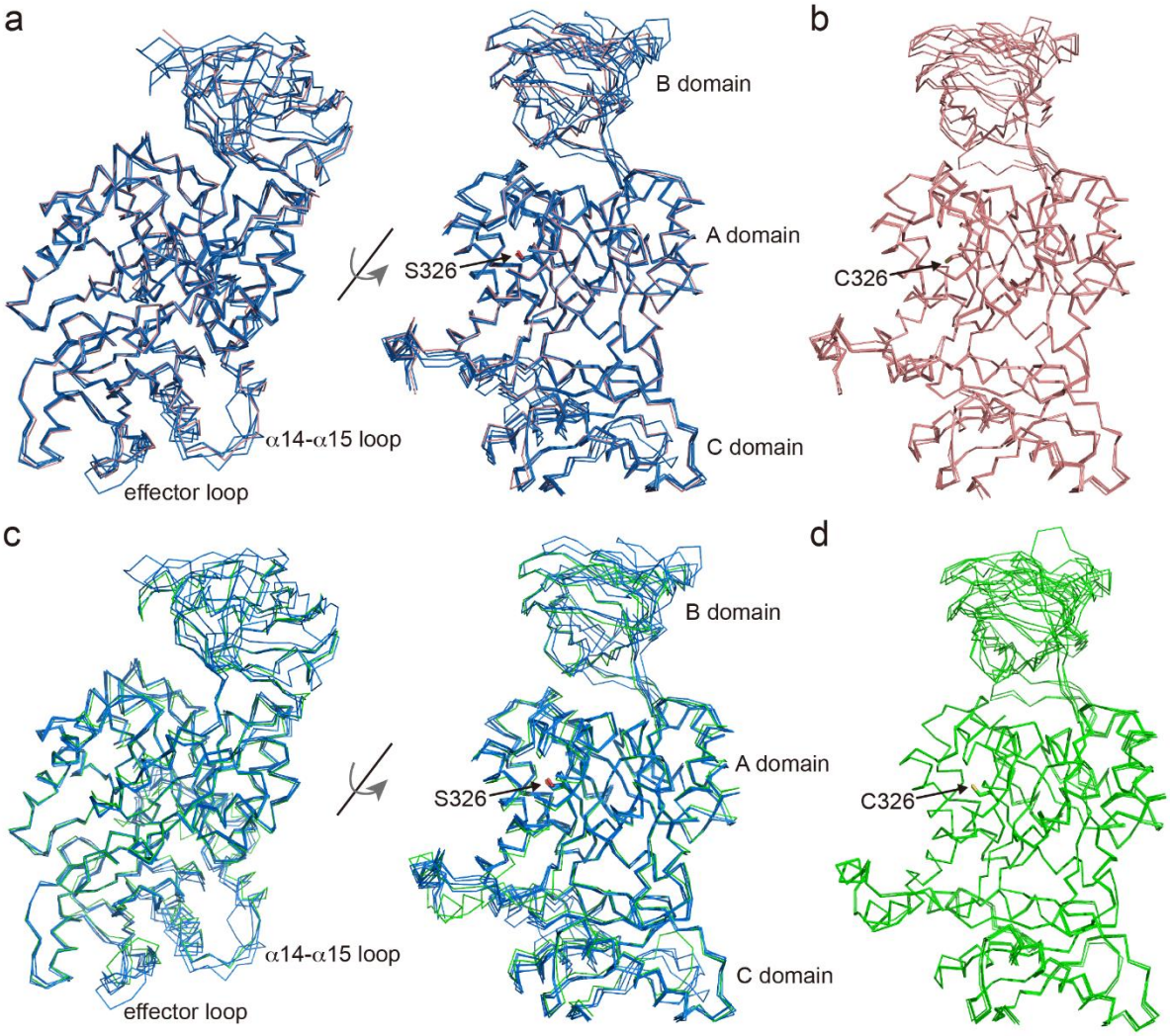


Figure S3. PKM2 is sulfhydrated by H_2S, notably cysteine 326. (a) Left: The cell lysate from MDA-MB-231 cells transfected with HA-tagged PKM2 or PKM2^{C326S} mutants was treated with 100 μM NaHS for 30 min at 37°C. The modified biotin switch assay was then applied to precipitate sulfhydrated protein. The biotin-labeled protein was analyzed by immunoblotting with anti-HA antibodies to detect sulfhydration of transfected PKM2. Right: The SSH-labeled HA-PKM2 was normalized with the level of total HA-PKM2. Data are presented as the means ± SEM (n=3 biological replicates). One-way ANOVA followed by Tukey's multiple comparisons test was used for the statistical analysis (*p<0.05; **p<0.01). Immunoblotting experiments were repeated at least 3 times with similar results. **(b)** Left: Purified recombinant PKM2 or PKM2^{C326S} mutant protein was treated with 100 μM NaHS for 30 min at 37°C. The modified biotin switch assay was then applied to precipitate sulfhydrated protein. The biotin-labeled protein was analyzed by immunoblotting with anti-PKM2 antibody to detect sulfhydration of PKM2. Right: The SSH-labeled PKM2 was normalized with the level of total PKM2. Data are presented as the means ± SEM (n=3 biological replicates). One-way ANOVA followed by Tukey's multiple comparisons test was used for the statistical analysis (*p<0.05; **p<0.01). Immunoblotting experiments were repeated at least 3 times with similar results. Source data are provided as a Source Data file.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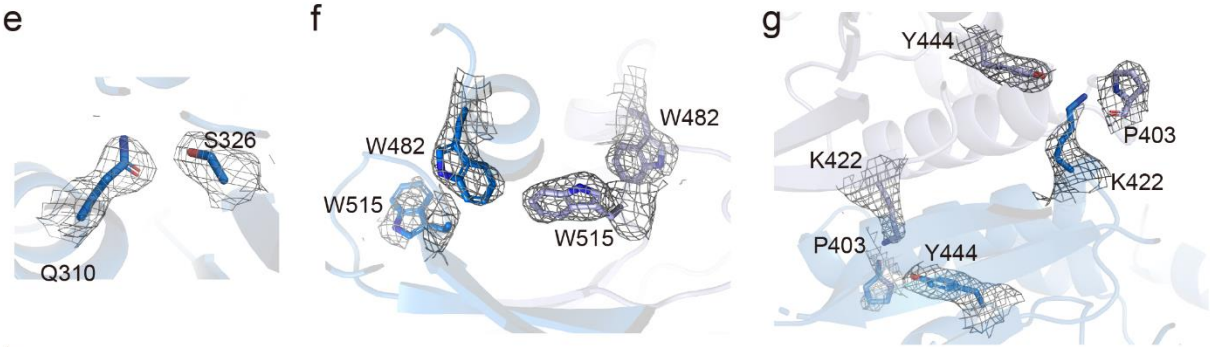
B domain
A domain
S326
C domain
α14-α15 loop
effector loop
b
C326
c
B domain
A domain
S326
C domain
α14-α15 loop
effector loop
d
C326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Y444

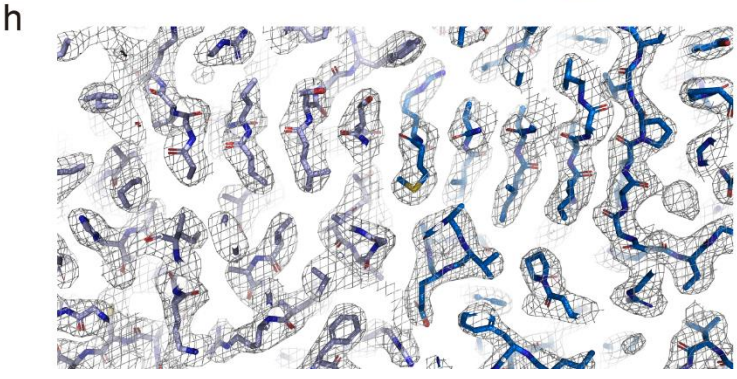

h

Figure S4. Structural analyses of PKM2^{C326S}, compared to the T and R states. (a) Structural overlay of PKM2^{C326S} protomers on one of the T-state protomers. **(b)** Structural overlay of PKM2 T-state protomers (PDB: 4FXJ). **(c)** Structural overlay of PKM2^{C326S} protomers on one of the R-state protomers. **(d)** Structural overlay of PKM2 R-state protomers (PDB: 4B2D). PKM2^{C326S} protomers are in blue, T-state protomers in pink, and R-state protomers in green. **(e-g)** 2Fo-Fc electron density map (gray) contoured at 1 σ for the designated residues. **(h)** 2Fo-Fc electron density map (gray) at 1σ for the A (blue) and D (light blue) protomer interface.

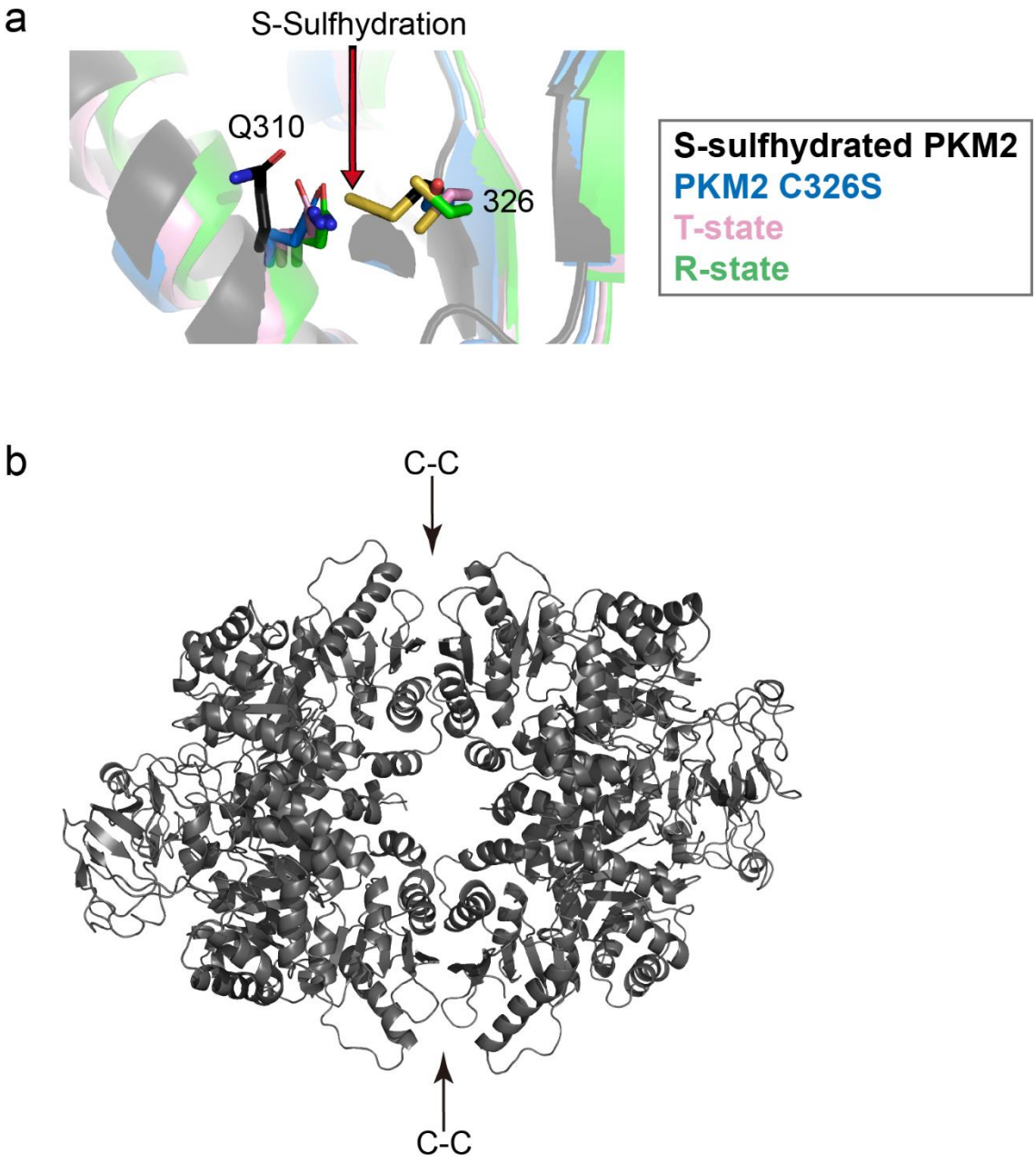
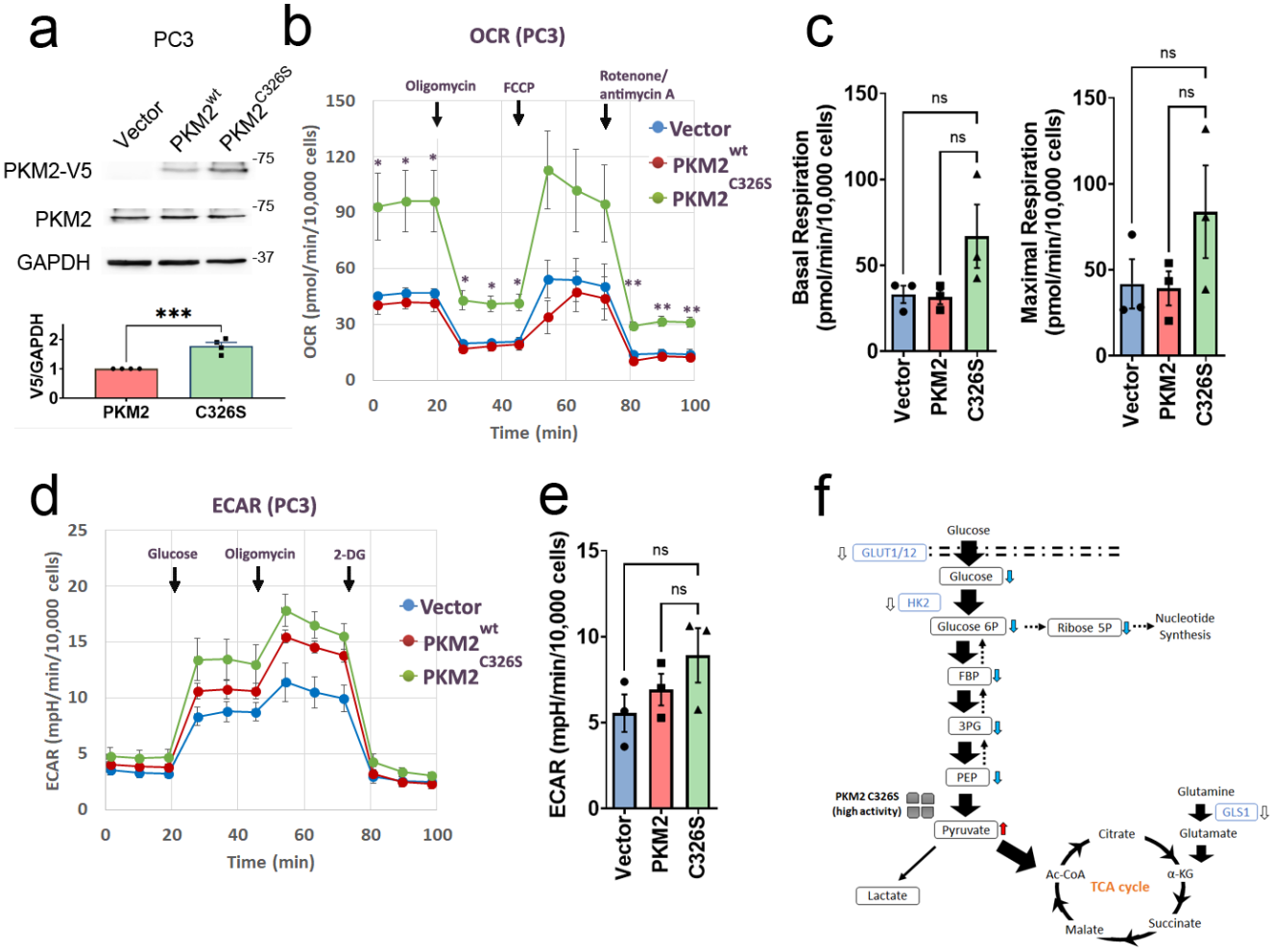


Figure S5. Simulation of PKM2 structure with S-sulfhydration at C326. (a) S-sulfhydration at C326 is shown for the most stable conformer (black), aligned with the C326S mutant (blue), T-state (pink), and R-state (green) conformations. **(b)** Simulation structure of the PKM2 tetramer with S-sulfhydration at C326.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PC3
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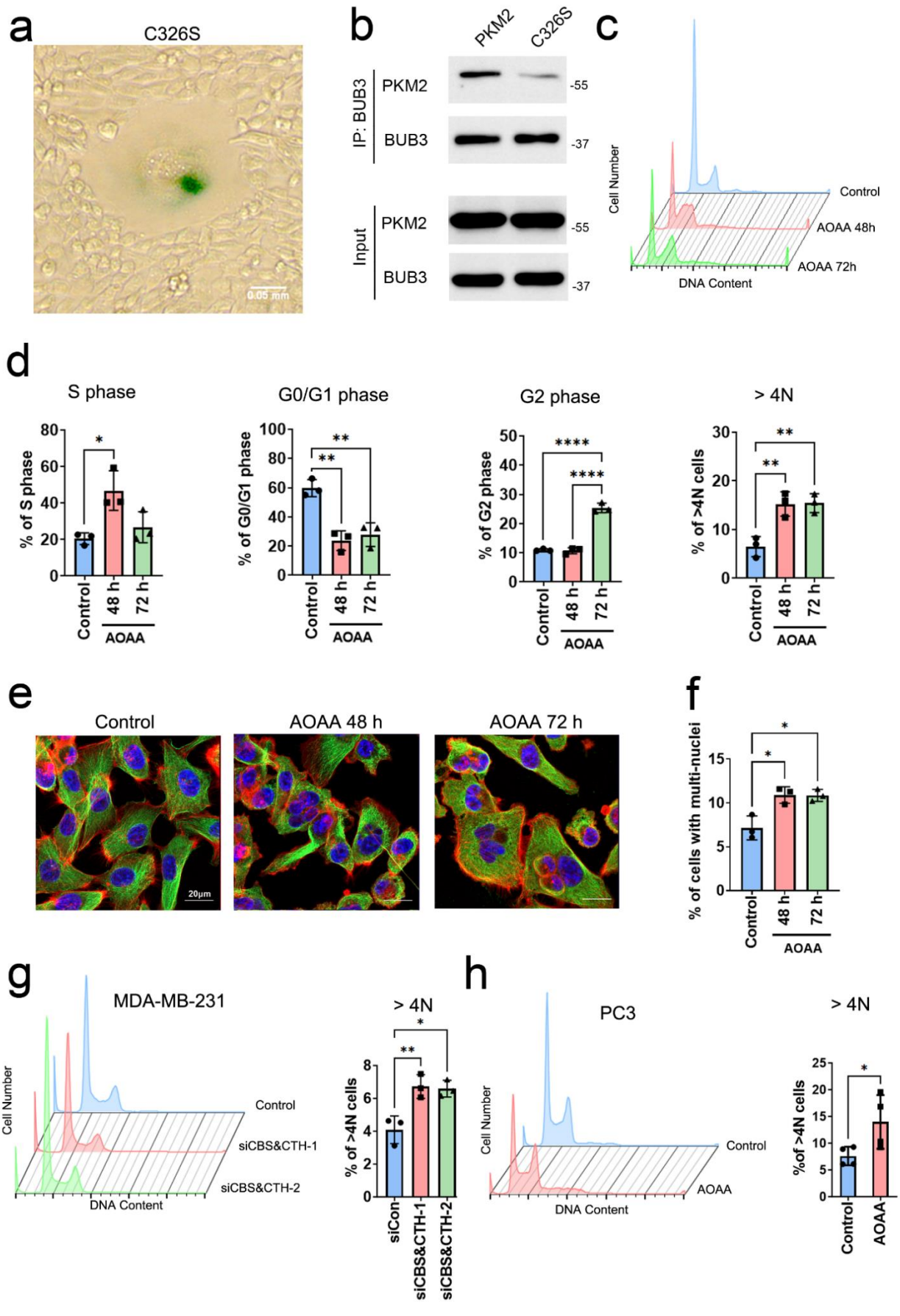
PKM2wt
PKM2C326S
PKM2-V5
PKM2
GAPDH
-75
-75
-37
V5/GAPDH
***
PKM2
C326S
b
OCR (PC3)
Oligomycin
FCCP
Rotenone/
antimycin A
OCR (pmol/min/10,000 cells)
Time (min)
Vector
PKM2wt
PKM2C326S
c
Basal Respiration
(pmol/min/10,000 cells)
Maximal Respiration
(pmol/min/10,000 cells)
ns
Vector
PKM2
C326S
d
ECAR (PC3)
Glucose
Oligomycin
2-DG
ECAR (mpH/min/10,000 cells)
Time (min)
e
ECAR (mpH/min/10,000 cells)
f
Glucose
GLUT1/12
HK2
Glucose 6P
Ribose 5P
Nucleotide
Synthesis
FBP
3PG
PEP
PKM2 C326S
(high activity)
Pyruvate
Lactate
Glutamine
GLS1
Glutamate
Citrate
Ac-CoA
TCA cycle
α-KG
Malate
Succinate

Figure S6. Blockade of PKM2 C326 sulfhydration enhances mitochondrial oxidative phosphorylation in PC3 cells. (a) Upper: Western blot analysis of stable expression of V5-tagged PKM2 (wild type or C326S mutant) in PC3 cells. The samples derive from the same experiment but different gels for V5, and another for PKM2 and GAPDH were processed in parallel. GAPDH is the internal control. Bottom: The V5-tagged PKM2 was normalized with the level of GAPDH. Data are presented as the normalized means ± SEM (n=3 biological replicates). The two-tailed student's t-test was used for the statistical analysis (***p<0.001). Immunoblotting experiments were repeated at least 3 times with similar results. **(b)** The oxygen consumption rate (OCR) curves in PC3 cells expressing vector alone, $PKM2^{wt}$, or $PKM2^{C326S}$. Cells were treated with oligomycin, FCCP, and rotenone/antimycin A, respectively. Data are presented as means ± SD (n=3 biological replicates). The one-tailed student's t-test was used for the statistical analysis (*p<0.05, **p<0.01; C326S compared to the vector or PKM2 group). **(c)** The level of basal OCR and maximal OCR normalized to the cell numbers in PC3 cells expressing vector alone, $PKM2^{wt}$, or $PKM2^{C326S}$ (n=3 biological replicates). One-way ANOVA followed by Tukey's multiple comparisons test was used for the statistical analysis (ns: not significant). **(d)** The ECAR curves in PC3 cells expressing Vector alone, $PKM2^{wt}$, or $PKM2^{C326S}$. Cells were treated with glucose, oligomycin, and 2-DG, respectively. Data are presented as means ± SD (n=3 biological replicates). **(e)** Glycolysis normalized to the cell numbers in PC3 cells expressing Vector alone, $PKM2^{wt}$, or $PKM2^{C326S}$ (n=3 biological replicates). One-way ANOVA followed by Tukey's multiple comparisons test was used for the statistical analysis (ns: not significant). **(f)** Schematic diagram of how blocking PKM2 sulfhydration at C326 facilitates the TCA cycle, resulting in the shortage of glycolytic intermediates for rapid DNA synthesis and enhanced oxidative phosphorylation. Source data are provided as a Source Data file.


a
C326S
0.05 mm
b
PKM2
C326S
IP: BUB3
PKM2
BUB3
Input
PKM2
BUB3
-55
-37
-55
-37
c
Cell Number
Control
AOAA 48h
AOAA 72h
DNA Content
d
S phase
% of S phase
G0/G1 phase
% of G0/G1 phase
G2 phase
% of G2 phase
> 4N
% of >4N cells
Control
48 h
72 h
AOAA
e
Control
AOAA 48 h
AOAA 72 h
20μm
f
% of cells with multi-nuclei
g
MDA-MB-231
Cell Number
Control
siCBS&CTH-1
siCBS&CTH-2
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siCon
siCBS&CTH-1
siCBS&CTH-2
h
PC3
Cell Number
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AOAA
DNA Content
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Control
AOAA

Figure S7. Blockade of PKM2 sulfhydration at C326 or depletion of H_2S causes defects in cell cycle progression and cytokinesis failure. (a) SA-β-gal activity staining of MDA-MB-231 cells expressing $PKM2^{C326S}$. Scale bar: 0.05 mm. **(b)** MDA-MB-231 cells transfected with wild-type PKM2 or $PKM2^{C326S}$ were synchronized by double thymidine block (2 mM) and then with RO-3306 (2.5 μg/mL) for 12 hr. After being released for 6 h, cells entering metaphase were harvested, immunoprecipitated with anti-BUB3 antibody, and immunoblotted with PKM2 and BUB3 antibodies. Immunoblotting experiments were repeated at least 3 times with similar results. **(c, d)** MDA-MB-231 cells were treated with 0.25 mM AOAA for 48 or 72 h. The cell cycle was examined by the PI staining method and measured by flow cytometry. **(c)** The representative image is shown. **(d)** The percentage of cells at different cell cycle phases was analyzed. Data are presented as the means ± SD (n=3 biological replicates). One-way ANOVA followed by Tukey's multiple comparisons test was used for the statistical analysis (*p<0.05; **p<0.01; ****p<0.0001). **(e, f)** Immunostaining of actin filaments (Red), α-tubulin (Green), and DNA counterstaining with DAPI (Blue) of MDA-MB-231 cells with 0.25 mM AOAA treatment for 48 or 72 h. **(e)** Representative images are shown. Scale Bar: 20μm. **(f)** The percentage of poly-nuclei cells ($n \geqq 2$) was analyzed. Data are presented as the means ± SD (n=3 biological replicates). One-way ANOVA followed by Tukey's multiple comparisons test was used for the statistical analysis (*p<0.05). **(g)** MDA-MB-231 cells were transfected with siCon, siCBS&CTH-1, and siCBS&CTH-2 for 72 h. The cell cycle was examined by the PI staining method and measured by flow cytometry. Left: The representative image is shown. Right: The percentage of cells at different cell cycle phases was analyzed. Data are presented as the means ± SD (n=3 biological replicates). One-way ANOVA followed by Tukey's multiple comparisons test was used for the statistical analysis (*p<0.05; **p<0.01). **(h)** PC3 cells were treated with 0.25 mM AOAA for 48h. The cell cycle was examined by the PI staining method and measured by flow cytometry. Left: The representative image is shown. Right: The percentage of cells at different cell cycle phases was analyzed. Data are presented as the means ± SD (n=4 biological replicates). The one tailed student's t-test was used for the statistical analysis (*p<0.05). Source data are provided as a Source Data file.

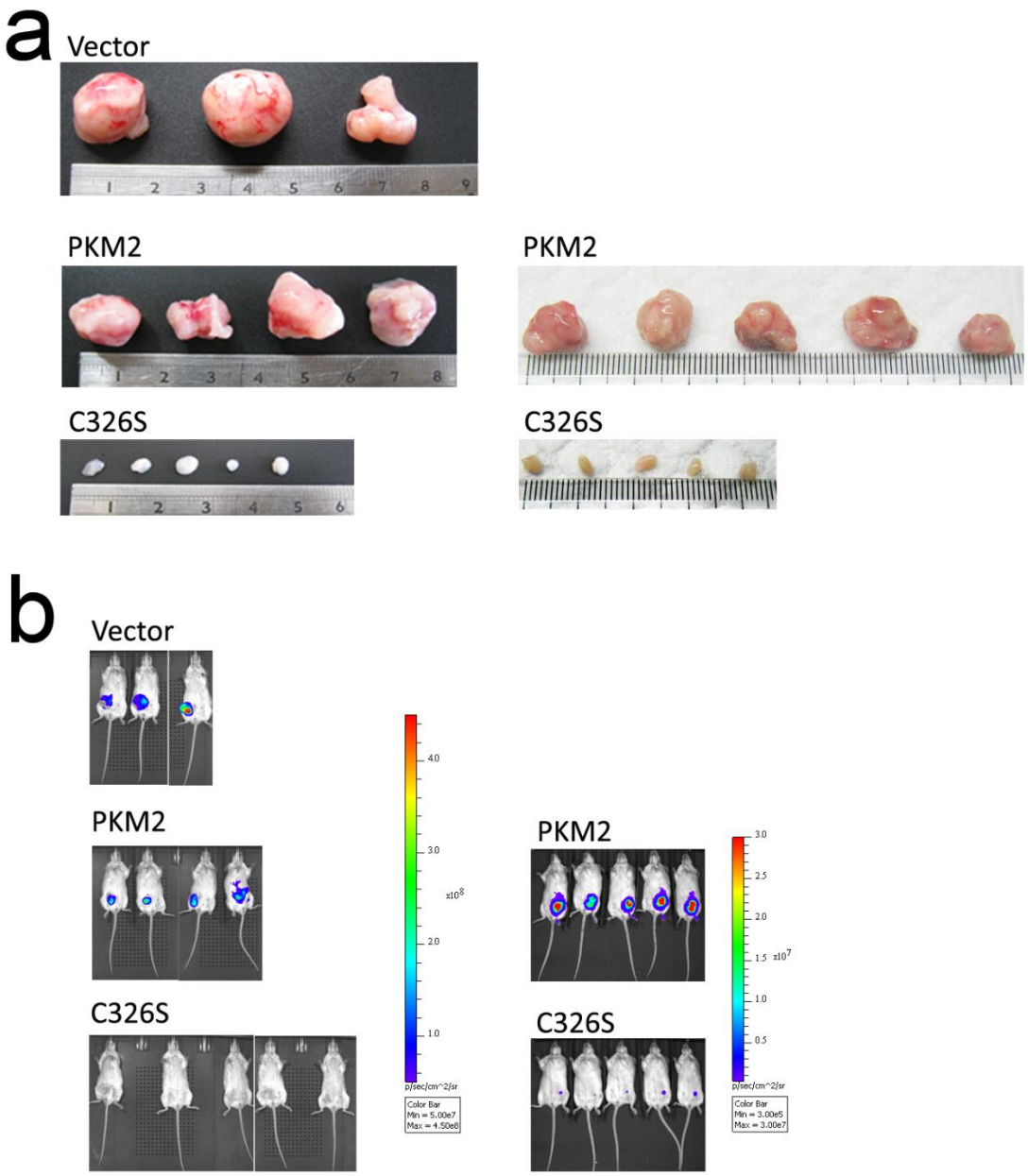

Figure S8. Blockade of PKM2 sulfhydration at C326 reduces tumor growth in the mouse xenograft model. (a) Pictures of all xenograft tumors of MDA-MD-231 cells expressing vector, $PKM2^{wt}$ or $PKM2^{C326S}$. **(b)** All animal bioluminescence images after implantation of MDA-MD-231 cells expressing vector, $PKM2^{wt}$ or $PKM2^{C326S}$ for 7 weeks.