Supplementary Material

Glucose triggered ZEB1 O-GlcNAcylation determines mesenchymal pancreatic cancer cell ferroptosis sensitivity

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Figure S1. Glucose enhanced mesenchymal pancreatic cancer cells ferroptosis sensitivity and O-GlcNAcylation level. (**A**) Western blot showed the basic expressions of ZEB1, E-Cadherin and N-Cadherin in PANC1, PaTU8988, BXPC3, ASPC1 and PANC02 cells. β-Tubulin protein expression was detected as a loading control. (**B**) The cell viability of PANC1 and PaTU8988 cells with the treatments DMSO, Erastin (4, 15 μM) or RSL3 (0.5, 5 μM) for 48 h combined with Ferrostain-1 (5 μM), Z-VAD-FMK (1 μM) or Necrosulfonamide (0.5 μM) were monitored using a CCK-8 assay. The relative viability was normalized to DMSO control. (**C**) MDA level in PaTU8988 cells treated with DMSO or Erastin (15 μM) for 48 h in glucose concentration gradient medium (4500, 2750, 1875, 1000 mg/mL). (**D**) Western blot analyzed the ZEB1 and SLC7A11 expression at the protein level in PANC1 and PaTU8988 cells cultured with glucose concentration gradient medium (4500, 3625, 2750, 1875, 1000 mg/mL). β-Tubulin protein expression was detected as a loading control. (**E-F**) Western blot analyzed the O-GlcNAc expression at the protein level in PANC1 (**E**) and PaTU8988 (**F**) cells cultured with glucose concentration gradient medium (4500, 3625, 2750, 1875, 1000 mg/mL). β-Tubulin protein expression was detected as a loading control. (**G**) Western blot showed the O-GlcNAcylation and SLC7A11 level in HPDE6 and PANC1 cells cultured with glucose or glucose-depletion medium for 48 h. β-Tubulin was used as a loading control. (**H**) MDA level in PANC1 and HPDE6 cells treated with DMSO or Erastin (4 μM) for 48 h in glucose present or depletion medium. (**I**) Western blot showed the O-GlcNAcylation level in PANC1, MCF7 and HT1080 cells cultured with glucose present medium for 48 h. β-Tubulin was used as a loading control. (**J**) CCK-8 assay assessed the cell viability of HT1080 and MCF7 cells treated with Erastin concentration gradient for 48h in glucose present medium, respectively. RSL3 represents for (1S,3R)-RSL3. MDA represents for malondialdehyde. O-GlcNAc represents for

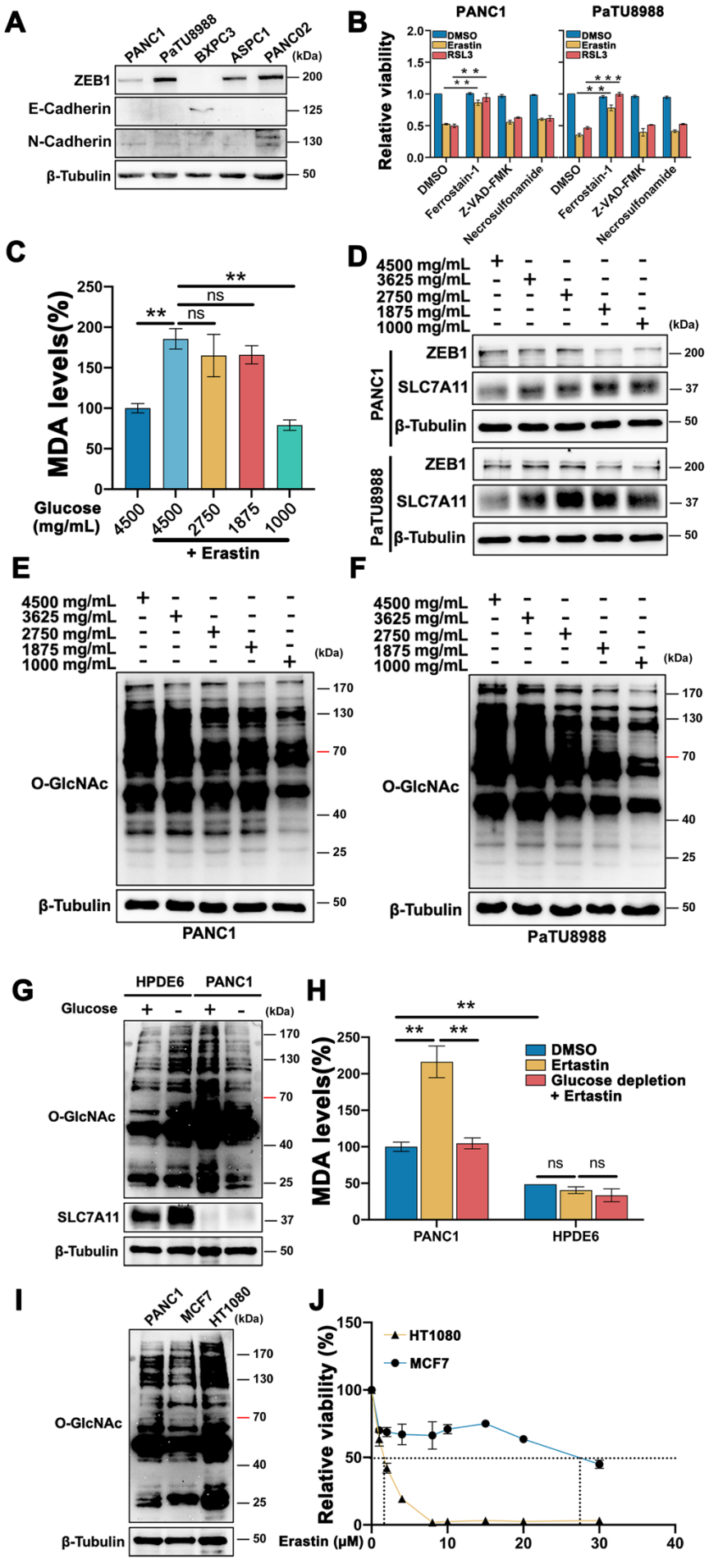
O-GlcNAcylation. Experiments were repeated three times and the data are expressed as the mean ± SEM. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$. **** $P < 0.0001$.

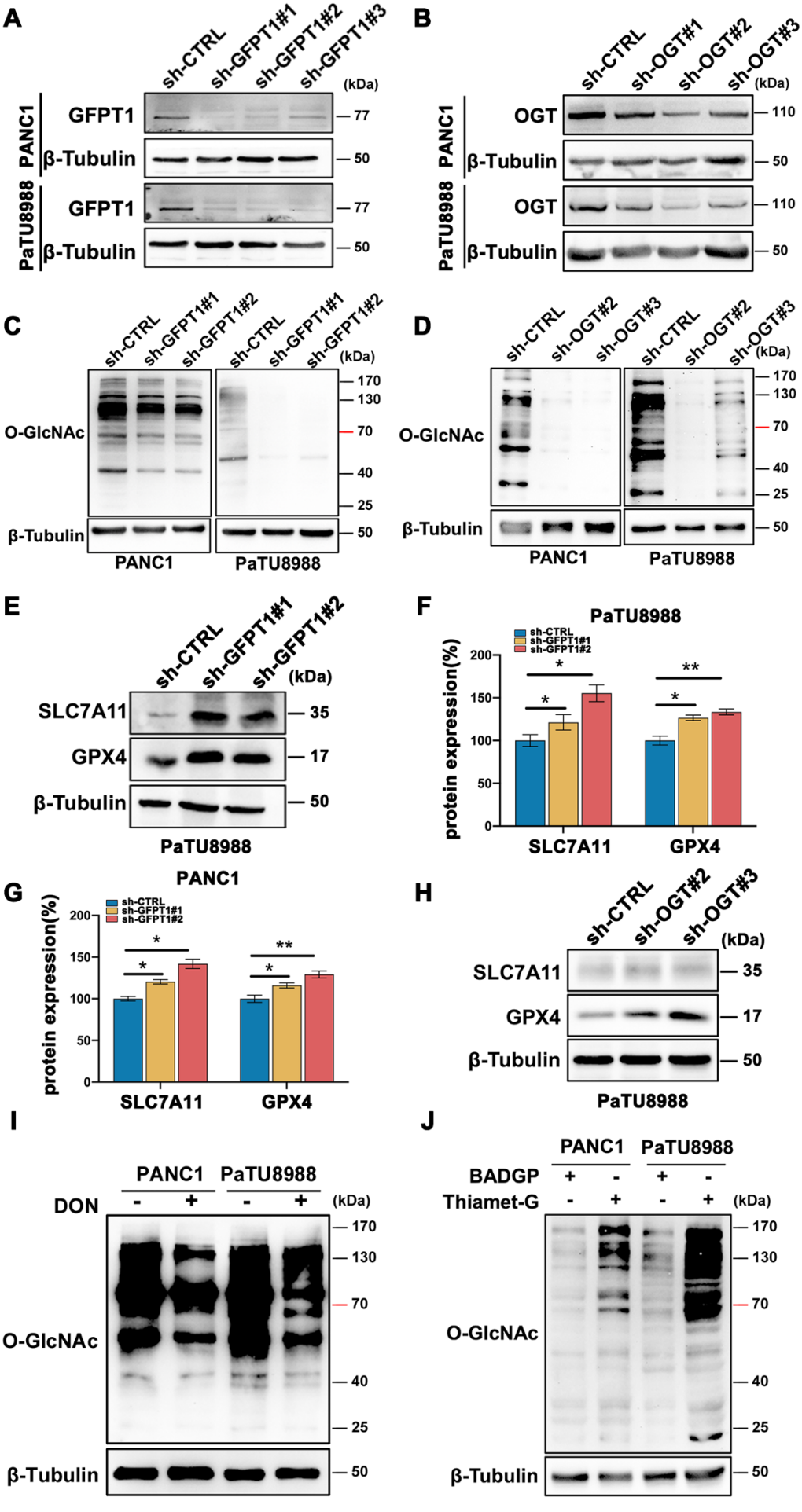
Figure S2. Glucose induced O-GlcNAcylation promoted mesenchymal pancreatic cancer cells ferroptosis. (**A**) PANC1 and PaTU8988 cells were transfected with sh-CTRL or sh-GFPT1 plasmids and GFPT1 protein expression were measured by immunoblotting analysis. β-Tubulin protein expression was detected as a loading control. (**B**) PANC1 and PaTU8988 cells were transfected with sh-CTRL or sh-OGT plasmids and OGT expression were measured by immunoblotting analysis. β-Tubulin protein expression was detected as a loading control. (**C**) Immunoblotting images of O-GlcNAcylation in sh-CTRL or sh-GFPT1 PANC1 and PaTU8988 cells. β-Tubulin protein expression was detected as a loading control. (**D**) Immunoblotting images of O-GlcNAcylation in sh-CTRL or sh-OGT PANC1 and PaTU8988 cells. β-Tubulin protein expression was detected as a loading control. (**E**) Western Blot assessed the SLC7A11 and GPX4 levels in GFPT1-silenced (sh-GFPT1) or control (sh-CTRL) PaTU8988 cells. β-Tubulin was used as a loading control. (**F**) The panel showed the ratios between SLC7A11/GPX4 and β-Tubulin protein levels of Figure S2E, and the sh-CTRL cells were set to 100%. (**G**) The panel showed the ratios between SLC7A11/GPX4 and β-Tubulin protein levels of Figure 2C, and the sh-CTRL cells were set to 100%. (**H**) Western Blot assessed the SLC7A11 and GPX4 levels in OGT-silenced (sh-OGT) or control (sh-CTRL) PaTU8988 cells. β-Tubulin was used as a loading control. (**I**) Immunoblotting images of O-GlcNAcylation in PANC1 and PaTU8988 cells with or without DON treatment for 48h. β-Tubulin protein expression was detected as a loading control. (**J**) Immunoblotting images of O-GlcNAcylation in PANC1 and PaTU8988 cells with BADGP (5 mM)

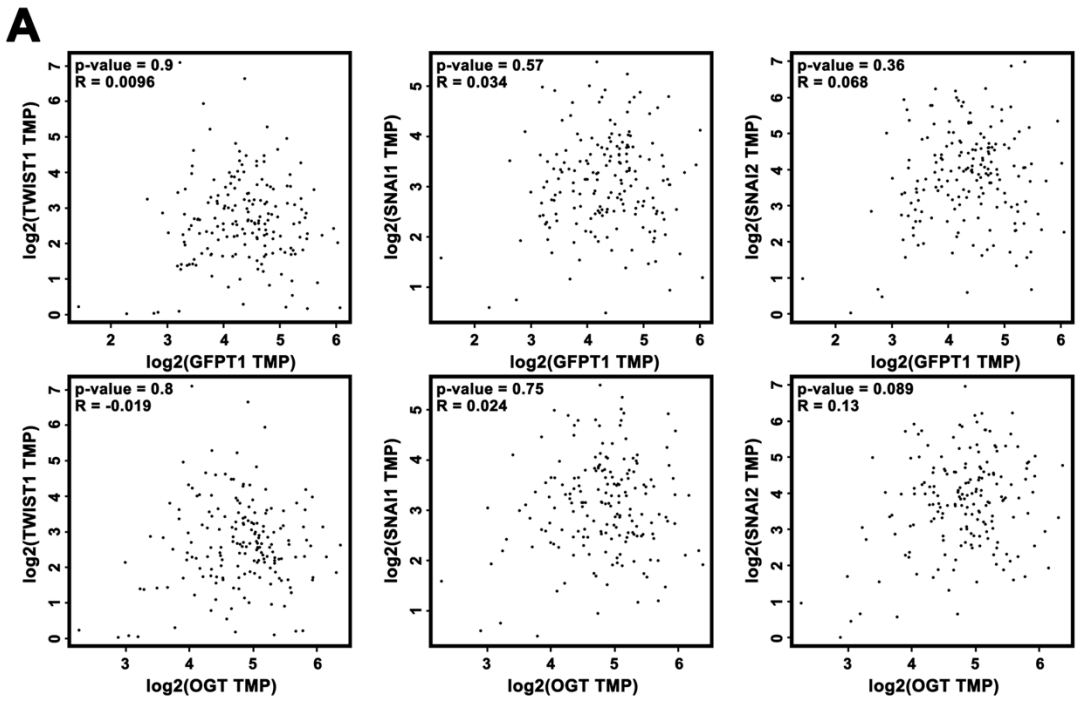
or Thiamet-G (20 μM) for 48h. β-Tubulin protein expression was detected as a loading control. O-GlcNAc represents for O-GlcNAcylation. DON represents for 6-Diazo-5-oxo-L-nor-Leucine. BADGP represents for Benzyl 2-acetamido-2-deoxy-a-D-galactopyranoside.

Figure S3. ZEB1 O-GlcNAcylation is involved in glucose regulated mesenchymal pancreatic cancer cells ferroptosis sensitivity. (**A**) The correlation between EMT-TFs and O-GlcNAcylation-associated genes expression was detected from the Cancer Genome Atlas (TCGA) PDAC samples. The data were analyzed by GEPIA. (**B**) PaTU8988 cells were transfected with sh-CTRL or sh-ZEB1 plasmids. Cell lysates were prepared to analyze the expression levels of the ZEB1 by Western blot. β-Tubulin protein expression was detected as a loading control. (**C**) Western blot assessed SLC7A11 and GPX4 levels in ZEB1-silenced (sh-ZEB1) or control (sh-CTRL) PaTU8988 cells. β-Tubulin was used as a loading control. (**D**) MDA level in ZEB1-silenced (sh-ZEB1) or control (sh-CTRL) PaTU8988 cells treated with Erastin (15 μM). (**E**) ZEB1 O-GlcNAcylation was determined by Co-IP in PANC1 cells. (**F**) RT-qPCR showed the mRNA expression of ZEB1 in PANC1 and PaTU8988 cells with or without OGT silenced. ACTB mRNA expression was detected as a loading control. (**G**) PANC1 and PaTU8988 cells were treated with BADGP (5 mM), Thiamet-G (20 μM) or DMSO for 48 h. Cell lysates were prepared to analyze the expression levels of the ZEB1 by Western blot. β-Tubulin protein expression was detected as a loading control. EMT-TFs represents for Epithelial-mesenchymal transition- associated transcription factors. PDAC represents for Pancreatic Ductal Adenocarcinoma. BADGP represents for Benzyl 2-acetamido-2-deoxy-a-D-galactopyranoside. Experiments were repeated three times and the data are expressed as the mean ± SEM.

Figure S4. FASN-FADS2 axis regulated polyunsaturated fatty acid biosynthesis was involved in ZEB1 O-GlcNAcylation driven ferroptosis sensitivity. (**A**) Co-IP analysis of ZEB1 O-GlcNAcylation in PANC1 and PaTU8988 cells transfected with Vector, ZEB1-WT-Flag, ZEB1-T678A-Flag and ZEB1-S555A-Flag plasmids. β-Tubulin protein expression was detected as a loading control. (**B**) PaTU8988 cells were transfected with indicated plasmids, followed by Erastin (15 μM) treatment for 48h. Relative lipid peroxidation levels was measured through MDA assay kit. (**C**) PANC1 and PaTU8988 cells were cultured in normal or glucose-depletion medium. Cell lysates were prepared to analyze the expression levels of ACLY, ACSS2, FASN, SCD1 and FADS2 proteins by Western blot. β-Tubulin protein expression was detected as a loading control. (**D**) Western blot assessed the expression level of ACLY, ACSS2, FASN, SCD1 and FADS2 in sh-CTRL or sh-OGT PaTU8988 cells. β-Tubulin was used as a loading control. (**E**) Western blot assessed the expression level of ACLY, ACSS2, FASN, SCD1 and FADS2 in sh-CTRL or sh-ZEB1 PaTU8988 cells. β-Tubulin was used as a loading control. (**F**) Western blot analyzed the FASN and FASD2 in ZEB1-WT and ZEB1-S555A PaTU8988 cells. β-Tubulin was used as a loading control. O-GlcNAc represents for O-GlcNAcylation. MDA represents for malondialdehyde. Experiments were repeated three times and the data are expressed as the mean ± SEM. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$. **** $P < 0.0001$.


A
PANC1
PaTU8988
BXPC3
ASPC1
PANC02
(kDa)
ZEB1
E-Cadherin
N-Cadherin
β-Tubulin
200
125
130
50
B
PANC1
PaTU8988
DMSO
Erastin
RSL3
Relative viability
DMSO
Ferrostain-1
Z-VAD-FMK
Necrosulfonamide
C
MDA levels(%)
Glucose (mg/mL)
4500
4500
2750
1875
1000
+ Erastin
ns
D
4500 mg/mL
3625 mg/mL
2750 mg/mL
1875 mg/mL
1000 mg/mL
ZEB1
SLC7A11
β-Tubulin
PANC1
PaTU8988
E
O-GlcNAc
β-Tubulin
PANC1
F
PaTU8988
G
HPDE6
PANC1
Glucose
SLC7A11
H
DMSO
Ertastin
Glucose depletion
+ Ertastin
I
PANC1
MCF7
HT1080
J
HT1080
MCF7
Relative viability (%)
Erastin (μM)


A
sh-CTRL
sh-GFPT1#1
sh-GFPT1#2
sh-GFPT1#3
(kDa)
PANC1
GFPT1
β-Tubulin
PaTU8988
GFPT1
β-Tubulin
77
50
77
50
B
sh-CTRL
sh-OGT#1
sh-OGT#2
sh-OGT#3
(kDa)
PANC1
OGT
β-Tubulin
PaTU8988
OGT
β-Tubulin
110
50
110
50
C
sh-CTRL
sh-GFPT1#1
sh-GFPT1#2
sh-CTRL
sh-GFPT1#1
sh-GFPT1#2
(kDa)
O-GlcNAc
β-Tubulin
170
130
70
40
25
50
PANC1
PaTU8988
D
sh-CTRL
sh-OGT#2
sh-OGT#3
sh-CTRL
sh-OGT#2
sh-OGT#3
(kDa)
O-GlcNAc
β-Tubulin
170
130
70
40
25
50
PANC1
PaTU8988
E
sh-CTRL
sh-GFPT1#1
sh-GFPT1#2
(kDa)
SLC7A11
GPX4
β-Tubulin
35
17
50
PaTU8988
F
PaTU8988
sh-CTRL
sh-GFPT1#1
sh-GFPT1#2
protein expression(%)
200
150
100
50
0
*
*
**
*
SLC7A11
GPX4
G
PANC1
sh-CTRL
sh-GFPT1#1
sh-GFPT1#2
protein expression(%)
200
150
100
50
0
*
*
**
*
SLC7A11
GPX4
H
sh-CTRL
sh-OGT#2
sh-OGT#3
(kDa)
SLC7A11
GPX4
β-Tubulin
35
17
50
PaTU8988
I
PANC1
PaTU8988
DON
-
+
-
+
(kDa)
O-GlcNAc
β-Tubulin
170
130
70
40
25
50
J
PANC1
PaTU8988
BADGP
+
-
+
-
Thiamet-G
-
+
-
+
(kDa)
O-GlcNAc
β-Tubulin
170
130
70
40
25
50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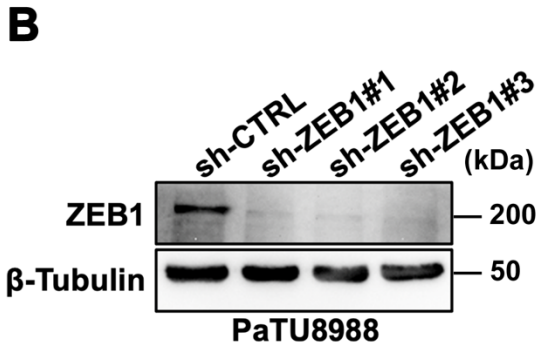
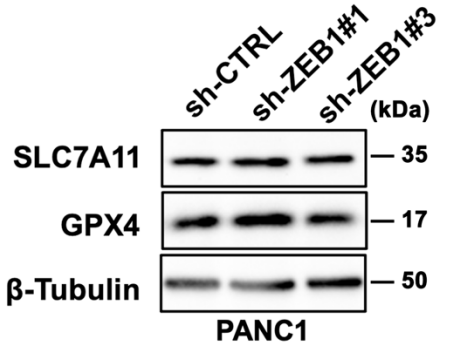
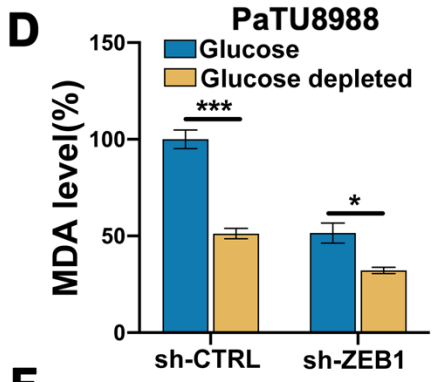
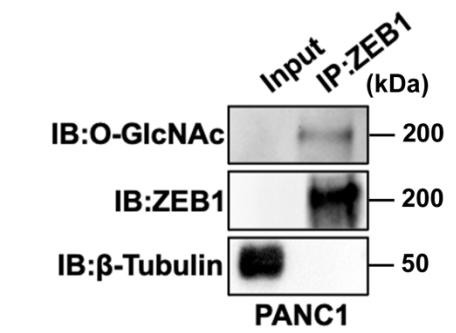
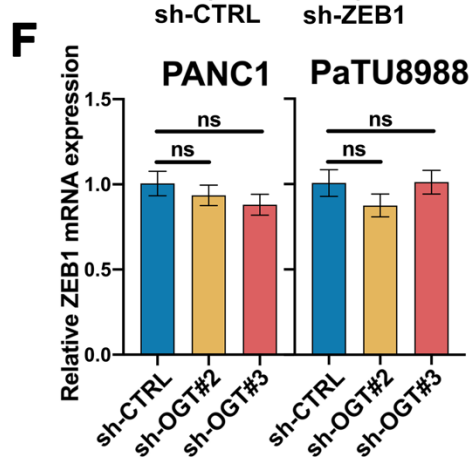
p-value = 0.9
R = 0.0096
log2(TWIST1 TMP)
log2(GFPT1 TMP)
p-value = 0.57
R = 0.034
log2(SNAI1 TMP)
log2(GFPT1 TMP)
p-value = 0.36
R = 0.068
log2(SNAI2 TMP)
log2(GFPT1 TMP)
p-value = 0.8
R = -0.019
log2(TWIST1 TMP)
log2(OGT TMP)
p-value = 0.75
R = 0.024
log2(SNAI1 TMP)
log2(OGT TMP)
p-value = 0.089
R = 0.13
log2(SNAI2 TMP)
log2(OGT TMP)

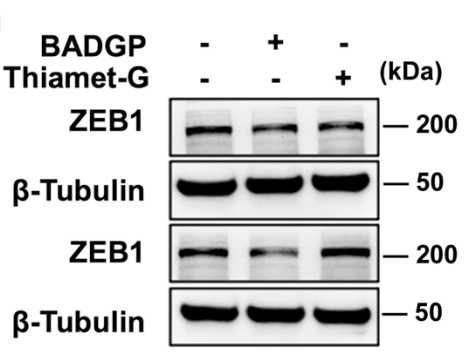

B
sh-CTRL
sh-ZEB1#1
sh-ZEB1#2
sh-ZEB1#3
(kDa)
ZEB1
200
β-Tubulin
50
PaTU8988


C
sh-CTRL
sh-ZEB1#1
sh-ZEB1#3
(kDa)
SLC7A11
35
GPX4
17
β-Tubulin
50
PANC1


D
PaTU8988
Glucose
Glucose depleted
MDA level(%)
150
100
50
0
***
*
sh-CTRL
sh-ZEB1


E
Input
IP:ZEB1
(kDa)
IB:O-GlcNAc
200
IB:ZEB1
200
IB:β-Tubulin
50
PANC1


F
PANC1
PaTU8988
Relative ZEB1 mRNA expression
1.5
1.0
0.5
0.0
ns
ns
ns
ns
sh-CTRL
sh-OGT#2
sh-OGT#3
sh-CTRL
sh-OGT#2
sh-OGT#3


G
BADGP
Thiamet-G
(kDa)
PANC1
ZEB1
200
β-Tubulin
50
PaTU8988
ZEB1
200
β-Tubulin
50

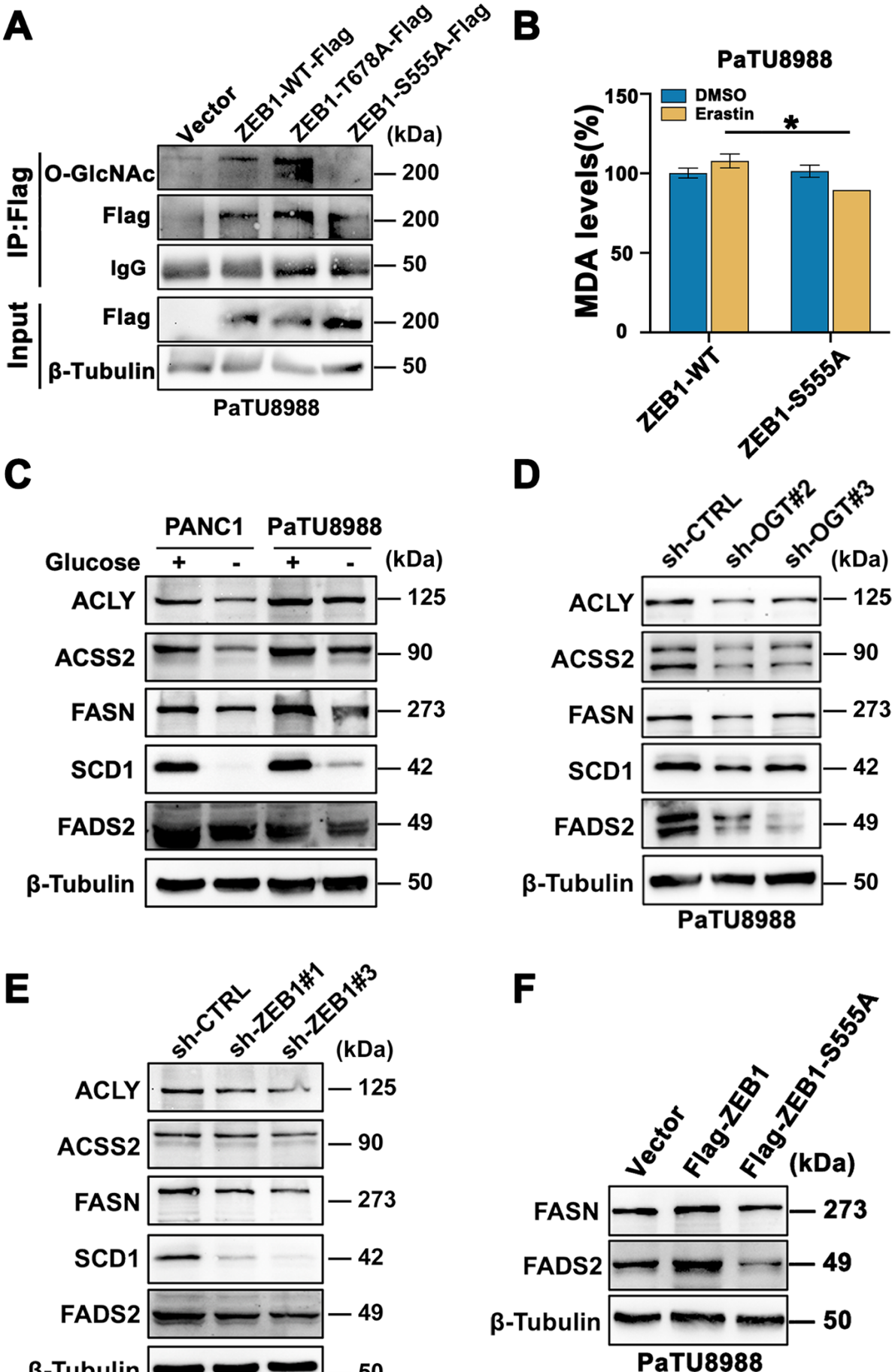

A
Vector
ZEB1-WT-Flag
ZEB1-T678A-Flag
ZEB1-S555A-Flag
(kDa)
IP:Flag
O-GlcNAc
Flag
IgG
Input
Flag
β-Tubulin
200
200
50
200
50
PaTU8988
B
PaTU8988
DMSO
Erastin
MDA levels(%)
150
100
50
0
*
ZEB1-WT
ZEB1-S555A
C
PANC1
PaTU8988
Glucose
+
-
+
-
(kDa)
ACLY
ACSS2
FASN
SCD1
FADS2
β-Tubulin
125
90
273
42
49
50
D
sh-CTRL
sh-OGT#2
sh-OGT#3
(kDa)
ACLY
ACSS2
FASN
SCD1
FADS2
β-Tubulin
125
90
273
42
49
50
PaTU8988
E
sh-CTRL
sh-ZEB1#1
sh-ZEB1#3
(kDa)
ACLY
ACSS2
FASN
SCD1
FADS2
β-Tubulin
125
90
273
42
49
50
PaTU8988
F
Vector
Flag-ZEB1
Flag-ZEB1-S555A
(kDa)
FASN
FADS2
β-Tubulin
273
49
50
PaTU8988

Table S1 Primers for knockdown plasmids

Gene	Sequence
sh-CTRL	5`-GTTCTCCGAACGTGTCACGTT-3`
sh-GFPT1#1	5`-GCAGATACTTTGATGGGTCTT-3`
sh-GFPT1#2	5`-CGTCTTTCTATCCATCGAATT-3`
sh-GFPT1#3	5`-CCTCTGGCTTTGGTGGATAAA-3`
sh-OGT#1	5`-TTTAGCACTCTGGCAATTAAA-3`
sh-OGT#2	5`-GCTGAGCAGTATTCCGAGAAA-3`
sh-OGT#3	5`-GCCCTAAGTTTGAGTCCAAAT-3`
sh-ZEB1#1	5`-GCGGTAGATGGTAATGTAATA-3`
sh-ZEB1#2	5`-GCAAGTGTTGGAGAATAATCA-3`
sh-ZEB1#3	5`-GCATACACCTACTCAACTACG-3`

Table S2 Primers for overexpression and mutant plasmids

Gene	Target sequence
ZEB1-WT-Flag	F:5`-TCCTCGAGACTAGTTgccaccatgaaagttacaaattataatactgtggtag-3` R:5`-GTCCATTCCGCGGCCGCTggcttcatttgtcttttcttcagacac-3`
ZEB1-T678A-Flag	F:5`-tcCTCGAGACTAGTTgccaccatgaaagttacaaattataatactgtggtag-3` R:5`-GTCCATTCCGCGGCCGCTggcttcatttgtcttttcttcagac-3`
ZEB1-S555A-Flag	F:5`-TCCTCGAGACTAGTTgccaccatgaaagttacaaattataatactgtggtag-3` R:5`-GTCCATTCCGCGGCCGCTggcttcatttgtcttttcttcagacac-3`

Table S3 Primers for RT-qPCR

Gene	Sequence
ACTB	F: 5`-CACCATTGGCAATGAGCGGTTC-3`
	R: 5`-AGGTCTTTGCGGATGTCCACGT-3`
ZEB1	F:5`-GGCATACACCTACTCAACTACGG-3`
	R:5`-TGGGCGGTGTAGAATCAGAGTC-3`
ACLY	F: 5`-ATCGGTTCAAGTATGCTCGGG-3`
	R: 5`-GACCAAGTTTTCCACGACGTT-3`
ACSS2	F: 5`-AAAGGAGCAACTACCAACATCTG-3`
	R: 5`-GCTGAACTGACACACTTGGAC-3`
FASN	F:5`-TTCTACGGCTCCACGCTCTTCC-3`
	R:5`-GAAGAGTCTTCGTCAGCCAGGA-3`
SCD1	F: 5`-CCTGGTTTCACTTGGAGCTGTG-3`
	R: 5`-TGTGGTGAAGTTGATGTGCCAGC-3`
FADS2	F:5`-TGCAACGTGGAGCAGTCCTTCT-3`
	R:5`-GGCACATAGAGACTTCACCAGC-3`

Table S4 Antibodies for Western blot, IP and IHC

Antibody	Source	Catalog number	Application (dilution)
O-Linked N-Acetylglucosamine	Abcam	ab201995	WB (1:1000) IHC (1:200)

β-Tubulin	ABclonal	AC030	WB (1:5000)
ZEB1	CST	3396S	WB (1:1000)
ZEB1	Proteintech	21554-1-AP	IP (1:200), IHC (1:50)
E-Cadherin	Proteintech	20874-1-AP	WB (1:1000)
N-Cadherin	Proteintech	22018-1-AP	WB (1:1000)
SLC7A11	CST	12691S	WB (1:1000)
GFPT1	Proteintech	14132-1-AP	WB (1:1000)
GPX4	Abcam	ab125066	WB (1:1000)
OGT	Proteintech	11576-2-AP	WB (1:1000)
HDAC	GeneTex	GTX100513	WB (1:1000)
FASN	CST	3189S	WB (1:1000)
ACLY	CST	4332S	WB (1:1000)
ACSS2	Santa cruz	SC-398559	WB (1:1000)
FADS2	Proteintech	28034-1-AP	WB (1:1000)
SCD1	Abcam	ab236868	WB (1:1000)
COX2	Abcam	ab283574	IHC (1:50)
Flag	ABclonal	AE005	IP (1:200)

Abbreviations: WB, western blotting assay; CST, Cell Signaling Technology.