

1 **Supplementary information for**
2 **FTO O-GlcNAcylation downregulates NLRP3 inflammasome and**
3 **pyroptosis in macrophages**

4

5

6

7

8 **The PDF file includes:**

9 Supplementary Figs. 1 to 4

10 Legends for data files 1 to 4

11 Supplementary Tables. 1 to 7

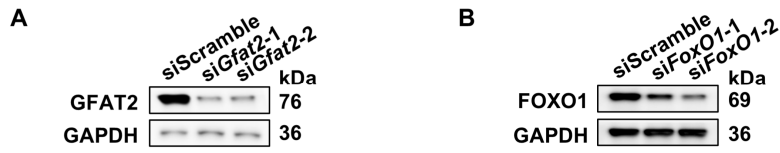
12

13

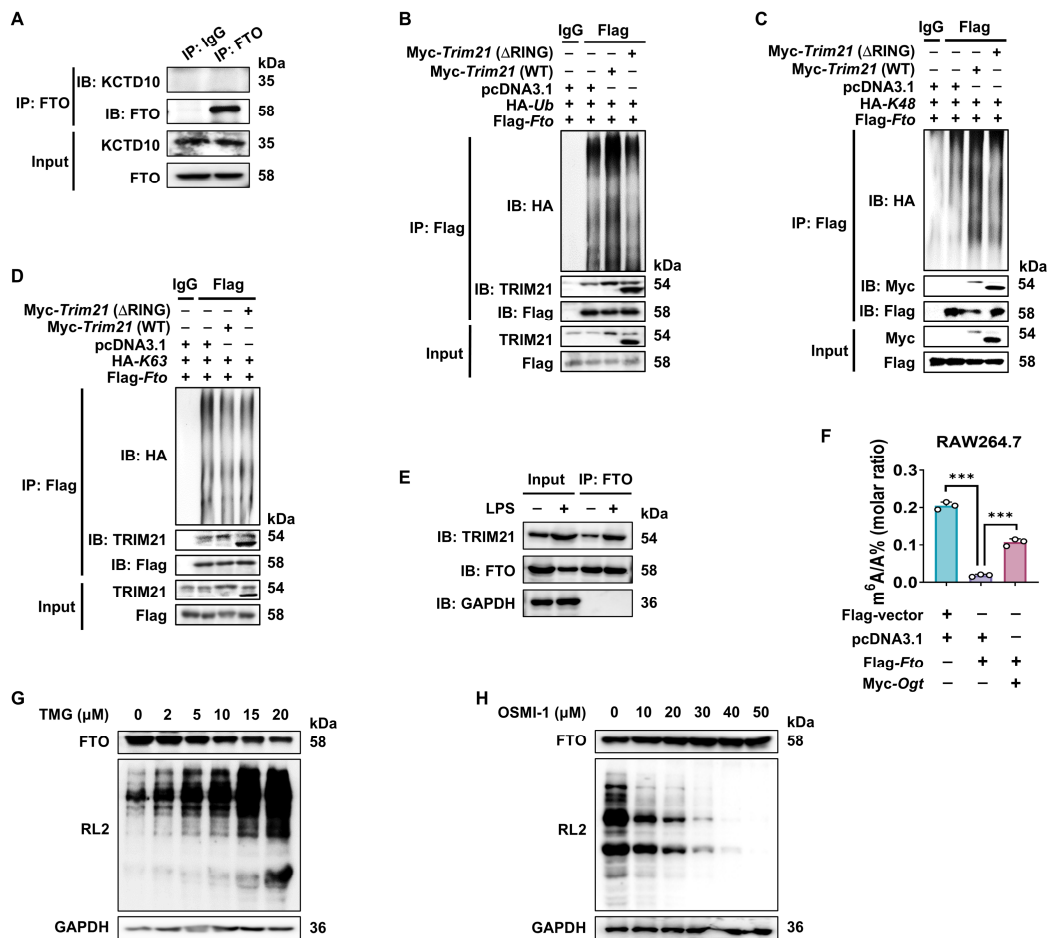
14

15

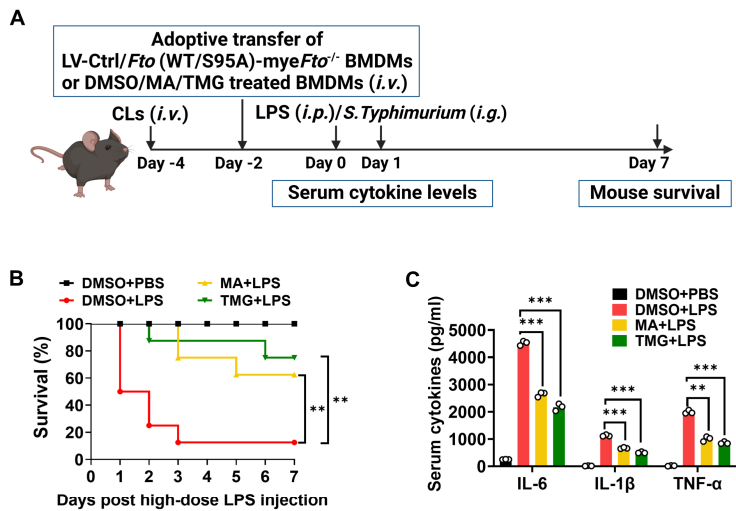
16 **Supplementary Figures**
17



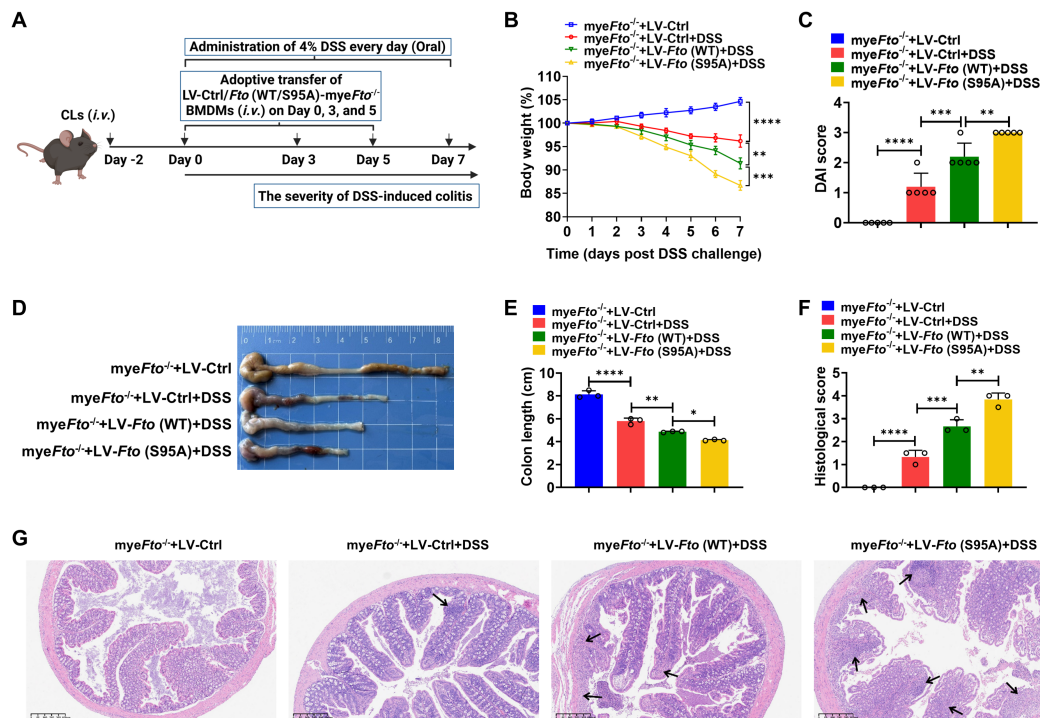
18 **Supplementary Fig. 1. Validation the knockdown effect of *siGfat2* and *siFoxO1*.** A-B, Immunoblot
19 analysis of *siGfat2-1/siGfat2-2* (A) and *siFoxO1-1/siFoxO1-2* (B) silencing efficiencies in BMDMs. GAPDH
20 served as a loading control for (A-B). Data are representative of three independent experiments with similar
21 results.
22
23
24



25
 26 **Supplementary Fig. 2. FTO O-GlcNAcylation facilitates TRIM21-mediated FTO K48-ubiquitination**
 27 **degradation.** **A**, Co-IP and immunoblot analysis of FTO binding with KCTD10. Total FTO was IP with anti-
 28 FTO Ab from RAW264.7 cells, followed by immunoblotting with anti-FTO and anti-KCTD10 Abs. **B-D**,
 29 Co-IP and immunoblot analysis of the effects of TRIM21 on FTO ubiquitination. HEK293T cells were co-
 30 transfected with *Flag-Fto* and *HA-Ub* (B)/*K48* (C)/*K63* (D) plus *Myc-Trim21* (WT), *Myc-Trim21* (Δ RING)
 31 mutant vectors. **E**, Co-IP and immunoblot analysis of FTO binding with TRIM21 in the presence or absence
 32 of LPS stimulation. BMDMs were exposed to PBS or LPS for 12 h, and then cell lysates were subjected to
 33 Co-IP with an anti-FTO and immunoblot with Abs against FTO and TRIM21. **F**, LC-MS/MS detection of
 34 m⁶A/A (%) of total RNA in individual RAW264.7 cells transfected with *Flag-Fto* or *Myc-Ogt*. **G-H**,
 35 Immunoblot analysis of the FTO expression in RAW264.7 cells treated with TMG (G) or OSMI-1 (H) for 24
 36 h. GAPDH served as a loading control for (E) and (G-H). Data are representative of three independent
 37 experiments with similar results. Data represent the mean \pm SD with significance determined by one-way
 38 ANOVA followed by the Dunnett multiple comparison tests for (F). *** $p < 0.001$.
 39



40
 41 **Supplementary Fig. 3. Promotion of O-GlcNAcylation or inhibition of FTO alleviates LPS-induced**
 42 **inflammatory responses and septic shock in mice.** **A**, The illustration of macrophage depletion and
 43 reconstitution procedure. CLs: clodronate liposomes. **B**, Kaplan–Meier survival curves of macrophage-
 44 depleted mice reconstituted with mouse BMDMs treated with DMSO or MA or TMG, followed by PBS or
 45 LPS (25 mg/kg, mouse) challenge. Log-rank (Mantel-Cox) test was used to assess the statistical difference.
 46 8 mice were in each group. **C**, ELISA of serum IL-6/IL-1 β /TNF- α concentrations from mice (**B**) injected
 47 with PBS or LPS (25 mg/kg, mouse) for 24h. Data are representative of three independent experiments with
 48 similar results. Data represent the mean \pm SD with significance determined by one-way ANOVA followed by
 49 Tukey’s multiple comparison tests for (**C**). ** $p < 0.01$; *** $p < 0.001$.
 50



51
 52 **Supplementary Fig. 4. FTO O-GlcNAcylation protects against DSS-induced inflammatory bowel**
 53 **diseases.** **A**, The illustration of macrophage depletion and reconstitution procedure. **B-C**. Comparison of
 54 body weights (B) and Disease Activity Index (DAI) scores (C) between each group of mice as indicated. $n =$
 55 5 mice/group. **D**. Macroscopic picture of the colon between each group of mice as indicated. $n = 3$ mice/group.
 56 **E**. Comparison of colon length between each group of mice as indicated. $n = 3$ mice/group. **F**.
 57 Histopathological score of colon tissues from each group of mice. $n = 3$ mice/group. **G**. HE staining of colon
 58 tissues from each group of mice. $n = 3$ mice/group. Data are representative of three independent experiments
 59 with similar results. Data represent the mean \pm SD with significance determined by two-way repeated
 60 measures ANOVA followed by Tukey's multiple comparison tests for (B), and by one-way ANOVA followed
 61 by Tukey's multiple comparison tests for (C) and (E-F). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

62

63 **Supplementary Tables**

64

65

66

Supplementary Table 1. E3 ubiquitin ligases from mass spectrometry

67

Protein/Accession	Description	Score	Coverage
TRIM21/Q3U7K7	RING-type E3 ubiquitin-protein ligase	4.02	3.89
KCTD10/Q922M3	The substrate-specific adapter of E3 ligase	8.81	16.19

68

69

70

Supplementary Table 2. Primers used in RT-qPCR

71

Genes	Forward (5'-3')	Reverse (5'-3')
<i>Fto</i>	CCTCAATGACTCAGACGATGG	GAGCACCGCATTGTCATGC
<i>Gfat2</i>	GTATGATTGGCCGACCCTGG	ATGCTAGCCGGAGAGCTGAA
ChIP- <i>Gfat2</i>	AAGTGCTCTACTACTGAGCTAAGTCC	GCTACCACTGCTGAGGAATCTAAC
<i>FoxO1</i>	CCCAGGCCGGAGTTTAACC	GTTGCTCATAAAGTCGGTGCT
<i>Gapdh</i>	CAGGAGAGTGTTTCCTCGTCC	GATGGGCTTCCCCTTGATGA

72

73

74
75

Supplementary Table 3. Antibodies used in this study

Antibodies	Source	Identifier
FTO	Abcam	Cat. No. ab92821, RRID: AB_10565042
O-GlcNAc (RL2)	Abcam	Cat. No. ab2739, RRID: AB_303264
OGT	Cell Signaling Technology	Cat. No. 24083, RRID: AB_2716710
OGA	ABclonal Biotech	Cat. No. A24124, RRID: AB_3668909
FOXO1	ABclonal Biotech	Cat. No. A2934, RRID: AB_2764748
GFAT2	ABclonal Biotech	Cat. No. A15374, RRID: AB_2762279
TRIM21	ABclonal Biotech	Cat. No. A18027, RRID: AB_2861823
KCTD10	Proteintech	Cat. No. 27279-1-AP, RRID: AB_2880827
p-Ser/Thr	ABclonal Biotech	Cat. No. AP1067, RRID: AB_2863939
p-P65	Cell Signaling Technology	Cat. No. 3033, RRID: AB_331284
P65	Cell Signaling Technology	Cat. No. 3034, RRID: AB_330561
Rabbit anti-Flag	ABclonal	Cat. No. AE092, RRID: AB_2940847
Mouse anti-Myc	Proteintech	Cat. No. 60003-2-Ig RRID: AB_2734122
Mouse anti-HA	Abcam	Cat. No. ab18181, RRID: AB_444303
Ubiquitin	Proteintech	Cat. No. 10201-2-AP, RRID: AB_671515
K48-ubiquitin	Abcam	Cat. No. ab140601, RRID: AB_2783797
K63-ubiquitin	Abcam	Cat. No. ab179434, RRID: AB_2895239
NLRP3	R & D systems	Cat. No. MAB7578, RRID: AB_2889405
ASC rabbit mAb	Cell Signaling Technology	Cat. No. 67824, RRID: AB_2799736
ASC mouse mAb	Proteintech	Cat. No. 67494-1-Ig, RRID: AB_2882718
Caspase-1	Cell Signaling Technology	Cat. No. 2225, RRID: AB_2243894
GSDMD	Abcam	Cat. No. ab219800, RRID: AB_2888940
GAPDH	Santa Cruz Biotechnology	Cat. No. sc-365062, RRID: AB_10847862
Rabbit IgG	Cell Signaling Technology	Cat. No. 3900, RRID: AB_1550038
Mouse IgG	Proteintech	Cat. No. 66360-1-Ig, RRID: AB_2827991
Alexa Fluor 488- anti-mouse	Cell Signaling Technology	Cat. No. 4408, RRID: AB_2130165
Alexa Fluor 594- anti-rabbit	Cell Signaling Technology	Cat. No. 8889, RRID: AB_2716249
HRP conjugate anti- Mouse IgG	Proteintech	Cat. No. SA00001-1, RRID: AB_2722565
HRP conjugate anti- Rabbit IgG	Proteintech	Cat. No. SA00001-1, RRID: AB_2722565

76
77

78

Supplementary Table 4. All siRNAs used in this study

79

Genes	Sense (5'-3')	Antisense (5'-3')
<i>siFoxO1-1</i>	GAAAGAGUUCUUGGUGGAUGCUGAA	UUGAGCAUCCACCAAGAACUCUUUC
<i>siFoxO1-2</i>	CAAGUUACGGAGGAUUGAACCAGUA	UACUGGUUCAAUCCUCCGUAACUUG
<i>siGfat2-1</i>	CCGAGAGCUCCAAGUUUGCAUAUAA	UUUAUAUGCAAACUUGGAGCUCUCGG
<i>siGfat2-2</i>	CAAAGGCUACGAGUUUGAGUCAGAA	UUCUGACUCAAAACUCGUAGCCUUUG
<i>siTrim21-1</i>	CAACAGUUUCUGCUCCGAATT	UUCGGAGCAGAAACUGUUGTT
<i>siTrim21-2</i>	GGACAUGUUGGGUUCAUAUTT	AUAUGAACCCAACAUGUCCTT

80

81

82
83

Supplementary Table 5. Primer sequences for molecular clonings

Primer names	Primer sequences (5'-3')
pFlag-CMV- <i>Fto</i>	F: TACAAAGACGATGACGACAAGCTTATGAAGCGCGTCCAGACCGCGG R: CGATATCAGATCTATCGATGAATCCTAGGATCTTGCTTCCAGCAG
pFlag-CMV- <i>Fto</i> -N	F: CCAAGCTTATGAAGCGCGTCCAGACCGCGGAGGA R: CGGAATTCCTACTCTGCCACACGGTGAGTGGAACATAAC
pFlag-CMV- <i>Fto</i> -C	F: CCAAGCTTATGTGCTCAACAGGCACCTTGGAT R: CGGAATTCCTAGGATCTTGCTTCCAGCAGCTGGCCCTCT
pFlag-CMV- <i>Fto</i> (S95A)	F2: GCTCACCCAGTGGCTCGCATCCTCAT R2: ATGAGGATGCGAGCCACTGGGGTGAGC
pFlag-CMV- <i>Fto</i> (T92A)	F2: CAAAGATGTGCTGCCCCAGTGTCTCGC R2: GCGAGACTGGGGCAGCACATCTTG
pFlag-CMV- <i>Fto</i> (S226A)	F2: GAAGATGGCGGTGGCTGGCATCACGAT R2: ATCGTGATGCCAGGCCACCGCCATCTTC
pFlag-CMV- <i>Fto</i> (S237A)	F2: CTGGTGGACAGGGCAGCCGTGGCAGT R2: ACTGCCACGGCTGCCCTGTCCACCAG
pFlag-CMV- <i>Fto</i> (K213R)	F2: ATGCCCTACTTGAGAGAGGAGCCCTAT R2: ATAGGGCTCCTCTCTCAAGTAGGGCAT
pcDNA3.1-Myc- <i>Ogt</i>	F: TTGGATCCATGGCGTCTTCCGTGGGCAACGTG R: CCAAGCTTGCTGACTCGGTGACTTCAACAGGCTTA
pcDNA3.1-Myc- <i>Ogt</i> (K908A)	F2: ATTTTCTCACCTGTGGCTCCTGCAGAGGAGCATGTCAGGAGAG R2: ACCTCTCCTGACATGCTCCTCTGCAGGAGCCACAGGTGAGAAA
pcDNA3.1-Myc- <i>Ogt</i> -F1	F: CACCACACTGGACTAGTGGATCCACAGAATCCCCTTCTAGCAG R: CCAAGCTTGCTGACTCGGTGACTTCAACAGGCTTA
pcDNA3.1-Myc <i>Ogt</i> F2	F: AGAATCCCCTTCTAGCAGAAAATCCTGATTTGACTGTGTTTCGCAGT R: CACTGCGAACACAGTACAAAATCAGGATTTTCTGCTAGAAGGGGATT
pcDNA3.1-Myc- <i>Ogt</i> F3	F: GATTTGACTGTGTTTCGCAGTGACCCTTGACCCAAATTTTCTGGATGC R: CCAGAAAATTTGGGTCAAGGGTCACTGCGAACACAGTACAAAATCAG
pcDNA3.1-Myc- <i>Gfat2</i>	F: AGCGTTTAAACGGGCCCTCTAGAATGTGCGGAATCTTTGCCTACAT R: AGCACAGTGGCGGCCGCTCGAGATTCCACAGTGACAGACTGGCT
pcDNA3.1-Myc- <i>FoxO1</i>	F: GCTAGCGTTTAAACGGGCCCTCTAGAATGGCCGAAGCGCCCCAGGTG R: CACAGTGGCGGCCGCTCGAGAGCCTGACACCCAGCTGTGTGTTGTAG
pcDNA3.1-Myc- <i>Trim21</i>	F: CCCTCGAGGATGTCACCCTCTACAACCTC R: CGGAATCCGCATCTTTAGTGGACAGAGCTT
pcDNA3.1-Myc- <i>Trim21</i> (Mut)	F: CCCTCGAGGCTGCTCCGAAACCTCAGGCC R: CGGAATCCGCATCTTTAGTGGACAGAGCTT
pLV- <i>Fto</i>	F: AGAAGATTCTAGAGCTAGCGAATTCATGAAGCGCGTCCAGACCGCGG R: CGCAGATCCTTCGCGGCCGCGGATCCCTAGGATCTTGCTTCCAGCAG
pLV- <i>Fto</i> (S95A)	F2: GCTCACCCAGTGGCTCGCATCCTCAT R2: ATGAGGATGCGAGCCACTGGGGTGAGC

84
85

86

Supplementary Table 6. Plasmids used in this study

87

Recombinant plasmids	SOURCE	IDENTIFIER
pcDNA-HA-Ub (WT)	Drs. Xuetao Cao and Baoxue Ge	N/A
pcDNA-HA-Ub (K6)	Drs. Xuetao Cao and Baoxue Ge	N/A
pcDNA-HA-Ub (K11)	Drs. Xuetao Cao and Baoxue Ge	N/A
pcDNA-HA-Ub (K27)	Drs. Xuetao Cao and Baoxue Ge	N/A
pcDNA-HA-Ub (K29)	Drs. Xuetao Cao and Baoxue Ge	N/A
pcDNA-HA-Ub (K33)	Drs. Xuetao Cao and Baoxue Ge	N/A
pcDNA-HA-Ub (K48)	Drs. Xuetao Cao and Baoxue Ge	N/A
pcDNA-HA-Ub (K63)	Drs. Xuetao Cao and Baoxue Ge	N/A

88

89

Supplementary Table 7. All Reagents used in this study

Reagent	Source	Identifier
Lipopolysaccharide (LPS)	Sigma-Aldrich	Cat. No. L2630
GalNAz	Ruixibio	Cat. No. R-C-1225
GlcN	Sigma-Aldrich	Cat. No. G4875
UDP-GlcNAc	Sigma-Aldrich	Cat. No. U4375
DAPI	Sigma-Aldrich	Cat. No. D9542
Fetal bovine serum (FBS)	Gibco	Cat. No. 10099141
M-CSF	Peptotech	Cat. No. 315-02-50
Cycloheximide (CHX)	MCE	Cat. No. HY-12320
MG-132	MCE	Cat. No. HY-13259
3-Methyladenine (3-MA)	MedChemExpress	HY-19312
Chloroquine (CQ)	MedChemExpress	HY-17589A
Thiamet G (TMG)	ApexBio	Cat. No. B2048
OSMI-1	Sigma-Aldrich	Cat. No. SML1621
Meclofenamic acid (MA)	MCE	Cat. No. HY-117275
RNase Inhibitor	Takara	Cat. No. 2313B
Nuclease P1	Sigma-Aldrich	Cat. No. N8630
SAP	NEB	Cat. No. M0371S
Protein A+G magnetic beads	MCE	Cat. No. HY-K0202
NEOFECTION™ DNA transfection reagent	Neofect Biotech	Cat. No. TF201201
Lipofectamine 2000	Thermo Fisher	Cat. No. 12566014
PMSF	Roche	Cat. No. 10837091001
TRIzol Reagent	Invitrogen	Cat. No. 15596026
ReverTra Ace® qPCR RT Kit	Toyobo Life Science	Cat. No. FSQ-101
SYBR® Green Realtime PCR Master Mix	Toyobo Life Science	Cat. No. QPK-201
Liposomes and clodronate liposomes (CLs)	FormuMax	Cat. No. F70101C-A
Mouse TNF- α ELISA kit	Dakewe Biotech	Cat. No. 1217202
Mouse IL-6 ELISA kit	Dakewe Biotech	Cat. No. 1210602
Mouse IL-1 β ELISA kit	Dakewe Biotech	Cat. No. 1210122
Cell lines		
RAW264.7	The China Center for Type Culture Collection (CCTCC)	Cat. No. GDC0143
HEK293T	The China Center for Type Culture Collection (CCTCC)	Cat. No. GDC0187