Supplemental Information

for

Muscular G9a regulates muscle-liver-fat axis by musclin under overnutrition in female mice

Short title: female muscular G9a-musclin signaling prevents obesity

Wenquan Zhang[1], Dong Yang[2], Yangmian Yuan[1], Chong Liu[1], Hong Chen[2], Yu Zhang[2], Qing Wang[1], Robert B. Petersen[3], Kun Huang[2*] & Ling Zheng[1,4*]

[1]Hubei Key Laboratory of Cell Homeostasis, College of Life Sciences, Wuhan University, Wuhan, China, 430072

[2]Tongji School of Pharmacy, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, 430030

[3]Foundational Sciences, Central Michigan University College of Medicine, Mount Pleasant, MI, USA. 48859

[4]Frontier Science Center for Immunology and Metabolism, Wuhan University, Wuhan, China, 430072

Corresponding authors

Ling Zheng, Ph.D.
College of Life Sciences
Wuhan University
Wuhan, China 430072
lzheng@whu.edu.cn

Kun Huang, Ph.D.
Tongji School of Pharmacy
Huazhong Univ. of Sci. & Tech.
Wuhan, China, 430030
kunhuang@hust.edu.cn

Table S1. Primers used in the present study.

Gene	Forward	Reverse
(1) qPCR primers		
Ehmt2	GGTGAAGCCATCTAGAAAACGG	GAACCTCCATGTACTCACTGG
Ehmt1	CAGACAGCTTGCTTGCCTTT	GCCCACAGTGGAATGGGTTA
Atrogin-1	GAGGCAGATTCGCAAGCGTTTGAT	TCCAGGAGAGAATGTGGCAGTGTT
MuRF-1	AGTGTCCATGTCTGGAGGTCGTTT	ACTGGAGCACTCCTGCTTGTAGAT
Myostatin	TCACGCTACCACGGAAACAA	AGGAGTCTTGACGGGTCTGA
Myf5	GGGACCAGTTTGAGCCAAGA	GCCGATCCATGGTAGTGGAC
Myh7	TTACTTGCTACCCTCAGGTGG	CTCCTTCTCAGACTTCCGCA
Myh2	GGAGGCTGAGGAACAATCCA	GGGACAGCCTTACTCTTCGC
Myh4	ACCTGGCCAAGTTCCGTAAG	TCTCCTGTCACCTCTCAACAGA
Cd36	TTGAAAAGTCTCGGACATTGAG	TCAGATCCGAACACAGCGTA
Srebp1c	GTGTTGGCCTGCTTGGCTCT	GAGCAGCCTGGGGGAAATCTA
Acly	GGCCAGAGAGCTGGGTTTGA	CCCGAGCACAGATGATGGTG
Fasn	TCTGGGCCAACCTCATTGGT	GAAGCTGGGGGTCCATTGTG
Acc	CCAGCTGATCCTGCGAACCT	GAACATTCCCGCAAGCCATC
Acadm	AGGTTTCAAGATCGCAATGG	CATTGTCCAAAAGCCAAACC
Scd1	TTCTCAGAAACACACGCCGA	AGCTTCTCGGCTTTCAGGTC
Ppara	ACTACGGAGTTCACGCATGTG	TTGTCGTACACCAGCTTCAGC
Cpt1a	CCGATCATGGTTAACAGCAA	TGCAGCAGAGATTTGGCATA
Cpt1b	TCTTCTTCCGACAAACCCTGA	GAGACGGACACAGATAGCCC
Pnpla2	CATGATGGTGCCCTATACTC	GTGAGAGGTTGTTTCGTACC
Lipe	AGCGCTGGAGGAGTGTTTT	CCGCTCTCCAGTTGAACC
Mgll	CGGACTTCCAAGTTTTTGTCAGA	GCAGCCACTAGGATGGAGATG
Ppargc1a	GGACATGTGCAGCCAAGACTCT	CACTTCAATCCACCCAGAAAGCT
Ucp1	AACAGAAGGATTGCCGAAAC	AGAGGCAGGTGTTTCTCTCC
Prdm16	ACAGGCAGGCTAAGAACCAG	CGTGGAGAGGAGTGTCTTCAG
Cidea	ATCACAACTGGCCTGGTTACG	TACTACCCGGTGTCCATTTCT
Dio2	AGTCAAGAAGGTGGCATTCGATC	ACAGCTTCCTCCTAGATGCCT
Cox8b	TGTGGGGATCTCAGCCATAGT	AGTGGGCTAAGACCCATCCTG
Acadl	GCTTATGAATGTGTGCAACTCC	CCGAGCATCCACGTAAGC
Atp5a	GCTGAGGAATGTTCAAGCAGA	CCAAGTTCAGGGACATACCC
Cox4	TACTTCGGTGTGCCTTCGA	TGACATGGGCCACATCAG
Cox7a	CGAAGAGGGGAGGTGACTC	AGCCTGGGAGACCCGTAG
Acox1	CCGCCACCTTCAATCCAGAG	CAAGTTCTCGATTTCTCGACGG
Musclin	CCCCTTGACAGACTCTCAGC	GCCGGTTTCTACCAATCCGA
Il6	CACTTCACAAGTCGGAGGCT	CTGCAAGTGCATCATCGTTGT
Il15	CATCCATCTCGTGCTACTTGTG	GCCTCTGTTTTAGGGAGACCT
Irisin	AGTGAGCCTGTGCTCTTCAA	AGAGAGCTATAACACCTGCCC
Igf1	AGAGCCTGCGCAATGGAATA	TGCTGATTTTCCCCATCGCT

Fgf2	GCGACCCACACGTCAAACTA	CCGTCCATCTTCCTTCATAGC
Foxo1	TTCCGTCCATCGGTGTTTCC	ATGCAAACCAGGCCTTCTCA
Rn18s	CACCATCATGCAGAACCCACGAC	AGCCTCTCCAGGTCCTCACGC

(2) ChIP-qPCR primers

Musclin-P1	GACAGAATGCAACCATGGGC	CACCTAAGTGCACTGCCAGA
Musclin-P2	GGCGACTCTAACCTCTGCAT	CACTGTGCTGTGTCCCTGAA
Musclin-P3	CAACGACAGGGGTTGGAGTA	TGCCTGCAGCTTATCCCAAA

(3) Primers for genotyping

G9a-Loxp	TGTGAGTTCCAGGTAGTGGC	GAATGCCACACAGCAGTGAC
Ckmm-Cre	TAAGTCTGAACCCGGTCTGC	GTGAAACAGCATTGCTGTCACTT
HSA-Cre	GAACCTGATGGACATGTTCAGG	AGTGCGTTCGAACGCTAGAGCCT

(4) Sequences of shRNA

shG9a#1	5’- GCCTGTACTATGATGCGTA -3’
shG9a#2	5’- GCAGCTCAATCGAAAGCTT -3’

Table S2. Antibodies used in the present study.

Antibodies	Vendor	**Catalog number**	**Dilution**
G9a (WB)	Abcam	#31874	1:5000
G9a (ChIP)	Abcam	#185050	1:50
GLP (WB)	Bioss	#16789	1:1000
H3K9me2 (WB)	Abcam	#1220	1:10000
H3K9me1 (WB)	Abcam	#9045	1:10000
H3K9me3 (WB)	PTM Biolab	#PTM616	1:10000
Histone H3 (WB)	Cell Signaling Technology	#9715	1:1000
Foxo1 (WB)	Cell Signaling Technology	#2880	1:1000
p-Foxo1 (WB)	Cell Signaling Technology	#84192	1:1000
α-Tubulin (WB)	Beyotime	#AF0001	1:5000
Musclin (WB)	R&D systems	#MAB2620	1:500
MyHC Ⅰ (IF)	DSHB	#BA-F8	1:500
MyHC Ⅱa (IF)	DSHB	#SC-71	1:500
MyHC Ⅱb (IF)	DSHB	#BF-F3	1:500
Ucp1 (IHC)	Abcam	#10983	1:1000

Table S3. Biochemical analysis of serum from female WT and *Ehmt2*^{Ckmm} mice.

	WT-NC	*Ehmt2*^{Ckmm}-NC	WT-HFD	*Ehmt2*^{Ckmm}-HFD
Leptin (ng/mL)	ND	ND	13.3 ± 10.0	7.6 ± 2.8
Insulin (ng/mL)	ND	ND	1.0 ± 0.4	1.1 ± 0.2
TG (mg/dL)	41.7 ± 12.8	43.9 ± 16.9	78.1 ± 20.6*	85.0 ± 24.2*
TC (mg/dL)	69.7 ± 5.2	62.5 ± 6.7	124.1 ± 16.1*	111.8 ± 14.4*

ND, not determined. TG, triglycerides; TC, total cholesterol. Values are means ± SD. n = 4-6 animals per group. *$P < 0.05$ compared to the WT-NC group.

Table S4. Biochemical analysis of serum from male WT and *Ehmt2*^{Ckmm} mice.

	WT-NC	*Ehmt2*^{Ckmm}-NC	WT-HFD	*Ehmt2*^{Ckmm}-HFD
Leptin (ng/mL)	ND	ND	27.7 ± 9.0	32 ± 11.2
Insulin (ng/mL)	ND	ND	1.5 ± 0.9	2.5 ± 0.4
TG (mg/dL)	74.4 ± 3.4	73.1 ± 11.0	108.9 ± 10.8*	104.6 ± 11.9*
TC (mg/dL)	79.3 ± 9.6	88.2 ± 15.2	153.7 ± 22.5*	155.4 ± 22.4*

ND, not determined. Values are means ± SD. n = 5-8 animals per group. *P < 0.05 compared to the WT-NC group.

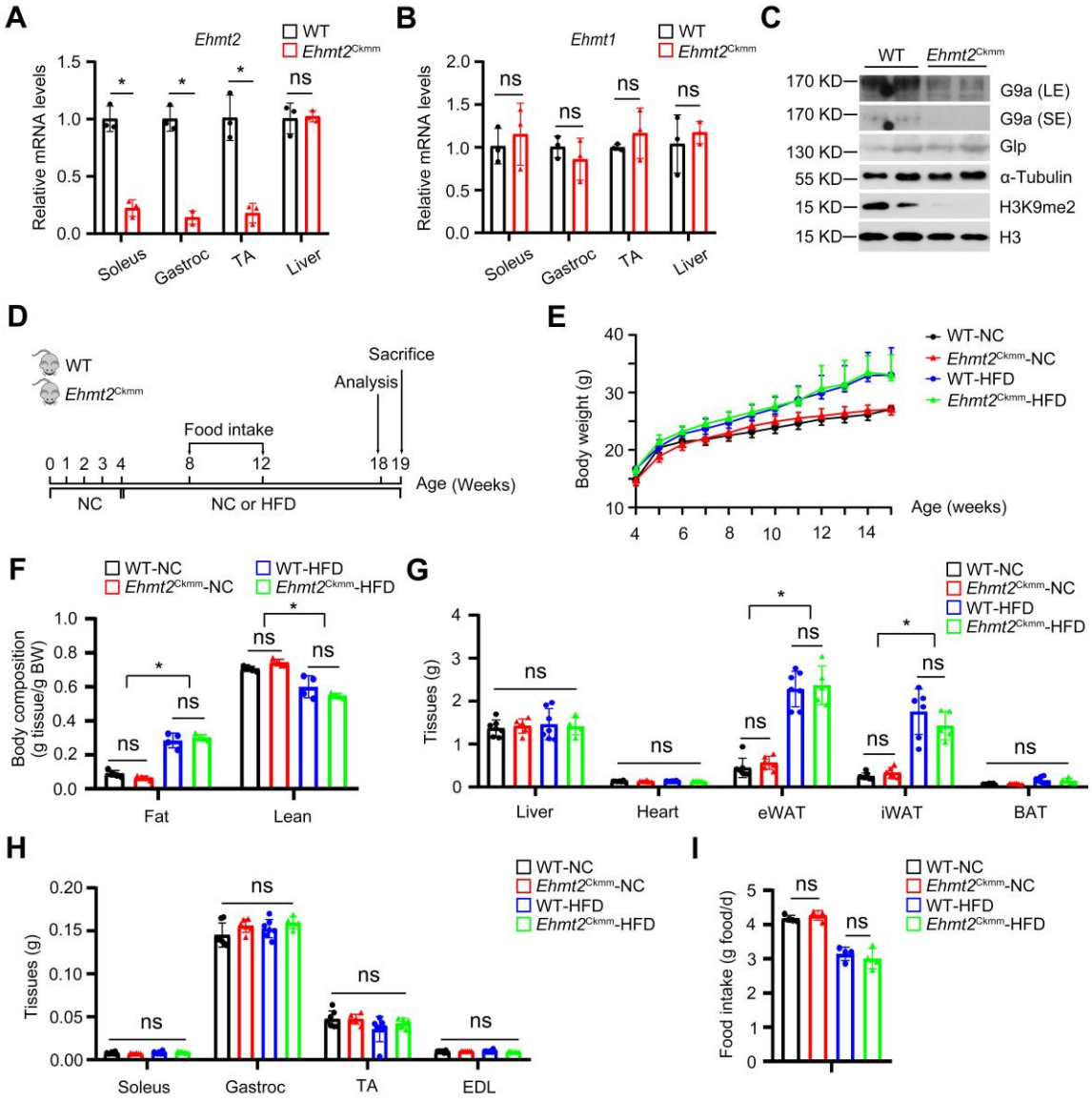

Figure S1. Phenotypes of NC- or HFD-fed *Ehmt2*^{Ckmm} male mice. (A-B) qPCR results of *Ehmt2* (A) and *Ehmt1* (B) levels in soleus, gastroc, TA and liver of WT or *Ehmt2*^{Ckmm} male mice. (C) Western blot of G9a, Glp and H3K9me2 in the TA of WT or *Ehmt2*^{Ckmm} male mice. (D) Experimental design for male and female mice. (E-F) growth curves (E), body mass (F) of WT or *Ehmt2*^{Ckmm} males. (G-I) Tissue weights in liver, heart, eWAT, iWAT and BAT (G), different muscle parts (H), and food intake (I) of WT or *Ehmt2*^{Ckmm} males. Gastroc, gastrocnemius; TA, tibialis anterior; EDL, extensor digitorum longus; NC, normal chow; HFD, high fat diet; n = 3-7 per group; ns, no significance; *P < 0.05.

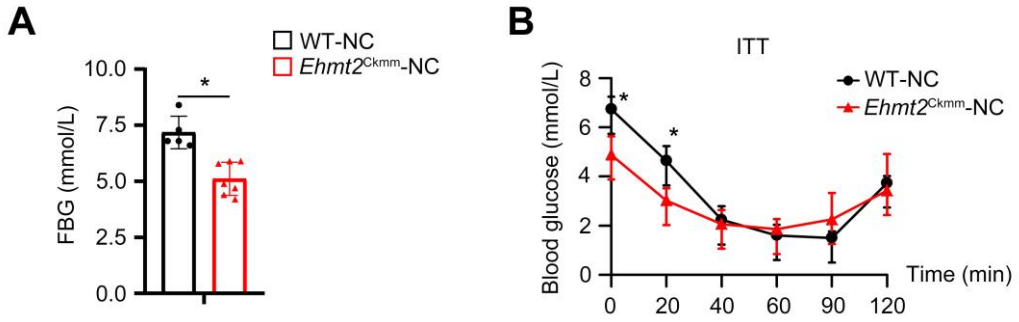

Figure S2. Glucose homeostasis of NC-fed *Ehmt2*^{Ckmm} female mice. (A-B) FBG (A) and ITT (B) of NC-fed female WT or *Ehmt2*^{Ckmm} mice. FBG, six-hour fasting blood glucose; ITT, insulin tolerance test; n = 5-7 per group; $^{*}P < 0.05$.

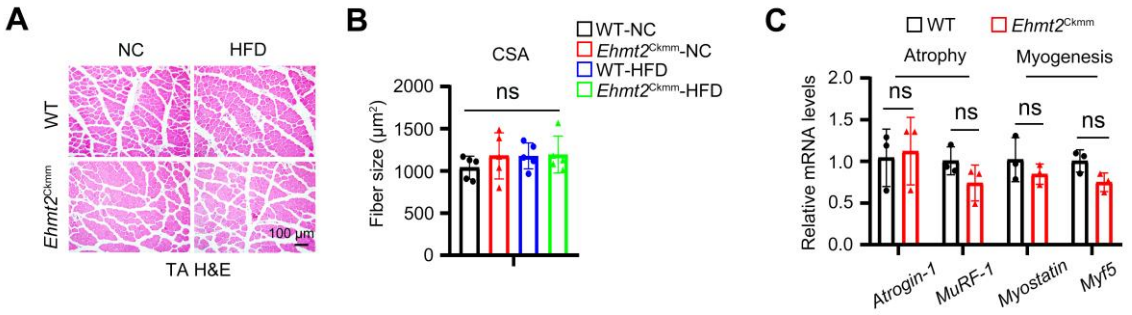

Figure S3. *Ehmt2*^{Ckmm} male mouse shows no obvious change in muscle development. (A-B) H&E staining (A) and quantitative analysis of myofiber size (B) in the TA of NC- or HFD-fed WT or *Ehmt2*^{Ckmm} male mice. (C) qPCR results of genes involved in muscle atrophy and myogenesis in the TA of NC-fed WT or *Ehmt2*^{Ckmm} male mice. TA, tibialis anterior; CSA, cross sectional area. n = 3-5 per group; ns, no significance; $^{*}P < 0.05$.

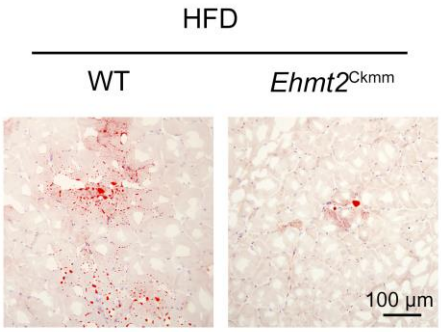

Figure S4. HFD-fed *Ehmt2*^{Ckmm} female mouse shows reduced lipid accumulation in muscle. Representative oil red O staining on the TA sections of HFD-fed WT or *Ehmt2*^{Ckmm} female mice. TA, tibialis anterior; n = 5-6 per group.

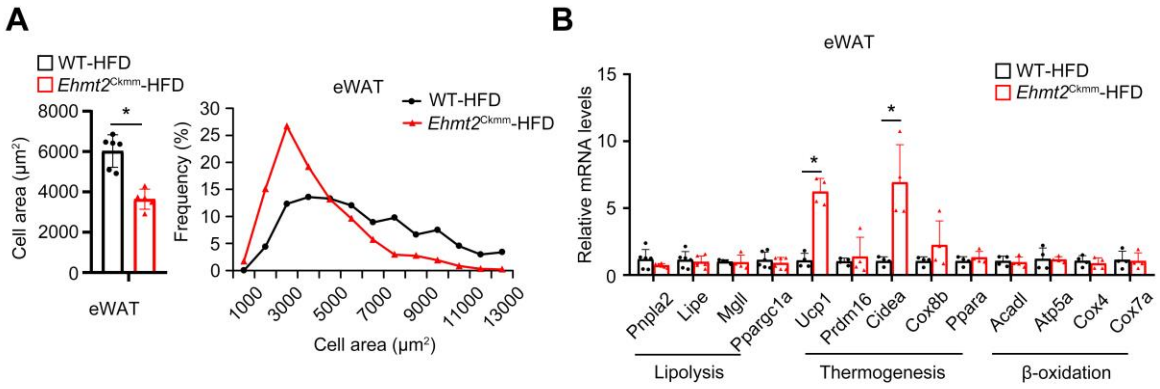

Figure S5. *Ehmt2*^{Ckmm} female mouse shows reduced adipocyte hypertrophy in eWAT. (A) Average cell area (left) and adipocyte size distribution (right) in eWAT of HFD-fed WT or *Ehmt2*^{Ckmm} female mice. (B) qPCR results of indicated genes in eWAT of HFD-fed WT or *Ehmt2*^{Ckmm} female mice. n = 4-6 per group; ns, no significance; *P < 0.05.

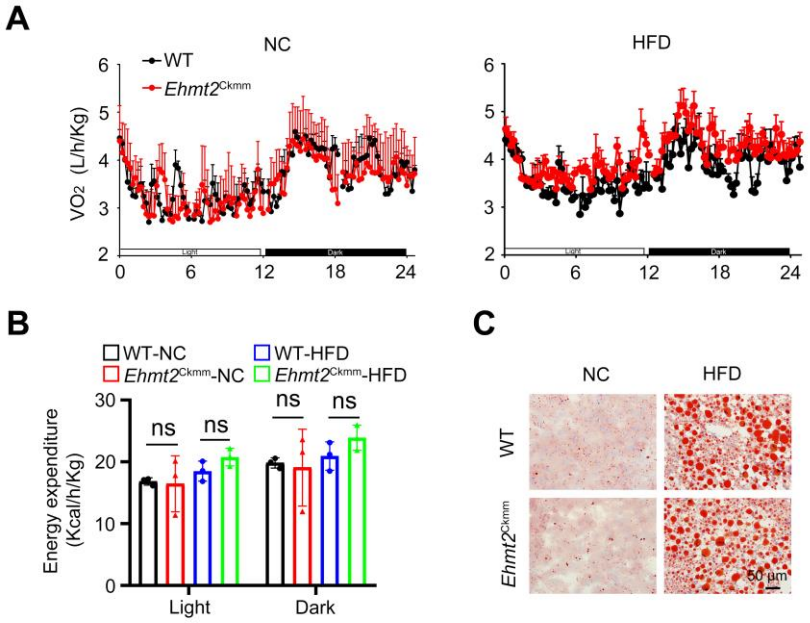

Figure S6. *Ehmt2*^{Ckmm} male mouse shows no obvious changes in energy expenditure and hepatic lipid. (A-B) Oxygen consumption (A) and energy expenditure (B) of NC- or HFD-fed WT or *Ehmt2*^{Ckmm} male mice. (C) Representative oil red O staining on the hepatic sections of NC- or HFD-fed WT or *Ehmt2*^{Ckmm} male mice. n = 3-6 per group; ns, no significance; $^{*}P < 0.05$.

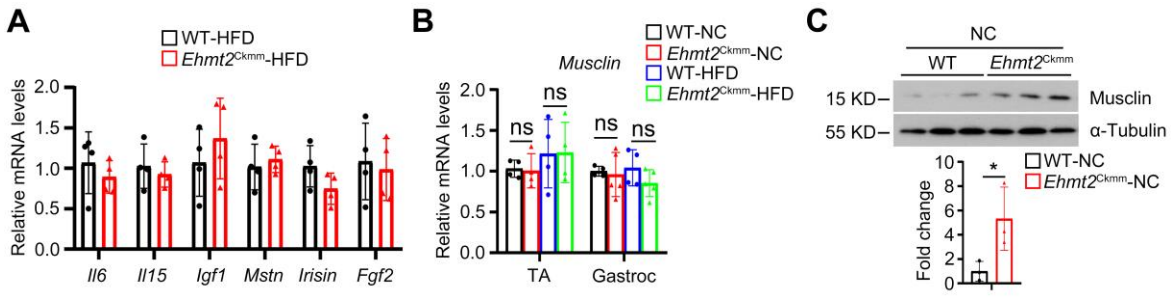

Figure S7. Significant increase in muscular musclin in the *Ehmt2*^{Ckmm} female, but not male mice. (A) qPCR results of several known myokines in the TA of HFD-fed WT and *Ehmt2*^{Ckmm} female mice. (B) qPCR results of *musclin* levels in TA and Gastroc of NC or HFD-fed WT and *Ehmt2*^{Ckmm} male mice. (C) Western blot (up) and quantitative analysis (down) of musclin in the TA of NC-fed WT or *Ehmt2*^{Ckmm} female mice. n = 3-5 per group; ns, no significance; $^{*}P < 0.05$.

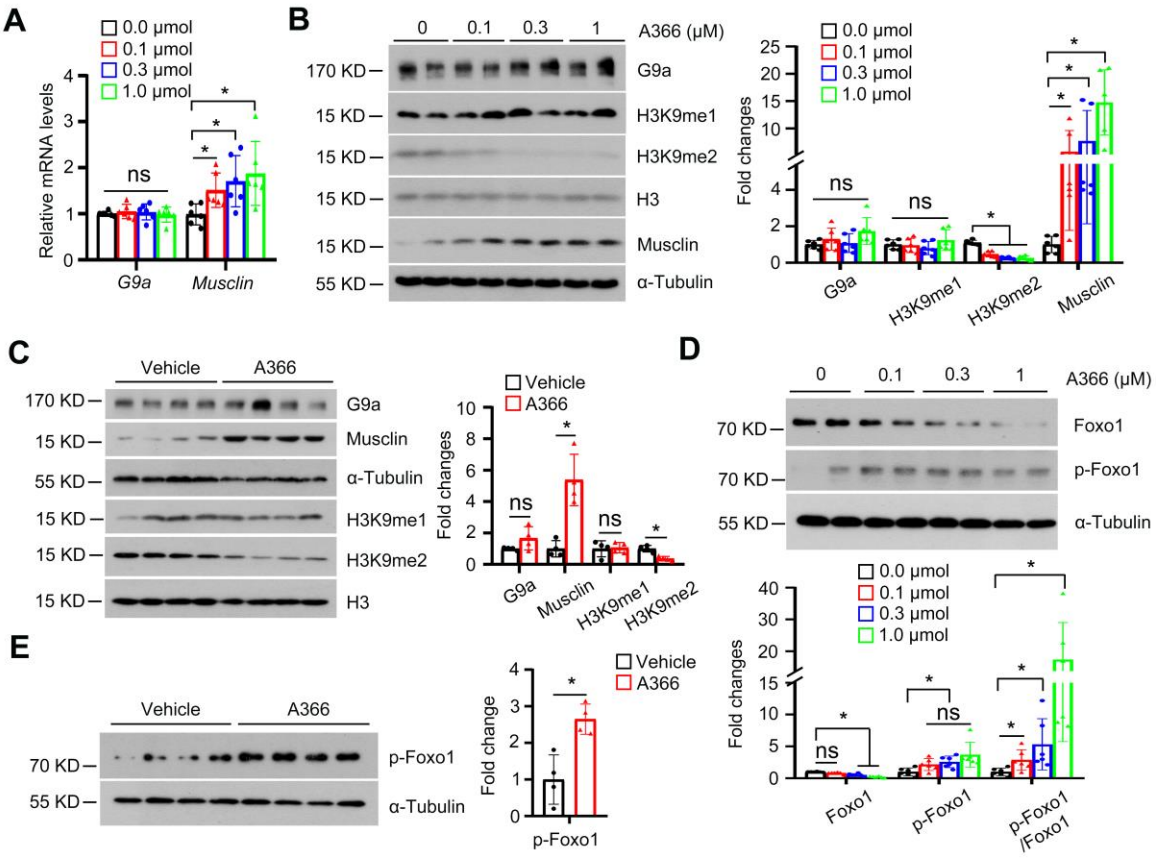

Figure S8. A366 upregulates musclin level *in vivo* and *in vitro*. (A-B) qPCR results of *G9a* and *Musclin* (A), Western blots (B, left) and quantitative analysis (B, right) of G9a, musclin and H3K9me1/2 in A366-treated C2C12 cells. (C) Western blot (left) and quantitative analysis (right) of G9a, musclin and H3K9me1/2 in WT female mice treated with A366. (D) Western blot (up) and quantitative analysis (down) of Foxo1 and p-Foxo1 in A366-treated C2C12 cells. (E) Western blot (left) and quantitative analysis (right) of p-Foxo1 in A366-treated WT female mice. n = 4 per group for animal studies; n = 6 per group for cultured cell experiments; ns, no significance; *P < 0.05.

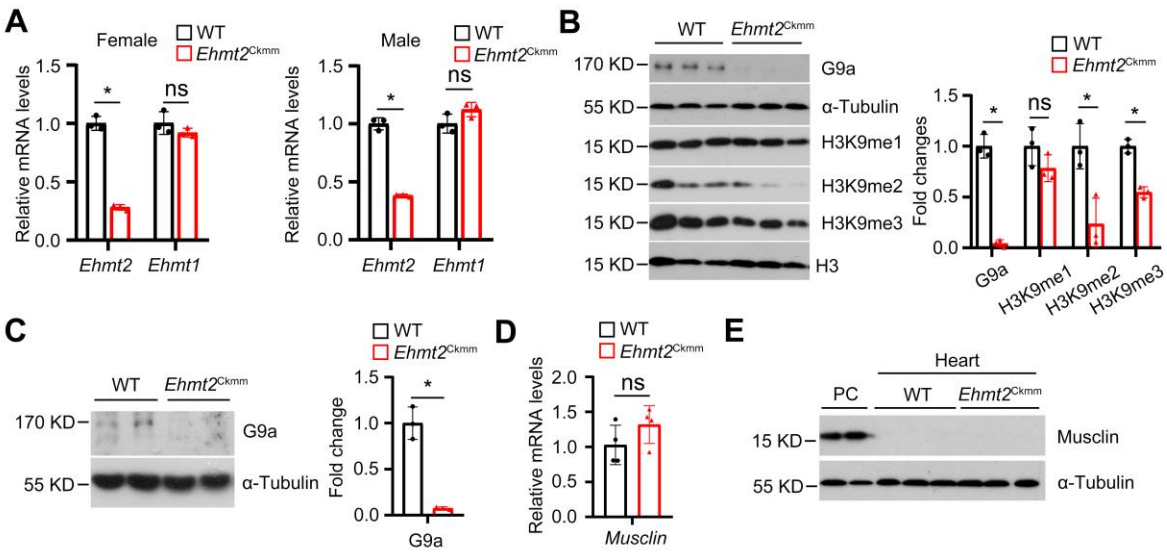

Figure S9. Low musclin levels in the heart of *Ehmt2*^{Ckmm} female mouse. (A) qPCR results of *Ehmt2* and *Ehmt1* levels in the heart of WT or *Ehmt2*^{Ckmm} female (left) and male (right) mice. (B) Western blot (left) and quantitative analysis (right) of G9a, Glp and H3K9me1/2/3 in the heart of WT or *Ehmt2*^{Ckmm} females. (C) Western blot (left) and quantitative analysis (right) of G9a level in the heart of WT or *Ehmt2*^{Ckmm} males. (D-E) *Musclin* mRNA level (D) and protein level (E) in the heart of WT or *Ehmt2*^{Ckmm} females. PC, whole tissue lysate protein samples from skeletal muscle; n = 3-4 per group; ns, no significance; *P < 0.05.

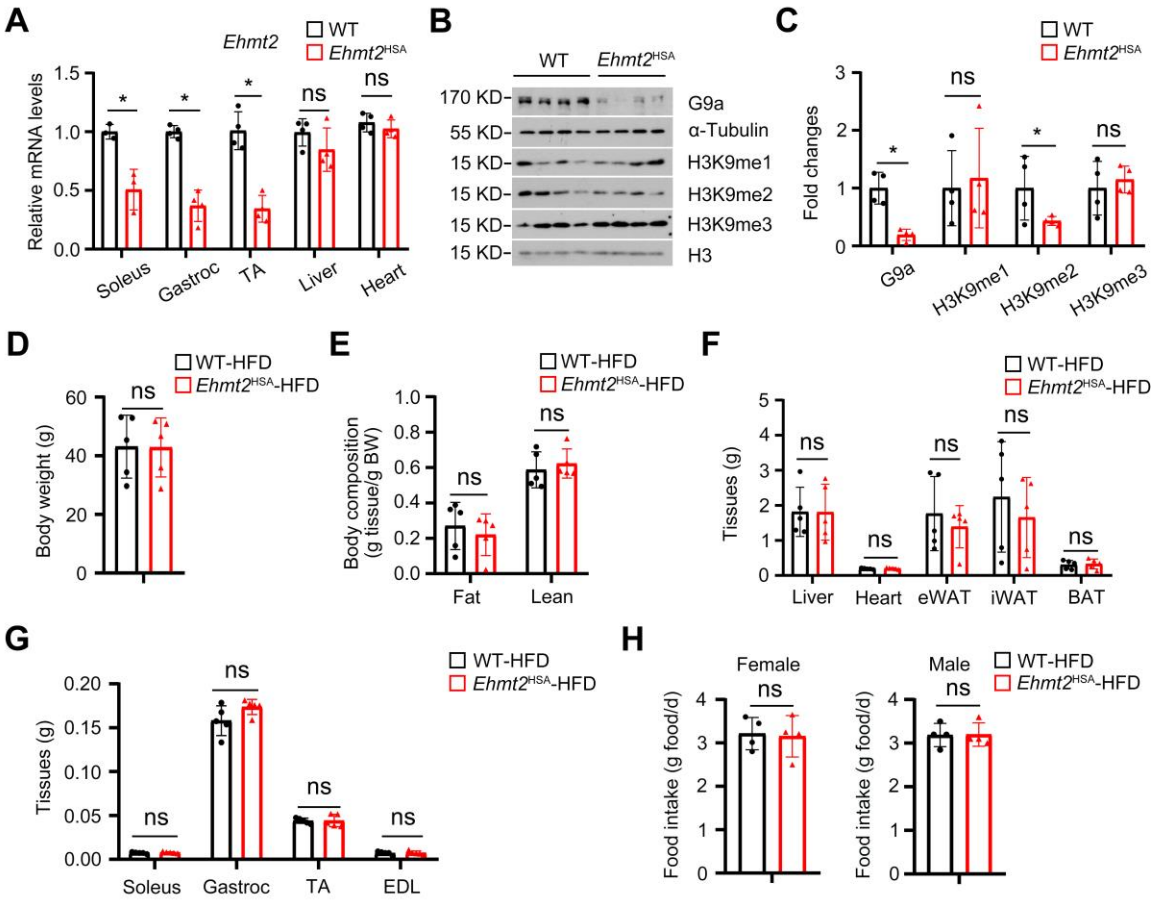

Figure S10. Phenotypes of HFD-fed *Ehmt2*^{HSA} male mice. (A) qPCR results of *Ehmt2* level in soleus, gastroc, TA, liver and heart of WT or *Ehmt2*^{HSA} male mice. (B-C) Western blot (B) and quantitative analysis (C) of G9a and H3K9me1/2/3 levels in TA of WT or *Ehmt2*^{HSA} male mice. (D-G) Body weight (D), body mass (E), tissue weights of liver, heart, eWAT, iWAT, BAT (F) and different muscle parts (G) of HFD-fed WT or *Ehmt2*^{HSA} male mice. (H) Food intake of HFD-fed WT or *Ehmt2*^{HSA} female and male mice. Gastroc, gastrocnemius; TA, tibialis anterior; EDL, extensor digitorum longus; n = 3-5 per group; ns, no significance; *P < 0.05.

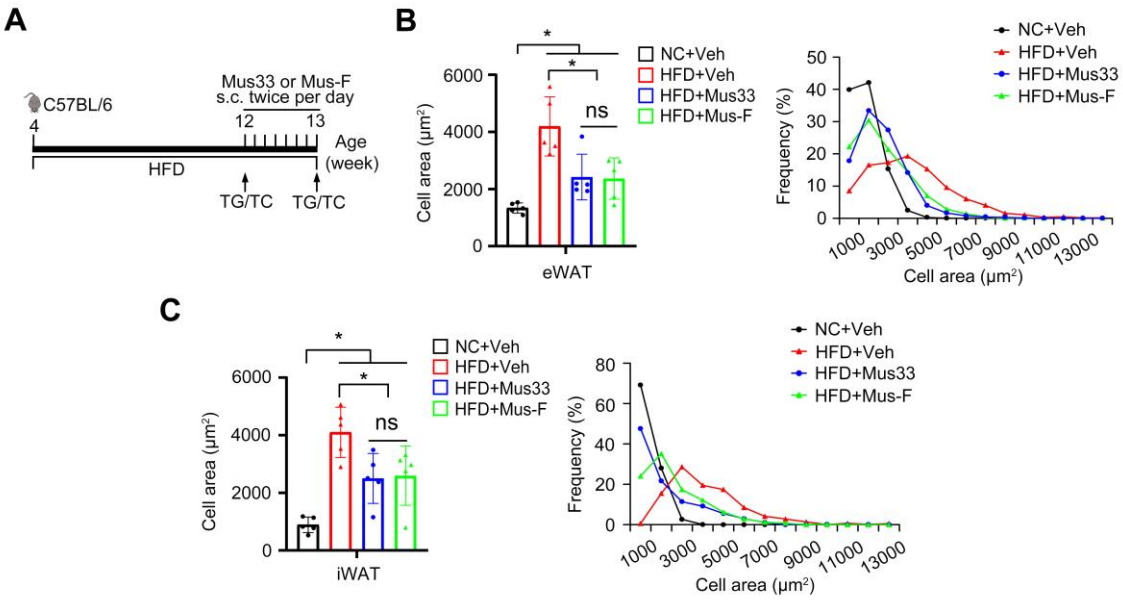

Figure S11. Administration of musclin prevents HFD-induced adipocyte hypertrophy in female mice. (A) Experimental design for musclin treatment in C57BL/6 mice. (B-C) Average cell area (left) and adipocyte size distribution (right) in eWAT (B) and iWAT (C) of HFD-fed WT female mice, with or without musclin treatments. n = 5 per group; ns, no significance; *P < 0.05.

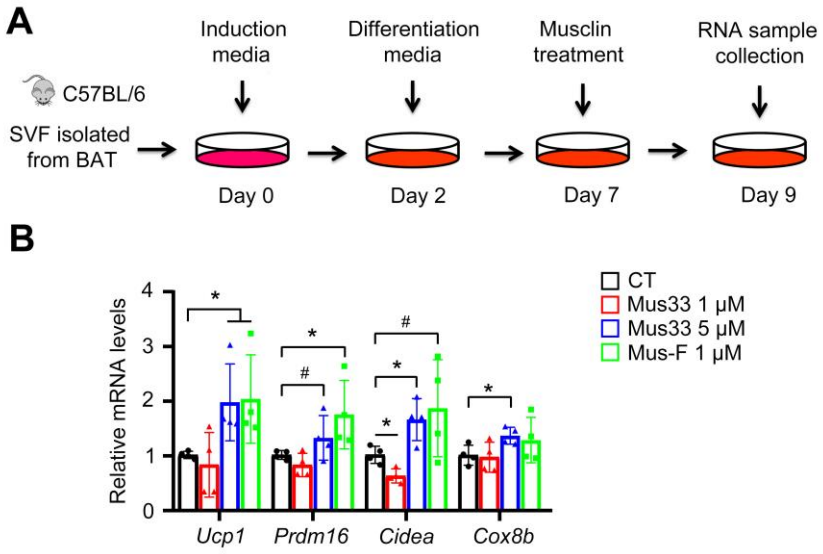

Figure S12. Administration of musclin upregulates thermogenic genes expression in primary brown adipocytes. (A) Experimental design for musclin treatment in primary brown adipocytes. (B) Expression of several thermogenic genes in primary brown adipocytes with musclin treatment. n = 4 per group; $^{*}P < 0.05$; $^{\#}P < 0.08$.