Methionine and S-Adenosylmethionine levels are critical regulators of PP2A activity modulating lipophagy during steatosis

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Supplementary Table 1: Specifications of the Methionine deficient diet (Ssniff® EF R/M acc. AIN 76A 0.15% L-methionine).

(SSNIFF, Soest, Germany)	
ssniff® EF R/M acc. AIN 76A 0.15% L-methionine	
	Minerals [%]
Calcium	0.63
Phosphorus	0.55
Sodium	0.11
Magnesium	0.05
Potassium	0.36
Ca:P	1.14
	Fatty acids [%]
C 8:0	---
C10:0	---
C12:0	---
C14:0	0.01
C16:0	0.57
C16:1	0.01
C18:0	0.1
C18:1	1.31
C18:2	2.77
C18:3	0.05
C20:0	0.02
C20:1	---
C20:5	---
C22:6	---
	Amino acids [%]
Lysine 1.43	1.43
Methionine	*0.15*
Cystine	0.08
Met+Cys	0.9
Threonine	0.77
Tryptophan	0.22
Arginine	0.63
Histidine	0.55
Valine	1.18
Isoleucine	0.9
Leucine	1.7
Phenylalanine	0.93
Phe+Tyr	1.85
Glycine	0.36
Glutamic acid	3.9
Aspartic acid	1.29
Proline	2
Alanine	0.57
Serine	1.03
	Vitamins per kg
Vitamin A	4,000 IU
Vitamin D3	1,000 IU
Vitamin E	57 mg
Vitamin K (as menadione)	5 mg
Vitamin C	---- mg
Thiamin (B1)	3 mg
Riboflavin (B2)	5 mg
Pyridoxine (B6)	6 mg
Cobalamin (B12)	10 µg
Nicotinic acid	29 mg
Pantothenic acid	15 mg
Folic acid	2 mg
Biotin	20 µg
Choline-Chloride	1,040 mg
	Trace elements per kg
Iron	46 mg
Manganese	59 mg
Zinc	35 mg
Copper	7 mg
Iodine	0.22 mg
Selenium	0.14 mg
Cobalt	0.02 mg
	Crude Nutrients [%]
Dry matter	96.8
Crude protein (N x 6.25)	17.4
Crude fat	5.1
Crude fibre	5
Crude ash	2
N free extracts	67.4
Starch	13.9
Sugar	51
	Energy [MJ/kg]
Gross Energy (GE)	19

Supplementary Table 2: Specifications and conditions of the antibodies used.

Antibody	Supplier	Western blot	Immunofluo -rescence	Cat. N
β-ACTIN	SIGMA	1/5000	-	A 5315
α-TUBULIN	Abcam	-	1/100	Ab18251
ATG3	Cell Signaling Technology	1/1000	-	3415
ATG5	Cell Signaling Technology	1/1000	-	8540
ATG7	Cell Signaling Technology	1/1000	-	2631
ATG12	Cell Signalling Technology	1/1000	-	4180
BECLIN1	Cell Signalling Technology	1/1000	-	3495
GAPDH	Abcam	1/1000	-	Ab8245
LC3	Cell Signalling Technology	1/1000	1/50	2775
pS6 (Ser 235/236)	Cell Signalling Technology	1/1000	-	4857
S6 Ribosomal protein	Cell Signalling Technology	1/1000	-	2317
4E-BP1 (53H11)	Cell Signalling Technology	1/1000	-	9644
4E-BP1	Cell Signalling Technology	1/1000	-	9452
p4E-BP1	Cell Signalling Technology	1/1000	-	9451
pMTOR (Ser 2448)	Cell Signalling Technology	1/1000	-	2971
MTOR	Cell Signalling Technology	1/1000	-	2983
LAMP2	Abcam	1/1000	1/100	Ab13524
Methyl-PP2A	Upstate	1/100		05546
Demethylated PP2A	Upstate	1/1000		05577
PP2A A Subunit	Upstate	1/1000		7250
LAMP2	Abcam	1/1000	1/100	Ab13524

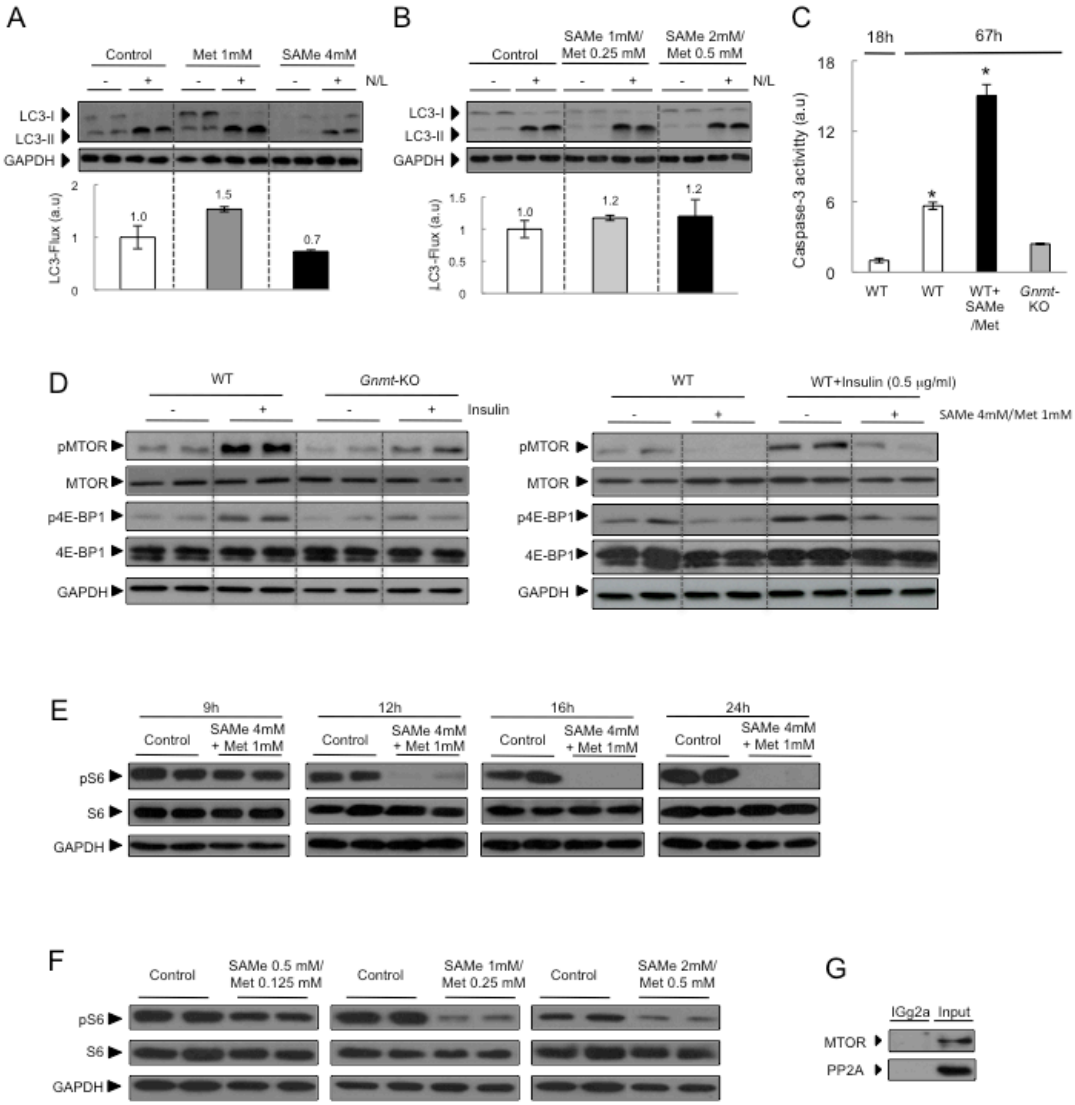
Material and Methods

PP2A C Subunit Methylation Assay. Hepatocytes were pre-treated with 3-Deazaadenosine (Deaza) (20 μM) during 6h, and then cultured in SAMe and methionine supplemented medium during 12h. 20 μl of protein lysate (1 μg/μl) were treated with either 50 μl of preneutralized base solution (80 mM NaOH, 80 mM HCl, and 200 mM Tris pH 6.8) or with 20 μl of base (200 mM NaOH) during 20 minutes to completely demethylate PP2Ac and then neutralized with 30 μl of neutralization buffer (133.3 mM HCl and 333.3 mM Tris pH 6.8). Samples were compared by immunoblot using unmethylated PP2A antibody (Upstate). Percentage of PP2A methylation was calculated by subtracting the densitometry value of unmethylated PP2A normalized with Actin, in the sample treated with base solution by the densitometry value in the control sample. Percentage was calculated by considering WT control samples as 100%.

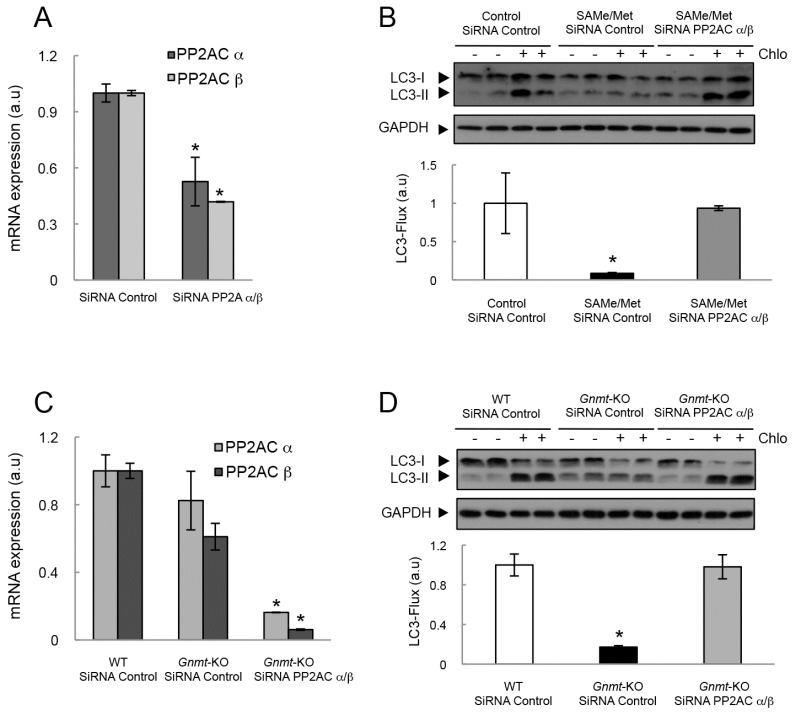
Gene Silencing. Primary hepatocytes were transfected with 84 nM of each small interfering RNA (siRNA) constructs (SIGMA, SASI_Mm01_00126453 for PP2A catalytic subunit β, and SASI_Mm01_00038232 for PP2A catalytic subunit α) using DharmaFECT Transfection Regeant (Dharmacon). MLP cells were transfected with 100 nM of each small interfering RNA (siRNA) using Lipofectamine 2000 (Invitrogen). In both cell types, transfection was done twice during a 48-hour period (once every 24 hours). Silencing mix was left overnight, and afterwards, the cultured medium was replaced with fresh medium.

Caspase-3 activity. Caspase-3 activity was assayed by using a fluorescent substrate (Ac-DEVD-AFC) (Enzo Life Sciences). Hepatocytes were homogenized in 200 μl of lysis buffer (10 mM HEPES, 0.1% CHAPS, 2mM EDTA, 5mM dithiothreitol). 30 μg of protein lysates were incubated with 2.5 μl of Ac-DEVD-AFC in 500 μl of reaction buffer (10 mM PIPES pH 7.4, 2 mM EDTA pH8, 0.1% of CHAPS and 5mM of dithiothreitol) at 37°C. Enzymatic activity was measured at time 0 and after 2 hours

in a luminescence spectrophotometer at an excitation of 390 nm and emission of 510 nm.



Supplementary Figure 1: Methionine and SAMe effect on MTOR activity and autophagy flux. (**A,B**) Hepatocytes were treated with SAMe and methionine during 24h. When indicated, hepatocytes were incubated in the presence or absence of N/L for the last 4 hours of culture and LC3 levels were analyzed by WB. (**C**) Hepatocytes were cultured the indicated times in the presence or absence of SAMe (4mM) and methionine (1mM). Then the activity of Caspase-3 was analyzed (as described in supplementary Material and Methods). (**D**) After seeding, hepatocytes were medium changed, and treated when indicated with SAMe and methionine during 18h. Then hepatocytes were treated for 20 minutes with insulin (0.5mg/ml). (**E**) Hepatocytes cultured with SAMe and methionine at the indicated times. (**F**) Hepatocytes were cultured with SAMe and methionine at the indicated doses during 16h. **(G)** WB analysis of PP2A and MTOR in control (IgG2a) and MTOR-immunoprecipitated extracts from WT hepatocytes. Data are represented as mean +/- SEM (*p<0.05 N/L Vs. Control).



Supplementary Figure 2: PP2A silencing effect on autophagy flux. (**A,B**) MLP-29 cells were co-transfected twice during 48h every 24h, with siRNA constructs for PP2A catalytic subunit α, and for PP2A catalytic subunit β (as described in supplementary Material and Methods). (**A**) Transfection efficiency was study by analyzing the mRNA levels of *PP2A catalytic subunit α and subunit β* and values were normalized with *GAPDH m*RNA expression. (**B**) After silencing, MLP cells were medium changed, treated with SAMe and methionine during 18h, and then incubated with chloroquine for the last 6 hours of culture. (**C,D**) Hepatocytes were co-transfected twice during 44h every 22h, with siRNA constructs for PP2A catalytic subunit α and the PP2A catalytic subunit β (as described in supplementary Material and Methods). After silencing, hepatocytes were medium changed, and incubated with chloroquine during 4 hours. (**C**) mRNA expression levels of *PP2A catalytic subunit α and β* were were normalized with *18S* ribosomal RNA expression. (**D**) LC3 levels were analyzed by WB. Data are represented as mean +/- SEM (*p<0.05 N/L Vs. Control).

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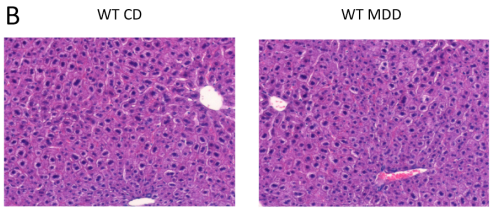
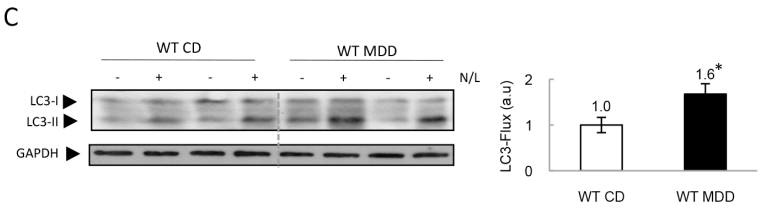
Mouse Genotype	Met (pmol/mg)	SAMe (pmol/mg)	SAH (pmol/mg)	AST (U/L)	ALT (U/L)	TG (nmol/mg prot)
WT	86.6 ± 11.3	72.9 ± 3.5	8.3 ± 0.9	51 ± 2	26 ± 0.3	43.3 ± 6.7
WT + MDD	52.6*± 10.0	47.0*± 4.0	15.7* ± 0.8	54 ± 2	15* ± 1	37.7 ± 7.1





Supplementary Figure 3: Effect of Methionine Deficient Diet (MDD) in WT livers. (**A**) Hepatic SAMe, methionine, SAH, TG and serum transaminases levels from WT mice fed with a normal diet or MDD. (**B**) Haematoxylin/Eosin staining of WT mice fed with a normal diet or MDD. (**C**) WB analysis showing LC3-flux in liver sections, obtained from WT mice fed with a normal diet or MDD, which were incubated during 2h in the presence or absence of protease inhibitor leupeptin (200 μM) and ammonium chloride (40 mM). Data are represented as mean +/- SEM of at least duplicate experiments (t-test, *p<0.05 Group Vs. WT).