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Supplemental Material to:

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Autophagy: Resetting glutamine-dependent metabolism and oxygen consumption

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Figure S1. Complete metabolomic profile of WT and *atg5*$^{-/-}$ MEFs under glutamine depletion. Hierarchical cluster (single dendrogram clustered by biomolecules) analysis of 204 analyzed biomolecules with complete linkage algorithm is shown. Signal fold change was normalized to WT at 0 h set as 1. All data points were $\log_2$ transformed and used to calculate the Self-Organizing Map. Transformed fold changes are shown in green (decreasing) and red (increasing).

Figure S2. *Atg5* regulates the metabolic response to glutamine depletion in m5-7 cells. (A) Lower intracellular glutamine levels in Dox-treated "ATG5-off" m5-7 cells. The m5-7 cells, which were derived from *atg5*$^{-/-}$ MEFs and stably express an inducible tetracycline-off *Atg5* construct[1], were cultured in the presence and absence of Dox. The repression of ATG5 expression was confirmed by Western blot (right panel). Intracellular glutamine levels were measured and normalized to cell number (left panel). Cells were harvested at the end of glutamine deprivation (0, 6 and 24 h). (B–E) Glutamine depletion increases glucose consumption and lactate production. Dox^{+} "ATG5-off" m5-7 cells exhibited lower glucose consumption and reduced ability to produce lactate upon glutamine depletion. The amounts of (B) glucose, (C) lactate, (D) sodium and (E) potassium ions in the medium, collected individually at the end of glutamine deprivation (0, 6 and 24 h), were analyzed, normalized (5 x 10^6 cells), and plotted. The data were collected from three independent experiments. Dox: doxycycline; $*p < 0.05$.

Figure S3. Glutamine depletion decreased the level of γ-glutamyl amino acids and non-essential amino acids in WT MEFs but increased them in *atg5*$^{-/-}$ MEFs. (A) Alterations of alanine, glutamine, glutamate, aspartate, proline and asparagine levels in glutamine-starved WT and *atg5*$^{-/-}$ MEFs. Signal fold change was normalized to WT at 0 h set as 1 and $\log_2$-transformed. Transformed fold changes are shown in green (decreasing) and red (increasing). (B) Profiles of γ-glutamyl-leucine, γ-glutamyl-isoleucine, γ-glutamyl-valine and γ-glutamyl-threonine in WT and *atg5*$^{-/-}$ MEFs subjected to glutamine withdrawal. Cell preparation, quantitation and data

analyses were performed as described in Figure 2. *p < 0.05; **p <0.01; ***p < 0.001; Gln: glutamine.

Figure S4. Glutamine supplementation rescued cell proliferation. (A) Cell proliferation was inhibited by glutamine depletion and 2-DG treatment. WT and *atg5*$^{-/-}$ MEFs were seeded in complete medium to reach 10% confluence on the day of treatment, and then grown in serum-supplemented DMEM in the presence and absence of glutamine (4 mM) or in full medium with 2-DG (10 mM) for the indicated time periods. Relative cell proliferation (determined by the cell proliferation assay in methods) with each treatment was calculated by comparing to the 0 h time point, set as 1. Results from three independent experiments are shown as the mean ± S.D. The student's *t*-test was performed to determine the difference between (a) control (24/48 h) and -Gln (24/48 h); (b) control (24/48 h) and 2-DG (24/48 h). *p < 0.05. (B) WT and *atg5*$^{-/-}$ MEFs were seeded in complete medium to reach 10% confluence on the day of treatment, and then grown in glutamine-depleted DMEM with the indicated supplements. Cell proliferation assays were performed at the end of indicated time periods. Results from three independent experiments are shown as the mean ± S.D. Gln: glutamine (4 mM); dNTP: deoxynucleotides triphosphate (10 μM); dNMP: deoxynucleotides monophosphate (10 μM); IMP: inosine monophosphate (10 μM); MP: methylpyruvate (10 mM).

Figure S5. Lack of autophagy induction by glutamine deprivation in tfLC3-m5-7 cells. The m5-7 cells, which were derived from *atg5*$^{-/-}$ MEFs and stably express an inducible tetracycline-off *Atg5* construct[1], were transiently transfected with mRFP-GFP tandem fluorescent-tagged MAP1LC3 (tfLC3). (A) Suppression of ATG5 expression by Dox-treatment in m5-7 cells. ATG5 was expressed in the absence of Dox (B, D, F and H) or suppressed in the presence of doxycycline (Dox; 20 μg/mi; (C, E, G and I)). The cells were maintained in (B and C) complete medium, (D and E) EBSS for 6 and 24 h, (F and G) medium supplemented with 2-DG (10 mM) for 6 and 24 h or (H and I) glutamine-depleted medium for 6 and 24 h. Gln, glutamine; 2-DG, 2-deoxyglucose; Scale bar, 10 microns.

Reference

1. Hosokawa N, Hara Y, Mizushima N. Generation of cell lines with tetracycline-regulated autophagy and a role for autophagy in controlling cell size. FEBS letters 2006; **580** (11):2623-2629.

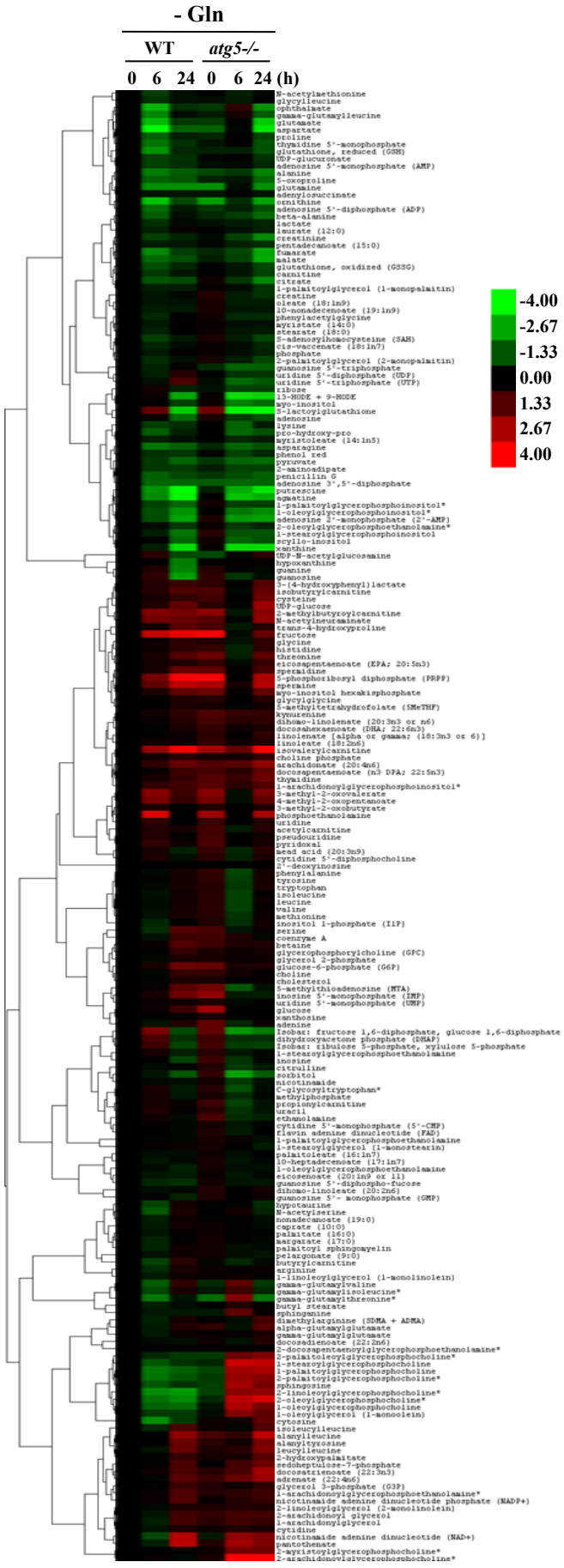

- Gln
WT
atg5-/-
0 6 24 0 6 24 (h)
-4.00
-2.67
-1.33
0.00
1.33
2.67
4.00
N-acetylmethionine
glycylleucine
ophthalmate
gamma-glutamylleucine
glutamate
aspartate
proline
thymidine 5'-monophosphate
glutathione, reduced (GSH)
UDP-glucuronate
adenosine 5'-monophosphate (AMP)
alanine
5-oxoproline
glutamine
adenylosuccinate
ornithine
adenosine 5'-diphosphate (ADP)
beta-alanine
lactate
laurate (12:0)
creatinine
pentadecanoate (15:0)
fumarate
malate
glutathione, oxidized (GSSG)
carnitine
citrate
1-palmitoylglycerol (1-monopalmitin)
creatine
oleate (18:1n9)
10-nonadecenoate (19:1n9)
phenylacetylglycine
myristate (14:0)
stearate (18:0)
S-adenosylhomocysteine (SAH)
cis-vaccenate (18:1n7)
phosphate
2-palmitoylglycerol (2-monopalmitin)
guanosine 5'-triphosphate
uridine 5'-diphosphate (UDP)
uridine 5'-triphosphate (UTP)
ribose
13-HODE + 9-HODE
myo-inositol
S-lactoylglutathione
adenosine
lysine
pro-hydroxy-pro
myristoleate (14:1n5)
asparagine
phenol red
pyruvate
2-aminoadipate
penicillin G
adenosine 3',5'-diphosphate
putrescine
agmatine
1-palmitoylglycerophosphoinositol*
1-oleoylglycerophosphoinositol*
adenosine 2'-monophosphate (2'-AMP)
2-oleoylglycerophosphoethanolamine*
1-stearoylglycerophosphoinositol
scyllo-inositol
xanthine
UDP-N-acetylglucosamine
hypoxanthine
guanine
guanosine
3-(4-hydroxyphenyl)lactate
isobutyrylcarnitine
cysteine
UDP-glucose
2-methylbutyroylcarnitine
N-acetylneuraminate
trans-4-hydroxyproline
fructose
glycine
histidine
threonine
eicosapentaenoate (EPA; 20:5n3)
spermidine
5-phosphoribosyl diphosphate (PRPP)
spermine
myo-inositol hexakisphosphate
glycylglycine
5-methyltetrahydrofolate (5MeTHF)
kynurenine
dihomo-linolenate (20:3n3 or n6)
docosahexaenoate (DHA; 22:6n3)
linolenate [alpha or gamma; (18:3n3 or 6)]
linoleate (18:2n6)
isovalerylcarnitine
choline phosphate
arachidonate (20:4n6)
docosapentaenoate (n3 DPA; 22:5n3)
thymidine
1-arachidonoylglycerophosphoinositol*
3-methyl-2-oxovalerate
4-methyl-2-oxopentanoate
3-methyl-2-oxobutyrate
phosphoethanolamine
uridine
acetylcarnitine
pseudouridine
pyridoxal
mead acid (20:3n9)
cytidine 5'-diphosphocholine
2'-deoxyinosine
phenylalanine
tyrosine
tryptophan
isoleucine
leucine
valine
methionine
inositol 1-phosphate (I1P)
serine
coenzyme A
betaine
glycerophosphorylcholine (GPC)
glycerol 2-phosphate
glucose-6-phosphate (G6P)
choline
cholesterol
5-methylthioadenosine (MTA)
inosine 5'-monophosphate (IMP)
uridine 5'-monophosphate (UMP)
glucose
xanthosine
adenine
Isobar: fructose 1,6-diphosphate, glucose 1,6-diphosphate
dihydroxyacetone phosphate (DHAP)
Isobar: ribulose 5-phosphate, xylulose 5-phosphate
1-stearoylglycerophosphoethanolamine
inosine
citrulline
sorbitol
nicotinamide
C-glycosyltryptophan*
methylphosphate
propionylcarnitine
uracil
ethanolamine
cytidine 5'-monophosphate (5'-CMP)
flavin adenine dinucleotide (FAD)
1-palmitoylglycerophosphoethanolamine
1-stearoylglycerol (1-monostearin)
palmitoleate (16:1n7)
10-heptadecenoate (17:1n7)
1-oleoylglycerophosphoethanolamine
eicosenoate (20:1n9 or 11)
guanosine 5'-diphospho-fucose
dihomo-linoleate (20:2n6)
guanosine 5'- monophosphate (GMP)
hypotaurine
N-acetylserine
nonadecanoate (19:0)
caprate (10:0)
palmitate (16:0)
margarate (17:0)
palmitoyl sphingomyelin
pelargonate (9:0)
butyrylcarnitine
arginine
1-linoleoylglycerol (1-monolinolein)
gamma-glutamylvaline
gamma-glutamylisoleucine*
gamma-glutamylthreonine*
butyl stearate
sphinganine
dimethylarginine (SDMA + ADMA)
alpha-glutamylglutamate
gamma-glutamylglutamate
docosadienoate (22:2n6)
2-docosapentaenoylglycerophosphoethanolamine*
2-palmitoleoylglycerophosphocholine*
1-stearoylglycerophosphocholine
1-palmitoylglycerophosphocholine
2-palmitoylglycerophosphocholine*
sphingosine
2-linoleoylglycerophosphocholine*
2-oleoylglycerophosphocholine*
1-oleoylglycerophosphocholine
1-oleoylglycerol (1-monolein)
cytosine
isoleucylleucine
alanylleucine
alanyltyrosine
leucylleucine
2-hydroxypalmitate
sedoheptulose-7-phosphate
docosatrienoate (22:3n3)
adrenate (22:4n6)
glycerol 3-phosphate (G3P)
1-arachidonoylglycerophosphoethanolamine*
nicotinamide adenine dinucleotide phosphate (NADP+)
2-linoleoylglycerol (2-monolinolein)
2-arachidonoyl glycerol
1-arachidonylglycerol
cytidine
nicotinamide adenine dinucleotide (NAD+)
pantothenate
2-myristoylglycerophosphocholine*
2-arachidonoylglycerophosphocholine*

Figure S1

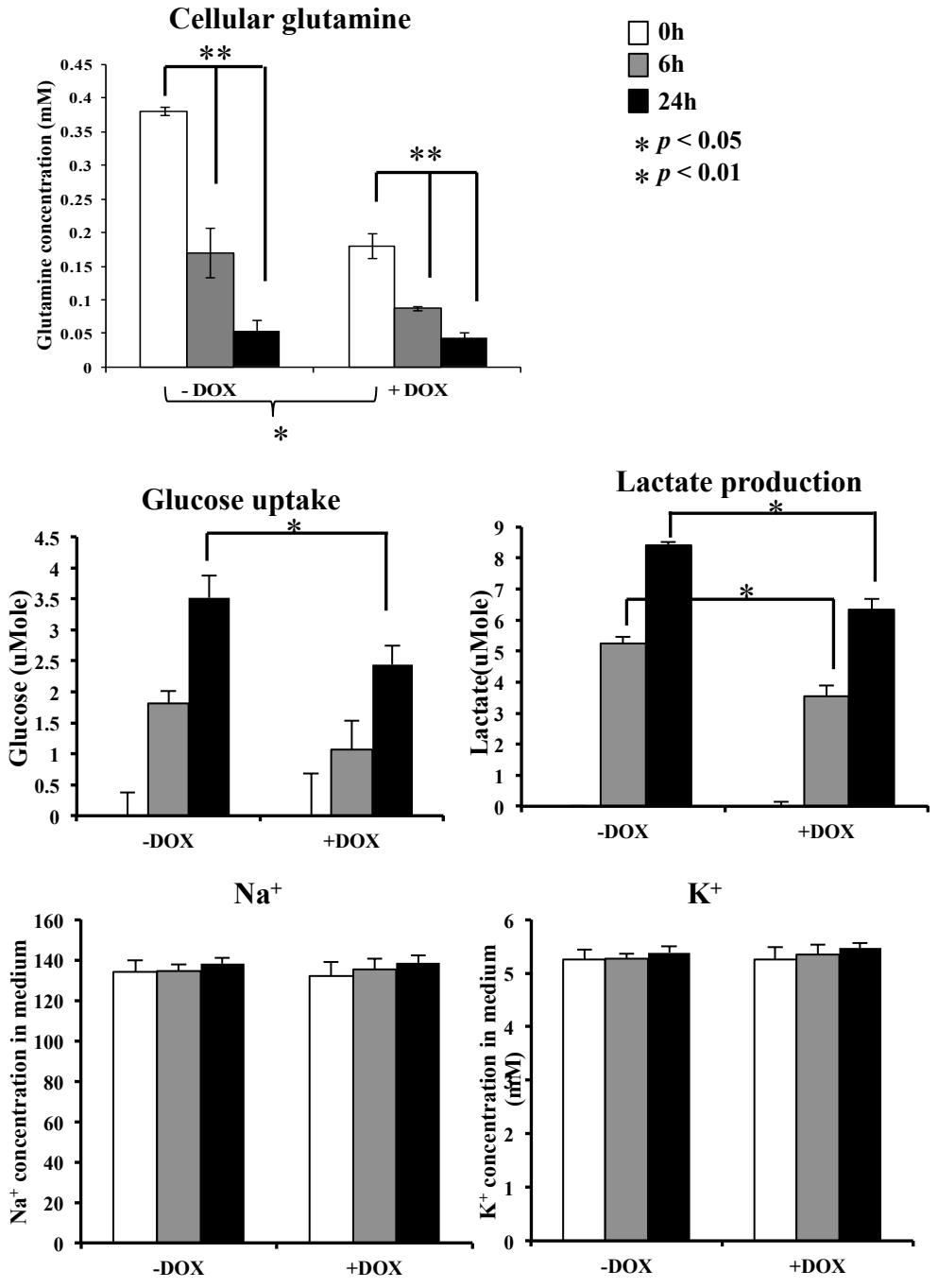
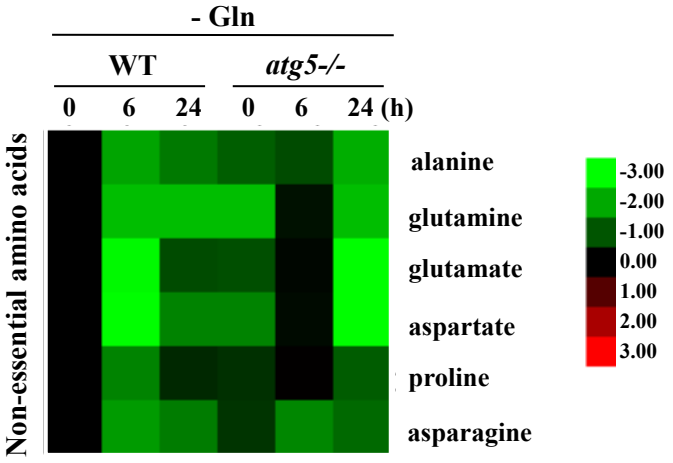

Cellular glutamine
0h
6h
24h
* p < 0.05
* p < 0.01
Glutamine concentration (mM)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
**
**
- DOX
+ DOX
*
Glucose uptake
Glucose (uMole)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
-DOX
+DOX
Lactate production
Lactate(uMole)
9
8
7
6
5
4
3
2
1
0
*
*
-DOX
+DOX
Na+
Na+ concentration in medium
160
140
120
100
80
60
40
20
0
-DOX
+DOX
K+
K+ concentration in medium (mM)
6
5
4
3
2
1
0
-DOX
+DOX

Figure S2

A


- Gln
WT
atg5-/-
0 6 24 0 6 24 (h)
Non-essential amino acids
alanine
glutamine
glutamate
aspartate
proline
asparagine
-3.00
-2.00
-1.00
0.00
1.00
2.00
3.00

B

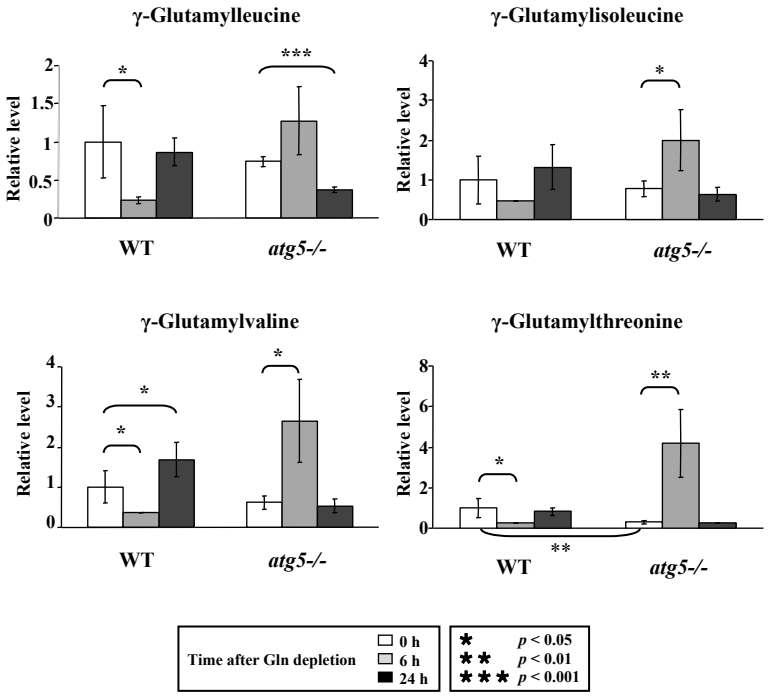
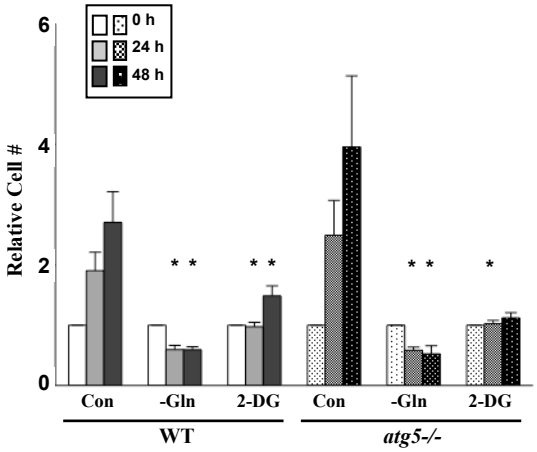

γ-Glutamylleucine
γ-Glutamylisoleucine
γ-Glutamylvaline
γ-Glutamylthreonine
Relative level
WT
atg5-/-
Time after Gln depletion
0 h
6 h
24 h
p < 0.05
p < 0.01
p < 0.001

Figure S3

A


0 h
24 h
48 h
Relative Cell #
6
4
2
0
* *
* *
* *
*
Con
-Gln
2-DG
Con
-Gln
2-DG
WT
atg5-/-

B

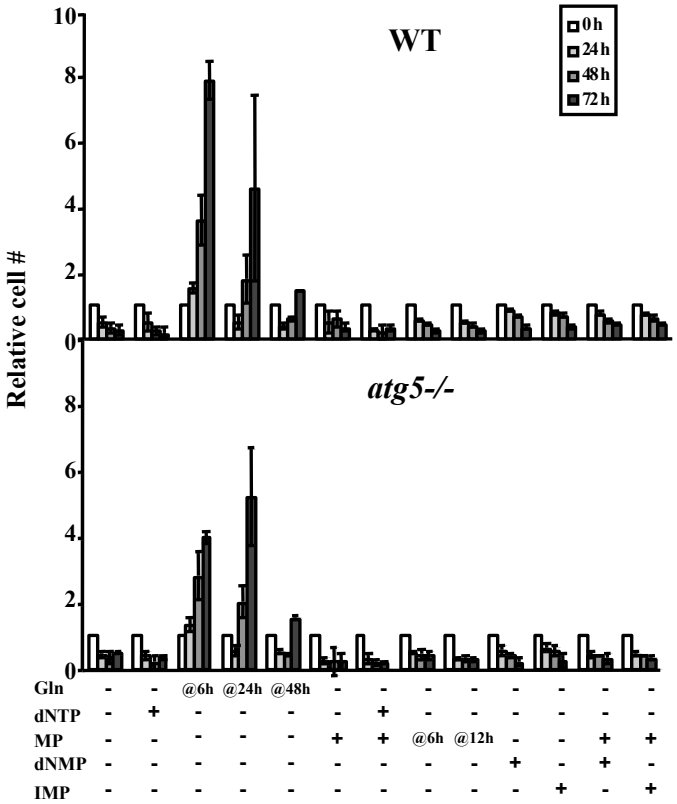

WT
0h
24h
48h
72h
Relative cell #
10
8
6
4
2
0
atg5-/-
Gln - - @6h @24h @48h - - - - - - - -
dNTP - + - - - - + - - - - - -
MP - - - - - + + @6h @12h - - + +
dNMP - - - - - - - - - + - + -
IMP - - - - - - - - - - + - +

Figure S4

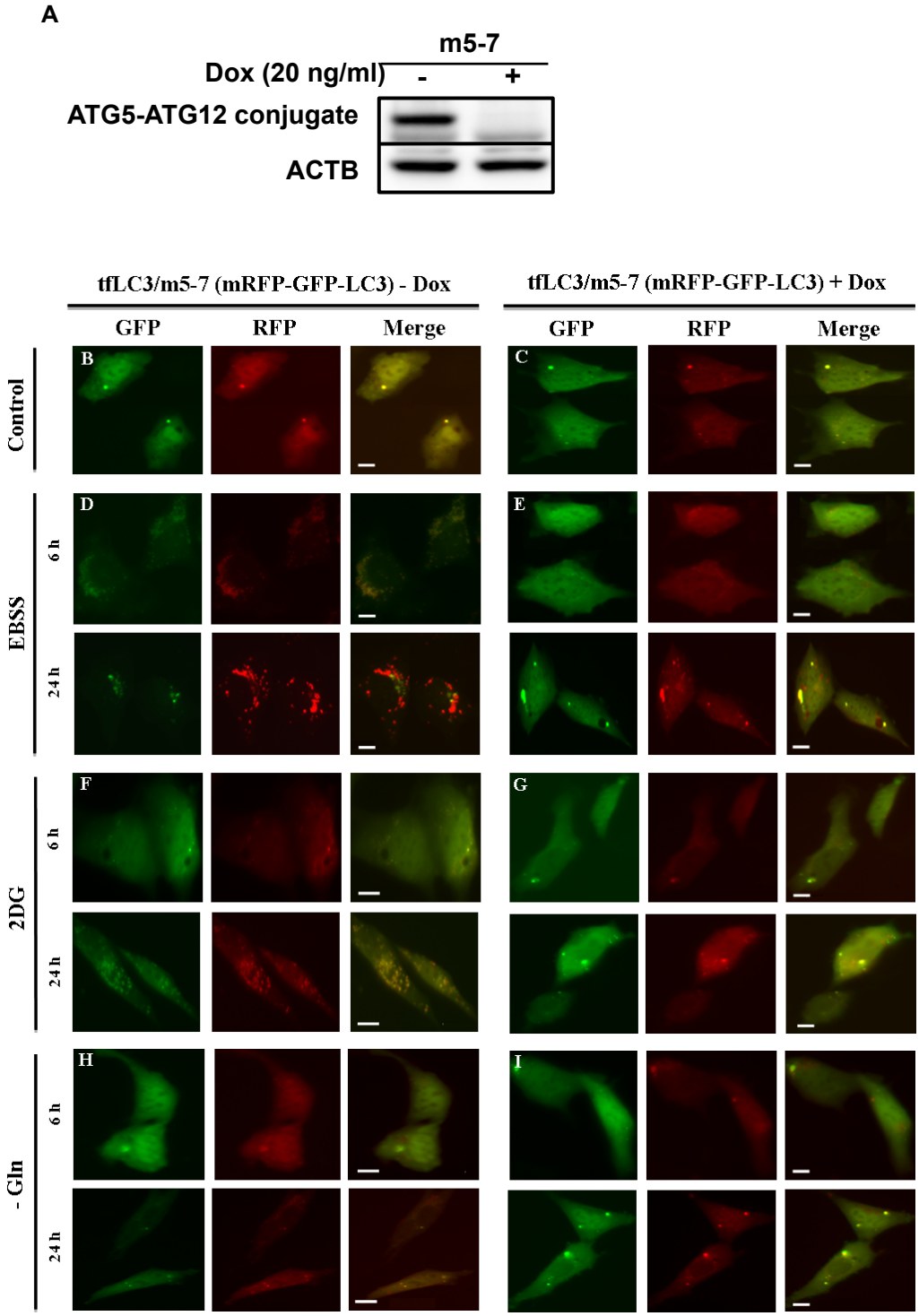

A
m5-7
Dox (20 ng/ml)
-
+
ATG5-ATG12 conjugate
ACTB
tfLC3/m5-7 (mRFP-GFP-LC3) - Dox
tfLC3/m5-7 (mRFP-GFP-LC3) + Dox
GFP
RFP
Merge
GFP
RFP
Merge
Control
EBSS
6 h
24 h
2DG
6 h
24 h
- Gln
6 h
24 h
B
C
D
E
F
G
H
I

Figure S5

Supplementary Table S1. Compounds with significant differences between WT MEFs and *atg5-/-* MEFs.

Super Pathway	Sub Pathway	Biochemical Name	Ratio of basal *atg5-/-* / WT)
Upregulated			
Amino Acid	Creatine metabolism	creatine	1.53
	Cysteine, methionine, SAM, taurine metabolism	cysteine	2.05
	Cysteine, methionine, SAM, taurine metabolism	S-adenosylhomocysteine (SAH)	1.34
	Cysteine, methionine, SAM, taurine metabolism	methionine	1.52
	Glycine, serine and threonine metabolism	glycine	1.43
	Glycine, serine and threonine metabolism	serine	2.24
	Glycine, serine and threonine metabolism	threonine	3.23
	Glycine, serine and threonine metabolism	betaine	1.96
	Histidine metabolism	histidine	1.82
	Phenylalanine & tyrosine metabolism	phenylalanine	1.67
	Phenylalanine & tyrosine metabolism	tyrosine	1.68
	Phenylalanine & tyrosine metabolism	3-(4-hydroxyphenyl)lactate	1.64
	Polyamine metabolism	spermine	6.43
	Polyamine metabolism	spermidine	2.89
	Polyamine metabolism	5-methylthioadenosine (MTA)	3.37
	Tryptophan metabolism	C-glycosyltryptophan*	1.52
	Tryptophan metabolism	tryptophan	1.54
	Urea cycle; arginine-, proline-, metabolism	citrulline	1.46
	Valine, leucine and isoleucine metabolism	3-methyl-2-oxovalerate	4.37
	Valine, leucine and isoleucine metabolism	isoleucine	1.52
	Valine, leucine and isoleucine metabolism	leucine	1.59
	Valine, leucine and isoleucine metabolism	valine	1.61
	Valine, leucine and isoleucine metabolism	4-methyl-2-oxopentanoate	3.52
	Valine, leucine and isoleucine metabolism	2-methylbutyroylcarnitine	4.53
	Valine, leucine and isoleucine metabolism	isovalerylcarnitine	6.16
Carbohydrate	Aminosugars metabolism	N-acetylneuraminate	2.53
	Nucleotide sugars, pentose metabolism	5-phosphoribosyl diphosphate (PRPP)	16.16
Cofactors and vitamins	Folate metabolism	5-methyltetrahydrofolate (5MeTHF)	1.27
	Pantothenate and CoA metabolism	pantothenate	1.44
	Pyridoxal metabolism	pyridoxal	2.27
	Riboflavin metabolism	flavin adenine dinucleotide (FAD)	1.42
	Pantothenate and CoA metabolism	coenzyme A	2.08
Lipid	Carnitine metabolism	acetylcarnitine	2.03

	Essential fatty acid	linolenate [alpha or gamma; (18:3n3 or 6)]		1.65
	Essential fatty acid	dihomo-linolenate (20:3n3 or n6)		2.15
	Essential fatty acid	docosapentaenoate (n3 DPA; 22:5n3)		2.18
	Essential fatty acid	docosahexaenoate (DHA; 22:6n3)		1.73
	Essential fatty acid	eicosapentaenoate (EPA; 20:5n3)		1.8
	Fatty acid metabolism (also BCAA metabolism)	propionylcarnitine		1.89
	Glycerolipid metabolism	glycerol 3-phosphate (G3P)		1.44
	Glycerolipid metabolism	phosphoethanolamine		5.52
	Glycerolipid metabolism	glycerophosphorylcholine (GPC)		1.36
	Glycerolipid metabolism	cytidine 5'-diphosphocholine		1.57
	Glycerolipid metabolism	choline		1.73
	Inositol metabolism	inositol hexaphosphate (IP6)		3.21
	Long chain fatty acid	linoleate (18:2n6)		1.74
	Long chain fatty acid	10-heptadecenoate (17:1n7)		1.26
	Long chain fatty acid	10-nonadecenoate (19:1n9)		1.33
	Long chain fatty acid	oleate (18:1n9)		1.3
	Long chain fatty acid	dihomo-linoleate (20:2n6)		1.44
	Long chain fatty acid	mead acid (20:3n9)		1.81
	Long chain fatty acid	palmitate (16:0)		1.18
	Long chain fatty acid	arachidonate (20:4n6)		2.45
	Lysolipid	1-arachidonoylglycerophosphoinositol*		2.62
	Lysolipid	1-stearoylglycerophosphoethanolamine		1.55
	Lysolipid	1-arachidonoylglycerophosphoethanolamine*		1.48
	Monoacylglycerol	2-linoleoylglycerol (2-monolinolein)		1.37
	Monoacylglycerol	2-arachidonoyl glycerol		1.73
	Monoacylglycerol	2-palmitoylglycerol (2-monopalmitin)		1.3
	Sphingolipid	palmitoyl sphingomyelin		1.24
	Sterol/Steroid	cholesterol		1.32
Nucleotide	Purine metabolism, (hypo)xanthine/inosine containing	inosine 5'-monophosphate (IMP)		4.83
	Purine metabolism, adenine containing	adenine		2.96
	Purine metabolism, guanine containing	guanosine 5'- monophosphate (GMP)		1.4
	Purine metabolism, guanine containing	guanosine 5'-diphospho-fucose		1.24
	Pyrimidine metabolism, cytidine containing	cytidine 5'-monophosphate (5'-CMP)		1.26
	Pyrimidine metabolism, uracil containing	pseudouridine	2.13	
	Pyrimidine metabolism, uracil containing	uridine	1.88	
	Pyrimidine metabolism, uracil containing	uridine 5'-monophosphate (UMP)	1.43	

Downregulated			
Amino acid	Alanine and aspartate metabolism	alanine	0.46
	Alanine and aspartate metabolism	aspartate	0.34
	Glutamate metabolism	glutamine	0.21
	Glutamate metabolism	glutamate	0.52
	Glutathione metabolism	5-oxoproline	0.7
	Glutathione metabolism	glutathione, reduced (GSH)	0.69
	Urea cycle; arginine-, proline-, metabolism	ornithine	0.18
	Urea cycle; arginine-, proline-, metabolism	proline	0.67
Energy	Krebs cycle	malate	0.72
Nucleotide	Purine metabolism, adenine containing	adenosine 5'-monophosphate (AMP)	0.88
	Purine metabolism, adenine containing	adenosine 3',5'-diphosphate	0.37
	Purine metabolism, adenine containing	adenylosuccinate	0.8
Peptide	gamma-glutamyl	gamma-glutamylthreonine*	0.31

*indicates a biochemical that has not been confirmed with an authentic standard but whose spectral properties strongly support its identity

Supplementary Table S2. Real-time PCR primer pairs

Gene	Forward primer	Reverse primer
Aco1	CCATCCGTGATGTTAGGAGCAG	GACAGGTAAGGCATGACTCCAC
Atg5	CAGAAGGTTATGAGACAAGAAGATG	TGGATGGACAGTGTAGAAGGTC
Cs	ATGCAGAGGGAATGAACCGAGC	GAGTCAATGGCTCCGATACTGC
Flnb	GAACTCACACGCAGGATGCTGT	GGCGGCTTTTATTCTCACCATCG
Gapdh	CCCCTTCATTGACCTCAACTA	CTCCTGGAAGATGGTGATGG
Gpt2	CATTGCGGCAAGCCAAAGACCA	GCTTCTCTTCCCAGGCAAAGTG
Idh1	GCTGGCTTTGTATCTCA	CGCCCATCATACTTCTTCA
Idh2	GGCTGTCAAGTGTGCCACAATC	TTGGCTCTCTGAAGACGGTTCC
Ldhb	CCTCAGATCGTCAAGTACAGCC	ATCCGCTTCCAATCACACGGTG
Mdh1	TTCTGGACGGTGTCCTGATGGA	TAGGACAGCCACATCCAGGTCT
Me1	ATGGAGAAGGAAGGTTTATCAAAG	GGCTTCTAGGTTCTTCATTTCTTC
Me2	GGCTAAGAGCTGTTACCACTCC	CGTAAACGCCATTCCCTTGTT
Myc	TCGCTGCTGTCCTCCGAGTCC	GGTTTGCCTCTTCTCCACAGAC
Ogdh	GGTGTCGTCAATCAGCCTGAGT	ATCCAGCCAGTGCTTGATGTGC
Sdha	GAGATACGCACCTGTTGCCAAG	GGTAGACGTGATCTTTCTCAGGG
Sdhb	TGCGGACCTATGGTGTTGGATG	CCAGAGTATTGCCTCCGTTGATG
Slc1a5	CTGCCTGTGAAGGACATCTCCT	CTCGGCATCTTGGTTCGATCCA
Slc3a2	GAGCGTACTGAATCCCTAGTCAC	GCTGGTAGAGTCGGAGAAGATG
Slc7a5	GGTCTCTGTTCACGTCCTCAAG	GAACACCAGTGATGGCACAGGT
Sucla2	GGTGTCTCTGTTCCCAAAGGCT	TTTCCTCTGCCGCCAGCCAAAA
Suclg1	GTCTTACACAGCCTCTCGGAAAC	ACTCCAAAGCCTGCTGACTGTG
Suclg2	AGCTCAAGGTGCCACTGGTAGT	GCTTTCTTGGCTGCATCCTCCA
Cdkn1a	TCGCTGTCTTGCACTCTGGTGT	CCAATCTGCGCTTGGAGTGATAG
Bbc3	ACCGCTCCACCTGCCGTCAC	ACGGGCGACTCTAAGTGCTGC
Pmaip1	GGAAGTCGCAAAAGAGCAGGATG	CTGCCGTAAATTCACTTTGTCTCC