RE: GENESDEV/2018/320481

Autophagy Modulates Lipid Metabolism to Maintain Metabolic Flexibility for *Lkb1*-Deficient *Kras*-Driven Lung Tumorigenesis

Vrushank Bhatt[1], Khoosheh Khayati[1], Zhixian Sherrie Hu[1], Amy Lee[1], Wali Kamran[1], Xiaoyang Su[1,2], Jessie Yanxiang Guo[1,2,3 #]
[1]Rutgers Cancer Institute of New Jersey, New Brunswick, NJ 08901;
[2]Department of Medicine, Rutgers Robert Wood Johnson Medical School, New Brunswick, NJ 08901;
[3]Department of Chemical Biology, Rutgers Ernest Mario School of Pharmacy, Piscataway, NJ 08854 USA;
[#]Corresponding Author

Running Title: Autophagy Supports *Lkb1*-Deficient Lung Tumorigenesis

Keywords: Autophagy, LKB1, non-small cell lung cancer, lipid metabolism, energy metabolism

Corresponding Author:
Jessie Yanxiang Guo, Ph.D.
Rutgers Cancer Institute of New Jersey
RBHS-Robert Wood Johnson Medical School
Rutgers, The State University of New Jersey
195 Little Albany Street
New Brunswick, NJ 08903-2681
yanxiang@cinj.rutgers.edu
Voice: 732-235-9657

Supplementary Data

Supplemental materials and methods

Metabolomic analysis by LC-MS

Metabolites extraction for LC-MS analysis.

To extracting metabolites from cells, *Atg7*$^{+/+}$ and *Atg7*$^{-/-}$ TDCLs cultured in 6-cm dishes in triplicate were treated with RPMI, HBSS and HBSS with glutamine for 1 hour and then quickly washed twice with PBS; subsequently, cells were incubated with 1 mL of 40:40:20 methanol:acetonitrile:water with 0.5% formic acid solution on ice for 5 minutes followed by neutralization with 50μL of 15% ammonium bicarbonate. The cells were then scrapped from the plates using a cell lifter, transferred to 1.5 mL freshly labeled tubes on ice and centrifuged at 4°C for 10 min at 13,000 rpm. The supernatants were transferred to LC–MS autosampler vials (on ice) and sent for LC-MS analysis.

To extract metabolites from lung tumors, at 18 weeks of post-Lenti-Cre infection, the mice bearing *Atg7*$^{+/+}$ and *Atg7*$^{-/-}$ KL lung tumors was euthanized and lung tumors were quickly dissected and snap frozen in liquid nitrogen with pre-cooled Wollenberger clamp. Subsequently, frozen tissue samples were first weighed (~20-30 mg each sample) and ground using a Cryomill (Retsch, Newtown, PA) in liquid nitrogen at 25Hz for 2 min. The resulting powder was then mixed with −20°C 40:40:20 methanol:acetonitrile:water with 0.5% formic acid, followed by 10 sec vortexing, 10min incubation on ice, neutralization with 50μL/mL of 15% ammonium bicarbonate, and 10 min centrifugation at 4°C and 16,000*g*. The supernatants were transferred to LC–MS autosampler vials (on ice) and sent for LC-MS analysis.

LC−MS analysis of the cellular metabolites was performed on the Q Exactive PLUS hybrid quadrupole-orbitrap mass spectrometer (Thermo Scientific) coupled to hydrophilic interaction chromatography (HILIC). The LC separation was performed on UltiMate 3000 UHPLC system with an XBridge BEH Amide column (150 mm × 2.1 mm, 2.5 µM particle size, Waters, Milford, MA) with the corresponding XP VanGuard Cartridge. The liquid chromatography used a gradient of solvent A (95%:5% H2O:acetonitrile with 20 mM ammonium acetate, 20 mM ammonium hydroxide, pH 9.4), and solvent B (20%:80% H2O:acetonitrile with 20 mM ammonium acetate, 20 mM ammonium hydroxide, pH 9.4). The gradient was 0 min, 100% B; 3 min, 100% B; 3.2 min, 90% B; 6.2 min, 90% B; 6.5 min, 80% B; 10.5 min, 80% B; 10.7 min, 70% B; 13.5 min, 70% B; 13.7 min, 45% B; 16 min, 45% B; 16.5 min, 100% B. The flow rate was 300 µl/min. Injection volume was 5 µL and column temperature 25 °C. The MS scans were in negative ion mode with a resolution of 70,000 at m/z 200. The automatic gain control (AGC) target was 3×10^6 and the scan range was 75−1000. Metabolite features were extracted in MAVEN (Cite: PMID 21049934).

Analysis of saponified FAs by LC-MS

To extract saponified FAs for LC-MS analysis, *Atg7*$^{+/+}$ and *Atg7*$^{-/-}$ TDCL clones cultured in 6-cm dishes were assessed in triplicate. TDCLs were cultured in normal RPMI and HBSS starvation conditions for 4 hours; subsequently cells were rinsed twice with 2 mL of 37°C PBS, followed by adding 1 mL of -20°C 90% methanol with 0.3M potassium hydroxide. The resulting liquid and cell debris were scraped into 4 mL glass tubes. The samples were heated at 80°C for 1 hour to saponify FAs, followed by acidification with

100μL of formic acid. The mixture was then vortexed for 1 min and the samples were extracted twice with 1 mL of hexane, dried under N2, and dissolved in 1:1:1 methanol:chloroform:isopropranol to a final concentration of 2μL of cell volume per milliliter for LC-MS analysis.

The LC separation of FAs was performed on UltiMate 3000 UHPLC system with an Agilent InfinityLab Poroshell 120 EC-C18 (2.1*150mm, 2.7um) column. The liquid chromatography used a gradient of solvent A (90%:10% H2O:Methanol with 1 mM ammonium acetate, 35 mM acetic acid), and solvent B (2%:98% H2O:isopropanol with 1 mM ammonium acetate, 35 mM acetic acid). The gradient was 0 min, 25% B; 2 min, 25% B; 5.5 min, 65% B; 9.5 min, 100% B; 13.5 min, 100% B; 14 min, 25% B; 20 min, 25% B. The flow rate was 200 μl/min. Injection volume was 2 μL and column temperature 45 °C. The MS scans were in negative ion mode with a resolution of 70,000 at m/z 200. The automatic gain control (AGC) target was 3×10^6 and the scan range was 200−1000. Metabolite features were extracted in MAVEN. The ^{13}C isotope natural abundance and impurity of labeled substrate was corrected using AccuCor written in R (Cite: PMID 28471646).

Lipidomic analysis by LC-MS

To extract lipids for LC-MS analysis, cells were cultured in 6-cm dishes in RPMI. The following day, cells were rinsed twice with 2 mL of 37°C PBS, followed by 1 mL of cold (-20°C) 0.1M HCl:methanol (1:1). The resulting liquid and cell debris were scraped into 4 mL glass tubes. 500μL of cold chloroform was added. The mixture was vortexed for 1 min

and centrifuges at 4C for 5 min at 18,000g. The lower chloroform phase was transferred to a freshly labeled glass vial, dried under N2, and dissolved in 1:1:1 methanol:chloroform:isopropranol to a final concentration of 2μL of cell volume per milliliter for LC-MS analysis.

The LC separation of lipids was performed on UltiMate 3000 UHPLC system with an Agilent InfinityLab Poroshell 120 EC-C18 (2.1*150mm, 2.7um) column. The liquid chromatography used a gradient of solvent A (90%:10% H2O:Methanol with 1 mM ammonium acetate, 35 mM acetic acid), and solvent B (2%:98% H2O:isopropanol with 1 mM ammonium acetate, 35 mM acetic acid). The gradient was 0 min, 25% B; 2 min, 25% B; 5.5 min, 65% B; 12.5 min, 100% B; 16.5 min, 100% B; 17 min, 25% B; 30 min, 25% B. The flow rate was 200 μl/min. Injection volume was 2 μL and column temperature 45 °C. The MS scans were in positive ion mode with a resolution of 70,000 at m/z 200. The automatic gain control (AGC) target was 3×10^6 and the scan range was 200−1000. Metabolite features were extracted in MAVEN.

Supplemental Figure Legends

S1. Loss of Lkb1 causes KL tumors to be selective against autophagy-defective tumor growth. A. PCR shows mouse genotyping. B. IHC of Lkb1, Atg7, p62 and LC3 shows that Lkb1 was deleted in KL tumors, but Atg7 was incompletely deleted in KL tumors in mice infected with Adenoviral-Cre. Accumulation of p62 and LC3 indicates autophagy blockade. C. IHC of Lkb1 and Atg7 in $Lkb1^{+/+}$;$Kras^{G12D/+}$ (K) tumors to show Atg7 was homogenously deleted in K tumors induced by Adenoviral-Cre. D. WB of Atg5-

12 and LC3 of KL tumor samples shows that autophagy is active in one *Atg7*$^{-/-}$ KL tumor marked in red (mouse ID 8136). E. Kaplan-Meier survival curve of mice bearing KL tumors that were intranasally infected with Adenoviral-Cre (P= 0.05, log-rank test).

S2. Autophagy ablation impairs KL lung tumorigenesis in mice intranasally infected with Lenti-Cre.

A. Representative histology of scanned lung sections of KL and KP mice at the indicated times post Lenti-Cre infection. B. Representative IHC of Lkb1 in low magnification to show that Lkb1 is homogeneously deleted in all tumors and Atg7 deletion impairs KL tumor growth. C & D. Representative IHC of Atg7 at high (C) and low (D) magnification at the indicated times. E. Representative IHC of p62 and LC3 to show autophagy ablation in *Atg7*$^{-/-}$ KL tumors at 14 weeks post-infection (Red arrow indicates autophagosome in *Atg7*$^{+/+}$ tumors; black arrow indicates LC3 aggregates in *Atg7*$^{-/-}$ tumors).

S3. Autophagy deficiency impairs oncogenic signaling.

A. Representative H&E of KL lung tumors at low magnification at the indicated times. B. Representative H&E of *Atg7*$^{-/-}$ KL lung tumors at 29 weeks post-Lenti-Cre infection: I. scanned lung section (I: inflammation; A: adenocarcinoma; S: squamous cell carcinoma). II. Representative H&E of adenocarcinoma (ADC). III. Representative H&E of lung inflammation (INF). IV. Representative H&E of squamous cell carcinoma (SCC). C-E. Representative IHC of pS6 (C), pERK (D) and Ki67 (E) at low magnification at the indicated time points.

S4. Characterization of KL TDCLs.

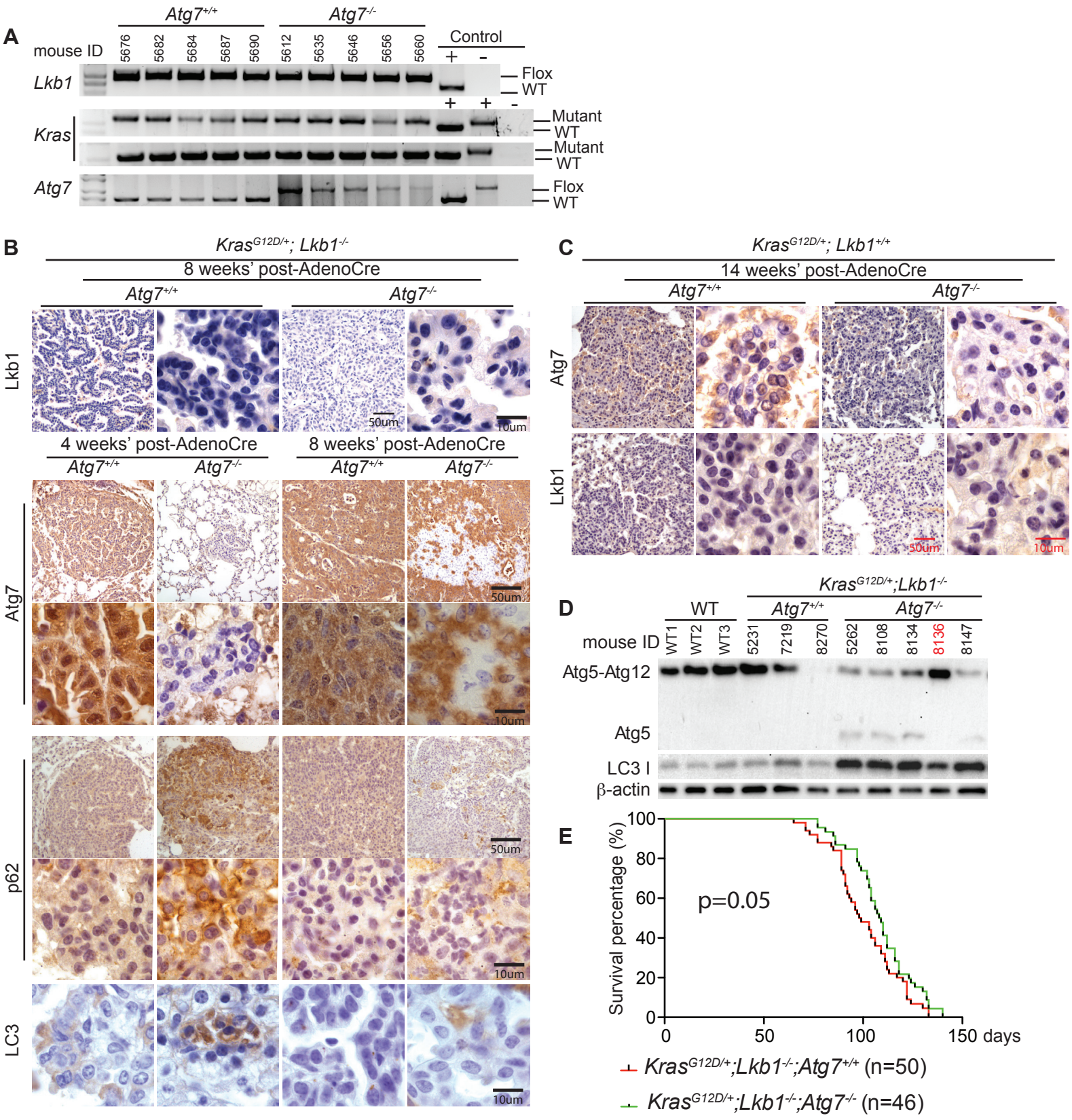
A. WB of Atg7, Atg5-Atg12 and LC3 shows autophagy activity was blocked by Atg7 deletion in KL TDCLs. Red indicates Atg7 was not deleted in "*Atg7*$^{-/-}$" TDCL clones. B. Characterization of TDCLs by PCR. Red indicates that *Flox* of *Atg7* was not deleted in "*Atg7*$^{-/-}$" TDCL clones. C. WB of Atg7 shows that Atg7 was deleted in *Atg7*$^{flox/flox}$ KL cells treated with Adenoviral-Cre. D. Relative cell proliferation of KL cells without (*Atg7*$^{flox/flox}$) or with acute Atg7 deletion (*Atg7*$^{del/del}$) by Adenoviral-Cre in nutrient-rich RPMI medium. E. Clonogenic survival assay shows Atg7 deletion by Adenoviral-Cre increased the sensitivity of KL TDCLs to HBSS starvation-induced cell death, which was rescued by glutamine (2mM) or BSA-Pal (20μM). F. WB of Atg5-12, LC3 and β-actin of KL TDCLs. G. Relative cell proliferation of Atg7$^{+/+}$ KL TDCLs without or with Atg5 knockdown in nutrient-rich RPMI medium. H. Clonogenic survival assay of *KL* TDCLs without or with Atg5 knockdown in HBSS or HBSS supplemented with glutamine (2mM) or BSA-Pal (20μM).

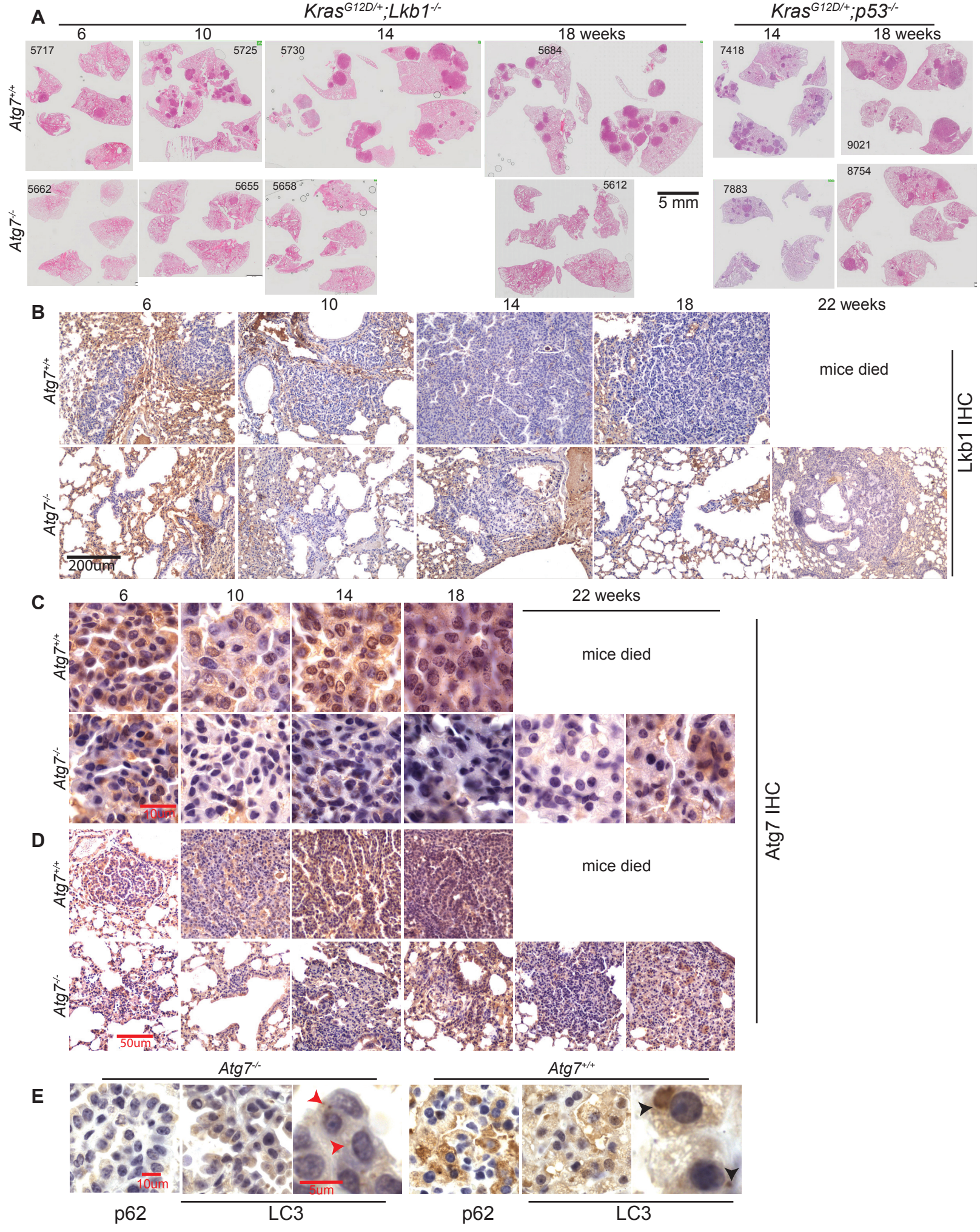
S5. Autophagy mediates metabolites recycling in starvation. A. Levels of metabolites in normal RPMI and HBSS starvation in *Atg7*$^{+/+}$ and *Atg7*$^{-/-}$ TDCLs after 1 hour treatment. B. Amino acids consumption of TDCLs in nutrient-rich conditions shows Atg7 depletion upregulates the consumption of serine, methionine and threonine. Each bar represents the average of four clones from *Atg7*$^{+/+}$ or *Atg7*$^{-/-}$ TDCLs. (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

S6. Autophagy deficiency promotes nucleotide degradation in starvation.

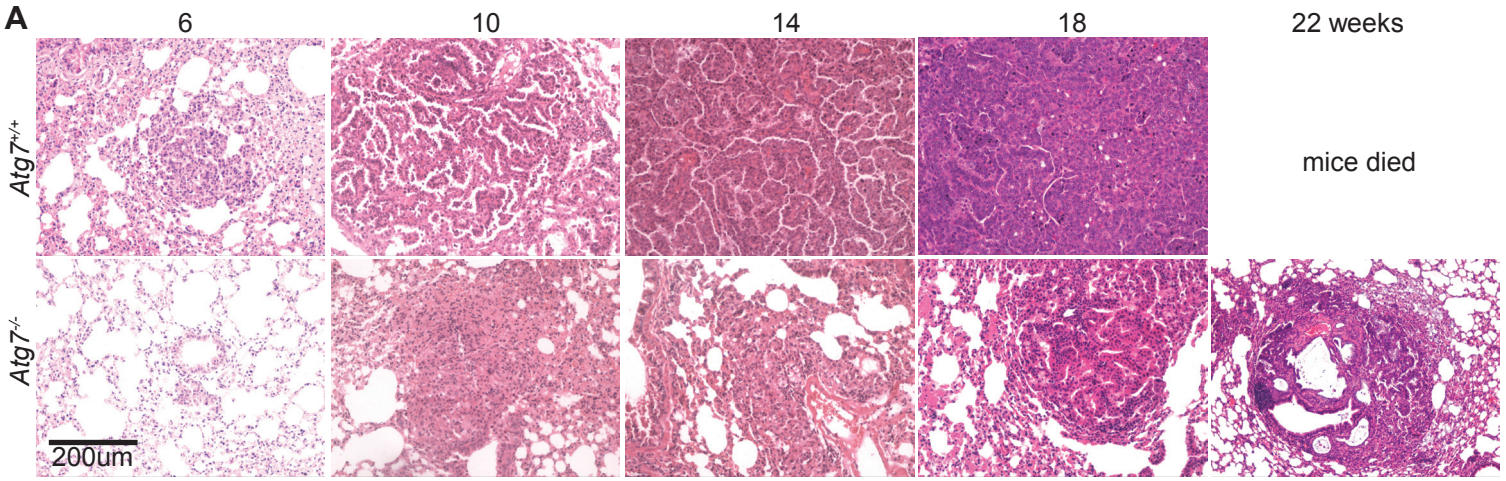
A. Schematic of nucleotide degradation. B. The levels of substrates from nucleotide degradation in normal and HBSS starvation in TDCLs show *Atg7* depletion promotes nucleotide catabolism. C. Clonogenic survival assay of TDCLs in HBSS supplemented with nucleosides (2mM). (* P<0.05, *** P< 0.001).

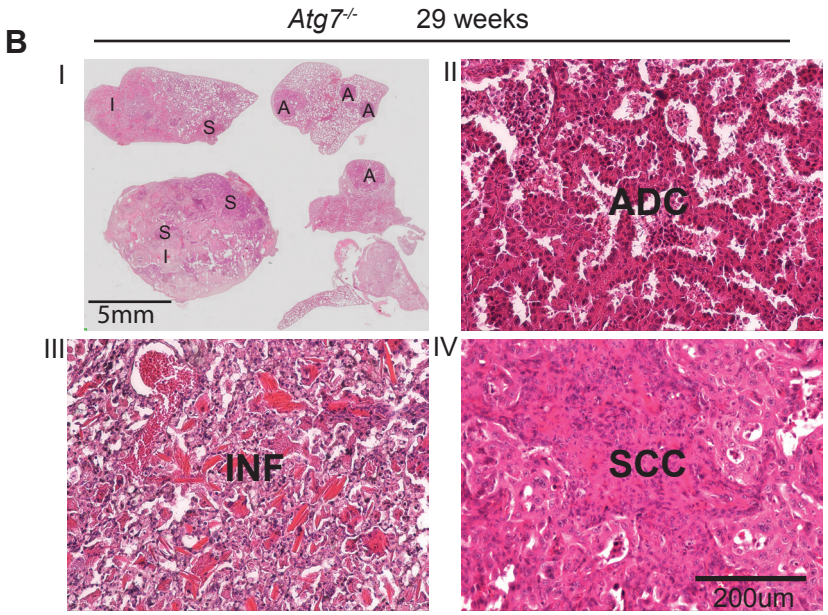
S7. Autophagy deficiency alters the lipid metabolism of *Lkb1*-deficient cells. A. [$U^{13}C6$] glucose tracer labeling to FAs of KL TDCLs shows that *Atg7* depletion promotes *de novo* FA synthesis and inhibits elongation. B. Clonogenic survival assay of $Atg7^{+/+}$ and $Atg7^{-/-}$ KL TDCLs in nutrient-rich RPMI condition supplemented without or with Fasnall (50μM). C. Cell proliferation of $Atg7^{+/+}$ and $Atg7^{-/-}$ cells in nutrient-rich RPMI condition treated without or with ETO (50μM). (* P<0.05, ** P<0.01, *** P< 0.001). D. WB of ATG5-12, LC3 and β-actin of human lung cancer cells without or with ATG5 knockdown. E. Relative cell proliferation of *LKB1*-mutant human lung cancer cells without or with ATG5 knockdown in nutrient-rich RPMI medium. F. Clonogenic survival assay of *LKB1*-mutant human lung cancer cells without or with ATG5 knockdown in HBSS or HBSS supplemented with glutamine (2mM), BSA or BSA-Pal (20μM).

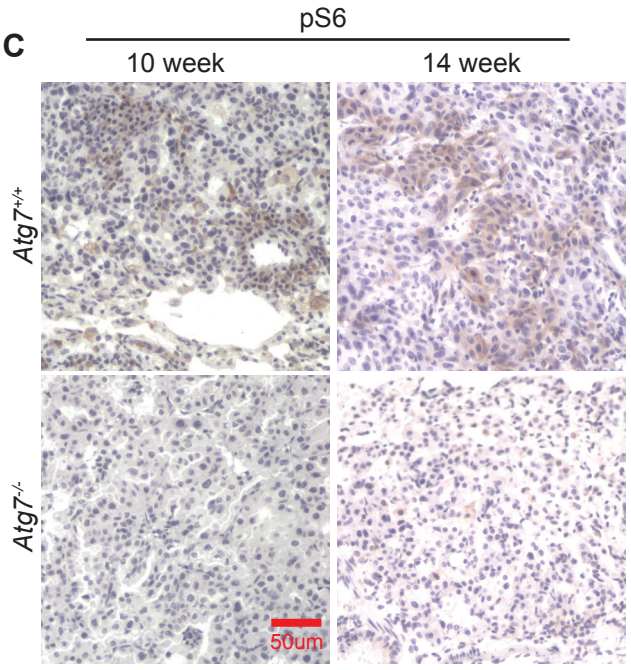

A
Atg7+/+
Atg7-/-
mouse ID
5676
5682
5684
5687
5690
5612
5635
5646
5656
5660
Control
+
-
Lkb1
Flox
WT
Kras
Mutant
WT
Atg7
Flox
WT
B
KrasG12D/+; Lkb1-/-
8 weeks' post-AdenoCre
Atg7+/+
Atg7-/-
Lkb1
50um
10um
4 weeks' post-AdenoCre
8 weeks' post-AdenoCre
Atg7
p62
LC3
C
KrasG12D/+; Lkb1+/+
14 weeks' post-AdenoCre
Atg7
Lkb1
D
KrasG12D/+;Lkb1-/-
WT
WT1
WT2
WT3
5231
7219
8270
5262
8108
8134
8136
8147
Atg5-Atg12
Atg5
LC3 I
β-actin
E
Survival percentage (%)
p=0.05
150 days
KrasG12D/+;Lkb1-/-;Atg7+/+ (n=50)
KrasG12D/+;Lkb1-/-;Atg7-/- (n=46)

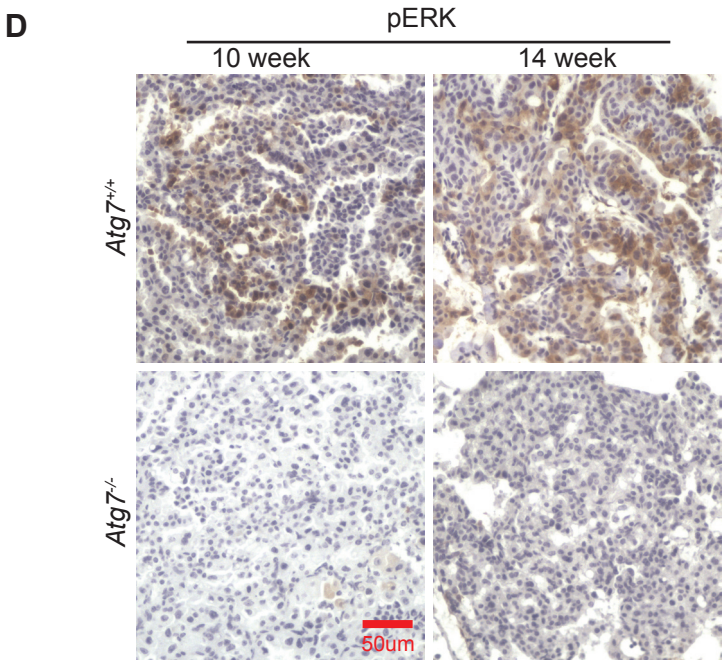

A
Kras^G12D/+;Lkb1^-/-
Kras^G12D/+;p53^-/-
6
10
14
18 weeks
14
18 weeks
Atg7+/+
Atg7-/-
5717
5725
5730
5684
7418
9021
5662
5655
5658
5612
7883
8754
5 mm
B
6
10
14
18
22 weeks
mice died
Lkb1 IHC
200um
C
6
10
14
18
22 weeks
mice died
10um
D
mice died
50um
Atg7 IHC
E
Atg7-/-
Atg7+/+
10um
5um
p62
LC3
p62
LC3

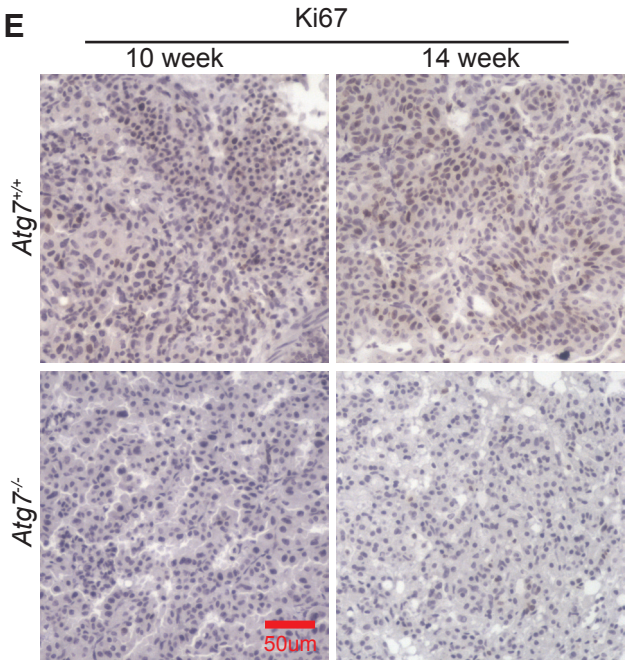
Supplemental_Fig_S3

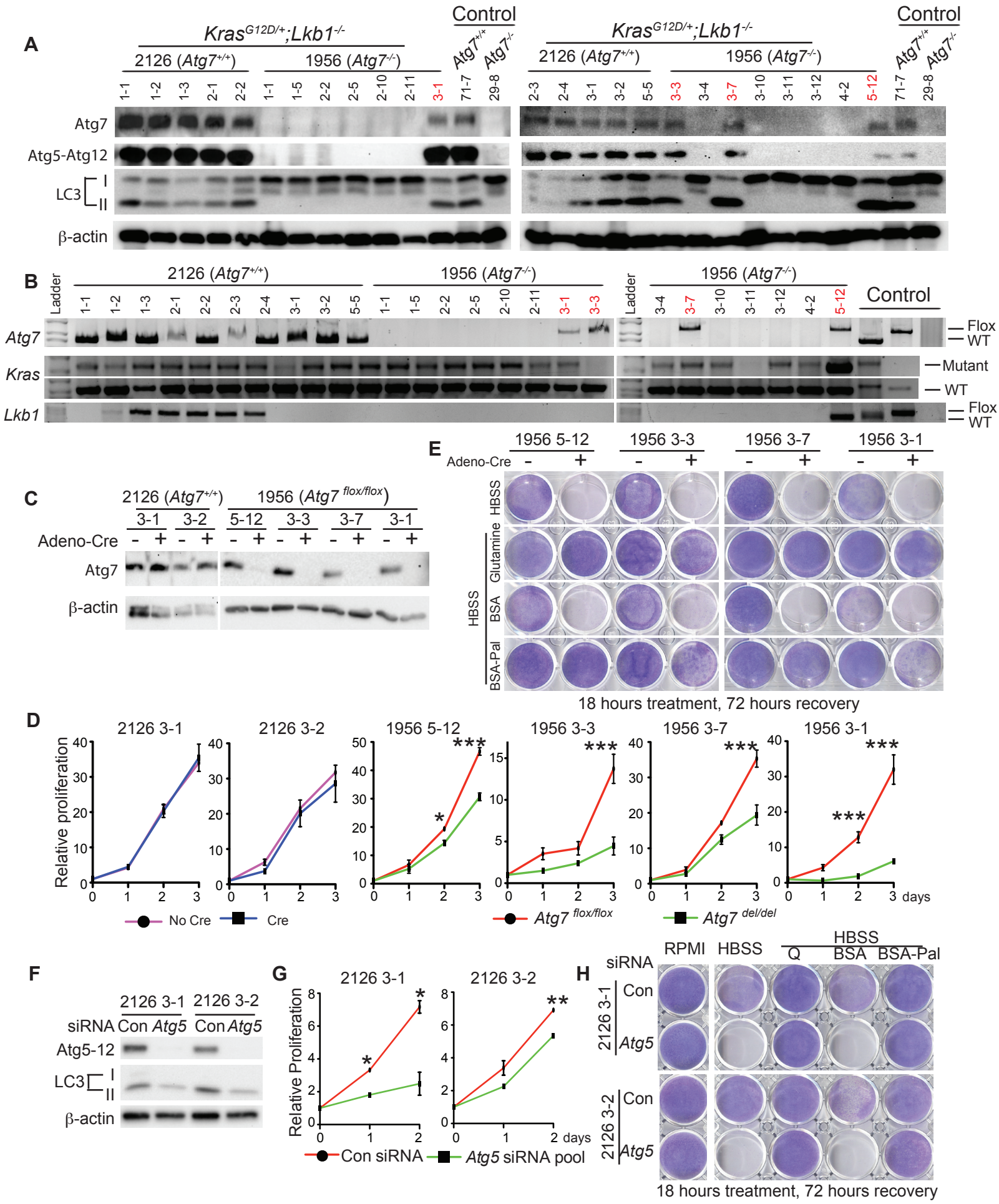


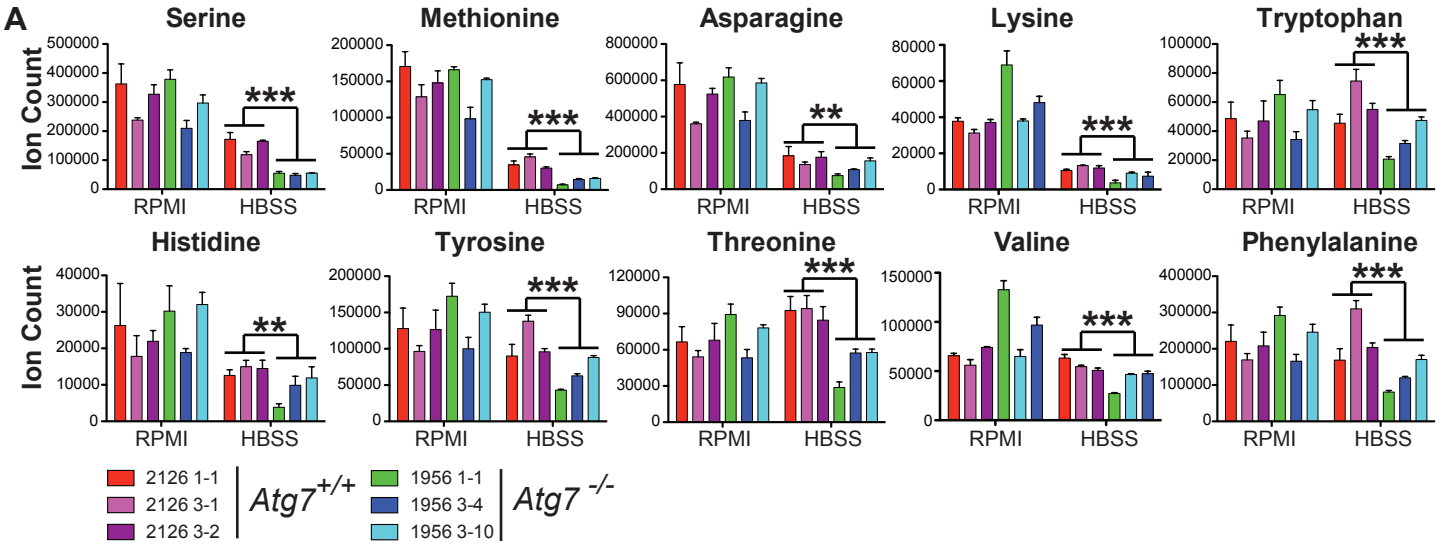


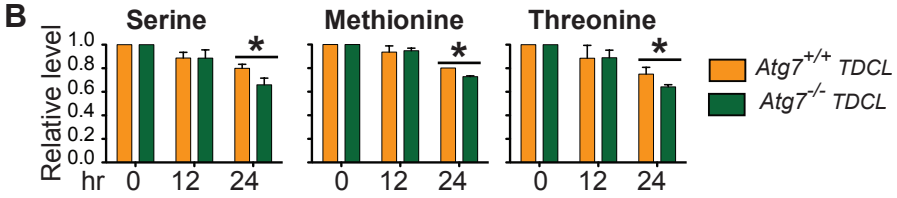


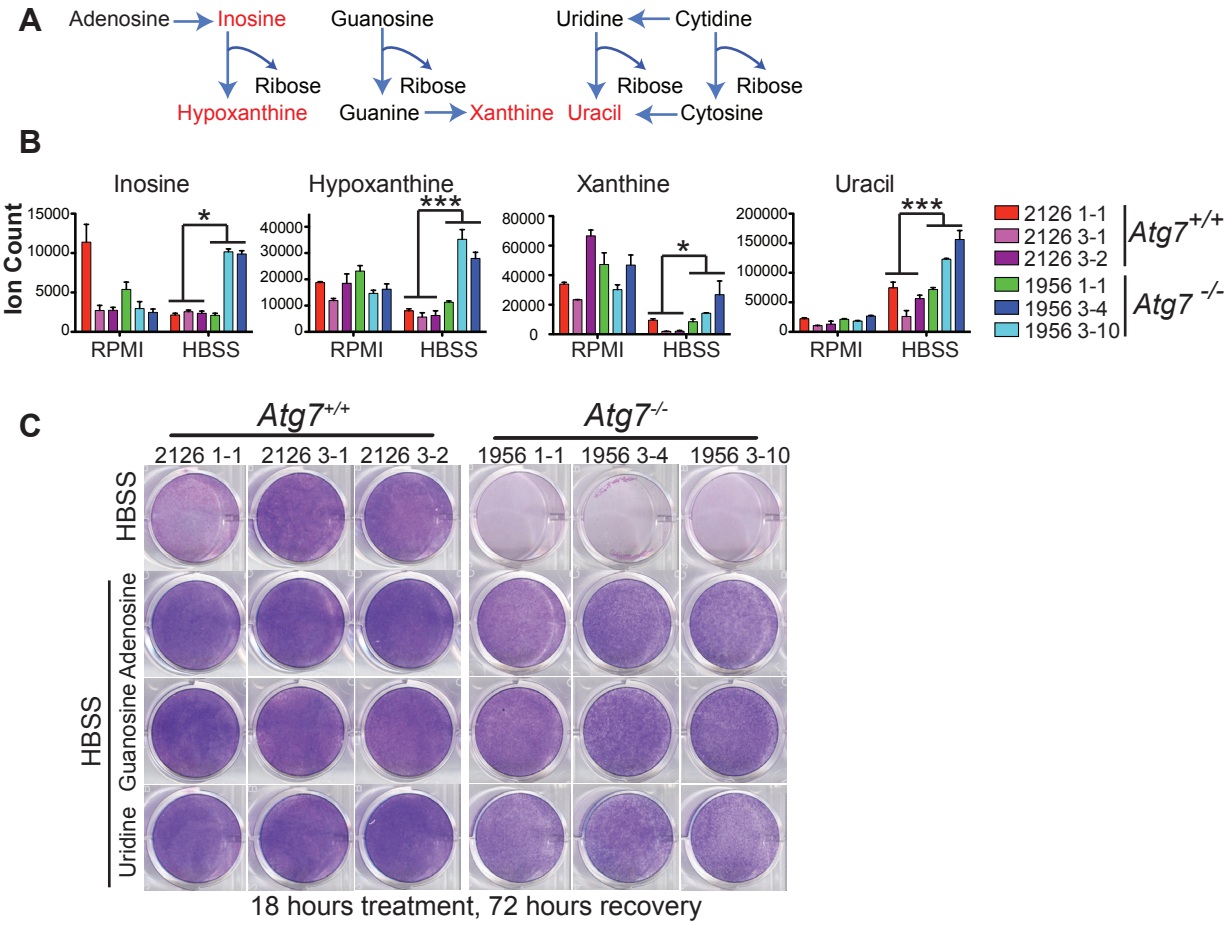


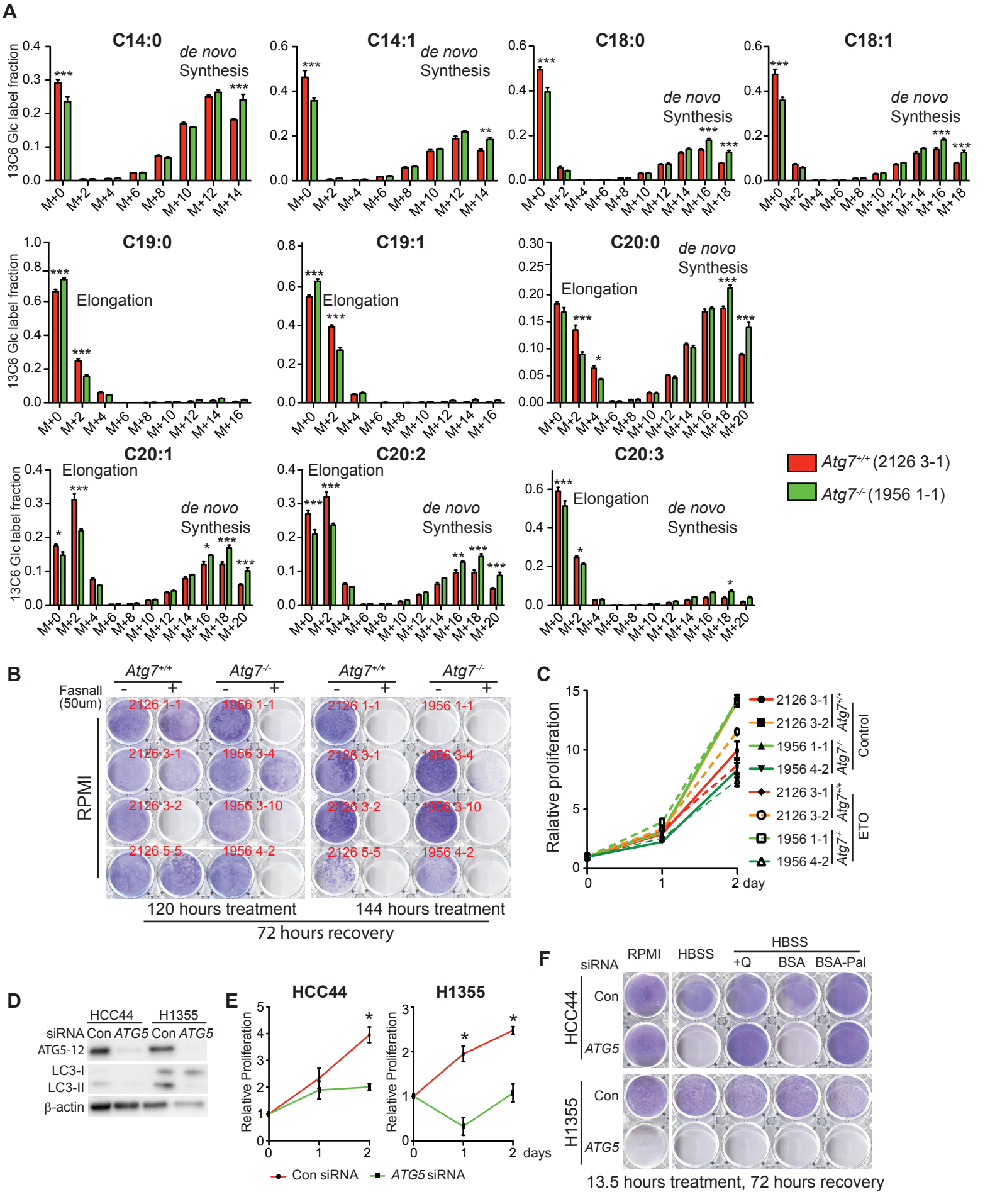



A
Control
Kras G12D/+;Lkb1 -/-
2126 (Atg7 +/+)
1956 (Atg7 -/-)
Atg7 +/+
Atg7 -/-
Atg7
Atg5-Atg12
LC3 I II
β-actin
B
Ladder
Atg7
Kras
Lkb1
Flox
WT
Mutant
C
2126 (Atg7 +/+)
1956 (Atg7 flox/flox)
Adeno-Cre
Atg7
β-actin
D
2126 3-1
2126 3-2
1956 5-12
1956 3-3
1956 3-7
1956 3-1
Relative proliferation
No Cre
Cre
Atg7 flox/flox
Atg7 del/del
days
E
Adeno-Cre
HBSS
Glutamine
BSA
BSA-Pal
18 hours treatment, 72 hours recovery
F
siRNA Con Atg5
Atg5-12
LC3 I II
β-actin
G
Relative Proliferation
Con siRNA
Atg5 siRNA pool
H
RPMI
HBSS
Q
BSA
BSA-Pal
siRNA
Con
Atg5
18 hours treatment, 72 hours recovery


A
Serine
Methionine
Asparagine
Lysine
Tryptophan
Histidine
Tyrosine
Threonine
Valine
Phenylalanine
Ion Count
RPMI
HBSS
2126 1-1
2126 3-1
2126 3-2
Atg7+/+
1956 1-1
1956 3-4
1956 3-10
Atg7 -/-


B
Serine
Methionine
Threonine
Relative level
hr
0
12
24
Atg7+/+ TDCL
Atg7-/- TDCL


A
Adenosine
Inosine
Guanosine
Uridine
Cytidine
Ribose
Ribose
Ribose
Ribose
Hypoxanthine
Guanine
Xanthine
Uracil
Cytosine
B
Inosine
Hypoxanthine
Xanthine
Uracil
Ion Count
15000
10000
5000
0
40000
30000
20000
10000
80000
60000
40000
20000
200000
150000
100000
50000
*
***
*
***
RPMI
HBSS
2126 1-1
2126 3-1
2126 3-2
1956 1-1
1956 3-4
1956 3-10
Atg7+/+
Atg7 -/-
C
Atg7+/+
Atg7-/-
2126 1-1
2126 3-1
2126 3-2
1956 1-1
1956 3-4
1956 3-10
HBSS
HBSS
Adenosine
Guanosine
Uridine
18 hours treatment, 72 hours recovery


A
C14:0
C14:1
C18:0
C18:1
C19:0
C19:1
C20:0
C20:1
C20:2
C20:3
13C6 Glc label fraction
de novo Synthesis
Elongation
Atg7+/+ (2126 3-1)
Atg7-/- (1956 1-1)
B
Fasnall (50um)
RPMI
2126 1-1
1956 1-1
2126 3-1
1956 3-4
2126 3-2
1956 3-10
2126 5-5
1956 4-2
120 hours treatment
144 hours treatment
72 hours recovery
C
Ralative proliferation
2 day
Control
ETO
D
HCC44
H1355
siRNA Con ATG5 Con ATG5
ATG5-12
LC3-I
LC3-II
β-actin
E
HCC44
H1355
Relative Proliferation
days
Con siRNA
ATG5 siRNA
F
siRNA
RPMI
HBSS
HBSS
+Q
BSA
BSA-Pal
Con
ATG5
13.5 hours treatment, 72 hours recovery