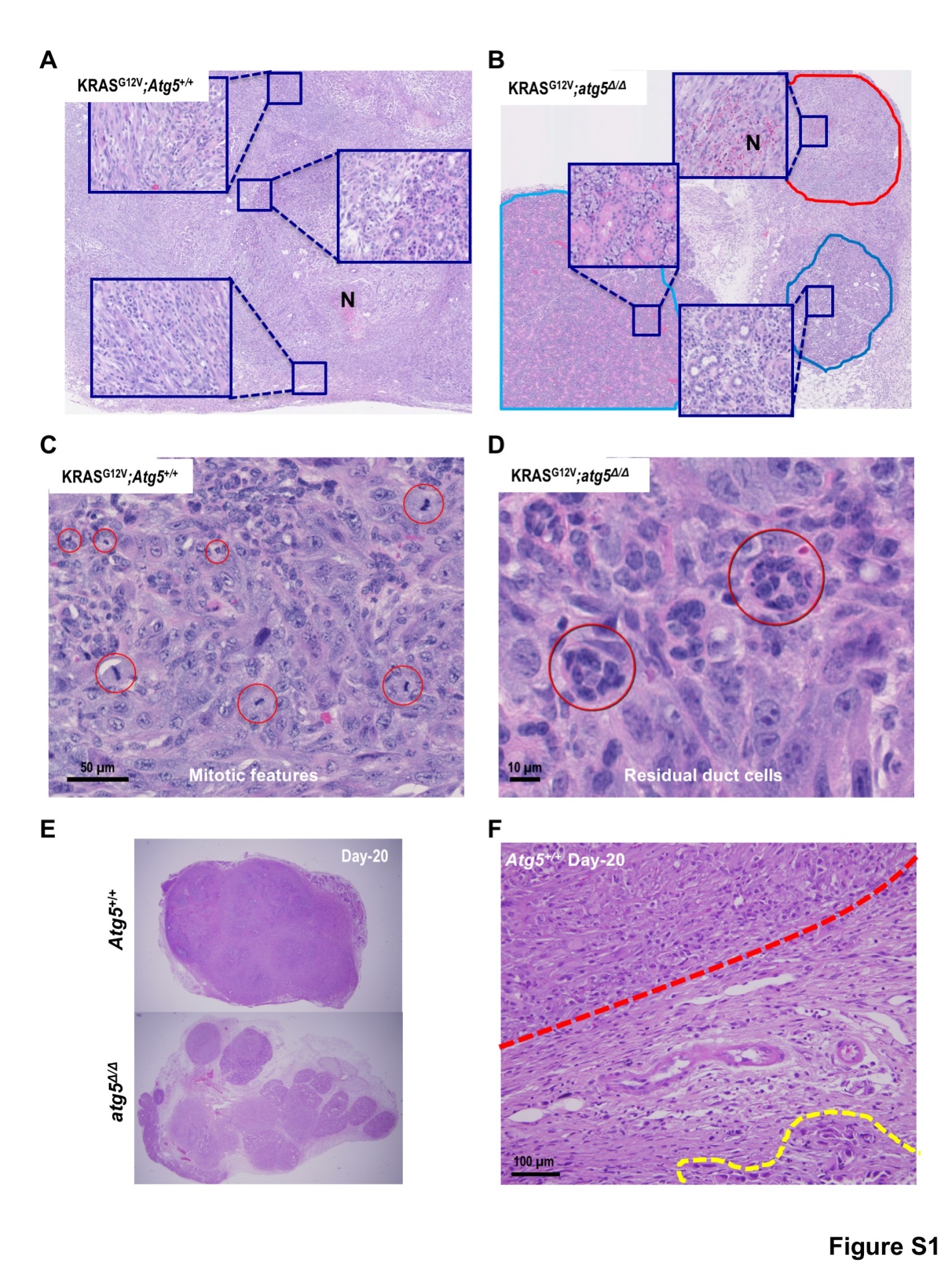
**Autophagic Reliance Promotes Metabolic Reprogramming in Oncogenic KRAS-driven Tumorigenesis**

H. Helen Lin, Yiyin Chung, Chun-Ting Cheng, Ching Ouyang, Yong Fu, Ching-Ying Kuo,

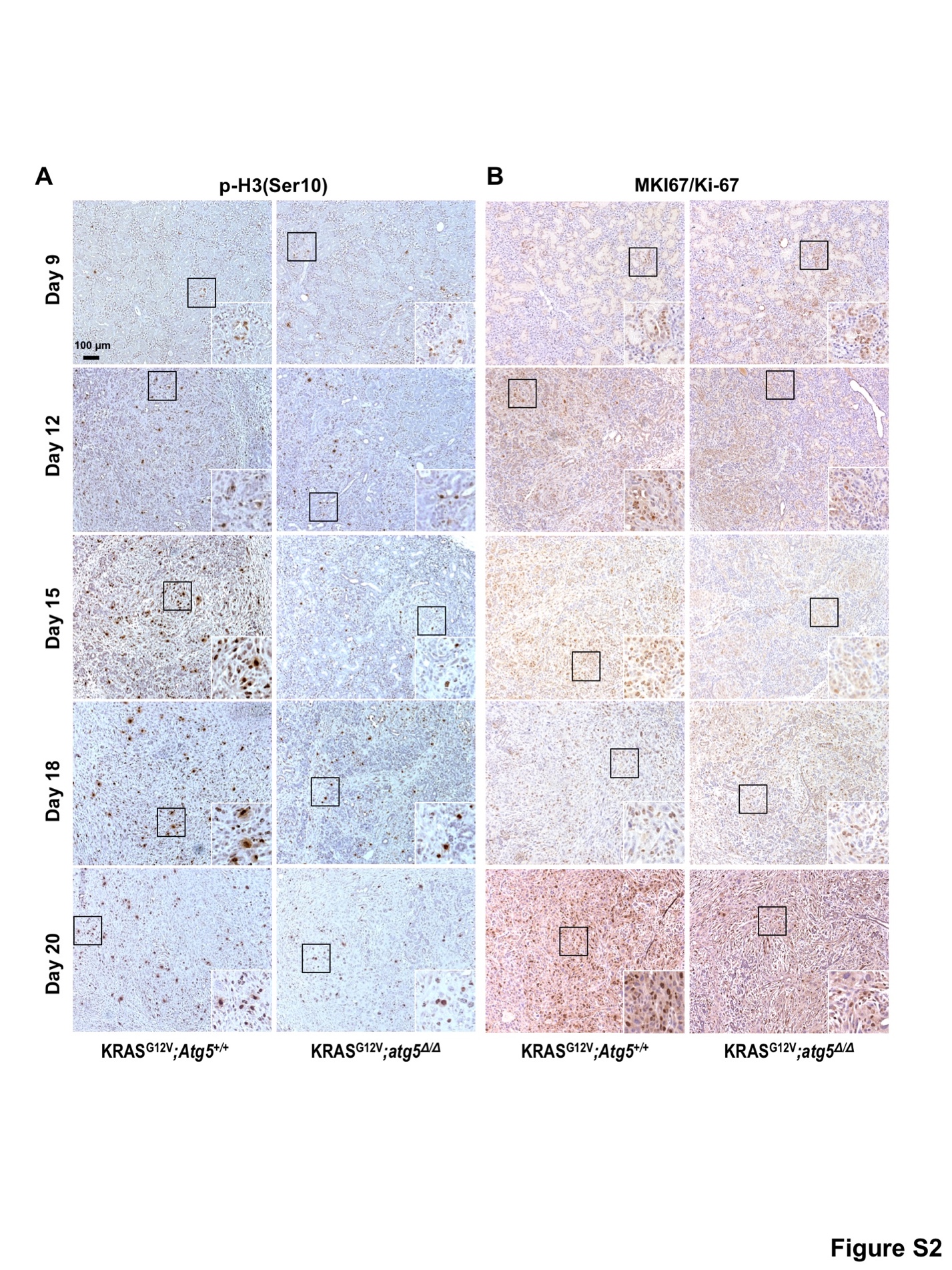
Kevin K. Chi, Maryam Sadeghi, Peiguo Chu, Hsing-Jien Kung, Chien-Feng Li,

Kirsten H. Limesand and David K. Ann

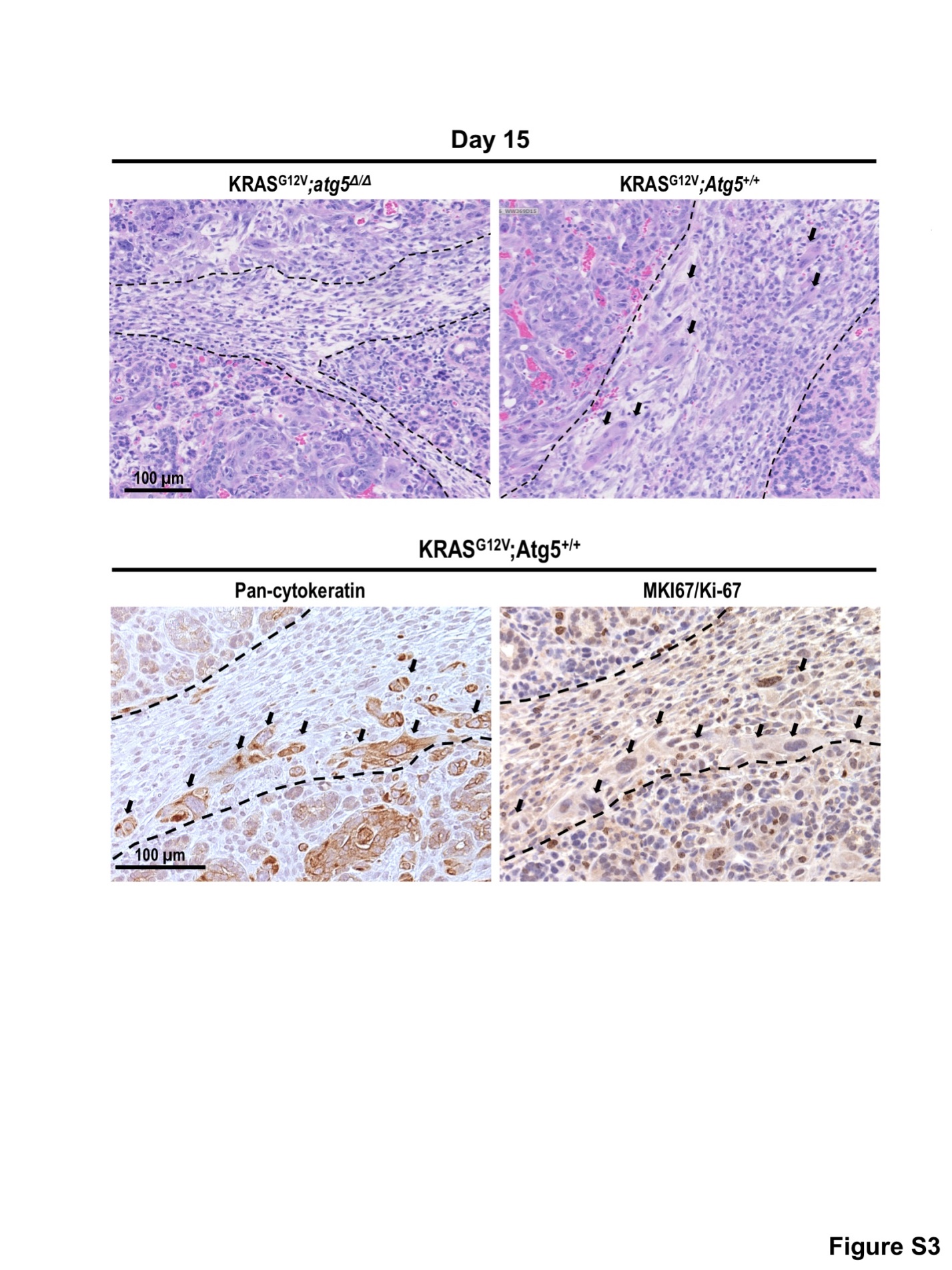
**Supplementary Information**

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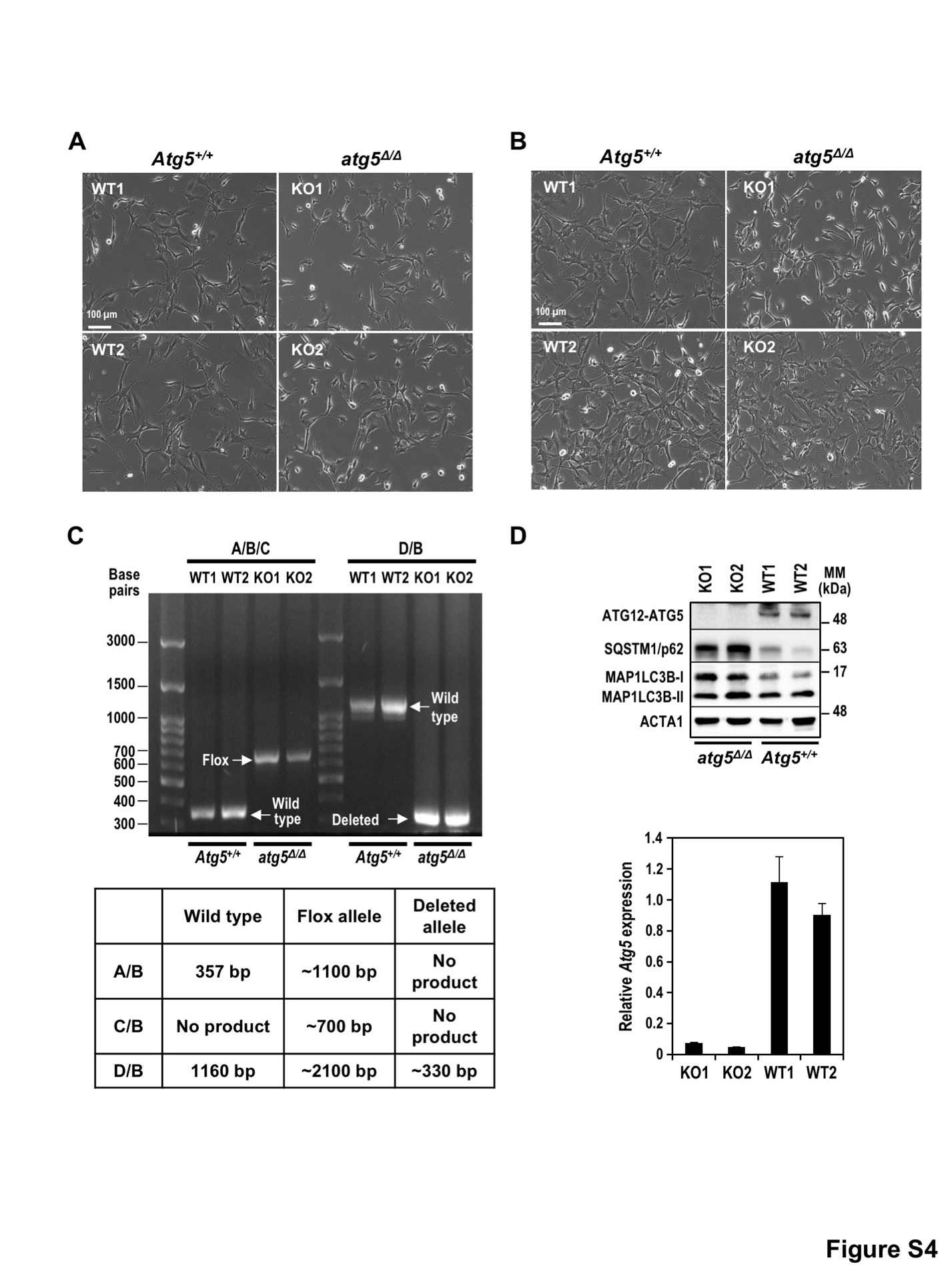
**Figure S1.** Compromised autophagy reduces the tumor burden in the Ela-CreERT;LGL-KRASG12V model. (**A** and **B**)Representative histological analyses (hematoxylin and eosin staining) of submandibular glands of KRASG12V*;Atg5+/+* (**A**)and KRASG12V*;atg5Δ/Δ* (**B**)mice at day 24 post-tumor induction. Tumors in *Atg5+/+* mice (**A**) appear more aggressive than in *atg5Δ/Δ* mice (**B**). N, necrotic area. (**C**)Mitotic figures (*red circles*) and apoptotic changes (cell shrinking, chromatin condensation, and nuclear fragmentation) were seen in tumor cells from KRASG12V*;Atg5+/+* mice. Scale bar: 50 μm. (**D**)Residual ducts (*red circles*) are visible in the tumor areasin KRASG12V*;atg5Δ/Δ* mice. Scale Bar: 10 μm (**E**)Representative tiling images from histological analyses (hematoxylin and eosin staining) of submandibular glands of KRASG12V*;Atg5+/+* (*upper panel*)and KRASG12V*;atg5Δ/Δ*(*lower panel*)mice at day 20 post-tumor induction showing that the tumor diffusely invaded into the peri-salivary gland fat in *Atg5+/+* mice (fullness of tumor) than in *atg5Δ/Δ*mice (presence of fibro-adipose tissue between tumor nodules). (**F**)Extra-salivary gland invasion in KRASG12V*;Atg5+/+* mice at day 20. Note the infiltrating edge of the tumor (*red dotted line*) and scattered nests of tumor cells (*yellow dotted line*). Scale bar: 100 μm.

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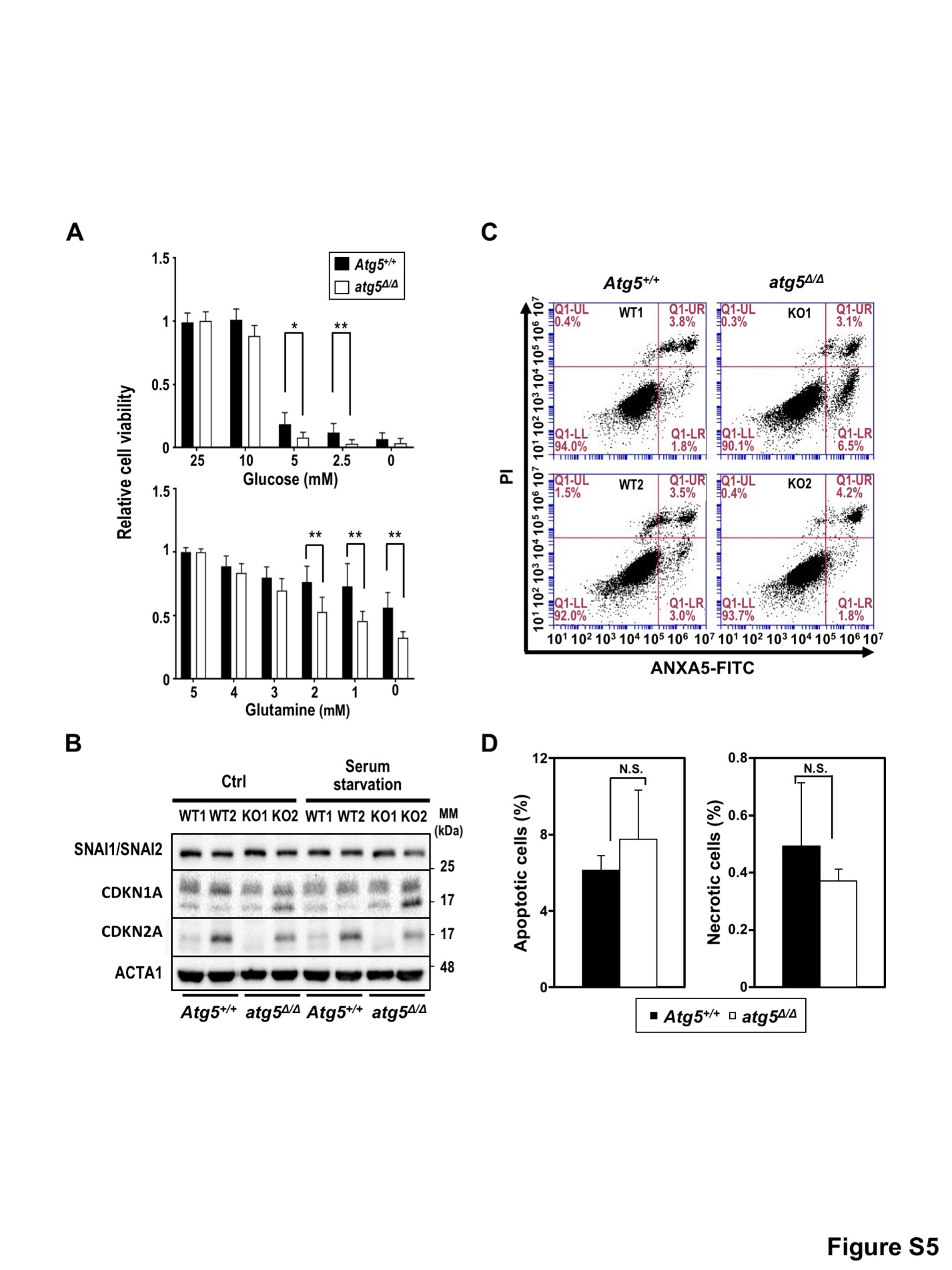
**Figure S2.** SDC tumors of KRASG12V*;Atg5+/+* mice grow more aggressively starting at day 15 post-tumor induction. Representative IHC of phosphorylated serine 10 of histone H3 (p-H3[Ser10])-stained (**A**) and MKI67/Ki-67-stained (**B**) SDC tumors from KRASG12V*;Atg5+/+*and KRASG12V*;atg5Δ/Δ* mice at day 9, 12, 15, 18 and 20 after tumor induction. Quantification of number of p-H3(Ser10)-positive and MKI67/Ki-67-positive cells was shown on **Figs. 2F** and **2H**, respectively. Three random images were taken from each mouse tumor sample. At least 4 mice were included in each group. Scale bar: 100 μm.

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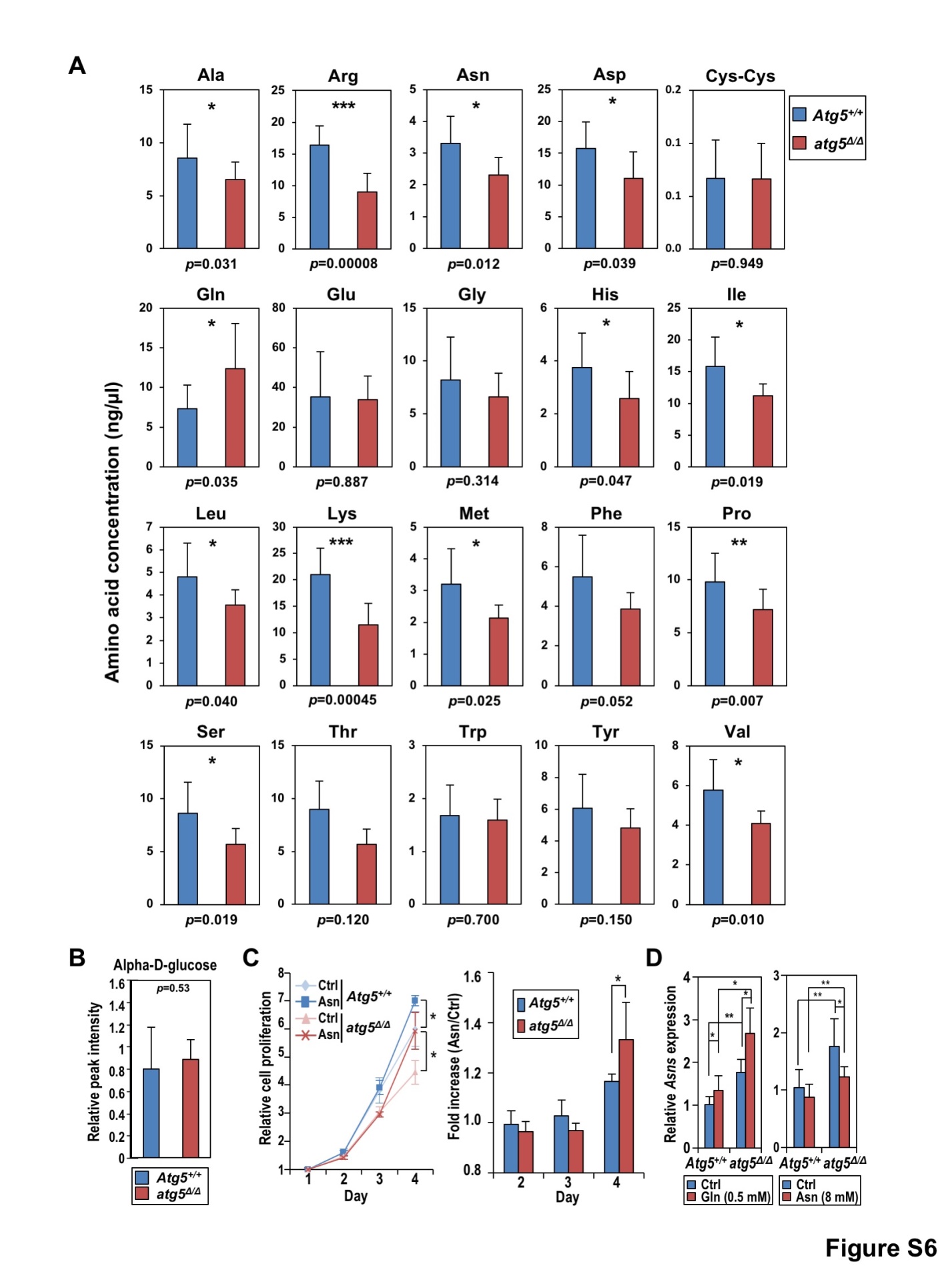
**Figure S3.** Stromal invasion.Representative histological analyses (hematoxylin and eosin staining) of the submandibular glands from KRASG12V*;atg5Δ/Δ* (*upper left panel*) and KRASG12V*;Atg5+/+* (*upper right panel*)mice at day 15 post-tumor induction. Tumor cells invading into peri-lobular stroma were observed in KRASG12V*;Atg5+/+* mice (black arrows). Representative IHC of pan-cytokeratin (*lower left panel*) highlights tumor cells and MKI67/Ki-67 (*lower right panel*), shows that invading tumor cells are not proliferating, using consecutive sections. Black arrow, invading tumor cell; black dotted line, margin of tumor regions. Scale bar: 100 μm.

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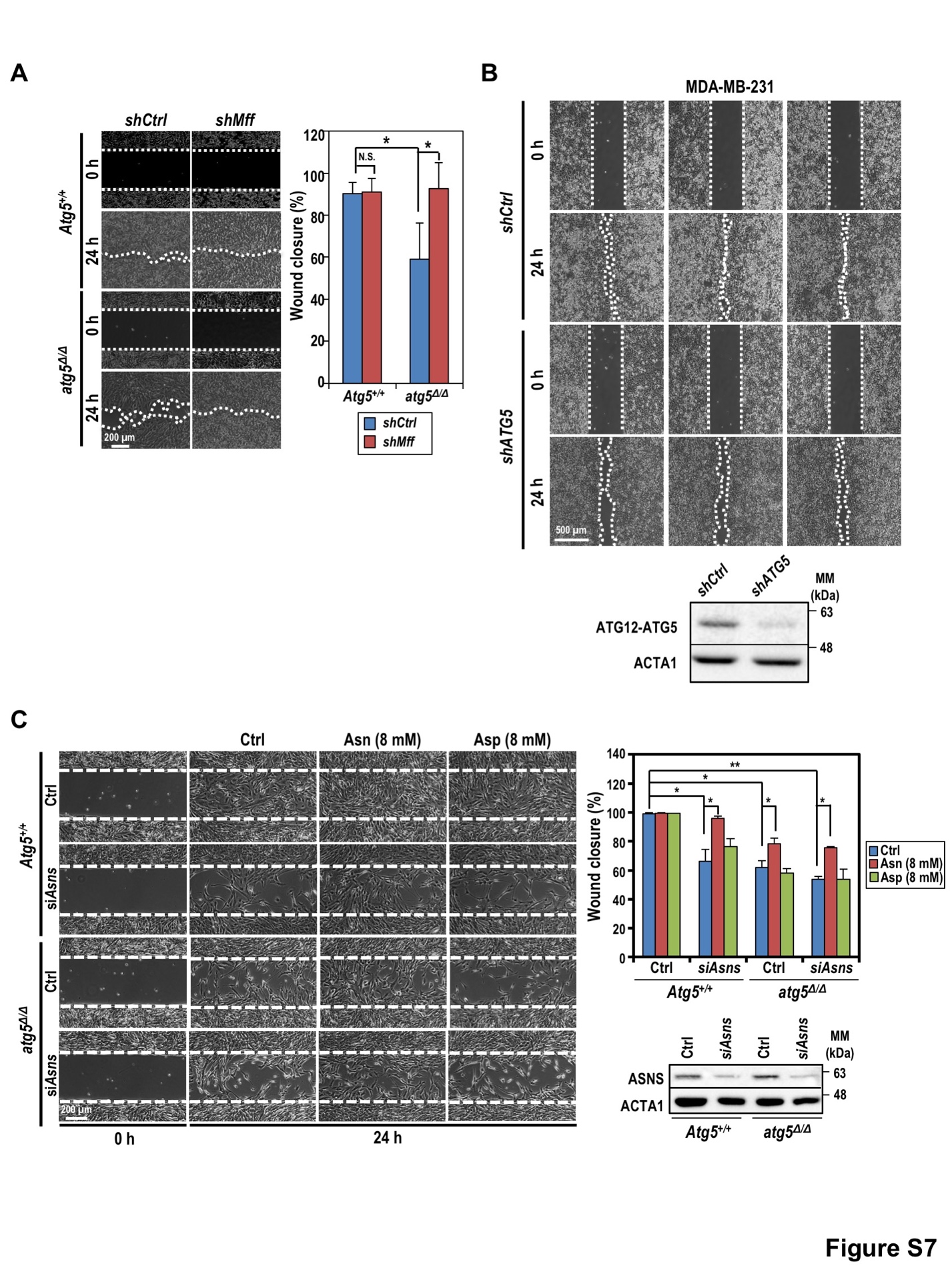
**Figure S4.** Characterization of KRASG12V*;Atg5+/+* and KRASG12V*;atg5Δ/Δ* SDC tumor cells. (**A** and **B**) Live-cell images of SDC cells isolated from 2 mice in each genotype group at low density **(A)** and high density (**B**). All 4 cells demonstrated similar sarcomatoid morphology. (**C**) Genotyping analyses confirmed the disruption of the *Atg5* gene in isolated KRASG12V*;atg5∆/∆* tumor cells. Knockout of *Atg5* in KRASG12V*;Atg5+/+* and KRASG12V*;atg5Δ/Δ* cells is validated by PCR. Primer sets A, B, C, and D were designed for genotyping *Atg5*. Genomic DNA isolated from 2 KRASG12V*;Atg5+/+* and 2 KRASG12V*;atg5Δ/Δ* cells were used as PCR templates. Specific PCR product sizes for individual *Atg5* genotypes are summarized in the table. WT:KRASG12V*;Atg5+/+* cells; KO: KRASG12V*;atg5Δ/Δ* cells. (**D**)Western and qRT-PCR analyses confirming *Atg5* genetic disruption in KRASG12V*;atg5Δ/Δ* tumor cells. Equal amounts of whole cell lysates from isolated KRASG12V*;Atg5+/+* and KRASG12V*;atg5Δ/Δ* cells were subjected to western blot analysis, and a representative image is shown (n = 3) (*upper panel*). Undetectable ATG12–ATG5, accumulation of SQSTM1 and abundant LC3B-I with a lower LC3B-II to LC3B-I ratio, a hallmark of compromised autophagy, were observed in KRASG12V*;atg5Δ/Δ* tumor cells. ACTA1 is shown as a loading control. Relative *Atg5* mRNA abundance was calculated by designating the average ΔCt of WT1 and WT2 as 1 after normalizing to *Rn18s* rRNA (*lower panel*).

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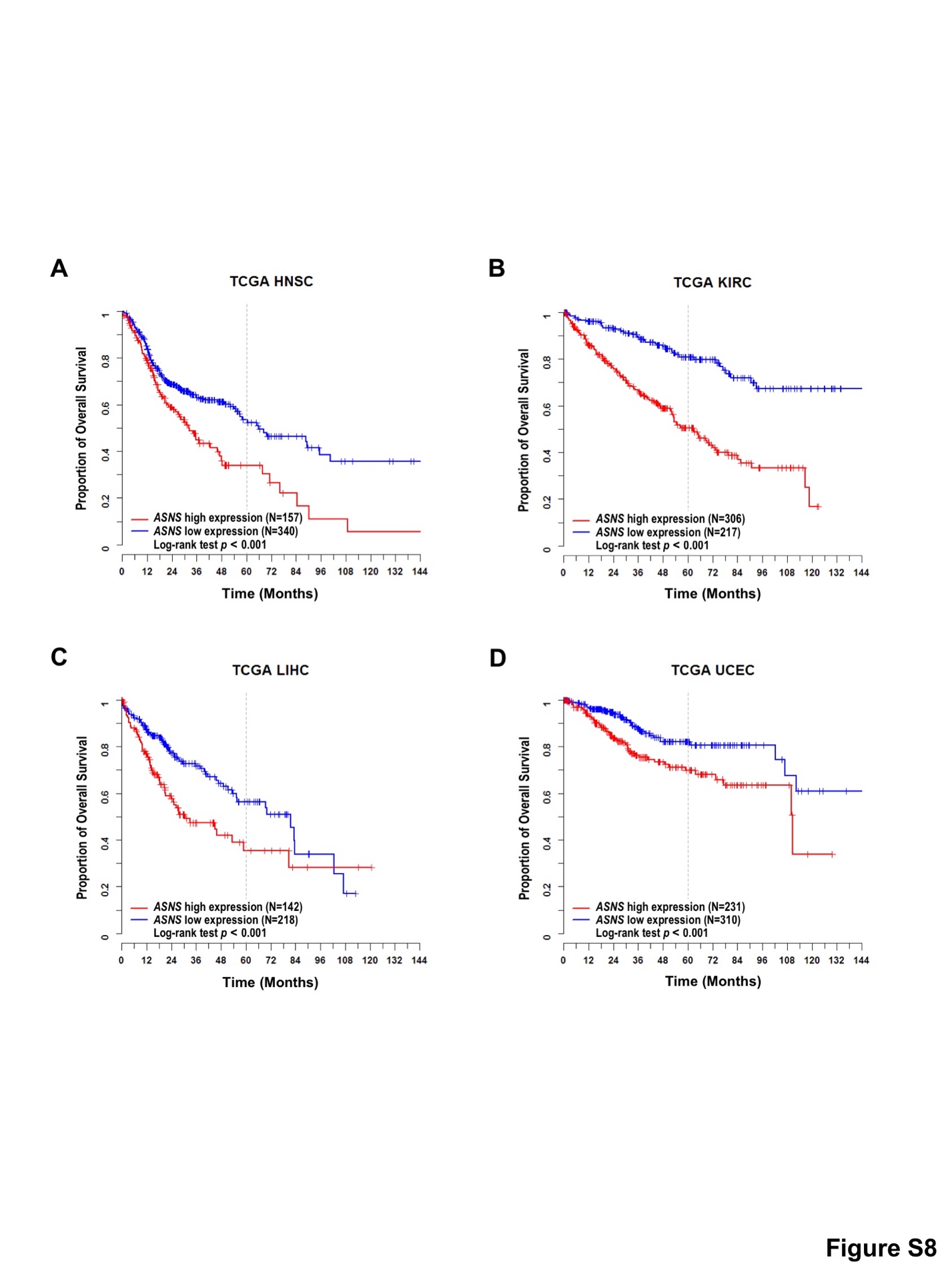
**Figure S5.** Compromised autophagy affects the response to metabolic stress, but not the senescence markers and the apoptotic cell population between autophagy-competent and -deficient SDC-tumor cells. (**A**) KRASG12V*;atg5Δ/Δ* cells are more sensitive than KRASG12V*;Atg5+/+* cells to the deprivation of glucose (*upper panel*) and glutamine (*lower panel*). Cells were incubated in the indicated concentrations of glucose and glutamine for 48 h, prior to acid phosphatase (ACP) cell viability assays.(**B**)Equal amounts of whole cell lysates from 2 KRASG12V*;Atg5+/+* and 2 KRASG12V*;atg5Δ/Δ* tumor cells were subjected to western blot analysis. The protein levels of senescence markers CDKN1A and CDKN2A varied among individual cells, but no overall differences between autophagy-competent and -compromised cells were observed. EMT was also examined by probing with an antibody recognizing both SNAI1 and SNAI2. The protein level of SNAI1 and SNAI2 was not affected by autophagy competency. ACTA1 is shown as a loading control.(**C** and **D**) KRASG12V*;Atg5+/+* and KRASG12V*;atg5Δ/Δ* cells were harvested and stained with ANXA5-FITC and PI for 20 min at room temperature and then subjected to flow cytometry analysis. (**C**)Representative flow cytometry data showing both ANXA5 binding and PI uptake of the cells. The percentages of cells that were ANXA5-positive, PI-positive, or double positive for both ANXA5 and PI were as indicated. (**D**)Percent of ANXA5-positive cells (*left panel*) and PI-positive (ANXA5-negative) cells (*right panel*) were not different based on autophagy competency. N.S.: not significant.

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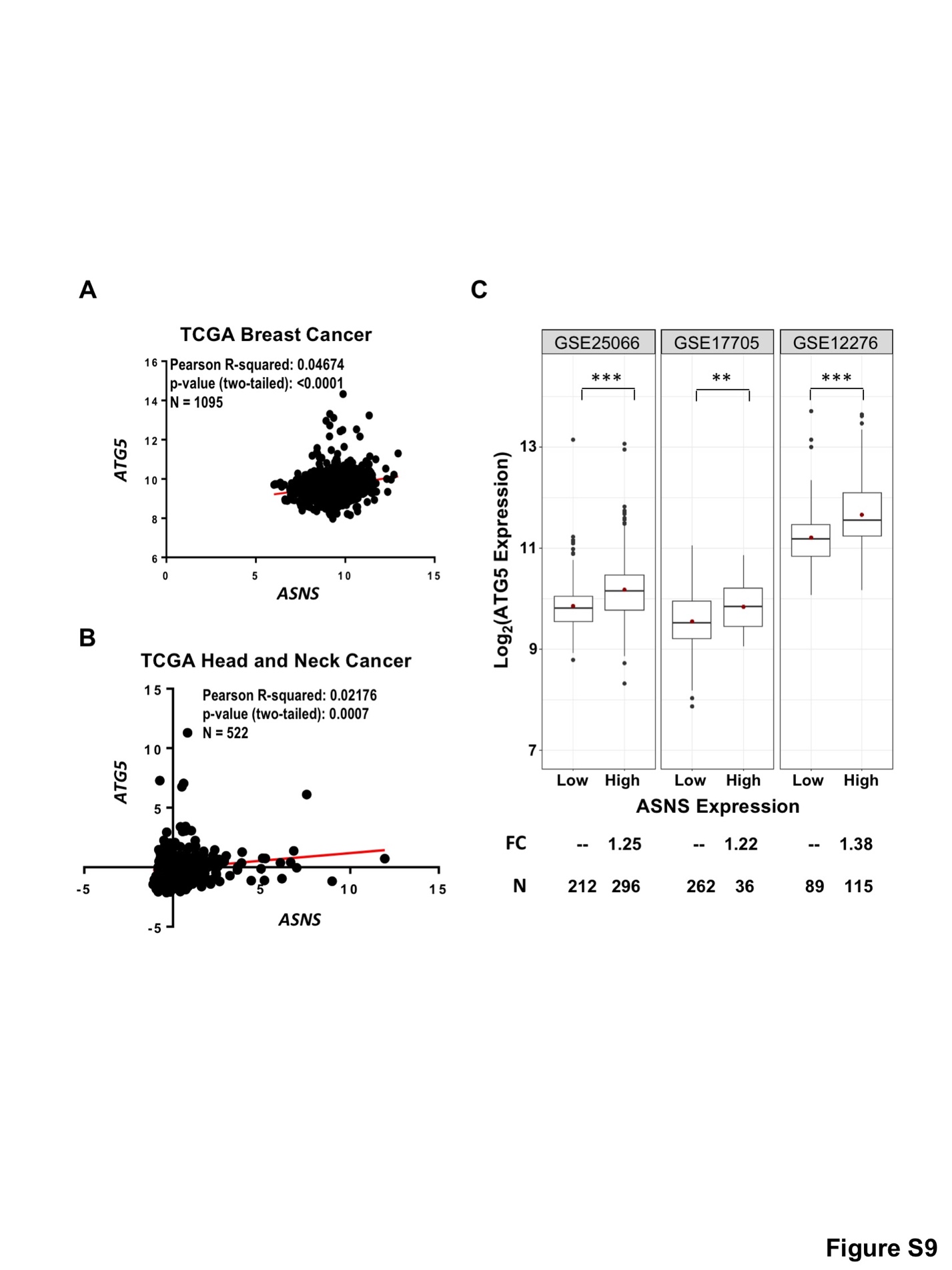
**Figure S6.** Twelve of 20 intracellular amino acid levels are reduced in autophagy-deficient SDC-tumor cells. (**A**) Amino acid profiles were determined by HPLC/MS as described in Methods. Data are shown as mean ± SD; n = 5. \*: *p* < 0.05; \*\*: *p* < 0.01; \*\*\*: *p* < 0.001 (Student’s *t*-test, two-tailed, unpaired). (**B**) Intracellular glucose level was not affected by autophagy capacity. Intracellular α-D-glucose level was determined by LC-MS followed by searching for matches in METLIN database (<https://metlin.scripps.edu/>). Data is shown as mean ± SD; n = 7. (**C**) Asparagine supplementation promotes cell proliferation. Cells were seeded at 20% confluency in full media with or without asparagine (Asn, 8 mM). Cell viability was measured daily by ACP assays for 4 consecutive days. Growth curve (*left panel*) and fold increase in cell proliferation (Asn over control; *right panel*) are shown. Data are shown as mean ± S.D.; n = 3. \*: *p* < 0.05 (ANOVA with Bonferroni multiple comparisons test). (**D**) Nutrient-regulated endogenous *Asns* (asparagine synthetase) expression. Total RNAs were isolated from cells of both genotypes cultured in glutamine-depleted medium (Gln, 0.5 mM), control medium (Gln, 5 mM), or asparagine supplemented control medium (Gln, 5 mM; Asn, 8 mM) for 24 h. *Asns* mRNA abundance was measured by qRT-PCR and normalized to *Rn18s* RNA. Data were analyzed using 2ΔΔCt method and the *Asns* mRNA level in KRASG12V*;Atg5+/+* cells grown in control medium is designated as 1. Data are shown as mean ± S.D.; n = 3. \*: *p* < 0.05; \*\*: *p* < 0.01 (Student’s *t*-test, two-tailed, unpaired).

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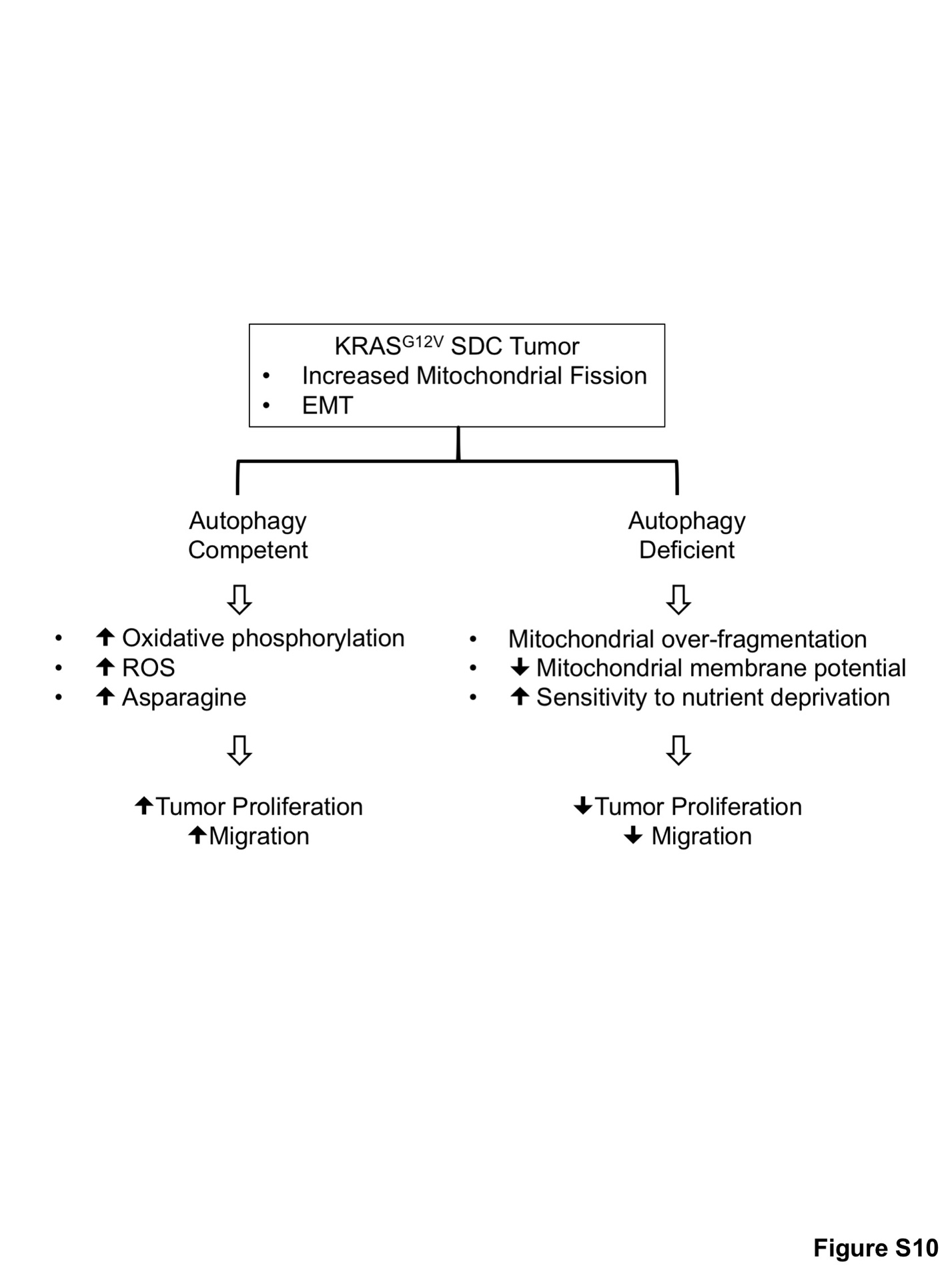
**Figure S7.** Knockdown of MFF, ATG5, or ASNS differentially affected the motility in oncogenic KRAS-driven tumor cells. (**A**) Wound healing assays show that knockdown of MFF restored cell migration in KRASG12V*;atg5Δ/Δ* cells. A set of representative images and quantification of wound closure are shown.\*: *p* < 0.05; N.S.: not significant (Student’s *t*-test, two-tailed, unpaired).(**B**)Wound healing assay of control MDA-MB-231 (*shCtrl*) and *ATG5*-knockdown MDA-MB-231 (*shATG5*) cells. Images were taken immediate after the wounds were created (0 h) and at 24 h post-wounding. Images from 3 independent experiments are shown. Knockdown of ATG5is confirmed by western blot analysis with ACTA1 as a loading control (*bottom panel*). (**C**) Knockdown of ASNS reduced KRASG12V*;Atg5+/+* cell motility while that of KRASG12V*;atg5Δ/Δ* cells was not affected. The effect of ASNS-KD can be reversed by supplementation of Asn (8 mM) but not Asp (8 mM). Cells were transfected with siRNA against mouse *Asns* (*siAsns)* orcontrol siRNA (ctrl) and incubated for 48 h before wound healing assay. Representative images were shown (*left panel*). Quantification of percent of wound closure is shown (*upper right panel*). Data was shown as mean ± S.D.; n=3. Knockdown of ASNSwas confirmed by western blot analysis (*lower right panel*). ACTA1 serves as a loading control. \*: *p* < 0.05; \*\*: *p* < 0.01 (Student’s *t*-test, two-tailed, un-paired).

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**Figure S8.** Kaplan–Meier survival analyses based on *ASNS* expression across multiple TCGA cancer types.Survival curves were analyzed for the following human cancers; (**A**)head and neck squamous cell carcinoma (HNSC), (**B**)kidney renal clear cell carcinoma (KIRC), (**C**)liver hepatocellular carcinoma (LIHC), and (**D**)uterine corpus endometrial carcinoma (UCEC).

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**Figure S9.** A positive correlation between expression levels of *ATG5* and *ASNS* in human cancer. Scatter plots show positive correlation between *ATG5* and *ASNS* gene expression in (**A**) Breast Cancer (*p* < 0.0001) and (**B**) Head and Neck Cancer (*p* < 0.001) inTCGA datasets. Pearson correlation and *p*-value were calculated using GraphPad Prism 7. (**C**) *ATG5* expression levels were compared between the high and low *ASNS* expression groups that exhibited differential distal metastasis-free survival, as shown in **Figs. 7E, 7F** and **7G**. Fold change (FC) of normalized expression values and the number (N) of samples are labeled at the bottom.  Statistical *p*-values between groups were determined using Welch’s *t*-test (\*\*: *p* <0.01; \*\*\*: *p* <0.001).

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**Figure S10.** Summary of key characteristics of autophagy-competent and autophagy-deficient oncogenic KRAS-induced salivary duct carcinoma.The diagram summarizes our findings on differential mitochondrial biogenesis, nutrient dependency, and epithelial-to-mesenchymal transition (EMT) progression based on autophagy capacity. These factors converge to render reduced tumor proliferation and migration during autophagy-impaired tumorigenesis and extended survival of the respective tumor-bearing mice.