

1 **The HMGB1-RAGE axis modulates the growth of autophagy-deficient hepatic tumors**

2 Bilon Khambu^{1*}, Honghai Hong^{1,2}, Sheng Liu^{3,4}, Gang Liu¹, Xiaoyun Chen¹, Zheng Dong⁵, Jun
3 Wan^{3,4,6}, Xiao-Ming Yin¹

4
5 Department of ¹Pathology & Laboratory Medicine, Indiana University School of Medicine,
6 Indianapolis, Indiana, USA; ²The third affiliated hospital of Guangzhou Medical University,
7 Guangzhou, Guangdong, China; ³Department of Medical and Molecular Genetics, ⁴Center for
8 Computational Biology and Bioinformatics, Indiana University School of Medicine,
9 Indianapolis, Indiana, USA; ⁴Department of Cellular Biology and Anatomy, Medical College of
10 Georgia at Augusta University and Charlie Norwood VA Medical Center, Augusta, GA, USA;
11 ⁶School of Informatics and Computing, Indiana University – Purdue University at Indianapolis,
12 Indianapolis, IN 46202

13
14 *. Correspondence to:

15 Bilon Khambu, PhD

16 Department of Pathology and Laboratory Medicine

17 Indiana University School of Medicine, Indianapolis, IN 46202

18 Phone: 317-274-1789, Fax: 317-491-6639

19 Email: bikhambu@iupui.edu

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21 **Running title:** Role of HMGB1 in tumor development in the absence of autophagy.

22

23 **Abstract**

24 Autophagy is an intracellular lysosomal degradative pathway important for tumor
25 surveillance. Autophagy deficiency can lead to tumorigenesis. Autophagy is also known to be
26 important for the aggressive growth of tumors, yet the mechanism that sustains the growth of
27 autophagy-deficient tumors is not known. We previously reported that progression of hepatic
28 tumors developed in autophagy-deficient livers required high mobility group box 1 (HMGB1)
29 that is released from autophagy-deficient hepatocytes. However, the mechanism by which
30 HMGB1 promotes hepatic tumorigenesis is not understood. In this study we examined the
31 pathological features of the hepatic tumors and the mechanism of HMGB1-mediated
32 tumorigenesis using liver-specific autophagy-deficient (*Atg7*^{-/-}) and *Atg7*^{-/-}/*Hmgb1*^{-/-} mice. We
33 found that in *Atg7*^{-/-} mice the tumors cells were still deficient in autophagy and could also
34 release HMGB1. Histological analysis using cell-specific markers suggested that fibroblast and
35 ductular cells were present only outside the tumor whereas macrophages were present both
36 inside and outside the tumor. Genetic deletion of HMGB1 or one of its receptors, receptor for
37 advanced glycated end product (*Rage*), retarded liver tumor development. In addition, we found
38 that expression of RAGE was only on ductular cells and Kupffer's cells but not on hepatocytes,
39 which suggested that HMGB1 might promote hepatic tumor growth through a paracrine mode
40 that altered the tumor microenvironment. Furthermore, HMGB1 and RAGE enhanced the
41 proliferation capability of the autophagy-deficient hepatocytes and tumors. Finally, RNAseq
42 analysis of the tumors indicated that HMGB1 induced a much broad changes in tumors. In
43 particular, genes related to mitochondrial structures or functions were enriched among those
44 differentially expressed in tumors in the presence or absence of HMGB1, revealing a potential

45 key role of mitochondria in sustaining the growth of autophagy-deficient liver tumors via
46 HMGB1 stimulation.

47

48 **Introduction**

49 Autophagy is an important mechanism regulating tumorigenesis. Its dysfunction due to
50 external stress or genetic inactivation may lead to tumorigenesis. Indeed, liver-specific deletion
51 of *Atg5* or *Atg7* causes spontaneous development of liver tumors¹⁻⁴. Similarly, reduced
52 autophagy from constant activation of mammalian target of rapamycin complex 1 (mTORC1)
53 also promotes hepatic neoplastic transformation. For example, deletion of phosphatase and tensin
54 homolog (PTEN) or tuberous sclerosis complex 1 (TSC1) leads to constitutive activation of
55 mTORC1, and a decrease in autophagic activity, causing spontaneous HCC^{5,6}. These studies
56 suggest that hepatocytes require the tumor-suppressive function of autophagy for maintaining its
57 homeostasis.

58 Excessive reactive oxygen species (ROS) generated due to autophagy-deficiency is
59 implicated in tumor development^{7,8}. Consequently, pharmacological inhibition of ROS
60 formation by the antioxidant N-acetylcysteine results in a strong suppression of tumor
61 development in *Atg5*-deficient liver⁸. Moreover, there is a persistent activation of an anti-
62 oxidative stress-related transcription factor NFE2L2/ NRF2(nuclear factor, erythroid 2 like 2) to
63 limit the oxidative injury⁹. Paradoxically, codeletion of *Nrf2* gene also prevents tumorigenesis in
64 the autophagy-deficient liver^{1,3}.

65 On the other hand, autophagy could also regulates hepatic tumorigenesis by modulating
66 the release of a damage-associated molecular pattern (DAMP) molecule, high mobility group

67 box 1 (HMGB1). Our previous work discovered that defective autophagy leads to the release of
68 HMGB1, which promotes hepatic tumorigenesis². HMGB1 is an extensively studied non-
69 histone nuclear protein that facilitates binding of regulatory proteins to DNA and typically
70 enhances transcriptional activation¹⁰. It is known that nuclear HMGB1 can be released into the
71 extracellular environment and acts as an immune mediator in sterile inflammation. However,
72 codeletion of *Hmgb1* in the autophagy-deficient liver results in delayed tumor development via a
73 mechanism independent of its usual role in injury, inflammation, and fibrosis². It is thus unclear
74 how HMGB1 mechanistically promotes hepatic tumorigenesis in autophagy-deficient liver.

75 In the present study, we have characterized the cellular and molecular context of the
76 hepatic tumors driven by autophagy deficiency. We observed that the tumors were originated
77 mainly from the autophagy-deficient hepatocytes that had already released HMGB1.
78 Furthermore, we showed that HMGB1 and its dominant receptor RAGE positively affect the
79 proliferation of tumor cells. RNA sequencing analysis identified expressional differences in
80 multiple genes and signaling pathways in tumors derived from *Atg7*^{-/-} liver and in tumors
81 derived from *Atg7*^{-/-}/*Hmgb1*^{-/-} livers. Our data, therefore, identify a key role of HMGB1 in
82 autophagy-deficient tumor growth. HMGB1 could thus be a potential therapeutic target.

83

84 **Results**

85 **1. Hepatic tumor cells in autophagy-deficient livers have features consistent with** 86 **autophagy deficiency.**

87 Autophagy possesses both antitumorigenic and protumorigenic role, depending on
88 whether it occurs before or after the onset of tumorigenesis. Autophagy-deficient livers develop

89 tumors, confirming the surveillance role of autophagy in the liver. The tumor first appears at the
90 9-month of the age and the tumor size and the number gradually increase as the mice get older^{2,3}.
91 The tumors in *Atg5*- or *Atg7*- conditional knock out livers seem to be hepatic adenoma, which
92 does not progress to carcinoma or metastasis stage³. However, the molecular and cellular nature
93 of these tumors had not been fully characterized.

94 We thus examined hepatic tumor from autophagy-deficient mice(*Atg7*^{-/-}) mice. Lack of
95 the ATG7 expression was confirmed in tumor and non-tumor liver tissue from *Atg7*^{-/-} mice
96 when compared to age-matched *Atg7* *F/F* mice (**Figure 1A**). The level of the autophagy
97 substrate SQSTM1 was also higher in *Atg7*^{-/-} tumor as in the non-tumor tissue. Analysis of
98 LC3B, an autophagy-specific marker showed no formation of LC3B-II in the *Atg7*^{-/-} tumor as in
99 the non-tumor samples (**Figure 1A**). These results indicated that the tumor tissues were also
100 deficient in autophagy and thus they would have arisen from the autophagy-deficient
101 hepatocytes. We further confirmed this notion by examining the expression of SQSTM1 and
102 ubiquitin(UB) in the liver. Immunohistological and immunofluorescence analysis was performed
103 by taking images of eight different regions covering the non-tumor, peri-tumor, and the tumor
104 regions as shown in **Figure 1B**. A clear accumulation of SQSTM1 and UB in the tumor region of
105 the autophagy-deficient liver was observed, which was at the level similar to that in the non-
106 tumor tissues (**Figure 1C-D**), suggesting that the tumor tissues were defective in autophagy and
107 had defective protein quality control. In addition, the tumor tissues were all positive for the
108 hepatocyte-specific marker, HNF4 α , which were colocalized in the same cells that had elevated
109 SQSTM1 and UB staining (**Figure 1F**).

110 We next analyzed whether the accumulation of SQSTM1 in tumor tissue could activate
111 the anti-oxidative response-related NRF2 transcription factor as in non-tumor tissues^{1,9}. We

112 found that one NRF2 target protein, NQO1, were drastically elevated in the tumor tissues of the
113 *Atg7*^{-/-} mice (**Figure 1A**). The mRNA level of the NRF2 target genes, *Nqo1* and *Gstm1* were
114 also significantly elevated in the *Atg7*^{-/-} samples whether they were from non-tumor or tumor
115 tissue (**Figure 1G**). These observations indicated that hepatic tumors in autophagy-deficient
116 livers arise from the autophagy-deficient hepatocytes with alterned NRF2 and SQSTM1 levels.

117

118 **2. Hepatic progenitor cells are localized exclusively in the non-tumor region but not inside** 119 **the tumor.**

120 Hepatic progenitor cells(HPC), also known as oval cells or ductular cells, expand during
121 chronic liver injury in patients and in rodents^{11,12}. The expansion of HPCs is significant in the
122 autophagy-deficient livers². HPC has been noted to possess the capacity to become tumorigenic
123 in vivo when transduced with H-ras and SV40LT¹³. We thus explored the relationship of these
124 cells to the tumor in autophagy-deficient livers by examining their spatial interactions.

125 H-E staining showed that the distribution of HPCs was mostly around the tumor-adjacent
126 region (**Figure 2A**). In the area of tumor tissues, the normal tissue architecture, such as bile duct,
127 and portal tract formation, was completely lost. Moreover, the tumor region was composed of
128 irregular hepatic plates with tumor cells showing large nuclear-cytoplasmic ratio and
129 occasionally nuclear atypia (**Figure 2A**). Immunostaining for CK19, a common marker for
130 expanded HPC showed that the hepatic tumors were negative for CK19 (**Figure 2B**). Instead,
131 most of the CK19 positive cells appear to form a compact sheet surrounding the tumor (**Figure**
132 **2B**). Further analysis of the HPC distribution using Sox9 as another marker revealed a similar
133 pattern of distribution exclusively in the peritumor and non-tumor regions (**Figure 2C**). Some of

134 the HPCs were positive for SQSTM1 aggregates although many did not show elevated
135 SQSTM1(**Supplementary Figure S1A-B**). The possibility that some of this SQSTM1 positive
136 HPC may be derived from the autophagy-deficient hepatocytes cannot be excluded as such
137 transdifferentiation had been reported previously ^{11,14}.

138 Interestingly, HPC and liver cancer stem cells (CSC) also share several cellular markers
139 ¹⁵. Markers such as EpCAM, CD133, and CD24 have been used for isolating CSC with stem cell
140 features ^{15,16}. HPC in the context of chronic liver injury has also been considered as one possible
141 origin of liver CSC. We thus analyzed the expression of these CSC markers in the non-tumor and
142 tumor tissues of the autophagy-deficient liver. Real-time PCR analysis showed that the
143 expression of *Cd133*, *Cd200*, *Cd34*, *Cd44*, *Ly6a/Sca1*, and *Ly6d* were significantly upregulated
144 in *Atg7*^{-/-} liver tissues compared to control *Atg7* *F/F* mice (**Supplementary Figure S2A**). The
145 elevation of these CSC markers in the tumor tissues also suggested that tumors have a
146 precursor/stem-cell phenotype. Such induction was not observed with *Cd24a*, and *Cd90*,
147 suggesting the possible heterogeneity in the CSC in the tumor and non-tumor tissues of *Atg7*^{-/-}
148 mice (**Supplementary Figure S2A**). Interestingly, most of the stemness-related transcription
149 factors such as *Oct4*, *Nanog*, *Klf4* and *Sox2* were significantly downregulated in *Atg7*^{-/-} livers as
150 compared to *Atg7**F/F* livers (**Supplementary Figure S2B**). The lack of expression of Nanog has
151 been linked to the adenoma nature of the tumor ¹⁷. These changes were not more significant in
152 the tumor tissue than in the non-tumor tissues, and thus may not be the mechanisms
153 discriminating the two types of tissues.

154 HPCs that are mostly detected in peritumoral areas has been reported to express multiple
155 angiogenic paracrine factors such as vascular endothelial growth factor(VEGF), platelets-derived
156 growth factor(PDGF), and angiopoietin (ANGPT) in pediatric hepatoblastoma and HCC ¹⁸.

157 These HPCs could interact with pro-tumorigenic cells heterotypically via mitogenic factors. We
158 thus examined the expression of several angiogenic markers. Real-time PCR analysis indicated
159 that expression of angiogenic factor *Angpt2* and *Pdgfb* were significantly upregulated in liver
160 tumor and non-tumor tissues compared to wild-type *Atg7 F/F* mice (**Supplementary Figure S3**).
161 Such induction was not observed with *Vegfa* and *Angpt1* (**Supplementary Figure S3**),
162 suggesting the possible heterogeneity in the angiogenic factors acting within the peritumoral
163 niche of *Atg7*^{-/-} mice. Taken together, the distinct separation of the HPC and tumor cells in the
164 *Atg7*^{-/-} liver suggests that the HPC may not evolve into the tumor cells but could contribute to a
165 tumor microenvironment that affects the tumor development.

166

167 **3. Fibrosis is present in the peri-tumor region and encapsulates the tumor.**

168 Development of hepatic tumors are strongly associated with the status of liver fibrosis,
169 with 80-90% of HCCs developing in the fibrotic or cirrhotic livers¹⁹. On a cellular level,
170 fibrogenesis is most significantly mediated by the activation of hepatic stellate cells (HSCs) that
171 transdifferentiate from Vitamin A-storing pericyte-like cells to alpha-smooth muscle actin (α -
172 SMA)-positive, collagen-producing myofibroblasts in response to liver injury. Since liver
173 fibrosis is one of the earliest events that occur in the autophagy-deficient liver², we examined
174 how closely the tumor cells were associated with liver fibrosis.

175 Immunostaining analysis indicated that the number of desmin-expressing HSCs was
176 remarkably increased in *Atg7*^{-/-} mice (**Figure 2D**). Unlike the distribution of macrophages, the
177 desmin positive HSC were absent inside the tumor but were present in the non-tumor and peri-
178 tumor regions of the liver (**Figure 2D**). Consistent with the increased desmin positive HSCs in

179 peri-tumor and non-tumor region, increased fibrillar collagen deposition was detected by Sirius
180 Red and Trichome stain in the non-tumor and peri-tumor region (**Figure 2E-F**). Collagen
181 deposition was notably absent inside the tumor of the autophagy-deficient liver (**Figure 2E-F**).
182 Taken together, the peri-tumoral desmin positive HSCs may be responsible for the production of
183 the fibers that encapsulated and demarcated the tumor tissue. It is possible that fibrosis in the
184 autophagy-deficient liver may play an inhibitory role against tumor infiltration into normal
185 tissues, thus contributing to the more benign presentation of the tumorigenesis in this setting.

186

187 **4. Macrophages but not other immune cells can be found inside the tumor tissue.**

188 Hepatocellular neoplasia often occurs in the setting of chronic injury and inflammation,
189 which is present in autophagy-deficient livers^{2,20}. Persistent inflammation is also known to
190 promote and exacerbate malignancy. Among many different types of inflammatory cells, the
191 tumor-associated macrophage (TAM) is a key component involved in the initiation and
192 maintenance of tumor cells²¹. Factors secreted by the TAM are thought to contribute to the
193 initiation and promotion of tumors. Since the identification of inflammatory and immune cells
194 assists in characterizing the nature of the hepatic tumor and their potential contribution to the
195 tumor growth, we next examined the distribution of inflammatory and immune cells in the
196 tumor-bearing *Atg7*^{-/-} mice.

197 Immunohistological staining for the hepatic kupffer's cells (F4/80⁺) showed their
198 presence in both tumoral and non-tumoral regions (**Figure 3A**). In contrast, most of the
199 myeloperoxidase (MPO)-positive neutrophils, CD3-positive T cells, and CD45R-positive B cells
200 were absent from the tumoral region but present exclusively in the non-tumor region (**Figure 3B-**

201 **D).** Quantitative RT-PCR also found a strong upregulation of F4/80 and Ly6c expression in 12-
202 month old *Atg7*^{-/-} livers as compared to age-matched *Atg7*^{F/F} livers, and there was a further
203 elevation in tumor tissues (**Figure 3E**). The CD4 mRNA level was modestly elevated but the
204 CD8 mRNA level was significantly suppressed in the tumor-bearing *Atg7*^{-/-} liver (**Figure 3E**).

205 Macrophages can play important roles in regulating hepatocytes proliferation and
206 survival by secreting cytokines such as tumor necrosis factor- α (TNF α), IL-6, and IL-1 β and
207 growth factors such as VEGF, hepatocytes growth factors(HGF) and transforming growth
208 factors(TGFs). TNF α produced by infiltrating macrophages could activate TNF receptor
209 1(TNFR1)-NF-kB signaling in hepatocytes, resulting in enhanced tumor growth²². In contrast
210 to the presence of infiltrating F4/80 macrophages and elevated expression of F4/80 and Ly6c, the
211 mRNA expression of a set of inflammatory cytokines such as *TNF α* , *IL-6*, *IL-1 β* , and *IL-17* were
212 strongly downregulated in the 12-month old tumor-bearing *Atg7*^{-/-} liver (**Supplementary Figure**
213 **S4**). These data suggest that there is ongoing non-resolving inflammation in tumor and non-
214 tumor tissue of autophagy-deficient mice but their contribution to tumor growth has yet to be
215 fully determined.

216

217 **5. Autophagy deficient hepatic tumor cells release HMGB1.**

218 Autophagy-deficient livers manifest multiple pathological changes, including liver injury,
219 inflammation, fibrosis, ductular reaction and tumor development^{1,2,23}. Moreover, the autophagy-
220 deficient hepatocytes continuously release HMGB1, an intracellular nuclear DAMP protein, to
221 impact the expansion of HPC². Given that HMGB1 as a secretory factor could recruit other
222 hepatic cells such as inflammatory cells or fibrotic cells to the tumor, favoring the buildup of

223 permissive microenvironment^{24,25}, we sought to determine whether the tumor tissues also release
224 HMGB1 similar to the non-tumor autophagy-deficient liver tissue.

225 As anticipated, the immunoblot analyses found that less HMGB1 proteins are present in
226 tumor and non-tumor tissue of the *Atg7*^{-/-} liver, as compared to the *Atg7*^{F/F} liver (**Figure 4A**).
227 Co-immunofluorescence staining of HMGB1 and SQSTM1 also showed that the tumor cells
228 with the accumulated SQSTM1 were devoid of both nuclear and cytosolic HMGB1 (**Figure 4B**).
229 The mRNA level of HMGB1 was comparable between the liver tissues of *Atg7*^{F/F}, and *Atg7*^{-/-}
230 mice (**Figure 4C**). Thus, the loss of HMGB1 protein in hepatic tumor cells supports further the
231 notion that the nature of these cells are autophagy-deficient, and suggests that these tumor cells
232 had released HMGB1, which could affect the microenvironment.

233

234 **6. HMGB1 promotes hepatic proliferation.**

235 HMGB1 has a mitogenic effect in many cell lines including in vitro human HCC cell
236 lines²⁶. HMGB1 released by autophagy-deficient hepatocytes could affect the growth of
237 tumorigenic hepatocytes, thus promoting hepatocarcinogenesis. Indeed we had found that
238 HMGB1 was important for the tumorigenesis in the autophagy-deficient liver².

239 Consistently, we now found that *Atg7*^{-/-} livers had a remarkably increased number of the
240 hepatocytes positive for Proliferation of cell nuclear antigen (PCNA) (**Figure 5A**). PCNA
241 positive cells seem to be present in both non-tumor and tumor regions without much differences
242 in the level. This observation was confirmed by immunostaining for Ki67 positive cells
243 (**Supplementary Figure S5A-B**). Ki67 positive cells were elevated in non-tumor and intra-

244 tumor regions of the *Atg7*^{-/-} liver without differences in the number of proliferating cells
245 (**Supplementary Figure S5A**).

246 To determine the role of HMGB1 in the proliferation of hepatocyte, we compared the
247 cellular proliferation in 15-month old *Atg7*^{-/-} and *Atg7*^{-/-}/*Hmgb1*^{-/-} mice because both genotypes
248 developed a notable but different number of tumors at this age². Immunostaining analysis for
249 PCNA showed a lower number of proliferating hepatocytes in the tumor of *Atg7*^{-/-}/*Hmgb1*^{-/-}
250 livers than those in the *Atg7*^{-/-} livers (**Figure 5A**). Interestingly, the number of PCNA positive
251 cells was also lower in the non-tumor region of *Atg7*^{-/-}/*Hmgb1*^{-/-} livers when compared to the
252 non-tumor region of *Atg7*^{-/-} livers (**Figure 5A**).

253 Because the increased cellular proliferation is associated with increased expression of
254 cyclin protein, we next examined the expression of Cyclins. First, immunostaining found that
255 the expression of Cyclin D1 was more up-regulated in the tumor samples of *Atg7*^{-/-} liver than in
256 the tumors of the *Atg7*^{-/-}/*Hmgb1*^{-/-} livers (**Figure 5B, Supplementary Figure S6**). Second,
257 immunoblot analysis of Cyclin E, similar to PCNA protein, also showed a higher level in tumor
258 and non-tumor regions of the *Atg7*^{-/-} livers than that in the *Atg7*^{-/-}/*Hmgb1*^{-/-} livers (**Figure 5C-**
259 **D**). Real-time PCR analysis demonstrated that hepatic expression of *CCND1*, *CCNA1*, and
260 *CCNB1* were significantly up-regulated in *Atg7*^{-/-} mice, compared to *Atg7* *F/F* mice(**Figure 5E**).
261 The expression of *CCND1* and *CCNA1* was even more pronouncedly elevated in the tumor
262 region than in the non-tumor tissues from *Atg7*^{-/-} mice (**Figure 5E**). Such induction was not
263 observed in tumor tissues from *Atg7*^{-/-}/*Hmgb1*^{-/-} mice (**Figure 5E**), suggesting that *Hmgb1*
264 deletion retarded cell cycle progression via the downregulation of the expression of cyclins in
265 *Atg7*^{-/-} mice. These results indicate that hepatic tumors of *Atg7*^{-/-}/*Hmgb1*^{-/-} are less proliferative

266 than the tumors in *Atg7*^{-/-} mice. Thus HMGB1 had an impact on cell proliferation in the
267 autophagy-deficient liver.

268 We then examined the phosphatidylinositol 3-kinase(PI3K)/AKT signaling pathway that
269 regulates various cellular responses in HCC proliferation and survival^{27,28}. Intriguingly,
270 immunoblot analysis showed that phospho-AKT was detected at higher levels in *Atg7*^{-/-}/*Hmgb1*^{-/-}
271 *-/-* livers compared to *Atg7*^{-/-} livers regardless the sample type (**Supplementary Figure S7A**).
272 The expression level of phospho-glycogen synthase kinase 3 (GSK3) β , one of the downstream
273 target of AKT was also markedly increased in the *Atg7*^{-/-}/*Hmgb1*^{-/-} livers (**Supplementary**
274 **Figure S7A**). However, the protein levels of total and phospho-PDK, an upstream regulator of
275 AKT were comparable between the two genotypes (**Supplementary Figure S7A**). In addition,
276 we found an increased level of phospho-JNK in *Atg7*^{-/-}/*Hmgb1*^{-/-} livers as compared to that in
277 *Atg7*^{-/-} livers regardless the sample type (**Supplementary Figure S7B**). JNK can be a dominant
278 effector of mitogen-activated protein kinase in the liver²⁹. JNK catalyzes the phosphorylation of
279 numerous substrate proteins including the c-Jun transcription factor to regulate the gene
280 expression. However, the protein expression level of phospho-c-Jun and total-c-Jun were
281 comparable between the two genotypes (**Supplementary Figure S7B**). The mammalian target of
282 rapamycin complex 1 (mTORC1) signaling pathway, the mitogen-activated protein
283 kinase(MAPK)/ERK signaling pathway, and the Janus activated kinase/Signal Transducer and
284 Activator of Transcription Family or transcription factors (JAK/STAT3) signaling pathway, all
285 have been associated with cell growth³⁰⁻³³. However, we did not detect significant differences in
286 the activation of these pathways between *Atg7*^{-/-} mice and *Atg7*^{-/-}/*Hmgb1*^{-/-} mice
287 (**Supplementary Figure S7C-E**). Taken together while the reason for the paradoxical elevation
288 of AKT and JNK phosphorylation in *Atg7*^{-/-}/*Hmgb1*^{-/-} livers is not clear these events do not

289 seem to be tumor specific and may not be related to the reduced proliferation status of tumors
290 from in these livers. Alternativley, it is notable that the hepatocytes could offer a very different
291 cellular context in which the conventional oncogenes or tumor suppressor genes can act in
292 opposite ways^{34,35}.

293

294 **7. RAGE deletion impairs proliferation and retards liver tumor development.**

295 HMGB1 is a non-histone nuclear protein that facilitates the binding of regulatory proteins
296 to DNA and typically enhances transcriptional activation¹⁰. When released extracellularly,
297 HMGB1 can binds to one of its receptors, such as RAGE or TLR4³⁶. In our previous study,
298 *Atg7*^{-/-} mice develop hepatic tumors at 9-month old in the liver, which could be inhibited by the
299 deletion of either *Hmgb1* or *Rage*². While *Atg7*^{-/-}/*Hmgb1*^{-/-} at the age of 12-month old were still
300 largely devoid of tumors in the liver², we now found that 12-month old *Atg7*^{-/-}/*Rage*^{-/-} mice
301 developed a significant presence of tumors albeit at a level slightly lower than that in the *Atg7*^{-/-}
302 livers (**Figure 6A**). However, hepatic tumors developed in the 12-month old *Atg7*^{-/-}/*Rage*^{-/-}
303 mice were significantly smaller in size compared to those in the *Atg7*^{-/-} mice (**Figure 6B**). The
304 data indicate that deletion of *Rage* still delayed the development of tumors in older *Atg7*^{-/-} mice
305 although in a less prominent manner than *Hmgb1* deletion. Notably, the number of PCNA-
306 positive cells and the expression of cyclin D1 were also remarkably decreased in *Atg7*^{-/-}/*Rage*^{-/-}
307 livers compared to that in the *Atg7*^{-/-} livers(**Figure 6C-D**). These data suggest that the loss of
308 RAGE in autophagy-deficient livers reduced tumor cell proliferation and tumor expansion in the
309 liver. HMGB1 interaction with the RAGE receptor can thus mediate a significant level of cell
310 proliferation and tumor development in the autophagy-deficient liver.

311 To determine whether HMGB1 released by the autophagy-deficient hepatocytes or
312 hepatic tumor cells could act as an autocrine fashion to promote cellular proliferation, we
313 examined whether hepatocytes could express RAGE. Immunofluorescence staining was
314 performed in frozen tissue from *Atg7 F/F* and *Atg7-/-* liver. We found that RAGE was almost
315 exclusively expressed by cells other than hepatocytes based on cell morphology, but it was
316 detected on the cell surface, consistent with its being a receptor molecule (**Figure 7A**). To
317 examine which non-hepatocytes expressed RAGE, double immunofluorescence staining for
318 RAGE, together with CK19 or SOX9 (for ductular cells), F4/80 (for Kupffer cells), or Desmin
319 (for stellate cells), was conducted. Colocalization of RAGE was evident in CK19 or SOX9-
320 positive ductular cells and F4/80-positive Kupffer cells, but not on the Desmin-positive stellate
321 cells in *Atg7-/-* liver (**Figure 7B**).

322 These findings indicate that RAGE was expressed on ductular cells and Kupffer cells but
323 not on hepatocytes nor stellate cells. Furthermore, these observations suggest that unlike the
324 possible direct effect of HMGB1 on the expansion of CK19-positive or SOX9-positive ductular
325 cells², the tumor-promoting effect of HMGB1 may not be mediated by a direct effect on the
326 autophagy-deficient hepatocytes, but possibly by an indirect effect through other RAGE-
327 expressing cells, such as the Kupffer's cells, which could then alter the microenvironment that
328 facilitate tumor development.

329

330 **8. RNA Sequencing revealed key molecular differences between tumors from *Atg7-/-* mice**
331 **and from *Atg7-/-/Hmgb1-/-* mice.**

332 Since the effect of HMGB1 in promoting tumor development may be mediated by an
333 altered microenvironment, there could be multiple alterations in tumor behaviors affected by this
334 process. We sought to investigate the transcriptomic profile of the tumor to better understand the
335 impact of HMGB1 on tumor development in autophagy-deficient livers. We chose to perform
336 RNA sequencing on tumor tissues obtained from *Atg7*^{-/-} and *Atg7*^{-/-}/*Hmgb1*^{-/-} mice at the age of
337 15 months old, when the tumor number and size were comparable in these mice.

338 The principal component analysis (PCA) on the RNAseq data indicated different
339 transcriptomic profiles in the tumor tissues of 15-month old *Atg7*^{-/-} and *Atg7*^{-/-}/*Hmgb1*^{-/-} mice
340 when compared with the non-tumor tissues (**Figure 8A**). The six non-tumor samples from the
341 two strains of mice were close to each other. In addition, two out of the three tumor samples
342 from *Atg7*^{-/-}/*Hmgb1*^{-/-} livers were also close to the non-tumor samples whereas tumor samples
343 from *Atg7*^{-/-} mice were separated the farthest from the rest of the samples. PCA thus suggests
344 that tumors from the two strains of mice were quite different with those from *Atg7*^{-/-}/*Hmgb1*^{-/-}
345 livers more similar to the non-tumor tissues in their transcriptomic profiles.

346 Differential expression analysis showed that 284 and 372 differentially expressed genes
347 (DEGs) were upregulated in tumors of *Atg7*^{-/-} and *Atg7*^{-/-}/*Hmgb1*^{-/-} livers, respectively,
348 whereas 326 and 300 genes were downregulated in tumors of these livers, respectively (**Figure**
349 **8B-C**). A complete list of these DEGs can be found in **Supplementary Tables S1-S5**. We then
350 focused on discovering unique molecular features in the tumours associated with the presence and
351 absence of HMGB1. When comparing the DEGs between *Atg7*^{-/-} and *Atg7*^{-/-}/*Hmgb1*^{-/-}, a small
352 number of up-regulated (28, **Figure 8B**) or down-regulated (12, **Figure 8C**) DEGs were found in
353 tumor tissues of both *Atg7*^{-/-} and *Atg7*^{-/-}/*Hmgb1*^{-/-} livers. The larger portions of DEGs were,

354 however, unique in *Atg7*^{-/-} and in *Atg7*^{-/-}/*Hmgb1*^{-/-} tumors, supporting that the tumors were
355 different in the presence or absence of HMGB1.

356 To understand the molecular features of these differences, we determined the Gene
357 Ontology (GO) terms and KEGG pathways that were significantly enriched in the unique DEG
358 sets. We found that many biological processes, particularly those associated with mitochondrial
359 structures or functions were significantly over-represented by the uniquely up-regulated DEGs
360 in *Atg7*^{-/-} tumors (**Figure 8D**). Notably, DEGs down-regulated uniquely in *Atg7*^{-/-}/*Hmgb1*^{-/-}
361 tumors were also enriched for those involved in the mitochondrial structures or functions,
362 (**Figure 8F**). Many genes related to mitochondrial oxidative phosphorylation (OXPHOS) or
363 electron transport chain (ETC) process were significantly downregulated in the tumors of *Atg7*^{-/-}/
364 *Hmgb1*^{-/-} liver. Particularly, the genes involved in the assembly or biogenesis of respiratory
365 complex I (NADH dehydrogenase complex) and complex III (Ubiquinol to Cytochrome c
366 electron transporter) were significantly downregulated. These observations suggested that a
367 major component of the tumor-promoting effects of HMGB1 could be related to mitochondrial
368 function and activity, which may impact the cellular bioenergetics and hence tumor growth in
369 autophagy-deficient liver.

370 Discussion

371 Autophagy is important for liver homeostasis and tumor surveillance. Deficiency of
372 hepatic autophagy, such as that caused by the liver-specific deletion of *Atg5* or *Atg7*, leads to
373 tumor development in aged mice¹⁻⁴. On the other hand, autophagy function is required for the
374 aggressive growth of tumors. The mechanism that sustains the growth of autophagy-deficient
375 tumors is not known. In this study, we examined the cellular and molecular nature of hepatic
376 tumors in the autophagy-deficient liver. Our findings support the following conclusions: 1) The

377 adenoma originates from the autophagy-deficient hepatocytes; 2) Hepatocyte-derived HMGB1
378 stimulates tumor cell proliferation; 3) HMGB1 mediates the proliferative signal at least in part
379 via RAGE in a paracrine mode; and 4) Tumors developed in the presence or absence of HMGB1
380 have significantly different transcriptomic profiles and mitochondria function could be an
381 important mechanistic linker to tumor promotion.

382

383 **1. HMGB1 may act in a paracrine model to stimulate tumor growth**

384 Hepatic tumors were histologically consistent with hepatocellular adenoma where the
385 benign tumor cells arrange in regular plates, usually one or two cells in thickness³. Here we
386 further showed that tumor cells were originated from the autophagy-deficient hepatocytes.

387 The composition of the tumor appears to be different from the non-tumor liver tissue. In
388 comparison to non-tumor tissues where different hepatic cells including hepatocytes,
389 inflammatory cells, fibrotic cells, and ductular cells coexist, the tumor tissue consists of mainly
390 the tumor cells (HNF4 α positive), and some macrophages (**Figure 1-3**)(**Supplementary Table**
391 **S6**). Other nonparenchymal cells are found only outside the tumor region. Fibrotic cells and
392 ductular cells seem to be responsible for the formation of a fibrous capsule that demarcates the
393 tumor from the non-tumor tissue. How the autophagy-deficient hepatocytes form the
394 adenomatous nodule, excluding the fibrotic cells and ductular cells but retaining some
395 macrophages, is intriguing. But macrophages could belong to those known as tumor-associated
396 macrophages(TAM) and may enter into the tumor tissue via tumor blood vessels³⁷.

397 HMGB1 is known to promote tumor development^{2,38,39}. However, how HMGB1 does
398 this, in particular for the autophagy-deficient hepatic tumors, is not clear. HMGB1 has been
399 shown to be important for expansion of ductular cells^{2,38}, , immune cells recruitment²⁵ and

400 activation of fibrotic cells²⁴. All these cellular events could favor tumorigenesis. Previously, we
401 showed that non-tumorigenic autophagy-deficient hepatocytes can release HMGB1². Here our
402 findings indicate that HMGB1 could be also released by the autophagy-deficient hepatic tumor
403 cells. Thus HMGB1 may act through an autocrine or paracrine mode to promote tumor growth.

404 We found that the proliferative effect of HMGB1 could be mediated via the RAGE
405 receptor as deletion of *Rage* also reduces tumor cell proliferation and delay tumor development
406 (**Figure 6**). We found that RAGE receptor was not expressed by hepatocytes and stellate cells at
407 the detectable level by immunostaining. But it could be readily detected on the surface of
408 Kupffer's cells and HPCs^{2 40}. Hence, the effect of HMGB1 in cell proliferation could be
409 mediated by a paracrine manner, although the autocrine mode could be not be completely
410 excluded (**Supplementary Figure S8**). In the paracrine mode, HMGB1 release by the
411 tumorigenic and non-tumorigenic autophagy-deficient hepatocytes could activate macrophages
412 or ductular cells, which then releases different cytokine factors that ultimately affect hepatocytes
413 growth and proliferation. From this aspect, interaction between tumorigenic hepatocytes and
414 TAM could generate an intratumoral microenvironment favoring cell growth and proliferation.
415 However, it seems that some of the well-defined proinflammatory cytokines such as TNF α , IL-
416 1 β , and IL-6 may not play the role as the expression of these cytokines are remarkably
417 downregulated in tumor tissues of *Atg7*^{-/-} mice (**Supplementary Figure S4**). On the other hand,
418 the RAGE-positive peri-tumoral ductular cells could possibly communicate with hepatocytes via
419 cytokines such as angiogenic factors ANGPT2, PDGFb to promote protumorigenic activities,
420 such as angiogenesis and invasiveness while inhibiting tumor infiltration into normal tissue. It is
421 also possible that the protumorigenic factors from TAM and/or ductular cells could be mediated
422 by extracellular vesicles, microRNAs and other cellular factors⁴¹.

423 The *Rage* deletion could not fully protect the *Atg7*^{-/-} mice from tumor development at
424 age of 12-month old although the tumor size still appeared significantly smaller (**Figure 6A-**
425 **B**). This is in contrast to *Atg7*^{-/-}/*Hmgb1*^{-/-} mice where *Hmgb1* deletion could significantly
426 inhibit tumor development in *Atg7*^{-/-} mice even at the 12-month time point (**Figure 6A-B**)². It is
427 possible that HMGB1 affect the tumorigenesis process not only via RAGE but also through other
428 receptors such as TLR4³⁶. Future studies can assess the potential role of TLR4 in this process.

429 2. The impact of HMGB1 on tumor cells can be broad.

430 Deletion of *Hmgb1* or *Rage* led to a significant reduction in the proliferative capability of
431 autophagy-deficient hepatocytes and tumors as demonstrated by the expression of PCNA, Ki67
432 and Cyclin D1. Thus the pro-proliferative effect by HMGB1 confers a generally stronger
433 capability of proliferation to autophagy-deficient hepatocytes, which would be beneficial to the
434 growth of tumors that are derived from these cells.

435 However, RNAseq analysis indicates that there are much more unique changes in the
436 molecular composition of the tumors caused by HMGB1. The enrichment of certain gene
437 expression related to mitochondrial structure and function in the presence of HMGB1 and lack
438 of such enrichment in the absence of HMGB1 are quite significant. *Hmgb1* deletion appears to
439 suppress the mitochondrial ETC in tumors of autophagy-deficient livers. Whether and how
440 downregulation of genes of mitochondrial ETC may suppress cell proliferation in *Atg7*^{-/-}/*Hmgb1*^{-/-}
441 tumors is unclear. But it is well known that mitochondrial ETC enables many metabolic
442 processes and is a major sources of ATP and building blocks for the proliferation of tumor cells.
443 As a consequence of ETC dysfunction, cell proliferation could be impaired due to bioenergetics
444 deficit. This notion is supported by the observation where pharmacological or genetic inhibition
445 of ETC caused impaired cell proliferation of cells in vitro^{42,43}. Interestingly, a recent study

446 suggest that ETC enables aspartate biosynthesis, a key proteogenic amino acid that is also a
447 precursor in purine and pyrimide synthesis and is required for tumor cells growth and survival
448 ^{44,45}. Thus tumors of *Atg7*^{-/-}*Hmgb1*^{-/-} liver may have defective ETC that could impair cell
449 proliferation by limiting an intracellular aspartate level besides causing bioenergetics deficits.
450 Many metabolic pathways including glycolysis, the TCA cycle, and β -oxidation produce the
451 electron donors that fuel the ETC. Hence, impairment or downregulation of ETC could limit the
452 regeneration of reducing equivalents, such as NAD⁺, which in turn suppresses glycolysis or the
453 TCA cycle. Future studies should address these possibilities for the understanding of how
454 HMGB1 sustains the growth of autophagy-deficient hepatic tumors. .

455 In conclusion, our findings demonstrate that hepatic adenoma originates from the
456 autophagy-deficient hepatocytes that release HMGB1. HMGB1, in turn, can stimulate
457 hepatocyte proliferation and hepatic tumorigenesis via RAGE in the autophagy-deficient liver.
458 The effect of HMGB1 on tumor cells are broad as revealed by transcriptomic analysis, which
459 offers mechanistic clues for future studies.

460 **Materials and Methods**

461 **Animal experiments:** *Atg7*^{F/F}, *Atg7*^{-/-}, *Atg7*^{-/-}*Hmgb1*^{-/-}, *Atg7*^{-/-}*Rage*^{-/-}, *Hmgb1*^{-/-}, and
462 *Rage*^{-/-} mice were used in this study. *Atg7*^{F/F} was obtained from Dr. Komatsu Masaaki (Nigata
463 University, Japan). These mice were backcrossed with C57BL/6J for another 10 generations as
464 described previously^{2,20}. Albumin-Cre mice were obtained from the Jackson Laboratory (Bar
465 Harbor, ME). *Hmgb1*^{F/F} and *Rage* mice were as described². Hepatic *Atg7*^{-/-} mice were further
466 crossed with *Hmgb1*^{F/F} or *Rage* to generate *Atg7*^{-/-}*Hmgb1*^{-/-} or *Atg7*^{-/-}*Rage*^{-/-} mice as
467 previously described². Both male and female mice were used in the study. All animals received

468 humane care, and all procedures were approved by the Institutional Animal Care and use
469 Committee(IACUC) of the Indiana University.

470 **Tumor sample collection:** The whole liver was carefully removed from the euthanized animals,
471 washed, and placed in cold PBS. The number of tumor nodules on the liver surface was counted
472 for all the liver lobes. Tumor nodules with >2mm in diameter were carefully removed and
473 examined as tumor tissue. Tissue without visible tumor nodules were sampled as non-tumor
474 tissues. All tissues were collected in separate tubes and stored at -80°C for future studies. Liver
475 tissues containing the tumor nodule and the surrounding non-tumor tissue were excised and fixed
476 in 10% neutral formalin or buffered with 4% PFA overnight for paraffin-embedding or for OCT
477 embedding. The tissue section was prepared from the frozen or paraffin blocks for general
478 histology, immunostaining, and immunohistochemistry analysis.

479 **General histological and immunological analysis:** General histology was examined on
480 paraffin-embedded sections stained with hematoxylin and eosin (H-E). Liver fibrosis was
481 determined by Sirius Red staining or Masson's Trichome staining. For immunostaining, liver
482 sections were subjected to heat-induced antigen retrieval using citrate buffer (pH 6.0) followed
483 by permeabilization and blockage with 10% goat or donkey serum in PBS containing 0.5%
484 triton-X for 1 hour. Sections were incubated overnight at 4°C with primary antibody diluted in
485 PBS. Primary antibodies used in this study are listed in **Supplementary Table S8**. Sections were
486 then incubated with Alexa-488 or Cy3-conjugated secondary antibodies. Images were obtained
487 using Nikon Eclipse TE 200 epi-immunofluorescence microscope. Hoechst 33342 was used for
488 nucleus staining. Images were analyzed using NIS-element AR3.2 software.

489 Immunoblot analysis was performed as described previously^{2,20} using primary
490 antibodies and respective secondary antibodies conjugated with horseradish peroxidase as listed

491 in **Supplementary Table S8**. The respective protein bands were visualized using the
492 immunobilon chemiluminescence system (Millipore, MA). The densitometry analysis of
493 immunoblotting images was performed using Quantity One Software (Bio-rad). Densitometry
494 values were normalized to the loading control (GAPDH) and then converted to units relative to
495 the untreated control.

496

497 **Total RNA isolation, reverse transcription, and quantitative real-time PCR analysis:** Total
498 RNA was isolated from liver tissues using a GeneTET RNA Purification Kit (Thermo Fisher
499 Scientific) according to the manufacturer's protocol. cDNA was synthesized using an M-MLV
500 Reverse Transcriptase Enzyme System (Life Technologies, Thermo Fisher Scientific) and
501 OligodT primers. The resulting cDNA products were subjected to qPCR reaction using SYBR
502 Green Master Mixes. qPCR was performed on a Quanta studio 3 PCR machine (Life
503 Technologies–Applied Biosystems, Thermo Fisher Scientific). The threshold crossing value(Ct)
504 was determined for each transcript and then normalized to that of the internal gene transcript(β -
505 actin). Fold change values were then calculated using the $2^{-\Delta\Delta Ct}$ method. Genes-specific primers
506 were designed using Integrated DNA Technologies (IDT) PrimerQuest software. Sequences of
507 the forward and reverse primers are listed in **Supplementary Table S7**.

508

509 **RNA-sequencing and bioinformatics analysis:** RNA was isolated as described above. RNAseq
510 was performed by The Center for Medical Genomics facility at Indiana University. The integrity
511 of RNA was determined using an Agilent Bioanalyzer 2100 (Agilent Technologies;Santa Clara,
512 CA). Extracted RNA was processed for rRNA removal using the Epicenter rRNA depletion kit
513 according to the manufacturer's instructions. rRNA-depleted RNA was subsequently used to

514 generate paired-end sequencing libraries using the Illumina RNA TruSeq Library Kit according
515 to the manufacturer's instruction. RNAseq was performed using Illumina HiSeq 4000 (Illumina,
516 San Diego, CA). For bioinformatics analysis, we first used FastQC
517 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>) to examine RNA-seq quality. Then
518 all high-quality sequences were mapped to the mouse genome (mm10, UCSC Genome Browser,
519 <https://genome.ucsc.edu/>) with the STAR, an RNA-seq aligner⁴⁶. The featureCounts was adopted
520 to assign uniquely mapped reads to genes according to UCSC refGene (mm10)⁴⁷. Those low-
521 expressed genes were not further analyzed if their raw counts were less than 10 in more than
522 three samples for each pairwise comparison. The gene expression was normalized cross all
523 samples based on trimmed mean of M (TMM) values implemented in EdgeR⁴⁸, followed by
524 differential expression analysis given comparisons between non-tumor and tumor tissues, in
525 either single knockout or double knockout mice. Genes with p values less than 0.01 after
526 multiple-test false discovery rate (FDR) correction were determined as differentially expressed
527 genes (DEGs) for specific comparisons. The gene ontology (GO) and KEGG pathways
528 significantly enriched in DEGs were identified by DAVID functional annotation analysis tools⁴⁹.

529

530 **Statistical Analysis:** Statistical analyses were performed with Sigma Plot. All experimental data
531 were expressed as Mean±SE. Student t-test was performed to compare values from two groups.
532 To compare values obtained from three or more groups, one way ANOVA analysis with the
533 appropriate post-hoc analysis was used. Statistical significance was taken at the level of P< 0.05.

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540

541 **Conflict of Interest:** The authors have declared that no conflict of interest exists.

542

543 **Authors contributions**

544 BK designed project and directed study, analyzed data and wrote the manuscript. BK, XC
545 conducted experiments, acquired data, and analyzed data. HH, GL, JW helped in gene expression
546 analysis. ZD gave critical discussions. XMY designed research experiments, analyzed data, and
547 edited the manuscript.

548

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667

Figure 1

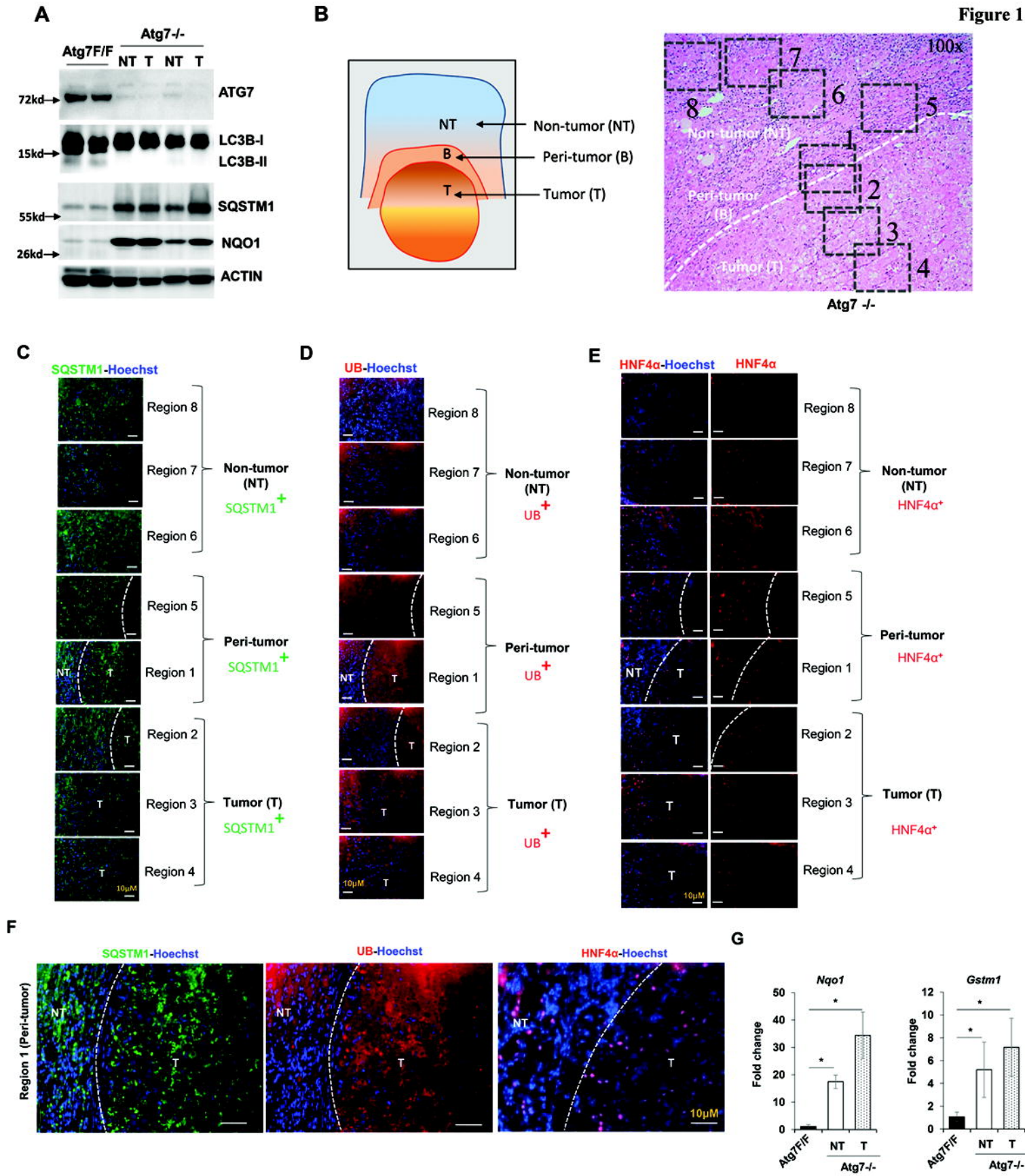
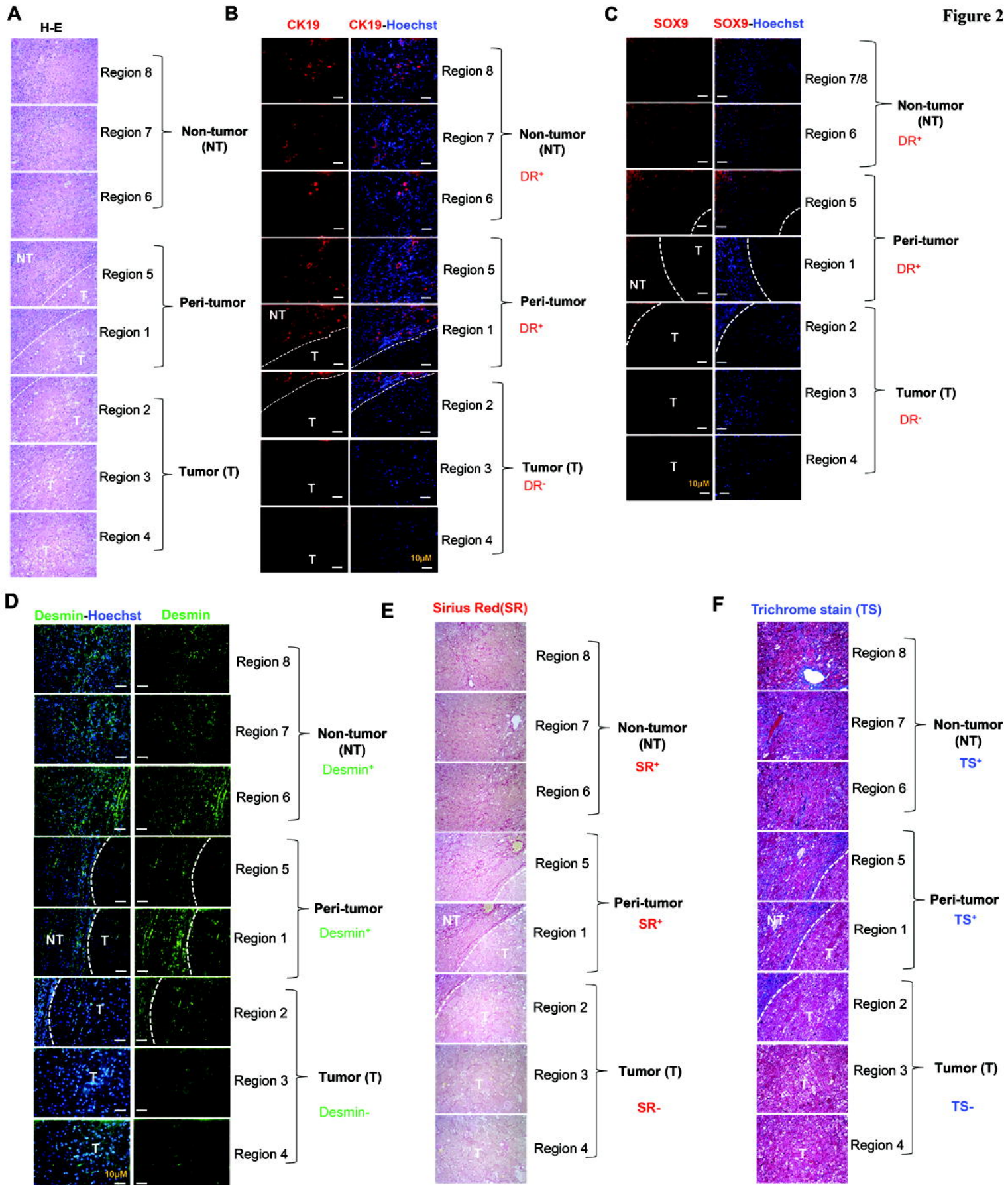
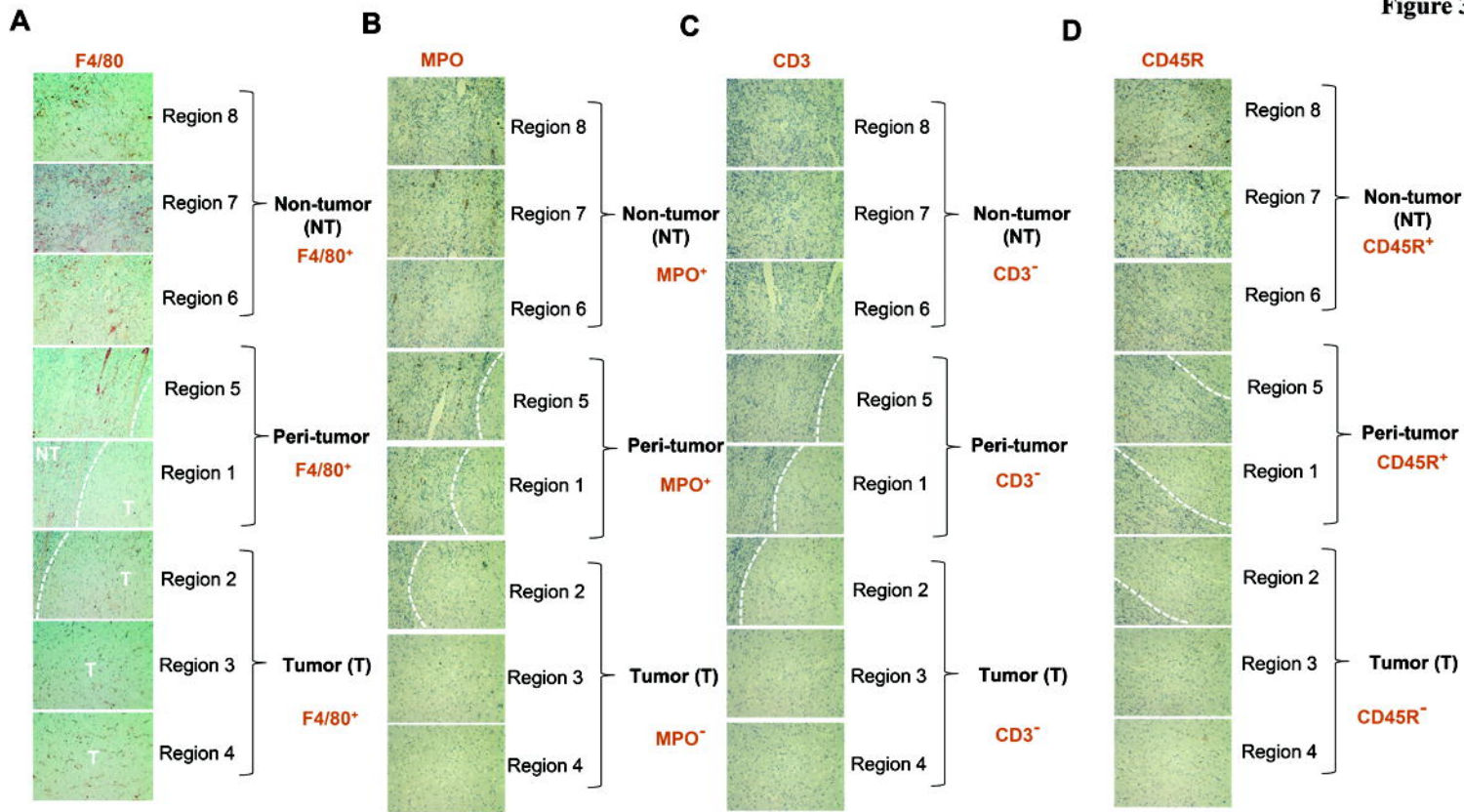
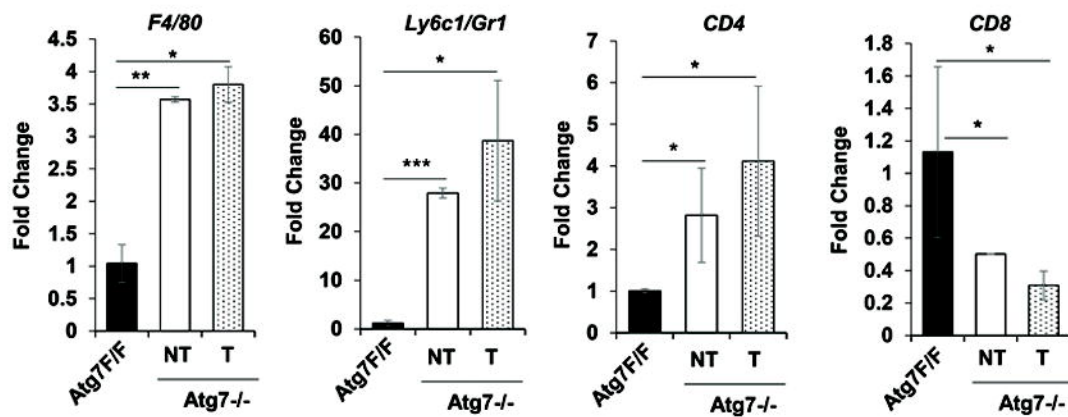
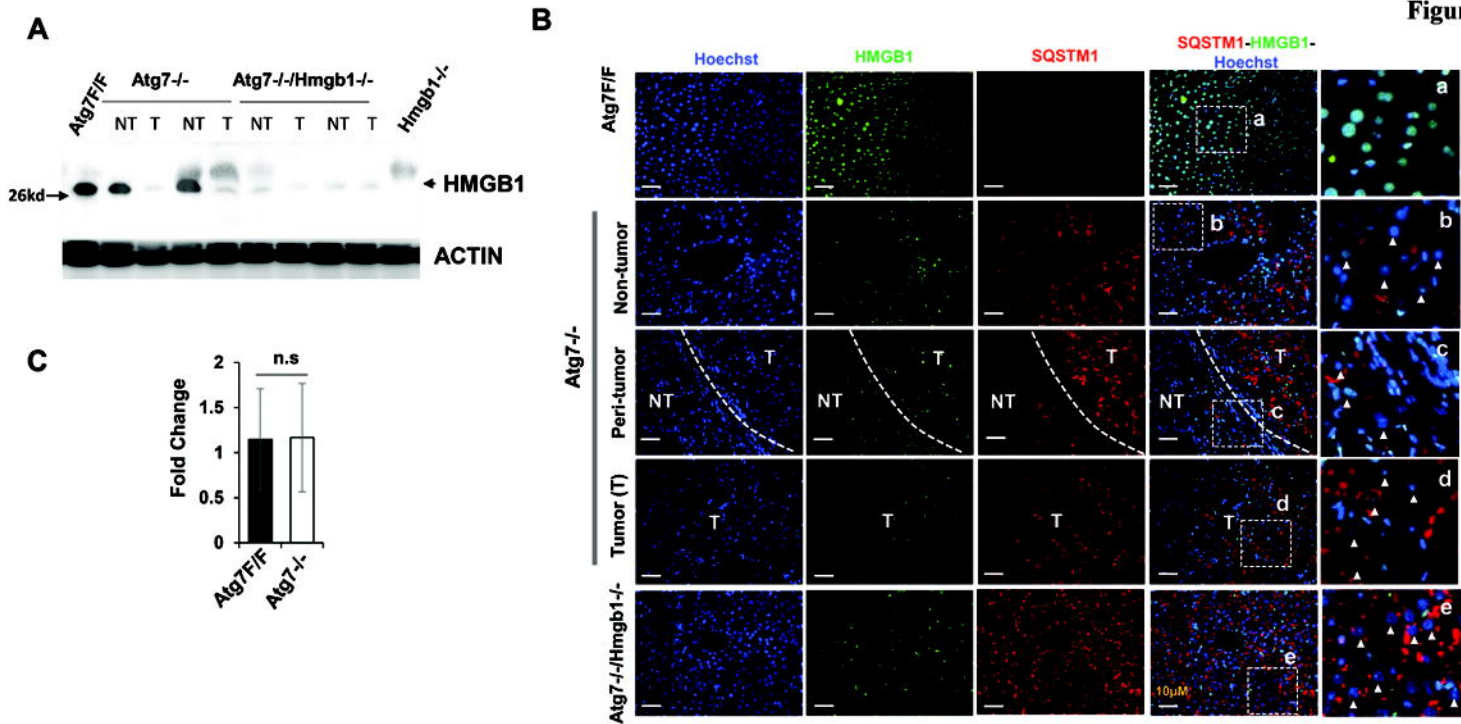
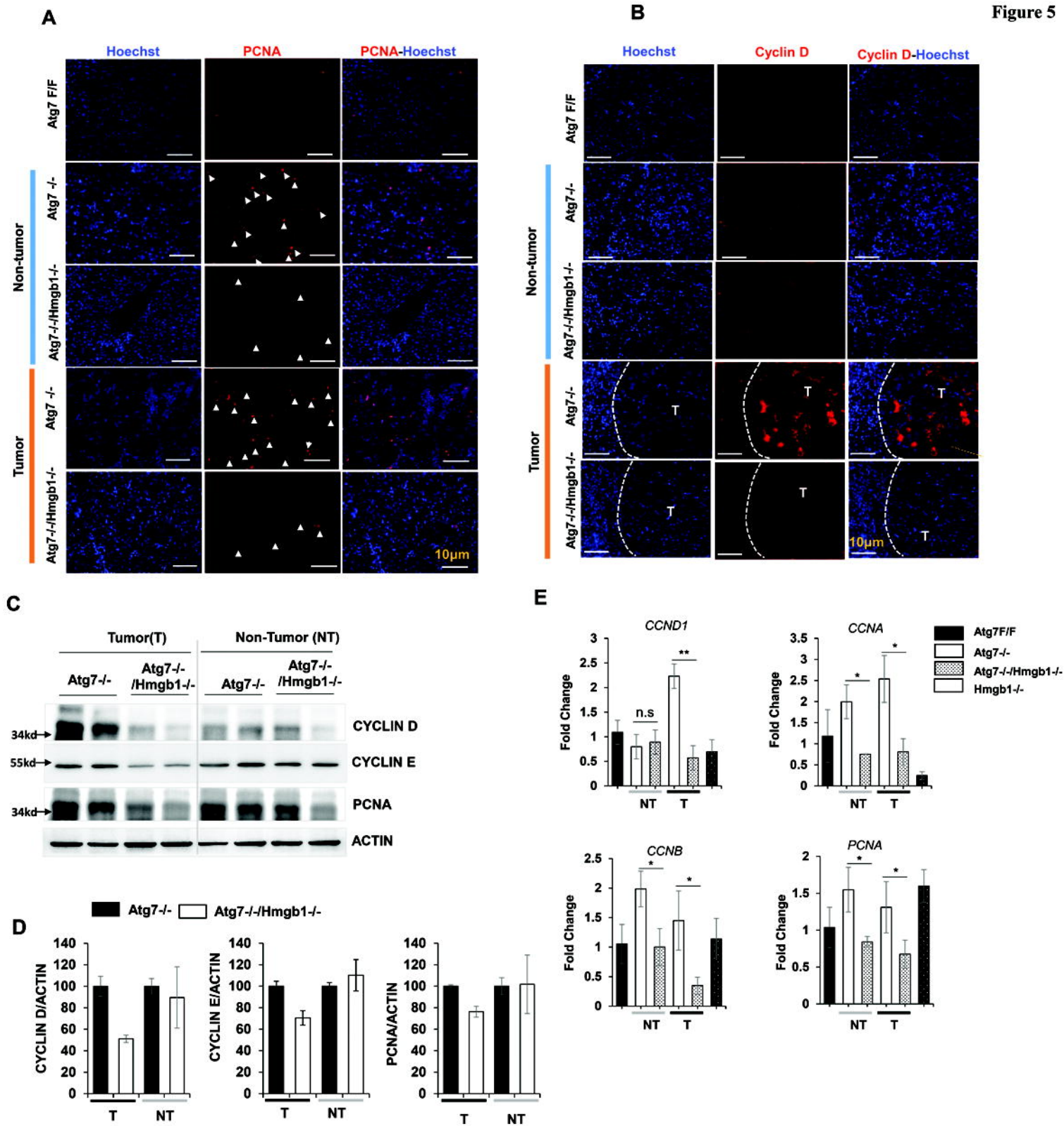


Figure 2

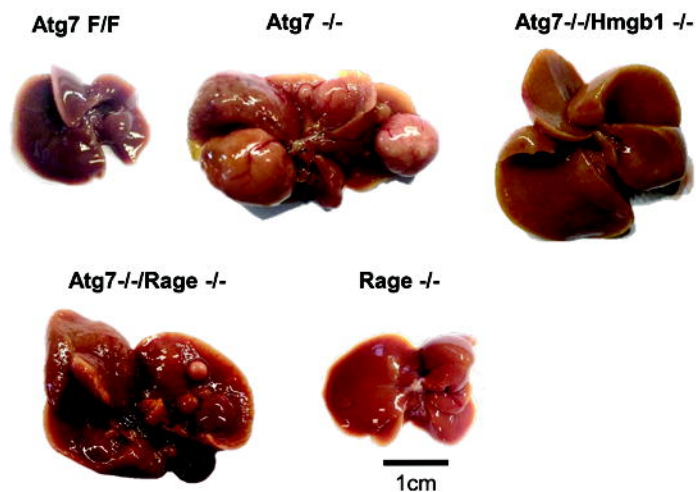


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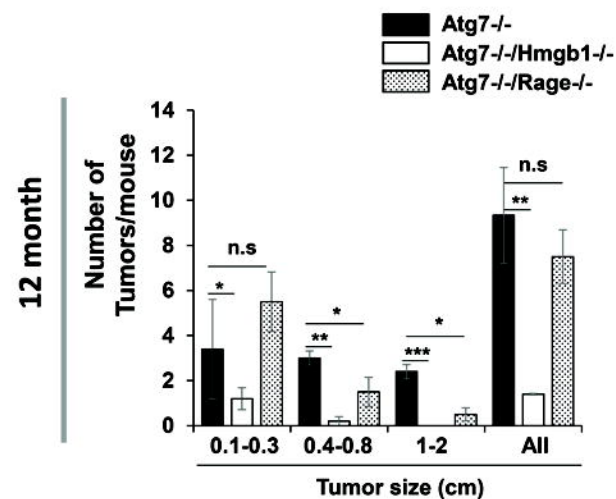




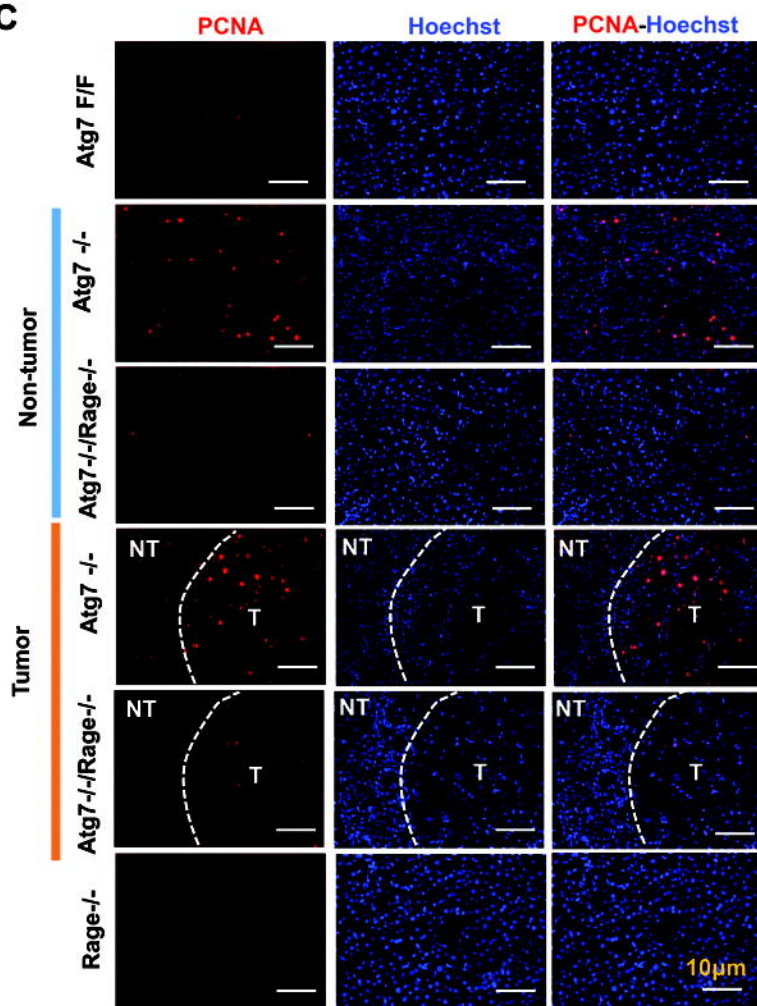
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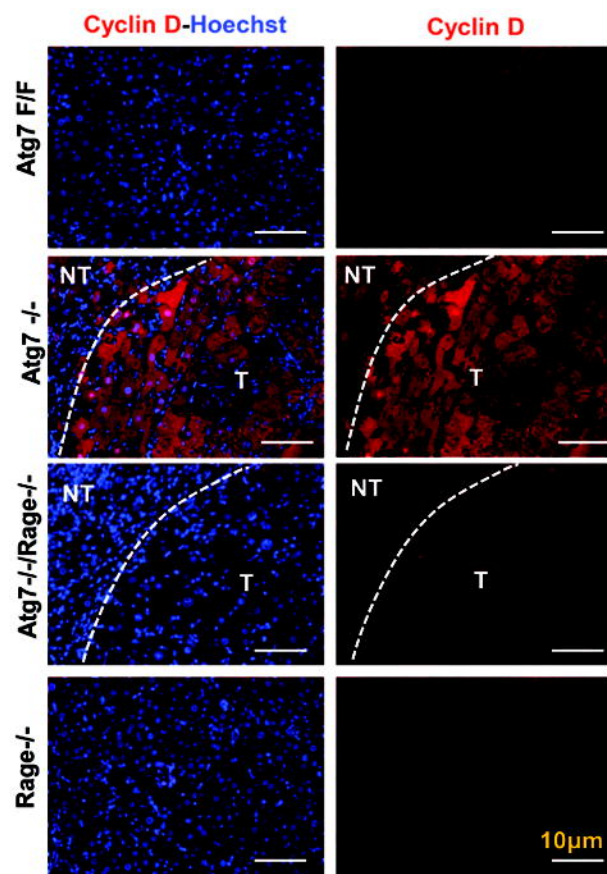
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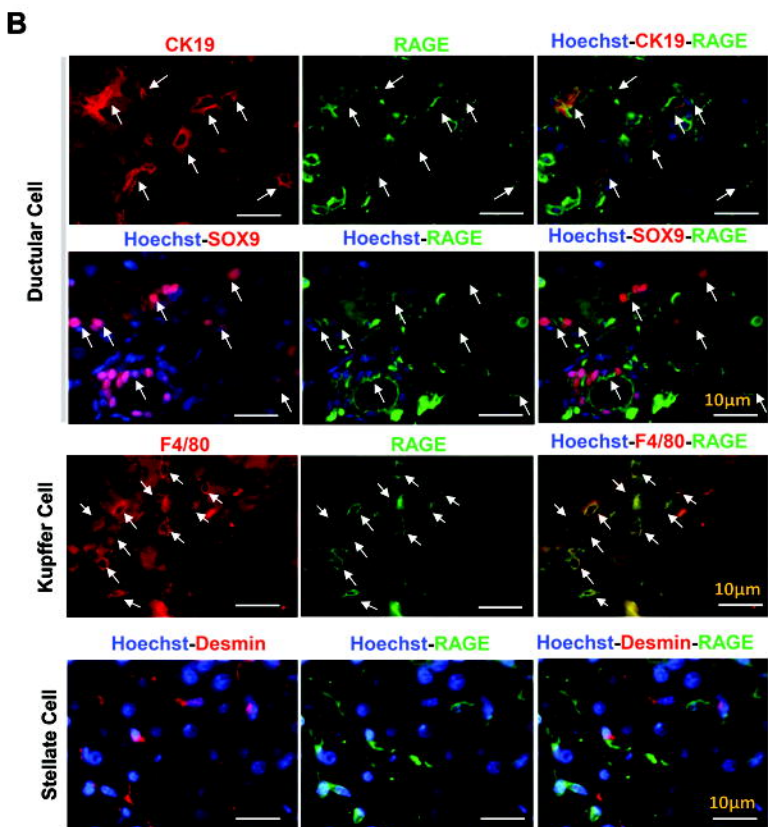
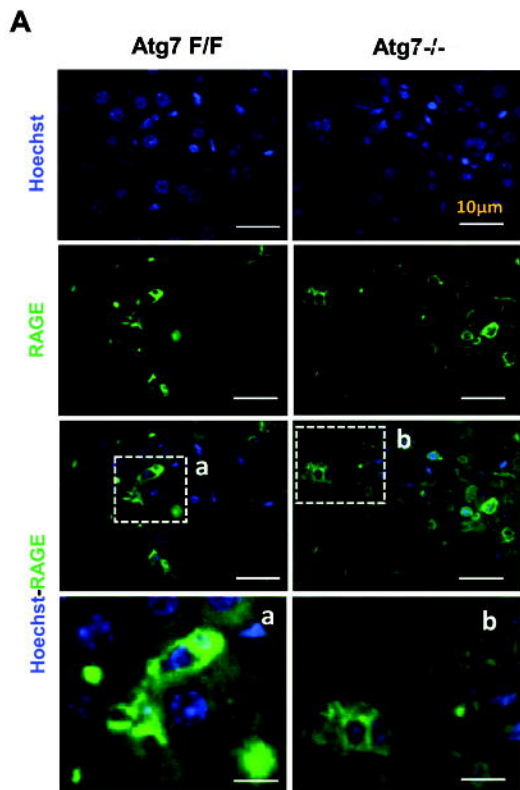


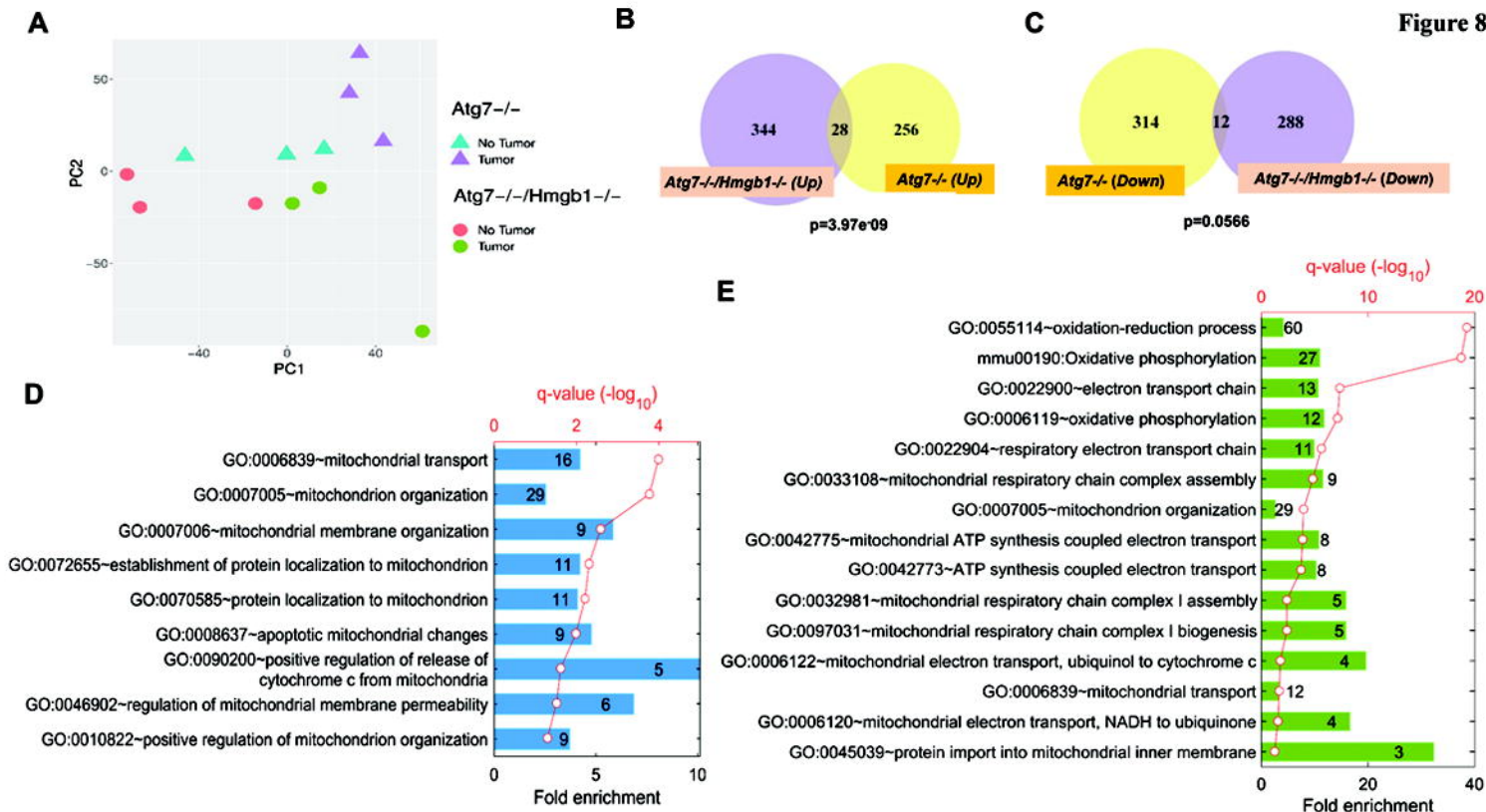
C



D







1 **Figure Legends**

2 **Figure 1:** Hepatic tumor in autophagy-deficient livers are derived from autophagy-deficient
3 hepatocytes. **(A)** Immunoblot analysis of autophagy function-related proteins (ATG7, SQSTM1,
4 LC3B-I/II) and NRF2 pathway-related proteins(NQO1) in whole livers isolated from 15-month
5 old *Atg7F/F*, and *Atg7-/-* mice. **(B)** Schematic representation of the non-tumor, peri-tumor, and
6 tumor region of the liver sections. Region 1 and Region 5: peri-tumor region, Region 2-Region
7 4: tumor region, and Region 6- Region 8: non-tumor region. **(C-E)** Livers from 12-month old
8 mice of *Atg7-/-* genotype were sectioned and immunostained with anti-SQSTM1(C), Anti-
9 Ubiquitin (UB) (D), or anti-HNF4 α (E). Dotted lines indicate the tumor border. **(F)** Magnified
10 image of the region 1(peri- and intra-tumor region) of panels C, D, & E. **(G)** The hepatic mRNA
11 expression level of NRF2 target genes, *Nqo1* and *Gstm1*, in the livers of 15-month old *Atg7F/F*,
12 and in the non-tumor and tumor samples from the liver of age-matched *Atg7-/-* mice. NT, non-
13 tumor, T, tumor. Data are reported as mean \pm SE,* $P<0.05$; n=3 mice per group.

14
15 **Figure 2.** Hepatic Progenitor Cells and fibrosis are localized exclusively in peri-tumor and non-
16 tumor regions but are absent inside the tumor. Liver sections from 12-month old mice of the
17 *Atg7-/-* genotype were subjected to H-E staining **(A)** (original magnification, X200) and
18 immunostaining for CK19 **(B)**, SOX9 **(C)**, Desmin immunostaining **(D)**, Sirius Red stain **(E)**, or
19 Trichrome stain **(F)** (original magnification, X200). Dotted lines indicate the tumor border. NT,
20 non-tumor, T, tumor.

21
22 **Figure 3.** Macrophages but not other immune cells are found within the tumor. Liver sections
23 from 12-month old mice of *Atg7-/-* genotype were subjected to immunohistochemistry staining

24 for F4/80 (A), Myeloperoxidase (MPO) (B), CD3 (C) and, CD45R (D) (original magnification,
25 X100). Dotted lines indicate the tumor border. (E) The hepatic mRNA expression level of
26 immune cell-associated genes in 15-month old *Atg7F/F* and *Atg7-/-* liver tissues. NT, non-tumor,
27 T, tumor. Data are reported as mean± SE, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, n.s.: no
28 significance; n=3 mice per group.

29
30 **Figure 4.** Hepatic HMGB1 is absent in the tumor of autophagy-deficient livers. (A) Livers of 15-
31 month old mice of different genotypes were examined for HMGB1 by immunoblotting assay.
32 (B) Liver sections from 15-month old mice of different genotypes were immunostained with
33 anti-HMGB1 and anti-SQSTM1. White dotted lines indicate the tumor border. White arrowhead
34 indicates the hepatocytes without nuclear HMGB1. (C) The hepatic mRNA expression level of
35 *Hmgb1* in 15-month old *Atg7F/F* and *Atg7-/-* mice, determined by real-time PCR. NT, non-
36 tumor, T, tumor. Data are reported as mean± SE, n.s., no significance; n=3 mice per group.

37
38 **Figure 5.** Loss of HMGB1 in hepatocytes correlates with reduced proliferation in the tumor. (A-
39 B) Liver sections from 15-month old mice of different genotypes were immunostained with anti-
40 PCNA(A), or anti-Cyclin D (B). White arrow indicated proliferating hepatocytes. White dotted
41 lines indicate the tumor border. (C) Immunoblot analysis of PCNA, cyclin D1, and cyclin E
42 proteins in the tumor or non-tumor sample of 15-month old *Atg7-/-* and, *Atg7-/-/Hmgb1-/-* mice.
43 (D) Densitometry qualification of the indicated proteins. (E) The hepatic mRNA level of
44 indicated genes were determined in the indicated tissues of 15-month old mice of different
45 genotypes, determined by real-time PCR. NT, non-tumor, T, tumor. Data are reported as mean±
46 SE, * $P < 0.05$, ** $P < 0.01$, n.s., no significance; n=3 mice per group.

47
48 **Figure 6.** Genetic loss of *Rage* inhibits tumorigenesis in autophagy-deficient livers. (A) Gross
49 images of representative livers of 12-month old *Atg7*^{-/-}, *Atg7*^{-/-}/*Hmgb1*^{-/-}, *Atg7*^{-/-}/*Rage*^{-/-}, and
50 *Rage*^{-/-} mice. (B) Average number and size distribution of the tumors observed in the livers of
51 12-month old mice of different genotypes. (C-D) Liver sections from 12-month old mice of
52 different genotypes were immunostained with anti-PCNA(C), or anti-Cyclin D (D). White dotted
53 lines indicate the tumor border. NT, non-tumor, T, tumor. Data are reported as mean± SE,*
54 $P<0.05$, ** $P<0.01$, *** $P<0.001$, n.s., no significance; n=3 mice per group. Size information of
55 the tumor from *Atg7*^{-/-}/*Hmgb1*^{-/-} livers is derived from what we has previously reported².

56
57 **Figure 7.** RAGE is expressed by ductular cells and Kupffer's cells but not by hepatocytes or
58 stellate cells. (A) Immunofluorescence staining for RAGE antigen in the livers of 9-week old
59 mice of *Atg7*^{F/F} and *Atg7*^{-/-} genotype. Framed ares are enlarged and shown in separate panels
60 (a,b). (B) Liver sections from 9-week old *Atg7*^{-/-} mice were coimmunostained with anti-RAGE,
61 together with anti-CK19 or SOX9 or F4/80 or Desmin. White arrows indcate cells with
62 colocalized signals.

63
64 **Figure 8.** RNAseq analysis indicates transcriptomic diffrences in the hepatic tumors of *Atg7*^{-/-}
65 mice and *Atg7*^{-/-}/*Hmgb1*^{-/-} mice. (A). PCA of transcriptomic data based on 12 RNA-seq
66 samples under the four indicated combinations of genotypes and tissue types. (B-C). Numbers of
67 DEGs that are significantly up-regulated (B) or down-regulated (C) ($p<0.01$) in the tumor
68 samples of *Atg7*^{-/-}/*Hmgb1*^{-/-} and/or *Atg7*^{-/-} mice. The p-values are indicated for the overlap
69 between the two groups of upregulated or downregulated DEGs, respectively. (D). GO biological

70 processes significantly over-represented in the non-overlapped 256 DEGs uniquely elevated in
71 the tumor samples of the *Atg7*^{-/-} mice. (E). GO biological processes and KEGG pathways
72 significantly enriched in the non-overlapped 288 DEGs uniquely repressed in the tumor sampels
73 of the *Atg7*^{-/-}/*Hmgb1*^{-/-} mice. For D and E, the heights of bars indicate the fold enrichment
74 compared to random selection, whereas the red dots represent the statistical significance, p-value
75 after FDR-adjusted multiple test correction. The numbers in the bars represent the numbers of
76 DEGs in the particular group which are associated with corresponding GO terms.

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