

1 **Dual targeting macrophages and microglia is a therapeutic vulnerability in**
2 **models of *PTEN*-deficient glioblastoma**

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29 **Abstract**

30 Tumor-associated macrophages and microglia (TAMs) are critical for tumor
31 progression and therapy resistance in glioblastoma (GBM), a type of incurable brain
32 cancer. We previously identified lysyl oxidase (LOX) and olfactomedin like-3 (OLFML3)
33 as essential macrophage and microglia chemokines, respectively, in GBM. Here,
34 single-cell transcriptomics and multiplex sequential immunofluorescence followed by
35 functional studies demonstrate that macrophages negatively correlate with microglia
36 in the GBM tumor microenvironment. LOX inhibition in *PTEN*-deficient GBM cells
37 upregulates OLFML3 expression via the NF- κ B-PATZ1 signaling pathway, inducing a
38 compensatory increase of microglia infiltration. Dual targeting macrophages and
39 microglia via inhibition of LOX and the CLOCK-OLFML3 axis generates potent anti-
40 tumor effects and offers a complete tumor regression in more than 60% of animals
41 when combined with anti-PD1 therapy in *PTEN*-deficient GBM mouse models. Thus,
42 our findings provide a translational triple therapeutic strategy for this lethal disease.

43 **Introduction**

44 Glioblastoma (GBM) is inevitably fatal and the most aggressive type of brain tumor in
45 adults, with a five-year survival rate of approximately 10% (1, 2). Although marked
46 progress has been achieved in understanding GBM pathogenesis, the prognosis of
47 GBM patients remains dismal and the median overall survival is still only 15–20 months
48 after initial diagnosis (3-6). The current standard of care for GBM includes maximal
49 safe surgical resection followed by radiation with concurrent temozolomide and
50 adjuvant temozolomide with the unfortunate development of treatment resistance (7-
51 9). In-depth studies of GBM genomics have yielded detailed atlases of oncogene and
52 tumor suppressor gene alterations (7, 10-12). *PTEN* loss occurs in about 30-40% of
53 GBM patients and 80-90% of GBM patients harbor alterations of the receptor tyrosine
54 kinase (RTK)/PI3K/PTEN pathway (10). Our recent studies have also identified
55 *CLOCK* as a potential oncogene that is amplified in about 5% of GBM cases (13).
56 Despite the substantial contribution of known genetic drivers in promoting GBM
57 development, targeted therapies such as those against RTK signaling have failed in
58 the clinic due to the intratumoral heterogeneity, which ensures the survival of
59 subpopulations of GBM cells in treated tumors (7, 14).

60

61 Increasing evidence shows that aberrant cancer-associated molecular
62 activities that result from gene alterations are not limited to cancer cells, but also
63 extend to stromal cells in the tumor microenvironment (TME) (15-17). Tumor-
64 associated macrophages and microglia (TAMs) are the most prominent immune cell
65 populations in the TME, which account for up to 50% of total cells in the GBM tumor
66 mass (18, 19). We have shown that *PTEN* inactivation and *CLOCK* overexpression in
67 GBM cells upregulate lysyl oxidase (LOX) and olfactomedin like 3 (OLFML3), which
68 trigger the infiltration of macrophages and microglia, respectively, into the GBM TME
69 (13, 20). Inhibition of LOX and *CLOCK*-OLFML3 axis markedly inhibits tumor growth
70 and suppresses macrophage and microglia infiltration in GBM mouse models (13, 20),
71 indicating that targeting LOX and *CLOCK*-OLFML3 axis are promising therapeutic

72 strategies for reducing the infiltration of immunosuppressive and tumor potentiating
73 macrophages and microglia into the GBM TME. However, the understanding of
74 functional relationship between macrophages and microglia in GBM is limited.

75

76 Immunotherapies, including immune checkpoint inhibitor (ICI) therapies, have
77 been shown to improve patient outcomes in multiple cancer types (21, 22). However,
78 emerging evidence demonstrates that such ICI therapies only produce modest clinical
79 benefit in GBM patients due to the presence of highly immunosuppressive cells (e.g.,
80 TAMs) in the TME (23-27). Genomic and transcriptomic analyses in GBM patient
81 tumors have shown that the presence of *PTEN* mutations and higher macrophage
82 abundance are associated with the lack of response to anti-PD1 therapy (28),
83 suggesting that TAMs might contribute to the development of anti-PD1 therapy
84 resistance in *PTEN*-deficient GBM. On the other hand, TAMs have been shown to
85 negatively impact the anti-tumor response of conventional therapies, such as
86 radiotherapy (29, 30). These findings support the importance of TAMs in affecting
87 therapy resistance; however, there is no effective therapeutic approach to target them
88 in the GBM TME.

89

90 In this study, we reveal that macrophages are negatively correlated with
91 microglia in the GBM TME. Specifically, suppressing macrophage infiltration in *PTEN*-
92 deficient GBM via LOX inhibition upregulates the expression of OLFML3 in GBM cells,
93 which induces a compensatory increase of microglia infiltration into the TME. We
94 hypothesized that blockade of macrophage infiltration and its compensatory effect on
95 microglia may result in a robust anti-tumor effect, which might be augmented when
96 combined with anti-PD1 therapy in *PTEN*-deficient GBM mouse models. Our
97 preclinical trials confirm that the triple therapy (LOX inhibition + CLOCK-OLFML3 axis
98 blockade + anti-PD1 therapy) leads to disease eradication in more than 60% of GBM-
99 bearing mice.

100

101 **Results**

102 **Targeting LOX improves the efficacy of anti-PD1 therapy in *PTEN*-deficient GBM.**

103 Our previous studies revealed that macrophage chemokine LOX is upregulated in
104 *PTEN*-deficient GBM cells (20). To confirm the expression pattern of LOX, we analyzed
105 the single-cell RNA sequencing (scRNA-Seq) data from GBM patient tumors (31) with
106 results showing that *LOX* was highly expressed in mesenchymal GBM cells, which
107 account for 29.23% of total malignant cells (**Figure 1, A-C**). *PTEN* deficiency is
108 common in mesenchymal GBM subtype, which harbors higher immunosuppressive
109 macrophages relative to classical and proneural GBMs (20, 32). To identify specific
110 immune cells linked to LOX expression in GBM, we audited the TCGA GBM tumors for
111 15 types of immune cells with validated gene set signatures (13, 20). Bone marrow-
112 derived macrophage (BMDM) and monocyte were identified as the top immune cell
113 types enriched in *LOX*-high tumors compared to *LOX*-low tumors. Conversely, an
114 activated CD8⁺ T cell signature was reduced in *LOX*-high tumors (**Figure 1D**). These
115 findings suggest a potential connection between LOX-regulated macrophages and
116 CD8⁺ T cells in GBM, which promoted us to explore the role of LOX inhibition in
117 regulating anti-tumor immune responses in *PTEN*-deficient GBM mouse models.

118

119 To confirm its role in regulating immune response *in vivo*, we developed GBM
120 mouse models by intracranial injection of CT2A (*PTEN*-deficient) or 005 GSC, a GSC
121 line harboring activated AKT (33, 34), and treated them with LOX neutralizing
122 antibodies or LOX inhibitor β -aminopropionitrile (BAPN), which showed an ability to
123 cross the blood-brain barrier (BBB) (**Supplemental Figure 1, A-C**).
124 Immunofluorescence (IF) staining demonstrated that treatment with BAPN or LOX
125 neutralizing antibodies in tumor-bearing C57BL/6 mice increased intratumoral CD8⁺ T
126 cells (**Supplemental Figure 1, D-I**) and activated CD8⁺ (CD8⁺CD69⁺) T cells (**Figure**
127 **1, E and F**). Given the role of PD-L1 in regulating immunosuppression in GBM, we
128 investigated whether LOX affects the expression of PD-L1 in *PTEN*-deficient GBM
129 cells (e.g., U87, CT2A, and *PTEN*CRISPR KO SF763 cells) and GSCs (e.g., 005 GSC,

130 GSC23, and GSC7-10) (20). The results showed that LOX inhibition genetically (e.g.,
131 shRNA-mediated LOX depletion) and pharmacologically (treatment with LOX inhibitor
132 BAPN) upregulated the expression of PD-L1 in *PTEN*-deficient GBM cells (**Figure 1,**
133 **G-J** and **Supplemental Figure 1, J-L**). Together, these findings led us to hypothesize
134 that LOX inhibition could improve the efficacy of anti-PD1 therapy in *PTEN*-deficient
135 GBM mouse models. Indeed, our results showed that BAPN treatment extended
136 survival of mice bearing CT2A and 005 GSC tumors and the anti-tumor effect was
137 further augmented when BAPN was combined with anti-PD1 therapy (**Figure 1, K** and
138 **L**).

139

140 **The negative association between macrophages and microglia in the GBM TME.**

141 Although our studies demonstrated that LOX inhibition alone and in combination with
142 anti-PD1 therapy can inhibit GBM progression, no mice cleared their tumors after the
143 treatment (**Figure 1, K** and **L**). We hypothesized that LOX inhibition-induced
144 impairment of macrophage infiltration might induce a compensatory change of other
145 immune cells in the GBM TME. To test this, we analyzed the scRNA-Seq data (31)
146 from glioma patient tumors with a focus on myeloid cells, which include macrophages,
147 microglia, monocytes, dendritic cells (DCs) and myeloid-derived suppressor cells
148 (MDSCs). Among them, macrophages and microglia are the dominant cell populations
149 (**Figure 2A**). By analyzing these myeloid cells in low-grade gliomas (LGG), newly
150 diagnosed GBM (ndGBM), and recurrent GBM (rGBM), we found that
151 macrophage/monocyte density was very low in LGG, increased in ndGBM, and highly
152 enriched in rGBM, whereas microglia showed the opposite expression pattern (**Figure**
153 **2B**), suggesting a negative correlation between them in glioma patient tumors. Further
154 analysis in GBM patient tumors revealed that the macrophage abundance was
155 negatively correlated with microglia in the TME (**Figure 2C** and **Supplemental Figure**
156 **2, A** and **B**). Next, we performed multiplex sequential immunofluorescence (SeqIF™)
157 to stain and image whole mount sections of tumors from IDH1-WT GBM patients in
158 continuity with the adjacent brain parenchyma (n = 3). The results showed that

159 P2RY12⁺ microglia were mostly distributed in the parenchyma and GBM margin,
160 whereas CD163⁺ macrophages were highly enriched in the tumors (**Figure 2D** and
161 **Supplemental Figure 2C**). Higher magnified view of tumor sections demonstrated that
162 CD163⁺ macrophages were distributed in the perivascular niches in the tumor and at
163 the brain interface (**Supplemental Figure 2, D and E**). More specifically in densely
164 cellular tumor regions, P2RY12⁺ microglia were absent when tumors harbor high
165 abundance of CD163⁺ macrophages (**Figure 2E**). Conversely, CD163⁺ macrophages
166 were relatively low when tumors have high infiltration of P2RY12⁺ microglia (**Figure**
167 **2F**).

168

169 **LOX inhibition reduces macrophage infiltration but upregulates OLFML3** 170 **expression and microglia infiltration in GBM.**

171 Given the critical role of PTEN-LOX signaling axis in triggering macrophage infiltration
172 (20), we investigated whether LOX inhibition can induce compensatory changes of
173 chemokines that might affect microglia infiltration. To this end, we performed RNA-Seq
174 profiling in U87 cells with *LOX* shRNA (sh*LOX*) versus shRNA control (shC). By
175 analyzing the RNA-Seq data as well as microarray data of SF763 cells with *PTEN*-KO
176 versus WT (20), we identified four genes (*OLFML3*, *LOXL1*, *ADAMTS9*, and *TGFA*)
177 that were upregulated by LOX knockdown and downregulated by *PTEN* KO in GBM
178 cells (**Figure 3, A and B**). Among them, OLFML3 attracted our attention since our
179 previous studies showed that OLFML3 is a microglia chemokine in GBM (13, 35).
180 Immunoblotting results confirmed that shRNA-mediated LOX knockdown in *PTEN*-
181 deficient GBM cells (e.g., U87 and *PTEN*-KO SF763 cells) and GSCs (e.g., GSC23
182 and GSC7-10) upregulated OLFML3 expression (**Figure 3C** and **Supplemental**
183 **Figure 3, A and B**). Similarly, pharmacologic inhibition of LOX using the inhibitor BAPN
184 increased the expression of OLFML3 in both human (e.g., U87, *PTEN*-KO SF763,
185 GSC23, and GSC7-10) and mouse (e.g., CT2A and 005 GSCs) GBM cells and GSCs
186 (**Figure 3, D and E** and **Supplemental Figure 3C**).

187

188 To confirm whether LOX inhibition-induced upregulation of OLFML3 could
189 affect microglia infiltration in the GBM TME, we first performed transwell migration
190 assays with results showing that the conditioned media (CM) from LOX-depleted or
191 inhibited U87 cells increased the migration ability of HMC3 microglia (**Figure 3, F and**
192 **G and Supplemental Figure 3, D and E**). Next, we overexpressed LOX in *PTEN*-WT
193 GL261 cells (**Figure 3H**) and then checked OLFML3 expression in control and LOX-
194 overexpressed (*Lox*-OE) cells and used CM from them to perform transwell migration
195 assay. The results showed that LOX overexpression downregulated OLFML3
196 expression in GBM cells (**Figure 3H**) and reduced the migration of SIM-A9 microglia
197 (**Supplemental Figure 3, F and G**). In addition to these *in vitro* studies, we analyzed
198 microglia and macrophage populations in control, LOX-inhibited, and LOX-
199 overexpressed tumors. The results from IF staining and flow cytometry showed that
200 BAPN-treated CT2A tumors had higher CX3CR1⁺ (IF) and CD45^{low}CD11b⁺CX3CR1⁺
201 (flow cytometry) microglia (**Figure 3, I-L**) and lower F4/80⁺ (IF) and
202 CD45^{high}CD11b⁺CD68⁺ (flow cytometry) macrophages (**Supplemental Figure 3, H-K**)
203 compared to control tumors. In contrast, LOX overexpression induced higher infiltration
204 of macrophages and lower OLFML3 expression and microglia infiltration in the GBM
205 TME (**Figure 3, M-P**). To further confirm whether this effect is dependent on GBM cells
206 or direct macrophage-microglia interaction, we used clodronate liposomes to deplete
207 macrophages in tumor-bearing mice. The results showed that LOX overexpression in
208 GL261 cells still downregulated OLFML3 expression and microglia infiltration in
209 macrophage-depleted tumors (**Supplemental Figure 3, L-O**).

210

211 Next, we aimed to examine whether inhibition of CLOCK-mediated OLFML3
212 expression and microglia infiltration will affect LOX expression and macrophage
213 biology. Immunoblotting results showed that shRNA-mediated depletion of CLOCK
214 and pharmacologic inhibition of CLOCK using the Rev-ErbA agonist SR9009 did not
215 affect the expression of LOX in QPP7 GSCs (*PTEN*-deficient), CT2A cells, and 005
216 GSCs (**Supplemental Figure 4, A-C**). Accordingly, the CM from SR9009-treated U87

217 cells did not change the migration ability of THP-1 macrophages (**Supplemental**
218 **Figure 4, D and E**). To confirm it *in vivo*, we developed GBM mouse model and
219 confirmed that SR9009 can cross the BBB (**Supplemental Figure 4, F-H**). Consistent
220 with our previous studies (13, 35), we found that inhibition of CLOCK using SR9009
221 reduced CX3CR1⁺ microglia (**Supplemental Figure 4, I and J**). However, SR9009
222 treatment did not affect F4/80⁺ macrophages (**Supplemental Figure 4, K and L**). As
223 evidenced by our recent publications (13, 20), here we further confirmed that SR9009
224 treatment impaired GBM cell proliferation, but LOX inhibition using BAPN had no such
225 effect (**Supplemental Figure 4, M-S**). Together, these findings encouraged us to
226 develop an effective therapeutic strategy by targeting the compensatory mechanism
227 between macrophages and microglia via simultaneously inhibiting LOX and the
228 CLOCK-OLFML3 axis. When we conducted the proof-of-principal combination of
229 BAPN and SR9009 in GBM-bearing mice, we observed a significant survival extension
230 relative to monotherapy in both CT2A and 005 GSC models (**Figure 4, A and B**). On
231 the histological level, proliferation marker Ki67 was dramatically decreased, whereas
232 apoptosis marker cleaved caspase 3 (CC3) was significantly increased in BAPN and
233 SR9009 combination treatment group compared to single treatment and control groups
234 (**Figure 4, C-E**).

235

236 **LOX affects OLFML3 expression via regulating the NF- κ B-PATZ1 signaling axis.**

237 To explore the potential mechanism for how LOX regulates OLFML3, we used GSEA
238 to catalog oncogenic signaling pathways modulated by LOX in U87 cells (shLOX
239 versus shC). The RELA_DN.v1_DN was identified as the top signature affected by
240 LOX (**Figure 5A and Supplemental Figure 5A**), suggesting the importance of LOX in
241 regulating NF- κ B pathway. The results from immunoblotting demonstrated that
242 shRNA-mediated depletion of LOX in U87 cells, *PTEN*-KO SF763 cells, GSC23, and
243 GSC7-10 significantly inhibited the NF- κ B subunit P65 and Phospho-P65 (**Figure 5B**
244 and **Supplemental Figure 5B**). To investigate the potential functional relevance of P65
245 in regulating OLFML3 expression in GBM cells, we treated shC and shLOX *PTEN*-KO

246 SF763 cells with the P65 inhibitor SC75741. The results showed that inhibition of P65
247 upregulated the expression of *OLFML3* in shC cells, but not in shLOX cells (**Figure**
248 **5C**).

249
250 To further identify LOX-regulated factors that can transcriptionally regulate
251 *OLFML3* in *PTEN*-null GBM cells, we overlapped the differential expressed genes
252 encoding human transcriptional factors (TFs) in U87 cells with shLOX versus shC and
253 in TCGA GBM tumors with *LOX*-low versus *LOX*-high. As a result, 22 potential TFs
254 were identified (**Figure 5D**), which were inserted into the JASPAR database (36) with
255 results showing that 10 of them can potentially bind to the *OLFML3* promoter. The
256 results from RT-qPCR assays in *PTEN*-null GBM cells, such as U87, *PTEN*-KO SF763
257 and U251 cells, revealed that *PATZ1* and *PRRX1* were upregulated upon shRNA-
258 mediated LOX depletion and the treatment with LOX inhibitor BAPN (**Figure 5, E and**
259 **F and Supplemental Figure 5, C and D**). Bioinformatics analyses in TCGA GBM
260 patient tumors demonstrated that *PATZ1* correlated negatively with *LOX*, whereas
261 *PRRX1* showed a positive correlation with *LOX* (**Supplemental Figure 5, E and F**).
262 The results from immunoblotting confirmed that depletion of LOX upregulated *PATZ1*
263 protein level in *PTEN*-KO SF763 cells and *PTEN*-deficient GSC23 and GSC7-10
264 (**Figure 5G and Supplemental Figure 5G**). Next, we aimed to confirm whether *PATZ1*
265 is regulated by P65 and whether *PATZ1* can bind to the promoter of *OLFML3* in *PTEN*-
266 null GBM cells. RT-qPCR demonstrated that P65 inhibition upregulated the expression
267 of *PATZ1* in shC, but not in shLOX *PTEN*-KO SF763 cells (**Figure 5H**), suggesting
268 that *PATZ1* is a downstream TF of the NF- κ B pathway. Based on the predicted binding
269 sites (**Figure 5I**), we designed 6 pairs of primers and performed ChIP-PCR assays
270 with results showing that *PATZ1* bound to the *OLFML3* promoter in *PTEN*-KO SF763
271 cells (**Figure 5J**). To further validate the function of NF- κ B-*PATZ1* signaling axis in
272 regulating *OLFML3* in GBM cells, we overexpressed *PATZ1* in *PTEN*-KO SF763 cells
273 (**Supplemental Figure 5H**) and found that *PATZ1* overexpression enhanced *OLFML3*
274 expression and abolished P65 activation-induced downregulation of *OLFML3* (**Figure**

275 **5K**). Conversely, shRNA-mediated PATZ1 depletion in *PTEN*-WT SF763 cells negated
276 P65 inhibition-induced upregulation of OLFML3 (**Figure 5L** and **Supplemental Figure**
277 **5I**). Given our previously studies have shown that OLFML3 can be transcriptionally
278 regulated by CLOCK in GBM, we investigated whether the regulatory effect of LOX-
279 NF- κ B-PATZ1 signaling axis on OLFML3 transcription is independent on CLOCK.
280 Immunoblotting results showed that LOX or P65 inhibition-induced OLFML3
281 upregulation was rescued by the treatment with SR9009 (**Supplemental Figure 5, J**
282 and **K**). Together, these findings suggest that inhibition of LOX upregulates OLFML3
283 via regulating the NF- κ B-PATZ1 signaling axis in *PTEN*-null GBM cells.

284

285 **Dual inhibition of LOX and CLOCK-OLFML3 axis activates anti-tumor immune**
286 **response and synergizes with anti-PD1 therapy.**

287 Similar to the survival benefits induced by LOX inhibition (**Figure 1, K** and **L**), we found
288 that CLOCK inhibition using SR9009 combined with anti-PD1 therapy resulted in
289 survival extension, but did not cure any tumor-bearing mice, in CT2A and 005 GSC
290 models (**Supplemental Figure 6, A** and **B**). Given the compensatory upregulation of
291 microglia upon LOX inhibition, we hypothesized that dual targeting macrophages and
292 microglia using BAPN and SR9009 would produce potent anti-tumor immunity in
293 *PTEN*-deficient GBM. IF staining demonstrated that intratumoral CD8⁺ T cells
294 (**Supplemental Figure 6, C** and **D**) and activated CD8⁺ (CD8⁺ CD69⁺) T cells (**Figure**
295 **6, A** and **B**) were increased upon the treatment with BAPN or SR9009, and these
296 enhancements were further heightened when these two treatments were combined.
297 Increases of activated CD8⁺ T cells induced by the treatment with BAPN, SR9009 and
298 their combination were confirmed by flow cytometry for CD45⁺CD3⁺CD8⁺CD69⁺ and
299 CD45⁺CD3⁺CD8⁺IFN- γ ⁺ activated T cells in both CT2A and 005 GSC tumors (**Figure**
300 **6, C-F** and **Supplemental Figure 6, E-I**). In preclinical trials, the triple therapy with
301 BAPN, SR9009 and anti-PD1 resulted in a significant survival extension in both CT2A
302 (**Figure 6G**) and 005 GSC models (**Figure 6H**). Notably, 63% and 67% of CT2A and
303 005 GSC tumor-bearing mice cleared their tumors after the therapy (**Figure 6, G** and

304 **H**). The triple therapy (BAPN + SR9009 + anti-PD1) activated T cell memory, as almost
305 all the mice that had previously cleared GBM tumors efficiently suppressed tumor
306 growth when re-challenged with CT2A cells or 005 GSCs and remained tumor-free
307 (**Figure 6, I and J**). Together, these findings suggest that the triple therapy targeting
308 macrophage and microglia infiltration, combined with anti-PD1 therapy, is a promising
309 therapeutic strategy for *PTEN*-deficient GBM.

310

311 Discussion

312 In this study, we uncover a mechanism underlying the negative correlation between
313 macrophages and microglia in the GBM TME, which provides the guidance for
314 designing an effective therapeutic strategy that involves dual targeting macrophages
315 and microglia, and in combination with anti-PD1 immunotherapy. We reveal that LOX
316 inhibition in *PTEN*-deficient GBM upregulates OLFML3 to induce a compensatory
317 increase of microglia infiltration into the GBM TME. Dual inhibition of LOX and CLOCK-
318 OLFML3 axis extends the survival of *PTEN*-deficient GBM-bearing mice and leads to
319 disease eradication in majority of tumor-bearing mice when combined with anti-PD1
320 therapy.

321

322 *PTEN* is a tumor suppressor gene that was originally isolated from a
323 homozygous deletion on chromosome 10q23 of human GBM (37, 38). *PTEN*
324 mutation/depletion is observed in about 30-40% of GBMs (10), which results in
325 PI3K/AKT pathway activation, contributing to tumor progression and radiotherapy
326 resistance (39). In addition to these cell intrinsic effects, recent studies revealed that
327 *PTEN* loss contributes to generate an immunosuppressive GBM TME through a variety
328 of mechanisms. For example, *PTEN* loss in GBM cells leads to immune escape via
329 inducing T cell apoptosis (40) and upregulating PD-L1 expression (41). Moreover, our
330 recent studies revealed that *PTEN* deletion in GBM cells results in upregulation of LOX,
331 which, in turn, triggers the infiltration of macrophages into the GBM TME (20). In this
332 study, we provide further evidence in *PTEN*-deficient GBM mouse models showing

333 that reducing macrophage infiltration via LOX inhibition enhances anti-tumor T cell
334 immunity and synergizes with anti-PD1 therapy. These *in vivo* results coupled with the
335 recent findings observed in GBM patients showing that *PTEN* mutations and
336 macrophage abundance are enriched in anti-PD1 therapy nonresponders compared
337 to responders (28), reinforces the importance of macrophages in regulating anti-PD1
338 therapy resistance and supports the treatment strategy of combining LOX inhibitors
339 and anti-PD1 therapy specifically in *PTEN*-deficient GBM. It will be important for future
340 studies to define the cut-points for *PTEN* deficiency in which these mechanisms are
341 operational to define a companion biomarker for clinical trial.

342

343 The robust infiltration of TAMs is one of the key GBM hallmarks (23, 42). Our
344 recent studies have identified *PTEN*-LOX and *CLOCK*-*OLFML3* axes as the key
345 factors responsible for the infiltration of macrophages and microglia, respectively (13,
346 20). However, the understanding of the relationship between macrophages and
347 microglia in the GBM TME is limited. Consistent with the recent findings (43), our
348 scRNA-Seq analysis in GBM patient tumors demonstrated that macrophages are
349 negatively correlated with microglia. In exploring the molecular mechanism underlying
350 this connection, we observed that macrophage chemokine LOX negatively regulates
351 the expression of microglia chemokine *OLFML3* in GBM cells by regulating the *NF-κB*-
352 *PATZ1* signaling axis. *In vivo*, suppressing macrophage infiltration via LOX inhibition
353 induces a compensatory increase of microglia, consistent with findings observed in
354 *Ccr2*-KO GBM tumors (43). These findings encouraged us to explore the possibility of
355 developing a combination therapy of suppressing the infiltration of both macrophages
356 and microglia via inhibition of LOX and *CLOCK*-*OLFML3* axis (13, 20) in *PTEN*-
357 deficient GBM mouse models. This hypothesis is supported by our data showing that
358 dual blockade of macrophage and microglia infiltration using BAPN and SR9009 (13,
359 20) generates higher anti-tumor activity relative to monotherapy. Given the known
360 immunosuppressive function of TAMs, increasing evidence shows that depleting and
361 reprogramming TAMs could synergize with ICIs in GBM (23, 25, 27, 35, 42, 44).

362 Previous efforts have centered on developing CSF1R inhibitors to deplete TAMs in
363 GBM, but the results showed that CSF1R inhibition only induces a transient anti-tumor
364 effect caused by the compensatory changes in macrophages after the treatment in
365 brain tumors (45, 46). Combined anti-CSF1R and anti-PD1 therapies in GBM mouse
366 models shows a modest effect to extend survival (47). Consistent with these preclinical
367 findings, a clinical trial with CSF1R inhibitor showed a minimal anti-tumor effect in
368 recurrent GBM (48). However, it should be noted that two mesenchymal GBM patients,
369 in tumors of which LOX expression and *PTEN* deficiency are high, showed extended
370 progression free survival in response to CSF1R inhibitor treatment (48). In this study,
371 our findings highlight that dual targeting macrophage and microglia infiltration using
372 BAPN and SR9009 coupled with anti-PD1 therapy produces robust anti-tumor effect
373 and leads to a sustained long-term anti-tumor memory response in *PTEN*-deficient
374 GBM mouse models.

375

376 In summary, our study not only reveals the molecular mechanism underlying
377 the macrophage-microglia connection in the GBM TEM, but also informs an effective
378 triple therapy for *PTEN*-deficient GBM. However, these findings should be interpreted
379 with caution given our study specifically focuses on *PTEN*-deficient GBM, which only
380 account for 30-40% of GBM cases. It will be interesting to determine whether the
381 conclusion of this study can be extended to *PTEN*-WT GBM. Moreover, the observed
382 effects of LOX and CLOCK inhibition on immune compartments (e.g., macrophages
383 and microglia) may relate to vascular changes in the GBM TME. Although our previous
384 studies have shown that LOX and CLOCK inhibition reduces tumor angiogenesis in
385 GBM (20, 49), further studies are needed to evaluate whether these treatments affect
386 vascular architecture and vessel leakage in GBM tumors.

387

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395

396 **Author Contributions**

397 Y.L. and J.W. performed most experiments. H.N. and A.B.H. performed multiplex
398 immunofluorescence. Y.L. performed single-cell sequencing data analysis. L.P. and
399 F.Z. helped with intracranial injection. F.K. and H.A. helped with immunoblotting and
400 ChIP-PCR experiments. P.C. conceived the project. Y.L. and P.C. wrote the manuscript.

401

402 **Competing Interest Statement**

403 Y.L. and P.C. are listed as inventors on a patent related to targeting the LOX and
404 CLOCK-OLFML3 signaling axis combining with or without anti-PD1 therapy or
405 radiotherapy. All other authors declare no competing interests.

406 **Methods and Materials**

407 **Sex as a biological variable**

408 Sex was not considered as a biological variable in this study.

409

410 **Cell culture**

411 The GBM cell lines U87, U251, SF763, and CT2A, as well as 293T cells were cultured
412 in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, #11995-065). The mouse
413 glioma cell line GL261 cells and SIM-A9 microglia were cultured in Dulbecco's Modified
414 Eagle Medium-Ham's F12 medium (Gibco, #10565-018). HMC3 microglia were
415 cultured in Eagle's Minimum Essential Medium (ATCC, #30-2003). THP-1
416 macrophages were cultured in RPMI 1640 medium (Gibco, #11875093). All cell lines
417 were cultured in the indicated medium containing 10% fetal bovine serum (FBS; Fisher
418 Scientific, #16140071) and 1:100 antibiotic-antimycotic (Gibco, #15140-122), and were
419 purchased from the American Type Culture Collection (ATCC). Human GSCs (GSC23
420 and GSC7-10) and mouse GBM tumor-derived 005 GSCs and QPP7 GSCs were
421 cultured in neural stem cell (NSC) proliferation media (Millipore, #SCM005) containing
422 20 ng/mL epidermal growth factor (EGF; PeproTech, #AF-100-15) and basic fibroblast
423 growth factor (bFGF; PeproTech, #100-18B). Human GSCs were gifted by Dr.
424 Frederick F. Lang from the Brain Tumor Center (The University of Texas MD Anderson
425 Cancer Center). 005 GSCs and QPP7 GSCs were provided by Dr. Samuel D. Rabkin
426 (Massachusetts General Hospital) and Dr. Jian Hu (The University of Texas MD
427 Anderson Cancer Center), respectively. We generated *PTEN* CRISPR KO in SF763
428 cells as described previously (20). All cells were confirmed to be mycoplasma-free and
429 were maintained at 37 °C and 5% CO₂. Cells were treated with BAPN (Sigma-Aldrich,
430 #B-A3134, 200 μM), SR9009 (Cayman, #11929, 5 μM), SC75741 (MedChemExpress,
431 #HY-10496, 5 μM), and/or NF-κB activator 1 (MedChemExpress, # HY-134476, 1 μM)
432 for 24 hrs for protein expression analysis or 8 hrs for mRNA expression analysis.

433

434 **Mice and intracranial xenograft tumor models**

435 Female C57BL/6 mice at 3 to 4 weeks of age were purchased from the Jackson
436 Laboratory (#0000664). All animals were grouped by 5 mice per cage and maintained
437 in IVC System (San Diego, CA) for a week before the experiment. The intracranial
438 xenograft tumor models were established as described previously (13, 49, 50). In brief,
439 mice were anesthetized by isoflurane through IMPAC6 Anesthesia System. Then a
440 dental drill was used to open a small hole in the skull of mice 1.2 mm anterior and 3.0
441 mm lateral to the bregma. Mice were placed into the stereotactic apparatus, and 5 μ L
442 005 GSC, CT2A, or GL261 cells in FBS-free culture medium were injected into the
443 right caudate nucleus 3.0 mm below the surface of the brain using a 10 μ L Hamilton
444 syringe with an unbeveled 30-gauge needle. The incision was closed using Vetbond
445 glue. Meloxicam (20 mg/kg, daily) was subcutaneously injected for pain relief for 3
446 days after surgery. Mice were assigned into different groups under blinded conditions
447 after a week of intracranial injection and received treatments with BAPN (2 g/L in
448 drinking water) on day 4, SR9009 (100 mg/kg/day, i.p.) for 10 days beginning at day 7
449 post-orthotopic injection, anti-PD1 (10 mg/kg body weight, i.p.) on days 11, 14, and
450 17 post-orthotopic injection, and/or clodronate liposomes (200 μ L, once every 3 days)
451 starting at day 4 post-orthotopic injection. Mice with neurological deficits or moribund
452 appearance were sacrificed according to the IACUC protocol. At the end of the
453 experiment, the brains of mice were collected, either fixed in 4% paraformaldehyde
454 (PFA) (ThermoFisher Scientific, #J61899.AK) after transcardiac perfusion with PBS for
455 optimal cutting temperature (OCT)-cryosectioning or processed using the percoll
456 density gradient cell separation method to isolate tumor-derived immune cells for flow
457 cytometry analysis.

458

459 **Mass spectrometry**

460 A high-performance liquid chromatography tandem mass spectrometry (LC-MS/MS)
461 assay was developed to quantify BAPN and SR9009 in plasma and brain of C57BL/6
462 mice. Specifically, the blood and brain tissues were collected after 1, 2, 4, and 8 hrs of
463 the administration of BAPN (2 g/L in drinking water) or SR9009 (100 mg/kg body weight,

464 i.p.). The plasma was generated using the standard centrifugation techniques, and the
465 brain tissues were pulverized by cryogenic grinding with liquid nitrogen. The plasma
466 and brain tissue samples were mixed with internal standards, deproteinized with MeOH,
467 and processed into LC-MS/MS to test the concentration of BAPN or SR9009. The
468 analysis was performed at the Mass Spectrometry Core in Research Resources
469 Center of University of Illinois at Chicago.

470

471 **Computational analysis of human GBM datasets**

472 For analysis of human GBM data, we downloaded the gene expression data of TCGA
473 datasets (Agilent-4502A and/or HG-U133A microarrays) from GlioVis:
474 <http://gliovis.bioinfo.cnio.es/>. The expression, correlation, and GSEA of interesting
475 genes and gene signatures in GBM patients were performed.

476

477 **GSEA analysis**

478 GSEA software 4.1.0 (http://www.broad.mit.edu/gsea/software/software_index.html)
479 from the Broad Institute was used. The gene expression data from microarray data of
480 public available GEO and our newly generated RNA sequencing data of U87 cells were
481 used for performing GSEA. The gene Ontology Biological Process (GOBP) signatures
482 were downloaded from the Molecular Signatures Database (51). The normalized
483 enrichment score (NES) and false discovery rate (FDR) were acquired by the analysis,
484 with FDR < 0.25 was considered statistically significant.

485

486 **Single-cell sequencing data analysis**

487 The scRNA-seq data of GEO, GSE131928 (31), were used to analyze expression
488 pattern of *LOX* in glioma cells and the distribution of myeloid cells, including
489 macrophages, monocytes, microglia, and DCs, in tumors of glioma patients (LGG,
490 ndGBM and rGBM). Based on their abundance in GBM tumors, the correlation
491 between macrophages and microglia was analyzed.

492

493 **Plasmids and viral transfections**

494 For gene knockdown, short hairpin RNA (shRNA) targeting human *LOX* and *PATZ1*
495 and mouse *Clock* in the pLKO.1 vector (Sigma-Aldrich, #SHC001) were used.
496 Lentiviral particles were generated as we described previously (13, 20). In brief, 8 µg
497 of the shRNA plasmid, 4 µg of the psPAX2 plasmid (Addgene, #12260), and 2 µg of
498 the pMD2.G plasmid (Addgene, #12259) were transfected into 293T cells plated in
499 100-mm dishes using Lipofectamine 2000 (Invitrogen, #13778150). Supernatant with
500 lentiviral particles was collected and filtered at 48 and 72 hrs after transfection. Cells
501 were infected with viral supernatant containing 10 µg/mL polybrene (Millipore, #TR-
502 1003-G). After 48 hrs, cells were selected by puromycin (10 µg/mL; Millipore, #540411)
503 and tested for the expression of *LOX*, *PATZ1*, and *CLOCK* by immunoblots. The
504 following human and mouse shRNA sequences (*LOX*: #3: TRCN0000286463 and #4:
505 TRCN0000286532; *PATZ1*: #4: TRCN0000274379 and #5: TRCN0000274416; and
506 *Clock*: #1: TRCN0000095686 and #2: TRCN0000306474) were selected for further
507 use following the validation.

508

509 For gene overexpression, plasmids of human Tagged Lenti ORF Clone of *PATZ1*
510 (Origene, #RC211869L4) and mouse Tagged Lenti ORF Clone of *Lox* (Origene,
511 #MR206463L4) were used. These plasmids were transformed into high-efficiency
512 chemically competent *Escherichia coli* cells (ThermoFisher Scientific, #C737303) and
513 recovered in Lysogenia broth (LB, Fisher BioReagents, # BP9723). Post-recovery, LB
514 containing *E. coli* transformants were plated on LB selection plates containing 34
515 µg/mL chloramphenicol (Fisher BioReagents, #BP904) for 16 hrs of incubation at 37 °C
516 to select clones containing the gene expression vectors. The selected colonies were
517 picked from the selection plates for inoculation in LB broth supplemented with 34
518 µg/mL chloramphenicol to maintain the selection, and then further purified for plasmid
519 DNA using a QIAprep Spin Miniprep Kit (Qiagen, #27106). Purified plasmids were
520 transfected into cells using lentiviral transfection methodology as previously described
521 (13, 20, 25).

522

523 **Immunofluorescence**

524 Immunofluorescence was performed using a standard protocol as previously
525 described (13, 20). In brief, slides from cryosections were kept at room temperature
526 for 30 min and fixed in 10% PFA for 30 min prior to permeabilization. Then, 0.25%
527 Triton X-100 (Sigma-Aldrich, #9036-19-5) in PBS was added for 30 min at room
528 temperature to permeabilize the cell membrane. After three times PBS washing,
529 sections were blocked by 5% goat serum for 30 min. Specimens were incubated with
530 primary antibody or PBS control for 1 hr at room temperature and then overnight at
531 4 °C. Then, the unbound primary antibodies were washed out by three times PBS for
532 3 min each and corresponding secondary antibody cocktails were prepared and added
533 to the sections for 1 hr incubation. Cell nuclear was counterstained with DAPI/anti-fade
534 mounting medium (Vector Laboratories, #H-1200-10). Immunofluorescence images
535 were captured using Nikon AX/AXR Confocal Microscope System with an apo 60 1.40
536 Oil 160/0.17 objective in the Center for Advanced Microscopy (CAM) at Northwestern
537 University. For one slide, 3-5 fields of images were captured randomly, and the
538 intensity of the protein signal was determined by Image J. The average quantified value
539 of these 3-5 fields was represented as the protein signal intensity of one sample and
540 presented as the individual point in the bar graph. The number of replicates for each
541 experiment is indicated in the figure legends. Antibodies specific to CD8 (Invitrogen,
542 #PA5-81344), CD69 (Santa Cruz Biotechnology, #sc-373799), CX3CR1 (Invitrogen,
543 #702321), F4/80 (Cell Signaling Technology, #30325S), OLFML3 (Invitrogen,
544 #702321), Ki67 (Cell Signaling Technology, #9129S), and CC3 (Cell Signaling
545 Technology, #9661S) were used.

546

547 **Sequential immunofluorescence (SeqIF™) multiplexing and microscopy**

548 GBM patients' formalin-fixed paraffin-embedded (FFPE) samples were collected at
549 Northwestern University, and pathologically segmented and graded by the study
550 neuropathologist (CMH). Automated multiplexed seqIF staining and imaging were

551 performed on these sections using the COMET™ platform (Lunaphore Technologies).
552 The multiplexed panel was comprised of 4 antibodies: GFAP (Abcam, #ab68428),
553 CD31 (Abcam, #ab182981), P2RY12 (Atlas Antibodies, #HPA014518), and CD163
554 (Abcam, # ab182422). The 4-plex protocol was generated using the COMET™ Control
555 Software, and reagents were loaded onto the COMET™ device to perform seqIF. All
556 antibodies were validated using conventional IHC and/or IF staining in conjunction with
557 corresponding fluorophores and DAPI (ThermoFisher Scientific, #D21490). For
558 optimal concentration and best signal-to-noise ratio, all antibodies were tested at 3
559 different dilutions: starting with the manufacturer-recommended dilution (MRD),
560 MRD/2, and MRD/4. Secondary Alexa fluorophore 555 (Invitrogen, #A32732) and
561 Alexa fluorophore 647 (Invitrogen, #A32733) were used at 1:200 or 1:400 dilutions,
562 respectively. The optimizations and full runs of the multiplexed panel were executed
563 using the technology integrated in the Lunaphore COMET™ platform (characterization
564 2 and 3 protocols, and seqIF™ protocols, respectively). The seqIF™ workflow was
565 parallelized on a maximum of 4 slides, with automated cycles of iterative staining of 2
566 antibodies at a time, followed by imaging, and elution of the primary and secondary
567 antibodies, with no sample manipulation during the entire workflow. All reagents were
568 diluted in Multistaining Buffer (BU06, Lunaphore Technologies). The elution step lasted
569 2 min for each cycle and was performed with Elution Buffer (BU07-L, Lunaphore
570 Technologies) at 37 °C. Quenching lasted for 30 sec and was performed with
571 Quenching Buffer (BU08-L, Lunaphore Technologies). Staining was performed with
572 incubation times set at 4 and 2 min for primary antibodies and secondary antibodies,
573 respectively. Imaging was performed with an integrated epifluorescent microscope at
574 20X magnification with Imaging Buffer (BU09, Lunaphore Technologies) and exposure
575 times set for DAPI 80 ms, Cy5 200 ms, TRITC 400 ms. Image registration was
576 performed immediately after concluding the staining and imaging procedures by
577 COMET™ Control Software. Each seqIF™ protocol resulted in a multi-layer OME-TIFF
578 file where the imaging outputs from each cycle were stitched and aligned. COMET™
579 OME-TIFF files contain a DAPI image, intrinsic tissue autofluorescence in TRITC and

580 Cy5 channels, and a single fluorescent layer per marker. The intrinsic tissue
581 autofluorescence signals were subtracted from the subsequent cycles and the markers
582 were subsequently pseudocolored for visualization of multiplexed staining results in
583 the Viewer from Lunaphore.

584

585 **Hematoxylin and Eosin (H&E) staining**

586 Staining was performed using the H&E staining kit (Abcam, #ab245880) according to
587 a standard protocol. In brief, the FFPE sections were baked at 65 °C for 2 hrs and then
588 were subjected to xylene and ethanol for deparaffinization and rehydration. After that,
589 the sections were incubated with hematoxylin, Mayer's (Lillie's Modification) for 5 min,
590 and then incubated with the Bluing Reagent and Eosin Y Solution (Modified Alcoholic)
591 for 15 s and 3 min, respectively. After washing, slides were dehydrated in three
592 changes of absolute alcohol and the images of tissue sections were captured using
593 TissueFAXS in the CAM at Northwestern University.

594

595 **Quantitative real-time PCR (RT-qPCR)**

596 Cells were pelleted, and RNA was isolated with the RNeasy Mini Kit (Qiagen, #74106),
597 as we previously described (13, 20). RNA was quantified by NanoDrop
598 spectrophotometers and then the All-In-One 5X RT MasterMix (Applied Biological
599 Materials, #G592) was used to reverse-transcribe RNA into cDNA in T100 Thermal
600 Cycler (Bio-Rad). RT-qPCR was performed with the use of SYBR Green PCR Master
601 Mix (Bio-Rad, #1725275) in CFX Connect Real-Time PCR Detection System (Bio-Rad).
602 Primers used for RT-qPCR were listed in **Supplemental Table 1**. The expression of
603 each gene was normalized to that of housekeeping gene *GAPDH*.

604

605 **ChIP-PCR**

606 ChIP-PCR was performed using the commercial Pierce™ Magnetic CHIP kit
607 (ThermoFisher Scientific, #26157) as we described previously (49). In brief, *PTEN*-KO
608 SF763 cells were cross-linked with 1% PFA for 10 min, and then reactions were

609 quenched using the glycine solution for 5 min at room temperature. Cells were then
610 lysed with membrane extraction buffer for 10 min on ice, and the chromatin
611 fragmentation was generated by Mnase digestion followed by sonication using three
612 20-sec pulses at 3-watt power. After that, the solubilized chromatin was incubated with
613 PATZ1 (Santa Cruz Biotechnology, #sc-393223 X) antibody overnight at 4 °C followed
614 by 2 hrs incubation with CHIP Grade Protein A/G Magnetic Beads with mixing. Immune
615 complexes were then washed with IP Wash Buffer I three times and IP Wash Buffer II
616 once. Elution Buffer was added to the sample for elution at 65°C for 30 min. Then
617 proteinase K (20 mg/mL) and NaCl (5M) were added for reverse-crosslinking at 65°C
618 for 1.5 hrs. Eluted DNA was purified using DNA Clean-Up Column and then used to
619 perform PCR. The *OLFML3* primers were designed according to the E-box of the
620 human *OLFML3* gene and were listed in **Supplemental Table 1**.

621

622 **Immunoblotting**

623 The protein expression of cells was tested by immunoblotting analysis as we described
624 previously (13, 20). In brief, cells were lysed on ice with RIPA lysis buffer (Thermo
625 Fisher Scientific, #89900) supplemented with Protease Inhibitor Cocktail (Thermo
626 Fisher Scientific, #78429). BCA Protein Assay Kit (ThermoFisher Scientific, #PI23225)
627 was used to measure protein concentration. Protein solution was mixed with the LDS
628 sample buffer and heated at 95 °C for 10 min. After that, protein samples were loaded
629 to SurePAGE gels (GenScript, #M00653) and then transferred to 0.2 µm nitrocellulose
630 (NC) membrane (Bio-Rad, #1620112) using a preprogrammed standard protocol for
631 30 min in the Trans-Blot Turbo system (Bio-Rad). NC membranes were blocked using
632 5% dry milk in TBST for 1 hr at room temperature and then incubated with primary
633 antibodies (1:1,000 dilution) overnight at 4 °C. After washing three times, membranes
634 were incubated with HRP-conjugated anti-mouse (Cell Signaling Technology, #7076S)
635 or anti-rabbit (Cell Signaling Technology, #7074S) secondary antibodies for 2 hrs at
636 room temperature. After washing, membranes were incubated with ECL substrate and
637 imaged under ChemiDoc Touch Imaging System (Bio-Rad). Antibodies were

638 purchased from the indicated companies, which include β -actin (Cell Signaling
639 Technology, #3700S), LOX (Abcam, #ab174316), CLOCK (Cell Signaling Technology,
640 #5157S), OLFML3 (Abcam, #ab111712), PD-L1 (Cell Signaling Technology, #64988S),
641 P-P65 (Cell Signaling Technology, #3033S), P65 (Cell Signaling Technology, #8242S),
642 and PATZ1 (Santa Cruz, #sc-393223 X).

643

644 **Migration assay**

645 HMC3 microglia (5×10^4) were suspended in serum-free culture medium and seeded
646 into 8.0 μm (Corning, #3422) inserts. SIM-A9 microglia (1×10^5) and THP-1
647 macrophages (1×10^5) were suspended in serum-free culture medium and seeded
648 into 5.0 μm (Corning, #3421) inserts. The CM from LOX-depleted or inhibited U87 cells,
649 LOX-overexpressed GL261 cells, or SR9009-treated U87 cells were added to the
650 receiver wells, respectively. After 10 hrs, migrated cells were fixed with 4% PFA
651 (ThermoFisher, #J61899.AK) for 30 min and stained with crystal violet (Sigma-Aldrich,
652 #C-3886) for another 30 min. The membrane inserts were washed with water and
653 imaged under an EVOS microscope. The number of transferred cells was counted
654 using ImageJ.

655

656 **Tumor-derived immune cells isolation**

657 Mice with neurologic deficits or moribund appearance were sacrificed to harvest their
658 brains. Immune cells in the brain tumors were isolated using the percoll density
659 gradient cell separation method as we previously described (35). In brief, after
660 perfusion with PBS, brains were homogenized on ice with pre-cold 10 ml HBSS. Then
661 cells were spun down at 1,500 rpm for 10 min at 4 °C, and were resuspended in 30%
662 Percoll (GE Healthcare, #17-0891-01). The solution was gently laid on top of the 70%
663 Percoll and centrifuged at 1,200 g for 30 min at 4 °C with accelerator 7 and breaker 0.
664 After removing myelin and debris, the interphase was collected and centrifuged at
665 1,500 rpm for 10 min at 4 °C. The cell pellet was resuspended for further analysis.

666

667 **Flow cytometry**

668 The single-cell suspensions were incubated with fixable viability dye (Invitrogen,
669 #5211229035) on ice for 10 min. After washing with FACS buffer (PBS with 1% BSA),
670 cells were incubated with the TruStain FcX (anti-mouse CD16/32) Antibody
671 (BioLegend, #103132) and True-Stain Monocyte Blocker (BioLegend, #426102) in 5%
672 BSA for 30 min on ice to block Fc receptors and non-specific binding of the cyanine
673 acceptor fluorophores. Different antibody cocktails, including PerCP/Cy5.5 anti-mouse
674 CD45 (BioLegend, #103132), AF488 anti-mouse CD3 (BioLegend, #100210), BV711
675 anti-mouse CD8 (BioLegend, #100747), PE/Cy7 anti-mouse CD69 (BioLegend,
676 #104512), APC/Cy7 anti-mouse IFN- γ (BioLegend, #505850), PE/Cy7 anti-
677 mouse/human CD11b (BioLegend, #101216), PE anti-mouse CD68 (BD Bioscience,
678 #566386), and BV421 anti-mouse CX3CR1 (BD Bioscience, #567531) were added to
679 the samples and incubated for 30 min on ice. After washing with FACS buffer, cells
680 were incubated with fixation buffer (BioLegend, #420801) overnight. Samples were
681 read through the BD FACSymphony or BD LSRFortessa flow cytometer and analyzed
682 in FlowJo v10.8.1.

683

684 **Proliferation (CFSE) assay**

685 Cell proliferation was assessed using the CellTrace carboxy fluorescein succinimidyl
686 ester (CFSE) Cell Proliferation Kit (Invitrogen, #C34554). Briefly, 1×10^6 cells were
687 collected and incubated with CFSE working solution (1:1,000) for 20 min at 37 °C. The
688 staining was stopped by adding complete cell culture media. After washing, cells were
689 cultured for 3 days with or without the treatment of BAPN (Sigma-Aldrich, #B-A3134,
690 200 μ M), or SR9009 (Cayman, #11929, 5 μ M) in the dark and used for flow cytometry
691 analysis. The percentage of CFSE positive peaks over the undivided peak (generation
692 0) was analyzed using FlowJo v10.8.1.

693

694 **Colony formation assay**

695 1×10^3 GBM cells were seeded in each well of 6-well plates with or without the

696 treatment of BAPN (Sigma-Aldrich, #B-A3134, 200 μ M), or SR9009 (Cayman, #11929,
697 5 μ M). After 7-10 days, cells were fixed and stained with 0.5% crystal violet in 25%
698 methanol for 1 hr. After three times washing by PBS, the plates were scanned, and the
699 colony number was counted using ImageJ.

700

701 **Patient samples**

702 Tumor samples from surgically resected IDH-WT GBMs were collected at
703 Northwestern Memorial Hospital. Three ndGBM patients (#ITA-13, male, 62-year-
704 old; #ITA-19, female, 50-year-old; and #ITA-26, female, 50-year-old) were
705 diagnosed according to the WHO diagnostic criteria. Formalin fixed paraffin
706 embedded blocks and slides were prepared and handled by the Northwestern
707 Central Nervous System Tumor Bank.

708

709 **Statistical analysis**

710 Statistical analyses were performed with one-way ANOVA tests for comparisons
711 among groups and Student *t*-tests for comparisons between two groups. Data were
712 represented as mean \pm SD. Correlation analysis was conducted using the Pearson
713 test to determine the Pearson correlation coefficient (*R*-value) and *P*-value. The
714 survival analysis for animal models was determined by conducting Log-rank (Mantel-
715 Cox) test. All statistical analyses were performed using GraphPad Prism 10 (GraphPad
716 Software, USA). The *P* values were designated as *, *P* < 0.05; **, *P* < 0.01; ***, *P* <
717 0.001; and n.s., not significant (*P* > 0.05).

718

719 **Study approval**

720 All animal experiments were performed with the approval of the Institutional Animal
721 Care and Use Committee (IACUC) at Northwestern University. The human tissue
722 protocol (STU00214485) was approved by the Institutional Review Board (IRB) at
723 Northwestern University.

724

725 **Data availability**

726 The scRNA-seq data of GBM patient tumors were obtained from the GEO database
727 (GSE131928). The human TCGA GBM data are available at GlioVis:
728 <http://gliovis.bioinfo.cnio.es/>. The microarray data of SF763 cells with *PTEN*-KO versus
729 WT were obtained from the GEO database (GSE122284). The RNA-Seq data of U87
730 cells with shLOX versus shC are provided in the Supplemental Table 2. Values for all
731 data points in graphs are reported in the Supporting Data Values file.

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846

847 **Figure Legends**

848 **Figure 1. LOX inhibition improves the efficacy of anti-PD1 therapy.**

849 (A) High-resolution uniform manifold approximation and projection (UMAP)
850 dimensional reduction of different subtypes, including mesenchymal-like (MES-like),
851 neural-progenitor-like (NPC-like), astrocyte-like (AC-like) and oligodendrocyte-
852 progenitor-like (OPC-like), of tumor cells from GBM patient tumors based on the single-
853 cell RNA sequencing (scRNA-Seq) dataset (GSE182109). (B) Pattern representing
854 single-cell gene expression of *LOX* in distinct subtypes of tumor cells based on above
855 scRNA-Seq dataset. (C) Percentage MES-like GBM cells out of total GBM cells, and
856 normalized *LOX* gene expression in different subtypes of malignant cells in GBM
857 patient tumors based on above scRNA-Seq dataset. (D) GSEA analysis for various
858 types of immune cells in *LOX*-high (n = 123) and *LOX*-low (n = 122) patient tumors
859 from the TCGA GBM database. (E and F) Immunofluorescence (E) and quantification
860 (F) of relative CD8⁺CD69⁺ T cells in tumors from CT2A tumor-bearing mice treated with
861 or without *LOX* inhibitor BAPN (2 g/L in drinking water) on day 4. Scale bar, 50 μm. n
862 = 3 independent samples. Student's t test. (G and H) Immunoblots for PD-L1 and *LOX*
863 in lysates of U87 (G) and *PTEN*-KO SF763 (H) cells expressing shRNA control (shC)
864 and *LOX* shRNAs (sh*LOX*). (I) Immunoblots for PD-L1 in lysates of U87 and *PTEN*-
865 KO SF763 cells treated with BAPN at indicated concentrations. (J) Immunoblots for
866 PD-L1 in lysates of CT2A cells and 005 GSCs treated with BAPN at indicated
867 concentration. (K and L) Survival curves of C57BL/6 mice implanted with CT2A cells
868 (2×10^4 cells/mouse, K) or 005 GSCs (2×10^5 cells/mouse, L). Mice were treated with
869 BAPN (2 g/L in drinking water) on day 4, and then received the treatment with IgG or
870 anti-PD1 (10 mg/kg body weight, i.p.) on days 11, 14, and 17. n = 5-7 mice per group.
871 Log-rank test. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

872

873 **Figure 2. Macrophages negatively related to microglia in GBM tumors.**

874 (A) High-resolution UMAP dimensional reduction of myeloid cells, including
875 macrophages, microglia, monocytes (Mono), dendritic cells (DCs), and myeloid-

876 derived suppressor cells (MDSCs), from GBM patient tumors based on the single-cell
877 RNA sequencing (scRNA-Seq) dataset (GSE182109). **(B)** Percentage of different
878 types of myeloid cells in tumors of low-grade gliomas (LGG), newly diagnosed GBM
879 (ndGBM) and recurrent GBM (rGBM) based on above scRNA-Seq data. **(C)**
880 Correlation between macrophages and microglia in the GBM TME based on the above
881 scRNA-Seq data. Pearson test. **(D)** Representative image of multiplex sequential
882 immunofluorescence showing the distribution of P2RY12⁺ microglia, CD163⁺
883 macrophages, GFAP⁺ tumor cells and CD31⁺ blood vessels in the tumor edges and
884 tumors from IDH1-WT GBM patients. Scale bar, 500 μ m. **(E and F)** Higher magnified
885 view of CD163⁺ macrophages and P2RY12⁺ microglia in the tumor edges and tumors
886 from IDH1-WT GBM patients. Scale bar, 100 μ m.

887

888 **Figure 3. LOX negatively regulates OLFML3 expression and microglia infiltration**
889 **in GBM.**

890 **(A and B)** Identification (A) and expression heatmap (B) of four overlapping PTEN-
891 LOX axis-regulated genes encoding secreted factors in *PTEN*-KO versus WT SF763
892 cells and in *LOX* shRNA (sh*LOX*) versus shRNA control (shC) U87 cells. Red signal
893 indicates higher expression, and blue signal denotes lower expression. **(C)**
894 Immunoblots for OLFML3 and LOX in lysates of U87 and *PTEN*-KO SF763 cells
895 expressing shC and sh*LOX*. **(D and E)** Immunoblots for OLFML3 in lysates of U87 and
896 *PTEN*-KO SF763 cells (D), and CT2A cells and 005 GSCs (E) treated with BAPN at
897 indicated concentrations. **(F and G)** Representative images (F) and quantification (G)
898 of relative migration of HMC3 microglia following stimulation with the conditioned
899 media (CM) from U87 cells pretreated with or without BAPN (200 μ M). Scale bar, 400
900 μ m. n = 3 independent samples. Student's t test. **(H)** Immunoblots for OLFML3 and
901 LOX in lysates of GL261 cells in the presence or absence of LOX overexpression (OE).
902 **(I and J)** Immunofluorescence (I) and quantification (J) of relative CX3CR1⁺ microglia
903 in tumors from CT2A-bearing mice treated with or without BAPN (2 g/L in drinking
904 water) on day 4. Scale bar, 50 μ m. n = 3 independent samples. Student's t test. **(K and**

905 **L)** Representative images (**K**) and quantification (**L**) of flow cytometry for the
906 percentage of intratumoral CD45^{low}CD11b⁺CX3CR1⁺ microglia in size matched tumors
907 from CT2A tumor-bearing mice treated with or without BAPN. n = 3 independent
908 samples. Student's t test. (**M-P**) Immunofluorescence (**M**) and quantification of relative
909 F4/80⁺ macrophages (**N**), CX3CR1⁺ microglia (**O**), and OLFML3⁺ cells (**P**) in tumors
910 from mice implanted with control and LOX-overexpressed GL261 cells. Scale bar, 50
911 μ m. n = 3 independent samples. Student's t test. *, $P < 0.05$; **, $P < 0.01$; and ***, $P <$
912 0.001.

913

914 **Figure 4. Dual Inhibition of LOX and CLOCK-OLFML3 axis exhibits a potent anti-**
915 **tumor effect in GBM mouse models.**

916 (**A and B**) Survival curves of C57BL/6 mice implanted with CT2A cells (2×10^4
917 cells/mouse, A) or 005 GSCs (2×10^5 cells/mouse, B). Mice were treated with BAPN
918 on day 4, and/or SR9009 (100 mg/kg/day, i.p) for 10 days beginning at day 7. n = 5-7
919 mice per group. Log-rank test. (**C-E**) Representative (**C**) and quantification (**D and E**)
920 of immunofluorescence staining of Ki67 and cleaved caspase 3 (CC3) in tumors from
921 CT2A-bearing mice treated with or without BAPN and SR9009. Scale bars, 50 μ m. n
922 = 3 independent samples. One-way ANOVA test. *, $P < 0.05$; **, $P < 0.01$; and ***, $P <$
923 0.001.

924

925 **Figure 5. LOX regulates OLFML3 expression through regulating the NF- κ B-**
926 **PATZ1 signaling axis.**

927 (**A**) GSEA analysis on RNA-seq data of U87 cells with LOX shRNA knockdown (shLOX)
928 versus shRNA control (shC) shows top 10 enriched oncogenic signaling pathways. (**B**)
929 Immunoblots for P-P65, P65, and LOX in lysates of U87 and PTEN-KO SF763 cells
930 expressing shC and shLOX. (**C**) Relative mRNA expression of OLFML3 in PTEN-KO
931 SF763 cells expressing shC and shLOX treated with or without P65 inhibitor (P65i)
932 SC75741 (5 μ M). n = 3 independent samples. Student's t test. (**D**) Identification of 22
933 overlapping transcription factors (TFs) in TCGA GBM tumors (LOX-low versus -high)

934 and U87 cells (shLOX versus shC). (E) Relative mRNA expression of 10 TFs in *PTEN*-
935 KO SF763 cells expressing shC and shLOX. n = 3 independent samples. One-way
936 ANOVA test. (F) Relative mRNA expression of the 10 TFs in U87 cells treated with or
937 without LOX inhibitor BAPN (200 μ M). n = 3 independent samples. Student's t test. (G)
938 Immunoblots for PATZ1 in lysates of *PTEN*-KO SF763 cells expressing shC and
939 shLOX. (H) Relative mRNA expression of *PATZ1* in *PTEN*-KO SF763 cells expressing
940 shC and shLOX and treated with or without P65i SC75741 (5 μ M). n = 3 independent
941 samples. Student's t test. (I) Schematic of designing CHIP-qPCR primers based on 3
942 potential binding sites. (J) Quantification of PATZ1 CHIP-qPCR in the *OLFML3*
943 promoter of *PTEN*-KO SF763 cells. IgG was used as the control. n = 3 independent
944 samples. Student's t test. (K) Immunoblots for OLFML3 in lysates of *PTEN*-KO SF763
945 cells with or without PATZ1 overexpression (OE) and treated with or without P65
946 activator (+). (L) Immunoblots for OLFML3 in lysates of *PTEN*-WT SF763 cells
947 expressing shC and sh*PATZ1* treated with or without P65i SC75741 (5 μ M). *, $P <$
948 0.05; **, $P <$ 0.01; ***, $P <$ 0.001; and n.s., not significant ($P >$ 0.05).

949

950 **Figure 6. Dual Inhibition of LOX and CLOCK-OLFML3 axis activates anti-tumor**
951 **immune response and synergizes with anti-PD1 therapy.**

952 (A and B) Immunofluorescence (A) and quantification (B) of relative CD8⁺CD69⁺ T
953 cells in tumors from CT2A model (2×10^4 cells/mouse) treated with or without BAPN
954 (2 g/L in drinking water) on day 4, and/or SR9009 (100 mg/kg/day, i.p.) for 10 days
955 beginning at day 7 post-orthotopic injection. Scale bar, 50 μ m. n = 3 independent
956 samples. One-way ANOVA test. (C and D) Representative images (C) and
957 quantification (D) of flow cytometry for the percentage of intratumoral CD8⁺CD69⁺ T
958 cells in size matched tumors from CT2A tumor-bearing mice treated with or without
959 BAPN (2 g/L in drinking water) on day 4, and/or SR9009 (100 mg/kg/day, i.p.) for 10
960 days beginning at day 7 post-orthotopic injection. n = 3 independent samples. One-
961 way ANOVA test. (E and F) Representative images (E) and quantification (F) of flow
962 cytometry for the percentage of intratumoral CD8⁺IFN- γ ⁺ T cells in size matched tumors

963 from CT2A tumor-bearing mice treated with or without BAPN (2 g/L in drinking water)
964 on day 4, and/or SR9009 (100 mg/kg/day, i.p.) for 10 days beginning at day 7 post-
965 orthotopic injection. n = 3 independent samples. One-way ANOVA test. **(G and H)**
966 Survival curves of C57BL/6 mice implanted with CT2A cells (2×10^4 cells/mouse, G)
967 or 005 GSCs (2×10^5 cells/mouse, H). Mice were treated with BAPN (2 g/L in drinking
968 water) on day 4, SR9009 (100 mg/kg/day, i.p.) for 10 days beginning at day 7 post-
969 orthotopic injection, and anti-PD1 (10 mg/kg, i.p.) on days 11, 14, and 17. n = 7-10
970 mice per group. Log-rank test. **(I and J)** Cured mice from the triple therapy were re-
971 challenged on day 70 with CT2A cells (2×10^4 cells/mouse, I) or on day 110 with 005
972 GSCs (2×10^5 cells/mouse, J). Similarly aged naive mice were implanted as controls.
973 n = 5 mice per group. Log-rank test. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

Figure 1.

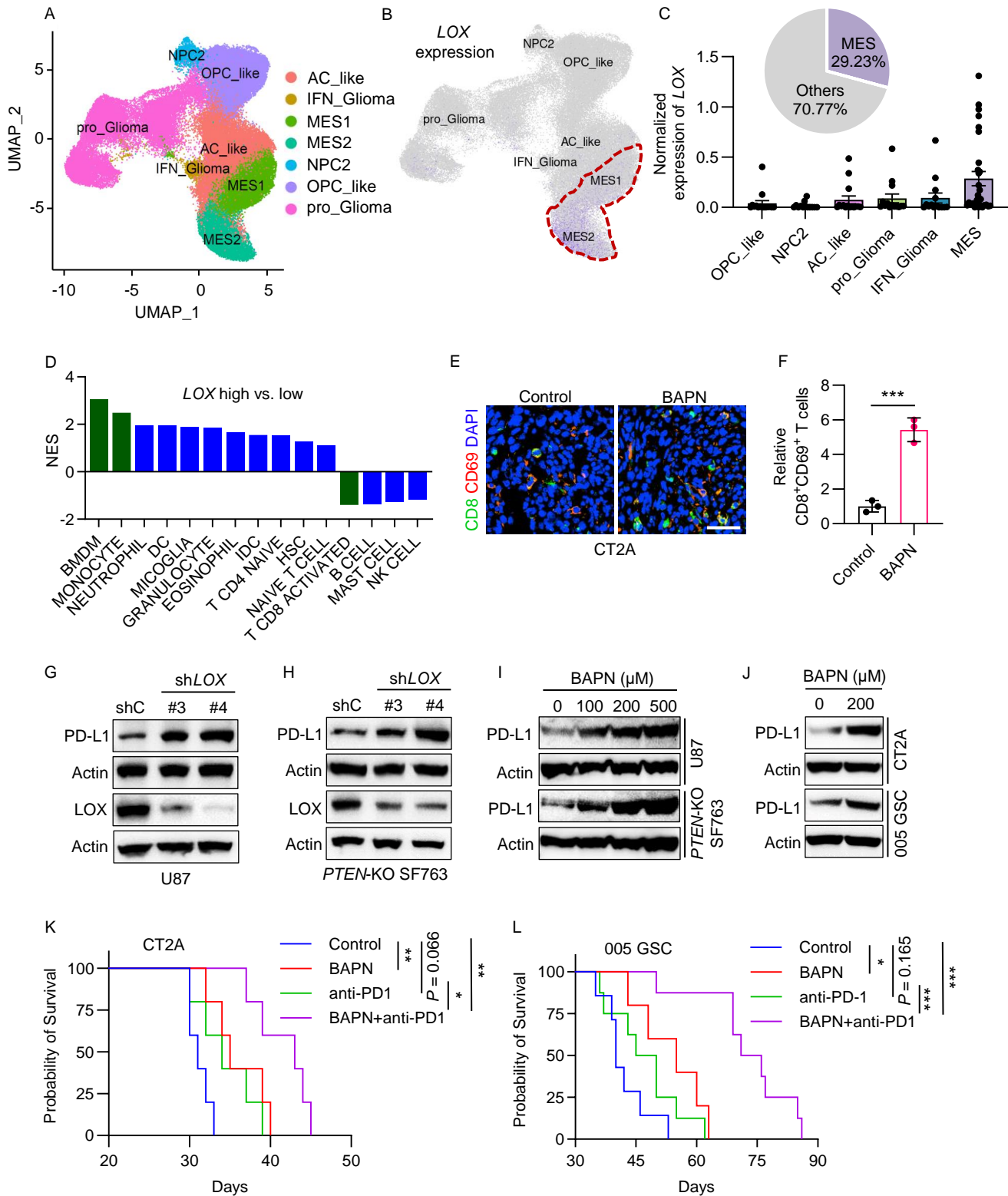


Figure 2

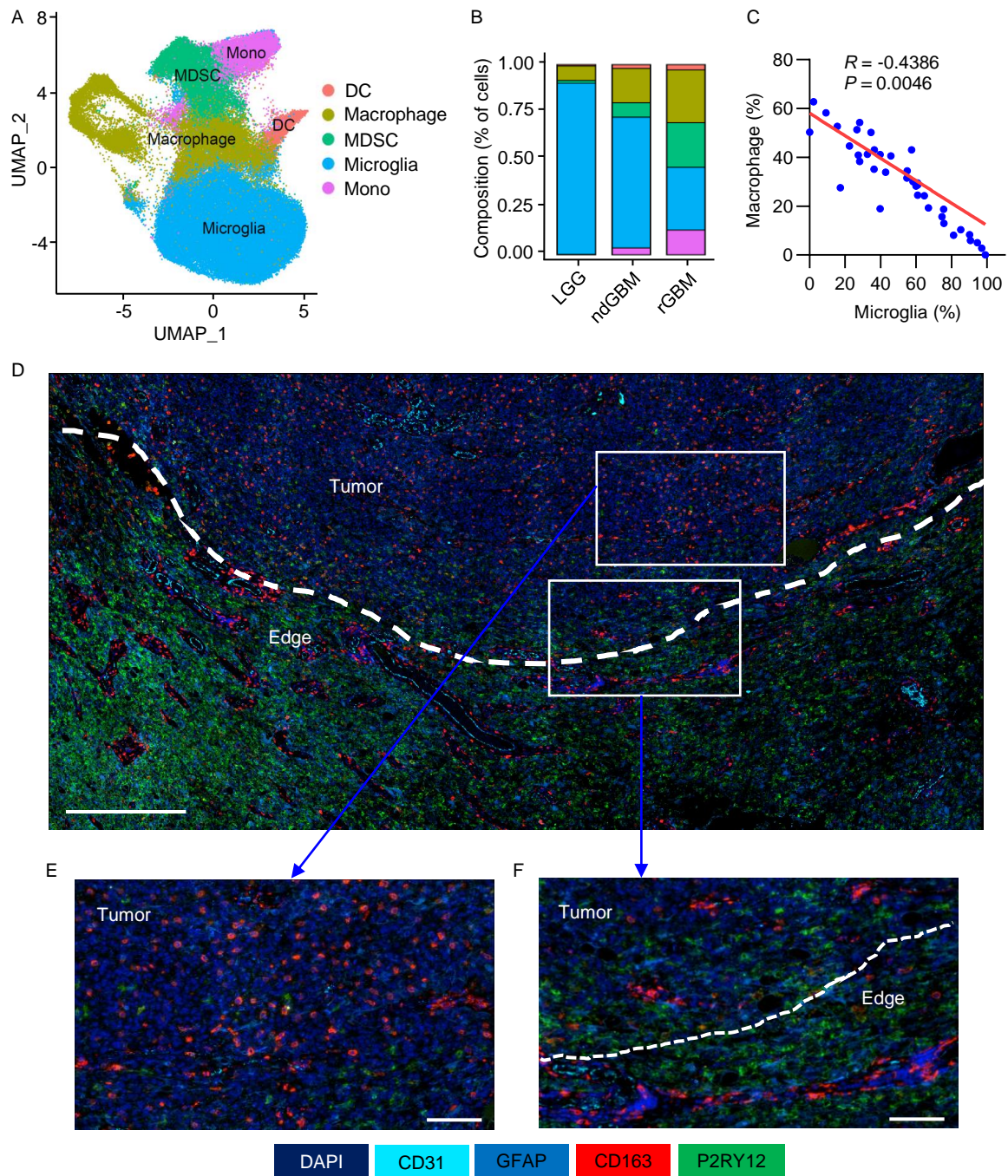


Figure 3

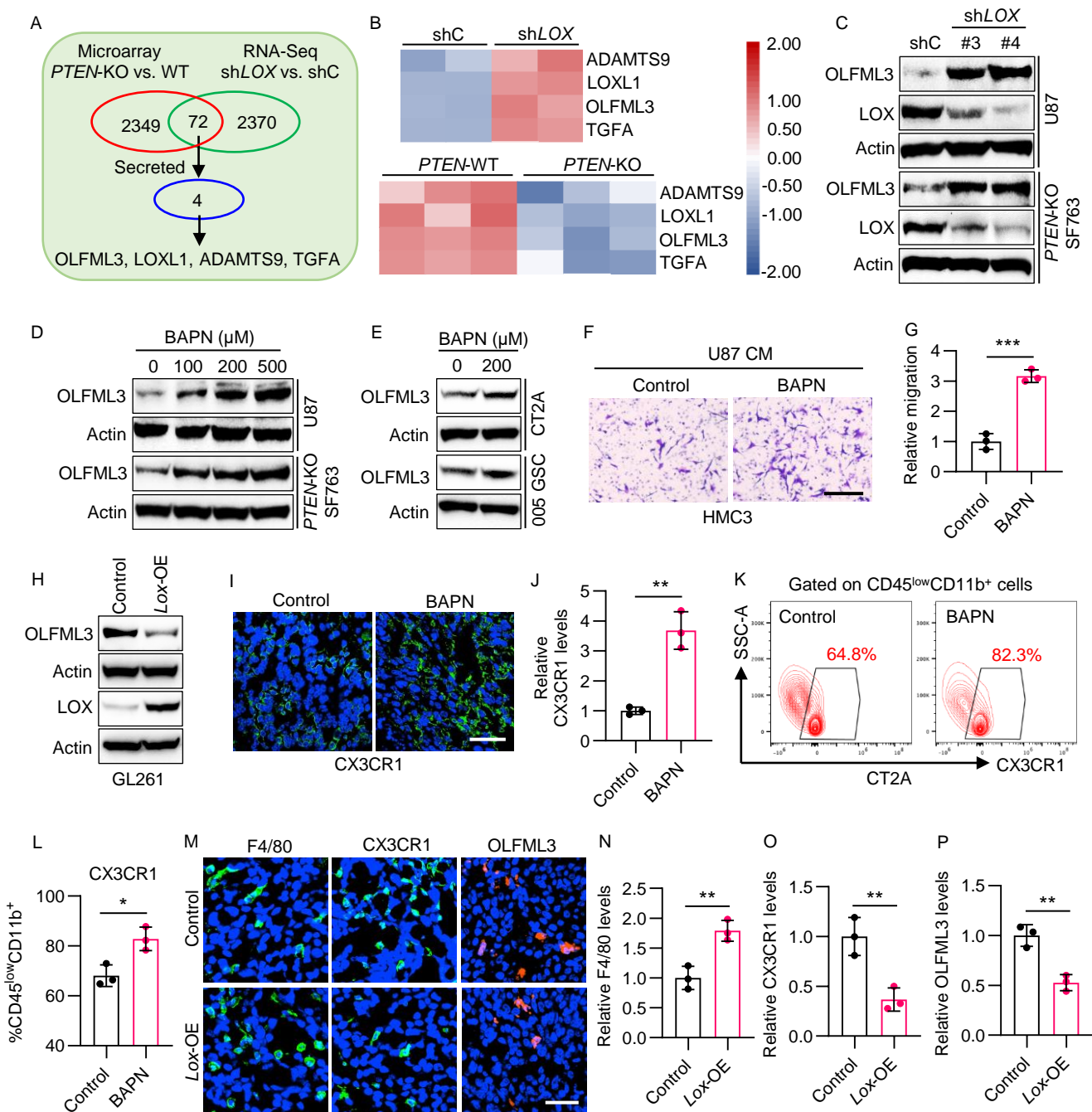


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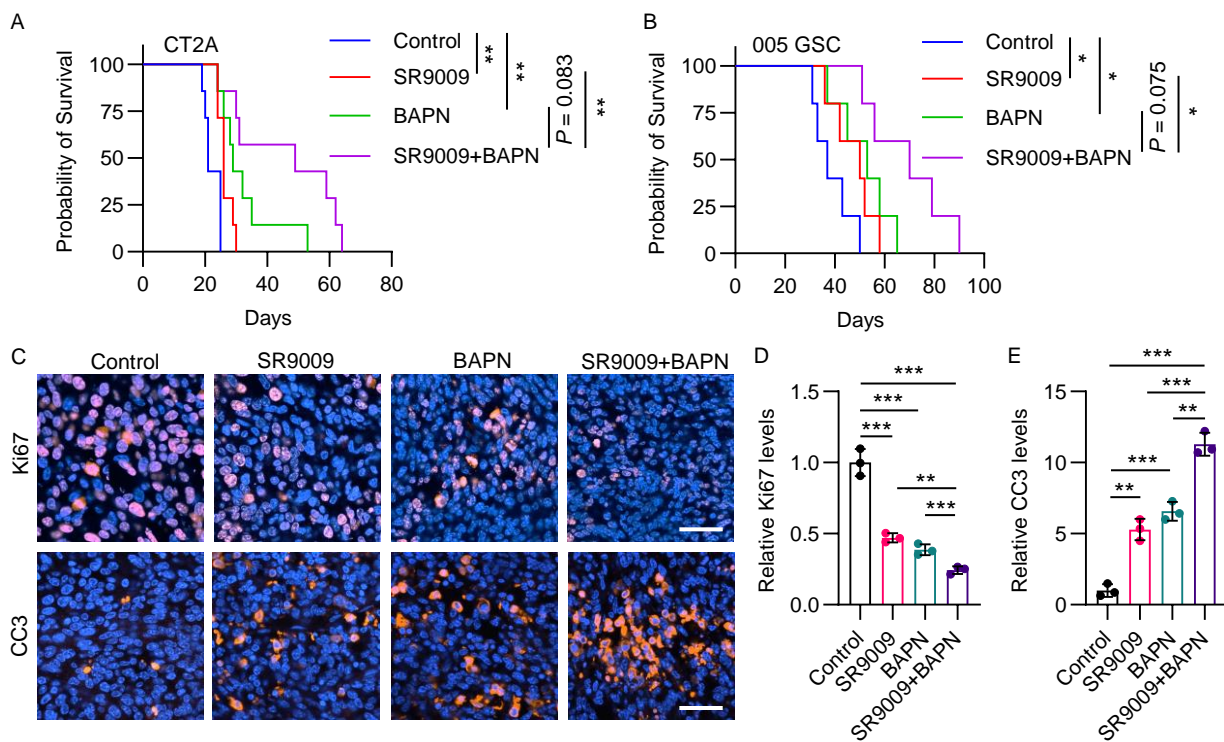


Figure 5

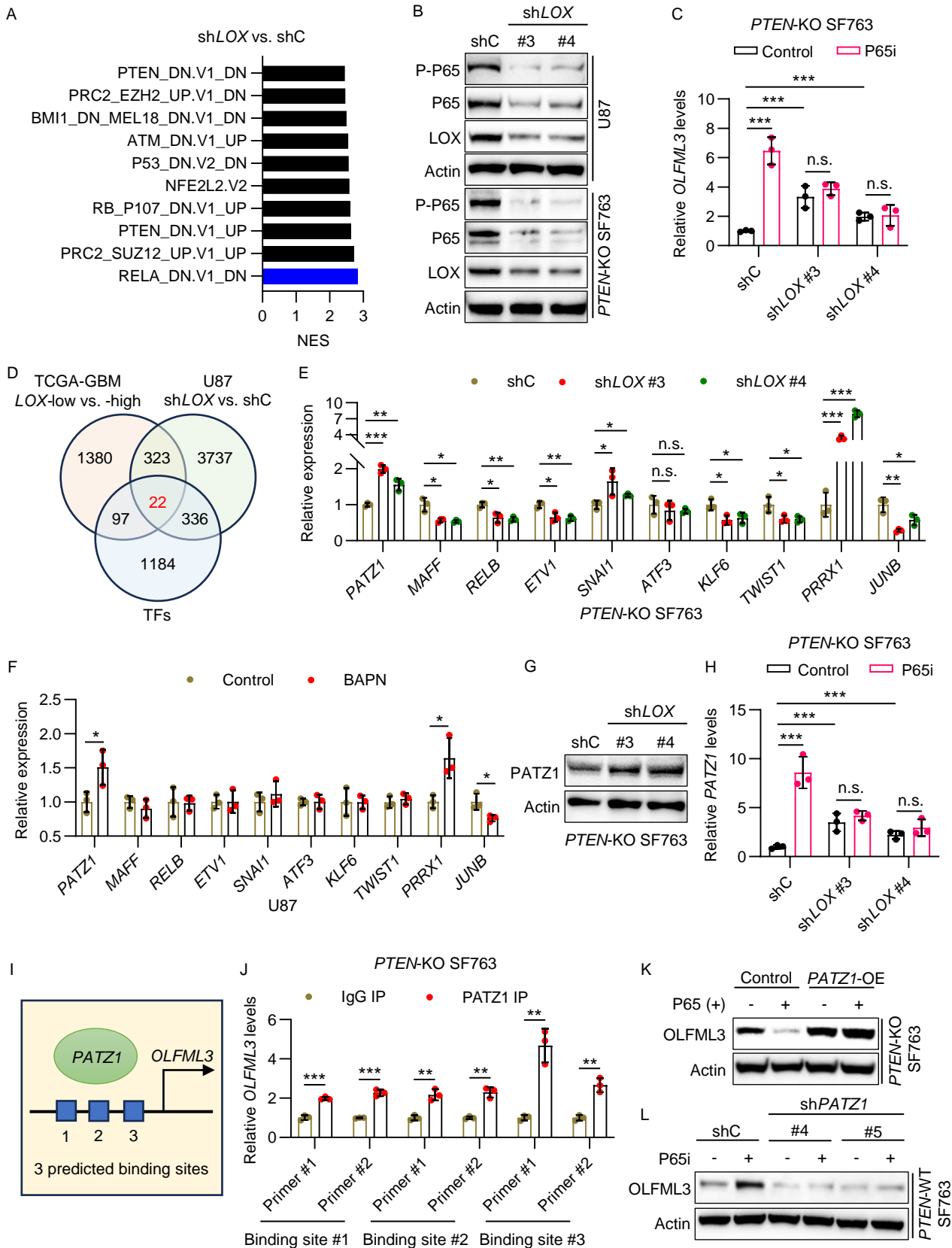


Figure 6

