

4 **Running title:** SMAP1 promotes hepatoblastoma cell proliferation

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6 **SMAP1 promotes hepatoblastoma cell proliferation by modulating the C-Kit-activated**
7 **ERK/MAPK pathway**

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9 Jie Liu^{1,2,#,*}, Ke Xu^{3,#}, Hongyue Wang^{3,#}, Yan Cheng³, Zihui Cheng³, Wenjie Bu², Feifei Wang^{4,5},
10 Xiaoxuan Zuo⁶, Xiaojian Li⁷, Nan Xia^{4,5,8}, Qian Dong⁴, Wenli Xiu⁴, Aiqin Lin^{2,*}
11

12 ¹Department of Pediatric Surgery, The First Affiliated Hospital of Wannan Medical University
13 (Yijishan Hospital of Wannan Medical University), Wannan Medical University, Wuhu, China;

14 ²Department of Medical Biology of Wannan Medical University, Wannan Medical University,
15 Wuhu, China; ³Department of Clinical Medicine of Wannan Medical University, Wannan Medical

16 University, Wuhu, China; ⁴Department of Pediatric Surgery, Affiliated Hospital of Qingdao

17 University, Qingdao University, Qingdao, China; ⁵Shandong Provincial Key Laboratory of Digital
18 Medicine and Computer-assisted Surgery, Affiliated Hospital of Qingdao University, Qingdao,

19 China; ⁶Department of Youth League Committee, Wannan Medical University, Wuhu, China; ⁷The
20 Third Department of Gastrointestinal Surgery, Yijishan Hospital of Wannan Medical University,

21 Wannan Medical University, Wuhu, China; ⁸Institute of Digital Medicine and Computer-assisted
22 Surgery of Qingdao University, Qingdao University, Qingdao, China
23

24 *Correspondence: linaiqin945@163.com; liujie19842020@163.com
25

26 #Contributed equally to this work.
27

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30 Hepatoblastoma (HB) is the most common pediatric liver malignancy. We investigated the role of
31 SMAP1 in HB proliferation and its mechanism. Sixty-seven HB tissues were analyzed for SMAP1
32 expression via immunohistochemistry and correlated with clinical outcomes. qRT-PCR quantified
33 SMAP1 mRNA in HB cell lines versus normal hepatocytes. SMAP1 was knocked down
34 (siRNA/lentivirus) or overexpressed (plasmid) in HB cells; proliferation was assessed by CCK-8
35 and colony formation. A nude-mouse xenograft model was used to evaluate tumorigenicity. Western
36 blotting determined C-Kit and ERK/MAPK pathway activity after SMAP1 modulation. Rescue
37 experiments used ERK activator C16-PAF, inhibitor U0126, or C-Kit inhibitor ISCK03. SMAP1
38 was upregulated in HB and predicted poor prognosis. Knockdown reduced C-Kit, p-C-Kit, and
39 p-ERK1/2, inhibited proliferation and colony formation, and suppressed xenograft growth.
40 Overexpression produced opposite effects. U0126 reversed ERK1/2 phosphorylation and p-Rb in
41 SMAP1-overexpressing cells, whereas C16-PAF restored ERK signaling and c-Myc/p-Rb in
42 knockdown cells. ISCK03 blocked C-Kit/ERK activation prompted by SMAP1 overexpression.
43 SMAP1 drives HB progression by activating C-Kit-ERK/MAPK signaling, representing a potential
44 therapeutic target.
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Key words: hepatoblastoma; SMAP1; C-Kit; ERK/MAPK

Hepatoblastoma (HB) is the most common malignant tumor of the liver in children. Although the incidence of HB is approximately 1.5 cases per one million children, the pathogenesis of the disease remains unclear [1]. Although the combination of neoadjuvant chemotherapy and surgery has greatly improved patient outcomes, the 5-year survival rate for patients with PRETEXT stage III/IV disease remains low [2-4]. Therefore, the precise diagnosis and treatment of childhood HB and the development of novel therapeutic strategies are urgent challenges [5]. To achieve these goals, a better understanding of the molecular mechanisms underlying the occurrence and development of HB is necessary.

Through exome sequencing, our previous research revealed that the *SMAP1* gene exhibits high-frequency copy number variations in HB [6]. The *SMAP1* gene is located at chr6q13, spanning 11 exons with a total length of 194,132 bp. *SMAP1* encodes a 467-amino-acid protein that includes an Arf-GAP domain, a C4-type zinc finger, and clathrin- and CALM-binding motifs [7]. *SMAP1* binds the clathrin heavy chain and regulates clathrin-dependent endocytosis of the transferrin receptor and E-cadherin. Although *SMAP1* mutations have been linked to several diseases [8-10], its role in HB has not been described. According to literature reports, C-Kit, also known as Kit or CD117, is a receptor for stem cell factor (SCF) and a protein that is frequently overexpressed in gastrointestinal stromal tumors, small cell lung cancer, hepatocellular carcinoma, and leukemia, and is closely associated with tumor development [11, 12]. Notably, hepatoblastoma cells have been shown to secrete stem cell factor (SCF), the ligand of C-Kit, supporting a paracrine role for this receptor in the hepatic microenvironment [11]. Moreover, C-Kit participates in the MAPK, PI3K, and JNK/SAPK cascades; the small GTPase Ras activates the MAPK cascade by promoting the phosphorylation of various transcription factors, after which activated extracellular signal-regulated kinase (ERK) translocates to the nucleus to regulate gene expression [13, 14]. The ERK/MAPK pathway, the first MAPK subfamily identified, transmits extracellular stimuli to intracellular targets through sequential phosphorylation thereby modulating cell survival, proliferation, differentiation, and invasion and is closely associated with tumorigenesis, progression, metastasis, and therapeutic response [15]. Building upon our previous research, the present study investigated whether *SMAP1* regulates cell proliferation and the related molecular mechanisms in HB by modulating the C-Kit-activated ERK/MAPK signaling pathway.

79 **Patients and Methods**

80 **Tissue collection.** Tumor tissue and adjacent tissue samples of pediatric HB patients who
 81 underwent surgical treatment were collected from the clinical sample banks of Qingdao University
 82 Affiliated Hospital and Yijishan Hospital of Wannan Medical University from February 2000 to
 83 February 2025. The patient inclusion criteria were as follows: 1) first pathological diagnosis of HB;
 84 and 2) available detailed clinical data and basic personal information. The exclusion criteria were as
 85 follows: 1) the presence of other cancers and 2) no detailed follow-up data available.
 86 Immunohistochemical staining was performed on the HB tumor tissue and adjacent normal liver
 87 tissue according to a previously reported method [16], according to which the expression of the
 88 SMAP1 protein was detected and scored. The staining score was determined according to the
 89 percentage of positively stained cells (0=less than 5%, 1=5-25%, 2=26-50%, 3=51-75%, and
 90 4=more than 75%) and the intensity of staining (0-3). The total score was obtained by multiplying
 91 the percentage score by the intensity score [6]. All tissue samples were obtained with the consent of
 92 the patient's family members, who signed informed consent forms. The present study was approved
 93 by the Ethics Committee of Qingdao University Affiliated Hospital (Approval No.: QYFY WZLL
 94 25776) and the Medical Ethics Committee of Wannan Medical University (Approval No.:
 95 LLSC-2022-16). The median IHC score of SMAP1 was used as the cut-off value to stratify patients
 96 into high-expression and low-expression groups. The cut-off value was set at 5, the median score of
 97 all 67 cases. Patients with a score ≥ 5 were assigned to the SMAP1 high-expression group, and
 98 those with a score < 5 were assigned to the low-expression group.

99 **Cell culture.** HuH-6 cells (#CC0114, CellCook) were cultured in modified DMEM medium
 100 (CM2007, CellCook) supplemented with 10% fetal bovine serum (#S711-001S, Lonsera), Hep-G2
 101 cells (#CC0101, CellCook) were cultured in MEM medium (#SH30024, HyClone) supplemented
 102 with 10% fetal bovine serum, and THLE-2 cells (#SC0659, Yuchicell) were cultured in THLE-2
 103 Cell-specific culture medium (#CX0123, Yuchicell). The cell lines were cultured at 37 °C in the
 104 presence of 5% carbon dioxide. When the cell density reached 80-90%, the cells were digested with
 105 0.25% trypsin (#25200056, Gibco) and placed in a six-well plate for subculture.

106 **siRNA transfection.** The st-h-SMAP1 (si-SMAP1) and siRNA-negative control (si-NC) were
 107 constructed by RiboBio biotechnology Company, Guangzhou. The sequences are shown in Table 1.

108 When the cell density in the six-well plate reached 30-50%, the culture medium was replaced with
109 fresh complete culture medium. si-*SMAP1* (20 μ M) and si-NC (20 μ M) were transfected into the
110 cells using transfection reagents (#C10511-05, Ribobio) in two wells each according to the
111 manufacturer's instructions; non-transfected cells in the other two wells served as blank controls.
112 RNA was extracted 24 h after transfection, and total protein was extracted 48 h after transfection.
113 The transfection efficiency was determined by RT-qPCR and Western blot assays.

114 **Plasmid transfection.** The *SMAP1* overexpression plasmid (OE-*SMAP1*) and its control empty
115 plasmid (OE-NC) were constructed by RiboBio biotechnology Company, Guangzhou. The
116 sequences are detailed in the Supplementary Table S1. When the cell density in the six-well plate
117 reached 80-90%, the culture medium was replaced with fresh complete culture medium. The
118 OE-*SMAP1* (1 μ g) and OE-NC (1 μ g) were transfected into the cells using transfection reagents
119 (#SL100568, GenMute) in two wells each according to the manufacturer's instructions;
120 non-transfected cells in the other two wells served as blank controls. RNA was extracted 24 h after
121 transfection, and total protein was extracted 48 h after transfection. The transfection efficiency was
122 determined by RT-qPCR and Western blot assays.

123 **RNA extraction and qRT-PCR.** Total RNA was extracted from the cells using TRIzol reagent
124 (#15596026, Invitrogen), and the RNA concentration and purity were determined using a micro
125 nucleic acid protein analyzer. First, the RNA concentration was adjusted uniformly to 0.5 μ g/ml.
126 RNA was reverse transcribed into cDNA using a reverse transcription kit (#M1701, Promega), and
127 relevant genes were amplified by fluorescence quantitative PCR using ACTB as an internal
128 reference. The $2^{-\Delta\Delta Ct}$ method was used to quantify gene expression. The sequences of the primers are
129 shown in Table 2.

130 **Protein extraction and western blot analysis.** The cells were lysed with Western and IP lysis
131 buffer (#P0013, Beyotime) on ice for 10 min, followed by centrifugation at 4 °C and 13,000 $\times$ g for
132 10 min. The total cell protein was then extracted, and the protein concentration was measured using
133 BCA kit (#P0012, Beyotime) and adjusted to be consistent in each group (10 μ g/ μ l). SDS-PAGE
134 protein loading buffer (5 $\times$) (#P0015, Beyotime) was added to the samples, after which they were
135 denatured in a metal bath at 100 °C for 10 min. The samples were stored at -20 °C until further use.
136 Protein samples (5 μ l) were separated by SDS-PAGE, then transferred onto Nitrocellulose
137 membranes (#HATF00010, Immobilon-NC) using the wet transfer method. The membranes were
138 blocked with TBST containing 5% skim milk powder (#BR010A, Biomiky) for 2 h. After washing,

139 the membranes were incubated with the primary antibodies overnight at 4 °C. The membranes were
140 then washed three times with TBST and incubated with the secondary antibodies. After washing the
141 membranes three times, the protein bands were detected using ECL luminous solution (#BMU102,
142 SuperKine) and a fluorescence and chemiluminescence gel imaging system (FluorChem E,
143 ProteinSimple, USA). The experimental results were analyzed using the AlphaView SA software.
144 The relevant antibody information is as follows: SMAP1 (#P103787, Yamei), p44/42 MAPK
145 (Erk1/2) (#4695T, CST), Phospho-p44/42 MAPK (#4370T, CST), c-Kit (#3074T, CST),
146 Phospho-c-Kit (Tyr703) (#3073T, CST), GAPDH (#AF1186, Beyotime), c-Myc (c-Myc, CST), and
147 Phospho-Rb (#8516S, CST). HRP-labeled secondary antibody (#A0208, #A0216, Beyotime).

148 **CCK-8 proliferation assay.** After the cells were transfected with the siRNA, overexpression
149 plasmid or respective control for 24 h, they were digested with 0.25% trypsin. A cell counter
150 (Celldrop BF, Denovix) was used to obtain a cell suspension of 50,000 cells/ml. Cell suspension,
151 PBS, or cell culture medium (100 µl/well) was added to each well in a 96 well plate (6
152 replicates/group), which was then incubated. After treatment, the cells were incubated with 10%
153 CCK8 reagent (#MK001B, Biomiky) every 24 h until 72 h at 37 °C for 1-2 h. Finally, the
154 absorbance of each well was measured at a wavelength of 450 nm using a microplate reader
155 (SYNERGY H1, BioTek) and analyzed.

156 **Colony-formation assay.** After the cells were transfected with the siRNA, overexpression plasmid
157 or respective control for 24 h, they were digested with 0.25% trypsin. A cell counter was used to
158 dilute the cell suspension to 500 cells/ml. In a new six-well plate, 2 ml of diluted cell suspension
159 was added according to the original grouping, with 6 replicates per group. The cells were observed
160 every 24 h, and the culture medium was replaced regularly for one week. At the end of this period,
161 when visible colonies had formed, the cells were fixed with methanol (1.5 ml) for 30 min. After the
162 methanol treatment, the sample was washed twice with PBS, then stained with crystal violet
163 staining solution (#C0121, Beyotime) at room temperature for 15 min, and finally rinsed with clean
164 water slowly until the background became clear. The cell colonies were imaged under the 4×
165 eyepiece to count the number of colonies.

166 **Xenotransplantation model for subcutaneous tumor formation in nude mice.** Ten 5-week-old
167 female BALB/c nude mice were obtained from Qinglongshan Experimental Animal Center
168 (Nanjing, China) and isolated in the SPF animal room for 3 days. The mice were randomly divided
169 into the following two groups, with 5 mice in each group: the stable *SMAP1* knockdown group (KD)

170 and the NC group (NC). The mice were given sterile water, food, and bedding. Stably transformed
171 *SMAP1* knockdown and NC HuH-6 cells were seeded into a 10 cm culture dish and allowed to
172 grow logarithmically until a cell density of 80-90% was reached. The cells were digested and
173 centrifuged, and the supernatant was discarded. The cells were resuspended in PBS (5×10^7
174 cells/ml) subcutaneously inoculated into the mice at a dose of 0.1 ml. The health, behavior and
175 weights of the animals were recorded every three days. Starting from the second week, the
176 subcutaneous tumor size was measured. The tumor volume was calculated using the following
177 formula: tumor volume=length $\times$ width²/2. At 3 weeks post-inoculation, the mice (n=10) were
178 anesthetized with 5% isoflurane and euthanized within 5 minutes by cervical dislocation. Before
179 subjected to euthanasia, the survival rate of the mice was 100%. All animals were fully anesthetized
180 before euthanasia: each mouse was placed in a sealed induction chamber and exposed to 5 %
181 isoflurane in 100 % O₂ at a flow rate of 1 L min⁻¹ until loss of the righting reflex (achieved within
182 ≤ 60 s). After confirming deep anesthesia (absence of toe-pinch and corneal reflexes), mice were
183 immediately removed from the chamber and euthanized by cervical dislocation within 5 min of the
184 initial anesthetic exposure. This sequence ensures the animals were completely unconscious and
185 insensitive to pain at the moment of sacrifice. The protocol follows the 2020 AVMA Guidelines for
186 the Euthanasia of Animals (accepted method: “inhaled anesthetic followed by cervical dislocation”)
187 and was approved by the Animal Ethics Committee of Wannan Medical University (Approval No.
188 LLSC-2022-065). The dissected tumors were weighed, and a portion of each tumor was fixed with
189 4% paraformaldehyde solution for subsequent immunohistochemical experiments. All the
190 experimental procedures were conducted in accordance with international guidelines.

191 **Immunohistochemistry for Ki-67 and cleaved caspase-3.** The paraffin-embedded tumor tissue
192 sections from the nude mice were routinely deparaffinized and immersed in different concentrations
193 of alcohol (100% $\rightarrow$ 90% $\rightarrow$ 80% $\rightarrow$ 70% $\rightarrow$ 50%, 3 min each), and they were then rinsed with
194 distilled water for 3 min. After treatment with citrate buffer (pH=6.0), the sections were subjected to
195 an antigen retrieval process using a microwave. The sections were then washed three times with
196 PBS (3 min each) and blocked with 5% goat serum at 36 °C for 1 h. The sections were incubated
197 with Ki-67 antibody (#GB111141, Servicebio, China; 1:500) and caspase-3 antibody (#19677-1-AP,
198 Proteintech, USA; 1:300) overnight at 4 °C. After washing the sections 3 times with PBS (3 min
199 each), they were incubated with human rabbit secondary antibodies at 37 °C for 1 h. The sections
200 were then washed 3 times with PBS (3 min each time), stained with DAB for 5 s, counterstained

201 with hematoxylin for 30 s, washed with tap water for 3 min, dehydrated with different
202 concentrations of alcohol (50% → 70% → 80% → 90% → 100%, each time for 3 min), air dried,
203 cleared with xylene for 5 min, and sealed. The corresponding scan results were observed and saved
204 on an immunohistochemical scanner. Ki-67-positive staining was indicated by brown staining in the
205 nucleus, while caspase-3-positive staining was indicated by brown staining in the cytoplasm. The
206 expression of Ki-67 and caspase-3 was evaluated by combining the comprehensive score of the
207 staining intensity and staining area. The IHC scoring criteria were the same as those above.

208 ***In vivo* bioluminescence imaging.** The nude mice were weighed before the experiment, and the
209 tumor size was measured. At 10-15 min before imaging, fluorescent dye stock solution (15 mg/ml,
210 #HY-12591B, MCE) was injected into the abdominal cavity according to the weight of the nude
211 mouse (final concentration 150 mg/kg), D-Luciferin was used as the substrate for *in vivo*
212 bioluminescence imaging. Approximately 5 min before imaging, the nude mouse was anesthetized
213 with 5% isoflurane. The tumor of the nude mouse was placed facing upward, and pictures were
214 acquired in a uniform position using the small animal live imaging device (IVIS Lumina LT, PE
215 Company, USA). The mouse was then returned to the cage, and the status of the nude mouse was
216 observed until autonomous activity was resumed.

217 **ERK/MAPK pathway rescue experiments.** After the cells were transfected with si-SMAP1 or NC
218 for 24 h, they were treated with the ERK activator C16-PAF (#HY-108635, MCE, at a final
219 concentration of 1 μM) for 24 h, and total protein was extracted. Two hours before transfection with
220 the SMAP1 overexpression plasmid or NC plasmid, the cells were treated with the ERK inhibitor
221 U0126 (#HY-12031, MCE, at a final concentration of 10 μM) was added. After 48 h of treatment,
222 total protein was extracted, and changes in the expression of related proteins after the addition of an
223 ERK activator and inhibitor were detected by Western blot analysis.

224 **C-Kit interference assay.** After the cells were transfected with the SMAP1 overexpression plasmid
225 or NC plasmid for 12 h, they were treated with the C-Kit inhibitor ISCK03 (#HY-101443, MCE, at
226 a final concentration of 10 μM) for 48 h, and total protein was extracted. The changes in the
227 expression of related proteins after the addition of a C-Kit inhibitor were detected by Western blot
228 analysis.

229 **Statistical analysis.** SPSS 26.0 and R 4.2.0 were used to analyze the data. The normality of the data
230 was assessed using the Shapiro-Wilk test. Categorical variables were compared using chi-square
231 tests or Fisher's exact tests when appropriate. Continuous variables were compared using Student's t

232 tests or Mann-Whitney U tests, depending on the distribution of the data. The data are presented as
233 the means±SDs. Comparisons were performed using two-tailed Student's t tests or one-way
234 ANOVA. Kaplan–Meier curves were analyzed by the log-rank test. A p-value of < 0.05 was
235 considered to indicate statistical significance. All data comes from at least three independently
236 repeated experiments.

237

238 **Results**

239 **Expression of the *SMAP1* gene in clinical samples and HB cell lines.** Immunohistochemical
240 staining of the 67 clinical HB tissue samples revealed that the expression of SMAP1 in HB tumor
241 tissue was significantly greater than that in normal liver tissue ($p < 0.05$; Figures 1A, 1B). To
242 investigate the relationship between SMAP1 expression and outcome in the 67 children with HB, a
243 Kaplan-Meier survival analysis was conducted. The event-free survival (EFS) and overall survival
244 (OS) rates of the high-SMAP1 expression group (N=38) were lower than those of the
245 low-expression group (N=29) (Figure 1C). Additionally, compared with the expression in THLE-2
246 normal human liver immortalized cells, *SMAP1* mRNA expression was significantly upregulated in
247 HB cells (HuH-6 and Hep-G2) ($p < 0.001$; Figure 1D).

248 **Cytological functional experiments of *SMAP1* in HB.** To investigate the specific role of SMAP1
249 in HB, CCK-8 and cell colony formation assays were used to detect the proliferation ability of
250 HuH-6 and HepG-2 cells after *SMAP1* knockdown and overexpression. Compared with the NC
251 group, *SMAP1* knockdown in HuH-6 and HepG-2 cells significantly inhibited tumor cell
252 proliferation (Figures 2A, 2B), whereas SMAP1 overexpression significantly increased tumor cell
253 proliferation ($p < 0.01$; Figures 2C, 2D).

254 **Knockdown of *SMAP1* inhibits subcutaneous xenograft growth in nude mice.** To further
255 investigate the effect of SMAP1 on tumor proliferation *in vivo*, stable *SMAP1* knockdown and NC
256 HuH-6 cells were subcutaneously inoculated into nude mice to establish a nude mouse
257 subcutaneous xenograft model. Tumor growth was monitored, and immunohistochemical staining
258 was performed on tumor samples from subcutaneous tumors in nude mice. The protein expression
259 of Ki-67, which is closely related to cell proliferation, and caspase-3, which is closely related to
260 apoptosis, was evaluated in the two groups. Compared with the NC group, *SMAP1* knockdown
261 inhibited subcutaneous xenograft growth in the nude mice (Figures 3A-3H). Compared with that in
262 the NC group, the expression of Ki-67 was significantly lower in the KD group, indicating

263 inhibition of tumor proliferation ($p < 0.01$). Compared with that in the NC group, the expression of
264 caspase-3 was significantly greater in the KD group, indicating that tumor cell apoptosis occurred
265 ($p < 0.001$; Figures 3I-3K).

266

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267 **SMAP1 regulates C-Kit activation of the ERK/MAPK pathway in HB cells.** The effect of
268 *SMAP1* knockdown on the regulatory-related proteins in HuH-6 and HepG2 HB cells was
269 investigated. Western blot analysis revealed that the protein expression levels of C-Kit, p-C-Kit, and
270 p-ERK1/2 were significantly reduced following *SMAP1* knockdown (Figure 4).

271 **SMAP1 regulates HB proliferation through activation of the ERK/MAPK pathway.** To further
272 clarify whether *SMAP1* participates in HB cell proliferation by regulating the ERK signaling
273 pathway, HuH-6 and HepG2 cells transfected with *SMAP1* overexpression plasmids were treated
274 with the U0126 ERK pathway inhibitor. Changes in the expression of ERK signaling
275 pathway-related proteins were detected by Western blot analysis. The *SMAP1* knockdown-induced
276 reduction of ERK1/2 phosphorylation level was restored by U0126 treatment, while the total
277 ERK1/2 protein level remained unchanged. In addition, the protein expression of p-Rb, a
278 downstream protein regulated by the ERK pathway, was also restored (Figure 5).

279 HuH-6 and HepG2 cells transfected with a *SMAP1*-targeted small interfering RNA were treated
280 with the C16-PAF ERK pathway agonist. Changes in the expression of ERK signaling
281 pathway-related proteins were detected by Western blot analysis. The *SMAP1* knockdown-induced
282 reduction in the ERK1/2 phosphorylation level was restored by C16-PAF treatment, whereas the
283 total ERK1/2 protein level remained unchanged. The expression of downstream proteins regulated
284 by the ERK pathway, such as c-Myc and p-Rb, was also restored (Figure 6).

285 **SMAP1 activates the ERK/MAPK pathway via C-Kit to regulate HB proliferation.** To clarify
286 whether *SMAP1* participates in HB cell proliferation by regulating ERK signaling through C-Kit,
287 HuH-6 and HepG2 cells transfected with the *SMAP1* overexpression plasmid were treated with the
288 ISCK03 C-Kit pathway inhibitor. Changes in the expression of ERK signaling pathway-related
289 proteins were detected by Western blot analysis. Treatment of *SMAP1*-overexpressing cells with
290 ISCK03 increased the C-Kit phosphorylation level and recovered the reduced ERK1/2
291 phosphorylation level, whereas the total ERK1/2 protein level remained unchanged (Figure 7).

292

293 Discussion

294 HB accounts for 80% of pediatric hepatic malignancies, and its incidence is increasing by 1-2%
295 annually [17]. The etiology of the HB is still unclear, and recurrent and metastatic disease remain
296 the main treatment challenge [18]. The molecular heterogeneity of HB and the scarcity of validated
297 druggable targets have hindered the development of precision therapies. Through whole-exome

sequencing of HB genomes, our previous study identified recurrent copy-number variations encompassing the *SMAP1* locus (chr6q13, spanning 11 exons and 194,132 bp) [6]. However, the functional contribution of *SMAP1* to HB pathogenesis had not been systematically investigated. The present study demonstrates, for the first time, that *SMAP1* is markedly upregulated in HB tissues and cell lines, where it drives tumor proliferation by attenuating C-Kit endocytosis and thereby sustaining ERK1/2 signaling. These findings establish the *SMAP1*/C-Kit/ERK axis as a novel signaling cascade in HB and nominate *SMAP1* as an independent prognostic biomarker and a potential therapeutic target.

The *SMAP1* gene was first identified and reported in 1998 [19]. A previous study has indicated that loss of *SMAP1* suppresses the migration of microsatellite-unstable colorectal cancer cells through an Arf6-dependent mechanism, although effects on cell proliferation and C-Kit regulation were not investigated [7]. Another study has reported that deletion of *SMAP1* leads to impaired proliferation of erythroid progenitor cells, but these observations were made in a hematopoietic system with a very limited sample size of less than 10 cases [20]. Based on analyses of 67 HB clinical specimens and two HB cell lines, the present study confirms the involvement of the *SMAP1*-related pathway in a pediatric solid tumor. The application of lentivirus-mediated stable knockdown, pharmacological rescue assays and nude mouse xenograft experiments provides solid causal evidence, with both sample size and depth of functional validation exceeding those of previous studies. A large body of evidence has demonstrated that ligand-activated C-Kit exerts tyrosine kinase activity and initiates downstream signaling cascades that modulate apoptosis, differentiation, proliferation, chemotaxis and cell adhesion, thereby facilitating tumor cell survival and tumorigenesis. A prior investigation has revealed the presence of a TGF- β /C-Kit positive-feedback loop in adult liver cancer, yet the upstream trigger responsible for C-Kit overactivation remains unclear [21]. Using a three-step siRNA-rescue-inhibitor strategy, we demonstrate for the first time that *SMAP1* resides upstream of C-Kit and directly regulates its phosphorylation, thus identifying an initiator of C-Kit overactivation. In terms of methodology, we combined exon copy number variation data with high-throughput IHC scoring to construct a *SMAP1* expression prognostic model, and we used lentiviral stable knockdown and transient transfection to provide direct translatable evidence for precise intervention targeting the *SMAP1*/C-Kit/ERK axis. The present findings confirmed that the ERK/MAPK pathway is closely linked to HB proliferation. *SMAP1* blocks C-Kit endocytosis and enriches it on the membrane, resulting in the hyperphosphorylation of ERK1/2 and the simultaneous activation of

329 downstream c-Myc and p-Rb; this process can be instantly switched off by U0126, suggesting the
330 synergistic effect of combining MEK inhibitors with SMAP1 silencing. To our knowledge, this is
331 the first report elucidating the role of SMAP1 in HB.

332 The present study had several limitations. First, although the number of patients in the present study
333 (67 patients) exceed that of most single-center studies, the sample size was modest for a rare disease,
334 and all specimens were obtained from two tertiary hospitals in eastern China; thus, geographic and
335 ethnic homogeneity may limit extrapolation to multiethnic cohorts worldwide. Second, the *in vitro*
336 experiments relied mainly on HuH-6 and Hep-G2 cell lines, both of which harbor TP53 deletion
337 and other specific alterations, which may result in overestimating the prominence of the
338 *SMAP1/C-Kit/ERK* axis in other molecular subtypes. Third, the animal model employed
339 subcutaneous xenografts, which lack the native hepatic microenvironment and immune infiltrates,
340 potentially leading to an underestimation of SMAP1-mediated tumor–host interactions. Future
341 efforts will expand along three lines. First, a multicenter, prospective HB registry (≥ 200 cases) will
342 be established, encompassing diverse ethnicities (European, East Asian and Middle Eastern), and
343 uniform CHIC-PRETEXT classification and follow-up protocols will be applied. Whole-exome
344 sequencing and single-cell RNA-seq will be integrated to systematically evaluate *SMAP1*
345 expression across different molecular subtypes and immune microenvironments, validating its
346 stability across populations. Second, Cre-loxP-driven, liver-specific *Smap1* conditional-knockout
347 mice and patient-derived xenograft (PDX) models will be generated to compare growth curves,
348 immune infiltration profiles and drug resistance features between subcutaneous and intrahepatic
349 tumors before and after *SMAP1* inhibition, providing a more accurate reflection of the efficacy and
350 therapeutic window of *SMAP1*-targeted strategies. Third, highly selective SMAP1 degraders
351 (PROTACs) or antisense oligonucleotides (ASOs) will be developed, and high-throughput drug
352 screening on organoid-microfluidic chips coupled with real-time imaging and multiparametric
353 metabolic readouts will be performed to rapidly identify synergistic drug pairs (e.g., C-Kit or MEK
354 inhibitors) to provide dosing and biomarker guidance for phase I/II clinical trials. These strategies
355 will compensate for the external validity limitations of the present study and offer a reproducible
356 technical roadmap and evaluation framework for targeting the endocytosis/RTK/MAPK axis in
357 other pediatric solid tumors.

358 In summary, the present findings revealed for the first time that SMAP1 drives HB proliferation by
359 activating the C-Kit/ERK/MAPK axis and established SMAP1 as an independent prognostic factor

and a novel, druggable target. Verification in multicenter cohorts, orthotopic models and precision combination regimens is needed to further establish SMAP1 as a key component for risk stratification and targeted therapy in HB, laying a solid theoretical and experimental foundation for improving survival outcomes in high-risk children.

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Supplementary data are available in the online version of the paper.

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454 Figure Legends

455

456 **Figure 1.** SMAP1 protein expression in hepatoblastoma, survival analysis, and screening of small
457 interfering RNA sequences. A, B) IHC analysis of clinical samples revealed that SMAP1 was
458 significantly overexpressed in hepatocellular carcinoma tumor tissue but had low expression in
459 normal liver tissue. Original magnifications, 200×. C) Kaplan-Meier survival analysis of SMAP1
460 expression in 67 pediatric HB patients. The median IHC score of 5 was used as the cut-off value to
461 define high-SMAP1 (score ≥ 5 , n=38) and low-SMAP1 (score < 5 , n=29) groups according to our
462 previous study [22]. D) Using actin as an internal reference, qRT-PCR experiments revealed that
463 compared to THLE-2 normal human liver immortalized cells, SMAP1 mRNA expression was
464 significantly upregulated in HB cells (HuH-6 and Hep-G2 cells) ($p < 0.001$). E) Hep-G2 cells were
465 transfected with three small interfering RNA sequences targeting the SMAP1 gene, namely,
466 st-h-SMAP1-1, st-h-SMAP1-2, and st-h-SMAP1-3. qRT-PCR was used to detect the knockdown
467 efficiency of SMAP1. The expression level of SMAP1 mRNA in the interference group was
468 significantly lower than that in the negative control and blank control groups. Among them, the
469 knockdown efficiency of st-h-SMAP1-1 was the highest, and this sequence was used in subsequent
470 experiments.

471

472 **Figure 2.** HuH-6 and Hep-G2 cell viability as detected by the CCK8 assay. A, C) The CCK-8
473 method was used to detect the proliferation of HuH-6 and Hep-G2 cells after knocking down the

SMAP1 gene. SMAP1 knockdown significantly inhibited the proliferation of HuH-6 and Hep-G2 cells in hepatoblastoma, with the effect being more significant after 72 h. Overexpression of SMAP1 increased tumor cell proliferation of HuH-6 cells and Hep-G2 cells after 24 h and 72 h, respectively. $**p < 0.01$; $***p < 0.001$. B, D) SMAP1 knockdown significantly reduced the proliferation and colony forming ability of HuH-6 and Hep-G2 cells. Overexpression of SMAP1 significantly increased HuH-6 and Hep-G2 proliferation and colony forming ability. $**p < 0.01$; $***p < 0.001$

481

Figure 3. Knockdown of SMAP1 inhibits subcutaneous xenograft growth in nude mice. A, B) *In vivo* imaging images of nude mice carrying tumors on days 14 and 21. C) Nude mice bearing tumors and xenografts. D) Growth rate of nude mouse xenografts. E) Changes in body weight of nude mice. F) Volume comparison of nude mouse xenografts. G) Weight comparison of nude mouse xenografts. H) Comparison of the body weights of nude mice. I) HE staining images (Original magnifications, 200 $\times$) and IHC images (Ki-67 and caspase-3) of xenografts in nude mice. Original magnifications, 100 $\times$. J, K) The expression of Ki-67 in the KD group was significantly lower than that in the NC group, indicating inhibited tumor proliferation. The expression of caspase-3 in the KD group was significantly higher than that in the NC group, indicating increased tumor cell apoptosis. Original magnifications, 100 $\times$. $*p < 0.05$; $**p < 0.01$; $****p < 0.0001$

492

Figure 4. Western blot analysis of protein expression levels in SMAP1 knockdown cells. After transfecting HuH-6 and Hep-G2 cells with SMAP1-targeted small interfering RNA, Western blot analysis was used to detect the effects of knocking down SMAP1 on the related proteins and phosphorylation levels in the C-Kit and ERK signaling pathways. SMAP1 knockdown significantly inhibited the total protein and phosphorylation level of C-Kit, as well as the phosphorylation level of ERK1/2 protein, but SMAP1 knockdown had no effect on the expression of total ERK1/2 protein. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$

500

Figure 5. Rescue experiment of SMAP1-mediated activation of the ERK/MAPK pathway in HB cells (ERK inhibitor). After transfecting HuH-6 and Hep-G2 cells with SMAP1 overexpression plasmids, they were treated with the U0126 ERK pathway inhibitor. Western blot analysis was used to detect changes in ERK signaling pathway-related proteins. The SMAP1 knockdown-induced

505 reduction in ERK1/2 phosphorylation level was restored with U0126 treatment, while the total
506 ERK1/2 protein level remained unchanged. The expression of the p-Rb downstream protein
507 regulated by the ERK pathway was also restored. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

508

509 **Figure 6.** Rescue experiment of SMAP1-mediated activation of the ERK/MAPK pathway in HB
510 cells (ERK agonist). After HuH-6 and Hep-G2 cells were transfected with the SMAP1-targeted
511 small interfering RNA, they were treated with the C16-PAF ERK pathway agonist. Western blot
512 analysis was used to detect changes in ERK signaling pathway-related proteins. The SMAP1
513 knockdown-induced reduction in ERK1/2 phosphorylation level was restored by C16-PAF
514 treatment, while the total ERK1/2 protein level remained unchanged. The expression of downstream
515 proteins regulated by the ERK pathway, such as c-myc and p-Rb, was also restored. * $p < 0.05$; ** p
516 < 0.01 ; *** $p < 0.001$

517

518 **Figure 7.** SMAP1 mediates the activation of the ERK/MAPK pathway via C-Kit in HB cells. After
519 HuH-6 and Hep-G2 cells were transfected with the SMAP1 overexpression plasmid, the cells were
520 treated with the ISCK03 C-Kit pathway inhibitor. The changes in ERK signaling pathway-related
521 proteins were detected by Western blot analysis. As the phosphorylation level of C-Kit protein
522 increased, the phosphorylation level of ERK1/2 protein was also restored, but the total ERK1/2
523 protein level remained unchanged. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

524

525 **Table 1.** The sequence of the SMAP1 small interfering RNA.

siRNA	positive-sense strand (5'-3')	antisense strand (5'-3')
si-SMAP1_001	CGAUGGGCUUCCUGGAAUA	UAUUCCAGGAAGCCCAUCG
si-SMAP1_002	GAUACAGUGCAUGCAAGAU	AUCUUGCAUGCACUGUAUC
si-SMAP1_003	CAGAUCAAGCAGUGGAAUU	AAUUCCACUGCUUGAUCUG

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527

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528 **Table 2.** The sequence of the primer.

Gene	Upstream primer sequence	Downstream primer sequence
ACTB	GCGTGACATTAAGGAAGC	CCACGTACACTTCATGTGG
SMAP1	CAGTGACCACGGGAACA	GCAGGTAAGGGATAGAAA

529

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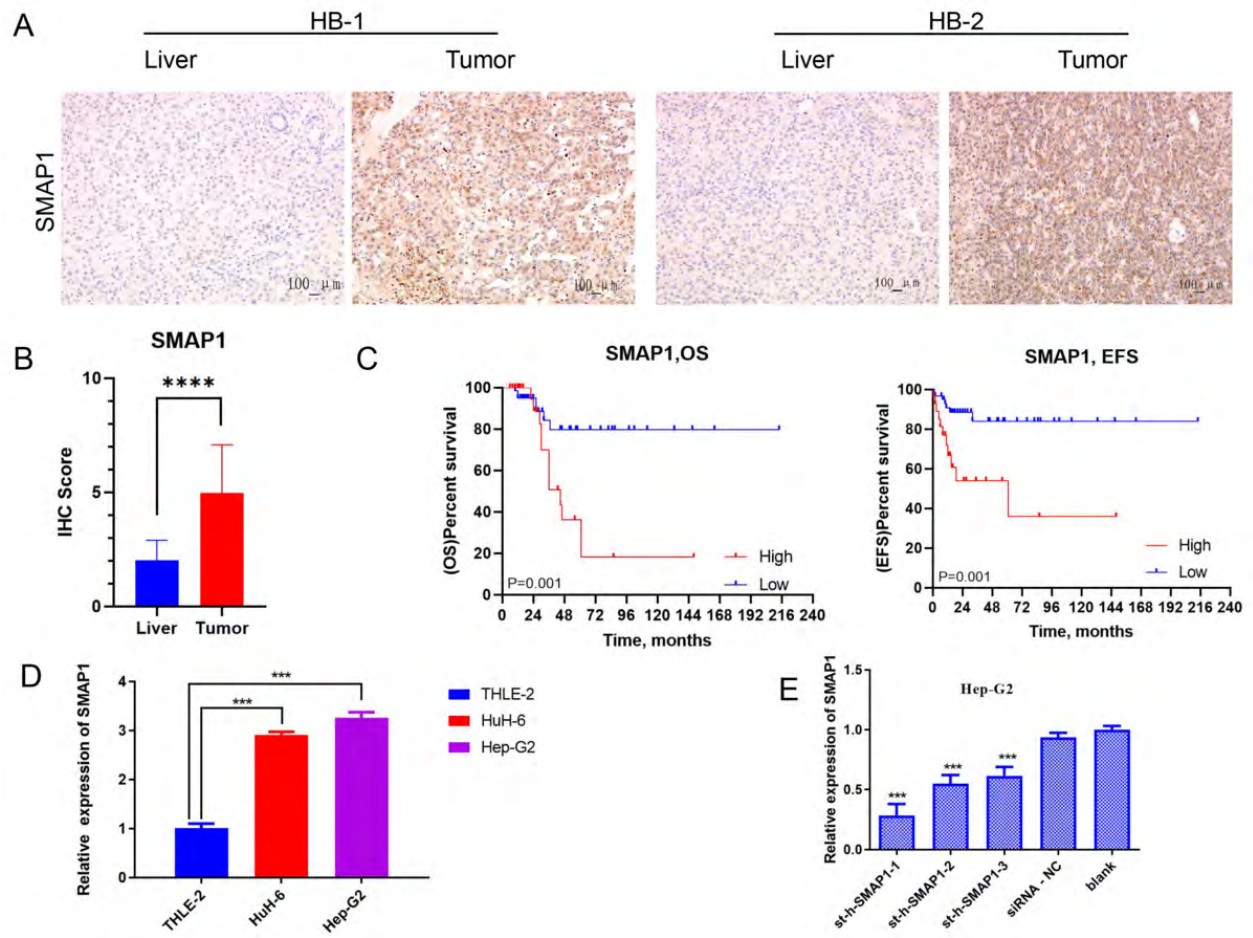


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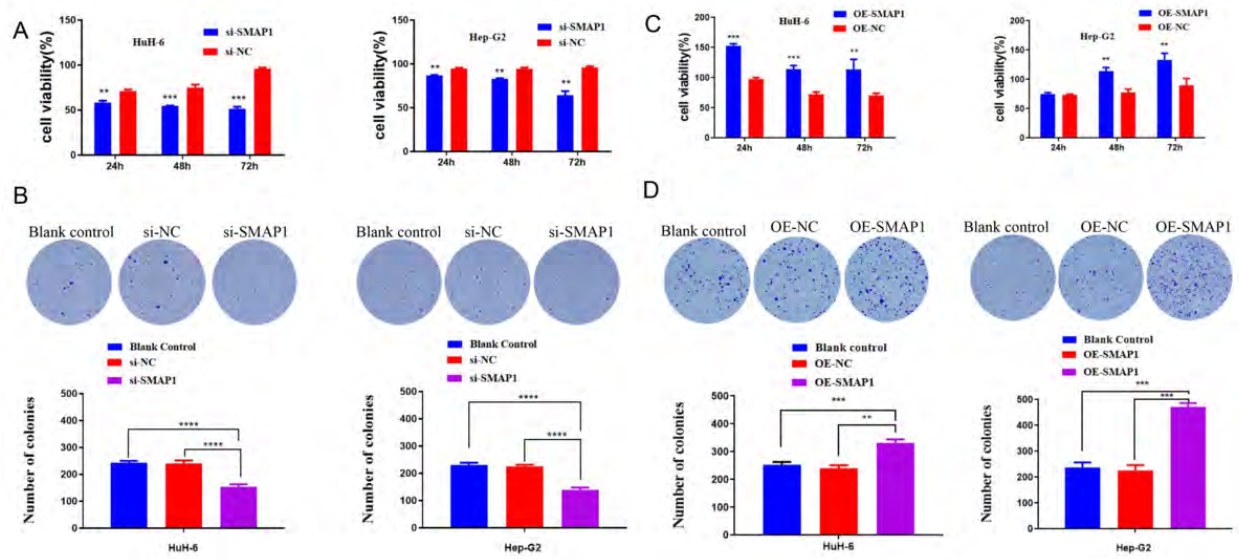


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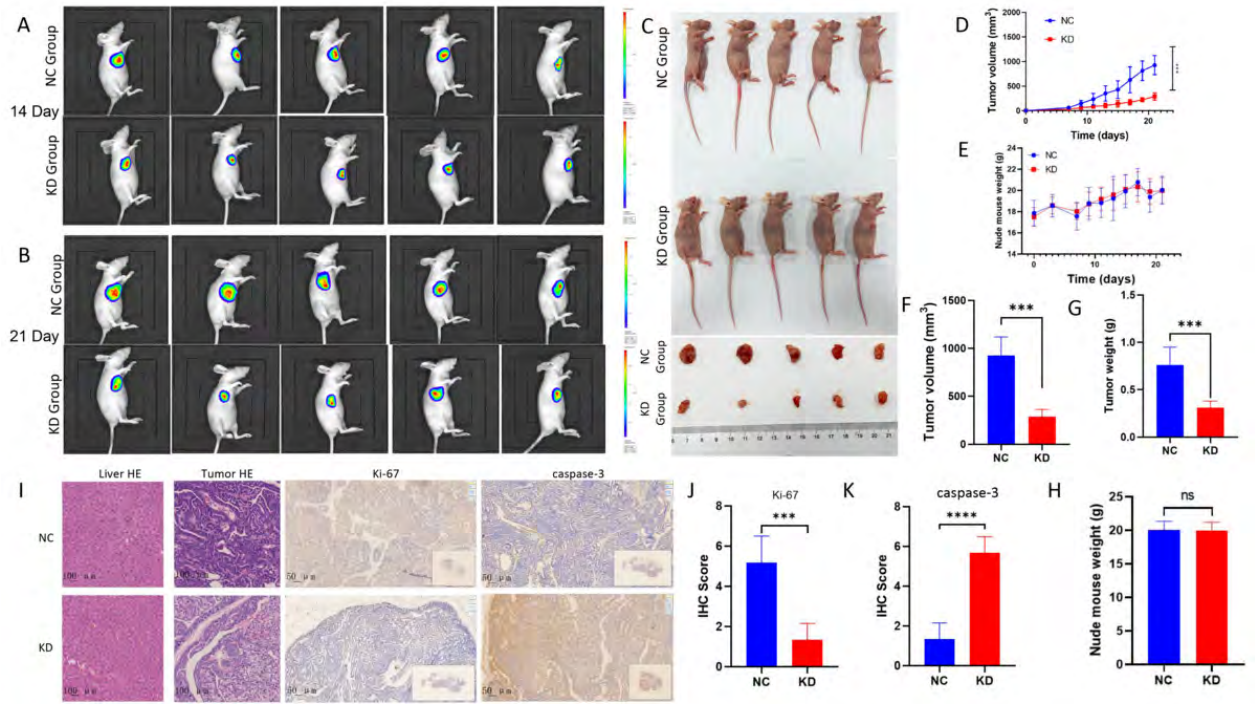


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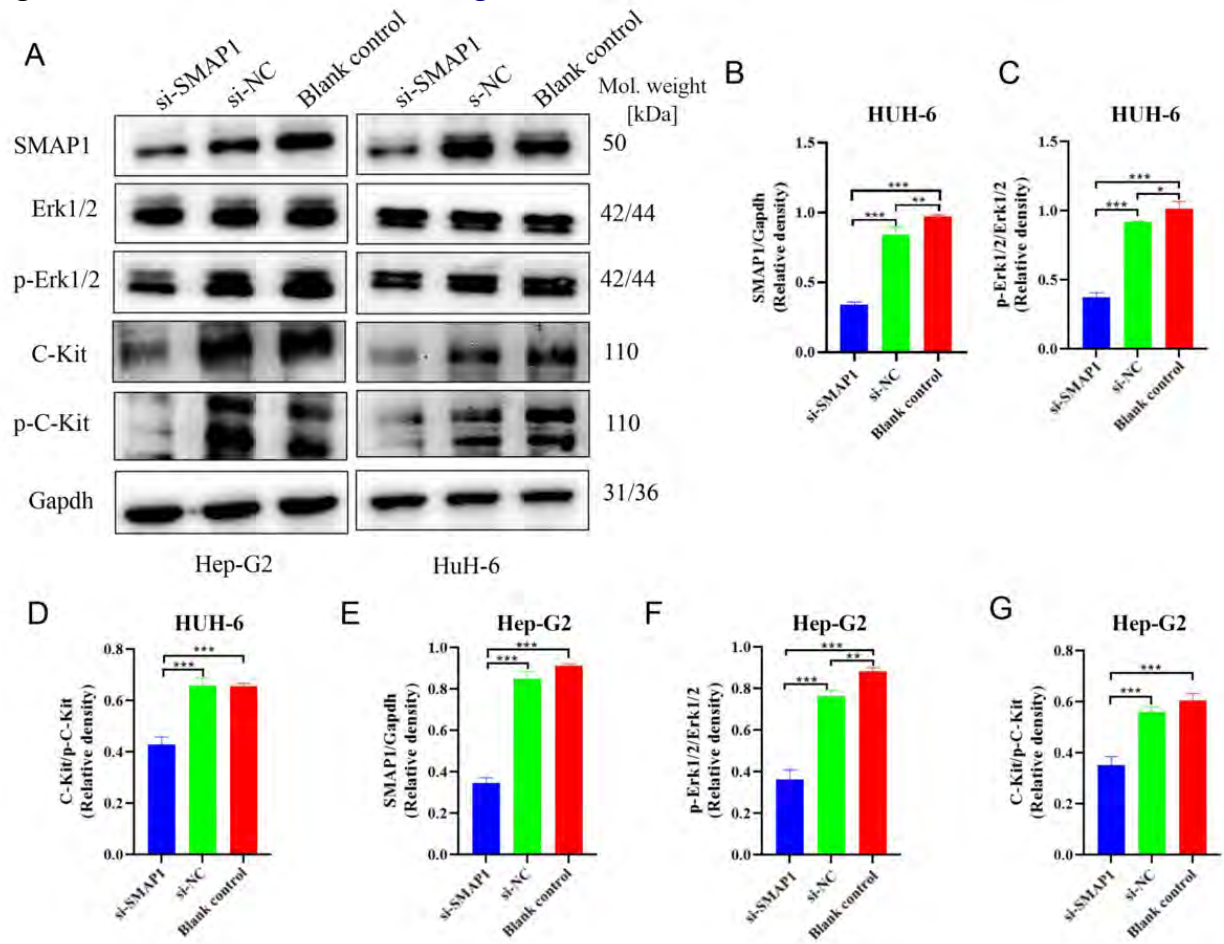


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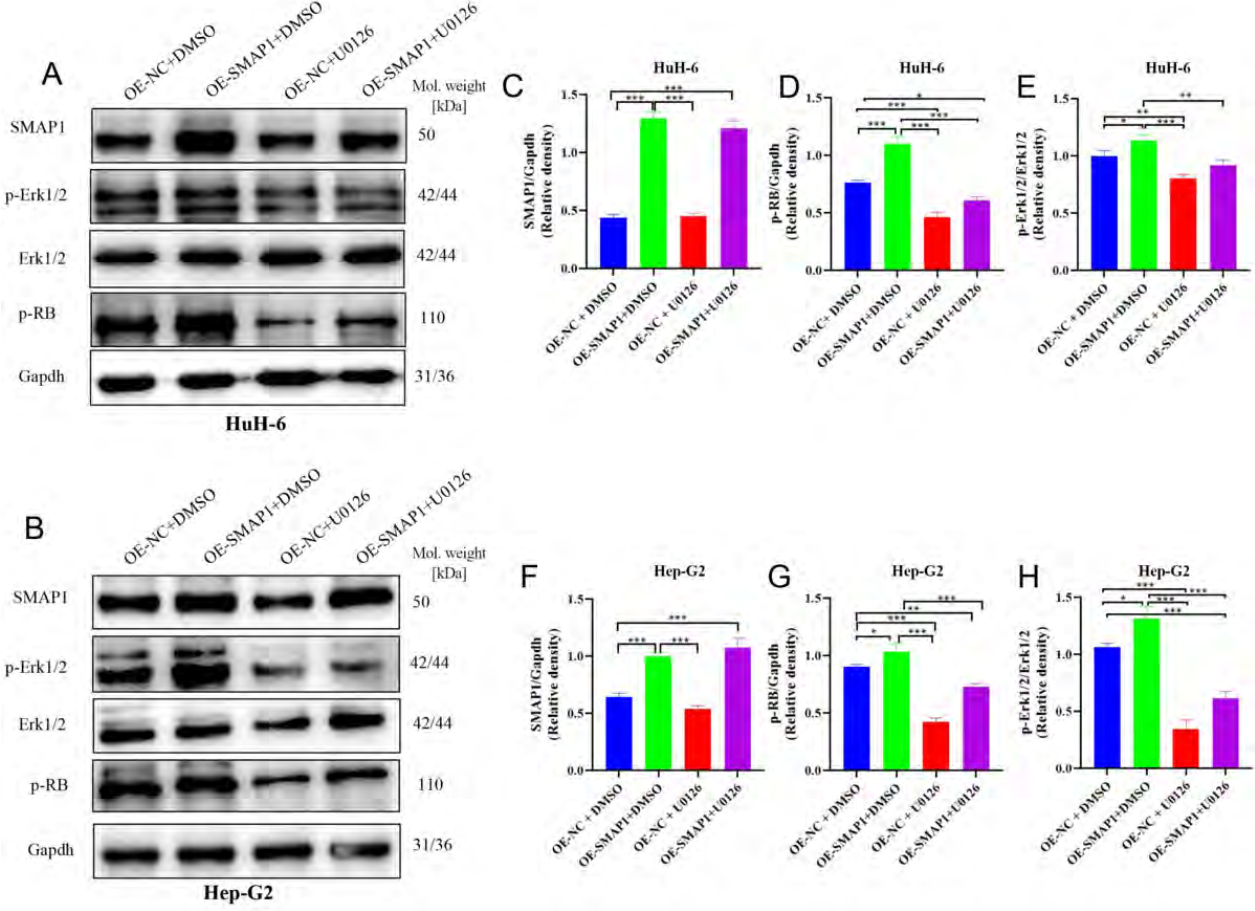


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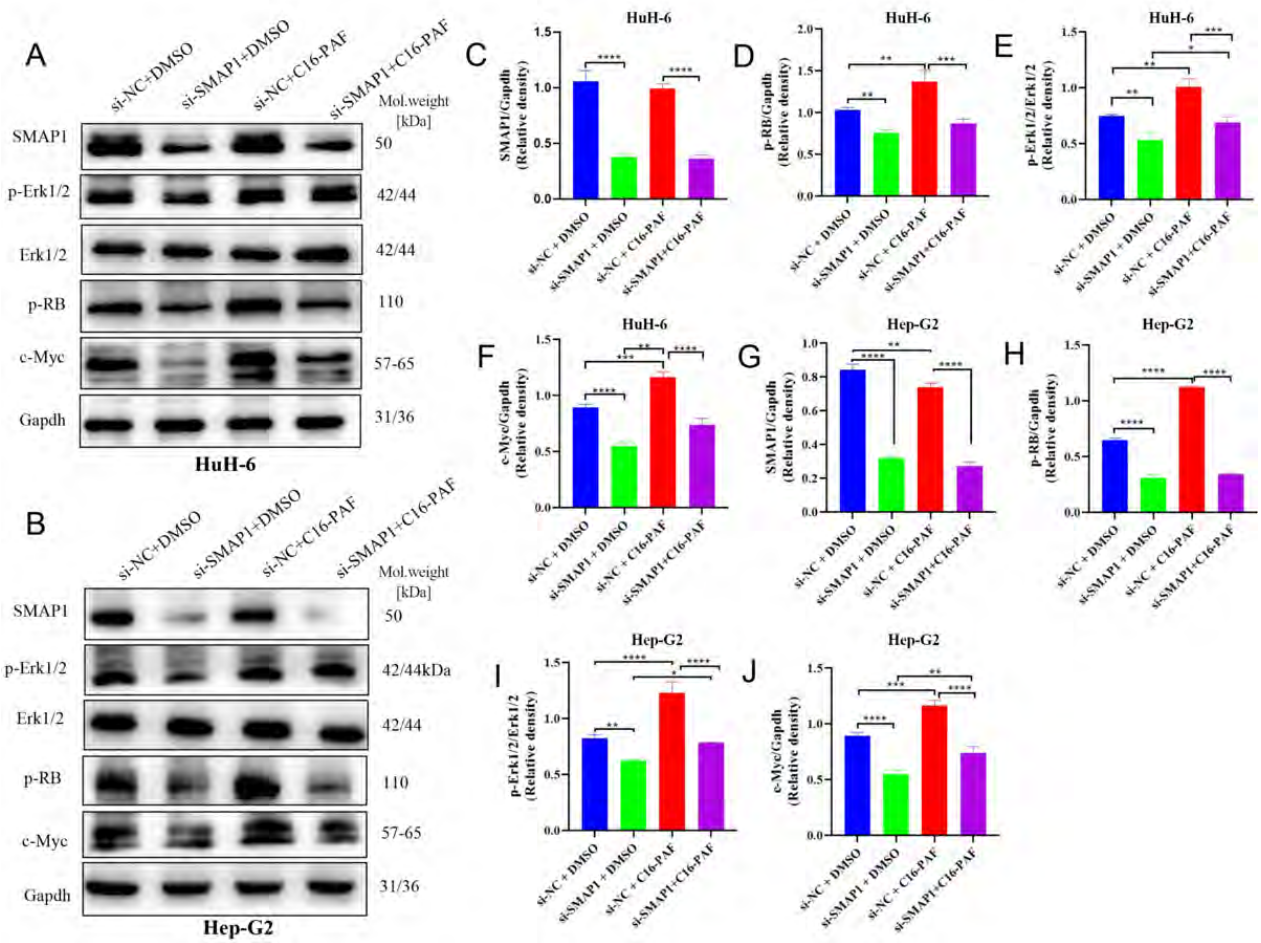


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