1 **KDM2B promotes cell viability by enhancing DNA damage response in canine**
2 **hemangiosarcoma**

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18

19   **Abstract**

20   Epigenetic regulators have been implicated in tumorigenesis of many types of cancer;
 21   however, their roles in endothelial cell cancers such as canine hemangiosarcoma (HSA) have
 22   not been studied. In this study, we found that lysine-specific demethylase 2B (Kdm2b) was
 23   highly expressed in HSA cell lines compared to normal canine endothelial cells. Silencing of
 24   Kdm2b in HSA cells resulted to increased cell death *in vitro* compared to the scramble control
 25   by inducing apoptosis through the inactivation of the DNA repair pathways and accumulation of
 26   DNA damage. Similarly, doxycycline-induced Kdm2b silencing in tumor xenografts resulted to
 27   decreased tumor sizes compared to the scramble control. Furthermore, Kdm2b was also highly
 28   expressed in clinical cases of HSA, and its expression levels was higher than in hemangioma, a
 29   benign counterpart of HSA. Based on these results, we hypothesized that pharmacological
 30   Kdm2b inhibition can also induce HSA cell death and can be used as an alternative treatment for
 31   HSA. We treated HSA cells with GSK-J4, a histone demethylase inhibitor, and found that
 32   GSK-J4 treatment also induced apoptosis and cell death. On top of that, GSK-J4 treatment in
 33   HSA tumor-bearing mice decreased tumor sizes without any obvious side-effects. In this study,
 34   we demonstrated that Kdm2b acts as an oncogene in HSA by enhancing DNA damage response
 35   and can be used as a biomarker to differentiate HSA from hemangioma. Moreover, we indicated
 36   that histone demethylase inhibitor GSK-J4 can be used as a therapeutic alternative to
 37   doxorubicin for HSA treatment.

38

39   **Keywords**

40   DNA repair, epigenetics, hemangiosarcoma, KDM2B, oncogene

41

42   **Introduction**

43   Canine hemangiosarcoma (HSA) is a highly malignant tumor of vascular endothelial
44   cells. It is an aggressive tumor with high rates of local recurrence and metastasis, and a low
45   overall survival time¹. Its high cellular heterogeneity has limited genomic and pathogenesis
46   studies in HSA. Genomic analyses have revealed that HSA cells have somatic coding mutations
47   in the *TP53*, *PIK3CA*, and *PIK3R1*. Furthermore, *CDKN2A/B* were found to be consistently
48   deleted and copies of *VEGFA*, *KDR* and *KIT* were gained². The oncogene involved in HSA,
49   however, is still unknown.

50   Epigenetic mechanisms are essential for reproduction, embryonic development, and
51   maintenance of normal cell function in eukaryotes³. Generally, the genetic alterations that cause
52   tumorigenesis are combined with epigenetic shifts, such as aberrant DNA methylation and
53   histone modifications, which may help oncogenic drivers improve cancer development,
54   metastasis, and resistance to therapies⁴. KDM2B (also known as NDY1 and FBXL10), an
55   H3K4me3, H3K36me2/3 and H3K79me3 demethylase, acts as a tumor suppressor in gastric
56   cancer by downregulating glycolysis, and tumor-derived mutation in Kdm2b enhances cell
57   proliferation through the inability of c-FOS degradation⁵. Alternatively, KDM2B can also act as
58   an oncogene in various types of cancers. In leukemia, Kdm2b is highly expressed and is
59   sufficient to transform hematopoietic progenitor cells⁶. In breast cancer, KDM2B regulates

60 polycomb complexes and controls self-renewal of breast cancer stem cells⁷. This bifunctional
61 activity means that the role of KDM2B in tumors is highly context dependent and must be
62 evaluated carefully⁸. While epigenetics is highly involved in pathogenesis of many cancers, its
63 role in HSA is still unknown.

64 Treatment of HSA is carried out by tumor excision and chemotherapy with
65 doxorubicin, cisplatin, or 5-fluorouracil^{9,10}. The mean survival time for surgical treatment is 3
66 months while the mean survival time for surgical treatment with chemotherapy is less than a
67 year^{11,12}. Chemotherapeutic drugs fail to improve survival times of cancer patients due to the
68 non-specificity of their cytotoxic effects in the cardiomyocytes, brain, liver and intestine^{13,14}. A
69 more effective, specific, and safer treatment for HSA is warranted.

70 Histone demethylase inhibitors are effective agents for anticancer treatment. In a
71 previous study, GSK-J4, a histone demethylase inhibitor, decreased AML disease progression by
72 downregulating DNA replication and cell cycle-related pathways through the enrichment of
73 H3K27me3¹⁵. GSK-J4 was initially developed as a KDM6 inhibitor, but it was found to inhibit
74 the catalytic activity of a wide range of JmjC domain-containing histone demethylases including
75 KDM2B¹⁶. Epigenetic therapy may provide additional treatment option, but no epigenetic drug
76 has tested for HSA.

77 In this study, we sought to establish the pathogenesis and to find a therapeutic

78 alternative for HSA by examining the role of epigenetic regulators.

79

80 **Results**

81 **Kdm2b is important for HSA cell survival**

82 We first performed RNA-sequencing analysis of canine aortic endothelial cells
83 (CnAOEC) and one HSA cell line, JuB2, to examine the expression levels of epigenetic
84 modifiers. Among the differentially expressed genes, histone methyltransferases/demethylases,
85 histone acetyltransferases/deacetylases, and DNA methyltransferase/demethylases were
86 dysregulated in JuB2 compared to CnAOEC (Fig. 1A, Supplementary Fig. 1A). Next, we
87 performed qRT-PCR to verify RNA-seq results of histone methyltransferases/demethylases in
88 CnAOEC and seven HSA cell lines. Some histone methyltransferases were incoherently
89 expressed in HSA cell lines compared to CnAOEC (Supplementary Fig. 1B). In contrast, three
90 histone demethylases were significantly upregulated in HSA cells compared to CnAOEC.
91 Moreover, one set of paralogs (*KDM2A* and *KDM2B*) were enriched, which implies their
92 importance in HSA (Fig. 1B). We then performed western blotting for Kdm1a, Kdm2a, and
93 Kdm2b and found that their protein levels were upregulated in all HSA cell lines compared to
94 CnAOEC (Fig. 1C and D). To provide further evidence, we performed qRT-PCR analysis in
95 ISO-HAS-B, a human angiosarcoma cell line, compared to Human Umbilical Vein Endothelial

96 Cells (HUVEC). We revealed that histone methyltransferases/demethylases were also
 97 dysregulated in ISO-HAS-B (Supplementary Fig. 1C and Fig. 1E). Furthermore, protein
 98 expression levels of KDM1A, KDM2A and KDM2B were also upregulated in ISO-HAS-B
 99 compared to HUVEC (Fig. 1F and G). These results suggest that epigenetic regulators,
 100 specifically Kdm1a, Kdm2a, and Kdm2b, are highly expressed in HSA and human
 101 angiosarcoma and, thus, may have a significant role in endothelial cell tumors.

102 To examine whether the overexpression of Kdm1a, Kdm2a and Kdm2b has important
 103 functional roles in HSA, we designed three shRNA sequences for each gene to knockdown
 104 *KDM1A*, *KDM2A* and *KDM2B* as well as two scramble RNA controls (scrRNA), and we
 105 expressed these shRNAs in HSA cell lines using a doxycycline (Dox)-inducible vector system.
 106 Knockdown was verified with western blotting after four days of Dox treatment (Fig. 2A).
 107 Knockdown of *KDM1A* and *KDM2A* did not have short term effects in JuB2 viability, whereas
 108 knockdown of *KDM2B* markedly decreased JuB2 viability within 4 days (Fig. 2B). To evaluate
 109 long term effects of Kdm1a, Kdm2a and Kdm2b on JuB2 cell survival, we performed colony
 110 formation assay and found that *KDM1A* and *KDM2A* knockdown could inhibit JuB2 colony
 111 formation, whereas *KDM2B* knockdown significantly reduced the number of colonies of JuB2
 112 (Fig. 2C and D, Supplementary Fig. 2A and B). Kdm2b knockdown with constitutively
 113 expressed shRNA vectors also induced the same phenotype in JuB2 cells and other HSA cell

lines such as JuB4, Re21, and Ud6 (Supplementary Fig. 2C and D). These results suggest that Kdm2b is an important histone demethylase in HSA cell survival, which encouraged us to further investigate its function. To examine whether Kdm2b can alter overall histone methylation levels in HSA, we performed western blotting for histone methylations that can be modified by Kdm2b. However, all possible histone methylations were not affected in JuB2 after *KDM2B* knockdown (Supplementary Fig. 2E). Next, to investigate which Kdm2b domain is responsible for HSA cell viability, we rescued Kdm2b function in *KDM2B* silenced HSA cells. We expressed wild type (WT) canine Kdm2b with silent mutations for the sequences which were targeted by the shRNAs and Kdm2b mutants with a point mutation for the JmjC domain (Kdm2b^{H283Y}), the CXXC domain (Kdm2b^{C586A}), or with PHD-finger domain deleted (Kdm2b^{ΔPHD})¹⁷⁻¹⁹. Overexpression of WT Kdm2b and Kdm2b^{C586A} mutant but not the Kdm2b^{H283Y} or the Kdm2b^{ΔPHD} mutants rescued the phenotype (Fig. 2E), which suggests that the JmjC and the PHD-finger domains are important for the Kdm2b function in HSA.

To verify our *in vitro* results and to know the effect of Kdm2b silencing *in vivo*, JuB2 cells with Dox-inducible shRNAs against *KDM2B* or the scramble shRNA were injected subcutaneously into nude mice and the tumor volumes over time were measured. Dox-containing food pellets were provided to the mice to induce shRNA expression when the largest tumor reached 150 mm³ in volume. Tumor xenografts started to decrease in volume four

132 days after induction of Kdm2b silencing, moreover, half of the xenografts bearing shKDM2B-A
133 and one out of eight xenografts bearing shKDM2B-C completely regressed 10 days
134 post-induction (Fig 3A and B). At the endpoint, tumor xenografts with silenced Kdm2b were
135 significantly smaller compared with the xenografts overexpressing the scramble RNA control
136 (Fig. 3C). We also xenografted JuB2 cells expressing the silencing vector for Kdm1a or Kdm2a.
137 Although tumor volume repression was initially observed post-doxycycline treatment, tumor
138 regrowth was observed 10 post-doxycycline treatment signifying that other histone
139 demethylases or proteins can rescue Kdm1a or Kdm2a function *in vivo* or some cell populations
140 were not affected by their knockdown (Fig. 3A).

141 These results suggest that Kdm2b is important for HSA cell survival both *in vitro* and
142 *in vivo* and its local catalytic activity is responsible for the phenotype.

143

144 **KDM2B knockdown induces apoptosis via accumulation of DNA damages**

145 To understand the mechanisms by which Kdm2b regulates HSA cell survival, we
146 performed RNA-seq followed by gene set enrichment analyses (GSEA) in JuB2 cells expressing
147 shKDM2B-A versus JuB2 cells expressing scrRNA-1. Silencing of Kdm2b showed negative
148 correlations with angiogenesis and glycolysis pathways, which might reflect decreased HSA cell
149 viability (Supplementary Fig. 3A). Interestingly, expressions of genes downregulated in

150 response to ultraviolet (UV) radiation (HALLMARK_UV_RESPONSE_DN) were decreased
151 by Kdm2b knockdown (Fig. 4A). Considering this correlation and the fact that UV response can
152 trigger DNA damage response²⁰, we speculated that DNA damage might be related to *KDM2B*
153 knockdown phenotypes. This speculation was further supported by positive enrichment of the
154 G2M checkpoint pathway and the interferon response pathway, which has been reported to be
155 associated with mitochondrial and nuclear DNA damage (Supplementary Fig. 3A)²¹⁻²⁴. These
156 results were validated by qRT-PCR (Fig. 4B, Supplementary Fig. 3B to E). To further dissect the
157 mechanism, we examined the expression levels of proteins involved in DNA repair pathway.
158 Total Atm, c-Fos, and γ H2A.X expressions were upregulated in HSA cell lines compared to
159 CnAOEC and their expressions were significantly decreased by *KDM2B* knockdown (Fig 4C
160 and D). Phosphorylated-Atm (pAtm), an active form of Atm, was also decreased by *KDM2B*
161 knockdown. Next, we carried out alkaline comet assay to detect DNA strand breaks in HSA cell
162 lines upon *KDM2B* silencing. Our results showed that *KDM2B* silencing drastically increased
163 tail DNA percentages, tail lengths, and tail momentums compared to the scramble control (Fig.
164 4E and F). Furthermore, flow cytometry analysis using propidium iodide (PI) revealed that JuB2
165 with silenced *KDM2B* increased aneuploid peaks although parental HSA cells also showed
166 aneuploidy (Fig 5A and B, Supplementary Fig. 4A and B). In contrast, overexpression of WT
167 Kdm2b in JuB2 HSA cells resulted to significantly decreased aneuploid cell population

168 compared to empty vector (EV) expressing JuB2 cells (Fig 5C and D). In addition, active
169 apoptosis markers, cleaved-caspase 3, Bax and phosphorylated Erk1/2, were increased in cells
170 with silenced *KDM2B* compared to the scramble control cells (Fig 5E). Furthermore, CnAOEC
171 overexpressing WT Kdm2b continued proliferating beyond day 20 while the EV expressing
172 CnAOEC showed decreased cell proliferation and cell death starting at day 11 (Fig 5F). In
173 addition, Kdm2b overexpression decreased the phosphorylation of Erk1/2 proteins at day 19
174 post-lentiviral Kdm2b overexpression (Fig. 5G and H) and repressed mRNA expressions of cell
175 cycle checkpoint genes such as *p15^{ink4b}*, *p16^{ink4a}*, and *ATR* (Fig. 5I). These results suggest that
176 *KDM2B* knockdown in HSA cells induces cell death via apoptosis caused by accumulation of
177 DNA damages due to low expression of proteins involved in DNA repair.

178

179 **Kdm2b is highly expressed in clinical cases of HSA**

180 We demonstrated that Kdm2b upregulation was important for HSA cell survival, but
181 Kdm2b expression in clinical HSA cases has not been investigated. To this end, we analyzed
182 seventeen clinical cases of HSA and compared the immunohistochemical Kdm2b expression
183 between HSA cells and normal endothelial cells in the same sample. Our results showed that
184 Kdm2b was highly expressed in HSA cells compared to normal endothelial cells in almost all
185 the cases examined regardless of proliferation patterns or degree of differentiation (Fig 6A and

186 B). The average Kdm2b score in HSA cells was significantly higher than that in normal
187 endothelial cells (Fig. 6C). We then also examined the benign endothelial tumor, hemangioma
188 (HMA), for Kdm2b expression. Interestingly, Kdm2b expression levels in hemangioma cells
189 were likely lower than normal endothelial cells in the same sample (Fig. 6D to F, $p=0.096$). The
190 average Kdm2b expression scores in HSA were significantly higher than in HMA cells when the
191 average Kdm2b scores in tumor cells were normalized to normal endothelial cells in the same
192 slides (Fig. 6G). Kdm2b expressions were also analyzed in histologically-similar tumors,
193 hemangiopericytoma (HPC), melanoma, and fibrosarcoma cells. Results showed that HPC,
194 melanoma, and fibrosarcoma cells had similar Kdm2b expression compared to normal
195 endothelial cells in the same slide (Supplementary Fig. 5A to C). These results suggest that
196 Kdm2b expresses in clinical HSA samples and can be used as a biomarker to differentiate HSA
197 and HMA. In addition, Kdm2b may also be used as a molecular marker to differentiate HSA
198 from HPC, melanoma, and fibrosarcoma.

199

200 **GSK-J4 inhibits HSA cell viability**

201 We demonstrated that KDM2B inhibition through shRNA could induce HSA cell death.
202 Based on this, we hypothesized that pharmacological Kdm2b inhibition could also induce HSA
203 cell death. To test this hypothesis, we treated HSA cell lines with different concentrations of

204 GSK-J4 and compared it to doxorubicin-treated cells. GSK-J4 inhibited HSA cell viability at a
205 lower concentration in JuB2, JuB4, and Ud6 cell lines compared to doxorubicin (Fig. 7A). HSA
206 cell lines treated with GSK-J4 have higher expressions of cleaved-caspase 3 compared to HSA
207 cells treated with doxorubicin. In contrast, GSK-J4 treated HSA cells have decreased t-Atm and
208 γ H2A.X expression compared to HSA cells treated with DMSO control (Fig.7B).

209 To determine whether GSK-J4 can be used as an alternative drug for HSA treatment,
210 we treated nude mice harboring JuB2 tumors with DMSO, GSK-J4 or doxorubicin. GSK-J4
211 treatment significantly decreased the tumor growth over time and tumor weights at the endpoint
212 compared to DMSO treatment (Fig 7C-E). Although Doxorubicin treatment also led to
213 decreased tumor growth, it had significant toxicity and led to 83% mortality in 21 days (Fig. 7F).
214 In contrast, GSK-J4 did not induce any death during the treatment period. Body and liver
215 weights decreased in doxorubicin-treated mice compared to DMSO or GSK-J4 treated mice
216 (Fig. 7G, Supplementary Fig. 6A). In addition, increased hematopoiesis in bone marrow, a sign
217 of myelosuppression, and dilation of intestines were only observed in mice treated with
218 doxorubicin (Supplementary Fig. 6B and C). These results suggest that GSK-J4 can selectively
219 inhibit HSA cell growth by inducing HSA cell apoptosis without any obvious side-effects. Thus,
220 GSK-J4 could be used as a therapeutic alternative to doxorubicin for HSA treatment.

221

222 Discussion

223 To our knowledge this is the first study which evaluated the role of epigenetic
 224 regulators in HSA. We demonstrated that Kdm2b upregulation in HSA cell lines and clinical
 225 samples is important for HSA cell survival by regulating DNA damage repair and apoptosis.
 226 There are three possible reasons for the high expression of Kdm2b in HSA. Firstly, *KDM2B*
 227 upstream regulators may be dysregulated. In bladder cancer, the upregulation of fibroblast
 228 growth factor-2 upregulated the KDM2B/EZH2-miR-101 pathway and promoted tumor cell
 229 proliferation, survival, and migration²⁵. In squamous cell and cervical carcinomas, increased
 230 copy number of *MYC* resulted to increased KDM2B expression²⁶. Secondly, the *KDM2B* gene
 231 itself may have acquired increased copy numbers. We demonstrated that parental HSA cell lines
 232 contain an aneuploid cell population. Since cell aneuploidy can cause genomic instability as a
 233 result of decreased DNA damage repair activity²⁷, some genes including *KDM2B* in HSA might
 234 have increased copy numbers. Lastly, *KDM2B* might be upregulated to compensate for DNA
 235 damage and to integrate genetic stability in HSA genomes. As we mentioned above, flow
 236 cytometry analysis revealed multiple aneuploid peaks in HSA cell lines. Aneuploidy has also
 237 been reported in clinical cases of HSA and in HSA cell lines, which implies that HSA cells are
 238 subject to high level cellular stress^{28–30}. In such conditions, HSA cells may bypass cell death
 239 caused by accumulated cellular stresses through modulation of gene expression. We showed that

240 knockdown of Kdm2b in HSA cell lines decreased DNA repair protein expressions and
241 increased DNA damage. In contrast, exogenous expression of Kdm2b in JuB2 and in CnAOEC
242 increased euploid population and inhibited apoptosis, respectively. Kdm2b may ease cellular
243 stress caused by DNA damage in endothelial cells by regulating the DNA repair system.

244 KDM2B and other histone demethylases are known to have a double-edged function
245 in cancer^{8,31}. A study showed that KDM2B suppresses tumorigenesis in gastric cancer by
246 enhancing c-Fos degradation, and that impairment of KDM2B through patient-derived
247 mutations enhances tumor cell proliferation³². In contrast, several studies showed that
248 degradation of c-FOS by KDM2B in prostate cancer and glioblastoma multiforme can increase
249 cancer cell resistance to chemotherapy^{33,34}. In HSA, we showed that positive regulation of these
250 genes by Kdm2b can enhance HSA cellular viability in contrast to a previous report where
251 KDM2B was shown to promote colon cancer cell survival by negatively regulating DNA
252 damage response-related genes such as ATM and ATR³⁵. These contrasting evidences support
253 the role of KDM2B in DNA damage response and suggest that the role of KDM2B in DNA
254 damage response in cancer cells is highly context dependent.

255 We have presented various evidences which demonstrate the role of Kdm2b as an
256 oncogene in HSA by regulating DNA repair system and apoptosis, and that Kdm2b can be used
257 as a biomarker to aid HSA diagnosis. We also demonstrated that a histone demethylase inhibitor

258 GSK-J4 can work as a new therapeutic alternative to doxorubicin treatment in HSA. These
259 findings shed light on the epigenetic pathology and provides a new insight for a novel therapy in
260 HSA.

261

262 **Materials and Methods**

263 **Cell culture**

264 The HSA cell lines were donated by Dr. Sakai (Gifu University)³⁶ and cultured as
265 described previously³⁷. CnAOEC was purchased from Cell Applications (CA, USA), and
266 ISO-HAS-B was received from Cell Resource Center for Biomedical Research Cell Bank in
267 Tohoku University (Sendai, Japan)³⁸. Both CnAOEC and ISO-HAS-B were cultured in
268 Endothelial Cell Growth Medium 2 Kit (Takara Bio, Inc. Kusatsu, Japan). All cells used were
269 routinely tested for *Mycoplasma* using PCR and were submitted to ICLAS Monitoring Center
270 (Kawasaki, Japan) for Mouse hepatitis virus testing^{39,40}.

271

272 **Mice**

273 All mouse experiments were performed under the AAALAC guidelines in Yale
274 University (protocol number: 2018-11286) and Hokkaido University (protocol number:19-0130).
275 Seven-week old female Balb/c Nude mice purchased from Charles River Laboratories (MA,

USA) were used for *KDM2B* knockdown experiments. Six-week-old KSN/Slc mice purchased from Japan SLC, Inc. (Shizuoka, Japan) were used for drug treatment experiments. Mice were kept in a temperature-controlled specific-pathogen-free facility on a 12 hr light/dark cycle. Animals in all experimental groups were examined at least twice weekly for tumorigenesis.

280

Tumor xenograft studies

A day before tumor inoculation, KSN/Slc mice were treated with 100 μ L of 2.5 mg/mL anti-asialo GM1 (Fujifilm Wako Pure Chemical Industries, Osaka, Japan) to increase the success rate of transplantation by depleting NK cells⁴¹. JuB2 parental HSA cells and JuB2 cells expressing the shRNAs or the scramble shRNA for *KDM2B* in the presence of doxycycline were cultured in 15 cm dishes without doxycycline. Mice were randomly assigned in each group. Three million HSA cells were resuspended in Corning® Matrigel® Basement Membrane Matrix (Corning Inc. NY, USA) and inoculated subcutaneously in mice anesthetized with 3% isoflurane or 100 mg/kg Ketamine and 10 mg/kg Xylazine. Tumor sizes were measured twice weekly one week after inoculation. When the largest tumor reached 150 mm³ in volume, mice were fed doxycycline-containing food to induce shRNA expression, or treated thrice weekly for three weeks with 50 mg/kg DMSO, 50 mg/kg GSK-J4 (Medchemexpress, NJ, USA), or 5 mg/kg doxorubicin (Fujifilm Wako Pure Chemical Industries) intraperitoneally. Mice were euthanized

294 with CO₂ when tumors reached 500 mm³ in volume or when mice exhibited abnormal behavior.
295 Tumors and major organs were weighed and fixed in 10% neutral buffered formalin for
296 histological examination.

297

298 **Western Blotting**

299 SDS lysis buffer (2% SDS, 50 mM Tris-HCl (pH6.8), 1mM EDTA (pH 8.0)) with
300 EDTA-free proteinase inhibitor cocktail (Sigma-Aldrich, MO, USA) was added in cultured cells.
301 Cell lysates were then sonicated using BRANSON Sonifier 450 (Branson Ultrasonics
302 Corporation, CT, USA) for two secs. Protein concentrations were measured with the Pierce™
303 BCA Protein Assay Kit (Thermo Fisher Scientific) before adding 4X Sample loading buffer
304 (200 mM Tris-HCl buffer (pH 6.8), 8% SDS, 40% Glycerol, 1% bromophenol blue, 20%
305 2-mercaptoethanol) and denaturing at 98°C for 10 mins. 3 µg proteins were separated by
306 SDS-PAGE and electrotransferred onto Immobilon®-P transfer membranes (Merck Millipore,
307 MA, USA), blocked with 5% skim milk in Tris-buffered saline with 5% Tween 20 (TBST), or
308 5% BSA in TBST for one hour at room temperature (RT) and incubated with primary antibody
309 in Can Get Signal Solution® 1 (TOYOBO, Osaka, Japan) overnight at 4°C. The membranes
310 were washed with TBST three times before incubating with the corresponding secondary
311 anti-mouse or anti-rabbit IgG antibody (GE Healthcare) in Can Get Signal Solution® 2

312 (TOYOBO). Signals were developed with Immobilon® Western Chemiluminescent HRP
313 substrate (Merck Millipore) and visualized in ImageQuant LAS 4000 mini luminescent image
314 analyzer (GE Healthcare). Captured data were processed using ImageJ software⁴². The list of
315 antibodies used in this study can be found as Supplementary Table 1.

316

317 **Quantitative RT-PCR (qRT-PCR)**

318 Total RNA was extracted with Nucleospin® RNA isolation kit (Macherey-Nagel
319 GmbH & Co. Düren, Germany) following the manufacturer's instructions. Synthesis of cDNA
320 was performed using the PrimeScript™ Reverse Transcriptase (Takara Bio, Inc.) according to
321 the manufacturer's instructions. qRT-PCR was performed with StepOne™ Real Time System
322 (Thermo Fisher Scientific). The oligos for qRT-PCR were designed as described elsewhere^{37,43},
323 and listed within Supplementary Table 2.

324

325 **RNA-sequencing**

326 CnAOEC or JuB2 HSA cells were cultured in 10 cm dishes in triplicate. Upon
327 reaching 80% confluency, RNA was extracted with Nucleospin® RNA isolation kit
328 (Macherey-Nagel GmbH & Co.) following the manufacturer's instructions. RNA samples were
329 submitted to Annoroad (Beijing, China) for further analyses. Quality testing was carried by

330 measuring RNA integrity (RIN), OD_{260/280} and OD_{260/230}. All samples had an RIN of 9.3 or better
331 and OD readings were within the range of 1.8-2.2. RNA-seq libraries were constructed using
332 NEBNext® Ultra RNA Library Prep Kit for Illumina® (New England Biolabs, MA, USA) and
333 sequenced with the Illumina HiSeq X-Ten platform (Illumina, CA, USA) to generate a
334 minimum of 20 million paired-end 150 bp reads. Sequencing reads were mapped to the canine
335 reference genome CanFam3.1 using STAR and aligned using RSEM^{44,45}. Differential expression
336 analyses were carried out using edgeR⁴⁶, and gene expression profiles were analyzed by GSEA
337 v4.03^{47,48}. The gene set database of h. all. v7.1. symbols. gmt (Hallmarks) was used.

338

339 **shRNA vector construction**

340 The shRNAs used in this study were designed using the Hannon lab shRNA design
341 tool (http://hannonlab.cshl.edu/GH_shRNA.html, Cold Spring Harbor Laboratory). Oligos were
342 inserted to pLKO.1-TRC or pInducer10-mir-RUP-PheS vector, a gift from David Root
343 (Addgene plasmid # 10878; <http://n2t.net/addgene:10878>; RRID:Addgene_10878)⁴⁹ and from
344 Stephen Elledge (Addgene plasmid # 44011; <http://n2t.net/addgene:44011>; RRID:Addgene
345 44011)⁵⁰, respectively. shRNA expressions were induced by supplementing 2 µM doxycycline in
346 the culture medium. The list of oligonucleotide sequences used for shRNA knockdown can be
347 found as Supplementary Table 3.

348

349 **Overexpression vector construction**

350   The coding sequence of canine *KDM2B* (ENSCAFT00000093772.1) was cloned from
351 cDNA synthesized from canine heart. Mutant Kdm2b (H283Y, C586A, ΔPHD) were
352 synthesized as described elsewhere^{15,51}. The amplicon tagged with FLAG sequences at its 3' end
353 was ligated into CSII-CMV-MCS-IRES2-Bsd vector, a gift from Dr. Miyoshi (RIKEN
354 BioResource Center, Ibaraki, Japan), with In-Fusion® HD Cloning Kit (Takara Bio, Inc.)
355 according to the manufacturer's instruction. The list of oligonucleotide sequences used for CDS
356 cloning are can be found as Supplementary Table 4.

357

358 **Lentivirus production**

359   Lentiviruses were produced following a protocol described elsewhere with a slight
360 modification that virus containing culture medium was used without concentration³⁷. Selection
361 of positive clones were done by culturing of cells in 10 µg/ml blasticidin- or 4 µg/ml
362 puromycin-containing cell medium.

363

364 **Alkaline comet assay**

365   JuB2 overexpressing shRNA for *KDM2B* or scrRNA were cultured in

366 doxycycline-containing medium for four days. 4×10^4 cells were used for alkaline comet assay
367 as described previously. 20 $\mu\text{g/mL}$ PI was used for staining and the comets were visualized with
368 BZ-9000 (BIOREVO) fluorescence microscope (Keyence, Osaka, Japan). Experiments were
369 performed at least three times with triplicates.

370

371 **Cell cycle analysis**

372 When HSA cells reach 70% confluency, they were stained with 1 μL of 0.1M BrdU for
373 45 mins at 37°C . Cells were washed with phosphate-buffered saline (PBS) and trypsinized
374 routinely. The cells were fixed in 70% ethanol overnight and washed with 0.5% Triton X-100 in
375 PBS (PBST) before resuspending the cells in 500 μL of 2N HCl-0.5% Triton X-100 for 30 mins
376 at RT and neutralizing with 500 μL of 0.1M $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ (pH 8.5) for 30 mins at RT. After
377 blocking with 1% BSA-0.3% Triton X-100 in PBS for 1 hr and washing with PBST, cells were
378 counted and divided into two tubes; 2.5×10^5 cells were used as controls and incubated in the
379 blocking buffer while 6×10^5 cells were incubated with anti-BrdU monoclonal antibody (1:100;
380 MOBU-1 clone, B35128, Thermo Fisher Scientific) for 1 hr at RT. Excess primary antibodies
381 were washed before staining with AlexaFluor 488 (1:1000; Thermo Fisher Scientific). DNA was
382 stained with 10 $\mu\text{g}/200 \mu\text{L}$ Propidium Iodide (Dojindo Molecular Technologies, Inc., Kumamoto,
383 Japan). Cell cycle and cell proliferation were analyzed in BD FACSVerse™ flow cytometer (BD

384 Biosciences, NJ, USA). Results were analyzed with FCS Express 4 software (De Novo
385 Software, CA, USA). Experiments were performed at least three times with triplicates.

386

387 **Cell viability analysis**

388 Cell viability was measured with Cell Counting Kit-8 (Dojindo Molecular
389 Technologies, Inc.) according to the manufacturer's instructions. The absorbance at 450 nm was
390 measured with NanoDrop™ 2000 (Thermo Fisher Scientific). Determination of IC₅₀ were
391 performed using Ky Plot 6.0 software (KyensLab, Inc., Tokyo, Japan) as described elsewhere⁵².
392 Experiments were performed at least three times with triplicates.

393

394 **Colony formation assay**

395 500 HSA cells were seeded in 6-well culture plates containing 2 mL normal medium
396 supplemented with DMSO or 2 µM doxycycline. Cells were cultured until the largest colony
397 reached 2 mm in diameter. Cells were fixed with 4% paraformaldehyde for 20 mins at RT,
398 stained with 0.01% Crystal Violet (Sigma-Aldrich, MO, USA) for 30 mins at RT, and the
399 images were captured with ChemiDoc XRS Plus (Bio-rad, CA, USA). Colony areas were
400 measured using ColonyArea plugin for ImageJ^{42,53}. Experiments were performed at least three
401 times with triplicates.

402

403 **Histopathology and immunohistochemistry (IHC)**

404   Written informed consents were obtained from the owners of the patient dogs, and the
405 samples were used only for research purposes. Hematoxylin and eosin staining was performed
406 as described previously⁵⁴. For IHC, tissue sections were deparaffinized, and heat-induced
407 antigen retrieval was performed in citric acid buffer (pH 6.8) with a pressure cooker.
408 Endogenous peroxidases were quenched with 0.3% H₂O₂ in methanol for 15 min at RT before
409 blocking the tissue sections with 10% normal rabbit serum (Nichirei biosciences, Tokyo, Japan)
410 for an hour at RT and incubating with KDM2B antibody (sc-293279, 1:50, Santa Cruz
411 Biotechnology, CA, USA) overnight at 4°C. Slides were washed with 0.01M PBS before
412 incubating with rabbit anti-mouse antibody (Nichirei biosciences) for 30 min at RT. Signals
413 were developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB; Dojindo).

414

415 **Quantification of IHC scores**

416   Histological slides were scanned with Nano Zoomer 2.0-RS (Hamamatsu Photonics,
417 Hamamatsu, Japan) and processed in QuPath ver 0.2.1⁵⁵. Scanned slides were opened as
418 Brightfield (H-DAB) in QuPath, and staining colors were automatically adjusted using Estimate
419 Stain Vectors function. Healthy tumor regions or normal blood vessels were randomly selected,

420 and cells were detected using Cell Detection function. Cells were annotated based on their
421 morphologies and location to allow QuPath to automatically classify each cell type correctly
422 using the Create Detection Classifiers function. The measured data was exported and used for
423 further analysis. We used Nuclear: DAB OD max scores for quantitative analysis because
424 Kdm2b signals were found only in heterochromatin regions in nuclei but they had abundant
425 euchromatin region. Furthermore, nonspecific signals from fibrin and erythrocytes were often
426 misinterpreted as nuclear or cytoplasmic signals especially in normal endothelial cells, thus, to
427 make the analysis as precise as possible, we decided to use max intensities in the nuclei.

428

429 **Statistical analysis**

430 Statistical analyses were performed with Microsoft Excel and R software (version
431 3.6.3). Student's *t*-tests was used to analyze difference between two groups while the Tukey's
432 test was used to analyze differences between multiple groups. The significance of the
433 differences in tumor volumes of DMSO, GSK-J4 or doxorubicin-treated mice were compared
434 using Dunnet's test while their survival post-treatment were compared using Log-rank test.
435 *p*-values less than 0.05 were considered statistically significant.

436

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446

447   **Availability of data and materials**

448   The RNA sequence data will be deposited in NCBI's Gene Expression Omnibus. All
449   other data supporting the findings of this study can be found within the supplementary files.

450

451   **Competing interests**

452   The authors declare no competing interests.

453

454   **Author contributions**

455   KG and KA designed the experiments. KG performed in vitro experiments. KG and KA

456 performed in vivo experiments. YS and HY assisted with the comet assay. KA analyzed the
457 RNA-sequencing data and performed other bioinformatics analyses. KG and KA were
458 responsible for the statistical analyses. QY, AK and TK supervised the experiments. KG and KA
459 wrote the paper.

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621

622 **Figure legends**

623

624 **Fig. 1 Epigenetic regulators are dysregulated in endothelial cell tumors.**

625 **A**, Transcripts per million (TPM) scores of histone demethylases in CnAOEC and JuB2 HSA
626 cell line. **B**, qRT-PCR verification of select histone demethylases in CnAOEC and HSA cell
627 lines. **C**, Western blotting for Kdm1a, Kdm2a, and Kdm2b in CnAOEC and HSA cell lines. **D**,
628 Quantitative analysis of Kdm1a, Kdm2a, and Kdm2b protein expressions in CnAOEC and HSA
629 cell lines. **E**, qRT-PCR analysis of *KDM1A*, *KDM2A*, and *KDM2B* mRNA expressions in
630 HUVEC and ISO-HAS-B. **F**, Western blotting for KDM1A, KDM2A, and KDM2B in HUVEC
631 and ISO-HAS-B. **G**, Quantitative analysis of KDM1A, KDM2A, and KDM2B protein
632 expressions in HUVEC and ISO-HAS-B. Data are presented as mean values $\pm$ s.d. * p <0.05
633 ** p <0.01 *** p <0.001, Student's *t*-test; ^c p <0.001, Tukey's test.

634

635 **Fig. 2 Kdm2b is important for HSA cell survival *in vitro*.**

636 **A**, Western blotting of Kdm1a, Kdm2a, or Kdm2b to verify the silencing efficiency of shRNA
637 vectors developed for Kdm1a, Kdm2a, and Kdm2b. **B**, Cell viability analysis after the induction
638 of the inducible shRNA vector using doxycycline. **C**, Colony formation assay of JuB2 cells after
639 Kdm1a, Kdm2a, or Kdm2b silencing. **D**, Quantitative analysis of **C**. **E**, Cell viability analysis

640 after rescue overexpression of WT Kdm2b, or dominant negative mutants for the JmjC
641 (Kdm2b^{H283Y}), CxxC (Kdm2b^{C586A}), or PHD (Kdm2b^{ΔPHD}) domains. Data are presented as mean
642 values ± s.d. ***p<0.001, Two-way ANOVA.

643

644 **Fig. 3 Kdm2b is important in HSA cell survival *in vivo*.**

645 **A**, Tumor growth at different time points before and after the induction of the shRNA
646 expression in JuB2 cells inoculated in nude mice. ***p<0.001, two-way ANOVA with Dunnett's
647 post-hoc test for tumor volumes after starting doxycycline treatments (arrow). **B and C**, Tumor
648 sizes and weights of tumors harboring scramble RNA or shRNA for Kdm1a, Kdm2a, or Kdm2b
649 78 days after tumor transplantation. Scale = 1 cm. **p<0.01 ***p<0.001, Dunnett's test. Data
650 are presented as mean values ± s.d.

651

652 **Fig. 4 Kdm2b positively regulates DNA repair pathway.**

653 **A**, GSEA enrichment plot for UV_Response_DN in shKDM2B-A versus scrRNA-1. **B**,
654 qRT-PCR verification of gene expressions listed in UV_Response_DN gene set. **C and D**,
655 Western blotting for Atm, c-Fos, and γH2A.X in normal endothelial cells and HSA cell lines (**C**)
656 and in JuB2 cells expressing shRNA control or shKDM2B (**D**). **E**, Representative images of
657 alkaline comet assays in scrRNA or shKDM2B JuB2 cells. **F**, Tail DNA percentages, lengths,

658 and momentums of JuB2 HSA cells harboring scrRNA or shRNA for Kdm2b. Data are
659 presented as mean values $\pm$ s.d. *** p <0.001, Tukey's test.

660

661 **Fig. 5 Kdm2b regulates aneuploidy and apoptosis in HSA.**

662 **A**, Histograms of PI intensities in scrRNA and shKDM2B JuB2 cells. **B**, Percentages of
663 aneuploid cells in shRNA and shKDM2B JuB2 cells. *** p <0.001, Tukey's test. **C**, Histograms
664 of PI intensities in EV infected JuB2 cells and WT Kdm2b JuB2 cells. **D**, Percentages of
665 aneuploid cells in EV JuB2 and WT Kdm2b JuB2. *** p <0.001, Student- t test. **E**, Western
666 blotting for cleaved-caspase 3, Bax and Erk 1/2 in JuB2 HSA cells expressing scrRNA controls
667 or shKDM2B RNAs. **F**, Phase contrast images of CnAOEC cells infected with EV or
668 overexpressing WT Kdm2b at day 1 and day 19. Scale = 100 μ m. **G**, Western blotting of Erk 1/2
669 in CnAOEC infected with EV or overexpressing WT Kdm2b. **H**, Quantification of Kdm2b and
670 phosphorylated Erk 1/2 expression in EV infected or WT Kdm2b expressing CnAOEC
671 normalized with Actin and total Erk 1/2, respectively. **I**, qRT-PCR analysis of cell cycle related
672 genes after overexpression of WT Kdm2B in normal endothelial cells. Data are presented as
673 mean values $\pm$ s.d. ** p <0.01 *** p <0.001, Student's t -test.

674

675 **Fig. 6 Kdm2b can be used as a differential biomarker for HSA.**

676 **A**, Representative images of hematoxylin-eosin staining (HE) and immunohistochemistry (IHC)
677 for clinical HSA samples. Proliferation patterns are indicated with case numbers. Scale = 100
678 μm . **B**, Max nuclear DAB intensities in individual endothelial cells (EC) and tumor cells
679 (Tumor) of each HSA case. **C**, Average max nuclear DAB intensities of EC and tumor cells in
680 each HSA case. **D**, Representative HE and IHC images of hemangioma (HMA), a benign
681 endothelial cell tumor. Scale = 100 μm . **E**, Max nuclear DAB intensities in individual EC and
682 tumor cells of each HMA case. **F**, Average max nuclear DAB intensities of EC and tumor cells
683 in each HMA case. **G**, Comparison of relative Kdm2b intensity of HSA and HMA cells
684 normalized by normal endothelial cells in their respective slides. Data are presented as mean
685 values $\pm$ s.d. $**p < 0.01$, Student's *t*-test.

686

687 **Fig. 7 GSK-J4 inhibits HSA cell proliferation *in vitro* and *in vivo*.**

688 **A**, Survival rates and IC_{50} values of GSK-J4 or doxorubicin-treated HSA cell lines. **B**, Western
689 blotting for $\gamma\text{H2A.X}$, Atm and cleaved-caspase 3 in HSA cells treated with GSK-J4 or
690 doxorubicin. **C**, Tumor growth curves of JuB2 HSA cell xenografts in nude mice. Treatment
691 with DMSO, GSK-J4, or doxorubicin started at day 45 (arrow). n indicates the number of mice.
692 $**p < 0.01$, two-way ANOVA with Dunnett's post-hoc test. **D**, Gross images of collected tumors
693 at day 64. n indicates the number of collected tumors. **E**, Tumor weights of DMSO, GSK-J4, or

694 doxorubicin-treated nude mice at necropsy. **F**, Kaplan-Meier survival curves of DMSO, GSK-J4,
695 or doxorubicin-treated nude mice after starting treatments. $**p < 0.01$, Log-rank test. **G**, Body
696 weights of DMSO, GSK-J4, or doxorubicin-treated nude mice at necropsy. Data are presented
697 as mean values $\pm$ s.d. $**p < 0.01$, Tukey's test.
698

Fig. 1

Fig. 2

A

B

C

Fig. 3

Fig. 4

A

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B **C** **D** **E** **F** **G** **H** **I**

Fig. 5

Fig. 6

Fig. 7