1 **KDM6 demethylases contribute to EWSR1-FLI1-driven oncogenic**

2 **transformation in Ewing Sarcoma**

3 Elisabet Figuerola-Bou[1‡], Carla Rios-Astorch[1‡], Enrique Blanco[2,3] , María Sánchez-

4 Jiménez[1] , Pablo Táboas[1], Guerau Fernández[4], Soledad Gómez[1], Oscar Muñoz[1], Pol

5 Castellano-Escuder[5], Sara Pérez-Jaume[1, 6], Marta García[1], Estela Prada[1], Silvia Mateo-

6 Lozano[1], Nicolo Riggi[7,8], Alexandra Avgustinova[1,9], Cinzia Lavarino[1,10], Luciano Di

7 Croce[2,3,11], Sara Sánchez-Molina[1*†] and Jaume Mora[1,10*†]

[1]Pediatric Cancer, Institut de Recerca Sant Joan de Déu (IRSJD); Esplugues de Llobregat (Barcelona), 08950, Spain.

[2]Center for Genomic Regulation (CRG), Barcelona Institute of Science and Technology; Barcelona, 08003, Spain.

[3]Universitat Pompeu Fabra (UPF); Barcelona, 08002, Spain.

[4]Molecular Genetics Department, Institut de Recerca Sant Joan de Déu; Esplugues de Llobregat (Barcelona), 08950, Spain.

[5]Biomarkers and Nutritional & Food Metabolomics Research Group, Department of Nutrition, Food Science and Gastronomy, University of Barcelona; Barcelona, 08028, Spain.

[6]Biostatistics Unit, Department of Clinical Practice, University of Barcelona; Barcelona, 08036, Spain.

[7]Institute of Pathology, Centre Hospitalier Universitaire Vaudois, Faculty of Biology and Medicine, University of Lausanne; Lausanne, 1004, Switzerland.

[8]Department of Cell and Tissue Genomics, Genentech Inc; South San Francisco, California, 94080, USA.

[9]Institute for Research in Biomedicine (IRB Barcelona), Barcelona Institute of Science and Technology; Barcelona, 08028, Spain.

[10]Pediatric Cancer Center Barcelona (PCCB), Hospital Sant Joan de Déu; Esplugues de Llobregat (Barcelona), 08950, Spain.

[11]Institucio Catalana de Recerca i Estudis Avançats (ICREA); Barcelona, 08010, Spain.

[‡]These authors contributed equally to this work.

[*]Corresponding author. Email: sara.sanchezm@sjd.es (S.S-M.); jaume.mora@sjd.es (J.M.)

[†]These authors contributed equally to this work as co-senior authors.

8   **Abstract**

9   Ewing Sarcoma (EwS) is an aggressive bone and soft tissue tumor driven by the fusion

10   oncoprotein EWSR1-FLI1. This aberrant transcription factor binds to GGAA

11   microsatellites, causing epigenetic reprogramming through the formation of active neo-

12   enhancers in a permissive cellular context. Inhibition of the oncogene remains

13   challenging and current efforts seek to exploit emergent epigenetic treatments targeting

14   EWSR1-FLI1 cofactors. Here, stemming from the genome-wide redistribution of

15   H3K27me3 upon expression of EWSR1-FLI1 in human pediatric mesenchymal stem

16   cells (hpMSC), we unravel the contribution of the H3K27me3 demethylases

17   KDM6A/UTX and KDM6B/JMJD3 in transcriptional activation at EWSR1-FLI1

18   enhancers. We found that KDM6A has a demethylase-independent role in recruiting

19   BRG1 at EWSR1-FLI1-primed enhancers containing single GGAA motifs, which is

20   critical for EwS tumor growth. Conversely, KDM6B demethylates H3K27me3 at specific

21   EWSR1-FLI1-active enhancers and co-localizes with BRG1 at GGAA repeats. KDM6B

22   knockout impairs tumor growth and its deletion synergizes with EZH2 inhibitors. Our

23   results highlight KDM6 demethylases as EWSR1-FLI1 functional partners with potential

24   for future targeted strategies.

25   **Teaser**

26   Unveiling KDM6 demethylases as key players in Ewing sarcoma.

Introduction

Ewing Sarcoma (EwS) is a deadly neoplasm that arises in the bones and soft tissues of children, adolescents and young adults (*1*). Whole-genome sequencing studies of EwS tumors reported a remarkably genome stability with very low mutational burden (*2, 3*). Like many developmental cancers, EwS is characterized by a differentiation block during development conferred by the expression of an oncogenic driver (*4*). The characteristic reciprocal chromosomal translocation most commonly involves the *EWSR1 RNA-binding protein 1* (*EWSR1*) gene in chromosome 11 and the *ETS transcription factor family member Fli-1 proto-oncogene* (*FLI1*) in chromosome 22 (*5*). The resulting fusion protein retains the transactivation domain of *EWSR1* fused to the DNA-binding domain of *FLI1* and acts as an aberrant transcription factor (*6, 7*). However, EWSR1-FLI1 can only achieve oncogenic transformation in the right cellular background (*8-10*). While EWSR1-FLI1 induces growth arrest or apoptosis in differentiated primary cells (*11, 12*), its expression in human mesenchymal stem cells (hMSC) recapitulates the EwS gene signature (*9, 13*). Nevertheless, stronger induction of oncogenic targets occurs in human pediatric MSC (hpMSC) as opposed to adult derived mesenchymal cells which highlights the need for a permissive molecular framework (*13*).

EWSR1-FLI1 exhibits a high affinity to bind DNA through GGAA motifs, a class of ETS specific response element, activating or repressing their targets based upon the number of motif repeats (*14, 15*). At GGAA repeats, multimers of EWSR1-FLI1 establish *de novo* active enhancers (neo-enhancers) by promoting chromatin opening through recruitment of chromatin-modifying complexes. Indeed, in EwS cell lines and primary tumors the vast majority of EWSR1-FLI1 binding sites are decorated by H3K27ac, a post-translational histone mark associated to active enhancers (*16, 17*). Consistently, EWSR1-FLI1 features scaffolding properties and recruits core members of the transcriptional

52 activator mammalian SWItch/Sucrose Non-Fermentable (SWI/SNF) chromatin or

53 BRG1/BRM-associated factor (BAF) complex, such as BAF155, at GGAA repeats that

54 are critical for transcriptional activation (*18*). Furthermore, we have described that

55 RING1B, a member of the Polycomb group (PcG) family of proteins (*19*), facilitates

56 EWSR1-FLI1 recruitment towards key enhancers (*20*). Although the mechanism behind

57 EWSR1-FLI1 gene repression is less well understood, it has been proposed that binding

58 of EWSR1-FLI1 monomers on single instances of the GGAA motifs causes displacement

59 of endogenous ETS transcription factors decreasing transcriptional activation (*16*).

60 Besides, a large number of transcriptional repressors such as *NKX2-2* are induced by

61 EWSR1-FLI1, highlighting indirect mechanisms to mediate gene silencing (*21*).

62 The post-translational histone mark trimethylation of H3K27 (H3K27me3) decorates

63 promoters and enhancers of repressed and bivalent genes (*22*). H3K27me3 mark restricts

64 cell fate by limiting chromatin accessibility to key developmental genes, thus proper

65 deposition or removal of this mark in a timely manner is critical during development (*23*).

66 EZH2, the core member of the Polycomb Repressive Complex 2 (PRC2), is the primary

67 enzymatic writer of H3K27me3 that maintains the silencing patterning fundamental for

68 cell identity and proper differentiation (*24, 25*). Removal of H3K27me3 concerted with

69 promoter activation is mediated by members of the KDM6 family of demethylases,

70 including KDM6A (UTX) and KDM6B (JMJD3), through its JmjC domain (*26, 27*), and

71 determine specification of human neural progenitor cells (*28*). KDM6A has been

72 described as a partner of the Set1/MLL complex which is responsible for active gene

73 expression by methylation of H3K4 at active enhancers (*29, 30*), while KDM6B has been

74 reported to cooperate with the transcription factor KLF4 in enhancer-driven

75 reprogramming and with SMAD3 in neural specific enhancers (*31, 32*).

76 Deregulation of the H3K27me3 balance that is governed by the coordinated enzymatic

77 activities of EZH2 and KDM6A/KDM6B leads to differentiation defects and cancer (*33*).

78 EZH2 has been involved in the tumor progression mechanisms of a variety of cancer

79 types (*34*), while KDM6A and KDM6B have been identified in numerous malignancies

80 with either oncogenic or tumor suppressor roles (*35-39*). We previously demonstrated

81 that EWSR1-FLI1 occupies weak repressed Polycomb chromatin states in hMSC (*20*).

82 Although EWSR1-FLI1-bound GGAA repeats are devoid of H3K27me3 both in EwS cell

83 lines and primary tumors (*16, 17*), in Human Umbilical Vein Endothelial Cells

84 (HUVECs) and hpMSC these regions are extensively decorated with H3K27me3 (*8, 40*).

85 EZH2 is a well-known directly activated target by EWSR1-FLI1 that blocks

86 neuroectodermal and endothelial differentiation in EwS (*9, 41*). However, the precise

87 mechanism behind the equilibrium of H3K27me3 deposition and removal and its impact

88 in EwS tumor development have not yet been addressed.

89 Here, we shed light on the dynamics of H3K27me3 removal by KDM6A and KDM6B

90 demethylases within the context of EwS. First, we show how H3K27me3 is redistributed

91 genome-wide during EwS tumorigenesis in hpMSCs. Next, we demonstrate that KDM6A

92 and KDM6B bind to the same genomic regions as EWSR1-FLI1, with KDM6A

93 decorating EWSR1-FLI1 primed enhancers containing GGAA single motifs and KDM6B

94 characteristically enriched at active enhancers containing multimeric GGAA repeats.

95 Importantly, both KDM6A and KDM6B demethylases are involved in EWSR1-FLI1-

96 related transcriptional activation in a demethylase-independent and dependent manner,

97 respectively, being critical for EwS tumor growth. Our findings provide deep knowledge

98 regarding specific functions of H3K27me3 KDM6 demethylases in EwS and support

99 epigenetic treatment strategies based upon the reversibility of the processes involved in

100 the H3K27me3 equilibrium.

101   **Results**

102   **H3K27me3 genome-wide redistribution upon EWSR1-FLI1 overexpression in**

103   **hpMSCs**

104   Several groups have documented a *bona fide* list of GGAA repeats bound by EWSR1-

105   FLI1 to activate transcription (*15, 16, 42*). Recently, our group and others have

106   demonstrated that overexpression of the oncogene triggers a loss of H3K27me3 at

107   promoters of genes that become transcriptionally activated in HUVEC cells and neural

108   crest stem cells (NCSC) (*20, 43*). In an attempt to determine the global extension of such

109   a decrease in H3K27me3, we analyzed published ChIP-seq data on H3K27me3 in hpMSC

110   overexpressing the fusion oncogene (*40*). We first examined the distribution of

111   H3K27me3 in genes decorated by this mark in the hpMSC control condition (5 kb around

112   TSS) and revealed a maximal decrease at 2 kb upstream the TSS upon EWSR1-FLI1

113   overexpression, concomitant with an increase in H3K27ac specific for TSS (fig. S1A).

114   Nevertheless, Western blot analysis reported similar H3K27me3 levels in both conditions

115   (Fig. 1A), suggesting an overall genome-wide redistribution following the introduction

116   of EWSR1-FLI1. In order to confirm this hypothesis, we segmented the genome into

117   3,069,655 bins of 1 kb and determined that averaged H3K27me3 signal strength along

118   bins was highly correlated between control and EWSR1-FLI1 samples confirming the

119   redistribution of this mark (fig. S1B). Specifically, we identified 103,766 bins with a

120   significant reduction of H3K27me3 (Down bins) and 110,845 bins with a significant gain

121   of H3K27me3 (Up bins) upon EWSR1-FLI1 overexpression (fig. S1C). Of note, as

122   previously proposed (*20*), most genomic regions presenting H3K27me3 down bins upon

123   expression of EWSR1-FLI1 in hpMSC are notably enriched over regulatory sequences of

124   bivalent genes (fig. S1D). Integration of H3K27me3 data with ChIP-seq data for EWSR1-

125   FLI1 reveals 67,224 bins where the oncogene was enriched. Importantly, we observed a

6

126   progressive loss of H3K27me3 along with a gain of the EWSR1-FLI1 signal in a fraction

127   of 4,080 overlapping bins upon EWSR1-FLI1 overexpression (Fig. 1B). To test whether

128   H3K27me3 redistribution occurs at the gene level, we searched for those genes in which

129   the gain/loss was significant (FC2, min>0.01; 2175 genes down and 2289 genes up) (Fig.

130   1C). Functional analysis of genes losing H3K27me3 correlated with neural processes and

131   metabolism (fig. S1E and table S1). For instance, *NKX2-2*, a well-known EWSR1-FLI1

132   target, exhibited a gain of H3K27ac with a concomitant moderate H3K27me3 decay along

133   its promoter and regulating enhancers (Fig. 1D). Association with the TGF-beta receptor

134   signaling pathway was obtained for genes gaining H3K27me3 (fig. S1E and table S1), in

135   agreement with the well-known repressed state of genes from this pathway in EwS (*44*).

136   For instance, *TGFBI* was one of the genes in which a gain of H3K27me3 after EWSR1-

137   FLI1 introduction was observed along the gene body (Fig. 1E). Altogether, these results

138   demonstrate that EWSR1-FLI1 targets regions decorated with H3K27me3 promoting its

139   redistribution.

140   Next, we hypothesized that KDM6A and KDM6B, which are the enzymes responsible

141   for the demethylation of H3K27me3 (*26, 27*), might be implicated in the loss of

142   H3K27me3 at promoters and enhancers of EWSR1-FLI1 targets. Indeed, when evaluating

143   the expression of KDM6A and KDM6B demethylases in a panel of cancer cell lines (*45*),

144   we found that while KDM6A expression was similar across tumor types, KDM6B was

145   more expressed in EwS cell lines than in other sarcomas (Fig. 1F). Similar trends were

146   reported in 184 EwS tumors at diagnosis (GSE17679, GSE34620 and GSE37371

147   accessions), where we confirmed that the expression of both KDM6A and KDM6B is

148   consistently higher than in MSC, while KDM6B expression in EwS tumors was

149   particularly high compared to other sarcomas (Fig. 1G). Analysis of KDM6A and

150   KDM6B by immunohistochemistry in 43 EwS primary tumor specimens from newly

151   diagnosed patients from our institution revealed that both demethylases were highly

152   expressed by semi-quantitative histoscore (H-score) analysis (Fig. 1H and fig. S1F). No

153   correlation between KDM6A and KDM6B expression in EwS tumors was found

154   according to the statistical analysis of their H-score (fig. S1G). Taken together, gene

155   expression data and immunohistochemical studies indicate that KDM6A and KDM6B are

156   highly expressed in EwS cell lines and in primary tumor samples.

157   **KDM6A and KDM6B co-localize genome-wide with EWSR1-FLI1 at primed and**

158   **active enhancers**

159   To shed light on whether KDM6A and KDM6B demethylases are involved in H3K27me3

160   to H3K27ac switches in enhancers regulated by EWSR1-FLI1, we carried out chromatin

161   immunoprecipitation followed by high-throughput sequencing (ChIP-seq) for KDM6A,

162   KDM6B and EWSR1-FLI1 in the EwS cell line A673. We identified 3,737 peaks for

163   KDM6A, 2,687 for KDM6B, and 4,800 for EWSR1-FLI1 (P value < 0.05 in all cases;

164   FDR < 10^{-3} for KDM6A and EWSR1-FLI1, FDR < 10^{-5} for KDM6B) (fig. S2A and table

165   S2). At the genomic level, we observed a clear preference for intergenic and genic regions

166   in all cases (Fig. 2A), which indicates a role at enhancers as previously described (*29,*

167   *31*). KDM6B peaks were enriched at promoter regions (10.1%), compared to EWSR1-

168   FLI1 and KDM6A peaks. We next categorized KDM6A, KDM6B and EWSR1-FLI1

169   peaks into active, primed or poised enhancers, and in active or poised promoters based

170   on Blanco *et al.* (*46*). Primed enhancers are regulatory regions that correlate with the

171   H3K4me1 mark in absence of H3K27ac (*47*). Remarkably, KDM6A is more abundant at

172   primed enhancers, whereas KDM6B mimics EWR1-FLI1 distribution and mainly

173   associates to active enhancers (Fig. 2B). In agreement with this, motif analysis found the

174   characteristic single instance of the GGAA consensus on KDM6A sites, while KDM6B

175   and EWR1-FLI1 peaks were enriched in both multiple and single GGAA motifs (fig.

8

176   S2B). Gene association to peaks retrieved 1,511 genes for KDM6A and 1,207 genes for

177   KDM6B, that were enriched in axon guidance, axonogenesis or nervous system

178   development GO categories for both demethylases (fig. S2C and table S3), indicating an

179   important role of these demethylases in promoting neuronal differentiation as previously

180   described (*28*). Primed enhancers define a state prior to activation that does not yield

181   RNA and correlates with cell type specificity (*47, 48*). Thus, the enrichment of KDM6A

182   in primed enhancers supports the involvement of this enzyme in neural cell specification.

183   Strikingly, when intersecting KDM6A and KDM6B peaks with the set of 697 EWSR1-

184   FLI1-superenhancers defined by Tomazou *et al.* (*17*), we found an overlap of 23% and

185   35%, respectively. To elucidate to which extend KDM6A and KDM6B co-localize with

186   EWSR1-FLI1, we intersected peaks from the three entities and found a strong overlap of

187   EWSR1-FLI1 with both demethylases (A-B-EF group) and with each demethylase

188   separately (A-EF and B-EF) (Fig. 2C). This result was validated in another EwS cell line,

189   TC71, where we found 1163 common peaks between EWSR1-FLI1 and

190   KDM6A/KDM6B (P value < 0.05; FDR < 10^{-5} in all cases) (fig. S2D). Of note, ChIP-seq

191   for EZH2 retrieves no intersection with EWSR1-FLI1 (fig. S2E), in agreement with

192   previous studies (*16, 20*). Motif analysis in A673 cell line revealed a strong enrichment

193   in GGAA multimeric repeats when EWSR1-FLI1 stands alone or together with KDM6B

194   (B-EF group), while the presence of KDM6A is linked to the single GGAA motif (A-B-

195   EF and A-EF groups), suggesting that KDM6A partners with a unique set of EWSR1-

196   FLI1 peaks (Fig. 2C). In agreement with our motif characterization, KDM6B together

197   with EWSR1-FLI1 (B-EF group) strongly overlap with high levels of H3K27ac and

198   H3K4me1 (i.e. active enhancers), while KDM6A is associated to lower levels of

199   H3K27ac and enrichment in H3K4me1 (i.e. primed enhancers) (Fig. 2, D and E).

200   Remarkably, KDM6B alone was found in a subset of transcriptionally active promoters

201 (H3K4me3 and H3K27ac) (Fig. 2E and fig. S2F), suggesting differential functionalities

202 for each demethylase. No significant enrichment of H3K27me3 was found in any

203 collection of peaks (fig. S2F). Previously we described RING1B co-localization genome-

204 wide with EWSR1-FLI1 at active enhancers of key target genes (20). Interestingly,

205 RING1B is enriched both in EWSR1-FLI1 enhancers containing KDM6A and KDM6B

206 (B-EF, A-EF and A-B-EF group (fig. S2G). Overall, our data support three classes of

207 enhancers according to their composition: (i) those where KDM6A and KDM6B co-

208 localize with the oncogene (A-B-EF) at single GGAA motifs (e.g. *CDH11* gene, Fig. 2F

209 and fig. S2I), (ii) KDM6A/EWSR1-FLI1 enhancers (A-EF) containing single GGAA

210 motifs (e.g. *SMYD3*, fig. S2H); and (iii) those containing the overlap of KDM6B and

211 EWSR1-FLI1 (without KDM6A, B-EF) and enrichment at multimeric GGAA repeats

212 (e.g. *NKX2-2* enhancer, Fig. 2F and fig. S2I). Altogether, the differential location and

213 partnership identified for each of the KDM6 demethylases in EwS suggest that KDM6B

214 specifies EWSR1-FLI1 for the most active regions and KDM6A signals for a specific set

215 of enhancers that commit to neural lineage.

216 **Knockdown of KDM6A and KDM6B downregulates EWSR1-FLI1-activated**

217 **targets**

218 To understand whether EWSR1-FLI1 changes the distribution of KDM6A and KDM6B

219 on chromatin, we performed KDM6A and KDM6B ChIP-seq in a HeLa cell model that

220 express EWSR1-FLI1 under the control of a doxycycline-inducible promoter (49). As

221 shown in Fig. 3A and 3B, although KDM6A and KDM6B are already present in EWSR1-

222 FLI1 bound positions before induction, both demethylases increase their enrichment at

223 these positions once the oncogene is overexpressed. These data suggest EWSR1-FLI1

224 might be able to cause changes in the genome-wide occupancy for these demethylases.

225 Our previous results suggested two different classes of EWSR1-FLI1 induced neo-

226 enhancers, when partnered with KDM6A -primed enhancers- or with KDM6B -active

227 enhancers-. Indeed, the expression of genes in the proximity of enhancers containing

228 KDM6A is lower than those with KDM6B (Fig. 3C). To gain insight into the role of both

229 demethylases on the EWSR1-FLI1-transactivation activity, we knocked-down KDM6A

230 and KDM6B in two EwS cell lines using a doxycycline-inducible system. We reported

231 the knockdown of the demethylases at the protein level with two different shRNA

232 sequences (KDM6A sh#1 and sh#2; KDM6B sh#1 and sh#2) in both A673 and TC71

233 cells (Fig. 3D and fig. S3A). In order to study genome-wide expression changes we

234 carried out global transcriptome analysis by RNA-seq upon knockdown of KDM6A (sh#1

235 and sh#2) and KDM6B (sh#1 and sh#2). Loss of each demethylase resulted in gene

236 downregulation in A673 (P adj < 0.01 and log FC cut-off 0.5 and FPKM > 10, fig. S3B,

237 fig. S3C and table S4) and TC71 cell lines (P adj < 0.1 and log FC cut-off 0.32 and FPKM

238 > 5, fig. S3C). Gene Set Enrichment Analysis (GSEA) in the A673 cell line found that

239 both KDM6A and KDM6B are necessary for the activation of Epithelial-to-Mesenchymal

240 Transition (EMT) genes (Fig. 3E and table S4). However, there is little overlap between

241 both sets of deregulated genes upon demethylase knockdown (86 up-regulated and 34

242 down-regulated genes in common between KDM6A and KDM6B), confirming different

243 functionalities for each demethylase. All these results support a selective active role of

244 KDM6A and KDM6B in the transcriptional activation network necessary for EwS

245 tumorigenesis.

246 To further understand the relevance of KDM6A and KDM6B in the transcriptional

247 activation program of EWSR1-FLI1, we intersected genes associated to the genomic

248 regions where these demethylases and EWSR1-FLI1 co-localize with deregulated targets

249 in A673. To associate genes to peaks we captured every gene in a distance of 100 kb of

250 both combinations of ChIP-seq peaks and obtained 3,134 genes and 3,326 genes in the

11

251   vicinity of EWSR1-FLI1/KDM6A and EWSR1-FLI1/KDM6B, respectively. We then

252   intersected these direct targets with the set of KDM6A and KDM6B significantly

253   downregulated or upregulated genes. We obtained 64 directly activated targets for

254   KDM6A and 139 for KDM6B with EWSR1-FLI1 peak (Fig. 3F and table S4), while 20

255   directly repressed targets for KDM6A and 101 for KDM6B were identified (fig. S3D).

256   GO analysis of activated-targets reported neural categories such as axonogenesis,

257   previously observed in figure S2C, and cell migration (fig. S3E). Among the list of

258   KDM6A/EWSR1-FLI1 activated targets we found *CDH11*, *IRS2* and *SYT1* (Fig. 3, F and

259   G and fig. S3F); while for KDM6B/EWSR1-FLI1 we found targets such as *IGF1, MMP9*

260   and *JARID2* previously described in EwS (Fig. 3, F and H and fig. S3G). Interestingly,

261   most of the direct targets of KDM6A contain peak for KDM6B although only few targets

262   were commonly downregulated. These results support the differential role of KDM6A

263   and KDM6B in transcriptional activation in EwS.

264   **KDM6A recruits BRG1 to EWSR1-FLI1-activated enhancers in a demethylase-**

265   **independent manner**

266   In order to avoid off-target effects and induce a permanent gene modification, we

267   generated knock-outs (KO) of KDM6 using CRISPR methodology. We confirmed that

268   our KO completely abolished KDM6A expression in A673 cells without causing changes

269   in EWSR1-FLI1 and KDM6B levels (Fig. 4A). Despite loss of the demethylase enzymatic

270   activity, global levels of H3K27me3 as well as H3K4me1 and H3K27ac were not altered

271   upon KO in A673 (fig. S4A). Further characterization of KDM6A KO by RNA-seq

272   showed that the percentage of downregulated genes (activated targets, 69.1 and 67.1%,

273   for sgRNA#1 and #2, respectively) was higher than for upregulated genes (repressed

274   targets, 30.9 and 32.9%, for sgRNA#1 and #2, respectively) (fig. S4B), supporting the

275   transcriptional activation role of KDM6A. Indeed, activated genes were enriched in

276   axonogenesis categories (fig. S4B) as already observed with shRNA (fig. S3E).

277   Intersection of KDM6A-EWSR1-FLI1 ChIP-seq direct targets (distance of 100 kb) with

278   significantly decreased targets for KDM6A KO, confirmed 260 direct activated targets (P

279   adj < 0.05 and log FC cut-off 1, Fig. 4B, Table S5). We further validated by RT-qPCR

280   the downregulation of genes containing KDM6A and EWSR1-FLI1 with KDM6B (A-B-

281   EF group), while the expression of targets containing KDM6B and EWSR1-FLI1 (B-EF

282   group) remained unchanged (Fig. 4C). The decrease in gene expression of KDM6A-

283   EWSR1-FLI1 targets identified by ChIP-seq (fig. S2D), was confirmed in a second EwS

284   cell line, TC71, upon KDM6A KO (fig. S4, C and D).

285   To elucidate genome wide the contribution of KDM6A demethylase activity in the

286   decreased expression of defined targets, we performed H3K27me3 ChIP-seq normalized

287   with spike-in in A673 control and KDM6A KO (sgCTRL and sgKDM6A A673 cells,

288   respectively). The resulting H3K27me3 peaks were divided into three classes: (i)

289   commonly found in control and KDM6A KO, (ii) only found in the control condition,

290   and (iii) only found in KDM6A KO. Quantification of signal strength for each class of

291   peaks showed no changes in the common group (sgCTRL+/sgKDM6A+), while a new

292   group of 1,616 peaks emerge upon deletion of the demethylase (sgCTRL-/sgKDM6A+,

293   Fig. 4D). Those regions gaining H3K27me3 upon KDM6A KO associated with 299 genes

294   (Fig. 4E). However, the average profile of H3K27me3 around the TSS of KDM6A-

295   activated genes (downregulated upon KDM6A KO) revealed no correlation between

296   changes on their gene expression and H3K27me3 levels (fig. S4E), exemplified with

297   *SYT1* in Figure 4F. To more accurately pinpoint the regions of the genome presenting

298   higher H3K27me3 changes, we segmented the genome in bins of 1 kb and determined

299   the average signal strength of the ChIP-seq for both conditions. This analysis retrieved

300   579,992 bins gaining (up) H3K27me3 and 142,556 bins losing H3K27me3 (down) (fig.

13

301   S4, F and G), supporting a general redistribution of the mark already observed by other

302   authors (*50*).

303   KDM6A demethylase-independent functions have been proposed in the Kabuki

304   syndrome, in the context of cell-induced differentiation with retinoic acid, mesoderm

305   differentiation of embryonic stem cells and in lung cancer (*30, 37, 51, 52*). To analyze

306   the contribution of KDM6A demethylase activity at KDM6A-EWSR1-FLI1 targets we

307   reintroduced KDM6A WT or a dead mutant enzyme (mutations in H1146A and E1148A)

308   in KDM6A KO cells (*53*). As shown in Figure 4G, both WT and dead mutant enzymes

309   recovered the expression on KDM6A-EWSR1-FLI1 target genes (*SYT1, IRS2, CDH11*

310   and *PCSK2*) confirming that the transcriptional activating function of KDM6A is

311   independent of its demethylase activity. This recovery of expression was also observed

312   in genes where only KDM6A is present with the oncogene, such as *CDH6* and *TCF7L1*

313   (Fig. 4G).

314   KDM6A has been described to physically associate with the BAF complex member

315   BRG1/SMARCA4 (*54-56*), which constitutes the central catalytic subunit that uses the

316   energy derived from ATP-hydrolysis to remodel nucleosomes and regulate transcription

317   (*57*). To elucidate whether KDM6A was mediating transcriptional activation at enhancers

318   through the recruitment of BRG1, first we evaluated whether they physically associate.

319   Using co-immunoprecipitation we observed that indeed, both proteins interact (Fig.

320   S4H). Next we evaluated BRG1 enrichment genome wide by ChIP-seq and observed that

321   intersection of KDM6A-EWSR1/FLI1 with BRG1 retrieved 1,808 common regions (Fig.

322   4H). Although peak signal intensity was unmodified in the global set of BRG1 peaks

323   upon KDM6A KO (fig. S4I), ChIP-seq signal significantly decreases for the common

324   BRG1-KDM6A regions (Fig. 4I). Validation of genome-wide results by ChIP-qPCR

325   showed that H3K27me3 levels were unmodified upon KDM6A KO at the enhancer and

326   promoter regions of two EWSR1-FLI1/KDM6A-activated target genes, *IRS2* and *SYT1,*

327   both in A673 and TC71 cell lines (Fig. 4J and fig. S4J), while BRG1 was evicted from

328   enhancer regions following KDM6A deletion (Fig. 4J). These results were also confirmed

329   upon KDM6A knockdown in the A673 cell line (fig. S4K). Altogether, our results suggest

330   that KDM6A contributes to the recruitment of BRG1 at EWSR1-FLI1 activated

331   enhancers facilitating transcriptional activation through a demethylase-independent

332   mechanism.

333   **KDM6A knockout decreases EwS tumor growth**

334   Based on the previous results where we showed KDM6A being actively collaborating

335   with EWSR1-FLI1 at enhancers, we aimed to investigate the dependency of EwS tumor

336   formation on KDM6A. First, to determine whether KDM6A affects EwS cell growth, we

337   studied cell proliferation *in vitro* of KDM6A KO cells. We observed a decrease of cell

338   proliferation on days 3 and 6 for both sgKDM6A#1 and #2 KO A673 cells (fig. S5A).

339   Moreover, KDM6A KO cells exhibited a significant decrease in their clonogenic capacity

340   compared to control and parental cells (Fig. 5A and fig. S5B). Next, we subcutaneously

341   injected sgCTRL and sgKDM6A #1 and #2 KO A673 cells into athymic nude mice and

342   monitored tumor growth. Xenografts of parental cells were included as an additional

343   control for tumor growth. Interestingly, we found KDM6A KO (#1 and #2) showed a

344   delay in tumor growth compared to control and parental-derived tumors (Fig. 5B and fig.

345   S5C). At 17 days post-injection tumors were significantly smaller for sgKDM6A#1

346   (mean tumor volume of 121 for sgKDM6A#1 and 252.5 and 280mm^3 for A673 and

347   sgCTRL tumors, respectively) (fig. S5D). We confirmed downregulation of KDM6A by

348   RT-qPCR and Western Blot (Fig. 5C and fig. S5E). Moreover, median survival increased

349   from 25 (control) to 35 days for sgKDM6A#1 and from 20.5 (control) to 55 days for

350   sgKDM6A#2, respectively (Fig. 5D). Tumor engraftment was significantly delayed in

both KDM6A KO clones, with a median tumor engraftment of 20 days for sgKDM6A#1 compared to 16 days for sgCTRL and parental tumors (Fig. 5E). Importantly, we confirmed a decreased expression of enhancer-bound KDM6A/EWSR1-FLI1 targets, *CDH11* and *IRS2* (Fig. 5C), in xenografted tumors, supporting the transcriptional activating role of KDM6A at these oncogenic targets. Immunohistochemical analysis of tumors confirmed the expression of the EwS marker CD99 and significantly lower expression of the proliferation marker Ki-67 in sgKDM6A-derived tumors (Fig. 5F and fig. S5F and G). The delay in tumor growth and the increase in survival compared to control were also observed when TC71 cells containing sgKDM6A #1 and #2 were used (fig. S5H and S5I). All these results confirm KDM6A as a critical factor for EwS tumor growth.

Previously, we found KDM6A targets enriched in categories related to neural differentiation (fig. S3D). Neurofilament (NF) proteins are cytoskeleton proteins that maintain neural axons and dendrites (*58*). Neoplastic cells of neural origin or those exhibiting neural differentiation express NF. EwS tumors show positivity to cell surface antigens related to neuroectodermal lineage (*59*) and intracellular markers such as neuron specific enolase (NSE) and NF proteins (*60, 61*). The heavy chain of neurofilament protein NF200 is highly expressed in EwS cells and tumors (*62, 63*). Considering that KDM6A targets developmental neural pathways in EwS, we hypothesized that neural markers such as NF200 could be perturbed in KDM6A-KO tumors. Indeed, NF200 is highly expressed in publicly available datasets of EwS tumors and cell lines (fig. S5, J and K). Furthermore, immunohistochemical analysis revealed lower levels of NF200 in KDM6A-KO tumors as compared with those from control group (Fig. 5F). Altogether, our data indicate KDM6A exerts its critical role on EwS tumor growth and engraftment likely regulating neural pathways *in vivo*.

Impact of KDM6B knockout in EWSR1-FLI1 targets

KDM6A and KDM6B in conjunction with EWSR1-FLI1 participate at enhancers of neural genes with a different configuration of GGAA motifs, suggesting that both demethylases do not necessarily play the same function in EwS. To understand whether the role of KDM6B in EwS cells is associated with its demethylase activity, we investigated the H3K27me3 dynamics in KDM6B depleted cells. First, we confirmed the KO of KDM6B in A673 cells, while levels of EWSR1-FLI1 and KDM6A remained unchanged (Fig. 6A). Next, we evaluated the levels of H3K27me3 by Western blot in KDM6B KO. We found that the depletion of the demethylase did not alter the global levels of H3K27me3 (fig. S6A). Further characterization of KDM6B KO by RNA-seq retrieved higher percentages of downregulated genes (32.1 and 71.7%, activated targets) than upregulated genes (17.9 and 28.3%, repressed targets) for sgRNA#1 and #2, respectively (fig. S6B). As previously shown for KDM6B, activated genes were enriched in cell migration (fig. S6B). By intersecting KDM6B-EWSR1-FLI1 ChIP-seq direct targets (distance of 100 kb) with significantly decreased targets for KDM6B KO, we defined 49 direct activated targets (P adj < 0.05 and log FC cut-off 0.5, Fig. 6B, Table S5). We confirmed by RT-qPCR that expression of targets potentially regulated by enhancers containing KDM6B and EWSR1-FLI1 (B-EF group) was exclusively dependent on KDM6B, while KDM6B KO did not affect expression of targets containing enhancers with KDM6A binding sites (A-B-EF group, Fig. 6C). Decreased expression in KDM6B and EWSR1-FLI1 targets inspected by ChIP-seq (fig. S2D), upon KDM6B KO was confirmed in a second cell line, TC71 (fig. S6C and D). To inspect for the demethylase activity of KDM6B genome wide, we performed H3K27me3 ChIP-seq normalized with spike-in in A673 control and KDM6B KO (sgCTRL and sgKDM6B, respectively). The obtained peaks were divided in three groups, as previously described

17

401   for KDM6A. Interestingly, in this case, we observed a decrease in the peaks from

402   common group (sgCTRL+/sgKDM6B+ group), which are repressed regions maintained

403   between both conditions. Although unexpected, these results are in concordance with the

404   decrease in EZH2 expression observed upon KDM6B KO (Fig. 6C). As shown in Figure

405   6D, H3K27me3 was increased in 2,366 peaks from the sgCTRL-/sgKDM6B+ group,

406   corresponding to 400 genes (Fig. 6E), while the average profile of H3K27me3 around the

407   TSS of KDM6B activated genes (downregulated upon KDM6B KO) showed no

408   correlation between changes on their gene expression and H3K27me3 levels (fig. S6E).

409   The intersection of the set of 400 genes gaining H3K27me3 with those gaining the

410   repressive mark upon KDM6A retrieves a minimal 5% of intersection, supporting the

411   differential contribution of each demethylase. Bin analysis of H3K27me3 in both

412   samples (sgCTRL and sgKDM6B#2) retrieves 380,371 bins gaining and 442,200 losing

413   H3K27me3, suggesting a redistribution of the repressive mark (fig. S6, F and G).

414   Although changes in expression did not correlate with increase of H3K27me3 at TSS

415   upon KDM6B KO, we observed moderate recoveries of the H3K27me3 levels genome-

416   wide at specific EWSR1/FLI1-KDM6B enhancers (Fig. 6F), whereas mild enrichment of

417   the repressive mark at these specific regions was validated by ChIP-qPCR (Fig. 6G).

418   Moreover, when we treated EwS cells with the KDM6A/B demethylase inhibitor GSK-

419   J4, we found that the expression of EWSR1-FLI1-activated targets was decreased

420   strongly in those targets containing only KDM6B, suggesting that GSK-J4 phenocopies

421   expression changes induced by KDM6B deletion (fig. S6H). In accordance with

422   published data describing BAF complex enrichment at GGAA repeats, we show

423   KDM6B-EWSR1-FLI1 co-localization with the BRG1 subunit (Fig. 6H), which is

424   enriched in both single and repeat GGAA and co-immunoprecipitated with KDM6B (fig.

425   S6, I and J). Overall, our results confirm KDM6B exerts its function as demethylase at

18

426 specific KDM6B/EWSR1-FLI1 targets, while genome-wide changes in H3K27me3 are

427 counterbalanced by EZH2 drop in KDM6B depleted cells.

KDM6B knockout decreases tumor growth and sensitizes EwS cells to the EZH2

428

429 **inhibitor GSK126**

430 Since KDM6B KO led to a lower clonogenic capacity of A673 and TC71 EwS cells, it

431 suggested that the demethylase activity in EWSR1-FLI1 targets could be essential for

432 tumor growth (Fig. 7A and fig. S7, A, B and C). To assess the relevance of KDM6B in

433 vivo, we generated xenografts by subcutaneously injecting sgCTRL and sgKDM6B #1

434 and #2 A673 cells into athymic nude mice. KDM6B KO cells had a slower tumor growth

435 and an increase in survival from 18.5 days in control to 30 and 33 in sgKDM&B #1 and

436 #2, respectively (Fig. 7, B and C). Immunohistochemical analysis of tumors confirmed

437 reduced levels of KDM6B and Ki67 (Fig. 7D).

438 The methyltransferase inhibitor GSK126 has been widely used to epigenetically de-

439 repress key targets in EwS (*64-66*). To further elucidate whether depletion of

440 KDM6A/KDM6B demethylases would prime EwS cells to EZH2 inhibition we analyzed

441 cell death in KDM6A/KDM6B KO EwS cells upon GSK126 treatment. GSK126

442 exposure was sufficient to increase apoptosis by Annexin V staining in KDM6B KO cells,

443 in contrast to KDM6A depletion where cells remained unaltered (fig. S7, D and E).

444 Furthermore, only KDM6B KO cells treated with the EZH2 inhibitor showed an increase

445 in cleaved PARP-1 (c-PARP) and the DNA damage marker γ-H2Ax by Western blot,

446 while H3K27me3 decreased accordingly upon GSK126 treatment in both KOs (Fig. 7E

447 and fig. S7F). Next, we assessed whether re-expression of WT KDM6B or its catalytic

448 inactive form (H1390A and E1392) in KDM6B KO cells influenced the induction of

449 apoptosis by GSK126. The KDM6B dead mutant recover viability even better that the

19

450 WT, indicating that the sensitization to GSK126 is not demethylase dependent (fig. S7G).

451 Consistently, we observed that depletion of KDM6B increased the expression of genes

452 related to double-strand break repair pathways (figure S7H and table S6). These results

453 would suggest that EZH2 inhibition synergizes with the DNA damage infringed by

454 KDM6B depletion in EwS cells.

455 **Discussion**

456 The importance of H3K27me3 redistribution in cancer has previously been reported in

457 high-grade glioma, lymphoma, and melanoma, concomitant with a gain of function of

458 EZH2 (*67, 68*). In this study, we performed an extensive genome-wide study of

459 H3K27me3 redistribution upon EwS overexpression. Although the maintenance of the

460 overall levels of H3K27me3 was previously described upon EWSR1-FLI1 knockdown,

461 published data on H3K27me3 and our results on KDM6 demethylases confirm locus

462 specific H3K27me3 changes are relevant for the tumorigenic process (*40*). Our findings

463 indicate a genome-wide gain of H3K27me3 in relevant genes from EWSR1-FLI1

464 pathways, supporting the important role of EZH2 in EwS tumorigenesis (*9, 41*). The lack

465 of intersection between EZH2 and the oncogene, as already proposed by other groups

466 (*16*), suggests an indirect repressor role for this Polycomb subunit. Further research on

467 this issue should clarify the functional interaction between EZH2 and EWSR1-FLI1

468 genome-wide. Importantly, we have previously described that EWSR1-FLI1 targets weak

469 Polycomb regions in hMSC (*20*). Here, we confirmed a set of regions in hpMSC highly

470 enriched in H3K27me3 before oncogene expression, where the loss of H3K27me3 is

471 concomitant to EWSR1-FLI1 binding, supporting the need for a demethylase at these

472 regions. Altogether, our results refine the understanding of the early steps of EwS

473 tumorigenesis and the role of the H3K27me3 mark in defining the transformed epigenome

474 of EwS.

475   We demonstrated that KDM6A and KDM6B promote the oncogenic process set by

476   EWSR1-FLI1. The depletion of both demethylases reduced the clonogenic capacity and

477   tumor growth in EwS xenografts. Importantly, although deletion of KDM6A and

478   KDM6B does not impede generation of neural progenitor cells from hESC (*28*), double

479   knockout of these demethylases in EwS is not viable, supporting their relevance in the

480   maintenance of the transformed phenotype. In agreement, KDM6A and KDM6B are

481   functional partners of EWSR1-FLI1 at distinct subsets of active enhancers in EwS.

482   Moreover, depletion of KDM6A/B alters pathways related to neural development and

483   correlate to phenotypical expression of genes involved in EMT, which is intimately linked

484   to the EwS metastatic processes (*69*). KDM6A has been identified as one of the 299

485   cancer driver genes in the cancer genome atlas project (TCGA) (*70*). Its role in cancer

486   development is cell-context specific, acting as a tumor suppressor or oncogenic factor.

487   Loss-of-function mutations in KDM6A have been identified in cancers affecting males

488   across multiple histiotypes, including T-ALL, pancreatic cancer, B-cell lymphoma, and

489   medulloblastoma (*39, 71-73*). Conversely, EwS is listed in the 12th position of cancer

490   malignancies harboring higher mRNA expression of KDM6A (*45*). Contrary to the dual

491   role of KDM6A in cancer, KDM6B has been only related to cancer progression and a

492   specific signature for EMT in lung and breast cancer (*74, 75*).

493   Most cancer studies deciphering the mechanisms behind KDM6A and KDM6B are based

494   on independent approaches for each demethylase or enzymatic inhibition by GSK-J4.

495   Here, we performed a genome-wide analysis of both KDM6A and KDM6B in two EwS

496   cell lines showing that both proteins co-localize with EWSR1-FLI1. Our study

497   demonstrates that KDM6A and KDM6B collaborate with EWSR1-FLI1 shaping two

498   different classes of enhancers that contribute distinctively to the oncogenic process:

499   KDM6A enhancers, enriched in single instances of the GGAA motif, and KDM6B

500 enhancers, which are characterized by multimeric GGAA repeats (Fig. 7F). Previous

501 work by Riggi *et al*. (*16*) described the single GGAA regions as EWSR1-FLI1-repressed

502 elements where endogenous transcription factors had been displaced by the oncogene.

503 Nevertheless, these regions are still decorated with low levels of H3K27ac and

504 transcription, in agreement with our observation of KDM6A linked to primed enhancers.

505 These type of enhancers constitute an intermediate state between active and repressed

506 enhancers (*47, 48*). Indeed, in our study KDM6A-associated genes containing GGAA

507 single motifs are less transcribed than those enriched with KDM6B. Although these

508 results might suggest an important role of KDM6A in determination of cell identity,

509 further analysis of the nature and implications of these primed enhancers and their

510 differential regulation is needed.

511 It has been shown that the participation of KDM6A in cancer progression is not restricted

512 to its enzymatic activity but to a demethylase-independent function (*37, 76*). Main

513 changes in H3K27me3 upon depletion of KDM6 demethylases have been described by

514 other groups in regions already containing this mark (*28, 32, 50, 77*). In our study, the

515 maintenance of H3K27me3 in the group of sgCTRL+/sgKDM6A+ peaks, reinforces the

516 KDM6A demethylase independent role. In contrast, the decrease of H3K27me3 observed

517 in the sgCTRL+/sgKDM6B+ group upon KDM6B KO might be due to reduction of

518 EZH2 expression. In agreement with our results, the expected H3K27me3 recovery upon

519 KDM6B inhibition is counterbalanced by the decrease in EZH2 expression in

520 neuroblastoma (*50*). A new set of peaks for both KDM6A and KDM6B (sgCTRL-

521 /sgKDM6A+ and sgCTR-/sgKDM6B+) was also observed, with no associated changes

522 in gene expression. Further research should be performed to explain their role. Overall,

523 all this data reinforces the differential behavior between both demethylases. Indeed,

524 treatment with the KDM6A/B demethylase inhibitor GSK-J4 has been reported to be

525 effective at the preclinical level for pediatric cancers like neuroblastoma and diffuse

526 intrinsic pontine glioma (DIPG) (*78, 79*). We have reported a mild increase of H3K27me3

527 at specific EWSR1-FLI1 enhancers upon KDM6B KO that are consistent with the

528 downregulation of these targets with GSKJ4 that we and others have observed (*80*).

529 Moreover, our results suggest that the effects of GSK-J4 described *in vitro* and *in vivo* by

530 Heisey *et al.* (*80*), might be due to the specific targeting of the demethylase activity of

531 KDM6B.

532 KDM6A recruitment of BRG1 was described at cardiac-specific enhancers as a key step

533 in activation of cardiac developmental programs (*56*). Our data reveal that KDM6A

534 recruits BRG1 at EWSR1-FLI1 enhancers in a demethylase-independent manner with

535 critical consequences on transcriptional activation of these targets (Fig. 7F). Nevertheless,

536 given that KDM6A is associated with the H3K4 methyltransferase MLL3/4 complex, a

537 reasonable possibility would imply that both might regulate gene expression through

538 coordinated histone modifications (*30*). In EwS, BAF155/SMARCC1 subunits of the

539 BAF complex are recruited by the fusion oncogene at enhancers containing GGAA

540 repeats to activate transcription (*18*). Interestingly, we found a strong overlap between

541 KDM6A and KDM6B with BRG1 at GGAA single motifs and repeats, respectively.

542 Although the interplay between KDM6B and BAF complex at GGAA repeats remain to

543 be elucidated, both might work in cooperation to support the strong transcriptional

544 activation orchestrated at GGAA repeats. Indeed, KDM6A and KDM6B as well as the

545 oncogene have been recently implicated in phase separation, adding another level of

546 regulation that might contribute to EWSR1-FLI1 chromatin remodeling (*81, 82*).

547 EZH2 sensitization upon KDM6A loss has been mostly described in cancers, including

548 multiple myeloma, lung cancer and bladder cancer, where KDM6A acts as tumor

549 suppressor. In such cases, EZH2 inhibition delays tumor onset and induces tumor

550 regression of KDM6A-null cells (*83-85*). Here, we observed sensitization to EZH2

551 inhibition in KDM6B-deleted cells. Nevertheless, contrary to the PRC2 amplification

552 demonstrated in multiple myeloma and bladder cancer upon KDM6A deletion, EZH2

553 expression decreased upon KDM6B KO and GSKJ4 treatment in EwS cells. In

554 agreement, we observed a decrease in the peaks from the common group

555 (sgCTRL+/sgKDM6B+). These results suggest that EwS mechanistically resembles

556 pediatric cancers like neuroblastoma, where both KDM6B and EZH2 are overexpressed

557 and constitute druggable targets (*50, 86*). It has been proposed that PcG proteins promote

558 survival of EwS cells in hypoxic conditions by repression of *KCNA5* (*66*). Indeed,

559 inhibition of EZH2 methyltransferase activity has no impact on cell survival under

560 physiological conditions but results in loss of cell viability under stress conditions (i.e.

561 hypoxia and nutrient deprivation) by induction of *KCNA5*. Our results suggest that

562 KDM6B KO, independent of its demethylase activity, induces a sufficient level of stress

563 to EwS cells, as shown by the increase in γH2AX, to activate apoptosis upon EZH2

564 inhibition. These results are in agreement with the specific induction of DNA repair

565 pathways observed in KDM6B-depleted EwS cells. Besides, the non-specific

566 demethylase inhibitor JIB-04 induces DNA damage in EwS cells (*87*). Future

567 experiments should address the differential contribution of each demethylase to DNA

568 damage and EZH2 functionality.

569 **Materials and methods**

570 **Mesenchymal stem cells and cell lines**

571 hpMSC were extracted from the healthy bone marrow of pediatric donors from HSJD and

572 characterized according to described protocols (*9, 88, 89*). Cells were cultured at low

573 confluence with Iscove's Modified Dulbeco's Medium (Gibco) supplemented with 10%

574   fetal newborn calf serum, 1% penicillin/streptomycin and 10 ng/mL of PDGF-BB

575   (PeproTech).

576   The EwS cell lines, A673 and TC71, were purchased from ATCC. Hela cells stably

577   infected with doxycycline-inducible EWSR1-FLI1-3xFlag were kindly provided by Dr.

578   de Álava and were induced as previously described (49). They were cultured in RPMI

579   1640 media (Gibco) and supplemented with 10% FBS, L-glutamine and

580   penicillin/streptomycin. Cells were maintained in a humidified chamber at 37ºC and 5%

581   CO_2, and split every 2-3 days when reaching confluence. EwS cells were treated with the

582   EZH2 inhibitor GSK126 and the demethylase inhibitor GSKJ4 (both from Selleckchem)

583   at 15 µM and 2.5 µM for 24 or 72 hours, respectively. DMSO was used as vehicle control.

584   **Patient samples**

585   This study was approved by the Institutional Review Boards. Written informed consent

586   was obtained from patients or their legal guardians before collection of samples. Biopsies

587   from 45 primary EwS tumors at diagnosis from the HSJD Biobank, integrated in the

588   Spanish Biobank Network of ISCIII and in the Xarxa de Tumors de Catalunya, were used

589   for experimental purposes in agreement with the ethical committee procedures. Two

590   samples were not appraisable for technical reasons and were excluded.

591   **Lentiviral and transient transfections**

592   Infection was performed as previously described (20). Infected cells were selected with

593   0.5-1 µg/mL puromycin for 72 hours and maintained in the first passages. Induction of

594   the shRNA was performed with doxycycline hyclate (Sigma Aldrich) at 2 µg/mL for 72

595   hours. Validation of the overexpression or knockdown was determined by Western blot.

596   Target sequences for the SMARTvector inducible Tet-On shRNA system (Dharmacon)

597   targeting KDM6A or KDM6B are described in table S7. Lentiviral plasmids pLV[Exp]-

598   Puro-EF1A for overexpressing KDM6A wild-type or a dead mutant enzyme with

599   H1146A and E1148A mutations (*53*) were constructed and packaged by VectorBuilder

600   and are described in table S7. pInducer-JMJD3 WT and pInducer-JMJD3 HE > AA

601   (H1390A/E1392A) were kindly gifted by Dr. Martínez-Balbás and were induced as

602   previously described (*81*). Empty and EWSR1-FLI1-pLIV vector was kindly provided by

603   Dr. Rivera and published in Boulay *et al.* (*18*).

604   **CRISPR/Cas9 genome editing**

605   KDM6A and KDM6B knockout cells were generated using the Gene Knockout Kit v2

606   (Synthego) containing a pool of three validated sgRNA and exogenous Cas9 following

607   manufacturers guidelines. CRISPR-edited cell pools were isolated by limit dilution in 96-

608   well plates. Isolated clones were expanded and cells were collected for subsequent

609   validation by Sanger sequencing of the PCR product. The Inference of CRISPR Edits

610   (ICE) analysis software (*90*) was used to analyze the obtained sequences of edited cells

611   (clones with a model fit $R^2 > 0.6$ were only considered). Knockout clones were further

612   validated by Western blot. Target sequences for the pool of three commercially

613   predesigned sgRNA targeting KDM6A or KDM6B (Synthego) are described in table S7.

614   **Cell viability, clonogenic assays and Annexin V staining**

615   Cell viability was determined by seeding EwS cell lines at $0.25 \cdot 10^5$ cells/mL in clear

616   bottom black walled 96-well Tissue Culture plates (Thermofisher) and incubated with 10

617   μL of Cell Titer Blue (Promega). Fluorescence was measured using the Infinite M Nano+

618   (Tecan) microplate reader at $560_{Ex}/590_{Em}$ nm. Clonogenic assays were performed by

619   seeding $0.12 \cdot 10^4$ cells/mL in 6-well plates and changing media every 2-3 days until

620   visible colonies were grown in wells. Cells were then fixed for 10 minutes with 4%

621   paraformaldehid (Santa Cruz Biotech), washed with PBS and then incubated with a

622　crystal violet solution (2% W/V, 20% methanol in PBS) for 5 minutes. Quantification of

623　the colony number was performed with the Image J (*91*) pluggin ColonyArea

624　(*92*).Cultured cells treated with the inhibitor were collected with trypsin, counted and re-

625　suspended in Annexin binding buffer from the Alexa Fluor® 488 Annexin V/Dead Cell

626　Apoptosis Kit (Thermofisher) following manufacturer's instructions. AnnexinV-stained

627　cells were immediately run in Cytek Aurora CS full spectrum flow cytometer (Cytek

628　Biosciences). FlowJo software version 10.2 was used to analyze collected data.

629　**RNA Extraction and Real-Time Quantitative RTqPCR**

630　Total RNA was isolated and purified using the RNeasy Mini Kit (Qiagen) following

631　manufacturer's instructions. Quantification of RNA samples was performed using

632　Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific). Reverse transcription

633　(RT) was performed using 1 µg of purified RNA and converted to cDNA with the

634　retrotranscriptase M-MLV Reverse Transcriptase, the RNasin Plus RNase inhibitor and

635　random primers (all from Promega). SYBR Green PCR Master Mix (Applied

636　Biosystems) was used to perform quantitative PCR (qPCR) with QuantStudio 6 Flex

637　(Applied Biosystems) using specific primer sequences (see table S7). Obtained data was

638　normalized to housekeeping gene and analyzed with the $2^{-\Delta\Delta CT}$ method relative to the

639　experimental control condition. Data was performed in at least three independent

640　biological experiments and expressed as mean ±SEM.

641　**RNA-seq and Functional Analysis**

642　RNA-seq libraries were prepared with 0,5-1 µg of total high quality RNA collected from

643　samples and the Illumina Stranded Total RNA Prep kit (Illumina) according to

644　manufacturer's instructions. Fastq files were analyzed using FastQC software (*93*) to

645　assess read quality. Adapters were removed and reads were trimmed using Cutadapt

646 software (*94*) according to per base Phred quality scores and minimum length. Reads

647 were pseudoaligned to GRCh37 using Kallisto (*95*). Gene read counts from Kallisto were

648 used to determine differential gene expression using R packages tximport (*96*) and

649 DESeq2 (*97*). ERCC spike-ins were used for sample normalization and batch effect was

650 removed using Limma package (*98*). Heatmaps were performed with heapmap2 R

651 package. Gene set enrichment analysis (GSEA) was used to determine biologically

652 relevant transcriptional events in gene sets from Hallmark Collection running each list

653 with the KDM6A/KDM6B KO gene expression phenotype (*99*). Reports of functional

654 enrichments of GO and other genomic libraries were generated using the EnrichR tool

655 (*100*). Additional RNA data was obtained from A673 shFLI1 (GSE61953), the GEO

656 repositories including 184 EwS tumors at diagnosis (GSE17679, GSE34620 and

657 GSE37371), and from the Cancer cell line Encyclopedia (*45*).

658 **Immunohistochemistry**

659 Immunohistochemical analyses were performed following standard protocols. Fixed

660 tumor xenografts were embedded in paraffin and cut in consecutive 2 μm thick sections.

661 Sections of paraffin tumors were deparaffinized, rehydratated in an alcohol battery and

662 incubated with antigen retrieval with Epitope retrieval solution pH 6.0 (Novocastra

663 Laboratories). Blocking with endogenous peroxidase was performed with Protein Block

664 (Novocastra Laboratories) for 5 min and subsequent steps were performed in DAKO

665 autostainer link 48 (Agilent Technologies, Inc.). Slides were counterstained in

666 hematoxylin-eosin, dehydrated with alcohol and xylene and finally cover slipped with

667 DPX. Nanozoomer 2.0 (Hamamatsu Photonics) was used to scan selected tumors for

668 digital image processing. For KDM6A and KDM6B stains in EwS tumors

669 immunohistochemical semi-quantification was scored by an independent pathologist. A

670 semiquantitative histoscore (H-score) value was calculated based on a linear combination

28

671   of the intensity and proportion of stained cells per camp that punctuates strongly stained

672   nuclei (SSN), the percentage of moderately stained nuclei (MSN), and the percentage of

673   weakly stained nuclei (WSN) following the following formula: H-score = 1 x WSN + 2

674   x MSN + 3 x SSN. The H-score value ranges possible scores from 0 to 300 (*101*). For

675   tumor xenografts H-score was calculated with IHC-Profiler (*102*) and ImageJ software

676   based on staining intensity and percentage of positive staining cells, and high positive

677   staining scored 3, positive staining scored 2, low positive scored staining 1, and negative

678   staining scored 0. The list of primary antibodies and dilutions used are listed in table S7.

679   **Protein Extract Preparation, immunoprecipitation and Western blotting**

680   Whole cell protein extracts were prepared in RIPA buffer (10 mM Tris-HCl pH 8, 1 mM

681   EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycolate, 0.1% SDS, and 140

682   mM NaCl) containing phosphatase and EDTA-free Protease Inhibitor Cocktail (Roche).

683   Cell lysates incubated on ice for 30 min were centrifuged at 12,000 rpm for 15 min at

684   4ºC. Histone extracts were isolated using the EpiQuick Histone Extraction kit (Epigentek)

685   following manufacturer's instructions. Protein supernatants were collected and quantified

686   by Bradford assay (Sigma Aldrich). Immunoprecipitation was performed with total

687   cellular extracts incubated at 4ºC overnight with primary antibody followed by incubation

688   with Dynabeads Protein A (Invitrogen). Immunoprecipitated samples, 50 μg of whole

689   cell extract or 5 μg of histone protein extracts were mixed with loading Laemmli buffer

690   with DTT for Western blot experiments following standard protocols. The primary

691   antibodies and dilutions are listed (see table S7) and secondary antibodies goat anti-rabbit

692   and goat anti-mouse IRDye (Li-COR Biosciences) were diluted 1:10,000 an incubated

693   for 1 h at room temperature to blotted membranes. Nitrocellulose membranes (Amershan

694   Protran) were scanned and visualized with Li-COR Odyssey Infrared Imaging System

695   (Li-COR Biosciences).

ChIP-qPCR

ChIP-qPCR assays were performed as previously described (*103*). Cultured cells were fixed using 1% of methanol-free formaldehyde (Thermo Fisher Scientific) for 10 min at room temperature, and crosslinking was stopped by adding 500 μL of glycine (1.25 M). Lysis was performed in soft lysis buffer 0.1% SDS, 0.15 M NaCl, 1% Triton X-100, 1 mM EDTA, and 20 mM Tris pH 8) supplemented with 1 mg/mL of protease inhibitors (Roche). Cell lysates were sonicated for 10 cycles with Bioruptor Pico (Diagenode) until chromatin was sheared to an average length of 200 bp. After centrifugation, a small fraction of eluted chromatin was measured with Qubit dsDNA HS kit (Thermofisher Scientific). Immunoprecipitations were prepared starting with 30 μg for each antibody and incubated overnight at 4ºC in a rotating wheel (see table S7). 50 μL of Dynabeads Protein A (Invitrogen) were added to samples, and the slurry was incubated for 2 hours to capture DNA fragments. Immunoprecipitates were washed with the following buffers: TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl pH 8, 150 mM NaCl), TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl pH 8, 500 mM NaCl), TSEIII (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris–HCl pH 8), and Tris-EDTA buffer. All incubation and washing steps were performed in a rotating wheel at 4˚C to avoid protein degradation. DNA captured by the beads was eluted by adding 120 μL of a solution containing 1% SDS, 0.1 M NaHCO3 and decrosslinked at 65˚C for 3 hours with gentle shaking. Genomic DNA fragments from ChIP samples were purified with QIAquick PCR Purification kit (Qiagen) and eluted in 50-100 μL of Tris-EDTA buffer. Differences in DNA content from ChIP assays were determined by qPCR using the SQ6 Real Time PCR System and SYBR Green master mix (Applied Biosystems). The reported data from at least three independent experiments

720   represent real-time PCR values normalized to input DNA and are expressed as percentage

721   of bound/input signal and presented as mean±SEM.

722   ChIP-seq and Bioinformatic Analysis

723   ChIP-seq libraries were prepared using 2-5 ng of input and ChIP samples and the kit

724   NEBNext Ultra DNA Library Prep for Illumina (New England Biolabs) following

725   manufacturer's protocol. All purification steps were performed using Agen Court

726   AMPure XP beads (Qiagen). NEBNext Multiplex oligonucleotides for Illumina (New

727   England Biolabs) was used for library amplification. Quality control and fragment size

728   was analyzed using Agilent High Sensitivity ChIP and quantified with KAPA Library

729   Quantification Kit (KapaBiosystems). ChIP-seq data from DNA and input samples were

730   sequenced with Hiseq 2500 Illumina sequencing system.

731   ChIP-seq samples were mapped against the hg19 human genome assembly using Bowtie

732   with the option -m 1 to discard those reads that could not be uniquely mapped to just one

733   region (*104*). ChIP-seq samples normalized by spike-in were mapped against a synthetic

734   genome constituted by the human and the fruit fly chromosomes (hg19 + dm3) using

735   Bowtie with the option -m 1 to discard reads that did not map uniquely to one region.

736   MACS was run with the default parameters but with the shift-size adjusted to 100 bp to

737   perform the peak calling against the corresponding control sample (*105*). DiffBind (*106*)

738   was run next over the union of peaks from each pair of replicates of the same experiment

739   to find the peaks significantly enriched in both replicates in comparison to the

740   corresponding controls (DiffBind 2.0 arguments: categories = DBA_CONDITION, block

741   = DBA_REPLICATE and method = DBA_DESEQ2_BLOCK).

742   We used SeqCode (*107*) for ChIP-seq downstream analysis across multiple stages: (i) the

743   genome distribution of each set of peaks was generated by counting the number of peaks

31

744   fitted on each class of region according to RefSeq annotations (*108*). Promoter is the

745   region between 2.5 kb upstream and 2.5 kb downstream of the transcription start site

746   (TSS). Genic regions correspond to the rest of the gene (the part that is not classified as

747   promoter) and the rest of the genome is considered to be intergenic. Peaks that overlapped

748   with more than one genomic feature were proportionally counted the same number of

749   times; (ii) aggregated plots showing the average distribution of ChIP-seq reads around

750   the TSS or along the gene body of each target gene were generated by counting the

751   number of reads for each region according to RefSeq and then averaging the values for

752   the total number of mapped reads of each sample and the total number of genes in the

753   particular gene set; (iii) heatmaps displaying ChIP-seq signal strength around the summit

754   of each peak were generated by counting the number of reads in this region for each

755   individual peak and normalizing this value with the total number of mapped reads of the

756   sample. Peaks on each heatmap were ranked by the logarithm of the average number of

757   reads in the same genomic region; (iv) boxplots showing the ChIP-seq level distribution

758   for a particular ChIP experiment on a set of genomic peaks were calculated by

759   determining the maximum value on this region at this sample, which was assigned

760   afterwards to the corresponding peak. To quantify genome-wide differences on

761   H3K27me3 gain/loss, we performed a similar approach over the full set of bins of 1 kb

762   (average value per bin, genome assembly: hg19) ; (v) BedGraph profiles were generated

763   from each set of mapped reads and uploaded into the UCSC genome browser to generate

764   the screenshots of tracks along the manuscript (*109*); (vi) the set of target genes of a

765   biological feature was found by matching all ChIP-seq peaks in the region 2.5 kb

766   upstream of the TSS until the end of the transcripts as annotated in RefSeq.

767   To build our collection of enhancers and promoters, we reanalyzed published ChIP-seq

768   samples of H3K4me1, H3K27ac, H3K27me3, and H3K4me3 in A673 cells (*16*). Each

769   class of enhancer and promoter categories were defined as in Blanco *et al.* (*46*). Promoters

770   were defined as ChIP peaks of H3K27 found up to 2.5 kb from the TSS of one gene and

771   enhancers on intergenic areas outside promoters or within gene introns. H3K4me3 was

772   required to be present in promoters but absent in enhancers. We defined five classes of

773   regulatory elements: active enhancers (H3K27ac), active promoters (H3K27ac +

774   H3K4me3), poised enhancers (H3K27me3), primed enhancers (H3K4me1 without

775   H3K27ac) and bivalent promoters (H3K27me3 + H3K4me3). To construct the list of

776   putative targets of KDM6A/KDM6B enhancers, we identified the list of genes in the

777   vicinity of overlapping KDM6A+EWSR1-FLI1 and KDM6B+EWSR1-FLI1 modules

778   (maximum distance between peaks and differentially regulated genes: 100 kb). Reports

779   of functional enrichments of GO and other genomic libraries were generated using the

780   EnrichR tool (*100*). Motif analysis of the sequences of ChIP-seq peaks was performed

781   with the MEME-ChIP tool, adjusting the MEME motif width between 5 and 15 bps (*110*).

782   ChIP-seq raw data from H3K27me3 in control and EWSR1-FLI1 hpMSC was kindly

783   provided by Dr Nicolo Riggi and Dr Miguel Rivera (accession number GSE106925). For

784   ChIP-seq experiments with EWSR1-FLI1 in EWS cell lines we used an antibody that

785   correlates 62% at peak level with published data (*16*) and nicely reproduce our previous

786   data with RING1B overlap (*20*) (see table S7).

787   **Murine xenograft studies**

788   *In vivo* studies were performed after the approval of the Institutional Animal Research

789   Ethics Committee and by the animal experimentation commission of the Catalan

790   government. $1 \cdot 10^6$ cells of parental, KDM6 knockout (sgRNA#1 and 2) and non-targeting

791   control (sgCTRL) A673 or TC71 cells in 200 μL of PBS with Matrigel (Becton

792   Dickinson) were subcutaneously injected in two flanks of Athymic Nude mice (Envigo)

793   (for A673 cells: n=11 for parental, n=11 sgKDM6A#1, n=9 sgCTRL; n=9 for parental,

794   n=7 sgKDM6A#2 and n=12 sgCTRL; n=8 for sgCTRL and n=11 sgKDM6B#1 and #2;

795   for TC71 cells: n=10 for sgCTRL, n= 9 sgKDM6A#1 and n= 9 sgKDM6A#2, n indicates

796   number of tumors). Tumor growth was monitored three times per week by measuring

797   growing tumors with a digital caliper. Mice were sacrificed when tumors reached a

798   volume of 1500mm^3 and tumors were excised. Collected tumors were divided in parts:

799   one part was frozen for protein experiments and the other was fixed in 10% formalin for

800   immunohistochemistry experiments. For RNA experiments 4 tumors of each

801   experimental group were dissociated with collagenase IV (50 mg/mL) (Sigma) and a

802   tissue chopper (Ted Pella, Inc). Then tissue homogenates were digested with 5 mg/mL of

803   DNAse I (Sigma) and trypsin/EDTA (0.25%) to subsequently separate mouse stroma

804   cells from human cells with Mouse Cell Depletion kit (Milteny Biotech) following

805   manufacturer's guidelines. Log-rank test was used to calculate significance of the groups

806   in Kaplan-Meier.

807   **Quantification and statistical analyses**

808   Data was analyzed using Graphpad Prism 9 software (San Diego, CA, USA) version

809   v.9.1.2 and expressed as mean ±SEM or SD as indicated in figure legends. Kruskal-Wallis

810   one-way analysis of variance (ANOVA) and two-way ANOVA with Tukey's correction

811   (for non-normally distributed data) were applied to determine differences between

812   multiple groups. Holm-Šídák or Dunn's tests were used for multiple comparison tests.

813   Student t-test and Mann-Whitney t-test (for non-normally distributed data) were used for

814   non-paired comparisons of two groups. Kaplan-Meier curves were compared with the

815   log-rank (Mantel-Cox) test. Statistically significant differences among groups are

816   annotated in each graph of the manuscript and the statistical tests applied can be found in

817   the figure legends. *$P<0.05$, **$P<0.01$, ***$P<0.001$, P****<0.0001.

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1261 **Figures and Tables**

1262 **Fig. 1**. **H3K27me3 genome-wide redistribution upon EWSR1-FLI1 overexpression**
1263 **in hpMSCs.** (**A**) Western blot showing levels of FLI1 and H3K27me3 in whole cell and
1264 histone extracts, respectively, in control (CTRL) and upon EWSR1-FLI1 overexpression
1265 in hpMSC (EF). Tubulin and histone H4 were used as loading controls. Numbers below
1266 represent band quantification of H3K27me3 normalized to H4 and relative to CTRL. (**B**)
1267 Heatmap depicting H3K27me3 and EWSR1-FLI1 ChIP-seq in 4,080 H3K27me3-FLI1
1268 coinciding bins for CTRL and EWSR1-FLI1 hpMSC. (**C**) Ranking of genes associated
1269 to H3K27me3 signal strength in bins (red for genes gaining and blue for genes losing
1270 H3K27me3) upon EWSR1-FLI1 overexpression in hpMSC. (**D**) University of California
1271 Santa Cruz (UCSC) genome browser signal tracks for H3K27me3, H3K27ac, and FLI1
1272 in CTRL and EWSR1-FLI1 hpMSC at the *NKX2-2* gene. Up or Down bins correspond to
1273 regions that gain or loss H3K27me3 upon EWSR1-FLI1 introduction, respectively, and
1274 are represented as black rectangles below tracks. Clusters of bins are highlighted in light
1275 blue. (**E**) Same as (**D**), at the *TGFBI* gene. (**F**) Violin plots representing mRNA levels of

KDM6A (above) and KDM6B (below) in a panel of EwS, osteosarcoma (OS), rhabdomyosarcoma (RMS), and synovial sarcoma (SS) cell lines extracted from Barretina *et al.*(*45*). (**G**) Same as (F) in primary sarcoma tumors from NCBI GEO public repository. MSCs derived from a healthy bone marrow were used as control tissue. (**H**) Immunohistochemical staining of KDM6A (above) and KDM6B (below) on sections of three representative primary EwS tumors from the cohort of 43 tumors from our institution counterstained with hematoxylin-eosin. White bar indicates 100μm scale. Statistical significance was determined by one-way analysis of variance (ANOVA) test with Holm-Šídák multiple comparison test (F) and (G) relative to EwS. **** P<0,0001, **$P < 0.01$, and *$P < 0.05$.

Fig. 2. KDM6A and KDM6B co-localize genome-wide with EWSR1-FLI1 at primed and active enhancers. (**A**) Pie chart showing genomic distribution of KDM6A, KDM6B, and EWSR1-FLI1 peaks relative to functional categories including promoters (±2.5kb from TSS), genic regions (intragenic regions not overlapping with promoter) and intergenic regions (rest of the genome) in A673 cells. (**B**) Bar plot depicting percentage of total regulatory elements in the genome (active/poised/primed enhancers and active/poised promoters) overlapping with KDM6A, KDM6B, and EWSR1-FLI1 peaks. (**C**) Venn diagram depicting the overlap of KDM6A, KDM6B, and EWSR1-FLI1 peaks in A673 cells. Table below shows top MEME DNA motifs and the corresponding E-value for every group of peaks. (**D**) Boxplots depicting the average ChIP-seq signal of H3K27ac (above) and H3K4me1 (below) on each subset of peaks. (**E**) Heatmap of KDM6A, KDM6B, EWSR1-FLI1, H3K27ac, H3K4me3, and H3K4me1 ChIP-seq signals for each group of peaks. (**F**) UCSC genome browser signal tracks for KDM6A, KDM6B, EWSR1-FLI1, H3K27ac, and H3K4me1 in A673 cells at the *CDH11* enhancer (above), and the *NKX2-2* enhancer (below). EWSR1-FLI1-KDM6B peaks with or without KDM6A (A-B-EF or B-EF, respectively) are represented as black bars below tracks. Statistical significance was determined by Wilcoxon signed-rank test relative to KDM6B-EF peaks (D). Error bars in (D) indicate SD. **** P<0,0001

Fig. 3. Knockdown of KDM6A and KDM6B downregulates EWSR1-FLI1-activated targets. (**A**) Boxplot depicting the average ChIP-seq signal of Flag, EWSR1-FLI1, KDM6A and KDM6B at EWSR1-FLI1 induced peaks before (HeLa) and upon induction of the oncogene (iHeLa). (**B**) Aggregated plot showing the average ChIP-seq signal of EWSR1-FLI1, KDM6A and KDM6B at EWSR1-FLI1 peaks in control and induced Hela cells. (**C**) Boxplot representing the RNA-seq levels in A673 cells of KDM6A, KDM6B, and EWSR1-FLI1 target genes from Riggi *et al*. (*16*). (**D**) Western blot showing levels of KDM6A and KDM6B in whole cell extracts upon KDM6A (left) or KDM6B (right) knockdown with two shRNA sequences (sh#1 and sh#2) at 72 hours doxycycline-induction in A673 cells. Tubulin was used as loading control. Numbers below represent band quantification of KDM6A or KDM6B normalized to tubulin and relative to shCTRL. (**E**) Gene Set Enrichment Analysis (GSEA) curves and Normalized Enrichment Scores (NES) for Hallmark collection of Epithelial to Mesenchymal Transition (EMT) in targets downregulated upon shKDM6A#2 (above) and shKDM6B#2 (below) knockdown

in A673 cells. False Discovery Rate (FDR) is also shown. (**F**) Heatmap and dendrogram showing expression levels of genes in the vicinity (100 kb) of KDM6A-EWSR1-FLI1 (left) and KDM6B-EWSR1-FLI1 (right) ChIP-seq peaks significantly downregulated upon knockdown of each demethylase in A673 cells. n, indicates number of deregulated direct targets upon knockdown. (**G**) UCSC genome browser signal tracks for KDM6A, KDM6B, EWSR1-FLI1, and H3K27ac at *SYT1* gene. RNA-seq tracks for shKDM6A (shRNA#2) and shKDM6B (shRNA#2) and the corresponding shCTRL in A673 are shown. (**H**) Same as (F) at *IGF1* gene. Statistical significance between groups was assessed by Wilcoxon signed-rank test (C). Error bars in (A and C) indicate SD of RPKMs. *** P<0,001

Fig. 4. KDM6A recruits BRG1 to EWSR1-FLI1-activated enhancers in a demethylase-independent manner. (**A**) Western blot of KDM6A, KDM6B, and EWSR1-FLI1 in whole cell extracts upon KDM6A KO with two sgRNA sequences (#1 and #2) in A673 cells. Tubulin was used as loading control. (**B**) Heatmap showing expression levels of significantly downregulated genes in the vicinity (100 kb) of KDM6A-EWSR1-FLI1 ChIP-seq peaks upon sgKDM6A#1 and #2 in A673 cells. (**C**) RT-qPCR of KDM6B-EWSR1-FLI1 targets with or without KDM6A ChIP-seq peaks in A673 sgKDM6A#1 and #2. (**D**) Boxplot depicting the average ChIP-seq signal of H3K27me3 at 4,369 peaks exclusive for sgCTRL or 1,616 peaks for sgKDM6A#1 ($sgCTRL^{+}/sgKDM6A^{-}$ and $sgCTRL^{-}/sgKDM6A^{+}$, respectively), and 1,610 peaks in common ($sgCTRL^{+}/sgKDM6A^{+}$). (**E**) Venn diagram depicting genes associated to H3K27me3 peaks in sgCTRL and sgKDM6A#1. (**F**) UCSC genome browser signal tracks for H3K27me3 at *SYT1* intronic enhancer in sgCTRL and sgKDM6A#1. (**G**) RT-qPCR of EWSR1/FLI1-KDM6A targets with (left) or without KDM6B (middle) in A673 sgKDM6A#1 upon overexpression of KDM6A WT or dead mutant (H1146A/E1148A). (**H**) Venn diagram depicting the overlap between KDM6A-EWSR1-FLI1 and BRG1 peaks in A673 cells. (**I**) Boxplot of BRG1 ChIP-seq signal on the common set of 1808 KDM6A-EWSR1-FLI1 and BRG1 peaks upon sgKDM6A#1. (**J**) ChIP-qPCR of BRG1, KDM6A, and H3K27me3 enrichment at the enhancer and promoter region of *IRS2* and *SYT1* KDM6A-activated targets upon sgKDM6A#1. *DICER* was used as a negative control region. Wilcoxon signed-rank test (D), Kruskal-Wallis test with Dunn's correction relative to sgCTRL (C) and (G), two-tailed Mann-Whitney test relative to sgKDM6A#1 (G), and ordinary two-way ANOVA with Holm-Šídák test relative to control (J) were applied. *GAPDH* was used as housekeeping gene (C) and (G). Error bars indicate SEM (C), (G) and (J) of three independent biological experiments and SD (D) and (I); ****$P<0.0001$, **$P < 0.01$, and *$P < 0.05$.

Fig. 5. KDM6A knockout decreases EwS tumor growth. (**A**) Colony formation assay of KDM6A KO cells (sgRNA#1 and 2) compared to parental cells and sgCTRL in A673. (**B**) Tumor growth curves of the average volume of xenografts derived from subcutaneous injection of KDM6A KO cells compared to parental and sgCTRL A673 cells in nude athymic mice (n=11 for parental, n=11 sgKDM6A#1, n=9 sgCTRL (left); n=9 for parental, n=7 sgKDM6A#2 and n=12 sgCTRL (right); n, indicates number of tumors).

(**C**) RT-qPCR determination of KDM6A targets *CDH11* and *IRS2* in RNA extracts from tumors excised from parental, sgCTRL and sgKDM6A (sgRNA#1) xenografts of A673 cells. *EWSR1-FLI1* and *KDM6A* expression is also shown. *GAPDH* was used as housekeeping gene. (**D**) Kaplan-Meier representing the percentage of xenograft tumors that reach a tumor volume of 1000 mm^3 within days post-injection in parental, sgCTRL and KDM6A KO (sgRNA#1 and 2) in A673 cells. (**E**) Same as (D) representing the percentage of xenograft tumors that reach a tumor volume of 150 mm^3. (**F**) Immunohistochemistry staining of CD99, KDM6A, Ki67, and NF200 on sections of tumors excised from sgCTRL and sgKDM6A (sgRNA#1) xenografts of A673 cells. CD99 was used as positive control for EwS cells, Ki67 for cell proliferation, and hematoxylin-eosin for histopathological evaluation of tissue. White scale bar represents 50 µm. Statistical significance was determined by Kruskal-Wallis test with Dunn's multiple comparison correction (C) relative to sgCTRL. Survival analysis was performed by Log-rank (Mantel Cox) test (D, E) relative to sgCTRL. Error bars indicate SEM of xenografts (B) and four independent xenograft tumors (C). ***P<0.001, **P < 0.01, and *P < 0.05.

Fig. 6. Impact of KDM6B knockout in EWSR1-FLI1 targets. (**A**) Western blot showing levels of KDM6A, KDM6B, and EWSR1-FLI1 in whole cell extracts upon KDM6B KO with two sgRNA sequences (#1 and #2) in A673 cells. Tubulin was used as loading control. (**B**) Heatmap showing expression levels of significantly downregulated genes in the vicinity (100 kb) of KDM6B-EWSR1-FLI1 ChIP-seq peaks upon sgKDM6B#1 and #2 in A673 cells. (**C**) RT-qPCR of KDM6B-EWSR1-FLI1 targets with or without KDM6A ChIP-seq peaks in A673 sgKDM6B#1 and #2. *GAPDH* was used as housekeeping gene. (**D**) Boxplot depicting the average ChIP-seq signal of H3K27me3 at 3,248 peaks exclusive for sgCTRL or 2,366 peaks for sgKDM6B#2 (sgCTRL$^+$/sgKDM6B$^-$ and sgCTRL$^-$/sgKDM6B$^+$, respectively), and 3,473 peaks in common (sgCTRL$^+$/sgKDM6B$^+$). (**E**) Venn diagram depicting the overlap between genes associated to H3K27me3 peaks in sgCTRL and sgKDM6B#2. (**F**) UCSC genome browser signal tracks for H3K27me3 at the intronic enhancer of *JARID2* in sgCTRL and sgKDM6B#2. (**G**) ChIP-qPCR of H3K27me3 enrichment at the enhancer region of *NKX2-2* and *CCND1* genes upon KDM6B KO with sgRNA#2. *ENC1* was used as negative control regions. (**H**) Venn diagram depicting the overlap of KDM6B-EWSR1-FLI1 and BRG1 peaks in A673 cells. Statistical significance was determined by Kruskal-Wallis test with Dunn's multiple comparison correction relative to sgCTRL (C), Wilcoxon signed-rank test (D), and ordinary two-way ANOVA with Dunnet multiple comparisons test relative to IgG (G). Error bars indicate SEM (C) and (G) of three independent biological experiments or SD (D); ****P<0.0001, **P < 0.01 and *P<0.05.

Fig. 7. KDM6B knockout decreases tumor growth and sensitizes EwS cells to the EZH2 inhibitor GSK126. (**A**) Colony formation assay of KDM6B KO cells (sgRNA#1 and 2) compared to parental and sgCTRL A673 cells. (**B**) Tumor growth curve of the average volume of xenografts derived from subcutaneous injection of KDM6B KO cells

1402   (sgRNA#1 and 2, n=11 each) compared to sgCTRL A673 cells (n=8, where n indicates
1403   the number of tumors) in nude athymic mice. (**C**) Kaplan-Meier representing the
1404   percentage of xenograft tumors that reach a tumor volume of 1000 mm^3 within days post-
1405   injection in sgCTRL and KDM6B KO (sgRNA#1 and 2) in A673 cells. (**D**)
1406   Immunohistochemistry staining of CD99, KDM6B, and Ki67 on sections of tumors
1407   excised from sgCTRL and sgKDM6B (sgRNA#1 and sgRNA#2) xenografts of A673
1408   cells. CD99 was used as positive control for EwS cells, Ki67 for cell proliferation, and
1409   hematoxylin-eosin for histopathological evaluation of tissue. Black scale bar represents
1410   50 μm. (**E**) Western blot showing levels of KDM6B, cleaved-PARP-1 (c-PARP), and
1411   phosphorylation of S139 in variant gamma-H2A.x (ɣ-H2Ax) in whole cell extracts
1412   (above) and H3K27me3 in histone extracts (below) from sgCTRL and sgKDM6B#2
1413   A673 cells treated with vehicle or GSK126 inhibitor (15 μM for 24 hours). Tubulin and
1414   histone H3 were used as loading controls, respectively. Numbers below represent band
1415   quantification of H3K27me3 normalized to H3 and relative to sgCTRL or sgKDM6B#2
1416   treated with vehicle. (**F**) Depletion of KDM6A, enriched at single GGAA regions bound
1417   by EWSR1-FLI1, perturbs BRG1 recruitment causing repression of target genes in a
1418   demethylase-independent manner (left). KDM6B co-localize with EWSR1-FLI1 and
1419   BRG1 at GGAA repeats, where its depletion results in repression and a mild accumulation
1420   of H3K27me3. Survival analysis was performed by Log-rank (Mantel Cox) test (C)
1421   relative to sgCTRL. Error bars indicate SEM of xenografts (B). ***P<0.001 and
1422   **P<0.01.

1423   **Supplementary Materials**

1424   **Figure S1. H3K27me3 genome-wide redistribution upon EWSR1-FLI1**
1425   **overexpression in hpMSCs.** (A) Metagene plot showing H3K27me3 (left) and H3K27ac
1426   (right) ChIP-seq signals in 6,150 and 15,810 target genes, respectively, at transcription
1427   start site (TSS) and transcription end sites (TES) within 5,000 kb window in control
1428   (CTRL) and upon infection with the oncogene (EWSR1-FLI1) in hpMSC. (B) Scatter
1429   plot of H3K27me3 signal in 3,069,655 bins of 1 kb in CTRL (x-axis) and EWSR1-FLI1
1430   (y-axis) hpMSC (R^2=0.582, slope=0.703). Bins that significantly gain (Up bins) or lose
1431   H3K27me3 (Down bins) are highlighted in red or blue, respectively. (C) Boxplot
1432   depicting the average ChIP-seq signal of H3K27me3 in Up and Down bins in CTRL and
1433   EWSR1-FLI1 hpMSC. (D) Bar plot depicting percentage of annotated regulatory
1434   elements (active/poised/primed enhancers and active/poised promoters) covered by at
1435   least one bin of the genome (1 kb) in up bins or down bins of H3K27me3 in CTRL and
1436   EWSR1-FLI1 hpMSC. (E) Bar chart representing the top five enriched signaling
1437   pathways of 2,879 genes from Down bins (above) and 2,621 genes from Up bins (below)
1438   from CTRL and EWSR1-FLI1 hpMSC and their associated P-value. (F) Boxplot
1439   depicting immunohistochemical score (H-score) mean values for KDM6A and KDM6B
1440   in our cohort of 45 EwS primary tumors (right). Individual H-score values are represented
1441   as dots. Table shows descriptive statistics information of the recruited samples (left). (G)
1442   Scatter plot of individual H-score values for KDM6A (x-axis) and KDM6B (y-axis)
1443   (r=0.000, CI=(-0.308,0.308), P-value=1.00). Statistical differences between groups were
1444   assessed by Wilcoxon signed-rank test (C). Error bars indicate SD (C) and (F).
1445   P***<0.001.

Figure S2. KDM6A and KDM6B co-localize genome-wide with EWSR1-FLI1 at primed and active enhancers. (A) Volcano plots of the significant peaks identified by DiffBind for KDM6A, KDM6B, and EWSR1-FLI1 (3,737, 2,687, and 4,800 respectively) (P value ≤ 0.05 for KDM6A and EWSR1-FLI1, and FDR $< 10^{-5}$ for KDM6B) in A673 cells. (B) Table showing the top MEME DNA motifs enriched for KDM6A, KDM6B and EWSR1-FLI1 peaks and the corresponding E-value for every set of peaks. (C) Bar chart representing the top five enriched gene ontology (GO) biological process of the 1,511 or 1,205 genes associated to KDM6A (above) or KDM6B peaks (below), respectively, and their associated P-value. (D) Venn diagram showing overlap of KDM6A, KDM6B, and EWSR1-FLI1 at peak level in TC71 cells. (E) Same as (D) overlap between EWSR1-FLI1 and EZH2 at peak level in A673 cells. (F) Boxplot depicting the average ChIP-seq signal of H3K4me3 (left) and H3K27me3 (right) in each set of peaks in A673 cells. (G) Same as (F) showing the average ChIP-seq signal of RING1B in each group of peaks in A673 cells. (H) UCSC genome browser signal tracks for KDM6A, KDM6B, EWSR1-FLI1, H3K27ac, and H3K4me1 at the *SMYD3* gene in A673. EWSR1-FLI1 and KDM6A peaks with or without KDM6B (A-B-EF or A-EF, respectively) are represented as black bars below tracks. (I) ChIP-qPCR of KDM6A and KDM6B in a set of EWSR1-FLI1-bound enhancer regions with both KDM6A and KDM6B (A-B-EF) or only KDM6B (B-EF) in A673 cells. *ENC1* was used as a negative control region. Statistical significance between groups was assessed by Wilcoxon signed-rank test (F) and (G) and Kruskal-Wallis test with Dunn's correction for multiple comparisons (I). Error bars indicate SD (F) and (G) or SEM of three independent biological experiments (I). ****P<0.0001, *P<0.05 and ns indicates not significant.

Figure S3. Knockdown of KDM6A and KDM6B downregulates EWSR1-FLI1-activated targets. (A) Western blot showing levels of KDM6A and KDM6B in whole cell extracts upon KDM6A (left) or KDM6B (right) knockdown with two doxycycline-inducible shRNA sequences (sh#1 and sh#2) at 72 hours in TC71 cells. Tubulin was used as loading control. Numbers below represent band intensity quantification of KDM6A or KDM6B normalized to tubulin and relative to shCTRL. (B) Volcano plots depicting log fold change (x-axis) and the associated log P-adjusted value (y-axis) of deregulated targets upon KDM6A (left) or KDM6B (right) knockdown with #sh1 or #sh2 sequences (above or below, respectively). Statistically significant deregulated targets according to set cut-off are highlighted in red. (C) Donut charts representing the percentage of significantly upregulated or downregulated targets upon KDM6A (above) or KDM6B (below) knockdown with sh#1 and sh#2 in A673 cells (red; 492 and 502 genes for shKDM6A#1 and #2, respectively; 1,090 and 1,369 genes for shKDM6B#1 and #2, respectively) or TC71 cells (orange; 583 and 731 genes for shKDM6A#1 and #2, respectively; 2,975 and 196 genes for shKDM6B#1 and #2, respectively). (D) Heatmap showing expression levels of genes in the vicinity of KDM6A-EWSR1-FLI1 and KDM6B-EWSR1-FLI1 ChIP-seq peaks (100 kb) that are significantly upregulated upon knockdown of each demethylase in A673 cells. n, indicates number of deregulated direct targets upon knockdown in each panel. (E) Bar chart representing the top five enriched gene ontology (GO) biological process and their associated P-value of the 64 genes in the vicinity (100 kb) of KDM6A-EWSR1-FLI1 peaks (above) and the 139 genes in the vicinity (100 kb) of KDM6B-EWSR1-FLI1 peaks (below) that are significantly downregulated upon knockdown of each demethylase in A673. (F) RT-qPCR determination of mRNA expression of EWSR1-FLI1 targets with active enhancers in shCTRL and shKDM6A (#1 and 2) in A673 cells. Values were normalized to *GAPDH* and relative to shCTRL. (G) Same analysis as in (F) for shCTRL and shKDM6B (#1 and

1495 2) in A673 cells. Statistical significance was determined by Kruskal-Wallis test with
1496 Dunn's multiple comparison correction related to sgCTRL. Error bars indicate SEM (F)
1497 and (G) of four independent biological experiments. **$P < 0.01$, and *$P < 0.05$.

1498 **Figure S4. KDM6A recruits BRG1 to EWSR1-FLI1-activated enhancers in a**
1499 **demethylase-independent manner.** (A) Western blot showing levels of H3K27ac,
1500 H3K4me1, and H3K27me3 in histone extracts upon KDM6A KO with two sgRNA
1501 sequences (#1 and #2) in A673 cells. Histone H3 was used as loading control. Numbers
1502 below represent band quantification of H3K27me3 normalized to H3 and relative to non-
1503 targeting control (sgCTRL). (B) Donut charts (up) representing the percentage of
1504 significantly upregulated or downregulated targets upon KDM6A KO (red indicates
1505 downregulated targets: 1,245 and 480 genes for sgKDM6A#1 and #2, respectively) in
1506 A673 cells. Bar chart (below) representing the top five enriched gene ontology (GO)
1507 biological process and their associated P-value of the set of differentially expressed genes
1508 for KDM6A KO. (C) Western blot showing levels of KDM6A, and KDM6B in whole
1509 cell extracts upon KDM6A KO with two sgRNA sequences (#1 and #2) in TC71 cells.
1510 Tubulin was used as loading control. (D) RT-qPCR determination of EWSR1-FLI1
1511 targets with both KDM6A and KDM6B ChIP-seq peaks (A-B-EF group) in TC71 KO
1512 cells (sgRNA #1 and #2). *GAPDH* was used as housekeeping gene. (E) Metagene plot
1513 showing H3K27me3 ChIP-seq signal of 1,245 KDM6A-activated targets from RNA-seq
1514 data at transcription start site (TSS) within 5,000 kb window in sgCTRL and
1515 sgKDM6A#1 in A673 cells. (F) Scatter plot of H3K27me3 ChIP-seq signal in 3,095,665
1516 bins of 1 kb in sgCTRL (x-axis) and sgKDM6A#1 (y-axis) (R^2=0.326, slope=0.631). (G)
1517 Boxplot depicting the average ChIP-seq signal of H3K27me3 in 1 kb bins in sgCTRL and
1518 sgKDM6A#1. Bin mapping analysis identified 579,992 and 142,556 bins that gained (up
1519 bins) or loss (down bins) H3K27me3 signal, respectively, upon KDM6A KO compared
1520 to control. (H) Western blot analysis of KDM6A and BRG1 protein levels in BRG1
1521 immunoprecipitated protein extracts. Lanes represent two input controls (i), the IgG
1522 control (IgG), empty lanes (-), and the BRG1 immunoprecipitated samples. (I) Boxplot
1523 depicting the average ChIP-seq signal of BRG1, EWSR1-FLI1, and KDM6A in sgCTRL
1524 and sgKDM6A#1 A673 cells. (J) ChIP-qPCR of H3K27me3 enrichment in the enhancer
1525 region of *CSRP1*, *PCSK2* and *SYT1* and in *SYT1* promoter region upon KDM6A KO
1526 sgRNA#1 in TC71 cells. *DICER* and *TAL1* were used as negative and positive control
1527 regions, respectively. (K) Same as (J) in the enhancer region of *TSPAN13, SYT1* and *IRS2*
1528 genes upon KDM6A knockdown with shRNA#2 in A673 cells. *DICER* and *TAL1* were
1529 used as negative and positive control regions, respectively. Statistical significance was
1530 determined by Kruskal-Wallis test with Dunn's multiple comparison correction related to
1531 sgCTRL (D), Wilcoxon signed-rank test (G and I) and ordinary two way ANOVA test
1532 with Holm-Šídák multiple comparisons correction (J and K) compared to control. Error
1533 bars indicate SD (G and I) or SEM (D, J and K) of three independent biological
1534 experiments. ****$P < 0.0001$, **$P < 0.01$, and *$P < 0.05$.

1535 **Figure S5. KDM6A knockout decreases EwS tumor growth.** (A) Bar chart showing
1536 cell viability upon KDM6A knockout (sgRNA #1 and 2) in A673 cells compared to non-
1537 targeting control (sgCTRL) at 3 and 6 days. Values are relative to day 0. (B) Bar charts
1538 showing number of colonies from Fig. 5A, in parental, sgCTRL and sgKDM6A (sgRNA
1539 #1 and 2) A673 cells. (C) Spaghetti plots showing tumor volume of xenografts from
1540 parental, sgCTRL, and sgKDM6A (sgRNA #1 and 2) in A673 cells. (D) Boxplot
1541 representing tumor volume at 17 days post-injection of parental, sgCTRL, and KDM6A
1542 KO cells (sgRNA#1) in A673. Each dot represents an individual tumor volume. (E)
1543 Western blot showing protein levels of KDM6A in 8 representative xenograft tumors

derived from sgCTRL and 4 from sgKDM6A (sgRNA#1 and 2) in A673. Tubulin was used as loading control. (F) Immunohistochemistry staining of CD99, KDM6A, Ki67, and NF200 on sections of tumors excised from sgCTRL and sgKDM6A (#2) xenografts of A673 cells. CD99 was used as positive control for EwS cells, Ki67 for cell proliferation, and hematoxylin-eosin for histopathological evaluation of tissue. White scale bar represents 50 µm. (G) Boxplot depicting H-score quantification of Ki67 in sgCTRL and sgKDM6A (#1 and #2) in immunohistochemistry sections from Fig. 5F and S5F. (H) Spaghetti plots showing tumor volume of xenograft tumors from sgCTRL and sgKDM6A (sgRNA #1 and 2) (n=9 each) in TC71 cells in nude athymic mice. (I) Kaplan-Meier representing the percentage of xenograft tumors that reach a tumor volume of 1000 mm3 within days post-injection in sgCTRL and KDM6A KO (sgRNA#1 and 2) in TC71 cells. (J) Boxplot representing mRNA levels of *NEFH* in a panel of 22 EwS cell lines extracted from from Barretina *et al.*(*45*). Each dot represents individual values for a given cell line with A673 cell line colored in red. (K) Violin plot representing mRNA levels of *NEFH* in primary tumors from GEO public data repositories including EwS among other primary sarcoma tumors including osteosarcoma (OS), rhabdomyosarcoma (RMS), and synovial sarcoma (SS). MSCs derived from the healthy bone marrow were included as control cells. Statistical significance was determined by ordinary two way ANOVA with multiple comparisons correction using Dunnett's test (A) relative to sgCTRL at day 0, Kruskal-Wallis test with Dunn's multiple comparison correction (B, D and G) relative to sgCTRL, survival analysis was performed by Log-rank (Mantel Cox) test (I) and ordinary one-way ANOVA with Holm-Šídák multiple comparison test (K) relative to EwS. Error bars indicate SEM; ****$P \leq 0.0001$, ***$P \leq 0.001$, **$P \leq 0.01$, *$P < 0.05$, ns indicates not significant.

Figure S6. Impact of KDM6B knockout in EWSR1-FLI1 targets. (A) Western blot showing levels of H3K27ac, H3K4me1, and H3K27me3 in histone extracts upon KDM6B KO with two sgRNA sequences (#1 and #2) in A673 cells. Histone H3 was used as loading control. Numbers below represent band quantification of H3K27me3 normalized to H3 and relative to non-targeting control (sgCTRL). (B) Donut charts (up) representing the percentage of significantly upregulated or downregulated targets upon KDM6B KO (red indicates downregulated targets: 354 and 377 genes for sgKDM6B#1 and #2, respectively) in A673 cells. Bar chart (below) representing the top five enriched gene ontology (GO) biological processes and their associated P-value of the set of differentially expressed genes for KDM6B KO. (C) Western blot showing levels of KDM6A, and KDM6B in whole cell extracts upon KDM6B KO with two sgRNA sequences (#1 and #2) in TC71 cells. Tubulin was used as loading control. (D) RT-qPCR of KDM6B-EWSR1-FLI1 targets in sgKDM6B#1 and #2 TC71 cells. *GAPDH* was used as housekeeping gene. (E) Metagene plot showing H3K27me3 ChIP-seq signal of 266 KDM6B-activated targets from RNA-seq data at transcription start site (TSS) within 5,000 kb window in sgCTRL and sgKDM6B#2 in A673 cells. (F) Scatter plot of H3K27me3 ChIP-seq signal in 3,095,665 bins of 1 kb in sgCTRL (x-axis) and sgKDM6B#1 (y-axis) (R^2=0.285, slope=0.501). (G) Boxplot depicting the average ChIP-seq signal of H3K27me3 in 1 kb bins in sgCTRL and sgKDM6B#2. Bin mapping analysis identified 380,371 and 442,200 bins that gained (up bins) or loss (down bins) H3K27me3 signal, respectively, upon KDM6B KO compared to control. (H) Western blot (above) showing levels of H3K27me3 in histone extracts of TC71 cells treated with vehicle or the demethylase inhibitor GSKJ4 at 2.5 and 5 µM (+ and ++, respectively) for 72h. Histone H4 was used as loading control. Numbers below represent band quantification of H3K27me3 normalized to H4 and relative to vehicle control. RT-qPCR (below)

determination of EWSR1-FLI1 targets with both KDM6A and KDM6B or with KDM6B ChIP-seq peaks (A-B-EF and B-EF groups, respectively) in TC71 cells treated with vehicle or GSK-J4 at 2.5 µM for 72 hours. *TBP* was used as housekeeping gene. (I) Table showing top MEME DNA motifs and the corresponding E-value for BRG1 ChIP-seq peaks in A673 cells. (J) Western blot analysis of KDM6B and BRG1 protein levels in BRG1 immunoprecipitated protein extracts. Lanes represent two input controls (i), the IgG control (IgG), empty lane (-), and the BRG1 immunoprecipitate samples. (I) Statistical significance was determined by Kruskal-Wallis test with Dunn's correction for multiple comparison (D), Wilcoxon signed-rank test (G) and Student t-test (H) related to control group. Error bars in (D and H) indicate SEM of three independent biological experiments; ****P<0.0001, **$P < 0.01$ and *$P < 0.05$.

Figure S7. KDM6B knockout decreases tumor growth and sensitizes EwS cells to the EZH2 inhibitor GSK126. (A) Bar charts representing colony number, from colony assay in Figure 7A of parental, sgCTRL and sgKDM6B (sgRNA #1 and 2) A673 cells. (B) Colony formation assay of KDM6B KO cells (sgRNA#1 and 2) compared to sgCTRL in TC71 cells. (C) Bar charts representing colony number, from colonies in Figure S7B in sgCTRL and sgKDM6B (sgRNA #1 and 2) TC71 cells. (D) Annexin V staining of sgCTRL and sgKDM6B#2 A673 cells treated with vehicle or GSK126 inhibitor at 15 µM for 24 hours. Numbers indicate percentage of cells in each quadrant. (E) Same as in (D) with sgKDM6A#1 in A673. (F) Western blot showing levels of KDM6A, cleaved-PARP-1 (c-PARP), and phosphorylation of S139 in variant gamma-H2A.x (ɣ-H2Ax) in whole cell extracts (above) and H3K27me3 in histone extracts (below) from sgCTRL and sgKDM6A#1 A673 cells treated with vehicle or GSK126 inhibitor (15 µM for 24 hours). Tubulin and histone H3 were used as loading controls for whole cell or histone extracts, respectively. Numbers below represent band quantification of H3K27me3 normalized to H3 and relative to sgCTRL or sgKDM6A#1 treated with vehicle. (G) Bar plot showing percent of viable, early and late apoptotic and necrotic cells by Annexin V staining, in sgCTRL, sgKDM6B#2 and cells overexpressing a wild-type (wt) or catalytic-dead (mut) form of KDM6B in A673 cells treated with vehicle or GSK126 (15 µM for 24h). (H) Heatmap and dendrogram showing expression of genes within the Hallmark collection of Double Strand Break Repair from GSEA analysis in shCTRL and shKDM6B#2, that are significantly upregulated upon KDM6B knockdown in A673 cells. n, indicates number of deregulated targets upon KDM6B knockdown. Statistical significance was determined by Kruskal-Wallis test with Dunn's correction for multiple comparison (A and C) relative to sgCTRL and ordinary two-way ANOVA test with Dunnett's multiple comparison correction relative sgCTRL treated with vehicle (G). Error bars indicate SEM of three independent biological experiments. **** $P \leq 0.0001$, ***P< 0.001, P** ≤ 0.01, P*≤ 0.05 and ns indicates not significant.
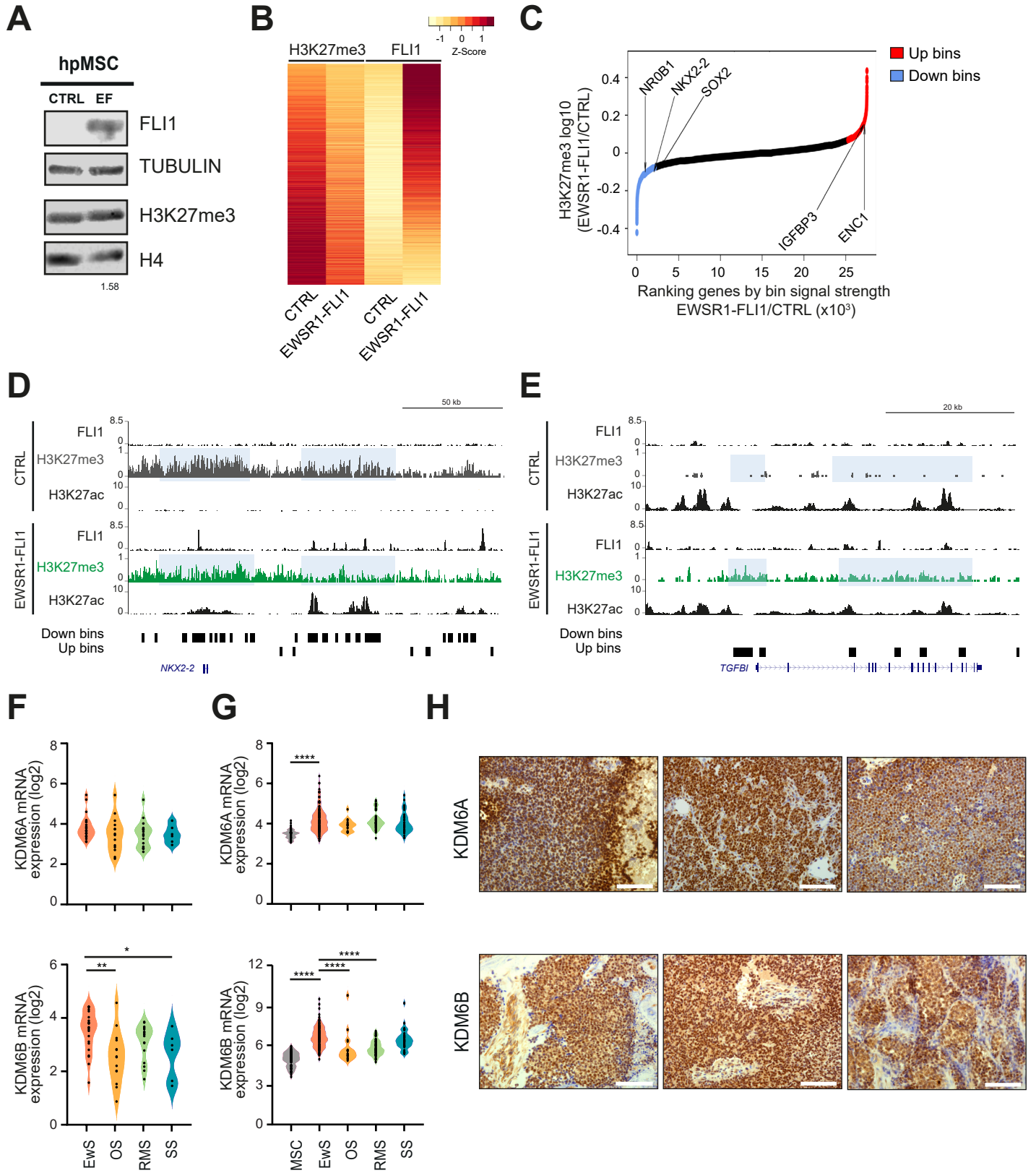
Other Supplementary Materials for this manuscript include the following:

Supplementary Table S1. Excel file showing genes associated to gain or loss of bins of H3K27me3 (up or down bins, respectively) following EWSR1-FLI1 introduction in hpMSC.
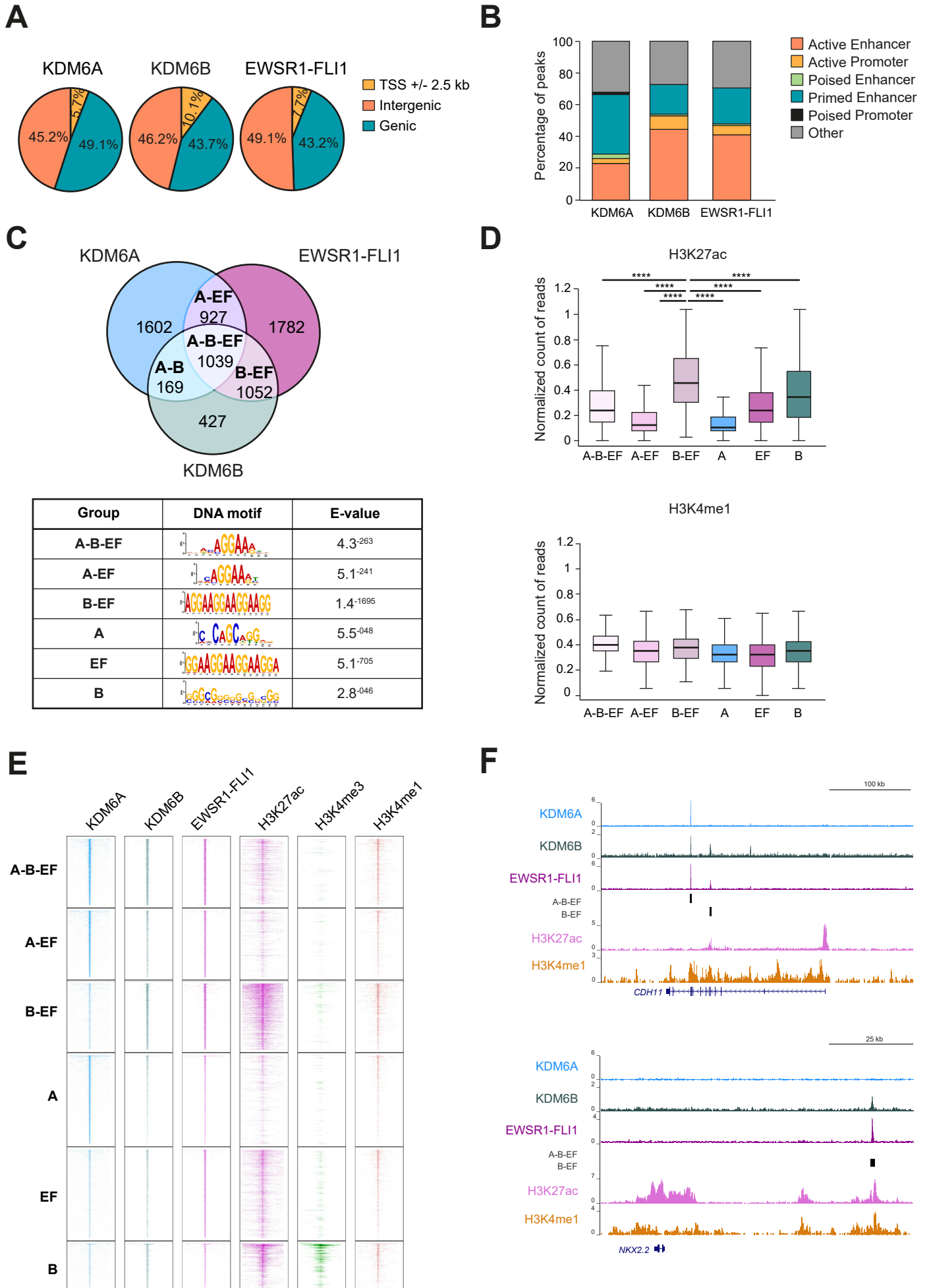
Supplementary Table S2. Excel file with summary of KDM6A, KDM6B and FLI1 Diffbind peaks from ChIP-seq data in A673 cells.

1637   **Supplementary Table S3.** Excel file with the associated list of genes for KDM6A and
1638   KDM6B ChIP-seq peaks in A673 cells.

1639   **Supplementary Table S4.** Excel file showing differential expressed genes of shKDM6A
1640   and shKDM6B with shRNA sequences #1 and #2 in A673 cells, the list of EWSR1-FLI1-
1641   KDM6A or EWSR1-FLI1-KDM6B targets downregulated upon KDM6A or KDM6B
1642   knockdown in A673 cells and the core enriched EMT gene sets upon knockdown of
1643   KDM6A or KDM6B.

1644   **Supplementary Table S5.** Excel file showing differential expressed genes of sgKDM6A
1645   and sgKDM6B with sgRNA sequences #1 and #2 in A673 cells, and the list of EWSR1-
1646   FLI1-KDM6A or EWSR1-FLI1-KDM6B targets downregulated upon KDM6A or
1647   KDM6B KO in A673 cells.

1648   **Supplementary Table S6.** Excel file with the list of genes from the Double Strand Break
1649   Repair category from GSEA analysis significantly upregulated upon KDM6B
1650   knockdown in A673 cells.

1651   **Supplementary Table S7.** Excel file with information of the antibodies, primers, and
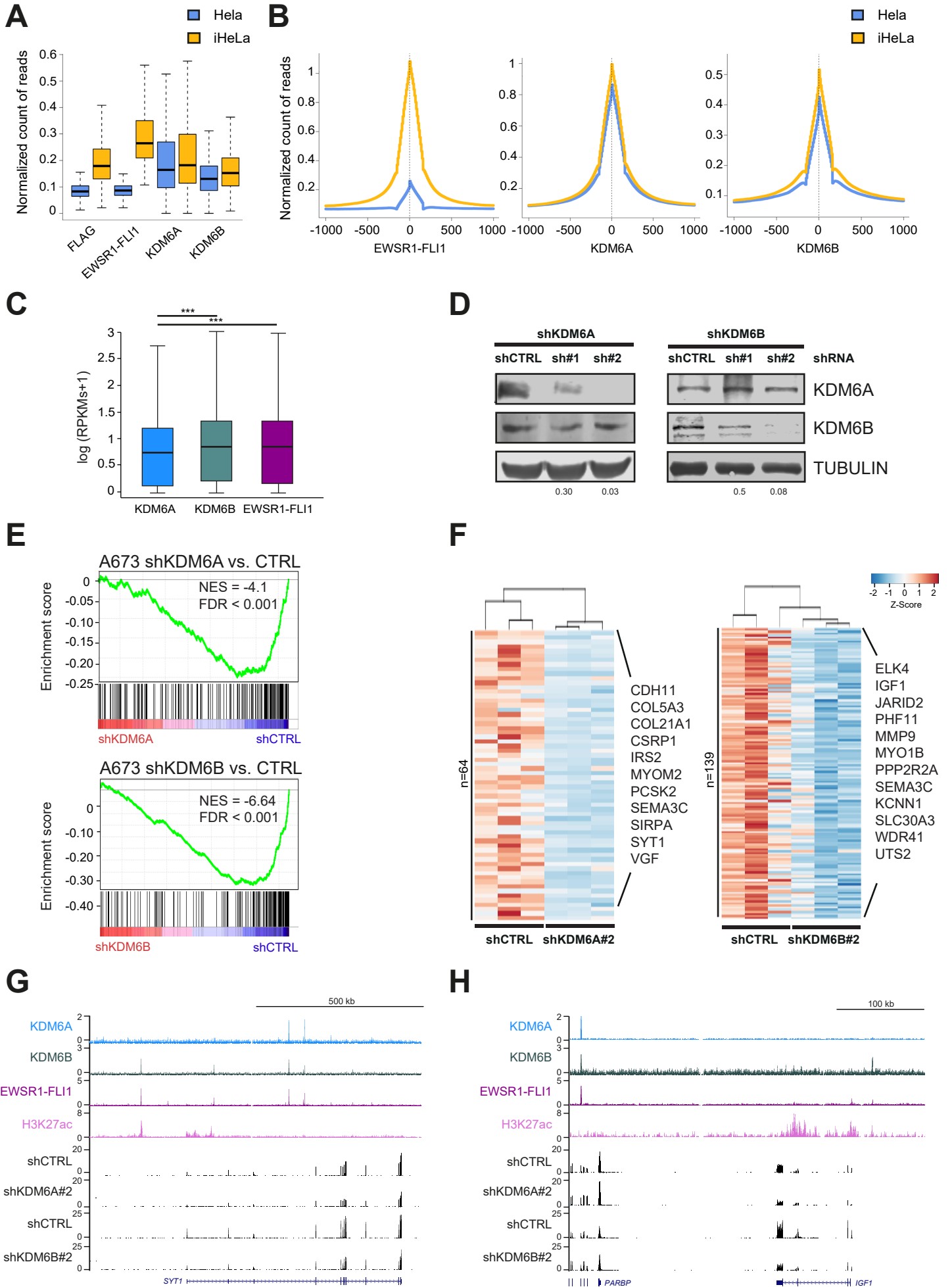1652   RNAi and CRISPR sequences used.

Figure 1

A hpMSC

CTRL EF

FLI1
TUBULIN
H3K27me3
H4

1.58

B

H3K27me3   FLI1

Z-Score

CTRL   EWSR1-FLI1   CTRL   EWSR1-FLI1

C

NR0B1 NKX2-2 SOX2

IGFBP3 ENC1

Up bins
Down bins

H3K27me3 log10 (EWSR1-FLI1/CTRL)

Ranking genes by bin signal strength
EWSR1-FLI1/CTRL (x10³)

D

50 kb

FLI1
CTRL   H3K27me3
H3K27ac

FLI1
EWSR1-FLI1   H3K27me3
H3K27ac

Down bins
Up bins

NKX2-2

E

20 kb

FLI1
CTRL   H3K27me3
H3K27ac

FLI1
EWSR1-FLI1   H3K27me3
H3K27ac

Down bins
Up bins

TGFBI

F

KDM6A mRNA expression (log2)

KDM6B mRNA expression (log2)

**
*

EwS   OS   RMS   SS

G

KDM6A mRNA expression (log2)

****

KDM6B mRNA expression (log2)

****   ****
****

MSC   EwS   OS   RMS   SS

H

KDM6A

KDM6B

Figure 2

Figure 3

Figure 4
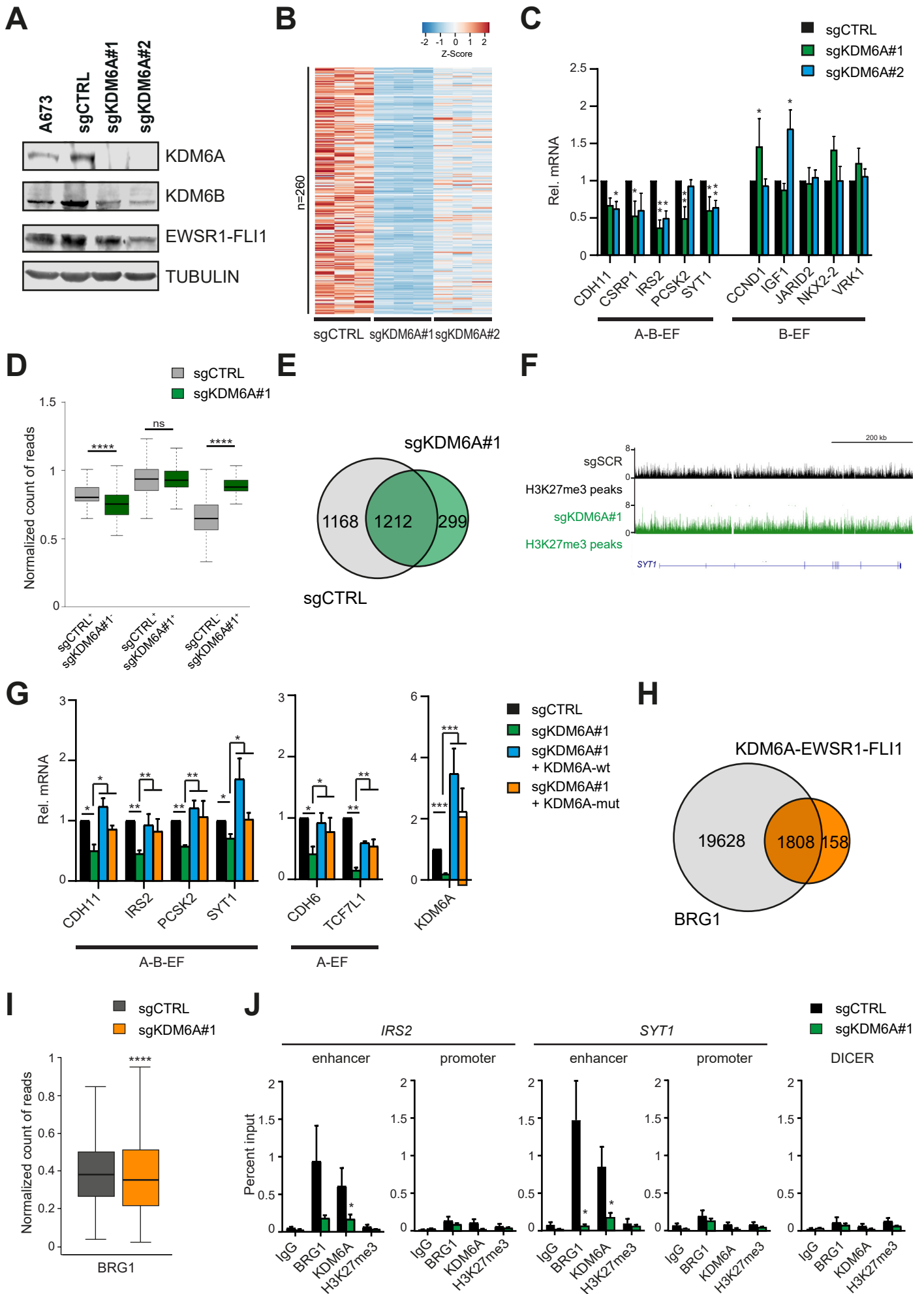
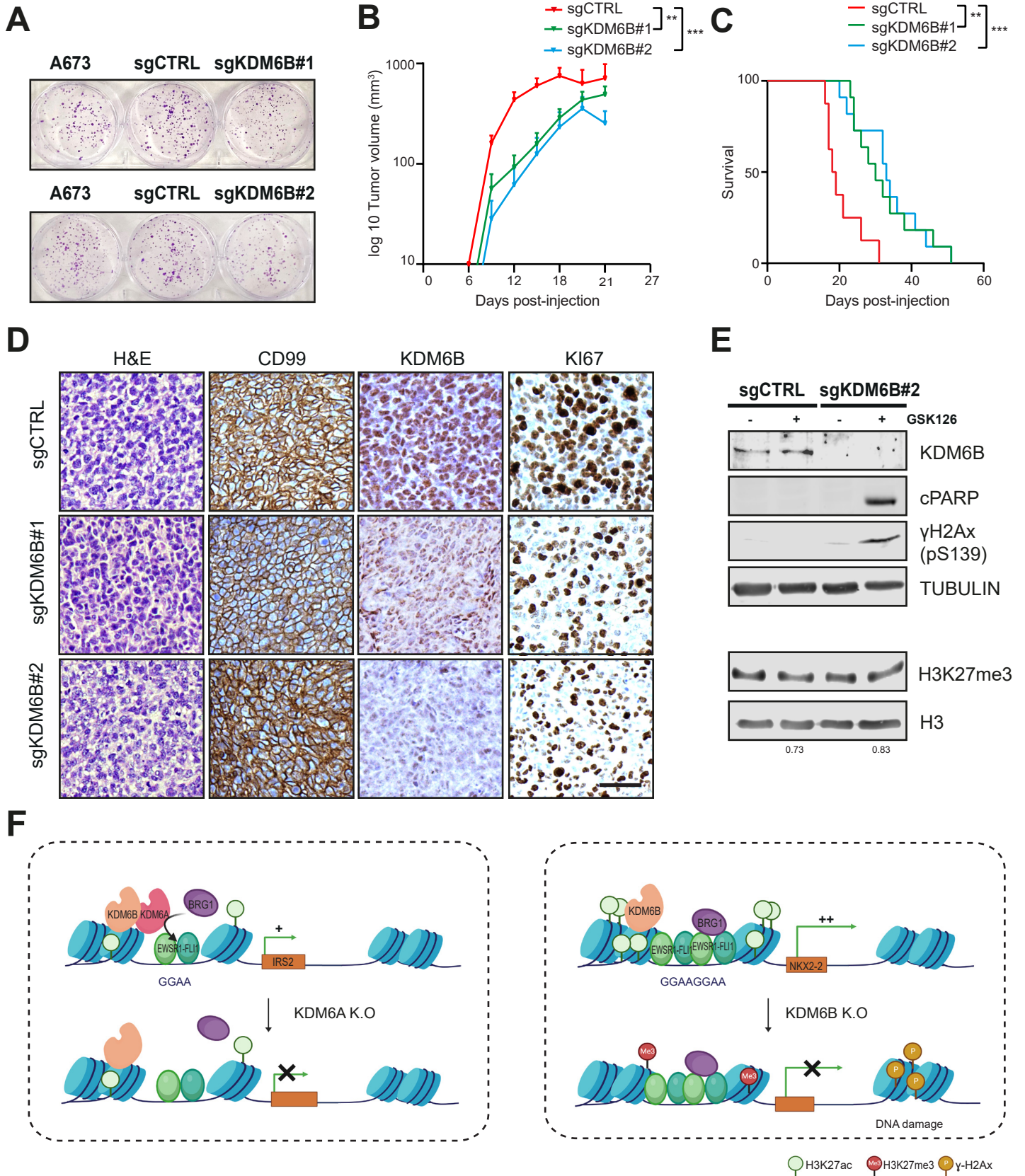# Figure 5
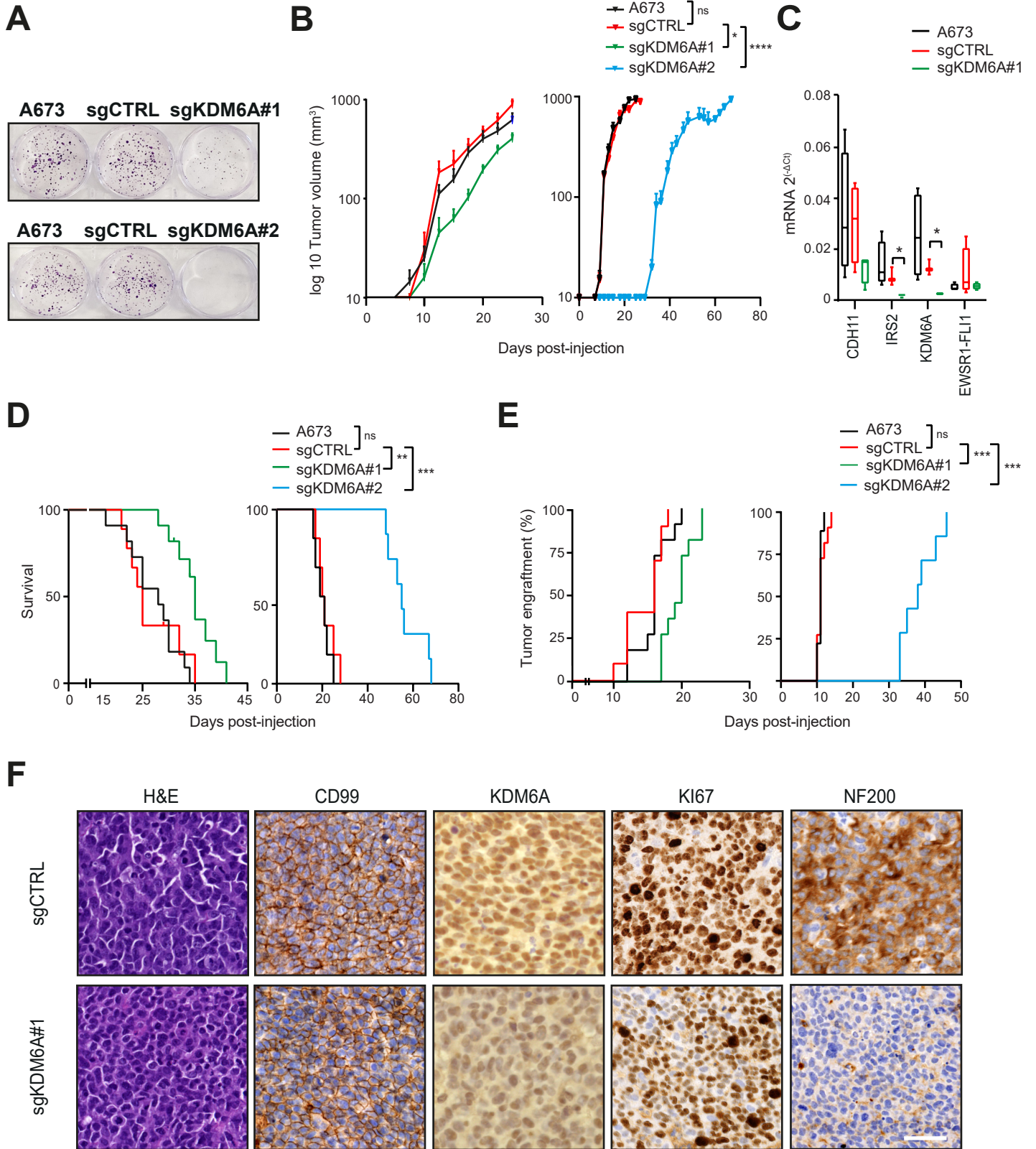
Figure 6
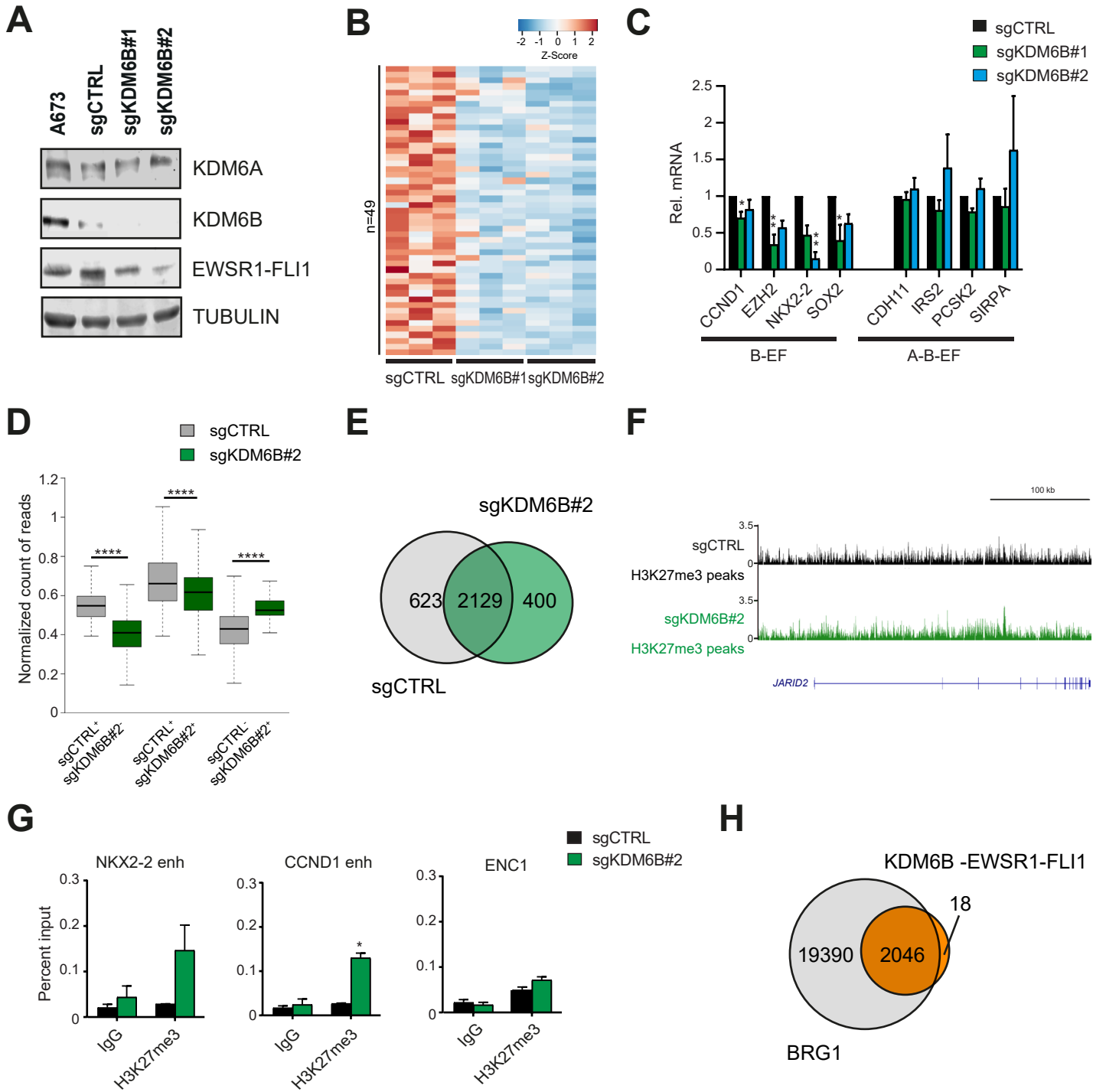
Figure 7

Supplementary Figure 1

A

H3K27me3

H3K27ac

CTRL
EWSR1-FLI1

B

Up bins
Down bins

C

Up bins
Down bins

*** ***

D

Active Enhancer
Active Promoter
Poised Enhancer
Primed Enhancer
Poised Promoter

E

Gene pathways associated with Down bins

Dopaminergic neurogenesis
Metapathway biotransformation phase I and II
Oxidation by cytochrome P450
Farnesoid X receptor pathway
Peptide GPCRs

- log10 (P value)

Gene pathways associated with Up bins

TGF-beta receptor signalling
Extracellular vesicle-mediated signalling in recipient cells
Cytokines and inflamatory response
Canonical and Non-canonical TGF-beta signalling
NOTCH1 regulation of human endothelial cell calcification

- log10 (P value)

F

	$n = 45$	n
H-score KDM6A	200 [100;300]	43
H-score KDM6B	210 [100;300]	43
Gender:		45
Female	16 (35.6%)	
Male	29 (64.4%)	

G

Supplementary Figure 2

A

B

Group	DNA motif	E-value
KDM6A		10^{-119}
KDM6B		10^{-482}
		10^{-89}
EWSR1-FLI1		10^{-600}
		10^{-315}

C

GO Biological process of KDM6A target genes

GO Biological process of KDM6B target genes

D

E

F

G

H

I

Supplementary Figure 3

Supplementary Figure 4

A

B

sgKDM6A#1   sgKDM6A#2

69.1%   67.1%

GO Biological process of KDM6A targets

Axon Guidance
Axonogenesis
Neuron Projection Guidance
Regulation of Anatomical Structure Morphogenesis
Regulation of Cell Migration

- log10 (P value)

C

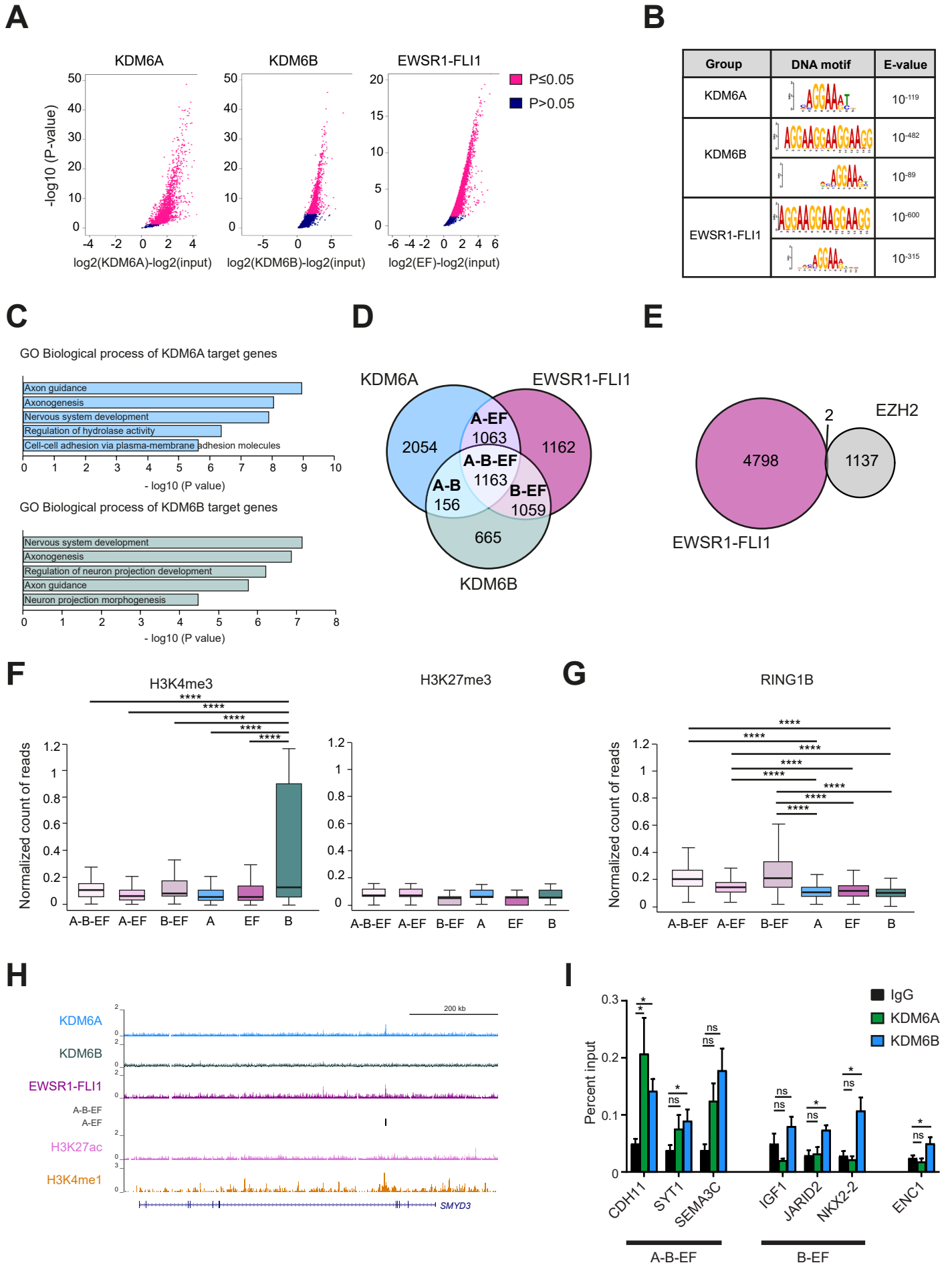**D**

E

F

G

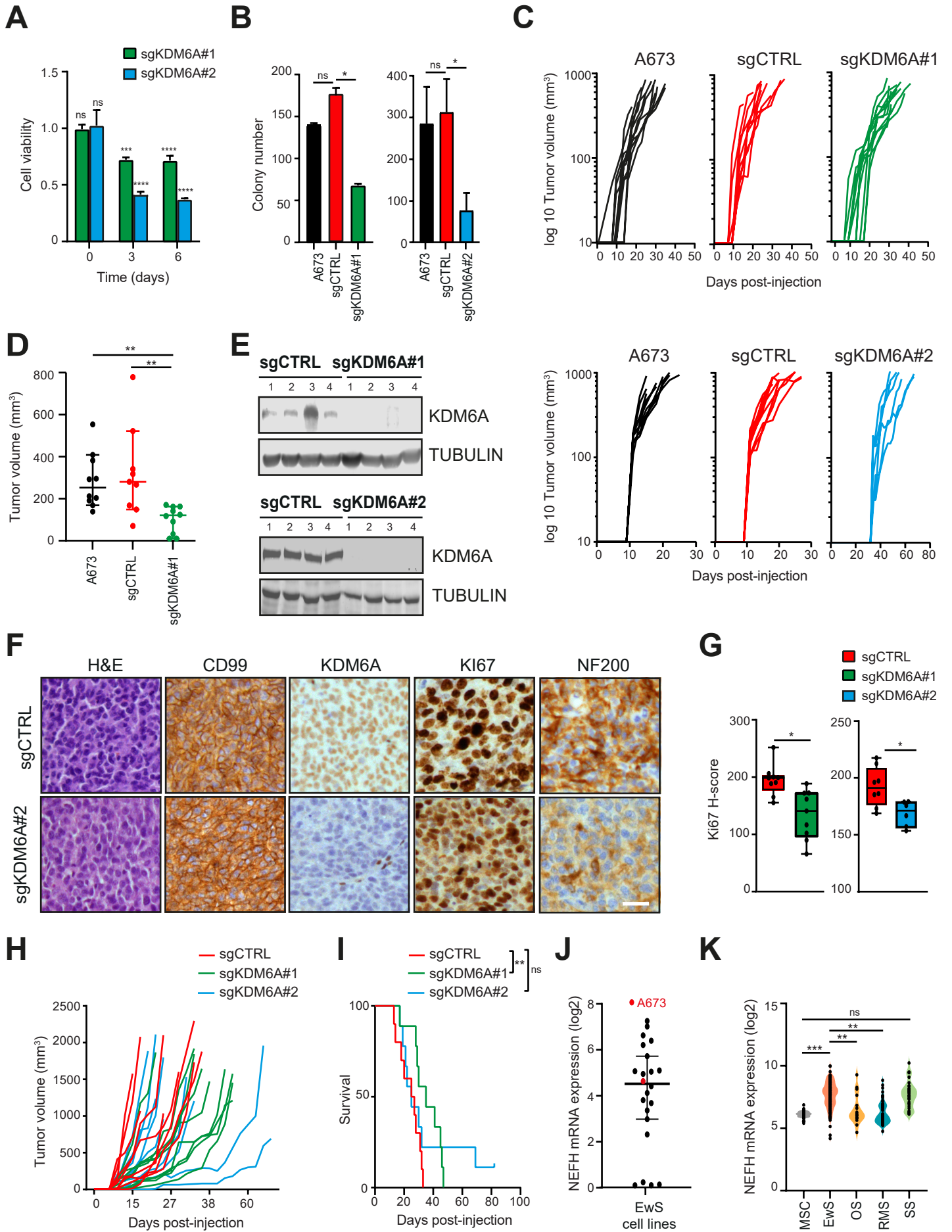

H

I

J

K

Supplementary Figure 5

Supplementary Figure 6

Supplementary Figure 7

A

B

C

D

E

F

G

H