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1 ***DLK1/DIO3* locus upregulation by a β -catenin-dependent enhancer drives cell**
2 **proliferation and liver tumorigenesis**

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30 **Short title:** The *DLK1/DIO3* locus in primary liver cancers

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51 **Abstract**

52 The *CTNNB1* gene, encoding β -catenin, is frequently mutated in hepatocellular carcinoma
53 (HCC, ~30%) and in hepatoblastoma (HB, >80%), in which *DLK1/DIO3* locus induction is
54 correlated with *CTNNB1* mutations. Here, we aim to decipher how sustained β -catenin
55 activation regulates *DLK1/DIO3* locus expression and the role this locus plays in HB and
56 HCC development in mouse models deleted for *Apc* (*Apc* ^{Δ hep}) or *Ctnnb1-exon 3* (β -
57 catenin ^{Δ Exon3}) and in human *CTNNB1*-mutated hepatic cancer cells. We identified an enhancer
58 site bound by TCF-4/ β -catenin complexes in an open conformation upon sustained β -catenin
59 activation (DLK1-WRE) and increasing *DLK1/DIO3* locus transcription in β -catenin-mutated
60 human HB and mouse models. DLK1-WRE editing by CRISPR/Cas9 approach impaired
61 *DLK1/DIO3* locus expression and slowed tumor growth in subcutaneous *CTNNB1*-mutated
62 tumor cell grafts, *Apc* ^{Δ hep} HB and β -catenin ^{Δ Exon3} HCC. Tumor growth inhibition resulted
63 either from increased FADD expression and subsequent caspase-3 cleavage in the first case,
64 or from decreased expression of cell cycle actors regulated by FoxM1 in the others.
65 Therefore, the *DLK1/DIO3* locus is an essential determinant of FoxM1-dependent cell
66 proliferation during β -catenin-driven liver tumorigenesis. Targeting the DLK1-WRE enhancer
67 to silence the *DLK1/DIO3* locus might thus represent an interesting therapeutic strategy to
68 restrict tumor growth in primary liver cancers with *CTNNB1* mutations.

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76 **Introduction**

77 The two most common primary liver tumors, hepatocellular carcinoma (HCC) in adults
78 and hepatoblastoma (HB) in children, are both characterized by mutations in the Wnt/ β -
79 catenin pathway. Somatic point mutations in the *CTNNB1* gene, encoding β -catenin, are
80 encountered in approximately 30% of HCC¹ and *CTNNB1* exon 3 deletions occur in more
81 than 80% of HB.^{2,3} *CTNNB1* mutations prevent β -catenin phosphorylation and its subsequent
82 proteasomal degradation orchestrated by a complex containing APC, AXIN1, GSK3 β , and
83 CK1. This leads to β -catenin stabilization and translocation into the nucleus, where, in
84 hepatocytes, it interacts primarily with TCF-4 before recruitment at Wnt responsive elements
85 (WRE).⁴ This recruitment allows the regulation of a specific gene repertoire acting on
86 metabolic and proliferative pathways.^{5,6} To finely tune its gene repertoire, β -catenin is able to
87 cooperate with a plethora of histone modifiers and chromatin remodelers,⁷ numbers of them
88 being mutated in HCC.⁸

89 In HB, additional mutations can also affect chromatin modifiers or long non-
90 coding(lnc)RNAs produced from parentally imprinted clusters, such as *H19* or *MEG3*.^{9,10} The
91 *DLK1/DIO3* locus encodes the largest cluster of non-coding(nc)RNAs, including 54
92 micro(mi)RNAs, several small nucleolar(sno)RNAs and lncRNAs (*e.g.*, *MEG3*, *MEG8/RIAN*,
93 *MEG9/MIRG*) expressed from the maternal allele, but also encoding paternally-expressed
94 RNAs such as *DLK1*, *RTL1* and *DIO3* (Fig.1A). The expression of the *DLK1/DIO3* locus is
95 mainly regulated by methylation of three differentially methylated regions (DMRs), named
96 *DLK1*-, *IG*-, and *MEG3*-DMRs, with different regulatory functions.¹¹ This imprinted locus is
97 crucial for cell pluripotency¹² and liver metabolic adaptation.¹³ RNAs produced from the
98 *DLK1/DIO3* locus are frequently under-expressed in cancers,¹⁴ either under- or over-
99 expressed in HCC,^{15,16} while *DLK1/DIO3* locus induction in HB is associated with poor
100 prognosis^{17,18} and *CTNNB1* mutations.¹⁸

101 In the present study, we decipher the molecular mechanisms whereby sustained β -
102 catenin activation affects gene expression at the *DLK1/DIO3* locus and the role that this
103 activation plays in the development of primary liver cancers. We used two mouse models that
104 develop HCC or HB following oncogenic activation of β -catenin signaling through either
105 inducible and liver-specific loss-of-function of *Apc* (*Apc* ^{Δ hep}),^{5,19-22} or deletion of *Ctnnb1-exon*
106 *3* (β -catenin ^{Δ Exon3}).²³ Using *in vivo* CRISPR/Cas9 editing, we identified a new regulatory site
107 upstream of *Meg3* (DLK1-WRE site) bound by oncogenic β -catenin/TCF-4 complexes and
108 responsible for *Dlk1/Dio3* locus induction. We also demonstrated the crucial pro-tumorigenic
109 role of the *DLK1/DIO3* locus in the regulation of apoptosis and FoxM1-driven cell cycle
110 progression during liver carcinogenesis mediated by β -catenin in both mouse models and in
111 hepatic cancer cell lines.

112

113 **Results**

114 **The *Dlk1/Dio3* locus is induced after sustained β -catenin activation in mouse livers and** 115 **tumors**

116 Our team has created two mouse models that recapitulate liver cancer development with
117 sustained β -catenin activation, either Cre-Lox-based *Apc* excision (*Apc* ^{Δ hep}) or CRISPR/Cas9
118 deletion of *Ctnnb1-exon 3* (β -catenin ^{Δ Exon3}) (Fig.S1A-C).²³ In both models, two tumor types
119 can emerge from healthy livers: either well differentiated HCC similar to human G5-G6 HCC,
120 or poorly differentiated tumors close to human HB. Human tumors and mouse tumors from
121 both models share dysregulated transcriptional programs.²³

122 RNAseq and small-RNAseq data showed that coding RNAs and ncRNAs within the
123 *Dlk1/Dio3* locus were ones of the most significantly overexpressed RNAs in preneoplastic
124 *Apc* ^{Δ hep} hepatocytes compared to wild-type (wt) (Tables S1-S2).^{5,21} Induction of *Meg3* and
125 miR-127 was confirmed by *in situ* hybridization in *Apc* ^{Δ hep} hepatocytes as well as in *Apc* ^{Δ hep}

126 and β -catenin ^{Δ Exon3} HCC and HB-like tumors (Fig.1B). Upregulation of *Mirg*, *Rian*, and miR-
127 127 was confirmed by RT-qPCR in *Apc* ^{Δ hep} tumors (TUM) relative to adjacent non-tumor
128 tissues (NT) (Fig.1C). It was found stronger in HCC compared to HB (Fig.1D) in agreement
129 with the maintenance of metabolic targets in HCC harboring hepatocyte features.^{23,24} The
130 locus induction appeared also higher in *Apc* ^{Δ hep} HCC compared to β -catenin ^{Δ Exon3} HCC
131 (compare Fig.1D/1E). It is also noteworthy that strong correlations between RNA expression
132 levels of *Rian*, *Mirg*, miR-127, and *Glul*, a canonical β -catenin target, were found both in
133 *Apc* ^{Δ hep} (Fig.S2A) and β -catenin ^{Δ Exon3} tumors (Fig.S2B). In DEN-induced livers tumors
134 without *Glul* induction, the expression of *Rian*, *Mirg*, and miR-127 was not modified (Fig.
135 1F) and no correlation with *Glul* was observed (Fig. S2C).

136 Using a Cre-GFP adenovirus, we sorted GFP+ *Apc* ^{Δ hep} hepatocytes during the earliest steps
137 of liver tumorigenesis (Fig.S1D). RNA-seq data showed that all RNAs within the *Dlk1/Dio3*
138 locus were induced between 6 and 15 days after *Apc* inactivation compared to non-activated
139 GFP- hepatocytes (Fig.S3A), similarly to canonical β -catenin targets such as *Glul* or *Axin2*
140 (Fig.S3B).

141 These results indicate that sustained β -catenin activation correlates with coordinated
142 upregulation of ncRNAs within the *Dlk1/Dio3* locus in preneoplastic hepatocytes and mouse
143 tumors.

144

145 **TCF-4/ β -catenin complexes bind upstream of *Meg3* promoting an enhancer activation**

146 Our next objective was to decipher how β -catenin activation promotes *Dlk1/Dio3* locus
147 expression. Our ChIP-seq data targeting the β -catenin cofactor TCF-4 in *Apc* ^{Δ hep} hepatocytes
148 showed that TCF-4 bound upstream of *Meg3* to a site containing two canonical WRE motifs
149 (named DLK1-WRE) (Fig.2A, Fig.S5A). TCF-4 binding was conserved in the human HepG2
150 cell line with activating *CTNNB1* mutations (public dataset GSM782122) (Fig.3A, Fig.S5H).

151 TCF-4 did not bind to the DLK1-WRE site in hepatocytes isolated from a mouse model
152 invalidated for β -catenin (β -cat ^{Δ hep} in Fig. 2A). Impaired expression of non-coding RNAs
153 within the *Dlk1/Dio3* locus was subsequently noticed in β -cat ^{Δ hep} hepatocytes (Tables S1-S2).
154 ChIP-qPCR targeting DLK1-WRE site confirmed increased binding of both TCF-4 (Fig.2B)
155 and β -catenin (Fig.S4A) in Apc ^{Δ hep} hepatocytes compared to wt hepatocytes. ATAC-seq
156 (Fig.2A) and ATAC-qPCR experiments (Fig.2C) indicated an open chromatin configuration
157 at the DLK1-WRE site in Apc ^{Δ hep} hepatocytes. Identical results were obtained from β -
158 catenin ^{Δ exon3} hepatocytes for TCF-4 binding and open chromatin conformation (Fig.S4B).

159 H3K4me1 and H3K27ac ChIP-qPCR experiments showed that these typical marks of
160 enhancers were both found significantly increased at the DLK1-WRE site in Apc ^{Δ hep} and β -
161 catenin ^{Δ exon3} hepatocytes compared to wt hepatocytes (Fig.2D and S4B). No significant
162 differences were found for the H3K4me3 mark associated with transcriptionally active
163 chromatin at transcription start sites (Fig.S4C). Increased TCF-4 binding was also detected at
164 the DLK1-WRE site in Apc ^{Δ hep} and β -catenin ^{Δ exon3} tumors compared to non-tumor tissues
165 (Fig. S4D). Remarkably, no modification in DNA methylation profiles and TCF-4 binding
166 was observed at the Ig-DMR in Apc ^{Δ hep} hepatocytes compared to wt hepatocytes (Fig.S4E-
167 F).²²

168 Interestingly, HNF4 α , a transcriptional factor involved in hepatocyte differentiation, bound
169 to the DLK1-WRE site in Apc ^{Δ hep} hepatocytes and independently of TCF-4 binding since its
170 binding was not affected in Apc ^{Δ hep} DLK1 ^{Δ WRE} hepatocytes (Fig.S4G). In addition, HNF4 α
171 was not co-immunoprecipitated with TCF-4 at the DLK1-WRE site in sequential ChIP
172 experiments (Fig.S4H). HNF4 α binding favored *Meg3* and *miR-127* expression since their
173 expression was impaired in HNF4 α ^{Δ hep} hepatocytes (Fig. S4I) - consistently with previous
174 data.²⁵ HNF4 α binding could therefore contribute in the higher expression of the locus

175 observed in HCC (Fig.1C), in which HNF4 α expression is maintained in contrast to HB
176 (PRJEB44400 dataset).²³

177 Globally, these results demonstrate that, upon sustained β -catenin activation, a DLK1-
178 WRE site located upstream of *Meg3*, bound by TCF-4/ β -catenin complexes, is in an open
179 configuration and marked by histone modifications typical of active enhancers. We can
180 assume that TCF-4/ β -catenin binding at this site promotes the formation of an active enhancer
181 favoring transcription of the entire *Dlk1/Dio3* locus.

182 Besides its transcriptional role, β -catenin is able to bridge distal DNA regions by chromatin
183 looping.⁷ The tridimensional organization of the *DLK1/DIO3* locus is also known to be highly
184 dynamic according to its expression patterns during embryonic development.^{26,27} We thus
185 investigated whether, following oncogenic TCF-4/ β -catenin binding, the activation of the
186 putative DLK1-WRE enhancer could affect the tridimensional organization of the
187 *DLK1/DIO3* locus. To this aim, we performed a 3C-qPCR analysis centered on the DLK1-
188 WRE site and covering the region between DLK1 and miR-136. The relative contact
189 frequencies measured all along the locus allow to determine specific interactions: the higher
190 the frequency, the closer the DNA region is relative to the DLK1-WRE site (vertical blue bar
191 in Fig.2E). In wt hepatocytes (Fig.2E, green dots), six major regions are interacting with the
192 DLK1-WRE site: one located upstream of *Dlk1*, two regions in the vicinity of the DLK1-
193 WRE site (sites 1 and 2), one site within the Ig-DMR (site 3), one within *Meg3* (site 4) and
194 one in the miR-136 region (site 6). In *Apc* ^{Δ hep} hepatocytes (Fig.2E, blue dots), binding of
195 TCF-4 to the DLK1-WRE site resulted in drastic DNA loop remodeling. Interactions with
196 sites 1, 2, and 4 were lost, while looping with the Ig-DMR (site 3) and the miR-136 region
197 (site 6) were reinforced. Remarkably, these two loops coincided with two open chromatin
198 regions observed by ATAC-seq in *Apc* ^{Δ hep} hepatocytes (sites 3 and 6 highlighted in pink in
199 Fig.2A).

200 We conclude that, in $Apc^{\Delta hep}$ hepatocytes, oncogenic β -catenin/TCF-4 complexes bound at
201 the DLK1-WRE site largely modify 3D chromatin organization and favor interactions with
202 coding regions located after the Ig-DMR rather than upstream regions. This important re-
203 organization may thus contribute to upregulate the expression of the non-coding RNAs
204 encoded in the downstream part of the *DLK1/DIO3* locus.

205

206 **The DLK1-WRE regulatory site is conserved in human hepatoblastoma**

207 Consistent with our observations in mouse models and as mentioned above, we identified a
208 TCF-4 binding site upstream of *MEG3* in HepG2 cells, a human hepatoblastoma cell line
209 harboring a *CTNNB1* deletion (Fig.3A and S5H) (GSM782122). Multiome single nucleus
210 ATAC-seq experiments conducted on samples from two HB patients harboring *CTNNB1*
211 mutations showed that the human DLK1-WRE site was in an open conformation in HB
212 compared to their paired adjacent non-tumor tissues (Fig.3A). The expression of miRNAs, as
213 well as lncRNAs and coding RNAs, from the *DLK1/DIO3* locus was increased in both 20
214 *CTNNB1*-mutated HB (yellow and orange squares in Fig.3B) and 2 non-mutated HB (white
215 square in Fig.3B) compared to their paired adjacent non-tumor tissues (Table S3).
216 Interestingly, a strong correlation was observed between expressions of the different ncRNAs
217 (Fig.3C) – supporting the idea of a global induction of this region in human HB.
218 Overexpression of these ncRNAs was confirmed in an independent larger collection of
219 primary (n=83) and recurrent HB (n=17) (Fig.3D, Table S4), and regardless of the HB
220 subgroups (Fig.3E). In this collection containing a higher number of non-mutated HB (n=7),
221 we found that *RIAN* and *DIO3OS* expression was even significantly higher in *CTNNB1*-
222 mutated HB compared to non-mutated HB, with the same tendency noticed for *MEG3*
223 (Fig.3F). Therefore, the upregulation of the *DLK1/DIO3* locus appears to be associated with
224 chromatin opening at the DLK1-WRE site in human HB with *CTNNB1* mutations.

225

226 **β -catenin/TCF-4 binding to DLK1-WRE is required for its optimal enhancer activity**

227 To decipher if the DLK1-WRE site is the key region underlying β -catenin regulation, we
228 designed two small guide RNAs (sgRNAs) to remove the DLK1-WRE site by CRISPR/Cas9
229 editing (DLK1/DIO3 ^{Δ WRE}) without potential off-targets (Fig.S5A). Then, sgRNAs were
230 integrated into plasmids containing the saCas9 sequence and inverted terminal repeats,
231 allowing *in vivo* editing using AAV8 particles.²⁸ Once subcloned, these constructs were
232 validated in the murine Hepa1-6 cell line with *Ctnnb1* mutation: DNA editing in one stable
233 clone (Fig.S5A) was successfully associated with a decrease in *Rian*, *Mirg*, *Meg3*, and *Rtl1*
234 expression (Fig.S5B), as well as down-regulation in DLK1 protein level (Fig.S5C).

235 Once validated, we genetically edited the DLK1-WRE site *in vivo* with AAV8 constructs
236 in *Apc* ^{Δ hep} mice, with the inactive Rosa26 locus as controls (Fig.S1A). As expected, DLK1-
237 WRE editing was only detected in *Apc* ^{Δ hep}-DLK1/DIO3 ^{Δ WRE} hepatocytes but not in non-
238 parenchymal cells (Fig.S5D-E). This resulted in a drastic decrease in all RNAs produced from
239 the *Dlk1/Dio3* locus in both RNA-seq and RT-qPCR experiments in *Apc* ^{Δ hep}-
240 DLK1/DIO3 ^{Δ WRE} hepatocytes (Tables S5-S6, Fig.S5F), with no impact in wt livers (Fig.S5G).
241 Additionally, impairment of TCF-4 binding at the DLK1-WRE site in *Apc* ^{Δ hep}-
242 DLK1/DIO3 ^{Δ WRE} hepatocytes (Fig.2F) was associated with a decrease in both H3K4me1 and
243 H3K27ac marks (Fig.2G) and less chromatin opening (Fig.2H). 3C experiments in *Apc* ^{Δ hep}-
244 DLK1/DIO3 ^{Δ WRE} hepatocytes showed that the 3D-chromatin organization at the DLK1-WRE
245 site got closer to that detected in wt hepatocytes: interactions between the DLK1-WRE site
246 and the site 2 were reinforced, while looping with the site 3 containing the Ig-DMR and the
247 site 6 near miR-136 was lost (pink triangles in Fig.2E).

248 Altogether, DLK1-WRE site suppression by CRISPR/Cas9 editing *in vivo* indicates that TCF-
249 4/ β -catenin binding at the DLK1-WRE site drives its enhancer activity and the subsequent
250 upregulation of the entire *Dlk1/Dio3* locus.

251

252 **DLK1-WRE site editing in $Apc^{\Delta hep}$ hepatocytes primarily affects regulators of mitotic**
253 **entry and progression**

254 Phenotypically, $Apc^{\Delta hep}$ -DLK1/DIO3 $^{\Delta WRE}$ livers demonstrated significant decrease in the
255 number of Ki-67+ hepatocytes (Fig.4A) associated with a reduced hepatomegaly²⁰ (Fig.4B)
256 compared to $Apc^{\Delta hep}$ -Rosa26 controls. Comparisons of RNA-seq data from $Apc^{\Delta hep}$ -
257 DLK1/DIO3 $^{\Delta WRE}$ versus $Apc^{\Delta hep}$ -Rosa26 hepatocytes showed that, among the top 50 most
258 significantly dysregulated genes, several genes implicated in cell proliferation and division
259 were downregulated: *Ccna2*, *Ccnb1*, *Kif20a*, *Kif20b*, *Ckap2*, *Top2a*, *Cdc2*, *Racgap1*, *Kif4a*,
260 *Hmnr*, *Nusap1*, and *Nuf2* (Table S7, some of which having been confirmed by RT-qPCR in
261 Fig.4C). These genes were upregulated in $Apc^{\Delta hep}$ hepatocytes compared to wt hepatocytes
262 (Table S8). Comparing $Apc^{\Delta hep}$ -DLK1/DIO3 $^{\Delta WRE}$ versus $Apc^{\Delta hep}$ -Rosa26 hepatocytes, gene
263 ontology identified genes related to microtubule cytoskeleton, mitotic spindle, cytokinesin
264 and cyclin B1/CDK1 complex (Fig.4D-E) and STRING analysis unveiled a hub of genes
265 related to mitotic sister chromatid segregation and cyclin-associated events during G2/M
266 transition (Fig.4F).

267 FoxM1, a typical proliferation-associated transcription factor, is known to regulate the
268 expression of genes involved in G2/M-transition and M-phase progression,²⁹ including
269 *Kif20a*, *Ccna2*, *Cdc2*, or *Cenpf*. Therefore, we hypothesized that FoxM1 might be the
270 keystone linking the *DLK1/DIO3* locus to proliferative actors. While we did not observe any
271 difference in *Foxm1* expression (Fig.5A) or nuclear localization (Fig.5B) between all
272 conditions, we noticed that FoxM1 binding was decreased at *Ccna2*, *Kif20a*, and *Cdc2*

273 promoters in $Apc^{\Delta\text{hep}}\text{-DLK1/DIO3}^{\Delta\text{WRE}}$ hepatocytes compared to $Apc^{\Delta\text{hep}}\text{-Rosa26}$ hepatocytes
274 (Fig.5C). We hypothesized that one lncRNA within the locus, either *Meg3*, *Rian*, or *Mirg*
275 could act as a guide for FoxM1 at these specific promoter regions. RNA immunoprecipitation
276 (RIP) experiments showed that *Meg3* was co-immunoprecipitated with FoxM1 in $Apc^{\Delta\text{hep}}\text{-}$
277 $Rosa26$ hepatocytes, while this association was undetectable in $Apc^{\Delta\text{hep}}\text{-DLK1/DIO3}^{\Delta\text{WRE}}$
278 hepatocytes (Fig.5D).

279 Altogether, these results indicate that the upregulation of the *DLK1/DIO3* locus, driven by
280 the β -catenin-dependent DLK1-WRE enhancer, favors FoxM1-mediated cell proliferation in
281 hepatocytes.

282

283 **DLK1-WRE site editing impairs $Apc^{\Delta\text{hep}}$ HB and HCC growth through cell autonomous** 284 **mechanisms and immune remodeling**

285 We studied the effect of DLK1-WRE editing on $Apc^{\Delta\text{hep}}$ and β -catenin $^{\Delta\text{exon3}}$ tumor
286 development (Fig.S1B-C). At sacrifice, we obtained 50% HCC and 50% HB-like tumors
287 (Fig.6A and S6A). Around 50% of tumors were not edited at the DLK1-WRE site as an
288 expected consequence of the probability rule for concomitant invalidation of both *Apc* and the
289 DLK1-WRE site in the same hepatocyte ($Apc^{\Delta\text{hep}}\text{-DLK1/DIO3}^{\text{WT}}$) (Fig.6B and S6B).
290 Retrospective analysis of tumor areas obtained by ultrasonography follow-up^{21,22} evidenced
291 that HB-like tumor growth was reduced for $Apc^{\Delta\text{hep}}\text{-DLK1/DIO3}^{\Delta\text{WRE}}$ subgroups (Fig.6C,
292 pink plots) compared to $Apc^{\Delta\text{hep}}\text{-Rosa26}$ (Fig.6C, blue plots) and $Apc^{\Delta\text{hep}}\text{-DLK1/DIO3}^{\text{WT}}$
293 (Fig.6C, green plots) HB-like tumors.

294 The slower growth of $Apc^{\Delta\text{hep}}\text{-DLK1/DIO3}^{\Delta\text{WRE}}$ HB-like tumors was associated with less
295 Ki-67+ staining (Fig.6D). *Rian*, *Mirg*, *Mki67*, *Ccna2*, *Nuf2*, *Top2a*, *Kif20b*, *Ckap2*, *Cenpf*, and
296 *Nusap1* were under-expressed in $Apc^{\Delta\text{hep}}\text{-DLK1/DIO3}^{\Delta\text{WRE}}$ HB-like tumors compared to
297 $Apc^{\Delta\text{hep}}\text{-Rosa26}$ HB-like tumors and, in most cases, compared to $Apc^{\Delta\text{hep}}\text{-DLK1/DIO3}^{\text{WT}}$ HB-

298 like tumors (Fig.6E-F). Moreover, *Axin2* was also decreased in $Apc^{\Delta hep}$ -DLK1/DIO3^{ΔWRE} HB-
299 like tumors compared to $Apc^{\Delta hep}$ -Rosa26 HB-like tumors (Fig.6F), which probably reflects
300 decreased β-catenin signaling in these tumors.

301 In both $Apc^{\Delta hep}$ and β-catenin^{Δexon3} HCC (Fig.S6C and S7C, respectively), we observed a
302 decrease in tumor growth for DLK1/DIO3^{ΔWRE} HCC (pink/purple plots) compared to Rosa26
303 HCC (blue plots). DLK1-WRE editing was associated with lower Ki-67 staining (Fig.S7C)
304 and decreased *Mki67*, *Top2a*, and *Kif20b* expression levels (Fig.S7D) in β-catenin^{Δexon3}-
305 DLK1/DIO3^{ΔWRE} HCC in agreement with the significant reductions in *Rian*, *Mirg*, and miR-
306 127 expressions (Fig.S7E). In $Apc^{\Delta hep}$ -DLK1/DIO3^{ΔWRE} HCC, DLK1-WRE editing in the
307 tumors tended to reduce *Rian* and *Mirg* expression (Fig.S6D), in a non-significant manner
308 probably because of the higher basal level observed in *Dlk1/Dio3* locus expression in $Apc^{\Delta hep}$
309 HCC (Fig.1C and E). This mild repression of the *Dlk1/Dio3* locus was associated with
310 comparable number of Ki67+-cells (Fig.S6E) and *Mki67*, *Ccna2*, *Top2a*, and *Kif20a*
311 expression levels (Fig.S6F). However, $Apc^{\Delta hep}$ -DLK1/DIO3^{WT} tumor size and growth were
312 significantly impaired compared to $Apc^{\Delta hep}$ -Rosa26 tumors (Fig.S6C, green plot).
313 Unfortunately, we could not obtain β-catenin^{Δexon3}-DLK1/DIO3^{WT} HCC to confirm this
314 observation, as a consequence of the concomitant injection and subsequent co-entry of
315 CRISPR/Cas9 constructs into hepatocytes. By comparing $Apc^{\Delta hep}$ and β-catenin^{Δexon3} models,
316 we nevertheless observed significant differences in *Mki67*, *Ccna2*, and *Top2a* expression,
317 which were drastically higher in β-catenin^{Δexon3} non-tumor tissues compared to $Apc^{\Delta hep}$ non-
318 tumor tissues (Fig.S7F). This could suggest that the proliferative index of the adjacent non-
319 tumor tissue could also influence β-catenin-driven HCC growth and efficacy of DLK1-WRE
320 editing.

321 Next, since oncogenic β-catenin is known to trigger an inflammatory response associated
322 with a significant infiltration of immune cells in $Apc^{\Delta hep}$ livers,³⁰ we analyzed immune cell

323 proportions by flow cytometry. In both $Apc^{\Delta hep}$ and β -catenin $^{\Delta exon3}$ $DLK1/DIO3^{\Delta WRE}$ tumors,
324 monocyte infiltration was reduced as compared to Rosa-26 controls (Fig.S8A and C), in
325 association with decreased expression in *Ccl2*, *Ccl5* and *Csf1* mRNAs, encoding three
326 chemokines essential for monocyte recruitment (Fig. S8B and D). This suggest that the
327 immune response fostered by oncogenic β -catenin signaling could arise in part downstream
328 from the activation of the *DLK1/DIO3* locus, and potentially contribute to its tumor-
329 promoting effect.

330 Altogether, our results show that β -catenin-driven HB growth is mainly dependent on the
331 level of activation of the *DLK1/DIO3* locus in the tumor itself while β -catenin-driven HCC
332 growth seems to be affected by the *DLK1/DIO3* level, not only in the tumor, but also in the
333 surrounding tissue. They also point to the activation of the *DLK1/DIO3* locus as a key
334 intermediate in the oncogenic β -catenin-dependent infiltration of monocytes in the tumors.

335

336 **Editing of the DLK1-WRE site impairs the pro-tumorigenic capacities of human and** 337 **mouse hepatic cancer cell lines harboring β -catenin-mutations**

338 Finally, we investigated the impact of DLK1-WRE editing in transformed cell lines from
339 mouse and human hepatic cancers harboring β -catenin mutations. The $DLK1/DIO3^{\Delta WRE}$
340 Hepa1-6 clones, characterized in Fig.S5A-C, were less proliferative than the Rosa26 clones
341 (Fig.7A). This was accompanied by a significant increase in the number of $DLK1/DIO3^{\Delta WRE}$
342 cells in G2/M phase (Fig.7B) together with a decrease in cyclin B1 protein level, a protein
343 required for mitotic initiation (Fig.7C). As observed in $Apc^{\Delta hep}$ - $DLK1/DIO3^{\Delta WRE}$ hepatocytes
344 (Fig. 5D), RIP experiments showed that *Meg3* co-immunoprecipitation with FoxM1 was
345 decreased in Hepa1-6- $DLK1/DIO3^{\Delta WRE}$ clones compared to Hepa1-6-Rosa26 clones (Fig.
346 7D). Consistent with *in vitro* data, Hepa1-6- $DLK1/DIO3^{\Delta WRE}$ clones also exhibited decreased
347 tumorigenic capacity compared to Hepa1-6-Rosa26 clones after subcutaneous allografting

348 into Nu/Nu mice. Tumor progression was significantly slower for Hepa1-6-DLK1/DIO3^{ΔWRE}
349 clones (Fig.7E) with 2.6-fold lower mean tumor volume and 4-fold lower weight at the time
350 of sacrifice (Fig.7F). Impaired DLK1/DIO3^{ΔWRE} tumor progression in Nu/Nu mice was
351 consistent with less Ki-67+ staining (Fig.7G) and more cells harboring cleaved caspase-3
352 (Fig.7H) compared to Rosa26 tumors. According to Targetscan and Dianalab algorithms,
353 FADD, a pro-apoptotic actor, is a potential target of miR-134 produced within the
354 *DLK1/DIO3* locus. We found that Fadd was increased in DLK1/DIO3^{ΔWRE} Hepa1-6 tumors at
355 both the mRNA and protein level (Fig.7I-J), and could favor caspase 3 cleavage.

356 The active DLK1-WRE site being conserved in human hepatic cell lines and HB tumors
357 (Fig.3A and S5H), we also generated DLK1/DIO3^{ΔWRE} clones from the Huh6 cell line, a
358 human hepatoblastoma cell line with a *CTNNB1* mutation (Fig.S5H). Editing of the DLK1-
359 WRE site led to reduced expression of *RIAN*, *MIRG*, *MEG3*, and *RTL-1* (Fig.S5I).
360 Proliferation of DLK1/DIO3^{ΔWRE} Huh6 clones was slower than those of control clones
361 (Fig.8A). In line, a significant increase in the number of DLK1/DIO3^{ΔWRE} cells in G2/M
362 phase was noticed (Fig.8B). DLK1/DIO3^{ΔWRE} clones had a significantly reduced capacity to
363 grow as spheroids (Fig.8C). *In vivo*, DLK1/DIO3^{ΔWRE} Huh6 clones exhibited decreased
364 tumorigenic capacity compared to control clones after subcutaneous xenografting into Nu/Nu
365 mice (Fig.8D). A 4-fold lower mean tumor volume and weight were observed at sacrifice
366 (Fig.8D-E). DLK1/DIO3^{ΔWRE} Huh6 tumors displayed a reduced number of Ki67+ cells
367 (Fig.8F) and decreased *MKI67* expression (Fig.8G). Importantly, as previously observed in
368 mouse *Apc*^{Δhep} hepatocytes (Fig.5C), FOXM1 was found to bind *CCNA2*, *CDC2* and *KIF20A*
369 promoters in human Huh6 clones (Fig.8H, blue squares), but was not recruited in
370 DLK1/DIO3^{ΔWRE} Huh6 clones (Fig.8H, pink dots). DLK1/DIO3^{ΔWRE} Huh6 tumors also
371 displayed a higher number of cells harboring cleaved caspase-3 (Fig.8I) and increased FADD
372 mRNA and protein levels (Fig.8J-K). In both DLK1/DIO3^{ΔWRE} Hepa1-6 (Fig.7K) and Huh6

373 grafted tumors (Fig.8L), *GLUL* expression was decreased, in agreement with inhibition of β -
374 catenin signaling in *DLK1/DIO3*^{AWRE} tumors, as previously noticed for mouse HB-like tumors
375 (Fig.6F).

376 In conclusion, the *DLK1/DIO3* locus contributes to the pro-tumorigenic capacities of
377 transformed human hepatic cancer cells harboring β -catenin mutations by enhancing their
378 proliferation through the FOXM1 axis and by decreasing FADD-dependent apoptotic
379 programs both *in vitro* and *in vivo*.

380

381 **Discussion**

382 Imprinted loci play major roles in cellular plasticity and cell reprogramming during cancer.
383 More particularly, the *DLK1/DIO3* locus, also known as the 14q32.2 cluster, is crucial for cell
384 proliferation and metabolic adaptation in the liver.¹³ In human and mouse HB, we found that
385 RNAs within the *DLK1/DIO3* locus are highly expressed and correlated with oncogenic
386 activation of β -catenin,^{3,31} as reported by others.¹⁸ Here, we have identified a regulatory site,
387 existing in mouse and human, responsible for the *DLK1/DIO3* locus induction driven by
388 oncogenic TCF-4/ β -catenin complexes and unveiled its functional impact on cell proliferation
389 and apoptosis (Fig.S9). We have also shown that dysregulation of the *Dlk1/Dio3* locus occurs
390 in mouse HCC emerging from healthy livers. Importantly, we have demonstrated that part of
391 the oncogenic role of the *DLK1/DIO3* locus involves the DLK1-WRE enhancer site *in vivo*.
392 Altogether, our work provides strong arguments for the therapeutic benefit of a targeted
393 repression of the DLK1-WRE enhancer.

394 In our study, we have identified the regulatory region activated in case of sustained β -
395 catenin activation during the very early steps of liver tumorigenesis and determined how this
396 region promotes the *DLK1/DIO3* locus transcription. This transcriptional regulation requires
397 the binding of oncogenic β -catenin/TCF-4 complexes at the DLK1-WRE site, which then

398 becomes an active enhancer. This site is also engaged in chromatin remodeling and long-
399 range chromatin interactions to bridge the enhancer region with other regulatory sites at the
400 Ig-DMR and in downstream regions. This DNA looping role for β -catenin/TCF-4 complexes
401 echoes two works published by Yochum *et al.* showing that β -catenin coordinates chromatin
402 looping at an enhancer site upstream of MYC, a canonical β -catenin target in colon
403 cancer.^{32,33} Furthermore, this regulatory mechanism is also reminiscent of chromatin looping
404 and enhancer-promoter bridging as a way to escape silencing for the imprinted *DLK1/DIO3*
405 locus.³⁴ Determining the allele of origin for the regulatory functions that we unveiled at the
406 DLK1-WRE site need to generate hybrids of our transgenic mice. Nevertheless, we did not
407 find any change in DNA methylation status at the Ig-DMR region in $Apc^{\Delta hep}$ hepatocytes,
408 which is rather in favor of an absence of imprinting loss.

409 Besides increasing knowledge on the regulation of the *DLK1/DIO3* locus, our editing
410 strategy has also deciphered the pro-tumorigenic events subsequent to its activation by β -
411 catenin signaling. Using a CRISPR/Cas9 strategy in mouse, we showed that editing of the
412 DLK1-WRE site inhibited HB and HCC growth in healthy livers. Nevertheless, the antitumor
413 activity of DLK1-WRE editing was the strongest in $Apc^{\Delta hep}$ HB-like tumors. We also
414 observed a decrease in HCC growth, notably in β -catenin ^{$\Delta exon3$} HCC with moderate activation
415 of the *Dlk1/Dio3* locus. In $Apc^{\Delta hep}$ HCC, it appears that tumor growth is highly dependent on
416 *Dlk1/Dio3* locus expression in the tumor microenvironment: inhibition of tumor growth of
417 $Apc^{\Delta hep}$ -DLK1/DIO3^{WT} HCC is similar to that of $Apc^{\Delta hep}$ -DLK1/DIO3 ^{ΔWRE} HCC, arguing that
418 an impairment of the *DLK1/DIO3* locus in non-tumor tissues could also modulate HCC
419 progression. HCC is the paradigm of inflammation-associated cancer emerging on cirrhotic
420 livers in 80% of cases. The *DLK1/DIO3* locus expression has been found to be upregulated in
421 response to several types of stresses, particularly in hepatocytes under metabolic disorders³⁵
422 and lipid overload³⁶. We have exploited public RNA-seq datasets generated on samples from

423 patients with diseased livers at high risk of HCC (GSE126848 and GSE142530).
424 Interestingly, we found that the expression of *RTL1*, within the locus, and *AXIN2*, a canonical
425 target of β -catenin, was upregulated during alcoholic hepatitis and metabolic dysfunction-
426 associated steatohepatitis (MASH). This presumes an activation of β -catenin signaling in
427 diseased livers, as reported by other studies showing that *CTNNB1*-mutated HCC are
428 significantly over-represented under MASH context.^{37,38} These results are also in agreement
429 with data obtained by others in db/db and ob/ob mice^{36,39}, as well as data generated in our lab
430 with mice fed with choline-deficient diet and methionine-choline deficient diets, showing an
431 increased expression in *Mirg*, *Rian*, and *Meg3* (unpublished data).

432 Besides their roles in hepatocytes, the dysregulation of ncRNAs within the locus has also
433 been reported in immune cells during inflammation, particularly in macrophages.^{40,41} Here,
434 we exemplified that DLK1-WRE editing in both *Apc*^{Ahep} and β -catenin ^{Δ exon3} tumors impaired
435 monocyte infiltration and expression of *Ccl2*, *Ccl5* and *Csfl* encoding chemokines essential
436 for monocyte recruitment. This could open new perspectives regarding the role played by the
437 locus in the control of immune cell populations, with the underlying molecular mechanisms
438 remaining to be deciphered. This also reinforces the interest to impair locus expression during
439 chronic liver diseases preceding HCC.

440 Our data obtained on DLK1/DIO3^{AWRE} hepatocytes and transformed hepatic cell lines
441 revealed that inhibition of cell proliferation and tumor progression was associated with a
442 decrease in the mRNA levels of several actors involved in cytokinesis and G2/M phase, the
443 cell phase during which the levels of β -catenin rise to a peak.⁴² Interestingly, a hub between
444 cell cycle actors and miRNAs within the *DLK1/DIO3* locus has also been inferred by
445 bioinformatics analysis of HB datasets.⁴³ Here, our study supports that the proliferative
446 impact of the *DLK1/DIO3* locus in preneoplastic hepatocytes is dependent on the β -catenin-
447 driven DLK1-WRE enhancer and the subsequent redistribution of FoxM1 at the promoters of

448 cell cycle actors. *Meg3* appears as a potential guiding partner for FoxM1 at these promoters to
449 regulate their transcription. FOXM1 is involved in the progression of several cancers
450 including hepatoblastoma⁴⁴ and compounds inhibiting FOXM1 have recently demonstrated
451 encouraging antitumor activities.⁴⁵ This could open new innovative treatment strategies
452 against FOXM1 as an alternative target for the treatment of *CTNNB1*-mutated cancers.

453 Altogether, our results unveil how a sustained activation of β -catenin signaling can
454 remodel the epigenetic and chromatin landscape of one of its key oncogenic targets with a
455 subsequent effect on proliferative gene signatures. We put forward the idea that targeting the
456 DLK1-WRE site represents a potent strategy to specifically repress the *DLK1/DIO3* locus in
457 liver tumors harboring β -catenin mutations. Regarding the crucial role of β -catenin in tissue
458 homeostasis and repair after injury, many drugs targeting this pathway have failed in
459 treatments because of their toxicities. Targeting downstream events, as we have outlined here
460 with the *DLK1/DIO3* locus, appears as a promising option. This could be performed by gene
461 editing therapies with zinc-finger nucleases, which gave promising results in haemophilia B
462 (NCT02695160 ongoing). The development of *in silico* approaches to predict genome wide
463 off-targets will open up new horizons for gene editing therapy, which could benefit to our
464 proposed strategy against the *DLK1/DIO3* enhancer in particular in HB, but also probably
465 during chronic liver diseases and HCC.

466

467 **Material and methods**

468 *In vivo CRISPR/Cas9 design and gene editing analysis.*

469 Small guides RNAs (sgRNAs) against the DLK1-WRE site were designed as previously for
470 β -catenin ^{Δ Exon3} (Table S9).²³ DNA were extracted from edited cells, livers and tumors and
471 analyzed as previously (Table S9).²³ PCR products were run on E-Gel 2% for 10 minutes
472 (Thermofischer, Waltham, MA) and bands quantified with ImageJ.

473

474 *Murine models*

475 The $Apc^{lox/lox}$ model was edited for the DLK1-WRE site with 3.6×10^{11} Vg of sg2 plus
476 1.9×10^{11} Vg of sg5 (DLK1/DIO3^{ΔWRE}), one month before β-catenin activation as reported
477 elsewhere.²³ Intraperitoneal injection of 2mg tamoxifen (MP Biomedicals, Irvine, CA) in
478 $Apc^{lox/lox}$ TTR-Cre mice resulted in Apc deletion in ≥90% of hepatocytes for preneoplastic
479 studies six days post-injection (pretumoral $Apc^{\Delta hep}$ model, Fig.S1A).¹⁹⁻²¹ A unique injection
480 of 1×10^9 Vg of an Ad5-Cre-GFP adenovirus led to tumors within 4-6 months,²⁰ which were
481 monitored by 2D-ultrasound (Vevo 2100, Visualsonics, Toronto, Canada), as previously
482 published (Fig. S1B).²¹ The tamoxifen procedure to inactivate β-catenin in mice carrying a
483 biallelic floxed *Ctnnb1* gene with loxP sites located between exons 2 and 6 and a TTR-Cre^{Tam}
484 ($\beta cat^{\Delta hep}$) was reported elsewhere.^{5,46,47} Diethylnitrosamine (DEN)-induced livers tumors were
485 obtained by intraperitoneal injection of 0.25mg DEN (Sigma Aldrich, Saint-Louis, MO) in
486 14-day-old male mice. The HNF4α^{Δ hep} model was obtained as described earlier.^{48,49}
487 For β-catenin^{Δ Exon3} model, AAV8 against the DLK1-WRE site and the exon 3 of *Ctnnb1*²³
488 were concomitantly administered and mice were monitored as described above (Fig. S1C).
489 Kinetic hepatocyte sorting was performed after retro-orbital injection of 1.5×10^9 particles of
490 Ad5-Cre-GFP adenovirus, liver perfusion with collagenase as described previously²¹ and GFP
491 sorting on an ARIA3 (BD, Franklin Lakes, NJ) (Fig.S1D).
492 Subcutaneous allografts were performed with 2×10^6 cells on both flanks from 5-weeks old
493 female Nu/Nu nude mice as reported elsewhere.⁵⁰ All animal procedures were carried out
494 according to French legal regulations and approved by an ethical committee (agreements 17-
495 082, 14009 and 16420).

496

497 *Human samples*

498 For HB cohort 1 (N=22, Table S3), RNA-seq was performed on total RNA extracted from
499 tumoral and adjacent non-tumor tissues surgically resected from 22 patients using the
500 *mirVana* kit (ThermoFischer, Waltham, MA) according to the supplier's protocol. The RNA
501 integrity number was evaluated by Agilent 2100 Bioanalyzer. Two μ g were used for
502 generation of each small RNA library with an Illumina TruSeq®Small RNA Sample Prep Kit
503 according to standard protocol (Illumina, San Diego, CA). Single read 50 nt sequencing was
504 performed by MGX-Montpellier GenomiX platform on an Illumina HiSeq 2000 using the
505 Sequence By Synthesis technique. Adapter sequences were trimmed from small RNA reads
506 using the Cutadapt (version 1.4.1) tool [<http://code.google.com/p/cutadapt/>], retaining reads of
507 the size 16-25 nt. Reads were then mapped to the human hairpin sequences (mirBase version
508 21) with Bowtie (v1.0). The number of reads mapping in the sense orientation to each hairpin
509 in each patient was used as an input for further analysis. Data were analyzed using DESeq2
510 package in R studio. The miRNA expression file was loaded in format .txt to obtain a matrix
511 with the value in i-th row corresponding to miRNA names and value in j-th column
512 corresponding to patient samples. We followed the DESeq2 manual,⁵¹ performed differential
513 miRNA expression analysis (DE) and produced output files including heatmaps and tables.
514 After the DE analysis, DESeq2 produces a set of values: base mean, log2 fold change and
515 adjusted pvalue for each miRNA between tumoral and non-tumor samples. Once exported,
516 our data were classified in an excel file and analyzed with the help of miRBase and UCSC
517 Browser [<https://genome.ucsc.edu/>].

518 For the HB cohort 2 (N=100, Table S4), RNA sequencing was performed as previously
519 described.^{3,52,53} Gene expression levels were calculated using the variance stabilizing
520 transformation (VERSUST) and the raw count matrix. Gene expression-based classification
521 of HB was done as previously described.³ Statistical analysis and data visualization were

522 performed using R software version 3.6.1 (R Foundation for Statistical Computing, Vienna,
523 Austria. <https://www.R-project.org>) and Bioconductor packages.

524 RNAseq data from a collection of livers with varying degrees of non-alcoholic fatty liver
525 disease compared with healthy livers from normal-weight individuals (GSE126848) and liver
526 biopsies from 28 patients with alcoholic hepatitis, cirrhosis compared to healthy controls
527 (GSE142530) were retrieved with GEO and analysed using the R package DESeq2 (v.1.38).⁵¹

528 *Cell culture*

529 Hepatocytes were isolated four days after tamoxifen injection in $Apc^{lox/lox}$ TTR-Cre mice and
530 maintained at 37°C in a humidified atmosphere containing 5% CO₂ as reported elsewhere.²¹

531 Hepa1-6 cells were obtained from the American Type Culture Collection and Huh6 cells from
532 C. Perret's lab.¹ They were grown in DMEM medium supplemented with 10% fetal bovine
533 serum (FBS) and 50U/mL penicillin-streptomycin at 37 °C in a humidified atmosphere
534 containing 5% CO₂ (Thermofischer, Waltham, MA). Stable clones were obtained following
535 co-transfections of sgRNA- and pmax-GFP plasmids, GFP-based sorting (ARIA3) and
536 amplification of selected clones.

537

538 *Proliferation/ Cell cycle analysis*

539 Cell proliferation was measured on 8,000 cells with the xCELLigence system (Agilent,
540 Santa Clara, CA) (N=3), as previously described.²¹ Cell cycle analysis was performed on
541 5×10^5 synchronized cells (with 24h FBS deprivation for Huh6 cells and 10µg/mL colchicine
542 for Hepa1-6 cells), fixed 48h later in PBS-20%/Ethanol-80% at -20°C for 15min and stained
543 with FxCycle PI/RNase solution (Thermofischer, Waltham, MA) for 30 min at room
544 temperature before analysis (Fortessa, BD).

545

546 *Sphere formation assay*

547 One thousand Huh6 clones were grown during 14 days onto ultra-low attachment 6-well
548 plates (Corning, Corning, NY) in DMEM/F12 medium supplemented with B27, 20ng/mL
549 EGF, 20ng/mL basic FGF and 100µg/mL gentamycin (Thermofischer, Waltham, MA) (N=3).

550

551 *RNA extraction and RT-qPCR*

552 Levels of miRNAs and mRNAs were determined on total RNA extracted with Trizol reagent
553 (Thermofischer, Waltham, MA), as previously reported (Table S9).²¹

554

555 *Quantitative chromosome conformation capture (3C-qPCR)*

556 3C-qPCR experiment was adapted from ^{26,54} and conducted on 5 million nuclei isolated from
557 hepatocytes lysed in homogenization buffer with an ultraturrax during 20min on ice and
558 centrifuged for 1h at 20,000xg. 3C assays were performed with EcoRI digestion, product
559 ligation and secondary XbaI digestion (N≥3).²⁶ Sample purity and DNA content were
560 determined with internal primers against *Gapdh* and digestion efficiency with three primer
561 sets (Table S9). For all 3C experiments, qPCR primers used were as previously published
562 with the anchor F16 located at the beginning of the DLK1-WRE site and thus not affected by
563 the CRISPR/Cas9 constructs used.⁵⁵ Their efficiencies were determined on equimolar
564 amounts of 3C-ligation products generated from BAC RPCI-23 clone 117C15
565 (Thermofischer, Waltham, MA) covering the genome segment between *DLK1* and *miR-136*
566 genes. 3C data were normalized to the “Basal Interaction Level” using the previously
567 published algorithm.²⁶

568

569 *ATAC-seq and ATAC-qPCR*

570 50,000 isolated hepatocytes were transposed for 30min in 50µL reaction mix containing
571 4.5µL transposase (kit #FC-121-103, Illumina, San Diego, CA) and 0.1% digitonin (adapted

572 from ⁵⁶) and the initial protocol was followed after transposition⁵⁷ for ATAC-seq and ATAC-
573 qPCR experiments (Table S9). For liver samples, omni-ATAC-seq was performed on 50,000
574 nuclei, isolated as for 3C assays, according to Corces *et al.*⁵⁸ in 50 μ L reaction mix with 2.5 μ L
575 transposase, 0.01% digitonin and 0.1% Tween20 for 30min. The following steps were
576 according to the initial protocol.⁵⁷

577 Libraries were controlled using a 2100 Bioanalyzer. and an aliquot of each library was
578 sequenced at low depth onto a MiSeq platform to control duplicate level and estimate DNA
579 concentration. Each library was then paired-end sequenced (2 \times 100 bp) on a HiSeq
580 instrument to get 40 million read pairs on average. As ATAC-seq libraries are composed in
581 large part of short genomic DNA fragments, and in order to reduce costs, we sequenced our
582 recent libraries on a Nextseq instrument (2 x 38bp). Our analysis showed that reducing read
583 length to 38bp does not affect mapping efficiency. Reads were first cleaned using
584 trimmomatic (removing of adaptors and low-quality bases). Trimmed reads were then aligned
585 to the mouse genome (mm9) using Bowtie2 with the parameter -X2000, and with 2-
586 mismatches permitted in the seed (default value). The -X2000 option allows the fragments <
587 2kb to align. Duplicated reads were removed with picard-tools. Resulted bam datasets were
588 then converted to BigWig, a coverage track adapted to visualize datasets in UCSC Genome
589 Browser or IGV. Conversion was performed using bamCoverage command from deepTools
590 with the parameters --binSize 10 --normalizeUsing RPKM --extendReads. The parameter --
591 normalizeUsing RPKM is used to normalize each dataset. We selected the normalization
592 method based on RPKM (Reads Per Kilobase per Million mapped reads), which calculates the
593 number of reads per bin / number of mapped reads (in millions). The parameter --extendReads
594 allows the extension of reads to fragment size. The default value is estimated from the data
595 (mean of the fragment size of all mate reads).

596 In human HB, Multiome approach was performed by Integragen SA (Evry, France) on
597 matched non-tumor livers (n=2) and hepatoblastomas (n=3) of two patients, according to the
598 commercial “Chromium Single Cell Multiome ATAC + Gene Expression” protocol. We used
599 10X Genomics Cell Ranger ARC 2.0.0 to align snATAC-seq reads to the human genome
600 (GrCh38/hg38) (Roerhig *et al.* in revision).

601

602 *Chromatin Immunoprecipitation (ChIP) and RNA immunoprecipitation (RIP)*

603 ChIP was performed as previously described²¹ on 25µg chromatin with 30µl protein
604 A/G(v/v)-dynabeads with antibodies of interest (Table S10). For tumors, samples were
605 homogenized with an ultra-turrax in 1% formaldehyde for 1 min. For RIP experiments, beads
606 were reverse crosslinked before RNA isolation by Trizol (Thermofischer, Waltham, MA).

607

608 *RNA-seq/smallRNA-seq*

609 RNA-seq and small-RNAseq were performed on 1µg total RNA extracted from Apc^{Δhep}-
610 ROSA26 versus DLK1/DIO3^{ΔWRE} hepatocytes (N≥4) respectively with TruSeq Stranded after
611 ribodepletion and TruSeq Small RNA and sequenced with Nextseq 500 (150b) (Illumina, San
612 Diego, CA).

613 Fastq files were then aligned using STAR algorithm (version 2.7.6a), on the Ensembl Mus
614 musculus GRCm38 reference release 96. Reads were then count using RSEM (v1.3.1) and the
615 statistical analyses on the read counts were performed with R (version 3.6.3) and the DESeq2
616 package (DESeq2_1.26.0) to determine the proportion of differentially expressed genes
617 between two conditions. We used the standard DESeq2 normalization method (DESeq2's
618 median of ratios with the DESeq function), with a pre-filter of reads and genes (reads
619 uniquely mapped on the genome, or up to 10 different loci with a count adjustment and genes
620 with at least 10 reads in at least 3 different samples). Following the package

621 recommendations, we used the Wald test with the contrast function and the Benjamini-
622 Hochberg FDR control procedure to identify the differentially expressed genes. R scripts and
623 parameters are available on GitHub ([https://github.com/BSGenomique/genomic-rnaseq-
624 pipeline/releases/tag/v1.0420](https://github.com/BSGenomique/genomic-rnaseq-pipeline/releases/tag/v1.0420)). For miRNA-Seq data analysis, Fastq files were uploaded on
625 Qiagen geneglobe analysis software for alignment and counting. Then, UMI matrix were used
626 as raw data for our R & DESeq2 pipeline.

627 Kinetic RNAseq was performed on HiSeq4000 in paired-end on at least three independent
628 samples of sorted GFP+ Apc^{Δhep} hepatocytes at day 6, 15, 21 after injection compared to GFP-
629 hepatocytes.

630

631 *Immunostaining/In situ hybridization*

632 Paraffin-embedded liver sections were treated and labeled as previously^{21,23} with antibodies
633 and probes of interest (Tables S9-S10).

634

635 *Western-blot*

636 Experiments were conducted on 20μg total proteins as reported elsewhere (Table S10).²¹

637

638 *Flow cytometry*

639 Livers and tumors were minced with scissors in DMEM containing collagenase IV (2.5
640 mg/mL, Sigma Aldrich, Saint-Louis, MO) and incubated for 30 min at 37°C. Cell suspensions
641 were passed through a 100μm filter and stained with appropriate antibodies for 30 min on ice
642 as previously (Table S10).⁵⁹ Data were acquired on a BD LSR Fortessa flow cytometer (BD
643 Franklin Lakes, NJ) and analyzed with FlowJo software. Absolute cell count was calculated
644 as previously with nonfluorescent beads and expressed as a number of cells per milligram of
645 tissue.⁵⁹

646

647 *Statistical analysis*

648 We assessed the significance of differences between two groups of samples using Mann-
649 Whitney tests and between three groups of samples using Kruskal-Wallis. $p < 0.05$ was
650 considered statistically significant. For human samples, difference in gene expression levels,
651 in two or more than two groups, was tested using Wilcoxon or Kruskal-Wallis tests,
652 respectively. Correlation analysis was performed using Pearson r correlation when both
653 variables were normally distributed with the assumptions of linearity and homoscedasticity or
654 Spearman's rank-order correlation.

655

656 *Data availability*

657 All data were deposited on GEO: RNAseq and smallRNAseq comparing $Apc^{\Delta hep}$
658 ROSA/DLK1/DIO3 ^{ΔWRE} hepatocytes on GSE206262, ATAC-seq on GSE211930, kinetic
659 RNAseq on GSE210482), MeDIP-seq on GSE239777. Others have been previously published
660 (Project: PRJNA150641 in ENA).^{5,21,23}

661

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685 formal analysis, investigation, validation; Isabelle Lagoutte: investigation; Stefano Caruso:
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687 Cécile Godard: investigation, methodology; Akila Iddir: investigation; Enzo Montmory:
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692 analysis, writing– original draft; Christophe F. Grosset: resources, formal analysis, Jessica
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694 and Angélique Gougelet: investigation, formal analysis, methodology, validation, writing –
695 original draft; conceptualization, funding acquisition and supervision.

696

697 **Competing interests:**

698 Two patents PCT/EP2023/053419 and EP22305162.4 have been deposited by JS, LP, SC and
699 AG. The other authors declare no conflict of interest.

700

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702 site, non-coding RNAs, targeted therapies

703

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931

932 **List of figure captions**

933

934 **Figure 1: The *Dlk1/Dio3* locus is induced in mouse HCC and HB-like tumors driven by** 935 **β -catenin**

936 **A:** Schematic representation of the *DLK1/DIO3* locus; **B:** *In situ* hybridization of *Meg3* and
937 miR-127 with staining of glutamine synthetase (GS) or active β -catenin in wt and *Apc* ^{Δ hep}
938 livers and in *Apc* ^{Δ hep} and β -catenin ^{Δ Exon3} HCC or HB-like tumors; CV: central vein, PV: portal
939 vein; **C-F:** Expression of *Rian*, *Mirg*, and miR-127 by RT-qPCR in *Apc* ^{Δ hep} tumors (TUM)

940 compared to adjacent non-tumor (NT) tissue (**C**); in $Apc^{\Delta hep}$ HCC and HB-like tumors (**D**); in
941 β -catenin ^{$\Delta Exon3$} tumors (**E**); in DEN tumors without β -catenin activation (**F**). Levels of
942 significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.0001$, ns: non-significant (Mann-
943 Whitney).

944

945 **Figure 2: β -catenin binding at the DLK1-WRE site opens chromatin and exerts**
946 **enhancer activity in $Apc^{\Delta hep}$ hepatocytes**

947 **A:** ChIP-seq targeting TCF-4 in wt, $Apc^{\Delta hep}$ and β -cat ^{Δhep} hepatocytes and ATAC-seq data in
948 wt and $Apc^{\Delta hep}$ hepatocytes. TCF-4 binding site is framed in the blue box (DLK1-WRE) and
949 sites common with 3C in pink; **B, D, F-G:** ChIP-qPCR analysis at the DLK1-WRE site for
950 TCF-4, H3K4me1 and H3K27ac relative to isotype control in $Apc^{\Delta hep}$ hepatocytes compared
951 to wt (**B, D**) and compared to $Apc^{\Delta hep}$ -DLK1/DIO3 ^{ΔWRE} hepatocytes (**F-G**); **C, H:** ATAC-
952 qPCR analysis at the DLK1-WRE site compared to wt (**C**) and to $Apc^{\Delta hep}$ -DLK1/DIO3 ^{ΔWRE}
953 hepatocytes (**H**); **E:** Relative contact frequencies in arbitrary unit (A.U.) between the DLK1-
954 WRE site (blue vertical bar) and 19 genomic sites (small vertical black bars on the map
955 below) measured in 3C experiments performed on wt, $Apc^{\Delta hep}$ -Rosa26 and $Apc^{\Delta hep}$ -
956 DLK1/DIO3 ^{ΔWRE} liver nuclei with error bars representing s.e.m. of 6, 5 and 3 biological
957 replicates, respectively. Regions of interest (highlighted in pink) are numbered from 1 to 6.
958 The lower panel illustrates the different chromatin loops distributed into six interaction zones:
959 the darkest the pink, the strongest the interaction. Figure made with Biorender. Levels of
960 significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.0001$, ns: non-significant (Mann-
961 Whitney).

962

963 **Figure 3: The *DLK1/DIO3* locus is induced in human hepatoblastoma in correlation with**
964 ***CTNNB1* mutations and DLK1-WRE opening**

965 **A:** Pseudo bulk snATAC-seq aggregated with Cellranger-atac of 3 human HB (T) and their
966 adjacent non-tumor tissue (N) at the DLK1-WRE site; the pink panel represents ChIP-seq data
967 targeting TCF-4 in the human hepatoblastoma HepG2 cell line (Gsm782122); **B:** RNAseq
968 expression data for the entire DLK1/DIO3 locus in HB normalized to their adjacent non-
969 tumor tissue (NT) (N=22, cohort 1). A white square in the β -catenin lane indicates HB with
970 intact *CTNNB1*, a yellow square HB with point mutation in *CTNNB1*, an orange square HB
971 with *CTNNB1* exon 3 deletion; **C:** correlation between *RIAN*, *MIRG*, *DIO3OS*, *DIO3*, *MEG3*,
972 miR-411 and miR-136 expressions in cohort 1; **D:** *RIAN*, *RTL-1*, and *MEG3* expression
973 determined by RT-qPCR in primary (N=83) and recurrent HB (N=17) *versus* non tumor liver
974 (NTL) (N=100, cohort 2); **E:** Expression of *DIO3OS*, *MEG3*, and *RIAN* determined by RT-
975 qPCR in the different subgroups of HB: embryonal (green), fetal hepatocytic 1 (pink), fetal
976 hepatocytic 2 (yellow) and mesenchymal (purple); **F:** Expression of *RIAN*, *DIO3OS*, and
977 *MEG3* determined by RT-qPCR in primary HB with *CTNNB1* mutations (*CTNNB1*^{mut}, N=76)
978 or with intact *CTNNB1* (*CTNNB1*^{wt}, N=7). Levels of significance: *p<0.05, ***p<0.005,
979 ****p<0.001, ns: non-significant (Kruskal-Wallis).

980

981 **Figure 4: DLK1-WRE editing affects $Apc^{\Delta hep}$ hepatocyte proliferation through inhibition**
982 **of mitosis and cytokinesis regulators**

983 **A:** Percentage of Ki-67+ hepatocytes in wt, $Apc^{\Delta hep}$ -Rosa26 and $Apc^{\Delta hep}$ -DLK1/DIO3 ^{ΔWRE}
984 livers; **B:** Percentage of liver to body weight ratio in wt, $Apc^{\Delta hep}$ -Rosa26 and $Apc^{\Delta hep}$ -
985 DLK1/DIO3 ^{ΔWRE} livers; **C:** Expression of *Top2a*, *Kif20b*, *Nuf2*, and *Nusap1* in $Apc^{\Delta hep}$ -
986 DLK1/DIO3 ^{ΔWRE} hepatocytes relative to $Apc^{\Delta hep}$ -Rosa26 hepatocytes; **D-F:** RNA-seq analysis
987 on $Apc^{\Delta hep}$ -DLK1/DIO3 ^{ΔWRE} and $Apc^{\Delta hep}$ -Rosa26 hepatocytes; **D:** The histograms summarize
988 ratio obtained with GSEA between the number of genes in the intersection of the query set
989 with a set from MSigDB (k/K), with p-value and FDR q-values for each item; **E:** Schematic

990 representation of the most significantly deregulated RNAs; **F**: Main hub obtained by STRING
991 analysis. Levels of significance: * $p < 0.05$, ** $p < 0.01$, **** $p < 0.001$, ns: non-significant
992 (Mann-Whitney).

993

994 **Figure 5: DLK1-WRE editing impairs FoxM1 binding at *Ccna2*, *Kif20a* and *Cdc2***
995 **promoters**

996 **A**: RT-qPCR analysis of *Foxm1* expression in $Apc^{\Delta hep}$ -Rosa26 and $Apc^{\Delta hep}$ -DLK1/DIO3 $^{\Delta WRE}$
997 hepatocytes compared to wt hepatocytes; **B**: Number of Foxm1+ nuclei in IHC; **C**:
998 Representative images of ChIP-PCR targeting FoxM1 at *Ccna2*, *Kif20a*, *Cdc2* and *Cenpf*
999 promoters compared to isotype control in $Apc^{\Delta hep}$ -Rosa26 and $Apc^{\Delta hep}$ -DLK1/DIO3 $^{\Delta WRE}$
1000 hepatocytes and inputs; the lower panel represents the PCR band quantification with ImageJ
1001 of all ChIP experiments against FoxM1 relative to isotype control; for the *cdc2* promoter in
1002 $Apc^{\Delta hep}$ -DLK1/DIO3 $^{\Delta WRE}$ hepatocytes, the cropped images are for two different mice
1003 analyzed on two gels with the same conditions of exposure. **D**: Quantification of *Meg3* RNA
1004 co-immunoprecipitated with FoxM1 in RIP-qPCR in $Apc^{\Delta hep}$ -DLK1/DIO3 $^{\Delta WRE}$ (n=4)
1005 compared to $Apc^{\Delta hep}$ -Rosa26 hepatocytes (n=2); data are represented as the relative binding
1006 compared to 18S. Figure made with Biorender. Levels of significance: * $p < 0.05$, ** $p < 0.01$,
1007 ns: non-significant (Kruskal-Wallis or Mann-Whitney).

1008

1009 **Figure 6: DLK1-WRE site editing slows tumor growth of $Apc^{\Delta hep}$ HB through decreased**
1010 **expression of mitotic entry regulators**

1011 **A**: Examples of glutamine synthetase staining of $Apc^{\Delta hep}$ HB showing a heterogeneous
1012 staining with many stromal cells; HB cells losing several metabolic features of mature
1013 hepatocytes express low level of glutamine synthetase compared to $Apc^{\Delta hep}$ HCC cells; **B**:
1014 Analysis of tumor editing by PCR band quantification with ImageJ in $Apc^{\Delta hep}$ HB. The upper

1015 panel is a representative image obtained from non-tumor tissue (NT) and tumors (T); **C:**
1016 Progression of cumulative tumor areas in $Apc^{\Delta hep}$ -DLK1/DIO3^{ΔWRE} HB and $Apc^{\Delta hep}$ -
1017 DLK1/DIO3^{WT} compared to $Apc^{\Delta hep}$ -Rosa26 HB with cumulative area at sacrifice indicated
1018 in the right panel; **D:** Representative images of ki-67 staining on $Apc^{\Delta hep}$ HB with high or low
1019 proliferation rate (left panel) and Quantification of Ki-67+ hepatocytes in percentage for all
1020 tumors (right panel); **E:** RT-qPCR analysis of *Rian* and *Mirg* in $Apc^{\Delta hep}$ HB compared to their
1021 NT tissues; **F:** RT-qPCR analysis of *Mki67*, *Ccna2*, *Nuf2*, *Top2a*, *Axin2*, *Kif20b*, *Nusap1*,
1022 *Cenpf*, and *Ckap2* relative to their NT tissues. Levels of significance: *p<0.05, ** p<0.01,
1023 ***p<0.005, ****p<0.001, ns: non-significant (Kruskal-Wallis).

1024

1025 **Figure 7: DLK1-WRE site editing impairs the protumorigenic capacities of murine**
1026 **hepatoma Hepa1-6 cells mutated for *Ctnnb1***

1027 **A-D:** Analysis of DLK1/DIO3^{ΔWRE} Hepa1-6 clones versus Rosa26 control clones;
1028 Proliferation rate at 48h (**A**); Percentage of cells in G2/M phase determined by flow
1029 cytometry (**B**); Cyclin B1 and A2 protein level determined by Western-blot (**C**);
1030 Representative quantification of *Meg3* RNA co-immunoprecipitated with FoxM1 in RIP-
1031 qPCR (n=2); data are represented as the relative binding compared to 18S (**D**); **E-K:** Analysis
1032 of DLK1/DIO3^{ΔWRE} Hepa1-6 tumors versus Rosa26 tumors; Tumor volumes measured every
1033 two days (**E**) and tumor weights at sacrifice (**F**); Percentage of Ki67+ tumor cells (**G**);
1034 Percentage of tumor cells with cleaved-caspase 3 (**H**); *Fadd* level determined by RT-qPCR
1035 (**I**); FADD protein level determined by Western-blot (**J**); *Glul* expression determined by RT-
1036 qPCR (**K**). Levels of significance *p<0.05, **p<0.01, ***p<0.005, ****p<0.0001 (Mann-
1037 Whitney).

1038

1039 **Figure 8: DLK1-WRE site editing impairs the protumorigenic capacities of human**
1040 **hepatoblastoma Huh6 cells mutated for *CTNNB1***

1041 **A-C:** Analysis of DLK1/DIO3^{ΔWRE} Huh6 clones versus non-edited clones; Proliferation rate
1042 at 48h (**A**); Percentage of cells in G2/M phase determined by flow cytometry (**B**); Number of
1043 spheroids (**C**); **D-L:** Analysis of DLK1/DIO3^{ΔWRE} Huh6 tumors versus non-edited tumors;
1044 Tumor volumes measured every two days (**D**); tumor weights at sacrifice (**E**); Percentage of
1045 Ki67+ tumor cells (**F**); *MKI67* level determined by RT-qPCR (**G**); Percentage of tumor cells
1046 with cleaved-caspase 3 (**H**); *FADD* mRNA level determined by RT-qPCR (**I**); *FADD* protein
1047 level determined by Western-blot (**J**), Representative FOXM1 binding at *CCNA2*, *KIF20A*
1048 and *CDC2* promoters normalized to isotype control in ChIP-qPCR experiments (n=2) (**K**);
1049 *GLUL* expression determined by RT-qPCR (**L**); *p<0.05, ** p<0.01, ***p<0.005,
1050 ****p<0.0001 (Mann-Whitney).

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