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

Abstract

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

Introduction

The two most common primary liver tumors, hepatocellular carcinoma (HCC) in adults and hepatoblastoma (HB) in children, are both characterized by mutations in the Wnt/β-catenin pathway. Somatic point mutations in the *CTNNB1* gene, encoding β-catenin, are encountered in approximately 30% of HCC[1] and *CTNNB1* exon 3 deletions occur in more than 80% of HB.[2,3] *CTNNB1* mutations prevent β-catenin phosphorylation and its subsequent proteasomal degradation orchestrated by a complex containing APC, AXIN1, GSK3β, and CK1. This leads to β-catenin stabilization and translocation into the nucleus, where, in hepatocytes, it interacts primarily with TCF-4 before recruitment at Wnt responsive elements (WRE).[4] This recruitment allows the regulation of a specific gene repertoire acting on metabolic and proliferative pathways.[5,6] To finely tune its gene repertoire, β-catenin is able to cooperate with a plethora of histone modifiers and chromatin remodelers,[7] numbers of them being mutated in HCC.[8]

In HB, additional mutations can also affect chromatin modifiers or long non-coding(lnc)RNAs produced from parentally imprinted clusters, such as *H19* or *MEG3*.[9,10] The *DLK1/DIO3* locus encodes the largest cluster of non-coding(nc)RNAs, including 54 micro(mi)RNAs, several small nucleolar(sno)RNAs and lncRNAs (*e.g.*, *MEG3*, *MEG8*/*RIAN*, *MEG9*/*MIRG*) expressed from the maternal allele, but also encoding paternally-expressed RNAs such as *DLK1*, *RTL1* and *DIO3* (Fig.1A). The expression of the *DLK1/DIO3* locus is mainly regulated by methylation of three differentially methylated regions (DMRs), named DLK1-, IG-, and MEG3-DMRs, with different regulatory functions.[11] This imprinted locus is crucial for cell pluripotency[12] and liver metabolic adaptation.[13] RNAs produced from the *DLK1/DIO3* locus are frequently under-expressed in cancers,[14] either under- or over-expressed in HCC,[15,16] while *DLK1/DIO3* locus induction in HB is associated with poor prognosis[17,18] and *CTNNB1* mutations.[18]

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

Results

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

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

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

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

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

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

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

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

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

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

Interestingly, HNF4α, a transcriptional factor involved in hepatocyte differentiation, bound to the DLK1-WRE site in Apc$^{\Delta hep}$ hepatocytes and independently of TCF-4 binding since its binding was not affected in Apc$^{\Delta hep}$ DLK1$^{\Delta WRE}$ hepatocytes (Fig.S4G). In addition, HNF4α was not co-immunoprecipitated with TCF-4 at the DLK1-WRE site in sequential ChIP experiments (Fig.S4H). HNF4α binding favored *Meg3* and *miR-127* expression since their expression was impaired in HNF4α$^{\Delta hep}$ hepatocytes (Fig. S4I) - consistently with previous data.[25] HNF4α binding could therefore contribute in the higher expression of the locus

observed in HCC (Fig.1C), in which HNF4α expression is maintained in contrast to HB (PRJEB44400 dataset).[23]

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

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

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

The DLK1-WRE regulatory site is conserved in human hepatoblastoma

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

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

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

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

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

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

Phenotypically, Apc$^{\Delta hep}$-DLK1/DIO3$^{\Delta WRE}$ livers demonstrated significant decrease in the number of Ki-67+ hepatocytes (Fig.4A) associated with a reduced hepatomegaly[20] (Fig.4B) compared to Apc$^{\Delta hep}$-Rosa26 controls. Comparisons of RNA-seq data from Apc$^{\Delta hep}$-DLK1/DIO3$^{\Delta WRE}$ *versus* Apc$^{\Delta hep}$-Rosa26 hepatocytes showed that, among the top 50 most significantly dysregulated genes, several genes implicated in cell proliferation and division were downregulated: *Ccna2*, *Ccnb1*, *Kif20a*, *Kif20b*, *Ckap2*, *Top2a*, *Cdc2*, *Racgap1*, *Kif4a*, *Hmmr*, *Nusap1*, and *Nuf2* (Table S7, some of which having been confirmed by RT-qPCR in Fig.4C). These genes were upregulated in Apc$^{\Delta hep}$ hepatocytes compared to wt hepatocytes (Table S8). Comparing Apc$^{\Delta hep}$-DLK1/DIO3$^{\Delta WRE}$ *versus* Apc$^{\Delta hep}$-Rosa26 hepatocytes, gene ontology identified genes related to microtubule cytoskeleton, mitotic spindle, cytokinesin and cyclin B1/CDK1 complex (Fig.4D-E) and STRING analysis unveiled a hub of genes related to mitotic sister chromatid segregation and cyclin-associated events during G2/M transition (Fig.4F).

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

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

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

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

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

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

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

In both $Apc^{\Delta hep}$ and β-$catenin^{\Delta exon3}$ HCC (Fig.S6C and S7C, respectively), we observed a decrease in tumor growth for $DLK1/DIO3^{\Delta WRE}$ HCC (pink/purple plots) compared to Rosa26 HCC (blue plots). DLK1-WRE editing was associated with lower Ki-67 staining (Fig.S7C) and decreased *Mki67*, *Top2a*, and *Kif20b* expression levels (Fig.S7D) in β-$catenin^{\Delta exon3}$-$DLK1/DIO3^{\Delta WRE}$ HCC in agreement with the significant reductions in *Rian*, *Mirg*, and miR-127 expressions (Fig.S7E). In $Apc^{\Delta hep}$-$DLK1/DIO3^{\Delta WRE}$ HCC, DLK1-WRE editing in the tumors tended to reduce *Rian* and *Mirg* expression (Fig.S6D), in a non-significant manner probably because of the higher basal level observed in *Dlk1/Dio3* locus expression in $Apc^{\Delta hep}$ HCC (Fig.1C and E). This mild repression of the *Dlk1/Dio3* locus was associated with comparable number of Ki67+-cells (Fig.S6E) and *Mki67*, *Ccna2*, *Top2a*, and *Kif20a* expression levels (Fig.S6F). However, $Apc^{\Delta hep}$-$DLK1/DIO3^{WT}$ tumor size and growth were significantly impaired compared to $Apc^{\Delta hep}$-Rosa26 tumors (Fig.S6C, green plot). Unfortunately, we could not obtain β-$catenin^{\Delta exon3}$-$DLK1/DIO3^{WT}$ HCC to confirm this observation, as a consequence of the concomitant injection and subsequent co-entry of CRISPR/Cas9 constructs into hepatocytes. By comparing $Apc^{\Delta hep}$ and β-$catenin^{\Delta exon3}$ models, we nevertheless observed significant differences in *Mki67*, *Ccna2*, and *Top2a* expression, which were drastically higher in β-$catenin^{\Delta exon3}$ non-tumor tissues compared to $Apc^{\Delta hep}$ non-tumor tissues (Fig.S7F). This could suggest that the proliferative index of the adjacent non-tumor tissue could also influence β-catenin-driven HCC growth and efficacy of DLK1-WRE editing.

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

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

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

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

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

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

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

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

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

Discussion

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

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

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

Besides increasing knowledge on the regulation of the *DLK1/DIO3* locus, our editing strategy has also deciphered the pro-tumorigenic events subsequent to its activation by β-catenin signaling. Using a CRISPR/Cas9 strategy in mouse, we showed that editing of the DLK1-WRE site inhibited HB and HCC growth in healthy livers. Nevertheless, the antitumor activity of DLK1-WRE editing was the strongest in $Apc^{\Delta hep}$ HB-like tumors. We also observed a decrease in HCC growth, notably in β-$catenin^{\Delta exon3}$ HCC with moderate activation of the *Dlk1/Dio3* locus. In $Apc^{\Delta hep}$ HCC, it appears that tumor growth is highly dependent on *Dlk1/Dio3* locus expression in the tumor microenvironment: inhibition of tumor growth of $Apc^{\Delta hep}$-$DLK1/DIO3^{WT}$ HCC is similar to that of $Apc^{\Delta hep}$-$DLK1/DIO3^{\Delta WRE}$ HCC, arguing that an impairment of the *DLK1/DIO3* locus in non-tumor tissues could also modulate HCC progression. HCC is the paradigm of inflammation-associated cancer emerging on cirrhotic livers in 80% of cases. The *DLK1/DIO3* locus expression has been found to be upregulated in response to several types of stresses, particularly in hepatocytes under metabolic disorders[35] and lipid overload[36]. We have exploited public RNA-seq datasets generated on samples from

patients with diseased livers at high risk of HCC (GSE126848 and GSE142530). Interestingly, we found that the expression of *RTL1*, within the locus, and *AXIN2*, a canonical target of β-catenin, was upregulated during alcoholic hepatitis and metabolic dysfunction-associated steatohepatitis (MASH). This presumes an activation of β-catenin signaling in diseased livers, as reported by other studies showing that *CTNNB*1-mutated HCC are significantly over-represented under MASH context.[37,38] These results are also in agreement with data obtained by others in db/db and ob/ob mice[36,39], as well as data generated in our lab with mice fed with choline-deficient diet and methionine-choline deficient diets, showing an increased expression in *Mirg, Rian,* and *Meg3* (unpublished data).

Besides their roles in hepatocytes, the dysregulation of ncRNAs within the locus has also been reported in immune cells during inflammation, particularly in macrophages.[40,41] Here, we exemplified that DLK1-WRE editing in both $Apc^{\Delta hep}$ and β-$catenin^{\Delta exon3}$ tumors impaired monocyte infiltration and expression of *Ccl2*, *Ccl5* and *Csf1* encoding chemokines essential for monocyte recruitment. This could open new perspectives regarding the role played by the locus in the control of immune cell populations, with the underlying molecular mechanisms remaining to be deciphered. This also reinforces the interest to impair locus expression during chronic liver diseases preceding HCC.

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

cell cycle actors. *Meg3* appears as a potential guiding partner for FoxM1 at these promoters to regulate their transcription. FOXM1 is involved in the progression of several cancers including hepatoblastoma[44] and compounds inhibiting FOXM1 have recently demonstrated encouraging antitumor activities.[45] This could open new innovative treatment strategies against FOXM1 as an alternative target for the treatment of *CTNNB1*-mutated cancers.

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

Material and methods

In vivo CRISPR/Cas9 design and gene editing analysis.

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

Murine models

The $Apc^{lox/lox}$ model was edited for the DLK1-WRE site with $3.6x10^{11}$ Vg of sg2 plus $1.9x10^{11}$ Vg of sg5 ($DLK1/DIO3^{\Delta WRE}$), one month before β-catenin activation as reported elsewhere.[23] Intraperitoneal injection of 2mg tamoxifen (MP Biomedicals, Irvine, CA) in $Apc^{lox/lox}$ TTR-Cre mice resulted in *Apc* deletion in ≥90% of hepatocytes for preneoplastic studies six days post-injection (pretumoral $Apc^{\Delta hep}$ model, Fig.S1A).[19-21] A unique injection of $1x10^{9}$Vg of an Ad5-Cre-GFP adenovirus led to tumors within 4-6 months,[20] which were monitored by 2D-ultrasound (Vevo 2100, Visualsonics, Toronto, Canada), as previously published (Fig. S1B).[21] The tamoxifen procedure to inactivate β-catenin in mice carrying a biallelic floxed *Ctnnb1* gene with loxP sites located between exons 2 and 6 and a $TTR\text{-}Cre^{Tam}$ ($\beta cat^{\Delta hep}$) was reported elsewhere.[5,46,47] Diethylnitrosamine (DEN)-induced livers tumors were obtained by intraperitoneal injection of 0.25mg DEN (Sigma Aldrich, Saint-Louis, MO) in 14-day-old male mice. The $HNF4\alpha^{\Delta hep}$ model was obtained as described earlier.[48,49]

For $\beta\text{-catenin}^{\Delta Exon3}$ model, AAV8 against the DLK1-WRE site and the exon 3 of *Ctnnb1* [23] were concomitantly administered and mice were monitored as described above (Fig. S1C).

Kinetic hepatocyte sorting was performed after retro-orbital injection of $1.5x10^{9}$ particles of Ad5-Cre-GFP adenovirus, liver perfusion with collagenase as described previously[21] and GFP sorting on an ARIA3 (BD, Franklin Lakes, NJ) (Fig.S1D).

Subcutaneous allografts were performed with $2x10^{6}$ cells on both flanks from 5-weeks old female Nu/Nu nude mice as reported elsewhere.[50] All animal procedures were carried out according to French legal regulations and approved by an ethical committee (agreements 17-082, 14009 and 16420).

Human samples

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

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

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

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

Cell culture

Hepatocytes were isolated four days after tamoxifen injection in $Apc^{lox/lox}$ TTR-Cre mice and maintained at 37°C in a humidified atmosphere containing 5% CO_2 as reported elsewhere.[21] Hepa1-6 cells were obtained from the American Type Culture Collection and Huh6 cells from C. Perret's lab.[1] They were grown in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 50U/mL penicillin-streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2 (Thermofischer, Waltham, MA). Stable clones were obtained following co-transfections of sgRNA- and pmax-GFP plasmids, GFP-based sorting (ARIA3) and amplification of selected clones.

Proliferation/ Cell cycle analysis

Cell proliferation was measured on 8,000 cells with the xCELLigence system (Agilent, Santa Clara, CA) (N=3), as previously described.[21] Cell cycle analysis was performed on $5x10^5$ synchronized cells (with 24h FBS deprivation for Huh6 cells and 10µg/mL colchicine for Hepa1-6 cells), fixed 48h later in PBS-20%/Ethanol-80% at -20°C for 15min and stained with FxCycle PI/RNAse solution (Thermofischer, Waltham, MA) for 30 min at room temperature before analysis (Fortessa, BD).

Sphere formation assay

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

RNA extraction and RT-qPCR

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

Quantitative chromosome conformation capture (3C-qPCR)

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

ATAC-seq and ATAC-qPCR

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

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

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

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

Chromatin Immunoprecipitation (ChIP) and RNA immunoprecipitation (RIP)

ChIP was performed as previously described[21] on 25µg chromatin with 30µl protein A/G(v/v)-dynabeads with antibodies of interest (Table S10). For tumors, samples were homogenized with an ultra-turrax in 1% formaldehyde for 1 min. For RIP experiments, beads were reverse crosslinked before RNA isolation by Trizol (Thermofischer, Waltham, MA).

RNA-seq/smallRNA-seq

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

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

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

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

Immunostaining/In situ hybridization

Paraffin-embedded liver sections were treated and labeled as previously[21,23] with antibodies and probes of interest (Tables S9-S10).

Western-blot

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

Flow cytometry

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

Statistical analysis

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

Data availability

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

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Author contributions

Julie Sanceau: investigation, formal analysis, methodology, validation, writing – original draft; Lucie Poupel: investigation, formal analysis, methodology, validation; Camille Joubel: formal analysis, investigation, validation; Isabelle Lagoutte: investigation; Stefano Caruso: formal analysis; Sandra Pinto: investigation, Christèle Desbois-Mouthon: investigation; Cécile Godard: investigation, methodology; Akila Iddir: investigation; Enzo Montmory: investigation, Cécile Dulary: investigation, Sophie Chantalat: investigation, methodology, formal analysis, Amélie Roehrig: investigation, formal analysis; Kevin Muret: formal analysis; Benjamin Saint-Pierre: formal analysis, Jean-François Deleuze: funding acquisition, Sophie Mouillet-Richard: resources, formal analysis; Thierry Forné: methodology, formal analysis, writing– original draft; Christophe F. Grosset: resources, formal analysis, Jessica Zucman-Rossi: resources; Sabine Colnot: conceptualization, funding acquisition, supervision

and Angélique Gougelet: investigation, formal analysis, methodology, validation, writing – original draft; conceptualization, funding acquisition and supervision.

Competing interests:

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

Keywords: primary liver cancers, transgenic mice, *in vivo* CRISPR/Cas9, β-catenin, enhancer site, non-coding RNAs, targeted therapies

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List of figure captions

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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