

1 **The mouse HP1 proteins are essential for preventing liver tumorigenesis**

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25 **Abstract**

26 Chromatin organization is essential for appropriate interpretation of the genetic
27 information. Here, we demonstrated that the chromatin associated proteins HP1 are
28 dispensable for hepatocytes survival but are essential within hepatocytes to prevent liver
29 tumor development in mice with HP1 β being pivotal in these functions. Yet, we found that the
30 loss of HP1 *per se* is not sufficient to induce cell transformation but renders cells more
31 resistant to specific stress such as the expression of oncogenes and thus *in fine*, more prone
32 to cell transformation. Molecular characterization of HP1-Triple KO pre-malignant livers and
33 BMEL cells revealed that HP1 are essential for the maintenance of heterochromatin
34 organization and for the regulation of specific genes with most of them having well
35 characterized functions in liver functions and homeostasis. We further showed that some
36 specific retrotransposons get reactivated upon loss of HP1, correlating with over-expression
37 of genes in their neighborhood. Interestingly, we found that, although HP1-dependent genes
38 are characterized by enrichment H3K9me3, this mark does not require HP1 for its
39 maintenance and is not sufficient to maintain gene repression in absence of HP1. Finally, we
40 demonstrated that the loss of TRIM28 association with HP1 recapitulated several
41 phenotypes induced by the loss of HP1 including the reactivation of some retrotransposons
42 and the increased incidence of liver cancer development. Altogether, our findings indicate
43 that HP1 proteins act as guardians of liver homeostasis to prevent tumor development by
44 modulating multiple chromatin-associated events within both the heterochromatic and
45 euchromatic compartments, partly through regulation of the corepressor TRIM28 activity.

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47 **Keywords:** chromatin; HP1; cancer; liver; transcriptional silencing; endogenous retrovirus

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49 Introduction

50 Chromatin organization is essential for the interpretation of genetic information in a cell-
51 type and tissue-specific manner ¹. Alteration of this organization can have devastating
52 consequences, as evidenced by the large number of diseases induced by mutations in
53 chromatin-associated proteins ^{2,3}, as well as by the dramatic changes in chromatin
54 organization observed in cancer cells ⁴. Although extensively studied in the past three
55 decades, it is still poorly understood how chromatin organization is regulated and involved in
56 tumorigenesis.

57 Chromatin can be divided according to its structural and functional features in
58 euchromatin and heterochromatin. Euchromatin displays low level of compaction, is highly
59 enriched in genes, and is transcriptionally competent. Conversely heterochromatin is highly
60 compacted, enriched in repetitive DNA sequences, and mostly silent ⁵. Heterochromatin
61 Protein 1 (HP1) proteins, first isolated as major heterochromatin components in *Drosophila*,
62 are highly conserved from yeast to mammals which express three isoforms (HP1 α , HP1 β
63 and HP1 γ) that are distributed in both eu- and heterochromatin ⁶. These proteins are
64 characterized by a chromodomain (CD) involved in recognition of H3 lysine-9 di- or
65 trimethylated (H3K9me_{2/3}), and a chromoshadow domain (CSD), which, through
66 dimerization, constitutes a platform for interaction with many protein partners. These two
67 domains are separated by the hinge domain involved in HP1 association with RNA and
68 recruitment to heterochromatin ^{7,8}. Accordingly, HP1 are important for heterochromatin
69 organization and silencing, chromosome segregation, gene expression, DNA repair, DNA
70 replication and for genome stability ^{9-11, 12,13}. Several studies suggested a correlation between
71 the level of HP1 expression and cancer development and/or metastasis. However, how HP1
72 are involved in these processes remains largely to be clarified ^{14,15}.

73 Liver chromatin organization has been well characterized in several physio-
74 pathological conditions ¹⁶. In addition, several known HP1 partners, including the
75 transcription cofactors TRIM24 and TRIM28, and the histone-lysine N-methyltransferase

76 SUV39H1 have been shown to play key roles in hepatocytes¹⁷⁻²¹. Together, this prompted
77 us to further characterize HP1 functions in liver through inactivation of all HP1 encoding
78 genes specifically in mouse hepatocytes. Here, we demonstrated that HP1 are dispensable
79 for hepatocytes survival but essential to prevent tumor development. We further identified
80 alterations of heterochromatin organization, of gene expression and of silencing of specific
81 ERVs through deregulation of the corepressor TRIM28 activity, all these features being most
82 likely key players in the process of tumorigenesis

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84

85 RESULTS

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87 **HP1 proteins are dispensable for hepatocyte survival but essential to prevent liver** 88 **tumor development**

89 To investigate HP1 *in vivo* functions, the HP1 β and HP1 γ encoding-genes (*cbx1* and *cbx3*,
90 respectively) were inactivated individually or together in the liver of either WT or HP1 α KO
91 mice²² using the Cre recombinase expressed under the control of the hepatocyte-specific
92 albumin promoter^{23,24}. This led to different liver-specific combinations of HP1 knockout (KO)
93 models as indicated in Figure 1A. Liver-specific excision of the *cbx1* and *cbx3* alleles was
94 confirmed by PCR and the level of HP1 proteins was checked by western blotting and
95 immunofluorescence (IF) as illustrated for animals having all HP1-encoding genes
96 inactivated in hepatocytes (thereafter called HP1-TKO) (Fig. 1B and Fig. S1A-B). Note that
97 because of the germ cell excision of *cbx5*, HP1 α was not expressed in any cells of mice
98 models carrying the *cbx5*^{-/-} alleles (Fig. 1B).

99 HP1-mutant mice were morphologically similar to their control counterparts throughout
100 their all life. However, autopsies of old animals revealed that all HP1-mutant animals
101 developed liver tumors with a higher incidence than control animals and with the loss of
102 HP1 β having the strongest effect as compared to the loss of HP1 α or HP1 γ (Figure 1C-D).
103 Analysis of the floxed *cbx1* and *cbx3* gene excision confirmed that tumors originated from
104 HP1-mutant hepatocytes (Fig. S1E). Since the loss of the HP1 isoforms display additive
105 effect on tumor incidence, we focus most of the following analysis on HP1-TKO livers.

106 Histological analysis of liver sections from young and middle-aged HP1-TKO animals
107 did not reveal any significant alteration of the structural organization of hepatocytes nor of the
108 liver parenchyma (Fig. S1C), whereas most old HP1-TKO animals developed tumor nodules
109 that could easily be distinguished from the rest of the liver parenchyma (Fig. 1E). These
110 nodules were characterized by the presence of well-differentiated hepatocytes but without

111 their specific trabecular organization, and thus, were identified as typical hepatocellular
112 carcinoma (HCC). Analysis of proliferation (Ki67), apoptosis (Activated caspase 3) and the
113 presence of DNA breaks (γ H2AX²⁵) by immuno-histochemistry (IHC) of Tissue Micro Arrays
114 (TMA) containing liver sections did not reveal any significant difference of proliferation
115 between young and middle-aged mutant and control animals (Fig. S1D) but a two-fold
116 increase in both the tumoral (TKOT) and non-tumoral (TKON) parts of HP1-TKO livers
117 compared with controls. No change was detected in the number of apoptotic- nor γ H2AX-
118 positive cells at any age.(Fig. S1D-E).

119 We then tested by RT-qPCR the expression of several genes frequently altered in human
120 HCC ²⁶. *Arid1A*, *Trp53*, *E2f1* and *E2f7* were significantly over-expressed in HP1-TKO (Fig.
121 1F). Moreover, α -fetoprotein (*Afp*), a marker of human HCC ²⁷, was strongly over-expressed
122 exclusively in the tumor tissue of three of the five tested tumors (Fig. 1F). Altogether, these
123 data demonstrated that HP1 proteins are dispensable within hepatocytes but they are
124 additionally involved to prevent tumor development, with HP1 β being pivotal in this function.

125 **Loss of HP1 increases intrinsic transformation potential of BMEL cells**

126 To unambiguously test the viability of hepatic cells in absence of any HP1 isoform, bipotential
127 hepatic BMEL (Bipotential Mouse Embryonic Liver) cell lines were established according to
128 the protocol described by Strick-Marchand & Weiss ²⁸. All HP1 encoding genes were
129 inactivated in these cells as illustrated in Figure 2A. These cells, thereafter called HP1-TKO
130 cells, were morphologically similar but had a tendency to proliferate faster than control cells
131 (Fig. 2B). To determine whether the loss of HP1 intrinsically induced tumorigenic properties
132 in BMEL cells, we first tested the ability of single cells to form colonies in presence or
133 absence of HP1. As illustrated in Figure 2C, HP1-TKO cells formed more colonies than
134 control cells. However, none of these cells were able to grow on soft agar (data not shown).
135 Because oncogenic stress is a well-known factor of tumorigenesis and since that several
136 genes with putative oncogenic activities were over-expressed in HP1-TKO livers (*BMyc*,
137 *Ect2*, *Mas1*, *Mycl*, *Rab27a*, *Rabl2* and *Src*), we tested the response of control and HP1-TKO

138 cells to the expression of the oncogenes H-RasV12 and SV40 either alone or in combination.
139 H-RasV12 alone or with SV40 lead to massive cell death as well as to senescence as
140 measured by the expression of β -galactosidase, in control but not HP1-TKO cells (Fig. 2D
141 and Fig. S2A-B). Conspicuously, both control and HP1-TKO BMEL cells expressing H-
142 RasV12 and SV40 but not H-RasV12 alone were able to form colonies in soft agar however,
143 HP1-TKO cells formed more colonies than control cells (Fig. 2E and data not shown).
144 Altogether, these data indicated that the loss of HP1 *per se* did not lead to cell transformation
145 but increased the potential of BMEL cells to get transformed upon expression of oncogenes.

146 Since we identified HP1 β as essential for preventing liver tumor development (Fig.1C-
147 D), we wondered whether its expression was sufficient to restore the phenotype induced by
148 HP1 inactivation. We thus established BMEL HP1-TKO lines expressing HP1 β in fusion with
149 the fluorophore YFP and found that it was indeed sufficient to decrease the ability of HP1-
150 TKO cells to form colonies in soft agar in response H-RasV12 and SV40 expression (Fig. 3B,
151 lanes 6-7 and Fig. S2C).

152 **Heterochromatin organization is altered in HP1-TKO hepatocytes**

153 We next initiated molecular characterization of both HP1-TKO pre-malignant livers and
154 HP1-TKO BMEL cells. As shown in Figure 3A, H3K9me3 and H4K20me3, two marks of
155 constitutive heterochromatin, were strongly decreased in the liver of young and middle-aged
156 HP1-TKO mice compared with age-matched controls. Conversely, no change of H3K27me3
157 nor of H3K9me2, H4K20me2 and H4K20me1 was observed in these same samples (Fig.
158 3A). The specific decrease of H3K9me3 and H4K20me3 was also observed in HP1-TKO
159 BMEL cells (Fig. 3B lanes 1 to 5) and importantly, it was restored by expression of HP1 β -
160 YFP (Fig. 3B lanes 6-7). Further, IF analysis in BMEL cells, indicated that only H3K9me3
161 associated with chromocenters (i.e., DAPI-dense structures that contain structural
162 components of heterochromatin) was drastically reduced in HP1-TKO cells, whereas the
163 labeling within euchromatin was not significantly affected (Fig. 3C). The level and distribution
164 of 5-methyl cytosine (5mC) were not altered in HP1-TKO BMEL cells (Fig. 3C). Although

165 HP1-TKO chromocenters clustered, quantification of the intensity of DAPI staining indicated
166 that it was roughly homogeneously distributed throughout control nuclei, whereas it was
167 increased progressively from the inner to the external part in HP1-TKO nuclei (Fig. 3D). Yet,
168 this was not associated with any significant change in the level nor distribution of laminB1
169 (Lamb1) (Fig. 3C).

170 Finally, we tested whether the decrease of pericentromeric H3K9me3 was associated
171 with a decrease of this mark on Major Satellite repeats, the main component of
172 pericentromeric heterochromatin. To this end, we performed H3K9me3 and H3K27me3
173 chromatin immunoprecipitation (ChIP) in BMEL cells and found that H3K9me3, but not
174 H3K27me3, was completely lost on Major Satellites (Fig. 3E). Yet, we did not observe any
175 over-expression of these repeats which even had a tendency to be down-regulated in
176 absence of HP1 in both liver and BMEL cells (Fig. 3F). Similarly, there was no change in the
177 number of these repeats within the liver genome (Fig. 3G). These data demonstrated that
178 HP1, notably HP1 β , are essential in hepatocytes for the maintenance of constitutive
179 heterochromatin histone marks and for the sub-nuclear organization of chromocenters, but
180 not for the expression or the stability of major satellites.

181 **HP1 proteins are involved in the regulation of specific gene expression programs**

182 To investigate the role of HP1 in the regulation of gene expression in liver, we
183 performed an unbiased RNA-seq transcriptomic analysis of libraries prepared from 7 week-
184 old control and HP1-TKO liver RNA. We found that 1215 genes were differentially expressed
185 (730 up-regulated and 485 down-regulated) between control and HP1-TKO livers (with a 1.5-
186 fold threshold difference and an adjusted $P \leq 0.05$) (Fig. 4A and supplementary Table 1).
187 Analysis of differentially expressed genes (thereafter called HP1-dependent genes) using
188 David Gene Ontology (<https://david.ncifcrf.gov/>) and Gene Set Enrichment Analysis (GSEA;
189 <http://software.broadinstitute.org/gsea/index.jsp>) softwares revealed that several biological
190 processes were significantly affected in HP1-TKO livers. First, there was a very high

191 enrichment of genes encoding for the Krüppel Associated Box (KRAB) domain within up-
192 regulated genes ($P = 5.8E-26$) (Fig. 4B & Supplementary Tables 1; 2 & 3) which was-
193 validated by RT-qPCR (Fig. 4C). Up-regulated genes were also enriched in genes belonging
194 to the GO terms signal peptide, immunity, guanylate-binding protein and response to virus
195 (Fig. 4B), suggesting activation of an inflammatory response in HP1-TKO livers (Fig. 4B-C &
196 Supplementary Table 4). Genes encoding for members of the p450 cytochrome (CYP) family
197 were also strongly enriched in HP1-dependent genes with 7 up-regulated and 18 down-
198 regulated amongst the 79 CYP genes detected in the present RNAseq analysis. In particular,
199 11 *cyp2* family genes were involved in Endoplasmic Reticulum (ER) and redox functions that
200 are known to be essential for liver homeostasis ^{29,30,31} (Table 1). Moreover, *Nox4*
201 (nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4), a gene consistently
202 associated with ER and ROS in liver ³², was significantly down-regulated in HP1-TKO as
203 compared with control livers (Fig. 4C & Supplementary Table 1). In line with these results,
204 oxidation-reduction, ER, steroid hormone biosynthesis, lipid metabolic process were amongst
205 the most affected functions in HP1-TKO livers (Fig. 4B & Supplementary Tables 2; 3; 5 and
206 6). The differential expression of *Cyp2c29* and *Cyp2b10* (ER and redox), *Ifit2* (interferon γ
207 signature) and *Nox4* (ROS production) was validated by RT-qPCR in 7 week-old HP1-TKO
208 and control livers (Fig. 4C).

209 As mentioned above, the simultaneous loss of HP1 α and HP1 β also led to high
210 incidence of liver tumor development (Fig. 1C-D). To identify genes commonly deregulated in
211 HP1-TKO and HP1 $\alpha\beta$ -liverKO livers that might explain the tumor protective role of HP1, we
212 performed a RNAseq analysis in HP1 $\alpha\beta$ -liverKO (GSE84734). Only 18 genes (13- up and 5
213 down-regulated) were similarly deregulated in HP1 $\alpha\beta$ -liverKO and HP1-TKO livers
214 (supplementary table 7). Interestingly, on the 13 up-regulated genes, 5 belonged to the
215 family of transcriptional repressors KRAB-Zinc Finger Proteins (KRAB-ZFP). We thus
216 analyzed the expression of several of these *krabzfp* genes by RT-qPCR in HP1 $\alpha\beta$ -liverKO,
217 HP1 $\alpha\gamma$ -liverKO and control livers. As illustrated in Figure 4D, *zfp951* and *zfp992* were up-

218 regulated exclusively in HP1 $\alpha\beta$ -liverKO livers, *zfp345*, exclusively in HP1 $\alpha\gamma$ -liverKO livers
219 whereas *zfp984* and *5730507C01Rik* were up-regulated in both HP1 $\alpha\beta$ -liverKO and HP1 $\alpha\gamma$ -
220 liverKO livers. These results demonstrated specific and redundant functions for the different
221 HP1 isoforms and that HP1 β has specific functions for the regulation of *zfp951* and *zfp992*
222 genes that could thus be determinant in the HP1-dependent tumorigenesis process.

223 **HP1 loss lead to reactivation of specific retrotransposons and over-expression of** 224 **neighboring genes**

225 KRAB-ZFP are transcriptional repressors known to repress themselves and
226 retrotransposons of the endogenous retroviruses (ERV) family^{33,34}. To determine whether
227 the HP1-dependent deregulation of KRAB-ZFP was associated with altered ERV expression
228 in liver, we investigated the expression of DNA repeats in our RNA-seq dataset. Coordinates
229 of all annotated DNA repeats of the RepeatMasker database (mm10 assembly) were aligned
230 against the RNA-seq reads and only those that could be assigned unambiguously to a
231 specific genomic locus were analyzed. In total, 846 repeats were deregulated in HP1-TKO
232 compared with control livers with 71.3% being up-regulated and 28.7% down-regulated (Fig.
233 5A & Supplementary Table 8). Among up-regulated repeats, 59.4% were ERV, 19.2% long
234 interspersed nuclear elements (LINEs) and 9.3% short interspersed elements (SINEs)
235 supporting the hypothesis that HP1 were preferentially involved in ERV silencing in liver (Fig.
236 5B & Supplementary Table 8).

237 To assess the putative link between ERV and gene expression, we first mapped HP1-
238 dependent repeats located within 100kb surrounding HP1-dependent genes. This analysis
239 showed that a fraction of HP1-dependent genes (138 up-regulated and 94 down-regulated)
240 was associated with HP1-dependent repeats. Interestingly, this physical association
241 correlated with a functional association since 84% of repeats associated with up-regulated
242 genes were also up-regulated and 75.5% of repeats associated with down-regulated genes
243 were down-regulated (Fig. 5C & Supplementary Tables 9 & 10). Furthermore, up-regulated

244 repeats tended to be located closer to up-regulated genes than to down-regulated genes,
245 and vice versa (Fig. 5D). Altogether, this analysis strongly suggested a link between loss of
246 HP1, loss of KRAB-ZFP repressive activity, ERV reactivation and up-regulation of genes in
247 their neighborhood. Accordingly, several deregulated genes associated with deregulated
248 repeats such as *Mbd1*, *Bglap3*, *Obpa*, *Bmyc*, *Fbxw19* and *Zfp445* were already shown to be
249 controlled by ERVs (Fig. 5E and ^{35,36}).

250 **HP1 loss has gene-specific impact on H3K9me3 deposition**

251 KRAB-ZFPs regulate their targets through recruitment of the corepressor TRIM28 and
252 of the Histone Methyl Transferase (HMT) SETDB1 for the establishment of a
253 heterochromatin-like environment characterized by H3K9me3 enrichment ³⁷. To first test
254 whether the KRAB-ZFP/ERV pathway was also deregulated in HP1-TKO BMEL, we
255 measured the expression of *Zfp345*, *Zfp951*, *zfp992*, *Bglap3* and *Cyp2b10* in control and
256 HP1-TKO BMEL cells. As in HP1-TKO livers, all these genes were over-expressed in HP1-
257 TKO versus control BMEL cells (Fig. 5F). Control level of expression was rescued by HP1 β -
258 YFP for *Zfp951*, *zfp992*, *Bglap3* and *Cyp2b10* but not for *Zfp345* in agreement with our
259 observation that this latter gene was deregulated in HP1 α -liverKO but not HP1 α β -liverKO
260 livers (Fig. 4D and Fig. 5G). Several *krabzfp* genes were characterized by an enrichment of
261 H3K9me3 preferentially at their 3'UTR ³⁴. We therefore tested the enrichment of this mark at
262 the 3'UTR of *zfp345*, *zfp951* and *zfp992* as well as at three previously described positions of
263 *Bglap3* gene associated with a IAP ³⁵. In control BMEL cells, H3K9me3 was highly enriched
264 at the 3' UTR of all *krabzfp* genes, on the three positions associated with *Bglap3* as well as in
265 the promoter region of *Cyp2b10* as compared to the housekeeping gene *36B4* (Fig. 5H). In
266 HP1-TKO BMEL cells, H3K9me3 was significantly decreased at *zfp345* and *zfp992* genes.
267 However, in striking contrast it was unchanged at *zfp951*, at the P2 and IAP positions of the
268 *Bglap3* locus as well as at *Cyp2b10* and was even increased at the *Bglap3* p4 position (Fig.
269 5H). These results indicated that HP1-dependent loci are characterized by enrichment of

270 H3K9me3 which does not require HP1 for its maintenance and which is not sufficient to
271 repress the expression of the associated genes in absence of HP1.

272 **HP1 is necessary for TRIM28 activity within liver**

273 To better characterize the relationship between HP1, KRAB-ZFPs, TRIM28 and ERVs,
274 we first checked the expression of TRIM28 in HP1-TKO livers. This analysis showed that
275 TRIM28 expression was not significantly altered in HP1-TKO (Fig. 6A-B). We then used the
276 previously described mouse models in which either a mutated TRIM28 protein that cannot
277 interact with HP1 (T28HP1box) replaces TRIM28 or in which TRIM28 is depleted (T28KO)
278 specifically within liver^{17,38}. As expected, TRIM28 expression was strongly decreased in
279 T28KO livers, whereas it was only marginally decreased in T28HP1box livers (Fig. 6C). The
280 level of all three HP1 was not affected in these mouse strains (Fig. 6C). RT-qPCR analysis
281 showed that several HP1-dependent genes including *Nox4*; *Cypc29* and *Rsl1* were not
282 affected in T28HP1box and T28KO livers (Fig. 6D). Conversely, *Cyp2b10*; *Ifit2*; *Zfp345* and
283 *Zfp445* that were all over-expressed in HP1-TKO liver were also up-regulated in T28HP1box
284 and T28KO livers (Fig. 6D). To test whether the HP1-dependent ERV-associated genes
285 required TRIM28, the expression of *Mbd1* and *Bglap3* was assessed in T28KO and
286 T28HP1box livers. Like in HP1-TKO livers, both genes were over-expressed in T28KO and
287 T28HP1box livers, although to a lesser extent as compared to HP1-TKO livers (Fig. 6E).
288 Finally, analysis in old animals (Fig. 6G-H) showed that both genes were over-expressed in
289 the normal (TKON) and tumor (TKOT) liver parts from old HP1-TKO animals whereas *Mbd1*
290 was no longer over-expressed in old TRIM28 mutant mice livers. *Bglap3*, was slightly over-
291 expressed in T28KO but not in T28HP1box old animals (Fig. 6G-H).

292 As for HP1-TKO mice, old T28KO and T28HP1box mice developed more liver tumors
293 than controls, although with a lower incidence than HP1-TKO animals (32.4% and 33.5% for
294 T28KO and T28HP1box respectively, Fig. 6F). Altogether, these data suggested that HP1
295 prevented liver tumor development at least partially by sustaining TRIM28 repressive activity.

296

297 **DISCUSSION**

298 In this study, we demonstrated that HP1 proteins and in particular HP1 β are essential
299 within hepatocytes to prevent development of hepatocellular carcinoma (HCC) in mice. We
300 further showed that HP1 are pivotal within hepatocytes for appropriate expression of liver
301 specific genetic programs and for organization of pericentromeric heterochromatin. Last we
302 present several lines of evidence that HP1 are acting as barrier against liver tumor
303 development at least partially by keeping specific sets of retrotransposons silent through
304 regulation of TRIM28 corepressor activity.

305 The finding that HP1 proteins were not essential for neither cell viability nor liver
306 function was in contrast with many studies showing the fundamental functions of each HP1
307 isoform in various pluripotent and differentiated cellular systems ^{39,40} as well as during
308 embryonic development in various species, such as *Drosophila* ⁴¹, *C. elegans* ⁴². However, it
309 is important to note that liver has very specific properties, being mostly quiescent throughout
310 life but able to regenerate upon stress essentially through the re-entry of quiescent and fully
311 differentiated hepatocytes into cell cycle rather than via stem cell proliferation, as it is the
312 case in other tissues ^{43,44}. These specific properties of hepatocytes could rely on a peculiar
313 loose chromatin organization that might be less sensitive to the loss of HP1 as compared to
314 other cell types for cell viability but more prone to cell transformation as we observed in
315 BMEL cells. In support of this hypothesis, it is noteworthy that, among the 17 human cancers
316 tested in the database from Human Protein Atlas (<https://www.proteinatlas.org> and Fig. S3),
317 liver cancer is the one expressing the lowest levels of *cbx1*, 3 and 5. These data strongly
318 suggest that in Human, as in mice, low level of HP1 expression is determinant for the
319 initiation of liver tumorigenesis. Strikingly however, high levels of *cbx1*, 3 and 5 expression
320 are all unfavorable prognostic markers in liver cancer (<https://www.proteinatlas.org> and Fig.
321 S3), suggesting that the levels of HP1 have to be tightly regulated in liver and that HP1 could
322 have different functions at different stages of cancer development as it has been proposed
323 by Lee & Ann ⁴⁵ for breast cancer. Interestingly, we showed here, that the increased cellular

324 sensitivity to transformation induced by the loss of HP1 is reversible and can be rescued by
325 the sole re-expression of HP1 β suggesting that regulating the level of HP1 could be a
326 therapeutic strategy for liver cancer.

327 We further demonstrated that HP1 are essential within hepatocytes for the
328 maintenance of H3K9me3 and H4K20me3 at pericentromeric heterochromatin and for
329 appropriate localization of DAPI-dense structures. These results are in line with the
330 demonstration that in worms, inactivation of the two H3K9 methyltransferases Met-2 (me1
331 and me2) and SET-25 (me3) is necessary to trigger heterochromatin release from the
332 nuclear periphery whereas inactivation of either one or the other is insufficient⁴⁶. However
333 these results are in contrast with studies suggesting that HP1 is required to trigger
334 attachment of heterochromatin to the nuclear periphery⁴⁷. Furthermore, in contrast to the
335 results reported upon loss of H3K9me3 induced by inactivation of the histone
336 methyltransferases SUV39H1 and SUV39H2, the loss of H3K9me3 on major satellite repeats
337 in HP1-TKO hepatocytes did not result in neither decrease of H3K9me2 nor in over-
338 expression of these major satellite repeats^{48,49}. This observation suggests that in liver,
339 H3K9me2 maintenance is independent of HP1 and is sufficient to keep major satellite
340 repeats at a low level of transcription even in absence of HP1. SUV39H1 over-expression
341 has been reported to be associated with HCC development¹⁸ and HCC induced by a methyl-
342 free diet is also characterized by elevated SUV39H1 expression and increased H3K9me3 but
343 with reduced H4K20me3 deposition⁵⁰. This suggests that the global decreased level of
344 H4K20me3 rather than of H3K9me3 in HP1-TKO mice could be a key determinant of
345 tumorigenesis. In support of this hypothesis, H4K20me3 was reported to be essential for
346 genome integrity and for proper timing of heterochromatin replication whose deregulation has
347 recently been proposed to be involved in cancers⁵¹⁻⁵³. Our results highlight complex
348 interplay between heterochromatin components for the organization of this compartment that
349 is likely essential to prevent tumor development. As reported by others, we found that HP1
350 are involved in both repression and activation of gene expression^{7,54-56}. Interestingly,

351 although HP1-dependent genes are characterized by enrichment in H3K9me3, this mark
352 remains mostly unchanged upon HP1 inactivation but is not sufficient to maintain gene
353 repression. These results are reminiscent to those obtained in yeast showing that H3K9me3
354 can be maintained in absence of HP1 through association with a RNA-induced transcriptional
355 silencing complex⁵⁷.

356 Functionally, many HP1-dependent genes are involved in liver specific functions. In
357 particular, several belong to the p450 cytochrome family (Cyp) which is involved in liver
358 detoxification, in oxidative stress and homeostasis of the endoplasmic reticulum that are
359 three key factors in hepatocarcinogenesis⁵⁸. How these genes are regulated by HP1
360 remains to be determined. However nuclear receptors of the Peroxisome Proliferation-
361 Activated Receptors (PPAR) were shown to be important in this process and PPAR γ is
362 strongly down-regulated in HP1 mutant mice⁵⁹. It is thus tempting to speculate that this low
363 expression of PPAR γ underlies the deregulation of several *Cyp* genes. Furthermore, HP1-
364 TKO livers were also characterized by a transcriptional signature of an interferon γ response
365 strongly suggesting liver inflammation, a factor associated with 90% of hepatocarcinogenesis
366^{60 61 62}. Over-expression of retrotransposons *per se* was shown in some circumstances to
367 lead to inflammation through activation of the cGAS-STING pathway⁶³. As we have
368 demonstrated that the loss of HP1 reactivates specific retrotransposons, we can hypothesize
369 that they cause inflammation and thus a favorable environment for HCC development.

370 Finally, we demonstrated that the loss of HP1 lead to partial loss of TRIM28 activity
371 characterized by reactivation of a specific set of retrotransposons associated with over-
372 expression of genes in their neighborhood. Moreover, we showed that the loss of interaction
373 between TRIM28 and HP1 within hepatocytes is sufficient to partially recapitulate the
374 phenotypes induced by HP1 or TRIM28 inactivation. Consistent with our results, SETDB1,
375 the main H3H9 methyltransferase associated with TRIM28 and KRAB-ZFP for ERV silencing
376^{64,65} was identified as a human hepatic cancer driver gene (IntOgen,
377 <http://www.intogen.org/mutations/>). Together, these results strongly suggest that

378 deregulation of the KRAB-ZFP/TRIM28/SETDB1 pathway is determinant in HP1-dependent
379 tumorigenesis^{33,66,67}.

380 In conclusion, we identified HP1 proteins as key players to prevent liver tumorigenesis.
381 We further present evidence that this function of HP1 as guardian of liver homeostasis relies
382 on regulation of heterochromatin organization, gene expression and ERV silencing.

383

384 MATERIALS AND METHODS

385

386 Mouse models.

387 The Cbx5KO, T28KO (TRIM28KO) and T28HP1box (TRIM28-L2/HP1box) mouse strains
388 were described previously^{22,38,68}. Exons 2 to 4 within the *Cbx1* gene (HP1 β), and exon 3
389 within the *Cbx3* gene (HP1 γ) were surrounded by LoxP sites. Excision of the floxed exons
390 exclusively in hepatocytes by using mice that express the Cre recombinase under the control
391 of the albumin promoter (Alb-Cre mice,²³) led to a frameshift within the CSD-encoding
392 sequence of *Cbx1* and the CD-encoding sequence of *Cbx3*.

393 Mice were housed in a pathogen-free barrier facility, and experiments were approved by the
394 national ethics committee for animal warfare (n $^{\circ}$ CEEA-36).

395

396 Antibodies/oligonucleotides

397 The anti-TRIM28 and HP1 were previously described^{64, 65}. Anti-Casp3A (9661, Cell
398 Signaling); anti- γ H2AX (Ab11174, Abcam), anti-Ki67 (M3064, Spring Bioscience). Anti-5mC
399 (NA81, Calbiochem), anti-H3K9me3 (Active Motif, 39161), anti-H3-K27me3 (Milipore, 07-
400 449), H4K20me3 (Upstate, 07-463). Oligonucleotides are described in Supplementary Table
401 11.

402

403 Tissue processing for histology.

404 For fresh frozen tissues, 3mm sections of the liver large lobe were embedded in the OCT
405 compound (TissueTek) following standard protocols, and 18 μ m-thick sections were cut using
406 a Leica CM1850 cryostat and stored at -80°C .

407 For paraffin-embedded tissues, 3mm sections of the liver large lobe were fixed in 4% neutral-
408 buffered formalin (VWR Chemicals) at room temperature (RT) overnight, and stored in 70%
409 ethanol at 4°C . Fixed tissues were processed using standard protocols and embedded in
410 paraffin wax. Three- μ m-thick sections were cut using a Thermo Scientific Microm HM325
411 microtome, dried at 37°C overnight and stored at 4°C .

412

413 **Immunofluorescence analysis.**

414 Cryo-sections and cultured cells were fixed in formaldehyde (2%) at RT for 15min air dried at
415 RT for 20min and processed as described previously³⁸.

416

417 **Immunohistochemistry.**

418 Paraffin-embedded liver sections were processed for routine hematoxylin, eosin and Safran
419 or reticulin staining. For immunohistochemistry, sections were processed according to
420 standard protocols. Images were acquired with a Zeiss Apotome2 microscope and
421 processed using ImageJ.

422

423 **RNA extraction and RT-qPCR assays**

424 RNA was isolated from liver samples using TRIzol, according to the manufacturer's
425 recommendations (Life technologies). Reverse transcription was performed with Superscript
426 III according to the manufacturer protocol (Invitrogen). 1/100 of this reaction was used for
427 real-time qPCR amplification using SYBR Green I (SYBR Green SuperMix, Quanta).

428

429 **RNA-seq**

430 The details are described in supplementary methods. Data are available at GEO (accession
431 numbers: GSE84734 and GSE119244).

432

433 **ChIP**

434 ChIP were performed according to Abcam's protocol.

435

436 **Statistics and reproducibility.**

437 The Microsoft Excel or biostatgv (<https://biostatgv.sentiweb.fr>) softwares were used for
438 statistical analyses; statistical tests, number of independent experiments, and P-values are
439 listed in the individual figure legends. All experiments were repeated at least twice unless
440 otherwise stated.

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442

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463

464

465 **AUTHORS' CONTRIBUTIONS:**

466 NS and SH performed the analysis of mice and interpreted the data. MP and CB made the
467 libraries, generated and analyzed the RNA-seq data. AZ and EF performed the RT-qPCR
468 experiments. CG participated to mice analysis, NP supervised the histological core facility
469 and JYN performed the TMA. LK performed the pathological analysis of histological sections.
470 DG was involved in the establishment of BMEL cells. YP and EJ were involved in cell
471 transformation assays and chromatin analysis. FC designed, analyzed and interpreted the
472 data and wrote the manuscript with input from all co-authors.

473

474 **DECLARATION OF INTEREST:** No competing interests

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- 663
- 664

665 **LEGENDS FIGURES**

666 **Figure 1: HP1 proteins are essential to prevent tumour development in liver.** (A)
667 Schematic representation of the strategy to inactivate the two HP1-encoding genes (*Cbx1*, 3)
668 specifically in hepatocytes using the recombinase Cre expressed under the control of the
669 albumin promoter in WT or HP1 α KO mice. (B) Western blot analysis of whole-cell extracts
670 from liver samples confirmed the absence of HP1 α (the residual band is due to the very high
671 level of immunoglobulins in liver and thus the presence of light chains that are of the same
672 size as HP1 α) and the decreased expression of HP1 β and HP1 γ due to the hepatocytes-
673 specific excision of the corresponding genes in HP1-TKO as compared to age-matched
674 control mice. Ponceau staining was used as loading control. (C) Graph showing the
675 percentage of animals developing tumors (morphological and histological analysis). Controls
676 (Ctl; n=109; 6.4%), HP1 α KO (HP1a KO; n= 73; 8.5%), HP1 β -liverKO (HP1b KO; n=17;
677 52.9%), HP1 γ -liverKO (HP1g KO; n=37; 13.5%), HP1 α /HP1 β -liverKO (HP1ab KO; n=8;
678 50%), HP1 α /HP1 γ -liverKO (HP1ag; KO n=12; 25%) and HP1 α /HP1 β /HP1 γ -liverKO (HP1-
679 TKO, n=19; 78.9%). The exact Fisher statistical test was used for each genotype versus
680 control, **p value ≤ 0.01 ; ***p value ≤ 0.001 (D) Examples of morphology of livers with tumors
681 (arrows) in animals for each HP1 knockout combination. The liver morphology of a female
682 and a male age-matched controls are also shown (F Ctl and M Ctl, respectively). (E)
683 Histological analysis (hematoxylin-eosin-Safran staining) of one representative HP1-TKO
684 female (F-TKO) and one representative HP1-TKO male (M-TKO) livers. Upper panels:
685 tumor/liver parenchyma interface highlighted by arrowheads (low magnifications). Bottom
686 panels: magnification (x 100) of the boxes in the upper panels showing the tumor in the right
687 part of the images (thick plates of atypical hepatocytes). A venous tumor thrombus is also
688 present (asterisk).(F) RT-qPCR analysis of the expression of the indicated genes in control
689 (Ctl, n=5) and HP1-TKO (TKON: normal liver, n=6; TKOT: tumor, n=5) livers of animals older
690 than one year. RT-qPCR data were normalized to *Hprt* expression and are shown as the
691 mean \pm SEM. *p value < 0.05 ; ***p value < 0.001 , ns: not significant (Student's t-test).

692 **Figure 2 : HP1-TKO hepatic cells are viable and have increased potential to cellular**
693 **transformation upon oncogenic stress.** (A) Schematic representation of the strategy used
694 to establish BMEL cells from *Cbx5*^{-/-}; *Cbx1L2/L2*; *Cbx3L2/L2* fetal livers and to inactivate the
695 three HP1-encoding genes. Western blot analysis of whole-cell extracts from Het, Ctl and
696 HP1-TKO BMEL cells. "Het" were *Cbx5*^{+/-}; *Cbx1L2/L2* BMEL cells, "HP1-TKO" and "Ctl"
697 were established clones originating from one *Cbx5*^{-/-}; *Cbx1L2/L2*; *Cbx3L2/L2*; Cre-ERT
698 clone treated (KO1 and KO3) or not (C3 and C5) with tamoxifen respectively. HP1-TKO cells
699 were stably transfected with a plasmid (pCX, chicken β -actin promoter) allowing the
700 expression of YFP either alone (RCtl) or in fusion with HP1 β (RHP1b) (B) Proliferation curves
701 of 2 Control clones (C3 and C5) and 2 HP1-TKO clones (KO1 and KO3). The graph
702 represent the average of three independent experiments done in triplicates. Student t-test
703 analysis showed no significant difference between control and HP1-TKO clones (C) HP1-
704 TKO cells have a higher capacity of forming clones from isolated cells than control cells.
705 Representative pictures of clonogenic assays performed on the two control (C3 and C5) and
706 HP1-TKO (KO1 and KO3) clones with the graph representing the average of 5 independent
707 experiments. (D) HP1-TKO cells are more resistant than control cells to the expression of the
708 oncogene Ras-V12. Representative images of control (C3 and C5) and HP1-TKO (KO1 and
709 KO3) BMEL cells transduced with either an empty (control) or a Ras-V12 (Ras-V12)
710 expressing lentivirus and grown for 8 days after the transduction. (E) HP1-TKO cells are
711 more prone to transformation than control cells in response to Ras-V12 and SV40
712 oncogenes expression. Representative images of control (C3 and C5) and HP1-TKO (KO1
713 and KO3) BMEL cells transduced with either an empty (control) or two Ras-V12 (Ras-V12)
714 and SV40 expressing lentivirus respectively (Ras-V12+SV40) grown for 8 days after the
715 transduction on culture dishes and then grown for 20 days in soft agar. Graph represented
716 the average of three independent experiments. Results are shown as the mean number of
717 colonies \pm SEM. *p value <0.05; (Student's t-test).

718 **Figure 3: HP1 are essential for the maintenance of heterochromatin marks but not to**
719 **regulate the expression of major satellites.** (A) Western blot analysis of nuclear extracts
720 from livers of 7-week-old and middle-aged (3-6-month-old) controls (Ctl: 1; 2; 5; 6) and HP1-
721 TKO (TKO: 3; 4; 7; 8) mice with antibodies against the indicated histone marks. Ponceau
722 staining was used as loading control. (B) Western blot analysis of whole cell extracts of one
723 Het (lane 1), two (lanes 2-3) controls, two (lanes 4-5) HP1-TKO and two (lanes 6-7) HP1-
724 TKO clones expressing HP1 β -YFP (RHP1b) fusion protein with the indicated antibodies. (C)
725 IF analysis of H3K9me3, H3K27me3, 5mC and LamB1 in BMEL cells. (D) Loss of HP1 leads
726 to a partial relocation of DAPI-dense regions towards the nuclear periphery. Representative
727 images of paraffin-embedded liver tissue sections from 7-week-old control (Ctl) and HP1-
728 TKO (TKO) mice stained with DAPI (63x magnification). To select mostly hepatocytes, only
729 the largest nuclei with a size comprised between 70 and 150 μm^2 and with a circular shape
730 were selected for this analysis. 2D sections of nuclei were divided in four concentric areas (1
731 to 4) and DAPI staining intensity was quantified using the cell profiler software. The mean
732 fractional intensity at a given radius was calculated as the fraction of the total intensity
733 normalized to the fraction of pixels at a given radius in n=584 control and n=762 HP1-TKO
734 (TKO) nuclei. Data are the mean \pm SEM. ***p value <0.001. (E) ChIP analysis of H3K9me3
735 and H3K27me3 enrichment on major satellite repeats within control (Ctl, C3 and C5) and
736 HP1-TKO (TKO, KO1 and KO3) BMEL cells. Graphs represent the average of 5 independent
737 experiments and are shown as the mean \pm SEM. ***p value <0.001, ns: not significant
738 (Student's t-test). (F) Loss of the three HP1 proteins did not affect the expression of major
739 satellites in neither liver nor BMEL cells. qPCR assays were performed using total RNA from
740 livers of 7-week-old control (n=4) and HP1-TKO mice (n=4) and on control (Ctl, C3 and C5)
741 and HP1-TKO (TKO, KO1 and KO3) BMEL cells. data were normalized to *Hprt* expression
742 and are shown as the mean \pm SEM. ns: not significant (Student's t-test). (G) Satellite repeats
743 were quantified by qPCR on genomic DNA from the same animals as those used for (F).

744

745 **Figure 4: HP1 are essential regulators of gene expression in liver.** (A) MA plot after
746 DSeq2 normalization of RNA-seq data from 7-week-old control (n=3) and HP1-TKO (n=4)
747 liver RNA samples. Red dots represent genes that are differentially expressed between
748 control and HP1-TKO mice (adjusted p-value $p < 0.05$). (B) Functional clustering of HP1-
749 dependent genes using the DAVID Gene Ontology software. (C) RT-qPCR analysis of the
750 expression of the indicated genes. RNA was extracted from livers of 7-week old control (Ctl)
751 and HP1-TKO (TKO) mice. (D) RT-qPCR analysis of the expression of 4 *krabzfp* genes
752 (*zfp951*, *zfp992*, *zfp984* and *5730507C01Rik* (573Rik)) whose expression was found to be
753 deregulated in both HP1 $\alpha\beta$ -liverKO and HP1-TKO livers by RNA-seq analysis and 1 *krabzfp*
754 gene (*zfp345*) deregulated in both HP1 $\alpha\gamma$ -liverKO and HP1-TKO livers by RNA-seq analysis.
755 cDNA were prepared from livers of 5-weeks old controls (Ctl), HP1 $\alpha\beta$ -liverKO (HP1 $\alpha\beta$ KO)
756 and HP1 $\alpha\gamma$ -liverKO (HP1 $\alpha\gamma$ KO). RT-qPCR data were normalized to *Hprt* expression and are
757 shown as the mean \pm SEM. Statistical analysis were made between expression in controls
758 and expression in the different HP1-mutant samples. *p value < 0.05 ; ***p value < 0.001 , ns:
759 not significant (Student's t-test).

760 **Figure 5: HP1 are required for silencing specific endogenous retroviruses (ERVs) in**
761 **hepatocytes.** (A) MA-plot after DSeq2 normalization of RNA-seq reads including repeats
762 aligned against the Repbase database. Red dots represent genes and repeats that are
763 differentially expressed between controls and HP1-TKO liver samples ($p < 0.05$). (B)
764 Distribution of the different families of retrotransposons amongst repeats that are up-
765 regulated upon loss of HP1 (Repeat_Up) compared to repeats that are down-regulated
766 (Repeat_Down) and to the genome-wide distribution of repeats according to the
767 RepeatMasker database (All). (C) Repeats over-expressed in HP1-TKO liver samples
768 compared with controls (Repeat_Up) are over-represented in regions (\pm 100kb) around
769 genes over-expressed in HP1-TKO (genes_up). Conversely, repeats down-regulated in HP1-
770 TKO liver samples compared with controls (Repeat_Down) are over-represented in regions
771 (\pm 100kb) around genes repressed in HP1-TKO (genes_down). (D) Repeats that are up-

772 regulated or down-regulated upon HP1 loss tend to be closer to genes that are up- or down-
773 regulated in HP1-TKO, respectively. The absolute distance (in base pairs) was measured
774 between the gene transcriptional start site and the beginning of the repeat, according to the
775 RepeatMasker annotation. (E) Representative Integrative Genomic Viewer snapshots of the
776 indicated up-regulated genes associated with up-regulated repeat sequences. (F) RT-qPCR
777 analysis of the expression of the indicated genes in Control (Ctl) and HP1-TKO (TKO) BMEL
778 cells. (G) RT-qPCR analysis of the expression of the indicated genes in Control rescue (RCtl)
779 and HP1 β rescue (RHP1b) BMEL cell lines. RT-qPCR data were normalized to *Hprt* and
780 *36B4* expression and are shown as the mean \pm SEM. **p* value <0.05; ****p* value <0.001, ns:
781 not significant (Student's t-test). (H) ChIP analysis of H3K9me3 enrichment in the 3'UTR
782 regions of *zfp345*, *zfp951* and *zfp992*, in the P2, IAP and p4 regions of the *Bglap3* gene
783 locus³⁵. **p* value <0.05; ***p* value <0.01, ****p* value <0.001, ns: not significant (Student's t-
784 test).

785 **Figure 6: The loss of association between HP1 and TRIM28 partially recapitulates the**
786 **phenotypes induced by the loss of HP1.** (A) TRIM28 expression is independent of HP1
787 proteins. RT-qPCR quantification of TRIM28 expression in total RNA from livers of 7-week-
788 old control (Ctl; n=4) and HP1-TKO (TKO; n=4) mice. Data were normalized to *Hprt*
789 expression and are shown as the mean \pm SEM. (B) Western blot analysis of 50 μ g of whole
790 cell extracts from 7-week-old control (1 and 2) and HP1-TKO (3 to 5) livers using an anti-
791 TRIM28 polyclonal antibody. Tubulin was used as loading control. (C) The loss of interaction
792 between TRIM28 and HP1 does not significantly alter the level of expression of neither
793 TRIM28 or HP1. 50 μ g of whole liver extracts from 7-week-old controls (1; 2), TRIM28KO
794 (T28KO; 3-5) and TRIM29HP1box (T28HP1box; 6-8) mice were analyzed by western blotting
795 using the anti-TRIM28 polyclonal and anti-HP1 α , β and γ monoclonal antibodies. GAPDH
796 and Ponceau staining were used as loading controls. (D) TRIM28 is involved in the
797 regulation of the expression of some but not all HP1-dependent genes. RT-qPCR analysis
798 using liver RNA samples from 5 week-old control (n=5), T28KO (n=5) and T28HP1box (n=5)

799 mice. (E) TRIM28 is involved in the regulated expression of HP1- and ERV-dependent
800 genes. Analysis of *Mbd1* and *Bglap3* expression by RT-qPCR using liver RNA samples from
801 7-week-old control (Ctl) and HP1-TKO (TKO) mice, and 5-week-old control (n=5), T28KO
802 (n=5) and T28HP1box (n=5) mice. (F) TRIM28 and its association with HP1 is essential to
803 prevent liver tumor development. Representative morphological aspect of TRIM28 mutant
804 livers. (G) *Bglap3* and *Mbd1* are over-expressed in HP1-TKO livers of old (>1year) mice. RT-
805 qPCR was performed using RNA from old control (n=7), and HP1-TKO liver samples (TKON
806 for normal part, TKOT for tumor part) (n=7). (H) The alteration of *Mbd1* and *Bglap3*
807 expression upon loss of the association between TRIM28 and HP1 proteins was not
808 maintained in old animals. RT-qPCR analysis using RNA from control (n=5), T28KO
809 (T28KON for normal part, T28KOT for tumor part) (n=5) and T28HP1box (T28HP1boxN for
810 normal part, T28HP1boxT for tumor part) livers (n=5). All expression data were normalized to
811 *Hprt* expression and are shown as the mean \pm SEM. ns, no significant difference **p* value
812 <0.05; ***p* value <0.01; ****p* value <0.001 (Student's t-test).

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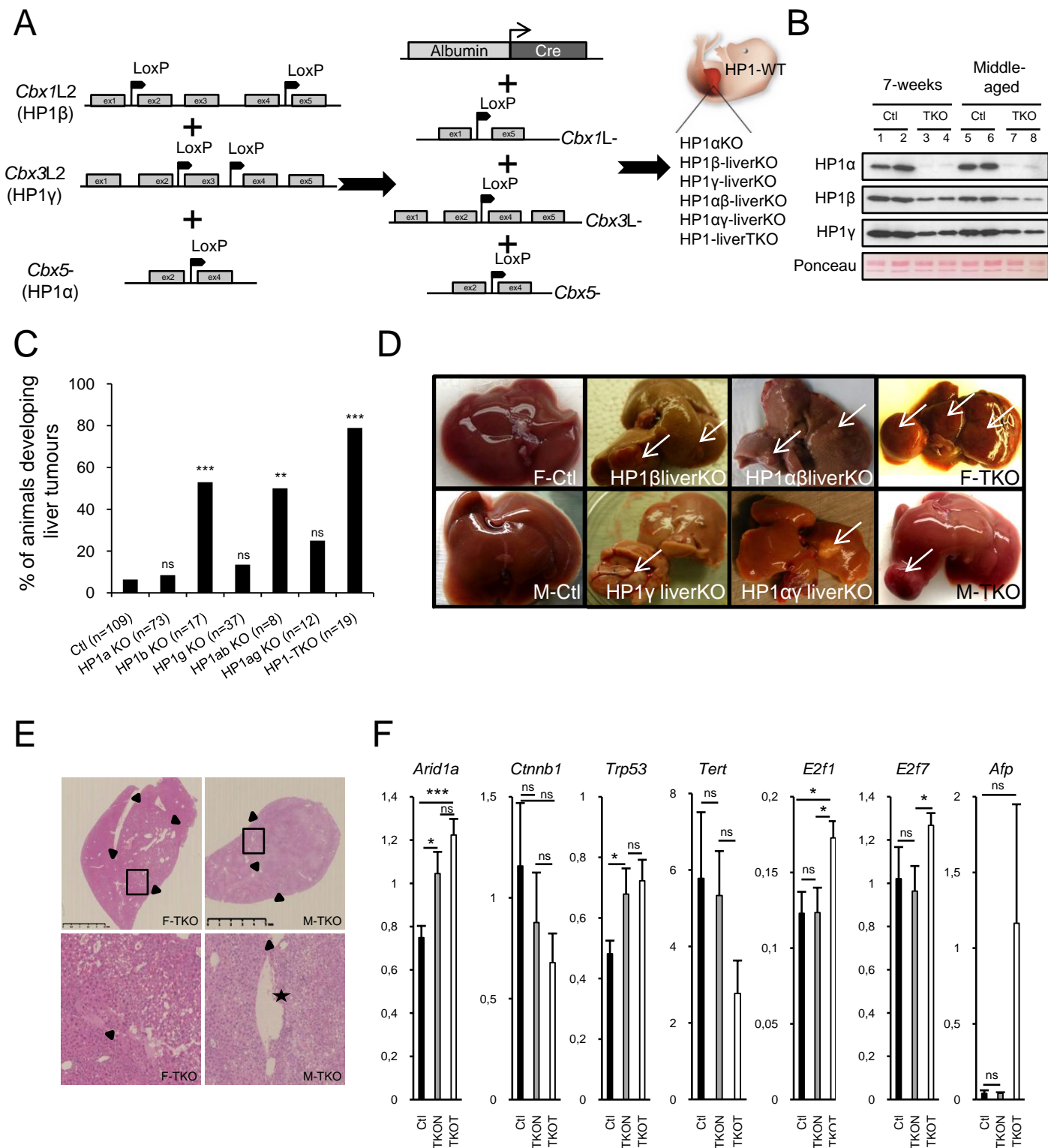


Figure 1, Saksouk & Hajdari et al,

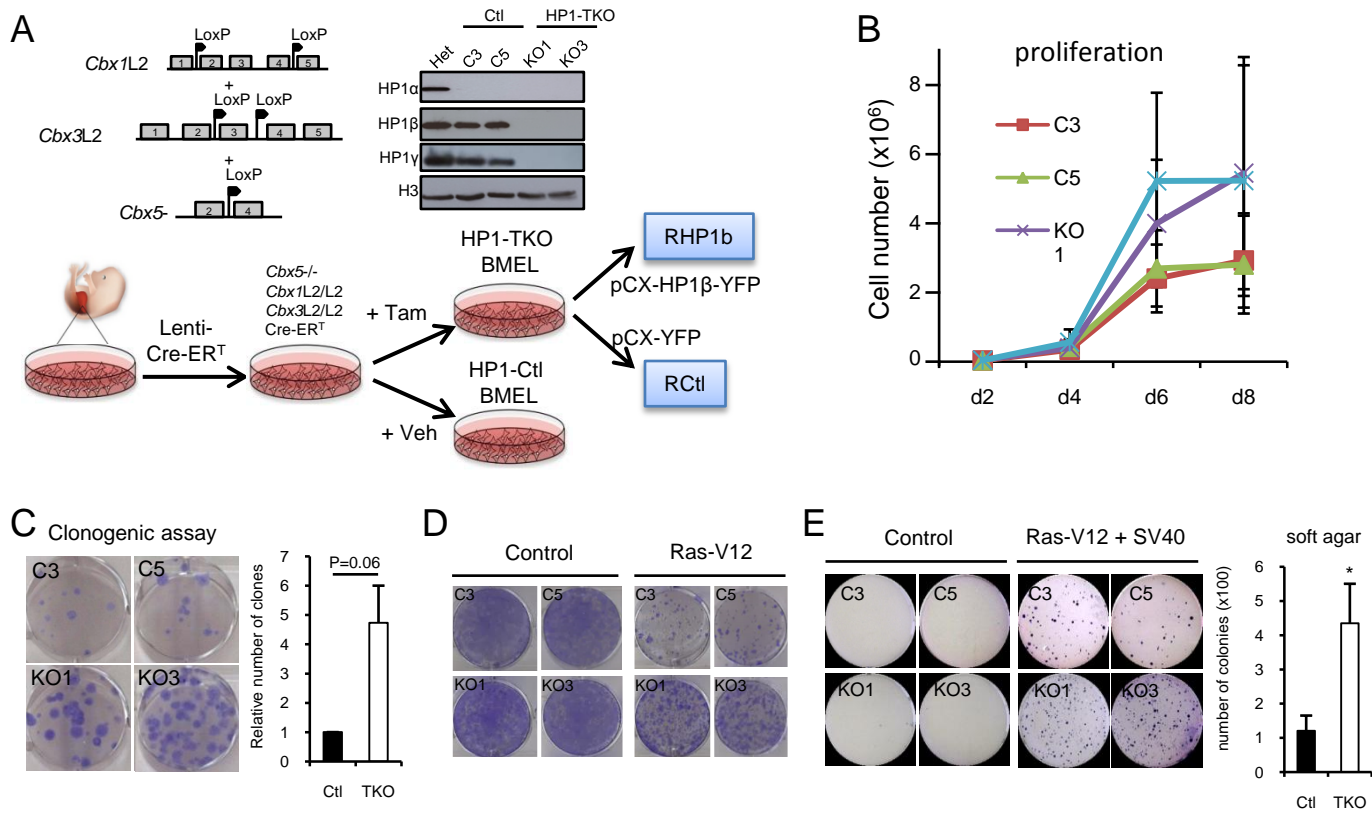


Figure 2. Saksouk & Hajdari et al.

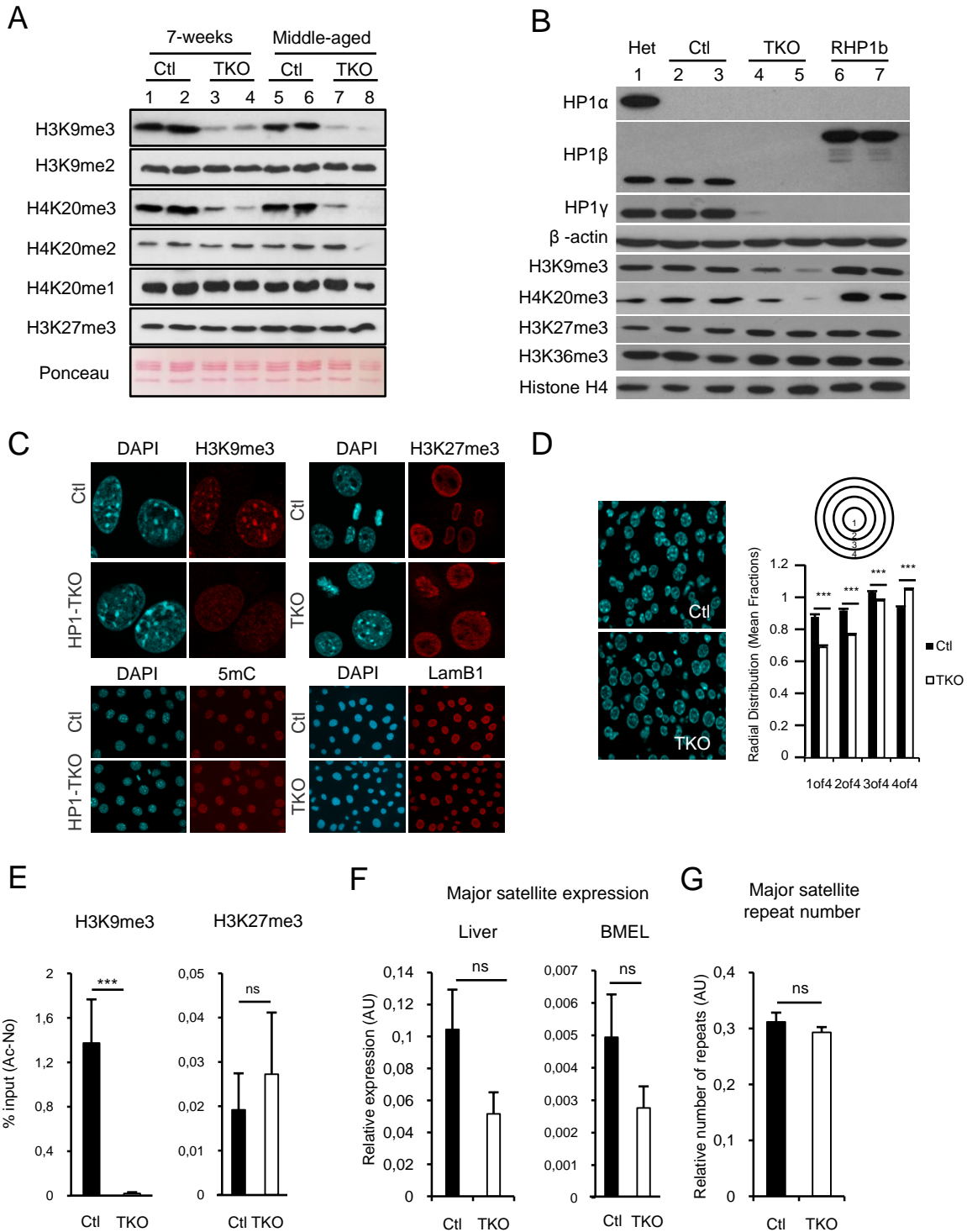


Figure 3, Saksouk, Hajdari et al,

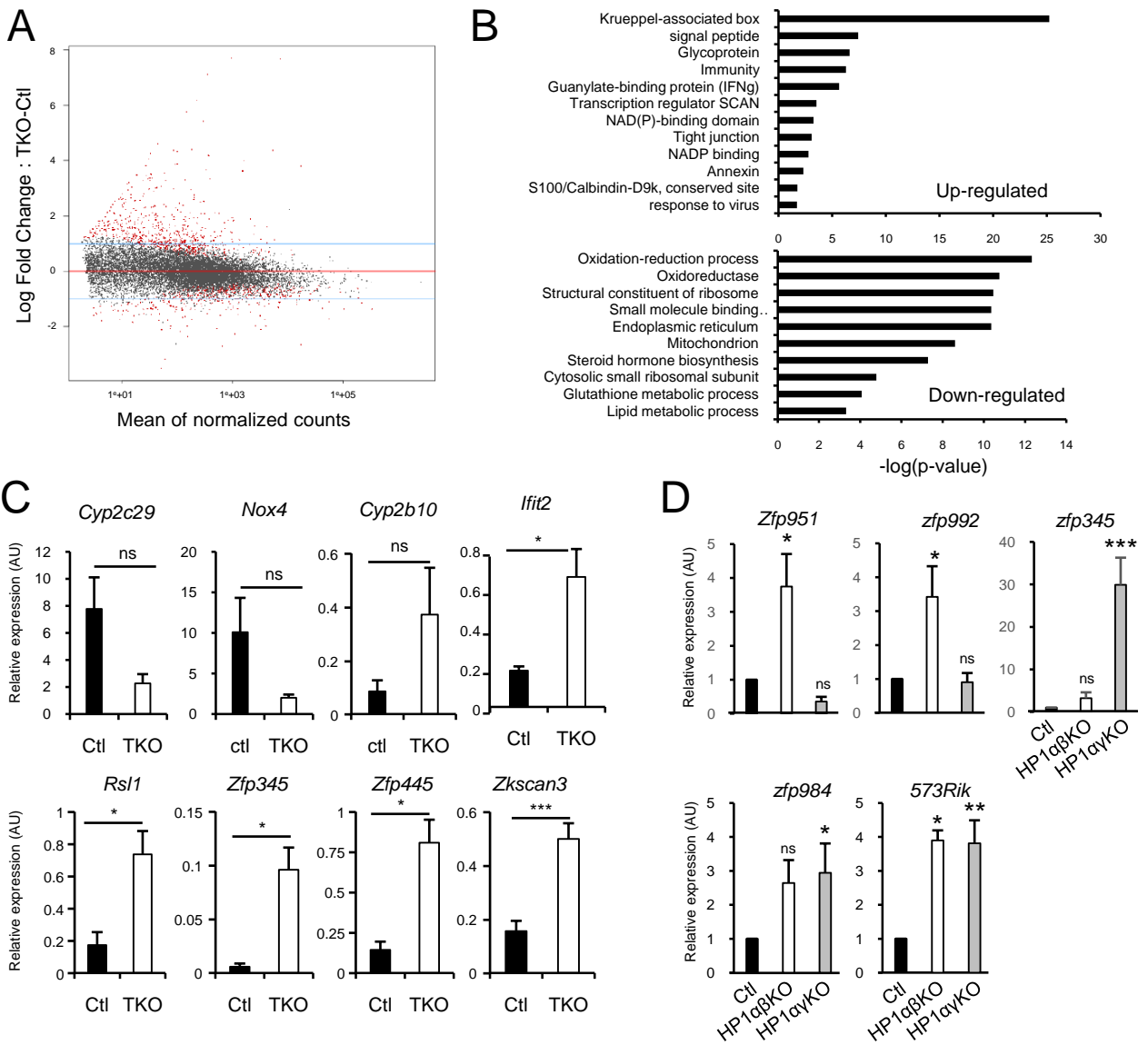


Figure 4 Saksouk & Hajdari et al,

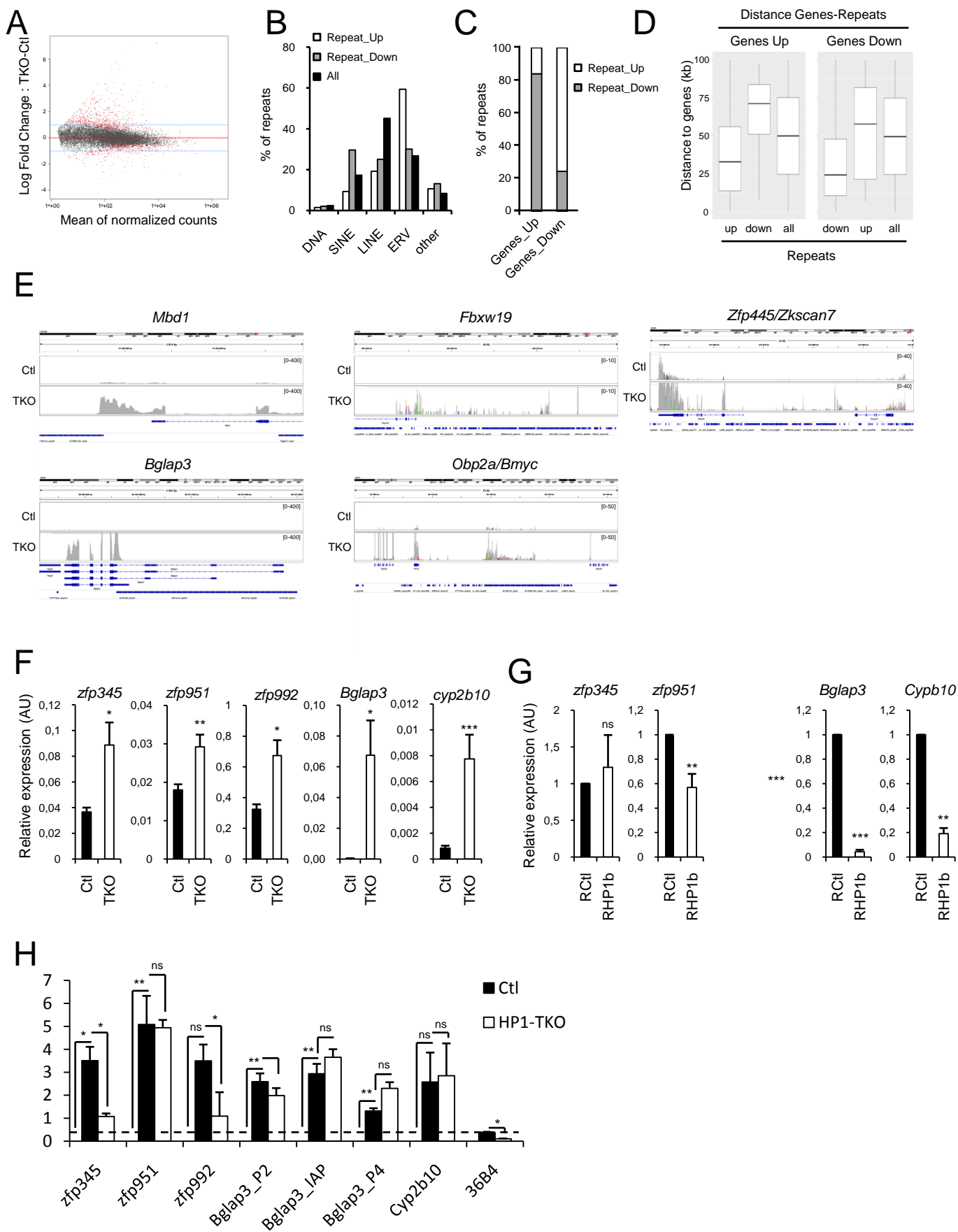


Figure 5 Saksouk & Hajdari et al,

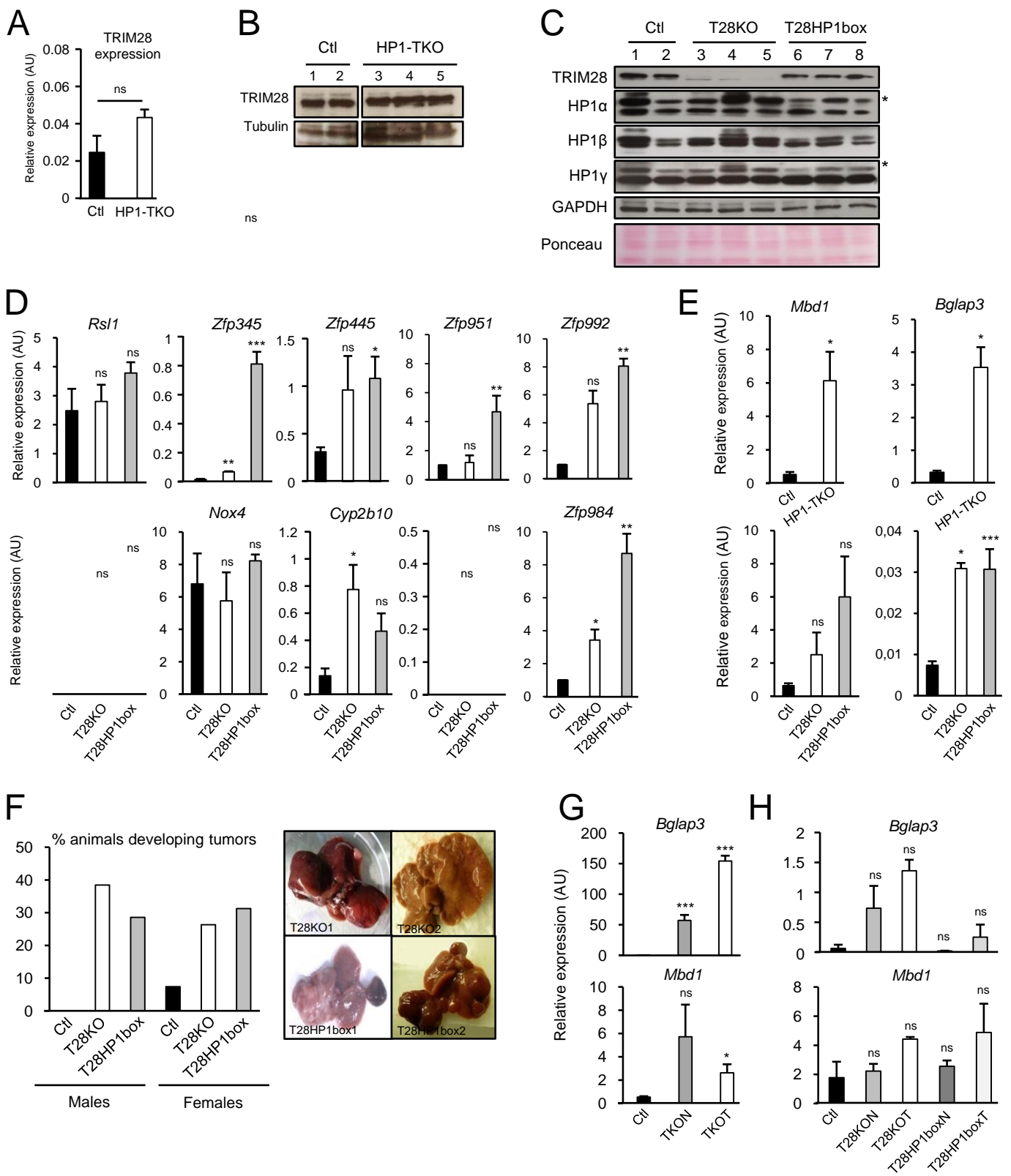


Figure 6, Saksouk & Hajdari et al,

Table 1: HP1-dependent p450 genes.

| Gene name | Log2 fold-change | padj | Redox | Endoplasmic reticulum | Drug metabolism | Lipid metabolism | Steroid synthesis |
|------------|------------------|----------|-------|-----------------------|-----------------|------------------|-------------------|
| Cyp2b10 | 4.06 | 1.41E-38 | 1 | 1 | 0 | 0 | 1 |
| Cyp2b9 | 2.68 | 4.51E-10 | 1 | 1 | 0 | 0 | 1 |
| Cyp2b13 | 1.80 | 0.000120 | 0 | 0 | 0 | 0 | 1 |
| Cyp4f16 | 1.63 | 1.36E-09 | 0 | 0 | 0 | 0 | 0 |
| Cyp2d12 | 1.38 | 0.000468 | 0 | 0 | 0 | 0 | 1 |
| Cyp2a4 | 1.09 | 0.0342 | 0 | 0 | 0 | 0 | 0 |
| Cyp2a22 | 1.06 | 0.000559 | 0 | 0 | 0 | 0 | 0 |
| Cyp2f2 | -0.65 | 0.00318 | 1 | 1 | 0 | 0 | 0 |
| Cyp4f13 | -0.67 | 0.0151 | 0 | 0 | 0 | 0 | 0 |
| Cyp2r1 | -0.72 | 0.0173 | 1 | 1 | 0 | 0 | 0 |
| Cyp27a1 | -0.83 | 1.30E-05 | 1 | 0 | 0 | 0 | 0 |
| Cyp2d37-ps | -0.83 | 0.0319 | 0 | 0 | 0 | 0 | 0 |
| Cyp3a25 | -0.85 | 0.00222 | 1 | 1 | 0 | 0 | 1 |
| Cyp39a1 | -0.88 | 0.00164 | 1 | 1 | 0 | 1 | 0 |
| Cyp2e1 | -0.94 | 7.81E-05 | 1 | 1 | 1 | 0 | 1 |
| Cyp2d26 | -0.95 | 2.57E-05 | 1 | 1 | 0 | 0 | 1 |
| Cyp2a5 | -0.97 | 0.00129 | 0 | 0 | 0 | 0 | 0 |
| Cyp2d13 | -1.00 | 0.00445 | 0 | 0 | 0 | 0 | 0 |
| Cyp1a2 | -1.25 | 3.65E-12 | 1 | 1 | 1 | 1 | 1 |
| Cyp2c53-ps | -1.31 | 0.01178 | 0 | 0 | 0 | 0 | 0 |
| Cyp2d40 | -1.42 | 6.83E-06 | 0 | 0 | 0 | 0 | 1 |
| Cyp46a1 | -1.64 | 0.000629 | 1 | 1 | 0 | 1 | 0 |
| Cyp3a59 | -2.15 | 3.62E-17 | 1 | 0 | 0 | 0 | 0 |
| Cyp2c44 | -2.20 | 2.18E-20 | 0 | 0 | 0 | 0 | 1 |
| Cyp2c29 | -2.60 | 7.65E-25 | 1 | 1 | 0 | 0 | 1 |

(1) found and (0) not found according to the David Gene Ontology software.