Transcription cofactors TRIM24, TRIM28, and TRIM33 associate to form regulatory complexes that suppress murine hepatocellular carcinoma

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TRIM24 (TIF1α), TRIM28 (TIF1β), and TRIM33 (TIF1γ) are three related cofactors belonging to the tripartite motif superfamily that interact with distinct transcription factors. TRIM24 interacts with the liganded retinoic acid (RA) receptor to repress its transcriptional activity. Germ line inactivation of TRIM24 in mice deregulates RA-signaling in hepatocytes leading to the development of hepatocellular carcinoma (HCC). Here we show that TRIM24 can be purified as at least two macromolecular complexes comprising either TRIM33 or TRIM33 and TRIM28. Somatic hepatocyte-specific inactivation of TRIM24, TRIM28, or TRIM33 all promote HCC in a cell-autonomous manner in mice. Moreover, HCC formation upon TRIM24 inactivation is strongly potentiated by further loss of TRIM33. These results demonstrate that the TIF1-related subfamily of TRIM proteins interact both physically and functionally to modulate HCC formation in mice.

Hepatocellular carcinoma (HCC) is the fifth most common type of cancer, with half a million newly diagnosed cases each year worldwide (1). Among the genetically engineered mice models, HCC spontaneously arises in only a few cases (2). We have previously shown that germ line inactivation of tripartite motif 24 (TRIM24) in mice results in HCC formation (3). TRIM24, first identified as transcriptional intermediary factor 1 alpha (TIF1α), is a cofactor that interacts with liganded nuclear receptors to modulate either positively or negatively their transcriptional activity (3–5). TRIM24 was also identified in murine HCC as a fusion partner of BRAF in the chimeric oncoprotein T18 (6). Despite the fact that TRIM24 is widely expressed, *Trim24*$^{-/-}$ mice develop tumors only in the liver, showing that TRIM24 is a key suppressor of the initiation and development of hepatocarcinogenesis (7).

In *Trim24*$^{-/-}$ mice, HCC develops as a result of a reproducible multistage process that parallels human HCC (8). Increased hepatocyte proliferation is observed as soon as postnatal wk 3, when normal hepatocytes enter into quiescence (3). At later times, a series of histological abnormalities (hypertrophic hepatocytes with enlarged nuclei), preneoplastic lesions [clear-cell foci of altered hepatocytes (FCA)] and neoplastic lesions (hepatocellular adenomas and carcinomas) become visible in mutant livers. Remarkably, the carcinogenic process was shown to correlate with increased RA-signaling and inactivation of a single allele of RARα receptor (*Rara*) is sufficient to restore the correct expression level of RA target genes, the normal rate of hepatocyte proliferation and to completely suppress hepatocarcinogenesis. These observations revealed an oncogenic function of RARα in the liver (3).

TRIM24 is the founding member of a conserved subfamily of TIF1α-related TRIM proteins with orthologues from *Drosophila* to human (9–13) that play crucial roles in many physiological processes including cell differentiation, development, and tissue homeostasis (12, 14–22). Members of this subfamily share a common N-terminal TRIM previously known as a RING–B-box–coiled-coil (RBCC) motif and a chromatin binding unit comprising a C-terminal plant homeo domain finger and bromodomain that act as a single functional unit to promote recognition of a combination of unmethylated lysine 4 of histone H3 (H3K4me0) and acetylated H3K23 (23). Tripartite motif 28 (TRIM28) [transcriptional intermediary factor 1 beta (TIF1β), KRAB-associated protein 1 (KAP1)] possesses an intrinsic silencing activity and also acts through chromatin via recruitment of chromatin modifiers (24–28).

Different members of the TIF1 family interact with distinct transcription factors; ligand activated nuclear receptors or p53 [TRIM24 (29, 30)], Krüppel-associated box domain-containing zinc finger proteins [TRIM28, (31)], and transforming growth factor (TGF)-β receptor-activated mothers against decapentaplegic (SMAD) and coSMAD proteins [tripartite motif 33 (TRIM33) also known as transcriptional intermediary factor 1 gamma (TIF1γ); (12, 17, 18)]. TRIM33 acts on the TGF-β pathway either as a monoubiquitin ligase for SMAD4 and/or as a cofactor for phosphorylated SMAD2/3 (17, 32), whereas it is also reported to act directly on the transcription initiation complex by promoting recruitment of positive transcription elongation factor b and the facilitates chromatin transcription (FACT) complex to counteract RNA polymerase II pausing (33).

To better understand the molecular function of TRIM24, we sought to identify its protein partners. Using cells expressing an epitope-tagged TRIM24, we show that TRIM28 and TRIM33 copurify with TRIM24 along with histone deacetylase 1 and 2 (HDAC1 and 2), and the heterochromatin proteins (HP). TRIM33 is a major interacting partner appearing almost stoichiometric with TRIM24 in the complex. Sequential immunoprecipitations show that TRIM24 exists in a major complex with TRIM33 and a lesser abundant complex with TRIM33 and TRIM28. Hepatocyte-specific inactivation of TRIM24, TRIM28, and TRIM33 all promote development of HCC, whereas simultaneous inactivation of both TRIM33 and TRIM24 potentiates

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HCC formation with respect to each of the corresponding single mutants. These results indicate that TRIM24, TRIM28, and TRIM33 interact both physically and functionally to modulate HCC in mice.

Materials and Methods

Cell Lines and Immunopurification and Protein Identification. Generation of HeLa cell lines expressing the N-terminal FLAG-HA epitope-tagged (E)-TRIM24 protein was performed by retroviral infection, extract preparation and tandem affinity purification using the FLAG and HA tags were all performed essentially as previously described (34). Purified complexes were analyzed on 4–12% Tris-Bis NuPage gels (Invitrogen) and stained with Coomassie blue. Protein identification was carried out by nanocapillary liquid chromatography-tandem MS (nanoLC–MS/MS) using a nanoACQUITY ultra performance liquid chromatography (UPLC) system (Waters), coupled to a hybrid electrospray quadrupole TOF mass spectrometer (SYNAPT HDMS, Waters). Detailed protocol for protein identification according to current guidelines is given in *SI Text*. Additional immunoprecipitations were performed on the purified complexes with antibodies against TRIM28 and TRIM33. Flowthroughs and immunoprecipitates were resolved by SDS-PAGE and analyzed by Western blot analysis with indicated antibodies. Antibodies against the endogenous TIF1-related TRIM proteins have been previously described (4, 24, 35).

Tissue Protein Extraction and Western Blot Analysis. Whole cell protein extracts were prepared as described (13) and protein concentration determined with Bio-Rad protein assay reagent (Bio-Rad). Equal amounts of protein (50–200 μg) were subjected to 8% SDS-PAGE. Immunoblots were carried out using the primary antibodies to each TRIM protein and the β-tubulin monoclonal antibody, and peroxidase-conjugated goat anti–mouse IgG and goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) were used as secondary antibodies.

Somatic Hepatocyte-Specific TRIM24, TRIM28, and TRIM33 Inactivation. The generation of mice harboring conditional *Trim24* alleles (*Trim24*$^{L2/L2}$ "floxed" mice) and conditional *Trim28* alleles have been previously described (3, 14). Mice with floxed *Trim33* alleles were engineered by inserting loxP sites in introns 1 and 4 of the *Trim33* gene such that Cre treatment deletes exons 2–4 creating a unique reading frame with a stop codon in exon 5 (36).

Constitutive hepatocyte gene inactivation was performed using transgenic Albumin (Alb)-Cre (*Alb-Cre*) mice (37) and tamoxifen inducible inactivation with *Alb-CreER*^{T2} mice that have been previously described (38). To generate hepatocyte-specific knockouts, *Trim*$^{L2/L2}$ and *Alb-Cre* or *Alb-CreER*^{T2} mice were bred to obtain mice homozygous for the floxed *Trim* locus and heterozygous for *Alb-Cre* or *Alb-CreER*^{T2}. These mice produce control and hepatocyte-specific knockout littermates.

Tamoxifen (Tam) induced inactivation was performed by intraperitoneal injection of 2-mo-old mice with 10 mg Tam dissolved in 100 μL of oil each day for five consecutive days. For long-term studies tamoxifen injections were repeated every 3 mo. The mice were maintained on a 12-h/12-h light/dark cycle and provided with access to water and standard rodent chow diet ad libitum. All animal procedures were approved by the French Ministry of Agriculture (agreement B67-218-5) according to guidelines in compliance with the European legislation on care and use of laboratory animals.

PCR Genotyping. Genomic DNA was isolated from mouse tail biopsies using standard procedures. Detection of recombination and presence of the Cre transgene was verified by PCR analysis. The primers used are listed in Table S1.

Histopathological Analysis. For analysis of tumorigenesis, at least 10 mice from each group were sacrificed >1 year after inactivation and at least five mice of each genotype were used for histopathological analysis at 3 mo after inactivation. After fixation in buffered formaldehyde, liver samples (left median lobes) including visible tumors were embedded in paraffin, serially sectioned (5 μm sections) and stained with haematoxylin and eosin. Lesions were classified into FCA, hepatocellular adenomas (HCA), and HCC according to established criteria (39).

Ki67 Labeling. Cell proliferation detected using the Ki67 antibody was carried out according to standard operating procedures (http://empress.har.mrc.ac.uk/browser/). The percentage of Ki67-positive cells (±SD) from five randomly chosen fields per section and three sections per liver from four to five animals of each genotype was determined and statistical significance of the results was analyzed using Student's *t*-test.

RNA Extraction and Real-Time Quantitative PCR (RT-qPCR). Total RNA was extracted from liver tissue samples using an RNeasy (QIAGEN) kit according to the manufacturer's instructions. 1.5 μg of RNA were reverse transcribed using SuperScript II RNase H-reverse transcriptase (Invitrogen) and 500 ng of oligo-dT22 primer according to the manufacter's instructions. The final product was diluted 80 times and 4 μL were mixed with forward and reverse primers (250 nM final concentration) listed in Table S1 and 5 μL of SYBR Green master mix (Qiagen). RT-PCR was performed using the LightCycler 1.5 system (Roche). Each cDNA sample was tested at least in triplicate. For quantification of gene expression changes, the δCt method was used to calculate relative fold changes normalized against the Hypoxanthine-guanine phosphoribosyltransferase gene (*Hprt*), as described in the manufacturer's protocol.

Results

Identification of TRIM24-Associated Proteins. To purify a native TRIM24 complex and identify its components, we generated a HeLa cell line stably expressing an N-terminal FLAG-HA E-TRIM24 (Fig. 1 *A* and *B*). E-TRIM24 localized in the nucleus with a diffuse euchromatin labeling, as previously described for wild-type TRIM24 (40). Nuclear extracts from the E-TRIM24 expressing and control untagged cells were purified by tandem immunoprecipitation with FLAG and HA antibodies. The precipitated fractions were resolved by SDS-PAGE (Fig. 1*C*) and the different subunits excised from the E-TRIM24 precipitate and identified by MS (Table S2). Almost no proteins were precipitated from the mock-infected cells. HDAC1, HDAC2, and HP1α, β, and γ were identified in the E-TRIM24 purified fraction confirming a previous report from our laboratory (24). The presence of the core histones indicated that the TRIM24 complex is associated, at least in part, with chromatin, a known feature of TRIM24 (23, 40). Importantly, MS-based proteomic analyses identified both TRIM28 and TRIM33 as TRIM24 partners. Although not quantitative, silver nitrate staining of the SDS gel of the purified complex suggests that TRIM33 is a major partner present in a close to stoichiometric ratio with TRIM24, whereas TRIM28 seems to be less abundant (Fig. 1*C*).

Immunoblotting confirmed the presence of all three TRIM proteins in the FLAG-HA immunoprecipitated E-TRIM24 eluate (Fig. 1*D*, lane 1). To determine if they were present in the same complex, we reprecipitated the eluate with antibodies against TRIM28 or TRIM33. Immunoprecipitation with TRIM33 depleted virtually all of the TRIM24 and TRIM28 and both were present in the bound fraction (lanes 2 and 3). In contrast, immunoprecipitation with TRIM28 antibody precipitated only a subfraction of TRIM24 and TRIM33 from the FLAG-HA eluate (lanes 4 and 5). These results are consistent with those of the silver nitrate staining and indicate the existence of a major TRIM33–TRIM24 complex and a second less abundant complex with all three TRIM proteins (Fig. 1*E*).

We next determined whether the endogenous TIF1-related TRIM proteins also interact in adult mouse liver. Immunoprecipitations with the antibodies against each TRIM protein resulted in coprecipitation of TRIM24 and TRIM33 (Fig. 1*F*). However, although the immunoprecipitation of TRIM33 efficiently coprecipitates TRIM24, less TRIM33 is coprecipitated when the TRIM24 antibody is used. These results suggest that although most cellular TRIM24 may be associated with TRIM33, the converse is not the case. Although these differences may result from the fact that each antibody may precipitate its cognate TRIM protein with differing efficiencies or with differing epitope accessibility, these observations clearly indicate interactions between the endogenous TIF1-related TRIM proteins in liver extracts.

Hepatocyte-Specific TRIM24 Inactivation Increases Proliferation in Pre- and Postquiescent Hepatocytes. The physical interaction of the TIF1-related TRIM proteins suggests that they may also cooperate functionally to suppress HCC. As the TRIM28 and TRIM33 germ line knockouts are embryonic lethal at day 8.5 and 9.5, respectively (14, 20), evaluation of their role in HCC

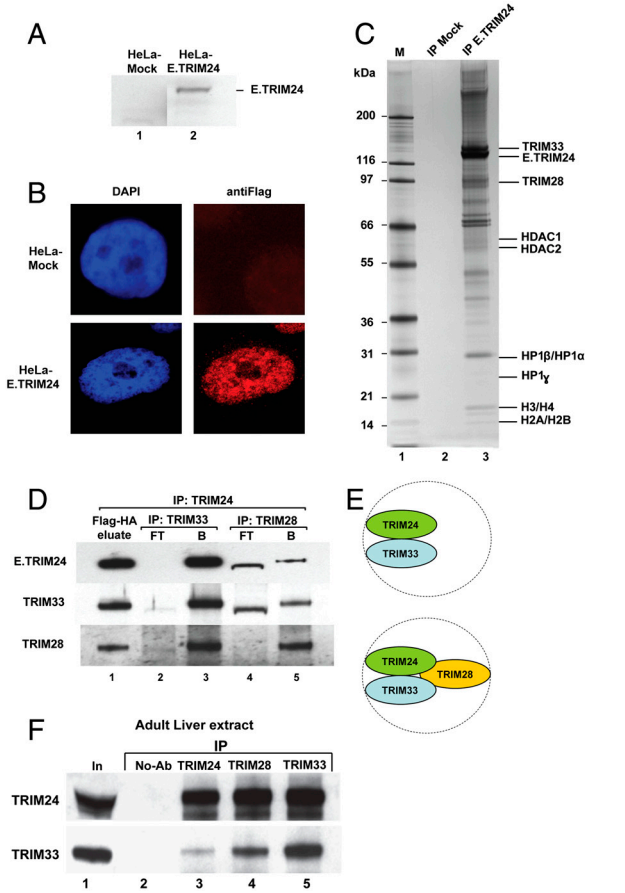


Fig. 1. Tandem affinity purification of TRIM24 containing complexes. (*A*) Anti-FLAG immunoblot to detect expression of E-TRIM24 in nuclear extracts from retroviral transduced or control HeLa cells. (*B*) Detection of E-TRIM24 expression and localization by immunofluorescence with anti-FLAG antibody. (*C*) SDS-PAGE and silver nitrate staining of tandem affinity purified TRIM24 and its partner proteins. The identity of proteins identified by MS is indicated. (*D*) Western blot analysis performed on the tandem immunopurified complex and the flowthrough (FT) and bound (B) fractions of the subsequent anti-TRIM33 or TRIM28 immunoprecipitations. (*E*) Schematic representation of the major TRIM24/TRIM33 and minor TRIM24/TRIM33/TRIM28 containing complexes. (*F*) Western blot analysis of immunoprecipitates of adult liver extracts. Immunoprecipitations were performed with antibodies against the endogenous TRIM proteins or only with protein G-sepharose beads, as indicated above each lane and the presence of the TRIM24 and TRIM33 proteins in the precipitated fractions visualized by immunoblot.

requires hepatocyte-specific inactivation. To assess the feasability of this approach, we first determined whether the HCC phenotype seen upon germ line inactivation of TRIM24 is cell-autonomous by generating a hepatocyte-specific knockout. Mice with floxed *Trim24* alleles were bred with mice expressing the Cre recombinase under the control of the albumin (*Alb*) promoter ($Trim24^{L2/L2}Alb\text{-}Cre^{+}$) allowing early postnatal recombination of the floxed allele. We further generated a mouse line in which *Trim24* could be inactivated in adult hepatocytes by Tam injection using a knock-in mouse line in which the $Cre\text{-}ER^{T2}$ fusion transgene is inserted into the 3′-UTR of the *Alb* gene (38). The use of this line allows investigation of the tumor suppressive properties of TRIM24 in adult liver following establishment of hepatocyte quiescence.

Western blot analysis of liver extracts from 5-wk-old $Trim24^{L2/L2}Alb\text{-}Cre^{+}$ mice revealed a strong reduction of TRIM24, whereas its levels were unchanged in testis extracts (Fig. 2*A*). Any residual TRIM24 is likely due to its expression in the nonhepatocyte cells of the liver. Histological examination of the livers of 3-mo-old $Trim24^{L2/L2}Alb\text{-}Cre^{+}$ mice by DAPI staining revealed TRIM24-deficient hepatocytes with enlarged polyploid nuclei (Fig. 2*C*). Labeling with Ki67 revealed a fourfold increase in proliferative cells (Fig. 2 *C* and *E*). Hepatocyte-specific inactivation of TRIM24 therefore results in abnormalities analogous to those observed in germ line mutant mice (3), indicating that TRIM24 restricts hepatocyte proliferation and predisposes to polyploidization in a cell-autonomous manner.

Similar results were observed in the $Trim24^{L2/L2}Alb\text{-}CreER^{T2+}$ mice where TRIM24 inactivation was induced in 2-mo-old animals by Tam injection. Selective loss of TRIM24 expression in liver extracts was observed 12 wk after Tam injection (Fig. 2*B*). Histological analysis revealed enlarged polyploid nuclei and a threefold increase in Ki67 labeled proliferative cells (Fig. 2 *D* and *F*). TRIM24 is thus not only required during the postnatal period when the hepatocytes normally enter into quiescence, but is continuously required to maintain the quiescent state.

We previously identified several genes whose expression is deregulated in germ line TRIM24 knockout livers (3). The expression of several of these TRIM24-regulated genes was compared in the livers of the germ line as well as the constitutive and inducible hepatocyte-specific TRIM24 knockouts 3 mo after inactivation (i.e., 3 mo of age in the germ line and constitutive hepatocyte strains, and 5 mo of age in the Tam-inducible strain). In germ line TRIM24 mutants, the expression of *Afp, Bmyc, GluR3, Lcn13, Mbd1*, and the uncharacterized *2610305D13Rik* transcript was reproducibly increased (Fig. 3). Deregulated expression of these genes was also observed in the constitutive and inducible hepatocyte-specific TRIM24-deficient livers (Fig. 3). Thus, similar to germ line inactivation, immediate postnatal or adult stage hepatocyte-specific TRIM24 inactivation both induce hepatocyte proliferation and deregulate the expression of TRIM24 target genes.

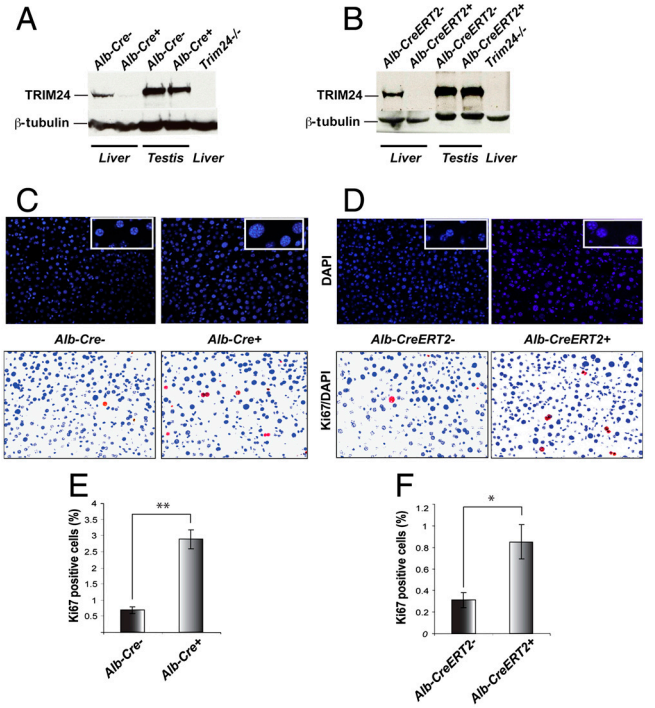


Fig. 2. Characterization of hepatocyte-specific TRIM24 knockout mice. (*A*) and (*B*) Western blot analysis with anti-TRIM24 and β-tubulin antibodies performed on liver and testis extracts from $Trim24^{L2/L2}$ mice whose genotypes with respect to the *Alb-Cre* and $Alb\text{-}CreER^{T2}$ transgenes are shown above each lane. In *B* all mice were injected with Tam. (*C*) and (*D*) Ki67 staining performed on liver sections from 3-wk-old $Trim24^{L2/L2}$ mice (*C*) or 5-mo-old Tam injected $Trim24^{L2/L2}$ animals (*D*) with the indicated transgenes. The inserts in the upper DAPI-stained panels show higher magnification images of enlarged polyploid nuclei. (*E*) and (*F*) Quantification of Ki67-positive hepatocytes in the indicated strains. Means $(N = 4) \pm$ SD $*P < 0.05$, $**P < 0.005$.

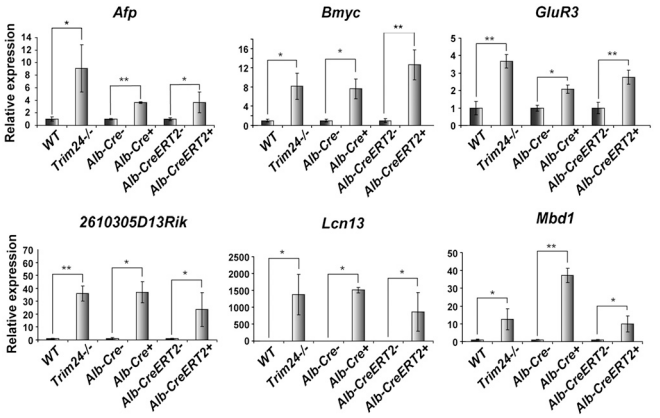
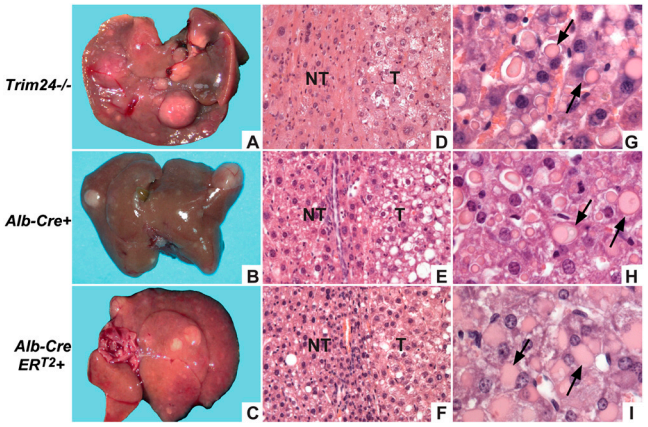


Fig. 3. Comparison of gene expression in TRIM24 germ line and hepatocyte-specific knockout mice. The expression of six representative genes was determined by RT-qPCR on whole total liver RNA from WT, *Trim24*$^{-/-}$ or *Trim24*$^{L2/L2}$ littermates with the indicated transgenes. Expression in the control mice was arbitrarily considered as = 1. Means $(N = 4) \pm$ SD $*P < 0.05$, $**P < 0.005$.

TRIM24 Inactivation Induces Hepatocarcinogenesis in Pre- and Post-quiescent Hepatocytes. Germ line TRIM24 inactivation leads to HCC as a consequence of a reproducible series of neoplastic transformations (7), characterized by the development of clear-cell FCA by 7 mo, then HCA by 9 mo, ending with HCC starting from 12 mo. We verified the presence of neoplastic lesions in the constitutive and Tam-induced hepatocyte-specific mutant livers approximately one year after TRIM24 depletion (i.e., 12 and 14 mo for *Trim24*$^{L2/L2}$*/Alb-Cre*$^{+}$ and Tam-injected *Trim24*$^{L2/L2}$*/Alb-CreER*$^{T2+}$ mice, respectively). Remarkably, all types of lesions previously observed in germ line TRIM24 knockout livers (FCA, HCA, HCC) were also observed in the hepatocyte-specific mutants (Fig. 4 *A–I*, summarized in Fig. 4*J*). The penetrance of tumor formation in the constitutive hepatocyte-specific line is similar to that seen in the germ line mutant, although it is slightly lower in the Tam-inducible strain (Fig. 4*J*). Together, these data indicate that TRIM24 acts as a cell-autonomous tumor suppressor in quiescent hepatocytes and validate the use of *Alb-Cre* mice to investigate the potential roles of TRIM28 and TRIM33 in hepatocarcinogenesis.

Hepatocyte-Specific Inactivation of TRIM28 and TRIM33 Induces Hepatocarcinogenesis. To determine whether TRIM28 and TRIM33 have a tumor supressor function in hepatocytes, mouse lines with floxed alleles of TRIM28 and TRIM33 were crossed with the *Trim24*$^{L2/L2}$*/Alb-Cre* line to obtain hemizygous progeny for *Trim24*$^{L2/+}$*/Trim28*$^{L2/+}$, or *Trim24*$^{L2/+}$*/Trim33*$^{L2/+}$ and the presence of the *Alb*-Cre transgene. These mice were intercrossed to obtain progeny with the *Alb-Cre* transgene and that were homozygous for the floxed alleles of each TRIM gene. We also generated *Alb-Cre* expressing mice with the compound floxed *Trim24* and *Trim33* or *Trim24* and *Trim28* alleles. Western blot analysis performed on liver extracts of 5-wk-old mice showed a strong decrease in the TRIM28 and TRIM33 proteins resulting from their hepatocyte-specific inactivation (Fig. 5 *A* and *B*).

Macroscopic examination of the livers of 14-mo-old mice from each strain clearly showed the presence of HCC. Statistical analysis of macroscopic tumors in each single and double-mutant strain distinguished weakly (1–3 tumors) and highly affected livers (>3 tumors) (Fig. 5*C*). Hepatocyte-specific inactivation of TRIM28 and TRIM33 promotes tumorigenesis, albeit with a lower penetrance compared to TRIM24, indicating that they act as hepatocyte tumor suppressors (Fig. 5*C*). Surprisingly, however, HCC incidence is lower in the TRIM24/TRIM28 double mutant compared to TRIM24 alone. In particular, the number of highly affected livers is reduced in the double mutants. Hence, although



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		Type of neoplasia			Total	
		FCA	HCA	HCC	neoplasias	%
Germ line	*Trim24-/-*	7/10	5/10	3/10	7/10	70
Constitutive	*Alb-Cre-*	0/10	0/10	0/10	0/10	0
	Alb-Cre+	7/10	5/10	3/10	7/10	70
Inducible	*Alb-CreERT2-*	0/14	0/14	0/14	0/14	0
	Alb-CreERT2+	7/14	5/14	2/14	7/14	50

Fig. 4. Spontaneous liver tumor formation in hepatocyte-specific TRIM24 knockout mice. (*A–C*) Representative macroscopic views of 14-mo-old *Trim24*$^{-/-}$ (*A*), *Trim24*$^{L2/L2}$*/Alb-Cre*$^{+}$ (*B*), and *Trim24*$^{L2/L2}$*/Alb-CreER*$^{T2+}$ (*C*) livers showing multiple tumor nodules, which were histologically classified as HCA or HCC. (*D–F*) Hematoxilin/Eosin staining of representative histology sections of HCA, the tumor is well circumscribed and causes compression of the surrounding nontumoral parenchyma. (*G–I*) Histology sections of solid HCC composed of hepatocytes with occasional eosinophilic cytoplasmic inclusions (arrows). (*J*) Statistics of neoplasias observed in germinal and hepatocyte-specific constitutive and Tam-inducible TRIM24 mutants. Neoplasias are classified as FCA, HCA, or HCC.

TRIM28 inactivation promoted HCC formation, its loss attenuates the tumorigenic effect of TRIM24 inactivation. In striking contrast, inactivation of both TRIM33 and TRIM24 led to a potent increase in tumor formation compared to each single mutant where almost all mice now showed highly affected livers.

We examined the expression of several TRIM24 target genes in the livers of the above mutant strains at 5 wk of age. Even though these genes were identified as deregulated in the germ line TRIM24 mutant liver (3), they are also up-regulated in the hepatocyte-specific TRIM24 knockout (Fig. 5 *E–G*). Most of these genes also showed deregulated expression in the TRIM24/TRIM33 and TRIM24/TRIM28 double mutant livers. However, the up-regulation of *Glur3* expression seen upon TRIM24 inactivation is lost when TRIM28 is also inactivated (Fig. 5*G*). The activation of this gene therefore appears to be TRIM28-dependent. Specificity is also seen in the single TRIM28 and TRIM33 mutants. The *Stat1* and *Tgm2* genes are deregulated upon TRIM24 inactivation only (Fig. 5*F*). On the other hand, expression of *Mbd1* and *Cyp2d9* is up-regulated by TRIM28 inactivation, but not upon loss of TRIM33, whereas the contrary is seen for *Lcn13* and *GluR3* (Fig. 5 *E* and *G*). Furthermore, the increase in *Lcn13* expression elicited by TRIM24 or TRIM33 inactivation is further augmented in the TRIM24/TRIM33 double mutant.

Discussion

TRIM24 is a Cell-Autonomous Suppressor of Hepatocarcinogenesis. We have previously shown that germ line inactivation of TRIM24 results in the development of HCC, however, this hepatocarcinogenesis could reflect a cell-autonomous or non-cell-autonomous effect. To address this issue, we inactivated TRIM24 specifically in hepatocytes. Loss of TRIM24 in postnatal hepatocytes or later in adult hepatocytes promoted a series of neoplastic lesions

including HCC. TRIM24 appears therefore to be required throughout the life span of the animal to maintain hepatocyte quiescence and repress HCC formation in a cell-autonomous fashion. Nevertheless, it should be noted that the penetrance of HCC was lower following adult hepatocyte-specific inactivation compared to hepatocyte-specific neonatal or germ line knockouts. Although this difference may simply reflect a less efficient inactivation in the Tam-inducible knockout, it could also reflect a more fundamental age-dependency of hepatocarcinogenesis. The lower penetrance may be explained by the lower cycling rate of adult hepatocytes that may render them intrinsically more resistant to induction of hepatocarcinogenesis (41).

One of the central questions concerning the mouse modeling of cancer is to which extent it parallels human pathologies. In this respect, our TRIM24 germ line knockout recapitulates the histological properties and sequential events of human hepatocarcinogenesis. Nevertheless, as human cancers mainly arise during adulthood, the effects of germ line or early postnatal inactivation are not necessarily an appropriate model for human cancers. The observation that inactivation of TRIM24 in adult hepatocytes also leads to HCC therefore reinforces the idea that these animals may be a relevant model for the study of some aspects of human HCC. Furthermore, as the tumorigenic effect of TRIM24 inactivation is linked to deregulated RA-signaling (3, 7), the knockout animals constitute an ideal model in which to screen unique RA-agonists or antagonists for their ability to modulate tumor formation in an in vivo context.

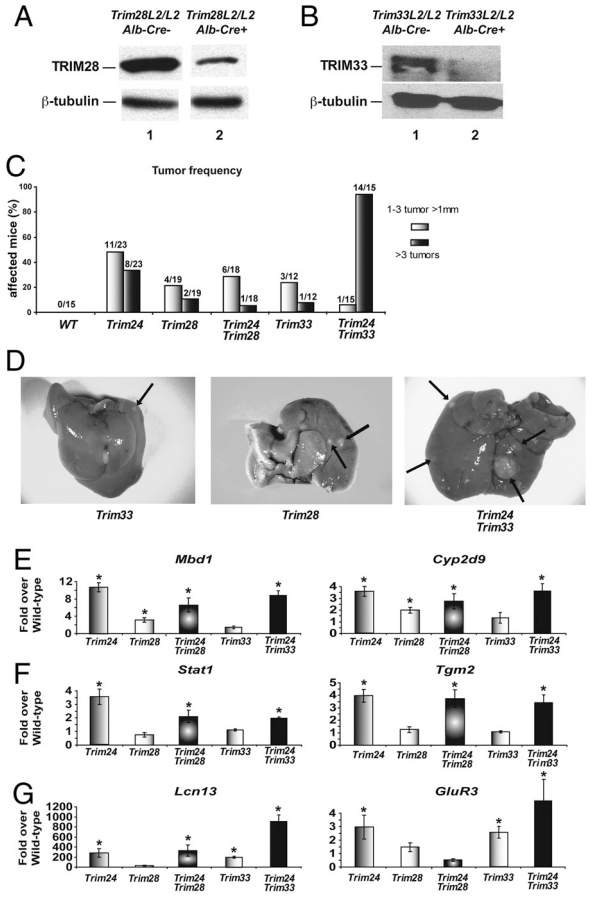

Fig. 5. Spontaneous HCC tumor formation in hepatocyte-specific TRIM28 and TRIM33 knockout mice. (*A*) and (*B*) Western blot analysis of TRIM28 and TRIM33 expression following hepatocyte-specific inactivation. (*C*) Statistics of macroscopic liver neoplasias in mice with the indicated genotypes. Livers were classified in two groups: Livers containing one to three tumors with a size >1 mm are indicated by the open bars and livers containing numerous tumors (>3) are indicated by the filled bars. (*D*) Macroscopic views of livers from mice with the indicated genotypes. Lesions are indicated by arrows. (*E–G*) Expression of TRIM24 target genes was quantified by RT-qPCR in all single and double-mutant mice. Expression in the control mice was arbitrarily considered as = 1. Means $(N = 4) \pm$ SD $^*P < 0.05$.

It is interesting to note that, in contrast to the HCC that arises upon TRIM24 loss of function in mice, overexpression of TRIM24 in human breast cancer is associated with poor prognosis (23). Indeed, although TRIM24 acts as a repressor of RA-signaling and tumorigenesis in hepatocytes (3), it coactivates estrogen receptor signaling in breast cancer cells and promotes their proliferation (23). TRIM24 appears therefore to differentially modulate carcinogenesis in a nuclear receptor and tissue-specific manner.

The TIF1-Related Subfamily of TRIM Proteins Interact to Form Regulatory Complexes that Modulate HCC. Previous studies indicated that TRIM28 and TRIM33 germ line knockouts elicit embryonic lethality at E8.5 and E9.5, respectively (14, 20). The distinct phenotypes of the TRIM24, TRIM28, and TRIM33 germ line knockouts demonstrates that each TRIM protein has distinct and nonredundant functions. In contrast, our present results reveal that they also associate with TRIM24 in at least two complexes and their somatic hepatocyte inactivation reveals a shared ability to repress HCC that could not be seen using the germ line knockouts. Together these observations show that TRIM28 and TRIM33 can cooperate with TRIM24 and act independently perhaps in other distinct protein complexes.

Purification of a native TRIM24 complex from HeLa cells revealed several partners such as the HP1 proteins that were previously shown to interact physically with TRIM24 via its HP1-box domain (24). The presence of HDACs is also in accordance with the observation that TRIM24 transcriptional repression could be abrogated by the HDAC inhibitor trichostatin A (24). More unexpectedly, we show that TRIM28 and TRIM33 are present in complexes with TRIM24. Both silver nitrate staining and sequential immunoprecipitations are consistent with the existence of two related complexes, a major TRIM33 and TRIM24 complex and a second less abundant complex additionally containing TRIM28. It has previously been shown that TRIM24 and TRIM33 can interact via their RBCC domain (42, 43), however it remains to be determined whether TRIM28 interacts directly with TRIM24 and/or TRIM33 or whether it associates with the complex via interactions with other subunits. Similarly, it is possible that the core histones, HP1 proteins and HDACs associate with the complex via interactions with TRIM24 or via a combination of all three TRIM subunits.

The observation that TRIM28 and TRIM33 associate with TRIM24 raised the question as to whether TRIM24 HCC suppression involved cooperative functional interactions with these partners. Postnatal hepatocyte-specific inactivation of TRIM28 leads to the formation of HCC, however, tumor formation is less frequent than in TRIM24-null hepatocytes perhaps reflecting the observation that TRIM28 inactivation results in a milder deregulation of only a subset of TRIM24 target genes. Loss of TRIM28 does not enhance HCC in TRIM24-mutant mice, but rather attenuates tumor formation showing that the presence of TRIM28 is required either to promote the tumor initiation or progression elicited by loss of TRIM24. In accordance with this idea, activation of *Glur3* expression upon loss of TRIM24 is abolished in the TRIM24/TRIM28 double-mutant. *Glur3* may therefore represent a class of genes whose activation requires TRIM28 accounting for the observation that TRIM28 inactivation attenuates tumorigenesis in TRIM24-mutant livers.

In contrast to the above, loss of TRIM33 significantly potentiates the tumor formation seen upon TRIM24 inactivation resulting in animals whose livers are characterized by numerous large tumors. These observations indicate a cooperative action of TRIM24 and TRIM33 in tumor suppression. This effect is also

reflected in the expression of target genes such as *Lcn13* that shows increased deregulation in the TRIM33/TRIM24 double-mutant. *Lcn13* may therefore represent a class of genes whose expression is repressed in a nonredundant manner by both TRIM24 and TRIM33. Interestingly, other members of the lipocalin family such as LCN2 have well-established roles in tumor progression (44, 45). It will be interesting to determine if LCN13 plays a role in HCC.

The additive effect of the compound TRIM24/TRIM33 mutant both on HCC formation and deregulation of gene expression suggests that TRIM24 and TRIM33 can affect distinct pathways that act together to suppress HCC. It is possible therefore that TRIM33 inactivation deregulates genes not affected by TRIM24, and that are perhaps synergistically regulated by both proteins. As TRIM24 represses RA-signaling and TRIM33 modulates the TGF-β signaling pathway (17, 32, 46), the enhanced tumorigenesis in the double-mutant mice may be a consequence of simultaneous alterations in both of these signaling pathways. Further comparative studies of the gene expression profiles of the tumors from each genotype may reveal additional evidence for cooperative modulation of RA and TGF-β signaling and the idea that the TRIM24-TRIM33 complex acts as an integrator of these two signaling pathways. It has previously been shown that TRIM33 inactivation potentiates precancerous lesions induced by oncogenic Kras to induce pancreatic tumors reminiscent of human intraductal papillary mucinous neoplasms (36). It will be interesting therefore to test whether further loss of TRIM24 would potentiate pancreatic tumor formation in the same way as loss of TRIM33 potentiates HCC in TRIM24 mutant animals.

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