Tripartite Motif 24 (Trim24/Tif1α) Tumor Suppressor Protein Is a Novel Negative Regulator of Interferon (IFN)/Signal Transducers and Activators of Transcription (STAT) Signaling Pathway Acting through Retinoic Acid Receptor α (Rarα) Inhibition*

Johan Tisserand 1, Konstantin Khetchoumian 2, Christelle Thibault, Doulaye Dembélé, Pierre Chambon 1, and Régine Losson 1

From the Department of Functional Genomics, Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), CNRS/INSERM/Université Louis Pasteur, BP 10142, 67404 Illkirch-Cedex, Communauté Urbaine de Strasbourg, France

Recent genetic studies in mice have established that the nuclear receptor coregulator Trim24/Tif1α suppresses hepatocarcinogenesis by inhibiting retinoic acid receptor α (Rara)-dependent transcription and cell proliferation. However, Rara targets regulated by Trim24 remain unknown. We report that the loss of Trim24 resulted in interferon (IFN)/STAT pathway over-activation soon after birth (week 5). Despite a transient attenuation of this pathway by the induction of several IFN/STAT pathway repressors later in the disease, this phenomenon became more pronounced in tumors. Remarkably, Rara haplo-deficiency, which suppresses tumorigenesis in Trim24−/− mice, prevented IFN/STAT overactivation. Moreover, together with Rara, Trim24 bound to the retinoic acid-responsive element of the Stat1 promoter and repressed its retinoic acid–induced transcription. Altogether, these results identify Trim24 as a novel negative regulator of the IFN/STAT pathway and suggest that this repression through Rara inhibition may prevent liver cancer.

Hepatocellular carcinoma (HCC)4 is the third leading cause of cancer death worldwide, causing over 660,000 deaths annually (1). Although much is known about the cellular changes that lead to HCC (2) as well as the etiological factors responsible for the majority of the cases (hepatitis B and C viruses infection, aflatoxin exposure, and alcohol abuse, 3), the molecular pathogenesis of HCC remains poorly understood (4, 5).

Initially identified as a fusion partner of B-Raf in T18 oncoprotein in the context of chemically induced HCC in mice (6), tripartite motif (Trim) 24 protein, also known as transcriptional intermediary factor 1α (Tif1α), was subsequently shown to modulate both positively and negatively the transcriptional activities of several nuclear receptors in a ligand-dependent fashion (7–13). Among them, retinoic acid (RA) receptors (Rara, -β, and -γ) control various biological processes, such as development, proliferation, and differentiation. Upon ligand binding (RA, the active derivative of vitamin A), Rars activate transcription of their target genes, including Cyp26a1 encoding an RA catabolic enzyme (for a review, see Ref. 14). Because many transcription factors and signaling molecules are targeted by Rars, the RA pathway can interfere with several other pathways. In particular, RA was demonstrated to synergize with interferon (IFN)/signal transducers and activators of transcription (STAT) signaling and thus to potentate inflammation (Ref. 15 and references therein).

The physiological significance of Trim24-mediated nuclear receptor repression has been demonstrated by recent genetic studies in mice revealing that Trim24 is a potent liver-specific tumor suppressor (16–18). Specifically, it was shown that Trim24−/− mutant mice are highly predisposed to the development of both spontaneous and chemically induced HCC. Hepatocyte-specific, inducible inactivation of Trim24 revealed that it acts as a cell–autonomous tumor suppressor, maintaining the quiescent state of hepatocytes (18). Similarly to models of liver cancer development in humans, the development of HCC in Trim24−/− mice is a multistage process, validating the utility of this mouse model for understanding basic mechanisms of hepatocarcinogenesis in humans (2, 16, 18, 19). As soon as 3
weeks after birth, while hepatocytes are terminating their postnatal development and are entering into quiescence, the first manifestations of the disease, namely increased hepatocyte proliferation, could be observed in Trim24-deficient mice. Increased hepatocyte ploidy (week 14) and preneoplastic (clear cell foci of cellular alterations) and neoplastic lesions (hepato-cellular adenomas and carcinomas) then appear with a highly reproducible kinetics (16–18). Remarkably, the whole process of HCC development in Trim24-deficient mice was shown to depend on overactivated RA signaling. Indeed, all pathological manifestations of Trim24-deficient liver disease described above are completely suppressed upon simultaneous loss of a single copy of the Rara gene (Trim24−/− Rara−/− mutant mice; Refs. 16 and 17). Consistent with this, administration of pharmacological doses of RA stimulates hepatocyte proliferation in wild-type mice, whereas injection of Rar antagonists restore normal hepatocyte proliferation rate in Trim24−/− mice (16, 17). These findings provided the first evidence that Rara can function as an oncogene in vivo and prompted us to examine the molecular mechanisms by which Rara overactivation stimulates hepatocyte proliferation. We have now characterized Trim24/Rara common downstream targets in hepatocytes and regulates hepatocyte proliferation. We have now characterized the molecular mechanisms by which Rara overactivation stimulates liver cell foci of cellular alterations) and hepatocellular proliferation. We have now characterized the molecular mechanisms by which Rara overactivation stimulated liver cell proliferation in wild-type mice, whereas injection of Rar antagonists restore normal hepatocyte proliferation rate in Trim24−/− mice (16, 17). These findings provided the first evidence that Rara can function as an oncogene in vivo and prompted us to examine the molecular mechanisms by which Rara overactivation stimulates hepatocyte proliferation. We have now characterized Trim24/Rara common downstream targets in hepatocytes and discovered that Trim24 is a novel negative regulator of the IFN/STAT signaling pathway, acting through Rara inhibition. Moreover, we found that the overactivation of IFN/STAT signaling pathway in Trim24−/− livers tightly correlates with RA-mediated hepatocarcinogenesis.

EXPERIMENTAL PROCEDURES

Mice and Tissue Samples—Generation of Trim24−/− and Trim24−/−/Rara−/− mice has been described previously (16). Colonies of both lines were maintained under specific pathogen-free conditions at the Mouse Clinical Institute of the Institut de Génétique et de Biologie Moléculaire et Cellulaire. Harvesting of mouse liver tissues was done as described by Khetchoumian et al. (16). Livers were rapidly excised and washed in PBS, and tissue portions were frozen in liquid nitrogen and stored at −80 °C until use for RNA extraction and Western blot analysis. A liver sample (left median lobe or a sample with a macroscopically visible tumor) was fixed in 4% paraformaldehyde and paraffin-embedded, and another sample was optimal cutting temperature-included and frozen for routine histology. All liver samples were subjected to histopathological evaluation before analysis. 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.

RNA Isolation and Quantitative RT-PCR—Total RNA was isolated from liver tissues and cell lines using an RNeasy kit (Qiagen) and TRizol reagent (Invitrogen), respectively. Five micrograms of RNA were used for cDNA synthesis using oligo(dT)24 primer and Superscript II reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. The final product was then diluted 80 times, and 4 μl were mixed with forward and reverse primers listed in supplemental Table S4 (each primer at 250 nM final concentration) and 5 μl of SYBR Green Master Mix (Qiagen). Real time PCR was performed using the LightCycler 1.5 system (Roche Applied Science). Each reaction was performed in triplicate, and the relative expression level of each gene was normalized to the hypoxanthine-guanine phosphoribosyltransferase (Hprt) level.

Microarray Experiments—Gene expression profiling was performed using the Affymetrix GeneChip® Mouse Genome 430 2.0 microarrays. Total RNA was isolated using an RNeasy kit (Qiagen) and quality-controlled by capillary electrophoresis (Agilent 2100 Bioanalyzer and RNA 6000 LabChip® kit). cDNA and cRNA synthesis and labeling as well as array hybridization were performed at the Affymetrix facility of the Institut de Génétique et de Biologie Moléculaire et Cellulaire according to the manufacturer’s instructions (Affymetrix). Raw microarray data were analyzed using the GeneChip Operating Software (GCOS) for normalization, detection calls, and expression values. A present, absent, or marginal call and a value of signal intensity were given for each gene. Changes in expression were first analyzed by analysis of variance and Newman–Keuls (for samples from 5-week-old mice) and Student’s t test (for samples from 14-week-old livers and tumors). Each gene was identified as up-regulated, down-regulated, or not changed based on significance (p < 0.01). We controlled the false discovery rate using sample permutations (false discovery rate <0.014). To eliminate unexpressed genes, a gene must have had a presence call in at least three of the four mutant (or wild-type (WT)) samples. Finally, the average signal intensity of replicates was used to calculate fold change. We selected the most significant changes with an arbitrary threshold of 1.7. In brief, the differentially expressed genes were selected based on p < 0.01, presence call >0.75, and -fold change >1.7. This set of genes was further filtered to eliminate genes without an Entrez Gene ID and to obtain a single entry for genes represented by multiple probe sets. Average linkage hierarchical clustering was performed using Cluster software. Biological process assignments were made using the DAVID bioinformatics tool and Ingenuity Pathway Analysis software.

Liver Protein Extraction and Western Blot Analysis—Liver whole-cell extracts were prepared as described previously (20). Protein concentration was determined using Bio-Rad protein assay reagent. Equal amounts of proteins (100−200 μg) were separated by SDS-PAGE and processed for Western blotting as described by Khetchoumian et al. (20). Rabbit polyclonal antibodies recognizing Stat1 (E-23) and Irf1 (C-20) from Santa Cruz Biotechnology (Santa Cruz, CA) and a monoclonal antibody against β-tubulin (1TUB1A2; a gift from Dr. M. Ould-Abedghani, Institut de Génétique et de Biologie Moléculaire et Cellulaire) were used as primary antibodies. Peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) were used as secondary antibodies. Detection was carried out using an enhanced chemiluminescence (ECL) kit (Amersham Biosciences).

Cell Culture and Transfection—The murine non-transformed hepatocyte cell line AML12 was described previously (21) and obtained from the European Collection of Cell Cultures. Cells were grown at 37 °C in a humidified incubator containing 5% CO2, 95% air in Dulbecco’s minimal essential medium (DMEM)/F-12 medium (1:1) supplemented with 10% fetal bovine serum, 100 μM dexamethasone, 50 μg/ml gentamicin sulfate, and a mixture of insulin, transferrin, and selenium.
(5 μg/ml). Quantitative RT-PCR and chromatin immunoprecipitation (ChIP) experiments were carried out using cells treated with vehicle (ethanol) or 1 μm all-trans-retinoic acid (Sigma) in medium containing delipidated serum.

AML12 cells (2 × 10⁸) were transfected using the Amazxa Nucleofector reagent (Amazxa Biosystems, Lonza) with pSG5-based expression vectors for Rara (0.125 μg) and Rxra (0.125 μg) together with a control empty vector, FLAG-tagged Trim24 wild type (F-Trim24), or Trim24L730A/L731A (8) (4.75 μg) according to the manufacturer’s instructions.

ChIP Assays—ChIP assays were performed as described previously (16) except for some modifications. In brief, cells (2 × 10⁸) were cross-linked with 1% formaldehyde (v/v) for 10 min at 37 °C, resuspended in 450 μl of SDS lysis buffer (Upstate), and incubated at 37 °C for 10 min. Cell lysates were sonicated to obtain DNA fragments of 200–500 bp using the Bioruptor from Diagenode. Sonicated samples were then cleaned by centrifugation at 13,000 rpm for 15 min at 4 °C, and 20% of the cleared supernatant was kept for subsequent purification of input genomic DNA. The remaining chromatin (350 μl) was first pre-cleared overnight with 50 μl of Protein G-Sepharose beads at 4 °C. The beads were removed by centrifugation at 2,000 rpm for 2 min, and 50 μg of sonicated chromatin were then incubated with 25 μl of M2 FLAG affinity agarose beads (Sigma) or non-conjugated beads as a negative control. ChIP was performed by incubation at 4 °C for 2 h in 1 ml of ChIP dilution buffer (16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl, 1.2 mM EDTA, 1% Triton X-100, 0.01% SDS). The immunoprecipitates were collected by centrifugation and sequentially washed in 3 × 1 ml of ChIP low salt buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS), 3 × 1 ml of ChIP high salt buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS), 3 × 1 ml of LiCl buffer (10 mM Tris-HCl, pH 8.0, 250 mM LiCl, 1 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate), and in 2 × 1 ml of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) before being eluted in two consecutive steps by adding 250 μl of elution buffer (1% SDS, 100 mM NaHCO₃) at each step. Eluates and input chromatin were incubated at 65 °C for a minimum of 4 h in the presence of 0.2 mM NaCl to reverse cross-linking of the chromatin. Proteins were digested by addition of 10 μl of 1 M Tris-HCl, pH 6.8; 10 μl of 0.5 M EDTA; and 20 μg of proteinase K and incubation at 42 °C for 1 h. The DNA was recovered by phenol/chloroform extraction and coprecipitation with GlycoBlue (Ambion), dissolved in 100 μl of TE buffer, and analyzed by quantitative PCR with primers flanking the RA-responsive element (RARE) regions of the Stat1 and Cyp26a1 promoters (22, 23) and the Hprt promoter as a control (see supplemental Table S4). Trim24 occupancy of the RARE-containing regions of Stat1 and Cyp26a1 promoters was quantified by normalizing the PCR signals from the immunoprecipitation samples to the PCR signals obtained from both the input DNA and the promoter region of the Hprt housekeeping gene, which showed no change in expression in Trim24⁻/⁻ livers or F-Trim24-transfected AML12 cells and therefore was used as a negative control. Normalization in this way is an effective method to significantly reduce interassay variation arising from extensive manipulations of the chromatin during the experimental processes. Statistical significance was determined by using the Student’s t test on three independent experiments, each performed in triplicate.

**RESULTS**

In an effort to identify Trim24 and Rara downstream targets that may play a role in liver carcinogenesis, we analyzed transcriptomic changes that take place in early precancerous livers of Trim24⁻/⁻ mutants that occurred in a Rara gene dosage-dependent manner. Gene expression profiling experiments were performed comparing liver transcriptomes of week 5 Trim24⁻/⁻ knock-out (KO) or Trim24⁻/⁻/Rara⁻/⁻ “rescued” (R) mice with transcriptomes of WT littermates using the Affymetrix Mouse Genome 430 2.0 microarrays (GSE19675). At this age, no overt liver pathology was detectable in Trim24 KO mice (supplemental Fig. S1), but these mice were characterized by an increased hepatocyte proliferation and an overexpression of numerous RA-responsive genes (16, 17). Because no such defects were observed in age-matched Trim24⁻/⁻/Rara⁻/⁻ rescued mice that are not prone to tumor development (17), we expected to identify oncogenic downstream Rara targets among genes deregulated in KO but not R livers. To identify differentially expressed transcripts, gene lists from different samples were filtered according to multiple criteria as described under “Experimental Procedures.” Briefly, transcripts detectable in at least three of the four samples showing a -fold change ≥ 1.7 with a p value ≤ 0.01 were extracted, giving rise to 194 and 80 unique genes differentially expressed between KO/WT and R/WT, respectively (Fig. 1, A and B). Hierarchical clustering allowed distinguishing six clusters from these gene lists (Fig. 1A). Cluster 5 was by far the largest, containing 122 overexpressed genes in Trim24 KO livers only, including HCC markers Afp and H19 (3.2- and 1.75-fold, respectively; supplemental Table S1). 145 of the 194 genes deregulated in Trim24 KO livers exhibited no significant

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change in Trim24−/− Rara+/− livers, indicating that their deregulation in the absence of Trim24 is reversed upon deletion of a single copy of Rara (Fig. 1, B and C). These genes were therefore referred to as Trim24/Rara-dependent. Of the 80 deregulated genes in R livers, 31 genes did not show any significant deregulation in Trim24 KO livers and were referred to as Trim24-independent/Rara-dependent, whereas the remaining 49 genes were deregulated in both Trim24 KO and Trim24−/− Rara+/− R livers and were referred to as Trim24-dependent/Rara-independent. The complete gene lists are presented in

FIGURE 1. Transcriptional analysis at week 5. A, average linkage hierarchical clustering representing the expression profiles of genes deregulated in week 5 Trim24−/− (KO) and Trim24−/− Rara+/− (R) livers. Each column represents a liver sample, and each row represents a gene. Red and green represent up- and down-regulation, respectively. Six major clusters (1−6) are highlighted at left (see supplemental Table S1 for the complete list). B, Venn diagram indicating the relationships between gene alterations in week 5 KO and R livers with respect to Trim24 and Rara dependence. C, scatter graph showing the extent of the rescue: average (Avg) expression of Trim24/Rara-dependent genes in mutants (blue, KO; red, R) is a function of their expression level in WT. D, Ingenuity Pathway Analysis identification of Trim24/Rara-dependent genes. The bars and the curve represent the number of genes and −log(p value), respectively. Pathways with −log(p) over the base line are significantly altered (p < 0.05). Ag, antigen; IRF, INF Regulated Factor; PKR, Protein kinase R; RIG1, Retinoic Acid inducible Gene 1; ddx58, Asp-Glu-Ala-Asp (DEAD) box protein 58; IL, Interleukin.

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supplemental Table S1. Taken together, these results identify Rara as a major target of Trim24 repression in the liver.

**IFN/STAT Activation at Week 5**—The DAVID bioinformatics tool was used to identify biological processes that could be controlled by Trim24/Rara-dependent genes and revealed a significant enrichment in genes involved in immune-related processes (supplemental Table S2). Using the Ingenuity Pathway Analysis software, we subsequently identified the IFN/STAT signaling pathway ($p < 1.5 \times 10^{-5}$) as well as several IFN/STAT-associated pathways (IFN Regulatory Factor, RIG1, immunodeficiency, antigen presentation, Protein Kinase R IFN induction) as being the most significantly altered (Fig. 1D). This signaling pathway plays key roles in mediating the antiviral, antigrowth, and immunomodulatory activities of IFNs (for a review, see Ref. 24). The transduction of IFN signals in target cells involves activation of Type I and Type II IFN receptors and receptor-associated Janus-activated kinase (JAK), which in turn leads to activation and nuclear translocation of STAT proteins and results in the transcription of IFN-stimulated genes (ISGs).

Comparison of our list of Trim24/Rara-dependent genes with the ISG Database (25) showed 67 overlapping genes with 66 ISGs (1.8-fold) and numerous ISGs, such as Ift1 (10.5-fold), Ifti3 (9.3-fold), Oas1 (5.0-fold), and Isg15 (7.5-fold). Activation of the IFN/STAT pathway was confirmed by quantitative RT-PCR (qPCR) in all Trim24 KO liver samples. qPCR analysis also revealed up-regulation of Ifi1, an important regulator of the IFN pathway (26), and confirmed that expression of Ifi1, Stat1, Oas1, and Ifi1 is not altered in the livers of age-matched Trim24-Rara mutants, indicating that their deregulation in the absence of Trim24 is correlated with Rara activity (Fig. 2A). Importantly, overexpressions of Stat1 (2.2-fold) and Ifi1 (2.3-fold) in Trim24 KO livers were also confirmed at the protein level (Fig. 2B). Knowing that phosphorylation of Stat1 at Ser-727 is a key event of IFN/STAT activation, we investigated this phosphorylation level by Western blotting. We showed an accumulation of phosphorylated Stat1 (Ser-727) in Trim24 KO livers but not in Trim24-Rara livers at week 5 (2.1-fold; Fig. 2B). Taken together, all these data provide compelling evidence for a Rara-dependent overactivation of the IFN/STAT pathway in early pre-cancerous livers of Trim24 KO mice.

**Adaptive Responses at Week 14**—We next investigated whether the activation of the IFN/STAT pathway in Trim24 KO livers is maintained at a later stage of the disease. At week 14, Trim24 KO mice were characterized by increased hepato-cellular proliferation and ploidy (16–18). Liver transcriptomes of week 14 Trim24 KO and control WT littermates ($n = 5$) were determined using the Affymetrix Mouse Genome 430 2.0 microarrays (GSE19675). Only 90 genes were found to be deregulated in the Trim24 KO liver samples (fold change $\geq 1.7$, $p \leq 0.01$); 65 genes were up-regulated and 25 were down-regulated (Fig. 3A). Surprisingly, although HCC markers Afp and H19 remained overexpressed, several up-regulated genes correspond to well documented growth inhibitors, such as Cdkn1a (p21; 7.0-fold; Ref. 27), Bmhc (2.7-fold; Ref. 28), and Hnf6/One-cut1 (4.5-fold; Refs. 29 and 30). Overexpression of these growth inhibitors in hyperproliferating week 14 Trim24-deficient hepatocytes suggests the existence of an adaptive mechanism activated by the liver to fight against hyperproliferation. However, ectopic expression of growth inhibitors is known to alter hepatocyte homeostasis and in particular to lead to increased ploidy (31, 32).

Overall, qPCR analyses revealed that only 14.6% of genes deregulated in week 14 KO livers were Rara-dependent (74.7% at week 5; supplemental Fig. S2A). Moreover, whereas 32.6% of week 5 Trim24-dependent/Rara-independent genes remained deregulated at week 14, only 3.4% of Trim24/Rara-dependent genes were still deregulated at this later stage (supplemental Fig. S2B). Considering that KO but not R livers are prone to tumor development, these results suggest that adaptive mechanisms occur to specifically fight against deleterious Trim24/Rara-dependent deregulations. Supporting this idea, RA pathway hyperactivation that has been described to promote hepatocarcinogenesis was markedly attenuated at week 14 as revealed by the overlapping of our list of deregulated genes with the Balmer and Blomhoff (33) RA target gene database (supplemental Fig. S2C). Likewise, of the 90 aberrantly expressed genes in week 14 KO livers, only 10 genes were present in the ISG Database (Ref. 25 and Fig. 3B), including Usp18/Ubhp43 recently described as a negative regulator of IFN signaling (34). We carried out qPCR validation for Usp18 (Fig. 3C) and investigated the expression levels of four additional negative regulators of the IFN/STAT pathway: Socs1, Socs2 (35), Pias1, (36), and Pp2ac (37). Contrary to the situation at week 5, Socs1 and Socs2 both exhibited increased expression in KO livers (2.0- and 1.8-fold) and may thus be responsible for the marked attenuation of the IFN/STAT pathway observed at week 14 as compared with week 5 Trim24 KO livers (Table 1). Taken together, these results show that the RA and IFN/STAT pathways are synchronized and strongly suggest the activation of adaptive response in reaction to Trim24/Rara-dependent tumorigenic deregulations at week 14. If confirmed and generalized, this feed back mechanism (occurring while HCC marker overexpressions still allow diagnosis of HCC predispositions) could be targeted (and promoted) in future cancer-preventive therapies.

**IFN/STAT Activation in HCC**—This attenuation led us to examine the status of the IFN/STAT pathway in HCCs from Trim24 KO mice (GSE9012; Ref. 17). 129 components of the IFN/STAT pathway, including the IFNγ receptors 1 (Ifngr1; 3.2-fold) and 2 (Ifngr2; 6.1-fold), Jak2 (2.3-fold), Stat1 (4.7-fold), and Stat2 (2.1-fold) as well as numerous ISGs common to week 5 Trim24/Rara-dependent genes were found to be deregulated in HCCs (Table 1 and supplemental Table S3). We validated a subset of these genes by qPCR and identified a decrease in expression of the negative regulators Socs1 and Socs2 (2.1- and 1.7-fold; supplemental Fig. S3). Therefore, the progression of Trim24-deficient hepatocytes toward HCC seems to require reactivation of the IFN/STAT pathway (Fig. 3D). In agreement with this idea, a transcriptomics analysis of 43 human HCCs revealed that 65% of tumors (28 HCCs) exhibit overexpression of ISGs (38). Interestingly, we noticed that Trim24 down-regu-
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| Gene symbol | Description | Affy ID | Week 5 | Week 14 | Tumors |
|-------------|-------------|---------|--------|---------|--------|
| 2810417H13Ri | RIKEN cDNA 2810417H13 gene | 1419153_at | 1.87 | — | — |
| A630077B13Ri | RIKEN cDNA A630077B13 gene | 1436576_at | 2.81 | — | — |
| Agrn | Agrin | 1426670_at | 1.87 | — | — |
| Apol9b | Apolipoprotein L, 9b | 1424518_at | 3.27 | — | — |
| BCO31672 | cDNA sequence BC031672 | 1451777_at | 4.29 | — | — |
| Blnk | B-cell linker | 1419780_at | 4.14 | 4.92 | — |
| Bta2 | Bone marrow stromal cell antigen 2 | 1429215_at | 2.35 | — | — |
| Cflr1 | Complement factor H-related 1 | 1419436_at | 2.08 | — | — |
| Csrp1 | Cysteine- and glycine-rich protein 1 | 1425811_at | 1.81 | — | — |
| Cxcl10 | Chemokine (C-X-C motif) ligand 10 | 1418930_at | 4.37 | — | — |
| Ddx58 | DEAD (Asp-Glu-Ala-Asp) box polypeptide 58 | 1365662_at | 1.91 | — | — |
| Ddx58 | DEXH (Asp-Glu-X-His) box polypeptide 58 | 1451426_at | 4.33 | — | — |
| Eps1l | Epithelial stromal interaction 1 | 1454169_at | 3.19 | — | — |
| Fbn1 | Fibronectin 1 | 1460208_at | 2.70 | — | — |
| Ggpt4 | Guanylate nucleotide-binding protein 1 | 1420549_at | 2.79 | — | — |
| Ggpt2 | Guanylate nucleotide-binding protein 2 | 1435906_at | 4.00 | — | — |
| Ggpt3 | Guanylate nucleotide-binding protein 3 | 1418392_at | 5.16 | — | — |
| Gloss | Glutamate-ammonia ligase (glutamine synthetase) | 1420635_at | 0.58 | 0.12 | — |
| Hcr5 | Hect domain and RLD 5 | 1438037_at | 5.99 | — | — |
| Ifi27 | Interferon-α-inducible protein 27 | 1426278_at | 3.58 | — | — |
| Ifi35 | Interferon-induced protein 35 | 1445897_at | 2.14 | — | — |
| Ifi44 | Interferon-induced protein 44 | 1423555_at | 6.47 | 5.64 | — |
| Ifi47 | Interferon-γ-inducible protein 47 | 1417292_at | 3.13 | — | — |
| Ifi1h | Interferon-induced with helicase C domain 1 | 1426276_at | 2.29 | 1.99 | — |
| Ifi12l | Interferon-induced protein with tetratricopeptide repeats | 1450783_at | 15.90 | 6.13 | — |
| Ifi2 | Interferon-induced protein with tetratricopeptide repeats | 1418293_at | 5.28 | — | — |
| Ifit3 | Interferon-induced protein with tetratricopeptide repeats | 1440025_at | 9.31 | — | — |
| Igf1 | Interferon-γ-induced GTPase | 1417411_at | 2.73 | — | — |
| Igf2 | Interferon-γ-induced GTPase 1 | 1419042_at | 1.87 | — | — |
| Igf2 | Interferon-γ-induced GTPase 2 | 1417793_at | 2.82 | — | — |
| Igf7 | Interferon regulatory factor 7 | 1417344_at | 4.67 | — | — |
| Igf9 | Interferon regulatory factor 9 | 1421322_at | 1.72 | — | — |
| Iggn | Immunity-related GTPase family, M | 1418825_at | 2.37 | — | — |
| Igf15 | ISG15 ubiquitin-like modifier | 1431591_at | 7.54 | — | — |
| Igfl3 | Lectin, galactose-binding, soluble 3 | 1426088_at | 2.79 | — | — |
| Lgals3 | Lectin, galactose-binding, soluble, 3-binding protein | 1448380_at | 2.20 | — | — |
| Ly6a | Lymphocyte antigen 6 complex, locus A | 1417185_at | 5.24 | — | — |
| Mbnm | Moloney leukemia virus 10 | 1416380_at | 1.73 | 2.12 | — |
| Mek44c | Membrane-spanning 4 domains, subfamily A, member 4 | 1450291_at | 5.70 | — | — |
| Mmyh10 | Myosin, heavy polypeptide 10, non-muscle | 1452740_at | 1.94 | — | — |
| Nmi | N-myric (and STAT) interactor | 1423519_at | 1.88 | — | — |
| Nuna1 | Nuclear mitotic apparatus protein 1 | 1438027_at | 3.59 | — | — |
| Oas1a | 2,5-Oligoadenylate synthetase 1A | 1424775_at | 5.05 | 6.02 | — |
| Oas1l | 2,5-Oligoadenylate synthetase-like 1 | 1424339_at | 5.53 | — | — |
| Parp12 | Poly(ADP-ribose) polymerase family, member 12 | 1426774_at | 2.33 | — | — |
| Phf11 | PHD finger protein 11 | 1438868_at | 1.86 | — | — |
| Plac8 | Placenta-specific 8 | 1415335_at | 1.87 | — | — |
| Plcl1 | Placenta 1 | 1452178_at | 1.75 | — | — |
| Ppp2r2 | Phosphoribosyl pyrophosphate synthetase 2 | 1420638 at | 1.79 | — | — |
| Pomp10 | Proteasome (prosome, macropain) subunit, β type 10 | 1448632_at | 1.81 | — | — |
| Pomp8 | Proteasome (prosome, macropain) subunit, β type 8 | 1429692_at | 2.08 | — | — |
| Pomp9 | Proteasome (prosome, macropain) subunit, β type 9 | 1405066_at | 2.84 | — | — |
| Rtp4 | Receptor transporter protein 4 | 1418580_at | 3.57 | — | — |
| S100a11 | S100 calcium-binding protein A11 (calgizzarin) | 1460351_at | 2.51 | 3.66 | 17.15 |
| Samd4f | Sterile α motif domain-containing 9-like | 1460603_at | 2.66 | — | — |
| Samd1d | SAM domain and HD domain, 1 | 1418131_at | 1.74 | — | — |
| Scotin | Scotin gene | 1423986_at | 2.44 | — | — |
| Stat1 | Signal transducer and activator of transcription 1 | 1450033_at | 3.00 | — | 4.72 |
| Tap1 | Transporter 1, ATP-binding cassette, subfamily B (MDR) | 1416016_at | 2.90 | — | — |
| Tgb | Transforming growth factor, β induced | 1448123_at | 1.71 | — | — |
| Tgtp | T-cell-specific GTPase | 1449009_at | 5.74 | — | — |
| Tlr2 | Toll-like receptor 2 | 1419132_at | 1.92 | — | — |
| Traf1 | TRAF type zinc finger domain-containing 1 | 1428346_at | 1.90 | — | — |
| Trim26 | Tripartite motif protein 26 | 1424929_at | 1.72 | — | — |
| Trim34 | Tripartite motif protein 34 | 1421530_at | 3.95 | — | 2.88 |
| T乏力 | Thymidylate family kinase LPS-inducible member | 1450484_at | 4.44 | — | — |
| Ubel1 | Ubiquitin-activating enzyme E1-like | 1426970_at | 2.61 | — | — |
| Usp18 | Ubiquitin-specific peptidase 18 | 1418191_at | 4.88 | 2.40 | 5.72 |

*Fold change values correspond to the average of the values for all the mutant (or tumor)/control sample comparisons (n = 4 or 5); —, does not meet the criteria of statistical significance. HD, Hydroxyl Domain; MDR, MultiDrug Resistance; PHD, Plant Homeodomain; SAM, Sterile Alpha Mot; TRAF, Tumor Necrosis Factor (TNF) Receptor Associated Factor; ISG, IFN Stimulated Gene; RLD, Regulator of Chromosome condensation 1 (RCCC1)-like Domain.

The overexpression of the IFN/STAT pathway is an important evolutionarily conserved component of hepatocarcinogenesis (Fig. 3D).
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A

Relative mRNA expression (arbitrary units)

Stat1

Oas1

WT

Trim24−/−

Trim24−/− Rara+/−

Ifit1

Irf1

B

Stat1

p-Stat1

Irf1

β-tubulin

WT

Trim24−/−

Trim24−/− Rara+/−

FIGURE 2. Validation of deregulated expression of genes involved in IFN/STAT signaling pathway. A, qPCR experiments confirming that Stat1, Oas1, Ifit1, and Irf1 are up-regulated in livers of week 5 Trim24−/− but not Trim24−/− Rara+/− mice. Expression was analyzed in triplicate and is relative to Hprt (Error bars represent standard error; *, p < 0.01; **, p < 0.001). B, immunoblot analysis of liver protein extracts from week 5 Trim24−/−, Trim24−/− Rara−/−, and control (WT) mice using the indicated antibodies against Stat1, phosphorylated Stat1 (p-Stat1) (Ser-727), and Irf1; β-tubulin was used as a loading control (see “Experimental Procedures”). Western blots were performed in triplicate with liver samples from three different animals.

To further investigate the mechanism of this inhibition, we next studied the recruitment of Trim24 and Rara to the Stat1 promoter region using ChIP technology. As a control, we examined in parallel the binding of Trim24 and Rara to a well characterized RA target, the Cyp26a1 promoter (17). Although Rara was shown to occupy RARE of the endogenous Stat1 promoter both in the presence and absence of RA, we observed a ligand-dependent recruitment of Trim24 to Stat1 RARE (Fig. 4B). This recruitment was not observed for Trim24L730A/L731A whose capacity to interact with Rara was abolished (Fig. 4B). Similar results were obtained on the Cyp26a1 RARE, validating the reliability of the technology used and the promoter occupancies (Fig. 4B). Taken together, these results show that Trim24 is a direct repressor of RA-mediated transactivation of the Stat1 gene and therefore a negative regulator of the IFN/STAT signaling pathway through Rara inhibition.

DISCUSSION

In the present study, we aimed to further characterize the molecular functions of the Trim24 tumor suppressor protein as well as to identify Trim24 targets genes. Among them, Rara-dependent targets should likely include those controlling hepatocyte proliferation and hepatocarcinogenesis because deletion of a single Rara allele in a Trim24-null background completely reverses spontaneous liver tumor development (16, 17). Transcriptomics analyses at different stages of the disease revealed Rara-dependent overexpression of 66 and 129 components of the IFN/STAT pathway upon loss of Trim24 at week 5 and in HCC, respectively. By performing ChIP experiments in a murine hepatic cell line (AML12), we demonstrated that Rara is bound to a RARE element of the Stat1 promoter. Hence, upon RA treatment, Rara can directly activate Stat1 expression and consequently IFN/STAT signaling pathway in hepatic cells. However, in the presence of RA, Trim24 was also recruited to the Stat1 RARE and attenuated this RA-mediated activation. This recruitment and consequent inhibition of transcription required the ability of Trim24 to interact with Rara. Therefore, we identified Trim24 as a novel negative regulator of the IFN/STAT signaling pathway acting through Rara inhibition (see the model in Fig. 5). Our model is based on the presence of an active IFN/STAT signaling pathway in the liver cells, which was confirmed by the detection of Ser-727 phosphorylated Stat1 as well as Ifna and Ifng expression in livers of every studied genotype (Fig. 2B). Although it has been established that RA can synergize with IFNs to induce the expression of Stat1 targets, it is to our knowledge the first time that an activation of RA signaling has been described to be sufficient to activate the IFN/STAT signaling pathway.

Molecules inducing IFN/STAT antiviral activity are administered to treat viral hepatitis and are thought to participate to antitumoral defense in HCC. However, four observations emphasize the need to carefully re-evaluate the use of these drugs in regard to their possible involvement in liver tumor promotion. (i) Overactivation of the IFN/STAT pathway was very pronounced in Trim24 KO HCC. In total, 129 genes of the pathway were found to be deregulated in the sense of the acti-

regulate Stat1 transcription, an RARE was identified in the promoter region of Stat1 (22). This led us to examine the effect of RA on Stat1 expression in the murine non-transformed hepatocyte cell line AML12, which was transiently transfected with an expression vector for Rara and its heterodimeric partner Rxra. Similarly to the well known direct RA target genes Cyp26a1 and Stra6, Stat1 was rapidly up-regulated after 3 h of treatment with RA, whereas longer treatment was required for the induction of Stat1 downstream targets Irf1, Oas1, and Ifit1 (Fig. 4A). Coexpression of Trim24 led to a significant attenuation of the RA-induced increase of mRNA levels of all genes tested (Fig. 4A). Importantly, a Trim24 mutant that does not interact with Rara (Trim24L730A/L731A; Ref. 8) lost its ability to repress RA-mediated transactivation (Fig. 4A). These results convincingly show that Trim24 can inhibit the RA-dependent activation of the IFN/STAT pathway in hepatic cells and dem-

onstrate that this down-regulation requires the full capacity of Trim24 to directly bind to Rara.
vation of the pathway (i.e. up-regulation of activators and target genes and down-regulation of repressors). (ii) This overactivation took place very early in tumorigenesis (5 weeks) and is highly correlated to the tumor-promoting activation of RA signaling as revealed by the time course of gene deregulations in Trim24−/− livers. (iii) Tumor suppressors and growth inhibitors (p21, Bmyc, and Hnf6/Onecut) were induced together with IFN/STAT pathway repressors (Usp18, Socs1, and Socs2) following initial activation of the pathway. This resulted in a marked attenuation of the IFN/STAT pathway (week 14) and strongly suggests the existence of protective mechanisms to fight against deleterious effects of these overactivations on liver.

FIGURE 3. Evolution of transcriptomes of Trim24-deficient mice during hepatocarcinogenesis and comparison with human HCCs. A, average linkage hierarchical clustering of the 90 genes that were significantly deregulated in week 14 Trim24−/− (KO) liver samples. Each column represents a liver sample, and each row represents a gene. Red and green represent up- and down-regulation, respectively. B, genes involved in the IFN response deregulated in week 14 Trim24 KO livers. A list of genes up- or down-regulated by a factor >1.7 (p ≤ 0.01) found in the IFN-regulated gene database is shown. Fold change values (FC) correspond to averages of all the KO/WT sample comparisons (n = 5). C, expression analysis of Usp18, Socs1, Socs2, PIAS1, and Pp2ac by qPCR in livers of week 14 WT and Trim24−/− mice (n = 4). Expression was analyzed in triplicate and normalized to Hprt level (Error bars represent standard error; *, p < 0.005; **, p < 0.001). D, IFN/STAT overactivation during hepatocarcinogenesis in mice and humans. A summarizing scheme representing the activation levels of the IFN/STAT pathway relative to normal hepatic tissue at different stages of liver disease in Trim24-deficient mice (upper part) and in 28 cases of human HCCs (lower part) described by Breuhahn et al. (38). Footnote 1, according to Breuhahn et al. (38).
FIGURE 4. Trim24 functions as a repressor of IFN/STAT pathway induction by RA in liver-derived AML12 cells. A, forced expression of Trim24 in AML12 cells inhibits RA-dependent transactivation of IFN pathway genes. AML12 cells (2 × 10⁶) were transfected using the Amaxa Nucleofactor (Amaxa Biosystems, Lonza) with pSG5-based expression vectors for Rara (0.125 μg) and Rxra (0.125 μg) together with a control empty vector, F-Trim24, or F-Trim24L730A/L731A defective in Rara interaction (F-Trim24LL/AA) (4.75 μg). Cells were treated with RA or vehicle for 3 (top) or 24 h (bottom). Expression was analyzed by qPCR in triplicate and normalized to Hprt level (Error bars represent standard error; *, p < 0.05; **, p < 0.005). B, ligand-dependent recruitment of Trim24 to the RARE region of Stat1 promoter. Left, schematic representation of Cyp26a1 and Stat1 promoters. Right, anti-FLAG ChIP from AML12 cells transfected with either a control empty vector (−), F-Trim24 (+), F-Trim24L730A/L731A, or F-Rara together with Rara and Rxra. Immunoprecipitated and input materials were analyzed by qPCR using primers specific for the RARE-containing regions of Cyp26a1 (positive control) and Stat1. Results shown as relative enrichments as compared with Hprt (negative control; horizontal line) are the mean of three independent experiments, each performed in triplicate (Error bars represent standard error; *, p < 0.05).
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FIGURE 5. Model of Trim24 limiting IFN/STAT pathway through inhibition of Rara activity. RXR, retinoid X receptor.

homeostasis. (iv) The early up-regulation of the IFN/STAT pathway was not observed in Trim24+/− Rara+/− livers, which are not prone to tumor development. All these results suggest that the IFN/STAT pathway could favor hepatocarcinogenesis. In agreement with this idea, interferon treatment of some human patients has been shown to result in the rapid growth of HCC (40). Altogether, these data suggest that the IFN/STAT pathway is an evolutionarily conserved promoter of hepatocarcinogenesis. In support to this, the IFN/STAT pathway has been proposed to be oncogenic (41–45). In particular, in gastric and colorectal cancer models, restoring a normal activation level of Stat1 results in reduction or suppression of carcinogenesis (44, 45).

Several other correlative studies demonstrate the involvement of the IFN/STAT pathway in hepatocarcinogenesis. The human HCC marker Fat10 was demonstrated to be induced by TNFα/IFNγ synergy (46). More strikingly, Yoshikawa et al. (47) first reported that SOCS1 is often silenced by methylation in human HCC and shows growth-suppressive activity, suggesting the requirement of a constitutively activated STAT pathway in the development of human HCC. These discoveries are consistent with the down-regulation of Socs1 that we observed in Trim24 KO HCC as well as with its overexpression at week 14 as a probable feedback. Furthermore, a genetic study revealed that IFNγ is critically involved in the initiation stage of chemically induced hepatocarcinogenesis (48). Most importantly, gene expression profiling of 43 human HCCs revealed that 65% of these tumors exhibit overexpression of ISGs (38). Interestingly, we noticed that Trim24 down-regulation is part of a highly discriminative transcriptional signature of this tumor subgroup. The correlation between IFN/STAT activation and Trim24 underexpression in these HCCs strongly supports our proposal and once more suggests that basic mechanisms of hepatocarcinogenesis are evolutionarily conserved.

However, it still remains elusive which steps of carcinogenesis are controlled by IFN/STAT pathway. Mice lacking Socs1 display an increased Stat1 activation level and spontaneously develop colorectal carcinomas, a phenotype suppressed by concomitant Ifng inactivation (44). The observation that these animals display an elevated intestinal epithelium proliferation level even in non-tumoral tissues (44) suggests that overactivation of IFN/STAT pathway could be mitogenic. On the other hand, recent works on aggressive tumor clones revealed that IFN/STAT activation promotes metastatic potential and resistance to chemotherapy and radiation (49, 50). Indeed, stable knockdown of Stat1 reversed the aggressive phenotype and decreased both colonization and resistance to therapy (50). Therefore, the IFN/STAT pathway seems to be involved in different stages of carcinogenesis. This is in good agreement with our discoveries showing IFN/STAT activation in both pretumoral hyperproliferating livers (week 5) and HCCs and underlines the crucial importance of a balanced IFN/STAT signaling.

Molecular mechanisms leading to tumorigenesis following abnormal IFN/STAT activation remain to be clarified, but several mediators could be proposed based on very recent reports. The Isg15/Herc5/Usp18 pathway is activated by IFN/STAT signaling and modulates various cellular processes, including immune response by deubiquitination and prevention of degradation (51). In tumors, activation of this pathway is a marker of chemo- and IFN resistance (51). In the liver it is exploited by hepatitis C virus to evade the immune response and was demonstrated to inhibit the apoptotic effect of Type I IFN (51, 52). Interestingly, all three IFN/STAT-inducible components of this pathway were overexpressed in a Rara-dependent manner in week 5 Trim24−/− livers (Isg15, 7.54-fold; Herc5, 5.98-fold; and Usp18, 4.88-fold) and tumors (10.17-, 4.79-, and 3.69-fold, respectively). Therefore, it is tempting to speculate that activation of this pathway might be established in Trim24 KO HCC to circumvent IFN/STAT antitumoral activity. Moreover, Usp18 has been recently demonstrated to enhance epidermal growth factor receptor (EGFR) mRNA translation, thus promoting tumorigenic activity, cancer cell survival, and cell proliferation (53). This discovery hence makes Usp18 an interesting candidate for mediating IFN/STAT-induced hepatocarcinogenesis in Trim24 KO mice.

Additional genetic experiments are required to fully establish IFN/STAT pathway as oncogenic in the liver. Mice lacking Stat1 show hypersensitivity to opportunistic infections (especially mycobacterial infection) but are otherwise normal, do not present any developmental defect, and are indistinguishable from their WT littermates in a pathogen-free environment (54, 55). The generation of Trim24/Stat1 double knock-out mice (work in progress) and the investigation of their liver phenotype should represent a decisive step to characterize the oncogenic potential of the IFN/STAT signaling pathway in the liver. Interestingly, an anti-IFNγ antibody that is being tested in patients for Crohn disease has been proposed as a candidate molecule for the inhibition of the IFN/STAT pathway in colorectal can-
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