Arginine methylation regulates c-Myc–dependent transcription by altering promoter recruitment of the acetyltransferase p300

Protein arginine methyltransferase 1 (PRMT1) is an essential enzyme controlling about 85% of the total cellular arginine methylation in proteins. We have shown previously that PRMT1 is an important regulator of innate immune responses and that it is required for M2 macrophage differentiation. c-Myc is a transcription factor that is critical in regulating cell proliferation and also regulates the M2 transcriptional program in macrophages. Here, we sought to determine whether c-Myc in myeloid cells is regulated by PRMT1-dependent arginine methylation. We found that PRMT1 activity was necessary for c-Myc binding to the acetyltransferase p300. PRMT1 inhibition decreased p300 recruitment to c-Myc target promoters and increased histone acetyltransferase p300 recruitment to c-Myc target promoters. PRMT1 inhibition decreased the transcription of several target genes, including peroxisome proliferator-activated receptor γ (PPARG) and mannose receptor C-type 1 (MRC1), suggesting that PRMT1 is necessary for c-Myc function in M2 macrophage differentiation. Of note, in primary human blood monocytes, p300–c-Myc binding was strongly correlated with PRMT1 expression, and in liver sections, PRMT1, c-Myc, and M2 macrophage levels were strongly correlated with each other. Both PRMT1 levels and M2 macrophage numbers were significantly lower in livers from individuals with a history of spontaneous bacterial peritonitis, known to have defective cellular immunity. In conclusion, our findings demonstrate that PRMT1 is an important regulator of c-Myc function in myeloid cells. PRMT1 loss in individuals with cirrhosis may contribute to their immune defects.

Protein arginine methylation is a common posttranslational modification that plays a role in multiple pathways, including cell cycle control, RNA processing, and DNA replication. PRMT1 is responsible for about 85% of the total cellular arginine methylation. It methylates both histone and non-histone proteins; however, many protein targets have not yet been defined. Arginine methylation impacts gene transcription and splicing as well as upstream signal transduction including a number of innate immunity pathways. The abnormal function of PRMT1 is closely associated with several types of cancer and cardiovascular disease, suggesting a relationship between arginine methylation and both inflammation and cell growth.

c-Myc is a proto-oncogene that is associated with tumor development and is involved in pathways important for cell growth and survival. c-Myc, together with its partner, Max, can induce expression of a large number of genes. In addition, c-Myc can suppress the expression of genes through binding to Miz1. More recently, a number of studies have reported a key role of c-Myc in myeloid cell survival and function.

We recently discovered that the protein arginine methyltransferase PRMT1 reversibly regulates a broad array of Toll-like receptor-dependent innate immune signaling pathways via methylation of TRAF6 (11) and regulation of PPARγ expression (12). This latter function is necessary for survival in vivo models of septic shock, but the detailed mechanism of PRMT1-dependent PPARγ regulation was not studied. PPARγ is induced during M2 macrophage differentiation and is regulated by a number of transcription factors including STAT6, IRF4, and c-Myc. We found that c-Myc and PRMT1 expression. This PRMT1–c-Myc axis thus

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2 The abbreviations used are: PPARγ, peroxisome proliferator-activated receptor γ; SBP, spontaneous bacterial peritonitis; PLA, proximity ligation assay.

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appears to be a factor altering innate immune responses both \textit{in vitro} and \textit{in vivo}.

\textbf{Results}

\textbf{Arginine methylation is required for c-Myc–p300 interaction}

We recently discovered that the protein arginine methyltransferase PRMT1 regulates innate immune signaling pathways in monocytes and macrophages via regulation of the expression of a number of target genes (11, 12). However, the detailed mechanism of PRMT1-dependent regulation of gene expression was not entirely clear. Some studies suggest that PRMT1 is recruited to the promoters of target genes by a transcription factor, where it then modifies both histones and transcription factors to facilitate gene expression (18, 19). c-Myc is an important transcription factor in regulating myeloid cell functions (8–10). Previous studies on c-Myc and N-Myc regulation by PRMT1 suggest that PRMT1 regulates Myc gene expression (20, 21) and function (21).

To determine how PRMT1 might affect c-Myc, we analyzed c-Myc expression in THP-1 cells by immunofluorescence in the presence of the PRMT1 inhibitor AMI-1. AMI-1 treatment did not change the expression or subcellular localization of c-Myc (Fig. 1A). Next we examined the effect of AMI-1 on the binding of c-Myc to the transcriptional cofactors Max, Miz-1, p300, and HDAC1. We found that the c-Myc–p300 interaction in THP-1 cells was reduced by AMI-1 (Fig. 1, B and C). We also observed a decrease in c-Myc–Max binding, but there was no effect of AMI-1 on the c-Myc–HDAC1 interaction (Fig. 1B). Fig. 1D shows detection of the PRMT1–c-Myc interaction using a proximity ligation assay (PLA), seen as brownish red dots throughout the cell. Similar to the immunoprecipitation results, the PLA signal for c-Myc–p300 and c-Myc–Max interactions was abolished by AMI-1 (Fig. 1E). The c-Myc–Miz1 PLA signal was not changed. Additionally, we found that c-Myc–p300 and c-Myc–Max but not c-Myc–HDAC1 interactions were reduced in peritoneal macrophages isolated from PRMT1 myeloid KO mice (Fig. 1F).

These results suggest that arginine methylation is required for c-Myc–p300 binding. To determine whether c-Myc is a target of PRMT1, we purified c-Myc protein and analyzed it by mass spectrometry. We found that c-Myc is arginine-methylated on two residues that are located in the C-terminal portion of its transactivation domain and involved in the c-Myc–p300 interaction (Fig. 2, A and B).

To determine whether PRMT1 levels actually correlate with the c-Myc–p300 interaction in primary monocytes and confirm the relevance of the above findings to humans, we measured p300–c-Myc binding in human primary monocytes using a modified sandwich enzyme-linked immunosorbent assay (ELISA). We observed wide variations in the PRMT1 levels among the tested individuals, with a significant correlation between p300–c-Myc binding and PRMT1 expression in these samples (Fig. 2C).

\textbf{Arginine methylation is required for c-Myc–p300 interaction at target gene promoters}

Next we tested the effects of PRMT1 inhibition on p300 binding to c-Myc target gene promoters. Loss of methylation did not affect c-Myc promoter binding to its target genes but decreased p300 and, to a lesser extent, Max recruitment to \textit{CCNB1} and \textit{CDK4} promoters, as evidenced by p300 chromatin immunoprecipitation (ChIP) (Fig. 3A). The loss of p300 promoter binding was due to its failure to bind c-Myc; this was shown by ChIP–re-ChIP experiments with c-Myc ChIP followed by p300 re-ChIP (Fig. 3B). Reduced binding of p300 might allow for binding of other factors such as HDAC1, similar to the previously described switch from Myc-p300 to Myc-HDAC1 at the SNAIL, ZEB1, and ZEB2 promoters (22). Indeed, AMI-1 did increase HDAC1 binding at the c-Myc target gene promoters \textit{CCNB1} and \textit{CDK4} (Fig. 3, A and B). This combination of a decrease in p300 and an increase in HDAC1 recruitment resulted in reduced levels of histone H3K27 acetylation (H3ac) at these promoters (Fig. 3A). Similarly, c-Myc also bound to \textit{PPARG}, \textit{STAT6}, and \textit{MRC1} promoters, consistent with previously published data (10) (Fig. 3C). We also detected a significant ability of AMI-1 to reduce c-Myc–p300 recruitment to the \textit{PPARG} and \textit{MRC1} promoters but not to the \textit{STAT6} promoter (Fig. 3, C and D).

\textbf{Arginine methylation regulates c-Myc transcripational activity}

To assess the effect of methylation-dependent changes in p300/HDAC1 recruitment and histone acetylation on c-Myc target gene mRNA expression, we performed a series of experiments in which we measured target gene mRNA after overexpression of c-Myc before and after inhibition of methylation with AMI-1. Upon initial characterization of the effects of c-Myc overexpression in this system, we unexpectedly observed that overexpression of c-Myc led to a decrease in PRMT1 expression both at the protein and mRNA levels (Fig. 4A). This ability of c-Myc to suppress PRMT1 complicated the use of c-Myc overexpression as a tool to determine how c-Myc effects are modulated by its own arginine methylation.

To overcome this problem, we compared the effects of the methylation inhibitor on c-Myc overexpression–driven gene expression with and without the simultaneous overexpression of PRMT1. Fig. 4B demonstrates these results. c-Myc was overexpressed in either untreated THP-1 cells or in cells treated with AMI-1. c-Myc overexpression stimulated the transcription of \textit{CCNB1}, \textit{CDK4}, and the \textit{PPARγ} target gene, \textit{CD209} (Fig. 4B), but the magnitude of this stimulation was small. The combination of PRMT1 and c-Myc co-expression resulted in a significantly higher increase in the mRNA levels for all genes except \textit{STAT6}. Because \textit{CD209} is not a direct target of c-Myc, the effect on its expression could be due to \textit{PPARγ} up-regulation. In all cases this stimulation was mediated by PRMT1 enzymatic activity, as the addition of AMI-1 abolished the effect (Fig. 4B). Because c-Myc regulates M2 macrophage differentiation through \textit{PPARγ} and other M2 gene expression including \textit{MRC1} (10), we measured the expression of MRC1 in THP-1 macrophages expressing c-Myc, PRMT1, or both in the presence or absence of the PRMT1 inhibitor AMI-1 (Fig. 4C). We found that c-Myc overexpression induced MRC-1 to a higher level in the presence of PRMT1 overexpression, and induction was abolished in the presence of the methylation inhibitor (Fig. 4C).
Figure 1. Arginine methylation regulates c-Myc cofactor binding. A, representative images of c-Myc immunofluorescence analysis in untreated or AMI-1–treated (50 μM for 16 h) THP-1 cells. B, Western blot analysis of immunoprecipitation (IP) using anti-c-Myc antibody or IgG as a negative control in untreated or AMI-1–treated (10 and 50 μM for 16 h) THP-1 cells. Lower panels show the densitometry quantification of the p300/Max/HDAC1 signal from three independent immunoprecipitation experiments. Data are presented as mean ± S.D. **, p < 0.01. n.s., not significant. C, Western blot analysis of immunoprecipitation using anti-p300 antibody or IgG as a negative control in untreated or AMI-1–treated (50 μM for 16 h) THP-1 cells. D, representative images of PLAs in THP-1 cells. c-Myc interaction with PRMT1 was detected using anti-PRMT1 and anti-c-Myc antibodies. Negative control, signal in the absence of primary antibodies. E, representative immunoblots of PLAs for c-Myc interaction with p300, Max, or Miz1 in THP-1 cells untreated or treated with 50 μM AMI-1 were detected using anti-p300, anti-Max, or anti-Miz1 and anti-c-Myc. The average number of PLA-positive THP-1 cells is shown for three independent experiments (right). Data are presented as mean ± S.D. **, p < 0.01. F, Western blot analysis of immunoprecipitation using anti-c-Myc antibody or IgG as a negative control in wild-type and PRMT1 knock-out macrophages.
An R299K-R346K mutant c-Myc is deficient in the ability to recruit p300 to CCNB1 and CDK4 promoters

To verify the functional role of c-Myc arginine methylation at Arg-299 and Arg-346, we mutated both residues to lysine and tested single and double mutants for function. First, we tested whether these mutations abolish c-Myc arginine methylation as measured by PLA. Fig. 5A shows that these mutations had no effect on the PLA signal resulting from the proximity of c-Myc and Max or c-Myc and HDAC1 but did show reduced p300 binding when both sites were mutated (Fig. 5A).

Next we tested the ability of the mutants to recruit p300 to c-Myc target promoters. All three mutants showed normal binding to the CCNB1 and CDK4 promoters (Fig. 5B). However, ChIP–re-ChIP experiments showed that unlike the wild-type protein, the R299K-R346K double mutant lost the ability to recruit p300 to both promoter sites. Consistent with this result, double mutant c-Myc was less efficient in inducing the transcription of CCNB1 and CDK4 mRNA, especially in the presence of PRMT1 (Fig. 5C), although the mutation did not abolish the ability of PRMT1 to induce those genes completely. Taken together, these data suggest that PRMT1-dependent methylation of c-Myc at Arg-299 and Arg-346 controls both the recruitment of p300 to c-Myc target gene promoters and the expression of these genes.
PRMT1 is down-regulated in liver cells from individuals with decompensated cirrhosis

Individuals diagnosed with cirrhosis have a number of well-known abnormalities in their response to bacterial infections (23). Spontaneous bacterial peritonitis is an extreme example of an infectious complication that results, in part, from the immune system defects in individuals with advanced cirrhosis. To evaluate whether PRMT1-dependent regulation correlates with innate immune defects in persons with liver disease, we examined PRMT1 levels in the livers of those with cirrhosis with or without a history of SBP. PRMT1 protein levels were decreased significantly in both hepatocytes and non-parenchymal cells from individuals with a history of SBP (Fig. 6A). Reduced PRMT1 expression correlated with reduced numbers of MRC1-positive M2 macrophages in those livers (Fig. 6, B and C). Co-staining with anti-PRMT1 and anti-CD206 (MRC1) antibodies revealed that the majority of M2 macrophages are PRMT1-positive. Co-staining with anti-c-Myc and anti-MRC1 antibodies showed that high levels of c-Myc expression in the cirrhotic livers are found only in M2 macrophages (MRC1-positive cells) (Fig. 6D). Fig. 6E shows that a number of M2 macrophages correlated with both PRMT1 expression and c-Myc expression. Moreover, we found that the number of M2 macrophages was significantly higher when both PRMT1 and c-Myc were high ("high high" group) compared with a situation in which either of them is low (Fig. 6F). Taken together, these data suggest that in human liver, PRMT1 and c-Myc cooperatively regulate M2 macrophage numbers.

Discussion

The work presented in this study has identified a novel interaction between c-Myc and PRMT1 in which arginine methylation is necessary for c-Myc to recruit p300 to target gene promoters and serve as a transcriptional activator (Fig. 7). Recent studies have shown that c-Myc posttranslational modifications can serve to switch c-Myc from a positive to a negative regulator of gene promoters (24). Our results suggest that arginine methylation may be such an activation/inactivation switch. This effect is important at a number of gene promoters and is required for M2 macrophage differentiation, possibly as a result of its regulation of the expression of PPARγ.

We found that c-Myc is arginine-methylated at Arg-299 and Arg-364, and these sites control c-Myc binding to p300. Mutation of those sites to lysine abolishes p300 binding without affecting binding to other co-factors such as HDAC1 and Max. Interestingly, the binding of c-Myc to Max is also inhibited by the...
PRMT1 inhibitor AMI-1, suggesting that this binding is controlled by other arginine methylation sites on c-Myc, or possibly by Max itself, that are not identified in this study.

The regulation of gene transcription is often mediated by the recruitment of various chromatin-remodeling enzymes to promoter sites. c-Myc recruits several of these enzymes through its transactivation domain including p300 and p400 (part of the acetyltransferase complexes), SKP2 (a histone ubiquitination enzyme), HDAC1 (a histone deacetylase), DNMT1, and others (5). We showed that recruitment of two of those factors, p300 and HDAC1, is regulated by PRMT1. Previous studies have also reported PRMT1-mediated regulation of p300 recruitment by other transcription factors such as HNF-4α (18), p53 (19), and others (25). The mechanism of this regulation involved both arginine methylation of the transcription factor itself and the methylation of histones at the target gene promoters. We propose that a similar mechanism regulates c-Myc function. However, we observed only partial inhibition of gene activation in c-Myc methylation-deficient mutants, suggesting that other factors such as histone methylation or additional methylation sites might also play a role.

Previously we showed that PRMT1 expression is inhibited in the livers of individuals with cirrhosis (26).
reason for low PRMT1 levels is still unclear. We have observed that overexpression of c-Myc by itself is able to decrease the expression of PRMT1 (Fig. 4A). Because reduced PRMT1 could subsequently decrease c-Myc methylation, the possibility of a positive-feedback loop exists where an increase in c-Myc could perpetuate a decrease in PRMT1 expression. Whether a mechanism of this nature occurs in cirrhosis with SBP is uncertain.

In this study we showed further that PRMT1 controls macrophage phenotype in the livers of persons with cirrhosis, likely through its regulation of c-Myc transcriptional activity at M2 gene promoters. In individuals with cirrhosis, particularly those most immunocompromised with a history of SBP, the levels of PRMT1 protein in the liver tissue were extremely low, which correlated with low M2 macrophage numbers. Lack of M2 macrophage differentiation can result in enhanced susceptibility to bacterial infections. Thus PRMT1 deficiency may contribute to the immune defects seen in those persons.

In addition to its association with M2 differentiation, c-Myc activation in myeloid and other cells has been associated with the development and progression of cancer (10, 27–30). Because PRMT1 is necessary for c-Myc gene activation, its inhibition in this regard might be beneficial. It is known that overexpression of PRMT1 is associated with a number of cancers and that PRMT1 promotes cancer cell proliferation (1, 4). The data presented in this study suggest that the inhibition of PRMT1 in cirrhosis might play a beneficial or tumor suppressor role due to inhibition of c-Myc-dependent gene activation. A full understanding of the role of c-Myc–PRMT1 interactions in tumor cell growth will require further studies.
In summary, this study has demonstrated a novel mechanism by which arginine methylation via the methyltransferase PRMT1 regulates the activity of c-Myc. Low levels of PRMT1 are a characteristic of multiple cell types derived from patients with advanced, decompensated cirrhosis, and a further understanding of these interactions could lead to new therapeutic approaches in dealing with immune defects and cell growth abnormalities in these persons.
Materials and methods

Antibodies

Primary antibodies—Anti-PRMT1 (F339), acetyl-histone H3 (Lys-27) (D5E4), and HDAC1 (10E2) mouse monoclonal antibodies were from Cell Signaling. Anti-lamin B (C20), ChIP-grade anti-Max (C-17), anti-p300, anti-Miz1 (H-190), and monoclonal anti-MRC1 anti-H9252-actin were from Santa Cruz Biotechnology. Anti-mono- and dimethyl arginine (clone 7E6), rabbit anti-PRMT1 antibody (against amino acid residues 300–361), ChIP-grade anti-Max antibody (73C5a), mouse ChIP-grade anti-c-Myc antibody (9E11), and rabbit anti-c-Myc antibody (Y69) were from Abcam. Anti-asymmetric dimethylarginine antibodies were from Active Motif. Mouse anti-H9252-actin, mouse monoclonal anti-PRMT1 antibody clone 171 (against amino acid residues 1–361), and anti-FLAG (M2) antibodies were from Sigma-Aldrich, and anti-GAPDH was from Ambion.

Secondary antibodies—IRDye 800CW goat anti-mouse IgG and IRDye 680RD goat anti-rabbit IgG were from Li-COR. General HRP-conjugated secondary antibodies were from Southern Biotechnology Associates (Birmingham, AL).

Cell culture

THP-1 cells were from InvivoGen and were maintained according to recommended procedures. AMI-1 was obtained from EMD4 Biosciences and used at 10 μM for 16–24 h prior to harvest. Cells were transfected using Lipofectamine LTX transfection reagent (Invitrogen) according to the manufacturer’s protocol.

Isolation of mouse peritoneal macrophages

Myeloid-specific PRMT1 knock-out mice were described previously (12). Primary peritoneal macrophages were isolated from PRMT1 fl/fl LysM Cre or PRMT fl/fl wild-type littermates as described previously (31). 8–10-week-old mice were killed by CO₂ asphyxiation. Briefly, 10 ml of sterile PBS was injected into the caudal half of the peritoneal cavity using a 25-gauge needle (beveled side up) followed by gently shaking the entire body for 10 s. Saline containing resident peritoneal cells was collected, and cells were plated on uncoated tissue culture plates and incubated for 60 min at 37 °C. Nonadherent cells were removed by washing five times with warm PBS. Macrophages were maintained in RPMI medium (Invitrogen) containing 10% FBS.

Vectors

The pcDNA3-c-myc plasmid was provided by Wafik El-Deiry via Addgene (Addgene plasmid 16011). pCMV6-PRMT1 was from Origene. The Myc point mutations and C-terminal FLAG tag were generated by site-directed mutagenesis (Q5® site-directed mutagenesis kit, New England Biolabs).

Human specimens

De-identified human liver specimens from liver explants were obtained from the Liver Center Tissue Bank at the University of Kansas Medical Center. All studies using human tissue samples were approved by the Human Subjects Committee, University of Kansas Medical Center.

Peripheral blood mononuclear cells were isolated from the PBMC fraction using MACS beads (human CD14, Miltenyi Biotec, catalog no. 130-050-201) according to the manufacturer’s instructions.

LC/MS analysis

Ten 15-cm dishes of THP-1 cells were seeded at 1 × 10⁷ cells/dish and transiently transfected with 30 μg of Myc-FLAG plasmid/dish. Cells were harvested 48 h post-transfection. Myc-FLAG was purified using a FLAG purification kit from Sigma according to the manufacturer’s instructions. Identification of methyl-Arg and acetyl-Lys on Myc was done using in-gel
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digestion with endoproteinase trypsin; LC-MS/MS and a database search were performed by MS Bioworks LLC, Ann Arbor, MI.

Real-time PCR
RNA was extracted from cultured cells using the RNeasy Mini Kit (Qiagen). cDNA was generated using the RNA reverse transcription kit (Applied Biosystems, catalog no. 4368814). Quantitative real-time RT-PCR was performed in a CFX96 real-time system (Bio-Rad) using specific sense and antisense primers combined with iQ SYBR Green Supermix (Bio-Rad) for 40 amplification cycles: 5 s at 95 °C, 10 s at 57 °C, and 30 s at 72 °C. The primers were as follows: ACTB, tgcctcgatggtgtacttccag and tgtcctggcactgggtttctcag; PRMT1, cagccggaaacactg-gagaa and gatgcacaaggtgctgattcag; CCNB1, tctggtgaaatgtggagaa-tggcaca and cgtatggcactcattggtttctcag; CDK4, acgggtgaagctggtggct-ctg and ttggtctggtctcagctgagggtaaag; STAT6, gtcctggataatggtga-agcagc and gctggtgtaagtgcccagtaca.

ChIP assay
Chromatin immunoprecipitation was performed as described previously (12, 32). THP-1 cells (1.5 × 10⁷) were cross-linked by the addition of 1% formaldehyde for 10 min. Cells were lysed with 10 mM Tris-HCl (pH 8.0), 10 mM NaCl, 3 mM MgCl₂, and 0.5% Nonidet P-40. Nuclei were collected by centrifugation, resuspended in 1% SDS, 5 mM/liter EDTA, and 50 mM/liter Tris-HCl (pH 8.0), and sonicated to generate chromatin to an average length of 100-500 bp. Next, samples in 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), and 150 mM NaCl were immunoprecipitated overnight at 4 °C with 4 μg of ChIP-grade antibody. 20 μl of magnetic beads (Dynabeads M-280, Invitrogen) were used to purify the immunocomplexes. Following purification, cross-links were reverted by incubation at 65 °C for 6 h. Samples were purified using a Qiagen kit. For ChIP re-ChIP analysis, DNA-protein complexes were eluted in 25 μl of Tris-EDTA buffer containing 10 mM DTT for 30 min at 37 °C. Eluates were diluted 1:20 with ChIP dilution buffer and used for a second immunoprecipitation. Primers were as follows:

- CDK4, agcaatgtcaagcggtcac and acaggccgcaagctagagag;
- CCNB1, tctggtgaaatgtggagaa-tggcaca and cgtatggcactcattggtttctcag;
- CDK4, acgggtgaagctggtggct-ctg and ttggtctggtctcagctgagggtaaag;
- STAT6, gtcctggataatggtga-agcagc and gctggtgtaagtgcccagtaca.

Proximity ligation assay
Proximity ligation assays were carried out using a PLA kit (Sigma) according to the manufacturer’s instructions. Prior to detection THP-1 cells were fixed with 4% paraformaldehyde, washed and permeabilized with 1% Triton in PBS, blocked with supplied PLA blocking buffer, and incubated with specific primary antibodies as indicated in the text. Interactions were visualized using Duolink Brightfield detection reagent (Sigma). The PLA assay omitting one or both primary antibodies was used as a negative control.

Immunofluorescence
Cells were fixed with 4% paraformaldehyde for 15 min at room temperature, washed with PBS, and permeabilized with 1% Triton X-100 for 15 min followed by blocking in immunofluorescence buffer (PBS containing 2.5 mM EDTA and 1% BSA) for 1 h. Cells were then incubated with primary antibody at 1:300 in PBS containing 2.5 mM EDTA, 1% BSA, and 0.1% Triton X-100 overnight at 4 °C. After washing with PBS, coverslips were incubated with Alexa Fluor-conjugated secondary antibody (1:500) in 0.1 μg/ml DAPI for 1 h in the dark at room temperature. Coverslips were washed and mounted with FluorSave reagent (Calbiochem). Slides were observed in a Nikon Eclipse 800 upright epifluorescence microscope (Nikon Instruments, Melville, NY). Images were acquired using a Nikon CoolSNAP camera.

Western blotting
Protein extracts (15 μg) were subjected to 10% SDS-PAGE, electrophoretically transferred to nitrocellulose membranes (Amersham Biosciences Hybrid ECL, GE Healthcare), and blocked in 3% BSA/PBS at room temperature for 1 h. Primary antibodies were incubated overnight at the manufacturer’s recommended concentrations. Immunoblots were detected with the ECL Plus Western blotting detection system (Amersham Biosciences) or using near-infrared fluorescence with the ODSYS Fc Dual-Mode imaging system (Li-COR). Expression levels were evaluated by quantifying the relative density of each band normalized to that of the corresponding β-actin or GAPDH band density.

ELISA
Modified ELISAs were carried out as follows. Plastic 96-well microtiter plates (Nunc-Immuno MaxiSorp plates) were coated overnight with an excess (0.5 μg) of the first primary antibody. Unbound protein was washed with PBS. Wells were blocked for 1 h with 0.3 ml of 3% BSA (Sigma, Cohn fraction V, essentially fatty acid–free) in PBS. After washing, samples containing the protein of interest (in 50 μl) were added, incubated for 2 h at room temperature, and then washed with PBS. The second primary antibody (0.1 μg in 50 μl) was added, incubated for 1 h at room temperature, washed, and then visualized by incubation with the secondary antibody conjugated with horse-radish peroxidase (The Jackson Laboratory) in the presence of 3% BSA for 0.5 h followed by reaction with 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (Sigma). The reaction was allowed to proceed for 20 min and then plates were quantitated spectrophotometrically at 410 nm.

Immunohistochemistry
Immunostaining on formalin-fixed sections was performed by deparaffinization and rehydration followed by antigen retrieval by heating in a pressure cooker (121 °C) for 5 min in 10 mM sodium citrate (pH 6.0). Peroxidase activity was blocked by incubation in 3% hydrogen peroxide for 10 min. Sections were rinsed three times in PBS/PBS-T (0.1% Tween-20) and incubated in Dako Protein Block (Dako) at room temperature for 1 h. After removal of the blocking solution, slides were
placed into a humidified chamber and incubated overnight with an antibody diluted 1:300 in Dako Protein Block at 4°C. Antigen was detected using the SignalStain Boost IHC detection reagent (catalog no. 8114, Cell Signaling Technology, Beverly, MA), developed with diaminobenzidine (Dako, Carpinteria, CA), counterstained with hematoxylin (Sigma-Aldrich), and mounted. Signal intensity was analyzed by Aperio ImageScope 12.1, and the intensity for hepatocytes and non-parenchymal cells was calculated using nuclear size cutoff: for hepatocytes, nuclear area >10 μm²; for non-parenchymal cells, nuclear area 5–10 μm².

Statistics
The results are expressed as mean ± S.D. Student’s t test, a paired t test, Pearson’s correlation, or one-way analysis of variance with Bonferroni post hoc test was used for statistical analyses. A p value < 0.05 was considered significant.

Author contributions—I. T. and S. A. W. designed the research, analyzed the results, and wrote the manuscript. I. T. and J. Z. performed the experiments. I. T. produced the figures, and S. K., B. B., and B. R. collected the human specimens. All authors analyzed the results and approved the final version of the manuscript.

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