

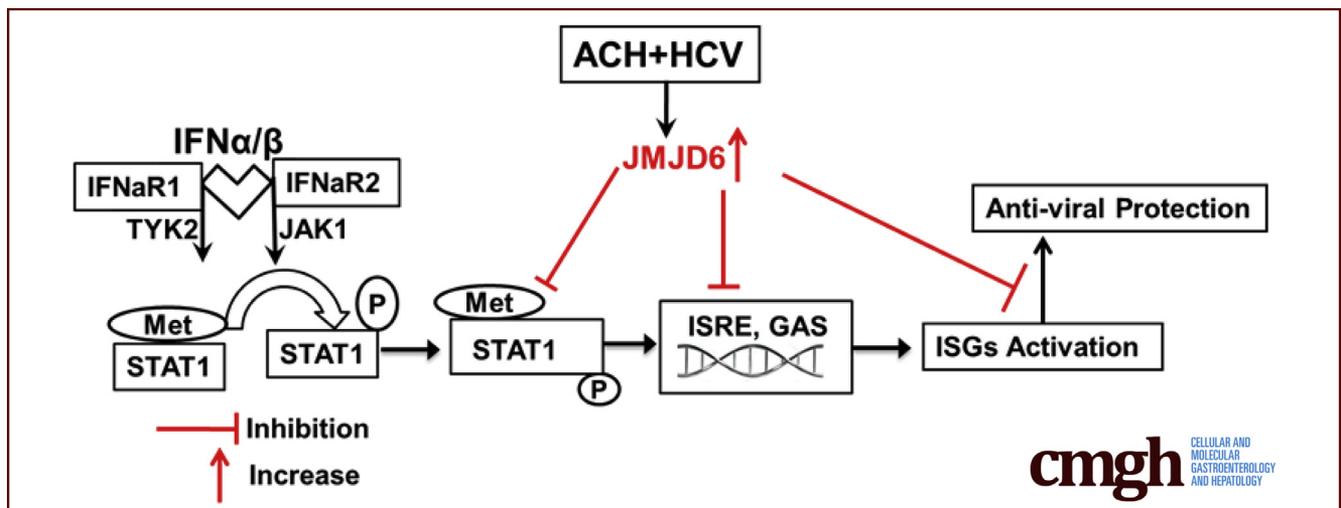
ORIGINAL RESEARCH

Demethylase JMJD6 as a New Regulator of Interferon Signaling: Effects of HCV and Ethanol Metabolism



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SUMMARY

Demethylase jumonji domain-containing protein 6 expression is enhanced by acetaldehyde and hepatitis C virus. Jumonji domain-containing protein 6 down-regulates signal transducer and activator of transcription 1 methylation in hepatocytes, thereby suppressing interferon α -induced signaling via the Janus kinase–signal transducer and activator of transcription pathway and interferon-sensitive gene activation. This leads to an increase in hepatitis C virus–RNA levels.

BACKGROUND & AIMS: Alcohol-induced progression of hepatitis C virus (HCV) infection is related to dysfunction of innate immunity in hepatocytes. Endogenously produced interferon (IFN) α induces activation of interferon-stimulated genes (ISGs) via triggering of the Janus kinase–signal transducer and activator of transcription 1 (STAT1) pathway. This activation requires protein methyltransferase 1–regulated arginine methylation of STAT1. Here, we aimed to study whether STAT1 methylation also depended on the levels of demethylase

jumonji domain-containing 6 protein (JMJD6) and whether ethanol and HCV affect JMJD6 expression in hepatocytes.

METHODS: Huh7.5-CYP (RLW) cells and hepatocytes were exposed to acetaldehyde-generating system (AGS) and 50 mmol/L ethanol, respectively. JMJD6 messenger RNA and protein expression were measured by real-time polymerase chain reaction and Western blot. IFN α -activated cells either overexpressing JMJD6 or with knocked-down JMJD6 expression were tested for STAT1 methylation, ISG activation, and HCV RNA. In vivo studies have been performed on C57Bl/6 mice (expressing HCV structural proteins or not) or chimeric mice with humanized livers fed control or ethanol diets.

RESULTS: AGS exposure to cells up-regulated JMJD6 expression in RLW cells. These results were corroborated by ethanol treatment of primary hepatocytes. The promethylating agent betaine reversed the effects of AGS/ethanol. Similar results were obtained in vivo, when mice were fed control/ethanol with and without betaine supplementation. Overexpression of JMJD6 suppressed STAT1 methylation, IFN α -induced ISG activation, and increased HCV-RNA levels. In contrast, JMJD6 silencing enhanced STAT1 methylation, ISG stimulation by IFN α , and attenuated HCV-RNA expression in Huh7.5 cells.

CONCLUSIONS: We conclude that arginine methylation of STAT1 is suppressed by JMJD6. Both HCV and acetaldehyde increase JMJD6 levels, thereby impairing STAT1 methylation and innate immunity protection in hepatocytes exposed to the virus and/or alcohol. (*Cell Mol Gastroenterol Hepatol* 2018;5:101–112; <https://doi.org/10.1016/j.jcmgh.2017.10.004>)

Keywords: Alcohol; HCV; JMJD6; STAT1.

See editorial on page 163.

Alcohol-induced progression of hepatitis C virus (HCV) infection is related to dysfunction of innate immunity in hepatocytes. Antiviral protection of hepatocytes requires induction of interferon α (IFN α) signaling via the Janus kinase–signal transducer and activator of transcription (JAK–STAT) pathway to activate interferon-stimulated genes (ISGs), which control viral replication. HCV and alcohol alter the IFN-induced JAK–STAT1 signaling by multiple mechanisms, including the prevention of STAT1 phosphorylation^{1–3} and impairment of STAT1 methylation.⁴ The latter is attributed to the changes in arginine methylation of STAT1. Indeed, in infected hepatocytes, methylation of STAT1 on Arg-31 is suppressed by HCV.^{5,6} Alcohol metabolites, especially acetaldehyde (Ach), further decrease STAT1 methylation, thereby dysregulating ISG activation and enhancing HCV replication.⁴

As shown earlier, STAT1 requires protein methyl transferase 1 (PRMT1)-mediated arginine methylation to attach to DNA and activate protective ISGs.⁷ In the liver, activities of many methyltransferases, including PRMT1, are regulated by the changes in the ratio between the methyl donor S-adenosylmethionine (SAM) and its toxic metabolite, S-adenosylhomocysteine (SAH). Alcohol and HCV decrease the SAM:SAH ratio^{8–10} in liver cells, suggesting suppression of SAM-dependent PRMT1 activity. We in fact observed the reduced STAT1 arginine methylation in HCV-infected hepatocytes exposed to the major ethanol metabolite Ach.⁴

In addition to methyltransferases, protein methylation levels may be regulated by demethylases. More recently, jumonji domain-containing protein 6 (JMJD6), a nonheme Fe(II) 2-oxoglutarate-dependent oxygenase with arginine demethylase and lysyl hydroxylase activities,^{11,12} has been shown to control arginine methylation of another innate immunity factor, TNF-receptor-associated factor 6.¹³ However, the role of JMJD6 in altering STAT1 methylation was not reported. In this study, we thus examined the effects of HCV and acetaldehyde on JMJD6 levels, which can decrease antiviral protection by impairing STAT1 methylation and subsequent IFN α -induced ISG activation in hepatocytes.

Materials and Methods

Reagents and Media

High-glucose Dulbecco's modified Eagle medium, Williams medium, and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA). Anti-mono-dimethyl arginine antibody (ab412) was obtained from Abcam, Inc (Cambridge, MA).

Anti-alcohol dehydrogenase (ADH) was a gift from Dr Michael Felder (University of South Carolina, Columbia, SC). Cytochrome P450 2E1 (CYP2E1) (AB1252) was from EMD Millipore (Temecula, CA). Antibody to the STAT-1 (sc-592) and β -actin (sc-47778) antibodies were from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). JMJD6 antibody (TA306835) and small interfering RNA (siRNA) (SR308094) were from OriGene (Rockville, MD); JMJD6 plasmid (plasmid 31358) was from Addgene (Cambridge, MA). Polymerase chain reaction (PCR) reagents, probes, and primers were from Life Technologies, Inc (Carlsbad, CA). Other reagents, all of analytical-grade quality, were from Sigma (St. Louis, MO).

Cells

Huh7.5 cells were transfected with pIV-G2 (CYP2E1) plasmid as previously described for other cell lines^{14,15} using Lipo TAXI (Invitrogen, Waltham, MA). Recombinant cells, designated *RLW cells*, were selected in culture medium containing G418 at 400 μ g/mL. Clones were expanded and screened for CYP2E1 expression and activity. The clones with the highest CYP2E1 activity were designated as RLW cells. Because we were unable to transfect RLW cells with the ADH plasmid, we used acetaldehyde that had been exogenously generated by a special in vitro system (acetaldehyde-generating system [AGS], see description later).

RLW Cell Treatments

RLW cells were infected by Japanese fulminant hepatitis virus-1 (HCV genotype 2a) virus at a multiplicity.¹⁶ On day 2 after infection, cells were exposed for up to 48 hours to either 50 mmol/L ethanol or to AGS that contained yeast ADH (0.02 U/mL), 2 mmol/L nicotinamide adenine dinucleotide (NAD), and 50 mmol/L ethanol. In the presence of RLW cells, the levels of generated Ach measured by gas chromatography in the medium fluctuated between approximately 250 (at 1–4 hours of exposure) and 50 μ mol/L (at 18–48 hours of exposure). These levels of Ach corresponded to the amounts of Ach produced by ADH-expressing liver cells and were equivalent to the physiological concentrations observed in the liver of ethanol consumers.^{15,17} Some treatments were performed in the presence or absence of protein

Abbreviations used in this paper: Ach, acetaldehyde; ADH, alcohol dehydrogenase; AGS, acetaldehyde-generating system; AMI-1, protein arginine N-methyltransferase inhibitor; BHMT, betaine-homocysteine-S-methyltransferase; cDNA, complementary DNA; CYP2E1, cytochrome P450 2E1; 4-MP, 4-methylpyrazole; HCV, hepatitis C virus; IFN, interferon; ISG, interferon-stimulated gene; JAK-STAT, Janus kinase–STAT, signal transducer and activator of transcription; JMJD6, jumonji domain-containing 6 protein; mRNA, messenger RNA; OA, okadaic acid; OAS-1, 2'-5'-oligoadenylate synthetase-1; OASL, 2'-5'-oligoadenylate synthetase-like protein; PCR, polymerase chain reaction; PP2A, protein phosphatase 2A; PRMT1, protein methyl transferase 1; RT, reverse-transcription; SAM, S-adenosylmethionine; siRNA, short interfering RNA; TK-NOG, thymidine kinase transgene-NOD/Shi-scld/IL-2R γ ^{null} mice.

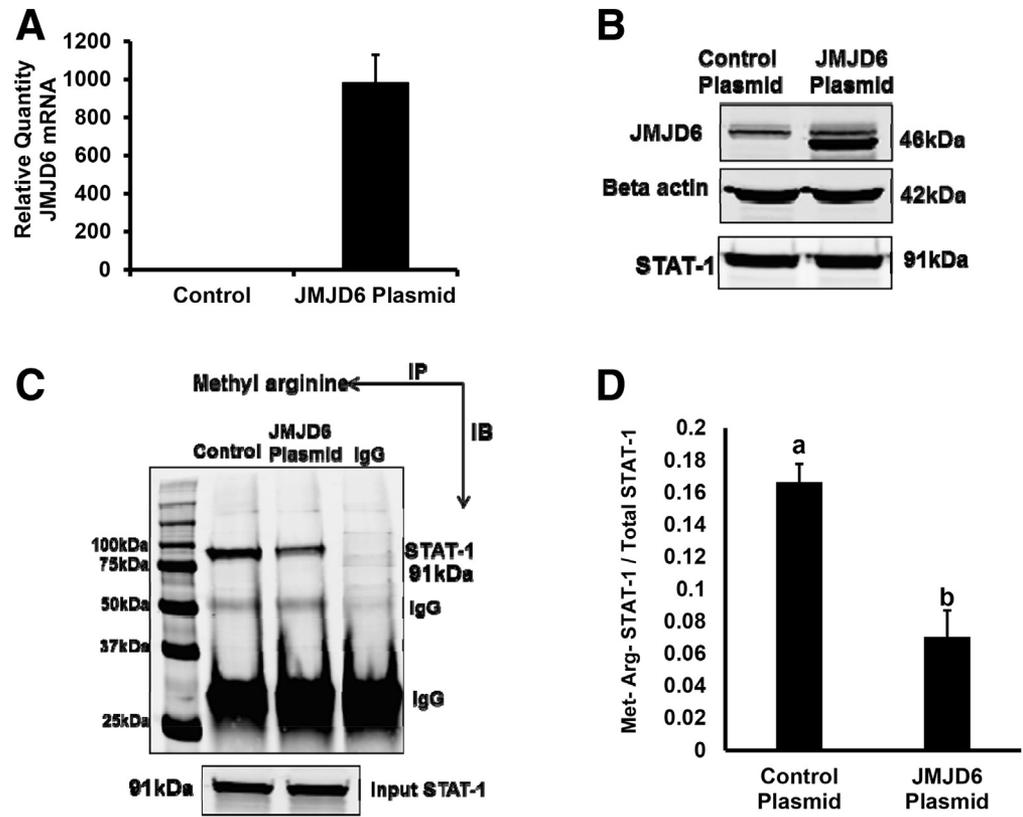
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Figure 1. JMJD6 over-expression suppresses STAT1 methylation in liver cells. Huh7.5 cells were transfected with JMJD6 plasmid and its vector (control plasmid). After 48 hours, efficiency of transfection was confirmed by JMJD6 expression: (A) mRNA by RT-PCR and (B) protein levels by WB. (C and D) In JMJD6-overexpressed samples, STAT1 arginine methylation was measured by immunoprecipitation of methyl arginine (or isotype IgG) with further probing for STAT1 in WB. Data are from 3 independent experiments presented as means \pm SD. Bars marked with the same letter are not significantly different from each other; bars with different letters are significantly different ($P \leq .05$). IB, immunoblotting; IP, immunoprecipitation.



arginine N-methyltransferase inhibitor, a PRMT1 inhibitor (100 μ mol/L), or 2 mmol/L betaine, a promethylating agent. The optimal concentration of betaine used for in vitro studies in liver cells was determined earlier.¹⁸

Human Hepatocytes and Treatments

Primary hepatocyte cells were obtained from the University of Pittsburgh Medical Center (Pittsburgh, PA) (the Liver Tissue Cell Distribution System was funded by National Institutes of Health contract HSN276201200017C). Cryopreserved hepatocytes (HUM 4100) were purchased from Triangle Research Labs (Durham, NC). Cryopreserved cells were thawed in hepatocyte thawing medium (MCHT50; Triangle Research Labs) and centrifuged at 100g for 8 minutes. Cell pellets were resuspended in hepatocyte culture William's E medium (Gibco, Gaithersburg, MD), supplemented with the HCM SingleQuots Kit (Lonza, Walkersville, MD), HEPES (1 mol/L; Sigma), and dexamethasone (10 mmol/L; Gibco), and plated on Matrigel-coated (Corning, Corning, NY) 6-well plates at a density of approximately 2×10^4 cells/cm². For attachment to the culture plate, 5% heat-inactivated fetal bovine serum was added during the 4- to 6-hour attachment period. Then cells initially plated on collagen (freshly isolated hepatocytes) or on Matrigel-coated plates (cryopreserved hepatocytes) were overlaid with Matrigel diluted with serum-free hepatocyte culture medium (at a final concentration of 240 μ g/mL) overnight. Hepatocytes were treated with either serum-free medium or exposed to 50 mmol/L ethanol in serum-free medium for 48 hours.

RNA isolation and real-time PCR. ISGs with antiviral activities, such as 2'-5'-oligoadenylate synthetase-like protein (OASL) (HS00984388_M1) and 2'-5'-oligoadenylate synthetase-1 (OAS-1) (Hs00973635_M1) were quantified by real-time PCR. Reagents used for RNA isolation, complementary DNA (cDNA) synthesis, and real-time PCR were from Life Technologies. Total RNA was isolated from cells using TRIzol (Applied Biosystems, Foster City, CA) reagent. A 2-step procedure was used in which 200 ng RNA was reverse-transcribed to cDNA using the high-capacity reverse-transcription kit. In the second step, the cDNA was amplified using the TaqMan (Applied Biosystems) Universal Master Mix-II with fluorescent-labeled primers (TaqMan Gene Expression Systems). These were incubated in a model 7500 quantitative reverse-transcription (RT)-PCR thermal cycler. The relative quantity of each RNA transcript was calculated by its threshold cycle, after subtracting that of the reference cDNA (glyceraldehyde-3-phosphate dehydrogenase). Data are expressed as the quantity of transcript. The relative HCV-RNA expression level in infected cells was quantified using the following primers, and probes for this consensus sequence were designed using PrimerExpress Software v2.0 (Applied Biosystems): 5'UTRF GACCGGGTCCT TTCTTGGAT; 5'UTRR CCAACACTACTCGGCTAGCAGTCT; probe FAM-ATTTGGGCGTGCCCCCG-NFQ. Glyceraldehyde-3-phosphate dehydrogenase (HS02786624_G1) was used as an internal control to normalize the gene of interest.

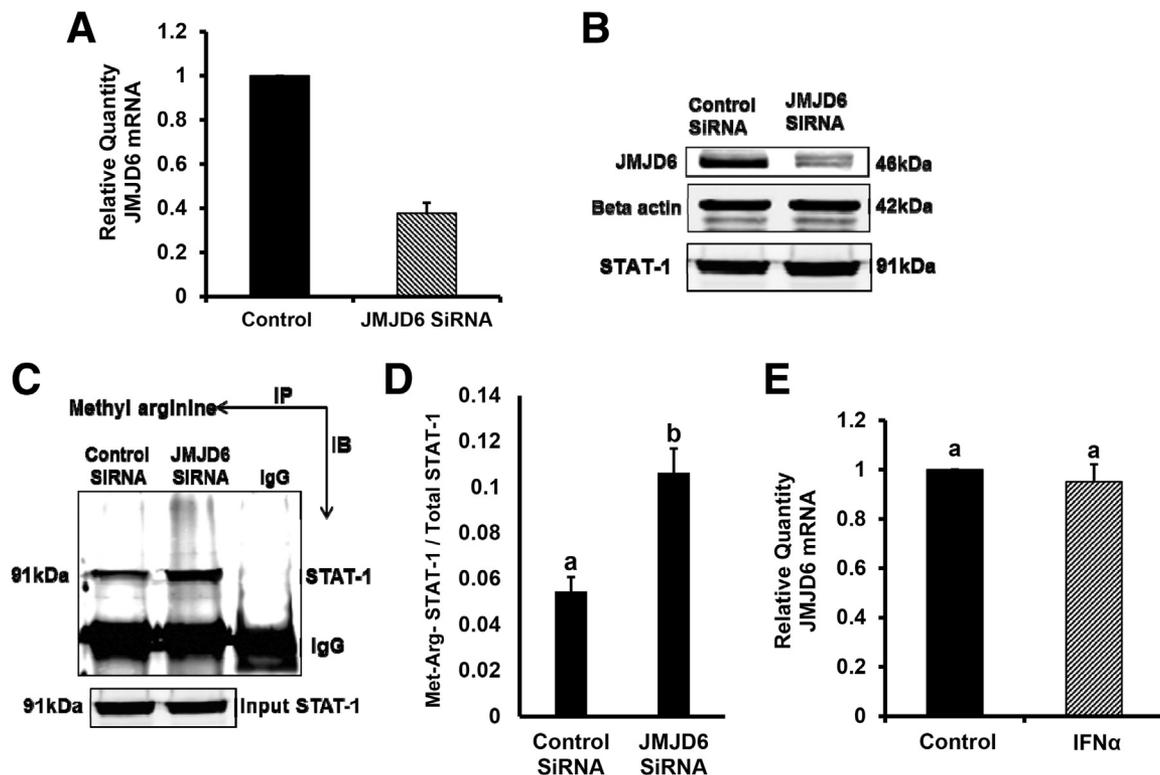


Figure 2. JMJD6 silencing up-regulates STAT1 methylation. Huh7.5 cells were transfected for 48 hours with scrambled (control) siRNA or JMJD6 siRNA to knock-down JMJD6 expression and then arginine methylation of STAT1 was measured as described in Figure 1. (A) JMJD6 mRNA, efficiency of mRNA suppression by siRNA transfection. (B) JMJD6 protein levels after control siRNA and JMJD6 siRNA transfection. (C and D) Arginine methylation of STAT1 after control and JMJD6 RNA transfection. (E) IFN α treatment does not affect JMJD6 mRNA levels. RLW cells were treated with 1000 IU of human IFN α for 1 hour and then JMJD6 RNA was measured by RT-PCR. Data are from 3 independent experiments presented as means \pm SD. Bars marked with the same letter are not significantly different from each other; bars with different letters are significantly different ($P \leq .05$). IB, immunoblotting; IP, immunoprecipitation.

Immunoblotting and immunoprecipitation. Cell lysates were prepared in 0.5 mol/L EDTA, 2 mol/L Tris, 20 mmol/L Na₃VO₄, 200 mmol/L Na₄P₂O₇, 100 mmol/L phenylmethylsulfonyl fluoride, 1 mol/L NaF, 20% Triton X-100 (Singh-Aldrich, St. Louis, MO), and aprotinin, pH = 7. Immunoprecipitations were performed by incubating each antigen-antibody complex with protein G sepharose (GE Healthcare Biosciences AB, Uppsala, Sweden) for overnight in a rotating shaker at 4°C, followed by washing and incubation with sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample solubilizing buffer at 95°C for 10 minutes. These subsequently were subjected to denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Immunoblotting was performed as described previously.¹⁹ β -actin was used as the loading control to normalize the proteins.

JMJD6 Plasmid Overexpression

Transfection was performed using the protocol from the manufacturer (OriGene) with empty vector or JMJD6 plasmid. The efficacy of transfection was monitored in 48 hours by Western Blot (WB) and real-time PCR.

JMJD6 siRNA Transfection

Transfection was performed using the protocol from the manufacturer (OriGene) with control (scrambled) or

specific siRNA. The efficacy of transfection was monitored in 48 hours by WB and real-time PCR.

Animal studies. All mice were treated according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals. All the animal protocols used in this study were approved by the Veterans Affairs Medical Center Institutional Animal Care and Use Committee. Briefly, C57Bl/6 mice were divided into the following 4 groups (n = 6 per group): control, ethanol, betaine, and betaine + ethanol. They were pair-fed control and ethanol Lieber DeCarli diets as described,²⁰ with or without 1% betaine (wt/vol). The effects of HCV structural proteins on JMJD6 expression was studied using HCV transgenic mice²¹ compared with their littermates, C57Bl/6 mice.

For this study, we also obtained liver tissue from humanized mice fed control and ethanol diets. We used TK-NOG chimeric mice provided by Dr Mamoru Ito (Central Institute of Experimental Medicine, Kawasaki City, Japan). Transgenic expression of herpes simplex virus thymidine kinase under albumin promoter allows conditional depletion of mouse hepatocytes after gancyclovir administration. Mice were transplanted with human hepatocytes at the Translational Mouse Model Core Facility (University of Nebraska Medical Center, Omaha, NE) as described.²² They were pair-fed control and ethanol diets as described for

C57Bl/6 mice earlier. We also used Severe combined immunodeficiency albumine -urokinase type plasminogen mice infected with HCV²³ and fed chow or chow plus 20% ethanol in water as detailed.²⁴ Importantly, after 5 weeks of feeding, control mice cleared HCV RNA, although it still persisted in ethanol-fed mice (1.71×10^4 – 4.75×10^5 HCV RNA copies/mL).

Statistical Analyses

Data from at least 3 independent experiments were expressed as mean values \pm standard error. Comparisons among multiple groups were determined by 1-way analysis of variance, using a Tukey post hoc test. For comparisons between 2 groups, we used the Student *t* test. A probability *P* value of 0.05 or less was considered significant.

Data

All authors had access to the study data and have reviewed and approved the final manuscript.

Results

Does JMJD6 Regulate STAT1 Methylation in Liver Cells?

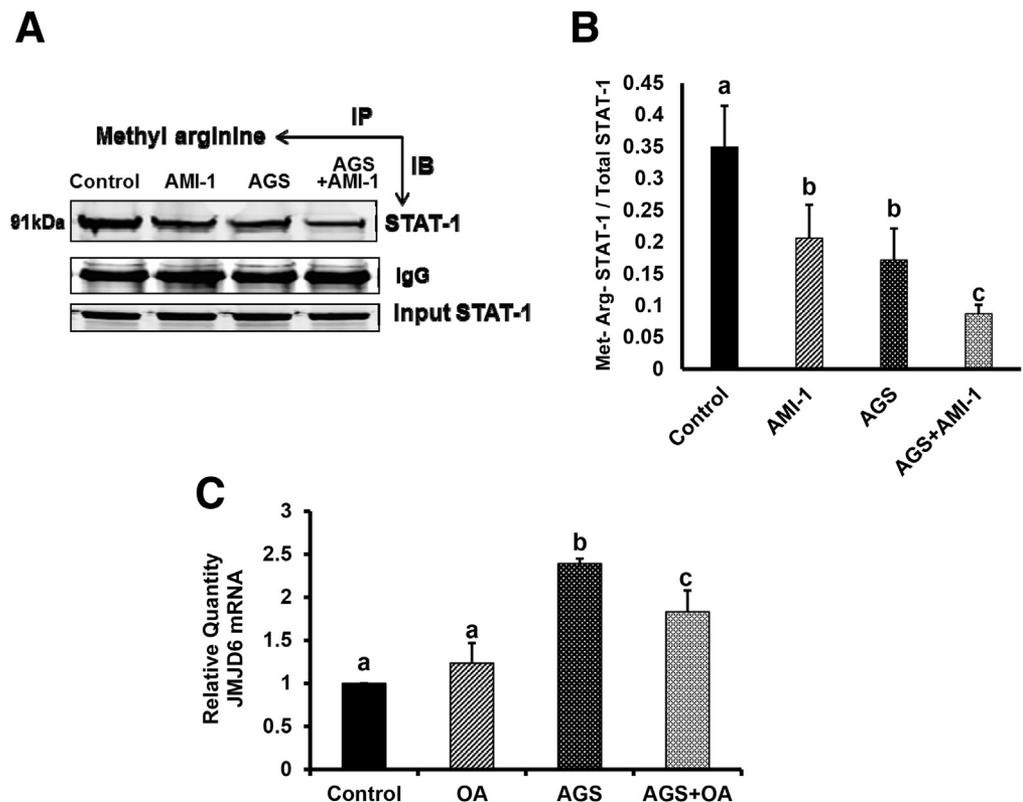
To answer this question, we either silenced JMJD6 by specific siRNA transfection or overexpressed it by plasmid transfection. Our overexpression studies successfully increased the levels of JMJD6, resulting in reduced STAT1 methylation (Figure 1). In contrast, silencing of JMJD6

enhanced STAT1 methylation (Figure 2A–D). Although STAT1 methylation is required for IFN-induced signaling through the JAK–STAT1 pathway, IFN α did not affect JMJD6 expression in liver cells (Figure 2E).

Acetaldehyde Increases JMJD6 Levels in Hepatoma Cells and Human Hepatocytes

Previously, we have shown that STAT1 methylation is regulated by the ethanol metabolite Ach, but not ethanol,⁴ presumably through suppression of PRMT1. This was observed in Huh7.5-CYP (RLW) cells exposed to an AGS as detailed in our earlier publications.^{1,4,25} To investigate whether AGS attenuated STAT1 arginine methylation only by suppressing PRMT1 or if additional mechanisms are involved, we treated RLW cells with AMI-1 (a specific inhibitor of PRMT1), AGS, and the combination of AMI-1 and AGS. Then, methyl arginine was immunoprecipitated and probed for STAT1. It appeared that both AMI-1 and AGS suppressed STAT1 methylation equally, whereas the combination of these factors decreased STAT1 methylation more than either treatment alone (Figure 3A and B). These results suggest that the effect of Ach is owing not only to PRMT1 suppression, but Ach-regulated demethylation also may play a role in the regulation of overall STAT1 methylation. Next, we measured JMJD6 messenger RNA (mRNA) in untreated and AGS-exposed RLW cells and found more than a 2-fold increase by AGS (Figure 3C). Because we previously reported that Ach-induced suppression of STAT1 methylation in RLW cells is protein phosphatase 2A (PP2A)-dependent,⁴

Figure 3. AGS increases JMJD6 expression in liver cells. (A and B) Effects of AMI-1 and AGS on arginine methylation of STAT1. RLW cells were treated either with AGS or PRMT1 inhibitor, AMI-1 (100 μ mol/L), or both, and then arginine methylation of STAT1 was measured as described in Figure 1C. (C) Effects of OA on AGS-induced expression of JMJD6 mRNA. Cells were treated with AGS, in the presence or absence of OA, and then JMJD6 mRNA was measured by RT-PCR. Data are from 3 independent experiments presented as means \pm SD. Bars marked with the same letter are not significantly different from each other; bars with different letters are significantly different ($P \leq .05$). IB, immunoblotting; IP, immunoprecipitation.



we also investigated whether the JMJD6 increase by AGS was regulated by PP2A. In this regard, RLW cells were treated with the PP2A inhibitor, okadaic acid (OA), in the presence or absence of AGS. Although OA exposure did not significantly affect JMJD6 mRNA levels in untreated cells, its up-regulation by AGS was attenuated by OA, indicating a partial PP2A dependence (Figure 3C). Surprisingly, we observed only a 21% increase in JMJD6 protein levels in RLW cells treated with AGS (Figure 4A and B).

Complementary studies also were conducted on human hepatocytes that were plated on Matrigel (3-dimensional culture). The advantage of this system is that hepatocytes continue to express ethanol-metabolizing enzymes CYP2E1 and ADH for at least 4 days (Figure 5A). These Matrigel-overlaid human hepatocytes were treated with 50 mmol/L ethanol for 48 hours and then JMJD6 mRNA and protein levels were measured by RT-PCR and WB, respectively. We observed a 100% increase in JMJD6 mRNA (Figure 5B). We also observed a 50% increase in protein levels by ethanol exposure, and the magnitude of JMJD6 increase was not dependent on IFN α treatment. The effect of ethanol was reversed by co-treatment with the ADH inhibitor 4-methylpirazole (4-MP), indicating the contribution of

ethanol metabolism and particularly acetaldehyde (Figure 5C and D). Importantly, ethanol exposure to hepatocytes also suppressed arginine methylation of STAT1, which was not affected by IFN α treatment, but was reversed by 4-MP (Figure 5E and F).

Does Betaine Prevent JMJD6 Increase?

A promethylating agent, betaine, prevents or attenuates the effects of Ach on STAT1 methylation in liver cells.⁴ To assess the role of betaine in JMJD6 regulation, RLW cells were treated with AGS in the presence or absence of betaine. We observed that betaine attenuates the effects of JMJD6 at both mRNA and protein levels (Figure 4A–C). Furthermore, in primary human hepatocytes plated on collagen (not Matrigel), betaine fully reversed the effect of AGS on JMJD6 mRNA expression (Figure 4D).

The mechanism by which betaine regulates the AGS-induced increase in JMJD6 is unknown. It is unlikely that it occurs via betaine-homocysteine-S-methyltransferase (BHMT), because the level of JMJD6 after RLW cell treatment with AGS and betaine was the same in the presence and absence of the BHMT inhibitor, dimethyl glycine (DMG) (Figure 4E). However, when AGS-induced oxidative stress is

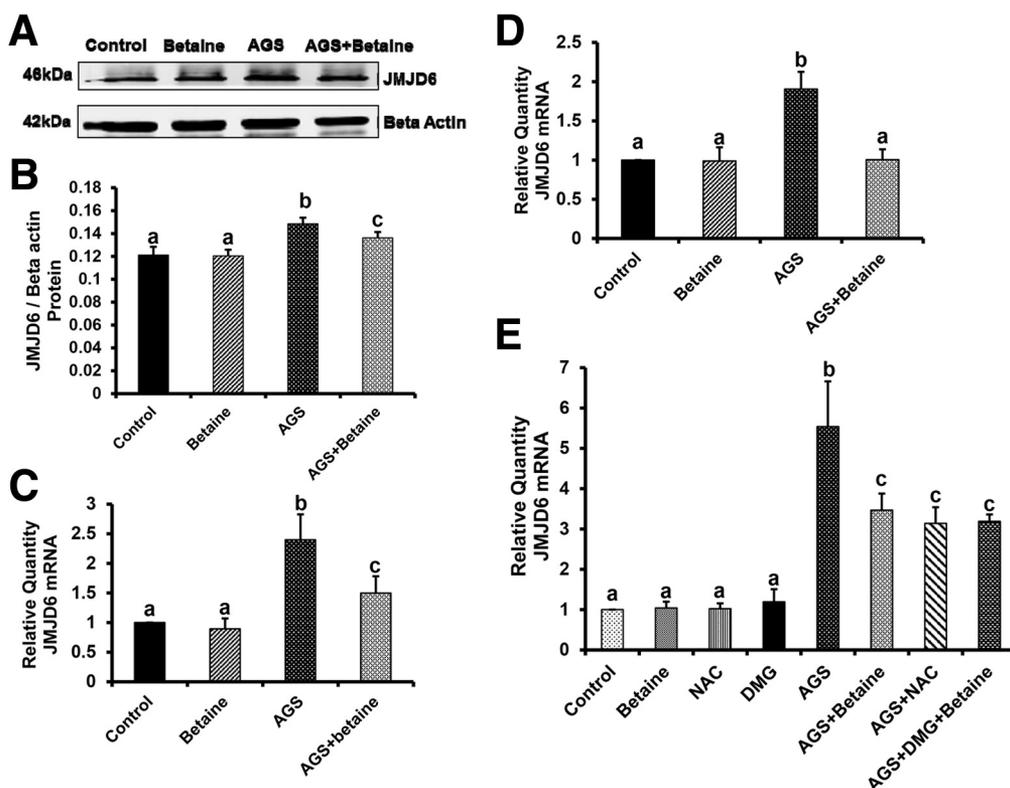


Figure 4. Betaine regulates JMJD6 expression in liver cells. (A–C) Effects of AGS and betaine on JMJD6 expression in RLW cells. Cells were treated with AGS in the presence or absence of 2 mmol/L betaine. (A and B) JMJD6 protein was measured by WB; and (C) JMJD6 mRNA was measured by RT-PCR. (D) Effects of ethanol on JMJD6 mRNA expression in human hepatocytes overlaid with Matrigel: hepatocytes were treated as in panel A and JMJD6 mRNA expression was measured by RT-PCR. (E) The effects of BHMT1 inhibitor and N-acetylcysteine (NAC) and betaine on AGS-induced JMJD6 expression. JMJD6 mRNA expression was induced in RLW cells treated with AGS for 48 hours in the presence or absence of BHMT inhibitor dimethyl glycine (DMG). Cells also were exposed to AGS in the presence or absence of NAC (5 mmol/L). Data are from 3 independent experiments presented as means \pm SD. Bars marked with the same letter are not significantly different from each other; bars with different letters are significantly different ($P \leq .05$).

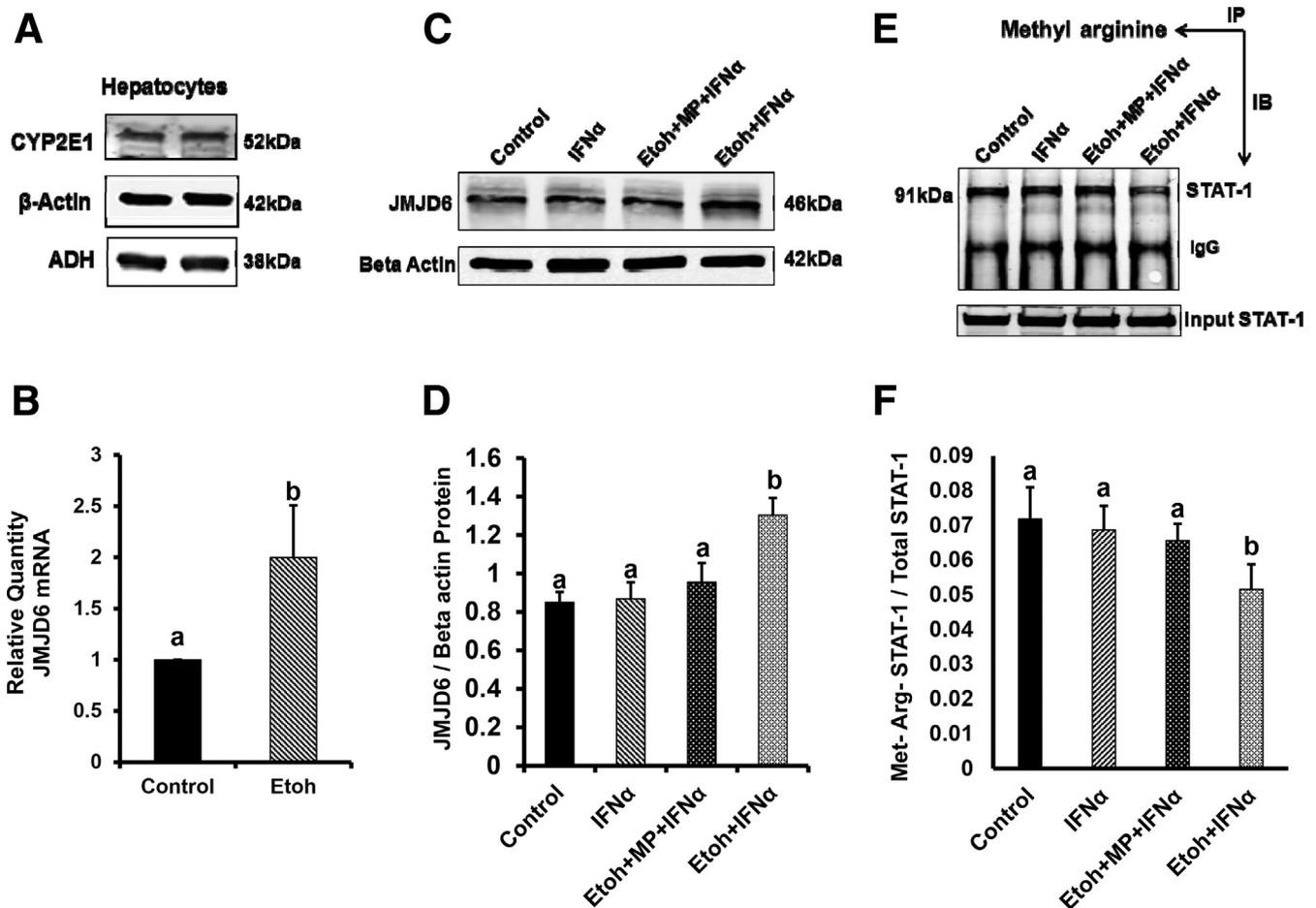


Figure 5. JMJD6 levels and STAT1 methylation in primary human hepatocytes. (A) Expression of CYP2E1 and ADH in Matrigel-overlaid human hepatocytes. Hepatocytes were overlaid in Matrigel and CYP2E1 and ADH expression was measured by WB after 4 days of culturing. (B) Effects of ethanol (EtOH) on mRNA JMJD6 expression: Matrigel-overlaid hepatocytes were exposed to 50 mmol/L ethanol for 48 hours and then JMJD6 mRNA expression was measured by RT-PCR. (C and D) Effects of ethanol on JMJD6 protein expression in human hepatocytes overlaid in Matrigel: hepatocytes were exposed to 50 mmol/L ethanol combined or not with 4-MP, in the presence or absence of IFN α . Then, JMJD6 expression was measured by WB. (E and F) Hepatocytes were treated as described in panel C, then STAT1 methylation was measured by immunoprecipitation WB (as in Figure 1C). Data are from 3 independent experiments presented as means \pm SD. Bars marked with the same letter are not significantly different from each other; bars with different letters are significantly different ($P \leq .05$). IB, immunoblotting; IP, immunoprecipitation.

inhibited by N-acetylcysteine, there is no JMJD6 mRNA increase by AGS. The same pattern was observed with betaine (Figure 4E), suggesting a possibility of an antioxidant effect of betaine.

In Vivo Regulation of JMJD6 by Ethanol

To test whether JMJD6 is up-regulated by ethanol feeding, we used liver tissue from 2 mouse models: C57Bl/6 mice and TK-NOG chimeric mice transplanted with human hepatocytes (humanized mice). These mice were fed control and ethanol liquid Lieber DeCarli diets in the presence or absence of betaine for 10 days followed by phosphate-buffered saline/ethanol gavage on day 11. Then, liver JMJD6 expression was measured by RT-PCR. In C57BL6 mice, ethanol feeding increased JMJD6 expression by 80% (Figure 6A), and the effect of ethanol was reversed by betaine co-administration. Betaine also

restored ethanol-induced suppression of glutathione (Figure 6B) and decreased thiobarbituric acid reactive substances TBARS levels as detected by the kit purchased from Cayman Chemicals (Ann Arbor, MI) (Figure 6C), indicating betaine's ability to reduce oxidative stress in ethanol-fed mice.

In humanized mice, ethanol feeding induced an approximately 120% increase in JMJD6 mRNA levels (Figure 6D). This increase was fully prevented by betaine supplementation in the ethanol diet. Because the JMJD6 primer probe is species-specific, we assume that the changes in JMJD6 levels by ethanol and betaine were reflective of the response of human hepatocytes to these agents.

JMJD6 and HCV

We tested whether infection of RLW cells with HCV affected the expression of JMJD6. Cells were infected with

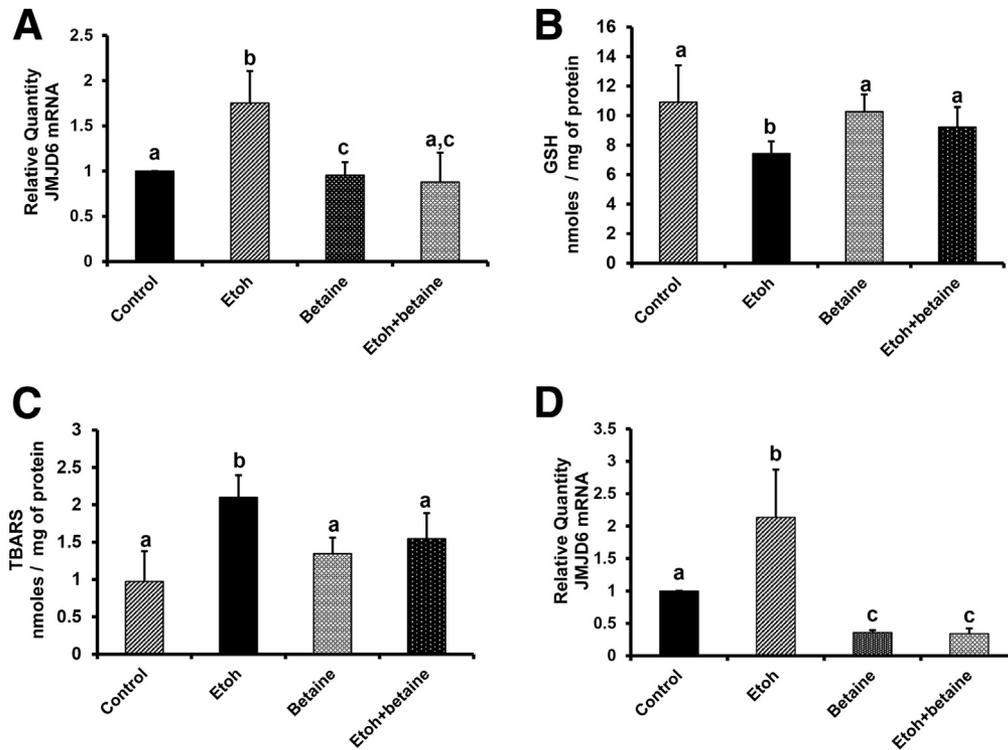


Figure 6. In vivo effects of ethanol on JMJD6 expression in the liver. (A) C57Bl/6 mice were fed ethanol in the presence or absence of betaine (6 mice/group) (see the [Materials and Methods](#) section), and then JMJD6 mRNA was measured by RT-PCR in liver tissue. (B) Glutathione was measured in the livers of C57Bl/6 mice fed as indicated in panel A. (C) Thiobarbituric acid reactive substances (TBARS) were measured in C57Bl/6 mice fed as indicated in panel A. (D) TK-NOG mice with humanized livers were fed ethanol in the presence or absence of betaine (4 mice/group), and JMJD6 mRNA was measured by RT-PCR in liver tissue (please note that human-specific primer probes, which do not cross-react with mouse probes, were used). Data are presented as means \pm SD. Bars marked with the same letter are not significantly different from each other; bars with different letters are significantly different ($P \leq .05$). EtOH, ethanol.

HCV for 48 hours, exposed to AGS for the next 48 hours, and then JMJD6 mRNA was measured by RT-PCR. The uninfected RLW cells treated with AGS were used as a negative control. We observed a 100% induction of JMJD6 mRNA in HCV-infected cells compared with uninfected RLW cells, which was further enhanced by 50% with AGS treatment (Figure 7A). Similarly, in HCV-infected humanized mice, ethanol feeding increased JMJD6 2-fold, which was comparable with the ethanol-mediated JMJD6 increase in the livers of ethanol-fed, noninfected, TK-NOG humanized mice (Figure 7B); HCV-infection increased JMJD6 mRNAs levels in hepatocytes compared with uninfected humanized mice fed even control diet. In HCV-transgenic mice, the level of JMJD6 was 35% higher than in their C57Bl/6 littermates (Figure 7C).

HCV impairs both STAT1 methylation and IFN-induced innate immunity protection in hepatocytes.²⁶ Thus, we tested whether JMJD6 expression regulates IFN α -induced activation of ISGs and affects HCV-RNA levels. To this end, JMJD6 was either silenced by siRNA or overexpressed by the plasmid transfection in IFN α -treated Huh7.5 cells. Then, the activation of representative ISGs, OASL, and OAS-1, as well as HCV RNA, were measured. Silencing of JMJD6 by siRNA increased OASL expression by 33% and OAS-1 by 80%, and decreased HCV RNA by 21%. However, JMJD6

overexpression suppressed OASL mRNA by 29% and OAS-1 by 40% and increased HCV RNA by 30% (Figure 7D–I). This indicates that JMJD6 is one of the factors involved in the regulation of protective IFN α -induced ISGs and HCV-RNA levels in liver cells.

Discussion

According to previously published studies, STAT1 methylation is regulated by PRMT1 and, thus, the impaired IFN α -induced JAK–STAT1 signaling in HCV-infected hepatocytes was attributed to PRMT1 dysfunction.^{5,7} Our earlier work showed that Ach impaired STAT1 methylation in uninfected and HCV-infected Huh7.5–CYP (RLW) cells. This ultimately disrupted IFN α signaling via the JAK–STAT1 pathway to attenuate ISG activation.⁴ Because the combination of PRMT1 inhibitor and Ach more potently suppresses STAT1 methylation than each treatment separately, we hypothesized that other factors in addition to PRMT1 regulate STAT1 methylation. One of the factors is a demethylase, JMJD6. We observed that alcohol metabolism, but not alcohol, is responsible for JMJD6 induction. Our in vitro studies used Huh7.5 cells, which do not metabolize ethanol. To overcome this, Huh7.5 cells were transfected with CYP2E1 (designated as RLW cells) and exposed to the AGS,

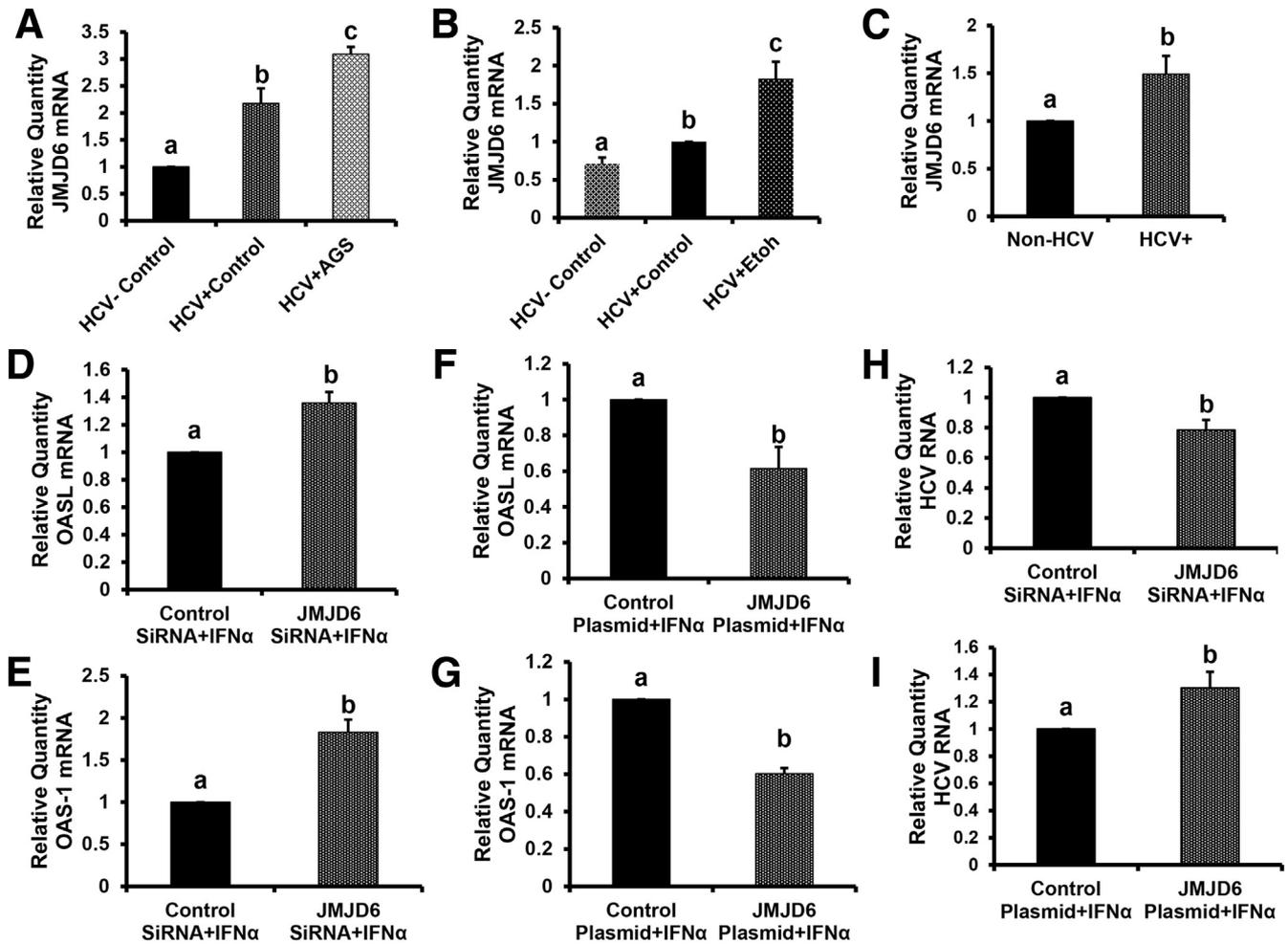


Figure 7. JMJD6 regulates IFN α -mediated innate immunity protection in HCV+ liver cells. (A) Effects of HCV and AGS on JMJD6 mRNA expression in RLW cells. Cells were infected with HCV for 4 days, in the presence or absence of AGS, and JMJD6 mRNA was measured by RT-PCR. (B) JMJD6 mRNA expression was increased by ethanol feeding in HCV-infected humanized SCID-Alb mice. HCV-infected humanized mice were fed by control or ethanol diet as described in the [Materials and Methods](#) section (3 mice/group), and JMJD6 mRNA was measured by RT-PCR. (C) JMJD6 mRNA expression in livers of HCV core-transgenic mice vs C57BL/6 (4 mice from each group). (D and E) Silencing of JMJD6 enhances activation of ISGs (OASL and OAS-1). HCV-infected cells were transfected with scrambled (control) or JMJD6 siRNA and pretreated with IFN α (200 IU, 4 h), and then OASL and OAS-1 mRNAs were measured by RT-PCR. (F and G) Overexpression of JMJD6 suppresses ISG (OASL and OAS-1) mRNA expression. Cells were transfected with JMJD6 plasmid or empty vector and in 48 hours were exposed to 200 IU IFN α for 4 hours; then OASL and OAS-1 mRNAs expression were measured. (H) JMJD6 silencing suppresses HCV-RNA levels. HCV-infected cells were treated as in panel D and HCV-RNA level was measured. (I) JMJD6 overexpression enhances HCV-RNA levels. Cells were treated as in panel F and HCV-RNA level was measured. Cell data are from 3 independent experiments presented as means \pm SD. Bars marked with the same letter are not significantly different from each other; bars with different letters are significantly different ($P \leq .05$). EtOH, ethanol.

which enzymatically generates physiologically relevant amounts of Ach as shown in several of our earlier publications.^{1,4,25,27} Remarkably, a possibility that ethanol metabolites and HCV decrease STAT1 arginine methylation by inducing demethylation of STAT1 has never been investigated. Here, we show that JMJD6 overexpression suppresses, whereas silencing it enhances, STAT1 methylation, indicating the direct role of JMJD6 in regulating STAT1 methylation. In HCV infection, STAT1 methylation dysfunction is induced via the PP2A mechanism and is accelerated by ethanol metabolism.^{4,6} Interestingly, an Ach-induced

increase in the demethylase expression also is regulated via PP2A, because its inhibition by OA partially prevented Ach-induced JMJD6 increase. Increased expression of JMJD6 in AGS-treated RLW cells was more robust at mRNA than at protein levels. This may be related to the fact that RLW cells are of hepatoma origin and high basal levels of JMJD6 protein in cancer cells is well documented.²⁸⁻³⁰ In fact, in Matrigel-overlaid primary human hepatocytes that express ethanol-metabolizing enzymes for more than 4 days in vitro, JMJD6 mRNA and protein levels are increased equally by ethanol exposure. Furthermore, the effects of ethanol on

JMJD6 was prevented by co-treatment with 4-MP, an ADH inhibitor, supporting the involvement of ethanol metabolism and, namely, its ADH-generated product, Ach, in the regulation of JMJD6 levels in hepatocytes. Up-regulation of JMJD6 was accompanied by ethanol-metabolism-induced suppression of arginine methylation of STAT1 detected in the same cells, thereby confirming the link between these parameters. In addition, an ethanol-induced increase in JMJD6 expression in the liver also was confirmed by *in vivo* feeding of C57Bl/6 and humanized mice.

In vitro and *in vivo* studies have shown that promethylating agents SAM and betaine restore HCV-impaired STAT1 methylation and suppress HCV RNA.^{4,26,31} However, it is not clear whether these agents also reverse ethanol-metabolism-enhanced JMJD6. Here, we show that betaine attenuates the *in vitro* effects of AGS (Ach). Protection by betaine seems to be related to antioxidant properties of this supplement rather than via the BHMT-mediated pathway because the BHMT inhibitor, dimethyl glycine, did not reverse the effects of betaine on Ach-increased JMJD6 expression. However, along with betaine, N-acetylcysteine attenuated the effects of AGS on JMJD6 overexpression, suggesting that antioxidants suppress ethanol-metabolism-induced JMJD6 increase. Importantly, in ethanol-fed C57Bl/6 mice, up-regulation of JMJD6 was prevented by betaine supplementation. We also observed the reduction of ethanol-induced oxidative stress by co-treatment with betaine because betaine supplementation enhances the level of glutathione and suppresses thiobarbituric acid-reactive substances in ethanol-fed mice. This may suggest that betaine works as an antioxidant and, thus, its suppressive effect on ethanol-increased JMJD6 expression may occur via this mechanism. Similar liver protection from oxidative stress by betaine was observed in our previously published study on ethanol-fed HCV-transgenic mice.²⁷ Thus, we cannot exclude that betaine indirectly affects oxidative stress and subsequent JMJD6 levels.

Modulation of JMJD6 levels by viruses already has been shown in the literature,^{32,33} but nothing is known about the role of JMJD6 in HCV infection. We found that HCV up-regulates JMJD6 in RLW cells and in the livers of HCV-infected humanized mice fed ethanol. Thus, HCV and ethanol metabolism both increase JMJD6 levels in the livers of ethanol-fed humanized mice. Ethanol feeding to humanized SCID mice definitely decreased antiviral protection in hepatocytes, leading to the persistence of HCV RNA, whereas mice on the control diet were able to clear the virus (see the [Materials and Methods](#) section and Osna et al²⁴). This lack of viral clearance in hepatocytes may be attributed to ISG suppression by impaired IFN signaling through the JAK-STAT1 pathway related to the JMJD6 increase-induced reduction in STAT1 methylation. Previously, we indeed observed almost equal suppression of many ISGs (viperin, OAS1, OASL, and Protein kinase R) by Ach in HCV-infected liver cells in response to IFN α -induced activation of the JAK-STAT1 pathway.⁴ Here, we studied whether JMJD6 regulates the expression of ISGs and subsequent HCV-RNA levels in hepatocytes. We would like to stress that we focused on IFN α -induced ISG activation, which is known to

be a result of triggering the JAK-STAT1 pathway. This is important because in HCV infection, the suppression of antiviral ISGs by ethanol in the absence of IFN α has been attributed to some other mechanisms, similar to the competition between ethanol and retinoic acid metabolisms in hepatocytes.³⁴ However, activation of these ISGs does not occur through the IFN α -induced JAK-STAT1 pathway, which serves as the next level of innate immunity regulation activated in hepatocytes by sensing of macrophage- and dendritic cell-secreted IFN α . In this study, we found increased activation of ISGs (using OASL and OAS-1 as representative genes) by IFN α and subsequent suppression of HCV RNA by JMJD6 silencing. In contrast, attenuation of ISG activation by IFN α accompanied by an increase in HCV RNA occurred in JMJD6-overexpressed HCV-infected Huh7.5 cells. This suggests that JMJD6 is one of the factors that regulates HCV infectivity levels in hepatocytes by suppressing IFN α -dependent ISG activation via the JAK-STAT1 pathway. Thus, by increasing JMJD6 expression, Ach promotes propagation of HCV in hepatocytes ([Figure 8](#)).

We conclude that arginine methylation of STAT1 is suppressed by JMJD6. Both HCV and acetaldehyde increase JMJD6 levels, thereby impairing STAT1 methylation and

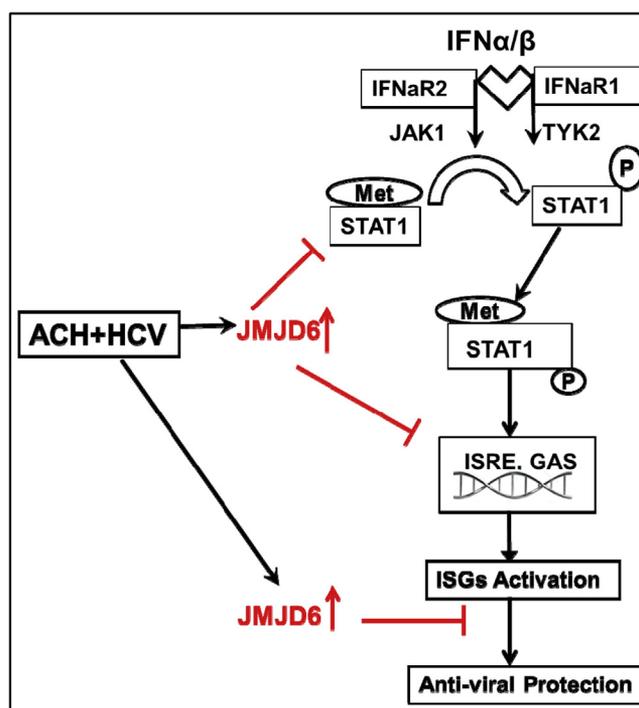


Figure 8. HCV and ethanol metabolism increase JMJD6 levels in liver cells (scheme). Normally, interferon type 1 induces IFN signaling in hepatocytes, leading to phosphorylation (p) of STAT factors and their translocation to nucleus, attachment to DNA, and activation of antiviral protective ISGs. Attachment of activated (p) STAT1 to DNA is regulated by STAT1 methylation (Met STAT1). Acetaldehyde and HCV activate demethylase JMJD6, which decreases STAT1 methylation, thereby attenuating STAT1 attachment to DNA, subsequent ISG activation, and antiviral protection. This results in a HCV-RNA level increase. ISRE, interferon stimulated response element.

innate immunity protection in hepatocytes exposed to virus and/or alcohol.

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- Author contributions**
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The authors disclose no conflicts.
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