Long noncoding RNA #32 contributes to antiviral responses by controlling interferon-stimulated gene expression

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Despite the breadth of knowledge that exists regarding the function of long noncoding RNAs (lncRNAs) in biological phenomena, the role of lncRNAs in host antiviral responses is poorly understood. Here, we report that lncRNA#32 is associated with type I IFN signaling. The silencing of lncRNA#32 dramatically reduced the level of IFN-stimulated gene (ISG) expression, resulting in sensitivity to encephalomyocarditis virus (EMCV) infection. In contrast, the ectopic expression of lncRNA#32 significantly suppressed EMCV replication, suggesting that lncRNA#32 positively regulates the host antiviral response. We further demonstrated the suppressive function of lncRNA#32 in hepatitis B virus and hepatitis C virus infection. lncRNA#32 bound to activating transcription factor 2 (ATF2) and regulated ISG expression. Our results reveal a role for lncRNA#32 in host antiviral responses.

lncRNA | innate immunity | interferon | ISG

Type I interferons (IFNs) up-regulate the transcription of hundreds of IFN-stimulated genes (ISGs) by activating the well-characterized Janus kinase/signal transducers and activators of transcription (JAK–STAT) pathway (1). Several in vitro studies have shown that ISGs induced by type I IFNs contribute to antiviral effects (2, 3). The treatment of chronic infections caused by hepatitis C virus (HCV) is currently based on a combination of PEGylated IFN-α (PEG–IFN-α) and ribavirin, and genetic variation in the *IL28B* region is associated with sustained virological responses (4). However, the positive response generated by the *IL28B* genotype is strongly associated with a lower level of ISG expression (5). The antiviral effect of type I IFN alone cannot account for the induction of protein-coding ISGs, suggesting that the identification of novel antiviral factors is required to elucidate the antiviral action of the IFN response.

The Encyclopedia of DNA Elements (ENCODE) project, which aims to catalog all of the biological functional elements in the human genome, has concluded that 80% of the human genome is functional and that the majority is transcribed into different types of RNAs, including noncoding RNAs (ncRNAs) (6). ncRNAs are classified as short ncRNAs, such as the well-characterized microRNAs, or long ncRNAs (lncRNAs). Most lncRNAs, as well as protein-coding mRNAs, are capped, polyadenylated, and spliced by cellular machinery (7). Recently, it was clearly elucidated that many lncRNAs have important roles in biological processes such as differentiation, apoptosis, development, and immune responses (8–11), and that they are tightly regulated by the cellular developmental or differentiation stage and by various biological stimuli (12, 13). In particular, several groups have reported the involvement of lncRNAs in many aspects of the adaptive or innate immune response.

Murine NeST governs microbial susceptibility by regulating the adaptive immune response in mice (14). The T helper 2 (Th2)-specific lincR-CCR2-5′AS regulates the migration of Th2 cells to the lungs (12). The stimulation of Toll-like receptors leads to the activation of lncRNAs, including lncRNA-Cox2, PACER, and NEAT1, which regulate the inflammatory response through specific interactions with cellular proteins (15–17). Moreover, recent studies have indicated that lncRNAs are involved in type I IFN signaling. A number of lncRNAs are induced by viral infection or IFN-β (18, 19). lncRNA-CMPK2, which is stimulated by IFN-α through the JAK–STAT pathway, suppresses the expression of ISGs such as IFIT3, ISG15, and IFITM1 (20). Influenza A virus-induced lncRNA (NRAV) inhibits the host response to viral infection by suppressing ISG expression (21). bone marrow stromal cell antigen 2 (BST2) IFN-stimulated positive regulator (BISPR) was identified as a positive regulator of BST2 expression (22, 23).

This study shows that lncRNA#32 has a critical role in ISG expression. The targeted depletion of lncRNA#32 leads to a reduction in the mRNA levels of several ISGs, including 2′-5′-Oligoadenylate Synthetase Like (OASL), Radical S-Adenosyl Methionine Domain Containing 2 (RSAD2), interferon gamma-induced protein 10 (IP-10), apolipoprotein B mRNA editing enzyme catalytic polypeptide 3A (APOBEC3A), and apolipoprotein B mRNA editing enzyme catalytic polypeptide 3G (APOBEC3G), and results in a significant increase in the replication of encephalomyocarditis virus (EMCV), hepatitis B virus (HBV), and HCV, all of which are human pathogens. In contrast, the overexpression of lncRNA#32 increases ISG mRNA levels. lncRNA#32 is stabilized by heterogeneous nuclear ribonucleoprotein U (hnRNPU) and functions through interactions with activating transcription factor 2 (ATF2). These results reveal that lncRNA#32 is a potential antiviral host factor that acts in concert with hnRNPU and ATF2.

Results

Identification of lncRNAs Involved in the Innate Immune Response. To identify lncRNAs involved in the innate immune response, we performed a cDNA microarray analysis. We first knocked out interferon regulatory factor (IRF)3 (IRF3-KO) in immortalized human hepatocytes (HuS cells) using the CRISPR method. Western

Significance

Here, we describe a key feature of the long noncoding RNA (lncRNA) involved in innate immunity. We identified 182 lncRNAs that were highly modulated in poly(I:C)-treated hepatocyte cells. Of these, lncRNA#32 regulated the level of IFN-stimulated genes under both unstimulated and type I IFN-stimulated conditions through interactions with hnRNPU and ATF2, and therefore plays an important role in antiviral immunity.

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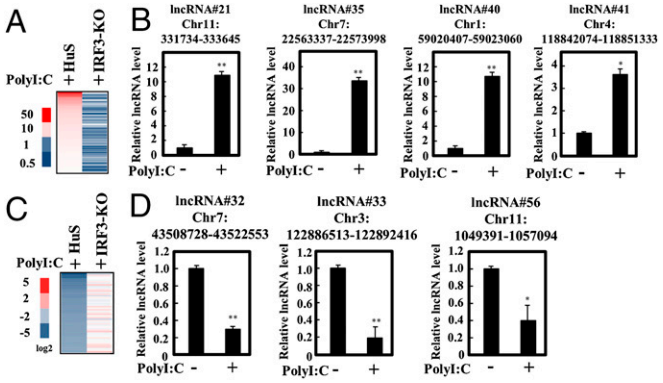

Fig. 1. Poly(I:C) induction of lncRNAs expression is IRF3 dependent. (*A* and *C*) Heat map showing greater than fourfold changes (*A*, up-regulated in HuS; *C*, down-regulated in HuS) in lncRNA expression following treatment with 50 ng of poly(I:C) for 18 h as determined by the cDNA microarray analysis of HuS or IRF3-KO HuS cells. (*B* and *D*) RT-qPCR analysis of lncRNAs following the treatment of HuS cells with poly(I:C) for 18 h. The data are normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. $*P < 0.05$; $**P < 0.01$.

blotting confirmed that the endogenous IRF3 protein was not detected in IRF3-KO-#1 cells following genomic editing by CRISPR (Fig. S1*A*) and that IRF3 deficiency resulted in the defective stimulation of IFN-β and ISG15 in response to poly(I:C) in IRF3-KO-#1 cells (Fig. S1*B*). In addition, the ectopic expression of IRF3 in IRF3-KO-#1 cells rescued the defect in response to poly(I:C) (Fig. S1*B*).

Wild-type (WT) and IRF3-KO cells were stimulated by poly(I:C) transfection for 18 h, and a gene expression microarray analysis was performed using the Agilent SurePrint G3 Human Gene Expression 8X60K V2 Microarray Kit. A total of 131 up-regulated and 51 down-regulated lncRNAs (fold change, >4) were detected in WT-HuS cells, but not in IRF3-KO cells, following poly(I:C) treatment (Fig. 1 *A* and *C*). The top 60 lncRNAs (48 up-regulated and 12 down-regulated lncRNAs) were selected, and lncRNA expression was verified by reverse transcription–quantitative PCR (RT-qPCR) (Fig. 1 *B* and *D*). A statistically significant correlation between the results of the RT-qPCR and microarray analysis was obtained for only 7 of the 60 lncRNAs [4 up-regulated lncRNAs (lncRNA#21, lncRNA#35, lncRNA#40, and lncRNA#41) and 3 down-regulated lncRNAs (lncRNA#32, lncRNA#33, and lncRNA#56)]. To identify lncRNAs functioning in the antiviral response, we examined the impact of deleting lncRNAs on the EMCV cytopathic effect. When lncRNA expression was silenced by siRNA (lncRNA#21, lncRNA#35, and lncRNA#32) or shRNA (lncRNA#33, lncRNA#40, lncRNA#41, and lncRNA#56) (Fig. S2*A*), no change in cell viability was observed (Fig. S2*B*). Each lncRNA knockdown cell line was treated with poly(I:C) for 24 h and then infected with EMCV for 48 h. As expected, we observed that cell viability of HuS cells treated with siControl and shControl was reduced by EMCV infection, and treatment with poly(I:C) before infection resulted in resistance to EMCV infection (Fig. 2). Similar to the control cells, the silencing of lncRNA#21, lncRNA#33, lncRNA#35, lncRNA#40, lncRNA#41, and lncRNA#56 in HuS cells had no effect on resistance to EMCV infection when the cells were treated with poly(I:C) (Fig. 2). In contrast, the silencing of lncRNA#32 using two different siRNAs to minimize the possibility of off-target effects dramatically decreased cell viability of EMCV-infected HuS cells despite treatment with poly(I:C).

Based on the above results, we focused on the role of lncRNA#32 in the antiviral response. Human lncRNA#32 (LOC100506895) is located on chromosome 7p13 and overlaps with the antisense strand of the HECW1 coding gene located within introns 21 and 22 and exon 22. Previously, 5′- and 3′-RACE studies revealed that the lncRNA#32 transcript was 2,946 nt in length (Fig. S1).

We performed RT-qPCR to assess the expression of lncRNA#32 in various human tissues and showed it was expressed at variable levels in a wide variety of tissues (Fig. S3). These results suggest that lncRNA#32 has a critical role in the innate immune response.

IFN-β Suppresses lncRNA#32 Expression. Because the silencing of lncRNA#32 expression strongly reduced the effect of poly(I:C) treatment, we next examined whether lncRNA#32 altered the antiviral effect of type I IFN in HuS cells. Treatment with IFN-β suppressed lncRNA#32 expression in HuS cells (Fig. 3*A*). Northern blot analysis using a specific probe confirmed that lncRNA#32 expression was significantly reduced in IFN-β–treated HuS cells (Fig. 3*B*). The expression of lncRNA#32 was reduced by IFN-β in a dose-dependent manner, whereas the expression of OASL, an ISG, was clearly induced by IFN-β (Fig. S4*A*). To address the relationship between the expression of ISGs and lncRNA#32, HuS cells were treated with IFN-β and a time/expression analysis was performed to determine OASL mRNA and lncRNA#32 expression over a 24-h period (Fig. S4*B*). The level of OASL mRNA reached a peak at 12 h after treatment with IFN-β and this increase was mirrored by a decrease in lncRNA#32 expression. These data strongly suggest the presence of negative-feedback regulation that controls the regulation of lncRNA#32 by IFN-β.

To verify the role of lncRNA#32 in the antiviral effect, we treated siControl- or silncRNA#32-transduced HuS cells and then measured cell viability after EMCV infection. Knockdown of lncRNA#32 reduced cell viability after EMCV infection in the absence of IFN-β (Fig. 3*C*), suggesting that lncRNA#32 contributes to EMCV-induced innate immune responses. The antiviral effect of IFN-β was also impaired by transfection with silncRNA#32 but not with siControl or silncRNA#35 (Fig. 3*D*). As expected, the viral titer of the supernatant from silncRNA#32-transduced HuS cells was significantly higher than that from siControl-transduced HuS cells (Fig. 3*E*). Moreover, the ectopic expression of lncRNA#32 decreased EMCV levels, even in the absence of IFN-β (Fig. 3 *F* and *G*).

These data indicate that lncRNA#32 has a critical role in antiviral responses.

lncRNA#32 Regulates the Expression of ISGs. To provide mechanistic insight into the antiviral action of lncRNA#32, we performed a

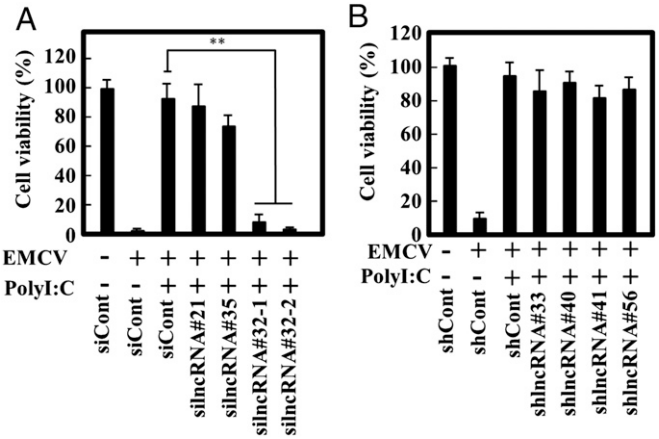

Fig. 2. lncRNA#32 has an important role in the antiviral effect of poly(I:C). HuS cells were transduced with the indicated siRNAs (*A*) or shRNAs (*B*). Twenty-four hours after transduction, the cells were treated with 10 ng/mL poly(I:C) for 24 h and then infected with EMCV [multiplicity of infection (MOI) = 0.1] for 48 h. Cell viability was determined using CellTiter-Glo (Promega). The data are shown as the mean ± SD of three independent experiments. $**P < 0.01$.

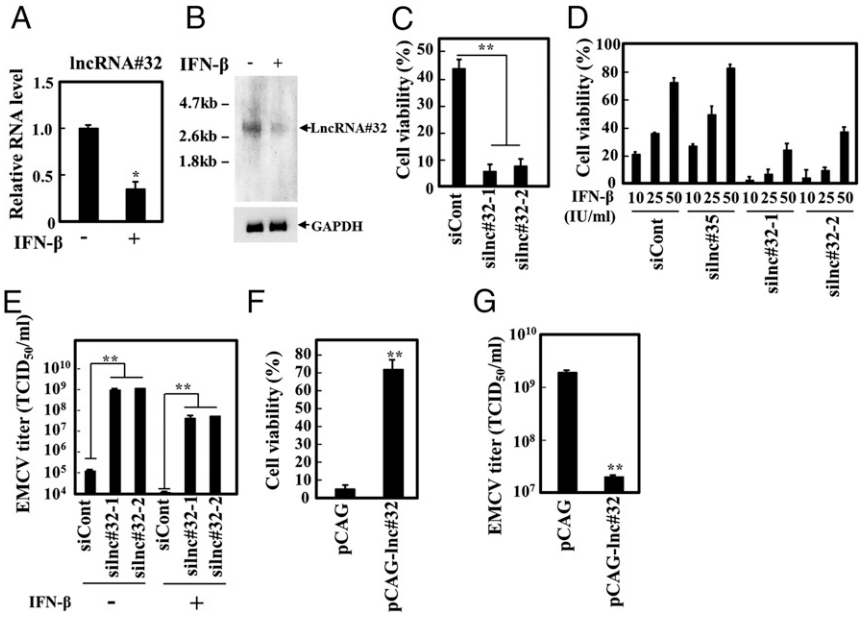

Fig. 3. lncRNA#32 regulates the antiviral effect of IFN-β. (*A* and *B*) HuS cells were treated with 50 IU/mL IFN-β for 12 h. The level of lncRNA#32 expression was determined by RT-qPCR (*A*) and Northern blotting (*B*). (*C*) HuS cells were transfected with siControl or silncRNA#32. Twenty-four hours after transfection, the cells were infected with EMCV (MOI = 0.02) for 48 h. Cell viability was determined using CellTiter-Glo. (*D*) HuS cells were transfected with the indicated siRNAs. Twenty-four hours after transfection, the cells were treated with different doses of IFN-β for 24 h and infected with EMCV (MOI = 0.1) for 48 h. Cell viability was determined using CellTiter-Glo. (*E*) HuS or lncRNA#32 knockdown HuS cells were treated with 10 IU/mL IFN-β for 24 h and then infected with EMCV (MOI = 0.1). Forty-eight hours after infection, the viral titer in the supernatant was measured using the 50% tissue culture infective dose ($TCID_{50}$) bioassay. (*F* and *G*) HuS or lncRNA#32-overexpressing HuS cells were infected with EMCV (MOI = 0.1). Cell viability (*F*) and viral titers (*G*) were determined 48 h after infection. The data are shown as the mean ± SD of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

cDNA microarray analysis to profile gene expression in response to IFN-β treatment in lncRNA#32 knockdown HuS cells. The microarray data revealed that many known ISGs and chemokines, including IRF7, OASL, RSAD2, Chemokine C–C motif ligand 5 (CCL5), C–X–C motif chemokine 11 (CXCL11), and IP-10, were reduced by the silencing of lncRNA#32 (Fig. 4*A*). These findings were further confirmed by RT-qPCR (Fig. 4 *B* and *C*). Surprisingly, the silencing of lncRNA#32 decreased the level of ISG and chemokine expression in the absence of IFN-β, suggesting that lncRNA#32 itself regulates the level of ISG and chemokine expression under both unstimulated and IFN-stimulated conditions. Moreover, we observed that lncRNA#32 was significantly reduced after IFN-β treatment in PXB cells isolated from urokinase-type plasminogen activator transgenic/SCID mice bearing human primary hepatocytes (Fig. 4*D*). The disruption of lncRNA#32 impaired the expression of IP-10 mRNA in PXB cells (Fig. 4*E*). Importantly, these effects were also observed in THP-1 macrophages (Fig. S5). In addition, the

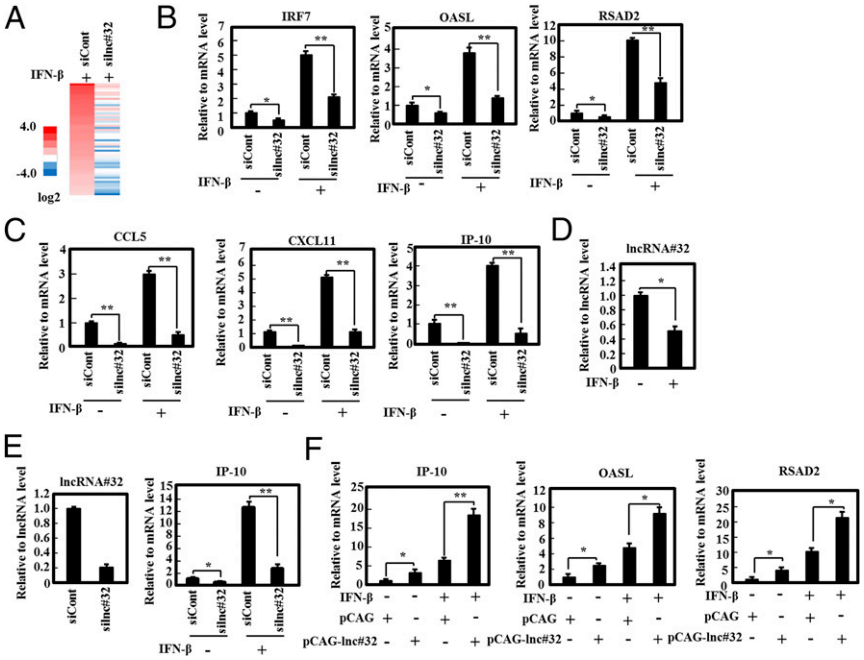

Fig. 4. lncRNA#32 regulates ISG expression. (*A*) Heat map representation of differentially regulated genes in siControl- or silncRNA#32-transfected HuS cells stimulated with 50 IU/mL IFN-β for 12 h. (*B* and *C*) HuS cells were transfected with siControl or silncRNA#32 for 24 h and then treated with 20 IU/mL IFN-β for 24 h. The mRNA levels of antiviral ISGs (*B*) and IFN-inducible chemokines (*C*) were determined by RT-qPCR and normalized to GAPDH. (*D*) PXB cells were treated with 50 IU/mL IFN-β for 18 h. The level of lncRNA#32 was determined by RT-qPCR. (*E*) PXB cells were transfected with siControl or silncRNA#32 for 24 h and then treated with 50 IU/mL IFN-β for 18 h. The level of lncRNA#32 or IP-10 mRNA was determined by RT-qPCR. (*F*) HuS cells were transfected with pCAG or pCAG-lncRNA#32. Forty-eight hours after transfection, the cells were treated with 20 IU/mL IFN-β. The mRNA levels of the indicated genes were determined by RT-qPCR. The data are shown as the mean ± SD of three independent experiments. The data are normalized to GAPDH expression. $^{*}P < 0.05$; $^{**}P < 0.01$.

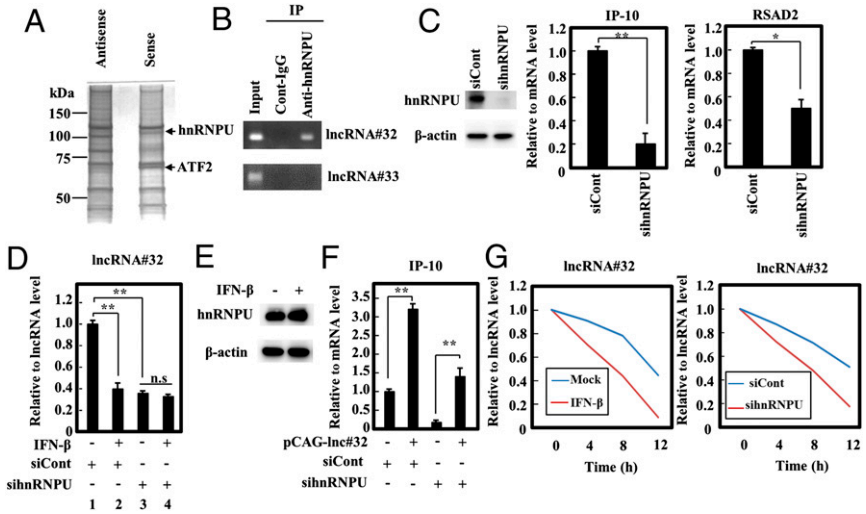

Fig. 5. hnRNPU and ATF2 interact with lncRNA#32, and hnRNPU stabilizes lncRNA#32. (*A*) Sense and antisense lncRNA#32 RNA were biotinylated by in vitro transcription and incubated with HuS total cell lysate. Silver staining was performed to visualize biotinylated lncRNA#32-associated proteins after SDS/PAGE. The lncRNA#32-specific bands (arrows) were excised and analyzed by mass spectrometry. (*B*) HuS total cell lysates were subjected to immunoprecipitation with control IgG or an anti-hnRNPU antibody. The resulting immunocomplexes were then analyzed for the presence of lncRNA#32 or lncRNA#33 by RT-PCR. (*C*) HuS cells were transfected with siControl or sihnRNPU. Forty-eight hours after transfection, the levels of IP-10 and RSAD2 mRNA were determined by RT-qPCR. The data are normalized to GAPDH expression. Knockdown efficiency of hnRNPU was determined by Western blotting. (*D*) HuS cells were transfected with siControl or sihnRNPU. Forty-eight hours after transfection, the cells were treated with 50 IU/mL IFN-β. Twelve hours after treatment, the level of lncRNA#32 expression was determined by RT-qPCR and normalized to GAPDH. (*E*) HuS cells were treated with 50 IU/mL IFN-β for 24 h. The level of hnRNPU expression was determined by Western blotting. (*F*) HuS cells were transfected with siControl or sihnRNPU for 24 h and then transfected with pCAG or pCAG-lncRNA#32. Forty-eight hours after transfection, the level of IP-10 mRNA was determined by RT-qPCR and normalized to GAPDH. (*G*) IFN-β–stimulated HuS (*Left*) or hnRNPU-knockdown HuS (*Right*) cells were treated with actinomycin D. The cells were harvested for RNA extraction at the indicated time points, and the level of lncRNA#32 was determined by RT-qPCR and normalized to GAPDH. The data are shown as the mean ± SD of three independent experiments. $*P < 0.05$; $**P < 0.01$.

overexpression of lncRNA#32 in HuS cells led to the induction of the basal level expression of these genes (Fig. 4*F*).

lncRNA#32 Binds to hnRNPU. Many lncRNAs physically bind to other cellular factors to mediate their functions (8, 9, 14, 15, 17, 21). To identify the functional binding partners of lncRNA#32, we performed an RNA pull-down assay using biotinylated lncRNA#32. Biotinylated sense lncRNA#32 (or antisense lncRNA#32 as a negative control) was incubated with total protein extract from HuS cells and pulled down with streptavidin magnetic beads. The associated proteins were analyzed by SDS/PAGE and silver staining (Fig. 5*A*). Two distinct bands specifically present in the sense lncRNA#32 pull-down samples were analyzed by mass spectrometry and identified as heterogeneous nuclear ribonucleoprotein U (hnRNPU) and ATF2.

To confirm that hnRNPU physically binds to lncRNA#32 in HuS cells, we performed RNA immunoprecipitation. lncRNA#32, but not control lncRNA#33, was precipitated with an anti-hnRNPU antibody (Fig. 5*B*). hnRNPU has been shown to bind to several lncRNAs, including XIST and Firre (24, 25), and it broadly inhibits the replication of RNA viruses (26). Moreover, hnRNPU promotes the stabilization of Toll-like receptor-induced cytokine mRNAs (27). We next investigated whether hnRNPU was involved in the expression of lncRNA#32-regulated genes. Under unstimulated conditions, the knockdown of hnRNPU resulted in a decrease in IP-10 and RSAD2 expression, which was regulated by lncRNA#32 (Fig. 5*C*). We also determined whether hnRNPU affected the expression of lncRNA#32. Consistent with the results in Fig. 2*A*, the level of lncRNA#32 expression was inhibited by IFN-β treatment in siControl cells (Fig. 5*D*, compare lane 1 to lane 2). Moreover, the siRNA-mediated knockdown of hnRNPU significantly reduced the level of lncRNA#32 (Fig. 5*D*, compare lane 1 to lane 3). Interestingly, IFN-β treatment had no effect on lncRNA#32 expression in hnRNPU knockdown cells (Fig. 5*D*, compare lane 3 to lane 4), suggesting that decreased lncRNA#32 expression caused by IFN-β treatment was hnRNPU dependent. In addition, the decrease of lncRNA#32 expression by IFN-β treatment was not caused by changes in hnRNPU expression. Indeed, there was no effect on hnRNPU expression by IFN-β treatment (Fig. 5*E*). Notably, the overexpression of lncRNA#32 in hnRNPU knockdown cells rescued IP-10 expression (Fig. 5*F*). To gain further insight into the mechanism by which lncRNA#32 expression is reduced by IFN-β treatment or hnRNPU knockdown, we analyzed the half-life of lncRNA#32 mRNA by inhibiting RNA PolII transcription with actinomycin D. The level of lncRNA#32 was significantly decreased in IFN-β–treated or hnRNPU-knockdown cells compared with that in control cells. Curves showing the decline in lncRNA#32 levels as a function of time were similar for both the IFN-β–treated and hnRNPU-knockdown cells (Fig. 5*G*). These results suggest that hnRNPU regulates the stabilization of lncRNA#32.

ATF2 Is Required for the Function of lncRNA#32. A pull-down assay using biotinylated sense lncRNA#32 identified hnRNPU and ATF2 as interacting proteins (Fig. 5*A*). Thus, we investigated whether lncRNA#32 physically binds to ATF2 in HuS cells. We observed an enrichment of lncRNA#32 in the anti-ATF2 antibody precipitates compared with the control IgG precipitates (Fig. 6*A*). To define the region of lncRNA#32 that interacts with ATF2, we conducted an immunoprecipitation experiment using partial fragments of lncRNA#32 and found that a fragment of the 3′-half of lncRNA#32, which consists of nucleotides 2000–2946, specifically bound to recombinant ATF2 (Fig. S6*A*). Moreover, a deletion mutant of the ATF2-binding region of lncRNA#32 did not induce IP-10 expression (Fig. S6*B*).

ATF2 is a member of the leucine zipper family of DNA-binding proteins and is involved in regulating the expression of various genes, including those encoding inflammatory factors (28). To address whether ATF2 participated in lncRNA#32-mediated ISG expression, ATF2 was silenced in lncRNA#32-overexpressing cells

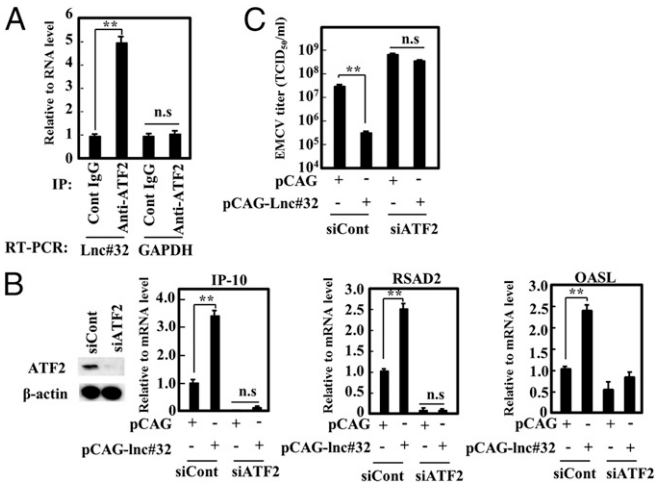

Fig. 6. ATF2 is required for function of lncRNA#32. (*A*) HuS total cell lysates were subjected to immunoprecipitation with control IgG or an anti-ATF2 antibody. The resulting immunocomplexes were then analyzed for the presence of lncRNA#32 and GAPDH by RT-qPCR. (*B*) HuS cells were transfected with siControl or siATF2 for 24 h and then transfected with pCAG or pCAG-lncRNA#32. Forty-eight hours after the second transfection, the level of expression of the indicated genes was determined by RT-qPCR and normalized to GAPDH. Knockdown efficiency of ATF2 was determined by Western blotting. (*C*) HuS cells were transfected with siControl or siATF2 for 24 h and then transfected with pCAG or pCAG-lncRNA#32. Forty-eight hours after the second transfection, the cells were infected with EMCV (MOI = 0.1). Forty-eight hours after infection, the viral titer in the supernatant was measured using the $TCID_{50}$ bioassay. The data are shown as the mean ± SD of three independent experiments. $^{**}P < 0.01$.

(Fig. 6*B*). Consistent with our findings described above, the ectopic expression of lncRNA#32 induced IP-10, RSAD2, and OASL in siCont-transfected cells. The knockdown of ATF2 reduced the levels of these lncRNA#32-regulated genes. Importantly, the ectopic expression of lncRNA#32 had no effect on the expression levels of these genes in ATF2-knockdown cells. Moreover, these effects were correlated with the antiviral effect produced by lncRNA#32 (Fig. 6*C*). To investigate whether IFN-stimulated gene factor 3 (ISGF3), a transcriptional activator of ISGs, affects lncRNA#32 function, STAT2, an ISGF3, was silenced in HuS cells. We confirmed that the depletion of STAT2 reduced RSAD2 expression in IFN-β–treated cells (Fig. S7*A*). Importantly, overexpression of lncRNA#32 induced RSAD2, IP-10, and IRF7 expression in STAT2-depleted cells similar to control cells, suggesting that ISGF3 is not necessary for lncRNA#32 function (Fig. S7*B*). ATF2 binds to the cAMP-responsive element [consensus sequence: 5′-TGACGT(A/C)(A/G)-3′]. Therefore, we searched the cAMP-responsive element in the surrounding promoter region of lncRNA#32-regulated ISGs, and we identified a cAMP-responsive element in intron 1 of IRF7 variant a (Fig. S8*A*), which was regulated by lncRNA#32. To investigate whether ATF2 binds to intron 1 of IRF7 variant a, we performed a chromatin immunoprecipitation assay using anti-ATF2 antibody. We found that ATF2 was present on intron 1 of IRF7 variant a (Fig. S8*B*). Moreover, the silencing of lncRNA#32 impaired the binding of ATF2 to intron 1 of IRF7 variant a (Fig. S8*C*). These results suggest that lncRNA#32 is required for the binding of ATF2 to intron 1 of IRF7 variant a.

lncRNA#32 Contributes to Anti-HBV and -HCV Responses in Primary Hepatocytes. We next demonstrated whether lncRNA#32 contributed to innate immune responses against human pathogenic viruses. Using microarray analysis, we observed that RSAD2 (encoding Viperin) (Fig. 4) and members of the APOBEC3 family of genes (Fig. S9) that exhibit anti-HCV and -HBV activity (29, 30), respectively, were regulated by lncRNA#32 in HuS cells. We therefore examined whether lncRNA#32 regulated the anti-HCV and -HBV response in primary human hepatocytes (PHHs) derived from fresh human tissue. The knockdown of lncRNA#32 in PHH cells was significantly increased during HCV replication under unstimulated conditions [Fig. S10*A*, IFN-β (−)]. Moreover, the anti-HCV activity of IFN-β was impaired by knockdown of lncRNA#32 [Fig. S10*A*, IFNβ (+)]. Similar results were also observed for anti-HBV activity of IFN-β in PHH cells (Fig. S10*B*). We further determined whether ATF2 and hnRNPU, which are lncRNA#32-binding proteins, also affected anti-HCV responses of IFN-β and observed that knockdown of ATF2 or hnRNPU reduced anti-HCV responses in PXB cells under unstimulated and IFN-β–stimulated conditions (Fig. S11 *A* and *B*). In addition, anti-HBV responses were impaired in ATF2 or hnRNPU-knockdown PXB cells under unstimulated and IFN-β–stimulated conditions (Fig. S11 *C* and *D*). These results clearly indicate that lncRNA#32 positively regulates the antiviral response by controlling ISG expression.

Discussion

The ENCODE project revealed that a large portion of the human genome is transcribed into ncRNA, some of which function to regulate biological processes (1). Some lncRNAs have a critical role in adaptive and innate immunity (31). NeST is one such lncRNA that is well characterized in regard to its role in immunity. NeST is expressed in Th1 $CD4^+$ T cells, $CD8^+$ T cells, and NK cells, and controls microbial susceptibility by regulating IFN-γ production in mice (14). Other antiviral-related lncRNAs such as NRAV and lncRNA-CMPK2 are also negative regulators of antiviral gene expression, including ISGs (20, 21). Moreover, previous studies reported that BISPR positively regulates the expression of BST2, which is a protein-coding ISG (22, 23). Both BST-2 and BISPR are induced by IFN-α, and overexpression or knockdown of BISPR resulted in an increase and decrease in BST2 expression, respectively. In addition, EZH2, a component of the polycomb repressive complex 2, negatively regulated BST2 and BISPR expression (23). However, it is unclear how BISPR regulates BST2 expression (23).

We found that lncRNA#32 acts as a positive transcriptional regulator of antiviral-related ISGs and chemokines. lncRNA#32 positively regulates the expression of ISGs and chemokines under both unstimulated and IFN-stimulated conditions through its interaction with ATF2 (Figs. 4 and 6). Treatment with IFN-β destabilizes lncRNA#32 (Fig. 5*G*). The suppression of lncRNA#32 by IFN-β treatment may protect the cell from excess inflammation caused by the high expression of ISGs. ATF2 participates in the regulation of the expression of various genes, including those involved in inflammation. ATF2 activation stimulates the transcription of various genes such as proinflammatory cytokines and chemokines (32). Moreover, ATF2-deficient mice show an impaired response to lipopolysaccharide compared with control mice (28). Consistent with these reports, we observed that knockdown of the ATF2-binding lncRNA#32 reduced the expression of chemokines such as IP-10 and CCL5 (Fig. 4*C*). Taken together, these data suggest that lncRNA#32 has an important regulatory role in inflammation.

Recent studies reported that hnRNPs bind to several lncRNAs and that the hnRNP–lncRNA complex regulates the expression of target genes by binding to the promoter regions of genes that are regulated dependent upon lncRNA–hnRNP (9, 15, 33). We also observed that hnRNPU is part of a positive regulatory mechanism that maintains the expression of ISGs and chemokines by binding to and stabilizing lncRNA#32 (Fig. 5). hnRNPU has been implicated in many levels of gene regulation including transcription, RNA splicing, and DNA repair (34–36). Moreover, hnRNPU binds to both Xist RNA and the X chromosome and contributes to X-chromosome inactivation (24). The possibility that the lncRNA#32–hnRNPU complex may target ISG promoters was not clearly addressed by the present study. However, the overexpression of lncRNA#32 rescued IP-10 expression in hnRNPU-knockdown

cells (Fig. 5*F*), suggesting that hnRNPU does not directly contribute to the regulation of ISG expression.

Cytidine deaminases are APOBEC3 family members that have important roles in antiviral responses against HIV-1, HBV, AAV, and HPV infection. In addition, type I IFN induces APOBEC3A, APOBEC3B, APOBEC3C, APOBEC3F, and APOBEC3G expression (37). In the current study, we found that lncRNA#32 regulates APOBEC3A and APOBEC3G expression (Fig. S9). Importantly, the silencing of lncRNA#32 enhanced HBV and HCV replication in IFN-stimulated cells, suggesting that lncRNA#32 has an important role in the antiviral activities mediated by ISGs (Fig. S10). However, IFN-β inhibits lncRNA#32 expression, indicating the presence of a negative-feedback regulatory pathway involving the regulation of lncRNA#32 expression by IFN-β. The prevention of an excessive IFN response is critical for protection against inflammatory damage. Indeed, the deregulated expression of APOBEC3A correlates with genomic DNA mutations (38).

In conclusion, our results demonstrate that lncRNA#32 positively regulates the expression of ISGs and the antiviral effect of type I IFN. hnRNPU stabilizes lncRNA#32, and ATF2 is required for lncRNA#32 function. Therefore, we conclude that lncRNA#32 is important in the innate immune response and has an important role in antiviral immunity.

Materials and Methods

For details of materials and methods, please see *SI Materials and Methods*.

Cell Culture. The immortalized human hepatocyte cell line HuS was cultured as described previously (39). PXB cells were purchased from PhoenixBio. PHH cells were purchased from VERITAS.

Microarray. The SurePrint G3 Human Gene Expression 8X60K Microarray, version 2 (Agilent Technologies), was used to analyze differences in lncRNA or mRNA expression levels in HuS cells after treatment with poly(I:C) or IFN-β. Total RNA was prepared using an RNeasy Mini Kit (Qiagen).

siRNA Transfection. siRNA transfections were carried out using the Lipofectamine RNAiMAX Reagent (Life Technologies) according to the manufacturer's protocol. The duplex siRNA nucleotides specific to the mRNA and the Mission siRNA Universal Negative Control were purchased from Sigma. The sequences of the siRNAs were as follows: lncRNA#32-1-sense, CCA GAG AAC UUU GGU AAU UTT; lncRNA#32-1-antisense, AAU UAC CAA AGU UCU CUG GTT; lncRNA#32-2-sense, GCU GCU GAA UUC UGA CUA ATT; lncRNA#32-2-antisense, UUA GUC AGA AUU CAG CAG CTT; hnRNPU-sense, GUC ACU AAC UAC AAG UGG ATT; hnRNPU-antisense, UCC ACU UGU AGU UAG UGA CTT; ATF2-sense, CAA ACC CUU UCU AUG UAC UTT; ATF2-antisense, AGU ACA UAG AAA GGG UUU GTT; STAT2-sense, GGC UCA UUG UGG UCU CUA ATT; and STAT2-antisense, UUA GAG ACC ACA AUG AGC CTG.

5′- and 3′-RACE. The 5′- and 3′-RACE analyses were performed using the SMARTer RACE cDNA amplification Kit (Takara) according to the manufacturer's instructions. RACE PCR products were cloned into pGEM-T easy (Promega) and sequenced.

Statistical Analysis. Comparisons between groups were performed using Student's *t* test. Data are shown as the mean ± SEM. The differences were considered statistically significant when $P < 0.05$.

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