

HTLV-1 bZIP Factor HBZ Promotes Cell Proliferation and Genetic Instability by Activating OncomiRs

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Abstract

Viruses disrupt the host cell microRNA (miRNA) network to facilitate their replication. Human T-cell leukemia virus type I (HTLV-1) replication relies on the clonal expansion of its host CD4⁺ and CD8⁺ T cells, yet this virus causes adult T-cell leukemia/lymphoma (ATLL) that typically has a CD4⁺ phenotype. The viral oncoprotein Tax, which is rarely expressed in ATLL cells, has long been recognized for its involvement in tumor initiation by promoting cell proliferation, genetic instability, and miRNA dysregulation. Meanwhile, HBZ is expressed in both untransformed infected cells and ATLL cells and is involved in sustaining cell proliferation and silencing virus expression. Here, we show that an HBZ–miRNA axis promotes cell proliferation and genetic instability, as indicated by comet assays that showed increased numbers of DNA-strand breaks. Expression profiling of miRNA revealed that infected CD4⁺ cells, but not CD8⁺ T cells, overexpressed oncogenic miRNAs, including miR17 and miR21. HBZ activated these miRNAs via a posttranscriptional mechanism. These effects were alleviated by knocking down miR21 or miR17 and by ectopic expression of *OBFC2A*, a DNA-damage factor that is downregulated by miR17 and miR21 in HTLV-1–infected CD4⁺ T cells. These findings extend the oncogenic potential of HBZ and suggest that viral expression might be involved in the remarkable genetic instability of ATLL cells. *Cancer Res*; 74(21); 6082–93. ©2014 AACR.

Introduction

Human T-cell leukemia virus type I (HTLV-1) is associated with adult T-cell leukemia/lymphoma (ATLL; ref. 1) that regularly occurs after a prolonged period of viral latency. HTLV-1 can also cause inflammatory diseases, such as tropical spastic paraparesis (TSP)/HTLV-1–associated myelopathy (HAM; ref. 2), uveitis (3), and infective dermatitis (ID; ref. 4). *In vivo*, HTLV-1 preferentially infects both CD4⁺ and CD8⁺ T

cells, and triggers the persistent clonal expansion of these two host cell subsets, although cellular transformation is often restricted to the CD4⁺ subset (5). Although ATLL occurs in only 1% to 3% of infected individuals, the nonimmortalized infected CD4⁺ T cells derived from carriers without malignancy accumulate numerous preleukemic features, including multinuclearity, impaired cytokinesis, chromatin bridges, suboptimal telomerase activity, and increased cell proliferation (6–8). In contrast, the clonal expansion of infected CD8⁺ T cells was found to rely on delayed cell death in a cIAP-2–dependent manner (6–8).

Genome-wide transcriptome reprogramming is a hallmark of HTLV-1 infection (5, 8, 9) that relies mainly on Tax and HBZ, two HTLV-1–encoded proteins that can hijack host transcription factors and chromatin modifiers to positively or negatively modulate cellular gene expression (10, 11). The pleiotropic effects of Tax activity include cell proliferation, prevention of apoptosis, and promotion of genetic instability (12). Accordingly, Tax functions to promote aneuploidy, gene mutations, telomere attrition, and clastogenic DNA damage, all within the context of activated cell proliferation (13). Because the Tax gene is inactivated in ATLL cells (14–16), its pleiotropic cellular effects are thought to be mainly associated with tumor initiation rather than tumor maintenance.

In contrast, one consistent feature of ATLL cells is the constitutive expression of HBZ, a b-ZIP factor encoded by the minus-strand RNA of the HTLV-1 provirus (17, 18). HBZ knockdown suppresses the proliferation of HTLV-1–transformed cell lines, emphasizing the key role of HBZ in the

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Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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continuous expansion of ATLL cells (19). HBZ is also involved in the nonmalignant clonal expansion of infected cells, as its expression is correlated with proviral loads, inflammatory markers, and disease severity in TSP/HAM (20). In line with this activity, transgenic mice expressing HBZ develop symptoms similar to those observed in HTLV-1 carriers, including T-cell lymphomas and inflammatory lesions (21). These data indicate that, in addition to Tax, HBZ might play a significant role in early leukemogenesis through molecular mechanisms that remain to be elucidated. To date, the effect of HBZ on genetic instability has not been investigated, even though ATLL cells express HBZ and display a dynamic spectrum of both complex cytogenetic abnormalities and somatic mutations *in vivo* (13, 22–24).

microRNAs (miRNA) are evolutionarily conserved, small (~21 nucleotides), noncoding RNAs that are encoded within the genomes of almost all eukaryotes from plants to mammals. Most organisms express hundreds of miRNAs that are integral to almost all known biologic processes. In general, miRNAs post-transcriptionally regulate protein synthesis by base pairing to partially complementary sequences in the 3'-untranslated regions (UTR) of target mRNAs (25–29). In human diseases, particularly cancer, epigenetic and genetic defects in miRNAs and their processing machinery are common hallmarks of disease. ATLL cells exhibit global repression of miRNA expression (30), including the loss of miR31 expression that has been shown to activate NF- κ B (30). At the molecular level, both Tax and the viral RNA-binding protein Rex modulate miRNA expression in immortalized cell lines by altering NF- κ B activation or the RNAi machinery (12, 31–33). To our knowledge, no study to date has assessed the effects of HBZ on the biogenesis and activity of miRNAs. Furthermore, whereas human lymphocyte subsets are known to possess specific miRNA signatures involved in T-cell differentiation and activation (34, 35), little is known about the role of miRNA dysregulation in the clonal expansion of untransformed infected CD4⁺ and CD8⁺ T cells *in vivo*, including its role, if any, in viral persistence, inflammation, genetic instability, and early leukemogenesis.

To assess the effect of HTLV-1 infection on the miRNA expression profiles of host cells *in vivo*, we performed an integrated analysis of miRNA- and mRNA-expression profiles of cloned CD4⁺ and CD8⁺ T cells derived from infected individuals without malignancy. When compared with their uninfected counterparts, each T-cell subset displayed specific

changes in miRNA expression. Accordingly CD4⁺ T cells, but not CD8⁺ nonimmortalized T cells, overexpressed known oncogenic miRNAs, such as miR17 and miR21, which target the DNA-damage effector OBFC2A-hSSB2 in these cells. Surprisingly, in CD4⁺ T cells derived from infected individuals, the expression of these miRNAs strongly correlated with HBZ expression, but not Tax expression. In fact, miR17 and miR21 were found to be posttranscriptionally upregulated by HBZ, while HBZ-miRNA-mediated downregulation of OBFC2A expression triggered both cell proliferation and genomic instability. Together, our results reveal a close association of HBZ expression, miRNA dysregulation, genetic instability, and abnormal cell proliferation.

Materials and Methods

Materials and Methods are detailed in the Supplementary Data. Peripheral blood mononuclear cells (PBMC) were obtained from HTLV-1-infected individuals after obtaining written informed consent according to the principles expressed in the Declaration of Helsinki. Pertinent clinical data accompanying the samples are given in Table 1. The study was approved by the Institutional Review Board of the Hospices Civils de Lyon (Lyon, France). HeLa cells were obtained from the American Type Culture Collection.

Cell culture and microarray analysis

HeLa cells were cultured in Dulbecco Modified Eagle Medium (DMEM), supplemented with 10% heat-inactivated FCS, L-glutamine, penicillin (5 U/mL), and streptomycin (5 μ g/mL) at 37°C in a humidified atmosphere with 5% CO₂. PBMCs were cultured by limiting dilution cloning as previously described (6) and detailed in the Supplementary Data. During the growth period, cellular clones were not immortalized and required IL2 and stimulation with phytohemagglutinin (PHA) every 2 weeks for continued growth. RNA expression profiling of 24 cloned T cells (6 infected CD4⁺, 6 infected CD8⁺, 6 uninfected CD4⁺, and 6 uninfected CD8⁺ T-cell clones) was carried out using an Agilent V3 miRNA microarray (Agilent) and a GeneChip Human Exon 1.0 ST array (Affymetrix) according to the manufacturer's instructions. The miRNA and mRNA array data have been submitted to Gene Expression Omnibus (GEO) #GSE46345 and #GSE46518, respectively.

Table 1. Patient characteristics

Patient	Gender	Age	Native country	Status	EDSS	Duration of illness, y
Patient 06	F	71	Martinique	HAM/TSP	5	11
Patient 11	M	52	France	HAM/TSP	5.5	6
Patient 19	F	48	Martinique	HAM/TSP	6.5	3
Patient 28	M	61	France	HAM/TSP	6	5
Patient 33	F	44	Martinique	HAM/TSP	4.5	4

Abbreviations: EDSS, expanded disability status score; F, female; M, male.

Quantitative RT-PCR and luciferase reporter assay

Quantitative RT-PCR (qRT-PCR) assays are detailed in the Supplementary Data. Oligonucleotide sequences are available upon request. Reporter assays using the 3'-UTR-OBFC2A target region fused to the 3'-UTR of the luciferase gene in the *pMIR-REPORT* miRNA expression vector (Invitrogen) were used to test the suppressive capacity of miR17 and miR21. The p3'-OBFC2A reporter vectors are detailed in the Supplementary Data. The luciferase activity was assessed 24 hours after transfection using the Dual-Luciferase Reporter Assay System (Promega).

Cell transfection and Western blotting

HeLa cells were transfected using the calcium phosphate precipitation method with the plasmid pcDNA-HBZ-Myc encoding the spliced isoform of HBZ (18). miRNA inhibitors (LNA technology; miRCURY microRNA Power inhibitor; Exiqon) were delivered using the Lipofectamine RNAiMAX (Invitrogen). For Western blotting, samples were fractionated by electrophoresis on 10% denaturing polyacrylamide gels, transferred to polyvinylidenedifluoride (PVDF) membranes, and probed with antibodies recognizing hSSB2 (Bethyl Laboratories); phospho-Ser139-H2AX (Cell Signaling Technology), β -actin (Cell Signaling Technology), and signals were visualized with the ECL Plus Western Blotting Detection Reagent (GE, Amersham).

In silico analysis of miRNA targets

MiRWalk computational analysis (<http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/>) was used to identify the putative target genes of miR17, miR21, miR23b, and miR27b. The minimum number of nucleotides for a miRNA response element (MRE) was set to 7 ($P = 0.05$). The search for MREs was restricted to the 3'-UTR of mRNAs. Using Access software, *in silico* predicted miRNA targets were cross-matched with downregulated mRNAs (fold change ≥ 1.5 ; $P = 0.05$) of infected CD4⁺ T cells compared with those of uninfected cells.

Comet assay

Transiently transfected HeLa cells were grown for 24 hours and exposed to 5 ng/mL of neocarzinostatin or control medium without neocarzinostatin for 3 hours. After 24 hours of additional growth, cells were collected and resuspended in cold PBS at a density of 10^5 cells/mL and a comet assay was performed, according to the manufacturer's protocol (Trevigen). Experiments were carried out in triplicates and DNA damage was quantified for 50 cells for each experimental condition by determining the tail moment, a function of both the tail length and intensity of DNA in the tail relative to the total DNA amount, using the software Comet Score (TriTek).

MTT proliferation assay

Cell proliferation rates were assessed by the MTT assay (CellTiter 96 Non-Radioactive Cell Proliferation Assay; Promega). Fifty thousand cells were plated and cultured for 24 hours in 100- μ L medium in 96-well plates. The MTT dye (Promega, 15 μ L) was added to each well for the last hour of culture incubation. Supernatants were decanted and 100 μ L of

the Solubilization Solution/Stop Mix was added to each well, and cells were incubated for 1 hour at room temperature. The OD value was measured using an ELISA reader at 570 nm, with 650 nm as a reference. Experiments were carried out in triplicate and repeated at least three times. MTT-predicted cell proliferation was also confirmed by cell counting using Trypan blue dye (not shown).

Results

miRNA signature of nonimmortalized HTLV-1-positive T cells

Human lymphocyte subsets possess specific miRNA signatures that can be modified upon viral infection (34, 35). To address which changes in miRNA expression accompany cell-associated replication of HTLV-1, we carried out miRNA expression profiling of infected and uninfected CD4⁺ and CD8⁺ T cells derived from naturally infected individuals without malignancy (Table 1). Twenty-four T-cell clones were assayed for miRNA microarray analysis as described in Materials and Methods. They included six uninfected CD4⁺ T-cell clones, six infected CD4⁺ T-cell clones, six uninfected CD8⁺ T-cell clones, and six infected CD8⁺ T-cell clones. The complete microarray dataset has been deposited *en toto* into the GEO database and is available under record number GSE46345. HTLV-1-positive CD4⁺ T cells expressed higher levels of the miRNAs miR27b, miR23b, and miR34b compared with uninfected CD4⁺ T cells (fold change >1.5 ; $P < 0.05$). In contrast, infected CD8⁺ T cells expressed higher levels of miR34b, let-7a, and let-7f compared with their uninfected counterparts. Additional miRNA gene families such as miR17, miR21, miR15, and let-7 were found to be differentially expressed among infected clones. The differential expression of these miRNAs was confirmed in T-cell clones used for microarray analysis using miRNA-specific real-time qRT-PCR (not shown). We next assessed the significance of the microarray data by quantifying the expression level of some miRNAs using qRT-PCR analysis of 65 additional T-cell clones. Figure 1 shows that infected CD4⁺ clones expressed significantly higher levels of miR17 ($P = 0.0056$, Mann-Whitney test), miR21 ($P = 0.011$), miR23b ($P = 0.0063$), and miR27b ($P = 0.023$) than uninfected CD4⁺ clones. Infected CD4⁺ clones exhibited lower expression levels of miR374b than their CD8⁺ counterparts; however, this difference was not statistically significant between the infected and uninfected cell categories (Supplementary Fig. S1). In contrast, qRT-PCR analysis revealed a wide range of expression level of other miRNAs within infected cells and failed to demonstrate any significant changes across the different subtypes (Supplementary Fig. S1). Together, these results indicated that significant high levels of miR17, miR21, miR23b, and miR27b constitute a miRNA signature of nonimmortalized HTLV-1-infected CD4⁺ cells but not CD8⁺ T cells *in vivo*.

HBZ triggers the expression of miR17, miR21, miR23b, and miR27b

As previously described, infected CD4⁺ and CD8⁺ T cells harbored a wide range of Tax/Rex and HBZ transcript levels without showing a significant difference between the

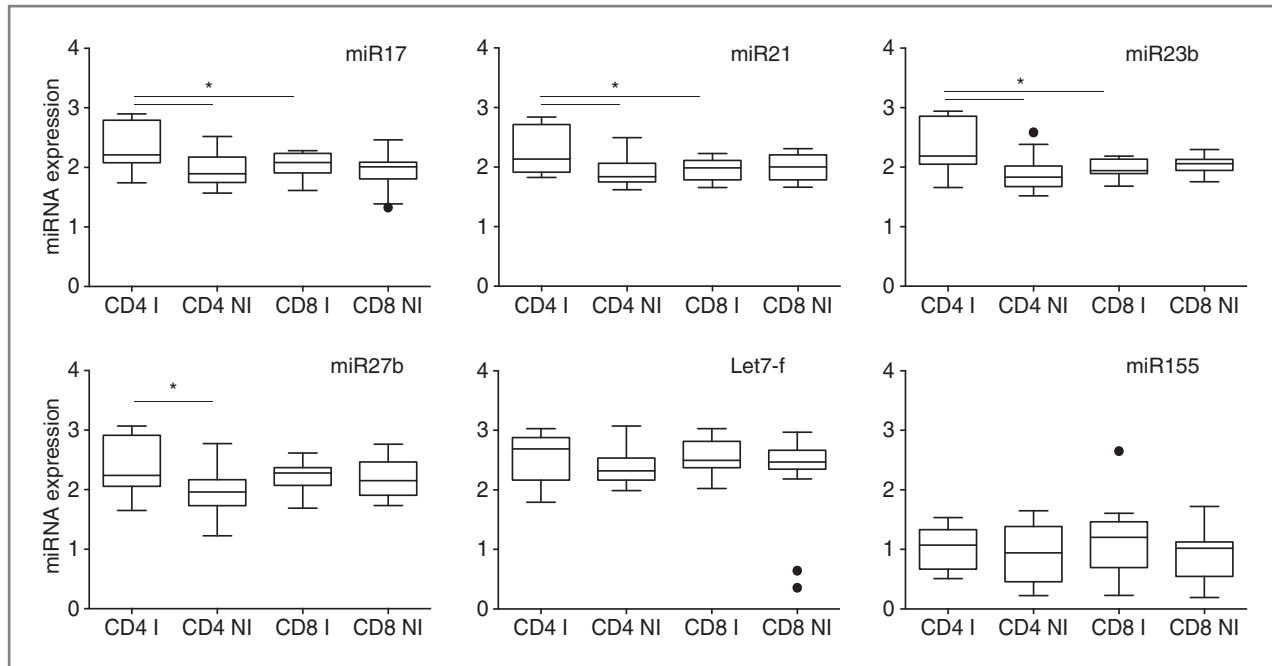


Figure 1. miRNA expression in cloned T cells derived from HTLV-1-infected individuals without malignancies. Box-whisker plot showing qRT-PCR results of miRNA expression in 65 T-cell clones (ICD4, $n = 16$; UICD4, $n = 17$; ICD8, $n = 15$; UICD8, $n = 17$) derived from patients without malignancy. Additional data are presented in Supplementary Fig. S1. Results are based on technical triplicate measurements of each sample and multiple independent samples for each T-cell category. The variation depicted by the box-whisker plot therefore reflects technical as well as biologic variation within each sample category. The line represents the median and outliers are indicated by black circles. *, $P < 0.05$, Mann-Whitney test.

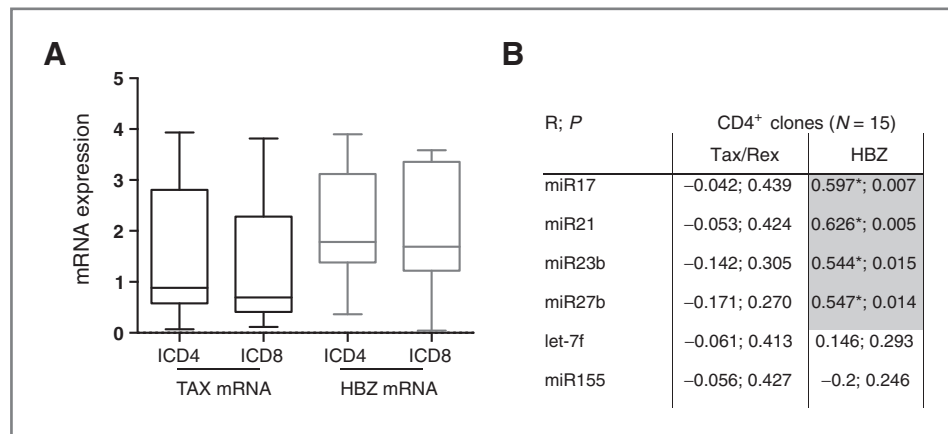
two cell subtypes (Fig. 2A; refs. 5–8). Figure 2B shows that a significant positive correlation linked HBZ to miRNA expression for miR17, miR21, miR23b, and miR27b in CD4⁺ T cells. In contrast, no correlation was observed between HBZ, Let-7f, and miR155 expression. Similarly, there was no correlation between the expression of Tax/Rex mRNA and that of the six analyzed miRNAs (Fig. 2B). To more confidently assess the role of HBZ in miRNA expression, we performed transient transfection assays of HeLa cells with increasing amounts of an HBZ-expressing vector and quantified the expression of endogenous miR17, miR21, miR23b, and miR27b by qRT-PCR. As shown in Fig. 3A, HBZ was

capable of upregulating the expression of these miRNAs in a concentration-dependent manner. In contrast, transfection with a vector expressing Tax/Rex did not significantly affect the expression of the six miRNAs (data not shown).

HBZ stimulates the expression of miR17 and miR21 at a posttranscriptional level

The miR21 and miR17 are two of the best characterized oncogenic miRNAs and are involved in numerous malignancies (36–39). The gene miR21, along with its own promoter, is located in an intronic region of a coding gene, *TMEM49*. Meanwhile, miR17 is encoded by the polycistronic miRNA

Figure 2. Oncogenic miRNA expression correlates with HBZ expression in CD4⁺ T-cell clones. A, qRT-PCR analysis of Tax and HBZ mRNA in 30 infected clones (15 CD4⁺) derived from HTLV-1 carriers. B, the expression of miR17, miR21, miR23b, and miR27b parallels that of HBZ in CD4⁺ T-cell clones. Results were analyzed by the Spearman correlation coefficient test between miRNA levels and Tax/Rex and HBZ mRNA expression.



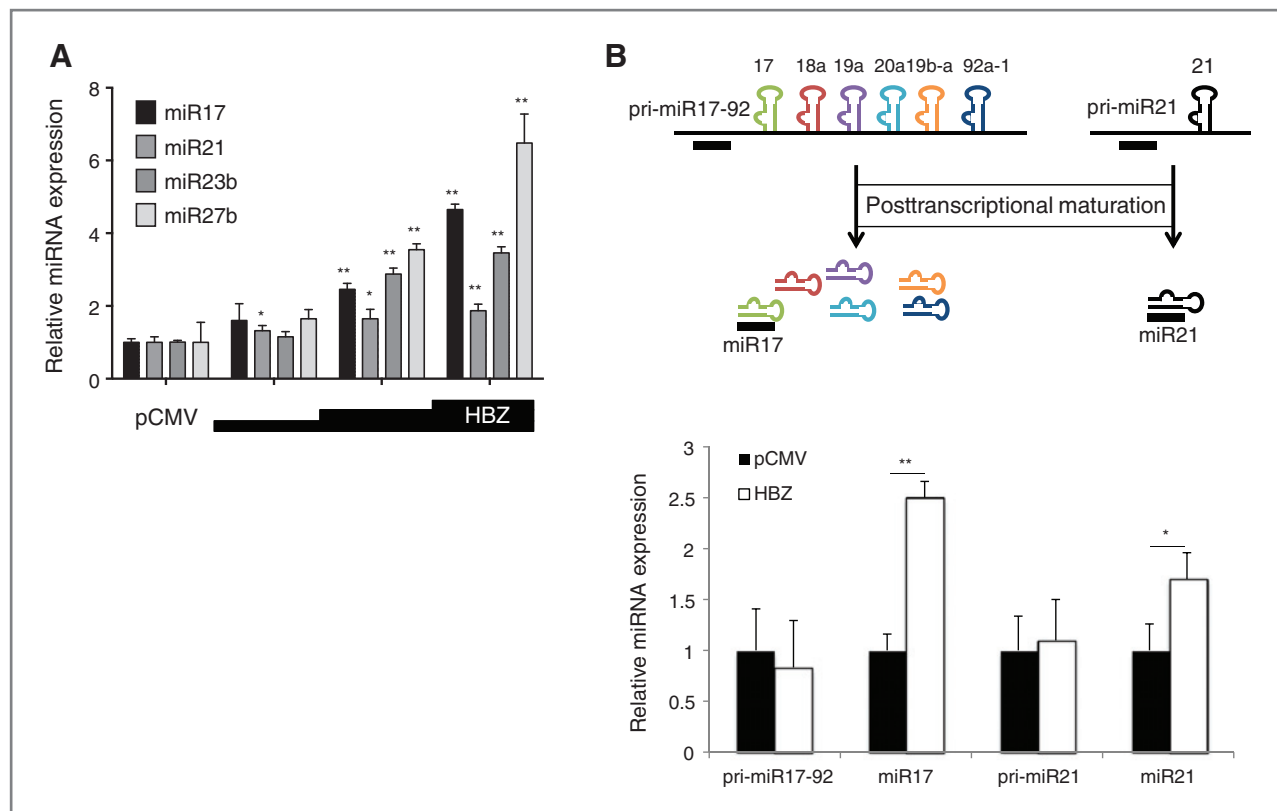


Figure 3. HBZ promotes the expression of oncogenic miRNAs at a posttranscriptional level. **A**, ectopic expression of HBZ increases endogenous miR17, miR21, miR23b, and miR27b expression in a dose-dependent manner. HeLa cells were transiently transfected with either the control plasmid (pCMV) or increasing amounts of HBZ plasmid. Endogenous miRNAs were quantified by qRT-PCR as detailed in Materials and Methods. **B**, HBZ stimulates miRNA expression without affecting pri-miRNA transcription. Bold lines represent TLDA (TaqMan Low Density Array) probes used for quantifying miRNA or pri-miRNA transcripts. pri-miR17-92 and pri-miR21 were quantified using qRT-PCR in HeLa cells expressing HBZ as well as those without ectopic HBZ expression. *, $P < 0.05$; **, $P < 0.01$, Mann-Whitney test.

cluster miR17-92, also called oncomiR-1 (36). The primary transcript (pri-miR) of this cluster contains six tandem stem-loop hairpin structures that encode six mature miRNAs: miR17, miR18a, miR19a, miR20a, miR19b-1, and miR92-1 (40). Our microarray data indicated that these miRNAs did not follow the same expression pattern upon infection, thereby suggesting that miR17 upregulation likely occurs at a post-transcriptional level. Having found that HBZ can stimulate miR17 expression, we examined the effects of HBZ on the expression of the endogenous pri-miRNA that encodes miR17 in HeLa cells (Fig. 3B). In contrast to mature miR17, pri-miR17-92 expression was not significantly altered by HBZ expression. This result confirmed that HBZ stimulates the biogenesis of miR17 at a posttranscriptional level. Similar results were obtained for miR21 (Fig. 3B). In line with this result, pri-miR17 and pri-miR21 expression was not significantly different between infected and uninfected CD4⁺ clones (Supplementary Fig. S2). Collectively, these data suggest that HBZ can promote the posttranscriptional maturation of certain miRNAs.

HTLV-1-associated miRNAs from CD4⁺ T cells regulate genes involved in genetic disorders

To identify the mRNA targets of miRNAs that are deregulated in HTLV-1-infected cells, we examined the entire

genome expression profile for the 12 CD4⁺ clones used for miRNA analysis. The data are available online in the GEO database (GSE46518). Overall, 224 genes showed modified expression levels in infected cells as compared with their uninfected counterparts (fold change >1.5; $P < 0.01$). In parallel, MirWalk computational analysis was used to identify the putative target genes of miR17, miR21, miR23b, and miR27b, as detailed in Materials and Methods. Among 117 repressed genes, 63 (53%) were computationally predicted to be targeted by miR17, miR21, miR23b, or miR27b. Forty-two, 37, 29, and 45 transcripts harbored at least one MRE for miR17, miR21, miR23b, or miR27b, respectively. Eighty percent (51 of 63) of putative mRNA targets possessed at least one MRE, whereas 20% harbored all four MREs in their 3'-UTR. The mRNAs that contained all four MREs included BET1L, C18orf25, CCNL2, CXorf57, KIAA0323, MESDC2, OBFC2A, PAFAH2, PDE7B, USP28, VCL, and ZNF614, and were categorized as major targets of HTLV-1-dependent miRNAs in infected CD4⁺ T cells clones. qRT-PCR analysis confirmed the microarray data for five of these genes (Supplementary Fig. S3).

Pathway analysis was carried out using the Ingenuity Pathway Analysis (IPA; Ingenuity Systems). Of the 224 genes that were found to be dysregulated in HTLV-1-positive CD4⁺ T cells, the majority are involved in genetic disorders (45%) and

neurologic diseases (33%; Supplementary Fig. S4). These two dominant pathways were over-represented, even when the analysis was restricted to the 63 genes targeted by miR17, miR21, miR23b and miR27b (41% and 23%, respectively). In contrast, the remaining pathways had varying representations between the two groups of genes.

HBZ promotes genomic instability in a miR17- and miR21-dependent manner

We next wanted to assess whether HBZ had a significant effect on genomic integrity. To this end, we used an alkaline comet assay to determine whether HBZ expression triggered DNA-strand breaks. Cells were assayed as shown in Fig. 4A and as detailed in Materials and Methods. These experi-

mental conditions had no significant impact on cell death (Supplementary Fig. S5). As shown in Fig. 4B, 24 and 48 hours after transfection, HBZ-expressing HeLa cells exhibited a significantly higher frequency of DNA-strand breaks than control cells. This frequency decreased at 48 hours in control cells that were treated with or without the DNA-damaging agent neocarzinostatin, whereas DNA-strand break frequency increased in HBZ-expressing cells (Fig. 4B). These results demonstrated, for the first time, that HBZ triggers DNA-strand breaks.

To assess the role of HBZ-inducible miRNAs in genetic instability, we examined whether the functional inhibition of miR17 or miR21 could alleviate the DNA damage induced by HBZ. As shown in Fig. 4C, LNA-anti-miRNA treatment

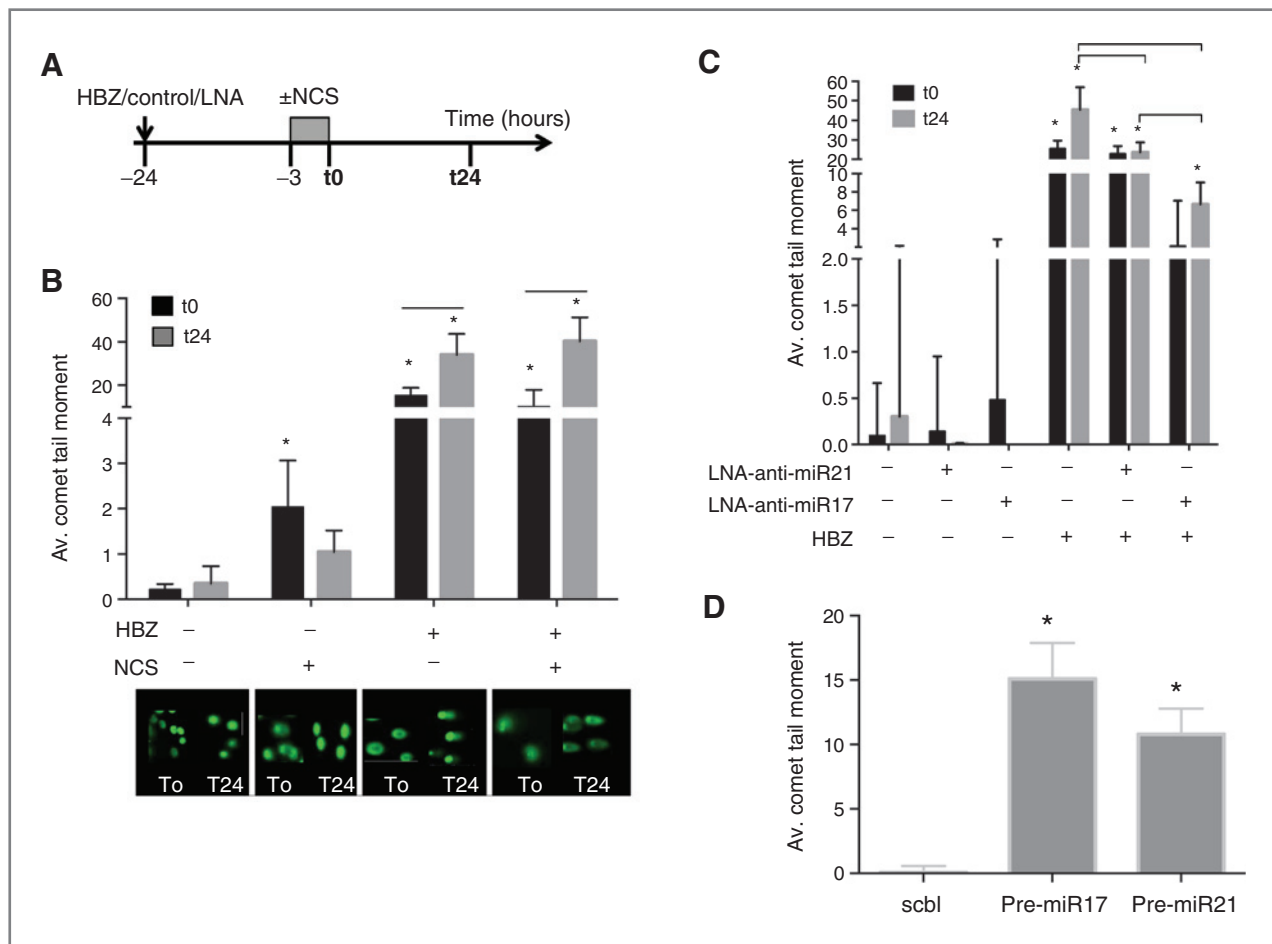


Figure 4. HBZ triggers DNA-strand breaks in a miR17- and miR21-dependent manner. A, schematic of the workflow to analyze the DNA-damage response in the presence of HBZ expression. HeLa cells were transfected with HBZ or control vector and exposed to neocarzinostatin (NCS) for 3 hours, 24 hours after posttransfection as indicated. Comet and MTT assays were carried out immediately after neocarzinostatin exposure (t0) and after 24 hours of recovery (t24). Assays were also performed with cells withdrawn from neocarzinostatin exposure. B, bar graphs showing the effects of HBZ as measured with the comet assay. Quantitative analysis of tail moment measurements were carried out in HeLa cells expressing HBZ or control vector and exposed to low doses of neocarzinostatin (5 ng/mL) as indicated. For each category, 50 comet assays were quantified. C, bar graphs showing the effects of LNA-anti-miR17 and LNA-anti-miR21 in comet assays. Assays were carried out as described above, with HeLa cells transiently cotransfected with an HBZ vector and LNA-anti-miRNAs or control vectors. D, bar graphs showing the effects of pre-miR17 and pre-miR21 in comet assays. HeLa cells were transiently cotransfected with either scramble (scbl) or pre-miR17 and pre-miR21. Comets were quantified at t24 as described in Materials and Methods. In B–D, data are shown as mean \pm SD of three independent experiments. *, statistically different from the control cells at the same time point or between different assays (horizontal bar), $P < 0.05$, Student *t* test.

caused no significant difference in DNA-strand break frequency in the control cells, but significantly reduced DNA damage in HBZ-expressing cells. With LNA-anti-miR21, the effect was noticeable at 24 hours, whereas LNA-anti-miR17 dramatically decreased the frequency of DNA-strand breaks at both the initial time point and at 24 hours. Conversely, transient overexpression of either miR17 or miR21 increased the proportion of HeLa cells with DNA-strand breaks (Fig. 4D).

HBZ downregulates the expression of the DNA-damage response factor hSSB2 in a miR17- and miR21-dependent manner

To elucidate the mechanism by which HBZ induced genomic instability and DNA breaks in a miRNA-dependent

manner, we searched for candidate genes that have repressed expression in infected CD4⁺ T cells. Among the genes that were found to be repressed in HTLV-1-positive CD4⁺ T-cell clones, *OBFC2A*, which encodes hSSB2, a recently identified single-stranded DNA-binding protein involved in protecting genome stability (41, 42), had four putative MREs for miR17, miR21, miR23b, and miR27b. We next used qRT-PCR analysis to confirm *OBFC2A* expression in T-cell clones derived from patients. As shown in Fig. 5A, *OBFC2A* mRNA levels were significantly lower in HTLV-1-positive cells compared with those in HTLV-1-negative CD4⁺ T-cell clones (0.6630 vs. 0.9023; $P = 0.0178$, Mann-Whitney test). This difference remained significant when the outlier in the control group was withdrawn from the analysis ($P = 0.03$, Mann-Whitney test). We then examined

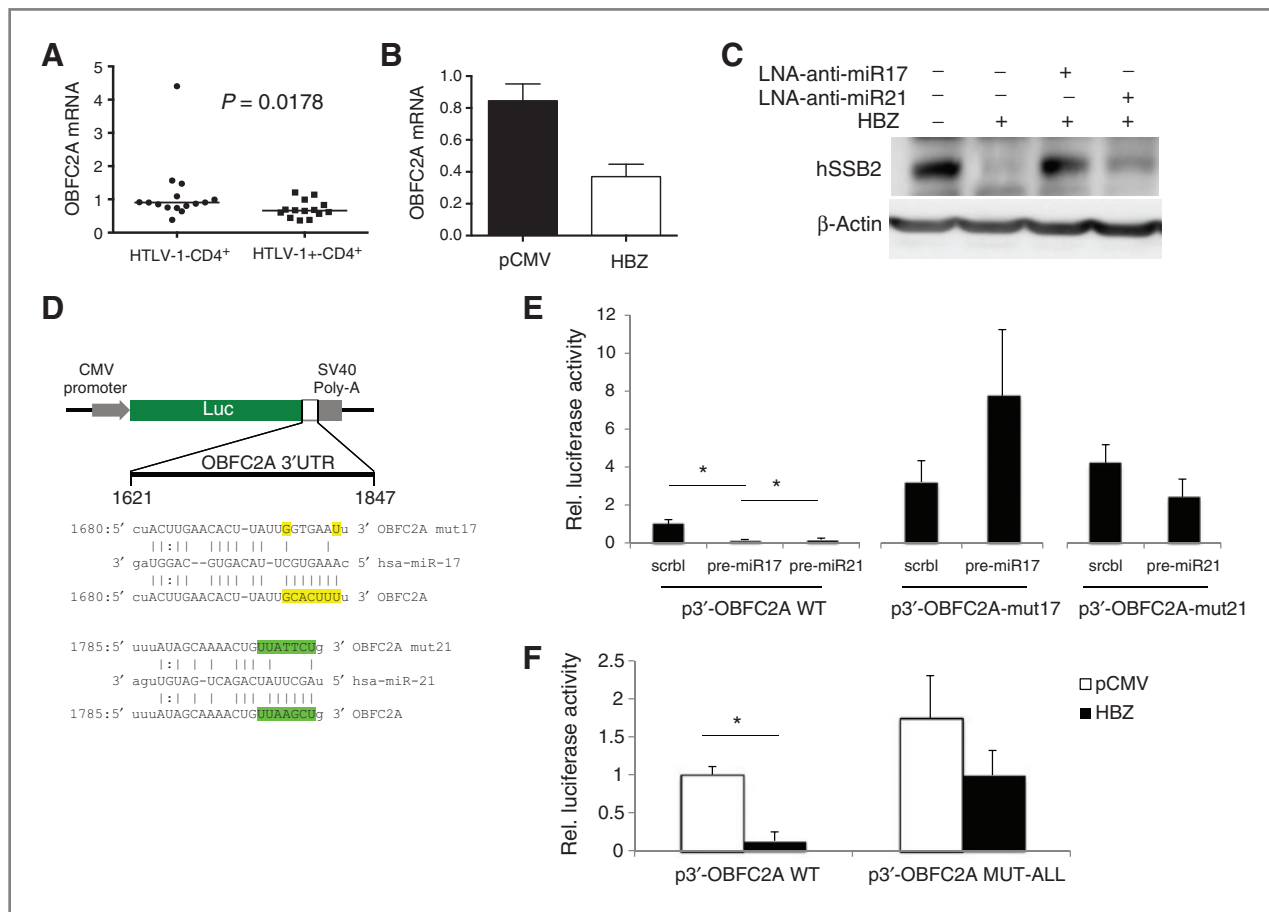


Figure 5. HBZ represses *OBFC2A* expression via miR17 and miR21. **A**, qRT-PCR analysis of *OBFC2A* mRNA in cloned CD4⁺ T cells carrying or not carrying HTLV-1. **B**, qRT-PCR analysis of *OBFC2A* mRNA in HeLa cells transiently transfected with either an HBZ-expressing or control vector. Quantification was performed 48 hours after transfection. **C**, Western blot analysis of hSSB2 protein in HeLa cells expressing HBZ with or without LNA-anti-miRNAs. HeLa cells were cotransfected with an HBZ vector along with either LNA-anti-miR17, LNA-anti-miR21, or scrambled anti-miR control. hSSB2 expression was assessed 48 hours after transfection. **D**, *OBFC2A* DNA sequence encompassing the MREs for miR17 and miR21 (fragment 1621–1847) was cloned into the pMIR-REPORT vector (p3'-*OBFC2A*-WT). p3'-*OBFC2A*-17 and p3'-*OBFC2A*-21 carry mutations in the target sites for miR21 or miR27. **E**, p3'-*OBFC2A*-WT, p3'-*OBFC2A*-17, and p3'-*OBFC2A*-21 were transfected into HeLa cells together with synthetic pre-miR17, pre-miR21, or scrambled miRNA control. After 24 hours, firefly luciferase activity was measured and normalized to *Renilla* expression levels ($n = 3$). *, $P < 0.05$ Mann-Whitney test. **F**, the effects of HBZ on *OBFC2A* expression with a wild-type 3'-UTR or one with both miR17 and miR21 MREs mutated were measured with luciferase assays as described in **E** by cotransfecting HeLa cells with an HBZ-expressing vector along with either p3'-*OBFC2A*-WT or p3'-*OBFC2A*-ALL vectors, respectively. *, $P < 0.05$ Mann-Whitney test.

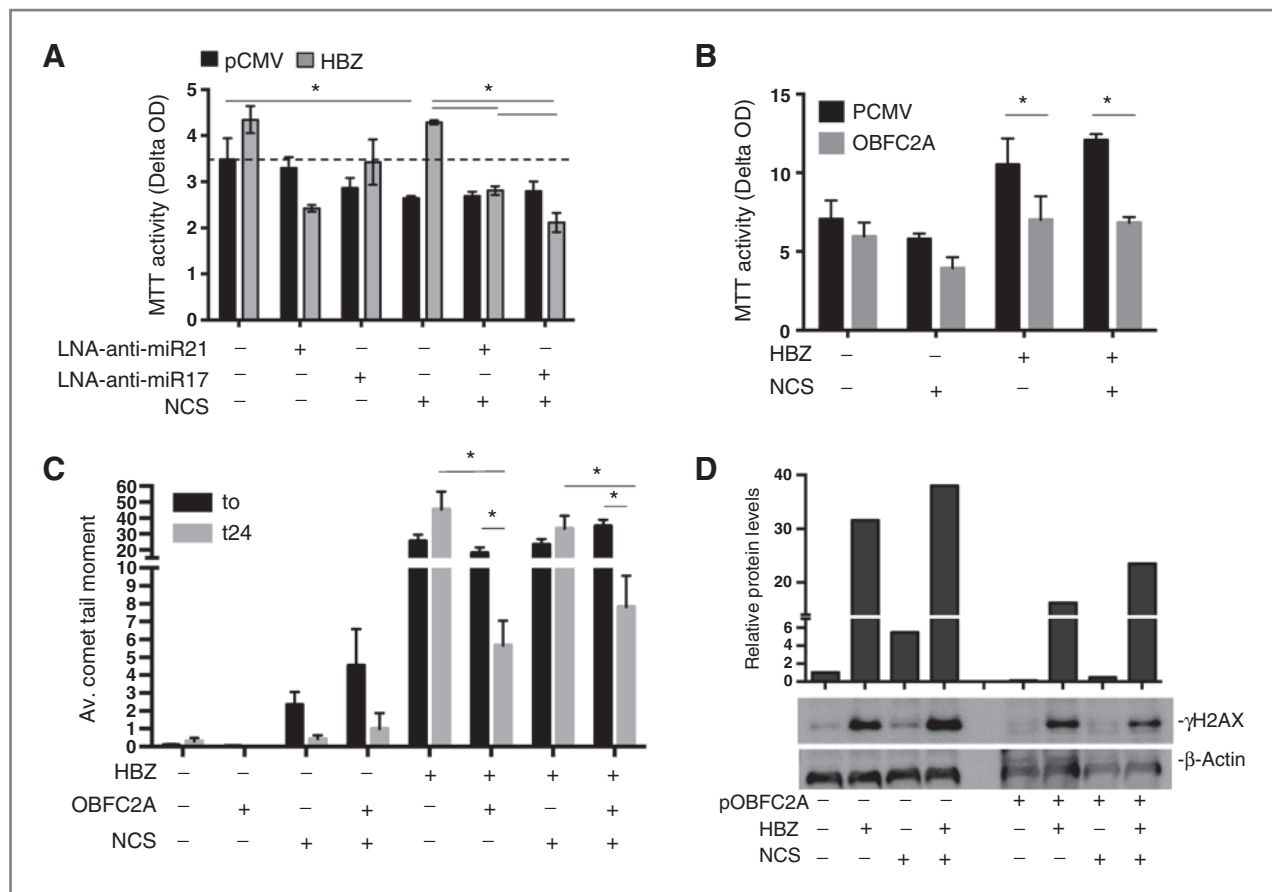


Figure 6. OBFC2A counteracts the DNA-damage effects of HBZ. **A**, LNA-anti-miR17 and LNA-anti-miR21 alleviate the effect of HBZ on cell growth. The effects of neocarzinostatin (NCS) treatment (as described in Fig. 4A) on cell proliferation were determined using the MTT assays. MTT activity (Delta OD) is presented as the ratio (t24/t0) of optical density measured at t24 to that measured at t0. HBZ expression as well as miR17 and miR21 suppression caused similar effects at 48 hours posttransfection (Supplementary Fig. S8). The data are expressed as mean \pm SD of three independent experiments, with each performed in triplicate. *, $P < 0.05$ Mann-Whitney test. **B**, OBFC2A alleviates the effect of HBZ on cell growth. HeLa cells were transiently cotransfected with vectors encoding an OBFC2A mRNA unresponsive to miRNA-mediated modulation in the presence or absence of neocarzinostatin (NCS). Cell proliferation was assayed using the MTT assays. *, $P < 0.05$ Mann-Whitney test. **C**, transient overexpression of OBFC2A decreases both the average tail moment of HBZ-expressing cells (*, $P < 0.05$, Student *t* test). **D**, the level of H2AX phosphorylation at serine 139 (γ H2AX) at the t24 time point. Histograms represent the quantification of γ H2AX signals normalized against β -actin.

the effect of HBZ expression on endogenous OBFC2A gene expression in HeLa cells. As shown in Fig. 5B and C, HBZ expression decreased OBFC2A expression at both the mRNA ($P = 0.0095$, Mann-Whitney test) and protein (hSSB2) level.

To clarify the role of miRNAs in the regulation of OBFC2A by HBZ, we cotransfected HeLa cells with an HBZ-expressing vector alongside LNA-anti-miR17, LNA-anti-miR21, or a scrambled miRNA control. As shown in Fig. 5C, the functional inactivation of miR17 and miR21 alleviated the repressive effect of HBZ on hSSB2 expression, whereas ectopic overexpression of miR17 and miR21 resulted in a reduced level of endogenous OBFC2A-hSSB2 mRNAs (Supplementary Fig. S6). Taken together, these results suggested that OBFC2A-hSSB2 might be a *bona fide* target of HBZ-induced miRNAs. To test this hypothesis, we constructed pMIR-REPORT constructs that express a luciferase RNA fused with either the wild-type sequence of the OBFC2A 3'-UTR (p3'-OBFC2A-WT) or a 3'-

UTR sequence mutated for the MREs of miR17 (p3'-OBFC2A-mut17), miR21 (p3'-OBFC2A-mut21), or both (p3'-OBFC2A-ALL; Fig. 5D). A significant decrease in luciferase activity was observed in cells cotransfected with the p3'-OBFC2A-WT and miRNA mimics (either synthetic pre-miR17 or pre-miR21), but not in cells cotransfected with p3'-OBFC2A-mut17 and p3'-OBFC2A-mut21, suggesting that OBFC2A is a direct target of miR17 and miR21 (Fig. 5E). Consistent with this result, increased luciferase activity was detected in cells transfected with p3'-OBFC2A-WT along with LNA-anti-miR17 or LNA-anti-miR21 (Supplementary Fig. S6). As shown in Fig. 5F, ectopic HBZ expression significantly decreased luciferase activity in cells cotransfected with p3'-OBFC2A-WT, whereas it had no significant effect in cells cotransfected with p3'-OBFC2A-ALL. Taken together, these data suggest that HBZ represses OBFC2A mRNA expression in a miR17- and miR21-dependent manner.

HBZ–miRNA–mediated downregulation of hSSB2 triggers abnormal cell proliferation and genomic instability

Because HBZ promotes both cellular growth (17) and DNA damage (present results), we next assessed whether these cellular events rely on the repression of *OBFC2A* expression by HBZ via miR17 and miR21. We therefore tested whether miRNA-induced downregulation of hSSB2 could alter the genomic integrity of HBZ-expressing cells. As shown in Fig. 6A and Supplementary Fig. S8, HBZ expression enhanced the growth of HeLa cells, whereas cell proliferation was significantly decreased in the presence of LNA–anti-miR17 or LNA–anti-miR21 despite HBZ expression. This result suggests that HBZ-mediated cell proliferation depends on miR17 and miR21.

We then investigated the proliferation rate of HBZ cells exposed to a low dose of neocarzinostatin, which was sufficient to cause a significant reduction in MTT activity in control cells (Fig. 6A). Interestingly, compared with control cells, neocarzinostatin-treated HBZ-expressing cells were unaffected in their proliferation capacity, suggesting that HBZ enabled cells to tolerate DNA damage. This tolerance was significantly attenuated when cells were transfected with either LNA–anti-miR17 or LNA–anti-miR21, indicating that the elevated expression of functional miR17 and miR21 help HBZ-expressing cells to bypass DNA-damage checkpoints and allows proliferation despite the presence of DNA breaks.

To determine the role of hSSB2 in HBZ–miRNA–mediated cell proliferation in the presence of DNA damage, we cotransfected HeLa cells with the HBZ vector and an hSSB2-expressing vector. The latter construct lacked the *OBFC2A*-3'-UTR, rendering it unresponsive to miRNA-mediated modulation. Ectopic hSSB2 expression induced a mild decrease in MTT activity in untreated control cells, whereas it significantly reinforced the growth inhibition in neocarzinostatin-treated cells (Fig. 6B). The largest difference was observed in HBZ-expressing cells wherein ectopic hSSB2 expression caused a decrease in the proliferative capacity of these cells by 44% and 33% in the presence and absence of neocarzinostatin, respectively (Fig. 6B).

We next investigated whether repression of the proliferation of HBZ-expressing cells by hSSB2 was correlated with a decrease in DNA-damage levels. As shown in Fig. 6C, hSSB2 overexpression significantly reduced the frequency of DNA-strand breaks in neocarzinostatin-exposed HBZ-expressing cells after 24 hours of recovery. A similar effect was observed in the absence of neocarzinostatin exposure (Fig. 6C). In addition, H2AX (γ H2AX) phosphorylation was also decreased in cells expressing ectopic hSSB2, which confirmed the decrease in DNA damage, as H2AX is a sensitive indicator of both DNA damage and DNA replication stress (Fig. 6D). Taken together, these results strongly suggest that HBZ–miRNA–mediated downregulation of hSSB2 contributes, at least in part, to abnormal cell proliferation and genetic instability.

Discussion

Our miRNA expression analysis in the context of HTLV-1 infection revealed an HBZ–miRNA axis that promotes cell

proliferation and genetic instability. The integrated analysis of miRNA- and mRNA-expression profiles of CD4⁺ T cells derived from HAM/TSP patients determined that the HTLV-1-infected cells expressed higher levels of certain oncogenic miRNAs. The expression of these miRNAs correlated with HBZ expression, while transient transfection experiments demonstrated that HBZ activated miR17 and miR21 at a posttranscriptional level. The putative miRNA target genes expressed in HTLV-1⁺ CD4⁺ T cells were enriched for those that are involved in genetic disorders, suggesting that HBZ might promote genetic instability via miRNA dysregulation. Accordingly, we identified *OBFC2A* as a *bona fide* target of miR17 and miR21, and subsequently demonstrated that HBZ–miRNA–mediated downregulation of *OBFC2A* triggered abnormal cell proliferation and genomic instability of HeLa cells. Although the HBZ–miRNA axis might have multiple consequences in CD4⁺ T cells beyond that seen in HeLa cells, these results significantly expand our understanding of the oncogenic role of HBZ, which was previously considered to be limited to promoting cell proliferation (43).

In vivo, T cells persistently infected with HTLV-1 acquire numerous phenotypic changes with respect to cell morphology, apoptosis, proliferation, DNA content, and telomere homeostasis (5–8, 23, 24). Several studies have suggested roles for specific miRNAs in influencing the phenotypes of lymphoid T cells (35). For example, miR181 modulates the T-cell antigen receptor sensitivity (44); miR150 regulates the transcription factor c-Myb13 and miR155 affects the differentiation of CD4⁺ T lymphocytes into T helper type I (Th1) cells, regulatory T-cell development (45, 46), and the promotion of T-cell-dependent tissue inflammation (46). HTLV-1 was previously shown to modify the pattern of miRNA expression in cell lines and ATLL samples (30–33, 47–50). Our results here add to these findings, as the miRNA expression profile of nonimmortalized infected T cells derived from carriers without malignancies was significantly distinct from that of uninfected cells. Remarkably, infected CD4⁺ T cells overexpressed known oncogenic miRNAs, such as miR17 (36), miR21 (37–39), miR23b (51, 52), and miR27b (Fig. 1; refs. 51, 53, 54). Interestingly, we found that the predicted targets for the oncogenic miRNAs overexpressed in these HTLV-1⁺ CD4⁺ T cells were significantly enriched for genes involved in genetic disorders, neurologic diseases, cellular growth and proliferation, gene expression, and cellular function and maintenance (Fig. 2). Together, these differences in miRNA expression are consistent with the known differences in the effects of the virus on these T-cell subsets and fit well with the preleukemic phenotype of HTLV-1–positive nonimmortalized CD4⁺ T cells (5, 6).

The pleiotropic effects of Tax were previously thought to govern HTLV-1–associated miRNA dysregulation. By targeting Drosha to the proteasome, Tax influences global miRNA biogenesis (49). Furthermore, Tax activates the transcription of miR130b (33) and miR146a (32), and downregulates miR149 and miR873, which both directly target the chromatin-remodeling factors p300 and p/CAF that are known to play a critical role in HTLV-1 pathogenesis (47). Nonimmortalized HTLV-1–

positive clones express a wide range of Tax RNA levels, likely modifying numerous cellular pathways (5–8). Here, we found no relationship between Tax and miRNA expression in these cells. Instead, we found that the level of HBZ expression strongly correlated with that of four oncomiRNAs that are overexpressed in CD4⁺ T cells (Fig. 2). Transient transfection assays in HeLa cells confirmed these relationships and characterized HBZ as a new player in miRNA homeostasis. Interestingly, although HBZ interferes with numerous transcription factors, it did not appear to stimulate miR17 and miR21 expression via transcriptional activation, as is the case for Tax and miR146a expression via NF- κ B (31). Rather, our results demonstrated that HBZ increased the expression of miR17 and miR21 without increasing the transcription of their pri-miRNA transcripts. This HBZ-dependent posttranscriptional deregulation represents a novel mechanism that allows the activation of a restricted number of miRNAs belonging to a polycistronic miRNA cluster, such as the miR17-92 cluster (ref. 36 and present results). As such, future investigations will be important to address the molecular mechanisms underlying the posttranscriptional effect of HBZ.

Genetic instability is a hallmark of HTLV-1 infection that helps the infected cells to escape the strong anti-HTLV-1 cytotoxic T-lymphocyte (CTL) response. Genetic instability can also protect infected cells from clonal dominance and further transformation by promoting neo-antigen formation (5, 23, 24). On the other hand, HTLV-1-triggered gene alterations have been proposed to mount a mutator phenotype that propitiates leukemogenesis (5, 23, 24). So far, Tax has been recognized as the main source of HTLV-1-associated genetic instability. Here, we found that HBZ promotes oncomiRNA expression as well as DNA-strand breaks. These effects relied, at least in part, on the downregulation of *OBFC2A* via the HBZ-dependent posttranscriptional activation of miR17 and miR21 (Figs. 5 and 6). *OBFC2A* encodes hSSB2, which intervenes with ATM signaling and promotes subsequent activation of DNA repair and cell-cycle checkpoints (55, 56). Importantly, our results have also shown that HBZ expression does not promote growth arrest in DNA-damaged cells. On the contrary, HBZ-expressing cells continued to proliferate, even in the presence of neocarzinostatin (Fig. 6). This phenotype was reversed by ectopic expression of *OBFC2A*, which decreased the proliferation rate and restored the DNA-damage response in the same HBZ-expressing cells (Fig. 6).

Given this novel source of genetic instability, to what extent can it be exploited, if at all, by the infected cell? During nonmalignant stages of infection when HBZ is expressed (43), we can propose that, similar to Tax, HBZ helps persistent clonal expansion in the face of host immune response. However, in sharp contrast to Tax, the HBZ DNA sequence is regularly spared from epigenetic silencing *in vivo* (43). Taken together with our results, this suggests that HBZ might be more influential than Tax in sustaining persistent clonal expansion and promoting early leukemogenesis through its dual cellular effect, namely proliferation and genetic instability. At the malignant stage, our results indicate that HBZ expression might be implicated in the plethora of genetic defects that characterize ATLL cells. These

cells regularly display somatic mutations (23, 24) and complex cytogenetic abnormalities with evidence of frequent clonal cytogenetic evolution during disease progression (22, 57–60). These acquired genetic abnormalities and their clonal evolution impact on disease aggressiveness, relapse risk, and resistance to treatment (22, 57–60). Given that ATLL cells are Tax-negative but regularly express HBZ (43), the intraclonal genetic drift that characterizes ATLL cells was thought to result only from the tumor genotype, as is the case for virus-unrelated leukemias (61). However, our results indicating that HBZ triggers DNA-strand breaks in proliferating cells suggest that HBZ expression in tumor cells might contribute to the dynamic spectrum of complex cytogenetic abnormalities and somatic mutations that characterize ATLL cells (22, 57–60). However, these cells also have low levels of numerous miRNAs, including miR17 (30), suggesting that the HBZ-miRNA axis might be impaired at the late tumor stage. These data argue for a role of the HBZ-miRNA axis, and subsequent genetic instability, in initiating rather than promoting HTLV-1-associated leukemogenesis. Interestingly, Fig. 4C shows that HBZ remains capable of triggering DNA damage in the absence of functional miR17 and miR21, suggesting that additional mechanisms connect HBZ to genetic instability.

In conclusion, our results suggest that the preleukemic phenotypes of HTLV-1-positive CD4⁺ T cells include an oncogenic miRNA profile that is promoted by HBZ. We show that HBZ inactivates *OBFC2A* via oncomiRNAs, and thereby promotes cell proliferation and genetic instability. Thus, the notion that Tax is the unique generator of genetic instability may require revision. In addition, as in the case of DNA tumor viruses (62), HTLV-1 expression in tumor cells might be an important factor in triggering clonal evolution. As a corollary, targeting HBZ might be a promising approach for preventing and treating ATLL.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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HTLV-1 bZIP Factor HBZ Promotes Cell Proliferation and Genetic Instability by Activating OncomiRs

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