STLV-1-infected clones are thought to contribute to the formation of the tumor.

Treatment with anti-CCR4 antibody decreased proviral load in STLV-1-infected Japanese macaques

ATL cells express high levels of CC chemokine receptor 4 (CCR4) [28]. Recently, mogamulizumab, a humanized IgG1 monoclonal antibody against CCR4 [29], was approved in Japan for the treatment of relapsed ATL patients. HTLV-1-infected cells of healthy carriers also express CCR4, which indicates that mogamulizumab likely reduces the proviral load in HTLV-1-infected asymptomatic individuals [30]. High proviral load has been reported to be associated with HAM/TSP, HTLV-1 uveitis, and risk of ATL, indicating that mogamulizumab may potentially be used for the treatment of HTLV-1-associated diseases and the prevention of ATL. However, it is not clear whether mogamulizumab can reduce the proviral load in HTLV-1-infected individuals. We confirmed that mogamulizumab also recognizes macaque CCR4 by staining Japanese macaque PBMCs *in vitro* with the fluorescently labeled antibody (see Additional file 3). Then, we tested the efficacy of mogamulizumab to reduce the proviral load in STLV-1-infected Japanese macaques. Mogamulizumab was administered to two monkeys with high proviral load (Mf-6 and Mf-7), once a week for 4 weeks. As shown in Figure 7A, nearly half of the $CD4^+$ T cells expressed CCR4 before the treatment (week 0). After the treatment, the CCR4 positivity decreased to 1.62% and 12.4% respectively. We also

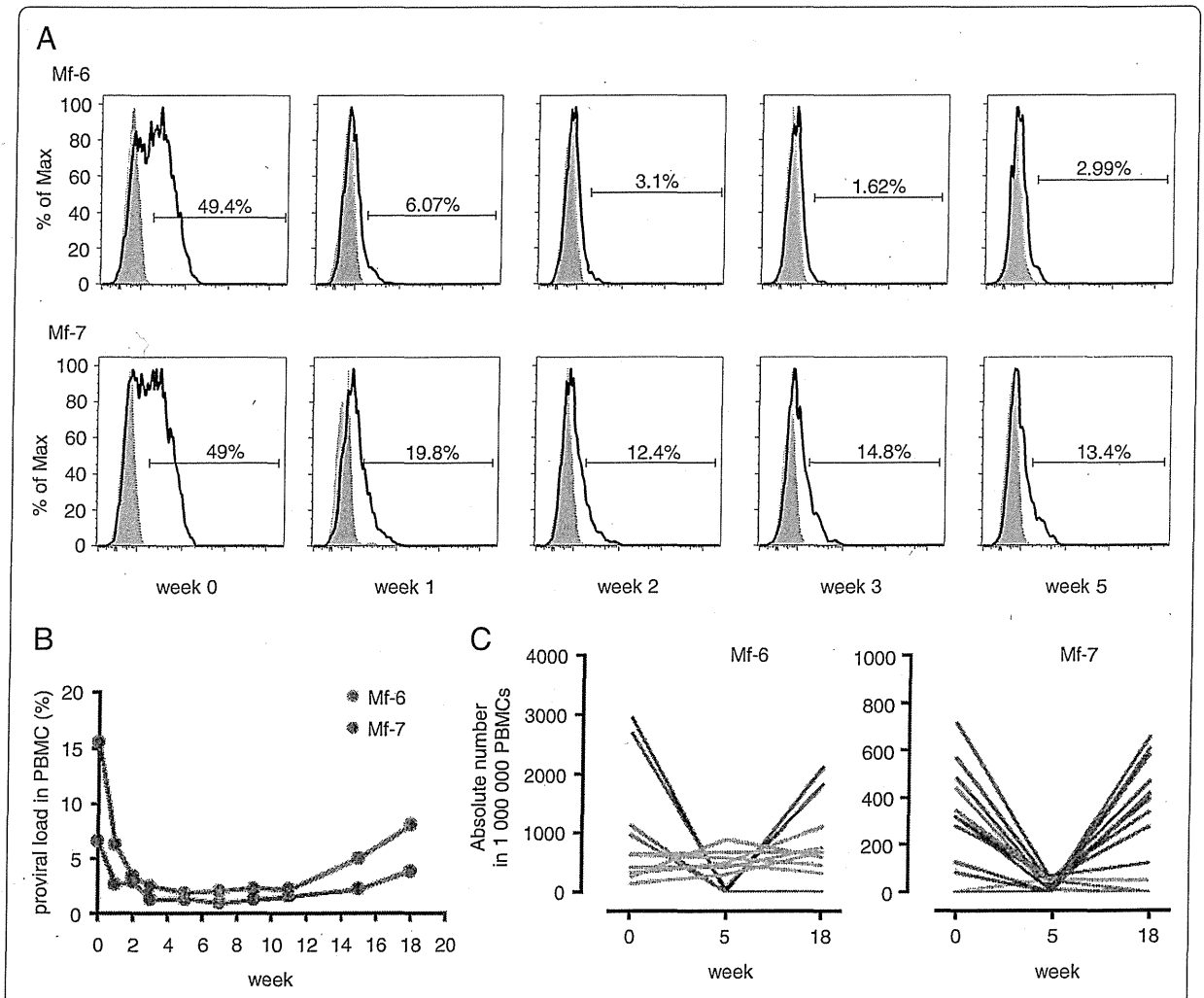

Figure 7 Effect of anti-CCR4 antibody on STLV-1 dynamics *in vivo*. **(A)** $CD3^+CD4^+$ T cells were gated and the expression of CCR4 was analyzed by flow cytometry. **(B)** Changes in STLV-1 proviral load in two monkeys treated with anti-CCR4 antibody until week 3. **(C)** Absolute cell numbers of the five most abundant clones in 1,000,000 PBMCs at weeks 0, 5 and 18 are shown.

measured proviral load over the course of the treatment and found that it decreased dramatically within 2 weeks (Figure 7B). Thus, this demonstrates that mogamulizumab can indeed reduce the number of STLV-1-infected cells *in vivo*.

Eight weeks after the final administration of mogamulizumab, the proviral load started to recover (Figure 7B). To investigate whether mogamulizumab influences the clonality of STLV-1-infected cells, we evaluated the absolute number of each clone by high-throughput sequencing of provirus integration sites. Figure 7C shows changes of the five most abundant clones at weeks 0, 5 and 18. The major clones before the treatment (week 0) recovered at week 18 (red lines in Figure 7C), while some clones were present constantly during the treatment (grey lines) or diminished after the treatment (blue lines). Interestingly, some clones (green lines) that emerged in a monkey after treatment were rare or even not detected before treatment (Figure 7C).

Discussion

HTLV-1 is thought to originate from STLV-1. In STLV-1-infected monkeys, investigators found clonal proliferation of STLV-1-infected cells and the preferential infection of $CD4^+$ T cells by the virus [15,31]. Moreover, several groups reported the development of lymphomas in STLV-1-infected monkeys [16,17,32-35]. Monoclonal integration of STLV-1 in the lymphoproliferative disease of African green monkeys was detected by Southern blot [16,33], demonstrating the direct causative role of STLV-1. Thus STLV-1-infected non-human primates have been thought to be a useful animal model for HTLV-1 research. The dynamics of infected cells after treatment with histone deacetylase inhibitors and reverse transcriptase inhibitors has been analyzed in STLV-1-infected baboons, and it was found that this combination significantly decreased proviral load in treated animals [36]. However, there have been no detailed studies on functions of STLV-1-encoded genes. Analyses of the functions of its accessory and regulatory proteins are necessary if we are to use STLV-1-infected monkeys as a model of HTLV-1 infection. In the present study, we focused on Japanese macaques naturally infected with STLV-1.

The amino acid sequence of STLV-1 Tax is closely homologous to that of HTLV-1 Tax, and this study demonstrated that their functions on various transcriptional pathways are similar as well. This study was the first to identify SBZ as an antisense transcript of STLV-1 and a homolog of HBZ. SBZ and HBZ share only approximately 73% identity at the amino acid level. Nevertheless, for all the functions we examined, SBZ behaves similarly to HBZ. In particular, SBZ expression could induce Foxp3 expression like HBZ expression does. This might be attributed to the following reasons. First, the N-terminal region, as well as the heptad repeats of hydrophobic amino acids in the basic leucine zipper domain, are conserved between HBZ and SBZ. This may allow SBZ to interact with and suppress NF-κB, AP-1 and other transcription factors with basic leucine zipper motifs [37,38]. Second, the LXXLL-like region (Leu27, Leu28, Leu48 and Leu49), which is critical for the interaction with p300 and Smad3 protein, is also conserved between HBZ and SBZ [22,39]. Some lysine residues present in HBZ are substituted with different amino acids in Japanese macaque SBZ. This study showed that SBZ has similar functions compared with HBZ, suggesting that these lysine residues are not critical for their functions. However, further studies are necessary for deep understanding of implication of these amino acid sequences.

HTLV-1 increases the number of infected cells by clonal proliferation of infected cells, which likely facilitates cell-to-cell transmission of this virus. Clonal proliferation of STLV-1-infected cells in Celebes macaques was demonstrated by the conventional inverse PCR method [15]. However, this technique could detect only a limited population of the clones because of its limited sensitivity or the stochastic amplification of the integration sites. In the present study, we investigated more comprehensively the clonal proliferation of infected cells in Japanese macaques naturally infected with STLV-1 by massively sequencing the unique integration sites of the provirus. The finding that STLV-1-infected cells proliferated clonally in the monkeys with higher proviral loads resembles the finding for HTLV-1. Furthermore, one monkey had lymphoma in the brain, showing that STLV-1 induces lymphoma in Japanese macaques. Analyses of STLV-1 integration sites in this T-cell lymphoma showed that one of the major clones in the brain was unique to this tumor, suggesting that this clone played an important role in the lymphomagenesis of this tumor.

This study also revealed a remarkable difference in STLV-1 seroprevalence between Japanese macaques (320/533: 60%) and rhesus macaques (1/163: 0.6%). Previous studies showed that the seroprevalence in rhesus macaques was 25%, and that in Japanese macaques was quite high [40-42]. Similarly, high seroprevalence was reported in baboons [43]. Furthermore, many studies reported the development of lymphoma in baboons [17,44,45]. The high seroprevalence and the development of lymphomas in Japanese macaques and baboons may suggest a higher susceptibility of these species to STLV-1 infection. Japanese macaques and baboons infected with STLV-1 may be suitable models for HTLV-1 research.

In this study, we also demonstrated that mogamulizumab strongly suppressed proviral load in STLV-1-infected Japanese macaques. Proviral load was suppressed for 4 weeks after the final administration of mogamulizumab, which seems reasonable when considering that the half-life of the

antibody administered at 1.0 mg/kg is approximately 18 days as measured in a clinical trial [46]. Some STLV-1-infected major clones recovered after the treatment, while other clones were still suppressed or even not detected. In HTLV-1-infected individuals, HTLV-1 proviral load is relatively constant in the chronic phase, although some minor clones fluctuate [25]. This study is the first to report that most of the major clones recover after the withdrawal of mogamulizumab. This observation suggests that the major clones may have some growth advantages that allow them to proliferate robustly *in vivo*. These growth advantages may be due to the integration site of the provirus, accumulation of genetic mutations, or epigenetic changes. The population of some clones remained constant over the course of the treatment. We speculate that these clones are negative for CCR4 expression. High proviral load is associated with risk of ATL and inflammatory diseases. Therefore, suppression of proviral load by mogamulizumab is a possible treatment for HTLV-1-associated inflammatory diseases such as HAM/TSP.

Conclusions

In summary, this study is the first to show that STLV-1 Tax and SBZ have activities similar to those of Tax and HBZ, activities which likely induce clonal proliferation and T-cell lymphoma in infected monkeys. STLV-1-infected Japanese macaques appear to be a good model for studying the effects of anti-viral drugs and the immunological aspects of HTLV-1 infection.

Methods

Biological samples of macaques

Japanese macaques (*Macaca fuscata*) and rhesus macaques (*Macaca mulatta*) used in this study were reared in the Primate Research Institute, Kyoto University. Blood samples were obtained from the macaques (for routine veterinary and microbiological examination) under ketamine anesthesia. All animal studies were conducted in accordance with the protocols of experimental procedures that were approved (2011–095) by the Animal Welfare and Animal Care Committee of the Primate Research Institute of Kyoto University, Inuyama, Japan.

Antibody screening and measurement of proviral load

Plasma samples were screened for the presence of antibodies against HTLV-1 by particle-agglutination test using SERODIA-HTLV-1 (Fujirebio). Proviral load was measured by real-time PCR quantifying the copy number of *tax* and *RAG1* as previously described [47]. Primers and probes are available in Additional file 4.

Detection of STLV-1 transcripts

Total RNA was extracted from STLV-1-infected Japanese macaque cell line Si-2 [48] with Trizol (Invitrogen), then cDNA was synthesized with SuperScript III (Invitrogen) using oligo dT primer. STLV-1 *tax* and *SBZ* was detected by PCR using primers (see Additional file 4) from the synthesized Si-2 cDNA: for STLV-1 *tax*, 2 min at 95°C, followed by 35 cycles of 20 seconds at 95°C, 10 seconds at 61°C, and 30 seconds at 72°C, and additional 5 min at 72°C; for SBZ, 2 min at 95°C, followed by 35 cycles of 20 seconds at 95°C, 10 seconds at 58°C, and 30 seconds at 72°C, and additional 5 min at 72°C. For comparison, HTLV-1 *tax* and *HBZ* were also amplified by PCR using cDNA of HTLV-1-infected cell lines (MT-1 or MT-2) with the same conditions. The primers used are shown in Additional file 4.

Plasmids

The PathDetect pNFκB-Luc, pAP-1-Luc and pNFAT-Luc plasmids were purchased from Stratagene. The 3TP-Lux, TopFlash reporter plasmids and WT-Luc were described previously [22,49]. The coding sequences of STLV-1 Tax and SBZ were amplified from STLV-1 provirus using oligos (see Additional file 4) and cloned into pME18Sneo to generate expression plasmids of STLV-1 Tax and SBZ. HTLV-1 tax was amplified using flanking primers (see Additional file 4) from pCGTax [50] and subcloned into pME18Sneo. The expression vector of HBZ cloned into pME18Sneo was described previously [11]. For the reporter assay, Jurkat cells or HepG2 cells were co-transfected with the reporter plasmid and the viral protein expression plasmids specified in each experiment, as previously described [22,24,51]. The activity of firefly luciferase was represented by normalizing to that of Renilla luciferase.

Retroviral vectors

The SBZ coding fragment was inserted into pGCDNSamI/N utilizing the NotI and SalI sites and SBZ-expressing retroviral vector was prepared as described previously [22].

Transduction of primary T-cells with retroviral vectors

$CD4^+CD25^-$ mouse T lymphocytes were stimulated and transduced with SBZ-expressing retroviral vector as previously described [22]. Forty-eight hours after the transduction, cells were harvested and analyzed by flow cytometry.

Flow cytometry

Antibodies used in this study were as follows: anti-human CD4 (OKT4), anti-Tax MI-73 [52], anti-mouse CD4 (RM4-5), anti-human CD271 (NGFR) (C40-1457), anti-mouse Foxp3 (FJK-16s), anti-human CD3 (SP34-2) and anti-human CCR4 (1G1, which recognizes a different epitope from that recognized by mogamulizumab). Intracellular staining was performed as previously described for Tax [52] and Foxp3 [22]. Cells were analyzed

by BD FACSCanto II with FACS Diva Software (BD Biosciences) or BD FACSVerse with FACSuite software (BD Biosciences).

Deep sequencing of provirus integration sites

The provirus integration sites in the Japanese macaque genome were amplified by linker-mediated PCR as previously described [27], with some modifications. Japanese macaque PBMC genomic DNA (3 μg) was sheared by sonication with a Bioruptor UCD-200 TM to obtain DNA fragments of approximately 200–500 bp. The ends of the DNA fragments were repaired to generate blunt ends using 18 units of T4 DNA polymerase, 5.3 units of DNA Klenow Polymerase I and 18 units of T4 polynucleotide kinase (TOYOBO) in T4 DNA ligase buffer (NEB) supplemented with 300 μM each of dNTP (TAKARA Bio). Adenine nucleotides were added to the blunt ends, and then linkers were ligated using 24 units of T4 DNA ligase (TOYOBO) in T4 DNA ligase buffer (NEB) utilizing the overhang of one thymidine nucleotide at the 3' end of the linker. The linker was generated by annealing two oligonucleotides (see Additional file 4). The first round of PCR was performed with the primers, STLV-1 Bio5 and Bio4. STLV-1 Bio5 anneals to the sequence within LTR of the STLV-1 provirus and Bio4 is the sequence present in the linker (see Additional file 4). Then, nested PCR was performed with the primers, Ion A-Bio7 and P1. In Ion A-Bio7, uppercase letters denote the sequence that anneals to the viral LTR downstream of STLV-1 Bio5, whereas the sequence in lowercase letters represents a tag specific for the Ion Torrent Personal Genome Machine (Ion PGM). P1 is also a tag specific for Ion PGM, which appears in the linker sequence (see Additional file 4). The amplification conditions of both the first and second PCR were 96°C for 30 sec, 7 cycles of 94°C for 5 sec and 72°C for 1 min, 23 cycles of 94°C for 5 sec and 68°C for 1 min, followed by additional 68°C for 9 min. Amplified fragments of approximately 150–300 bp were size-selected with E-Gel SizeSelect Agarose Gel (Life Technologies) and used as a DNA library in subsequent deep sequencing. Template beads to be sequenced with Ion Torrent Personal Genome Machine (Ion PGM) were prepared with the DNA library using the Ion PGM 200 Xpress Template Kit (Applied Biosystems) and subjected to sequencing on Ion Torrent 314 or 316 semiconductor chip using Ion PGM 200 Sequencing Kit (Applied Biosystems).

Deep sequencing data analysis

The host genomic sequences, located between the region immediately adjacent to the viral 3' LTR (ACACA) and the linker sequence (AGATCG), were extracted from the reads. Reads that started with GTTGGG (viral 5' LTR) were removed. Remaining reads were mapped to the reference genome of *Macaca mulatta* (MMUL 1.0) using the Burrows-Wheeler Aligner (BWA) [53]. Reads that were mapped only to single sites were analyzed. In order to obtain the absolute frequency of each provirus clone (the number of sister cells of the clone), the end position of each mapped read was obtained from the start position and cigar code in the SAM file generated by BWA. The reads with an identical start position and end position (integration site and shear site) were judged to derive from a single DNA fragment amplified by PCR, while reads with identical integration sites but distinct shear sites were judged to derive from different cells in a clone. In other words, the number of reads in the second category reflects the absolute frequency of each clone. Relative frequency represents the proportion of the absolute frequency of a clone to the number of all the sister cells observed. In order to minimize the distortion of relative frequencies of major clones, 6,000 reads that were mapped only to single sites were randomly selected for each specimen and analyzed (see Additional file 2).

Treatment of STLV-1$^+$ Japanese macaques with humanized anti-CCR4 antibody

Two Japanese macaques infected with STLV-1 were treated with mogamulizumab, which is an antibody against CCR4 and is approved in Japan as a drug to treat relapsed ATL. Mogamulizumab was provided by Kyowa Hakko Kirin Co Ltd. One mg/kg mogamulizumab was diluted in 40 ml saline and infused into each monkey intravenously for 20 min. Administration was performed once a week for 4 times. Before each administration, a 10 ml of blood sample was obtained. After the fourth administration, blood samples were collected every 2 weeks until week 11. Extra samples were collected on week 15 and week 18. The two monkeys were observed for any adverse effects during the experiment.

Additional files

Additional file 1: Phylogenetic analyses of HTLV-1 subtypes and Japanese macaque STLV-1.

Additional file 2: Deep sequencing data analysis.

Additional file 3: In vitro staining of Japanese macaque PBMCs with mogamulizumab.

Additional file 4: Primers and oligonucleotides.

Competing interests

Kyowa Hakko Kirin provided us the monoclonal antibody (mogamulizumab) that was used in this study.

Authors' contributions

JY and M. Matsuoka conceived of this study. JT carried out antibody screening and proviral load measurement. M. Miura, KS, GM and TZ carried out the molecular experiments and the reporter assays. AK, AW, AS and HA coordinated the macaque experiments and collected the macaque specimens. PM analyzed viral protein and surface marker expression. KO carried out immunohistochemistry and pathological analyses. M. Miura carried out massive sequencing and its data analysis. M. Miura, JY and M.

Matsuoka prepared the manuscript. All the authors approved the final manuscript.

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Current Opinion in Virology

Human T-cell leukemia virus type 1: replication, proliferation and propagation by Tax and HTLV-1 bZIP factor

Masao Matsuoka and Jun-ichirou Yasunaga

Human T-cell leukemia virus type 1 (HTLV-1) spreads primarily by cell-to-cell transmission. Therefore, HTLV-1 promotes the proliferation of infected cells to facilitate transmission. In HTLV-1 infected individuals, the provirus is present mainly in effector/memory T cells and Foxp3+ T cells. Recent study suggests that this immunophenotype is acquired by infected cells through the function of HTLV-1 bZIP factor (HBZ). Tax, which is encoded by the plus strand, is crucial for viral replication and *de novo* infection, while HBZ, encoded by the minus strand, is important for proliferation of infected cells. Importantly, HBZ and Tax have opposing functions in most transcription pathways. HBZ and Tax cooperate in elaborate ways to permit viral replication, proliferation of infected cells and propagation of the virus.

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Introduction

Transmission of human T-cell leukemia virus type 1 (HTLV-1) is confined to three routes; mother-to-infant, sexual parenteral transmission, and blood transfusion/needle sharing [1]. A striking feature of this virus is that HTLV-1 is transmitted primarily in a cell-to-cell fashion, and infectivity of free virions is very poor. By contrast, another human retrovirus, human immunodeficiency virus (HIV), transmits by both cell-free and cell-to-cell contact. The transmission of HTLV-1 requires living infected cells in breast milk, semen and blood products. To facilitate its transmission, this virus increases the number of infected cells *in vivo* by stimulating their proliferation.

HTLV-1 was discovered in 1980 as the first human retrovirus [2,3]. Thereafter, this virus was found to be linked with a human disease, adult T-cell leukemia (ATL) [4]. Subsequently it was found that this virus also causes another disease, HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP), as well as HTLV-1 uveitis, infective dermatitis, and myopathy [1]. These diseases are thought to be associated with the fact that infected host immune cells proliferate *in vivo*. In this review, we summarize recent findings on the replication of HTLV-1, the proliferation of infected cells, and HTLV-1 propagation — matters which are closely related for this virus.

Virus entry and cell-to-cell transmission

Unlike HIV, HTLV-1 can infect a variety of cells; its receptor is thought to be a commonly expressed molecule [5]. It has been reported that HTLV-1 envelope protein interacts with three cellular molecules, heparan sulfate proteoglycan (HSPG) [6], neuropilin-1 [7], and a glucose transporter, GLUT1 [8], for entry into cells. Conformational changes of the complex consisting of the HTLV-1 virion and these molecules are thought to occur sequentially during the entry step. First, the HTLV-1 envelope attaches to HSPG, and it then forms complexes with neuropilin-1, which results in stabilization of the complex. Thereafter, GLUT1 is associated with the complex, and finally triggers the fusion process necessary to viral entry [9•].

In vitro experiments showed that free virions had poor infectivity, while co-culture of uninfected cells with HTLV-1 infected cells easily established HTLV-1 infected cells [10]. It has been reported that cell-mediated infection of HTLV-1 is 10,000 times more efficient than cell-free infection, while cell-to-cell infection by HIV-1 is only twice as efficient as cell-free infection [11]. Three models for the mechanism of cell-to-cell infection by HTLV-1 have been proposed: (1) virological synapse [12] and (2) biofilm [13••], and (3) cellular conduits [14]. HTLV-1 infected cells form a virological synapse with uninfected cells; ICAM-1 and LFA-1 are implicated in this synapse formation. Tax is also implicated, specifically in microtubule reorientation [15]. Indeed, Tax enhances cell-to-cell infection [11]. On the other hand, there is evidence to support the biofilm model as well. HTLV-1-infected T cells retain viral particles with virally-induced extracellular matrix components, including collagen, agrin, tetherin and galectin-3 [13••]. By cell contact, these viral assemblies adhere to other cells, resulting in infection with HTLV-1.

An increased number of infected cell augments the chances of transmission. Indeed, for mother-to-infant

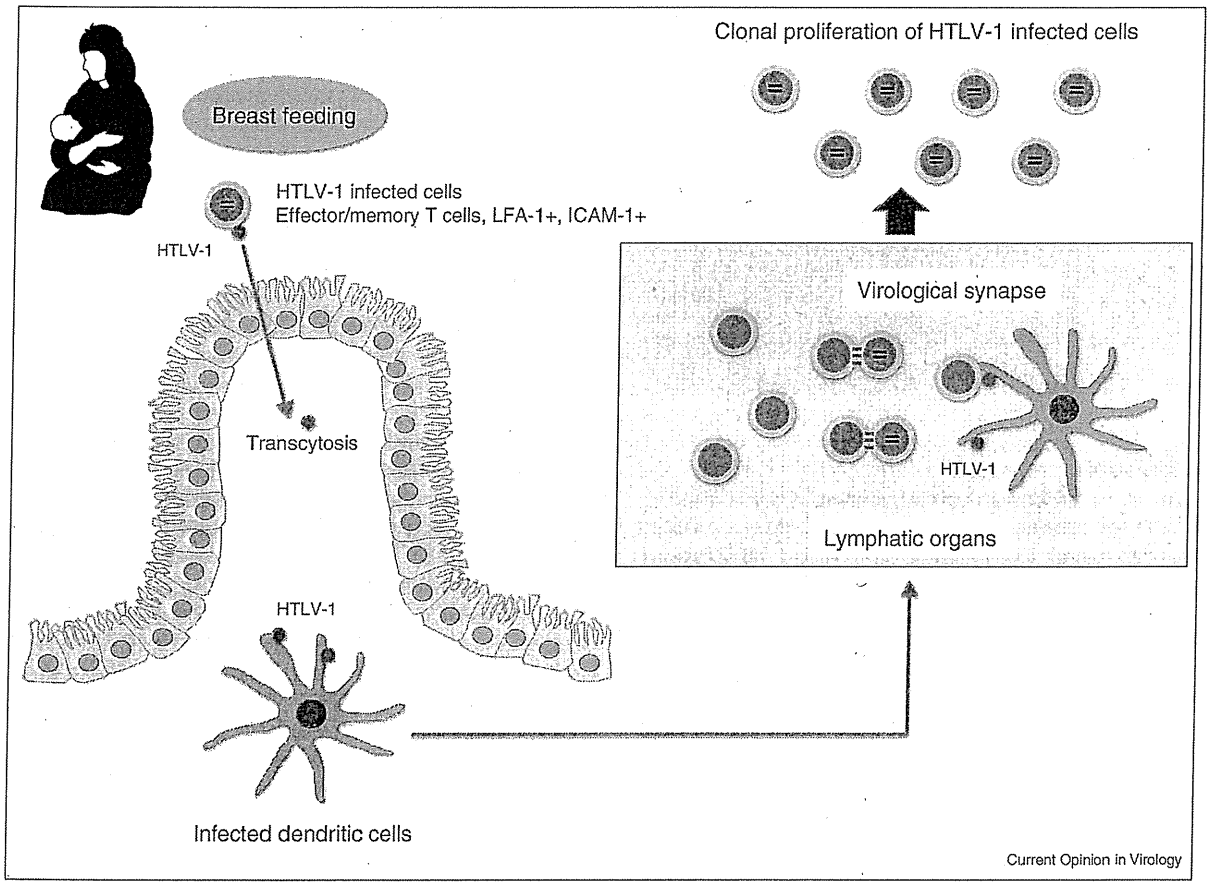
transmission, it has been reported that infants have higher chances of getting infected from mothers with higher proviral loads [16].

Transmission of HTLV-1

HTLV-1 can be transmitted by breast-feeding, sexual contact and blood transfusion. This transmission requires living infected cells, since this virus transmits mainly by cell-to-cell contact (Figure 1). Therefore HTLV-1 infected cells are hypothesized to have attributes that promote their entry into breast milk and semen. It has been reported that breast milk contains T-cells, most of which are effector/memory T cells expressing LFA1 and ICAM-1 [17], and HTLV-1 provirus has been detected in such effector/memory T cells [18]. These findings suggest that HTLV-1 may confer a phenotype to infected cells that facilitates their entry into breast milk. What component of HTLV-1 confers this effector/memory phenotype to HTLV-1 infected cells? Transgenic expression of HBZ in CD4+ T cells increased the number of effector/memory T cells and regulatory T cells, while transgenic mice expressing Tax had no change in the phenotype of CD4+ T cells [19••]. This clearly demonstrates that the immunophenotypes of ATL cells and HTLV-1 infected cells are conferred by HBZ, not by Tax. This conferred phenotype, which involves high levels of expression of adhesion molecules, enables HTLV-1 infected cells to enter into breast milk and semen (Figure 1).

Next, the virus must override epithelial barriers. How does HTLV-1 cross the alimentary tract? Recently, it has been shown that free infectious HTLV-1 virions could cross the epithelial barrier via a transcytosis mechanism [20•]. HTLV-1 virions could then infect human dendritic cells (DCs) that exist in the epithelial barrier [21]. Infected DCs likely migrate to draining lymph nodes and then form virological synapses with T cells (Figure 1). It is difficult to infect T cells by free virus *in vitro*.

Figure 1



Transmission and *de novo* infection with HTLV-1. HTLV-1 is transmitted via breast feeding, sexual intercourse, and blood transfusion. For any of these routes, living infected cells are essential. HTLV-1 infected cells have the immunophenotypes of effector/memory T cells or regulatory T cells. These cells tend to enter breast milk. HTLV-1 enters into the alimentary tract by transcytosis, and infects dendritic cells. Infected DCs transmit virus to uninfected T cells via virological synapses. Then infected T cells expand clonally *in vivo*.

However, free virus can infect DCs, and infected DCs can propagate HTLV-1 infection, suggesting that DCs are the spreader of this virus *in vivo* [21]. Expression of adhesion and co-stimulatory molecules is crucial for immunological synapses between T cells and DCs [22]. Thus, the immunophenotypes (effector/memory T cells, regulatory T cells, and enhanced expression of adhesion molecules) conferred by HBZ are crucial for the further spread of this virus *in vivo*. Thus, using HBZ, HTLV-1 induces infected cells to acquire certain immunophenotypes that facilitate its entry into the body and its subsequent spread within the body.

Clonal proliferation of HTLV-1 infected cells

After infection, HTLV-1 spreads by cell-to-cell infection and DC mediated infection. This *de novo* infection of cells is thought to form a pool of infected cells at an early phase of infection. In an experiment using immunodeficient mice with human lymphocytes, administration of reverse transcriptase inhibitors, tenofovir disoproxil fumarate (TDF) or azidothymidine (AZT) beginning after one week of infection could neither block nor decrease proviral load of HTLV-1, while TDF or AZT could block infection when they were injected at the same time of infection [23]. These results suggest that a pool of HTLV-1 infected clones is generated at very early phase of infection, and after that time, clonal proliferation of infected cells is predominant. This notion is also supported by clinical findings that reverse transcriptase inhibitors or integrase inhibitors did not alter proviral load in HTLV-1 infected individuals [24,25].

After this early stage of *de novo* infection, HTLV-1 infected clones are subject to selection by both host immunological attack and viral gene expression. In seroconvertors, the clonality of HTLV-1 infected cells was not stable at an early phase, but then stabilized at the chronic carrier state phase [26], indicating that HTLV-1 infected clones are selected at early phase of infection, and then, selected clones survive *in vivo*.

Since the HTLV-1 provirus integrates at random sites within the host genome, the clonality of HTLV-1 infected cells can be analyzed by studying these integration sites. Inverse PCR has been used to identify the integration sites and determine the clonality of infected cells [27,28]. Recently, high-throughput sequencing has been shown to be capable of detailed analysis of clonality [29••]. It is well known that HAM/TSP patients possess higher proviral loads compared with asymptomatic carriers. Analysis of clonality using high-throughput sequencing revealed that the abundance of each clone did not differ, but the number of different clones increased in HAM/TSP patients compared with asymptomatic carriers [29••]. By contrast, the abundance of certain clones increased in patients coinfected with HTLV-1 and strongyloides, and in infective dermatitis patients with HTLV-1 infection (IDH patients) [30•]. It is noteworthy that ATL develops relatively frequently in IDH patients and HTLV-1 carriers coinfected with strongyloides, while the occurrence of ATL is not so frequent in HAM/TSP patients [31]. Thus the enhanced abundance of clones and increased cell division might promote the development of ATL.

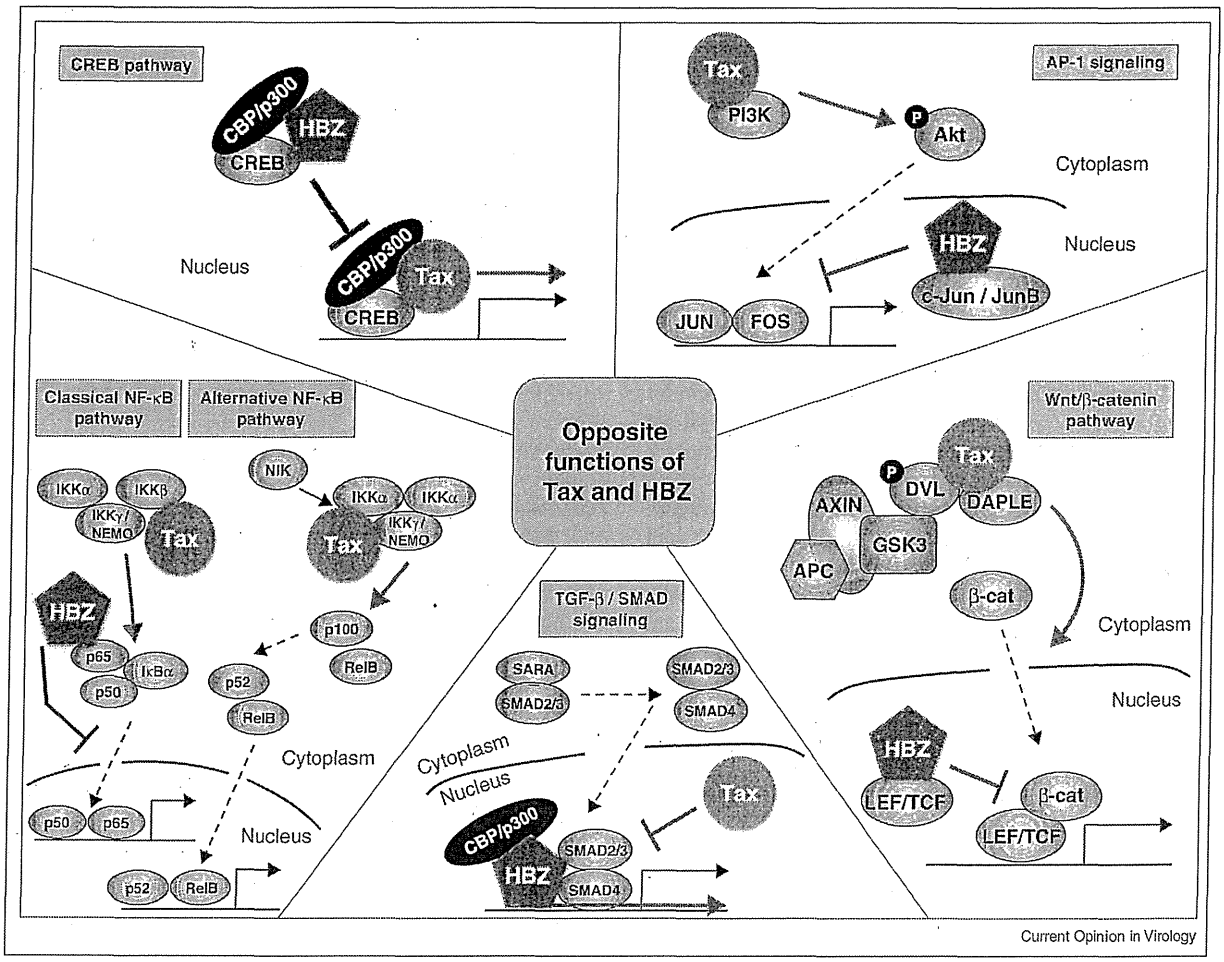
What drives cell division of HTLV-1 infected cells? HBZ is ubiquitously expressed in ATL cells and HTLV-1 infected cells *in vivo*, and promotes their proliferation [32]. In addition, Tax enhances mitogenic antigen-receptor signals [33,34]. The details of the mechanisms by which HBZ and Tax stimulate cell proliferation are complex and fascinating. In fact, HBZ and Tax have opposite effects on most signaling pathways [35] (Figure 2). For example, Tax activates the AP-1, NFAT, and CREB pathways while HBZ suppresses them [36,37]. Conversely, Tax inhibits TGF-β/Smad pathway whereas HBZ activates it [38]. Tax activates both the canonical and non-canonical NF-κB pathways [39]. HBZ inhibits only the canonical NF-κB pathway by interacting with p65. Expression of Tax promotes cell proliferation and simultaneously induces cellular senescence by induction of p21 and p27. HBZ prevents Tax induced cellular senescence by inhibiting p65 [40]. Thus, the elaborate interactions of various signaling pathways with Tax and HBZ control the proliferation of HTLV-1 infected cells. In addition to this relationship between HBZ and Tax, it has been reported that HBZ mRNA has growth-promoting activity [32], indicating another complex connection of HBZ as RNA and protein.

Furthermore, we have reported that HBZ suppresses the canonical Wnt pathway by inhibiting DNA binding by TCF-1/LEF-1 transcription factors, while Tax activates canonical Wnt signaling [41]. By contrast, HBZ enhances the transcription of Wnt 5a, which is a ligand for the non-canonical Wnt pathway. The canonical Wnt pathway is predominant during the development of T cells in the thymus, while non-canonical Wnt signaling is activated in peripheral T cells. These findings suggest that HBZ modulates the intra-cellular environment of peripheral T cells, which are natural target of this virus.

Control of transcription of viral genes

The HTLV-1 provirus encodes the regulatory genes (*tax* and *rex*) and the accessory genes (*p12*, *p13*, *p30*, and *HBZ*) in pX region; these genes regulate viral replication and the proliferation of infected cells [1]. For their transcription, the LTRs at each end of the provirus are used as promoters: the 5′LTR and 3′LTR control the transcription of the viral genes encoded in the plus and minus strands of the provirus, respectively (Figure 3). Since the plus strand of the provirus encodes all structural proteins and the viral genomic RNA, 5′LTR-mediated transcription is required for viral replication and transmission. Tax

Figure 2

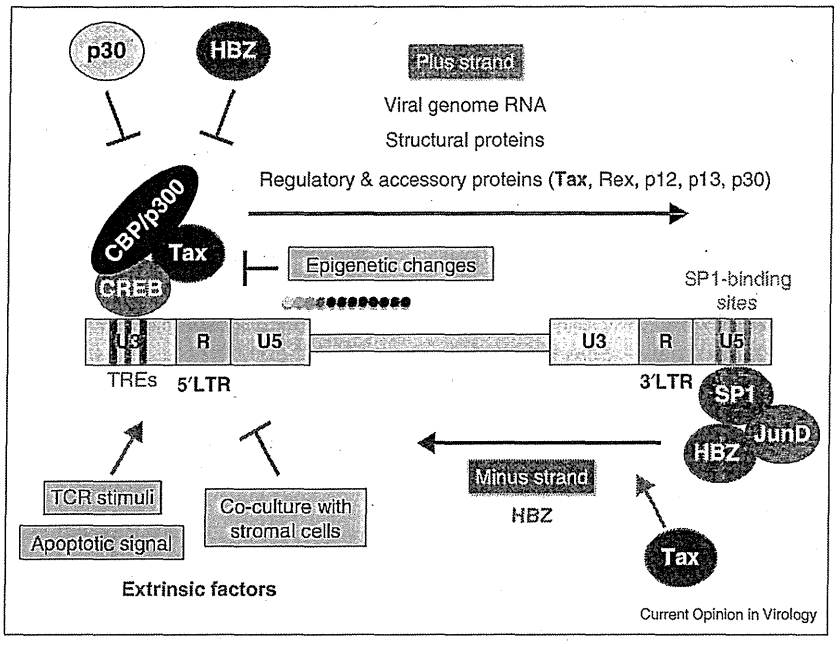


Opposite functions of Tax and HBZ. Tax and HBZ have opposite functions in many signaling pathways. Tax activates the CREB pathway by recruiting CREB and CBP/p300 to the promoters of target genes, whereas HBZ also interacts with the same proteins, suppressing Tax-mediated transcription. Tax activates both the classical and the alternative NF-κB pathways, and HBZ selectively suppresses classical signaling by targeting p65. Tax activates PI3K and induces the transcription of AP-1 target genes. HBZ negatively regulates this pathway by its inhibitory interactions with c-Jun and JunB through their bZIP domains. Tax forms a complex with DAPLE and DVL, and activates the canonical Wnt pathway. HBZ interacts with LEF-1/TCF-1 at point further downstream in this pathway and suppresses the transcription of the target genes. Tax has a negative effect on the TGF-β/SMAD pathway; however, HBZ activates it by interacting with SMAD2/3 and recruiting CBP/p300 to the promoters of the target genes.

is a potent activator of viral transcription through the 5′LTR. Tax does not bind to DNA, but activates the transcription of target genes by recruiting various transcription factors and modifying the epigenetic status of promoter regions [42]. The association between Tax and CREB is crucial for viral gene transcription. There are three 21-bp repeat elements, called Tax-responsive elements (TREs), located in 5′LTR, and the Tax-CREB complex recruits several histone acetyltransferaeses including CREB biding protein (CBP), p300, and p300/CBP-associated factor (PCAF) to the LTR, resulting in induction of viral expression. In addition to Tax, some cellular signaling machinery can enhance the activity of the 5′LTR. It has been shown that immune stimulation via T-cell receptor signaling activates the 5′LTR [34,43]. Another study showed that apoptotic signals induced viral transcription [44]. 5′LTR activation by these signals might be advantageous to efficient viral transmission and to viral 'escape' from a dying host cell.

Importantly, viral replication is actually suppressed *in vivo* [45], while viral antigens including Tax are quickly expressed in infected cells after they are transferred to *ex vivo* culture [46]. Host immune surveillance eliminates infected cells by targeting viral antigens. Among viral proteins, Tax is a major target of cytotoxic T-cells (CTLs)

Figure 3



Transcriptional control of HTLV-1 provirus. The 5′LTR is a promoter and enhancer of the plus strand transcripts that encode the viral genomic RNA, the structural proteins (Gag, Pol, and Env), and the regulatory/accessory proteins (Tax, Rex, p12, p13, and p30). Transcription via the 5′LTR is induced by recruiting the Tax-CREB-CBP/p300 complex to TREs in U3 region of 5′LTR, whereas the other viral factors (HBZ and p30) and epigenetic modifications on the 5′LTR suppress it. Some extrinsic factors are also associated with the activity of 5′LTR. By contrast, the 3′LTR is constitutively activated, and recruitment of SP-1 to its binding elements in U5 of the 3′LTR is important for 3′LTR activity. HBZ is encoded in the minus strand, and the HBZ-JunD complex enhances the transcriptional function of SP-1 on the 3′LTR.

[47]. It is well known that removal of CD8+ T-cells from PBMC allows infected cells to express Tax in the *ex vivo* cell culture [45], suggesting the presence of immune pressure against Tax *in vivo*. In addition, it was shown that, even in immunodeficient animal models, viral transcription from 5′LTR was suppressed, indicating that other mechanisms are involved in the silencing [48]. HTLV-1 can suppress its replication by its own proteins; p30 and HBZ are known to counteract Tax by competing for the binding to CREB, resulting in suppression of HTLV-1 replication [49]. p30 also inhibits the nuclear export of tax/rex mRNA [50]. Epigenetic changes, such as DNA methylation and histone modifications, are also involved in the silencing of HTLV-1. HTLV-1 differs from HIV in this respect. The LTR of HIV contains few CpG sites, while there are DNase hyper-sensitive regions, which explains the resistance of the HIV LTR to silencing [51,52]. On the other hand, the HTLV-1 LTR has many CpG sites, suggesting that HTLV-1 is susceptible to gene silencing mediated by DNA methylation. CpG methylation in the HTLV-1 provirus is observed in HTLV-1 carriers, and methylation tends to increase and to spread toward the 5′LTR during disease progression [53]. Indeed, Tax expression is frequently missing in ATL cells by epigenetic silencing of the 5′LTR as well as by genetic destruction of the 5′LTR or the *tax* gene [54,55]. Destruction of Tax expression enables ATL cells to escape from Tax-specific CTLs. Recently, it was reported that a histone deacetylase inhibitor, valproate (VPA), enhanced the expression of Tax and Gag in cultured HTLV-1-infected cells from asymptomatic carriers and HAM/TSP patients, suggesting that viral expression is suppressed by epigenetic mechanisms even in the carrier state [56•].

The 3′LTR functions as a promoter of the minus strand of the provirus [57]. It has been shown that the 3′LTR is conserved in all cases and CpGs are hypomethylated, suggesting that transcription through the 3′LTR is required for infected cells [53,58]. The *HBZ* gene is encoded in the minus strand, and alternative splicing makes the splice variants, the spliced and unspliced isoforms [59,60]. The spliced *HBZ* gene is transcribed from the 3′LTR, and the SP1 binding elements in 3′LTR are important for its transcription [57]. SP1 is a transcription factor ubiquitously expressed in a variety of cells, a fact which corresponds to the finding that HBZ is constitutively expressed in all ATL cases and HTLV-1 infected individuals [61]. It was also reported that SP1 forms a complex with HBZ and JunD and enhances the promoter

activity of HBZ [62], suggesting that SP1 is a key transcription factor for the activity of the 3′LTR. Interestingly, it was shown that Tax positively regulates 3′LTR activity [57], although the significance of this observation remains unclear. Further studies need to be conducted for us to fully understand the regulation of transcription via the 3′LTR.

The host immune system and proliferation of infected cells

After infection, provirus load (the number of infected cells) and clonality are determined by the balance between viral gene expression and the host immune response [63]. As described, Tax is highly immunogenic, while the immunogenicity of HBZ protein is very low [64••]. However, provirus load is well correlated with the immune response to HBZ; a low immune response to HBZ is associated with a high provirus load in HTLV-1 infected individuals. It is thought that HTLV-1 evolves to reduce the immunogenicity of HBZ, which is constitutively expressed and crucial for the proliferation of infected cells. Conversely, HTLV-1 infected cells express Tax more transiently. Tax is important for viral replication and *de novo* infection by HTLV-1. However, because of the high immunogenicity of Tax, HTLV-1 suppresses Tax expression *in vivo* by elaborately regulated mechanisms.

Conclusion

HTLV-1 evolved to propagate by cell-to-cell transmission. Therefore, this virus induces the proliferation of infected cells while under the pressure of host immune system. To this end, Tax and HBZ cooperate with each other in complicated ways to permit viral replication and promote the proliferation of infected cells. These phenomena are closely associated with the pathogenesis of this virus.

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Wip1 and p53 contribute to HTLV-1 Tax-induced tumorigenesis

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Abstract

Background: Human T-cell Leukemia Virus type 1 (HTLV-1) infects 20 million individuals world-wide and causes Adult T-cell Leukemia/Lymphoma (ATLL), a highly aggressive T-cell cancer. ATLL is refractory to treatment with conventional chemotherapy and fewer than 10% of afflicted individuals survive more than 5 years after diagnosis. HTLV-1 encodes a viral oncoprotein, Tax, that functions in transforming virus-infected T-cells into leukemic cells. All ATLL cases are believed to have reduced p53 activity although only a minority of ATLLs have genetic mutations in their p53 gene. It has been suggested that p53 function is inactivated by the Tax protein.

Results: Using genetically altered mice, we report here that Tax expression does not achieve a functional equivalence of p53 inactivation as that seen with genetic mutation of p53 (i.e. a $p53^{-/-}$ genotype). Thus, we find statistically significant differences in tumorigenesis between $Tax^{+}p53^{+/+}$ *versus* $Tax^{+}p53^{-/-}$ mice. We also find a role contributed by the cellular Wip1 phosphatase protein in tumor formation in Tax transgenic mice. Notably, $Tax^{+}Wip1^{-/-}$ mice show statistically significant reduced prevalence of tumorigenesis compared to $Tax^{+}Wip1^{+/+}$ counterparts.

Conclusions: Our findings provide new insights into contributions by p53 and Wip1 in the *in vivo* oncogenesis of Tax-induced tumors in mice.

Background

Human T-cell Leukemia Virus type 1 (HTLV-1) is the first identified human retrovirus. The virus belongs to the deltaretrovirus family and is the etiological agent of a highly aggressive neoplastic disease, Adult T-cell Leukemia/Lymphoma (ATLL), and inflammatory diseases including HTLV-1 Associated Myelopathy (HAM)/Tropical Spastic Paraparesis (TSP), uveitis, infective dermatitis and myositis [1-9]. HTLV-1 infects approximately 20 million individuals world-wide, and 1-5% of infected individuals will develop ATLL after a long latency period of 20 to 60 years [1].

HTLV-1 encodes a viral Tax oncoprotein. The singular expression of Tax is sufficient to transform primary rodent cells [10] and potentially human embryonic stem cells [11], immortalize human primary T lymphocytes [12,13], and induce tumors in transgenic mice [14-17]. Tax confers pro-proliferative and pro-survival properties to HTLV-1 infected cells [18-20] by pleiotropically activating effector proteins including the Cyclic AMP Responsive Binding Protein (CREB) and CBP/p300 [21-24], Nuclear Factor kappa-B (NF-κB) [25-29], Cyclin-Dependant Kinases (CDKs) [30-33], and Akt [34-36] amongst others. Tax also triggers DNA damage [37-42]. In transforming a normal T-cell into a leukemic cell, it is believed that Tax must also neutralize cellular checkpoints (e.g. p53 and mitotic spindle assembly checkpoint) that act to censor DNA damage [43,44] and aneuploidy [45,46].

p53 is a DNA-binding transcription factor that plays a key role in cell cycle regulation, apoptosis, and DNA repair [47]. The p53 gene is recognized as one of the most important tumor suppressor genes and is frequently mutated in human tumors including hematologic malignancies [48-50]. In many human malignancies, the frequency of p53 genetic mutation is ≥50% [51,52]; however, the frequency of mutated p53 in ATL patients is reported to be around 15% [53-58], suggesting that loss of p53 activity in ATL may largely arise through a

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mechanism other than genetic mutation. Several *in vitro* studies in different cell types have shown that Tax represses p53 activity [59-65]. Various mechanisms have been proposed for Tax-inactivation of p53. Indeed, it has been suggested that Tax inactivates p53 by acting through either the CREB [62] or the NF-κB [66,67] pathway; however, it has also been noted that neither mechanism satisfactorily explains Tax-p53 interaction [65], leaving the question of how Tax effectively disables p53 function incompletely answered.

Here, we have conducted *in vivo* experiments in mice to address two questions. First, we have assessed the effectiveness of Tax mediated inactivation of p53 *versus* inactivation of p53 by genetic mutations. Second, we have characterized Wip1 as a cooperating *in vivo* Tax co-factor in p53 inactivation. Using various genetically altered mice, we show that Tax inactivation of p53 is functionally less stringent than p53 inactivation by genetic mutation, and we report that the cellular Wip1 phosphatase protein collaborates functionally with Tax in inhibiting p53 activity.

Results

$Tax^{+}p53^{-/-}$ mice show reduced tumor free survival compared to $Tax^{+}p53^{+/+}$

In ATLs, p53 genetic mutations are less frequent than those seen in many other cancers [53,54,58]. It has been reasoned that the ability of Tax to inacti-vate p53 function [55] explains why ATL cells may not need to inactivate p53 by genetic mutation. Nevertheless, it has not been clearly characterized whether Tax inactivation of p53 is quantitatively equivalent to inactivation of p53 by genetic mutation. We sought to investigate this issue using gene-tically altered mice. Accordingly, we crossed Tax transgenic mice [15] with $p53^{-/-}$ mutant mice [68] to generate $Tax^{+}p53^{-/-}$, $Tax^{+}p53^{+/-}$ and $Tax^{+}p53^{+/+}$ progenies. We analyzed the genotypes (Figure 1) of the offsprings and monitored the animals over >300 days for tumor development (Figure 2). Tumor-free survival for $Tax^{+}p53^{-/-}$ mice (Figure 2A) was significantly worse compared to $Tax^{+}p53^{+/-}$ and $Tax^{+}p53^{+/+}$ counterparts ($p < 0.0001$; Gehan-Breslow-Wilcoxon test). There were no statistically significant differences in the levels of Tax expression between these two categories of Tax^{+} mice supporting that the difference in tumor-free survival was not due to levels of Tax expression (Additional file 1: Figure S1). Interestingly, no significant difference in tumor-free survival between $Tax^{+}p53^{+/-}$ and $Tax^{+}p53^{+/+}$ mice was found ($p = 0.7093$; Gehan-Breslow-Wilcoxon test); this finding agrees with our previous tumorigenesis study of $p53^{+/-}$ and $p53^{+/+}$ mice [69] that, in the context of our mice, we find no significant functional difference between homozygosity *versus* heterozygosity in wild type p53. Thus, our finding of a distinct difference in tumor-free survival of $Tax^{+}p53^{-/-}$ compared to $Tax^{+}p53^{+/+}$ mice indicates that Tax inactivation of p53 (i.e. $Tax^{+}p53^{+/+}$) is qualitatively less stringent than genetic inactivation of p53 (i.e. $Tax^{+}p53^{-/-}$).

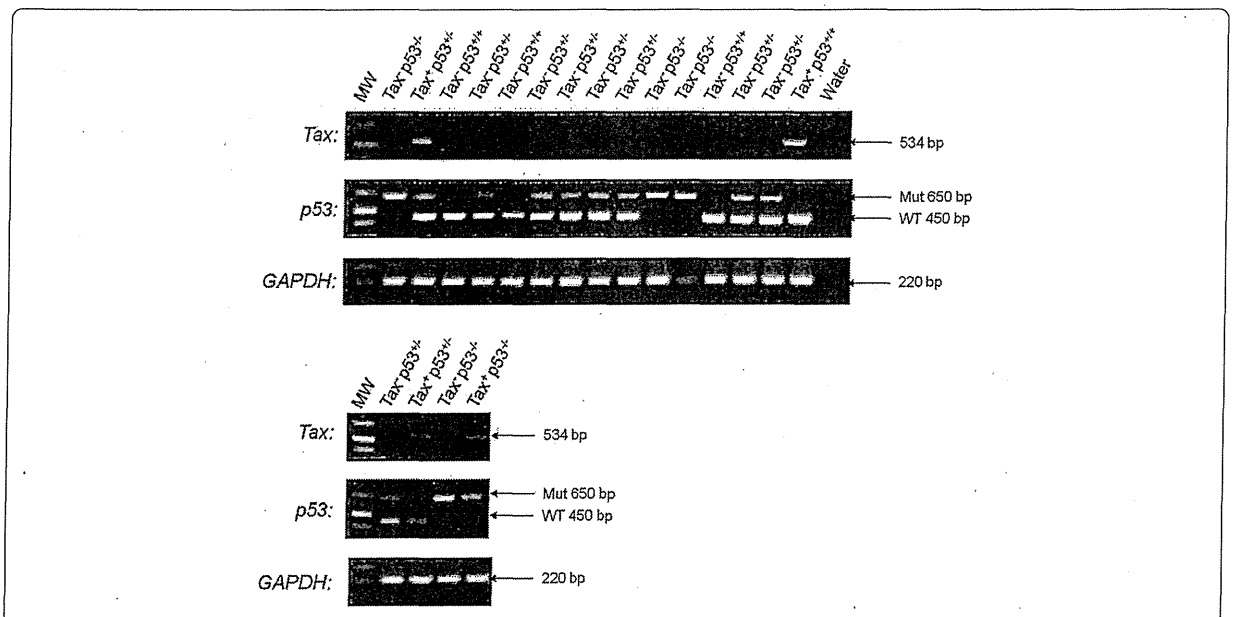

Figure 1 Genotyping of *p53KO/Tax Tg* mice. *p53* primers distinguish between WT and mutant p53 alleles with PCR products of 450 and 650 bp in size (top), respectively. Middle panel shows the detection of Tax DNA (534 bp), and the bottom panel shows PCR control detecting cell endogenous GAPDH gene (220 bp). Mut, mutant ; WT, wild type. Tax, p53, and GAPDH signals are as indicated.

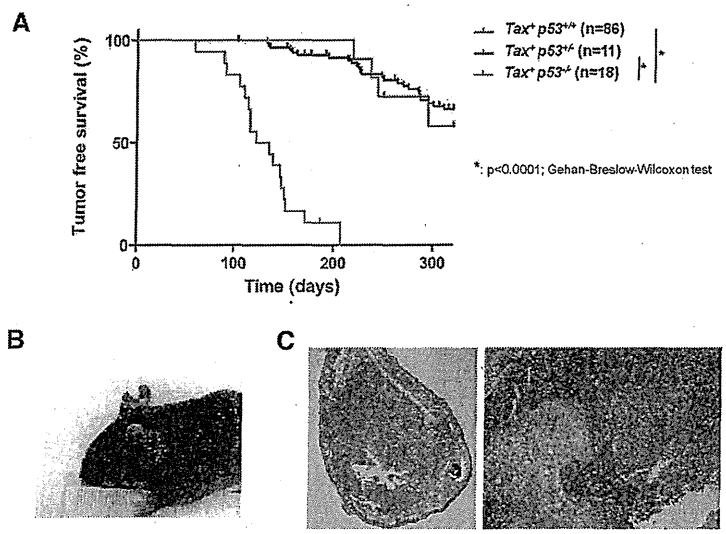

Figure 2 Statistically significant reduction of tumor-free survival in $Tax^{+}p53^{-/-}$ mice compared to $Tax^{+}p53^{+/+}$. (**A**) Tumor-free survival curves show decreased tumor free survival in $Tax^{+}p53^{-/-}$ mice compared to $Tax^{+}p53^{+/-}$ or $Tax^{+}p53^{+/+}$ animals. Statistical significance (*: p<0.0001) between Tax^{+} $p53^{-/-}$ and either $Tax^{+}p53^{+/-}$ or $Tax^{+}p53^{+/+}$ mice was determined using Gehan-Breslow-Wilcoxon test. (**B**) A Tax transgenic mouse with an ear tumor is shown for illustration. (**C**) Examples of tumor histology [hematoxylin and eosin (H&E) staining] from $Tax^{+}p53^{+/+}$ mice are shown. Example of a pleomorphic ear sarcoma from a $Tax^{+}p53^{+/+}$ mouse; (left): expansive soft tissue tumor located in peripheral connective tissue of the ear beneath the supportive cartilage; example of a hind leg pleomorphic histiocytic sarcoma and glandular adenocarcinoma with squamous hyperkeratosis from a $Tax^{+}p53^{+/+}$ mouse (right): the sarcoma consisted primarily of histiocytic tumor cells with dispersed round cells, sparse spindle cells and neutrophilic granulocytes. Similar tumors are also seen in $Tax^{+}p53^{-/-}$ mice.

Wip1 phosphatase modulates p53 activity

We wished next to understand how other non-genetic means of inactivating p53 might cooperate with Tax in cellular transformation. Wip1 (Wild-type p53-induced phosphatase 1) is a human protein phosphatase that has been shown to be amplified and over-expressed in multiple human cancers and has been suggested to exhibit oncogenic potential [70]. A plausible mechanistic scenario could be that Wip1 acts to inhibit p53 activity, thereby contributing to tumorigenesis. Through its ability to inhibit p53 tumor suppressor function, Wip1, like Tax, may reduce the selective pressure for *p53*-inactivating mutations during cancer progression [71,72]. To check the effect of Wip1 on p53, we assessed how its over-expression affects p53's transcriptional activity. Accordingly, we transfected human HCT-116 cells with a luciferase reporter plasmid containing 13 copies of a p53 consensus binding site (pG13-Luc; [73]) together with a Wip1 expression plasmid (Figure 3A and B), or we transfected pG13-Luc with a Tax expression plasmid-alone, or we transfected pG13-Luc with both Wip1 and Tax expression plasmids (Figure 3A and B). Under our transfection conditions, both Wip1-alone and Tax-alone with pG13-Luc robustly repressed the expression of the reporter plasmid by more than 40% ($p=1.496 \times 10^{-5}$ for Wip1-alone; $p=7.62\times10^{-5}$ for Tax-alone; t-test) (Figure 3A). Of note, the co-transfection of Wip1 with Tax repressed pG13-Luc expression by an additional 20% and 15% over that achieved with Tax-alone ($p=0.0025$; t-test) or Wip1 alone ($p=0.019$; t-test) (Figure 3A). When the transfections were performed in the presence of co-introduced exogenous p53, we again observed a statistically significant repression of p53 transcriptional activity; here, we saw >60% repre-ssion of pG13-Luc expression after transfection with Wip1-alone ($p=3.27 \times10^{-5}$; t-test) or Tax-alone ($p=2.22 \times10^{-5}$; t-test) (Figure 3B). In the presence of exogenously introduced p53, the co-transfection of Wip1 and Tax repressed pG13-Luc expression by more than 50% over that achieved with Tax-alone ($p=7.43\times10^{-5}$; t-test) or Wip1-alone ($p=1.25\times10^{-4}$ t-test) (Figure 3B). In Figure 3C, the expression of the transfected plasmids used in Figures 3A and 3B was checked by Western blotting. Taken together, these findings support that Wip1 and Tax cooperate in overall p53 inactivation.

Transient over-expression assays generally are imperfect reflections of physiological regulation. To ask in a more physiological manner how endogenous Wip1 expression regulates p53 activity, we independently isolated several primary MEF clones from $Wip1^{-/-}$ knock-out mice [74] and their $Wip1^{+/+}$ wild type siblings (genotyping examples of MEFs are shown in Figure 3D, top). We then compared cell endogenous p53 activity in several independently isolated $Wip1^{-/-}$ MEFs to other independently isolated control $Wip1^{+/+}$ MEFs employing either the pG13-Luc reporter assay (Figure 3D, bottom) or by determining the mRNA expression levels of a known p53-responsive target gene, $p21^{WAF1/CIP1}$ (Figure 3E). Notably,

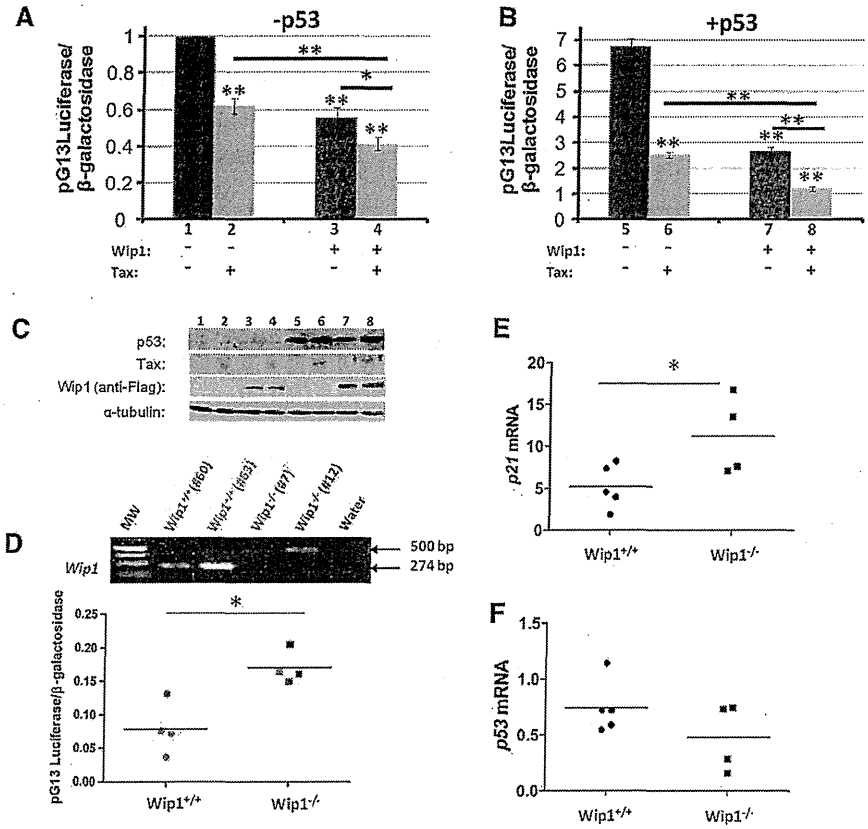

Figure 3 Wip1 phosphatase attenuates p53 activity. Wip1 and/or Tax expression reduces p53 transactivation of a pG13Luc-reporter in HCT-116 cells in the absence (**A**) or in the presence (**B**) of exogenous p53 (0.8 μg). HCT-116 cells were transfected with 0.2 μg of Tax and/or 0.75 μg of Wip1 expression plasmid (*: 0.01≤p≤0.05; **: p<0.05; t-test). (**C**) Cell lysates from a representative experiment were subjected to immunoblotting using anti-p53, anti-Tax, anti-Flag and anti-α-tubulin as indicated. The lane numbers of the samples in each case corresponds to the lane numbers indicated in panels (**A**) and (**B**). (**D**) Analysis of cell endogenous p53 activity was conducted using the pG13-Luciferase plasmid in *Wip1*$^{-/-}$ and *Wip1*$^{+/+}$ Mouse Embryonic Fibroblasts (MEF). Top panel shows PCR genotypic characterizations of two independent Wip1$^{+/+}$ (60, 63) and two independent Wip1$^{-/-}$ (7, 12) MEFs; each was assayed twice in pG13Luc-reporter assays. Bottom graph shows the luciferase assays. All luciferase activities were normalized to a co-transfected β-galactosidase reporter. Statistical significance was determined using t-test (*: p=0.0076). (**E**) Analyses of cell endogenous p21 and (**F**) p53 mRNAs in 5 independent Wip1$^{+/+}$ (left) and 4 independent Wip1$^{-/-}$ MEFs. Real-time RT-PCR analyses of *p53* and *p21* and *GAPDH* (internal standard) transcripts were performed in *Wip1*$^{-/-}$ and *Wip1*$^{+/+}$ MEFs. There was no statistically significant difference in p53 mRNA levels, while p21 mRNA levels were significantly different between *Wip1*$^{+/+}$ and *Wip1*$^{-/-}$ MEFs (*: p=0.0425; t-test).

the *Wip1*$^{-/-}$ MEFs showed statistically significant higher levels of pG13-Luc expression (p=0.0076; t-test) and higher levels of p21 mRNA (p=0.0425; t-test) than the *Wip1*$^{+/+}$ MEFs, suggesting that cell endogenous Wip1 does physiologically reduce p53 function in primary cells (Figures 3D and E). This regulation of p53 by Wip1, however, does not occur at the level of transcription because there was no statistically significant difference in the amounts of p53 mRNA in *Wip1*$^{+/+}$ *versus* *Wip1*$^{-/-}$ MEFs (Figure 3F).

Wip1 deficiency reduces Tax-tumorigenesis

The above results show that both Wip1 and Tax inactivate p53 function. Next, we asked how the two events might cooperate in tumorigenesis. To address their functional collaboration, we crossed Tax transgenic mice with *Wip1*$^{+/+}$ or *Wip1*$^{-/-}$ mice. Various genotypic offsprings were obtained from these crosses (genotyping examples are shown in Figure 4A), and the animals were monitored for tumorigenesis over 300 days (Figure 4B). Interestingly, *Wip1*$^{+/-}$ and *Wip1*$^{-/-}$ mice that express Tax showed significantly better tumor-free survival than *Wip1*$^{+/+}$ animals that express Tax (Figure 4B). Indeed, tumor-free survivals were statistically different between *Tax*$^{+}$*Wip*$^{-/-}$ (p=0.0319; Gehan-Breslow-Wilcoxon test) or *Tax*$^{+}$*Wip1*$^{+/-}$ mice (p=0.0396; Gehan-Breslow-Wilcoxon test) compared to *Tax*$^{+}$*Wip1*$^{+/+}$ mice. In view of findings above that p53 activity is higher in *Wip1*$^{-/-}$ MEFs

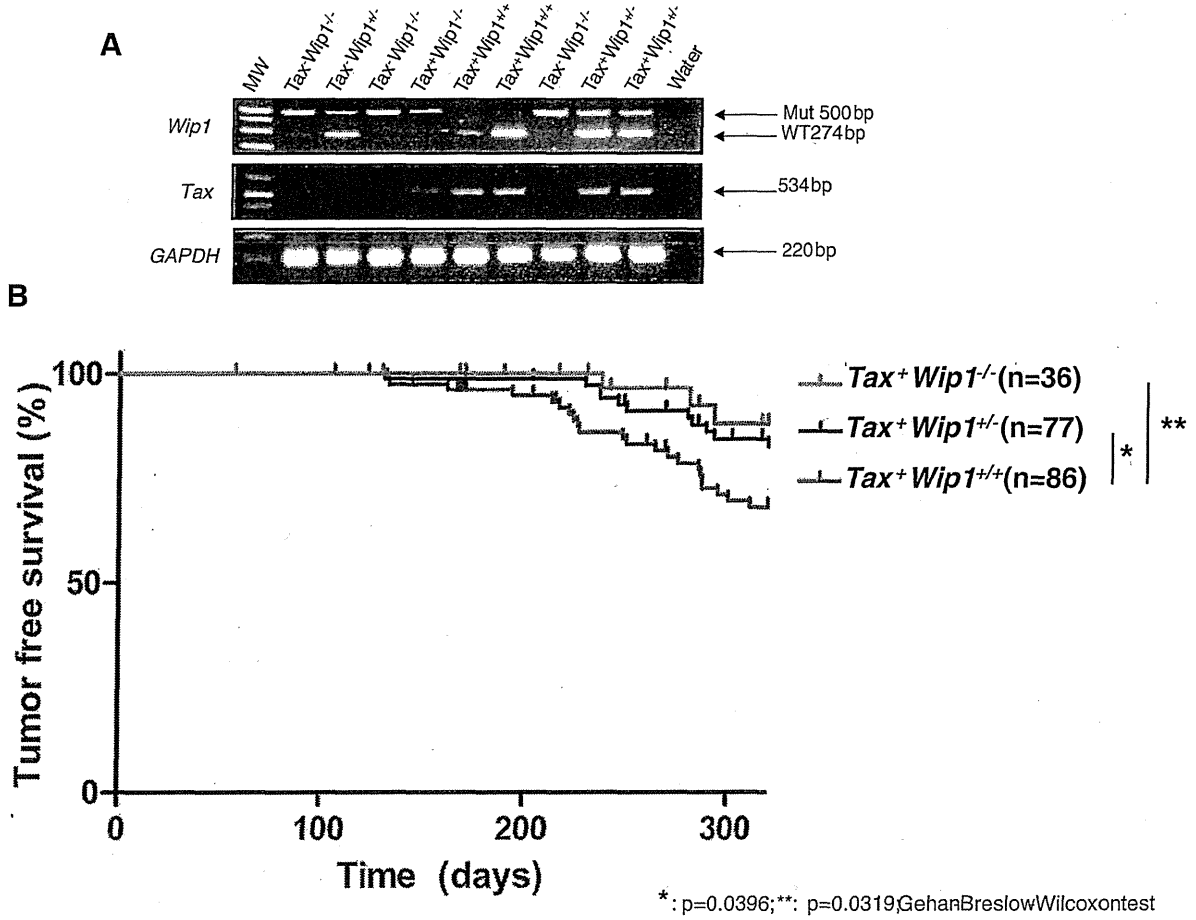

Figure 4 Statistically significant increase in tumor free survival in $Tax^{+}Wip1^{-/-}$ mice compared to $Tax^{+}Wip1^{+/+}$ mice. (**A**) Genotyping of *Wip1/Tax Tg* mice. The primers for Wip1 distinguish WT and mutant Wip1 alleles with PCR products of 274 and 500 bp, respectively. The GAPDH gene serves as an internal PCR control (220 bp). (**B**) Tumor-free survival curves show increased tumor free survival of Tax^{+} $Wip1^{-/-}$ and Tax^{+} $Wip1^{+/-}$ mice compared to Tax^{+} $Wip1^{+/+}$ animals. Statistically significant (*=p=0.0396 ; **=0.0319) differences between Tax^{+} $Wip1^{-/-}$ or Tax^{+} $Wip1^{+/-}$ and Tax^{+} $Wip1^{+/+}$ mice were determined using Gehan-Breslow-Wilcoxon test.

compared to $Wip1^{+/+}$ MEFs; one interpretation of these *in vivo* tumor results is that homozygous loss of Wip1 (i.e.$Tax^{+}Wip1^{-/-}$) reduces the level of p53-inactivation in Tax expressing cells compared to counterpart cells that expresses both Wip1 and Tax (i.e. $Tax^{+}Wip1^{+/+}$); this reduced inactivation of p53 could explain the increased tumor-free survival observed in the $Tax^{+}Wip1^{-/-}$ over the $Tax^{+}Wip1^{+/+}$ mice.

Tax expression does not increase Wip1 transcription

Figure 4B shows that when Tax and Wip1 are expressed together overall *in vivo* transforming potential is increased. Tax is known to activate or repress the transcription of various genes [75-80]; thus a possibility is that Tax expression affects Wip1 transcription. To address this possibility, RNA was isolated from Tax-expressing HTLV-1–transformed MT2, MT4, C8166 cells and compared to RNAs from HTLV-1–negative $CD4^{+}$ T-cell lines, CEM, Jurkat and H9; specific transcripts were quantified by real-time RT-PCR (Figure 5A). The real-time RT-PCR results showed no correlation between *Tax* expression and *Wip1* expression in these cells. To check in a different way that Tax has no effect on *Wip1* transcription, we transiently transfected $p53^{-/-}$HCT116 (Figure 5B), $p53^{+/+}$HCT116 (Figure 5C), or HeLa cells (Figure 5D) with various amounts of a Tax expression plasmid and measured Wip1 mRNA. $p53^{-/-}$ HCT116 and $p53^{+/+}$ HCT116 cells [81] have been commonly used to study p53 function. In these cells, we observed no statistically significant change in *Wip1* mRNA upon Tax expression. We also transfected MEFs and HCT-116 cells with a Tax expression plasmid and immunostained the cells for Tax and Wip1 proteins. Based on visualization by confocal microscopy, no difference in

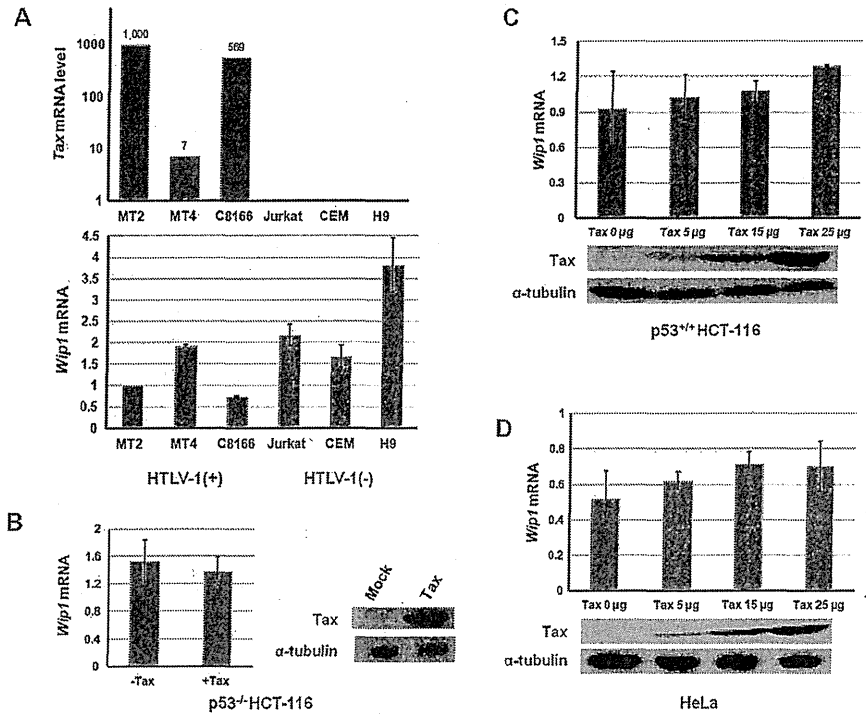

Figure 5 Analysis of *Wip1* mRNA expression in Tax-expressing and Tax-non-expressing cells. (**A**) Total RNAs from HTLV-1–transformed MT-2, MT4, C8166 T-cell lines and HTLV-1-negative $CD4^+$ control T-cell lines (Jurkat, CEM, and H9) were extracted and reverse transcribed. The cDNAs were used for real-time RT-PCR analyses of *Wip1*, *Tax*, and *GAPDH* (internal standard) transcripts. The mRNA relative expression levels of *Wip1* and *Tax* mRNA were determined and normalized as multiples of the *GAPDH* mRNA. The columns represent the average results from 3 experiments; the error bars are mean errors. (**B**) Real-time RT-PCR analyses of *Wip1* and *GAPDH* (internal control) transcripts were performed in $p53^{-/-}$ HCT116, (**C**) $p53^{+/+}$ HCT116 and (**D**) HeLa cells after transfection with a control vector or a Tax-expression vector. To detect Tax protein, immunoblots were stained using Tax and α-tubulin specific monoclonal antibodies. Tubulin was used as a loading control.

Wip1 signal intensity was seen in Tax-expressing cells *versus* Tax-negative cells (Figure 6A and Additional file 2: Figure S2A). These findings demonstrate that Tax expression does not change ambient Wip1 protein level and agree with the RNA measurement results that Tax expression does not alter Wip1 mRNA expression (Figure 5).

In our immunostainings, we did note that Tax and Wip1 colocalize in the nucleus (Figure 6A and Additional file 2: Figure S2A). Moreover, additional immunostainings also show that Wip1 and p53 colocalize in the nucleus (Figure 6B and Additional file 2: Figure S2B). Thus, conceivably, Tax, p53, and Wip1 interaction occurs through intranuclear contacts. Currently, we do not have sufficient data to fully understand whether the colocalization of Tax, Wip1, and p53 manifests in direct protein-protein interactions or the proteins interact through bridging by additional factors. Experiments are in progress to define better these mechanistic interactions.

Discussion

Colloquially known as the guardian of the genome, p53 is an important player in cancer biology, as exemplified by its ubiquitous loss of function in cancers. Thus, approximately 50% of human cancers are genetically mutated in p53 [29,82-85], and the other 50% show attenuated or abrogated p53 activity through means other than mutation [86]. In the case of ATLL, the frequency of p53 gene deletion and mutation is lower than in many other types of cancers and has been reported to approximate 15% [54]. Indeed, our own anecdotal findings are consistent with this low prevalence; in a recent survey of 7 primary ATLL cells, we found no evidence for any of the 11 most frequent p53 somatic gene mutations that have been described for lymphoid neoplasms (Zane, data not shown).

Cancers that retain wild-type p53 gene, nevertheless, can have attenuated p53 activity *via* other mechanisms. For example, Mdm2, an E3 ubiquitin ligase that promotes p53 degradation, is a major negative regulator of p53 [87-89]. Another example of negative regulation arises from the Twist1 protein. Twist1 accumulates in sarcomas that are genotypically p53 wild-type; it dysregulates p53 phosphorylation promoting its degradation [90]. Additional examples come from DNA tumor