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THE ROLE OF SMALL NON-CODING RNAs IN HUMAN T-CELL LEUKEMIA VIRUS TYPE 1 (HTLV-1) INFECTION AND TRANSFORMATION

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PH.D. THESIS

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LIST OF ABBREVIATIONS

4-1BB	tumour necrosis factor receptor superfamily, member 9
ACTB	beta-actin
ATLL	adult T-cell leukaemia/ lymphoma
Bcl-2	B-cell lymphoma 2
bp	base pairs
CDK4	Cyclin-Dependent Kinase 4
CDKN1A	Cyclin-Dependent Kinase Inhibitor 1A
cDNA	complementary DNA
CREB	cAMP response element-binding
DLL-1	delta-like 1
DMSO	dimethylsulfoxide
DTT	dithiothreitol
EBV	Epstein-Barr virus
FACS	fluorescence-activated cell sorter
FAM	6-carboxyfluorescein
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
HAM/TSP	HTLV-1-associated myelopathy/tropical spastic paraparesis
HCl	hydrochloric acid
HTLV-1	human T cell leukemia virus type 1
IκB	inhibitor of NF-κB
IKK	IκB kinase
IKKα/β/γ	components of IKK (IκB kinase) complex
IL-2	interleukin 2
JAG1	jagged 1
KSHV	Kaposi's sarcoma-associated herpesvirus
LTR	long terminal repeat
Mdm2	Mouse Double Minute 2
mRNA	messenger RNA
miRNA	micro-RNA
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
nt	nucleotide
OX40	tumour necrosis factor receptor superfamily, member 4
OX40L	tumor necrosis factor (ligand) superfamily, member 4
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PMA	phorbol myristate acetate
RelA	NF-κB, p65 subunit
rev	reverse
RISC	RNA-induced silencing complex
RNAi	RNA interference
RPMI	Rosewell Park Memorial Institute 1640 medium
RT-PCR	reverse transcriptase PCR
SDS	sodium dodecyl sulfate
shRNA	small hairpin RNA

siRNA	small interfering RNA
SIRT1	Sirtuin 1
sncRNA	small non-coding RNA
SP1	Specificity Protein 1
SRF	serum response factor
T reg	regulatory T cell
TP53INP1	Tumor Protein P53 Inducible Nuclear Protein 1
tRF	tRNA fragment
TAMRA	tetramethylrhodamine
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
U	unit
VEGF-α	vascular endothelial growth factor alpha
YY1	Yin Yang 1

ABSTRACT

Human T-cell leukemia virus type 1 (HTLV-1) is the causative agent of two distinct pathologies, adult T-cell leukemia/lymphoma (ATLL), an aggressive neoplasm of mature CD4+ T-cells, and tropical spastic paraparesis/HTLV-1-associated myelopathy (TSP/HAM), a demyelinating neurodegenerative disease. The emerging importance of small noncoding RNAs in normal cell physiology and disease has prompted studies of their role in T-cell activation and transformation. The work described in the present thesis was aimed at understanding the role of small noncoding RNAs, in particular microRNAs and tRNA fragments (tRFs), in HTLV-1 infection and ATLL pathogenesis.

The laboratory generated small RNA libraries to identify the repertoire of small noncoding RNAs expressed in two HTLV-1-infected T-cell lines (C91PL and MT-2) compared to normal CD4+ T-cells. Results revealed upregulation of miR-34a in the cell lines. Many tRFs were identified in both uninfected and infected cells. One of the most abundant tRFs (tRF-3019) was derived from the 3' end of tRNA-proline, which is considered to be the primer for HTLV-1 reverse transcriptase. Results of an *in vitro* reverse transcriptase assay verified that tRF-3019 was capable of priming HTLV-1 reverse transcriptase. Both tRNA-proline and tRF-3019 were detected in HTLV-1 virus particles. tRF-3019 may thus play an important role in HTLV-1 reverse transcription and could represent a target to control HTLV-1 infection.

Data from a microarray-based analysis of microRNA expression in ATLL samples compared to normal CD4+ T-cells revealed 21 downregulated microRNAs and 6 upregulated microRNAs. Upregulated microRNAs included miR-34a, which is a member of the highly conserved miR-34 family that acts as a tumor suppressor induced by p53 in other cell types. However, p53 is known to be functionally inactivated or mutated in ATLL cells and HTLV-1-infected cell lines. Treatment of infected cell lines with nutlin-3a, a drug that restores p53 activity by interfering with MDM2, resulted in an upregulation of miR-34a and strong downregulation of several of its predicted targets. These findings indicate that unblocking the p53 pathway in HTLV-1-infected cells promotes engagement of the miR-34a/mRNA regulatory network.

The final aim of the project was to identify microRNAs regulated by the viral regulatory protein Tax. To this end the HTLV-1-negative T-cell line Jurkat was transfected with a Tax expression plasmid and assayed for changes in mRNA and microRNA expression by quantitative RT-PCR. Results revealed significant alterations in the levels of 7 microRNAs in the presence of Tax. These included let-7g, whose levels were reduced in the Tax-expressing cells. Let-7g was also found to be downregulated in ATLL samples compared to normal CD4 cells analysed by microarrays, suggesting that this microRNA might play a tumor suppressor role in HTLV-1-mediated transformation. Experiments are currently underway to identify targets of let-7g in infected cells using as a starting point 14 genes identified by integrating results from microRNA target prediction programs with expression profiles for microRNAs and mRNAs in ATLL cells vs. CD4 controls.

RIASSUNTO

Il virus T-linfotropico umano di tipo 1 (HTLV-1) è l'agente eziologico della leucemia/linfoma a cellule T dell'adulto (ATLL, Adult T-cell leukemia/lymphoma), un'aggressiva neoplasia a carico dei linfociti T CD4+ maturi, e della paraparesi spastica tropicale/mielopatia associata ad HTLV (TSP/HAM, Tropical spastic paraparesis/HTLV-associated myelopathy), una patologia degenerativa del sistema nervoso centrale.

L'interesse crescente nello studio e nella comprensione della funzione degli "small non-coding RNA" in cellule normali e tumorali ci ha spinto ad uno studio del loro ruolo nell' attivazione e nella trasformazione delle cellule T. Il lavoro descritto nella presente tesi mira a comprendere il ruolo degli "small non-coding RNA" (sncRNA), in particolare microRNA e frammenti tRNA (tRFs), nell' infezione da HTLV-1 e nella patogenesi dell'ATLL.

Nel nostro laboratorio sono state generate librerie di "small RNA" per identificare il repertorio di sncRNA espressi in due linee cellulari infettate con HTLV-1 (C91PL e MT-2) rispetto alle cellule T CD4 + normali. I risultati hanno rivelato un'aumentata espressione del miR-34a nelle linee cellulari infettate. Molti frammenti di tRNA (tRFs) sono stati identificati sia nelle cellule infettate che non infettate. Uno dei tRFs più abbondanti (tRF-3019) è derivato dall' estremità 3' del tRNA-prolina, che è considerato il primer per la trascrittasi inversa dell'HTLV-1. I risultati ottenuti da un saggio di trascrittasi inversa *in vitro* hanno dimostrato che il tRF-3019 è in grado di funzionare da primer nella trascrizione inversa di HTLV-1. La presenza sia del tRNA-prolina che del tRF-3019 è stata evidenziata nelle particelle virali. Il tRF-3019 potrebbe quindi svolgere un ruolo importante nella retrotrascrizione del virus e potrebbe rappresentare un "target" terapeutico nell'infezione da HTLV-1.

I dati ottenuti dall' analisi con microarray sull' espressione di microRNA in campioni di ATLL e in campioni di cellule T-CD4 + normali ha rivelato una diminuzione nell'espressione di 21 microRNA e un'aumentata espressione di 6 microRNA.

I microRNA sovraespressi comprendono anche il miR-34a, che è un membro della famiglia dei miR-34, altamente conservati, che agiscono come oncosoppressori indotti da p53 in diversi tipi cellulari. Tuttavia, p53 è inattiva o mutata in cellule ATLL e in linee cellulari HTLV-1-infettate. Il trattamento di linee cellulari infettate con Nutlin-3a, un farmaco che ripristina l'attività di p53 legandosi a MDM2, ha rivelato un aumeto di espressione di miR-34a e una forte riduzione dell'espressione di alcuni dei suoi target. Questi risultati suggeriscono che attivando il pathway di p53 in cellule HTLV-1-infettate si potrebbe promuovere l'ingaggio del network regolatorio del miR-34a.

Infine, ci siamo proposti di identificare i microRNA regolati dalla proteina virale Tax. A tal fine la linea cellulare T non infetta, Jurkat, è stata transfettata con un plasmide di espressione per Tax e sono state testate le variazioni di espressione di mRNA e microRNA mediante RT-PCR. I risultati hanno rivelato che in presenza di Tax ci sono alterazioni significative nei livelli di espressione di 7 microRNA. Queste variazioni includono il microRNA let-7g, i cui livelli sono ridotti nelle cellule che esprimono Tax. Da studi effettuati su microrrays, let-7g risulta sottoespresso in campioni ATLL rispetto alle cellule CD4 normali, suggerendo che questo microRNA potrebbe svolgere un ruolo di oncosoppressore nella trasformazione mediata da HTLV-1. Gli esperimenti, attualmente in corso, permetteranno di identificare i target di let-7g in cellule infettate utilizzando come punto di partenza 14 geni ottenuti dall'integrazione dei risultati dei programmi di predizione dei target dei microRNA con i profili di espressione di microRNA e mRNA in cellule ATLL rispetto ai controlli CD4.

1 INTRODUCTION

1.1 Human T-cell leukemia virus type 1: taxonomy, epidemiology and pathogenesis

Human T-cell leukemia virus type 1 (HTLV-1) was the first human retrovirus to be identified and acknowledged with oncogenic properties (Poiesz et al., 1980). Approximately 20 million people are infected worldwide, with geographical prevalence especially in regions of south-western Japan, Central Africa, the Caribbean Basin, Central and South America and the Melanesian Islands (Figure 1). Sporadic infection occurs in Europe and North America. Transmission of the virus may occur in a "vertical" manner from mother to newborn (e.g. mainly through breastfeeding and in few cases during gestation or peripartum), or "horizontally" through exchange of biological fluids (e.g. sexual contact and parenteral transmission) (Proietti et al., 2005; Goncalves et al., 2010).

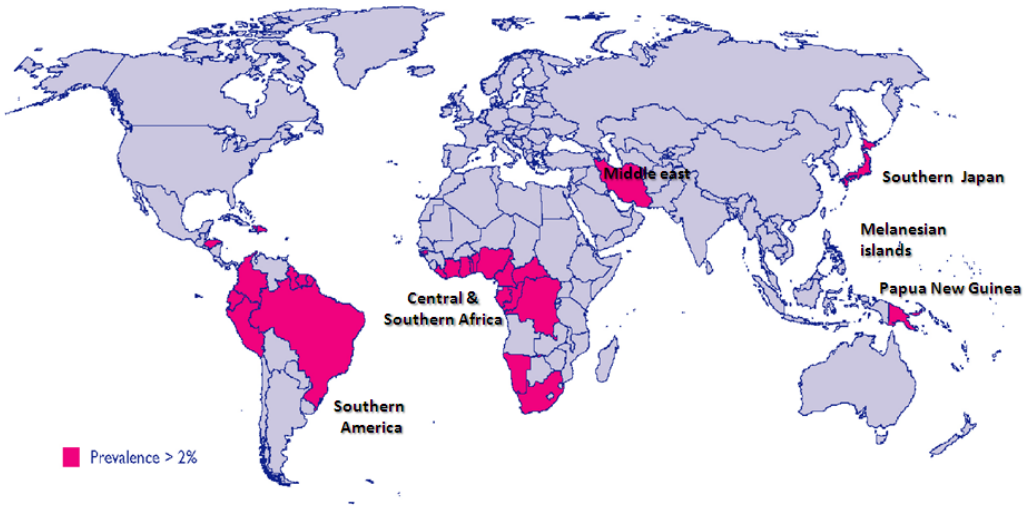


Figure 1. Worldwide prevalence of HTLV-1 (modified Proietti et al., 2005).

HTLV-1 is a member of the Retroviridae family, Oncovirinae sub-family, Deltaretrovirus genus, which also includes HTLV-2, -3, -4, the simian T-lymphotropic viruses (STLVs), and bovine leukemia virus (BLV). STLV and BLV infections are associated with neoplastic diseases, while the pathogenicity of HTLV-2, -3, -4 has not been clearly established (Araujo et al., 2004; Mahieux et al., 2009).

Deltaretroviruses are considered to be "complex"retroviruses, as their genomes contain extra open reading frames (ORFs) in addition to the gag, pol, pro and env genes common to all retroviruses (Cavallari et al., 2011). In the case of HTLV-1, the extra ORFs code for a transcriptional activator named Tax, a post-transcriptional regulatory protein named Rex and four accessory proteins named HBZ, p30, p13 and p12/p8 (Lairmore et al., 2011).

HTLV-1 is the etiologic agent of two pathologies, adult T-cell leukemia/lymphoma (ATLL), an aggressive malignancy of mature CD4+ T-cells that is extremely refractory to current therapies (Uchiyama et al., 1977; Tsukasaki et al., 2009), and tropical spastic paraparesis/HTLV-1-associated myelopathy (TSP/HAM), a demyelinating neurodegenerative disease (Gessain et al., 1985; Osame et al., 1986). In addition, accumulating evidence supports an association between HTLV-1 infection and a number of chronic inflammatory diseases such as uveitis, arthropathy and infective dermatitis.

Most HTLV-1-infected individuals remain asymptomatic throughout life; only 2-5% develop ATLL or TSP/HAM after a latency period of decades or several years, respectively.

ATLL is classified into four clinical forms: acute, chronic, smouldering and lymphoma (Tsukasaki et al., 2009). The prognosis of acute ATLL is extremely poor with an overall survival of a few months. ATLL cells possess multi-lobulated nuclei and are called “flower cells” (Figure 2); they are usually CD3+ CD4+ CD8- CD25+ and frequently accumulate in peripheral blood as well as in lymphoid organs and skin (Matsuoka, 2005). ATLL cells express very little, if any, viral protein and frequently carry defective proviral copies integrated in the host genome.

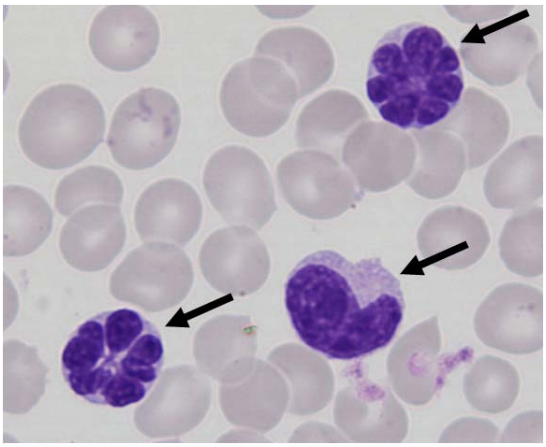


Figure 2. Typical "flower cell" in the peripheral blood of an acute ATLL patient (from Matsuoka, 2005).

HTLV-1 infection is accompanied by a high frequency of Forkhead Box P3 positive (FoxP3+) T-cells (Kohno et al., 2005; Chen et al., 2006). FoxP3 is a marker of regulatory T cells (Treg), which play a critical role in suppressing the immune response. The increased frequency of FoxP3+ cells results from the HTLV-1-mediated expression of the chemokine CCL22, which binds the CCR4 receptor on Treg cells, favouring their migration and survival. The FoxP3+ T cells suppress the growth of autologous ATLL clones, retarding the progression of ATLL; on the other hand, they suppress the host's CTL response, which normally limits HTLV-1

replication and reduces the risk of HTLV-1-associated diseases (Toulza et al., 2010; Bangham et al., 2011).

TSP/HAM is characterized by a slowly progressive spastic paraparesis, associated with bladder dysfunction and sensory disorders. Parenchymal and perivascular infiltration of mononuclear cells occurs in the white and gray matter of the spinal cord, resulting in demyelization and fibrosis. The presence of infiltrating T-cells in the spinal cord lesions and of Tax-specific CTL in the cerebrospinal fluid and in the peripheral blood suggests that TSP/HAM might have an autoimmune basis. This hypothesis is consistent with the association between the human leukocyte antigen (HLA) haplotype and the risk of developing TSP/HAM (Jeffery et al., 1999; Barmak et al., 2003).

1.2 Infection and virus propagation

The HTLV-1 virion consists of a core that contains the viral-encoded enzymes reverse transcriptase, integrase and protease, the single-stranded diploid RNA genome surrounded by capsid and matrix proteins. A lipoproteic envelope, composed of a plasma membrane-derived lipid bilayer and the gp21 and gp46 envelope glycoproteins, surrounds the viral core (Figure 3). HTLV-1 presents a broad cell tropism in vitro (monocytes, microglial cells, epithelial cells, B- and T-lymphocytes), but it is mainly detected in CD4+ T-lymphocytes and dendritic cells of infected individuals. So far, three cellular receptors of HTLV-1 have been identified: the glucose transporter GLUT-1, heparan sulphate proteoglycan (HSPG), and neuropilin-1. Current models suggest that the virus may first contact HSPG on the cell surface, and then form complexes with neuropilin-1 through the viral envelope protein (Jones et al., 2005; Lambert et al., 2009). Afterwards, the

interaction between the viral envelope protein gp46 and GLUT-1 would favour membrane fusion and entry into the cell (Manel et al., 2003). The gp46-GLUT-1 interaction allows the envelope protein gp21 to mediate cellular membrane fusion with the formation of the virological synapse. The virological synapse is an organized contact area whose assembly results from the polarization of the cytoskeleton of the infected cell and the accumulation of HTLV-1 core complexes and genome at the cell junction (Ikagura et al., 2003; Majorovits et al., 2008).

In addition to the formation of the virological synapse, two other mechanisms have been proposed to be involved in the cell-to-cell transmission of HTLV-1. The first involves the storage of viral particles from HTLV-1-infected cells in extracellular biofilm-like structures, composed of collagen, agrin, and linker-proteins. When infected cells attach to uninfected cells, those structures are rapidly transferred to the surface of the target cells, favouring infection (Pais-Correia et al., 2010). Another mechanism of virus spread involves the activity of the viral accessory protein p8. p8 enhances T-cell contact by interacting with LFA-1 and ICAM-1 and mediates formation of intracellular conduits among T-cells, through which virions may be transmitted (Van Prooyen et al., 2010). All these mechanisms are consistent with the fact that cell-free HTLV-1 particles are usually undetectable in the serum of HTLV-1 infected subjects and cell-free blood products are not infectious (Fan et al., 1992; Derse et al., 2001).

After virus entry into the target cell, the viral genome is reverse-transcribed by the viral reverse transcriptase (RT), producing an RNA-DNA hybrid. The ribonuclease H (RNAse H) component of viral RT degrades the RNA strand, while the DNA strand is used as a template by RT, which also has DNA-polymerase-DNA-

dependent activity, to synthesize a complementary DNA strand. The double-stranded DNA circularizes and transfers to the nucleus, where it integrates randomly in the host genome. Integration is mediated by the viral enzyme integrase and by the long terminal repeats (LTRs) located at both ends of the viral genome. Viral genes are then transcribed and translated by the cellular machinery. Virion assembly occurs through the interactions between the nucleocapsid and the genomic RNA, and the matrix and the host plasma membrane. Viral particles incorporate two copies of the single stranded RNA genome along with tRNA, RT, protease and integrase.

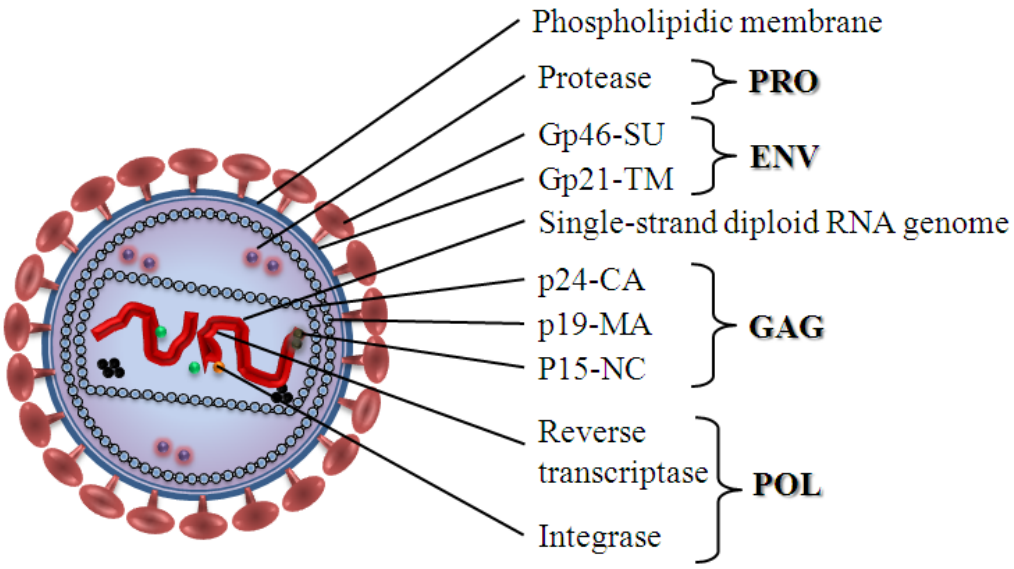


Figure 3. Schematic representation of the HTLV-1 virion (modified from Le Blanc et al., 2001)

1.2.1 Propagation of HTLV-1 *in vivo*

After an individual has been infected with HTLV-1, the propagation and persistence of the infected cells in the host relies mainly on "mitotic transmission" of the integrated viral genome to daughter cells, rather than on de novo infection of new host cells (Overbaugh et al., 2001).

1.2.2 *In vitro* infection of T-cells by HTLV-1

Many studies of HTLV-1 employ T-cell lines that are chronically infected with the virus. While some cell lines were derived by direct culture of PBMC from patients with ATLL or TSP/HAM, many others were generated by cocultivating normal mitogen-stimulated PBMC or umbilical cord blood cells with cells from infected patients; the donor cells are exposed to a lethal dose of gamma irradiation or are treated with a cytotoxic drug to ensure that they will not propagate in the co-culture. The newly infected cells are initially dependent on exogenous IL-2 for growth. After several months of culture, it is possible to detect mono- or oligoclonal provirus integration in the recipient cells. This profile results from a selection process of one or few major clones that carry several genetic alterations and acquire the capability to grow in an interleukin-2 (IL-2)-independent manner. These cells usually show a CD3+ CD4+ IL-2R+ (IL-2 receptor, CD25), or, rarely, a CD3+ CD8+ IL-2R+ phenotype (Lairmore et al., 2007).

1.3 HTLV-1 genetic organization and gene expression

The genome of HTLV-1 reflects the basic structure of the Deltaretrovirus genus: at the 5' and 3' ends are located the LTRs which flank the partially overlapping open reading frames (ORFs) of the gag, pro, pol and env genes that code for enzymes and structural proteins of mature virus particles (Figure 4). The region between the end of the env gene and the 3' LTR is termed the X region, and contains at least four partially overlapping ORFs, termed x-I through x-IV, coding for regulatory and accessory proteins (Figure 4A). The minus strand of HTLV-1 also contains an ORF located in the pX region (antisense orientation) (Larocca et al.,

1989) which codes for the HBZ protein (HTLV-1 bZIP factor) (Figure 4B) (Gaudray et al., 2002).

Expression of the highly condensed HTLV-1 genetic information is achieved through (i) ribosomal frameshifting, which generates a Gag-Pro-Pol polyprotein from the full-length transcript; (ii) alternative splicing, which produces distinct mRNAs coding for the Env and pX region genes; (iii) polycistronic translation, which produces the Tax and Rex proteins from the same mRNA; and (iv) minus-strand transcription, to produce HBZ.

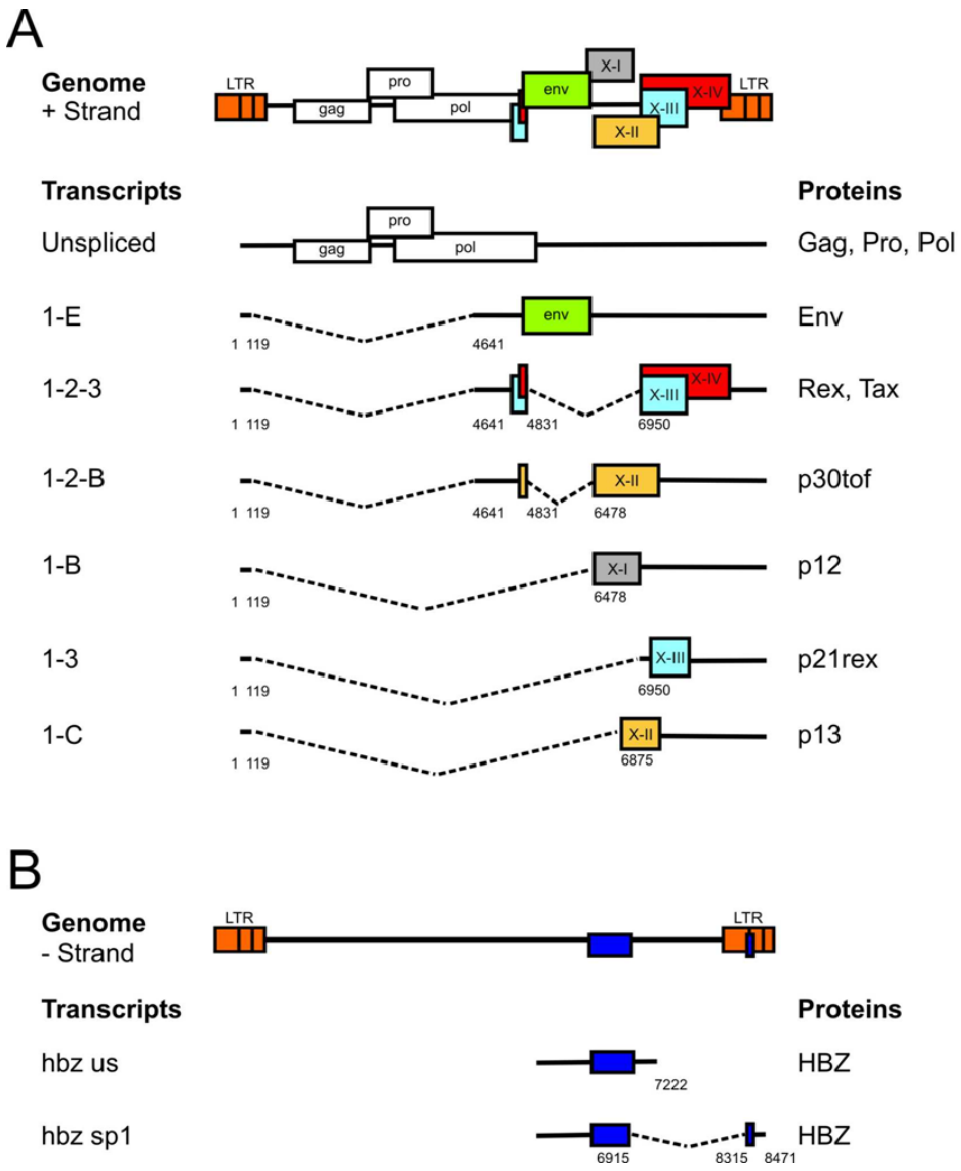


Figure 4. Organization and expression of the HTLV-1 genome. A: Plus-strand ORFs, transcriptional map and proteins coded by each mRNA are shown. The numbering indicates splicing sites used for the generation of the mature mRNAs. Resulting exons are: 1 (1-119), 2 (4641-4831), 3 (6950-8493), B (6478-8493), C (6875-8493) and E (4641-8493). mRNAs are named according to their exonic composition. B: For the minus-strand, the ORF, transcriptional map and proteins coded by each mRNA are shown. The numbering indicates the start sites used for the generation of the mature mRNAs. Resulting exons are: hbz us (7222-4834) and hbz sp1 (8471-8315 and 6915-4834) (Adapted from Rende et al., 2011).

1.3.1 Gag-Pro-Pol.

An unspliced mRNA codes for Gag-Pro-Pol and also serves as genomic RNA. The gag gene codes for the 19 kDa matrix (MA), 24 kDa capsid (CA) and 15 kDa nucleocapsid (NC) structural proteins. The pro gene encodes the viral protease. The 5' portion of the pol gene encodes the reverse transcriptase (RT) protein, while sequences downstream code for Integrase. These genes are translated as polyproteic precursors (Gag, Gag-Pro and Gag-Pro-Pol) generated through ribosomal frameshifting at the gag-pro and/or gag-pro-pol junction. The precursors are post-translationally modified by myristylation at the N-terminus, an essential step for their insertion in the internal side of the plasma membrane of the infected cell. After anchoring to the plasma membrane the precursors are cleaved by the viral protease to generate the single mature polypeptides.

1.3.2 Env.

A singly-spliced mRNA contains the env gene. It codes for a 68-kDa precursor which is post-translationally modified by glycosylation and cleavage into two proteins named gp46-SU, localized at the surface of virions and responsible for the binding to the GLUT-1 receptor, and gp21-TM, the transmembrane protein that mediates membrane fusion and formation of the virological synapse.

1.3.3 Tax

A doubly-spliced mRNA codes for two essential regulatory proteins, Tax and Rex. Tax is a 353-amino acid (40-kDa), mainly nuclear, phosphoprotein that transcriptionally controls the expression of plus strand viral genes and a large number of cellular genes. Functional domains in Tax include an N-terminal nuclear

localization signal (NLS) and zinc finger domains that mediate binding to transcription factors (CREB/ATF and SRF; see below), a central domain and C-terminal domain that allow binding to transcriptional coactivators CBP/p300 and P/CAF, respectively, and a central dimerization domain (reviewed by Romanelli et al., 2013).

1.3.3.1 Effects of Tax on the CREB pathway

Tax was initially described as an activator of LTR-directed transcription (Felber et al., 1985). Three Tax responsive elements (TRE), within the U3 region of the LTR, are sufficient to confer Tax responsiveness (Brady et al., 1987). Each element contains an octamer motif TGACG(T/A)(C/G)(T/A) flanked by a GC stretch at the 5' and 3' ends (Jeang et al., 1988). The octamer motif shares homology with the consensus cAMP responsive element (CRE) (5'-TGACGTCA-3'). Tax binds indirectly to the TRE element by interacting with members of the CREB/ATF family (Giam et al., 1989). Tax enhances the dimerization of CREB/ATF factors, increasing their affinity for the TRE, and further stabilizes the ternary complex through direct contact of the GC-rich flanking sequences (Kimzey et al., 1998; Lundblad et al., 1998). Tax also recruits coactivators (CBP/p300 and P/CAF) to facilitate transcription initiation. Physiologically, the CREB-CBP/p300 interaction is controlled by CREB phosphorylation in response to different signal transduction pathways. Through its ability to bind both CREB and CBP/p300, Tax triggers CREB activation even in the absence of phosphorylation. In this way, viral gene transcription becomes independent from cellular signals.

Through its interactions with CREB/ATF proteins, Tax can activate a variety of cellular genes, including interleukin 17 (IL-17) and c-fos (Alexandre et al., 1991;

Dodon et al., 2004) and repress the expression of other genes such as cyclin A, p53 and c-myb (Mulloy et al., 1998; Nicot et al., 2000; Kibler et al., 2001).

1.3.3.2 Effects of Tax on the NF-κB pathway

The oncogenic properties of Tax are due in large part to its ability to activate NF-κB, a major survival pathway engaged by HTLV-1 infection (Saggioro et al., 2009). The mammalian NF-κB proteins consist of five structurally related members: p65/RelA, RelB, c-Rel, NF-κB1 (p50/and its precursor p105) and NF-κB2 (p52/and its precursor p100). NF-κB proteins form homo-heterodimers that regulate the expression of target genes bearing a NF-κB-responsive element in their promoters (Hayden et al., 2012). In unstimulated cells, NF-κB dimers are sequestered in the cytoplasm by inhibitory proteins called IκBs (comprising p105, p100, IκBα, IκBβ, IκBγ, IκBζ and Bcl-3) that mask the nuclear localization signal of NF-κB. Upon cell stimulation, IκB proteins are rapidly phosphorylated and degraded by the proteasome, and NF-κB translocates into the nucleus to regulate the expression of target genes coding for cytokines, chemokines, adhesion molecules, inhibitors of apoptosis, and other proteins (Hayden et al., 2012).

Two main signalling pathways lead to NF-κB activation: the canonical (or classical) and the non-canonical (or alternative) pathways. The canonical NF-κB pathway is induced by a variety of innate and adaptive immunity mediators, such as pro-inflammatory cytokines (TNF-α, IL-1β), and engagement of Toll-like receptors (TLRs) and antigen receptors (TCR, BCR) (Pahl, 1999; Bonizzi et al., 2004). The crucial step in the canonical NF-κB pathway is the activation of the IκB-kinase (IKK) complex, which consists of the two kinases, IKKα and IKKβ (Zandi et al., 1997), and of one regulatory subunit IKKγ, also known as NF-κB essential

modulator (NEMO) (Yamaoka et al., 1998) (Figure 5A). Activated IKK phosphorylates IκBα on serines 32 and 36, triggering its ubiquitination and proteasomal degradation. This unmasks the DNA binding activity of the p50/RelA dimer, and allows its translocation into the nucleus where it activates the transcription of target genes (Beinke et al., 2004) (Figure 5A).

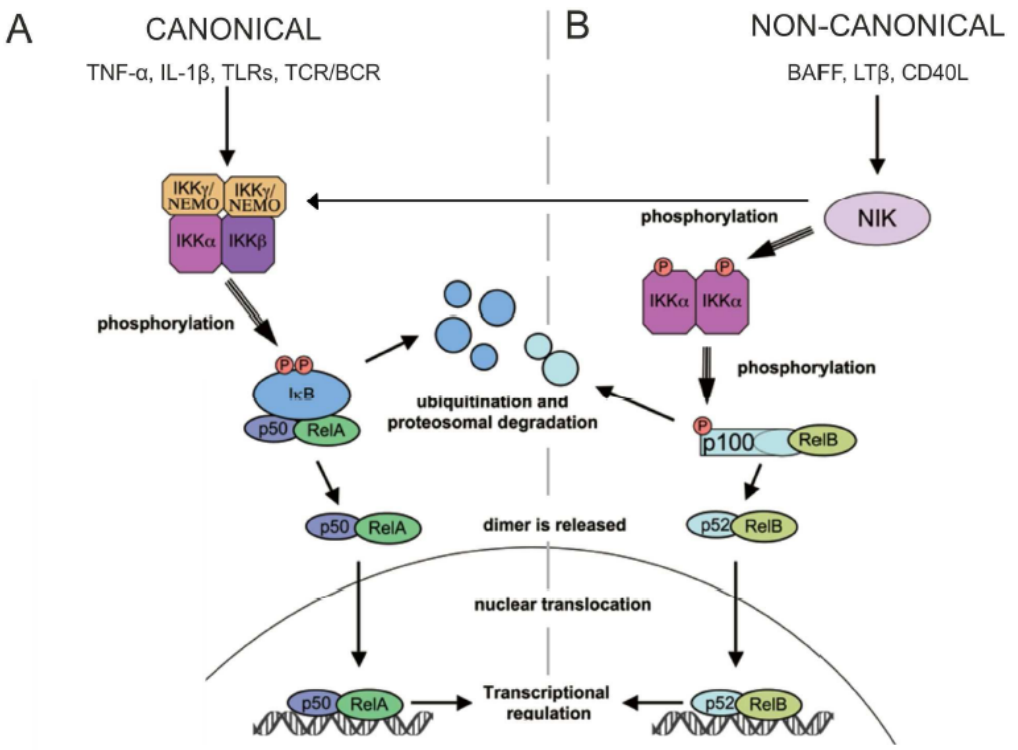


Figure 5. NF-κB activation. The figure summarizes the steps involved in activation of the canonical and non-canonical pathways (see text). (Adapted from Morgan et al., 2011).

Tax intervenes at multiple levels to activate the canonical NF-κB pathway. In the cytoplasm, Tax directly binds to NEMO and recruits the IKK complex to the perinuclear compartment, where it is phosphorylated and activated (Harhaj et al, 1999; Xiao et al., 2000). Tax also activates kinases upstream to the IKK complex, including MAPK/ERK kinase kinase 1 (MEKK1) and TGF-β activating kinase 1 (TAK1) (Wu et al., 2007), thus enhancing IKKα and IKKβ phosphorylation, and

IκBα and IκBβ degradation (Harhaj et al., 1999). In addition, Tax binds IKKα and IKKβ and activates their kinase activity independently of the upstream kinases (Chu et al., 1998). The binding of Tax to IκBs also enhances their degradation independently of IKK phosphorylation (Suzuki et al., 1995). Tax thus promotes IκB degradation at multiple levels, allowing nuclear translocation of NF-κB independently of external stimuli. In the nucleus, Tax recruits RelA, CBP/p300 and PCAF (Bex et al., 1998) into discrete transcriptional hot spots termed Tax nuclear bodies, leading to NF-κB transcriptional activation (Semmes et al, 1996; Bex et al., 1997).

The non-canonical NF-κB pathway is important for secondary lymphoid organ development and homeostasis. It is induced by B-cell activating factor (BAFF) (Claudio et al., 2002), lymphotoxin β (LTβ) (Dejardin et al., 2002), and CD40 ligand (Xiao et al., 2001a; Coope et al., 2002). The crucial step in this pathway is the processing of p100 into p52 by IKKα, thus allowing p52 DNA binding in association with its partner RelB. The phosphorylation and activation of IKKα is mediated by the upstream NF-κB inducing kinase (NIK) (Figure 5B) (Xiao et al., 2001b; Xiao et al., 2004). Interestingly, NIK can also induce long-term activation of the IKK complex and IκBα degradation, thus activating also the canonical NF-κB pathway (Zarnegar et al., 2008) (Figure 5A and B).

The induction of the non-canonical pathway is a hallmark of NF-κB activation by HTLV-1 infection, because this arm of the pathway usually is not active in normal T cells (Xiao et al., 2001a). Tax induces the non-canonical NF-κB pathway by promoting the processing of p100 to p52 (Xiao et al., 2006). Tax-mediated processing of p100 requires NEMO as an adaptor protein in the assembly

of the Tax/IKK complex, and is independent of the NIK kinase. In contrast to the canonical Tax/NEMO/IKK complex, which contains both the IKKα and β, the non-canonical complex contains only IKKα (Xiao et al., 2001b), which phosphorylates p100 leading to its processing to p52 (Qu et al., 2004).

Almost all steps of the NF-κB pathway can be terminated through feedback inhibition mechanisms. Among the physiological NF-κB termination mechanisms, the most rapid and essential is mediated by PDZ-LIM domain-containing protein 2 (PDLIM2). This protein shuttles RelA to the nuclear matrix, and ubiquitinates it, thus targeting it for proteasomal degradation (Tanaka et al., 2007). Tax directly shuts off this feedback inhibition mechanism by binding to PDLIM2, resulting in reduced RelA degradation, although this process results in the proteasomal degradation of Tax itself (Yan et al., 2009).

Tax–mediated activation of the NF-κB pathway results in increased survival through the transcriptional activation of the anti-apoptotic factors Bcl-XL, Bfl1 and HIAP-1 (Kawakami et al., 1999, Tsukahara et al., 1999; Nicot et al., 2000; De La Fuente et al., 2003) and downregulation of the pro-apoptotic protein Bax (Brauweiler et al., 1997). In addition, Tax induces a physical interaction between RelA and p53 that inhibits p53 transcriptional activity (Jeong et al., 2004). Furthermore, Tax represses p53 at the protein level via NF-κB using two different mechanisms: (i) activated IKK directly phosphorylates p53 to trigger p53 ubiquitination and proteasomal degradation by the b-TrCP ubiquitin ligase (Xia et al., 2009); (ii) activated NF-κB induces expression of MDM2, a ubiquitin ligase for p53 ubiquitination and degradation (Busuttil et al., 2010).

1.3.3.3 Effects of Tax on the SRF/AP-1 pathway

Tax increases the expression of the transcription factor AP-1 (activator protein -1) a homo- or heterodimeric complex of Fos (c-Fos, FosB, Fra1 and Fra2) and Jun (c-Jun, JunB and JunD) (Fujii et al., 1991; Fujii et al., 2000). Fos and Jun transcription is positively regulated by the serum responsive factor (SRF) in response to various stimuli such as cytokines, growth factors, stress signals and oncogenes. SRF binds to the Fos/Jun promoters through two SRF responsive elements (SRE): a CArG box (CC(A/T)6GG) and an upstream Ets box (GGA(A/T)). Once SRF occupies the CArG box, the ternary complex factor (TCF) establishes protein interactions with SRF and subsequently binds the upstream Ets site. This complex then recruits the co-activators P/CAF and CBP/p300 to activate transcription.

Tax activates the transcription of promoters under the control of SRE motifs through the interaction with transcription factors associated with the SRF pathway (Fujii et al., 1991; Alexandre et al., 1991). This interaction results in increased binding of SRF to the SRE (Dittmer et al., 1997). Once the complexes are stabilized, Tax recruits the coactivators CBP/p300 and P/CAF and mediates transactivation (Shuh and Derse, 2000).

1.3.3.4 Non-transcriptional effects of Tax

Tax also controls the cell cycle by interacting with cyclins-D1, -D2 and -D3 as well as with cyclin-dependent kinases (CDKs) 4 and 6 (Neuveut et al., 1998; Haller et al., 2002). Through these interactions, Tax stabilizes the cyclin D2/CDK4 complex and enhances its kinase activity, leading to hyperphosphorylation of the retinoblastoma protein (Rb). Tax also associates with the CDK inhibitors (CDKI)

p15INK4b and p16INK4a and counteracts their CDK4-inhibitory activity (Suzuki et al., 1997; Suzuki et al., 1999). In addition, Tax binds to Rb and enhances its proteosomal degradation (Kehn et al., 2005).

Tax also interacts with Tax1 binding protein (TAX1BP2) and Ran/Ran binding protein 1 (RanBP1), which control centrosome amplification during mitosis (Peloponese et al., 2005; Ching et al., 2006). Through these interactions, Tax induces supernumerary centrosomes and causes multipolar mitosis, thus contributing to aneuploidy, a hallmark of HTLV-1-infected cells (Marriott et al., 2002; Boxus et al., 2009). Furthermore, Tax interacts with the anaphase promoting complex (APC), which controls the metaphase-anaphase transition (Liu et al., 2005). APC directs the ubiquitination and proteosomal degradation of cyclin B1 and Pds1p/securin. Securin and cyclin B1 inhibit separase; a protease that destroys the connection links of sister chromatids. In normal cells, chromosomes start to segregate only after the kinetochore is subjected to the mechanical tension generated by the mitotic spindle (Nasmyth, 2005). Tax activates APC in the S phase, before the cell enters mitosis, decreasing the levels of cyclin B1 and securin, and resulting in the premature activation of separase, thus leading to unequal chromosomal separation between cells (Liu et al., 2005).

Tax also causes DNA damage generating double strand breaks both by modulating the timing of activation of the replication origins (Boxus et al., 2012) and by inducing oxidative stress (Kinjo et al., 2010). In addition, Tax attenuates the DNA damage response through sequestration and/or inhibition of critical proteins such as ATM, DNA-PK, CHK1-2 and p53 (Boxus et al., 2012).

Although Tax is a major target of CTLs, ATLL cells frequently lose the expression of Tax. Therefore, Tax is proposed to play an important role in the persistent proliferation of HTLV-1-infected cells mainly during the healthy carrier state. In this phase, the mutator phenotype conferred by Tax promotes accumulation of genetic and epigenetic changes that finally lead to Tax-independent proliferation and, following the silencing of Tax, escape from the host immune system (Yasunaga et al., 2007).

1.3.4 Rex

Rex is a 189-amino acid, 27 kDa nuclear/nucleolar phosphoprotein that is able to shuttle between the nucleus and the cytoplasm (Palmeri et al., 1996; Narayan et al., 2003), allowing the nucleo-cytoplasmic export of incompletely spliced viral RNA, controlling in this way viral gene expression at the post-transcriptional level. This function is mediated through direct interaction with a 254-nucleotide stem-loop cis-acting RNA element termed the Rex-responsive element (RxRE) (Grone et al., 1994), present in the U3/R region of the 3' LTR of all HTLV-1 transcripts (Ahmed at al., 1990; Bogerd et al., 1992). Rex contains an N-terminal NLS that also functions as an RNA binding domain, and a nuclear export signal (NES) flanked by 2 multimerization domains (reviewed by Narayan et al., 2003). The NES interacts with the protein chromosome region maintenance interacting protein 1 (CRM1/exporting 1) and allows export of the Rex-viral mRNA complexes from the nucleus to the cytoplasm (Bogerd et al., 1995). Although Rex is not required for cellular immortalization *in vitro*, it is necessary for infectivity and viral persistence *in vivo* (Ye et al., 2003), since expression of the unspliced and singly-spliced viral RNAs encoding structural proteins is necessary for the assembly of virions. The fact that

these mRNAs depend on Rex for expression suggests that the Rex-RxRE interaction may function as a molecular switch controlling the transition between productive and latent phases of HTLV-1 infection.

1.3.5 Accessory proteins

p21rex. p21rex is a truncated isoform of Rex lacking the N-terminal NLS/RNA binding domain of the full-length protein. p21Rex might act as a repressor of full-length Rex, thereby inhibiting the expression of transcripts coding for structural proteins, enzymes and accessory proteins (Heger et al., 1999). This would favor entry of the virus into latency.

p30tof. p30tof is a 241-amino acid, nucleolar-nuclear non-shuttling protein (Ciminale et al., 1992; D'Agostino et al., 1997). p30tof functions at the post-transcriptional level by inhibiting the nuclear export of the tax/rex mRNA; this effect results in a global inhibition of viral gene expression, suggesting that p30tof might act as a latency factor (Nicot et al., 2004). p30tof also interacts with the RNA-binding domain of Rex and thereby prevents Rex from interacting with the RxRE (Baydoun et al., 2008). p30tof also affects transcription from promoters with cellular CRE and viral TRE sequences by interacting with the co-activator CBP/p300 (Zhang et al., 2000; Zhang et al., 2001), and can disrupt the assembly of the CREB–Tax–p300/CBP complex on TREs. This may result in decreased transcription of the viral genome, thereby facilitating viral latency. By recruiting the co-activator Tat-interacting protein 60 (TIP60) p30tof promotes the formation of the Myc/TIP60 transcription complex on Myc-response E-box elements and thereby transactivates Myc-driven transcription (Awasthi et al., 2005). p30tof expression results in

alteration of the cell cycle events that would promote early viral spread and T cell survival (Datta et al., 2007).

p13. p13 an 87-amino acid protein that corresponds to the C-terminal portion of p30tof. However, the two proteins are expressed from distinct mRNAs (Figure 4) and have different activities. p13 is targeted to the inner mitochondrial membrane and induces specific alterations in mitochondrial morphology (Ciminale et al., 1999; D'Agostino et al., 2002; D'Agostino et al., 2005). Functional studies of p13 revealed that it inhibits proliferation of HeLa cells and Jurkat T cells and sensitizes Jurkat T cells to apoptosis triggered by ceramide and Fas ligand (Silic-Benussi et al., 2004; Hiraragi et al., 2005). p13 also interferes with the ability of HeLa cells and Ras/Myc-transformed primary fibroblasts to grow as tumors in nude mice, suggesting that it may exert tumor-suppressor-like activity (Silic-Benussi et al., 2004). p13 reduces the uptake of calcium into mitochondria (Biasiotto et al., 2010), reduces mitochondrial membrane potential (Biasiotto et al., 2010), induces an influx of potassium ions into the mitochondrial matrix (Silic-Benussi et al., 2010a), and raises the levels of mitochondrial reactive oxygen species, which favors activation of primary T-cells (Silic-Benussi et al., 2010b).

p12. p12 localizes in the endoplasmic reticulum (ER) and in the Golgi apparatus (Koralnik et al., 1993; Ding et al., 2001; Johnson et al., 2001). p12 interacts with the β and γc chains of the interleukin-2 receptor (IL-2R), resulting in reduced surface expression (Mulloy et al., 1996). The binding to the cytoplasmic domain of the β chain, involved in the recruitment of Jak1 and Jak3, determines an increase in the transcriptional activity of STAT-5, providing a proliferative advantage to T cells (Nicot et al., 2001). p12 also sequesters free MHC class I heavy

chains (MHC-I-Hc), preventing their binding to β2-microglobulin, favouring escape from CTL recognition and clearance by the immune system of infected cells (Johnson et al., 2001). Furthermore, p12 causes a reduction in the expression of ICAM-1 and ICAM-2, which mediate adhesion of natural killer (NK) cells to the infected cells, resulting in the protection from NK cell-mediated cytotoxicity (Banerjee et al., 2007). p12 interacts with calreticulin and calnexin (Ding et al., 2001), two ER-resident proteins that regulate Ca++ storage and release, suggesting a p12-mediated Ca++ leakage from the ER (Ding et al., 2002). Moreover, p12 stimulates nuclear factor of activated T-cells (NFAT) (Albrecht et al., 2002), by interacting with calcineurin, a Ca++ -responsive protein phosphatase that controls NFAT activity (Kim et al., 2003). These effects decrease the threshold for T-cell activation (Nicot et al., 2005). A proteolytic cleavage product of p12 named p8 induces the formation of small tubular structures that facilitate cell-to-cell transmission of the virus (Van Prooyen et al., 2010).

1.3.6 HBZ

The HBZ protein contains an N-terminal transcriptional activation domain, a central domain and a C-terminal basic ZIP domain (bZIP) and three NLS (Gaudray et al., 2002; Hivin et al., 2005). HBZ localizes in the nucleus with a speckled pattern and interacts with a number of transcription factors, including CREB-2, p300/CBP, Jun family members, and NF-κB (Matsuoka et al., 2009). Binding of HBZ to JunB and c-Jun decreases their DNA binding activity by preventing their interaction with Fos, leading to repression of the AP-1 complex; on the contrary the interaction of HBZ with Jun-D stimulates its transcriptional activity (Thebault et al., 2004), and results in the activation of JunD-dependent cellular genes including human

telomerase reverse transcriptase (hTERT) (Kuhlmann et al., 2007). HBZ inhibits the classical NF-κB pathway by inhibiting the DNA binding of the NF-κB subunit p65 and by increasing the expression of PDLIM2, the E3 ubiquitin ligase of p65, leading to enhanced ubiquitination and degradation of p65 (Zhao et al., 2009). HBZ expression is associated with proliferation of ATLL cells *in vivo* and *in vitro* (Satou et al., 2006; Arnold et al., 2008). Mutational analyses of the hbz gene showed that hbz mRNA, rather than HBZ protein, has a growth-promoting effect on T-cells (Satou et al., 2006) possibly by up-regulating transcription of the E2F1 gene and its downstream targets. A major quota of HBZ RNA is retained in the nucleus, supporting a noncoding role (Rende et al., 2011). Transgenic expression of HBZ in mice leads to the development of T-cell lymphomas and systemic inflammatory diseases (Satou et al., 2011).

1.4 MicroRNAs and tRNA fragments (tRFs)

1.4.1 MicroRNAs

MicroRNAs (miRNAs) are single-stranded RNA molecules of about 22 nt which play important roles in regulation of gene expression at the post-transcriptional level by hybridizing to complementary sequences on target transcripts, leading to the silencing of the mRNA's expression (Krol et al., 2010; Libri et al., 2013). miRNAs play vital roles in various physiological and pathological processes, including tumorigenesis (Zhu et al., 2010).

miRNAs are evolutionary conserved across broad phylogenetic distances. Most mammalian miRNAs are encoded by multiple genes (paralogues) with distinct genomic positions, probably the result of gene duplications. As an example,

members of the the let-7 family of miRNAs are coded by 12 different loci in the human genome (Guo et al., 2010). Approximately 50% of mammalian miRNA loci are found in clusters in the genome. Such clusters are generally transcribed as a single polycistronic transcription unit (Lee et al., 2002), although some individual miRNAs are transcribed from separate promoters. Approximately 40% of miRNA loci are located in introns, and about 10% are located in exons of non-coding transcripts (Kim et al., 2009).

The first microRNA was discovered in 1993 in nematodes (Lee et al., 1993; Wightman et al., 1993). Since then, the number of known miRNAs has continually increased (Lau et al., 2001; Lee et al., 2001; Lagos-Quintana et al., 2001). The recent development of deep sequencing technologies (Lu et al., 2005; Margulies et al., 2005) and computational prediction methods (Lai et al., 2003; Nam et al., 2005; Li et al., 2006; Huang et al., 2007) has accelerated the discovery of new small RNA sequences. miRNAs have been identified in protozoa, plants, metazoan animals and viruses. The sequences and genomic locations of the known miRNAs are catalogued in the Sanger miRBase at http://www.mirbase.org/. The current miRBase (miRBase version 20, released in June 2013) contains 2578 human miRNAs. Global miRNA profiling studies (e.g., Landgraf et al., 2007) indicate that some miRNAs are specific for a particular cell lineage or differentiation stage, while others are expressed in many cell types and thus probably play broader roles in cell physiology.

1.4.1.1 Biogenesis of microRNA

Transcription and processing in the nucleus. Most miRNA genes are transcribed by RNA polymerase II (Pol II) (Lee et al., 2004; Cai et al., 2004) and a few are transcribed by Polymerase III (Pol III) (Borchert et al., 2006).

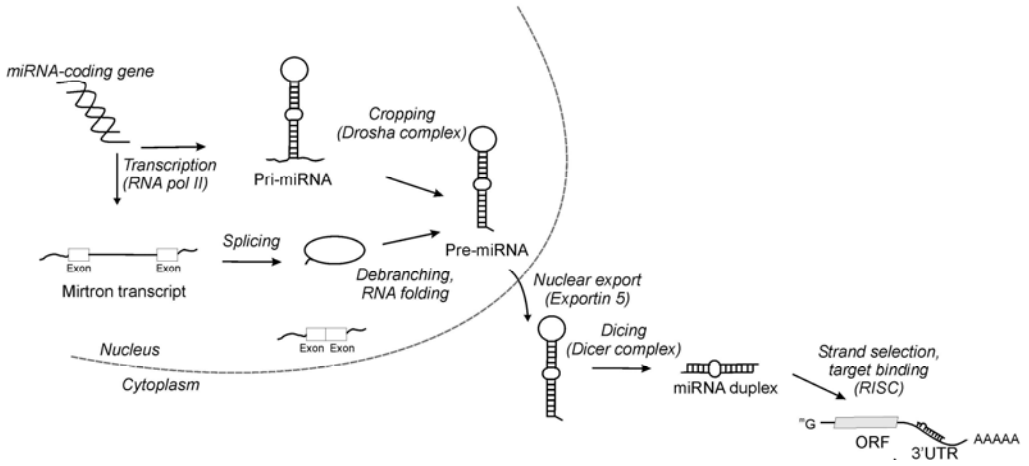


Figure 6. microRNA biogenesis. Shown are the canonical and mirtron pathways of miRNA production from RNA pol II-derived transcripts. As described in the text, other types of RNAs may also give rise to small RNAs with miRNA activity. From D'Agostino et al., 2012.

The primary transcript produced by RNA Pol II, termed the pri-miRNA, is usually several thousand nucleotides long. The mature miRNA sequence is located in the stem portion of a local hairpin structure in the pri-miRNA (Figure 6). The first step of miRNA maturation (termed "cropping") consists of the cleavage at the base of the stem of the hairpin structure by a complex containing the ribonuclease Drosha and a single-stranded RNA binding protein named DGCR8. DGCR8 interacts with the ssRNA tails and the stem of the pri-miRNA, while Drosha cleaves the stem ~11 bp from its base (Han et al., 2006).

Most miRNAs coded within introns are processed before splicing in a co-transcriptional event (Kim et al., 2007). The splicing commitment complex is thought to bind the intron and interact with the Drosha-DGCR8 complex which cleaves the intronic miRNA before the intron is excised. The pre-miRNA possesses a ~2-nt 3′ overhang that is specifically recognized by the nuclear export factor exportin 5 (EXP5) (See below).

A small number of miRNAs are located in short introns and do not require Drosha processing for their biogenesis (Ruby et al., 2007; Okamura et al., 2007; Berezikov et al., 2007) ("mirtrons"; Figure 6). Following splicing, the lariat-shaped intron is debranched and folds into a hairpin structure that resembles pre-miRNA. Some mirtrons contain extended tails at either the 5′ or 3′ end and undergo exonucleolytic trimming in order to become a substrate for nuclear export. In addition, small RNAs can also derive from other non-coding RNAs, such as tRNA (see below) or small nucleolar RNA (snoRNA) (Ender et al., 2008). Multiple non-canonical pathways can therefore generate, through Drosha-independent processes, miRNA precursors that finally enter the common miRNA pathway (Kim et al., 2009).

Nuclear export and cytoplasmic maturation. Exportin 5 is a member of the nuclear transport receptor family that exports pre-miRNAs from the nucleus to the cytoplasm (Kim, 2004; Lund et al., 2004; Bohnsack et al., 2004; Yi et al., 2005). Exportin 5 binds cooperatively to the pre-miRNA and the GTP-bound form of the cofactor Ran in the nucleus, and releases the pre-miRNA in the cytoplasm following the hydrolysis of GTP to GDP. Exportin 5 is able to specifically recognize and bind the characteristic pre-miRNA structure, presenting a >14 bp dsRNA stem with a short 3′ (Gwizdek et al., 2003; Basyuk et al., 2003; Lund et al., 2004; Zeng et al., 2004).

In the cytoplasm, a complex containing a ribonuclease named Dicer cleaves the pre-miRNAs near the terminal loop, releasing a ~22 nt miRNA duplex (Bernstein et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001; Knight et al., 2001) (Figure 6). Similar to Drosha, human Dicer acts in association with the dsRNA-binding proteins TRBP (TAR RNA-binding protein, also known as TARBP2) (Chendrimada

et al., 2005) and PACT (also known as PRKRA) (Lee et al., 2006), which seem to contribute to the formation of the RNA-induced silencing complex (RISC; see below). Some miRNAs (e.g. miR-451) are cleaved from a pre-miRNA by Ago2 rather than Dicer (Cheloufi et al., 2010; Cifuentes et al., 2010).

Argonaute loading. The ~22 nt RNA duplex generated by the action of Dicer is loaded onto the effector complex, RISC (Figure 6). The RISC core contains proteins of the Argonaute (AGO) family. The human AGO family includes 4 members named AGO 1-4, also known as EIf2C1-4. AGO proteins are composed of four domains: the amino-terminal domain; the PAZ domain, which binds the 3′-end of miRNAs; the MID domain, which binds the 5′-phosphate of miRNAs; and the PIWI domain, which adopts an RNase H-like fold and has endonucleolytic activity in some AGOs (Jinek et al., 2009). One strand of the ~22 nt RNA duplex is bound by AGO and is retained in the RISC as a mature miRNA (the guide strand), whereas the other strand (the passenger strand) is degraded. The relative thermodynamic stability of the two ends of the duplex contributes to determine which strand is incorporated into the RISC, with the strand having more unstable base pairs at the 5′ end typically retained while the other strand is degraded. However, the stringency of strand selection may differ for different miRNA duplexes (Khvorova et al., 2003; Han et al., 2006). Together with AGO proteins, in humans Dicer and TRBP (and/or PACT) contribute to effector complex assembly by forming the RISC loading complex (RlC). The RlC seems to bind to RNA duplexes and facilitates mature miRNA loading on AGO. Another important component of the RISC complex is GW182 (glycine-tryptophan protein of 182 kDa), which plays a role in both translational repression and mRNA degradation (see below).

1.4.1.2 MicroRNA function

The majority of animal miRNAs bind to imperfect complementary sequences in the 3′-UTR of target mRNAs, leading to either translation repression or mRNA deadenylation and subsequent degradation.

Silencing of an mRNA target by a miRNA usually requires nearly perfect base-pairing between nt 2-8 of the microRNA (the 'seed sequence') and a segment on the target mRNA (Bartel, 2009; Krol et al., 2010; Zhu et al., 2010); other nucleotides in the miRNA may or may not base-pair with the target. Given its importance in miRNA function, many miRNA target prediction algorithms are based on searches for matches between seed sequences and 3'UTRs.

miRNA-mRNA interactions that involve partial base-pairing typically lead to translational repression of the mRNA. Increasing evidence suggests that translational repression occurs predominantly at the initiation step (Huntzinger et al., 2011). Figure 7A depicts an mRNA that is ready to be translated. The poly(A) tail is bound by poly(A)-binding protein (PABPC), which interacts with eukaryotic translation-initiation factor 4G (eIF4G), which in turn is associated with the cap structure through interaction with the cap-binding protein eIF4e. This interaction closes the mRNA in a loop structure that is efficiently translated and protected from degradation.

GW182 (glycine-tryptophan protein of 182 kDa) contained in RISC is able to bind to PABPC. The interaction of AGO-GW182 with PABPC blocks the formation of the eIF4F complex and the closed conformation of the mRNA, thus inhibiting translation initiation (Figure 7C).

The AGO-GW182 interaction also directs mRNAs to the cellular 5′-to-3′ mRNA decay pathway (Figure 7B). mRNAs are first deadenylated by the CAF1–CCR4–NOT complex, and then decapped by the decapping complex DCP1-DCP2. Finally, decapped mRNAs are degraded by the major cytoplasmic 5′-to-3′ exonuclease XRN1. The relative contributions of translational repression versus mRNA degradation by miRNAs remain to be understood (Filipowicz et al., 2008; Huntzinger et al., 2011; Huntzinger et al., 2013).

When miRNAs bind to target mRNA with nearly perfect sequence complementarity, the mRNA can be cleaved endonucleolytically and degraded by RISC containing AGO 2 (Bartel, 2009; Carthew et al., 2009; Chekulaeva et al., 2009; Fabian et al., 2010). This is the common way of miRNA action in plants but is instead rare in animals. In the case of fully perfect or nearly miRNA-mRNA complementarity, target mRNA cleavage occurs between nucleotides 10 and 11, opposite the miRNA strand, and is catalysed by AGOs. The resulting mRNA fragments are degraded from the newly generated 3′ and 5′ ends.

miRNAs may also exert a repressive function by binding in the 5′ UTRs or coding regions of mRNAs (Lytle et al., 2007). In particular contexts, translational activation (Filipowicz et al., 2008) and heterochromatin formation (Kim et al., 2008) have also been described.

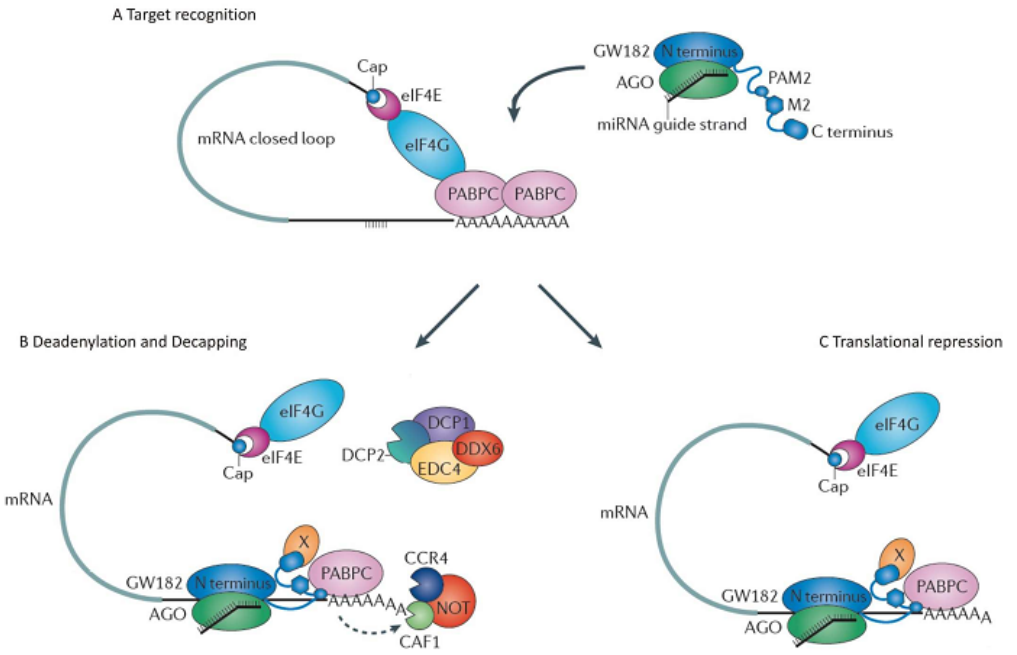


Figure 7. Mechanisms of miRNA-mediated gene silencing in animals. A) Shown on the left is an mRNA that is closed in a loop structure mediated by interactions between eIF4G and PABPC and is ready to be translated. An mRNA that is recognized by a miRNA within a RISC does not form the closed loop due to binding of PABPC to the RISC component GW182 (shown in C). This leads to a block in translation or destruction of the mRNA through deadenylation, decapping and cleavage by the major 5′-to-3′ exonuclease XRN1 (shown in B). From Huntzinger et al., 2011.

miRNA sequences can be subjected to editing carried out by ADARs (adenosine deaminases that act on RNA), which catalyse the conversion of adenosine to inosine in dsRNA, thereby altering the base-pairing and structural properties of target RNA. Both pri-miRNAs and pre-miRNAs can be targeted by ADARs, and the modifications can affect Drosha and Dicer activity and also prevent the export of pre-miRNAs. A differential editing that affects the biogenesis process may in part be responsible for tissue- specific miRNA expression. On the other hand, editing events leading to seed sequence changes can have an important impact on the target specificity of the miRNA (Kawahara et al., 2007; Heale et al., 2009). miRNA

sequences often show heterogeneous ends due to addition or deletion of 1-2 nt (Azuma-Mukai et al., 2008; Seitz et al., 2008). The 3′ ends tend to be much more variable than the 5′ ends because changes in the 5′ terminus result in shifts of the seed sequences, which alter the target specificity of the miRNA. The mechanisms of the variations are unknown but they might be explained by imprecise or alternative processing by RNase III enzymes or by deletions due to exonucleolytic activities. The 3′ ends of miRNA can often present untemplated nucleotides (mostly uracil and adenine), likely added by unknown terminal uridyl/adenyl transferases (Kim et al., 2009).

A single miRNA has the potential to regulate hundreds of different target genes and a gene generally contains several target sites for different miRNAs, thus leading the generation of an extremely complex miRNA regulatory network. Bioinformatic predictions estimated that 60% of all 3'UTRs of human protein-coding genes contain perfect binding sites for miRNA seed sequences (Friedman et al., 2009). Consequently, the unique combination of miRNAs in each cell type regulates the expression of thousands of mRNAs. miRNAs are thus likely to regulate most normal biological processes, including developmental timing, cell differentiation, cell proliferation, cell death, metabolic control, transposon silencing and antimicrobial defense. Not surprisingly, aberrant miRNA expression or function contributes to the pathogenesis of many diseases, including cancer (Sayed et al., 2011).

The first direct evidence for the importance of miRNAs in human cancer came from a study of chronic lymphocytic leukemia (CLL), which revealed a tumor suppressor function for miR-15a and miR-16-1 (Calin et al., 2002). Subsequent

studies have identified many additional miRNAs with oncogenic or tumor suppressor activities in the context of solid and hematopoietic tumors (Croce, 2009).

1.4.1.3 MicroRNAs and viruses

All viruses rely on the host gene expression machinery for their replication and may therefore be affected by the host miRNA network at some level. In turn, viruses have evolved mechanisms that exploit the miRNA network to impinge on host cell turnover and immune defenses to promote expansion and persistence of infected cells (Umbach et al., 2009; Zhuoa et al., 2013). In addition, some viruses express their own miRNAs which in some cases are homologous to host miRNAs. For example, BLV (bovine leukemia virus) produces a viral miRNA named BLV-miR-B4 that is very similar to human miR-29 and shares common targets (Kincaid et al., 2012; Zhuoa et al., 2013). KSHV (Kaposi's Sarcoma Associated Herpesvirus) expresses a viral miRNA named miR-K12-11 that resembles cellular miR-155 (Zhuoa et al., 2013), a miRNA that exhibits oncogenic properties in several solid tumors and haematological malignancies. In addition, some viruses produce RNAs or proteins that suppress the RNAi pathway and thereby may have general effects on miRNA expression (Strebel et al., 2009). Interestingly, HTLV-1 Rex has been shown to interact with Dicer, inhibiting its activity and thereby reducing the efficiency of the conversion of shRNA to siRNA (Abe et al., 2010). This finding opens the possibility that HTLV-1 could control microRNA biogenesis through the action of Rex.

1.4.1.4 MicroRNAs in normal CD4+ T-cells

miRNA profiles in T-cell development. The first experimental evidence for an important role of miRNAs in T-cell development and homeostasis came from experiments performed in mice demonstrating that conditional deletion of Dicer at early stages of thymocyte development reduced the populations of peripheral CD4+ and CD8+ cells (Cobb et al., 2006) and impaired the ability of peripheral CD4+ T cells to differentiate into mature helper cells (Muljo SA et al., 2005). A detailed study of miRNAs in human T-cell development showed that the miRNA profile in DP cells was distinct from those of CD4 SP and CD8 SP cells, while the SP populations showed important similarities; a general upregulation of miRNAs from the DP to the SP stage was noted (Ghisi et al., 2011). Maturation of thymocytes into peripheral T cells was characterized by progressive upregulation of miR-150, miR-146a, and miR-146b and downregulation of miR-128; miR-181 was less abundant in mature peripheral T lymphocytes compared with DP thymocytes (Ghisi et al., 2011).

miRNAs in activated T cells. A study of human T lymphocytes following in vitro activation indicated a trend toward upregulation of miRNA expression (Grigoryev et al., 2011). The top 5 upregulated miRNAs were miR-221, miR-210, miR-98, miR-29b and miR-155, and the top 5 downregulated miRNAs were miR-181a, miR-199a, miR-223, miR-224 and miR-127-3p, most of which had not been previously described as being involved in the regulation of immune activation. The investigators identified targets for miR-155 and miR-221 and proposed that these miRNAs participate in a negative-feedback loop inhibiting cell proliferation and regulating survival in response to activation (Grigoryev et al., 2011).

miRNAs in Tregs. The early studies of mice with deletion of Dicer at the stage of thymocytes also implicated the miRNA pathway in Treg development (Cobb et al., 2006); deletion of Dicer after Treg lineage commitment (at the time of Foxp3 induction) resulted in a profound impairment of Treg suppressor function and fatal systemic autoimmune disease (Liston et al., 2008; Zhou et al., 2008). A comparison of human Tregs (CD4+, CD25+, Foxp3+) vs naïve T-cells (CD4+CD25-) indicated that Tregs can be distinguished on the basis of their increased levels of miR-21, miR-181c and miR-374 and reduced levels of miR-31 and miR-125a (Rouas et al., 2009). This study also demonstrated the regulation of Foxp3 by miR-31, which downregulated expression of Foxp3 through direct targeting of its 3'UTR, and miR-21, which upregulated its expression through an indirect mechanism (Rouas et al., 2009).

A detailed analysis of 17 lymphocyte subsets isolated from human peripheral blood identified specific patterns of miRNas in naïve, memory and Treg CD4+ T cells and showed that miR-125b controls a network of target genes involved in CD4+ T cell ontogenesis; forced expression of miR-125b resulted in a block in differentiation and favored a naive phenotype of CD4+ T cells (Rossi et al., 2011).

1.4.1.5 Cellular microRNA expression in HTLV-1-infected cell lines and ATLL samples

The first study of miRNAs in the context of HTLV-1 was published in 2008 by Pichler et al. The study employed quantitative RT-PCR to detect miR-21, miR-24, miR-146a, miR-155, miR-191, miR-214 and miR-223 in cell lines derived from ATLL patients and TSP/HAM patients, cell lines generated by cocultivating umbilical cord blood cells with ATLL cells, and a T-cell line that expresses Tax in

repressible manner; controls included uninfected PBMC, CD4+ T-cells and uninfected T-cell lines. Results demonstrated that miR-21, miR-24, miR-146a and miR-155 were significantly upregulated in the HTLV-1-transformed cell lines, while miR-223 was downregulated. It is noteworthy that all 4 upregulated miRNAs are also upregulated in EBV-infected B-cells during latency III, the viral growth program that drives B-cell proliferation (Cameron et al., 2008). Pichler et al. (2008) showed that Tax is able to upregulate miR-146a through the NF-κB pathway; this was later confirmed by Tomita et al. (2012).

Yeung et al. (2008) used microarrays to examine miRNAs in PBMC from patients with acute ATLL compared to pooled control PBMC, as well as HTLV-1-transformed cell lines compared to umbilical cord blood cells. The ATLL samples and infected cell lines shared 6 upregulated miRNAs (i.e., miR-18a, 9, 17-3p, 130b, 20b, and 93) and 9 downregulated miRNAs (i.e., miR-1, 130a, 199a*, 126, 144, 335, 337, 338, 432). Yeung et al. also examined PBMC exposed to the tumor-promoting agent phorbol-12-myristate 13-acetate (PMA) compared to untreated PBMC, and identified 3 miRNAs that were upregulated in ATLL cells, HTLV-1-infected cell lines and PMA-treated cells, namely miR-93, miR-130b, and miR-18a. Yeung et al. showed that miR-93 and miR-130b targeted a cellular tumor suppressor protein named tumor protein 53–induced nuclear protein 1 (TP53INP1), and that Tax was able to upregulate miR-130b through the NF-κB pathway (Yeung et al., 2008).

Bellon et al. (2009) also used microarrays to compare miRNA expression in ATLL cells versus control PBMC and CD4+ T-cells. miRNAs with altered expression levels included miR-150, miR-155, miR-223, miR-142-3p and miR142-5p (upregulated) and miR-181a, miR-132, miR-125a and miR-146b (downregulated).

Examination of these miRNAs in HTLV-1-infected cells yielded similar data, with the exception that miR-150 and miR-223 were downregulated instead of upregulated. The investigators also showed that treatment of HTLV-1-infected cell lines with an inhibitor of NF-κB (pathenolide) or JNK (JNK II) resulted in reduced levels of the miR-155 precursor.

A microarray-based study by Yamagishi et al. (2012) that compared a large panel of ATLL samples and control CD4+ T-cells revealed downregulation of 59 miRNAs and upregulation of only 2 miRNAs in the tumor samples. The most strongly downregulated miRNA was miR-31, which had been previously identified as a tumor suppressor and/or metastasis-associated miRNA in breast cancer (Valastyan et al., 2009; Schmittgen, 2010). The investigators demonstrated that loss of miR-31 expression was due to epigenetic silencing mediated by recruitment of repressor polycomb complexes on the miR-31 promoter. The mRNA coding for NIK (NF-κB-inducing kinase), a positive regulator of the noncanonical NF-κB pathway, was identified as a target of miR-31. Forced expression of either miR-31 or a NIK-specific shRNA in ATLL cells reduced cell proliferation and suppressed expression of anti-apoptotic genes such as Bcl-xl, XIAP, and FLIP, thus supporting a tumor suppressor function for miR-31 and an pro-survival role for NIK in the context of ATLL (Yamagishi et al., 2012).

It is striking how little the results of the studies described above overlap with each other. This is highlighted in Table 1, which lists the miRNAs that were identified in at least two studies of miRNAs in ATLL samples or infected cell lines. The next section summarizes interesting features of some of these miRNAs.

Table 1. miRNAs identified in at least two studies of ATLL samples or HTLV-1-infected cell lines

ATLL samples		Infected cell lines	
miRNA	**References**	**miRNA**	**References**
↑ miR-150	1,2	↑ miR-146a	4,5
↑ miR-155	1,2	↑ miR-155	2,4
↓ miR-31	1,3	↓ miR-150	1,2
↓ miR-125a	2,3	↓ miR-223	1,2,4
↓ miR-126	1,3		
↓ miR-130a	1,3		
↓ miR-146b	2,3		
↓ miR-181a	2,3		
↓ miR-335	1,3		

References: 1, Yeung et al., 2008; 2, Bellon et al., 2009; 3, Yamagishi et al., 2012; 4, Pichler et al., 2008; 5, Tomita et al., 2012. ↑Upregulated, ↓Downregulated.

miR-150. This miRNA is was found to upregulated in ATLL but downregulated in HTLV-1-infected cell lines (see Table 1). As mentioned in Section 1.4.1.5, miR-150 is gradually upregulated during T-cell development and downregulated in activated CD4+ T-cells, and is downregulated in Tregs through the action of Foxp3 (Cobb et al., 2006). Overexpression of miR-150 was shown to inhibit the proliferation of B-lymphoma cell lines (Chang et al., 2008) and reduce proliferation and induce apoptosis of NK cell lines (Watanabe et al., 2011) and T-ALL cell lines (Ghisi et al., 2011), indicating its possible function as a tumor suppressor. miR-150 is also downregulated in Sezary syndrome (Ballabio, et al., 2010), in NK/T-cell lymphomas (Watanabe et al., 2011), and in several other hematological tumors including pediatric acute leukemia (Zhang et al., 2009),

anaplastic large-cell lymphoma (Merkel et al., 2010), diffuse large B-cell lymphoma (Roehle et al., 2011), mantle cell lymphoma (Zhao et al., 2010; Di Lisio et al., 2010), chronic myeloid leukemia (Flamant et al., 2010). Cellular genes known to be targeted by miR-150 include c-Myb, a transcription factor that is overexpressed in some human leukemias and in tumors of the breast and colon (Ramsay et al., 2008), and NOTCH3 (Ghisi et al., 2011). The interaction of miR-150 with a component of the Notch pathway is particularly interesting, as this pathway plays a key role in the development of the T-cell compartment (Sultana et al., 2010). It would be of interest to investigate the influence of miR-150 on the Notch pathway in ATLL cells given the finding of a high rate of activating Notch mutations and constitutive activation of the Notch pathway in ATLL patients (Pancewicz et al., 2010).

miR-155. This miRNA is highly expressed in Tregs and activated T cells as well as in activated B cells, activated macrophages and dendritic cells (Faraoni et al., 2009). Mice deficient in miR-155 show impairments in T, B and dendritic cell functions (Rodriguez et al., 2007). CD4+ T-cells from miR-155-deficient mice tend to differentiate into Th2 cells and show reduced IL-2 and IFN-γ production in response to antigen stimulation (Thai et al., 2007). miR-155 expression in Tregs depends on the activity of Foxp3; miR-155 in turn blocks expression of SOCS1, a negative regulator of IL-2R signalling, thus maintaining Tregs highly sensitive to IL-2 (Lu et al., 2009). Overexpression of miR-155 in CD4+ T-cells renders them resistant to Treg-mediated suppression (Stahl et al., 2009). In addition to ATLL, Overexpression of miR-155 has been documented in several other hematological malignancies and solid tumors (e.g., lung, thyroid, pancreas, breast, colon, and cervix) (Faraoni et al., 2009).

In mice, forced expression of miR-155 induces polyclonal pre-B-cell tumors (Costinean et al., 2006). miR-155 may also be important in the mechanisms of B-cell transformation driven by EBV, which induces miR-155 expression, and KSHV, which codes for a miR-155 orthologue with target specificity similar to that of the cellular miRNA (Gottwein et al., 2007; Skalsky et al., 2007). The EBV LMP-1 protein upregulates cellular miR-155 through NF-κB (Gatto et al., 2008; Lu et al., 2008; Rahadiani N et al., 2008; Forte et al., 2012). miR-155 in turn targets IKKε (Lu et al., 2008), a transcriptional target of the NF-κB pathway that is involved in the interferon antiviral response, suggesting that The NF-κB pathway might be responsible for high levels of miR-155 in HTLV-1-infected cells. The oncogenic properties of miR-155 can in part be explained by its ability to block expression of tumor protein 53-induced nuclear protein 1 (TP53INP1) (Gironella et al., 2007), which, as described above, is also targeted by miR-93 and miR-130b in HTLV-1-infected cells.

miR-146a. miR-146a is overexpressed in various solid cancers (He et al., 2005; Volinia et al., 2006) and in pediatric AML and B-ALL (Zhang et al., 2009). It was first identified as a miRNA that fine-tunes the innate immune response, as its expression is induced by NF-κB after Toll-like and IL-1 receptor (TIR) engagement, and exerts a negative feedback control on TIR signalling by targeting the adaptor proteins IRAK1 and TRAF6 (Taganov et al., 2006). miR-146a is upregulated in CD4+ T-cells after activation through the T-cell receptor (TCR) (Cobb BS et al., 2006), resulting in a reduction of IL-2 production by impairing AP-1 transcriptional activity, leading to an attenuation of the IL-2 signal (Curtale et al., 2010; Rusca et al., 2011). miR-146a also targets FADD (Fas-Associated Death Domain) and thereby

protects T-cells from AICD (Activation Induced Cell Death), an apoptotic response that follows TCR stimulation and is crucial for the termination of the immune response and for peripheral tolerance to self-antigens (Curtale et al., 2010). Although in some cell contexts miR-146a can downregulate NF-κB activity (Bhaumik et al., 2009) and therefore silence its own expression, this feedback mechanism appears to be blocked in HTLV-1-infected cells, likely through the action of Tax.

miR-223. As described above, miR-223 is downregulated in HTLV-1-infected cell lines and is instead upregulated in murine Tregs (Cobb et al., 2006). miR-223 is a key modulator of promeylocyte-to-granulocyte differentiation (Fazi et al., 2005). miR-223-deficient mice exhibit an increase in granulocytic progenitors and neutrophils with an unusual morphology and hypersensitivity to activating stimuli, accompanied by a spontaneous lung inflammation with extensive neutrophil infiltration (Johnnidis et al., 2008). A study by Sun et al. suggests that miR-223 is a hematopoietic-specific miRNA which has crucial functions in myeloid lineage development (Sun et al., 2010). miR-223 is upregulated in bladder cancer (Gottardo et al., 2007), esophageal adenocarcinoma (Mathe et al., 2009) and in recurrent ovarian cancer (Laios et al., 2008), but is downregulated in hepatocellular carcinoma (Wong et al., 2008) and gastric cancer (Kang et al., 2012). miR-223 is also significantly down-regulated in acute lymphoblastic leukemia compared with acute myeloid leukemia and together with other miRNAs may represent a differential diagnostic signature for these tumors (Mi et al., 2007). miR-223 downregulation is also included in miRNA signatures predicting poor prognosis in CLL (Calin et al., 2004; Fulci et al., 2007; Stamatopoulos et al., 2009). miR-223 therefore appears to exert either oncogenic or tumor suppressor properties depending on the cell context.

Deregulation of miR-223 expression is observed during infection with influenza virus and hepatitis B virus, and in inflammatory bowel disease, type 2 diabetes, leukaemia and lymphoma. Evidence suggests that miR-223 may limit inflammation and prevent collateral damage during infection and oncogenic myeloid transformation (reviewed by Haneklaus et al., 2013).

1.4.2 sncRNAs derived from tRNAs

tRNAs are fundamental components of the translation machinery that function as carriers that transport amino acids to the growing polypeptide chain during the translation of mRNA. Mature tRNAs are approximately 73 nt in length and contain an amino acid attached to their 3'ends. The tRNAs contain a D loop, T loop, and anticodon loop, which interact with each other via conserved nucleotides to give a compact L-shaped tertiary structure. tRNA genes are transcribed by RNA polymerase III to produce precursor transcripts ending with a poly U tail. The precursor transcripts undergo a series of processing events including splicing (for some tRNAs), removal of a 5' segment by the endonuclease RNase P, removal of a 3' segment by the endonuclease RNase Z, addition of a 3'CCA that accepts the amino acid, and modification of several bases.

In 1970, Yudelevich's group observed that a fragment derived from $tRNA^{leu}$ was expressed in *Escherichia coli* shortly after bacteriophage T4 infection (Dube et al., 1970; Yudelevich, 1971). Almost 30 years later in 1999, Shing's group isolated fragments of tRNAs from human urinary bladder carcinoma and studied their function for the first time (Zhao et al., 1999). Over the last decade, advanced techniques in cloning and high-throughput sequencing have led to the identification of many sncRNAs derived from tRNAs in several organisms. These fragments are

17-55 nt in length and originate from different portions of the tRNA molecule (reviewed by Garcia-Silva et al., 2013). Deep sequencing and cloning approaches are now starting to address the questions surrounding small tRNA fragment biogenesis and function (reviewed by Martens-Uzunova et al., 2013).

tRNA fragments are divided in two major classes: tRNA halves and small tRNA fragments (tRFs) (reviewed by Sobala et al., 2011; Garcia-Silva et al., 2013).

1.4.2.1 tRNA halves

tRNA halves (also known as tsRNAs) are derived from the cleavage of tRNAs at the anticodon loop in response to oxidative stress, hypoxia, or apoptotic inducers. tRNA halves have a size of 30–35 nt and are generated from both the 3' and 5' portions of the parent tRNA (Figure 8). Biogenesis of tRNA halves is not yet clear, and many models are proposed by various research groups.

In prokaryotes, anticodon nucleases are responsible for cleavage of tRNA, while in higher eukaryotes cells and fission yeast stress-induced tRNA cleavage is catalyzed by Angiogenin (ANG) and Rny1 respectively. Cleavage seems to occur on mature tRNAs, since they usually have a mature 5' end and 3'CCA (reviewed by Martens-Uzunova et al., 2013).

1.4.2.2 tRNA-derived RNA fragments (tRFs)

tRFs were recently identified by high-throughput sequencing technology. tRFs are about 19 nt in length and are derived from mature tRNA or tRNA precursors (Lee et al., 2009; Mosher et al., 2010; Loss-Morais et al., 2013). tRFs are the products of tRNA cleavage at specific positions, which have been associated with

stress responses, development, alteration of tRNA structural stability and other biological processes (Li et al., 2008; Thompson et al., 2009; Yamasaki et al., 2009).

Cole et al. performed high-throughput sequencing analysis of HeLa cells and observed that the most abundant tRNA-derived small RNAs are products of processing of tRNA-Arginine, tRNA-Glutamine, tRNA-Lysine, and tRNA-Valine. These tRNAs were almost exclusively processed from the 5' end, with cleavage by Dicer at the D-loop, resulting in small RNAs of approximately 19 nt (Cole et al., 2009).

A deep-sequencing analysis of sncRNA in prostate cancer cell lines revealed 135 sncRNA derived from tRNAs (Lee et al., 2009). The fragments corresponded to the 3' end of tRNA precursors or to the 5' end or the 3' end of mature tRNAs. The authors named the 3 classes tRF-1, tRF-5 and tRF-3, respectively (Figure 8). Garcia-Silva et al. (2013) proposed the names 3'U tRFs, 5'tRFs, and 3' CCA tRFs for the 3 classes identified by Lee and coauthors. This more complicated naming system refers to the fact that tRF-1 sequences terminate with a poly (U) tract generated by RNA Pol III run-off, and tRF-3 sequences terminate with a 3'CCA that is added to all mature tRNAs.

The biogenesis of tRFs has not yet been fully elucidated, and may vary among organisms. However, it is clear that various molecules associated with the tRNA maturation machinery and miRNA/siRNA pathways play key roles in biogenesis of tRFs. The tRF-1 class is generated through cleavage of tRNA precursors by RNAse Z. This tRNA maturation step is known to occur in the nucleus. Since 3' U tRFs are abundant in the cytoplasm, it seems that newly generated 3' U tRFs need to be exported from nucleus. In alternative, a cytoplasmic

form of tRNAseZ may give rise to tRF-1 sequences in the cytoplasm by cleaving tRNA precursors that have escaped the nucleus (reviewed by Garcia-Silva et al., 2013).

Production of tRF-5 and tRF-3 sequences must occur after cleavage of the tRNA precursor by RNAse P and RNAse Z and the addition of CCA (for tRF-3s). Dicer was shown to be responsible for cleavage to produce a tRF-5 (Cole et al., 2009) and a tRF-3 (Maute et al., 2013) in mammalian cells. However, another study of sncRNA in mammalian cells indicated that most tRF-5 and tRF-3 sequences are produced through a mechanism that does not require Dicer (Li et al., 2012).

1.4.2.2.1 Biological function of tRFs.

Although the exact roles of tRFs are yet to be elucidated, evidence suggests that some tRFs play an important role in cellular stress responses and cell proliferation. In stress conditions like oxidative stress or starvation, often as a prelude to apoptosis, the expression of tRNA-derived fragments rises proportionally (Thompson et al. 2008; Thompson et al. 2009). In normal cells, it is possible that tRNA-derived fragments function as essential apoptotic signals or cause apoptosis indirectly, such as through inhibition of protein translation. Stress-related tRNA cleavage pathways are believed to play major roles in malignant cells by inducing proliferation or escape from apoptosis (reviewed by Martens-Uzunova et al., 2013). Recent studies that employed deep sequencing and bioinformatics have demonstrated the role of tRFs in the life cycle of a wide range of human pathogens, including *Escherichia coli, Aspargillus fumigates, Giardia lamblia,* and *Trypanosoma cruzi* as well viruses.

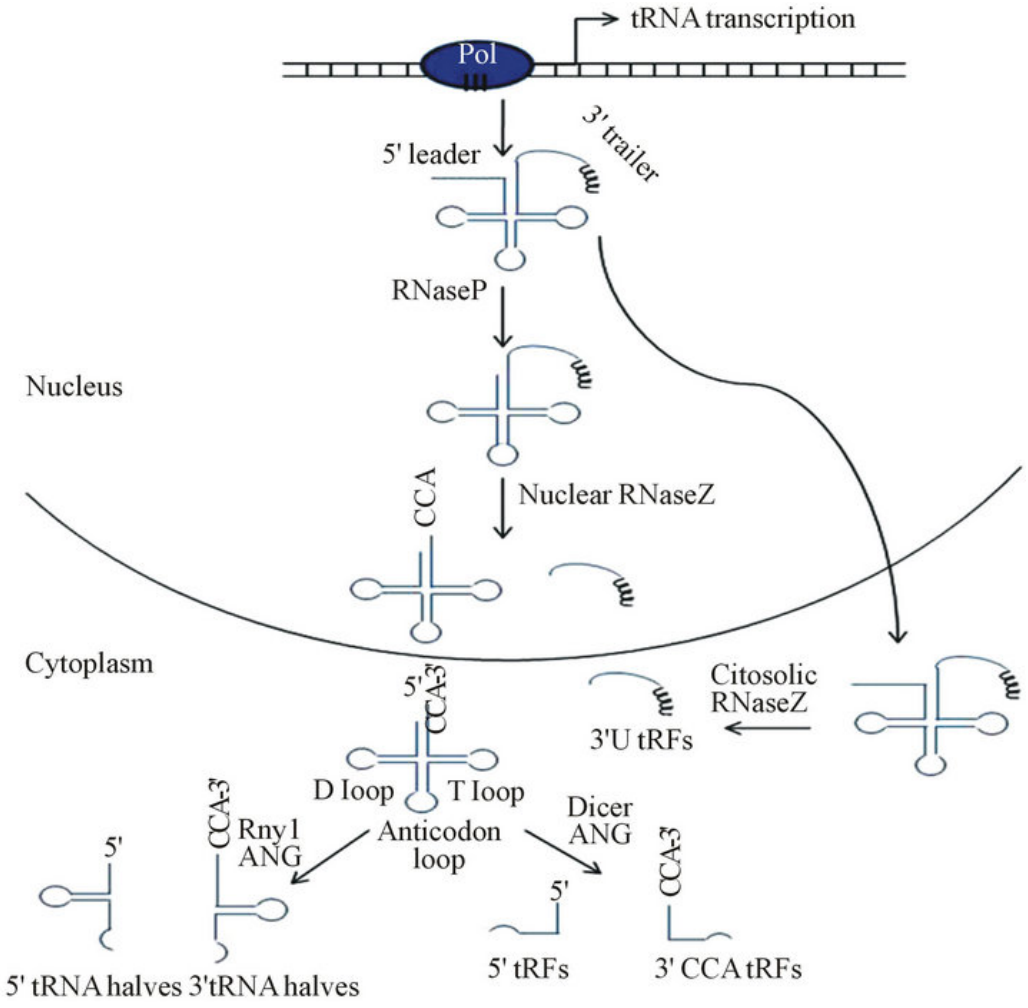


Figure 8. Biogenesis of small noncoding RNAs from tRNAs; tRNA halves and tRFs. These sequences are generated through multi-step processes mediated by RNaseZ, Angiogenin, Rny1 or Dicer (from Garcia-Silva et al., 2013).

2 Identification of tRFs in HTLV-1-infected cells

2.1 Background aims of the study

Our laboratory compared the profiles of sncRNAs in normal CD4+ T-cells and the HTLV-1-transformed cell lines C91PL and MT-2 by high-throughput sequencing of small RNA libraries. A search for tRFs highlighted the presence of tRF-3019, which is derived from the 3' end of tRNA-proline. This was of interest, as tRNA-proline is considered to be the primer for HTLV-1 reverse transcriptase (Seiki et al., 1982). tRF-3019 exhibited perfect sequence complementarity to the primer binding site of HTLV-1. This raised the possibility that the tRF could be fully sufficient as a primer for reverse transcription.

The study described in Section 2 was aimed at determining whether tRF-3019 is incorporated into virus particles and can function as a primer for viral reverse transcriptase.

2.2 MATERIALS AND METHODS

2.2.1 Cell culture

The HTLV-1 infected T-cell lines C91PL and MT-2 (Popovic et al., 1983) were maintained in RPMI (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 2 mM glutamine (Invitrogen), 100 units/ml penicillin and 20 units/ml streptomycin (complete RPMI). Total RNA was isolated using TRIzol (Invitrogen). RNA concentration was measured using a Nanodrop spectrophotometer.

2.2.2 Small RNA libraries

The construction and analysis of small RNA libraries derived from normal CD4+ cells (resting and in vitro-stimulated) and cell lines C91PL and MT-2 are described in Ruggero et al., J. Virol., in press. Excel tools were used to search sequence reads for the 135 tRFs described by Lee et al. (2009).

2.2.3 RT assay using tRF-3019

Preparation of RNA template. A DNA fragment corresponding to nt 721-822 of the HTLV-1 ATK reference sequence was amplified by PCR. We used HTLV-1 molecular clone ACH (Kimata et al., 1994) as a template and primers U5-s and Gag-as. A 20-nt tail was added to the 5' end of the product with a second round of PCR using primers Tail-U5-s and Gag-as. The 129-nt fragment was cloned into vector pSG5E, which is a modified version of pSG5 (Stratagene) containing the polylinker of pBluescript (Stratagene) 3' to the T7 promoter. The resulting plasmid (pSG-U5-PBS) was linearized 3' to the insert and *in vitro*-transcribed with T7 RNA

polymerase (Invitrogen). After digestion with DNase 1 to eliminate the plasmid, the mixture was extracted with phenol-chloroform and ethanol-precipitated to recover RNA. The resulting pellet was resuspended in water and stored at -80°C.

Preparation of virus particle lysates containing HTLV-1 reverse transcriptase. For preparation of viral particle lysates, we used confluent cultures of C91PL cells (HTLV-1-infected T-cell line). The suspension cultures were centrifuged at low speed to remove cells. The supernatant was passed through a 0.45 micron filter (Sartorius) and centrifuged at 24,000 rpm in an SW28 rotor for 2 hours. Pelleted material was resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5% Nonidet-P40; 10 µl per 10 ml centrifuged supernatant) and stored at -80°C.

RT assay. The RT assay was performed by using a method based on a published protocol (Balestrieri et al., 2011; Frezza et al., 2014). For each RT assay, a 100-ng aliquot of in vitro-transcribed RNA was combined with 10 pmol of either tRF-3019 RNA, miR-150-5p RNA (negative control), tRF-3019 DNA (positive control) or water instead of primer in a 10.5-µl volume and annealed at 70°C for 10 min and then cooled on ice. The mixtures were brought to a final volume of 20 µl containing 1 mM each dNTP, 10 U RNase inhibitor, RT buffer (25 mM Tris-HCl, pH 8.3, 5 mM MgCl2, 50 mM KCl, 2 mM DTT) and 2 µl virion lysate and incubated at 37°C for 1 hour followed by 95°C for 5 min.

For PCR amplification, we used a 2.5-µl aliquot of the cDNA product in a final volume of 25 µl containing 1X Taq Gold PCR buffer, 2 mM MgCL2, 200 µM dNTPs, 5 pmol each of primer Tail-s and U5-as and 0.5 U AmpliTaq Gold DNA polymerase (Life Technologies). The PCR method consisted of a denaturation step at

94°C for 1 min followed by 25 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 45 sec. Products were analyzed on a 6% polyacrylamide gel and stained with ethidium bromide. Images were obtained using a BioRad Gel Doc XRS system.

RT-PCR to detect tRFs and tRNAs. To eliminate contamination of cultures with exosomes that might be present in FBS, complete RPMI containing 20% FBS was centrifuged at 24,000 rpm for 4 hours using a Beckman-Coulter SW28 rotor to pellet any exosomes. Supernatant medium was then passed through a 0.2-micron filter and brought to 10% FBS by adding an equal volume of RPMI containing antibiotics and glutamine.

C91PL cells were cultured to confluence in the exosome-depleted medium and virus particles were recovered by ultracentrifugation as described above. RNA was isolated from pelleted particles and the producer C91PL cells using TRIzol LS (Life Technologies) according to the manufacturer's protocol (see Section 3.2.4 for details). As summarized in Figure 9, aliquots of the RNA were subjected to denaturing PAGE through a 15% polyacrylamide gel to separate species in the size range of full-length tRNAs from small RNAs, with tRNAs visible in the cellular RNA sample and 5 pmol synthetic miR-150-5p serving as size markers for the 2 fractions. The gel was stained with ethidium bromide and the regions containing tRNAs and small RNAs (about 15-30 nt) were excised, crushed and incubated with gentle mixing in elution buffer (300 mM sodium acetate, pH 5.2, 1 mM EDTA) overnight at 4°C. RNA was ethanol-precipitated and resuspended in dH_2O.

Primer sets used amplify tRF-3019, tRF-3003 and the tRNAs from which they are derived (tRNA-Pro and tRN-Ala, respectively) are listed in Table 2 and

depicted in Figure 9. RT-PCR to detect tRF-3019 and tRF-3003 was based on a protocol for detecting microRNAs (Sharbati-Tehrani et al., 2008). Size-fractionated RNA (2 µl) was annealed with 2 pmol primer RT7-tRF-3019 or RT8-tRF-3003 at 70°C for 10 min in a 7.5-µl volume. The mixture was brought to 10 µl with the addition of 1 mM dNTP, 1X RT Buffer, and 5 U AMV reverse transcriptase (Finnzymes) and reverse-transcribed at 40°C for 1 hr. Two-microliter aliquots of the resulting cDNAs were PCR-amplified in a final volume of 25 µl containing 1X Taq Gold PCR buffer, 1.5 mM MgCL2, 200 µM dNTP, 0.1 pmol of primer Short-tRF-3019 or Short-tRF-3003, 2.5 pmol each of primers PCR-tRF-s and PCR-tRF-as, and 0.5 U AmpliTaq Gold DNA polymerase. The PCR method consisted of a denaturation step at 95°C for 10 min, 5 cycles of denaturation at 95°C for 30 sec, annealing at 40°C for 45 sec and extension at 72°C for 30 sec, followed by 22 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 45 sec and extension at 72°C for 30 sec.

To detect tRNA-Pro and tRNA-Ala, 1 µl of size-fractionated RNA was reverse-transcribed in a 10-µl reaction at 53°C for 50 min using the antisense primer and Superscript III (Life Technologies). Resulting cDNA (2.5 µl) was PCR-amplified using sense and antisense primers and AmpliTaq Gold with a denaturation step at 95°C for 8 min followed by cycles of denaturation at 95°C for 40 sec, annealing at 60°C for 40 sec and extension at 72°C for 40 sec (30 cycles for tRNA-Ala and 26 cycles for tRNA-Pro). To detect gag/pol RNA, 0.5 µl of RNA from virus particles or 200 ng total RNA from producer C91PL cells was reverse-transcribed using primer Gag-as and Superscript III and then PCR-amplified using primers U5-s and Gag-as and AmpliTaq Gold as described above for 30 cycles with an annealing

temperature of 59°C and a final extension step for 5 min at 72°C. PCR products were separated on 6% polyacrylamide gels.

Table 2. Primer sequences

Primer	Sequence
U5-s	CTCGGAGCCAGCGACAGC
Gag-as	gaagcttGCCTAGGGAATAAAGGGGC
Tail-U5-s	agagcggattaacggcctaaCTCGGAGCCAGCGACAGC
Tail-s	agagcggattaacggcctaa
tRF-3019 RNA	AUCCCGGACGAGCCCCCA
tRF-3019 DNA	ATCCCGGACGAGCCCCCA
miR-150 RNA	UCUCCCAACCCUUGUACCAGUG
U5-as	TGTGTACTAAATTTCTCTCCTG
RT7-tRF-3019	aacgtattcaccgtgagtggtTGGGGGC
Short-tRF-3019	cgtcagatgtccgagtagagATCCCGGACGAG
RT8-tRF-3003	aacgtattcaccgtgagtggtTGGTGGAG
Short-tRF-3003	cgtcagatgtccgagtagagTCCCCGGCACC
PCR-tRF-s	cgtcagatgtccgagtagag
PCR-tRF-as	aacgtattcaccgtgagtgg
tRNA-Pro-s	GGTCTAGGGGTATGATTCTCG
tRNA-Pro-as	GCTCGTCCGGGATTTGAACC
tRNA-Ala-s	GTGTAGCTCAGTGGTAGAGC
tRNA-Ala-as	TGGAGGTGCCGGGGATTG

Added tail sequences are indicated with lower case letters. Primers were purchased from Sigma-Aldrich.

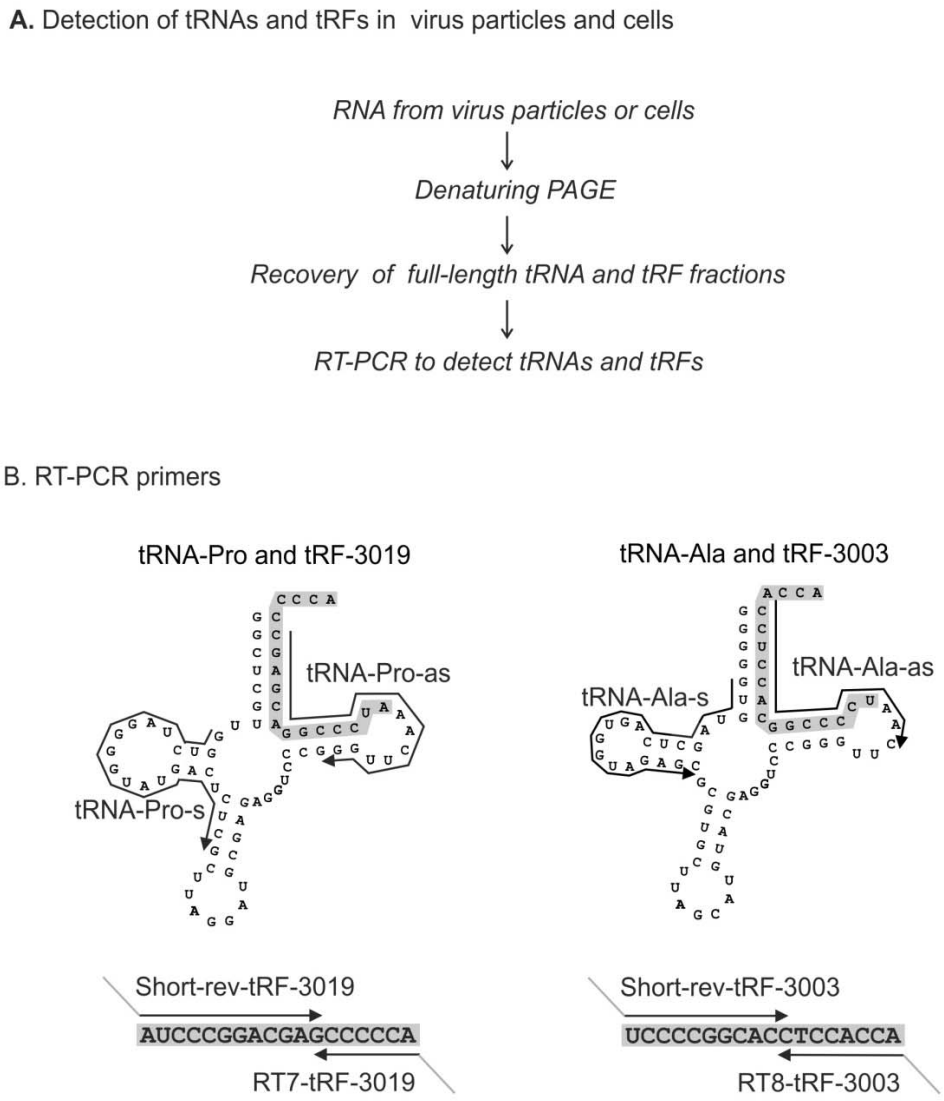


Figure 9. Method to detect tRNAs and tRFs in virus particles and C91PL cells. Panel A summarizes the method described in the Materials and Methods in which RNA isolated from virus particles and producer C91PL cells was subjected to denaturing PAGE to permit separation of tRNAs from small RNAs including tRFs. Panel B indicates positions of RT-PCR primers in tRNA-Pro, tRNA-Ala, tRF-3019 and tRF-3003 (Ruggero et al., J. Virol., in press).

2.3 RESULTS

2.3.1 tRFs expressed in HTLV-1-infected cells.

We analysed sequences from small RNA libraries for perfect matches to the 135 tRFs reported by Lee et al. in a study of prostate cancer cell lines (Lee et al., 2009). Overall, in both normal and HTLV-1-infected CD4 cells, fragments processed from the 3' end of mature tRNAs (tRF-3) were considerably more abundant than tRFs produced from the 3' end of tRNA precursors (tRF-1) or from the 5' end of mature tRNAs, (tRF-5) (Figure 10).

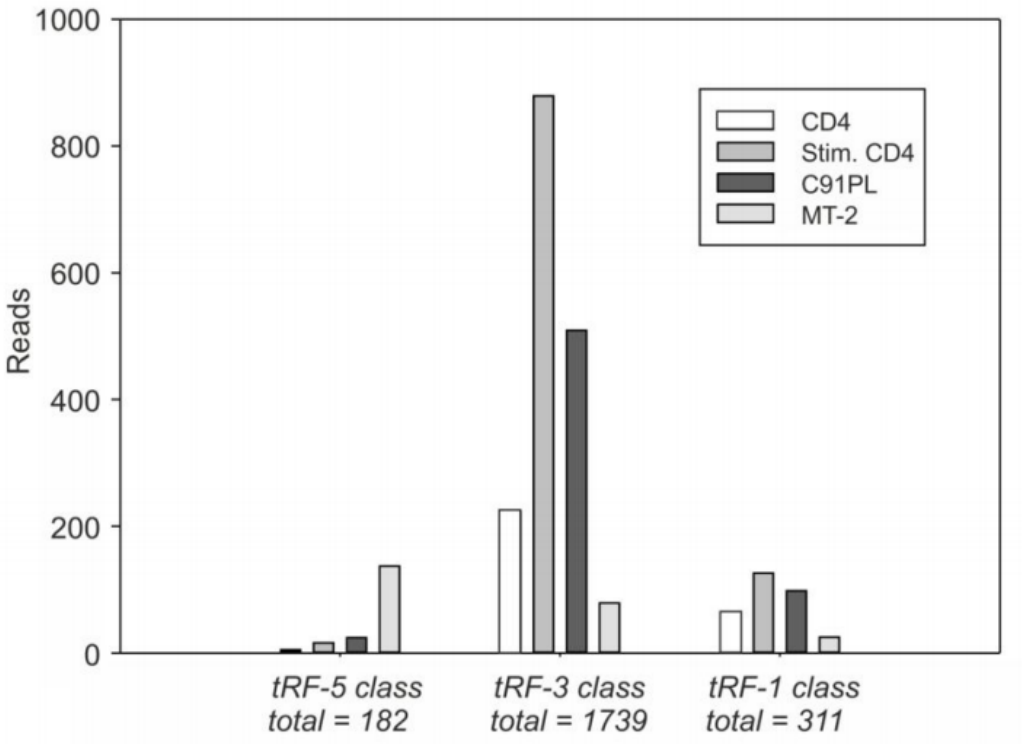


Figure 10. The graph shows the total numbers of sequence reads matching tRFs of the 3 classes (Ruggero et al., J. Virol., in press).

Analysis of the small RNA libraries indicated that many tRFs were upregulated in normal CD4 cells upon mitogenic stimulation. Among the 22

previously described tRF-1 sequences, tRF-1001 was the most abundant. tRF-3004 and tRF-3029 were more abundant in C91PL cells compared to stimulated CD4+ controls, and MT-2 cells yielded few tRF sequences compared to the other 3 cell types. Figure 11 shows the most abundant tRFs identified in the libraries.

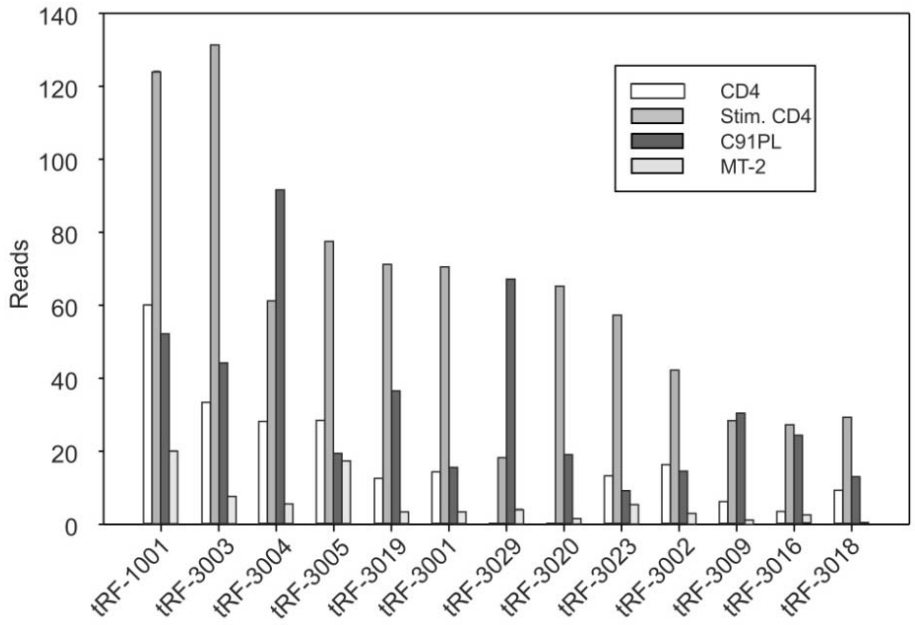
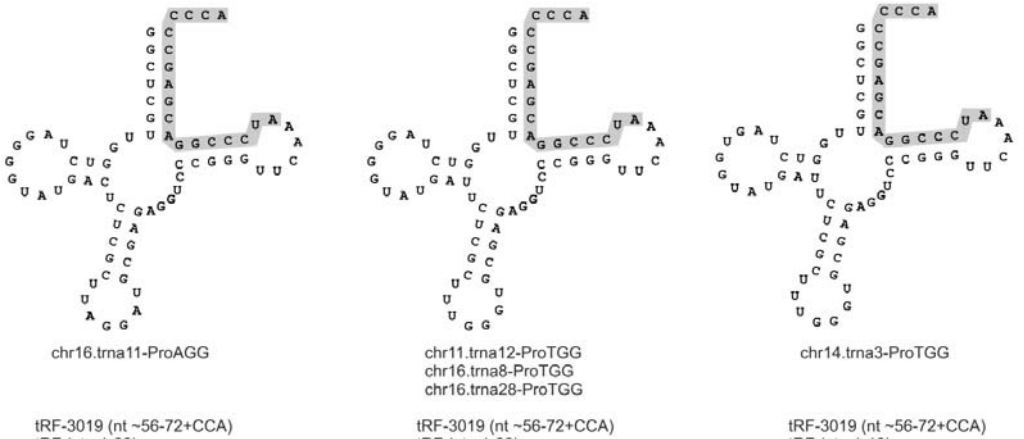


Figure 11. The graph compares the frequencies of tRFs with a total of at least 50 sequence reads summed among the 4 libraries (Ruggero et al., J. Virol., in press).

As described in the introduction to Section 2, tRF-3019 belongs to the tRF-3 class (Lee et al., 2009), and corresponds to the 3' end of tRNA-Pro, the tRNA considered to serve as the primer for HTLV-1 RT (Figure 12). tRF-3019 was the fifth most abundant tRF identified in our libraries, and was most abundant in stimulated CD4 cells. A BLAST search for tRNA genes able to produce tRF-3019 yielded 21 tRNA-Pro genes located on chromosomes 1, 5, 6, 11, 14, 16 and 17. The four libraries contained several tRF-3019 isoforms with additional nucleotides at the 5' end that matched perfectly to the human genome but were not complementary to the

viral genome. The libraries also contained a small number of reads corresponding to fragments derived from other portions of tRNA-ProTGG and tRNA-ProAGG.



	Sequence	CD4	STIM. CD4	C91PL	MT-2
tRF-3019 (nt ~56-72+CCA)	atcccggacgagccccca	12	71	36	3
tRF-3019 5' isoforms		1	11	13	1
tRF (nt ~1-18)	ggctcgttggtctagtgg	0	1	0	1
tRF (nt ~1-22)	ggctcgttggtctaggggtatc	0	2	2	9
tRF (nt ~8-26)	tggtctaggggtatgattc	3	2	0	3
tRF (nt ~32-53)	ttgggtgcgagaggtcccgggt	18	15	0	4

Figure 12. tRFs processed from tRNA-Pro. The top portion of the figure shows three examples of the 21 tRNA-Pro molecules that are able to produce tRF-3019 (highlighted in grey). The diagrams were obtained from the UCSC database and modified by adding the 3' CCA triplet which is present on mature tRNAs and tRF-3 sequences. The table indicates the sequences of the tRFs and the number of reads identified in each library.

2.3.2 tRF-3019 functions as a primer for HTLV-1 reverse transcriptase.

Interestingly**,** only the portion of tRNA-Pro corresponding to tRF-3019 is complementary to the HTLV-1 primer binding site (PBS) (Figure 13A), suggesting that the tRF would be fully sufficient as a primer for reverse transcription. We thus tested the primer activity of tRF-3019 in an *in vitro* reverse transcriptase assay carried out using a synthetic RNA template and the reverse transcriptase contained in HTLV-1 virus particles recovered from culture supernatant of C91PL cells. The RT

assays contained either no primer; synthetic tRF-3019 RNA; tRF-3019 DNA as a positive control, or miR-150-5p RNA as a negative control. The PCR reaction contained a sense primer specific for a tail sequence present in the synthetic RNA template and an antisense primer positioned immediately 5' to the PBS.

As depicted in Figure 13B, The RT assay performed using tRF-3019 RNA yielded the expected 87-bp PCR product, thus confirming that tRF-3019 can function as a primer for HTLV-1 RT. The assay carried out using tRF-3019 DNA primer yielded the 87-bp product along with a longer product indicated by the grey arrow in Figure 13B. This second band corresponded in size to an amplicon produced with the tail primer and residual tRF-3019 DNA present in the cDNA (i.e. 107 bp). Interestingly, trace amounts of the 87-bp product were also detected in the assays carried out using C91PL RT and miR-150-5p or no primer. This amplicon may have originated from cDNA primed by tRNA-Pro or tRF-3019 present in the viral particle lysate that was used as a source of RT.

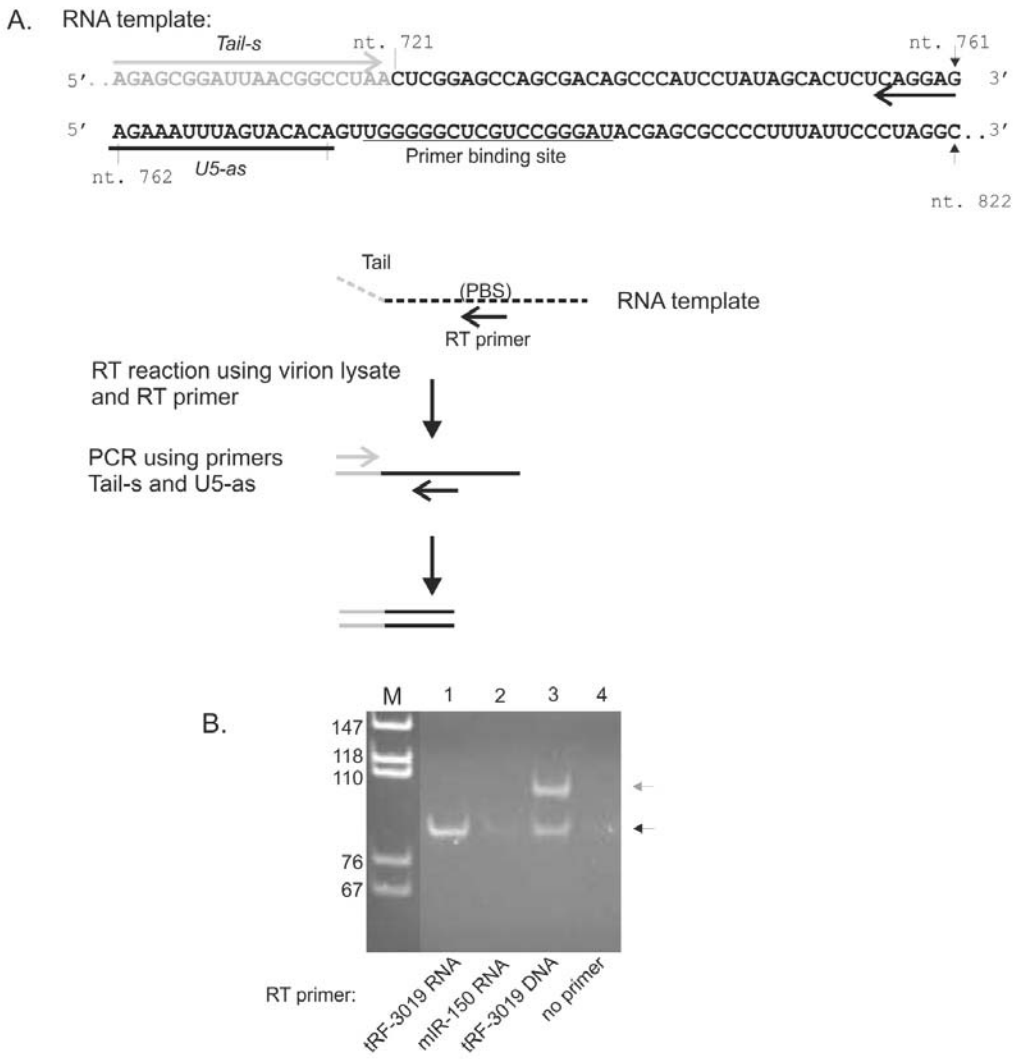


Figure 13. tRF-3019 acts as a primer for HTLV-1 reverse transcriptase. Panel A summarizes the RT assay. The template consisted of an *in vitro*-transcribed RNA spanning HTLV-1 nt 721-822 modified by the addition of a 20-nt tail at the 5'end. The template was incubated with HTLV-1 reverse transcriptase present in virus particles recovered from the culture supernatant of C91PL cells and either tRF-3019 RNA, miR-150-5p RNA (negative control), tRF-3019 DNA (positive control), or no primer. Products of the RT reactions were amplified by PCR using PCR primers Tail-s and U5-as and separated by PAGE in a 6% polyacrylamide gel along with Msp I-digested pBluescript as a size marker. Panel B shows a composite of the ethidium bromide-stained gel. The black arrow indicates the position of the 87-bp PCR product expected using primers Tail-s and U5-as. The additional band indicated by the grey arrow in lane 3 was consistent with a product amplified by Tail-s and residual tRF-3019 DNA primer added to the RT assay. Primer sequences are reported in Table 2.

2.3.3 HTLV-1-infected cells release particles containing tRF-3019

After confirming that tRF-3019 is capable of priming HTLV-1 reverse transcription, our efforts were aimed at determining whether the tRF and/or its partent tRNA-Pro were present in virus particles recovered from supernatants of C91PL cultures. As a control, we also assayed for tRF-3003, the most abundant tRF-3 detected in the 4 libraries, along with its parent tRNA-Alanine (tRNA-Ala). As outlined in Figure 9 and the Materials and Methods, RNA isolated from the virus particles and producer cells was subjected to denaturing PAGE to separate species in the tRF size range from full-length tRNAs. This was necessary as the tailed RT-PCR primers utilized to detect the tRFs also amplified the 3' ends of the full-length tRNAs.

As shown in Figure 14A, both tRNA-Ala and tRNA-Pro were readily detected in the C91PL cells. Interestingly, we observed that tRNA-Pro was enriched in virus particles compared to tRNA-Ala. The PCR products for both tRNAs were much more evident in the full-length tRNA fraction than in the tRF fraction, indicating that the denaturing PAGE step resulted in acceptable separation of the 2 size classes.

We subjected the same samples to RT-PCR with sets of primers that amplifed the tRFs present in the tRF fraction and the 3' ends of the tRNAs in the tRNA fraction (Table 2). As depicted in Figure 14B, results showed that tRF-3003 was much more abundant in the C91PL cells than in virus particles, while tRF-3019 was detected at comparable levels in virus particles and cells. Quantification of the intensities of the RT-PCR products confirmed that tRNA-Pro and tRF-3019 were enriched in virus particles compared to tRNA-Ala and tRF-3003 (Fig. 14C). As

shown in Figure 14D, RT-PCR assays on RNA isolated from the particles confirmed that they contain the HTLV-1 genomic gag/pol mRNA.

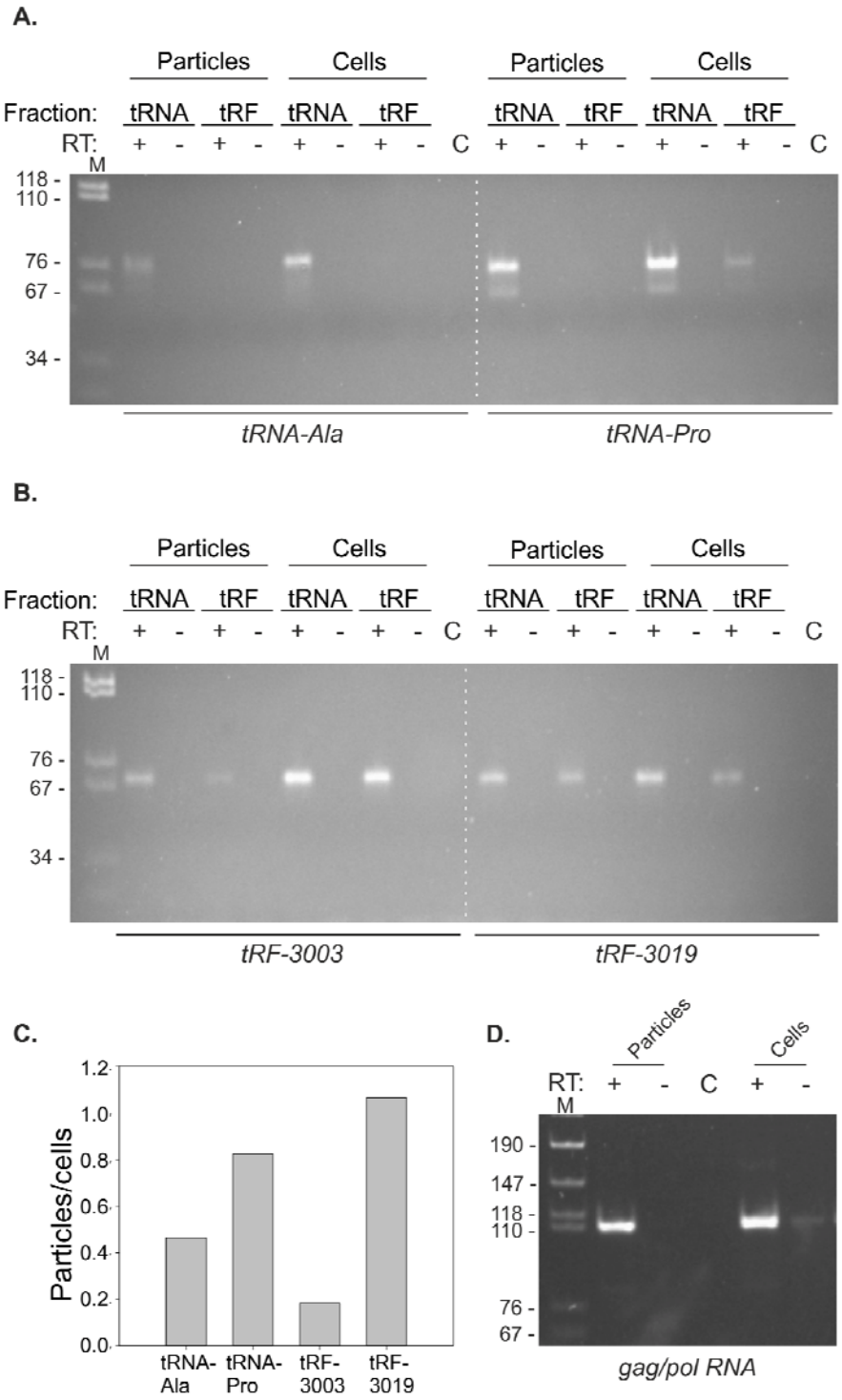


Figure 14. RT-PCR to detect tRNAs and tRFs in virus particles and C91PL cells. As described in the Materials and Methods and depicted in Figure 13, RNA from virus particles and producer C91PL cells was subjected to denaturing PAGE; regions of the gel containing tRNA and small RNA were excised and RNA was recovered by passive elution and ethanol precipitation. The resulting RNA fractions were subjected to RT-PCR to detect tRNA-Ala and tRNA-Pro (Panel A) and their tRF-3 sequences tRF-3003 and tRF-3019, respectively (Panel B). Shown are images of the products after separation on 6% polyacrylamide gels with Msp I-digested pBluescript as a size marker (M). Intensities of RT-PCR bands obtained for tRNAs and tRFs (measured in tRNA and tRF fractions, respectively) were measured using a BioRad Gel Doc XRS imager. Panel C shows a plot of ratios of band intensities obtained for virus particles vs. cells. Calculated ratios were tRNA-Ala (particles)/(cells)=0.46; tRNA-Pro (particles)/(cells)= 0.82; tRF-3003 (particles)/(cells) = 0.18; tRF-3019 (particles)/(cells) = 1.07. Panel D shows results of RT-PCR performed on RNA from the virus particles and producer cells to detect HTLV-1 genomic gag/pol RNA. RT-PCR was carried out using primers U5-s and Gag-as as (Table 2). RNA template was omitted from the RT reaction in lanes labelled C.

These findings indicate that both tRNA-Pro and tRF-3019 are incorporated into particles released in the supernatant of HTLV-1-infected cells. Although we cannot exclude the presence of exosomes in the particle preparations, our findings demonstrate that these particles contain reverse transcriptase activity (Fig. 12) and the viral genome (Fig. 14D), and are enriched for the PBS-specific tRNA-Pro and tRF-3019. Taken together these results strongly suggest that tRF-3019 is likely to contribute to HTLV-1 reverse transcription in newly infected cells (Ruggero et al., J. Virol., in press).

2.4 DISCUSSION

Our study of small RNA libraries revealed that normal CD4+ T-cells and HTLV-1-infected cell lines produce a variety of tRFs. The greater representation of tRF-3 sequences compared to tRF-1 and tR-5 classes detected in the libraries is in line with the preponderance of tRF-3 sequences found in prostate cancer cell lines (Lee et al., 2009) and in mature B cells (Mautea et al., 2013). Previous functional studies of tRF-1001, which was abundantly expressed in our libraries, revealed its elevated expression in cancer cell lines compared to normal tissue samples and indicated that it is required for cell proliferation (Lee et al., 2009). Among the tRF-3 sequences abundantly expressed in the four libraries, functional data are available for tRF-3018 in the context of B-cells. This tRF, named CU1276 in the B-cell study, was differentially expressed in different stages of B-cell maturation, with greatest expression found in the germinal center (GC) stage and absence in GC-derived lymphoma cells. Functional studies of tRF-3018/CU1276 verified its ability to associate with Argonaute proteins and repress expression of RPA1, a protein involved in DNA replication and repair (Mautea et al., 2013).

The present study focused on tRF-3019, as it corresponds to the 3' end of tRNA-Pro, which is considered to be the primer for HTLV-1 reverse transcriptase (Seiki et al., 1982). tRF-3019 was capable of priming HTLV-1 reverse transcription and was detected in virus particles. Taken together, these observations support a role for tRF-3019 in the life cycle of HTLV-1.

As shown in Figure 12, 12 of the 18 nucleotides of tRNA-Pro that are complementary to the HTLV-1 PBS are based-paired in the mature tRNA. This

positioning of the primer portion of the tRNA in a closed stem is a characteristic of all retroviral tRNA primers. These hydrogen bonds must be disrupted in order for the primer to bind to the PBS, which would not be necessary if a tRF is used as a primer.

The use of a tRNA as a primer of reverse transcription is a property of all retroviruses. The libraries examined in the present study contained a few sequence reads for tRF-3015, which represents the 3' end of tRNA-Lys, the primer for HIV-1. This tRF was identified in HIV-1-infected cells by Yeung et al. (2009). However, the investigators claimed that it was derived from a hybrid between HIV RNA and the 3' end of tRNA-Lys and provided evidence that it functioned to silence HIV-1 expression through an siRNA-like mechanism (Yeung et al., 2009).

Schopman et al. pointed out the possibility that tRFs may serve as primers for reverse transcriptase (Schopman et al., 2010), but also presented experimental evidence from studies of HIV-1 that did not support this proposal. Efficient HIV-1 reverse transcription requires interactions of tRNA-Lys with the PBS as well as other regions of the viral genome. Of particular importance is an 8-nt sequence termed the primer activation signal (PAS) located in the U5 region that binds to the third stem-loop (T-arm) of tRNA-Lys and promotes initiation of reverse transcription and elongation of the cDNA (Abbink et al., 2008). Although all retroviruses are predicted to contain a PAS (Beerens et al., 2002), the putative PAS in HTLV-1, which is positioned approximately 10 nucleotides 5' to the PBS, has not yet been functionally characterized.

The secondary structure of the tRNA primer must also be disrupted to allow nucleotides in the T-arm to interact with the primer activation signal (PAS). In HIV-1 the NC protein plays an important role in unfolding tRNA-Lys to allow its binding

to the HIV-1 PAS (Beerens et al., 2013). Interestingly, a study of NC proteins from several retroviruses indicated that the HTLV-1 NC protein possesses comparatively weak nucleic acid chaperone activity (Stewart-Maynard et al., 2008). It is possible that another mechanism is responsible for unfolding of tRNA-Pro or that the PAS interaction is not important to HTLV-1; in alternative tRF-3019 may serve as the major primer.

In fact, our *in vitro* assay showed that tRF-3019 permits reverse transcription of a segment of HTLV-1 RNA containing the PBS and predicted PAS. The detailed picture of the interactions between HIV-1 RNA elements and its tRNA primer raises the possibility that tRFs representing the 3' end of primer tRNAs might support the initiation of reverse transcription but not progressivity, with failure to proceed to the strand transfer step. In this case, tRF-3019 might inhibit the overall process of reverse transcription, thus acting as a restriction factor for HTLV-1 replication. Further studies will be necessary to test these hypotheses by comparing the ability of tRF-3019 and tRNA-Pro to prime and support strand transfer.

3 miR-34a and the p53 pathway in HTLV-1-infected cells

3.1 Background and aims of the study

The studies described in this section were built upon results of a previous analysis of microRNA expression in ATLL samples vs. normal CD4 T-cells carried out using microarrays. This analysis revealed 21 downregulated microRNAs and 6 upregulated microRNAs in the ATLL samples (Table 3). The list of upregulated microRNAs included miR-34a.

Table 3. Differentially expressed microRNAs in ATLL cells vs. control CD4+ cells

Downregulated microRNAs	Log2 fold change	q value (%)
miR-31	-8.53	0
miR-146b	-6.93	0
miR-194	-6.89	0
miR-193b	-6.76	0
miR-192	-6.73	0
miR-125b	-5.19	0
miR-125a	-5.02	0
miR-30b	-4.73	0
miR-212	-4.48	4.9
miR-99a	-4.41	4.9
miR-363	-3.01	4.9
miR-28	-2.43	0
miR-142-5p	-1.66	4.9
miR-29c	-1.43	4.9
miR-101	-1.42	4.9
miR-20b	-1.33	4.9
miR-26a	-1.20	0
miR-140	-1.18	0
Let-7g	-1.18	0
Let-7e	-1.15	0
miR-26b	-1.05	0

Upregulated microRNAs	Log2 fold change	q value (%)
miR-15b	1.48	0
miR-146a	1.85	0
miR-130b	2.17	0
miR-34a	2.49	0
miR-130a	5.33	0
miR-451	7.24	0

Reported are differentially expressed microRNAs identified through an analysis of 6 ATLL samples and 4 samples of resting CD4+ cells obtained using Agilent microRNA arrays. The *samr* package for R software from Bioconductor was employed, considering a false discovery rate threshold of 5%. Data are from the doctoral thesis of K. Ruggero.

Table 4 reports results of an analysis to identify differentially expressed known microRNAs in the small RNA libraries derived from normal CD4+ cells and HTLV-1-infected cell lines C91PL and MT-2 described in Section 2. We observed significant upregulation of miR-34a and downregulation of miR-146b and miR-150 in both infected cell lines compared to the controls.

Table 4. Differentially expressed microRNAs in infected cell lines vs. normal CD4+ T- cells

MT-2 vs. CD4	Log2 fold change	P value
miR-34a-5p	**6.15**	**0.00015**
miR-4448	4.89	0.00366
miR-7-5p	4.35	0.01564
miR-150-5p	**-10.08**	**0.00327**
miR-30c-5p	-8.05	0.01329
miR-146b-5p	**-7.52**	**0.03318**
miR-29c-3p	-6.87	0.03802

C91PL vs. CD4	Log2 fold change	P value
miR-34a-5p	**6.66**	**0.00001**
miR-92b-3p	3.82	0.01313
miR-23a-3p	3.15	0.01166
miR-150-5p	**-10.61**	**0.00127**
miR-342-5p	-7.02	0.02719
miR-26a-5p	-6.84	0.00776
miR-20b-5p	-6.74	0.03088
miR-146b-5p	**-4.76**	**0.02268**
miR-19b-3p	-3.83	0.03015
miR-16b-5p	-3.65	0.02867

MicroRNAs with statistically significant differences in expression are indicated in bold type. The analysis was performed using edgeR software.

Upregulation of miR-34a in ATLL cells and HTLV-1-infected cell lines was intriguing to us for 2 reasons: (i) as described below, miR-34a is considered to be a tumor suppressor; and (ii) p53, which is known to upregulate expression of miR-34a, is either inactive or mutated in HTLV-1-infected cells (see below).

qRT-PCR to detect miR-34a in 10 ATLL samples, in 3 chronically HTLV-1-infected T-cell lines, in 3 uninfected T-ALL cell lines, and in 11 resting CD4+ samples confirmed significant upregulation of miR-34a in all ATLL samples and the 3 infected cell lines (Figure 15). To our knowledge, this is the first description of upregulated miR-34a expression in a T-cell malignancy.

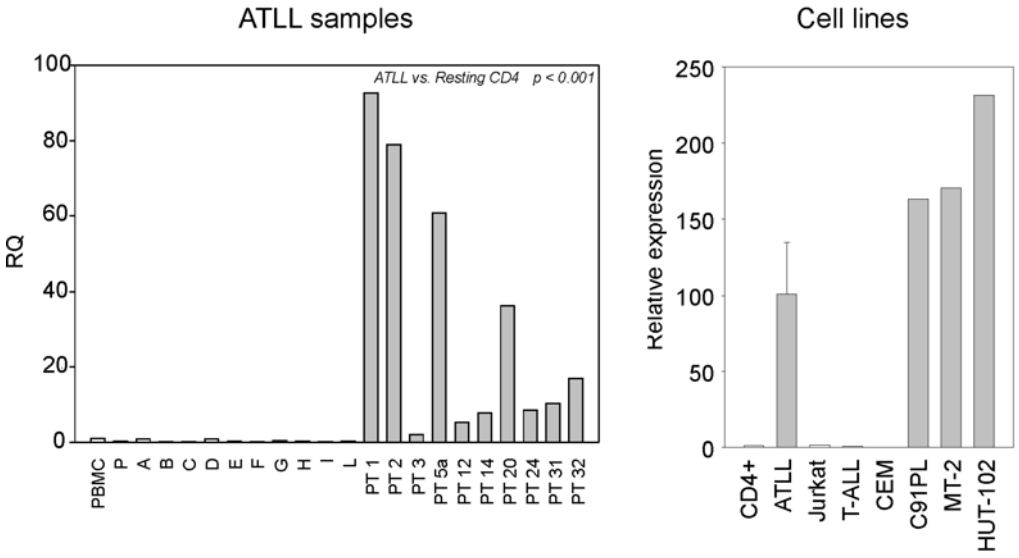


Figure 15. Quantitative RT-PCR to detect miR-34a in ATLL samples and cell lines. The left-hand panel shows qRT-PCR analyses carried out on 11 resting CD4+ controls (A-L and P) and 10 ATLL samples. Mean RQ values for ATLL samples and CD4+ T-cell controls were 31.91 and 0.316, respectively. The p value was calculated using the Mann-Whitney rank-sum test. The right-hand panel shows relative expression of miR-34a in T-cell lines. RQ values were normalized against the mean RQ of the microRNA measured in 11 CD4+ samples. The normalized mean expression levels of the microRNAs in the 10 ATLL samples are shown for comparison, with the standard error indicated. Cell lines C91PL, MT-2 and HUT-102 are chronically infected with HTLV-1. Cell lines Jurkat, T-ALL and CEM are derived from T- acute lymphoblastic leukemia and are not infected. Data are from the doctoral thesis of K. Ruggero.

miR-34a is known to silence the expression of SIRT1, a protein that inhibits the function of p53 by deacetylating it at crucial residues (see section 3.1.3.1) (Jang et al., 2011; Yamakuchi, 2012). Although one would expect SIRT1 to be silenced in cells expressing high levels of miR-34a, a recent report demonstrated that SIRT1 is upregulated in ATLL cells and HTLV-1-infected cell lines (Kozako et al., 2012). This finding suggested to us that miR-34a, although abundant, might not be functional in these cells. Experiments described in below tested whether treating HTLV-1-infected cells with drugs that activate p53 might also affect the function of miR-34a.

3.1.1 p53 structure and function

p53 is a 393-amino acid protein, with an apparent size of 53 kDa. It is encoded on the short arm of chromosome 17 (17p13.1) (Miller et al., 1986; McBride et al., 1986). The tetrameric form of p53 is a multidomain transcription factor, which binds to specific DNA response elements. p53 is integrated in various signaling networks by a multitude of protein–protein interactions, and is controlled by extensive posttranslational modifications (Hupp et al., 1994; Hupp et al., 1995; Tidow et al., 2007).

Under normal circumstances, p53 expression is maintained at low levels by the E3 ubiquitin ligase, murine double minute 2 (Mdm2). Mdm2 (also named Hdm2 in humans) binds p53 with high affinity in specific regions that are important for protein stabilization, activation, and retention within the cytoplasm (Vassilev et al., 2004). Mdm2-mediated ubiquitination targets p53 for nuclear export and proteasomal degradation (Honda et al., 1997). p53 indirectly regulates itself by transcriptionally inducing expression of Mdm2. This feedback system leads to low levels of cellular p53, so that inappropriate p53-mediated cell cycle arrest and apoptosis are prevented (Momand et al., 1992; Barak et al., 1993; Wu et al., 1993).

Activation of p53 is influenced by variety of stimuli including ionizing radiation (Kastan et al., 1991), UV radiation (Maltzman et al., 1984; Murphy et al. 2002), hypoxia (Hammond et al., 2002; Liu et al,. 2006) and reactive oxygen species (Messmer et al., 1996; Kim et al., 2002). Cellular stress signals result in upregulation of p53 and stimulate protein kinases that phosphorylate p53, which interferes with its binding to Mdm2 (Adler et al., 1997; Inoue et al., 2001). p53 then accumulates in the nucleus and induces cell cycle arrest and apoptosis if the cell is unable to repair

damaged DNA (Ko et al., 1996). Nuclear localization signals (NLS) and a nuclear export signal (NES) control the nuclear/cytoplasmic partitioning of p53. Three putative NLS domains, NLSI, NLSII and NLSIII have been identified on the C-terminus of p53 (Shaulsky et al., 1990; Ko et al, 1996; Mesaeli et al., 2004). NLSI (a.a.316–321) is the most conserved across different species and is mainly responsible for translocation of p53 to the nucleus (Dang et al., 1989; Shaulsky et al., 1990). Defects in p53 localization to the nucleus can lead to tumor formation (Schlamp et al., 1997; Mesaeli et al., 2004).

p53 is either mutated or functionally inactivated in the majority of cancers (Hollstein et al., 1991). ATLL is characterized by inactivation of p53 either as a result of inactivating mutations or more often through other mechanisms that lead to accumulation of the protein in an inactive state (Yamada et al., 2005; Tabakin-Fix et al., 2005). Tax plays an important role in p53 inactivation (Pise-Masison et al., 1998; Jeong et al., 2004).

3.1.2 p53 regulatory pathways and downstream targets

Key targets of p53 include genes for the cell cycle regulator and p21 (Wang et al., 2008; Millau et al., 2009; Poulsen et al., 2013) and for multiple pro-apoptotic proteins, such as; Bax, Noxa, Puma, Bid, Fas, DR5, APAF-1, p53AIP1, TP53INP1, FADD and FOXO1A (Miyashita et al., 1995; Wu et al., 1997; Muller et al., 1998; Oda et al., 2000; Moroni et al., 2001; Nakano et al., 2001; Sax et al., 2002; Fuhrken et al., 2007). As described above, p53 also targets its antagonizer Mdm2 (Toledo et al., 2007). Interestingly, p21 binds caspase-2 (Baptiste-Okoh N et al., 2008) and procaspase-3 (Suzuki A et al., 1999) with the effect of reducing apoptosis.

Recent discoveries revealed that p53 directly and indirectly regulates the expression of numerous genes via regulation of microRNAs in various cell types. These include miR-34a, miR-15a, miR-16-1, miR-23a, miR-26a, miR-103, miR-143, miR-145, miR-203, miR-206 and mir-605 (Suzuki et al., 2009; Xiao et al., 2011). p53-mediated microRNA regulation affects multiple cellular processes including cell cycle progression, migration, epithelial–mesenchymal transition, stemness, metabolism, differentiation and cell survival (Hermeking et al., 2012).

3.1.2.1 MDM2

MDM2/HDM2 is located on chromosome 12q13. As stated above, Mdm2 is a ring finger E3-ubiquitin ligase that acts as the predominant negative regulator of p53 (Oliner et al., 1993; Haupt et al., 1997; Momand et al., 2000, Sosin et al., 2012). Overexpression of MDM2 is associated with cancer development and progression in several tumor types and is often found in hematological malignancies including B-cell chronic lymphocytic leukemia (B-CLL) and non-Hodgkin's lymphoma (B-NHL) (Watanabe et al., 1994).

Mdm2 also affects the cell cycle, apoptosis and tumorigenesis through interactions with other molecules, such as ribosomal proteins, including ribosomal protein L26 (RPL26). RPL26 modulates Mdm2–p53 interactions by forming a ternary complex, which stabilizes p53 through inhibiting the ubiquitin ligase activity of Mdm2 (Zhang et al., 2010). Interestingly, Mdm2 can also ubiquitinate itself, which leads to its proteasomal degradation (Fang et al., 2000; Honda et al., 2000). Reports suggest that microRNAs play an important role to reactivate the p53 pathway in cancer cell types via repressing MDM2 expression or destabilizing Mdm2-p53 interactions. Mir-605 inhibits the p53-Mdm2 interaction by reducing

MDM2 expression; in a positive feedback loop, p53 regulates miR-605 expression (Xiao et al., 2011). Pichiorri et al. reported that miR-192, 194, and 215 are induced by p53 and that these microRNAs may affect the expression of MDM2 in myeloma cells (Pichiorri et al., 2010). Mir-143 and miR-145 are posttranscriptionally activated by upregulated p53. In vitro and in vivo, over expression of mir-143/mir-145 suppresses cellular growth and triggers the apoptosis of epithelial cancer, by enhancing p53 activity via MDM2 turnover (Zhang et al., 2013).

miR-18b stabilizes p53 in melanoma cells by targeting MDM2 (Dar et al., 2013). Expression of MDM2 and MDM4 are controlled by miR-661 (Hoffman et al., 2013).

Overexpression of MDM2 is associated with cancer development and progression in several tumor types and is often found in hematological malignancies including B-cell chronic lymphocytic leukemia (B-CLL) and non-Hodgkin's lymphoma (B-NHL) (Watanabe et al., 1994).

3.1.2.2 P21/CDKN1A

p21 (also called WAF1) is encoded by the CDKN1A gene located on chromosome 6 (6p21.2). p21 is a 165-amino acid residue protein, which belongs to the Cip/Kip family of cyclin-dependent kinase (cdk) inhibitors (Gu et al., 1993; Gartel et al., 1996, Abbas et al., 2009). p21 binds to and inactivates cyclin-CDK2 and -CDK4 complexes (Gu et al., 1993; He et al., 2005). This blocks the transition from G1 to S-phase and inhibits cell proliferation (Waldman et al., 1995; Knights et al., 2006; Noske et al., 2009). p21 is induced by p53 in response to DNA damage or

other stress signals and represents a surrogate marker for p53 activation (Suzuki et al., 2012; Kim et al., 2013).

p21/CDKN1A expression is controlled by various microRNAs including the miR-17-92 polycistron (Inomata et al., 2009), miR-106b and miR-93 (Ivanovska et al., 2008; Petrocca et al., 2008).

3.1.2.3 TP53INP1

TP53INP1 is a key participant in p53-mediated cell cycle arrest and death. TP53INP1 is a tumor suppressor gene located on the chromosome 8q22 (Nowak et al., 2002; Jiang et al., 2006a). The gene encodes two protein isoforms, TP53INP1α and TP53INP1β, which interact with p53 and modify its transcriptional activity of p53 on some target genes, such as CDKN1A and MDM2. This leads to cell cycle arrest in the G1-phase (D'Orazi et al., 2002; Tomasini et al., 2003; Ito et al., 2006).

TP53INP1 is down regulated in various types of cancer such as gastric, pancreatic (Jiang et al., 2006b; Gironella et al., 2007), liver (Ma et al., 2010), and breast cancer (Ito et al., 2006; Yamamoto et al., 2011). However, it is highly expressed in prostate cancer and anaplastic carcinoma of the thyroid (Ito et al., 2006). TP53INP1 expression is controlled by miR-155 in pancreatic cancer (Gironella et al., 2007; Seux et al., 2011) and by miR-130b and miR-93 in HTLV-1-transformed cells (Yeung et al., 2008). (See Section 1.4.1.5).

3.1.3 miR-34a and its downstream targets

The miR-34a gene is located on chromosome 1p. It was discovered as a tumor suppressor gene in neuroblastoma (Welch et al., 2007). Reduced levels of

miR-34a expression are also found in other tumors such as colon cancer, ovarian cancer, prostate cancer, liver cancer, chronic lymphocytic leukemia (CLL). Lodygin et al. reported downregulation of miR-34a in cancer due to aberrant CpG methylation of the promoter of miR-34a (Lodygin et al., 2008). p53 regulates the expression of miR-34a through binding to its promoter. Functionally active p53 is directly associated with miR-34a upregulation. The functional inactivation or mutation of p53 that characterizes more than 50% of cancers is a another potential cause of downregulation of miR-34a in transformed cells. Ectopic expression of miR-34a induces cell cycle arrest in the G1 phase and apoptosis in several experimental systems. Many potential targets of miR-34a have been identified (Yamakuchi et al. 2008; Hermeking, 2010; Atchison et al., 2011; Yamakuchi et al., 2012). The following sections summarize information on targets examined in the present study: SIRT1, SP1, CDK4, VEFGA, BIRC5, YY1, BCL2, MYC, and Notch pathway components.

3.1.3.1 SIRT1

Sirt1 is an NAD-dependent deacetylase that plays an important role in the maintenance of homeostasis and cell survival (Milner. 2009). Sirt1 deacetylase activity has been well documented in the regulation of several stress-induced transcription factors including p53 (Luo et al., 2001; Vaziri et al., 2001), HSF1 (Westerheide et al., 2009; Raynes et al., 2013) and NF-κB (Yeung et al., 2004; Salminen et al., 2008). Sirt1 is a 747–amino acid protein that contains 2 pairs of nuclear localization and nuclear export sequences that allow it to shuttle between the cytosol and nucleus (Lee et al., 2013).

Altered Sirt1 activity is associated with variety of human diseases such as metabolic syndrome, inflammation, neurodegeneration, and cancer (Saunders et al., 2007; Haigis et al., 2010; Stunkel et al., 2011; Yamakuchi et al., 2012). Recent reports suggest that SIRT1 may play dual roles in cancer promotion and suppression, depending on tissue contexts and the temporal and spatial distribution of SIRT1 upstream and downstream factors (Saunders et al., 2007; Yuan et al., 2013). Several studies found deregulation of Sirt1 in various tumor entities including breast cancer (Lee et al., 2011), ovarian epithelial tumours (Jang et al., 2009), non-small cell lung cancer (Noh et al., 2013), gastric carcinoma (Cha et al., 2009), thyroid cancer (Herranz et al., 2013), prostate cancer (Wang et al., 2011; Herranz et al., 2013), colorectal cancer (Nosho et al., 2009), and hepatocellular carcinoma (Choi et al., 2011).

Overexpression of SIRT1 is also reported in many leukemia/lymphoma cell types including B-cell lymphoma (Jang et al., 2008), acute myelogenous leukemia (Bradbury et al., 2005), chronic myelogenous leukemia (Li et al., 2012; Wang et al., 2013), and ATLL (Kozako et al., 2012). In HTLV-1 infected cells, SIRT1 knockdown induces apoptosis via activation of caspase-3 and PARP (Kozako et al., 2012).

miR-34a is a negative regulator of SIRT1 in various cancer cell types (Yamakuchi et al., 2008; Lou et al., 2013; Yuan et al., 2013). miRNA-29c is also reported to target oncogenic SIRT1 in hepatocellular carcinoma (Bae et al., 2013).

3.1.3.2 SP1

Sp1 is a ubiquitously expressed transcription factor that plays an important role in the regulation of a numerous genes (Black et al., 2001; Chu, 2012). Sp1 overexpression is reported in various cancer cell types including cancer of the breast (Zannetti et al., 2000), thyroid (Chiefari et al., 2002), liver (Lietard et al., 1997), pancreas (Abdelrahim et al., 2004), colon-rectum (Hosoi et al., 2004), stomach (Wang et al., 2003), and lung (Kong et al., 2010).

Sp1 can bind to at least 12,000 sites in the human genome (Cawley et al., 2004). Sp1 has been reported to control cell cycle progression and arrest (Abdelrahim et al., 2002), both pro- and anti-apoptotic factors, proteins involved in genomic stability (Kavurma et al., 2001; Kavurma et al., 2003), proto-oncogenes (e.g. *c-myc*) and tumor suppressors (e.g. p53) (DesJardins et al., 1993; Olofsson et al., 2007; Li et al., 2010).

Sp1 expression is controlled by numerous microRNAs that are linked to cancer including mir-29b (Garzon et al., 2009), miR-149 (Wang et al., 2013) and miR-34a and miR-93 (Li et al., 2011).

3.1.3.3 CDK4

In mammalian cells, the cell cycle is governed by two key classes of molecules, the regulatory cyclins and the catalytic CDKs that form active heterodimers leading to phosphorylation of target proteins. CDK4 and its close homolog CDK6 are serine/threonine kinases that form heterodimers with D-type cyclins and are central regulators of the G1–S transition of the cell cycle (Sheppard et al., 2013). Cyclin E–CDK2 complexes and cyclin D–CDK4/6 complexes together

phosphorylate the retinoblastoma protein (RB1), resulting in dissociation and thereby activation of E2F transcription factors and initiation of the S phase gene expression program (Malumbres et al., 2009). CDK4/6-mediated deactivation of RB1 is critical for cell-cycle progression. Tumor suppressor $p16^{INK4A}$ inhibits the assembly and activation of cyclin D-CDK4/6 complexes and thereby represses RB (Li et al., 1994).

CDK family members are activated by numerous oncogenic viruses including Kaposi's sarcoma-associated herpesvirus (KSHV) and HTLV-1 (Godden-Kent et al., 1997; Haller et al., 2002). Haller et al. (2002) reported that HTLV-1 Tax enhanced the cell cycle in the G_1 phase by modulating activaty of CDK4 and CDK6 holoenzyme complexes.

CDK4 has been shown to be regulated by miR-34a in prostate tumor cell lines (Fujita et al., 2008; Navarro et al., 2009; Hou et al., 2013).

3.1.3.4 VEGFA

VEGF-α is a 45-kDa homodimeric glycoprotein with a diverse range of angiogenic activities. It belongs to the VEGF-related gene family of angiogenic and lymphangiogenic growth factors which comprises six secreted glycoproteins (Ferrara et al., 2003; Gorski et al., 2003; Hicklin et al., 2005). VEGF-α plays an important role in a number of postnatal angiogenic processes such as wound healing, ovulation, menstruation, maintenance of blood pressure, and pregnancy (Brown LF et al., 1992), and it has also been linked to several pathologic conditions associated with increased angiogenesis, including cancer, arthritis, psoriasis, macular degeneration, and diabetic retinopathy (Inoki I et al., 2002; Ferrara et al., 2003; Hicklin et al., 2005).

The VEGFA gene is highly expressed in many cancer types, including cervical cancer (Zhu et al., 2013), lung cancer (Liu et al., 2009), liver cancer (Bi et al., 2012), cutaneous squamous cell carcinoma (Kanitz et al., 2012), colon cancer (Yamakuchi et al., 2011), and breast cancer (Cascio et al., 2010; Zhu et al., 2011). VEGF-α may participate in cell growth and angiogenesis in ATLL (Bazarbachi et al., 2004). VEGF-α is also potent competitor of HTLV-1 SU binding in HTLV-1 infected cells (Jones et al., 2008; Lambert S et al., 2009).

Several microRNAs reduce cell proliferation, motility, and angiogenesis by inhibiting the expression of VEGFA, including miR-20b (Cascio et al., 2010), miR-22 (Yamakuchi et al., 2011), miR-29a/b/c (Yang et al., 2013), miR-34a (Kumar et al., 2012), miR-125a (Bi et al., 2012), miR-126 (Liu et al., 2009), miR-128 (Shi et al., 2012), miR-192 (Geng et al., 2013), miR-199a-5p (Hsu et al., 2013), miR-200b (Choi et al., 2011), miR-200c (Chuang et al., 2012), miR-203 (Yang et al., 2013; Zhu et al., 2013), miR-297, miR-299 (Jafarifar et al., 2011) and miR-361-5p (Kanitz et al., 2012).

3.1.3.5 Survivin (BIRC5)

Survivin is a member of the inhibitor-of-apoptosis proteins (IAPs) family that is encoded by the BIRC5 gene, located on chromosome 17q25. It is a 16.5-kDa protein that contains a single 70-amino acid BIR (baculovirus repeat) domain and an extended α-helical coiled-coil C-terminus (Ambrosini et al., 1997; Waligórska-Stachura et al., 2012; Cheung et al., 2013). Survivin exhibits multiple pro-mitotic and anti-apoptotic functions that are conferred by differences in subcellular localization, phosphorylation, and acetylation (Cheung et al., 2013; Coumar et al., 2013). Survivin is ubiquitously distributed during embryonic and fetal developmental stages.

Survivin is overexpressed in various types of cancers including lung cancer (Kapellos et al., 2013), prostate cancer (Kishi et al., 2004), gastric carcinoma (Lu et al., 1998), colon cancer (Hernandez JM et al., 2011), bladder cancer (Margulis et al., 2008; Jeon et al., 2013) esophageal cancer (Kato et al., 2001), osteosarcomas (Trieb et al., 2003; Osaka et al., 2006), diffuse large B-cell lymphomas (Adida et al., 2000) and ATLL (Pise-Masison et al., 2009). Overexpression of survivin is associated with a poor prognosis and decreased survival rates in breast cancer (Nassar et al., 2008; Jha et al., 2012), oral carcinoma (Freier et al., 2007) and colorectal carcinoma (Sarela et al., 2000).

p53 suppresses BIRC5 expression, thereby favoring apoptosis (Waligórska-Stachura et al., 2012). Several microRNAs also inhibit expression of survivin in various cell types, including miR-203 (Wei et al., 2013), miR-218 (Alajez et al., 2011), miR-542-3p (Yoon et al., 2010) and miR-34a (Cao et al., 2013).

3.1.3.6 YY1

Yin Yang 1 (YY1) is a ubiquitous and multifunctional zinc-finger transcription factor with diverse and complex biological functions (Zhang et al., 2011; Atchison et al., 2011). YY1 influences transcriptional regulation, cell growth, apoptosis, large-scale chromosomal dynamics, X-chromosome inactivation and DNA repair, and it can act as a transcriptional activator, repressor, or initiator protein depending upon DNA binding site context or cell type (Galvin et al., 1997; Gordon et al., 2006; Atchison et al., 2011). One of its major roles is to recruit the Polycomb repressive complex (PRC) to DNA. Interestingly, YY1 is also a negative regulator of p53 via regulating Mdm2-mediated p53 ubiquitination through a direct physical

interaction mechanism, which reveals an important role in tumorigenesis (Sui et al., 2004).

Expression of YY1 has been reported to vary in different cell contexts, and some cancers show increased expression while other show reduced expression. Overexpression of YY1 is observed in cancers of the prostate, colon, breast, bone, liver, lung, bladder, cervix, skin, and blood (includes B and T acute lymphoblastic leukemia, diffuse large B-cell lymphoma, acute myeloid leukemia, chronic myeloid leukemia, chronic lymphocytic leukemia, Hodgkin lymphoma, Burkitt lymphoma, mantle cell lymphoma, and follicular lymphoma) (Erkeland et al., 2003; Pilarsky et al., 2004; de Nigris et al., 2006; Chinnappan et al 2009; Deng et al., 2009; Zaravinos et al., 2010; Castellano et al., 2010; Atchison et al., 2011). A recent study revealed that YY1 is important in the polycomb-mediated silencing of miR-31 expression in ATLL cells (Yamagishi et al., 2012) (see Section 1.4.1.5).

microRNAs that target and suppress the expression of YY1 include miR-7 (Zhang et al., 2013) and miR-34a (Chen et al., 2011).

3.1.3.7 BCL2

Bcl2 and its related family members play an important role in apoptosis. Abnormal regulation of Bcl2 (B-cell lymphoma/leukemia-2) family members is a frequent characteristic of malignant diseases and is associated with resistance to therapy (Frenzel et al., 2009; Willimott et al., 2010). The Bcl2-family includes proteins that promote cell survival such as Bcl-2, Bcl-XL, Mcl-1, A1, Bcl-W and others that promote cell death e.g. Bax, Bak, Bcl-XS, and Bok. The equilibrium between these pro- and anti-apoptotic proteins influences the susceptibility of cells to

a death signals (reviewed by Burlacu, 2003; Christodoulou et al., 2013). Bcl-2 is the prototypic pro-survival protein and was discovered by cloning of chromosomal translocations from cases of the human B-cell non-Hodgkin's lymphoma, follicular lymphoma (Willimott et al., 2010). Overexpression of the Bcl-2 protein is reported in many types of human cancers, including leukemias, lymphomas, and carcinomas (Sánchez-Beato et al., 2003). p53 is a negative regulator of BCL2 (Basu et al., 1998; Wu et al., 2013). Inactivation or mutation of p53 associated with upregulation of BCL2 expression drives cell survival pathway in cancers.

Various microRNAs directly or indirectly suppress the expression of BCL2. Willimott et al demonstrated that miR-125b and miR-155 repressed Bcl-2 mRNA expression, in human leukemic B-cells (Willimott et al., 2011). miR-15a, miR-16, miR-24-2, miR-30b, miR-34a, miR-125b, miR-129, miR-155, miR-181b, miR-182, miR-184, miR-195, miR-196b, miR-205, mR-210, miR-365-2, miR-449a, miR-497, miR-503 and miR-708, other microRNAs are validated to target and suppress the expression Bcl2 in various cancer types. miR-31 indirectly downregulates BCL2 expression via direct targeting of PRKCE (Koerner et al., 2013)

3.1.3.8 MYC

MYC, a proto-oncogene located on chromosome 8q24, encodes an evolutionarily conserved basic helix-loop-helix leucine zipper transcription factor that is commonly dysregulated in cancer. Deregulation of MYC results pleiotropic effects on cancer cell growth, proliferation, survival, angiogenesis, and metastasis. c-Myc is a widely expressed transcription factor. Myc uses distinct mechanisms for activating and repressing gene expression, and it is known that Myc binds to approximately 10–15% of genes, whether protein-encoding or transcribing non-

coding RNA (reviewed by Bui et al., 2010; Tripaldi et al., 2013). As a transcriptional regulator, Myc dimerizes with its binding partner Max and binds to genomic DNA directly upstream or within the first introns of target genes (Blackwood et al., 1991; Zeller et al., 2006; Chang et al., 2008).

In 2005, O'Donnell et al demonstrated that Myc directly activates transcription of the polycistronic miR-17-92 cluster by binding directly to an E-box within the first intron of the gene encoding the miR-17-92 primary transcript (O'Donnell et al., 2005). The miR-17-92 cluster consists of miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92a-1. Chang et al. observed that overexpression of Myc represses the maturation of Let-7 microRNA without affecting transcription of the pri-miRNA (Chang et al., 2008). In liver cancer cell lines, Myc upregulates miR-371-3 and inhibits miR-100, let-7a-2 and miR-125b-1. let-7, miR-23, miR-26, miR-29, and miR-30 families, and miR-150 were negatively regulated by Myc in hepatoblastoma (Buendia et al., 2012). Upregulation of miR-34a targets Myc expression and controls the cell cycle (Christoffersen et al., 2010).

3.1.3.9 Notch signaling pathway components

The Notch signaling pathway plays a vital role in the regulation of multiple cellular processes such as proliferation, differentiation and apoptosis. It is implicated in the maintenance of self-renewal potential in stem cells, binary cell-fate determination in progenitor cells, and induction of terminal differentiation in proliferating cells (Artavanis-Tsakonas et al., 1999; Katoh et al., 2006) In mammalian cells, there are four Notch receptors; NOTCH-1, NOTCH-2, NOTCH-3 and NOTCH-4, and five ligands; Jagged 1 (JAG1), Jagged 2 (JAG2), DLL1, DLL3 and DLL4 (reviewed by Katoh et al., 2007; Kume, 2009).

In many solid tumors and hematological malignancies, the expression of Notch receptors, ligands and target genes is deregulated (reviewed by Ranganathan et al., 2011; Jonusiene et al., 2013).

miR-34a can affect the regulation of Notch signaling by repressing the expression of NOTCH1, JAG1 and DLL1.

3.1.4 Modulation of p53 activity

The presence of functionally inactivated or mutant p53 is a major cause of resistance of tumor cells to death signals and makes them less sensitive or resistant toward chemotherapeutic agents and radiotherapy. Several strategies to activate p53 or 'cure' mutated p53 have been described (Wiman, 2006).

3.1.4.1 Nutlin-3a

A number of compounds target the p53-Mdm2 interaction, most notably RITA and Nutlin-3a (Issaeva et al., 2004; Shen et al., 2011). RITA (Reactivation of p53 and Induction of Tumor cell Apoptosis) binds to wild-type p53 and prevents its interaction with Mdm2, resulting in accumulation of p53. RITA induces p53 target gene expression and triggers massive apoptosis in various tumor cell types (Issaeva N et al., 2004).

Nutlin-3a is one of several cis-imadazoline analogues ('Nutlins') that were identified in a screen of synthetic chemicals for inhibitory effects upon p53-Mdm2 binding (Vassilev et al., 2004). Nutlin-3a competitively occupies the p53 binding site on Mdm2, which results in an increase in p53 activation (Vassilev et al. 2004; Ohnstad et al., 2011; Manfe et al., 2012). Nutlin-3a is non-genotoxic and appears to

be highly selective for the p53-Mdm2 binding site on Mdm2 with no direct activity on other proteins (Vassilev 2005; Shen et al., 2011) or upon MdmX (Hu et al., 2006). Antagonism of Mdm2 by Nutlin causes a post-translational increase in the activity of p53, p21, Mdm2 and the BH3 proteins, Noxa and Puma (Shen et al., 2011). The overall effect of p53 activation by Nutlin is to induce apoptosis and cause an increase in cell cycle arrest in the G1/S and G2/M phases. Nutlin-3a induced cell cycle arrest at the G1/S and G2/M boundaries and the depletion of S-phase cells in wild-type p53 cancer cell lines from different tumor types, including colon, breast, lung, prostate, melanoma, osteosarcoma, and renal cancer (Tovar et al., 2006). A study by Hasegawa et al. (2009) showed that Nutlin-3a induced cell-cycle arrest or apoptosis in ATLL cell lines containing wild-type p53 but did not affect cell lines containg mutated p53.

3.1.4.2 Etoposide

Etoposide (VP-16, 4′-dimethylepipodophylloxin-9-[4,6-O-ethylidene-beta-D-glucopyranoside]) is an antineoplastic drug that induces DNA damage via inhibiting topoisomerase II, which plays pivotal roles during both DNA replication and transcription. Etoposide stabilizes the complex formed by topoisomerase II and the 5′-cleaved ends of the DNA, thus forming stable (nonrepairable) protein-linked DNA double-strand breaks. Cells are able to recognize such DNA damage and, in turn, eliminate the injured cells by p53-mediated apoptosis (Karpinich et al., 2002; Grandela et al., 2008). Reports suggest that caspase-2 provides a connection between etoposide-induced DNA damage and the engagement of the mitochondrial apoptotic pathway (Robertson et al., 2002). Etoposide has been widely used for the treatment

of various types of cancers, but it also associated with increased risk of secondary leukemia (reviewed by Ratain et al., 1998; Ezoe, 2012).

3.1.4.3 Pifithrin-α

In 1999, Komarov et al. isolated a small molecule that inhibits p53 and named it pifithrin-α (Komarov et al., 1999). pifithrin-α can block p53-dependent transcriptional activity in normal cells and protect them from lethal side effects associated with anticancer treatment (Komarov et al., 1999; Strom et al., 2006; Suzuki K et al., 2011). The exact mechanism of action for pifithrin-α is not currently known. However, it has been hypothesised that pifithrin-α reduces nuclear translocation of p53 (Komarov et al., 1999).

3.1.5 Aim of the study

Differentially expressed microRNAs play important roles in the development and progression of cancers, and can act as tumor suppressors or oncogenes. Our laboratory's data from small RNA libraries, microarrays and real time RT-PCR revealed increased expression of miR-34a in ATLL samples and in HTLV-1 infected cell lines, compared to CD4+ T-cells.

p53 is known to regulate miR-34a but is functionally inactive or mutated in ATLL cells and in HTLV-1 infected cells. The experiments described below were aimed at investigating the influence of p53 on miR-34a activity in HTLV-1-infected cells.

3.2 MATERIALS AND METHODS

3.2.1 Cell culture

Cell lines C91PL and MT-2, chronically infected with HTLV-1, were maintained in as described in Section 2.2.1.

3.2.2 Drug treatments

Nutlin-3a (Sigma-Aldrich or Tocris Bioscience), Etoposide (Sigma-Aldrich) and Pifithrin-α (Sigma-Aldrich) were dissolved in dimethyl sulphoxide (DMSO; Sigma-Aldrich) to generate 10 mM stock solutions. C91PL and MT-2 cells ($3x10^5$cell/ml) were seeded in 6-well microplates (34.8-mm diameter) and exposed to Nutlin-3a (1 and 5 μM), Etoposide (5 μM), Pifithrin-α (10 μM), or DMSO (vehicle control). After 48 hours, cells were harvested for immunoblots and RNA analysis. Some cultures were also analyzed for cell death by staining with propidium iodide or Sytox Red (Invitrogen).

3.2.3 Immunoblotting

Cells to be analysed by immunoblotting were lysed in 20 mM Tris (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, 0.3% Nonidet-P40, and protease inhibitor mix (Complete, Roche). Protein concentrations were determined by a BCA assay (micro-BCA protein assay; Pierce). Samples were diluted with lysis buffer to reach equivalent total protein concentrations, denatured by adding Laemmli buffer and heated at 70°C for 5 min. Lysates were subjected to 12% (w/v) sodium dodecyl sulphate–polyacrylamide discontinuous gel electrophoresis (SDS-PAGE) and electrotransferred to Hybond-C Extra nitrocellulose membrane (GE Healthcare) for

90 minutes at 1 mAmp per cm^2 of membrane using a semi-dry blotter (GE Healthcare). Blots were blocked for 1 h in 2% blocking agent (Roche)-0.05% Tween 20-PBS, washed with 0.05% Tween 20-PBS and incubated for 90 minutes with goat anti-p53 polyclonal antibody (1:500; Santa Cruz), mouse anti-MDM2 monoclonal antibody (1:500; Santa Cruz), rabbit anti-SIRT1 polyclonal antibody (1:300; Santa Cruz), rabbit anti-YY1 polyclonal antibody (1:500; Santa Cruz) or rabbit anti-β-Actin (ACTB) polyclonal antibody (1:2000; Sigma-Aldrich) in 3% BSA-0.05% Tween-PBS followed by 45 minutes' incubation with horseradish peroxidase-conjugated anti-mouse, anti-rabbit or anti-goat secondary antibody (Pierce) diluted 1:5000 in 2% blocking agent-0.05% Tween-PBS. Blots were developed using chemiluminescence reagents (Supersignal, Pierce) and immunoreactive bands were visualized and quantified using a BioRad ChemiDoc XRS imager. Immunoblots shown in the thesis are representative of three experiments.

3.2.4 Extraction and quantification of RNA

Cells to be processed for RNA analysis were lysed in TRIZol (Invitrogen). Chloroform (200µl per 1ml of Trizol-sample) was added, mixed by vigorous shaking and incubated at room temperature for 5 minutes. Samples were then centrifuged at 12,000g for 15 minutes at 4°C to separate the mixture into a lower red phenol-chloroform phase, an interface and an upper colorless aqueous phase containing RNA. The upper aqueous phase was transferred to a 1.5-ml microfuge tube, combined with 0.7 volume isopropanol and centrifuged at 12,000g for 30 min at 4°C. The resulting RNA pellet was washed twice with 70% ethanol and centrifuged at 12,000g for 15 minutes at 4°C. The ethanol supernatant was removed and the RNA

pellet was air-dried and then resuspended in 30µl RNase free water. Samples were stored at -80°C.

To calculate the concentration and purity of RNA, samples, the absorbance was measured at 230 nm, 260 nm and 280 nm in NanoDrop 1000 Spectrophotometer V3.7 (Thermo Fisher Scientific). The wavelength 260 nm was used to measure the concentration of RNA; a wavelength of 1 being equal to 40µg/ml of RNA. A ratio of 260nm/280nm of ~2.0 is generally accepted as "pure" for RNA.

3.2.5 Elimination of contaminating DNA

RNA samples to be analysed for levels of mRNAs were combined with 0.1 volume 10X DNase I Buffer (Invitrogen) and 1 µL rDNase I (Invitrogen), mixed gently, and incubated at 37°C for 15 minutes. DNase was inactivated by adding EDTA (Invitrogen) and incubation at 70°C for 10 minutes.

3.2.6 Quantitative RT-PCR

RT-PCR for microRNAs. Total RNA was subjected to reverse transcription and quantitative PCR to detect miRNAs using Applied Biosystems Taqman microRNA assays and a 7900HT Fast Real-Time PCR System, according the manufacturer's protocol; RNU44 was used as an endogenous control. Fold changes reported in the thesis were calculated from three independent experiments. Differences in expression of miRNAs were calculated using formulas for relative quantification (RQ), where Ct is the threshold cycle.

$$\Delta Ct = Ct_{\text{miRNA of interest}} - Ct_{\text{RNU44}}$$

$$\Delta\Delta Ct = \Delta Ct_{\text{Drug treated cells}} - \Delta Ct_{\text{DMSO control}}$$

$$RQ = 2^{-\Delta\Delta Ct}$$

RT-PCR for mRNAs. For quantitative RT-PCR of MDM2, CDKN1A, TP53INP1, BCL2, BIRC5, CDK4, MYC, DLL1, SP1, JAG1, SIRT1, YY1 and VEGFA, total RNA was reverse transcribed using SuperScript II reverse transcriptase (Invitrogen) and random hexamers according to the manufacturer's protocol. Aliquots of the resulting cDNA were PCR-amplified by using specific primers listed in Table 5 and a PCR master mix containing SYBR Green (Roche or Thermo Scientific). The PCR reactions were performed in a LightCycler 480 (Roche) thermal cycler according the manufacturer's protocol; ACTB was used as an endogenous control. Fold changes reported in the thesis were calculated from three independent experiments. Differences in expression of miRNAs were calculated using formulas for relative quantification (RQ), where Cp the 'crossing point' indicated in the Light cycler analysis.

$$\Delta Cp = Cp_{\text{Gene of interest}} - Cp_{\text{ACTB}}$$

$$\Delta\Delta Cp = \Delta Cp_{\text{Drug treated cells}} - \Delta Cp_{\text{DMSO control}}$$

$$RQ = 2^{-\Delta\Delta Cp}$$

3.2.7 Primer design

Primer sequences were chosen by using the Primer3 online tool (Table 5). Gene specificity of primer sequences was confirmed with a Basic Local Alignment Search Tool (BLAST) assessment. Annealing temperatures were between 60°-62°C for all primers. Primers were purchased from Sigma-Genosys.

Table 5. Primers for RT-PCR.

Primer	Forward primer sequnce (Sense)	Reverse primer sequnce (Antisense)
MDM2	CTTCGGAACAAGAGACCCTG	TCTTTCACAGAGAAGCTTGGC
TP53INP1	CTTCCTCCAACCAAGAACCAG	CAAGCACTCAAGAGATGCCG
CDKN1A	AGACTCTCAGGGTCGAAAAC	TTCCAGGACTGCAGGCTTC
SIRT1	ACATAGACACGCTGGAACAGG	GATAGCAAGCGGTTCATCAGC
CDK4	GAAACTCTGAAGCCGACCAG	AGGCAGAGATTCGCTTGTGT
VEGFA	AAGGAGGAGGGCAGAATCATC	ACACAGGATGGCTTGAAGATG
DLL1	ACAGATTCTCCTGATGACCTC	TCACACACGAAGCGGTAGGA
SP1	ATGATGACACAGCAGGTGGAG	AGGTCTTGCCATACACTTTCC
BCL2	ATGTGTGTGGAGAGCGTCAA	GCCGTACAGTTCCACAAAGG
MYC	TGGATTTTTTTCGGGTAGTGG	CTCGTCGCAGTAGAAATACG
BIRC5	TTCTCAAGGACCACCGCATC	TGAAGCAGAAGAAACACTGGG
YY1	TCAGGGATAACTCGGCCATG	TGTGCGCAAATTGAAGTCCAG
JAG1	CGGCCTCTGAAGAACAGAAC	CAATGGGGTTTTTGATCTGG
ACTB	AGCACAGAGCCTCGCCTTTG	GGAATCCTTCTGACCCATGC

3.3 RESULTS

3.3.1 Effect of Nutlin-3a on viability of HTLV-1 infected cells.

The aim of this part of the study was to test the hypothesis that the modulation of p53 activity in HTLV-1 infected cells activates the p53 regulatory network and induces cell death. As shown in Figure 16, treatment of infected cell lines C91PL and MT-2 with nutlin-3a resulted in a dose-dependent decrease in cell viability in C91PL cells and MT-2 cells.

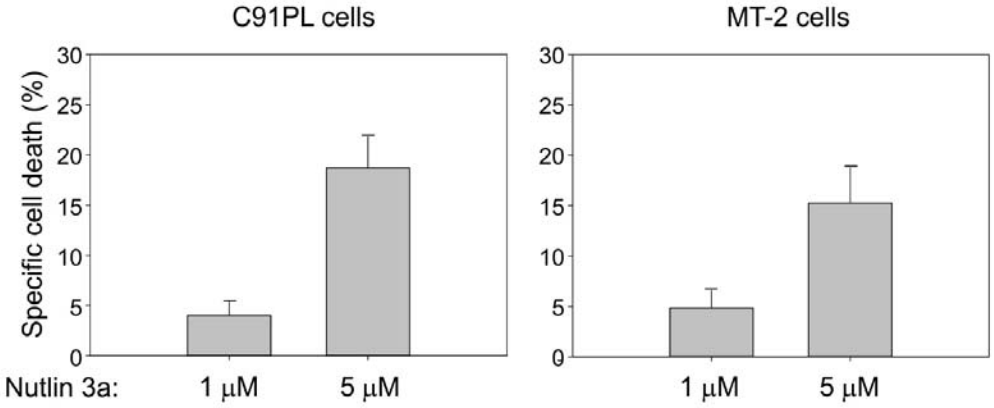


Figure 16. Cell death induced by treatment with nutlin-3a. C91PL cells and MT-2 cells that had been incubated with 1 μM or 5 μM nutlin-3a or with an equal volume of DMSO for 44-48 hrs were labelled with propidium iodide or Sytox Red for 20 min and then analysed using a BD FACSCalibur. Percentages of dead (i.e. stained) cells were used to calculate the specific cell death using the formula SCD = (% dead cells in treated culture-% dead cells in control culture/% living cells in control culture) X 100. The plots show mean SCD values with standard errors calculated from 6 experiments performed with C91PL cells and 5 experiments with MT-2 cells.

Cell-cycle analysis of C91PL cells and MT-2 cells revealed that Nutlin-3a treatment resulted in a block in the G1 phase (data not shown).

3.3.2 Nutlin-3a induces the stabilization of p53 protein in HTLV-1-infected cells.

Modulation of p53 levels by Nutlin-3a was assessed by immunoblotting. As depicted in Figure 17, we observed that Nutlin-3a upregulated expression of p53 protein in a dose-dependent manner in the HTLV-1 infected cell line C91PL. A similar trend of p53 upregulation was observed in Nutlin-3a- treated MT-2 cells (data not shown).

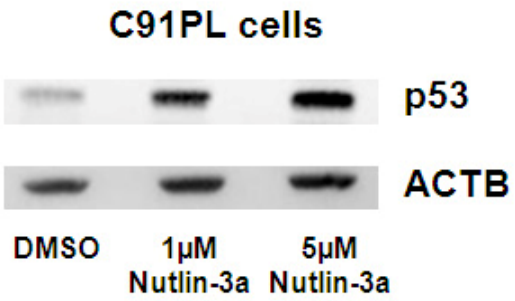


Figure 17. Stabilized p53 expression by Nutlin-3a in HTLV-1 infected cells. Immunoblot analysis of p53 protein expression in C91PL cells after treatment with 1- or 5 μM Nutlin-3a or an equal volume of DMSO for 48 hrs. β-actin (ACTB) was used as a loading control.

3.3.3 Increased expression of p21 (CDKN1A) and TP53INP1 through modulating p53 activity in HTLV-1 infected cells

3.3.3.1 Increased expression of p21 in Nutlin 3a-treated cells

The cyclin-dependent kinase inhibitor p21 (CDKN1A) is a known transcriptional target of p53. As shown in Figure 18, Nutlin-3a treatment resulted in a dose-dependent upregulation of p21 mRNA.

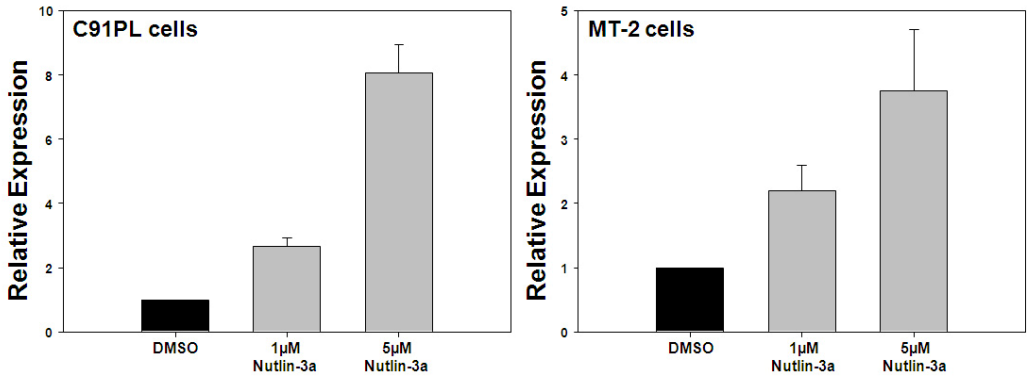


Figure 18. Induced expression of p21 (CDKN1A) in Nutlin-3a-treated cells. The plots show the fold induction of the p21 mRNA in C91PL cells and MT-2 cells after 48 hrs' treatment with Nutlin-3a compared to DMSO (set at 1). The RT-PCR method is described in the Materials and Methods. ACTB mRNA was used as an endogenous control.

3.3.3.2 Induction of TP53INP1 expression in Nutlin 3a-treated cells

The tumor suppressor protein TP53INP1 is a key factor in p53-mediated cell cycle arrest and death (Nowak J et al., 2002; Jiang et al., 2006). TP53INP1 expression is directly influenced by p53. As shown in Figure 19, we observed a substantial increase in the expression of TP53INP1 in Nutlin-3a-treated C91PL and MT-2 cells.

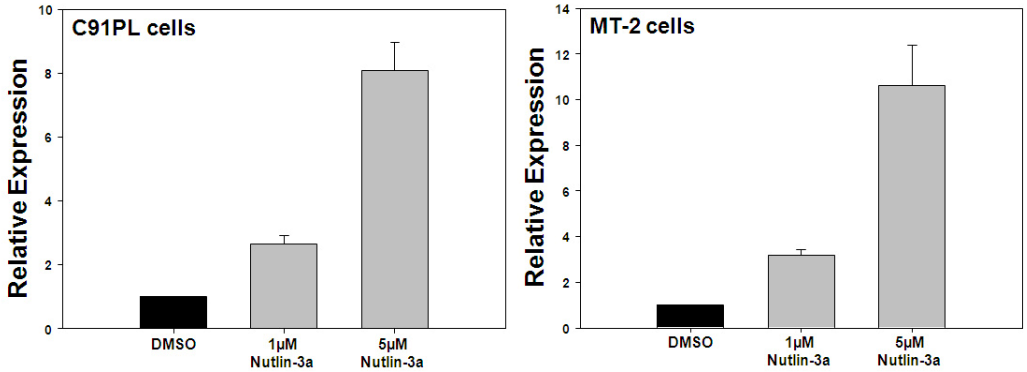


Figure 19. Induction of TP53INP1 expression in Nutlin-3a treated cells. The plots show the fold induction of the TP53INP1 mRNA in C91PL cells and MT-2 cells after 48 hrs' treatment with Nutlin-3a compared to DMSO (set at 1). ACTB mRNA was used as an endogenous control.

These observations showed that treatment of the infected cell lines with Nutlin-3a resulted in stabilization of p53 and induction of its activity as a transcription factor that upregulates expression of proteins that block the cell cycle and promote cell death.

3.3.4 Elevated expression of MDM2 after stabilization of p53 in HTLV-1 infected cells

As depicted in Figure 20, we also observed an increase in the expression of MDM2 in Nutlin-3a-treated cells. This result demonstrated that p53 was able to activate expression of its negative regulator Mdm2. However, as the activity of neosynthesized MDM2 would be blocked by Nutlin-3a, the p53-Mdm2 negative feedback loop will be interrupted.

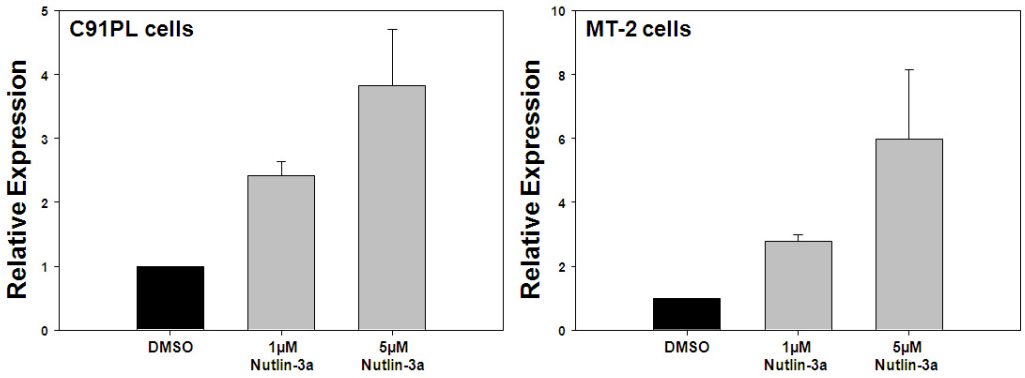


Figure 20. **Upregulation of MDM2 in Nutlin 3a-treated cells.** The plots report the fold induction of MDM2 mRNA in C91PL cells and MT-2 cells after 48 hrs' treatment with Nutlin-3a compared to DMSO (set at 1). ACTB mRNA was used as an endogenous control.

3.3.5 Nutlin-3a activates miR-34a expression and suppression of downstream targets

Having verified that Nutlin-3a is able to restore p53 function in the HTLV-1-infected cell lines, we next tested whether this would affect the expression of miR-34a and activate miR-34a regulatory pathways. Results of RT-PCR confirmed that miR-34a expression increased in Nutlin-3a-treated C91PL cells and MT-2 cells (Figure 21).

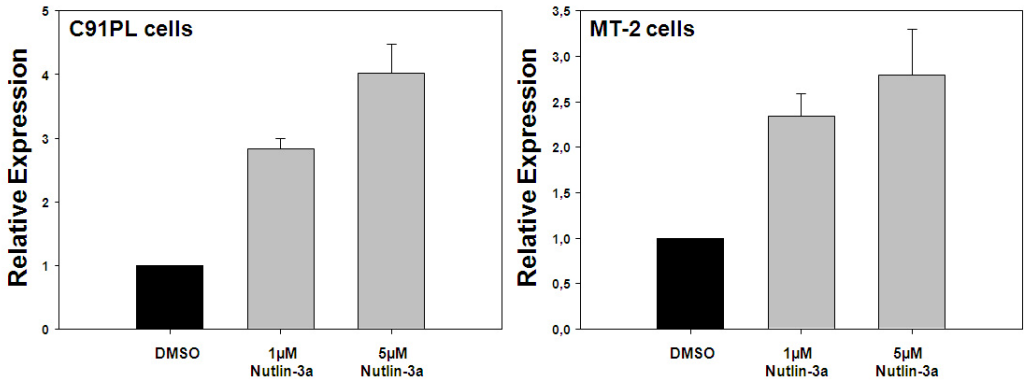


Figure 21. Upregulation of miR-34a through modulation of p53 activity in HTLV-1 infected cells. The results are shown as fold induction of miR-34a compared to DMSO-treated cells (set at 1). RNU44 was used as an endogenous control.

Studies carried out in other cell systems demonstrated that miR-34a inhibits the expression of the SIRT1 and YY1 mRNAs via binding to their 3' UTR regions (Yamakuchi et al. 2008; Lou W et al., 2013; Gordon S et al., 2006; Atchison M et al., 2011). We therefore performed immunobloting to identify the change in protein expression of SIRT1 and YY1. Immunoblot analysis revealed that the levels of SIRT1 and YY1 proteins were reduced in Nutlin-3a-treated cells, in comparison to the untreated cells (Figure 22).

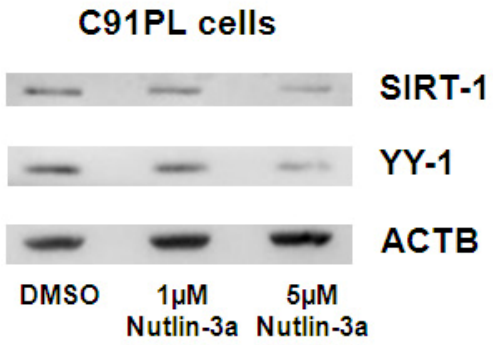


Figure 22. Reduced expression of SIRT1 and YY1 proteins as a consequence of miR-34a upregulation in Nutlin-3a treated cells. Shown is an immunoblot analysis of SIRT1 and YY1 expression in C91PL cells, after treatment with 1 and 5 μM Nutlin-3a or an equivalent amount of DMSO for 48 h. β-actin (ACTB) was used as a loading control**.**

We also analyzed the expression of the SIRT1 and YY1 mRNAs using quantitative RT-PCR. As shown in Figures 23 and 24, both mRNAs were downregulated in C91PL and MT-2 cells following Nutlin-3a treatment.

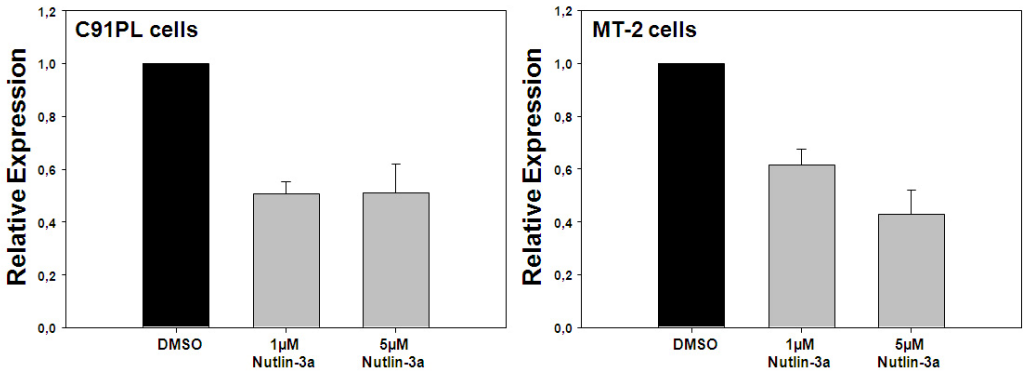


Figure 23. Downregulation of SIRT1 expression in Nutlin-3a-treated cells. The plots report show the fold reduction in the levels of SIRT1 mRNA after 48 hrs' treatment with Nutlin-3a compared to the DMSO control (set at 1). ACTB mRNA was used as an endogenous control.

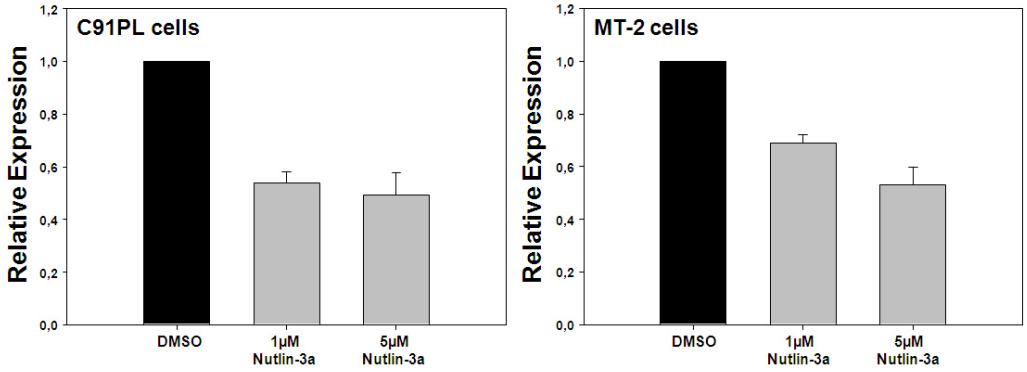


Figure 24. Downregulation of YY1 expression in Nutlin-3a treated cells. The plots report show the fold reduction in the levels of YY1 mRNA after 48 hrs' treatment with Nutlin-3a compared to the DMSO control (set at 1). ACTB mRNA was used as an endogenous control.

We next broadened our screening of miR-34a targets to BCL2, BIRC5, CDK4, DLL1, MYC, SP1, JAG1, and VEGFA mRNAs. As depicted in Figure 25, we observed downregulation of all of these transcripts in the Nutlin-3a-treated C91PL cells. A similar trend of BIRC5, CDK4, SP1, and VEGFA downregulation was observed in Nutlin-3a treated MT-2 cells (data not shown).

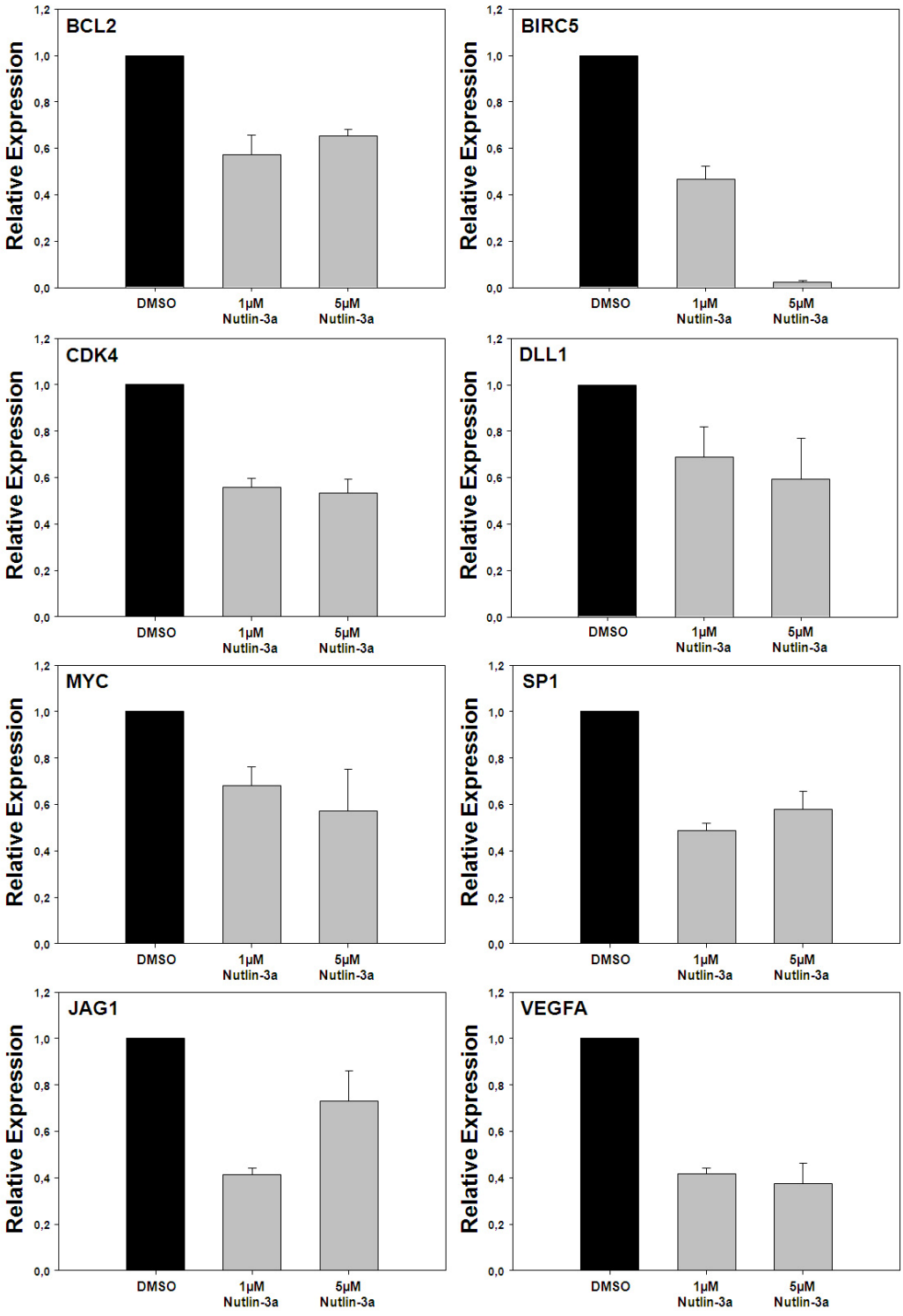


Figure 25. Expression profiles of additional miR-34a downstream targets in Nutlin-3a-treated cells. The plots report show the fold reduction in the levels of the indicated mRNAs in C91PL cells after 48 hrs' treatment with Nutlin-3a compared to the DMSO control (set at 1). ACTB mRNA was used as an endogenous control.

These observations showed that miR-34a is upregulated and is able to suppress its targets in response to Nutlin-3a treatment.

3.3.6 Changes in expression of other microRNAs in Nutlin-3a treated cells

In addition to miR-34a, we analyzed the expression of the following microRNAs in Nutlin3a-treated C91PL cells: miR-125a, miR-125b, miR-141, miR-142-5p, miR-142-3p, miR-106a, miR-107, miR-29c, miR-26a, miR-146a, miR-155, miR-221, miR-223, miR-93, miR-130b and let-7e. As depicted in Figure 26, miR-125a, miR-141, miR-142-5p, miR-142-3p, miR-106a, miR-93 and miR-130b were all downregulated, while miR-125b was upregulated in Nutlin-3a-treated cells.

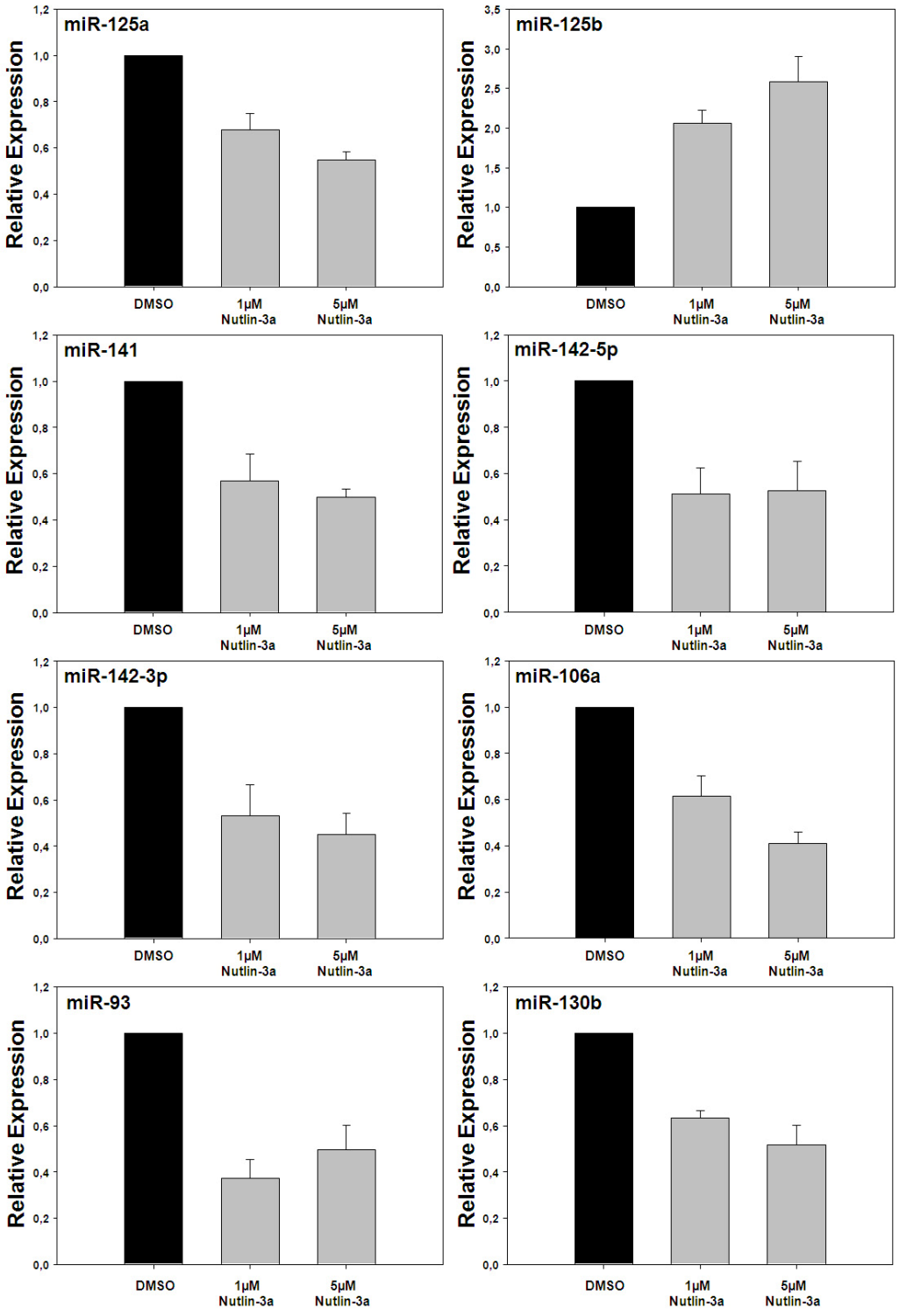


Figure 26. microRNA expression in Nutlin-3a-treated cells. The plots report fold changes in microRNA expression in C91PL cells after 48 hrs' treatment with Nutlin-3a compared to DMSO-treated cells (set at 1). RNU44 was used as an endogenous control.

3.3.7 Effect of etoposide and pifithrin-α on viability of HTLV-1 infected cells

To complement experiments performed using Nutlin-3a, we also treated the HTLV-1-infected cell lines with etoposide and pifithrin-α. As described in Section 3.1.2.2, etoposide is an antineoplastic drug that induces DNA damage via inhibiting topoisomerase II, which may lead to activation of the p53 pathway; pifithrin-α inhibits p53 through an unknown mechanism. We treated C91PL and MT-2 cells with either 5 µM etoposide, 10 µM pifithrin-α or an equal volume of DMSO (untreated) for 48h.

Treatment with etoposide or pifithrin-α resulted in decrease in cell viability in C91PL cells and MT-2 cells. Forty-eight hours after incubation with 5 µM etoposide, ~ 35% and ~25% specific cell death observed in C91PL cells and MT-2 cells, repectively. In both cell lines, ~ 10% specific cell death observed after treatment with 10 µM pifithrin-α (Figure 27).

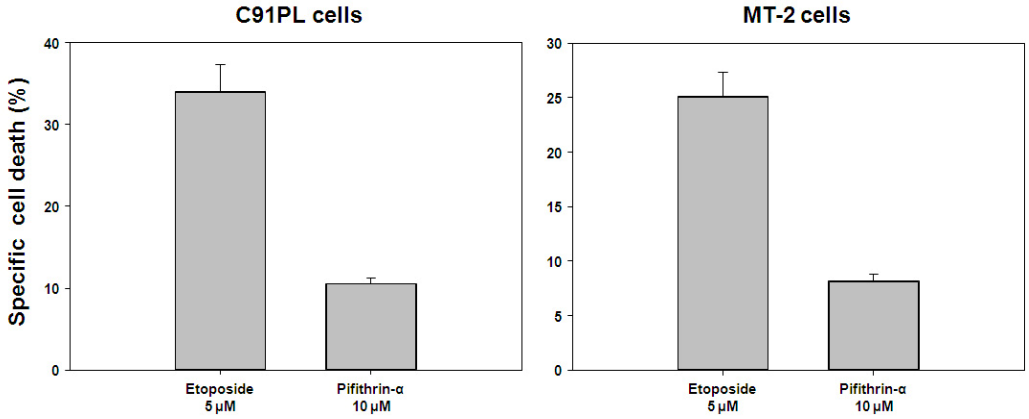


Figure 27. Cell death induced by treatment with etoposide or pifithrin-α. C91PL cells and MT-2 cells that had been incubated with 5 µM etoposide or 10 µM pifithrin-α or with an equal volume of DMSO for 48 hrs were labelled with propidium iodide for 20 min and then analysed using a BD FACSCalibur. Percentages of dead (i.e. stained) cells were used to calculate the specific cell death using the formula SCD = (% dead cells in treated culture-% dead cells in control

culture/% living cells in control culture) X 100. The plots show mean SCD values with standard errors calculated from 3 experiments performed on each cell line.

3.3.8 Effects of etoposide and pifithirin-α on p53 and its downstream targets

Immunoblotting results showed that p53 protein was upregulated in the etoposide-treated cells but not in the pifithrin-α-treated cells (Figure 28).

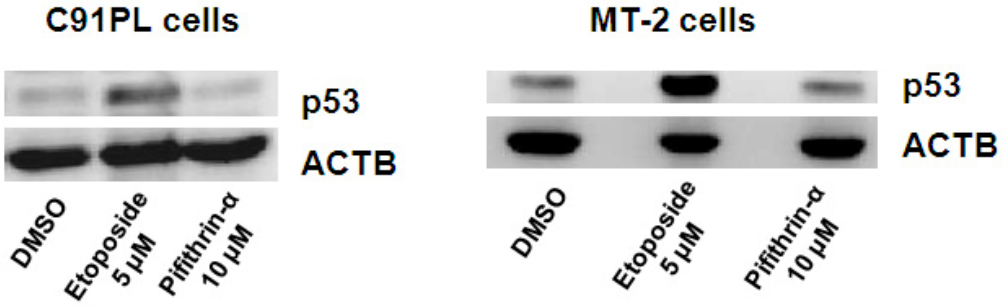


Figure 28. Modulation of p53 activity via etoposide and pifithrin-α in HTLV-1 infected cell lines. Shown are immunoblots to detect p53 and β-actin (ACTB) in lysates of cells treated with 5 µM etoposide or 10 µM pifithrin-α or an equivalent amount of DMSO (untreated) for 48 h. β-actin was used as a loading control.

3.3.9 Expression of p53 downstream targets in cells treated with etoposide

Figure 29 shows results of RT-PCR to compare the levels of MDM2, CDKN1A (p21), TP53INP1, miR-34a and miR-125b in etoposide-treated cells with respect to untreated cells.

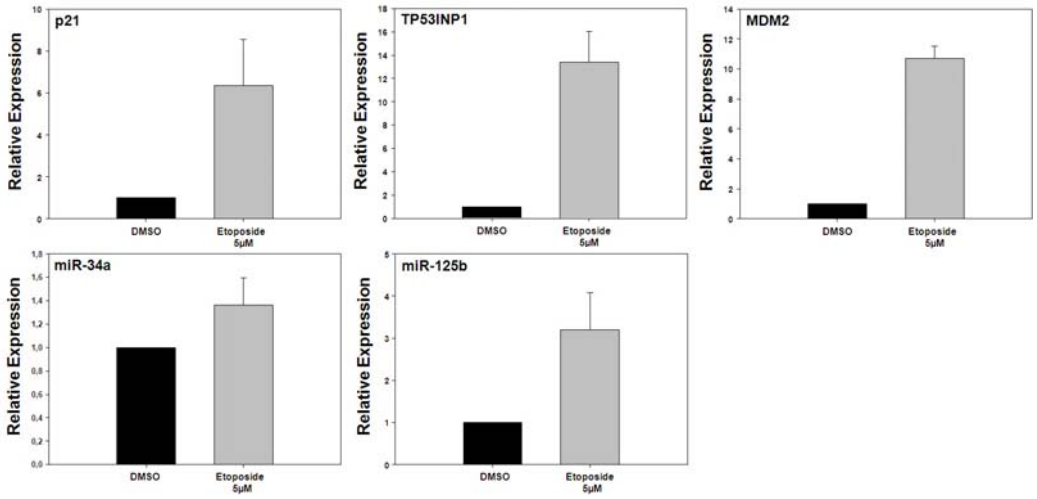


Figure 29. Expression pattern of p53 downstream targets induced by etoposide in HTLV-1 infected cells. The plots report fold changes in expression of the indicated mRNAs and microRNAs in MT-2 cells after 48 hrs' treatment with etoposide compared to DMSO-treated cells (set at 1). ACTB and RNU44 were used as endogenous controls for quantification of mRNAs and microRNAs, respectively.

3.3.10 Expression of miR-34a downstream targets in etoposide-treated cells

As shown in Figure 29D, treatment of MT-2 cells with etoposide resulted in a slight upregulation of miR-34a. Our next goal was to test the expression of the following miR-34a downstream targets: BCL2, BIRC5, CDK4, SIRT1, VEGFA and YY1. As depicted in Figure 30, the expression of mRNAs coding for BCL2, BIRC5 and CDK4 was reduced upon etoposide treatment. However, the expression of SIRT1, VEGFA and YY1 remained unchanged.

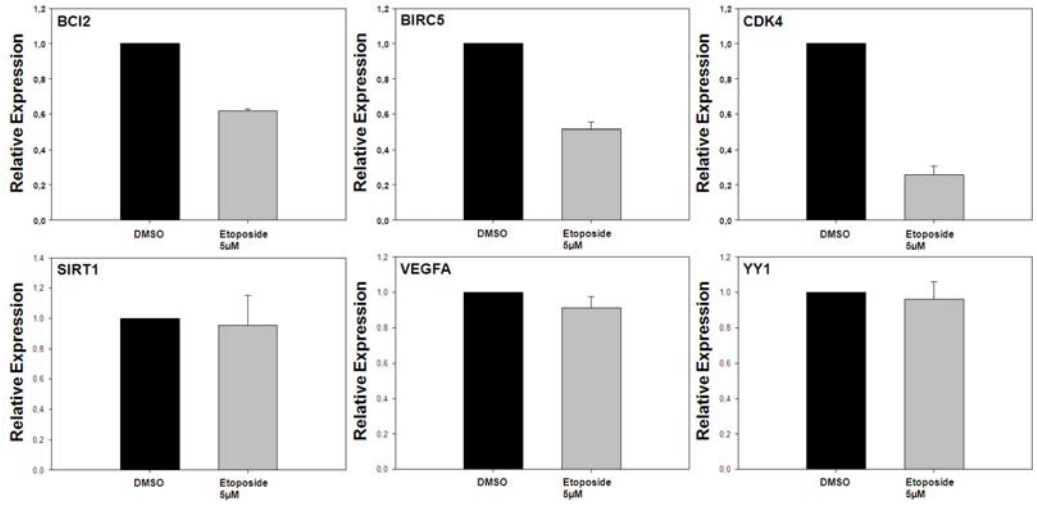


Figure 30. Expression of targets of miR-34a in etoposide-treated cells. The plots report fold changes in expression of the indicated mRNAs in MT-2 cells after 48 hrs' treatment with etoposide compared to DMSO-treated cells (set at 1). ACTB was used as an endogenous control.

3.3.11 Effect of pifithrin-α on p53 targets.

Results of RT-PCR assays on pifithrin-α–treated MT-2 cells revealed reduced expression of p53 downstream targets MDM2, CDKN1A, TP53INP1 and miR-34a (Figure 31). miR-125b expression was also downregulated in pifithrin-α-treated cells (Figure 31E).

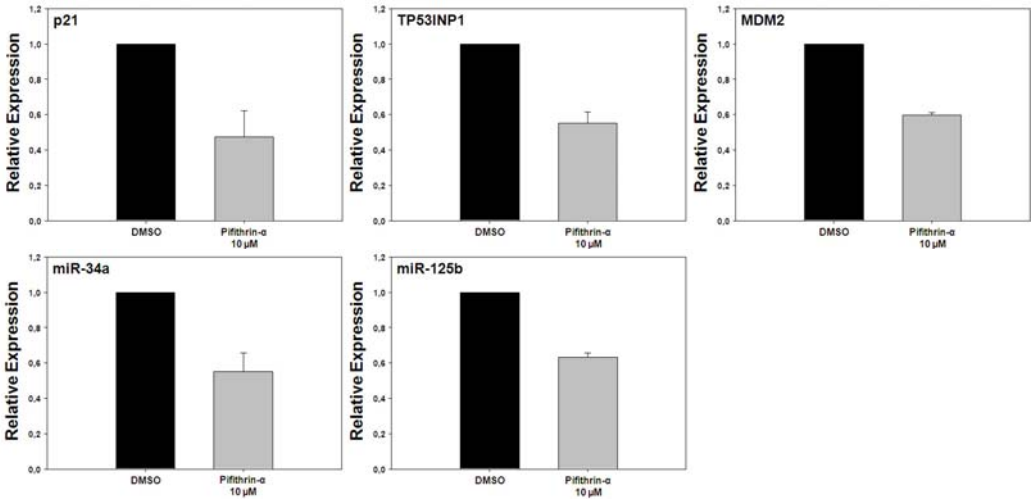


Figure 31. Expression pattern of p53 downstream targets in Pifithrin-α-treated cells. The plots report fold changes in expression of the indicated mRNAs and miRNAs in MT-2 cells after 48 hrs' treatment with pifithrin-α compared to DMSO-treated cells (set at 1). ACTB and RNU44 were used as endogenous controls for quantification of mRNAs and microRNAs, respectively.

3.3.12 Expression of targets of miR-34a in Pifithrin-α-treated cells

The observation that pifithrin-α resulted in downregulation of miR-34a in MT-2 cells led us to perform RT-PCR to detect the miR-34a downstream targets BCL2, BIRC5, CDK4, SIRT1, VEGFA and YY1. As shown in Figure 32, BCL2 and YY1 were upregulated in pifithrin-α-treated cells relative to untreated cells. However, we did not find changes in the expression of BIRC5, CDK4, SIRT1 or VEGFA (Figure 32).

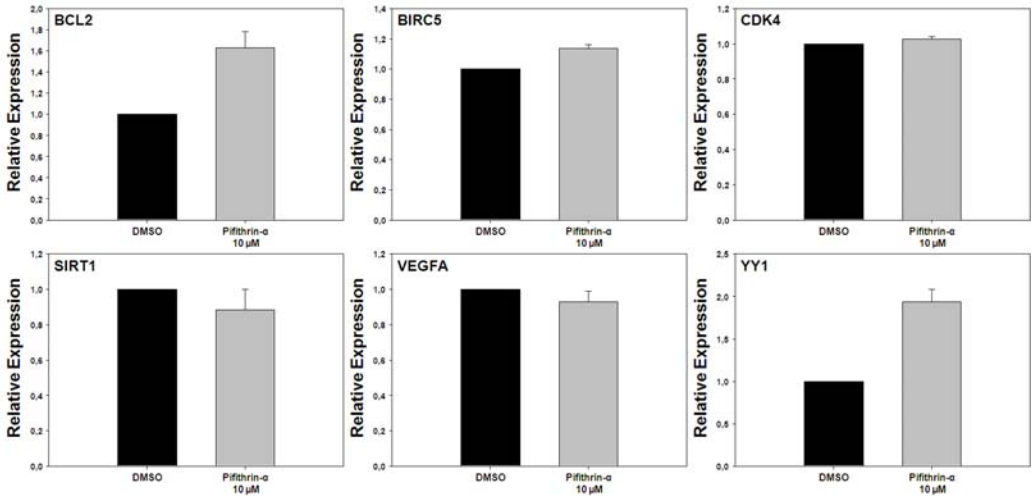


Figure 32. Expression profiles of miR-34a downstream targets in pifithrin-α-treated cells. The plots report fold changes in expression of the indicated mRNAs in MT-2 cells after 48 hrs' treatment with compared to DMSO-treated cells (set at 1). ACTB was used as an endogenous control.

3.4 DISCUSSION

This study investigated whether restoration of the p53 pathway in HTLV-1-infected cell lines might activate miR-34a, a tumor suppressor microRNA that is already expressed at high levels in ATLL cells and HTLV-1-infected cell lines (Figure 15) but appears unable to silence at least one confirmed target, i.e., the deacetylase SIRT1 (Kozako et al., 2012).

Treatment of C91PL cells and MT-2 cells for 48 hrs with Nutlin-3a, a drug that interferes with the ability of Mdm2 to destabilize p53, resulted in increased levels of p53 protein (Figure 17), and upregulation of the p53-regulated genes CDKN1A, TP53INP1 and MDM2 and miR-34a (Figures 18-21). These results indicated that that Nutlin-3a was effective rescuing p53 activity.

The further increase in miR-34a abundance was accompanied by a substantial reduction in expression of 9 genes that were previously identified as targets of miR-34a (SIRT1, BCL2, BIRC5, CDK4, MYC, YY1, DLL1, SP1, VEGFA; Figures 22-25). These findings lead us to conclude that induction of p53 by Nutlin-3a results in engagement of the miR-34a regulatory pathway (Sharma et al, Manuscript in preparation).

We can highlight the consequences of the loss of some of these miR-34a targets in ATLL cells and/or HTLV-1-infected cell lines:

- Loss of SIRT1 would allow p53 to remain in its active, acetylated form (Yamakuchi, 2009; Hermeking, 2010).
- Loss of Bcl-2 and Survivin would subtract important anti-apoptotic signals.

- Loss of CDK4 would interfere with progression through the cell cycle.
- Loss of Myc would interfere with the expression of many genes involved in cell proliferation.
- In the absence of YY1, miR-31 expression might be de-repressed; miR-31 would in turn block expression of NIK, a component of the NF-kB pathway that is upregulated in ATLL cells (Yamagishi et al., 2012) (see Section 1.4.1.5). Loss of YY1 would also interfere with the ability of MDM2 to ubiquitinate p53 (Gronroos et al., 2004; Sui et al., 2004).

Treatment of the cell lines for 48 hrs with the topoisomerase II inhibitor etoposide also increased the levels of p53 protein, especially in MT-2 cells (Figure 28). Further analyses of the effects of etoposide in MT-2 cells revealed substantial upregulation of the p53 targets CDKN1A, TP53INP1, MDM2 and miR-34a. However, the induction of miR-34a produced with etoposide was less evident than that produced with Nutlin-3a (about 1.4-fold vs. 3-fold, respectively). In line with obervations made using Nutlin-3a, treatment of MT-2 cells with etoposide resulted in downregulation of the miR-34a targets BCL2, BIRC5 and CDK4. However, unlike Nutlin-3a, etoposide did not affect the expression of SIRT1 or YY1. As described above, both SIRT1 and YY1 negatively affect p53 function. Therefore etoposide may have a less broad effect on p53 compared to Nutlin-3a.

Treatment of MT-2 cells with the p53 inhibitor Pifithrin-α caused a reduction the expression of downstream mRNA targets including MDM2, CDKN1A and TP53INP1 as well as miR-34a (Figure 31). This finding indicates that p53 is at least

in part responsible for the high levels of miR-34a detected in HTLV-1 infected cell lines.

miR-34a expression and regulation has not been studied extensively in hematological malignancies. Downregulation of miR-34a was reported in aggressive chronic lymphoid leukemia (CLL) with inactivated p53 (Mraz et al., 2009) but appears to be upregulated in acute myelogenous leukemia (AML) blasts compared to normal bone marrow cells or CD34+ hematopoietic progenitor cells (Isken et al., 2008). It is noteworthy that the upregulation of miR-34a was also indicated in an analysis of EBV-transformed B-cells (Mrazek et al., 2007). Subsequently, another study indicated the increased expression of pre-miR-34a and other cellular miRNAs during latency type III (the growth program phase) compared to latency I (Cameron et al., 2008).

Upregulation of miR-34a in HTLV-1-infected cells and ATLL cells might be a marker of persistent 'oncogenic stress' and/or engagement of the DNA damage repair pathway induced by HTLV-1 expression (Marriott et al., 2005). In alternative, a subset of activated T-cells expressing miR-34a may present a favourable environment for HTLV-1-replication and persistence. Future experiments will be aimed at understanding whether HTLV-1-infected cells may develop a high threshold of 'resistance' against miR-34a activity, or if some as yet unknown function of p53 is required for miR-34a to suppress its targets.

To gain more information on the interaction between p53 and microRNA regulation in HTLV-1 infected cells, we analyzed the expression of many additional microRNAs in Nutlin-3a-treated C91PL cells. Results demonstrated upregulation of miR-125b and downregulation of miR-125a, miR-141, miR-142-5p, miR-142-3p,

miR-106a, miR-93 and miR-130b (Figure 26). These observations suggest that p53 directly or indirectly regulates expression of these microRNAs.

The downregulation of miR-93 and miR-130b upon stabilization of p53 merits discussion in light of a study by Yeung et al. (2008), which showed that these microRNAs are highly expressed in ATLL cells and HTLV-1-infected cell lines(see Section 1.4.1.5). Yeung et al. observed that these microRNAs provide a survival advantage to HTLV-1-infected cells by suppressing the expression of TP53INP1, one of the p53 targets that was upregulated in cells treated with Nutlin-3a or etoposide (Figures 19 and 29, respectively). Yeung et al. (2008) also showed that Tax is able to upregulate miR-130b expression. Our experiments reveal a role for p53 in negatively regulating both microRNAs that overrides the effects of Tax.

miR-93 as well as miR-106a are also known to suppress the expression of CDKN1A; Petrocca et al. (2008) observed that these microRNAs are upregulated in gastric cancer and impair the TGFβ tumor suppressor pathway by inhibiting expression of p21. This finding suggests that the upregulation of CDKN1A seen in cells treated with Nutlin-3a may reflect both activation of p53 and suppression of miR-93a and miR-106a.

4 The impact of Tax on microRNA expression

4.1 Background and aim of the study

The transforming potential of HTLV-1 is attributed primarily to the viral protein Tax, which, in addition to transactivating the viral promoter, affects the expression and function of cellular genes controlling signal transduction, cell growth, apoptosis and chromosomal stability, resulting in clonal proliferation of infected cells (Satou Y et al., 2006; Boxus et al., 2009; Tang et al., 2013; Zhao et al., 2013) (see Section 1.3.3).

The aim of this study was to test the impact of Tax on microRNA regulation by expressing Tax in the T-cell line Jurkat followed by analysis of mRNA and microRNA expression using quantitative RT-PCR.

4.2 MATERIALS AND METHODS

4.2.1 Cell culture

The Jurkat T-cell line was maintained in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 2 mM glutamine (Invitrogen), 100 units/ml penicillin and 20 units/ml streptomycin (complete RPMI).

4.2.2 Transfection of Tax expressing plasmids

Jurkat cells were transfected with plasmid pcTAX, which expresses HTLV-1 Tax protein driven by the HCMV promoter (Smith et al., 1990). The transfection protocol was set up using the Neon transfection system (Life Technologies). Cells were counted and resuspended in aliquots of 5 x 10^6 cells in 100 µl of T buffer (Neon, Life Technologies) for each electroporation. The transfection mixture contained 1.5 µg of pcTAX or pBluescript, 1 µg of pMACS-LNGFR (Miltenyi Biotec), and pBluescript up to 4 µg total DNA. Transfections were carried out in 100 µl tip-electrodes of the Neon transfection system with one 1410-V, 30-msec pulse, following the manufacturer's recommendations. The cells were then seeded in 6-well microplates (34.8-mm diameter) containing complete RPMI lacking antibiotics. After 48 hrs aliquots of the cells were labeled with FITC-anti-LNGFR and analyzed by flow cytometry to check the efficiency of transfection. Transfected cells were then selected using magnetic beads conjugated with anti-LNGFR antibody (Miltenyi Biotec) and lysed for RNA analysis. The percentage of LNGFR-positive cells in the live-cell population ranged from 49.06 to 55.47%.

Cells were lysed in TRIZol (Invitrogen) for isolation of total RNA as described in Section 3.2.4.

4.2.3 Quantitative RT-PCR.

RT-PCR for microRNAs was carried out as described in Section 3.2.6. Differences in expression of miRNAs were calculated using formulas for relative quantification(RQ), where Ct is the threshold cycle.

$$\Delta Ct = Ct_{\text{miRNA of interest}} - Ct_{\text{RNU44}}$$

$$\Delta\Delta Ct = \Delta Ct_{\text{Tax positive}} - \Delta Ct_{\text{control (Tax negative)}}$$

$$RQ = 2^{-\Delta\Delta Ct}$$

mRNAs coding for OX40, OX40L and 4-1BB were detected by RT-PCR using a PCR mix containing SYBR Green as described in Section 3.2.6. Primer sequences are listed in Table 6A.

Tax expression was measured by quantitative RT-PCR using the custom-designed primers and probe listed in Table 6B (Rende et al., 2011). Probes were 5′ end-labeled with FAM and 3′end-labeled with TAMRA. As an internal control, GAPDH mRNA was analyzed in parallel by using the Endogenous Control Human GAPDH kit Reagents (Applied Biosystems). PCR reactions were performed with an ABI Prism 7900 HT Sequence Detection System by using 5 µl of each diluted RT sample (10 ng/µl) and 20 µl of diluted Taqman Universal PCR Master Mix (Applied Biosystems) and primers and probe ; each reaction was performed in duplicate. The cycling conditions comprised an initial step at 50°Cfor 2 min, denaturation at 95°C for 10 min, and 40 cycles at 95°C for 15 sec and 60°C for 1 min.

The absolute copy number of each transcript was determined and normalized (normalized copy number, NCN) for the copy number of the GAPDH mRNA in the transfected cells.

Table 6A. Primers to detect NF-κB-responsive genes and ACTB

Primer	Forward primer (Sense)	Reverse primer (Antisense)
OX40 (TNFRSF4)	CCTGCACAGTGGTGTAACCT	AGCGGCAGACTGTGTCCT
OX4OL (TNFSF4)	TTGCTGGTGGCCTCTGTAAT	TTGAATTCGAGGATACCGATG
4-1BB (TNFRSF9)	GCTCTCGATATCCGGTAGGA	GCCTGACCTAGCTAAGACACTTCT
ACTB	AGCACAGAGCCTCGCCTTTG	GGAATCCTTCTGACCCATGC

Table 6B. Primers and probe to detect Tax mRNA

TaxRex	**forward primer**	**Env s**	GTCCGCCGTCTAG^CTTCC
	reverse primer	**TaxRex as**	CTGGGAAGTGGG^CCATGG
	probe	**ENV-G**	(FAM)-CCCAGTGGATCCCGTGGAG-(TAMRA)

4.3 RESULTS

4.3.1 Identification of the expression of Tax in transfected Jurkat T-cells

To identify Tax-regulated microRNAs, the T-ALL cell line Jurkat was electroporated with a Tax-expressing plasmid or control plasmid along with a plasmid expressing LNGFR, which served as a marker to identify transfected cells. After evaluating transfection efficiency, LNGFR-positive cells were isolated using magnetic beads conjugated with anti-LNGFR antibody. RNA was isolated from the cells and subjected to quantitative RT-PCR to detect the Tax mRNA. As shown in Figure 33, results verified the expression of Tax in cells transfected with the Tax plasmid.

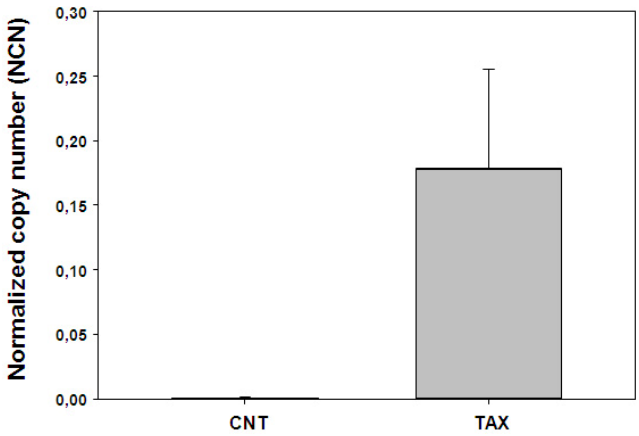


Figure 33. Expression of Tax in Jurkat T-cell line transfected with a plasmid expressing Tax. Panel shows the absolute copy number of the Tax mRNA measured using quantitative RT-PCR in Jurkat cells (Tax-positive) compared to controls (Tax-negative), 48 hrs after transfection. Mean values of six independent experiments and standard error bars are shown.

4.3.2 Elevated expression of OX40, OX40L and 4-1BB in Jurkat cells expressing Tax

To determine whether Tax was functionally active in the Jurkat cells, we measured the expression levels of 3 genes of the tumor necrosis factor receptor superfamily: TNFRSF4 (coding for OX40), TNFSF4 (coding for OX40L) and TNFRS9 (coding for 4-1BB). OX40 and 4-1BB are costimulatory receptors for TNF receptor-associated factor (TRAF) (Kanamaru et al., 2004), and OX40L (gp34 or TNFSF4) serves as a ligand for OX40. The ability of Tax to activate expression of OX40, OX40L and 4-1BB was demonstrated by Ohtani et al. (1998), Higashimura et al. (1996), and Pichler et al. (2008), respectively. Tax-mediated upregulation upregulation of these genes was proposed to promote survival and proliferation of long-lived T cell clones (Pichler et al., 2008; Kress et al.,2011).

Figure 34 shows that the cells transfected with Tax exhibited increased expression of OX40, OX40L and 4-1BB. The strongest upregulation was seen for OX40L (about 300-fold). These observations provided indirect evidence that the Tax protein was expressed and functionally active in the transfected Jurkat cells.

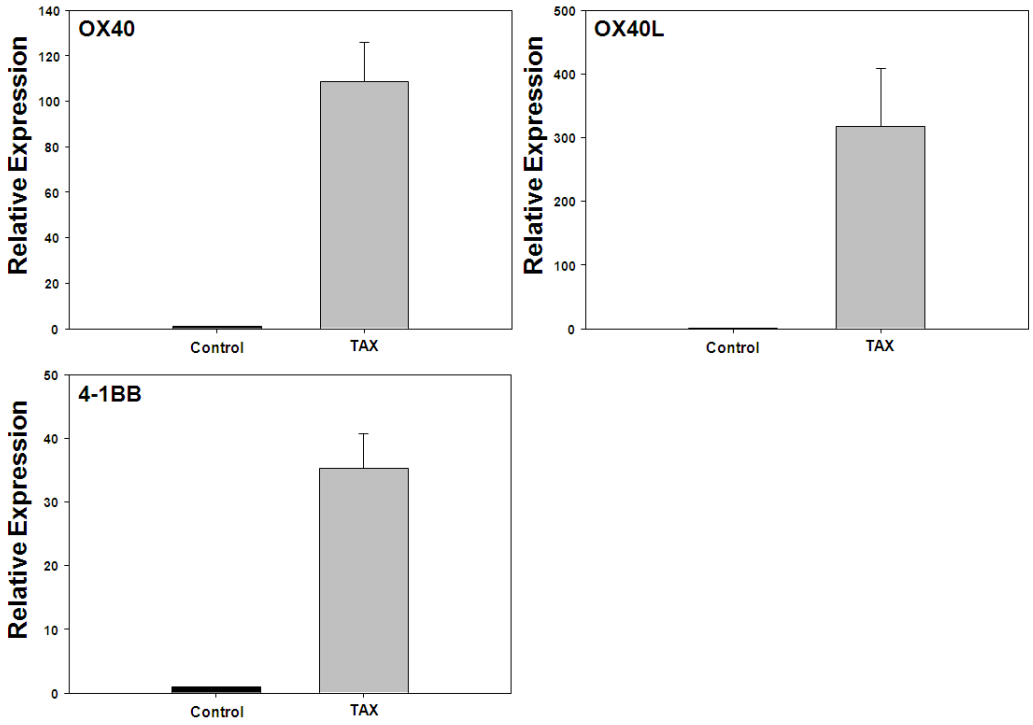


Figure 34. Upregulation of OX40, OX4OL and 4-1BB mRNAs in Jurkat T-cells expressing Tax. The plots show the fold induction measured by quantitative RT-PCR 48 hrs after transfection of Jurkat cells with a plasmid expressing Tax compared to controls (Tax negative). Shown are mean values from 6 independent experiments with standard error bars.

4.3.3 The impact of HTLV-1 Tax on microRNA regulation

As described in Section 3.1, our microarray-based analysis revealed 27 microRNAs that were differentially expressed in ATLL samples compared to normal CD4+ T-cells. To better understand the impact of Tax on microRNA regulation, we selected 19 microRNAs to analyze in Jurkat cells transfected with Tax compared to control cells (Table 7). Seventeen of these microRNAs were identified using array-based analysis from our lab (miR-18a, miR-20b, miR-26a, miR-29c, miR-30b, miR-31, miR-34a, miR-125a, miR-125b, miR-130b, miR-142-5p, miR-146a, miR-146b, miR-451, miR-212, let-7e and let-7g). The remaining 2 microRNAs were found to be upregulated in ATLL samples or HTLV-1 infected cells in previous studies; these

were miR-93 (Yeung et al., 2008) and miR-155 (Pichler et al., 2009; Bellon et al., 2009; Tomita, 2012).

Table 7. MicroRNAs analyzed in Jurkat cells transfected with Tax compared to control cells

Expression in ATLL cells or infected cell lines	Deregulated microRNAs
Upregulated	miR-18a, miR-34a, miR-93, miR-130b, miR-146a, miR-155, miR-451
Downregulated	miR-20b, miR-26a, miR-29c, miR-30b, miR-31, miR-125a, miR-125b, miR-142-5p, miR-146b, miR-212, let-7e, let-7g

4.3.4 Elevated expression of miR-146a and miR-155 in Jurkat T-cells expressing Tax

Findings from previous studies indicated the ability of Tax induce expression of miR-146a and miR-155 (Pichler et al., 2009; Tomita et al., 2009; Tomita, 2012). As shown in Figure 35, miR-146a and miR-155 were upregulated in Tax-expressing Jurkat cells by about 5.5-fold and 12-fold, respectively, compared to control cells.

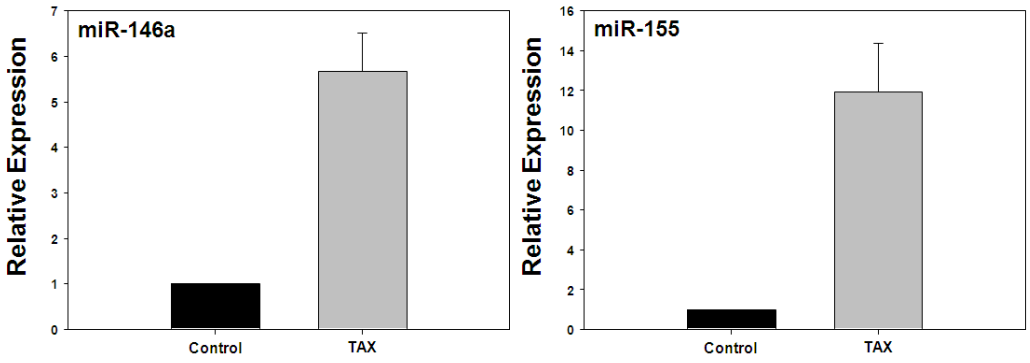


Figure 35. Induction of miR-146a and miR-155 expression in Jurkat T-cells expressing Tax. The plots show the fold induction of miR-146a and miR-155 in Jurkat cells (Tax-positive) compared to controls (Tax-negative), 48 hrs after

transfection. RNU44 was used as an endogenous control. The plots report mean values from 6 independent experiments with standard error bars.

4.3.5 The impact of Tax on the microRNA expression profile

Having verified the ability of Tax to upregulate miR-146a miR-155 in our experimental system, we proceeded with the analysis of the other microRNAs listed in Table 7. Results of RT-PCR revealed that Tax did not alter the expression of miR-26a, miR-29c, miR-30b, miR-31, miR-34a, miR-93, miR-125a, miR-125b, miR-130b, miR-451, miR-212 or let-7e. However, we observed a modest induction of miR-146b and miR-18a, a slight decrease in expression of miR-20b and miR-142-5p, and a more substantial decrease in expression of let-7g (Figure 36).

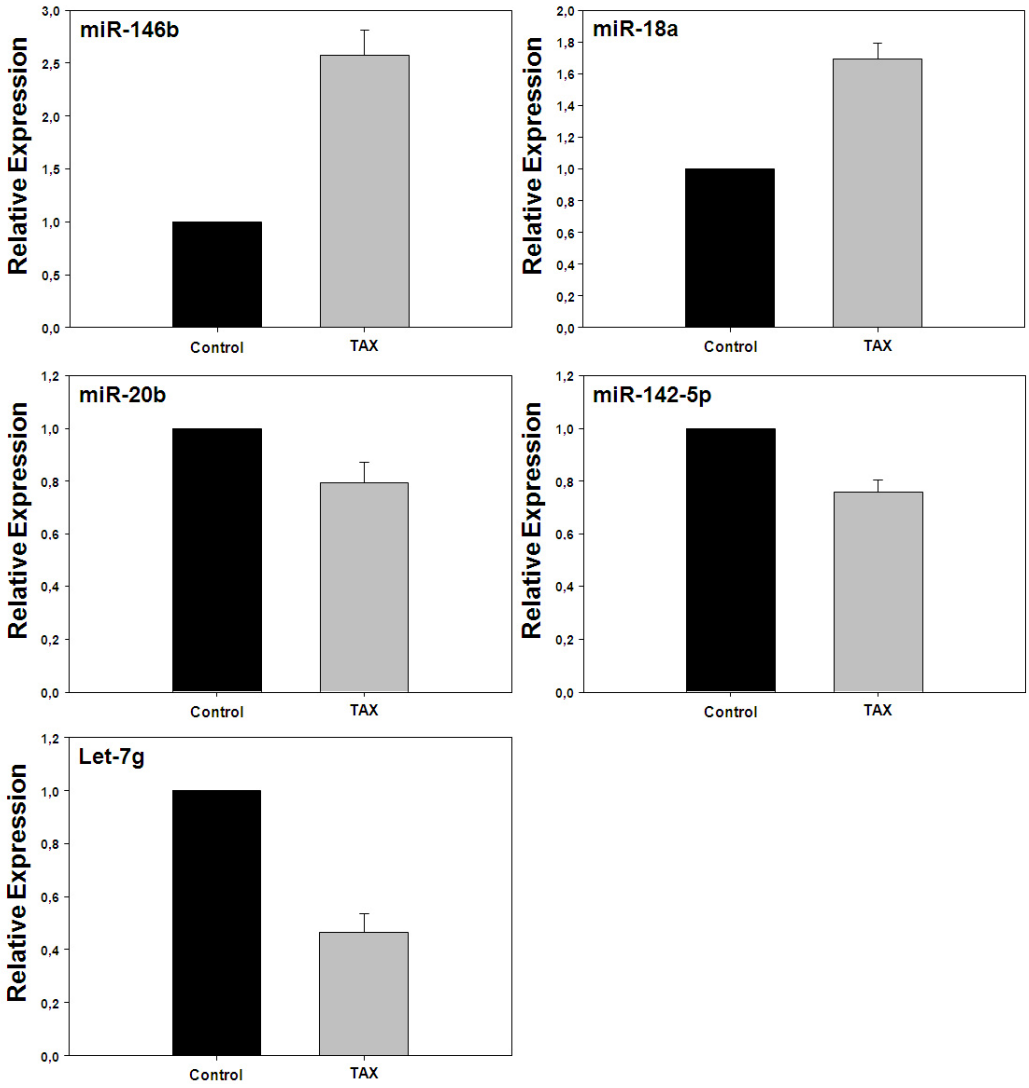


Figure 36: Changes in microRNA expression in Jurkat T-cells expressing Tax. The plots show the alterations in levels of the indicated microRNAs in Jurkat T-cells expressing Tax, compared to controls. RNU44 was used as an endogenous control. The plots report mean values from 6 independent experiments with standard error bars.

4.4 DISCUSSION

The aim of the present study was to identify microRNAs that are regulated by Tax in T-cells. The experimental setup consisted of transient transfection of the T-ALL cell line Jurkat with a plasmid expressing Tax, isolation of transfected cells, and analysis of microRNA levels by RT-PCR. This approach led to the identification of 4 microRNAs that were induced by Tax (miR-146a, miR-155, miR-146b, and miR-18a) and one microRNA that was downregulated in Tax-expressing cells (let-7g).

Among the 4 upregulated microRNAs, miR-146a and miR-155 showed the strongest induction (~6 fold and ~12 fold, respectively. The upregulation of these 2 microRNAs is in line with observations made by Pichler et al. (2008) (see Section 1.4.1.5). It is noteworthy that we did not observe an increase in the levels of miR-130b in the Tax-expressing Jurkat cells, as this microRNA was reported to be upregulated by Tax via the NF-κB pathway by Yeung et al. (2008).

Tax-mediated stimulation of the NF-κB pathway is a very important alteration induced by the virus that promotes survival and proliferation of infected T-cells and their transformation into tumor cells (Sun et al., 2005; Sun, 2011). Indeed, NF-κB activation is a hallmark of HTLV-1-infected cells (Watanabe et al., 2005) (see Section 1.3.3.2). However, Tax also influences several other transcription factors, including CREB, p53, AP-1, Myc, NFAT, SRF, p53, and TGF-β (reviewed by Hall and Fujji, 2005). It will therefore be important to identify the mechanism by which Tax up- or downregulates the individual microRNAs identified in the present study. This question could be approached by transfecting Jurkat cells with mutant forms of Tax that are unable to activate or repress specific transcription factors.

The upregulation of miR-146b and miR-18a by Tax (Figure 36) has not been reported before. miR-146b has the same seed sequence as miR-146a, and their entire sequences differ by only two nucleotides (Rusca et al., 2011). Given such a high degree of similarity, miR-146a and miR-146b should recognize the same targets. This is supported by studies on certain solid tumor cell lines which indicated that miR-146a and 146b interfere with invasion and metastasis (Bhaumik et al., 2008; Hurst et al., 2009).

miR-146a and miR-146b are processed from distinct precursors that are coded by different genes. Studies based on microarray analysis and quantitative RT-PCR revealed downregulation of miR-146b in ATLL cells versus normal CD4+ T-cells (Bellon et al. 2009; D'Agostino, unpublished). Further studies are needed to understand the mechansims regulating expression of miR-146a and miR-146b in Tax-expressing cells vs. ATLL cells.

miR-18a is a member of the oncogenic miR-17-92 cluster. In some types of cancer, upregulation of the miR-17-92 cluster promotes angiogenesis during tumor growth (Dews et al., 2006; Olive et al., 2010). miR-18a is known to suppress Dicer1 (Yao et al., 2012; Luo et al., 2013), and can thereby cause global downregulation of microRNA expression levels (Luo et al., 2013).

We observed reduced levels of let-7g in Tax-expressing Jurkat cells Let-7g is a member of the let-7 family. Let-7 was discovered in *C. elegans* and was shown to regulate development, cellular proliferation and differentiation (Pasquinelli et al., 2000). Let-7 family members control the expression of various targets such as BRCA1, BRCA2, FANCD2, PLAGL1, E2F6, E2F8, CHEK1, BUB1, BUB1B, MAD2L1, and CDC23 (Johnson et al., 2007)

Let-7g was reported to be upregulated in colon cancer (Nakajima et al., 2006), suggesting an oncogenic role. However, subsequent studies favored a tumor suppressor function for this microRNA. For example, let-7g represses the growth of lung cancer cells and lung tumor development (Kumar et al., 2007; Kumar 2008). In cancer cells, overexpression of let-7g alters cell cycle progression and reduces cell division (Charles et al., 2007). Let-7g expression is downregulated various cancers including breast cancer. Observations suggest that reduced expression of let-7g is associated with progression of cancer cells (Boyerinas et al., 2010; Qian et al., 2011).

Yan et al. (2013) reported that TNFRSF4 (OX40) is a target of let-7g. It will therefore be of interest to test whether let-7g is able to suppress TNFRSF4 expression in HTLV-1-infected cells.

As described in Section 1.4.1.2, microRNA-mRNA target interactions often result in degradation of the mRNA. Comparison of microRNA target predictions with expression profiles for microRNAs and mRNAs can therefore help in identifying mRNA targets for specific microRNAs that are biologically relevant in a particular cell system (Lionetti et al., 2009; Sales et al., 2010).

In collaboration with S. Bortoluzzi (University of Padova), our laboratory integrated Affymetrix gene expression data obtained for ATLL samples compared to CD4+ controls (Pise-Masison et al., 2009) with Agilent microRNA expression data to predict potential targets for differentially expressed microRNAs. Results revealed that let-7g is anticorrelated with 14 genes listed in Table 8. Future experiments will be aimed at validating targets of let-7g in infected cells using as a starting point these 14 genes.

Table 8. Lists of predicted targets of let-7g

Gene	Protein	Associated diseases	Expression in ATLL vs. CD4	References
SUOX	Sulfite oxidase	Hepatocellular carcinoma	Downregulated	Jin et al., 2012
DARS2	Aspartyl-tRNA Synthetase 2, Mitochondrial	-	-	-
CCNF	Cyclin F	Malignant germ cell tumors	Upregulated	Murray et al., 2013
SCD	Stearoyl-CoA Desaturase	Breast cancer and Prostate cancer	Upregulated	Hilvo et al., 2011; Kim et al., 2011
NEK3	NIMA-related kinase 3	Breast cancer	Upregulated	McHale et al., 2008
RFX5	Regulatory Factor X, 5	-	-	-
ATP8B4	ATPase, class I, type 8B, member 4	-	-	-
SEC14L1	SEC14-Like 1	Prostate cancer	Upregulated	Agell et al., 2012
PGRMC1	Progesterone Receptor Membrane Component 1	Breast cancer	Upregulated	Neubauer et al., 2013
RDX	Radixin	-	-	-
DPP3	Dipeptidyl-Peptidase 3	-	-	-
DCLRE1B	DNA Cross-Link Repair 1B	-	-	-
PTPN7	Protein Tyrosine Phosphatase, Non-Receptor Type 7	Childhood B-cell lymphoma	Downregulated	Fridberg et al., 2008
TTLL4	Tubulin Tyrosine Ligase-Like Family, Member 4	Pancreatic ductal adenocarcinoma cells	Upregulated	Kashiwaya et al., 2010

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6 APPENDIX

Manuscript in press

Ruggero K, Guffanti A, Corradin A, **Sharma VK,** De Bellis G, Corti G, Grassi A, Zanovello P, Bronte V, Ciminale V, D'Agostino DM. Small noncoding RNAs in cells transformed by human T-cell leukemia virus type 1: a role for a tRNA fragment as a primer for reverse transcriptase. J Virol. 2014 Jan 8. [Epub ahead of print]

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Small Noncoding RNAs in Cells Transformed by Human T-Cell Leukemia Virus Type 1: a Role for a tRNA Fragment as a Primer for Reverse Transcriptase

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ABSTRACT

The present study employed mass sequencing of small RNA libraries to identify the repertoire of small noncoding RNAs expressed in normal CD4$^+$ T cells compared to cells transformed with human T-cell leukemia virus type 1 (HTLV-1), the causative agent of adult T-cell leukemia/lymphoma (ATLL). The results revealed distinct patterns of microRNA expression in HTLV-1-infected CD4$^+$ T-cell lines with respect to their normal counterparts. In addition, a search for virus-encoded microRNAs yielded 2 sequences that originated from the plus strand of the HTLV-1 genome. Several sequences derived from tRNAs were expressed at substantial levels in both uninfected and infected cells. One of the most abundant tRNA fragments (tRF-3019) was derived from the 3′ end of tRNA-proline. tRF-3019 exhibited perfect sequence complementarity to the primer binding site of HTLV-1. The results of an *in vitro* reverse transcriptase assay verified that tRF-3019 was capable of priming HTLV-1 reverse transcriptase. Both tRNA-proline and tRF-3019 were detected in virus particles isolated from HTLV-1-infected cells. These findings suggest that tRF-3019 may play an important role in priming HTLV-1 reverse transcription and could thus represent a novel target to control HTLV-1 infection.

IMPORTANCE

Small noncoding RNAs, a growing family of regulatory RNAs that includes microRNAs and tRNA fragments, have recently emerged as key players in many biological processes, including viral infection and cancer. In the present study, we employed mass sequencing to identify the repertoire of small noncoding RNAs in normal T cells compared to T cells transformed with human T-cell leukemia virus type 1 (HTLV-1), a retrovirus that causes adult T-cell leukemia/lymphoma. The results revealed a distinct pattern of microRNA expression in HTLV-1-infected cells and a tRNA fragment (tRF-3019) that was packaged into virions and capable of priming HTLV-1 reverse transcription, a key event in the retroviral life cycle. These findings indicate tRF-3019 could represent a novel target for therapies aimed at controlling HTLV-1 infection.

AQ: A Adult T-cell leukemia/lymphoma (ATLL) is an aggressive neoplasm of mature CD4$^+$ cells that is etiologically linked to infection with human T-cell leukemia virus type 1 (HTLV-1). About 15 to 25 million people are infected with HTLV-1 worldwide, with infection most prevalent in southwestern Japan and the Caribbean basin. The virus is transmitted through blood, semen, and breast milk. While most infected individuals remain asymptomatic, about 3% eventually develop ATLL after decades of clinical latency. HTLV-1 also causes tropical spastic paraparesis/HTLV-associated myelopathy (TSP/HAM), a progressive demyelinating disease that targets mainly the thoracic spinal cord; similar to ATLL, TSP/HAM arises in about 3% of infected individuals, but after a latency period of years rather than decades (for reviews of HTLV-1 pathogenesis, see references 1 and 2).

HTLV-1 was the first human retrovirus to be identified and is the only one with a direct etiological link to cancer. HTLV-1 is classified as a "complex" retrovirus, as its genome contains extra open reading frames (ORFs), in addition to the *gag*, *pol*, *pro*, and *env* genes common to all retroviruses (reviewed in reference 3). The extra ORFs in HTLV-1 code for a transcriptional transactivator named Tax, a posttranscriptional regulatory protein named Rex, and four accessory proteins named HBZ, p30, p13, and p12/p8 (4).

HTLV-1 is found mainly in CD4$^+$ T cells *in vivo*. Infection of peripheral blood mononuclear cells (PBMC) with HTLV-1 yields interleukin 2 (IL-2)-dependent immortalized T cells, some of which progress to a fully transformed phenotype with IL-2-independent growth. The immortalizing potential of HTLV-1 is attributable primarily to the viral protein Tax. In addition to transactivating the viral promoter, Tax affects the expression and function of cellular genes controlling signal transduction, cell growth, apoptosis, and chromosomal stability (5) and is able to

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cause leukemia when expressed as a transgene in mice (6). HBZ also likely contributes to the oncogenic properties of HTLV-1; it is mitogenic for T cells (7) and is able to induce leukemia in transgenic mice (8). Other accessory proteins may affect viral transmission and persistence (9–11).

In the present study, we investigated the expression repertoire of small noncoding RNAs, in particular microRNAs (miRNAs) and tRNA fragments (tRFs), in HTLV-1-infected cells. MicroRNAs negatively regulate gene expression at the posttranscriptional level by base pairing to specific target mRNAs in RNA-induced silencing complexes (RISC) containing Argonaute proteins. Perfect base pairing leads to degradation of the mRNA in an RNA interference (RNAi)-like manner, while imperfect base pairing (the more frequent interaction) results in a block of translation (12). Posttranscriptional regulation of gene expression by microRNAs is of critical importance in normal cell physiology, and aberrant expression of microRNAs is emerging as a key component of a wide range of pathologies, including solid and hematological tumors (13).

Expression studies based on quantitative reverse transcriptase (RT) PCR and microarray analysis identified a number of cellular microRNAs that are downregulated or upregulated in infected cell lines and ATLL cells (14–17). Viruses may also produce microRNAs as a means of regulating the expression of viral or host genes (18). Recent studies of bovine leukemia virus (BLV), a retrovirus in the same subfamily as HTLV-1, revealed the expression of a cluster of viral microRNAs (19, 20). A computational analysis of the HTLV-1 genome identified 11 sequences with potential to form stem-loop structures that could yield viral microRNAs (21).

tRFs are produced from the 3′ ends of tRNA precursors or from the 5′ ends or the 3′ ends of mature tRNAs and are designated tRF-1, tRF-5, and tRF-3, respectively (22). tRFs have an average length of 19 nucleotides (nt) (23) and, similar to microRNAs, are produced by specific cleavage events rather than through degradation. tRF-1 sequences are cleaved from tRNA precursors by the RNase ELAC2, while Dicer was shown to be responsible for cleavage to produce tRF-5 (23) and tRF-3 (24). Although not much is known about the function of tRFs, tRF-5 and tRF-3 were shown to form complexes with Argonaute proteins (23, 24), and tRF-3 repressed expression of specific mRNA targets through a microRNA-like mechanism (24).

AQ: B We employed 454 massive sequencing to identify the repertoire of microRNAs and tRFs expressed in HTLV-1-infected cells compared to normal CD4$^+$ T cells. Comparison of the frequencies of known microRNAs in the libraries revealed 3 microRNAs that were differentially expressed in the infected cell lines compared to CD4 controls, 2 small RNAs that matched HTLV-1 sequences, and several abundant tRFs. We provide evidence that a tRF corresponding to the 3′ end of tRNA-proline is incorporated into virus particles and can function as a primer for viral reverse transcriptase.

MATERIALS AND METHODS

Cell culture. Cell lines C91PL and MT-2, chronically infected with HTLV-1 (25), were maintained in RPMI (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 2 mM glutamine (Invitrogen), 100 units/ml penicillin, and 20 units/ml streptomycin (complete RPMI). PBMC were isolated from buffy coat fractions obtained from healthy plasma donors attending the Transfusion Unit of Padua City Hospital by centrifugation through Ficoll-Hypaque (GE Healthcare). Half of the PBMC sample was immediately processed using the MACS CD4$^+$ T cell Isolation Kit II (Miltenyi Biotec), and the resulting CD4 cells were harvested for total RNA (see below). The other half of the PBMC preparation was placed in complete RPMI (1×10^6 cells/ml) supplemented with 100 μg/ml phytohemagglutinin (PHA) (Sigma-Aldrich) and cultured for 48 h. The culture was then supplemented with 50 U/ml IL-2 (Proleukin-Chiron), incubated for an additional 48 h, and harvested for isolation of CD4 cells with the MACS kit. Flow cytometry analysis revealed that the isolated cell preparations contained more than 99% and 94% CD4 cells in the unstimulated and stimulated preparations, respectively. Total RNA was isolated using TRIzol (Invitrogen). The quality of the RNA was assessed by electrophoresis using the RNA 6000 Nano Assay LabChip Kit and Agilent 2100 Bioanalyzer, and the RNA concentration was measured using a Nanodrop spectrophotometer.

Generation of small RNA libraries. Small RNA libraries were generated according to the method of Lau et al. (26) as follows. A 10-μg aliquot of each total RNA preparation was spiked with a ^{32}P-labeled 23-nt RNA tracer and then subjected to polyacrylamide gel electrophoresis (PAGE) through a 15% denaturing gel, along with a ^{32}P-labeled aliquot of Decade RNA ladder (Ambion); the presence of the RNA tracer permitted visualization of each modification and purification step after exposure of the gel to a phosphorimaging screen (Storm; GE Healthcare). Species migrating in the ~18- to 25-nucleotide size range were excised from the gel, eluted, and ethanol precipitated. The RNA was then modified by addition of a 17-nt oligonucleotide linker (miRNA Cloning Linker 1; IDT) at the 3′ end with RNA ligase (GE Healthcare), PAGE purified through a 12% denaturing gel, modified with a second 17-nt oligonucleotide linker at the 5′ end, and PAGE purified again through a 10% denaturing gel. The resulting modified RNA was reverse transcribed and PCR amplified in preparation for sequencing according to a protocol provided by G. Hannon (Cold Spring Harbor Laboratory), as described previously (27). The resulting samples were subjected to 454 massive sequencing using a Roche Life Sciences platform. The total numbers of sequence reads were 7,709 (freshly isolated CD4), 7,818 (stimulated CD4), 7,603 (C91PL), and 6,801 (MT-2). The library prepared from freshly isolated CD4$^+$ cells was described previously (27). Lists of sequence reads are available upon request.

Identification of microRNAs and tRFs by bioinformatics analysis. The sequence reads were trimmed from matches with sequencing primers or linker sequences and subjected to nonredundancy analysis with the NCBI nrdb program of the BLAST suite (28).

The sequences were compared with the mature microRNA subset of the mirBase database, version 18 (http://www.mirbase.org/) using the Smith-Waterman search program from the FastA sequence analysis suite (29), allowing a maximum of one mismatch between the sequence read and the reference mature miRNA. Read counts annotated with the mature microRNA names were tested for differential expression with the Bioconductor edgeR package (30). Reads that did not match mature microRNAs were subjected to a further comparison with mirBase precursor sequences. Sequences that did not map to known microRNA precursors were compared with the downloaded FastA sequences from the Genomic tRNA database (http://gtrnadb.ucsc.edu/) and tRNA-derived fragments (7) using the SHRiMP program (http://compbio.cs.toronto.edu/shrimp/). tRF-3019 and other tRFs were identified by searching sequence lists with Excel tools. The small RNA sequences that did not correspond to known microRNAs or tRFs were subjected to novel small RNA prediction with the mirDeep2 pipeline (31), but no suitable novel candidates were found. All the analyses were integrated with *ad hoc* written perl scripts.

To identify potential viral microRNAs, the sequence sets were compared to the HTLV-1 genome sequence ATK (GenBank accession no. J02029 and DDBJ accession no. M33896). Sequences with ≥90% identity AQ: C with HTLV-1 and ≤2 gaps and ≤2 mismatches were further analyzed as described in Results below.

RT assay using tRF-3019. (i) Preparation of RNA template. A DNA fragment corresponding to nt 721 to 822 of HTLV-1 ATK was PCR amplified using the HTLV-1 molecular clone ACH (32) as a template and

primers U5-s and Gag-as (see Table S1 in the supplemental material). A 20-nt tail was added to the 5′ end of the product with a second round of PCR using primers Tail-U5-s and Gag-as. The 129-nt fragment was cloned into vector pSG5E, which is a modified version of pSG5 (Stratagene) containing the polylinker of pBluescript (Stratagene) 3′ to the T7 promoter. The resulting plasmid (pSG-U5-PBS) was linearized 3′ to the insert and *in vitro* transcribed with T7 RNA polymerase (Invitrogen). After a DNase 1 digestion to eliminate the plasmid, the mixture was extracted with phenol-chloroform and ethanol precipitated to recover the RNA. The resulting pellet was resuspended in water and stored at −80°C.

(ii) Preparation of virus particle lysates containing HTLV-1 reverse transcriptase. Confluent cultures of C91PL cells were centrifuged at low speed to remove the cells. The supernatant was passed through a 0.45-μm filter (Sartorius) and centrifuged at 24,000 rpm in an SW28 rotor for 2 h. The pelleted material was resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5% Nonidet-P40; 10 μl per 10 ml centrifuged supernatant) and stored at −80°C.

(iii) RT assay. The RT assay was based on a published method (33, 34). For each RT assay, a 100-ng aliquot of *in vitro*-transcribed RNA was combined with 10 pmol of either tRF-3019 RNA, miR-150-5p RNA (negative control), tRF-3019 DNA (positive control), or water instead of primer in a 10.5-μl volume; annealed at 70°C for 10 min; and then cooled on ice. The mixtures were brought to a final volume of 20 μl containing 1 mM each deoxynucleoside triphosphate (dNTP), 10 U RNase inhibitor, RT buffer (25 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$, 50 mM KCl, 2 mM dithiothreitol [DTT]), and 2 μl virion lysate and incubated at 37°C for 1 h, followed by 95°C for 5 min. A 2.5-μl aliquot of the cDNA product was amplified in a final volume of 25 μl containing 1× *Taq* Gold PCR buffer, 2 mM $MgCl_2$, 200 μM dNTPs, 5 pmol each of primers Tail-s and U5-as, and 0.5 U AmpliTaq Gold DNA polymerase (Life Technologies). The PCR method consisted of a denaturation step at 94°C for 1 min, followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 45 s. The products were analyzed on a 6% polyacrylamide gel and stained with ethidium bromide. Images were obtained using a Bio-Rad Gel Doc XRS system.

RT-PCR to detect tRFs, tRNAs, and gag/pol RNA. FBS may contain exosomes carrying small RNAs; to avoid this possible source of contamination, complete RPMI containing 20% FBS was centrifuged at 24,000 rpm for 4 h using a Beckman-Coulter SW28 rotor to pellet any exosomes. The supernatant medium was then passed through a 0.2-μm filter and brought to 10% FBS by adding an equal volume of RPMI containing antibiotics and glutamine. C91PL cells were cultured to confluence in the exosome-depleted medium, and virus particles were recovered as described above. RNA was isolated from the pelleted particles and the producer C91PL cells using TRIzol LS (Life Technologies). Aliquots of the RNA were subjected to denaturing PAGE through a 15% polyacrylamide gel to separate species in the size range of full-length tRNAs from the small RNAs, with tRNAs visible in the cellular RNA sample and 5 pmol synthetic miR-150-5p serving as size markers for the 2 fractions. The gel was stained with ethidium bromide, and the regions containing tRNAs and small RNAs (about 15 to 30 nt) were excised, crushed, and incubated with gentle mixing in elution buffer (300 mM sodium acetate, pH 5.2, 1 mM EDTA) overnight at 4°C. RNA was ethanol precipitated and resuspended in distilled H_2O (dH_2O) (10 μl per 19 ml of original culture supernatant) (see Fig. 6, where the fractions are labeled tRNA and tRF).

The primer sets used to amplify tRF-3019, tRF-3003, and the tRNAs from which they are derived (tRNA-Pro and tRN-Ala, respectively) are listed in Table S1 in the supplemental material (also see Fig. 6A). RT-PCR to detect tRF-3019 and tRF-3003 was based on a protocol for detecting microRNAs (35). Size-fractionated RNA (2 μl) was annealed with 2 pmol primer RT7-tRF-3019 or RT8-tRF-3003 at 70°C for 10 min in a 7.5-μl volume. The mixture was brought to 10 μl with the addition of 1 mM dNTP, 1× RT buffer, and 5 U avian myeloblastosis virus (AMV) reverse transcriptase (Finnzymes) and reverse transcribed at 40°C for 1 h. Two-microliter aliquots of the resulting cDNAs were PCR amplified in a final volume of 25 μl containing 1× *Taq* Gold PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTP, 0.1 pmol of primer Short-tRF-3019 or Short-tRF-3003, 2.5 pmol each of primers PCR-tRF-s and PCR-tRF-as (specific for tails added by the RT primer and Short-tRF primer), and 0.5 U AmpliTaq Gold DNA polymerase. The PCR method consisted of a denaturation step at 95°C for 10 min and 5 cycles of denaturation at 95°C for 30 s, annealing at 40°C for 45 s, and extension at 72°C for 30 s, followed by 22 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 45 s, and extension at 72°C for 30 s.

To detect tRNA-Pro and tRNA-Ala, 1 μl of size-fractionated RNA was reverse transcribed in a 10-μl reaction mixture at 53°C for 50 min using the antisense primer and Superscript III (Life Technologies). The resulting cDNA (2.5 μl) was PCR amplified using sense and antisense primers and AmpliTaq Gold, with a denaturation step at 95°C for 8 min, followed by cycles of denaturation at 95°C for 40 s, annealing at 60°C for 40 s, and extension at 72°C for 40 s (30 cycles for tRNA-Ala and 26 cycles for tRNA-Pro). To detect gag/pol RNA, 0.5 μl of RNA from virus particles or 200 ng total RNA from producer C91PL cells was reverse transcribed using primer Gag-as and Superscript III and then PCR amplified using primers U5-s and Gag-as (see Table S1 in the supplemental material) and AmpliTaq Gold as described above for 30 cycles with an annealing temperature of 59°C and a final extension step for 5 min at 72°C. The PCR products were separated on 6% polyacrylamide gels.

RESULTS

Generation, sequencing, annotation, and quantification of small RNA libraries. Total RNA was isolated from the HTLV-1-infected cell lines C91PL and MT-2 (25), which have a $CD4^+$ phenotype, and from control normal unstimulated and *in vitro*-stimulated $CD4^+$ T cells. RNA species of ~18 to 25 nt were separated by polyacrylamide gel electrophoresis and modified with tags at the 5′ and 3′ ends according to the method of Lau et al. (26) and sequenced using a Roche 454 Life Sciences platform. Sequence reads were mapped against mirBase release 18 to identify the populations of mature known microRNAs in the investigated cell lines, and the edgeR Bioconductor statistical package (see Materials and Methods) was employed to identify statistically significant differences in the frequencies of known microRNAs.

The graphs in Fig. 1 illustrate the 10 most abundant known microRNAs identified in the libraries. miR-142-3p, a marker of hematopoietic cells, stood out as the most abundant microRNA in freshly isolated $CD4^+$ T cells and in the infected cell line MT-2, with a frequency more than triple that of the other microRNAs. miR-21, a microRNA known to be linked to T-cell activation and transformation (36), became nearly as frequent as miR-142-3p upon *in vitro* stimulation of $CD4^+$ cells and was also abundant in the two infected cell lines. F1

To identify the microRNAs connected with HTLV-1 infection, we calculated differences in the frequencies of microRNAs in infected cell lines versus resting and stimulated $CD4^+$ T cells. Three microRNAs were differentially expressed in both infected cell lines compared to control CD4 cells (indicated in boldface in Table 1: miR-34a-5p was upregulated, and miR-150-5p and miR-146b-5p were both downregulated). An analysis performed using the miRDeep2 software did not yield any putative new microRNA candidates among the sequence reads detected in this study. AQ:D/T1

Small RNAs expressed by HTLV-1. With the aim of identifying viral microRNAs, the reads obtained from MT-2 and C91PL cells were aligned to the HTLV-1 genome. This analysis yielded 25 sequences with ≥90% identity with HTLV-1 and ≤2 gaps and ≤2 mismatches (data not shown). Two sequences shown in Fig. 2A perfectly matched the primary plus-strand HTLV-1 transcript. Both sequences were present only in the MT-2 library. Sequence F2

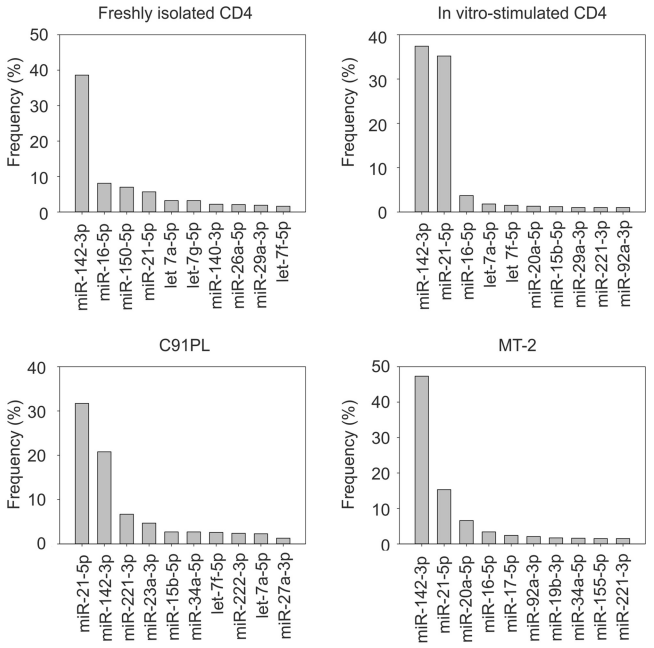


FIG 1 Relative abundances of microRNAs identified by 454 sequencing. Shown are the 10 most abundant microRNAs in normal CD4 T cells and HTLV-1-infected cell lines. Frequencies were calculated by dividing the number of sequence reads for each microRNA by the total number of sequence reads for all known microRNAs.

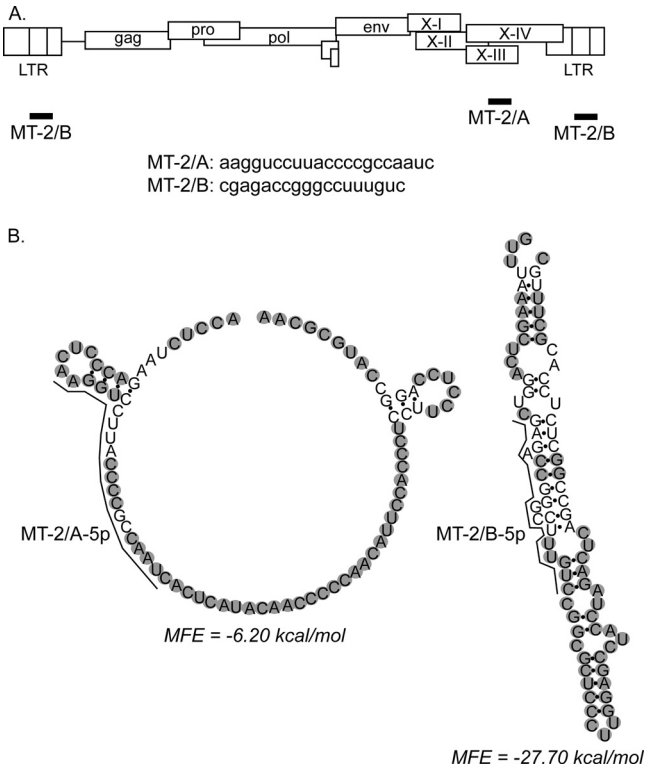


FIG 2 Small RNAs expressed by HTLV-1. (A) Positions and nucleotide sequences of the two small RNA species identified in MT-2 cells. MT-2/A corresponded to nt 7582 to 7602 in exon 3 of the HTLV-1 ATK sequence. MT-2/B corresponded to nt 513 to 530 of the 5′ R region and nt 8792 to 8808 of the 3′ R region of ATK. (B) Secondary structure predicted by RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi; University of Vienna) for HTLV-1 sequences containing MT-2/A and MT-2/B with 15 nt added at the 5′ end and 50 nt at the 3′ end to simulate a pre-miR. The optimal secondary structures and their minimum free energy (MFE) values are indicated. AQ: H

MT-2/A was positioned in exon 3, between the stop codons for p30/p13 and Rex. Sequence MT-2/B was located in the R region, in a position within stem-loop D of the Rex response element (RXRE) (37). This segment of the HTLV-1 genome was predicted to form a pre-miR-like structure and thus to have the potential to give rise to a viral microRNA (21). Figure 2B shows the predicted secondary structures of genomic regions containing MT-2/A or MT-2/B with 5′ and 3′ flanking sequences to simulate their position in the 5′ portion of a pre-miR. Results showed that sequence MT-2/A is likely to be present mostly in an unstructured region, while sequence MT-2/B has a high probability to be positioned in a stem.

AQ: E

TABLE 1 Differentially expressed microRNAs[a]

Cells	MicroRNA	Log_2 FC	*P* value
MT-2 vs. CD4	**miR-34a-5p**	**6.15**	**0.00015**
	miR-4448	4.89	0.00366
	miR-7-5p	4.35	0.01564
	miR-150-5p	**−10.08**	**0.00327**
	miR-30c-5p	−8.05	0.01329
	miR-146b-5p	**−7.52**	**0.03318**
	miR-29c-3p	−6.87	0.03802
C91PL vs. CD4	**hsa-miR-34a-5p**	**6.66**	**0.00001**
	hsa-miR-92b-3p	3.82	0.01313
	hsa-miR-23a-3p	3.15	0.01166
	hsa-miR-150-5p	**−10.61**	**0.00127**
	hsa-miR-342-5p	−7.02	0.02719
	hsa-miR-26a-5p	−6.84	0.00776
	hsa-miR-20b-5p	−6.74	0.03088
	hsa-miR-146b-5p	**−4.76**	**0.02268**
	hsa-miR-19b-3p	−3.83	0.03015
	hsa-miR-16b-5p	−3.65	0.02867

[a] MicroRNAs with statistically significant differences in expression are indicated in boldface.

tRFs expressed in HTLV-1-infected cells. We next tested the sequences identified in the libraries for perfect matches to the 135 tRFs reported by Lee et al. in a study of prostate cancer cell lines (22). Table S2 in the supplemental material lists the number of sequence reads for each tRF, as well as isoforms showing variations at the 5′ or 3′ end. Overall, in both normal and HTLV-1-infected CD4 cells, fragments processed from the 3′ ends of mature tRNAs (tRF-3) were considerably more abundant than tRFs produced from the 3′ ends of tRNA precursors (tRF-1) or from the 5′ ends of mature tRNAs (tRF-5) (Fig. 3B). F3

Figure 3C shows the most abundant tRFs identified in the libraries. Among the 22 previously described tRF-1 sequences, tRF-1001 was the most abundant. tRF-1001, as well as the other tRFs, were upregulated in normal $CD4^+$ cells upon mitogenic stimulation. tRF-3004 and tRF-3029 were more abundant in C91PL cells than in stimulated $CD4^+$ controls, and MT-2 cells yielded few tRF sequences compared to the other 3 cell types.

The tRF-3 class also includes tRF-3019 (22). This tRF corresponds to the 3′ end of tRNA-Pro, the tRNA considered to serve as the primer for HTLV-1 RT (38). tRF-3019 was the fifth most abundant tRF identified in our libraries and was most abundant in

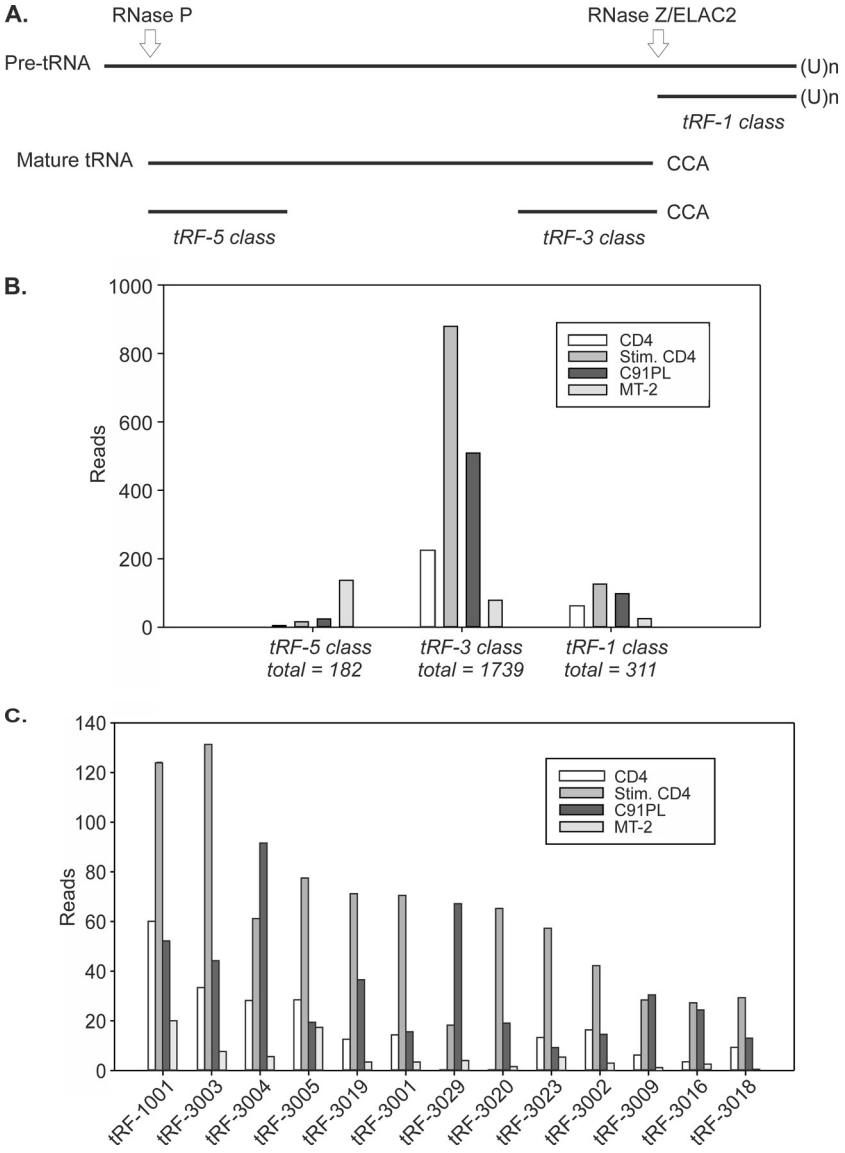
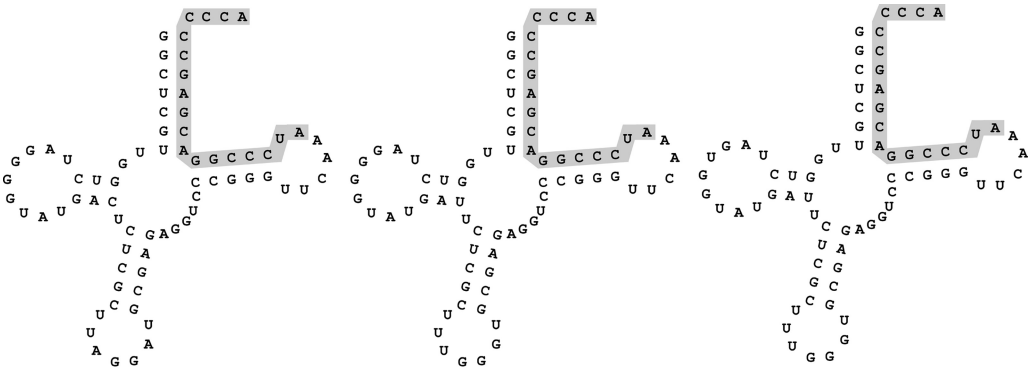


FIG 3 Relative abundances of tRFs. (A) The 3 classes of tRFs aligned to the tRNA precursor. (B) Total numbers of sequence reads with perfect matches to each of the tRF classes, together with 5′ and 3′ isoforms (see Table S2 in the supplemental material). (C) Sequence reads for tRFs with a total of at least 50 sequence reads summed among the 4 libraries.

stimulated $CD4^+$ cells. A BLAST search for tRNA genes able to produce tRF-3019 yielded 21 tRNA-Pro genes located on chromosomes 1, 5, 6, 11, 14, 16, and 17 (see Table S3 in the supplemental material). The four libraries contained several tRF-3019 isoforms with additional nucleotides at the 5′ end that perfectly matched the human genome but were not complementary to the viral genome (Fig. 4). The libraries also contained a small number of reads corresponding to fragments derived from other portions of tRNA-ProTGG and tRNA-ProAGG.

tRF-3019 functions as a primer for HTLV-1 reverse transcriptase. It is noteworthy that only the portion of tRNA-Pro corresponding to tRF-3019 is complementary to the HTLV-1 primer binding site (PBS) (Fig. 5A), suggesting that the tRF would be fully sufficient as a primer for reverse transcription. We thus tested the primer activity of tRF-3019 in an *in vitro* reverse transcriptase assay carried out using a synthetic RNA template and the reverse transcriptase contained in HTLV-1 particles recovered from the culture supernatant of C91PL cells (Fig. 5A). The RT assay mixtures contained either no primer, synthetic tRF-3019 RNA, or tRF-3019 DNA as a positive control or miR-150-5p RNA as a negative control. The PCR mixture contained a sense primer specific for a tail sequence present in the synthetic RNA template and an antisense primer positioned immediately 5′ to the PBS. Figure 5B shows the results of the assays. The RT assay performed using tRF-3019 RNA yielded the expected 87-bp PCR product, thus confirming that tRF-3019 can function as a primer for HTLV-1 RT. The assay carried out using a tRF-3019 DNA primer yielded the 87-bp product, along with a longer product indicated by the gray arrow in Fig. 5B. This second band corresponded in size to an amplicon produced with the tail primer and residual tRF-3019 DNA present in the cDNA (i.e., 107 bp). Interestingly, trace amounts of the 87-bp product were also detected in the assays



	Sequence	CD4	STIM. CD4	C91PL	MT-2
tRF-3019 (nt ~56-72+CCA)	AUCCCGGACGAGCCCCCA	12	71	36	3
tRF-3019 5′ isoforms		1	11	13	1
tRF (nt ~1-18)	GGCUCGUUGGUCUAGUGG	0	1	0	1
tRF (nt ~1-22)	GGCUCGUUGGUCUAGGGGUAUC	0	2	2	9
tRF (nt ~8-26)	UGGUCUAGGGGUAUGAUUC	3	2	0	3
tRF (nt ~32-53)	UUGGGUGCGAGAGGUCCCGGGU	18	15	0	4

FIG 4 tRFs processed from tRNA-Pro. (Top) Three examples of the 21 tRNA-Pro molecules that are able to produce tRF-3019 (shaded). The diagrams were AQ: I obtained from the UCSC database and modified by adding the 3′ CCA triplet that is present on mature tRNAs and tRF-3 sequences (Fig. 3). (Bottom) Sequences of the tRFs and number of reads identified in each library.

carried out using C91PL RT and miR-150-5p or no primer. This amplicon may have originated from cDNA primed by tRNA-Pro or tRF-3019 present in the viral particle lysate that was used as a source of RT (see below).

HTLV-1-infected cells release particles containing tRF-3019. Having established that tRF-3019 is capable of priming HTLV-1 reverse transcription, we next tested for the presence of tRNA-Pro and tRF-3019 in virus particles recovered from supernatants of C91PL cultures. As a control, we also assayed for tRF-3003, the most abundant tRF-3 detected in the 4 libraries, along with its parent, tRNA-Ala. As described in Materials and Methods, RNA isolated from the virus particles and producer cells was subjected to denaturing PAGE to separate species in the tRF size range from full-length tRNAs. This was necessary, as the tailed RT-PCR primers utilized to detect the tRFs also amplified the 3′ ends of the F6 full-length tRNAs (Fig. 6A). RT-PCR products were separated by PAGE; the intensities of the resulting bands were measured to estimate relative abundances of the tRNAs and tRFs in virus particles versus cells.

Figure 6B shows PAGE analyses of the RT-PCR products obtained for tRNA-Ala and tRNA-Pro. As expected, both tRNA-Ala and tRNA-Pro were readily detected in the C91PL cells. However, a plot of the ratios of the band intensities (Fig. 6D) revealed that tRNA-Pro was enriched in virus particles compared to tRNA-Ala. The RT-PCR products for both tRNAs were much more evident in the full-length tRNA fraction than in the tRF fraction, indicating that the denaturing PAGE purification step resulted in acceptable separation of the 2 size classes.

Figure 6C shows the results of RT-PCR carried out on the same samples using primer sets that amplified the tRFs present in the tRF fraction and the 3′ ends of the tRNAs in the tRNA fraction. Calculation of band intensity ratios (Fig. 6D) showed that tRF-3003 was much less abundant in the virus particles than in the cells, while tRF-3019 was detected at comparable levels in virus particles and cells. Therefore, we concluded that tRF-3019 was enriched in virus particles compared to tRF-3003.

These findings indicate that both tRNA-Pro and tRF-3019 are incorporated into particles released in the supernatant of HTLV-1-infected cells. It is necessary to point out that these particles may also contain exosomes, which may also package proteins and RNA. However, the observation that the particles were enriched for tRNA-Pro and tRF-3019 compared to tRNA-Ala and tRF-3003 supports a specific packaging process directed by interaction of the tRNA/tRF with the HTLV-1 PBS. As shown in Fig. 6E, RT-PCR assays on RNA isolated from the particles confirmed that they contain the HTLV-1 genomic gag/pol mRNA. Therefore, although we cannot exclude the presence of exosomes in the particle preparations, our findings demonstrate that these particles contain reverse transcriptase activity (Fig. 5) and the viral genome and are enriched for the PBS-specific tRNA-Pro and tRF-3019. Taken together, these results strongly suggest that tRF-3019 is likely to contribute to HTLV-1 reverse transcription.

DISCUSSION

In the present study, we employed mass sequencing to identify the repertoire of small noncoding RNAs expressed in normal T cells compared to cells transformed with HTLV-1. The results revealed a distinct pattern of microRNA expression in HTLV-1-infected cells, two virus-encoded small RNAs, and a number of tRFs. Interestingly, the tRNA fragment tRF-3019 was detected in virus

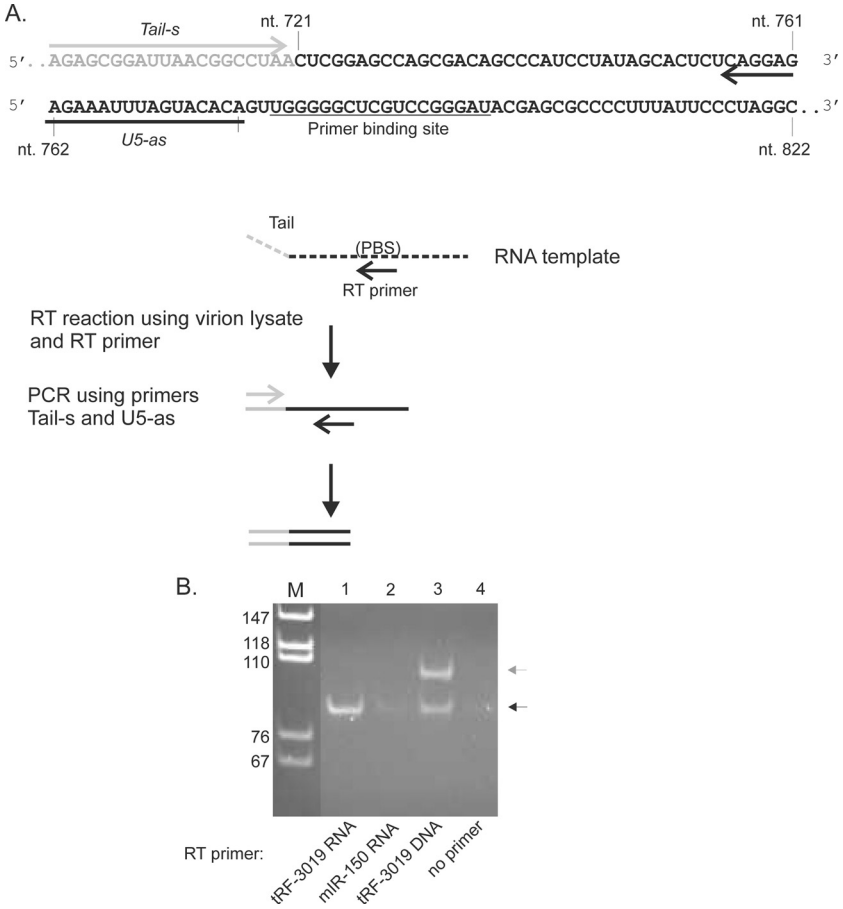


FIG 5 tRF-3019 acts as a primer for HTLV-1 reverse transcriptase. (A) Summary of the RT assay. The template consisted of an *in vitro*-transcribed RNA spanning HTLV-1 nt 721 to 822 modified by the addition of a 20-nt tail at the 5′ end. The template was incubated with HTLV-1 reverse transcriptase present in virus particles recovered from the culture supernatant of C91PL cells and either tRF-3019 RNA, miR-150-5p RNA (negative control), tRF-3019 DNA (positive control), or no primer. The products of the RT reactions were amplified by PCR using PCR primers Tail-s and U5-as and separated by PAGE in a 6% polyacrylamide gel, along with MspI-digested pBluescript as a size marker. (B) Composite of the ethidium bromide-stained gel. The black arrow indicates the position of the 87-bp PCR product expected using primers Tail-s and U5-as. The additional band in lane 3 indicated by the gray arrow was consistent with a product amplified by Tail-s and residual tRF-3019 DNA primer added to the RT assay. The primer sequences are reported in Table S1 in the supplemental material.

particles and was capable of priming HTLV-1 reverse transcription.

Three microRNAs were differentially expressed in both infected cell lines compared to control $CD4^+$ cells: miR-34a-5p was upregulated, and miR-150-5p and miR-146b-5p were both downregulated. Their shared pattern of regulation in the two infected cell lines suggests that these microRNAs play important roles in HTLV-1 infection/transformation, rather than representing markers of T-cell activation, which are also present in HTLV-1-infected cells.

The observation that miR-150-5p expression is reduced in HTLV-1-infected cell lines is consistent with other studies (15, 16). miR-150-5p undergoes upregulation during T-cell development (27) but is diminished upon stimulation of normal murine $CD4^+$ T cells (39). Expression of miR-150-5p is increased in several hematological tumors, including ATLL samples (15, 16), but is downregulated in the cutaneous $CD4^+$ T-cell lymphoma Sézary syndrome (40), in NK/T-cell lymphomas (41), and in several other hematological malignancies (reviewed in reference 42). Forced expression of miR-150-5p in B-lymphoma cell lines (43), T-acute lymphoblastic leukemia (T-ALL) cell lines (27), and NK cell lines (41) produced antiproliferative and/or proapoptotic effects. Validated targets of miR-150-5p include the oncogenes c-Myb (44) and NOTCH-3 (27), as well as the HIV-1 3′ untranslated region (UTR) (45). It is noteworthy that the minus-strand HTLV-1 transcripts coding for HBZ contain 2 potential binding sites for miR-150-5p (46).

miR-146b-5p is gradually upregulated during T-cell development from the double-positive $CD4^+$ $CD8^+$ to the single-positive $CD4^+$ or $CD8^+$ stage (27). The sequence of miR-146b-5p is almost identical to that of miR-146a, which was identified as upregulated through the action of Tax in previous studies of HTLV-1-infected cell lines (14, 47). miR-146b-5p mRNA targets, therefore, likely overlap those identified for miR-146a, which include the Toll-like receptor signaling pathway proteins TRAF6 and IRAK1 (48), the apoptosis signaling protein FADD (49), and the chemokine receptor CXCR4 (50). miR-146b-5p is downregulated in ATLL (16, 17), Sézary syndrome (40), and several other hematological malignancies but is upregulated in mycosis fungoides (51) and pediatric acute myeloid leukemia (52; reviewed in reference 42).

miR-34a-5p is known to be upregulated by p53 in response to

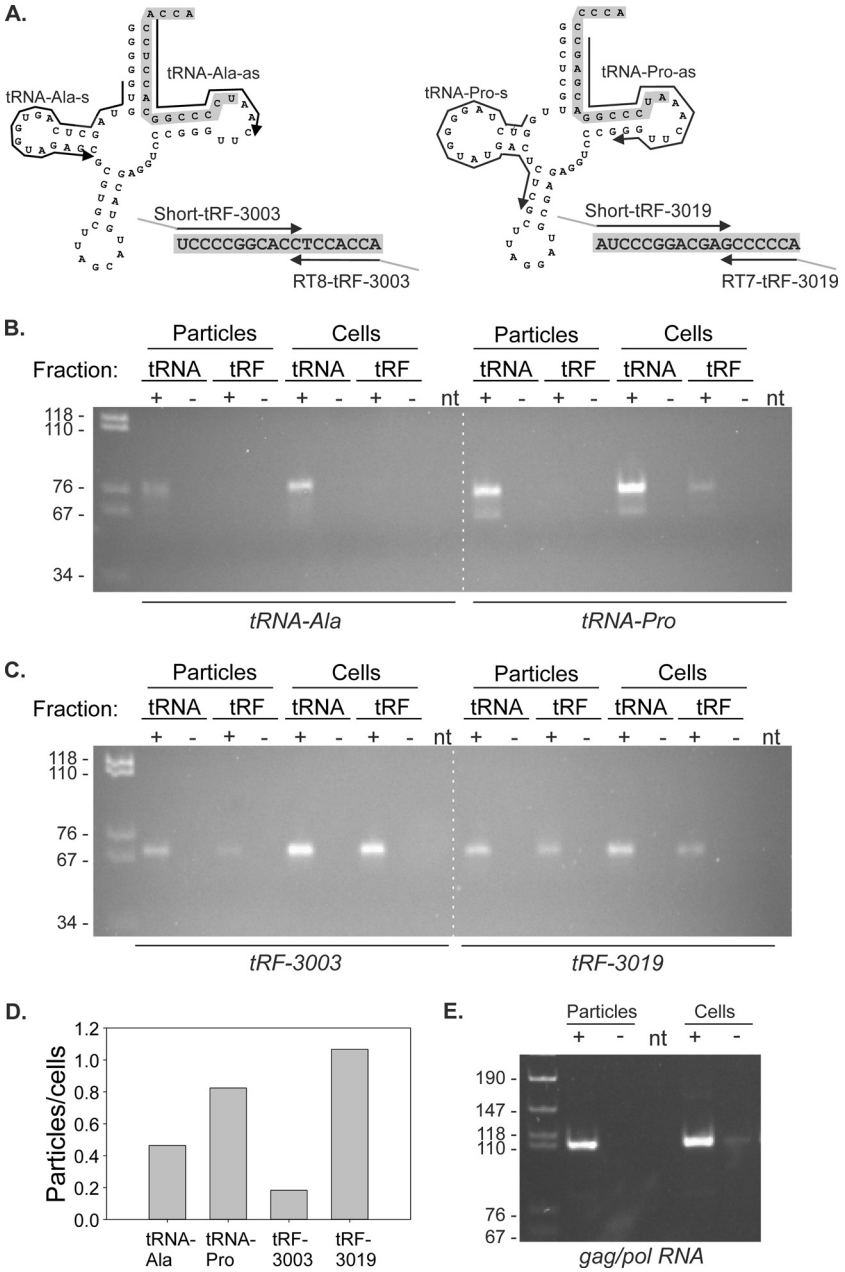


FIG 6 RT-PCR to detect tRNAs, tRFs, and gag/pol RNA in virus particles and C91PL cells. (A) As described in Materials and Methods, RNA from virus particles and producer C91PL cells was subjected to denaturing PAGE; regions of the gel containing tRNA and tRFs were excised, and RNA was recovered by passive elution and ethanol precipitation. The resulting fractions were subjected to RT-PCR to detect tRNA-Ala, tRNA-Pro, and their tRF-3 sequences, tRF-3003 and tRF-3019, respectively. (B and C) Images of the RT-PCR products after separation on 6% polyacrylamide gels. The intensities of RT-PCR bands obtained for tRNAs and tRFs (measured in tRNA and tRF fractions, respectively) were measured using a Bio-Rad Gel Doc XRS imager. (D) Plot of ratios of band intensities obtained for virus particles versus cells. The calculated ratios were as follows: tRNA-Ala, particles/cells = 0.46; tRNA-Pro, particles/cells = 0.82; tRF-3003, particles/cells = 0.18; tRF-3019, particles/cells = 1.07. (E) Results of RT-PCR performed on RNA from the virus particles and producer cells to detect HTLV-1 genomic gag/pol RNA. RT-PCR was carried out using primers U5-s and Gag-as as described in Materials and Methods. The dashed white lines were added to panels B and C to aid in their alignment. The first lane on each gel contained MspI-digested pBluescript as a size marker; band sizes in basepairs are indicated on the left. The plus and minus signs above the lanes indicate RT reactions carried out in the presence (+) or absence (−) of reverse transcriptase. RNA template was omitted from the RT reaction in lanes labeled nt.

genotoxic and oncogenic stresses. miR-34a-5p targets genes affecting cell proliferation and survival, resulting in growth arrest, senescence, and apoptosis; its downregulation in several solid tumors suggests a tumor suppressor role (53). miR-34a-5p was found to be more abundant in memory versus naive $CD4^+$ T cells (54) and is upregulated in Epstein-Barr virus-transformed B cells (55) during latency type III (56) and in hepatitis B virus-associated hepatocellular carcinoma (57) and might thus exert diverse effects depending on the cell context (58). The results of RT-PCR assays indicated strong upregulation of miR-34a-5p

in primary samples from ATLL patients (D. M. D'Agostino, AQ: G ●●●, unpublished data).

Recent studies revealed that BLV, a complex oncogenic retrovirus related to HTLV-1, encodes a cluster of viral microRNAs (19, 20). Our deep-sequencing analysis also revealed two virus-encoded small RNA species (MT-2/A and MT-2/B). However, the fact that MT-2/A and MT-2/B were detected with only one sequence read each suggests that, in contrast to BLV, HTLV-1 may not rely on viral microRNAs as a mechanism of posttranscriptional regulation. Alternatively, the production of viral microRNAs might not be favored in cells that are chronically infected, such as MT-2 and C91PL. Therefore, before concluding that HTLV-1 does not produce microRNAs, it will be important to measure their levels of expression in the context of primary samples obtained from infected patients.

The greater representation of tRF-3 sequences than of tRF-1 and tR-5 classes in the libraries is in line with the preponderance of tRF-3 sequences found in prostate cancer cell lines (see Table S2 in reference 22) and in mature B cells (24). Previous functional studies of tRF-1001, which was abundantly expressed in our libraries, revealed its elevated expression in cancer cell lines compared to normal tissue samples and indicated that it is required for cell proliferation (22). Among the tRF-3 sequences abundantly expressed in the four libraries, functional data are available for tRF-3018 in the context of B cells (24). This tRF, named CU1276 in the B-cell study, was differentially expressed in different stages of B-cell maturation, with the greatest expression found in the germinal center (GC) stage while it was absent in GC-derived lymphoma cells. Functional studies of tRF-3018/CU1276 verified its ability to associate with Argonaute proteins and to repress expression of RPA1, a protein involved in DNA replication and repair (24).

The present study focused on tRF-3019, as it corresponds to the 3′ end of tRNA-Pro, which is generally considered to be the primer for HTLV-1 reverse transcriptase (38). tRF-3019 was capable of priming HTLV-1 reverse transcription (Fig. 5) and was enriched in virus particles (Fig. 6). Taken together, these observations support a role for tRF-3019 in the life cycle of HTLV-1.

As shown in Fig. 4, 12 of the 18 nucleotides of tRNA-Pro that are complementary to the HTLV-1 PBS are based paired in the mature tRNA. This positioning of the primer portion of the tRNA in a closed stem is a characteristic of all retroviral tRNA primers. These hydrogen bonds must be disrupted in order for the primer to bind to the PBS, which would not be necessary if a tRF is used as a primer.

The libraries examined in the present study contained a few sequence reads for tRF-3015, which represents the 3′ end of tRNA-Lys, the primer for HIV-1 (see Table S2 in the supplemental material). Schopman et al. (59) pointed out the possibility that tRFs may serve as primers for reverse transcriptase but also presented experimental evidence from studies of HIV-1 that did not support this proposal. Efficient HIV-1 reverse transcription requires interactions of tRNA-Lys with the PBS, as well as other regions of the viral genome. Of particular importance is an 8-nt sequence termed the primer activation signal (PAS) located in the U5 region that binds to the third stem-loop (T arm) of tRNA-Lys and promotes initiation of reverse transcription and elongation of the cDNA (reviewed in reference 60). Although all retroviruses are predicted to contain a PAS (61), the putative PAS in HTLV-1, which is positioned approximately 10 nucleotides 5′ to the PBS, has not yet been functionally characterized.

The secondary structure of the tRNA primer must also be disrupted to allow nucleotides in the T arm to interact with the PAS. In HIV-1, the NC protein plays an important role in unfolding tRNA-Lys to allow its binding to the HIV-1 PAS (62). Interestingly, a study of NC proteins from several retroviruses indicated that the HTLV-1 NC protein possesses comparatively weak nucleic acid chaperone activity (63). It is possible that another mechanism is responsible for unfolding of tRNA-Pro or that the PAS interaction is not important for HTLV-1.

Alternatively, tRF-3019 may serve as the major primer. In fact, our *in vitro* assay showed that tRF-3019 permits reverse transcription of a segment of HTLV-1 RNA containing the PBS and predicted PAS. The detailed picture of the interactions between HIV-1 RNA elements and its tRNA primer raises the possibility that tRFs representing the 3′ ends of primer tRNAs might support the initiation of reverse transcription, but not progressivity, with failure to proceed to the strand transfer step. In this case, tRF-3019 might inhibit the overall process of reverse transcription, thus acting as a restriction factor for HTLV-1 replication. Further studies will be necessary to test these hypotheses by comparing the abilities of tRF-3019 and tRNA-Pro to prime and support strand transfer.

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AQD—Au/(Table 1) Please define/spell out "FC."

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AQE—Au/Please clarify: what exactly is a "miR"? Up to this point in the text, the term/abbreviation has been used only as part of a designation and was never introduced.

AQF—Au/Please define tRNA-ProTGG and tRNA-ProAGG.

AQG—Au/Please provide all author names.

AQH—Au/(Fig. 2B) Reference to color coding was deleted because the figure is in black and white.

AQI—Au/(Fig. 4) Please spell out "UCSC."