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Role of *miR-34a* in HTLV-1-infected cells

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ABSTRACT

Human T-cell leukaemia virus type 1 (HTLV-1) is the etiological agent of an aggressive neoplasm of $CD4^+$ T-cells termed adult T-cell leukaemia/lymphoma (ATLL) and a neurodegenerative disease termed tropical spastic paraparesis/HTLV-associated myelopathy (TSP/HAM). To better understand the pathways controlling HTLV-1 infection, persistence and transformation, our laboratory is investigating the interplay between the virus and the cellular microRNA (miRNA) network.

This study was focused on *miR-34a*, which is known as a tumor suppressor in other contexts, but is highly expressed in HTLV-1 infected cell lines, newly infected PBMCs and ATLL samples.

Further studies of HTLV-1-infected cell lines C91PL and MT-2 treated with the NF-κB inhibitor Bay 11-7082 indicated that the NF-κB pathway contributes to sustain *miR-34a* expression in this cellular context.

Identification of the primary *miR-34a* precursor (pri-miRNA) produced in C91PL cells through the 5'RACE technique revealed that the main pri-*miR-34a* species present in this cell line corresponded to a two-exon transcript previously identified in cells of different lineage. The presence of an NF-κB binding site near the transcription start site of the pri-*miR-34a* provided further evidence that *miR-34a* expression is driven by this pathway in HTLV-1-infected cells.

Treatment of C91PL and MT-2 cells with the MDM2 inhibitor Nutlin-3a resulted in stabilization of p53, a further increase in the levels of *miR-34a* and downregulation of several of its targets, including SIRT1, a deacetylase whose substrates include p53, the inhibitor of apoptosis protein (IAP) BIRC5, which codes for Survivin, and the transcription factor E2F3, which is involved in controlling cell cycle. Interestingly, although Nutlin-3a induced G1 arrest in both cell lines, MT-2 cells entered late apoptosis, while C91PL cells showed signs of senescence.

The last part of this project was aimed at investigating the effects of Nutlin-3a on viral gene expression and directly assessing the impact of *miR-34a* in C91PL cells by electroporating a *miR-34a* mimic.

We observed that Nultin-3a treatment of C91PL cells increased levels of the majority of the alternatively spliced HTLV-1 transcripts. Although the introduction of the *miR-34a*-mimic into C91PL cells resulted in a reduction in the expression of key targets of the miRNA, it did not induce cell cycle arrest, death, senescence, or alterations in viral gene expression, suggesting that other factors come into play in the response to Nutlin-3a treatment.

RIASSUNTO

Il virus T-linfotropico umano di tipo 1 (HTLV-1, Human T-cell leukaemia virus type 1) è l'agente eziologico della leucemia/linfoma a cellule T dell'adulto (ATLL, adult T-cell leukaemia/lymphoma) e della paraparesi spastica tropicale/mielopatia associata ad HTLV (TSP/HAM, Tropical spastic paraparesis/HTLV-associated myelopathy), una patologia degenerativa del sistema nervoso centrale.

Al fine di comprendere i meccanismi volti a regolare l'infezione da HTLV-1, la sua persistenza e la capacità di indurre trasformazione cellulare, il nostro laboratorio ha investigato le relazioni tra virus e microRNA (miRNA).

In particolar modo, il presente lavoro si è incentrato sull'analisi dell'espressione di *miR-34a*, noto oncosoppressore in altri contesti tumorali, il quale risulta espresso ad alti livelli in campioni derivanti da pazienti ATLL, linee cellulari stabilmente infettate da HTLV-1 e PBMC infettati *de novo*.

Uno studio approfondito effettuato su linee cellulari infettate da HTLV-1, C91PL e MT-2, trattate con un inibitore di NF-κB, Bay 11-7082, ha rivelato come il pathway di NF-κB sia coinvolto nell'aumento di espressione di *miR-34a* in questo contesto cellulare.

Inoltre, la determinazione del precursore di *miR-34a* (pri-miRNA) effettuata in cellule C91PL mediante la tecnica denominata 5'RACE, ha permesso di identificare un trascritto costituito da due esoni, precedentemente descritto in una diversa linea.

Inoltre, trattamenti delle linee cellulari C91PL e MT-2 con un inibitore di MDM2, Nutlin-3a, hanno portato alla stabilizzazione di p53, ad un incremento dei livelli di *miR-34a* e alla riduzione dei suoi mRNA bersaglio, tra cui SIRT1 (una deacetilasi tra i cui substrati è annoverato anche p53), BIRC5 (codificante Survivin, una proteina inibitrice dell'apoptosi) ed E2F3 (un fattore di trascrizione implicato nel controllo del ciclo cellulare).

È interessante notare come Nutlin-3a, nonostante causi l'arresto del ciclo cellulare in fase G1 in entrambe le linee, induca un'apoptosi tardiva in cellule MT-2, e porti invece le cellule C91PL ad uno stato di senescenza.

Infine, il progetto si è rivolto a investigare gli effetti di Nutlin-3a sull'espressione genica virale, nonchè l'impatto diretto di *miR-34a* su cellule C91PL mediante elettroporazione con un "*miR-34a* mimic".

I nostri esperimenti ci hanno permesso di osservare come cellule C91PL trattate con Nutlin-3a presentino livelli aumentati dei trascritti di HTLV-1. Tuttavia, l'introduzione di *miR-34a*-mimic in cellule C91PL, pur confermando una riduzione nei livelli di espressione dei bersagli di *miR-34a*, non ha mostrato induzione di arresto del ciclo cellulare, morte o senescenza, così come non ha alterato l'espressione dei geni virali, suggerendo che anche altri fattori possano agire in risposta ai trattamenti con Nutlin-3a.

1. INTRODUCTION

1.1 HTLV-1: Human T-cell leukaemia virus type 1

Human T-cell leukaemia virus type 1 (HTLV-1) belongs to the *Deltaretrovirus* genus of the Orthoretrovirinae subfamily, which also includes HTLV-2, -3, -4, simian T-lymphotropic virus (STLV) and bovine leukaemia virus (BLV). HTLV-1, STLV and BLV are associated with neoplastic diseases, while the pathogenicity of HTLV-2, -3, -4 has not been clearly established *(Araujo and Hall, 2004; Feuer and Green, 2005; Mahieux and Gessain, 2009*).

HTLV-1 was first isolated from a patient with a T-cell lymphoma in 1980 and represents the first human retrovirus with established oncogenic properties (*Poiesz et al., 1980; Hinuma et al., 1981*). Approximately 10-20 million people are infected with HTLV-1, with most cases found in the Caribbean, Southern Japan, Africa, South America and the Pacific islands.

Transmission of the virus occurs from mother to new-born through breastfeeding or perinatally, or through exchange of biological fluids (sexual contact and parenteral transmission) (*Proietti et al., 2005*).

The virus integrates its genome into host cells and therefore establishes a permanent infection. While most HTLV-1-infected subjects do not present any major clinical manifestation, about 2 to 5% will develop adult T-cell leukaemia/lymphoma (ATLL), an aggressive malignancy of mature $CD4^+$ T-cells that is extremely refractory to current therapies (*Tsukasaki et al., 2009; Uchiyama et al., 1977*), or a demyelinating neurodegenerative disease termed tropical spastic paraparesis/HTLV-associated myelopathy (TSP/HAM) (*Gessain et al., 1985; Osame et al., 1986*), after a latency period of years to decades. HTLV-1 infection is also associated with arthritis, uveitis, dermatitis, lymphadenitis, and other inflammatory disorders (*Mochizuki et al., 1996; Sato et al., 1991; Proietti et al., 2005*).

The factors that determine whether HTLV-1 infection will produce ATLL or other pathologies are not well understood. ATLL development has been correlated to the perinatal transmission from infected mothers to children, whereas TSP/HAM is prevalent when the infection is transmitted to adults as in the case of transfusion of infected blood products (*Uchiyama, 1997*).

It was demonstrated that *in vitro* infection of peripheral blood mononuclear cells (PBMC) with HTLV-leads to immortalization T-cells that eventually acquire the capability to divide indefinitely in an interleukin-2 (IL-2)-independent manner. Indeed, after several months of culture it is possible to detect a mono- or oligoclonal profile of provirus integration resulting from a process that selects for one or few clones that carry several

genetic alterations and acquire the capability to grow in the absence of IL-2. These cells usually show a $CD3^+$ $CD4^+$ IL-$2R^+$ (interleukin-2 receptor) phenotype, or, rarely, $CD3^+$ $CD8^+$ IL-$2R^+$ (*Green and Chen, 2001*).

After transformation, the neoplastic phenotype is maintained regardless of viral expression and the levels of viral proteins are low in these clones. Indeed, viral promoter methylation, accumulation of mutations in the viral Tax gene or deletions of the proviral 5' LTR lead to the silencing of viral gene expression (*Miyazaki et al., 2007; Takeda et al., 2004*).

ATLL cells express very few viral proteins and frequently carry defective proviral copies integrated in their genome.

Furthermore, the propagation and persistence of the infected cells in the host relies mainly on "mitotic transmission" of the integrated viral genome to daughter cells (*Overbaugh and Bangham, 2001*).

1.1.1 Infection and virus propagation

The HTLV-1 virion consists of a core that contains 2 copies of the single-stranded RNA genome and the viral enzymes (reverse transcriptase, integrase and protease), a capsid and a lipoproteic envelope containing the gp21 and gp46 envelope glycoproteins (*Manel et al., 2005; Lairmore and Franchini, 2007*) (**Figure 1**).

Although the cell tropism of HTLV-1 *in vitro* is quite broad and includes monocytes, microglial cells, epithelial cells, and B- and T- lymphocytes, *in vivo* it is mainly detected in $CD4^+$ T-lymphocytes and dendritic cells (*Manel et al., 2005; Lairmore et al., 2007; Macatonia et al., 1992*).

HTLV-1 transmission requires cell-to-cell contact and cell-free HTLV-1 viral particles are poorly infectious (*Kataoka. et al., 2015*); indeed, cell-mediated infection is estimated to be 100,000-fold more efficient than cell-free infection (*Mazurov et al., 2010*).

HTLV-1 uses three main cellular receptors to enter target cells: the glucose transporter GLUT-1 (*Manel et al., 2003*), heparan sulphate proteoglycan (HSPG), and neuropilin-1. GLUT-1, interacting with the viral protein gp46, mediates the cell-to-cell contact required for efficient viral spread.

During the binding phase, the virus contacts HSPG on the cell surface and forms complexes with neuropilin-1 through the viral envelope protein (*Jones et al., 2005; Lambert et al., 2009*). The gp46-GLUT-1 interaction allows p21-mediated cellular membrane fusion with the formation of the virological synapse, an essential structure necessary for cell-to-cell transmission of HTLV-1. The engagement of intracellular adhesion molecule-1 (ICAM-1) (*Barnard et al., 2005)* and the activation of the Ras-MEK-ERK pathway (*Nejmeddine et al., 2009*) induce the polarization of the microtubule-

organizing centre (MTOC) of the infected cell leading to the accumulation of HTLV-1 core complexes and genome at the cell junction. The virion components are then transferred to the uninfected cell as enveloped particles (*Igakura et al., 2003; Majorovits et al., 2008)*.

Tax also takes part in the formation of the virological synapse. Tax accumulates in the contact region between infected and target cells, enhancing MTOC formation by stimulating the CREB pathway (*Nejmeddine et al., 2005; Nejmeddine et al., 2009*). Other proteins involved in HTLV-1 binding and entry into the target cells (e.g. hDlg, neuropilin-1 and heparan sulphate proteoglycans) mediate antigen recognition and cell adhesion and are part of the virological synapse (*Pinon et al., 2003; Blot et al., 2004; Ghez et al., 2006*). HTLV-1 also uses an alternative method of transmission through an extracellular biofilm-like structure that stores viral particles, facilitating virus spread after cell-to-cell contact (*Pais-Correia et al., 2010*).

Viral proteins are also detected in exosomes, suggesting a role for cell-derived microvesicles in HTLV-1 infection (*Jaworski et al., 2014*). These exosomes might assist in virus transfer to naive cells, deliver bioactive viral proteins, or sequester immune-reactive viral proteins (*Giam et al., 2016*).

All the transmission pathways described above are consistent with the fact that cell-free HTLV-1 particles are usually undetectable in the serum of HTLV-1-infected subjects and that cell-free blood products are not infectious (*Fan et al., 1992; Derse et al., 2001*).

After virus entry, the viral RNA genome is converted to double-stranded DNA through a complex process directed by the viral reverse transcriptase (RT), producing an RNA-DNA hybrid.

Subsequently, the RNA strand is degradated by the ribonuclease H (RNAse H) activity of RT, while the DNA strand is used as a template to synthesize a complementary DNA strand through RT's DNA-polymerase-DNA-dependent activity.

The double-strand DNA circularizes and is transferred to the nucleus, where it integrates randomly in the host genome. Integration is mediated by the viral enzyme integrase and the long terminal repeats (LTRs). Viral genes are transcribed and translated by the cell machinery and progeny virions are assembled in the cytoplasm through the interaction between the viral nucleocapsid and the plasma membrane.

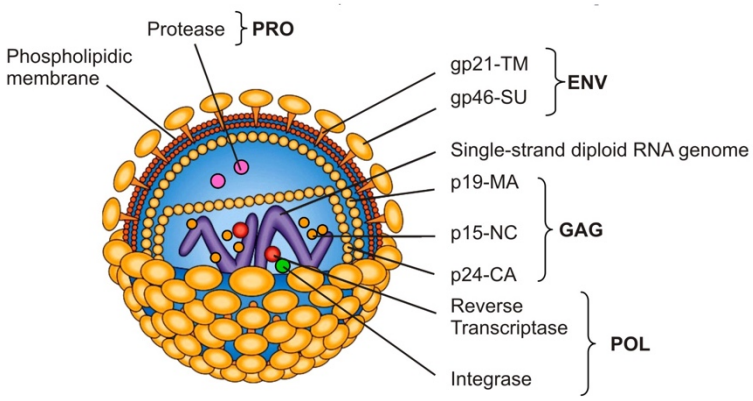


Figure 1. Schematic representation of the HTLV-1 virion (*Le Blanc et al., 2001*).

1.1.2 Genetic organization and gene expression

HTLV-1 has a genome of about 9 kb and expresses multiple gene products coded on both strands of its proviral genome by using two promoters. The HTLV-1 genome contains genes coding for structural proteins and enzymes (gag, pro, pol and env) and two long terminal repeats (LTRs) typical of retroviruses, which flank the partially overlapping open reading frames (ORFs) of the gag, pro, pol and env genes. The LTRs are subdivided into three regions (U3, R and U5) that contain the *cis*-acting elements essential for viral gene expression: transcription factor-binding sites, polyadenylation and splicing sites.
A region called pX is located between the env gene and the 3' LTR and contains at least four partially overlapping ORFs, termed x-I through x-IV, that encode the post-transcriptional regulator Rex, the transactivator Tax and the accessory proteins (p12, p13, p30). The complementary strand of the genome also contains an ORF located in the pX region (antisense orientation) (*Larocca et al., 1989*) which codes for the HBZ protein (*Gaudray et al., 2002; Boxus et al., 2009*).
HTLV-1 is able to express its highly condensed genetic information through three different mechanisms: ribosomal frameshifting (which generates a Gag-Pro-Pol polyprotein from the full-length transcript), alternative splicing and polycistronic translation (which produces distinct mRNAs coding for the Env and pX region genes), minus-strand transcription (which generates at least 2 different transcripts encoding 2 isoforms of the HBZ protein).

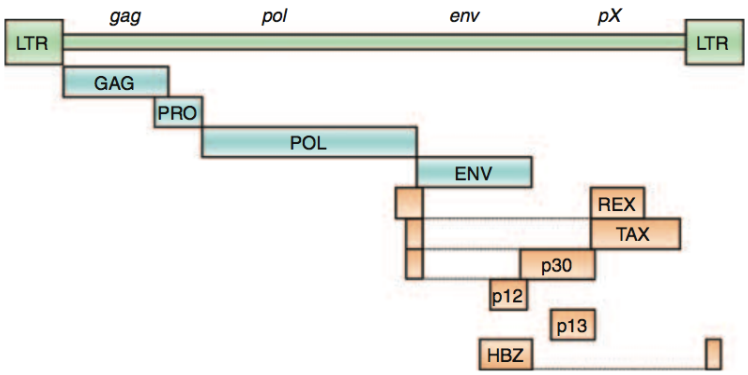


Figure 2. The HTLV-1 proviral genome (*Boxus et al., 2009*).

Figures 3 and **4** show the four major classes in which HTLV-1 transcripts are subdivided, based on their splicing.

a) unspliced (US) mRNA, coding for Gag-Pro-Pol, and used as genomic RNA;
b) singly-spliced (SS) mRNAs, coding for the envelope glycoproteins (Env) and for the accessory proteins p21rex, p12 and p13;
c) doubly-spliced (DS) mRNAs, coding for the regulatory proteins p40 (Tax) and p27 (Rex), and for the regulatory/accessory protein p30tof;
d) mRNAs generated from the negative strand, coding for HBZ protein.

The structural protein Gag and the enzymes Protease and Polymerase of mature virus particles are derived from the full-length US mRNA (8.6 kb), which is also packaged into virions as the genomic RNA (*Lairmore and Franchini, 2007*).

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, which converts the viral single-stranded RNA genome into double stranded DNA through its DNA polymerase and RNaseH activities. Sequences downstream code for Integrase, which is responsible for the integration of the reverse-transcribed viral genome in the host cell genome.

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 (**Figure 3**). 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.

A 4.2 kb SS mRNA is translated into a 68-kDa Env precursor, which is post-translationally modified by glycosylation and cleavage into two proteins named gp46-

SU, localized at the surface of the infected cells and virions and responsible for the binding to the GLUT-1 receptor, and gp21-TM, a transmembrane protein that mediates membrane fusion and formation of the virological synapse.

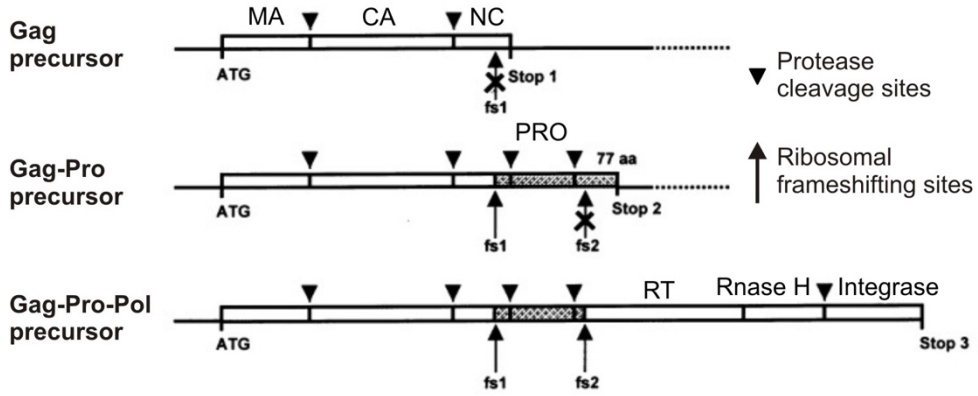


Figure 3. Schematic representation of the Gag-Pro-Pol polyprotein precursors of HTLV-1. Fs1 and fs2: ribosomal frameshifting sites; MA: matrix, CA: capsid; NC: nucleocapsid, PRO: protease, RT: reverse transcriptase (*Le Blanc et al., 2001*).

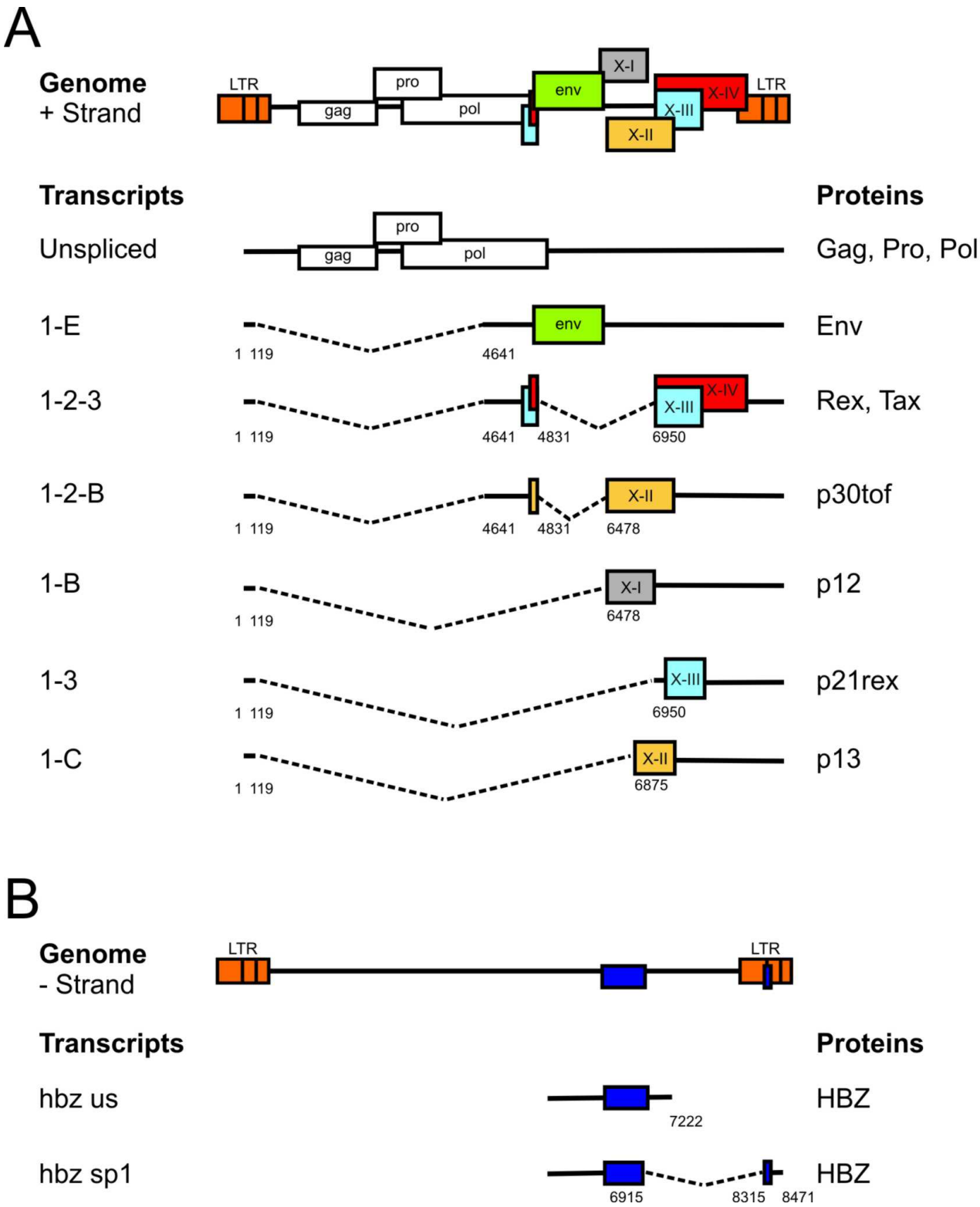


Figure 4. Organization and expression of the HTLV-1 genome. Plus-strand ORFs, transcriptional map and proteins coded by each mRNA are shown. The numbering indicates splicing sites used for the generation of the mature mRNAs. Resulting exons are: 1 (1-119), 2 (4641-4831), 3 (6950-8493), B (6478-8493),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

1.1.3 Functions of the pX region proteins

The presence of the pX region between the *env* gene and the 3' LTR is a characteristic of the *Deltaretroviruses*.

The pX region of the HTLV-1 genome contains four partially overlapping ORFs (x-I, x-II, x-III, x-IV) that code for the non-structural proteins Tax, Rex, p21rex, p30tof, p13 and p12. The expression of these proteins is regulated by alternative splicing and polycistronic translation.

All of the pX region mRNAs contain exon 1, which is non coding. Singly spliced mRNAs contain exon 1 and different 3' exons, and code for the accessory proteins p13 (mRNA 1-C, translated from a start codon located in the x-II ORF) and p12 (mRNA 1-B, translated from a start codon in the x-I ORF) and for the accessory/regulatory protein p21rex (mRNA 1-3, translated from a start codon located in the x-III ORF) *(Ciminale et al., 1992; Koralnik et al., 1992*). Doubly spliced mRNAs contain exons 1 and 2, and different 3' exons. Exon 2 contains 2 start codons (AUGs). mRNA 1-2-3 is a bicistronic transcript coding for the regulatory proteins Rex and Tax; Rex initiates at the first AUG in exon 2 and continues in the x-III ORF in exon 3, while Tax initiates at the second AUG and continues in the x-IV ORF in exon 3. The 1-2-B mRNA codes for the accessory/regulatory protein p30tof, which is translated from the second start codon of exon 2 and continuing in frame with the x-II ORF in exon B (*Ciminale et al., 1992; Koralnik et al., 1992*).

The negative strand of the HTLV-1 genome contains one ORF located in the pX region (antisense orientation) which generates at least 2 different transcripts, one spliced (HBZ sp1) and the other unspliced (HBZ us) (*Cavanagh et al., 2006; Murata et al., 2006; Satou et al., 2006*). These transcripts code for 2 isoforms of HBZ protein that differ by 7 amino acids at the N-terminus due to the presence of the first exon only in the former transcript. HBZ sp1 has multiple transcriptional initiation sites in the U5 and R regions of the 3' LTR, whereas the HBZ us transcript initiates within the Tax gene. Both HBZ sp1 and HBZ us have TATA-less promoters (*Yoshida et al., 2008*).

Tax

Tax is a 353-amino acid, 40-kDa phosphoprotein that transcriptionally regulates the expression of viral genes and many cellular genes.

Tax contains three main domains:

- a hydrophobic N-terminal domain, containing the CREB-binding region that interacts with the CRE-binding/activating transcription factor (CREB/ATF) and the serum responsive factor (SRF) to allow the modulation of transcription, cell cycle and cell signalling, and the nuclear localization signal (NLS);

- a central region, containing two leucine zipper motif regions (LZRs) for protein dimerization and binding of cellular factors, the kinase-inducible domain (KID) that interacts with the kinase-inducible exchange (KIX) domain of the transcriptional co-activators CREB binding protein (CBP) and p300, and binding domains for proteins that induce NF-κB activation;
- a C-terminal domain, containing the PDZ-binding motif (PBM) involved in the interaction of Tax with cellular factors.

Tax was initially described as an activator of LTR-directed transcription (*Felber et al., 1985*). The U3 region of the LTR contains three repeat sequences, TRE (Tax Responsive Elements), necessary and sufficient to confer Tax responsiveness (*Brady et al., 1987*). In addition to activating the viral LTR promoter, Tax stimulates transcription of a large number of cellular genes through interactions with the cellular transcription factors CREB/ATF, NF-κB, SRF and AP-1.

Post-translational modifications of Tax, including phosphorylation, acetylation, ubiquitination and sumoylation influence its cellular localization and its ability to activate the NF-κB pathway. In particular, NF-κB activation is dependent on the presence of lysines K6 and K8.

One of the main properties of Tax is the ability to immortalize primary human T-cells; indeed, studies by Hasegawa et al. demonstrated that the presence of Tax in transgenic mice was sufficient to induce an ATLL-like malignancy (*Hasegawa et al., 2006*). Tax also mediates the inactivation of p53 through a mechanism that seems to involve the NF-κB and CREB pathways.

Tax constitutively activates both the canonical and non-canonical NF-κB pathways, through its association with RelA and the IκB kinase complex or inducing by p100 processing and translocation in the nucleus of p52, respectively (*Rende et al., 2012; Romanelli et al., 2013*).

Through NF-κB activation, Tax induces 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*).

Furthermore, Tax is able to interact with proteins stimulating the G1-S phase transition of the cell cycle through different mechanisms including transcriptional activation or repression, post-translational modifications and protein-protein interactions (*Jeang et al., 2004*).

Interestingly, tumour cells from about 50% of ATLL patients express little or no Tax, suggesting that this protein may be important during the early stages of infection and T-cell transformation, but dispensable once the transformed state has been established.

Rex

Rex is a 189-amino acid, 27-kDa nuclear/nucleolar phosphoprotein that contains three main domains:

- an N-terminal region rich in arginines, containing a nuclear localization signal (NLS) and an overlapping RNA binding domain (RBD), that mediates binding to viral RNAs;
- a region rich in leucines, containing an activation domain (AD) and a nuclear export signal (NES) that interacts with the protein chromosome region maintenance interacting protein 1 (CRM1/exporting 1) and allows export of the Rex-viral mRNA complex from the nucleus to the cytoplasm;
- sequences flanking the NES that are needed for formation of Rex multimers.

Rex localizes to the nucleus and mediates the nucleo-cytoplasmic export of incompletely spliced viral RNA, controlling in this way viral gene expression at the post-transcriptional level. Rex directly interacts with a 254-nucleotide stem-loop cis-acting RNA element termed the Rex-responsive element (RxRE) in the U3/R region of the 3' LTR of all HTLV-1 transcripts.

The first step for the nuclear export of viral mRNA requires the formation of Rex multimers on the RxRE. After Rex-RxRE binding and Rex multimerization, CRM1 binds to RanGTP, along with the Rex-mRNA complex, and translocates this complex across the nuclear pore by interacting with phenylalanine-glycine rich nucleoporins. In the cytoplasm RanGTP is then converted to RanGDP and is released from the Rex-mRNA complex (*Rende et al., 2011*).

In addition to its role in RNA trafficking, Rex inhibits splicing *in vitro*, during the initial phase of spliceosome formation and to increase mRNA stability in the nucleus. Experiments performed with protein kinase inhibitors indicated that Rex function is modulated by phosphorylation on serine/threonine residues *(Adachi et al., 1990; Kesic et al., 2009*), a property that highlights the importance of cellular factors in the multi-level regulation of HTLV gene expression (*Younis and Green, 2005*).

Contrary to Tax, Rex is not necessary for cellular immortalization, while it is required for infectivity and viral persistence (*Ye et al., 2003*), since expression of the US and SS 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.

Rex was recently shown to inhibit Dicer activity and thereby reduces the efficiency of the conversion of shRNA to siRNA (*Abe et al., 2010*).

p21rex

p21rex is a truncated isoform of Rex that lacks the N-terminal arginine-rich domain. It was suggested that p21rex interferes with the activity of full-length Rex, inhibiting the expression of transcripts coding for structural proteins, enzymes and accessory proteins (*Heger et al., 1999*), playing a role as a latency-inducing factor in the HTLV-1 life cycle.

p30tof

p30tof is a 241-amino acid nucleolar-nuclear non-shuttling protein (*Ciminale et al., 1992; D'Agostino et al., 1997*) that contains a Nucleolar Retention Signal (NoRS; aa 73-78) and 2 arginine-rich NLS (*D'Agostino et al., 1997; Ghorbel et al., 2006*). At the post-transcriptional level p30tof inhibits the nuclear export of the Tax/Rex mRNA, resulting in a global inhibition of viral gene expression, suggesting a role as a latency factor (*Nicot et al., 2004*). p30tof interacts with the RNA-binding domain of Rex when it is bound to RNA, preventing its association with the RxRE. However, as a low level of virus expression is required during the infection, Rex permits the export of residual Tax/Rex mRNA bounds to p30tof (*Sinha-Datta et al., 2007*).

On the other hand, Rex induces a conformational change in p30tof or in the local RNA structure, releasing p30tof from its p30 responsive element (RE) and allowing export of some Tax/Rex mRNA to the cytoplasm.

p30tof also affects transcription from CRE sequences depending on its concentration: at low levels, p30tof activates transcription from the HTLV-1 LTR promoter, while high levels repress LTR promoter activation. Cellular genes that are modulated by p30tof include adhesion molecules and genes involved in T-cell activation and apoptosis (*Michael et al., 2004; Taylor et al., 2009*).

Interactions between p30tof and the kinase-inducible exchange domain (KIX) of p300/CBP, which also binds CREB and Tax, disrupts the assembly of the CREB-Tax-p300/CBP complex on TRE sequences, leading to decreased transcription of the viral genome, which favors viral latency (*Zhang et al., 2000; Zhang et al., 2001*).

The ability of p30tof to affect the expression of cellular genes involved in the cell cycle and apoptosis (*Awasthi et al., 2005*) and promote early viral spread and T cell survival (*Michael et al., 2004; Taylor et al., 2009; Sinha-Datta et al., 2007*), point to a role for p30tof in HTLV-1-induced transformation.

p13

p13 is an 87-amino-acid, 13-kDa protein that corresponds to the C-terminal portion of p30tof (*Berneman et al., 1992*). p13 contains a functional Mitochondrial Targeting Sequence (MTS) corresponding to amino acids 21-31 near the N-terminus that

targets it to the mitochondrial inner membrane (*Ciminale et al., 1999; D'Agostino et al., 2002*).

Introduction of inactivating mutations in p30tof and p13 in the HTLV-1 genome does not affect productive infection or immortalization of human T-cells in tissue culture (*Robek et al., 1998*). However, these viruses replicate poorly in a rabbit model, suggesting a role for p30tof and/or p13 in viral propagation and persistent infection (*Bartoe et al., 2000, Hiraragi et al., 2006*).

Cells expressing p13 have isolated clusters of round-shaped, swollen mitochondria (*Ciminale et al., 1999*). These effects depend on the presence of four arginines at positions 22, 25, 29 and 30 in the MTS, which constitute the charged face of an amphipathic α-helix (*Silic-Benussi et al., 2004*). Changes in morphology are accompanied by modifications in mitochondrial permeability to Ca^{2+} and K^{+}, loss of mitochondrial membrane potential, and an increase in the activity of the respiratory chain, which leads to accumulation of mitochondrial reactive oxygen species (ROS) *(Silic-Benussi et al., 2004; Silic-Benussi et al. 2009; Biasiotto et al., 2010)*. These ROS reduce the threshold for opening of the permeability transition pore (PTP), a channel that regulates apoptosis (*Rasola and Bernardi, 2007*). These properties may be responsible for the ability of p13 to sensitize cells to apoptosis induced by Fas and ceramide (*Hiraragi et al., 2005; Chiami et al., 2006*) and to interfere with the ability of HeLa cells and Myc+Ras-expressing fibroblasts to grow as tumours in nude mice (*Silic-Benussi et al., 2004*).

Studies performed in T-cells indicated that the effects of p13 on cell death depend on the cells' basal ROS setpoint: in normal T-cells, which have low basal ROS levels, p13 induces activation, while in transformed T-cells, which have higher basal ROS levels, p13 induces apoptosis (*Silic-Benussi et al., 2010*). These observations suggest a dual role for p13: stimulating the expansion of the population of untransformed infected cells while promoting elimination of transformed cells, thus supporting viral persistence.

p13 becomes more stable and partially re-localizes to the nucleus when co-expressed with Tax (*Ghorbel et al., 2006*). In the nucleus, p13 interferes with the ability of Tax to bind to CBP/p300, leading to decreased Tax-mediated viral gene transcription (*Andresen et al., 2011*).

p12

p12 is a 99-amino acid protein that localizes in the endoplasmic reticulum (ER) and in the Golgi apparatus *(Koralnik et al., 1993; Ding et al., 2001; Johnson et al., 2001*). Although p12 is not required for HTLV-1 replication *in vitro*, it plays a key role in the stabilization of a productive viral infection *in vivo (Albrecht et al., 2000; Collins et al., 1996; Derse et al., 1997; Robek et al., 1998*).

p12 interacts with the interleukin-2 receptor (IL-2R), resulting in reduced surface expression (*Mulloy et al., 1996*) and in the recruitment of the Janus-associated kinases 1

and 3 (Jak1 and Jak3), leading to an increase in the transcriptional activity of the signal transducers and activators of transcription-5 (STAT-5), and then 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 and leading to a decrease in functional MHC-I on the surface of HTLV-1 infected cells. In addition, p12 reduces the expression of ICAM-1 and ICAM-2, resulting in the protection of HTLV-1-infected primary $CD4^+$ T cells from attack by NK cells (*Banerjee et al., 2007*). These effects allow infected cells to evade the immune system (*Johnson et al., 2001*).

In the ER, p12 interacts with calreticulin and calnexin (*Ding et al., 2001*), regulating Ca^{2+} storage and release, leading to a reduction in calcium available for release from the ER stores and an increase in cytosolic Ca^{2+} levels *(Ding et al., 2002*).

p12 induces nuclear factor of activated T-cells (NFAT) activation (*Albrecht et al., 2002*), by interacting with calcineurin, a Ca^{2+}-responsive protein phosphatase that controls NFAT activity (*Kim et al., 2003*). Taken together these effects decrease the threshold for T-cell activation (*Nicot et al., 2005*).

The modulation of Ca^{2+} signalling is a shared mechanism for different viruses to facilitate their infection *(Chami et al., 2006; Zhou et al., 2009*).

A proteolytic cleavage product of p12 named p8 accumulates at the cell surface, where it is recruited to the immunological synapse, resulting in downregulation of TCR signalling (*Fukumoto et al., 2007*). Cell-surface accumulation of p8 also changes the adhesion properties of cells and mediates the formation of tubular structures ('conduits') that permit exchange of material between cells (*Van Prooyen et al., 2010*).

HBZ

The HBZ protein localizes in the nucleus, thanks to the presence of two regions rich in basic amino acids and a DNA binding domain. HBZ also contains an N-terminal transcriptional activation domain (AD), a central domain (CD), and a C-terminal basic ZIP domain (bZIP) (*Gaudray et al., 2002*).

HBZ interacts with cellular transcription factors, including CREB-2, p300/CBP, Jun family members, and NF-κB (*Matsuoka and Green, 2009*).

Through interactions mediated by its bZIP domain, HBZ inhibits the binding between CREB-2 and the TRE in the HTLV-1 LTR, suppressing Tax-mediated transcription from the 5' LTR (*Gaudray et al., 2002*).

HBZ also binds proteins of the Jun family, in particular JunB, c-Jun and JunD. cJun/JunB undergo proteasomal degradation or become sequestered within nuclear bodies, with consequent reduction in target gene transcription.

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*).
Interestingly, HBZ inhibits the activation of the NF-κB pathway by blocking the binding of p65 to DNA and by increasing the expression of PDLIM2, which enhances ubiquitination and degradation of p65.
HBZ is not necessary for viral replication or immortalization *in vitro*, but increases infectivity and viral persistence *in vivo* (*Arnold et al., 2006*). HBZ transcripts are found in ATLL samples, suggesting that its expression plays a critical role in the development of this disease (*Mesnard et al., 2006; Raval et al., 2015*).
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.

1.2 Role of HTLV-1 in the development of ATLL

ATLL is a highly malignant and intractable T-cell neoplasm with a very heterogeneous clinical course. The Japan Clinical Oncology Group (JCOG) has proposed four clinical subtypes (acute, lymphoma, chronic, and smouldering) based on the prognostic factors, clinical features, and the natural history of the disease (*Tsukasaki et al., 2009*). The acute form of ATLL is extremely aggressive and refractory to the current therapies. The patients show in their peripheral blood atypical T-cells with multilobulated nuclei (flower cells) (*Boxus et al, 2009*).
Transformation of HTLV-1-infected T-cells to ATLL arises from the interplay between numerous host factors and viral-encoded genes, with HTLV-1 Tax and HBZ playing central roles. In fact, Tax and HBZ each exhibit oncogenic potential when expressed in transgenic mice and influence senescence induction, viral latency and persistence, genome instability and cell proliferation.
The transforming activity of Tax is due mainly to its stimulation of the NF-κB pathway, with resulting to upregulation of the expression of cellular genes that mediate lymphocyte proliferation and resistance to apoptosis (*Bangham and Ratner, 2015*).
There is evidence for the existence of a pre-ATLL state characterized by the presence of peripheral lymphocytes that display abnormal morphology resembling ATLL cells with poly- or oligo-clonal integration of the viral genome.
The acute subtype of ATLL has an extremely poor prognosis, with an overall survival of a few months. It is characterized by the presence of proliferating infected T-cells, resulting in long-lived clones in the circulation. Each clone presents a unique site of

integration of the HTLV-1 provirus in the host genome; the majority carry a single copy, while 10% of ATLL clones have two copies, some of which may be defective (*Cook et al., 2014*).

Usually, ATLL cells are $CD3^+$ $CD4^+$ $CD8^-$ $CD25^+$ and frequently accumulate in peripheral blood as well as in lymphoid organs and skin.

Furthermore, HTLV-1 infection is accompanied by a high frequency of T-cells expressing the surface marker Forkhead Box P3 ($FoxP3^+$) (*Chen et al., 2006; Kohno et al., 2005*).

The presence of FoxP3 is typical of regulatory T cells (T_{reg} cells). In HTLV-1 infection the upregulation of the CCL22 chemokine by Tax-expressing cells engages the CCR4 receptor on the functional T_{reg} cell population, resulting in an enhancement of the migration and survival of $FoxP3^+$ cells *in vitro*. This condition may both delay the progression of ATLL and HTLV-1-associated inflammatory diseases and contribute to the immune suppression observed in ATLL (*Toulza et al., 2010*).

Another important characteristic of ATLL is hypercalcemia, due to the induction by Tax of the transcriptional activation of a parathormone-like peptide.

1.2.1 Current therapies for ATLL

Clinical features, the presence of circulating anti-HTLV-1 antibodies and ATLL cell morphology are the parameters on which ATLL diagnosis is currently based. The identification of the monoclonal integration of HTLV-1 proviral DNA is analysed in malignant cells to confirm the diagnosis.

Although the quantification of HTLV-1 sites is not used as a diagnostic criterion of ATLL, its analysis has recently been accomplished through deep sequencing (*Laydon et al., 2014*), as a high proviral load in HTLV-1 carriers is suggested to be associated with the development of ATLL.

Four clinical subtypes of ATLL (acute, lymphoma, chronic, and smouldering) were defined in 1991 by the JCOG, based on the presence of organ involvement, leukemic manifestation, high lactate dehydrogenase (LDH) and hypercalcemia that altogether reflect the prognosis and natural history of the disease.

The treatment strategy for ATLL patients is based on these clinical subtypes (**Table 1**).

1. Indolent-type ATLL: Smoldering- or favorable chronic-type
(1) Watchful waiting for asymptomatic patients
(2) Interferon-α (IFN-α)/zidovudine (AZT) or watchful waiting for symptomatic patients
(3) Skin lesion: Local therapy; Topical steroids, Ultraviolet light, Radiation Systemic therapy; Steroids, Oral retinoids, Single agent chemotherapy
2. Aggressive-type ATLL: Unfavorable chronic-, lymphoma- or acute-type
(1) Chemotherapy: VCAP-AMP-VECP CHOP or less-toxic regimen for elderly patients
(2) VCAP-AMP-VECP + mogamulizumab
(3) Allogeneic hematopoetic stem cell transplantation (allo-HSCT)
(4) IFN-α/AZT (except for lymphoma-type)
3. Relapse or refractory ATLL
(1) Mogamulizumab
(2) Allo-HSCT
(3) New agents under clinical trial: Brentuximab vedotin, Bortezomib, Lenalidomide, Panobinostat, Forodesine Pralatrexate, Denileukin diftitox
(4) Vaccine (autologous dendritic cells with tax-peptide)

VCAP-AMP-VECP: vincristine, cyclophosphamide, doxorubicin, and prednisolone (VCAP); doxorubicin, ranimustine, and prednisolone (AMP); and vindesine, etoposide, carboplatin, and prednisolone (VECP). CHOP: doxorubicin, cyclophosphamide, vincristine and prednisone.

Table 1. Treatment strategy for adult T-cell leukaemia/lymphoma (ATLL) (From *Kato et al., 2015*).

Watchful waiting. For patients affected by indolent ATLL (chronic and smouldering types, displaying few if any symptoms), a watchful waiting approach is recommended, while patients presenting symptoms are advised to utilize watchful waiting combined with the treatment with interferon-α and Zidovudine (IFN-α/AZT). Recent studies showed that a cohort of patients with indolent ATLL, treated with watchful waiting and/or chemotherapy, displayed a poorer prognosis than initially expected with a median survival time of 4.1 years, suggesting that indolent ATLL should be closely monitored in the clinical setting with practices other than watchful waiting (*Takasaki et al., 2010*).

Chemotherapy. The most common regimen is CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone), given twice weekly, but a 2007 phase III clinical study suggested that treatment with high doses of VCAP-AMP-VECP (vincristine, cyclophosphamide, doxorubicin, and prednisone; doxorubicin, ranimustine, and prednisone; vindesine, etoposide, carboplatin, and prednisone) was more effective in newly diagnosed patients with aggressive ATLL subtypes (*Tsukasaki et al., 2007*).
However, these combined chemotherapies are very complex and include drugs with limited availability in many countries. An alternative treatment with similar efficacy is DA-EPOCH (dose-adjusted etoposide, prednisone, vincristine, cyclophosphamide, and doxorubicin).

Antiviral therapy. Several clinical studies tested the combination of zidovudine (AZT) and interferon-alpha (IFN-α), with different degrees of success. Results from a worldwide meta-analysis of 254 ATLL patients treated with AZT/IFN-α (*Bazarbachi et al., 2010*) support the continued use and development of AZT/IFN-α therapy for acute and indolent subtypes of ATLL, taking into account that patients treated with first-line AZT/IFN-α therapy had a higher 5-year overall survival rate compared to a 5-year overall survival rate for first-line chemotherapy.

alloHSCT. Allogeneic Hematopoietic Stem Cell Transplantation (alloHSCT) is an emerging treatment for aggressive ATLL. A Japanese retrospective study described a 3-year overall survival rate of 33% for a cohort of 386 ATLL patients who received alloHSCT from different graft sources (*Hishizawa et al., 2010*). This study highlighted that several factors significantly decreased survival rates, including the age of patient/recipient (>50), sex, status other than complete remission, and use of unrelated cord blood compared to HLA-matched grafts. Overall, alloHSCT remains a hopeful treatment option for young patients with aggressive subtypes of ATLL.

Targeted therapies. Targeted therapies against ATLL include the proteasome inhibitor, Bortezomib, HDAC inhibitors (such as vorinostat, romidepsin, and panobinostat), the JAK1/2 inhibitor Ruxolitinib, arsenic trioxide and IFN-α, monoclonal antibodies directed against ATLL/T-cell surface molecules (anti-PD-1, anti-CD25, anti-CD2, anti-CD52, and CCR4), novel anti-folates, the purine nucleotide phosphorylase inhibitor Forodesine, antiangiogenic therapy and the immunomodulatory drug Lenalidomide (*Ishitsuka et al., 2014*). The HTLV-1 envelope glycoproteins seemed to be an attractive candidate for an anti-HTLV-1 vaccine, because they mediate infection and trigger both humoral and cellular immune responses, but unfortunately early studies demonstrated that this approach only provided partial protection to animals challenged with HTLV-1-infected cells (*Rodriguez et al., 2011*).
Several studies have recently suggested that Tax or HBZ peptides may have a potential therapeutic role to provide protective immunity against HTLV-1 infection and/or ATLL development (*Suehiro et al., 2015; Sugata et al., 2015*).

1.3 MicroRNAs

MicroRNAs (miRNAs) are single-stranded noncoding RNAs of about 22 nt. miRNAs hybridize to complementary sequences on target transcripts, leading to reduced expression of the mRNA target. The miRNA-mRNA interaction almost always involves

nt 2-8 of the miRNA (the 'seed sequence') and is often reinforced by base-pairing of additional sequences toward its 3' end. miRNA binding sites are generally found in the 3'UTRs of target mRNAs; a single mRNA may contain sites for many miRNAs, and most miRNAs have the potential to interact with many mRNAs.

Many miRNAs are phylogenetically conserved, indicating that they have important roles throughout evolution (*Ibanez-Ventoso et al., 2008)*.

In mammals, miRNAs are predicted to control the activity of more than 60% of all protein-coding genes and thus participate in the regulation of many cellular processes (*Fabian et al., 2010*). Therefore, it is not surprising that aberrant miRNA expression or function takes part in the pathogenesis of many diseases, including cancer (*Sayed et al., 2011*).

Many mammalian miRNAs are encoded by multiple genes (paralogues) with distinct genomic positions, probably due to gene duplications. As an example, the let-7 family miRNAs are coded by 12 different loci in the human genome. Paralogues often have identical seed sequences and are thought to target the same mRNAs. However, because the 3′ sequences of miRNAs also contribute to target binding and because paralogue miRNAs often present distinct expression patterns, members of the same seed family might have distinct roles (*Ventura et al., 2008*). Approximately 50% of mammalian miRNA loci are found in clusters in the genome. Such clusters are generally transcribed as a single polycistronic transcription unit (TU) (*Lee et al., 2002*), although there may be exceptional cases in which individual miRNAs are transcribed from separate promoters. Approximately 40% and 10% of miRNA loci are located in introns or in exons of non-coding transcripts, respectively. miRNAs can be also found in protein-coding TUs, in exons or more commonly in intronic regions, which account for ~40% of all miRNA loci. 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*) have accelerated the discovery of new small RNA sequences.

The sequences and genomic locations of the known miRNAs are catalogued in the Sanger miRBase at http://www.mirbase.org/ (*Griffiths-Jones et al., 2008*). The current miRBase (version 21, released in June 2014) contains 1881 precursors and 2588 mature human miRNAs. Global miRNA profiling studies (*Basso et al., 2009; Ghisi et al., 2011; Landgraf et al., 2007; Morin et al., 2008*) 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 fine-tuning gene expression.

Some aspects of miRNA biogenesis and function are shared by the RNA interference (RNAi) pathway, an evolutionarily conserved mechanism that triggers sequence-specific degradation of complementary mRNAs.

1.3.1 miRNA biogenesis

Transcription of miRNA genes. The majority of miRNA-coding genes are transcribed by RNA polymerase II (Pol II) (*Lee et al., 2004; Cai et al., 2004*). A range of Pol II-associated transcription factors regulates the transcription of miRNA genes depending on specific conditions and cell types (*Lee et al., 2008*). RNA Pol II-driven transcription generates a primary transcript (pri-miRNA) that contains a hairpin structure consisting of a stem of ~33 nt and a loop of ~10 bp; the stem portion harbors the mature miRNA sequence. A few miRNAs that are associated with Alu repeats are transcribed by RNA Polymerase III (Pol III).

Nuclear processing by Drosha. The first step of miRNA maturation (termed "cropping") occurs in the nucleus and consists of the cleavage of the pri-miRNA, resulting in release of the ~65 nt hairpin, termed the pre-miRNA (*Lee et al., 2002*).
The cleavage reaction is driven by Drosha, a nuclear RNAse III-type enzyme, in association with the DiGeorge syndrome critical region gene 8 (DGCR8) cofactor to form the Microprocessor complex, a protein complex of ~650 kDa in humans (*Lee et al., 2003; Han et al., 2004; Denli et al., 2004; Gregory et al., 2004; Landthaler et al., 2004*).
DGCR8 interacts with the 5' and 3' ends and the stem of the pri-miRNA, while Drosha cleaves ~11 bp from the base of the stem of the hairpin structure (*Han et al., 2006; Zeng et al., 2005*).
Canonical intronic miRNAs are processed before splicing in a co-transcriptional event (*Kim et al., 2007*) (**Figure 6B**). The microprocessor complex cleaves the intronic miRNA before the intron is excised. The hairpin-shaped pre-miRNA can enter the canonical miRNA pathway, whereas the rest of the transcript undergoes splicing.
A small group of non-canonical miRNAs termed "mirtrons" 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*) (**Figure 6C**). Following splicing, the lariat-shaped intron is debranched and folds into a hairpin structure that resembles a 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 form other non-coding RNAs, such as tRNA (*Babiarz et al., 2008*) and 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 by exportin 5. The pre-miRNA contains a short stem with a ~2-nt 3′ protrusion, which is specifically recognized by the nuclear export factor exportin 5

(EXP5). EXP5 is a member of the nuclear transport receptor family (**Figure 6A**) and binds cooperatively the pre-miRNA and the GTP-bound form of the cofactor Ran in the nucleus, releasing the pre-miRNA into the cytoplasm after the hydrolysis of GTP to GDP. (*Kim 2004; Lund et al., 2004; Bohnsack et al., 2004*)

Cytoplasmic processing by Dicer. The second processing step (termed "dicing") consists of cleavage of the pre-miRNA near the terminal loop, releasing a ~22 nt miRNA duplex (*Bernstein et al., 2001; Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001; Knight et al., 2001*) through the activity of the RNase III Dicer (**Figure 6A**). Dicer is a highly conserved protein produced by almost all eukaryotic organisms. It acts in the nucleus in association with dsRNA-binding proteins TRBP (TAR RNA-binding protein; also known as TARBP2) (*Chendrimada et al., 2005; Haase 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), although they are not necessary for processing activity itself.
The mature miRNA sequence is thus defined both by Drosha, which generates one end of the mature miRNA, and by Dicer, which measures ~22 nt from the pre-existing terminus of the pre-miRNA and generates the second end of mature miRNAs.

Argonaute loading. The ~22 nt RNA duplex generated by the action of Dicer is then loaded onto the effector complex, termed RNA-induced silencing complex (RISC). The RISC core is composed of proteins of the Argonaute (AGO) family, in turn divided into the AGO and the PIWI subfamilies. The human AGO family counts 4 members, AGO 1-4 (also known as EIf2C 1-4). 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 endonuclease activity in some AGOs (*Jinek et al., 2009*). Binding of AGO to one of the two strands of the ~22 nt RNA duplex results in its retention in the RISC as a mature miRNA (the guide strand), whereas the other strand (the passenger strand) is degraded. The mechanism of strand selection depends on the relative thermodynamic stability of the two ends of the duplex, selecting as guide strand the strand with more unstable base pairs at the 5′ end (*Schwarz et al., 2003*). However, some precursors have been demonstrated to produce miRNAs from both strands at comparable frequencies since strand selection is not a stringent process (*Han et al., 2006; Khvorova et al., 2003*). The association of AGO proteins with Dicer and TRBP (and/or PACT) constitutes the RISC loading complex (RLC), which binds RNA duplex and facilitates mature miRNA loading on AGO. After cleavage, Dicer releases the miRNA duplex and the guide strand binds the AGO protein, while the more stable end of the duplex is

retained by TRBP in the RLC (*Tomari et al., 2004; Preall et al., 2005*).

The endonuclease activity of AGO2 ("slicer activity") removes the passenger strand of siRNA duplexes and some miRNA duplexes with perfect sequence complementarities (*Matranga et al., 2005; Miyoshi et al., 2005; Leuschner et al., 2006; Diederichs et al., 2007*). On the other hand, "slicer activity" is absent in AGO1, AGO3 and AGO4, preventing the cleavage of the passenger strand, and most miRNA duplexes, which (unlike siRNA duplexes) contain mismatches in the middle. It has been proposed that the passenger strand is unwound by an RNA helicase activity and degraded after removal (*Kawamata et al., 2009; Yoda et al., 2010*).

Editing events at different stages in miRNA biogenesis can be responsible for tissue-specific miRNA expression or, if they affect the seed sequence, can have an important impact on the target specificity of the miRNA (*Heale et al., 2009; Kawahara et al., 2007*). ADARs (adenosine deaminases that act on RNA) catalyze the conversion of adenosine to inosine in dsRNA, which can alter the base-pairing and structural properties of the substrate RNA. In addition to altering the binding specificity of the mature miRNA, ADAR-mediated modification of pri-miRNAs and pre-miRNAs can affect the activity of Drosha and Dicer and prevent the export of pre-miRNAs.

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 5' ends are more conserved than the 3' ends, because 5'-end variations can shift the position of the seed sequence and thereby alter the target specificity of the miRNA. Variations at the extremities might be caused by imprecise or alternative processing by RNase III enzymes or by deletions due to exonucleolytic activities. Terminal uridyl/adenyl transferases may also add nucleotides to the 3'ends (*Kim et al., 2009*).

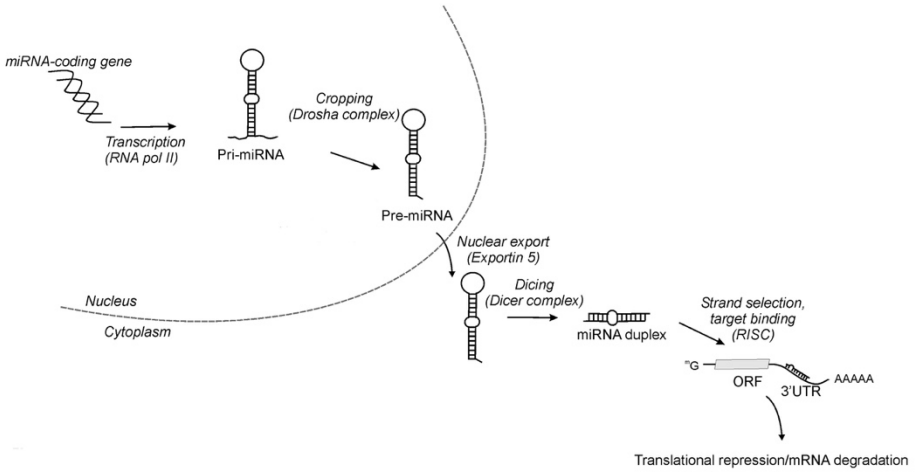


Figure 5: The miRNA pathway (modified from *D'Agostino et al., 2012*).

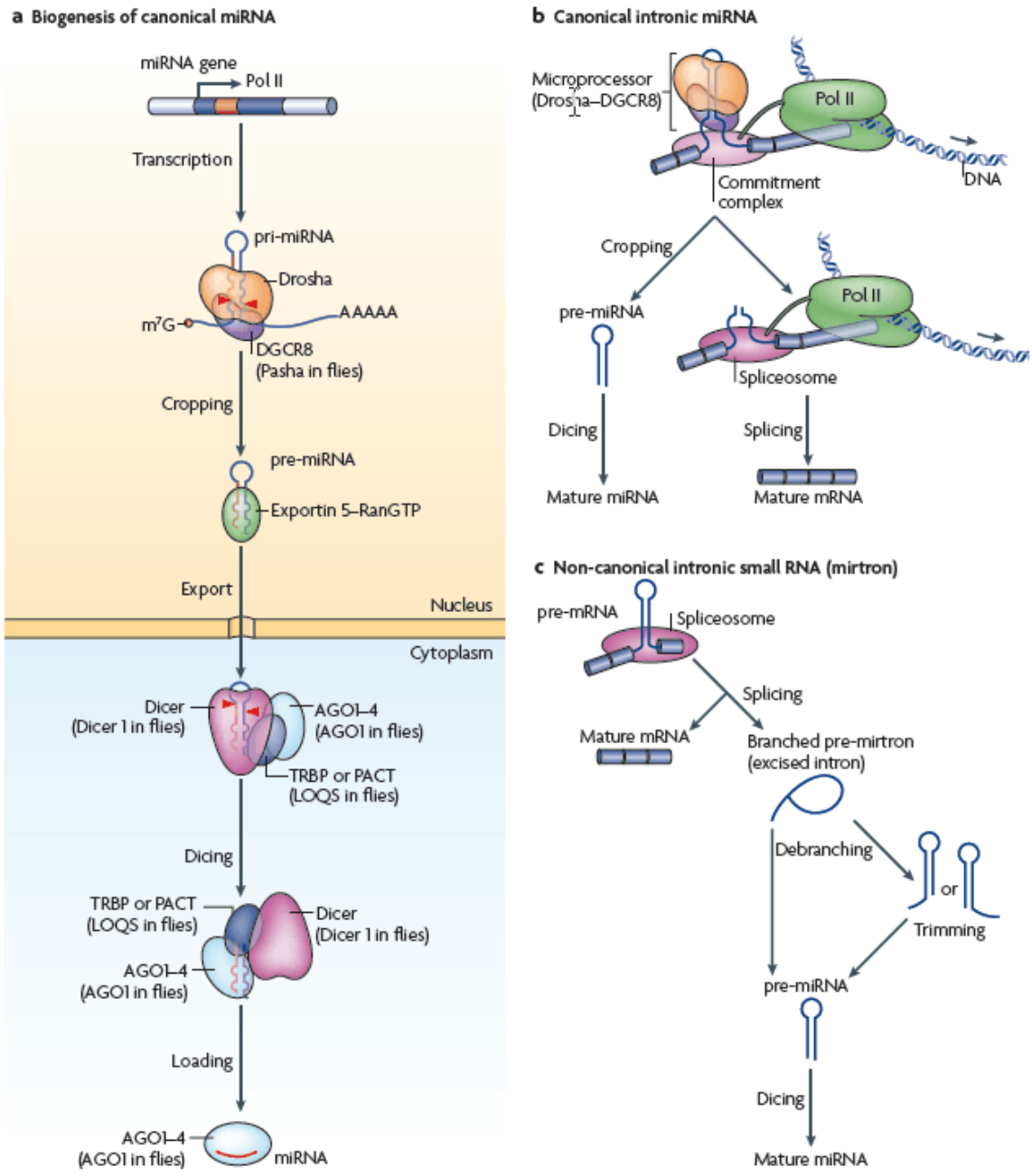


Figure 6: miRNA biogenesis. (A) RNA polymerase II (Pol II) transcribes the canonical miRNA genes as a primary transcript (pri-miRNA). The Drosha–DGCR8 complex (the Microprocessor complex) mediates the cleavage of the pri-miRNA into the ~65 nt miRNA precursor (pre-miRNA). The pre-miRNA structure (a short stem with a ~2-nt 3′ overhang) is recognized by the nuclear export factor exportin 5 (EXP5). After nuclear export, the cytoplasmic RNase III Dicer is responsible for the second processing step that produces the miRNA duplex. Dicer, in association with TRBP or PACT, interacts with Argonaute (AGO)1–4 to mediate the processing of pre-miRNA and the assembly of the RNA-induced silencing complex (RISC). One strand of the duplex is retained by the RISC as the mature miRNA, whereas the other strand is degraded. **(B)** Canonical intronic miRNAs are processed before splicing in a co-transcriptional event. The splicing commitment complex is thought to bind the Microprocessor compex which cleaves the intronic miRNA. The hairpin-shaped pre-miRNA can enter the canonical miRNA pathway, whereas the rest of the transcript undergoes splicing. **(C)** Non-canonical intronic small RNAs (mirtrons) are produced from spliced and debranched introns able to fold in a hairpin pre-miRNA. Mirtrons bypass the Drosha-processing step and a trimming step eliminates 5′ or 3′ extra tails before pre-miRNA export. m7G, 7-methylguanosine. (From *Kim et al., 2009*).

1.3.2 miRNA regulatory mechanisms

miRNAs are classified as regulatory molecules that are important in a variety of biological processes. Many diseases, and in particular cancer, present considerable variations in miRNA expression profiles that may be exploited as diagnostic and prognostic indicators.

miRNA expression is regulated at the transcriptional and post-transcriptional stages. Transcriptional regulation includes changes in the expression of a host gene and hypermethylation of the promoter of host or miRNA genes, while post-transcriptional mechanisms represent changes in miRNA processing and stability. Endogenous (hormones, cytokines) and exogenous (xenobiotics) compounds can also affect the expression of miRNAs (*Gulyaeva et al., 2016*).

The silencing activity of miRNAs occurs through the interaction between miRNAs and mRNAs, involving base-pairing of the seed sequence with a perfect or nearly-perfect complementary sequence usually in the 3' untranslated region (3'UTR) of the target mRNA. Many miRNAs reinforce the seed interaction by additional base-pairing of the rest of the miRNA sequence, which contributes to determine the target specificity.

miRNAs can regulate mRNAs through direct cleavage performed by Argonaute proteins, between the bases of the mRNA target that pair to bases 10 and 11 from the 5' end of the miRNA. The resulting product has a 5' phosphate and a 3' hydroxyl groups (*Martinez et al., 2004*; *Schwarz et al., 2004*). However, this process requires perfect base-pairing in the flanking regions of the cleavage site.

Figure 7 presents a model of miRNA-mediated mRNA silencing proposed by Huntzinger and Izaurralde (*Huntzinger et al., 2011*). As shown in **Figure 7A**, mRNAs poised to be translated form circular structures in which the cap is tethered to the polyA tail through interactions between eukaryotic translation-initiation factor 4G (eIF4G), the cap-binding protein eIF4E, and cytoplasmic poly(A)-binding protein (PABPC) (*Derry et al., 2006*). miRNAs bind to partially complementary sites in the target mRNA (usually in the 3'UTR) in association with an AGO protein bound to the glycine-tryptophan-repeat-containing protein GW182 (*Ketting, 2010*). Interaction of GW182 with PABPC (*Fabian et al., 2010; Huntzinger et al, 2010; Kozlov et al., 2010; Jinek et al., 2010*) exposes the 3' end of the mRNA to deadenylation by the CAF1-CCR4-NOT deadenylase complex. The mRNA can then be subjected to decapping by the DCP1-DCP2 decapping complex (**Figure 7B**), which then makes it a substrate for the 5'-3' exonuclease XRN1 (*Rehwinkel et al., 2005; Behm-Asmant et al., 2006; Wu et al., 2006; Eulaio et al., 2007; Eulaio et al., 2009; Piao et al., 2010*), resulting in its rapid degradation. The deadenylated mRNA may also persist in a translationally repressed form (not shown). As depicted in **Figure 7C**, loss of the closed-loop structure upon miRNA/AGO/GW182 binding may also lead to translational silencing in the absence of deadenylation.

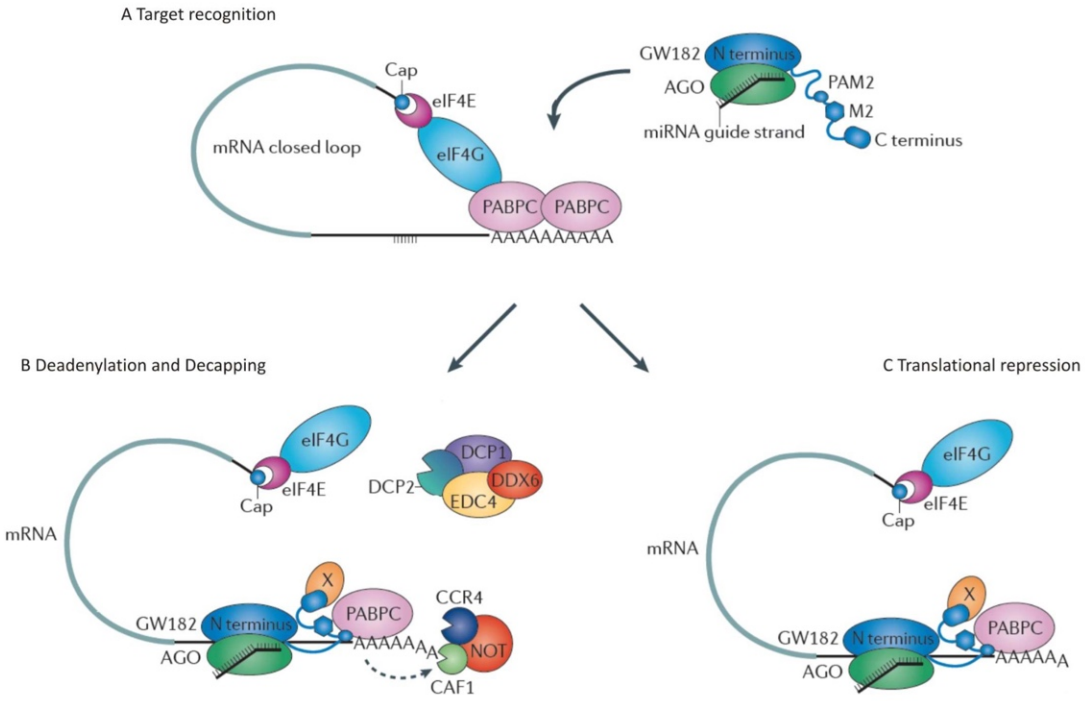


Figure 7: Mechanisms of miRNA-mediated gene silencing. **(A)** PABPC binds the mRNA poly(A) tail and interacts with eIF4G, associated with the cap structure through eIF4e, forming a circular mRNA structure that is efficiently translated and protected from degradation. miRNAs, linked to AGO, recognize their mRNA targets and the AGO-GW182 complex interacts with PABPC. **(B)** The AGO–GW182 complex recruits the deadenylase complex (CAF1, CCR4 and the NOT complex) that deadenylate mRNAs. Deadenylated mRNAs are then decapped by the decapping enzyme (DCP2, DCP1, EDC4 and DDX6) and degraded by the major 5′-to-3′ exonuclease XRN1. **(C)** Translation initiation is inhibiting by the interaction of AGO-GW182 with PABPC, which blocks the formation of the eIF4F complex and the closed conformation of the mRNA (modified from *Huntzinger and Izaurralde., 2011*).

1.3.3 miRNA in normal CD4⁺ T-cells

miRNA profiles in T-cell development. Studies from Cobb and colleagues demonstrated that miRNAs have an important role in T-cell development and homeostasis. Their experiments performed in mice showed that both $CD4^+$ and $CD8^+$ peripheral T-cell populations were lost if Dicer was deleted in thymocytes at the double-negative stage (DN) (*Cobb et al., 2006*), while deletion at the double positive transition (DN/DP) caused a great reduction in peripheral $CD8^+$ cells and the inability of peripheral $CD4^+$ T cells to differentiate into mature helper cells (*Muljo et al., 2005*). An analysis of global expression profiles of miRNAs in human T-cells revealed differences in DP, SP $CD4^+$ and SP $CD8^+$ cells, while the SP populations showed important similarities between them. In particular, miRNAs underwent a general upregulation from the DP to the SP stage (*Ghisi et al., 2011*), with a progressive upregulation of *miR-150*, *miR-146a*, and *miR-146b* and downregulation of *miR-128*, while *miR-181* was less abundant in mature peripheral T lymphocytes compared with DP thymocytes.

miRNAs in activated T-cells. Murine $CD4^+$ T-cells stimulated with anti-TCR and anti-CD28 showed 20 upregulated and 20 downregulated miRNAs, compared to the unstimulated counterpart (*Cobb et al., 2006*). An analysis of miRNA profiles in human $CD2^+$ T-lymphocytes stimulated with CD3/CD28 beads revealed a trend toward upregulation of miRNA expression upon activation (*Grigoryev, et al., 2011*). The most strongly upregulated miRNAs were *miR-221*, *miR-210*, *miR-98*, *miR-29b* and *miR-155*, while downregulated miRNAs included *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. Grigoryev and colleagues observed an increase in $CD4^+$ T-cell proliferation due to the silencing of *miR-155* or *miR-221* that resulted in increased expression of PIK3R1 and IRS2 (induced by knock-down of miR-155), and increased expression of the transcription factor FOS (induced by the knock-down of both miRNAs). The authors proposed that these effects provide a negative-feedback loop inhibiting cell proliferation and regulating survival in response to activation (*Grigoryev, et al., 2011*).

miRNAs in T_{reg}s. Deletion of Dicer in murine thymocytes disrupted T_{reg} development, leading to a reduction in the T_{reg} population in both the thymus and periphery (*Cobb et al., 2006*). Moreover, deletion of Dicer after T_{reg} lineage commitment (at the time of Foxp3 induction) resulted in a profound impairment of T_{reg} suppressor function and fatal systemic autoimmune disease (*Liston et al., 2008; Zhou et al., 2008*). Murine T_{reg}s ($CD4^+$, $CD25^+$, $GITR^+$) and non-T_{reg} $CD4^+$ naïve T-cells exhibit differential expression of many miRNAs. Naïve T cells stimulated *in vitro* produced a shift in their miRNA profile to that of T_{reg}s. T_{reg}s and activated T-cells showed upregulation of *miR-155*, *miR-214*, *miR-23b*, *miR-22*, *miR-21*, *miR-23a*, *miR-24*, *miR-27a*, *miR-103* and downregulation of *miR-29c*, *miR-142-5p*, *miR-142-3p*, *let-7* family members, *miR-30b*, *miR-30c*, *miR-25a*, *miR-26b* and *miR-150* compared to naïve cells (*Cobb et al., 2006*).
Furthermore, in humans, T_{reg}s ($CD4^+$, $CD25^+$, $Foxp3^+$) showed increased levels of *miR-21*, *miR-181c* and *miR-374* and reduced levels of *miR-31* and *miR-125a* compared to naïve T-cells ($CD4^+$, $CD25^-$) isolated from umbilical cord blood (*Rouas et al., 2009*). The same study revealed that Foxp3 was regulated by *miR-31*, which in turn downregulated the expression of Foxp3 by targeting its 3'UTR, and by *miR-21*, that used an indirect mechanism to upregulate FoxP3 expression.
An analysis of the miRNA signatures of 17 lymphocyte subsets purified from human peripheral blood revealed an upregulation of 20 miRNAs and a downregulation of 5 miRNAs in naïve $CD4^+$ T-cells compared to $CD4^+$ memory cells (*Rossi et al., 2011*). Interestingly, these miRNAs were not previously reported as differentially expressed in human T cells.
Among these, 4 miRNAs appeared to be specific for Th1 memory cells, 2 for Th17, and 4 were upregulated in Th2 memory cells. In vitro stimulation with anti-CD3 and anti-

CD28 antibodies resulted in the downregulation of 19 miRNAs that were expressed at high levels in naïve $CD4^+$ T-cells. Moreover, the study 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 favoured a naive phenotype of $CD4^+$ T-cells.

1.3.4 miRNAs in cancer

miRNAs are dysregulated in almost all solid and haematological malignancies. miRNA signatures allow the characterization of poorly differentiated tumours (*Lu et al., 2005*) and provide information about prognosis (*Garzon et al., 2010*).
miRNAs which are upregulated in cancer cells and contribute to carcinogenesis by inhibiting tumour suppressor genes, are considered oncogenic miRNAs (OncomiRs) (*Esquela-Kerscher et al., 2006*), while downregulated miRNAs, that normally prevent cancer development by inhibiting the expression of proto-oncogenes, are known as tumour suppressor miRNAs (*Dimopoulos et al., 2013*). For example, the tumour suppressor protein Pdcd4 is negatively regulated by *miR-21*, highly expressed in colorectal cancer, that consequently induces invasion, extravasation, or metastasis in colorectal cells (*Asangani et al., 2008*) indicating the oncogenic function of *miR-21*. On the other hand, the region encoding *miR-15* and *miR-16*, whose targets include the anti-apoptotic factor Bcl-2, is deleted in 65% of chronic lymphocytic leukaemia (CLL) and in other tumours (*Amodio et al., 2013*) leading to survival of these cells, suggesting that they function as tumour suppressors (*Cimmino et al., 2005*).
Furthermore, since miRNAs are stable and easily detectable, they are being investigated as candidate biomarkers to detect, classify tumours and predict disease outcome.
Expression analysis of 217 miRNAs is superior to genome-wide analysis of mRNAs for the purpose of classifying tumours (*Lu et al., 2005*).

1.3.5 miRNA in HTLV-1 infection and ATLL

All viruses use the host gene expression machinery for their replication and may therefore be affected by the host miRNA network at some level. On the other hand, viruses are able to influence host cell turnover and immune defences through mechanisms that exploit the miRNA network to promote expansion and persistence of infected cells (*Umbach and Cullen, 2009*). Moreover, some viruses express their own miRNAs which may be homologous to host miRNAs or can suppress the RNA interference (RNAi) pathway, thus affecting global miRNA expression (*Strebel et al, 2009*).

It is not surprising that some of the miRNAs that are linked with viruses are also involved in neoplastic transformation. For example, *miR-155*, which is overexpressed in several solid tumours and haematological malignancies, is upregulated by Epstein-Barr virus and expressed as a viral orthologue by Kaposi's sarcoma-associated herpesvirus (*Lin and Flemington, 2011*).

Viruses and miRNAs have reciprocal interactions: in fact, while viral gene expression can be affected by host miRNAs, viruses may also influence the expression of cellular miRNAs to block cell death and/or increase the expansion and persistence of infected cells.

In addition, some viruses have an impact on the miRNA maturation pathway. Interestingly, the viral proteins Tax and Rex are able to repress cellular miRNA production by influencing the activities of Drosha *(Van Duyne et al., 2012*) and Dicer (*Abe et al., 2010*), respectively.

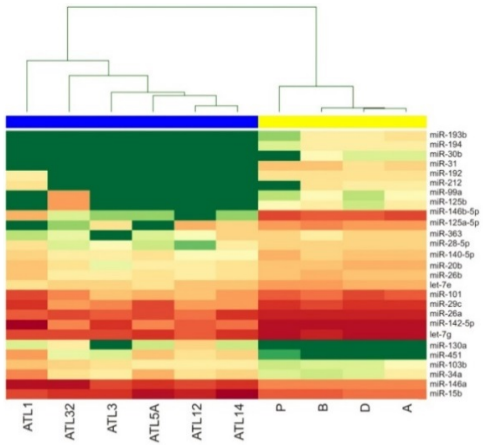
The emerging importance of miRNAs as fine-tuners of gene expression has prompted studies of the interplay between HTLV-1 and the miRNA network. The first such study showed that HTLV-1-infected cell lines express high levels of *miR-21*, *miR-24*, *miR-146a* and *miR-155* and reduced levels of *miR-223* compared to normal $CD4^+$ T-cells and uninfected T-cell lines (*Pichler et al., 2008*). ATLL cells also exhibit important differences in miRNA expression compared to normal peripheral blood mononuclear cells or $CD4^+$ T-cells (*Yeung et al., 2008; Bellon et al., 2009; Yamagishi et al., 2012).* The first miRNA profiling study of ATLL samples revealed elevated expression of *miR-93* and *miR-130b*, which target the mRNA coding for the pro-apoptotic protein TP53INP1 (*Yeung et al., 2008*). An analysis of a large panel of ATLL samples revealed an overall downregulation of miRNAs, including *miR-31*, an important target of which is NIK, an activator of the NF-κB pathway (*Yamagishi et al., 2012*). Downregulation of *miR-150* and *miR-223* in HTLV-1-transformed cells results in loss of control of their target STAT1 (*Moles et al., 2015*). On the other hand, Tax can upregulate expression of *miR-130b (Yeung et al., 2008*), *miR-146a* (*Pichler et al., 2008*) and *miR-155 (Tomita et al., 2009*). HBZ upregulates *miR-17* and *miR-21*, which target proteins that control chromatin remodelling (*Vernin et al., 2014*). Binding of the HTLV-1 genomic RNA by *miR-28-3p* interferes with reverse transcription, thereby blocking productive infection *(Bai et al., 2015*).

1.4 Upregulation of *miR-34a* in ATLL samples and HTLV-1-infected cell lines

In a study aimed at identifying known and potential new miRNAs, our laboratory sequenced small RNA libraries prepared from RNA isolated from the infected cell lines C91PL and MT-2 and from 2 samples of normal resting $CD4^+$ T-cells. Results showed

that the infected cell lines had a statistically significant increase in *miR-34a* sequence reads compared to the control CD4$^+$ cells (*Ruggero et al., 2014*). The upregulation of *miR-34a* stood out to us, as this miRNA is reported to be downregulated in many tumours and is considered to act as a tumour suppressor (*Hermeking, 2010; Spizzo et al., 2009*). As shown in **Figure 8**, results of a profiling analysis of cellular miRNA expression in 6 samples of ATLL cells compared to 4 samples of normal CD4$^+$ T-cells also indicated upregulation of *miR-34a* in the tumour samples.

A.



B.

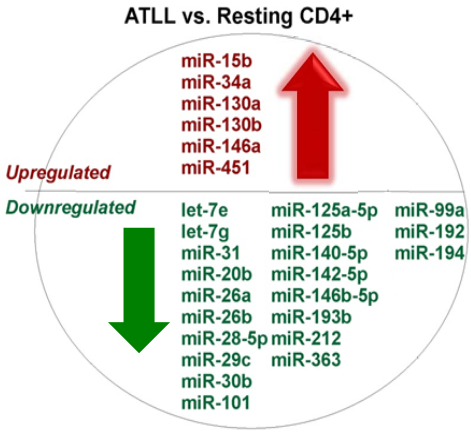


Figure 8: Analysis of miRNA expression in ATLL vs. normal CD4$^+$ T-cells with the use of microarrays. Samples consisted of total RNA extracted from PBMC of 6 ATLL patients (kindly provided by C. Pise-Masison, NIH, USA) and 4 samples of normal resting CD4$^+$ T cells (A, B, D, P). The array analysis was performed using *Agilent miRNA Microarray System version 1.0.*, which contained specific probes for 470 human miRNAs and 64 viral miRNAs. **(A)** Heat Map of miRNAs with a significant difference in expression in ATLL samples versus normal resting CD4$^+$ cells, using the *samr* package for R software from Bioconductor, considering a false discovery rate threshold < 0.01. Expression levels are represented by the colour key in which bright red corresponds to high expression levels and bright green to low levels. **(B)** Schematic representation of the data obtained from microarray analysis, summarizing the upregulated and downregulated miRNAs in ATLL samples compared to normal CD4$^+$ T-cells.

The expression of *miR-34a* in normal CD4$^+$ T-cells, ATLL samples and HTLV-1-infected cell lines was further investigated by quantitative RT-PCR. Results revealed considerable variation in the levels of *miR-34a* among samples of normal CD4$^+$ T-cells (**Figure 9A**) and ATLL samples (**Figure 9B**). Nevertheless, the expression of *miR-34a* was higher in all the ATLL cases compared to the CD4$^+$ controls, thus confirming its upregulation in ATLL.

miR-34a was also much more abundant in the HTLV-1-infected cell lines C91PL, MT-2 and HUT-102 (over 100-fold mean increase) compared to CD4$^+$ T-cells and non-infected T-cell lines Jurkat and CEM (**Figure 9C**).

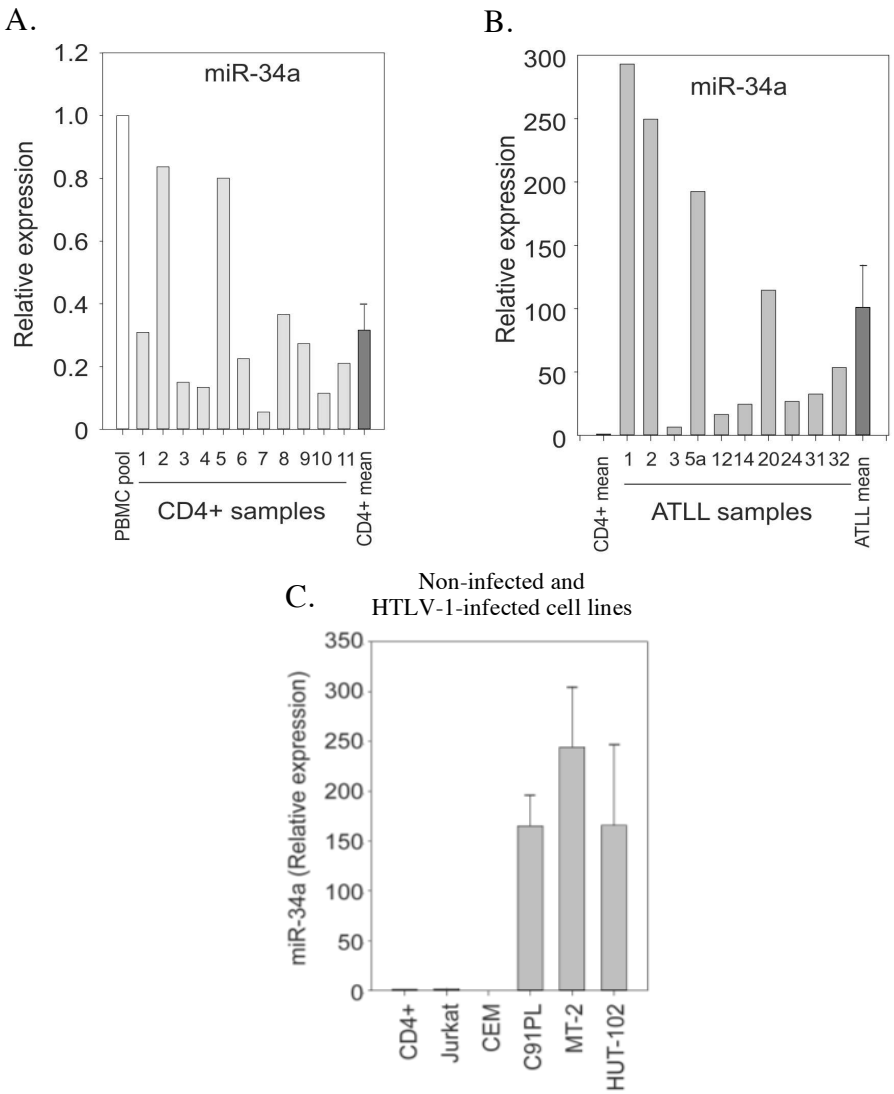


Figure 9: Expression of *miR-34a* in normal CD4⁺ cells and ATLL samples. Quantitative RT-PCR was performed to detect the expression of *miR-34a*. A pool of 4 PBMC samples was used as calibrator. **(A)** Expression of *miR-34a* in 11 samples of normal CD4⁺ T-cells (1-11). The dark grey bar shows the mean expression in the CD4+ samples normalized against the normal PBMC pool, set at 1. **(B)** *miR-34a* levels in 10 ATLL samples (1, 2, 3, 5a, 12, 14, 20, 24, 31, 32) scaled against the mean for the CD4⁺ cell samples (set at 1). *p*-value was calculated using the Mann-Whitney rank-sum test for ATLL vs. CD4⁺ ($p<0.001$). **(C)** Expression of *miR-34a* in HTLV-1-infected cell lines (C91PL, MT-2 and HUT-102) and non-infected T-cell lines (T-CD4⁺, Jurkat and CEM) scaled against the mean for the CD4⁺ cell samples (set at 1). The standard errors were calculated on the means from 3 samples for each cell line.

Interestingly, upregulation of *miR-34a* was also observed in B-cells transformed with Epstein-Barr virus (EBV) (*Mrazek et al., 2007; Cameron et al., 2008; Forte et al., 2012*) and Kaposi's sarcoma herpes virus (KSHV) (*Forte et al., 2012*), supporting previous evidence that *miR-34a* may exert diverse effects depending on the cell context (*Dutta et al., 2007*).

1.5 ***miR-34a***

miR-34a is encoded on chromosome 1p36, a region that is commonly deleted in neuroblastoma and other tumour types (*Welch et al., 2007*). In addition to miR-34a, mammals also express 2 related miRNAs named miR-34b and miR-34c which are processed from a common primary transcript coded on chromosome 11q23 (**Figure 10**) (*Misso et al., 2014*).

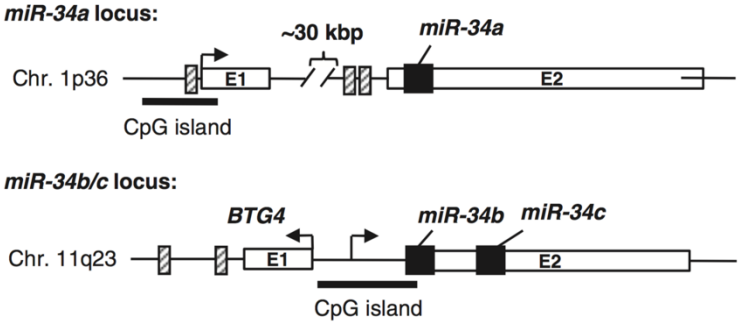


Figure 10: The *miR-34* family (from *Hermeking, 2010*).

In general, members of the *miR-34* family are frequently repressed in tumours (*Hermeking, 2012; Wang et al., 2013*) and *miR-34a* has been proposed as a tumour suppressor gene (*Hermeking, 2010*). Interestingly, *miR-34a* and *miR-34c* have identical seed sequences, whereas the *miR-34b* seed sequence is similar, but not identical, suggesting that *miR-34a* and *miR-34c* share similar mRNA targets, whereas *miR-34b* might have some distinct targets (*Rokavec et al., 2014*).

Validated targets of the *miR-34* family include mRNAs coding for proteins controlling the cell cycle, apoptosis and cell differentiation and senescence (*Chen and Hu, 2012*). Similar to other p53-target genes, *miR-34* genes may be the important targets for other signalling pathways involved in normal development.

Effects on cell proliferation and survival were observed following the ectopic expression of *miR-34* genes, in particular the cell-cycle arrest in the G_1 phase (*Tarasov et al., 2007*) and the inhibition of proliferation and colony formation in soft agar caused by ectopic *miR-34b/c* (*Corney et al., 2008*). Moreover, the introduction of *miR-34a* and *miR-34b/c* into primary human diploid fibroblasts induced cellular senescence (*He et al., 2007*).

1.5.1 *miR-34a* in cancer

As *miR-34a* is a known regulator of cell-cycle arrest, senescence and apoptosis, its inactivation can be considered a selective advantage for cancer cells.
Furthermore, as mentioned above, *miR-34a* has been proposed as tumour suppressor also because the chromosomal locus 1p36, where it resides, was found deleted in homozygosis in neuroblastoma and in other tumour types (*Cole et al., 2009*). Interestingly, a direct target of *miR-34a* is N-Myc, which is deregulated in neuroblastoma (*Wei et al., 2008*) and both up- and downregulates many target genes involved in cell cycle, DNA damage, differentiation and apoptosis (*Buechner et al., 2012*).
Moreover, the expression of *miR-34a* was low or undetectable in the majority of pancreatic cancer cell lines (*Chang et al., 2007*) and in experimental animal models of liver carcinogenesis (*Tryndyak et al., 2009*).
miR-34a is epigenetically downregulated in cell lines derived from many tumour types such as breast, lung, colon, kidney, bladder, pancreatic cancer and melanoma (*Lodygin et al., 2008*). CpG methylation of *miR-34b/c* was found in colorectal cancer (*Toyota et al., 2008*), in oral squamous cell carcinoma (*Kozaki et al., 2008*) and in malignant melanoma in which it correlated with metastatic potential (*Lujambio et al., 2008*). Aberrant CpG methylation of the promoter of *miR-34a* can decrease its expression in several types of cancer, including prostate carcinoma, melanoma and Hodgkin lymphoma (*Lodygin et al., 2008; Navarro et al., 2015*).
Inactivation of the *miR-34a* and *miR-34b/c* genes is thus a common event during tumorigenesis.
Studies by Zenz and Mraz showed that *miR-34a* expression was decreased in CLL and that it was associated with p53 mutations, chemotherapy-refractory disease, impaired the DNA damage response and decreased apoptosis (*Zenz et al., 2009; Mraz et al., 2009*). Furthermore, resistance to chemotherapy can be reversed through the restoration of *miR-34a* activity.

1.5.2 The activators of *miR-34a*: p53 and NF-κB

The *miR-34* genes contain several p53-responsive elements (*Raver-Shapira et al., 2007; Tarasov et al., 2007*) and are direct transcriptional targets of the tumour suppressor p53, contributing to its downstream effects.
Other inducers of *miR-34* expression include Nuclear Factor-kappa B (NF-κB), ELK1 and FoxO3a, which bind to the promoter regions of *miR-34a* and *miR-34b/c*, respectively (*Christoffersen et al., 2010; Kress et al., 2011*).

p53

p53 is a 393-amino acid transcription factor that contains four functional domains: an N-terminal transactivation domain, a central DNA binding domain, a tetramerization domain and a C-terminal nuclear localization domain.

In physiological conditions, the expression of p53 is negatively regulated by MDM2, an oncoprotein which binds p53 and keeps it at low levels by ubiqutination and consequent degradation, inhibition of p53 transactivation functions and alteration of its nuclear export. Transcription of the MDM2 gene is activated by p53 itself, so there is a negative feedback system that controls the level of p53. Several stimuli, e.g. DNA damage or other stress conditions, increase the level and the activity of p53 by inducing its phosphorylation on specific serine residues. These phosphate groups disrupt the p53-MDM2 interaction and contribute to p53 accumulation and activation. In response to this upregulation, p53, accumulated in the nucleus, activates or represses transcription of specific target genes. The products of these target genes induce cell cycle arrest or apoptosis, if the damage is too severe for repair (*Saito et al., 2015*).

p53 activity is also influenced by other post-translational modifications, including acetylation.

The TP53 gene is mutated in about 60% of tumours, and tumours with an intact TP53 gene commonly show a functional inactivation of the protein.

Only a small percentage of ATLL patients present mutated p53, however it is inactive in the leukemic cells of most ATLL patients and HTLV-1 transformed cells (*Yoshida et al., 2015*).

The inhibition of p53 by HTLV-1 is in part due to the activation of Tax, which mediates constitutive phosphorylation of p53 at specific serine residues that blocks its interaction with transcription factors. By inactivating p53, Tax can immortalize the HTLV-1-infected cells and destabilize their genome, leading to the accumulation of oncogenic mutations and chromosomal aberrations (*Saggioro et al., 2009*).

As the majority of HTLV-1 infected cells present wild type p53, its reactivation may be a therapeutic approach for ATLL.

Targets of p53 also include miRNAs. By influencing the expression of miRNAs, p53 can regulate many cellular processes, including the cell cycle, differentiation and cell survival (*Tabakin-Fix et al, 2006*).

Several studies have provided evidence that *miR-34a* expression is induced by DNA damage or genotoxic agent exposure through the action of p53. (**Figure 11**).

An induction of *miR-34a* was observed in human diploid fibroblasts treated with Nutlin-3, an MDM2 inhibitor (*Kumamoto et al., 2008*).

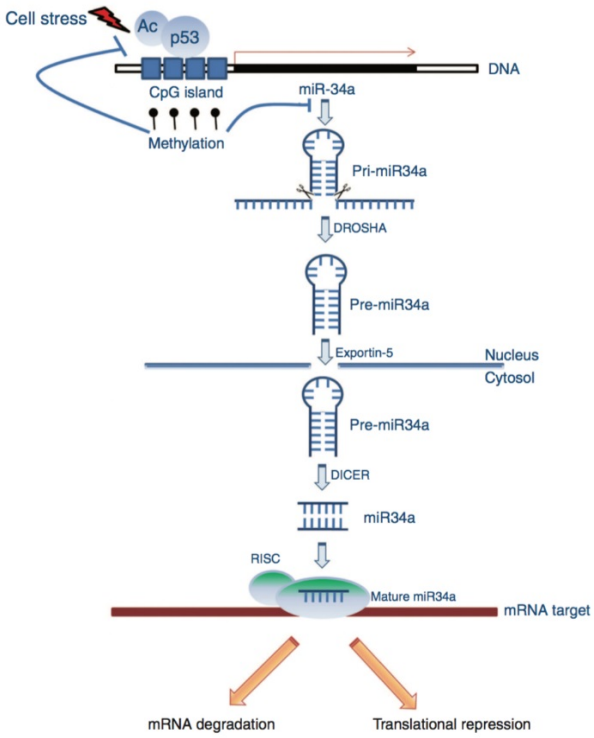


Figure 11: *miR-34a* biogenesis. p53, activated by DNA damage, binds the *miR-34a* promoter, which contains a binding sites for the p53 protein, enhancing its expression. Mutation in the DNA-binding domain of p53 reduces the expression of *miR-34a*. Hypermethylation of the CpG island in the *miR-34a* promoter silences *miR-34a* expression. This epigenetic silencing is dominant over its transactivation by p53 after DNA damage (from *Misso et al., 2014*).

It is known that p53 acts as a tumour suppressor through the activation of different transcriptional targets and *miR-34a* may support the inhibition of cell proliferation by engaging other effectors.

p53 can also influence miRNA biogenesis in a mechanism that involves its binding to DROSHA and results in enhanced pri-miRNAs/pre-miRNAs processing. Accordingly, mutations in the DNA-binding domain of p53 negatively affect this processing, reducing the expression of the related miRNAs.

In addition, *miR-34a* is able to upregulate p53-dependent apoptosis by targeting SIRT1, which deacetylates several targets, including p53. Deacetylation of p53 interferes with its activity as a transcription factor with consequent loss of induction of downstream targets, such as p21 and PUMA (*Yamakuchi et al., 2009*), inhibiting apoptosis. Therefore, *miR-34a*-mediated inhibition of SIRT1 expression favours p53 acetylation and activity (*Misso et al., 2014*).

However, there is not always a direct correlation between p53 alterations and low levels of *miR-34a*. Indeed, no significant correlation between p53 mutation and *miR-34a* dysregulation was observed in neuroblastoma and non-small-cell lung cancer (*Feinberg-Gorenshtein et al., 2009*).

Based on these observations, other mechanisms involved in *miR-34a* regulation must be postulated.

As it is known that p53 promotes *miR-34a* upregulation in many cellular contexts, we decided to test the effects of p53 activation on *miR-34a* expression in HTLV-1 infected cell lines C91PL and MT-2, which express wild-type but functionally inactivated p53. For this purpose, we used Nutlin-3a, which stabilizes p53 and increases its activity by inhibiting MDM2 (*Vassilev et al., 2004; Ohnstad et al., 2011; Manfe et al., 2012*).

NF-κB

Constitutive activation of the NF-κB pathway is a hallmark in HTLV-1 infection (*Ratner, 2005*). In mammals, the NF-κB family consists of five structurally related members, RelA, RelB (p65), c-Rel, NF-κB1 (p50/p105) and NF-κB2 (p52/p100), which form dimeric complexes that regulate the expression of target genes which contain NF-κB-responsive elements in their promoters or enhancers (*Hayden et al., 2012*).

The NF-κB pathway may be engaged by diverse stimuli such as antigens and cytokines. In non-activated cells, NF-κB dimers are sequestered in the cytoplasm by inhibitory proteins called IκBs (IκBα, IκBβ, IκBγ, IκBζ and Bcl-3), that mask the nuclear localization signal of NF-κB (*Siebenlist et al., 1994; Perkins, 2007*).

NF-κB activation involves phosphorylation of IκB inhibitors by the IκB kinase (IKK), which triggers their ubiquitination and subsequent proteasomal degradation, resulting in nuclear translocation of NF-κB dimers (*Karin and Ben Neriah, 2000*).

Moreover, the acetylation of RelA/p65 on specific lysine residues affects both the DNA-binding ability and transcriptional activity of the protein (*Kiernan et al., 2003*). Cellular processes such as adhesion, cell cycle, angiogenesis and apoptosis are regulated by NF-κB through its ability to drive expression of key gene products (*Karin and Lin, 2002*).

Overexpression of the p65 subunit of NF-κB is reported to increase *miR-34a* levels in the esophageal squamous cancer cell line EC109, while its inactivation through the transfection of dominant negative IκBα, led to a significant reduction in *miR-34a* expression (*Li et al., 2012*).

Bioinformatics analysis revealed three putative NF-κB binding sites in the promoter region of the *miR-34a* gene. Mutations in two of these sites impaired p65-induced *miR-34a* transcriptional activity, while the third one specifically binds NF-κB.

However, Li and colleagues showed that the increase in *miR-34a* levels induced by NF-κB p65 overexpression was reduced in cancer cell lines with mutant or inactivated p53.

A study performed in head and neck squamous cell carcinoma cell lines (HNSCC)

revealed a cross-regulatory relationship among NF-κB, p53 and *miR-34a*, suggesting an overlap between the majority of the predicted p53 targets and those for NF-κB, and hypothesizing that the two transcription factors may exert a concerted modulation on regulatory programs in tumour cells (*Yan et al., 2013*).

The NF-kB/Rel family of transcription factors plays a central role in the survival of HTLV-1-infected cells; in these cells NF-kB activation is largely attributable to the function of the Tax oncoprotein.

In the nucleus, Tax interacts with the NF-kB factors and increases their dimerization, which is essential for binding to target promoters (*Suzuki et al., 1993; Suzuki et al., 1994; Petropoulos et al., 1996*), resulting 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*).

1.5.3 Effects of *miR-34a* sequestration on cell viability

Based on the observation that *miR-34a* is upregulated in the context of ATLL samples and HTLV-1-infected cells lines (described in **paragraph 1.4**), our laboratory was interested in analyzing the influence of *miR-34a* on cell survival, using a functional knock-down approach.

For this propose, C91PL and MT-2 cells were transfected with either a "GFP-*miR-34a* sponge" construct expressing GFP with a synthetic 3'UTR containing 4 binding sites for *miR-34a* (*Rao et al., 2010*), or with a control GFP plasmid, without the specific binding sites. To detect the transfection efficiency, all transfections also included a plasmid expressing LNGFR as a transfection standard.

After 72 hours, transfected cells were co-labelled with FITC-anti-LNGFR and Live/Dead Red, a dye that specifically labels dead cells, and a flow cytometry analysis was performed.

Results showed that the expression of the GFP-*miR-34a* sponge was lower than the control in both cell lines, suggesting that the interaction with *miR-34a* occurred, as expected (**Figure 12A**). Moreover, it was observed that cells transfected with the *miR-34a* sponge showed an increase in cell death compared to cells transfected with the control (**Figure 12B**) (p-value=0.021 in C91PL and p-value=0.002 in MT-2). Taken together, these results suggest that *miR-34a* may represent a pro-survival factor in HTLV-1 infected cells and may contribute to the transformed phenotype, leading to the suggestion that targeting *miR-34a*, for example with drugs, might be effective for eliminating infected cells from the host either alone or in combination with other therapeutic agents.

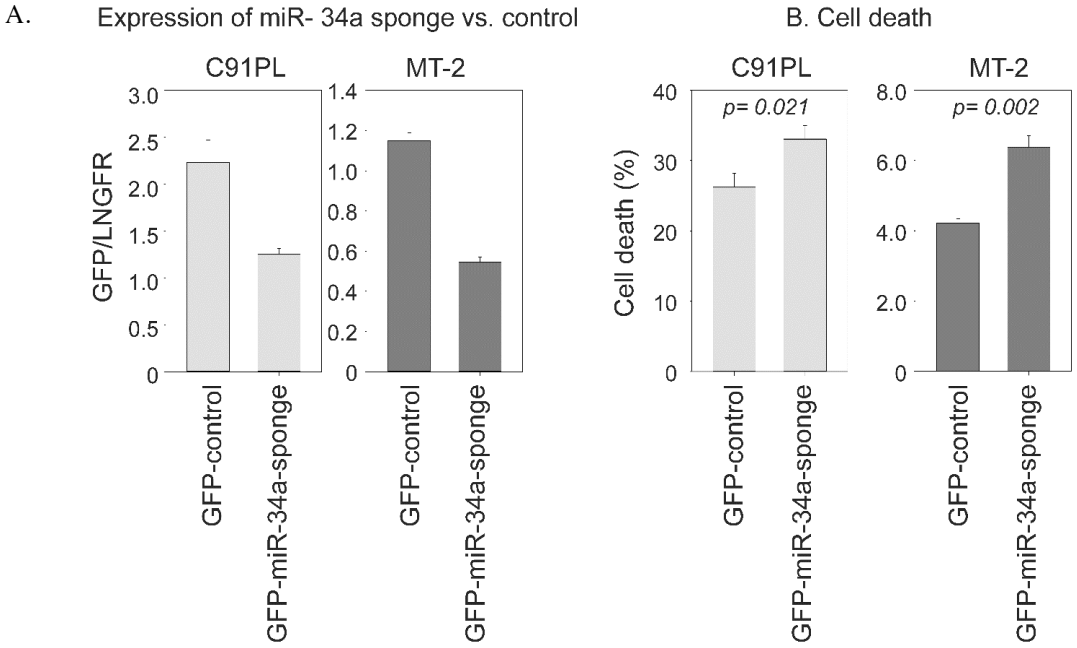


Figure 12: Sequestration of *miR-34a* in HTLV-1 infected cell lines. Cell lines MT-2 and C91PL were transfected with GFP-*miR-34a*-sponge or GFP-control plasmid together with pMACS-LNGFR (Miltenyi Biotec), as described in Material and Methods. **(A)** Flow cytometry was performed to detect GFP and LNGFR signals, and the percentage of positive cells was multiplyied by the mean fluorescence intensity (MFI). Bars indicated the means from 3 experiments with standard error bars. **(B)** C91PL and MT-2 cells were labelled with Live/Dead Red (Invitrogen) to identify dead cells and with anti-LNGFR antibody to identify transfected cells. The percent cell death was calculated by dividing the number of cells positive for both Live/Dead Red and LNGFR by the total number of LNGFR-positive cells. Plotted are mean % cell death values with standard errors. *p*-values were calculated using the Mann-Whitney rank-sum test (p=0.021 and p=0.002 for C91PL and MT-2, respectively).

The experiments described in the following sections were aimed at further understanding the regulation of *miR-34a* expression in HTLV-1-infected cells and its impact in cell proliferation/death.

2. MATERIALS AND METHODS

2.1 Cell culture

HTLV-1-infected T-cell lines C91PL and MT-2, the uninfected T-cell line Jurkat and the Jurkat-derived cell line JLR (see next section) were cultured in RPMI-1640 (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). HeLa cells were cultured in Dulbecco's modified Eagles medium (Sigma-Aldrich) supplemented with 10% FBS, 100 units/ml penicillin and 20 units/ml streptomycin (complete DMEM). Cultures were maintained in a 37°C, 5% CO_2 incubator.

2.2 Infection of PBMC with HTLV-1

Peripheral blood mononuclear cells (PBMC) were isolated from two healthy blood donors (Transfusion Unit of Padova City Hospital) by separation of the buffy coat fraction through Ficoll-Hypaque (GE Healthcare).

PBMC were cultured for 3 hrs in complete RPMI to deplete monocytes/macrophages by adhesion. $2x10^6$ C91PL cells were pelleted and lethally irradiated (69.5 Gy) and then placed in culture together with $2.5x10^6$ PBMC in a total volume of 4.5 ml complete RPMI supplemented with 500 μg/ml phytohemagglutinin (PHA, Sigma-Aldrich).

Irradiated C91PL cells or PBMC stimulated with PHA cultivated alone were used as controls. After 48 hours, cultures were supplemented with interleukin-2 (IL-2, 20 U/ml, Roche).

The cultures were then maintained in the presence of IL-2 and passaged periodically.

The control irradiated C91PL cells were dead after 2 weeks. The control PBMC cultures were harvested for RNA 8 days after stimulation and the co-cultures were harvested for RNA after 30 and 72 days.

Genomic DNA was also isolated and analysed by PCR with primers specific for the HTLV-1 U5-gag region (*Ruggero et al., 2014*). PCR with primers specific for the pre-*miR-34a* locus served as a control for the amplification reactions. PCR products were separated in non-denaturing polyacrylamide gels, stained with EuroSafe (Euroclone) and detected using a Cambridge UVTEC system. Genetic profiling was performed using the Promega PowerPlex 16 HS kit; resulting STR patterns were compared to that obtained for C91PL cells.

2.3 Quantitative RT-PCR for miRNAs and mRNA

Total RNA was isolated using TRIZol (Life Technologies) or TRI Reagent (Sigma-Aldrich) according to the manufacturer's protocol.
RNA was subjected to reverse transcription and quantitative PCR to detect individual miRNAs using Applied Biosystems Taqman miRNA assays according the manufacturer's protocol. Results were analysed by relative quantification using RNU-44 as an endogenous control.
RNA samples to be analysed for mRNA expression were treated with DNAse I (Invitrogen) for 15 minutes at room temperature. DNase I was inactivated by adding 25 mM EDTA and incubation for 10 minutes at 65°C.
Resulting DNA-free RNA was reverse-transcribed using SuperScript II reverse trascriptase (Invitrogen) and random hexamers, according to the manufacturer's protocol. For detection of cellular mRNAs, cDNA was subjected to a PCR reaction using specific primers and a PCR master mix containing SYBR Green (Roche). The templates were amplified in a LightCycler 480 (Roche) thermal cycler according the manufacturer's protocol; β-actin was used as an endogenous control for all samples.
HTLV-1 transcripts were quantified by RT-PCR using splice site-specific primers and custom Taqman probes as described previously (*Rende et al., 2011*).

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Pre-*miR-34a*	TGGCAGTGTCTTAGCTGGTTG	GGCAGTATACTTGCTGATTGCTT
TP53INP1	CTTCCTCCAACCAAGAACCAG	CAAGCACTCAAGAGATGCCG
SIRT1	ACATAGACACGCTGGAACAGG	GATAGCAAGCGGTTCATCAGC
BIRC5	TTCTCAAGGACCACCGCATC	TGAAGCAGAAGAAACACTGGG
E2F3	CACCCTGGACCTCAAACTGT	TCTGGAGGGGCTTTCACAAC
β-ACTIN	AGCACAGAGCCTCGCCTTTG	GGAATCCTTCTGACCCATGC

Table 2: Primers for quantitative RT-PCR

2.4 Drug treatments

C91PL and MT-2 cells were seeded at $3x10^5$ cells/ml in 6-well plates and treated with Nutlin-3a (Sigma-Aldrich, 1 μM or 5 μM, dissolved in DMSO), Bay 11-7082 (Sigma-Aldrich, 1 μM or 2.5 μM, dissolved in DMSO) or with DMSO as vehicle control.

2.5 Plasmids and transfections

Transfections of T-cell lines were performed using a Neon electroporator and kit (Life Technologies). After electroporation, the cells were incubated in complete RPMI medium without antibiotics for 4 hours, and then supplemented with medium containing penicillin/streptomycin.

Jurkat cells were transfected with plasmid pcTax (wild-type, mutant M22 or mutant M47, defective for activation of NF-κB and CREB, respectively (*Robek et al., 1999*) or with pBluescript as a negative control.

The JLR cell line was obtained by transfecting Jurkat cells with plasmid pLTR-mRFP, which expresses monomeric red fluorescent protein (mRFP) under the control of the HTLV-1 LTR promoter. The plasmid also contains an expression cassette for the Sh ble gene, which confers resistance to the antibiotic Zeocin (Life Technologies). Transfected cells were selected in the presence of Zeocin.

C91PL cells were transfected with 50 nM *mir*Vana *miR-34a*-mimic or *miR-34a*-inhibitor (Ambion).

HeLa cells were transfected using PolyJet (SignaGen Laboratories) with pcTax, M22 or M47 or with pBluescript as a negative control.

2.6 RLM - 5' RACE

RNA Ligase Mediated Rapid Amplification of cDNA Ends (RLM-RACE) represents an improvement to the classic RACE reaction (*Maruyama and Sugano, 1994, Shaefer, 1995*) that is specific for capped mRNA. For this assay we used the FirstChoice RLM RACE kit (Ambion).

Total RNA was extracted from C91PL cells using TRI Reagent. RNA (10 μg) was treated with DNAse I (Invitrogen) for 15 minutes at room temperature, then precipitated with ethanol and 300 mM Na-acetate and resuspended in nuclease-free H_2O. Resulting DNA-free RNA was treated with Calf Intestine Alkaline Phosphatase (CIP) at 37°C for 1 hour, in order to remove free 5'-phosphates from molecules such as ribosomal RNA, fragmented mRNA, tRNA, and contaminating genomic DNA; the cap structure found on intact 5' ends of mRNA is not affected by CIP. RNA was extracted again with TRI Reagent, and then treated with Tobacco Acid Pyro-phosphatase (TAP) at 37°C for 1 hour to remove the cap structure, leaving a 5'-monophosphate. An RNA Adapter oligonucleotide (**Table 3**) was then ligated to the RNA using T4 RNA ligase. The ligation reaction resulted in modification of the decapped mRNA with the adapter sequence at its 5' end, while other RNAs that had been de-phosphorylated with CIP were not competent

for ligation.
The adaptor-modified RNA was subjected to a random-primed reverse transcription reaction using M-MLV RT enzyme followed by nested PCR using Outer and Inner primers supplied with the kit and 2 primers located in the region of the *miR-34a* stem-loop (*Navarro et al., 2009*) (**Table 3**) to amplify the 5' end of the target transcript. PCR products were separated in a polyacrylamide gel, purified and sequenced using primer P2 and the Applied Biosystems BigDye kit. Sequences were aligned to the human genome using BLAST.

5' RACE Adapter	5'-GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA-3'
5' RACE Outer Primer	5'-GCTGATGGCGATGAATGAACACTG-3'
5' RACE Inner Primer	5'-CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG-3'
***miR-34a* P1 Primer**	5'-AGAGCTTCCGAAGTCCTGG-3'
***miR-34a* P2 Primer**	5'-TTGCTCACAACAACCAGCTAAGA-3'

Table 3: Primers for 5' RACE

2.7 Immunoblot

Cells were lysed in Cell Disruption Buffer (Ambion) supplemented with phosphatase inhibitors, protease inhibitors and nicotinamide, and the total protein content was measured using a modified Bradford assay (Pierce). Samples were balanced for equivalent protein content and subjected to SDS-PAGE followed by electrotransfer to supported nitrocellulose (GE Healthcare). Blots were saturated with 5% nonfat milk (Euroclone) and incubated first with the specific primary antibodies and then with horseradish peroxidase-conjugated secondary antibodies (Pierce).
The following primary antibodies were used: rabbit anti-β-actin polyclonal antibody (Sigma-Aldrich), rabbit anti-acetylated p53 antibody (Lysine-382, Cell Signaling), goat anti-p53 polyclonal antibody, rabbit anti-SIRT1 polyclonal antibody, mouse anti-Tubulin monoclonal antibody, rabbit anti-E2F3 antibody (Santa Cruz Biotechnology), rabbit anti-Survivin monoclonal antibody (Abcam), and mouse anti-Tax monoclonal antibody (*Lee et al., 1989*).
Immunoreactive bands were detected using chemiluminescence reagents (LiteAblot® TURBO, Euroclone) and visualized using a digital imager (Uvitec).

2.8 Analysis of cell death and cell cycle

Apoptotic cell death was measured using Annexin V conjugated to Alexa-647 (Molecular Probes) and propidium iodide (PI, Sigma-Aldrich).
Labelled cells were subjected to flow cytometry using a BD FACSCalibur to detect Annexin-V (FL4-H channel) and PI (FL2-H channel). The percentages of cells that were stained with Annexin-V or AnnexinV and PI (corresponding to early and late apoptosis, respectively) or PI alone (indicating necrosis) were measured.
Specific cell death (in reference to each staining pattern) was calculated using the following formula:

$$SCD = \frac{\%\ dead\ cells\ T - \%\ dead\ cells\ NT}{100 - \%\ dead\ cells\ NT} \cdot 100$$

T= treated cell culture
NT= non treated cell culture

For cell cycle analysis, cells were washed with PBS (phosphate-buffered saline) and fixed with 1 ml of ice-cold 70% ethanol for 30 minutes at -20ºC. Cells were then washed and incubated with 500 μl of staining solution (450 μl PBS + 50 μl of 1 mg/ml PI and 2 μl of 100 mg/ml RNAse A) for 1 hr at 37ºC. Cells were analyzed with a flow cytometer and data were processed with ModFit software.

2.9 MTT assay

After electroporation, C91PL cells were seeded at a density of $6x10^5$ cells/ml in complete RPMI medium. 24, 48 and 72 hours post-transfection, 250 μl of cells were pelleted and resuspended in 100 μl of MTT staining solution, consisting of 50 μl complete RPMI + 50 μl MTT reagent-Thiazolyl Blue Tetrazolium Bromide (stock 0.5 mg/ml, Sigma Aldrich). Cells were incubated for at least 4 hours at 37°C, 5% CO_2, to allow the conversion from MTT to Formazan crystals. Then, 100 μl of 10% SDS in 0.01 M HCl solution were added to the cells to solubilize the Formazan crystals. After at least 2 hours of incubation at 37°C, 5% CO_2, the optical density at 670 nm was measured using a Perkin-Elmer Victor X4 plate reader. The percent cell viability was calculated as the absorbance measured in the treated cultures divided by the absorbance measured the control cultures, multiplied by 100. Six independent experiments in triplicate were performed and the data were expressed as the mean ± standard deviation.

2.10 Immunofluorescence

Cells were isolated by centrifugation at 1200 rpm for 7 minutes and fixed with 3.7% formaldehyde in PBS for 20 minutes at room temperature. The cells were then rinsed two times with PBS and permeabilized in 0.1% NP40 in PBS for 10 minutes at room temperature. After centrifugation for 5 minutes at 2000 rpm and washing with PBS, cells were incubated with rabbit α-Survivin (Abcam, diluted 1:100 in PBS) and goat α-HSP60 (Santa Cruz, diluted 1:200 in PBS) for 1 hour at 37°C.
Following a centrifugation for 5 minutes at 2000 rpm and washing with PBS, cells were incubated with Alexa 488 α-rabbit antibody and Alexa 563 α-goat antibody (each diluted 1:500 in PBS) for 45 minutes at 37°C.
Cells were washed again, resuspended in 50% Glycerol-PBS and mounted on a microscope slide for analysis using a Zeiss LSM 510 laser scanning microscope.

2.11 β-galactosidase senescence assay

Senescent cells were detected using a cytochemical assay that measures β-galactosidase activity under acidic conditions (*Dimri et al, 1995*). Suspension cells were isolated by centrifugation at 1200 rpm for 7 minutes and fixed with 3.7% formaldehyde in PBS for 5 minutes at room temperature. The cells were then washed two times with PBS and incubated with SA-β-gal staining solution containing X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside; Sigma-Aldrich), whose cleavage by β-galactosidase results in the formation of a blue precipitate. Following incubation at 37°C for 16 hours in the dark, the cells were centrifuged at 1200 rpm for 7 minutes, rinsed twice with PBS and resuspended in PBS. Senescent (blue) cells were counted in microscope fields.

3. RESULTS

3.1 *miR-34a* upregulation in newly infected PBMCs

Having observed upregulation of *miR-34a* in ATLL samples and HTLV-1-infected cell lines, we set out to test the effect of the virus on expression of the miRNA in primary PBMC.

The experiment was performed in 2 cultures of PBMCs derived from healthy donors co-cultivated with lethally irradiated C91PL cells (as described in **paragraph 2.2** of Material and methods), in order to allow transmission of the virus.

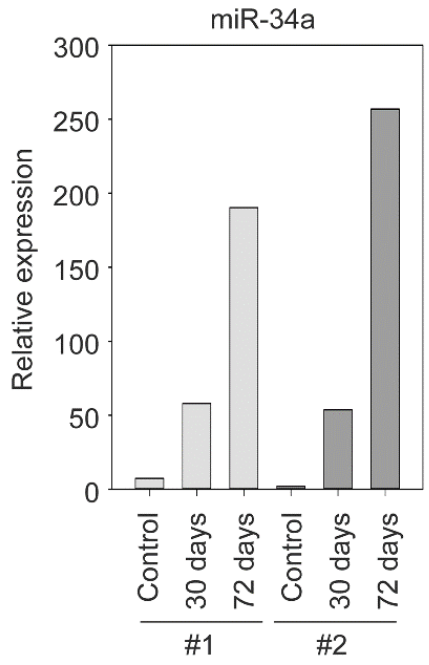
RNA was isolated from the co-cultures at 30 days and at 72 days, and from control samples of the PBMC cultured alone for one week.

Results of quantitative RT-PCR to detect *miR-34a* revealed that its expression underwent a progressive increase in both co-cultures compared to the uninfected PBMC (**Figure 13A**).

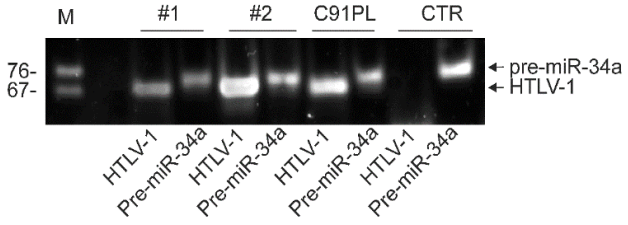
To confirm that the viral infection had occurred, we extracted genomic DNA from the cells at 72 days and performed PCR using primers specific for the HTLV-1 *gag* gene. Results confirmed that both PBMC cultures were HTLV-1-infected (**Figure 13B**).

To determine whether the cultures might contain residual C91PL cells, we performed a genotyping analysis of short tandem repeats (STR) on the genomic DNA from the infected cultures at 72 days and from an aliquot of C91PL cells. Results yielded distinct profiles for the infected cultures and the C91PL cells (**Figure 13C**), thus confirming that the cultures of newly infected PBMC no longer contained C91PL cells.

A.



B.



C.

Marker	#1	#2	C91PL
D3S1358	15,16	17,18	15,19
TH01	8	7,9.3	8,9
D21S11	31.2	28,29	29,31.2
D18S51	15	15,17	12,13
Penta E	7,10	7,17	16,20

Figure 13: Expression of *miR-34a* in newly infected PBMCs. (A) *miR-34a* levels were detected through quantitative RT-PCR in PBMCs from two healthy donors (indicated by light and dark grey bars) co-cultivated with lethally irradiated C91PL cells. Values were scaled against the mean for $CD4^+$ cell samples (set at 1). **(B)** PCR was performed on genomic DNA of infected PBMC cultures #1 and #2 at day 72 with specific primers to detect HTLV-1 *gag* and the pre-*miR-34a* genomic region (a positive control for the PCR amplification). Genomic DNA from C91PL cells and from non-infected PBMCs (Ctr) served as positive and negative controls for amplification of HTLV-1 DNA. The image shows the PCR products in a nondenaturing acrylamide gel. **(D)** The table indicates values for selected STR markers that distinguished the infected PBMC cultures from C91PL cells.

3.2 Identification of pathways responsible for *miR-34a* overexpression

The observation that, in the presence of HTLV-1 infection, *miR-34a* undergoes a strong upregulation led us to investigate the mechanism through which the virus regulates *miR-34a* expression.

Studies by Brommer and Tarasov (*Brommer et al., 2007; Tarasov et al., 2007*) indicated that *miR-34a* is a transcriptional target of p53. Other investigators showed that *miR-34a* can also be upregulated by phorbol esters in the erythroleukaemia cell line K562 (*Navarro et al., 2009*), by the NF-κB pathway in esophageal cancer (*Li et al., 2012*) and by Epstein-Barr virus (EBV) in transformed B-cells (*Forte et al., 2012*).

3.2.1 The NF-κB pathway sustains *miR-34a* upregulation in HTLV-1-infected cell lines

Activation of the NF-κB pathway is a hallmark of HTLV-1 infection (*Sun et al., 2005*). We therefore tested the effects of the NF-κB inhibitor Bay 11-7082 (*Pierce et al., 1997*) on *miR-34a* expression in infected cell lines C91PL and MT-2, both of which exhibit constitutive activation of the NF-κB pathway (*Nasr et al., 2005*).

Bay 11-7082 is a specific inhibitor of the NF-κB pathway that irreversibly prevents the phosphorylation and degradation of IκBα (*Garcìa et al., 2005*).

Several studies reported that in HTLV-1-infected T-cell lines, Bay 11-7082 led to apoptosis, reduced NF-κB binding to DNA and downregulation of the expression of the antiapototic gene BclxL. Furthermore, Bay 11-7082 treatments in immunodeficient mice negatively affected ATLL growth (*Kfoury et al., 2005*).

Inhibition of cell growth and apoptosis was observed after Bay 11-7082 treatment in cells with high NF-κB activity, but not in cells with low NF-κB activity.

In HTLV-1 infected cell lines, Bay 11-7082 is reported to be the most specific and effective NF-κB inhibitor, affecting survival and cell cycle promotion.

As NF-κB is considered a potential therapeutic target for hematopoietic malignancies, Bay 11-7082 may be useful as a therapeutic approach (*Mori et al., 2002; Rauert-Wunderlich, 2013*).

As shown in **Figure 14A**, treatment with Bay 11-7082 led to a dose-dependent reduction in *miR-34a* levels in both cell lines.

As Tax is a well-known NF-κB activator, we next transfected a Tax expression plasmid into Jurkat cells and measured the levels of *miR-34a* after 48 hours. Results showed that Tax upregulated *miR-34a*, although this increase was more modest than that observed for *miR-146a* (**Figure 14C**), which was previously reported to be upregulated by Tax through NF-κB stimulation (*Pichler et al., 2008; Tomita et al., 2009*).

We performed similar experiments in HeLa cells, with both wild-type Tax and Tax mutants M22 and M47, which are defective for activation of NF-κB and CREB, respectively (*Rimsky et al., 1988*). **Figure 14B** shows the results of this experiment, which indicated that wild-type Tax and TaxM22 mutant produced a modest increase in *miR-34a* expression (2-fold and 1.5-fold mean increase), compared to *miR-146a*, which was strongly upregulated by wildtype Tax and TaxM47.

Taken together, these results indicate that while the NF-κB pathway sustains *miR-34a* expression in C91PL cells and MT-2 cells, Tax has a modest effect on expression of this miRNA in Jurkat and HeLa cells, suggesting that theTax-NF-κB axis might not be the only driver of *miR-34a* expression.

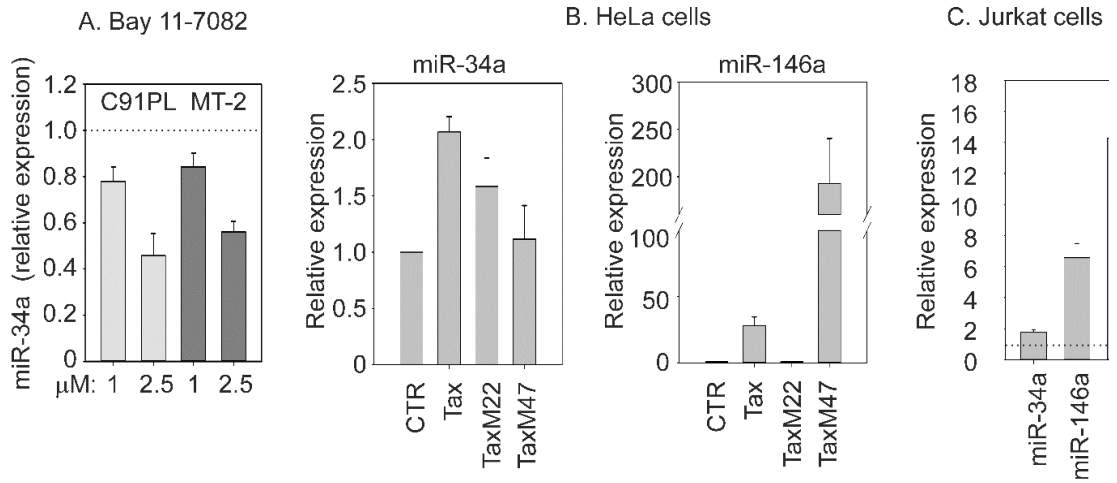


Figure 14: Changes in *miR-34a* levels upon inhibition of NF-κB or expession of Tax. **(A)** C91PL and MT-2 cells were treated for 48 hours with Bay 11-7082 (1 μM and 2.5 μM) and *miR-34a* expression was detected through quantitative RT-PCR. Both cell lines treated with the same volume of DMSO were used as control. The graph shows the mean relative expression of *miR-34a* in drug-treated cells compared to DMSO-treated controls, set at 1 (see dotted line). **(B)** HeLa cells were transfected with pBluescript (CTR) or with a plasmid expressing wild type Tax, Tax M22 or Tax M47. **(C)** Jurkat cells were transfected with pBluescript or with Tax expression plasmid. For both panels B and C, the relative expression of the indicated miRNAs (*miR-34a* or *miR-146a*) was measured by quantitative RT-PCR. Results were normalized against the pBluescript-transfected samples, set at 1 (see dotted line).

3.2.2 Identification of the primary *miR-34a* transcript in C91PL cells

Mature *miR-34a* is coded on the minus strand of the long arm of chromosome 1and can be processed from at least 4 spliced primary precursors (**Figure 15A**). Using 5' and 3' RACE (rapid amplification of cDNA ends), Tarasov et al. identified a pri-*miR-34a* consisting of 2 exons separated by about 30 kbp, with exon 2 containing the *miR-34a* stem-loop (*Tarasov et al., 2007*) in a lung cancer cell line induced to express p53 (**Figure 15A**, transcript EF570048.1). Another pri-*miR-34a* containing an extended exon 1 and a shorter exon 2 sequence was identified in Drosha-silenced HeLa cells (*Chang et al., 2007*) (**Figure 15A**, transcript EF592573.1). A subsequent analysis with 5' RACE showed that stimulation of a p53-null cell line with phorbol esters resulted in the production of precursors consisting of 2 upstream exons spliced to exons 1 and 2 (*Navarro et al., 2009*) (**Figure 15A;** pri-*miR-34a*-K1 and pri-*miR-34a*-K2).

Prompted by these observations, we were interested in identifying the pri-*miR-34a* expressed in HTLV-1-infected cells. For this purpose, we isolated total RNA from C91PL cells and performed a 5' RACE analysis, as described in Material and Methods.

Figure 15B shows the two main products that were obtained, of about 450 bp (band 1) and 200 bp (band 2). Sequencing analysis of the purified products revealed that product #1 corresponded to pri-miRNA EF592573.1, exons 1 and 2, with a distinct TSS compared

to previously identified TSS (**Figure 15C**; also see arrowhead and dots in **Figure 15A**). The sequence of band #2 corresponded to exon 2, with an intronic sequence upstream (data not shown) and without several nucleotides of the 5' RACE adaptor.

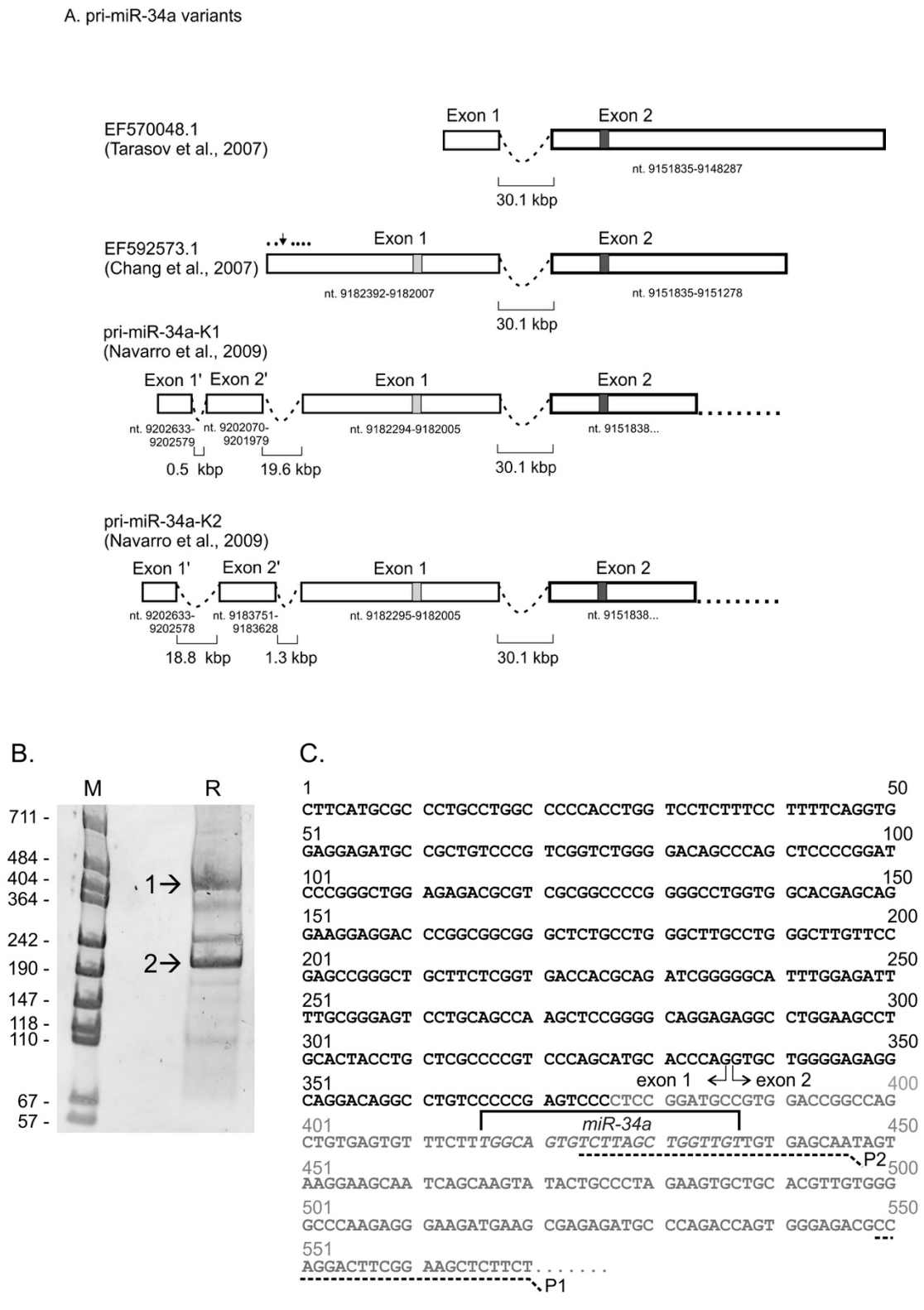


Figure 15: pri-*miR-34a* transcript produced in C91PL cells. Panel **A** shows pri-*miR-34a* variants described in previous studies (see text). Numbering is according to the GenBank GRCh38.p2 primary assembly (minus strand). The black box indicates the position of mature *miR-34a* (nt 9151756-9151735). Dots above exon 1 indicate different TSS identified by Chang et al. (*Chang et al., 2007*) and the arrow indicates the TSS for Band #1. Panel **B** shows PAGE-separated 5'RACE products generated from RNA isolated from C91PL cells (lane R, 5'RACE products, lane M, digestion of pBluescript with MspI as a marker). Bands 1 and 2 were excised, eluted, and sequenced using primer P2. Panel **C** shows the sequence obtained for Band #1 (reverse-complement). Band #2 was mapped to nt 9151920-9151796 (minus strand).

We thus concluded that the main pri-*miR-34a* present in C91PL cells is the two-exon p53-inducible transcript corresponding to EF592573.1, with an alternative TSS; the significance of the 200-bp product remains to be understood.

3.3 Role of *miR-34a* in HTLV-1 infected cells

In general, the expression of all members of the *miR-34* family is frequently repressed in tumours (*Hermeking, 2012; Wang et al., 2013*) and *miR-34a*, which is promoted by p53 and targets mRNAs coding for proteins controlling the cell cycle, apoptosis, cell differentiation and senescence (*Chen and Hu, 2012*), was proposed to act as a tumour suppressor (*Hermeking, 2010*).
However, in Epstein-Barr virus (EBV) infection of primary human B cells, Forte et colleagues (*Forte et al., 2012*) discovered a progrowth function for *miR-34a* in B cell transformation, in contrast with its canonical role as a tumour suppressor, thus highlighting the importance of studying miRNA functions in different cell types.
Based on this information, we decided to investigate the role of *miR-34a* in HTLV-1-infected cells.

3.3.1 Effects of Nutlin-3a on *miR-34a* expression

It is known that one of the main activators of *miR-34a* is p53. However, HTLV-1-infected cells and ATLL cells generally express either a wild-type but functionally inactivated p53, or less often, harbour inactivating TP53 mutations (*Tabakin-Fix et al., 2006*). To modulate *miR-34a* expression in HTLV-1 infected cell lines, in order to understand the functional significance of its upregulation, we first tested the effect of p53 activation on *miR-34a* levels in MT-2 and C91PL cells, both of which are reported to express wild-type p53 (*Hasegawa et al., 2009; Cereseto et al., 1996; Kamihira et al., 2001*).

To investigate the effects of p53 reactivation in HTLV-1 infected cells, in our study we employed Nutlin-3a, a *cis*-imidazoline analogue that selectively inhibits the p53-MDM2 interaction by competitively occupying the p53 binding site on MDM2. This results in stabilization of p53 (*Vassilev et al, 2004; Ohnstad et al, 2011; Manfe et al, 2012*). Nutlin-3a-induced activation of the p53 pathway can therefore induce apoptosis or senescence in tumour cells that express wild-type p53.

A study performed by Hasegawa and colleagues demonstrated that p53-wildtype ATLL cells and HTLV-1-infected cell lines can be effectively killed by Nutlin-3a. They identified several proteins whose expression was changed by the drug treatment, including Survivin (downregulated) and Bax, Puma and Noxa (upregulated) (*Hasegawa et al, 2009*).

Recent studies proposed that Nutlin-3a, by activating p53 in a selective and non-genotoxic manner, may represent an alternative to the current chemotherapy, in particular for pediatric tumours and hematological malignancies, which are frequently p53-wild-type at diagnosis (*Secchiero et al., 2011*).

MT-2 and C91PL cells were treated with 1 μM or 5 μM Nutlin-3a or with the same volume of DMSO (vehicle) for 48 hours.

Aliquots of the cultures were harvested for RNA, protein and viability assays. Results of immunoblots **(Figure 16A)** confirmed that p53 protein was stabilized in both cell lines by Nutlin-3a in a dose-dependent manner, and results of RT-PCR assays showed that the p53 target TP53INP1 increased, compared to the non-treated control **(Figure 16B)**.

As shown in **Figure 16C**, p53 activation also resulted in an increase in mature *miR-34a* and its precursor.

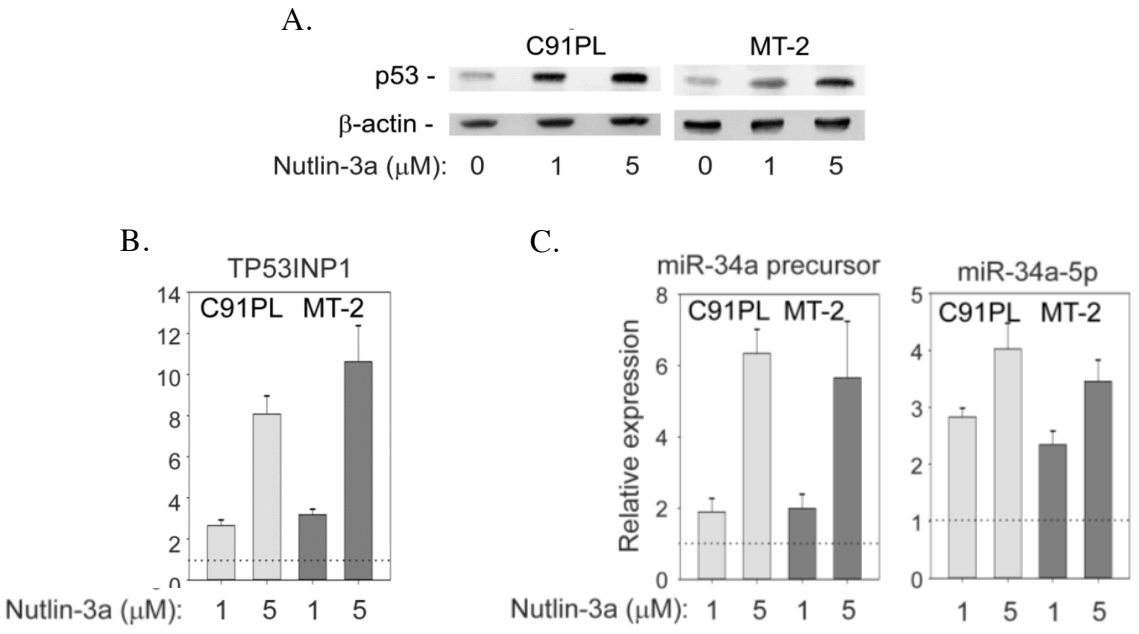


Figure 16: Nutlin-3a treatments activates p53 and increases miR-34a expression. C91PL and MT-2 cells were treated with DMSO (0), or Nutlin-3a at 1μM (1) and 5 μM (5) for 48 hours. **(A)** Immunoblot for evaluation of p53 expression; β-actin was used as loading control. **(B, C)** Quantitative RT-PCR was performed to calculate the relative expression of TP53INP1 mRNA and *miR-34a* (precursor and mature), comparing the data obtained for treated cells to DMSO-treated controls, set at 1 (see dotted line).

3.3.2 Effects of Nutlin-3a on cell turnover

Nutlin-3a-treated cells were also examined for viability and DNA content. Results from MTT viability assays indicated a reduction in cell viability of 20-40% in C91PL and 30-60% in MT-2 after 1 μM and 5 μM Nutlin-3a treatment, respectively (**Figure 17A**). Furthermore, there was also a cell cycle arrest in G1 phase in both cell lines, with a consequent decrease in the percentage of cells in S phase (**Figure 17B**). Apoptosis assays carried out by labelling with annexin V/propidium iodide (PI) revealed an induction of late apoptosis in MT-2 cells, indicated by an increase in annexin V/PI double-positive cells, and predominantly early apoptosis in C91PL cells, indicated by an increase in annexin V positive, PI-negative cells (**Figure 17C**).

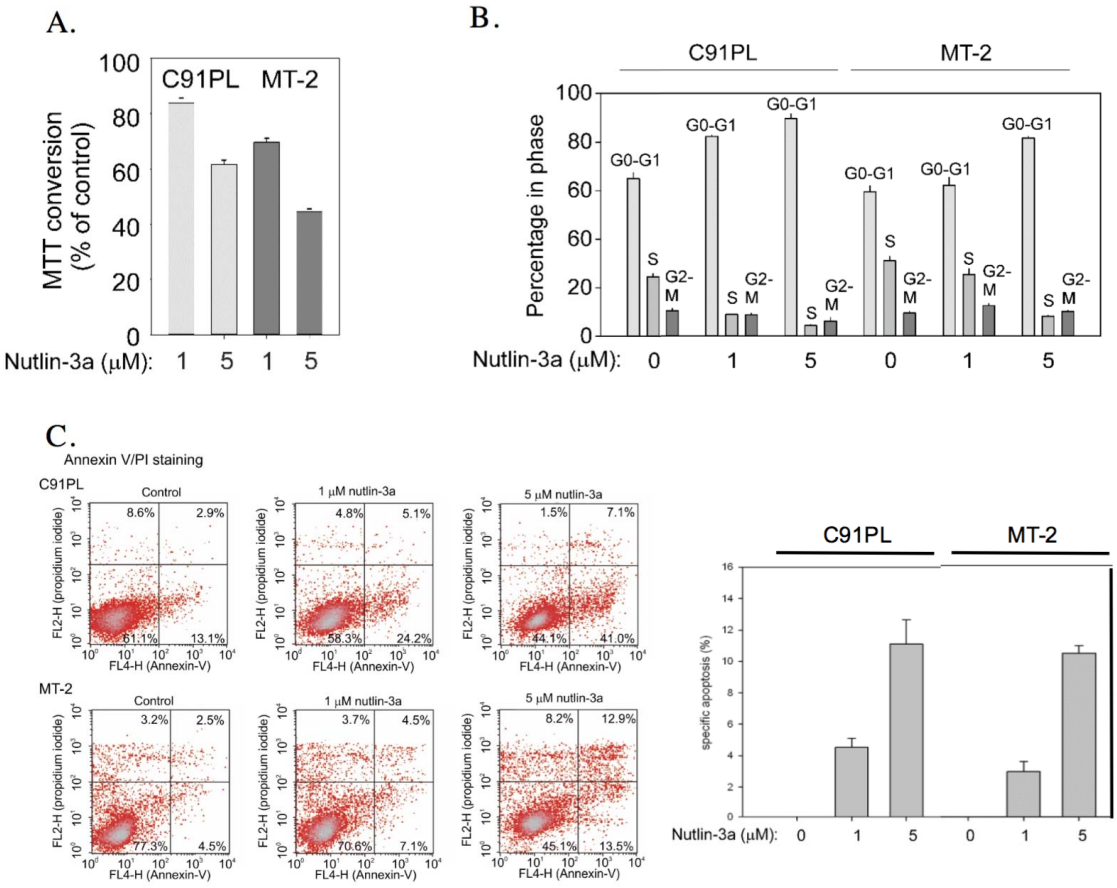


Figure 17: Effects of Nutlin-3a on cell viability, cycle cycle and death. C91PL and MT-2 cells were treated with Nutlin-3a (1 μM=1N or 5 μM=5N) or DMSO (NT) for 48 hours and analysed for viability, cell

cycle and apoptotic death. **(A)** MTT assay was performed to detect cell viability after the treatments in C91PL and MT-2 cells; the percentages of cell viability were normalized on DMSO-treated controls, set at 100%. **(B)** Cell cycle distribution was analysed by propidium iodide labelling as described in Material and methods. Bars indicate the means from 3 experiments with standard error. **(C)** Apoptotic cell death was analysed by Annexin-V/Propidium Iodide staining followed by flow cytometry. Specific cell death (SCD) was calculated using the sums of the percentages of cells stained with Annexin-V and AnnexinV/propidium iodide (in early and late apoptosis, respectively). Shown are representative scatter plots from one experiment and percentages of stained cells calculated using the specific cell death formula provided in the Materials and Methods.

3.3.3 Role of the p53-*miR-34a* axis on SIRT1 expression

The observation that Nutlin-3a-treated C91PL and MT-2 cells expressed increased levels of *miR-34a* prompted us to look at targets of *miR-34a* in these cells.

We therefore investigated the levels of the mRNA coding for the protein deacetylase sirtuin 1 (SIRT1), which contains 2 binding sites for *miR-34a* (**Figure 18A**) and was identified as a key target of *miR-34a* in other cell systems (*Yamakuchi et al., 2008*).

SIRT1 is a protein deacetylase that catalyses the deacetylation, by a NAD-dependent mechanism, of many proteins, including NF-κB and p53 (*Martinez-Redondo et al., 2013, Luo et al., 2013; Vaziri et al., 2001*), consequently modulating metabolism, inflammation, hypoxic responses, circadian rhythms, cell survival, and longevity. In cancer and in several other diseases, the activity of SIRT1 is altered. Its role in cancer promotion or suppression depends on tissue context, expression of up- and downstream factors and its temporal and spatial distribution (*Saunders et al., 2010; Yuan et al., 2013*).

Many studies provided evidence that SIRT1 has an important role in cancer (*Liu et al., 2009*); indeed, its expression is upregulated in prostate cancer, colon cancer, acute myeloid leukaemia and some skin cancers (*Bradbury et al., 2005; Huffman et al., 2007; Stunkel et al., 2007*). A study by Kozako and colleagues (*Kozako et al., 2012*) showed that ATLL cells and HTLV-1-infected cell lines also express high levels of SIRT1. It was proposed that SIRT1 promotes transformation by inhibiting p53 (*van Leeuwen and Lain, 2009*). However, in other tumour types, including ovarian cancer, glioblastoma, and bladder carcinoma, SIRT1 expression is decreased (*Deng, 2009*), suggesting a tumour suppressor activity. Thus, SIRT1 can act as either an oncogene or tumour suppressor, depending on the oncogenic pathways specific to particular tumours.

SIRT1 is regulated at the post-transcriptional level by two classes of molecules, RNA-binding proteins (RBPs) and miRNAs. More than 16 miRNAs, including *miR-34a*, modulate SIRT1 expression (*Yamakuchi et al., 2008*).

Quantitative RT-PCR assays and immunoblots confirmed that the levels of the SIRT1 mRNA and SIRT1 protein were reduced in C91PL and MT-2 cells upon treatment with Nutlin-3a (**Figures 18B** and **18C**, respectively). To further investigate the effects of Nutlin-3a on the *miR-34a*-SIRT1 regulatory loop, we measured the levels of total p53 and

p53 acetylated on lysine 382, which is a substrate for SIRT1 (*Nakamura et al., 2000*). Results showed that the levels of total p53 and acetylated p53 were increased in the drug-treated cells (**Figure 18D**). A calculation of the ratio of acetylated p53 versus total p53 confirmed that Nutlin-3a treatment enhanced acetylation on lysine 382; this modification is known to enhance p53 activity of the tumour suppressor (*Reed et al., 2014*). Therefore, by repressing SIRT1, *miR-34a* reinforces the p53 response in HTLV-1-infected cells exposed to Nutlin-3a.

Taken together, our findings indicate that treatment of HTLV-1-infected cell lines with Nutlin-3a engages the p53-miR-34a-SIRT1 feedback loop, leading to an increase in the p53 tumour suppressor response and cell death.

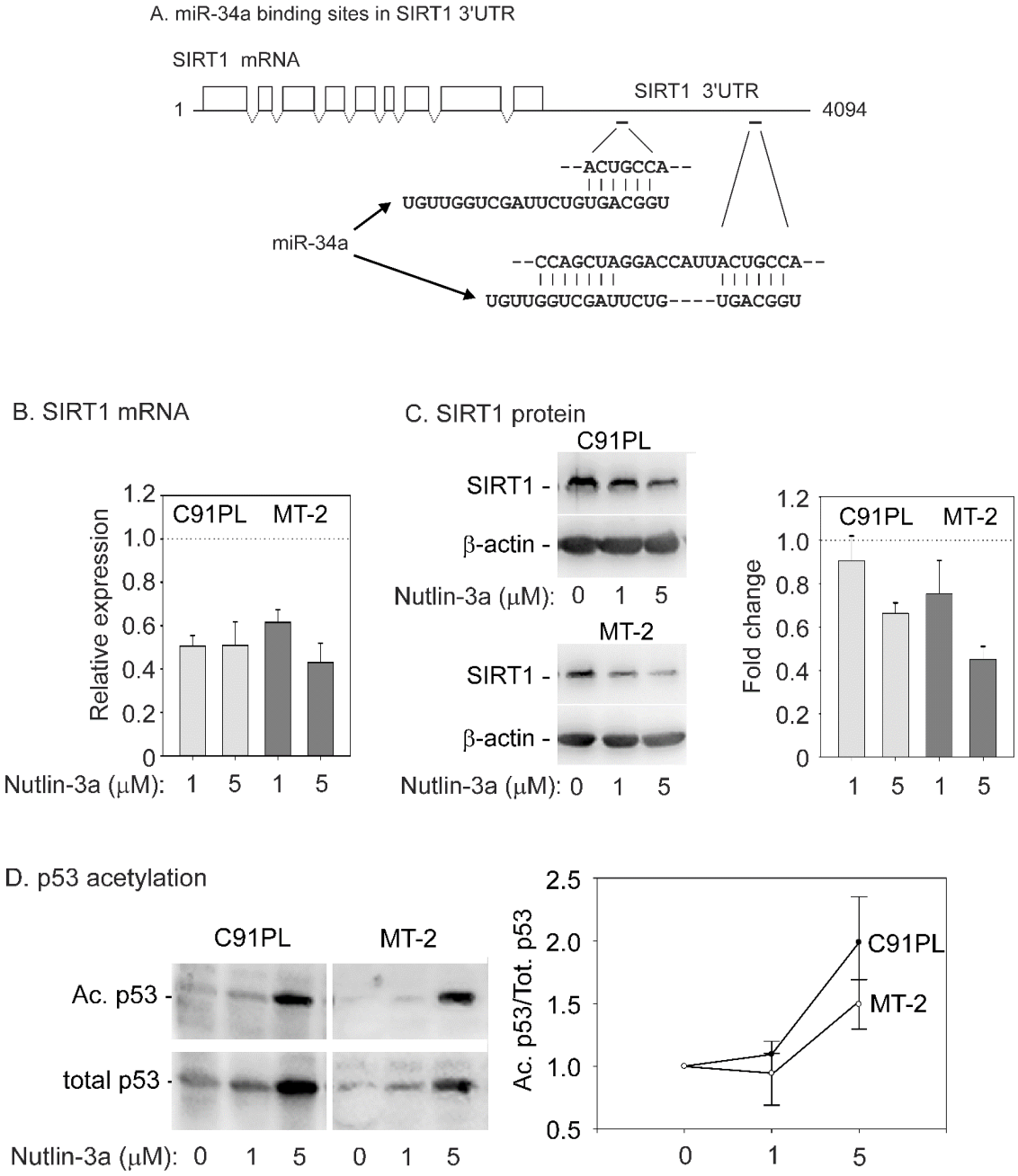


Figure 18: Reduced levels of SIRT1 in Nutlin-3a-treated cells and enhanced acetylation of p53. (A) The protein deacetylase SIRT1 contains 2 binding sites for miR-34a. **(B)** Quantitative RT-PCR results showed reduced levels of SIRT1 mRNA in C91PL and MT-2 cells treated with Nutlin-3a, compared with cells treated with DMSO, which SIRT1 relative expression was set at 1 (see dotted line). **(C)** Immunoblots to detect SIRT1 protein in the same samples showed comparable results; β-actin was used as loading control. **(D)** Immunoblots to detect total p53 and p53 acetylated on lysine 382 in Nutlin-3a-treated cells showed an increase in the acetylated p53/total p53 ratio. β-actin was used as loading control. The graphs show means and standard error bars from 3 experiments.

3.3.4 Effects of Nutlin-3a on other *miR-34a* targets: BIRC5 and E2F3

In the wake of the results obtained for SIRT1 in Nutlin-3a treated cells, we next investigated the levels of other mRNAs shown to be targets of *miR-34a* in other cell systems, in particular BIRC5 (coding for Survivin) and E2F3.

The BIRC5 (Baculoviral Inhibitor of apoptosis Repeat-Containing 5) gene codes for Survivin, a 16.5 kDa protein that belongs to the Inhibitor of Apoptosis Protein (IAP) family, and which suppresses apoptosis through the inhibition of caspase activation. Survivin is highly expressed in many types of cancer, including ATLL (*Kamihira et al., 2001*) and this is associated with a poor prognosis and decreased survival rates (*Nakayama et al., 2002*). For many years it was believed that, under physiological conditions, Survivin is expressed only in embryonic and fetal stages, but not in differentiated cells. However, recent studies reported that Survivin also has a role in normal cells including T-cells, hematopoietic progenitor cells, vascular endothelial cells, liver cells, gastrointestinal tract mucosa, erythroid cells, and polymorphonuclear cells (*Mobahat et al., 2014*). Nevertheless, high expression of Survivin in cancer cells suggests a pathological role for the protein and makes it a good potential target for cancer therapy. The molecular pathways controlling Survivin expression and function are still not well known, but its regulation is connected to p53 and *miR-34a* (*Waligòrska-Stachura et al., 2012; Cao et al., 2013*). A study by Shen et al. analysed the relationship between Survivin and *miR-34a* in larynx squamous cell carcinoma (LSCC) and reported the inhibition of proliferation and invasion following transfection of a *miR-34a* mimic (*Shen et al., 2012*). It is also known that Survivin plays important roles in apoptosis, cell proliferation, cell cycle, chromosome movement, mitosis and regulation of the response to cellular stress (*Altieri, 2013*).

E2F3 is a transcription factor of the E2F family. These proteins are involved in the control of the cell cycle, show activity in the induction of the S-phase during the transition from quiescence to proliferation and have oncogenic functions. In fact, the expression levels of E2F3 seem to be upregulated in transformed cells and its depletion inhibits the induction of S-phase in proliferating cells (*Noguchi et al., 2012; Leone et al., 1998*). The E2F3 locus encodes two proteins, E2F3a and E2F3b, that are alternative

splicing variants. These two isoforms share DNA binding, heterodimerization and pocket protein binding domains, but differ at their amino-terminal portions due to the presence of a longer and shorter first exon, respectively (*He et al., 2000; Leone et al., 2000*). The functions of E2F3a and E2F3b are not clearly understood, but data suggest that they may have partially opposing roles. Proliferating cells mainly express E2F3a with consequent transactivation of genes associated with DNA synthesis and cell-cycle progression (*Humbert et al., 2000*). Moreover, quiescent cells are stimulated to enter the cell cycle if E2F3a is overexpressed (*DeGregori et al., 1997*). On the other hand, E2F3b is constitutively expressed throughout the cell cycle, including the quiescent phase (*He et al., 2000; Leone et al., 2000*) and it represses the expression of the tumour suppressor $p19^{ARF}$ both in quiescent and normally proliferating cells (*Aslanian et al., 2004*).
The expression of high levels of E2F3 was found in prostate cancer cells, suggesting a role for E2F3 overexpression in carcinogenesis (*Foster et al., 2004*). These properties suggest an important role for E2F3 in the regulation of the cell cycle of proliferating cells and make it another hypothetical target for cancer therapy.

Results of quantitative RT-PCR shown in **Figure 19A** revealed that the BIRC5 mRNA was strongly downregulated in both C91PL and MT-2 cells treated with Nutlin-3a.
We next performed immunoblots to measure the levels of Survivin. As shown in **Figure 19B**, consistent with the results of mRNA analysis, the levels of Survivin were greatly reduced in both cell lines upon Nutlin-3a treatment.

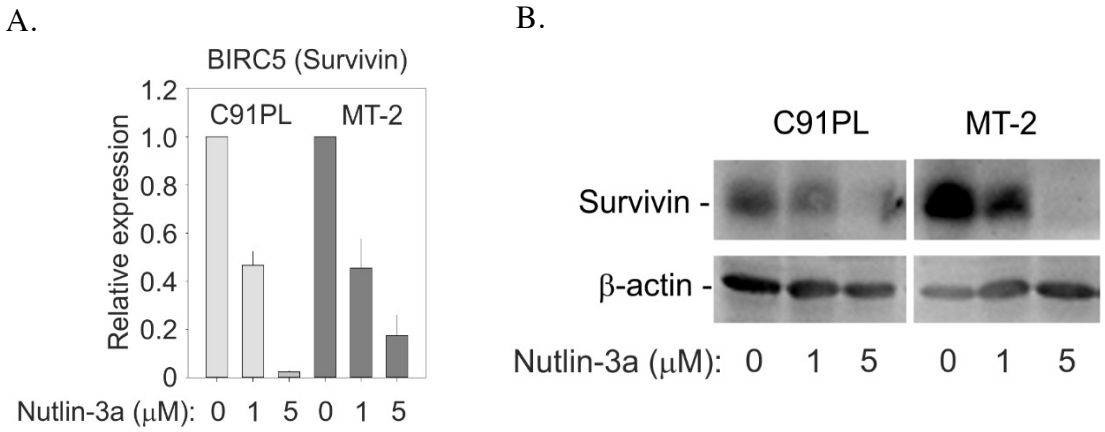


Figure 19: **(A) Analysis of BIRC5 expression**. The relative expression of BIRC5 mRNAs was calculated through quantitative RT-PCR analysis in C91PL and MT-2 cells treated with Nutlin-3a (1 μM or 5 μM) or DMSO. Results were compared with the data obtained for DMSO-treated controls, set at 1 (see dotted line). Bars indicated the means from 3 experiments with standard error bars. **(B) Immunoblot for evaluation of the expression of Survivin protein.** C91PL and MT-2 cells were treated with DMSO (0), Nutlin-3a 1μM (1) and 5 μM (5). Protein lysates were subjected to immunoblotting to detect Survivin; β-actin was used as loading control.

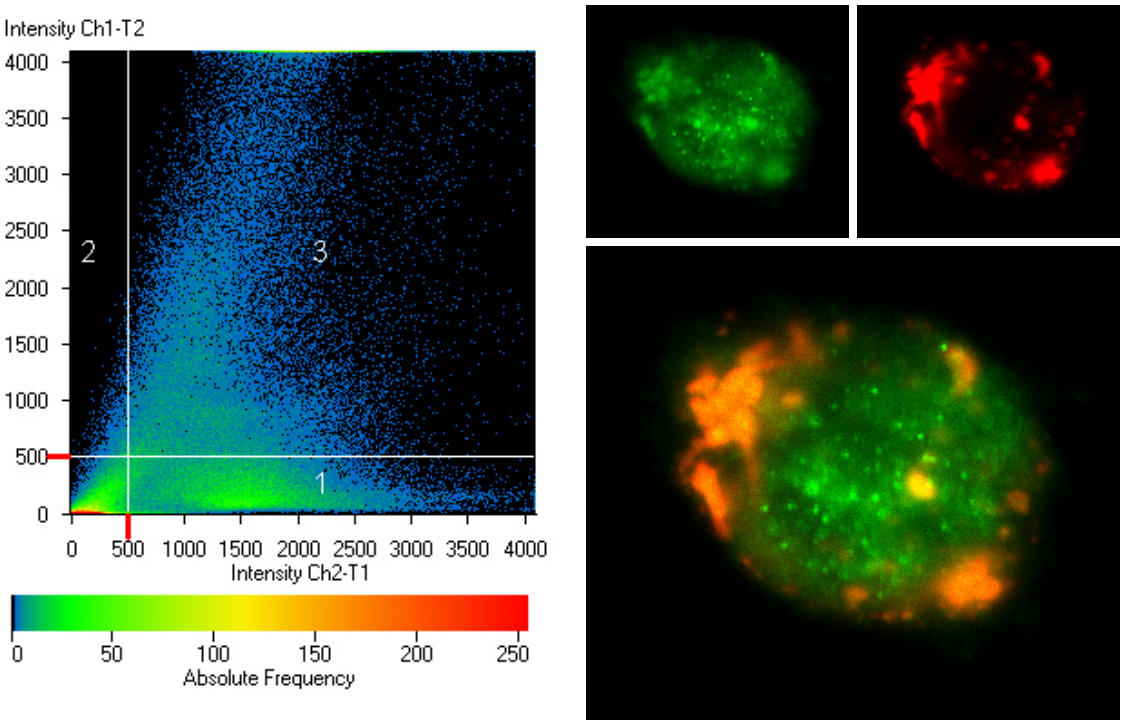
Furthermore, to test the effects of Nutlin-3a on Survivin in HTLV-1-infected cells, we set out to analyse its subcellular compartmentalization by an immunofluorescence assay.

We observed that in non-treated C91PL cells, Survivin showed a mixed staining pattern consisting of a diffuse cytoplasmic signal, small intense spots, and partial targeting to mitochondria, which were identified by staining with an antibody recognizing HSP60.

Treatment with Nutlin-3a led to an increase in the mitochondrial Survivin signal. Quantification of the co-localization index of HSP60 and Survivin revealed an increase in mitochondrial Survivin from 48% in untreated cells to 83% when cells were treated with Nutlin-3a (**Figures 20A** and **20B**).

In addition, mitochondria exhibited a more tubular morphology in the in Nutlin-3a-treated cells, suggesting a change the dynamics of the mitochondrial fission and fusion processes.

A.



B.

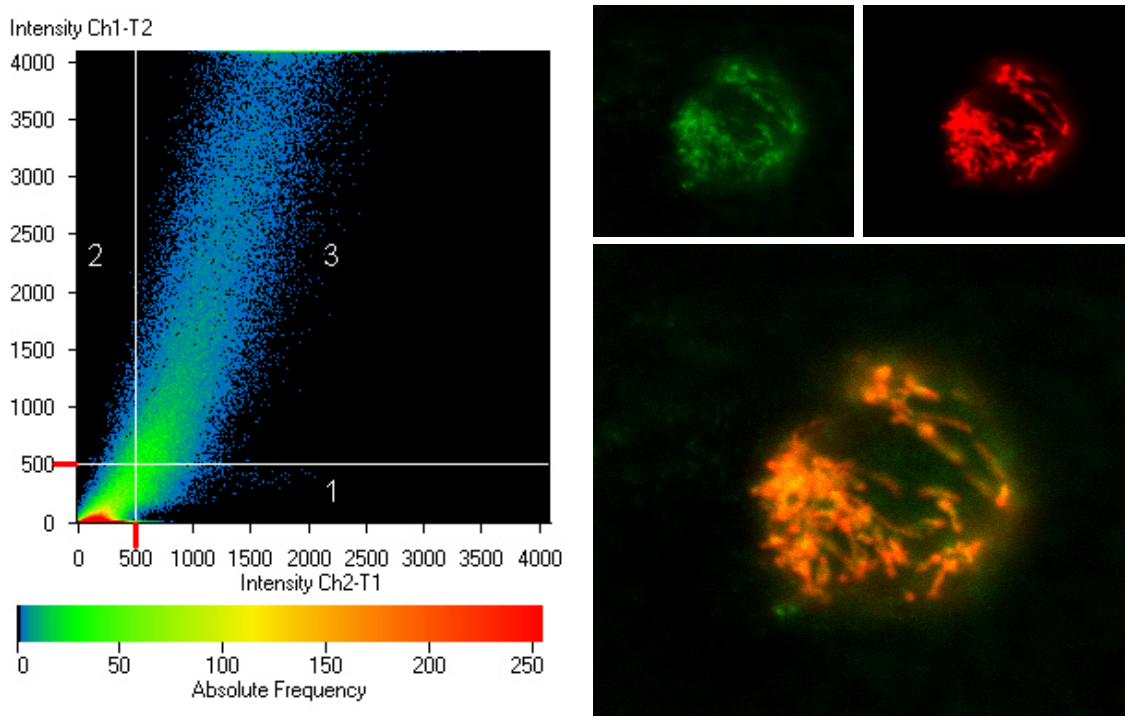


Figure 20: Survivin localization in mock (A) and Nutlin-3a (B) -treated C91PL cells. Images were detected with immunofluorescence and confocal microscopy analysis. Survivin is detected by Ch1-T2 (green) and HSP60 is detected by Ch2-T1 (red).

Based on these preliminary observational data, we plan to analyse Survivin localization by morphometric analysis to quantify changes in mitochondrial shape, and test the changes in the key regulators of mitochondrial dynamics (e.g. mitofusins, Drp1, Opa1).

We performed the same analysis for E2F3 and observed that E2F3 mRNA levels decreased in MT-2 cells, but, surprisingly, increased in C91PL cells (**Figure 21A**).
As shown in **Figure 21B**, the expression of E2F3 protein in C91PL and MT-2 cell treated with Nutlin-3a was complex: in C91PL cells we observed a switch from E2F3a to E2F3b, while MT-2 cells showed a general loss of E2F3 expression. It will be of interest to compare the expression of the mRNAs coding for the 2 isoforms by RT-PCR.

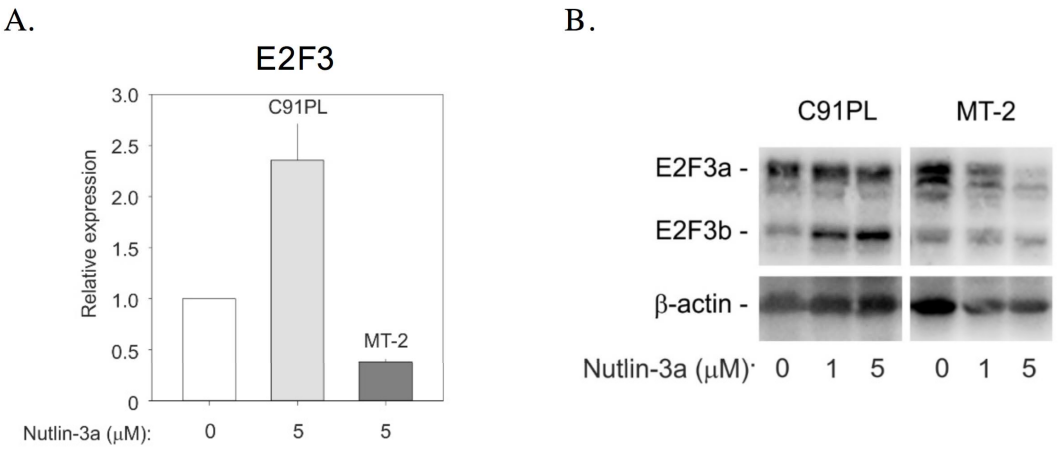


Figure 21: **(A) Analysis of E2F3 mRNA expression**. The relative expression of E2F3 mRNAs was calculated through quantitative RT-PCR analysis in C91PL and MT-2 cells treated with Nutlin-3a (1 **μ**M or 5 **μ**M) or DMSO. Results were compared with the data obtained for DMSO-treated controls, set at 1 (see dotted line). Bars indicated the means from 3 experiments with standard error bars. **(B) Immunoblot for evaluation of the expression of E2F3 protein.** C91PL and MT-2 cells were treated with DMSO (0), Nutlin-3a 1μM (1) and 5 μM (5). Protein lysates were subjected to immunoblotting to detect E2F3; β-actin was used as loading control.

Moreover, we further investigated the effects of Nutlin-3a on E2F3 function. Based on results obtained in C91PL and MT-2 cells treated with Nutlin-3a described above, we performed preliminary analysis for senescence in both cell lines. Previous studies reported that the E2F family of transcription factors controls cell cycle progression, but the two isoforms encoded by E2F3 may have partially opposing roles. Indeed, E2F3a facilitate cell cycle progression, while, on the contrary, E2F3b is classified as a repressor E2F and negatively controls the cell cycle, inducing cells in a quiescent state (*Leone et al., 2000; Hurst et al., 2008; Noguchi et al., 2012*).

Senescence in HTLV-1 infected cells was tested by detecting the expression of β-galactosidase that is active under acidic conditions, which is a marker of senescence. Senescent cells, which are stained blue in this assay, were counted in microscope fields (**Figure 22**). Results revealed a dose-dependent increase in the percentage of senescent cells in C91PL cultures treated with Nutlin-3a compared to non-treated cells (**Table 4**).

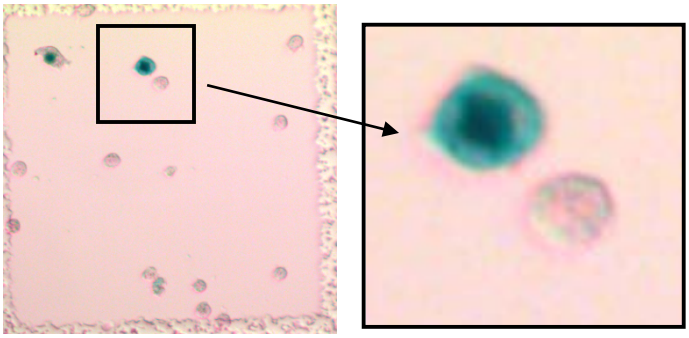


Figure 22: Senescent C91PL cells. Example of an observed microscope field, representative of the performed experiments. Senescent cells are stained blue as a result of β-galactosidase-mediated cleavage of the chromogenic substrate X-gal (5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside).

Treatment	% of senescent cells
DMSO	2.45
1 μM Nutlin-3a	8.56
5 μM Nutlin-3a	15.38

Table 4: Percentage of senescent cells in C91PL cultures in the absence and presence of Nutlin-3a.

In contrast, the MT-2 cultures did not show signs of senescence in the absence or presence of Nutlin-3a (data not shown). We plan to refine the senescence assay by performing time course analyses on cells using a specific fluorescent substrate for β-galactosidase that is suitable for flow cytometry analysis (C_{12}DFG, *Noppe et al., 2009*).

Taken together, our results suggested that the upregulation of *miR-34a* induced by Nutlin-3a suppressed Survivin expression, and caused the sequestration of this protein into the mitochondria of C91PL cells.
Furthermore, the switch from E2F3a to E2F3b and the engagement of senescence observed in Nutlin-3a-treated C91PL, but not in MT-2 cells, may justify the differences in cell death observed for two cell lines.

3.3.5 Effects of Nutlin-3a on HTLV-1 transcription

To gain more information on the impact of Nutlin-3a on HTLV-1-infected cells, we determined whether the drug affects the HTLV-1 transcript profile using C91PL cells as a model. Results of quantitative RT-PCR with splice junction-specific primer sets to

detect the expression of the HTLV-1 genes Gag, Tax/Rex, p13, p30, HBZ sp1 and HBZ US, showed that the majority of the transcripts analysed were upregulated, in a dose-dependent manner in response to Nutlin-3a (**Figure 23A**). In particular, we obtained a statistically significant increase with 5 **µ**M Nutlin-3a (*p*-value<0.002, indicated with *) for all the transcripts except for the p13 mRNA.
Results of immunoblotting showed that Tax protein levels were increased in the Nutlin-3a-treated cells (**Figure 23B**).

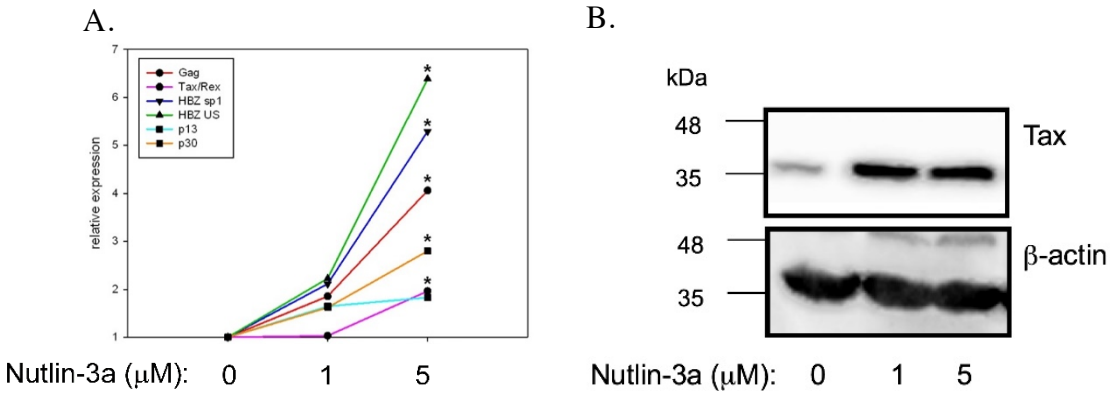


Figure 23: Expression of HTLV-1 transcripts in Nutlin-3a treated cells. C91PL cells were treated with DMSO or Nutlin-3a (1 µM or 5 µM) **(A)** Quantitative RT-PCR was performed to detect the relative expression of Tax/Rex, Gag, p13, p30, HBZ sp1 and HBZ US mRNAs. Each line represents the relative expression of a single HTLV-1 transcript in C91PL treated with Nutlin-3a; treatments with DMSO were set at 1. **(B)** Immunoblotting was performed to detect the expression of HTLV-1 Tax, which increased with the Nutlin-3a treatment, thus confirming the results obtained for mRNA expression.

We next investigated the effects of Nutlin-3a on the ability of C91PL cells to infect T-cells, using the Jurkat T-cell line as a model. For this purpose, we stably transfected Jurkat cells with a plasmid that expresses mRFP (monomeric red fluorescent protein) under the control of the viral LTR promoter, whose activity strictly depends on Tax. The resulting cell line, named JLR, can be used to detect LTR transactivation through the detection of mRFP fluorescence in flow cytometry.
To transmit HTLV-1 infection, JLR cells were placed in culture together with C91PL cells for 48 hours. Results of flow cytometric analysis showed a significant increase in the mean fluorescence intensity (MFI) in C91PL-JLR co-cultures compared to control cultures containing only JLR cells (*p*-value<0.001). These results indicated that the new infection and the activation of the 5' LTR by neo-synthesized Tax occurred (**Figure 24)** and confirmed the utility of our cell model.

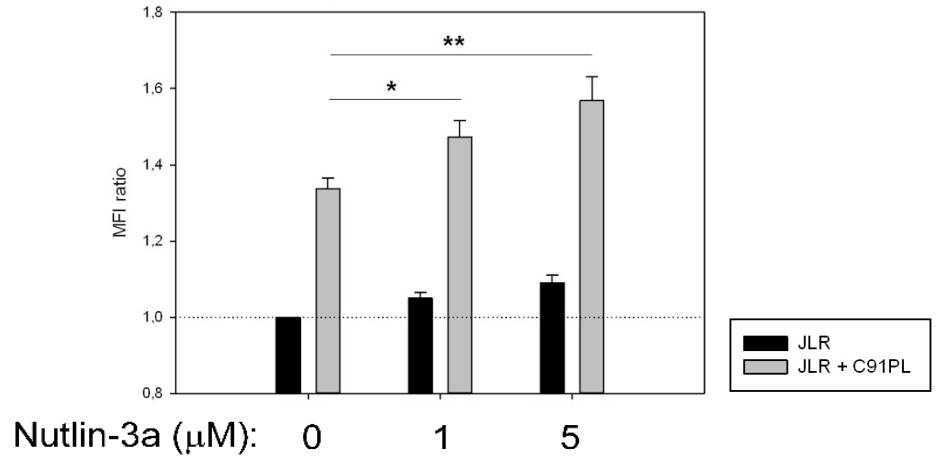


Figure 24: Transmission of HTLV-1 infection to JLR cells. JLR cells, which express mRFP under the control of the Tax-dependent LTR promoter, were co-cultivated with C91PL cells. Mean Fluorescence Intensity (MFI) of mRFP was detected by flow cytometry. Bars indicate the means from 9 experiments with standard error. * *p*-value=0.034; ** *p*-value=0.006

We then performed the C91PL-JLR infection assays in the absence and presence of Nutlin-3a. Nutlin-3a-treated C91PL-JLR co-cultures showed a modest, but significant, increase in LTR transactivation in the recipient JLR cells (measured as the MFI of mRFP fluorescence) compared to the controls treated with DMSO (**Figure 24**). Interestingly, control cultures of JLR cells alone also showed a small increase in mRFP fluorescence in the presence of Nutlin-3a, suggesting that Nutlin-3a may have an effect on basal activity of the viral LTR promoter. It is also noteworthy that the JLR cell line, like the parental Jurkat cells, are TP53-mutated. The effects of Nutlin-3a on HTLV-1 infection will be further investigated in future experiments using a p53-wildtype recipient T-cell line, e.g. MOLT3, or, preferably in primary T-cells.

3.3.6 Modulation of *miR-34a* activity with a *miR-34a*-mimic

In the context of HIV-1 infection, it was reported that *miR-34a* levels were increased in the presence of the viral regulatory protein Tat, and this overexpression promoted Tat-induced viral transactivation (*Zhang et al., 2012*). Furthermore, a study by Kapoor and colleagues demonstrated that *miR-34a* positively regulates HIV-1 replication (*Kapoor et al., 2015*). Encouraged by these observations in the context of another retrovirus, we set out to determine the impact of *miR-34a* in C91PL cells using a *miR-34a*-mimic or *miR-34a*-inhibitor (Ambion) introduced by electroporation. Results of quantitative RT-PCR showed that electroporation with the *miR-34a*-mimic produced a statistically significant increase (about 10-fold) in *miR-34a* levels compared to cells

transfected with a 'scramble' small RNA used as a control (**Figure 25**). In contrast, the *miR-34a*-inhibitor did not significantly affect the levels of *miR-34a*.

We next performed quantitative RT-PCR and immunoblots to detect the expression of SIRT1 and BIRC5, two *miR-34a* targets that are significantly downregulated in Nutlin-3a-treated C91PL cells (see above). Results confirmed a statistically significant decrease in both SIRT1 and BIRC5 mRNAs (**Figure 26A**) as well as SIRT1 protein (**Figure 26B**) and Survivin protein (data not shown) in cells treated with the *miR-34a*-mimic. These results provide evidence that the SIRT1 and BIRC5 mRNAs are direct targets of *miR-34a* in C91PL cells. Since we did not observe a statistically significant increase in SIRT1 or BIRC5 in cells treated with the *miR-34a*-inhibitor, we did not include it in subsequent analyses.

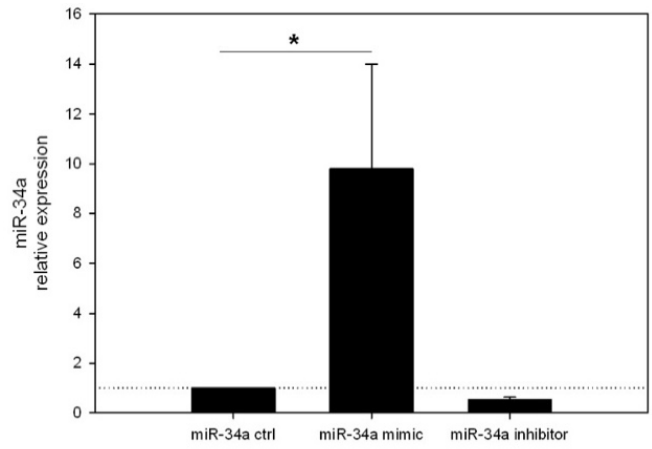
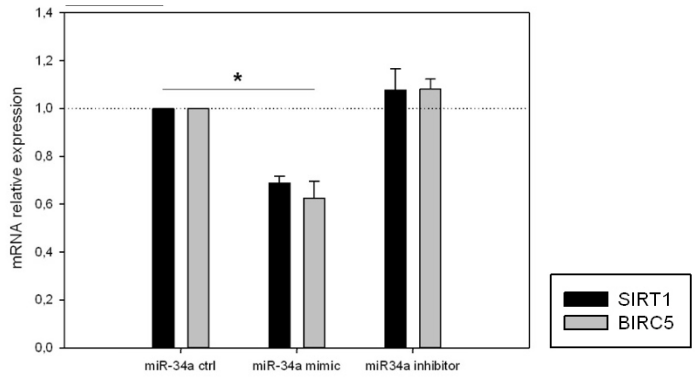


Figure 25: **Transfection of C91PL cells with a *miR-34a*-mimic or inhibitor.** C91PL cells were transfected with *miR-34a*-mimic/inhibitor or a control synthetic RNA. Quantitative RT-PCR was performed to detect *miR-34a* relative expression compared to the control, set at 1 (see dotted line). The bar indicates the mean from 6 experiments with standard error. *p*-value was calculated using the Mann-Whitney rank-sum test (***** *p*-value=0.026)

A.



B.

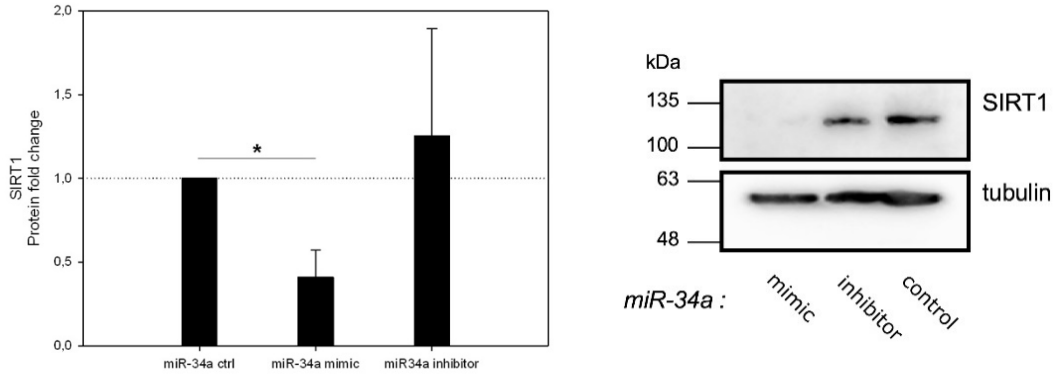


Figure 26: Analysis of *miR-34a* targets in C91PL cells transfected with a *miR-34a*-mimic or inhibitor. **(A)** The relative expression of SIRT1 and BIRC5 mRNAs was measured by quantitative RT-PCR. Results were compared with the data obtained for control-transfected cells, set at 1 (see dotted line). Bars indicate the means from 6 experiments with standard error. *p*-values were calculated using the Mann-Whitney rank-sum test (* *p*-values<0,002). **(B) SIRT1 protein expression is decreased in C91PL cells transfected with the *miR-34a*-mimic.** The image shows a representative composite immunoblot to detect SIRT1 and tubulin (used as a loading control) in C91PL cells transfected with *miR-34a*-mimic/inhibitor or control. The graph shows the mean expression of SIRT1 protein normalized to the control (set at 1, see dotted line) measured from 4 experiments with standard error. * *p*-value=0.029

We next investigated the impact of the *miR-34a*-mimic on cell death by labelling with Annexin V/PI. Results revealed that there was no change in the level of apoptotic cell death in C91PL cells transfected with the *miR-34a*-mimic, compared to the control (**Figure 27A**). The control and *miR-34a*-mimic-transfected cultures also exhibited similar cell cycle profiles (**Figure 27B**) and percentages of viable cells measured using the MTT assay (not shown).

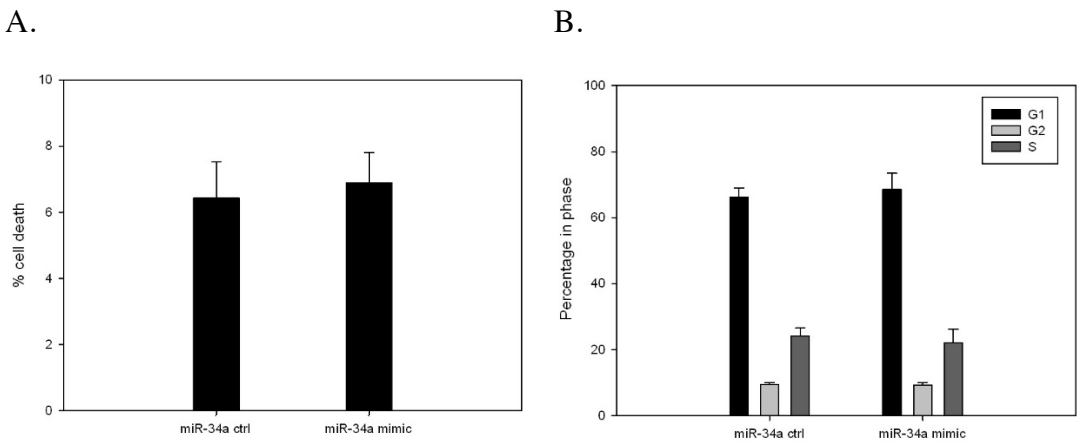


Figure 27: Effects of *miR-34a* on cell death and cycle cycle. 72 hours after transfection with a *miR-34a*-mimic or control RNA, C91PL cells were analysed for apoptotic cell death and cell cycle. **(A)** Results of annexin V/PI staining to detect apoptotic death. Specific cell death (SCD) was calculated using the sums of the percentages of cells stained with Annexin-V and AnnexinV/propidium iodide. **(B)**. Cell cycle distribution was analysed as described in Material and Methods. Bars indicate the means from 6 experiments with standard error.

Prompted by the results obtained in Nutlin-3a treated cells, we also performed quantitative RT-PCR to evaluate the expression of selected HTLV-1 mRNAs in C91PL cells transfected with the *miR-34a*-mimic.

However, in contrast with the effects observed with Nutlin-3a, we did not detect significant changes in the expression of viral transcripts (**Figure 28**).

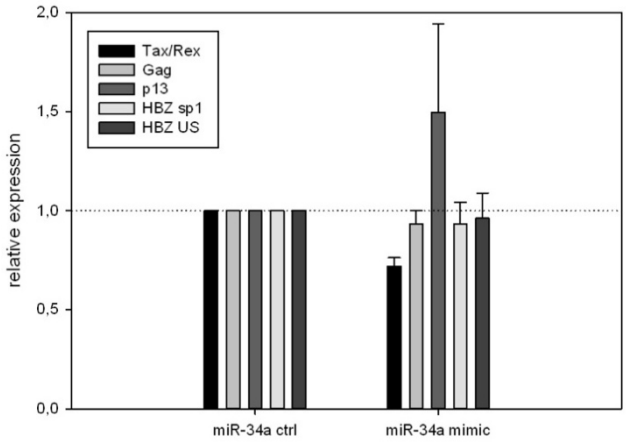


Figure 28: Expression of HTLV-1 transcripts in C91PL cells transfected with the *miR-34a*-mimic. Quantitative RT-PCR was performed to detect the relative expression of Tax/Rex, Gag, p13, p30, HBZ sp1 and HBZ US mRNAs at 72 hours post-transfection. Results were compared with the data obtained for the control, set at 1 (see dotted line). Bars indicate the means from 6 experiments with standard error.

Thus, although introduction of the *miR-34a*-mimic in C91PL cells resulted in downregulation of target mRNAs, this was not sufficient to influence cell turnover or to promote HTLV-1-gene expression, all key characteristics of Nutlin-3a-treated cells.

4. DISCUSSION

Increasing evidence suggests that in most cases miRNAs produce subtle reductions in the expression of target genes, suggesting that miRNAs function as "fine tuners" that increase the robustness of homeostatic networks by providing feedback or feedforward regulatory loops (*Ebert et al., 2012*). The present study provided insight into the expression and impact of *miR-34a* in HTLV-1 infection.

HTLV-1-infected cell lines, fresh ATLL samples and newly infected PBMC exhibit elevated levels of *miR-34a* compared to control $CD4^+$ T-cells (**Figures 9B and 13A**), a finding consistent with a previous analysis of small RNA libraries in the HTLV-1-infected cell lines C91PL and MT-2 (*Ruggero et al., 2014*).

Although in apparent contrast with the frequent downregulation of *miR-34a* in solid tumors, these observations are reminiscent of EBV-infected B-cells, where *miR-34a* is upregulated through LMP1-mediated stimulation of the NF-κB pathway and provides a survival advantage to the cells (*Forte et al., 2012*). The observed increase in death of C91PL and MT-2 cells upon functional knock down with a *miR-34a* "sponge" (**Figure 12**) indicated that upregulation of *miR-34a* likewise contributes a pro-survival advantage to HTLV-1-infected cells, rather than representing a functionally irrelevant side effect of infection.

The finding that Bay11-7082 reduced the levels of *miR-34a* in C91PL and MT-2 cells (**Figure 14A**) indicates that, similar to the mechanism described for EBV, the NF-κB pathway is also involved in driving high *miR-34a* expression in the context of HTLV-1 infection. Tax, a powerful NF-κB activator, was able to induce a modest increase in *miR-34a* levels in HeLa cells and Jurkat cells (**Figure 14B and 14C**). However, it is possible that upregulation of *miR-34a* in infected cells and ATLL cells might be sustained through additional mechanisms that influence the NF-κB pathway, e.g. through deregulation of the miR-31/NIK axis (*Yamagishi et al., 2012*), especially in light of the fact that Tax expression is often downregulated in ATLL cells (*Takeda et al., 2004*).

Results of a 5'RACE analysis indicated that the main *miR-34a* transcript produced in C91PL cells (**Figure 15**) is nearly identical to previously described 2-exon transcripts detected in HeLa cells (*Chang et al., 2007*). A study carried out in esophageal cancer showed that *miR-34a* is upregulated by NF-κB in a p53-dependent manner. It is noteworthy that an NF-κB binding site is positioned near the TSS of the pri-*miR-34a* produced in C91PL cells; this provides a further indication that *miR-34a* expression is driven by NF-κB in HTLV-1-infected cells.

Engagement of the p53 pathway in C91PL and MT-2 cells with Nutlin-3a further upregulated *miR-34a* expression (**Figure 16**), resulting in downregulation of SIRT1 (**Figures 18B and 18C**), cell cycle arrest (**Figure 17B**) and loss of cell viability (**Figures**

17A and 17C). These findings distinguish HTLV-1-infected cells from the EBV/B-cell system, in which stimulation of the p53 pathway did not substantially affect *miR-34a* levels (*Forte et al., 2012*). Our observation that the decrease in SIRT1 levels was accompanied by increased acetylation of p53 on lysine 382 (**Figure 18D**) indicates that SIRT1 may contribute to the functional inactivation of p53, a common finding in HTLV-1 infection/transformation.

SIRT1 is upregulated in HTLV-1-transformed cells, and particularly in acute ATLL *(Kozako et al., 2012*), an apparently paradoxical finding, given the high expression of *miR-34a* in ATLL cells. Furthermore, experiments carried out in chronically infected cell lines and ATLL cells showed that siRNA-mediated knockdown of SIRT1 expression or treatment with sirtuin inhibitors results in apoptotic death, suggesting that SIRT1 is important for survival of HTLV-1-transformed cells *(Tang et al., 2015; Kozako et al., 2015*); this possibility is consistent with the pro-death effect of Nutlin-3a in C91PL and MT-2 cells (**Figure 17C**).

Besides SIRT1, E2F3 and BIRC5 are also known targets *miR-34a*. Results of additional RT-PCR assays revealed downregulation of the E2F3 mRNA in Nutlin-3a-treated MT-2 cells, but upregulation in C91PL cells (**Figure 21A**).

Levels of the BIRC5 mRNA and its protein product Survivin were greatly decreased in Nutlin-3a-treated C91PL and MT-2 cells (**Figure 19**). Similar to SIRT1, Survivin is highly expressed in ATLL cells *(Pise-Masison et al., 2009; Kamihira et al., 2001; Mori et al., 2001*) and in HTLV-1-infected cells (*Mori et al., 2001*). In addition to being a target of transcriptional repression by p53 (*Hoffman et al., 2002*), the BIRC5 mRNA contains one binding site for miR-34 and was described as a target of miR-34a in melanoma cells (*Chen et al., 2010*).

Survivin suppresses apoptosis through the inhibition of caspase activation. Results of immunofluorescence assays performed in Nutlin-3a-treated C91PL cells showed that the residual Survivin protein accumulated in the mitochondria, which assumed a more tubular morphology compared to the mitochondria of untreated cells. We plan to further analyse Survivin localization in untreated and Nutlin-3a-treated cells by morphometric analysis to quantify changes in mitochondrial shape, and test the changes in the key regulators of mitochondrial dynamics (e.g. mitofusins, Drp1, Opa1).

Tax is known to contribute to inhibition of p53 (*Pise-Masison et al., 1998a, 1998b*) through a NF-κB-dependent mechanism (*Pise-Masison et a., 2000*). Interestingly, the small molecule inhibitor 9-aminoacridine simultaneously activates p53 and suppresses NF-κB in HTLV-1-transformed cells and renal carcinoma cells (*Jung et al., 2008; Gurova et al., 2005*), suggesting a mechanism of reciprocal regulation of the two transcription factors *(Webster et al., 1999*). Results of additional RT-PCR assays indicated that treatment of C91PL and MT-2 cells with Nutlin-3a did not decrease the

levels of the mRNA coding for 4-1BB, a known transcriptional target of NF-κB in HTLV-1-transformed cells (*Pichler et al., 2008*) (data not shown). This observation suggests that different inhibitors and different cell contexts lead to diverse outcomes in terms of p53 and NF-κB activity and *miR-34a* expression. It would thus be interesting to directly compare the effects of Nutlin-3a and 9-aminoacridine in HTLV-1-infected cell lines.

Taken together, our findings suggest that in the context of HTLV-1 infection/transformation, elevated *miR-34a* levels might play an important survival function by fine-tuning the expression of key factors such as SIRT1, E2F3 and Survivin. The recent finding that SIRT1 interferes with the ability of Tax to transactivate the LTR promoter (*Tang et al., 2015*) suggests that *miR-34a* might sustain viral expression by modulating SIRT1. A thorough understanding of the impact of *miR-34a* on HTLV-1-infected T-cells is worthy of further study, given the current interest in the use of *miR-34a* mimics, Nutlin-3a analogues and SIRT1 inhibitors to treat various cancers.

Studies of HIV-1 showed that the viral regulatory protein Tat stimulates expression of *miR-34a*, which in turn promotes Tat-induced viral transactivation (*Zhang et al., 2012*) and positively regulates HIV-1 replication (*Kapoor et al., 2015*). These findings prompted us to test the effects of Nutlin-3a on HTLV-1 expression. Results showed that the drug increased the levels of key viral transcripts in C91PL cells (**Figure 23A**). The effects of Nutlin-3a and other p53-activating drugs on expression and transmission of HTLV-1 therefore merits further investigation.

The observations made using the *miR-34a* sponge and Nutlin-3a suggested that *miR-34a* may exert a rheostat-like control on cell turnover depending on its expression levels. To further investigate this possible rheostat mechanism, we carried out experiments with a *miR-34a* mimic or a *miR-34a* inhibitor in C91PL cells. While the *miR-34a* inhibitor did not appear to function in our experimental system, transfection of the *miR-34a* mimic resulted in a reduction in the levels of SIRT1 and BIRC5, indicating that these mRNAs are bona fide targets of the miRNA in C91PL cells (**Figure 26**). However, unlike Nutlin-3a, the mimic did not significantly influence cell turnover (**Figure 27**) or expression of HTLV-1 transcripts (**Figure 28**), suggesting that activation of p53 may be a key component of the "pro-death" arm of the *miR-34a* rheostat.

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