

*Laboratory of General Pathology and Immunology  
Department of Surgical and Morphological Sciences  
University of Insubria, Varese, Italy*



***Human Tumor Retrovirus-Host-Interaction:  
Role of CIITA in the Functional Inhibition  
of HTLV-1 Tax-1 Oncogenic Protein***

Thesis of

**Rawan Abdallah**

***Ph.D. Program "Experimental Medicine and Oncology" XXVI Cycle***

Supervisors: Dr. Giovanna Tosi & Prof. Roberto Accolla

Coordinator: Prof. Antonio Toniolo

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*Dedicated to my beloved parents **Khodor & Samira***

*my brother **Abed el hafiz**, my sister **Racha**,*

*and my love **Radwan***

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# I. ABSTRACT

Human T-cell Lymphotropic Virus type-1 (HTLV-1) is the causative agent of an aggressive malignancy of CD4<sup>+</sup> T lymphocytes. Many evidences have shown that constitutive activation of NF- $\kappa$ B pathway by Tax-1 is crucial for T-cell transformation. Previous results demonstrated that CIITA, the master regulator of MHC class II gene transcription, inhibits HTLV-1 replication by blocking the transcription function of the viral transactivator Tax-1. Here we show that CIITA suppresses also Tax-1-mediated activation of the NF- $\kappa$ B pathway. CIITA interacts with and retains Tax-1 in the detergent insoluble cell fraction (cytoplasmic debris) and inhibits Tax-1-dependent nuclear translocation of RelA. Moreover, the overexpression of CIITA does not affect Tax-1 interaction with both RelA and IKK $\gamma$ . Nevertheless, the enzymatic activity of IKK kinase promoted by Tax-1 is impaired in the presence of CIITA. CIITA acts by suppressing at least the canonical NF- $\kappa$ B pathway, in that it also inhibits the activation of NF- $\kappa$ B by Tax-2, which is known to activate NF- $\kappa$ B through the canonical but not the non-canonical pathway. Overall, our results indicate that CIITA, beside acting as viral restriction factor against HTLV-1 infection, might counteract Tax-1 transforming activity. Thus, assessing the molecular basis of CIITA-mediated Tax-1 inhibition may be important in defining new strategies to control HTLV-1 spreading and oncogenic potential.

## II. INTRODUCTION

### II.1. HTLVs ( Human T cell Lymphotropic Viruses)

#### Discovery and classification

Human T-cell Lymphotropic Viruses type 1 (HTLV-1) and type 2 (HTLV-2) were the first discovered human retroviruses. HTLV-1 was identified in 1980 by Gallo and co-workers immediately after the discovery of the human T cell growth factor (TCGF or interleukin-2) that allowed long-term *in vitro* culture of T cells (Morgan et al., 1976) and the establishment of T cell lines from a patient with a cutaneous T cell lymphoma, who harbored type C retroviral particles (Poiesz et al., 1980). One year later, Hinuma and co-workers in Japan found that the MT-1 cell line, established from a patient with adult T-cell leukemia/lymphoma (ATLL), also harbored a retrovirus and produced antigens that reacted with sera from patients with ATLL (Hinuma et al., 1981). Subsequently, the viruses isolated by the two groups were shown to be identical, and the virus was called Human T cell Leukemia Virus type 1. In 1982, another retrovirus was isolated from the Mo-T cell line, which was derived from a patient with a variant hairy-T cell leukemia. The new virus was related to, but distinct from, HTLV-1, and was called Human T cell Leukemia Virus type 2 (Kalyanaraman et al., 1982). Recently, two new members of the HTLV family, HTLV-3 and HTLV-4, have been isolated, but for them no specific association with human diseases has been reported as yet (Calattini et al., 2005; Wolfe et al., 2005).

HTLVs belong to the *Deltaretrovirus* genus, Oncovirinae subfamily (type C) of the Retroviridae family. Several other retroviruses, including the simian T cell lymphotropic virus (STLV types-1, -2, -3 and -5) and the bovine leukemia virus (BLV), have been classified in this group, based upon genetic sequence and structural homologies (reviewed in Slattery et al., 1999). It is believed that HTLV-1 and HTLV-2 originated in humans by trans-species transmission of STLV-1 and STLV-2 viruses, respectively. Similar to HTLV-1, only STLV-1 infection associates with leukemia/lymphoma in non human primates (Hubbard 1993; Voevodin, 1996). STLV-5 strain is presently still devoid of a human counterpart (Liegeois et al., 2008).

## **Epidemiology, viral transmission and tropism**

Epidemiological studies show that HTLV-1 infects approximately 15 to 20 million people around the world and is endemic in certain geographic regions, including southern Japan, the Caribbean basin, central and western Africa, the southeastern United States, Melanesia and parts of South America (Ferreira et al., 1997).

The epidemiology of HTLV-2 is not as clearly defined as the one of HTLV-1. It has been reported that high HTLV-2 prevalence rates are found in intravenous drug users (IVDUs) and Native American populations (Lowis et al., 2002).

HTLV-1 and HTLV-2 viruses have common modes of transmission. Breast-feeding from mother to child is the primary method of transmission, but viruses can be transmitted also through blood or sexual contact.

HTLV-1 and HTLV-2 show a preferential tropism for CD4<sup>+</sup> and CD8<sup>+</sup> T cells, respectively, but they can also infect other populations, including monocytes, dendritic cells, B cells, microglial and endothelial cells (Lal et al., 1995; Casoli et al., 1995; Ijichi et al 1992; Wang et al. 2000; Hoffman et al., 1992; K. Jones et al., 2008). So far three cellular receptors of HTLV-1 have been identified: the glucose transporter GLUT-1, heparan sulphate proteoglycans (HSPG), and neuropilin-1. The precise role of HSPG and neuropilin-1 during virus entry is still unclear. According to a current model, the virus may first contact HSPG on the cell surface, and then form complexes with neuropilin-1 through the viral envelope protein (Ghez et al, 2010; Jones et al., 2005; Lambert et al., 2009). Afterwards, the gp46-GLUT-1 interaction allows the envelope protein gp21 to mediate cellular membrane fusion with the formation of the virological synapse (Manel et al., 2003; Coskun et al., 2005). The virological synapse is an organized contact area whose assembly results from the polarization of the cytoskeleton of the infected cell and the accumulation of HTLV-1 core complexes and genome at the cell junction. The virion components are then transferred to the uninfected cell as enveloped particles (Ikagura et al., 2003). In addition to the formation of the virological synapse, two other mechanisms have been proposed to be involved in the cell-to-cell transmission of HTLV-1. The first involves the storage of viral particles from HTLV-1 infected cells in extracellular biofilm-like structures, composed of collagen, agrin, and linker-proteins. When infected cells attach to uninfected cells, those structures are rapidly transferred

to the surface of the target cells, favouring infection (Pais-Correia et al., 2010). Another mechanism of virus spread involves the activity of the viral accessory protein p8. p8 enhances T-cell contact by interacting with LFA-1 and ICAM-1 and mediates formation of intracellular conduits among T-cells, through which virions may be transmitted (Van Prooyen et al., 2010). All these mechanisms are consistent with the fact that cell-free HTLV-1 particles are usually undetectable in the serum of HTLV-1 infected subjects and cell-free blood products are not infectious (Fan et al., 1992; Derse et al., 2001).

A notable exception to the cell-to-cell mode of viral transmission is represented by dendritic cells, which can be infected by cell-free HTLV-1 virions (Jones et al., 2008). HSPG and neuropilin-1 contribute to transmission of the virus to dendritic cells, but the major receptor is represented by CD209 also known as DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin) (Jain et al., 2009). HTLV-1-positive dendritic cells have been detected in the peripheral blood of infected individuals and they can efficiently transmit the virus to autologous primary CD4+ T-cells (Macatonia et al., 1992).

### **HTLVs-associated pathologies**

In 1977 Uchiyama and his collaborators described an unusual cluster of an aggressive form of adult T cell leukemia/lymphoma (ATLL) in Japan, which suggested the possible involvement of a transmissible agent in the disease (Uchiyama et al., 1977). In early eighties, after its discovery by the two groups mentioned above, the HTLV-1 virus has been defined as the etiologic agent of ATLL. But HTLV-1 is not only the causative agent of a human cancer; it also associates with a chronic progressive neurological disease called HTLV-1-associated myelopathy or tropical spastic paraparesis or HTLV-1-associated myelopathy (TSP/HAM) and with several other inflammatory disorders involving the eyes, the lungs or the skeletal muscles (Yoshida et al., 1982; Uchiyama, 1997; Mahieux and Gessain, 2003). Interestingly, the onset of the two diseases (ATLL and HAM/TSP) is mutually exclusive in HTLV-1-infected patients, who are life-long virus carriers. While the vast majority of them remain clinically asymptomatic, some (2 to 5%) develop ATLL usually many decades after the primary infection (Tajima and Kuroishi., 1985; Murphy et al., 1989). The disease associations of HTLV-2 are less clear. Similarly to HTLV-1, HTLV-2 infect primarily T lymphocytes, but in this case no clear epidemiological link to lymphoproliferative malignancies has been

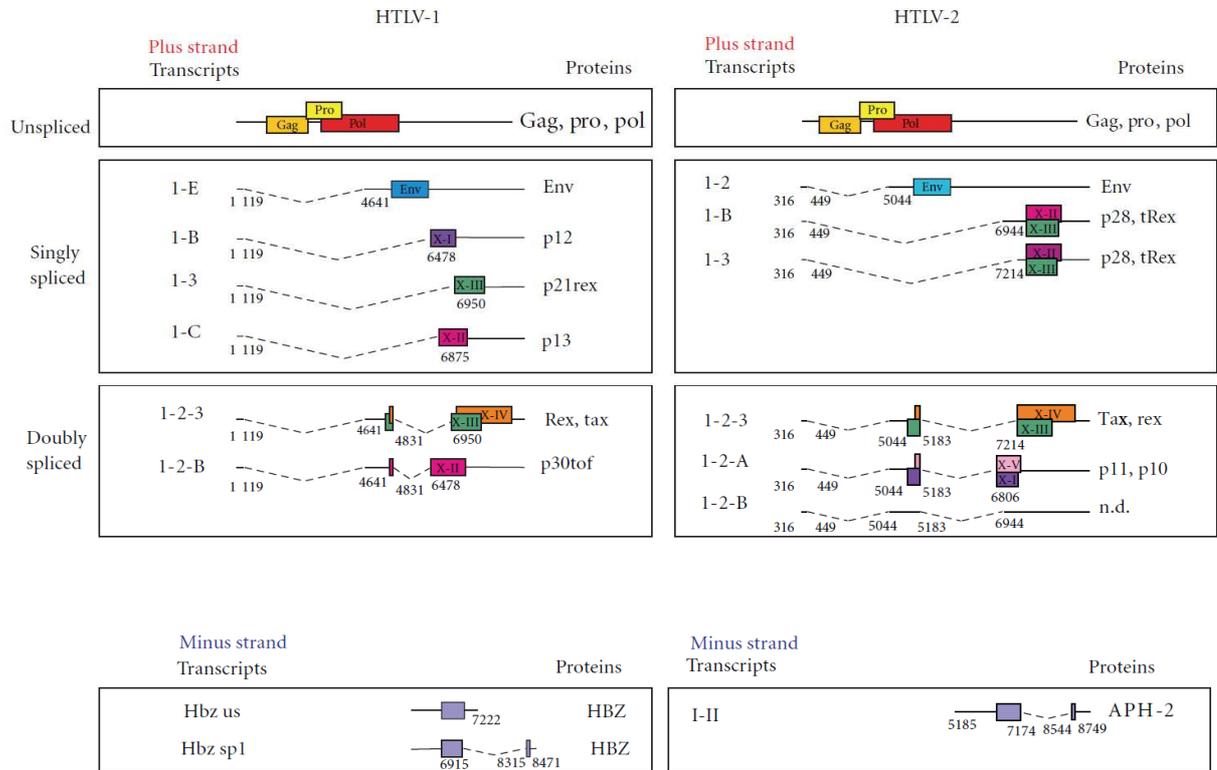
demonstrated. An association with few cases of atypical hairy T-cell leukemia and with an HAM/TSP “like” syndrome has been reported.

### **HTLV life cycle and genomic organization**

HTLV is a complex retrovirus. The virus particle contains two copies of single-stranded genomic RNA (Green et al., 1994). After the virus enters the host cell, viral RNA is used as a template to synthesize a double-stranded DNA molecule by using the viral reverse transcriptase (RT) and ribonuclease H (RNase H). The double-stranded viral cDNA is then randomly integrated by the viral enzyme integrase into the host genome establishing a latent infection (provirus). The provirus is transmitted from cell to cell during mitosis and remains quiescent until the transcription/translation of the viral genes occurs in activated host cell and the viral proteins are assembled with the genomic RNA into new virus particles which bud from the cell (productive infection).

HTLV-1 and HTLV-2 share approximately 70% nucleotide sequence homology and have a similar genomic structure (*5'LTR-gag-pol-env-pX-LTR3'*) with two long terminal repeats (LTRs) flanking the viral genes. Beside the common retroviral genes *gag-pol-env* coding, respectively, for essential proteins of the viral core, for the reverse transcriptase/integrase/protease enzymes and for the proteins of the envelope, the HTLV genome includes at its 3' the pX region, which contains several open reading frames for regulatory and accessory proteins that modulate viral expression and play an important role in viral pathogenesis. The coding potential of HTLV genome is greatly enhanced by different expression strategies that include ribosomal frame shifting and alternative splicing of mRNA. In addition, some of the alternatively spliced transcripts are polycistronic (Ciminale et al., 1992; Ciminale et al., 1995; Koralnik et al., 1992). Transcription from the 5' LTR promoter generates three major classes of mRNAs (Fig. 1): (a) full-length genomic mRNA (9 kb); (b) 4 kb singly spliced mRNAs, coding for the envelope glycoproteins (Env); (c) mRNAs of approximately 2 kb encoding proteins of the pX region. The pX regions of HTLV-1 and HTLV-2 encode, respectively, four and five, partially overlapping, major open reading frames (ORFs), termed x-I through x-V. The x-III and x-IV ORFs code for the regulatory proteins Rex and Tax, that are produced from a dicistronic doubly spliced mRNA. Rex allows the nuclear export of viral mRNA (Inoue et al., 1986; Hidaka et al., 1988), whereas Tax drives

transcription of the viral genome (Felber et al., 1985). In addition, Tax has an essential role in T cell transformation by deregulating the expression of a variety of cellular genes and signaling pathways (e.g. NF- $\kappa$ B pathway) involved in cell cycle progression, cell growth, DNA repair, and apoptosis (Yoshida, 2005). Other 2 kb transcripts encode the accessory proteins of the pX region. These transcripts include singly spliced mRNAs coding for p21<sup>rex</sup>, p12, and p13 (HTLV-1), tRex and p28 (HTLV-2) and doubly-spliced mRNAs, coding for the accessory proteins p30<sup>tof</sup> (HTLV-1), p10, and p11 (HTLV-2) (Ciminale et al., 1992; Ciminale et al., 1995; Koralnik et al., 1992). Most of the regulatory and accessory proteins of the two viruses share structural and functional homologies, while p13 and p8 (a cleaved form of p12), appear to be unique for HTLV-1 and p11 for HTLV-2. Recent studies showed that HTLV-1 and HTLV-2 also produce complementary-strand mRNAs, transcribed from the 3' LTR yielding spliced isoforms of the HBZ (HTLV-1 bZIP factor) and APH2 (anti-sense protein of HTLV-2) factors, respectively (Fig. 1). These proteins have a negative effect on Tax-dependent viral transcription (Gaudray et al., 2002; Clerc et al., 2008; Halin et al., 2009).



**Figure 1. Organization, alternative splicing, and coding potential of HTLV-1 and HTLV-2 mRNAs.** ORFs are indicated by colored boxes. Splice sites are indicated by numbers. n.d.: not determined (From Rende et al., 2012).

## **II.2 HTLV Regulatory Protein Tax**

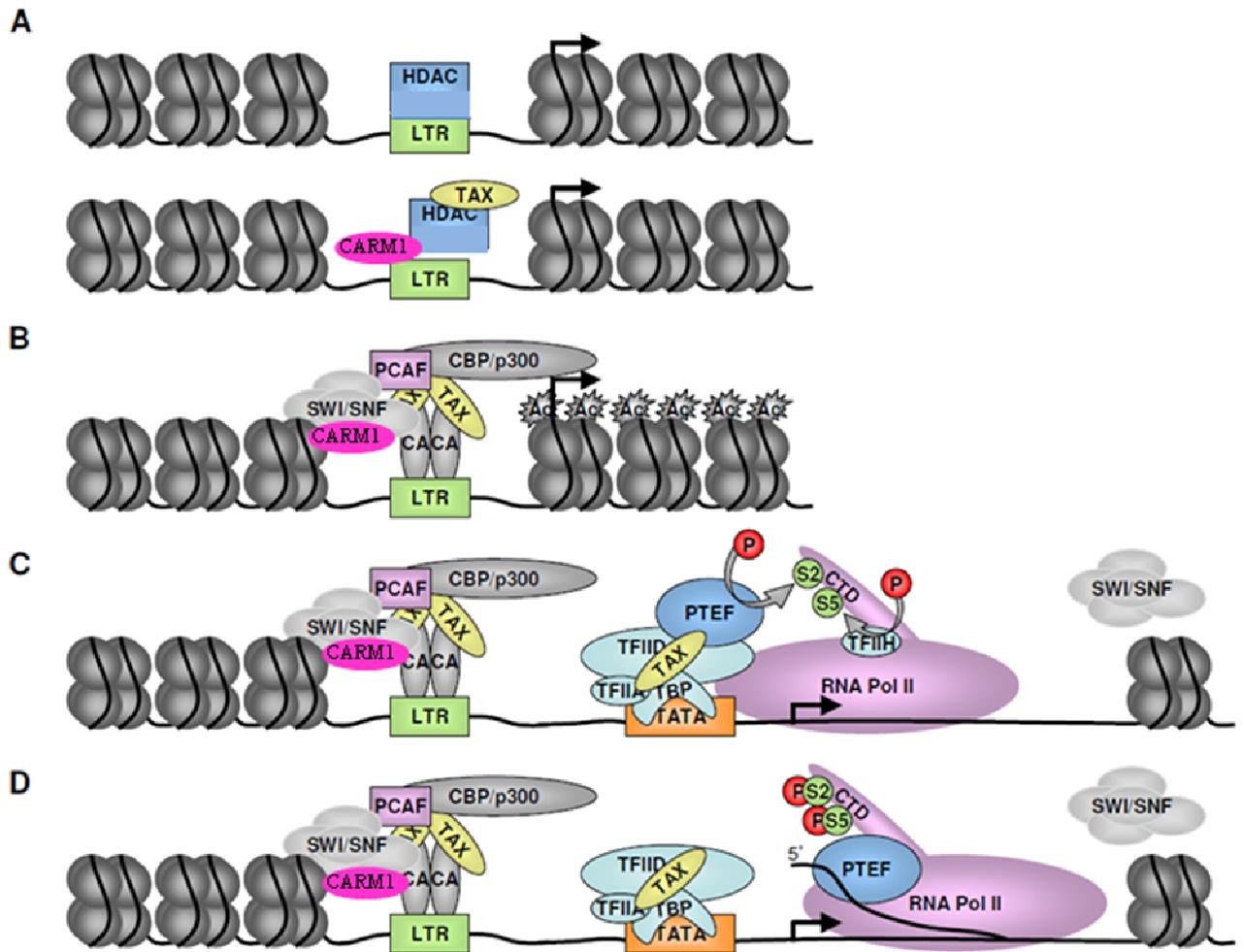
### **Tax promotes transcription of HTLV genome**

As mentioned above, Tax is the transcriptional activator of HTLV. It is expressed early during infection and drives transcription from the viral LTR promoter whose U3 region contains three conserved 21 base pair repeats, designated TxRE (Tax Responsive Element), including an imperfect CRE (cAMP-responsive element) sequence (5'-TGACGTCA-3'). Nevertheless, Tax is not a classic DNA-binding protein, but is recruited to the promoter via the interaction with the b-Zip domain of CREB/ATF (CRE-binding/activating transcription factors) or other transcriptional proteins such as AP-1, AP-2 and Sp-1 (Giam et al., 1989; Goren et al., 1995). Tax-1 enhances the dimerization of CREB/ATF factors, increases their affinity for the viral CRE and further stabilizes the ternary complex TxRE-CREB/ATF-Tax-1 through direct contact of the GC-rich flanking sequences (Kimzey et al. 1998; Lundblad et al. 1998). Tax-1 then interacts with and releases HDAC1 from the promoter (Lu et al., 2004) allowing the recruitment of transcriptional co-activators p300, CBP (CREB-binding protein) and P/CAF (p300/CBP associated factor) (Kwok et al., 1996; Kashanchi et al., 1998; Harrod et al., 1998; Jiang et al., 1999; Harrod et al., 2000; Georges et al., 2003). These cellular co-activators have an intrinsic acetyltransferase activity and by acetylating the histones in specific sites of the proviral genome allow the recruitment to the LTR promoter of the general transcription factors and the RNA Pol II, that initiate the transcriptional process. Moreover, Tax-1 associates with coactivator-associated arginine methyltransferase (CARM1), which preferentially induces arginine methylation of histone H3 and increases Tax-1-mediated transactivation of the LTR (Jeong et al., 2006). It has been demonstrated that Tax-1 binds BRG1 (Brahma Related Gene 1), a component of SWI/SNF (Switch/Sucrose Non Fermentable) complex, which uses the energy produced by the hydrolysis of ATP, to remodel the chromatin and increases the transcription (Wu et al., 2004). By cooperating with SWI/SNF and RNA Pol II, Tax-1 promotes nucleosome eviction during transactivation and, thus, the accessibility of DNA to transcription factors (Lemasson al., 2006). In addition, Tax-1 recruits the positive transcription elongation factor b (P-TEFb), composed by the Cyclin T1 and CDK9 kinase subunits, to the viral LTR promoter (Zhou et al., 2006). Phosphorylation of the RNA Pol II carboxyl-terminal domain by CDK9 is required to enable the switch from

initiation of transcription to elongation (Peterlin and Price, 2006). Thus, Tax-1 seems to play a role also downstream the initiation of transcription by preventing RNA Pol II stalling and permitting the elongation of transcription. (Fig. 2). The mechanism of action of HTLV-2 Tax-2 is assumed to be similar to that of Tax-1. However, several reports have shown that the two transactivators have distinct biological properties. In particular, we previously described a differential usage of co-activators with HAT activity between Tax-1 and Tax-2. CBP and p300, but not PCAF, cooperate with Tax-2 to promote the transcription from the viral LTR promoter (Tosi et al., 2006), whereas all three molecules are essential for optimal transactivation by Tax-1. This finding substantiates the existence of important differences between HTLV-2 Tax-2 and HTLV-1 Tax-1.

### **Structural and functional domains of Tax**

The different subtypes of HTLV-1 encode Tax-1 proteins of equal lengths (353 amino acids). In contrast, HTLV-2 has four distinct genetic subtypes, A, B, C and D, defined by phylogenetic analysis of their nucleotide sequences, the size and amino acid sequence of their Tax proteins. The Tax-2 proteins of HTLV-2 vary in length, with Tax 2B and -2C having similar lengths to Tax 1, 356 and 353 amino acids, respectively, although the C-terminal sequences of these proteins are divergent. Tax 2A lacks a 25 amino acid C terminal sequence, having a stop codon which truncates the protein at amino acid 331, and activates the LTR promoter less efficiently than Tax 2B (Sheehy et al., 2006; Pardi et al., 1993; Lewis et al., 2000.). Tax--2D is a Tax protein of 344 amino acids that as yet remains uncharacterized. The protein structure of Tax-1 and Tax-2B is presented in Figure 3. Tax-1 and Tax-2 share roughly 77% amino acid sequence homology and have conserved structural regions, even though the information for Tax-1 is by far more complete. Mutational analysis revealed several important functional domains in the Tax protein, including those mediating its transactivation capacity, its interaction with cellular factors or with DNA, its dimerization, or its subcellular localization (Smith et al., 1992; Adya et al., 1995; Yin et al., 1995; Goren et al., 1995; Harrod et al., 1998; Giebler et al., 1997; Kimzey et al., 1999; Tie et al., 1996; Semmes et al., 1995). Most portions of Tax-1 protein are indispensable for its activation function. Two activation domains have been mapped at the amino (aa 1-55) and carboxy termini (aa 289-322) (Semmes et al., 1995; Fujii et al., 1991; Boxus et al., 2008).

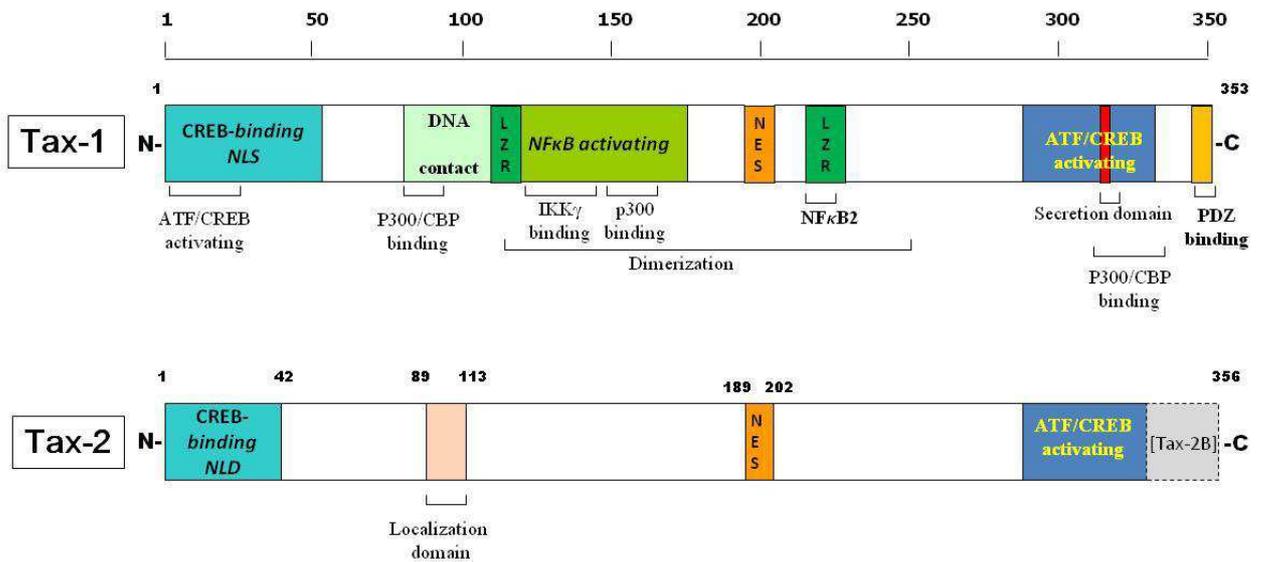


**Figure 2. Global model of Tax1 mediated transactivation.** Tax1 relieves transcriptional repression of the LTR through direct interaction with HDAC (i.e. HDAC1) (panel A). Tax1 recruits CREB/ATF transcription factors (CA in panel B), histone modifying enzymes and chromatin remodelers (SWI/SNF, P/CAF and CBP/p300, CARM1). Tax1 then allows binding of basal transcription factors on the TATA box (panel C). Once the initiation complex is formed, Tax1 recruits the P-TEFb factor, leading to CTD phosphorylation and processive elongation (panel D). Finally, interaction of Tax1 with SWI/SNF prevents stalling of transcription elongation. (Modified from Boxus et al., 2008).

Tax-1 functions optimally as a homodimer, and amino acid residues 123 to 204 form the Tax dimerization domain (Semmes et al., 1992; Jin et al., 1997). The interaction with cellular transcriptional factors/co-activators is critical for Tax function. It has been shown that amino acid residues 1 to 35 and amino acid residues 81 to 95 of Tax-1 are required for its interaction with ATF/CREB and CBP/p300, respectively (Adya et al., 1995; Yin et al., 1995; Goren et al., 1995; Harrod et al., 1998; Giebler et al., 1997). Tax-1 directly interacts with P/CAF through the amino acid residues 318 to 320 (Jiang et al., 1999) and may contact DNA through the amino acid residues 89 to 110 (Kimzey et al., 1999). The central part of Tax-1 contains two leucine zipper-like (LZR) motifs, required for DNA interaction and protein dimerization. Importantly, this region is also involved in NF- $\kappa$ B activation. Of note, Tax-1, but not Tax-2 contains a region (aa 225-232) responsible for the activation of p100 processing and p52 nuclear translocation. Thus, Tax-2 is unable to activate the non-canonical NF- $\kappa$ B pathway via the formation of a functional NF- $\kappa$ B2 complex (RelB/p52) (Bertazzoni et al., 2011 and references therein). The C-terminal region of both Tax-1 and Tax-2 contains an ATF/CREB activating domain, whereas the class I PDZ binding motif (PBM) is found only in Tax-1. This motif is critically involved in Tax-1-mediated transformation of rat fibroblasts and in promoting virus-mediated T-cell proliferation *in vitro* and persistence *in vivo* (Hirata et al., 2004; Xie et al., 2006). HTLV-2 Tax-2 protein which does not harbor a PBM has a lower transforming activity than Tax-1 (Endo et al., 2002).

### **Localization of Tax in the cell**

Because of its function as transcriptional activator, Tax has been thought to be a nucleoprotein. Indeed, Tax-1 predominantly localizes into the nucleus, but, depending on the cell type, it has been found in the cytoplasm as well (Alefantis et al., 2003). It has been suggested that Tax-1 localization in the cytoplasm is related to its capacity to activate the NF- $\kappa$ B pathway (Nicot et al., 1998). In the nucleus, Tax-1 is primarily located in inter-chromatin granules or spliceosomal speckles (Semmes and Jeang, 1996). In the cytoplasm, Tax-1 localizes to organelles associated with the cellular secretory process including the endoplasmic reticulum and Golgi complex (Nejmeddine et al., 2005; Alefantis et al., 2005).



**Figure 3. Structural and functional domains of Tax-1 and Tax-2B** The interaction domains with crucial cellular factors and the regions involved in transcriptional activation pathways are shown. NLS (nuclear localization sequence), NES (nuclear export sequence), NLD (nuclear localization determinant), LZR (leucine-zipper-like region). (From Bertazzoni et al., 2011).

The N-terminal region of Tax-1 spanning amino acids 1-48 contains the nuclear localization signal (NLS), which is distinguished from the short highly basic NLS of most nuclear proteins. Fusion to Tax-1 NLS re-targets a cytoplasmic protein to the nucleus (Smith et al., 1992). Various studies reported that Tax-1 shuttles between the nucleus and the cytoplasm, suggesting that Tax-1 also has a nuclear export signal (NES) (Burton et al., 2000). Recently, Tax-1 NES has been mapped to the region spanning amino acids 188 to 200 (Alefantis et al., 2003). It has been demonstrated that Tax-1 is also secreted in the supernatant of HTLV-1 infected cells isolated from HAM-TSP patients (Alefantis et al., 2005) and may behave as an extracellular cytokine. Tax-1 shuttling and secretion is mediated through interaction with cellular proteins involved in nuclear import (e.g. p62 nucleoporin), cytoplasmic export (e.g. CRM1 and calreticulin), ER to Golgi transport (e.g. the coat proteins  $\beta$ COP and COPII), Golgi to plasma membrane movement (e.g. SNAP23) and secretory pathways (e.g. SCAMP1 and SCAMP2) (Boxus et al., 2008, and references therein). Tax1 secretion involves a secretory signal located in the C terminal domain and requires interaction with SNAP23,

SCAMP1 and COPII (Jain et al., 2007). In contrast to Tax-1, Tax-2 is predominantly found in the cytoplasm and a 10-amino acid domain (positions 90 to 100) is responsible for the divergent subcellular distribution as compared to Tax-1 (Meertens et al., 2004). A functional but dispensable nuclear export signal located between amino acids 189-202 of Tax-2 has been identified by Chevalier and collaborators (Chevalier et al., 2005). Although Tax-2 protein accumulates prevalently in the cytoplasm, it contains a determinant for nuclear localization which is responsible for the presence of the transactivator in the nucleus (Orlandi et al., 2011). The sequence that contributes to nuclear import is present within the first 41 N-terminal residues of the Tax-2 protein (Turci et al., 2006; Bertazzoni et al., 2011).

### **Post-translational modifications of Tax**

Several post-translational modifications such as phosphorylation, ubiquitination, sumoylation and acetylation play a major role in Tax biology controlling its sub-cellular distribution, interaction with cellular proteins and activation of NF- $\kappa$ B.

The oncogenic potential of the HTLV-1 Tax protein involves activation of the NF- $\kappa$ B pathway, which depends on Tax phosphorylation, ubiquitination and sumoylation.

### **Tax-1 Phosphorylation**

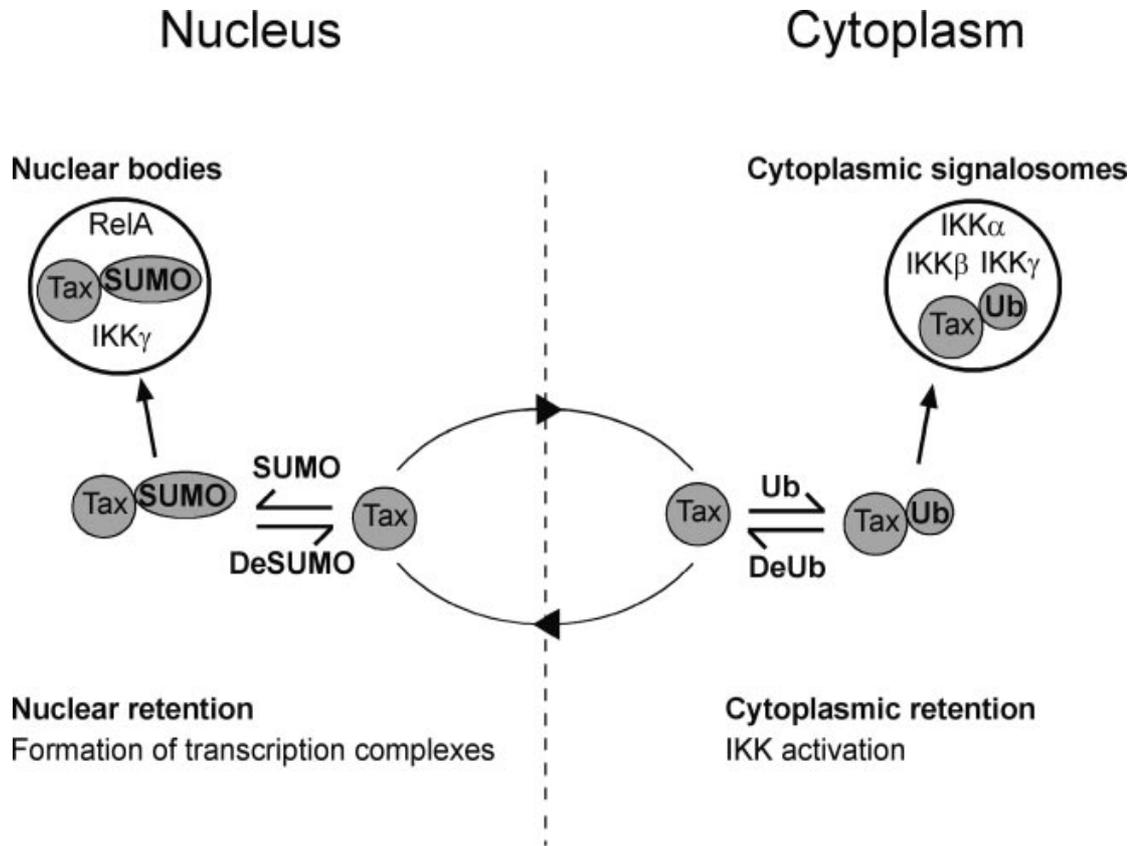
It has been shown that Tax is a phosphoprotein and that phosphorylation patterns differ depending on the cell types (Boehm et al., 1999). Tax-1 phosphorylation may occur on serine and threonine residues (Thr-48, Thr-184, Thr-215, Ser-300, Ser-301 and Ser-336) (Bex et al., 1999; Durkin et al., 2006), but serines 300 and 301 are the major targets. Phosphorylation of at least one of these two residues is required for Tax-1 nuclear localization and for Tax-mediated activation of gene expression (Bex et al., 1999). Moreover, phosphorylation at these residues is a prerequisite for Tax-1 ubiquitination, sumoylation and acetylation (Lodevick et al., 2009). Phosphomimicking mutation at Thr-215 resulted in Tax-1 inhibition and mutations at Thr-48 impaired activation of NF- $\kappa$ B pathway, whereas mutations at Thr-184 and Ser-336 were found to be silent. Recently, Jeong and collaborators provided indirect evidence that Tax-1 phosphorylation at Ser-160 stabilizes Tax-1 (Jeong et al., 2009).

### **Tax-1 Ubiquitination and Sumoylation**

Ubiquitination and sumoylation have been shown to play a critical role in the cellular localization, function and protein-protein interactions of Tax-1 (Avesani et al., 2010; Chiari et al., 2004; Harhaj et al., 2007; Peloponese et al., 2004). Five of ten lysine residues contained in Tax-1 sequence were found to be the major targets for these modifications. Lys189 (K4), Lys197 (K5), Lys263 (K6), Lys280 (K7) and Lys284 (K8), all contained within C-terminal region of Tax-1, strongly contribute to the ubiquitination of the protein, while sumoylation takes place on Lys280 (K7) and Lys284 (K8) (Lamsoul et al., 2005; Nasr et al., 2006). The role of ubiquitination and sumoylation in Tax-1 transactivating activity is clearly different. Ubiquitinated Tax-1 activates the NF- $\kappa$ B pathway through the binding with key factors such as IKK $\gamma$  and TAK-1 binding protein 2 (TAB2), with the subsequent RelA nuclear translocation (Kfoury et al., 2008; Yu et al., 2008). Whether the poly-ubiquitination targets Tax to the proteasome or enhances its activity is still not clear, although Yan and co-workers (Yan et al., 2009) demonstrated that poly-ubiquitination of K63 stabilizes Tax-1 and this form is predominant in the cytoplasm. They also found that poly-ubiquitination of K48 is predominant in the nucleus and constitutively targets Tax-1 for proteasomal degradation. On the other hand, Tax-1 sumoylation is required to recruit RelA and IKK $\gamma$  in Tax-1-related nuclear bodies, where Tax-driven transcription is promoted (Bex et al., 1997; Nasr et al., 2006; Chiari et al., 2004; Kfoury et al., 2011). These data suggest that Tax-1 ubiquitination is a key event for controlling Tax-1 cytoplasmic/nuclear functions, whereas Tax-1 sumoylation is essential for its nuclear activity (Fig. 4).

### **Tax-1 Acetylation**

It has been demonstrated that the nuclei of Tax-expressing cells, including HTLV-1 transformed T-lymphocytes, contain a pool of Tax molecules acetylated on lysine residue at position 346. Ser-300/301 phosphorylation is a prerequisite for Tax-1 localization in the nucleus and correlates with its subsequent acetylation. Sumoylation is also necessary for the formation of Tax nuclear bodies where acetylation of the Tax-1 is performed by the transcriptional coactivator p300. Overexpression of p300 markedly increased Tax acetylation and the ability of a wild type HTLV-1 provirus to activate gene expression from an integrated NF- $\kappa$ B-controlled promoter (Lodewick et al., 2009). Thus, Tax acetylation favors NF- $\kappa$ B activation and might play an important role in HTLV-1-induced cell transformation.



**Figure 4: Schematic diagram depicting a model for concerted ubiquitin and SUMO conjugation of the HTLV-1 Tax protein in Tax-mediated activation of the NF- $\kappa$ B pathway.** In the cytoplasm, ubiquitin-conjugated Tax activates the IKK signalosomes, resulting in the translocation of the RelA subunit of NF- $\kappa$ B to the nucleus. Deubiquitinated Tax migrates to the nucleus, in which it is sumoylated. Sumoylated Tax assembles nuclear bodies that include the RelA subunit of NF- $\kappa$ B and free IKK $\gamma$  along with other transcription and splicing factors, leading to the activation of specific Tax-responsive target genes. Tax can further be desumoylated, which reveals its nuclear export signal, leading to the exit of Tax from the nucleus (From Lamsoul et al., 2005)

### **II.3. HTLV-1-Mediated Transformation**

HTLV-1 is a transforming retrovirus. The oncogenic properties of HTLV-1 do not involve either the expression of a cell-derived viral oncogene (v-onc), or the activation of a cellular proto-oncogene (c-onc) through proviral integration. Instead, the oncogenicity of HTLV-1 is attributed mainly to the regulatory protein Tax. Tax-1 is essential for HTLV-1-mediated immortalization of primary human T cells ( Robek and Ratner, 1999), for the transformation of rat fibroblasts (Pozzatti et al., 1990, Tanaka et al. 1990) and for tumors induction in transgenic mice (Nerenberg et al.,1987, Hasegawa et al., 2006). The precise mechanism by which Tax initiates the malignant process is unclear, but it seems to involve the de-regulation of several steps both at transcriptional and post-transcriptional level. Tax activates transcription of many cellular genes, including interleukin-2 (IL-2) and IL-2Ra (Greene et al., 1986a; Greene et al., 1986b; Ballard et al., 1988; Marriott et al., 1992; Curtiss et al., 1996) and affects critical signal transduction pathways regulating cell cycle, cell growth, DNA repair and apoptosis (Yoshida, 2005, Kashanchi and brady, 2005, Hall et al., 2005). Many evidences indicate that the transcriptional activation of cellular genes is mediated by Tax-1-dependent activation of transcriptional factors, such as CREB/ATF, NF- $\kappa$ B and SRF (Serum Responsive Factor).

#### **Effects of Tax-1 on CREB pathway**

CREB/ATF members play a role in cell growth and apoptosis by regulating CRE-directed gene transcription. Physiologically, the CREB-CBP/p300 interaction is controlled by CREB phosphorylation which occurs in response to different environmental signals such as growth factors or stress. Through its ability to bind both CREB and CBP/p300, Tax-1 triggers CREB activation even in the absence of phosphorylation. In this way, CREB-mediated gene transcription becomes independent from cellular signals. By interacting with CREB/ATF proteins, Tax-1 can activate a variety of cellular genes, including the interleukin 17 (IL-17) and c-fos (Dodon et al., 2004; Alexandre and Verrier, 1991) and repress the expression of other genes such as cyclin A, p53 and c-myb (Kibler and Jeang, 2001; Mulloy et al., 1998; Nicot et al., 2000).

### **Effects of Tax-1 on the SRF/AP-1 pathway**

In HTLV-1-infected T cells expressing Tax-1, the expression of the oncogenic transcription factor AP-1 (activator protein-1) is increased (Fujii et al., 1991; Fujii et al., 2000). AP-1 is expressed as an homo- or heterodimer composed of Fos and Jun proteins. The serum responsive factor (SRF) binds to the Fos/Jun promoters via two SRF responsive elements (SRE) and activates transcription in response to various stimuli such as cytokines, growth factors, stress signals and oncogenes by recruiting the co-activators P/CAF and CBP/p300. It has been demonstrated that Tax-1 activates the transcription of promoters under the control of SRE motifs through the interaction with transcription factors associated with the SRF pathway (Alexandre and Verrier, 1991; Fujii et al., 1991; Alexandre et al., 1991). In particular, Tax-1 associates with SRF, stabilizes the binding of SRF to SRE and facilitates the recruitment of P/CAF and CBP/p300 (Shuh and Derse, 2000; Dittmer et al., 1997) increasing transcription of Fos and Jun.

### **Effects of Tax-1 on the NF- $\kappa$ B pathway**

The oncogenic properties of Tax-1 have been attributed, mostly, to its ability to activate NF- $\kappa$ B pathway (Saggioro et al., 2009). The mammalian NF- $\kappa$ B proteins consist of five structurally related members: p65/RelA, RelB, c-Rel, NF- $\kappa$ B1 (p50/and its precursor p105) and NF- $\kappa$ B2 (p52/and its precursor p100) which form homo-heterodimers that regulate the expression of target genes bearing NF- $\kappa$ B-responsive elements in their promoters (Sun and Yamaoka, 2005). In unstimulated cells, NF- $\kappa$ B dimers are sequestered in the cytoplasm by physical interaction with inhibitory proteins called I $\kappa$ Bs (comprising I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , I $\kappa$ B $\gamma$ , I $\kappa$ B $\zeta$ , and Bcl-3) that mask the nuclear localization signal of NF- $\kappa$ B. Upon cell stimulation, I $\kappa$ B proteins are rapidly phosphorylated and degraded by the proteasome, and NF- $\kappa$ B translocates into the nucleus to regulate the expression of target genes coding for cytokines, chemokines, adhesion molecules, inhibitors of apoptosis, and other proteins. The NF- $\kappa$ B precursor proteins, p105 and p100, contain I $\kappa$ B-like sequences in their C-terminal portion and also function as NF- $\kappa$ B inhibitors (Rice et al., 1992; Mercurio et al., 1993). Thus, the processing of these precursor proteins serves to both generate mature NF- $\kappa$ B subunits and disrupt their I $\kappa$ B-like function. Two main signalling pathways lead to NF- $\kappa$ B activation: the canonical (or classical) and the non-canonical (or alternative) pathways. The canonical NF- $\kappa$ B pathway is induced by a variety of innate and adaptive immunity mediators, such as pro-inflammatory

cytokines (TNF- $\alpha$ , IL-1 $\beta$ ), and engagement of Toll-like receptors (TLRs) and antigen receptors (TCR, BCR) (Pahl, 1999; Bonizzi et al., 2004). The crucial step in the canonical NF- $\kappa$ B pathway is the activation of the I $\kappa$ B-kinase (IKK) complex, which consists of the two kinases, IKK $\alpha$  and IKK $\beta$  (Zandi et al., 1997), and of one regulatory subunit IKK $\gamma$ , also known as NF- $\kappa$ B essential modulator (NEMO) (Yamaoka et al., 1998). Activated IKK phosphorylates I $\kappa$ B $\alpha$  on serines 32 and 36, triggering its ubiquitination and proteasomal degradation. This allows the translocation of p50/RelA dimer into the nucleus where it activates the transcription of target genes (Beinke and Ley., 2004) (Fig.5 A).

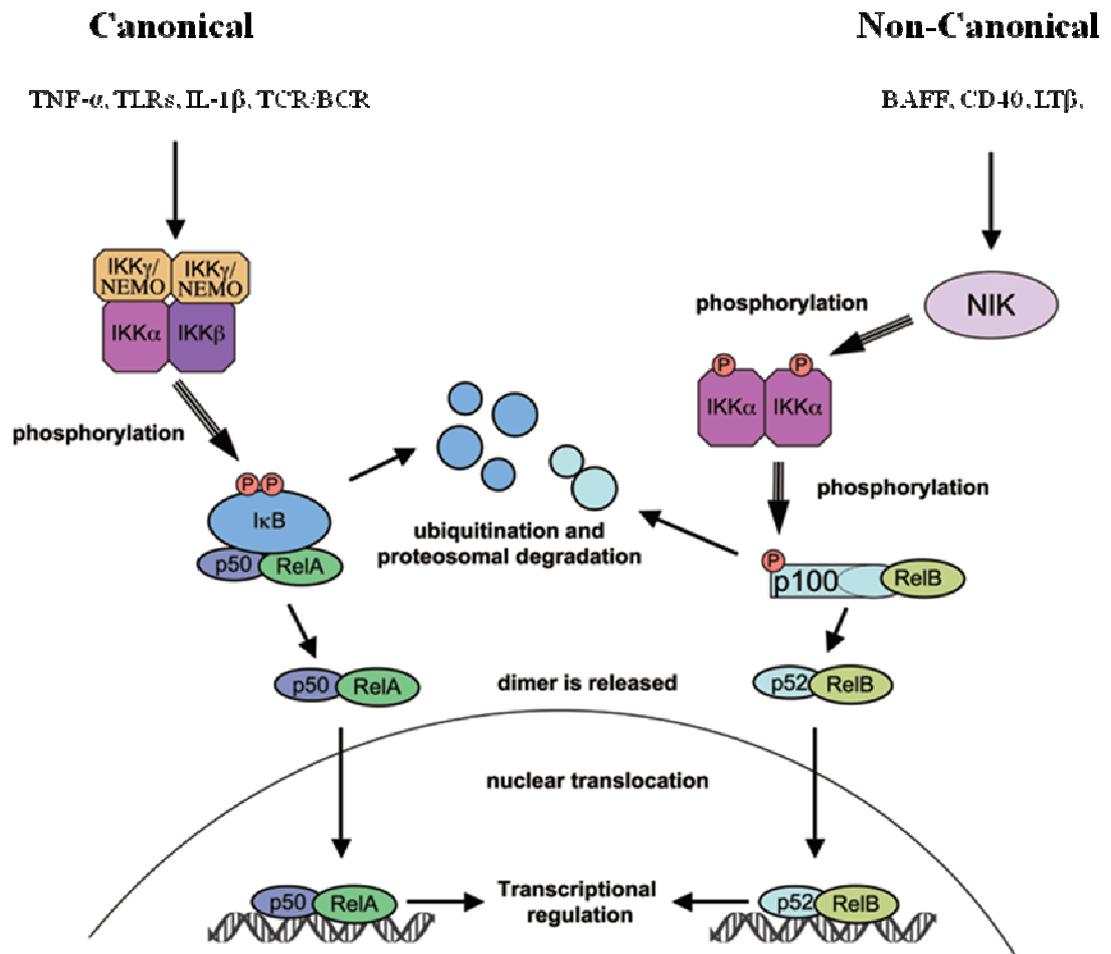
Tax-1 acts at multiple levels to activate the canonical NF- $\kappa$ B pathway. (1) In the cytoplasm, Tax-1 directly binds to NEMO and recruits the IKK complex to the perinuclear compartment, where it is phosphorylated and activated (Harhaj and Sun, 1999; Jin et al., 1999; Xiao et al., 2000). (2) Tax-1 also activates kinases upstream to the IKK complex, including MAPK/ERK kinase kinase 1 (MEKK1) and TGF- $\beta$  activating kinase 1 (TAK1) (Yin et al., 1998; Wu and Sun, 2007), enhancing IKK $\alpha$  and IKK $\beta$  phosphorylation and, thus, I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  degradation (Harhaj and Sun, 1999; Jin et al., 1999). (3) In addition, Tax-1 binds to IKK $\alpha$  and IKK $\beta$  and activates their kinase activity independently of the upstream kinases (Chu et al., 1998). (4) The binding of Tax-1 to I $\kappa$ Bs also enhances their degradation independently of IKK phosphorylation (Hirai et al., 1994; Suzuki et al., 1995). Tax-1 thus promotes I $\kappa$ Bs degradation at multiple levels, allowing nuclear translocation of NF- $\kappa$ B independently of external stimuli. (5) Tax-1 facilitates p105 degradation by interacting with HsN3 and HC9 subunits of 20S proteasome (6). In the nucleus, Tax-1 recruits RelA, CBP/p300 and PCAF (Bex et al., 1998; Bex and Gaynor, 1998) into discrete transcriptional hot spots termed Tax nuclear bodies, leading to NF- $\kappa$ B transcriptional activation (Semmes and Jeang, 1996; Bex et al., 1997). The non-canonical NF- $\kappa$ B pathway is important for secondary lymphoid organ development and homeostasis. Under physiological conditions, the non canonical NF- $\kappa$ B activation occurs primarily in B cells and lymphoid stromal cells but not in T cells. It is induced by B-cell activating factor (BAFF) (Claudio et al., 2002), lymphotoxin  $\beta$  (LT $\beta$ ) (Dejardin et al., 2002), and CD40 ligand (Coope et al., 2002; Xiao et al., 2001a). The crucial step in this pathway is the processing of p100 into p52 by IKK $\alpha$ , thus allowing p52 DNA binding in association with its partner RelB . The phosphorylation and activation of IKK $\alpha$  is mediated by the upstream NF- $\kappa$ B inducing kinase (NIK) (Xiao et al., 2001b; Xiao et al., 2004). Interestingly, NIK can also induce long-term activation of the IKK complex and I $\kappa$ B $\alpha$

degradation, thus activating also the canonical NF- $\kappa$ B pathway (Zarnegar et al., 2008) (Fig. 5B).

The induction of the non-canonical pathway is a hallmark of NF- $\kappa$ B activation by HTLV-1 infection, because this arm of the pathway usually is not active in normal T cells (Xiao et al., 2001a). Tax-1 induces the non-canonical NF- $\kappa$ B pathway by promoting the processing of p100 to p52 (Xiao et al., 2006). Tax-1-mediated processing of p100 requires NEMO as an adaptor protein in the assembly of the Tax/IKK complex, and is independent of the NIK kinase. In contrast to the canonical Tax/NEMO/IKK complex, which contains both the IKK $\alpha$  and  $\beta$ , the non-canonical complex contains only IKK $\alpha$  (Xiao et al., 2001b), which phosphorylates p100 leading to its processing to p52 (Qu et al., 2004).

Additional indirect mechanisms of constitutive NF- $\kappa$ B activation in HTLV-I infected cells has been proposed to rely on Tax-1-induced expression of various signaling molecules, such as the TNFR family molecules CD40, OX40, and CD30. It has been demonstrated that Tax induces an abnormal expression of CD40, further supporting the potential involvement of TNFRs in a positive feedback loop of HTLV-I-mediated NF- $\kappa$ B activation (Sun and Yamaoka, 2005 and references therein).

Almost all steps of the NF- $\kappa$ B pathway can be terminated through feedback inhibition mechanisms. Among the physiological NF- $\kappa$ B termination mechanisms, the most rapid and essential is mediated by PDZ-LIM domain-containing protein 2 (PDLIM2). These mechanisms are extremely important, since persistent activation of NF- $\kappa$ B can result in cellular damage, death or, in the opposite, to cellular transformation and cancer. PDLIM2 is an ubiquitously expressed cytoplasmic-nuclear shuttling protein that terminates NF- $\kappa$ B activation by shuttling RelA to the nuclear matrix, where PDLIM2 enhances RelA proteasome-mediated degradation through its E3 ubiquitin ligase activity (Fu et al., 2010; Tanaka et al., 2007).



**Figure 5. NF-κB activation: the canonical and non-canonical pathways.** A: The stimuli that induce the canonical NF-κB pathway converge on the activation of the IKK complex, which consists of the two kinases, IKKα and IKKβ, and one regulatory subunit IKKγ/NEMO. The IKK complex phosphorylates IκBα, triggering its proteasomal degradation. This allows the translocation of NF-κB1 (p50/RelA) in the nucleus, where it activates the transcription of target genes. B: The crucial step of the non-canonical NF-κB pathway is the processing of p100 into p52 mediated by IKKα, thus allowing NF-κB2 (p52/RelB) DNA binding. The phosphorylation and activation of IKKα is mediated by the upstream NIK kinase, which also induces IκBα degradation, thus activating the canonical NF-κB pathway (Modified from Morgan and Liu, 2011).

Tax-1 directly shuts off this feedback inhibition mechanism by binding to PDLIM2, resulting in reduced RelA degradation, although this process results in the proteasomal degradation of Tax itself (Yan et al., 2009). Tax-mediated activation of the NF-κB pathway results in

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

## **II.4. Host defenses against human retroviruses**

Adaptive and innate immune responses represent the most powerful tool used by the host to counteract infectious agents. Additional intrinsic defense systems against viral infections have been recently identified (Bieniasz et al., 2004). In addition to the conventional immune response many mammals, including humans, have evolved a complex of cellular proteins, named host restriction factors, that function as inhibitors of retroviral replication. These cellular factors are constitutively expressed or enhanced by interferons (Neil et al., 2009). Most of these anti-viral proteins were uncovered through the discovery of viral factors that counteract their function, implying that viruses are resistant to the restriction factors of their natural hosts. Although these findings suggested a cross-species restriction, further studies demonstrated that restriction factors may limit pathogenicity in vivo even in their specific host (Strebel et al., 2009; Goff, 2004; Rowland-Jones et al., 2001; Ross 2009).

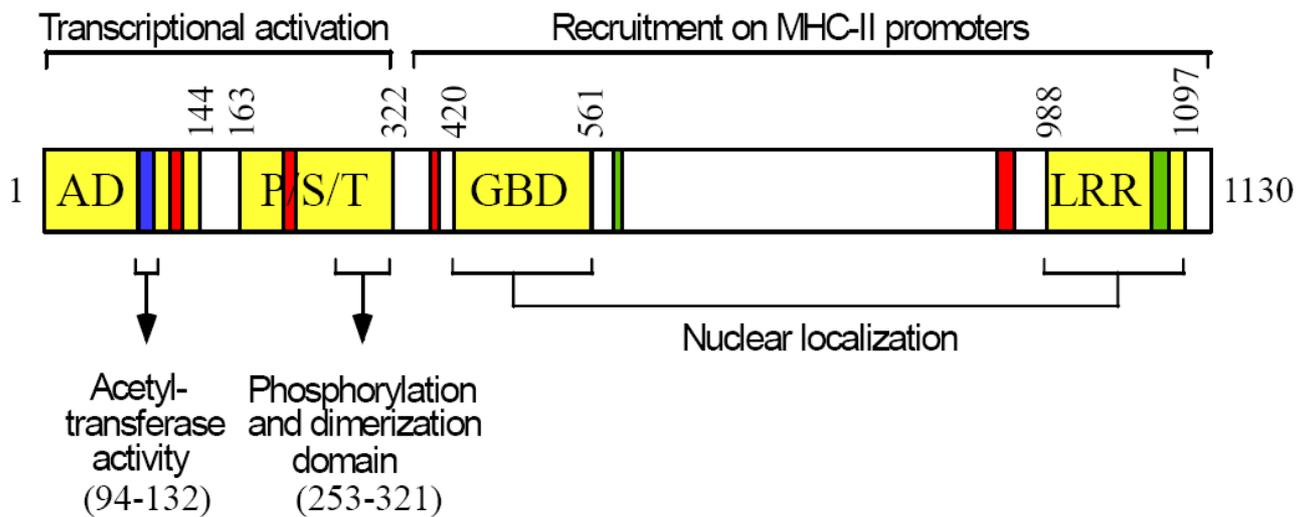
Recently, we discovered a new restriction factor against human retroviruses, the host cell transcription factor CIITA.

### **MHC Class II Transactivator (CIITA)**

The gene locus (AIR-1) and the function of class II transactivator (CIITA), were initially identified in our laboratory by a somatic cell genetic approach (Accolla, 1983; Accolla et al., 1985; Accolla et al., 1986). Several years later the CIITA cDNA was cloned by a

complementation strategy (Steimle et al., 1993) by using a HLA-II negative B cell line, RJ.2.2.5, obtained in our laboratory by mutagenesis and immunological-selection against HLA-II DR expression (Accolla et al., 1983).

CIITA is a complex molecule of 1130 aminoacids, composed of four essential functional domains (Fig. 6). Its N-terminal acid region forms the transcriptional activation domain that binds components of general transcriptional machinery and other co-factors to direct the initiation and elongation of MHC-II genes transcription (Kretsovali et al., 1998; Fontes et al., 1999; Kanazawa et al., 2000). The N- terminus of CIITA also contains an acetyltransferase activity (Raval et al., 2001). The proline-, serine- and threonine-rich region (P/S/T) is essential for the activity of CIITA (Chin et al., 1997) and contains the phosphorylation/dimerization domain (Tosi et al., 2002). The GTP-binding domain (GBD) and the C-terminal leucine-rich motif (LRR) are critical for the sub-cellular localization of CIITA (Harton et al., 1999; Hake et al., 2000). The function of CIITA can be modulated by post-translational modifications. For example, phosphorylation of certain residues of CIITA can increase its dimerization, its interaction with other key factors and its ability to trans-activate MHC class II promoters (Tosi et al., 2002; Sisk et al., 2003). In addition, ubiquitination of CIITA increases its ability to transactivate MHC class II genes (Greer et al., 2003). CIITA expression is tightly regulated at transcriptional level by four distinct promoters, named pI, pII, pIII and pIV. The function of pII is unknown. Promoters pI, pIII and pIV precede alternative first exons that are spliced to the shared downstream exons, and this gives rise to three types of CIITA mRNA, which differ at their 5' ends. pI promoter is used mainly by cells of myeloid origin, such as dendritic cells (DC) and by IFN- $\gamma$ -activated macrophages; pIII is a lymphoid-cell-specific promoter essential for CIITA expression in B cells and activated T cells; pIV drives the expression of CIITA in thymic epithelial cells and mediates induction by IFN- $\gamma$  in cells of non haematopoietic origin, such as fibroblasts, astrocytes, endothelial and epithelial cells (Muhlethaler-Mottet et al., 1997; Muhlethaler-Mottet et al., 1998; Reith et al., 2005).

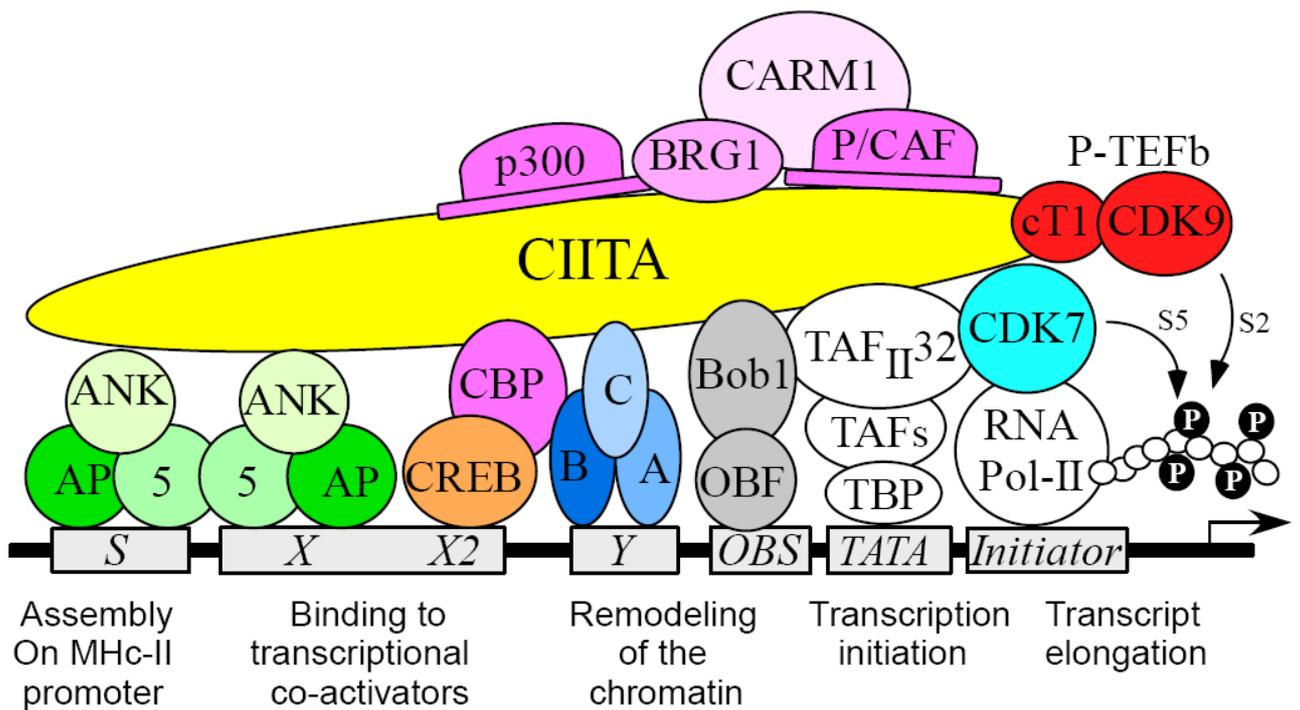


**Figure 6. Functional domains of CIITA protein.** CIITA is a protein of 1130 amino acids with distinct functional domains indicated in the scheme. The red bars represent the sequences NLS (nuclear localization signal), the green bars indicate sequences NES (nuclear export signal); AD, activation domain, P / S / T, region rich in proline / serine / threonine, GBD, GTP binding domain, LRR, repeated sequences rich in leucine.

### **CIITA as modulator of adaptive immunity and restriction factor against human retroviruses.**

The class II transactivator CIITA, is the master regulator of MHC-II gene expression. MHC class II molecules are cell-surface glycoproteins that are critical to the adaptive immune system because they present peptides, derived mainly from extracellular proteins, to the antigen receptor of CD4<sup>+</sup> T cells. MHC-class-II-mediated peptide presentation is essential for the positive and negative selection processes that shape the specificity of the T-cell-receptor repertoire of the CD4<sup>+</sup> T-cell population during its development in the thymus, for the homeostasis of the mature CD4<sup>+</sup> T-cell population in the periphery and for the initiation, amplification and regulation of protective immune responses to pathogens and tumors. To ensure tight control of these functions, MHC class II genes are themselves regulated in a precise cell-type-specific and developmental manner. Their expression is largely restricted to thymic epithelial cells and to antigen-presenting cells (APCs), that include B cells, cells of monocyte-macrophage lineage and dendritic cells (DC), specialized in the capture and presentation of extracellular antigens. MHC class II expression is mainly controlled at

transcriptional level. All MHC-II promoters have conserved upstream sequences (CUS), 150-300 base pairs upstream of the transcription-initiation site. CUS contain few boxes present in a tightly constrained order, orientation and spacing that are called S/W, X, X2 and Y boxes. The multiprotein complex, assembled at the SXY module forms with CIITA the so called MHC class II enhanceosome, which promotes MHC-II gene transcription (De Thé et al., 1996; Hoffman et al., 1992). CIITA is a non-DNA binding protein, recruited to the promoter via multiple synergistic interactions with DNA-bound factors, including the RFX and the NF-Y complexes (Caretta et al., 2000; Jabrane-Ferrat et al., 2002). CIITA acts as a transcriptional integrator by regulating different steps during gene transcription (Fig. 7). It interacts with transcriptional co-activators, general transcription factors, chromatin-modifying proteins such as CBP, p300, P/CAF, CARM1 and BRG1, as well as with the cyclin T1 subunit of the positive transcription elongation factor b (P-TEFb) to promote both initiation and elongation of MHC-II gene transcription (Kanazawa et al., 2000; Kretsovali et al., 1998). Due to this function, CIITA governs the triggering of CD4+ T cells, leading to optimal activation of downstream immune effector mechanisms, such as the antibody production by B cells. Antibody binding is a crucial event for neutralization of extracellular viruses, which cannot infect host cells. Beside this well known role of CIITA in adaptive immunity, we recently found that CIITA has evolved as a general defense mechanism of the host against human retroviruses. CIITA inhibits HIV-1 and HTLVs replication by targeting their respective viral transactivators Tat and Tax. CIITA competes with Tat for the binding to the cyclin T1 subunit of positive transcription elongation factor (P-TEFb) (Accolla et al., 2002) which is required for the elongation of HIV-1 viral transcripts (Wei et al., 1998). CIITA inhibits HTLV-2 replication by recruiting the cellular factor NF-Y, with a negative regulatory action on Tax-2 transcription function (Tosi et al., 2006). As far as HTLV-1 infection, it has been demonstrated that CIITA inhibits Tax-1-directed transactivation of the viral LTR, by competing with Tax-1 for cellular factors (CREB/ATF1 and P/CAF) used by the viral transactivator to direct HTLV-1 gene transcription (Tosi et al., 2011). Of note, it has been demonstrated that CIITA binds both Tax-1 and Tax-2 transactivators in vivo (Tosi et al., 2011; Orlandi et al., 2011). Taken together, these results definitely establish that CIITA, in addition to increasing the antigen-presenting function for pathogen antigens, behaves as an endogenous restriction factor with an intrinsic defensive role against human retroviruses infections.



**Figure 7. CIITA is a transcriptional integrator.** CIITA is recruited to the promoter of MHC-II genes via multiple interactions with proteins bound to DNA. All promoters upstream of the MHC-II genes present in the direction 5'-3' in the form of sequences highly conserved SX-X2-Y. Each of these sequences is the binding site for specific proteins. The heterotrimer RFX (Regulatory Factor X) binds X and S regions, the region X2 CREB/AP-1 binds the complex and NFY (nuclear factor Y), formed by three subunits NF-YA,-YB and-YC, binds the sequence Y. CIITA, once recruited to the promoter, functions as a transcriptional integrator by coordinating the various stages of the transcriptional process. CIITA promotes the recruitment of co-activators that alter the accessibility of chromatin inducing histone acetylation (CBP, p300, P / CAF) or methylation (CARM1) and chromatin remodeling factor, BRG-1, facilitates the 'initiation of transcription factors binding general transcription (TFIIB and TFIID) and supports the elongation of transcription by recruiting the RNA polymerase II into the complex P-TEFb (Positive transcription elongation factor b), formed by the cyclin T1 and the cyclin-dependent kinase CDK9 (Modified from Fontes et al., 1999).

### **III. AIM OF THE STUDY**

The work described in the present thesis was aimed at testing the hypothesis that the MHC class II transactivator CIITA, beside inhibiting the replication of HTLV-1 virus by targeting the viral Tax-1 transactivator, might also affect Tax-1-mediated deregulation of signaling pathways involved in cell growth and cell cycle progression. In particular, we focused on the NF- $\kappa$ B pathway that is constitutively activated by Tax-1 in HTLV-1-infected T cells, and is believed to contribute to the initiation of leukemogenesis in ATLL patients.

## IV. MATERIALS AND METHODS

### Plasmids

pNF- $\kappa$ B-Luc plasmid (Stratagene) contains five NF- $\kappa$ B binding sites (TGGGGACTTTCCGC)<sub>5</sub> upstream of the minimum promoter region that drives the expression of the firefly luciferase reporter gene. In phRL-CMV vector (Promega, Milan, Italy) the expression of Renilla luciferase is under the control of the CMV promoter. pLTR1-Luc vector containing 595 bp of HTLV-1 LTR promoter linked to the firefly luciferase gene, pCMV-Tax-1, and pCTax-2B were previously described (Tosi et al., 2011; Tosi et al., 2006). Tax-1 cDNA was excised from pJFETax (a kind gift from F. Bex) with BamHI digestion and cloned in pCDNA3.1 Hygro to obtain pCTax-1Hygro. pCfCIITA vector coding for flag-tagged full-length CIITA<sup>1-1130</sup> was a gift from Dr J. Ting, University of South Carolina. Expression vectors for all of the other flag-tagged deleted forms of CIITA were described elsewhere (Tosi et al., 2002).

### Cells

Human embryonic kidney 293T and Rat-1 fibroblast cells were grown in Dulbecco's modified Eagle medium supplemented with heat inactivated 10% fetal calf serum (FCS, Sigma) and 5 mM L-glutamine. Both cell lines do not express endogenous CIITA and, thus, MHC-II.

### NF- $\kappa$ B Luciferase assay and western blotting

293T cells were seeded in 60-mm-diameter plates and co-transfected with 0.15  $\mu$ g of the reporter plasmid pNF- $\kappa$ B-Luc, either with 12.5 ng of pCMV-Tax-1 or 50 ng of pCTax-2 with increasing amounts of CIITA expression vector (0.2, 0.4 and 0.8  $\mu$ g) by using Lipofectamine reagent (3  $\mu$ l/ $\mu$ g DNA) (Invitrogen). All transfections were carried out in the presence of 5 ng of phRL-CMV. Empty pcDNA3 vector was used as a stuffer DNA to maintain constant the total amount of transfected DNA. Cell extracts were prepared 24 h post-transfection and assayed for luciferase activities by using the dual luciferase reporter assay system (Promega) according to the manufacturer's instructions. Mean luciferase values, normalized to the *Renilla* values, of at least three independent experiments performed in duplicate are expressed

as percentages of Tax-1-dependent luciferase activity set to 100%. Error bars represent the standard deviation (SD). Cell lysates were analyzed for the expression of recombinant proteins by SDS-PAGE and Western blotting with the following antibodies: anti-CIITA 7-1H (Santa Cruz Biotechnology) or anti-Flag (Sigma-Aldrich) monoclonal antibodies to detect flagCIITA protein. Horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin secondary antibody was used. Blots were developed by chemiluminescence assay (SuperSignal West Pico, ThermoScientific).

### **Co-Immunoprecipitation assay**

For protein binding studies, 293T cells were seeded in 100 mm-diameter plates and transfected with expression vectors coding for flag-CIITA and Tax-1 using Lipofectamine. Empty pcDNA3 vector was used as a stuffer DNA. Twenty-four hours after transfection, cells were washed once with 1x PBS, lysed with 500 µl of lysis buffer (1% NP-40, 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA) supplemented with 0,1% protease inhibitor mixture containing Aprotin, Bestain, E-64, Leupetin and pepstain A (Sigma-Aldrich) incubating on ice for 45 min. The cell lysates were centrifuged at 14.000 rpm for 11 min at 4°C and pre-cleared with 20 µl of Protein A Sepharose 4 fast flow beads (GE Healthcare) for 20 minutes under rotation at 4°C. After the pre-clearing the cell extracts were incubated with 7 µl of rabbit anti-RelA or anti-IKKγ antibodies (Santa Cruz Biotechnology) on ice for 1hr and then added with 50 µl of Protein A Sepharose 4 fast flow. After overnight rotation at 4°C the immunocomplexes bound to the beads were washed six times with the lysis buffer and once with the lysis buffer containing 500 mM NaCl. Precipitated proteins were resolved by 8% SDS-PAGE and analyzed by Western blotting with specific antibodies. An aliquot, corresponding to 10% of the total cell extract was used for the detection of proteins expression by western blotting (input). The supernatant of the anti-Tax-1 hybridoma (clone 168A51-2) from the NIH AIDS Research and Reference Reagent Program, the mouse anti-CIITA 7-1H monoclonal antibodies (Sigma Aldrich) and the rabbit anti-RelA or anti-IKKγ antibodies (Santa Cruz Biotechnology) were used as primary antibodies for the immunoblot.

### **Immunofluorescence and confocal microscopy**

293T cells cultured on glass coverslips were transfected with 0.5  $\mu$ g of each plasmid expressing Tax-1 and flagCIITA full-length or the mutant 253-410. At 24 hours post-transfection, cells were fixed in methanol for 6 min at  $-20^{\circ}\text{C}$  and blocked with 0.5% BSA in 1x PBS for 1 hour at room temperature (RT). Slides were then incubated with the primary antibodies diluted in 0.1% BSA in 1x PBS overnight at  $4^{\circ}\text{C}$ . The following antibodies have been used: anti-Tax-1 hybridoma (clone 168A51-2), anti-RelA rabbit polyclonal antibody (Santa Cruz Biotechnology) and anti-flag M2 monoclonal antibody (Sigma). The slides were then washed five times with cold 1x PBS and incubated in the dark for 1 hr at RT with the following secondary antibodies (Life Technology): goat anti-mouse IgG2a conjugated to Alexa Fluor 488 to detect Tax-1, goat anti-rabbit IgG conjugated to Alexa Fluor 546 to detect RelA and goat anti-mouse IgG1 coupled to Alexa Fluor 633 to detect CIITA. Slides were then extensively washed with 1x PBS, mounted on coverslips by using the Fluor Save reagent (Calbiochem) and examined by a confocal laser-scanning microscope (Leica TCS SP5; objective lenses: HCX PL APO, 63X original magnification, numerical aperture 1.25). Images were acquired and analyzed by LAS AF software.

### **Subcellular Fractionation**

Cytoplasmic and nuclear fractions of 293T cells co-transfected with Tax-1 and flagCIITA expression plasmids, or Tax-1 plus the backbone vector were prepared using nuclear/cytosol fractionation kit according to manufacturer's instructions. (BioVision) Cytoplasm, nucleus, nuclear and cytoplasmic debris fractions were analyzed by 8% SDS-PAGE and Western blotting with the following primary antibodies: anti Tax-1 (clone 168A51-2), anti-CIITA 7-1H (Santa Cruz Biotechnology), anti-Tubulin (Sigma) and anti-CREB (Cell Signaling Technology). Blots were incubated with specific secondary antibodies conjugated with horseradish peroxidase and visualized by chemiluminescence.

### **Functional and phenotypic characterization of Rat-1 cells stably expressing Tax-1 and CIITA**

Rat-1 cells were stably transfected with the expression vectors for either Tax-1 or flag-tagged human CIITA by using Lipofectamine (6  $\mu$ l/ $\mu$ g DNA). Tax-1 and flag-CIITA-transfected cells were selected with medium containing Hygromycin (0.3 mg/ml) or Neomycin (0.5 mg/ml), respectively.

Rat-1/Tax-1 bulk transfectant was cloned by limiting dilution and several clones were selected. The presence of a functional Tax-1 was assessed by performing a gene reporter assay after transfection with the pLTR1-Luc vector expressing luciferase in a Tax-1 dependent manner (Tosi et al., 2011).

Rat-1/CIITA transfectants were stained with anti-rat MHC-II mouse monoclonal antibody RT1B (Becton Dickinson) for 30 min on ice, washed with 1x PBS and then incubated with a FITC-conjugated sheep anti-mouse antibody (Sigma) for 30 min on ice. After washings the cells were analyzed by cytofluorometry with a BD-FACSARIA II apparatus and data were elaborated with BDFACSdiva software.

To enrich the MHC-II positive Rat-1/CIITA population, cells growing under antibiotic selection were sorted by FACS and then cloned by limiting dilution. The expression of CIITA in two representative clones was analyzed. Ten millions of either Rat-1 parental and Rat-1/CIITA cells were lysed and cell extracts were incubated overnight at 4°C under rotation with anti-Flag M2 agarose beads (Sigma). Precipitated proteins were resolved by SDS-PAGE and analyzed by Western blotting with the anti-Flag M2 monoclonal antibody followed by a secondary (HRP)-conjugated anti-mouse antibody (GE Healthcare). Blots were developed by chemiluminescence assay (SuperSignal West Pico, ThermoScientific).

#### **Assay of colony formation in soft agar (CFSA)**

$2 \times 10^3$  Rat-1/Tax-1 or the control Rat-1 cells stably transfected with pCDNA3.1Hygro vector (Rat-1/pC) were seeded in 0.33% agarose in complete DMEM medium containing 0.3 mg/ml Hygromycin overlaid on 60 mm plates pre-coated with 0.5% agarose in DMEM with Hygromycin selection. After 2 weeks of culture, the formation of colonies was examined under a light microscope.

## V. RESULTS

### CIITA inhibits Tax-1-dependent NF- $\kappa$ B activation

To verify whether CIITA, beside inhibiting the LTR transactivation by Tax-1 (Tosi et al., 2011), is able to inhibit also the activation of NF- $\kappa$ B induced by Tax-1, 293T cells were co-transfected with the NF- $\kappa$ B luciferase reporter construct and the expression vector of Tax-1, alone or together with increasing amounts of a plasmid coding for CIITA full-length. These cells do not express endogenous CIITA and are MHC-II negative. At 24 h post-transfection, the cells were lysed, and the lysates were assessed for the NF $\kappa$ B-dependent luciferase activity. We found that CIITA inhibits Tax-1-mediated NF- $\kappa$ B activation in a dose dependent manner (Fig. 8A, columns 3 to 5 vs column 2). CIITA did not significantly affect the basal activation of NF- $\kappa$ B in 293T cells (data not shown).

In order to define the region of CIITA that mediates this inhibitory effect, several deletion mutants of CIITA, were also tested for their ability to inhibit Tax-1-dependent NF- $\kappa$ B activation. The expression of each mutant, assessed by western blotting, was similar to that of CIITA full length (Fig. 8A, bottom panels) The N-terminal 1-252 fragment inhibited Tax-1-dependent NF- $\kappa$ B activation, whereas the complementary C-terminal 253-1130 region exerted only a modest inhibition at the highest dose (Fig. 8A, columns 6 to 8 and columns 9 to 11 vs column 2, respectively). In addition, a CIITA fragment spanning amino acids 253 to 410 did not inhibit Tax-1-dependent NF- $\kappa$ B activation (Fig. 8A, columns 12 to 14 vs column 2). In contrast, CIITA  $\Delta$ 253-410, carrying an in-frame deletion of residues from positions 253 to 410 inhibited Tax-1-dependent NF- $\kappa$ B activation (Fig. 8A, columns 15 to 17 vs column 2). Interestingly, this mutant maintains all of the functional regions of CIITA, except the domain mediating the phosphorylation-dependent dimerization of the protein (Tosi et al., 2002), ). Taken together, these results indicate that CIITA inhibits Tax-1-dependent NF- $\kappa$ B activation independently of its dimerization and that the N-terminus of the molecule is necessary to mediate this inhibitory effect (Fig. 8B, black box).

## **CIITA affects Tax-1 subcellular localization**

We hypothesized that CIITA might inhibit Tax-1 function by affecting its distribution in the cell. To verify this hypothesis, we first analyzed Tax-1 subcellular localization by immunofluorescence (IF) and confocal microscopy in 293T cells transfected with the expression vector of Tax-1. At least two hundred Tax-1-expressing cells were analyzed and the results are shown in Fig 9A. We found that 70% of the cells express Tax-1 in nuclear punctate structures (nuclear bodies, NB), 20% express Tax-1 in both NB and cytoplasm and the remaining 10% present Tax-1 only in the cytoplasm, confirming previous reports (Lamsoul et al., 2005).

We then analyzed by confocal microscopy the localization of Tax-1 in 293T cells expressing also CIITA. The images relative to two distinct experiments and an enlarged field representative of the entire population are shown in Figure 9B and 9C, respectively. We observed a different subcellular distribution of Tax-1 in co-transfected cells compared with cells expressing Tax-1 alone (Fig. 9B and 9C vs 9A). In 60% of the co-transfected cells Tax-1 was found in the cytoplasm, whereas 40% of cells expressed Tax-1 both in the cytoplasm and nuclear bodies. Remarkably, no cells with exclusive localization of Tax-1 in nuclear bodies were observed. The overexpression of Tax-1, does not affect the subcellular distribution of CIITA, which exhibits its canonical nuclear and cytoplasmic localization (Fig. 9B and 9C).

Overall, these results indicate that the presence of CIITA alters the typical distribution of Tax-1, modifying the relative percentages of cells expressing Tax-1 in the different subcellular compartments.

## **CIITA causes Tax-1 accumulation in the detergent-insoluble cytoplasmic fraction**

In order to confirm the results of the immunofluorescence analysis described above, we performed the sub-cellular fractionation of 293T cells expressing Tax-1 alone (-) or Tax-1 plus CIITA (+). Total lysate, cytoplasm, nucleus, detergent-insoluble cytoplasmic and nuclear fractions (debris fractions) were analyzed by western blotting for the expression of Tax-1 and CIITA. The purity of each fraction was verified by assessing the presence of specific cytoplasmic and nuclear proteins (tubulin and CREB, respectively) (Fig. 10). We observed

that in the total cell lysate, the expression of Tax-1 was decreased in the presence of CIITA (lane 2 vs lane 1). In addition, in the presence of CIITA, Tax-1 was not found in both cytoplasm and nucleus (lane 4 vs 3 and lane 6 vs 5, respectively), but was exclusively accumulated in the cytoplasmic debris fraction (lane 8)

Taken together, both our IF and biochemical assays, demonstrate that CIITA traps Tax-1 in particulate cytoplasmic structures. This modified distribution of Tax-1 might explain CIITA-mediated inhibition of Tax-1 biological functions, including the impaired activation of NF- $\kappa$ B pathway.

### **Tax-1 retained in the cytoplasm by CIITA localizes in ER and Golgi apparatus**

It has been demonstrated that cytoplasmic Tax-1 localizes in ER and Golgi apparatus (Avesani et al., 2010; Alefantis et al., 2007; Turci et al., 2012). In order to assess whether Tax-1 trapped by CIITA in the cytoplasm localizes in the same compartments, we analyzed by IF and confocal microscopy the co-localization of Tax-1 with calreticulin and GM130, markers of ER and Golgi, respectively. We found that in 293T cells Tax-1 co-localizes with both calreticulin and GM130 (Fig. 11, panels A and B, respectively). CIITA, instead, is expressed in the ER but not in the Golgi apparatus. In addition, Tax-1 does not co-localize with vimentin, indicating that it is excluded from the intermediate filaments (data not shown). These results, show that CIITA traps Tax-1 in the cytoplasm, but does not affect its distribution in specific organelles.

### **CIITA prevents the nuclear translocation of p65/RelA induced by Tax-1**

It has been demonstrated that Tax-1 induces the translocation of p65/RelA in the nucleus and its accumulation in Tax-1-containing nuclear bodies.

In order to verify whether the ability of CIITA to trap Tax-1 in the cytoplasm and to inhibit Tax-1-mediated NF- $\kappa$ B activation, correlates with an impaired migration of RelA into the nucleus, we analyzed by IF the cellular distribution of endogenous RelA in 293T cells expressing Tax-1 with or without CIITA. As a control, Tax-1 was co-expressed with the deletion mutant 253-410 which, as shown in Figure 7, did not inhibit Tax-1-dependent NF- $\kappa$ B

activation. As shown in Figure 12, in cells that do not express Tax-1, RelA shows a diffuse cytoplasmic staining and was not detected in the nucleus (panel *a*). Expression of Tax-1 induces the translocation of RelA into the nucleus in Tax-1 containing nuclear bodies (panels *d-e*). Interestingly, the overexpression of CIITA does not affect the cytoplasmic distribution of endogenous RelA (panel *g*). Remarkably, in cells expressing both CIITA and Tax-1, RelA is retained in the cytoplasm (panel *j*). In contrast, the non-inhibiting CIITA fragment 253-410 does not impair the migration into the nucleus of both Tax-1 and RelA (panels *p-q*). These findings suggest that CIITA inhibits RelA nuclear translocation induced by Tax-1.

### **CIITA interacts with RelA and IKK $\gamma$ *in vivo* without affecting their association with Tax-1**

It has been demonstrated that Tax-1 interacts with RelA and with IKK $\gamma$  (Avesani et al., 2010; Harhaj and Sun, 1999). In addition, we recently demonstrated that CIITA binds Tax-1 *in vivo* (Tosi et al., 2011). To further detail the molecular mechanisms of CIITA-mediated inhibition of NF- $\kappa$ B activation by Tax-1, we asked whether this interaction affects the association of Tax-1 with RelA and IKK $\gamma$ .

To test this hypothesis, Tax-1 and flag-tagged CIITA were co-expressed in 293T cells. Cell lysates were immunoprecipitated with anti-RelA antibody, and RelA-bound proteins were assessed for the presence of Tax-1 and CIITA by western blotting. As a control of proteins expression, ten percent of total lysate was analyzed by Western blotting (Fig. 13A, input panels, lanes 1 to 4).

Results show that both CIITA (Fig. 13A,  $\alpha$ RelA IP,  $\alpha$ CIITA panel, lane 2) and Tax-1 (Fig. 13A,  $\alpha$ RelA IP,  $\alpha$ Tax-1 panel, lane 3) co-precipitated with endogenous RelA. Moreover, a very weak reduction of Tax-1-RelA interaction was observed in the presence of CIITA (Fig. 13A,  $\alpha$ RelA IP,  $\alpha$ Tax-1 panel, lane 4 vs 3).

In order to determine whether CIITA, like Tax-1, forms complexes with IKK $\gamma$  and potentially affects the interaction between Tax-1 and IKK $\gamma$ , lysates of 293T cells co-expressing either CIITA or Tax-1, or both proteins were coimmunoprecipitated with an anti-IKK $\gamma$  antibody. The immunoprecipitated complexes were analyzed for the presence of Tax-1 and CIITA by western blotting. We found that IKK $\gamma$  interacts with CIITA both in the absence and in the

presence of Tax-1 (Fig. 13B,  $\alpha$ IKK $\gamma$  IP,  $\alpha$ CIITA panel, lanes 2 and 4). This interaction does not affect the binding of Tax-1 to IKK $\gamma$  (Fig.13B,  $\alpha$ IKK $\gamma$  IP,  $\alpha$ Tax-1 panel, lane 3 vs 4).

Taken together, our results show that CIITA-mediated inhibition of NF- $\kappa$ B activation by Tax-1 cannot be attributed to an effect on the interaction between Tax-1 and two major components of the canonical NF- $\kappa$ B cascade, RelA and IKK $\gamma$ . In addition, the CIITA-RelA association does not modify the cellular distribution of RelA as shown in Figure 12, panel g, indicating that CIITA requires Tax-1 to mediate the observed delocalization of RelA.

### **CIITA suppresses Tax-1-induced phosphorylation of I $\kappa$ B**

To verify whether the kinase activity of IKK complex is impaired in the presence of CIITA, we analyzed by western blotting the phosphorylation of I $\kappa$ B inhibitor. In order to detect P-I $\kappa$ B, 293T cells transfected with pCDNA3 empty vector or with Tax-1 in the presence or in the absence of CIITA, were treated with the proteasome inhibitor MG132 to prevent P-I $\kappa$ B degradation. The expression of total I $\kappa$ B and P-I $\kappa$ B was assessed. As expected, Tax-1 increased the basal level of I $\kappa$ B phosphorylation (Fig. 14, middle panel, lane 5 vs 4). Interestingly, in the presence of CIITA any specific P-I $\kappa$ B band could be detected (Fig. 14, middle panel, lane 6).

We conclude that CIITA inhibits Tax-1-mediated nuclear translocation of RelA by inhibiting the phosphorylation, and thus the degradation, of its physiologic inhibitor I $\kappa$ B.

### **CIITA inhibits Tax-dependent activation of canonical NF- $\kappa$ B pathway**

It has been demonstrated that Tax-1 constitutively activates both the canonical and non canonical pathways of NF- $\kappa$ B (Bernal-Mizrachi et al., 2006), whereas Tax-2 activates only the canonical pathway (Huang et al., 2009; Journo et al., 2009 ; Meertens et al., 2004a).

In order to demonstrate whether CIITA-mediated inhibition of NF- $\kappa$ B activation by Tax-1 target the canonical pathway, we assessed the ability of CIITA to suppress Tax-2-mediated activation of NF- $\kappa$ B. 293T cells were co-transfected with the NF- $\kappa$ B luciferase reporter construct and Tax-1 or Tax-2 plasmids (Fig. 15, black bar and grey bar, respectively), plus increasing amounts of the plasmid coding for flag-tagged CIITA (Fig. 15, hatched bars). At 24 h posttransfection, the cells were lysed, and cell lysates were assessed for NF- $\kappa$ B-

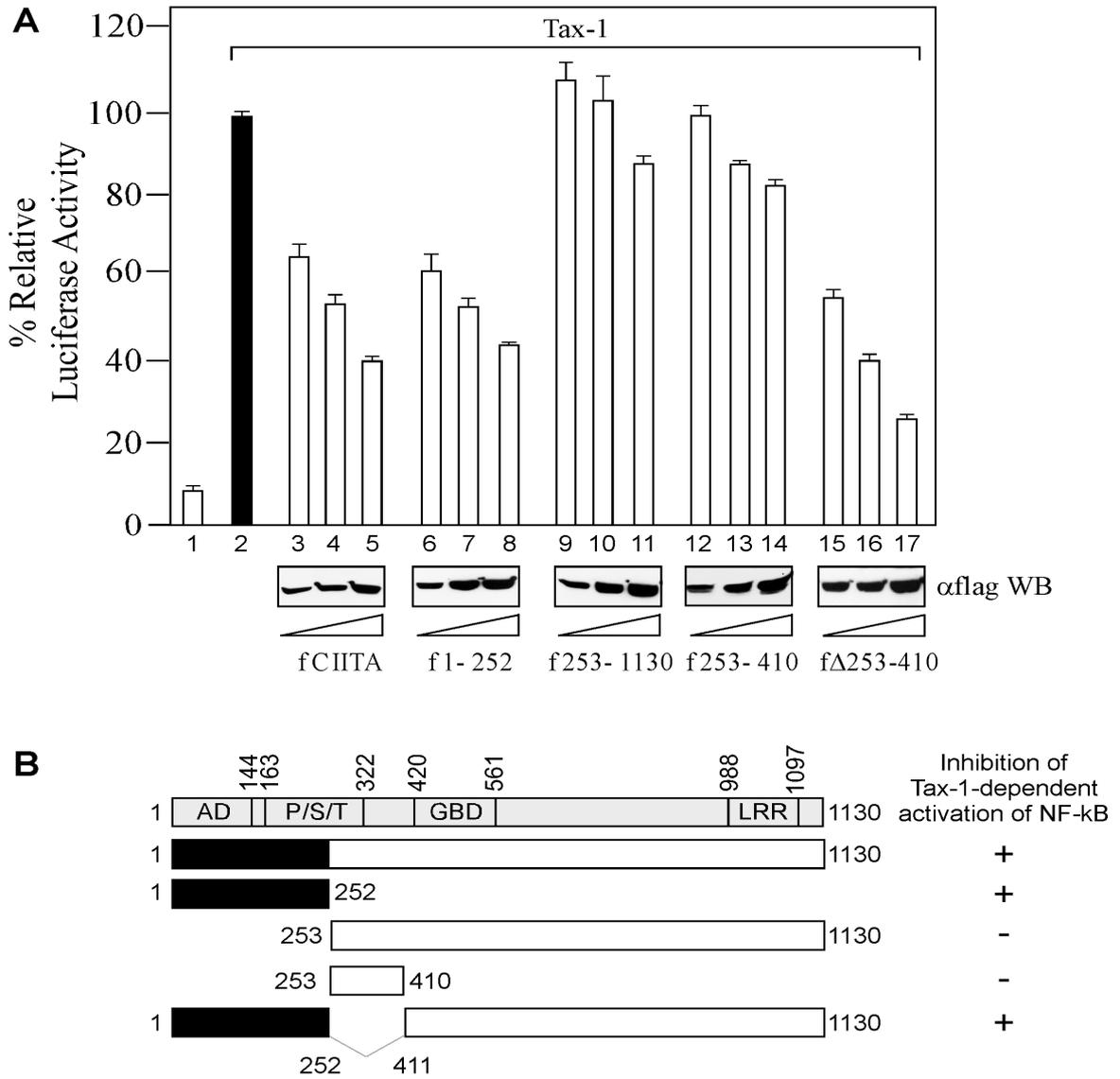
dependent luciferase activity. The expression of CIITA was detected by western blotting. We found that Tax-1 activates NF- $\kappa$ B pathway more efficiently than Tax-2 (Fig. 15, black bar vs grey bar). Nevertheless, CIITA inhibits both Tax-1 and Tax-2-mediated activation of NF- $\kappa$ B in a dose dependent manner (Fig. 15, lanes 3-5 vs lane 2 and lanes 7-9 vs lane 6, respectively). Taken together, these results indicate that CIITA inhibits Tax-dependent activation of NF- $\kappa$ B at least by suppressing the canonical activation pathway.

### **Rat-1-Colony formation soft agar assay (CFSA) as an approach to assess the capacity of CIITA to inhibit Tax-1 mediated transformation**

In order to assess whether the inhibition of Tax-1-dependent activation of NF- $\kappa$ B by CIITA is sufficient to inhibit the transformation capacity of Tax-1, we thought to take advantage of the CFSA assay which was previously used to examine the transformation of rat fibroblasts (Rat-1) by Tax-1. Endo and collaborators have shown that Tax-1-expressing Rat-1 cells cultured in soft agar form more and bigger foci as compared with parental Rat-1 cells (Endo et al., 2002). Thus we assessed whether the formation of the foci is impaired in Rat-1 cells expressing both Tax-1 and CIITA.

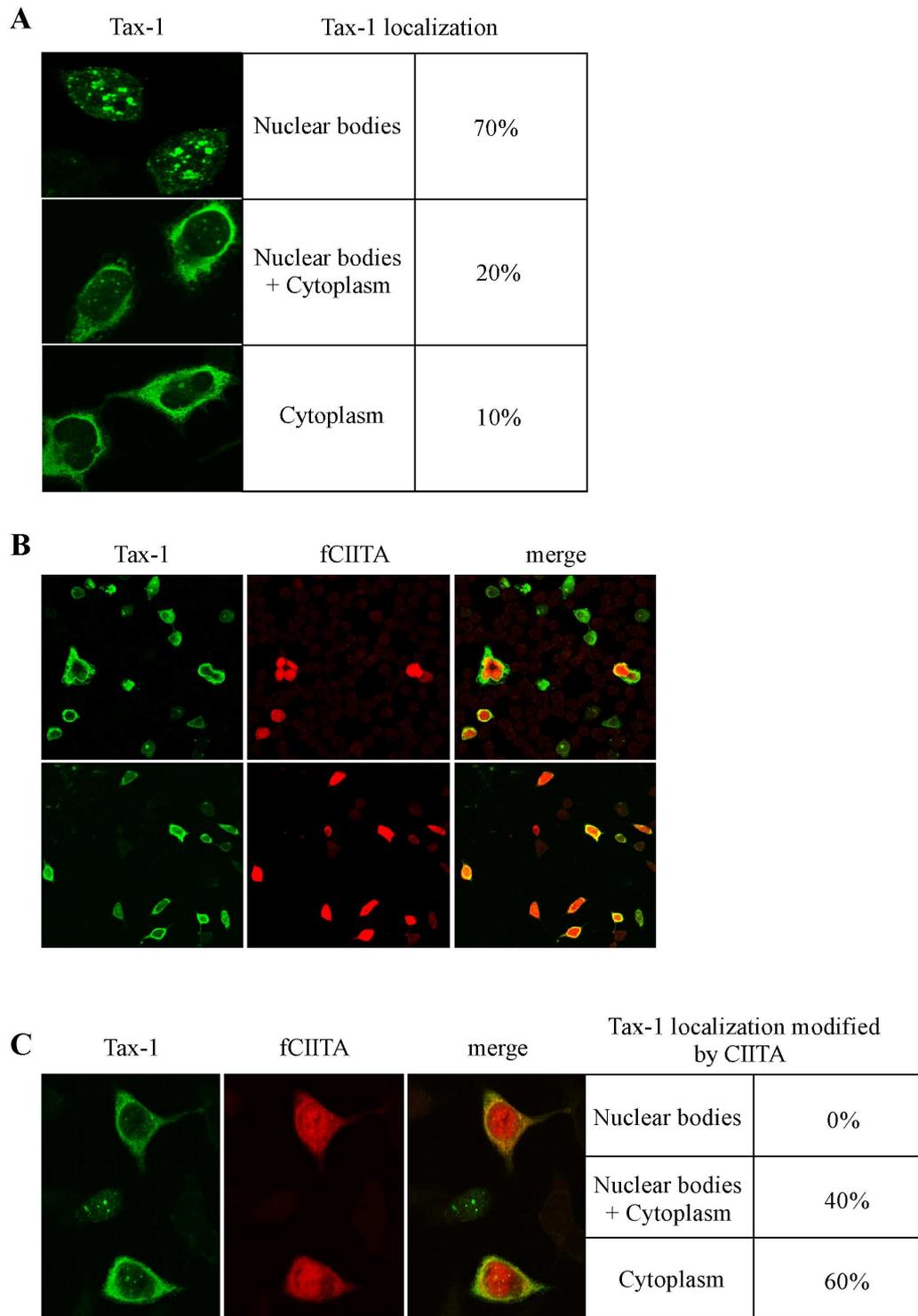
In order to establish Rat-1 cells stably expressing Tax-1, we transfected Rat-1 cells with the pTax-1 plasmid driving Tax-1 expression under the control of the CMV promoter. The original transfectant, selected on the basis of Hygromycin resistance, was then cloned by limiting dilution and assessed for the activation of the HTLV-1 LTR (LTR1) promoter. As shown in Fig. 16, among the tested clones (columns 4-6), only the G2 clone activates the viral promoter at level higher than the original transfectant and comparable to that observed in Rat-1 cells overexpressing Tax-1 (Fig. 16, lane 6 vs lanes 3 and 2, respectively). As a control, MHC-II negative Rat-1 cells expressing exogenous CIITA alone were generated and selected on the basis of Neomycin resistance and *de novo* MHC-II expression by FACS sorting and cloning. Two clones (B1 and D2) expressing functional CIITA were isolated (Fig. 17). By immunoprecipitation and Western blotting with anti-Flag antibody, we demonstrated that the two clones expressed detectable CIITA levels (Fig. 17 E, lanes 1-2). Notably, the amount of CIITA present in these two clones, and in particular in the D2 clone, was sufficient to inhibit the activation of HTLV-1 LTR by transiently expressed Tax-1 (Fig. 18, lanes 3 and 4 vs lane 2).

We first performed the CFSA assay with Rat-1/Tax-1 G2 clone. Surprisingly, and in contrast to other reports (Endo et al., 2002), no foci formation was observed compared with Rat-1 cells transfected with the empty pCDNA3 vector (Rat-1/pC) (Fig. 19) or CIITA (data not shown). This disappointing result might be explained with a toxic effect of Tax-1, expressed at too high levels under the control of the strong CMV promoter, in our Rat-1/Tax-1 G2 clone. According to this interpretation, it must be stressed that the growth rate of Rat-1/Tax-1 G2 clone was reduced compared with Rat-1/pC cells. These preliminary results suggest that a new Rat-1 clone expressing lower amount of functional Tax-1, will be preferable for our studies. Future efforts will be devoted to isolate such transfectant. This clone will be then co-transfected with CIITA cDNA in order to obtain the double Rat-1/Tax-1/CIITA transfectant. Once all the stable transfectants will be available, we will perform the CFSA assay to verify our hypothesis that CIITA might inhibit the oncogenic potential of Tax-1.

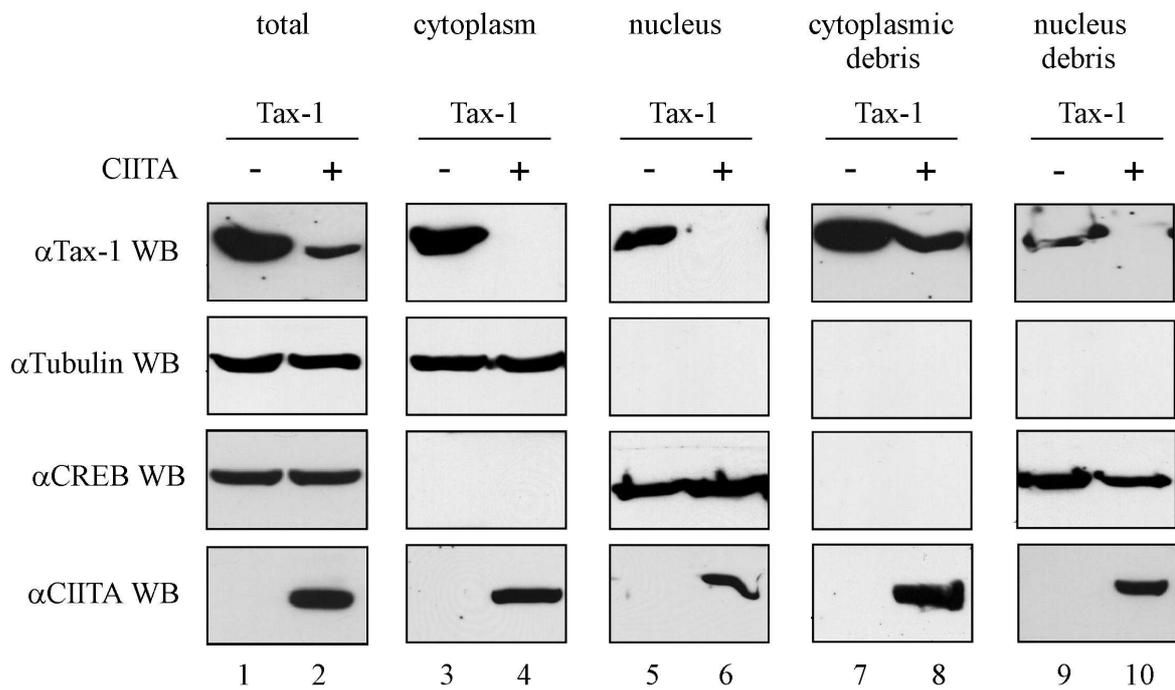


**Figure 8. The N-terminus of CIITA inhibits Tax-1-dependent NF-κB activation.**

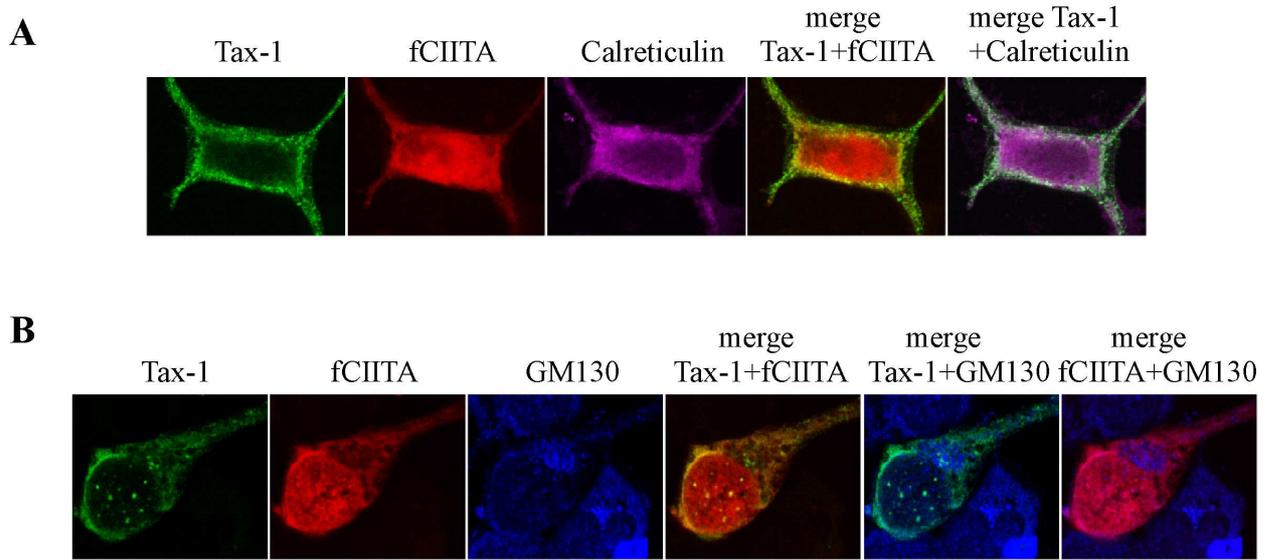
(A) 293T cells were co-transfected with fixed amounts of pNF-κB-Luc, phRL-CMV, and pTax-1 (column 2) and with increasing amounts (0.2, 0.4, and 0.8 μg) of the plasmid coding for either flag-tagged full-length CIITA1-1130 (lanes 3-5) or each of the deletion mutants, indicated at the bottom of panel A. The results of a representative experiment are shown. Mean luciferase activities, normalized to *Renilla* activity, are presented as percentages relative to activation by Tax-1 set to 100%. Column 1 represents the basal activity of cells transfected with the pcDNA3 vector. The expression of recombinant fCIITA proteins in cell extracts was evaluated by anti-flag western blotting. (B) Schematic representation of the results of the gene reporter assay illustrated in panel A. CIITA proteins used for the mapping are shown, along with their capacity to inhibit Tax-1-dependent activation of NF-κB pathway (+). The black box represents the domain from positions 1 to 252 that is necessary to inhibit Tax-1-dependent NF-κB activation. The endpoints of each CIITA mutant are indicated. At the top is a diagram of CIITA with its domains, labeled as follows: AD, activation domain; P/S/T, proline/serine/threonine-rich domain; GBD, GTP-binding domain; and LRR, leucine-rich repeats.



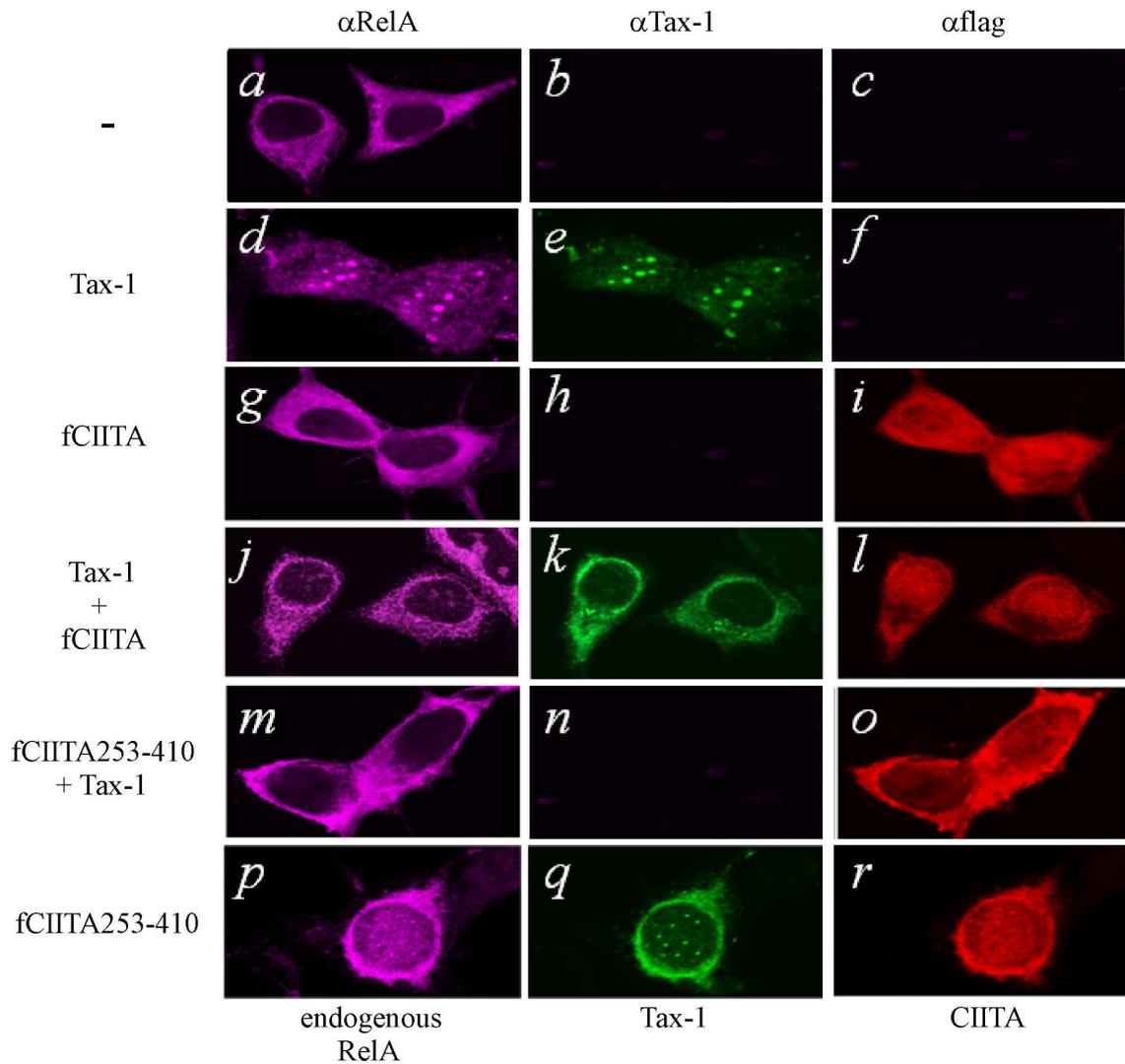
**Figure 9. The subcellular localization of Tax-1 is affected by CIITA.** 293T cells were transfected with Tax-1 expression vector alone (A) or in combination with pfCIITA plasmid (B and C) and analyzed by immunofluorescence and confocal microscopy for Tax-1 and CIITA sub-cellular localization.



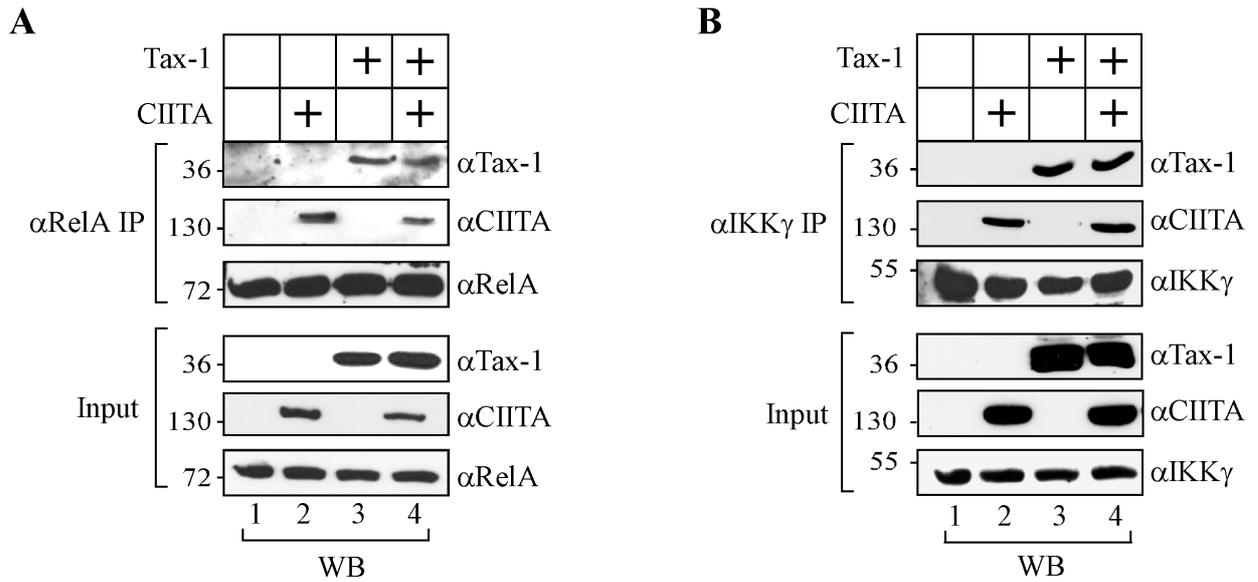
**Figure 10. CIITA causes the accumulation of Tax-1 in the cytoplasmic debris fraction.** 293T cells were co-transfected with Tax-1 and CIITA plasmids (+), or Tax-1 plus the backbone vector (-). Total cellular lysate and different sub-cellular fractions (cytoplasm, nucleus, cytoplasmic and nuclear debris) were analyzed by western blotting with the indicated antibodies. Anti-CREB and anti-tubulin antibodies were used to assess the purity of nuclear and cytoplasmic fractions, respectively.



**Figure 11. Tax-1 retained in the cytoplasm in the presence of CIITA localizes in calreticulin-containing structures and in the Golgi apparatus.** 293T cells were co-transfected with Tax-1 and CIITA expression vectors. By IF and confocal microscopy analysis, the co-localization of exogenous Tax-1 and CIITA with the endogenous calreticulin (A) and GM130 (B) was assessed. Single staining and merge images are shown, as indicated.



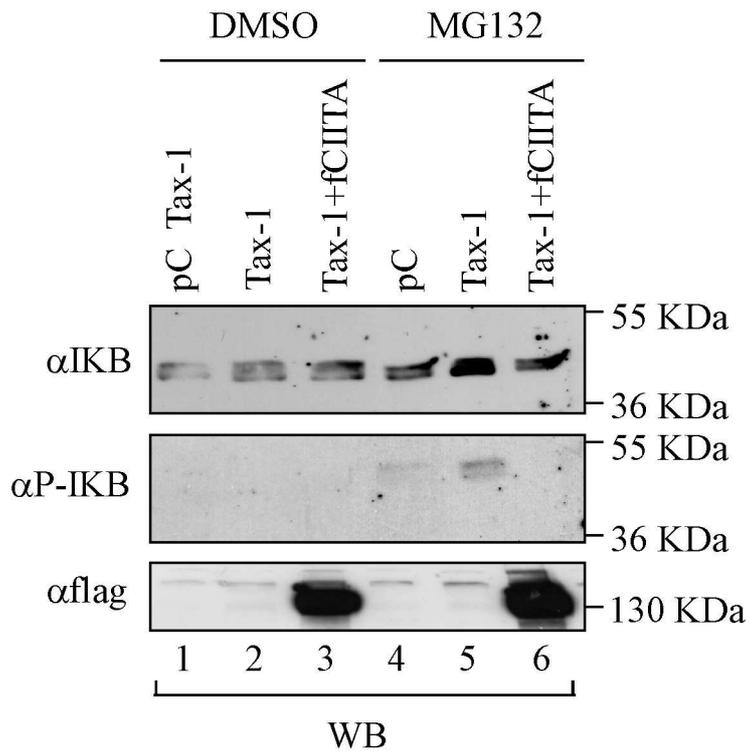
**Figure 12. CIITA blocks the Tax-1-dependent RelA nuclear translocation.** 293T cells were transfected with the expression vector of Tax-1 alone or with flag-tagged CIITA full length or the mutant CIITA253-410 plasmids. The cells were fixed, stained by triple immunofluorescence (anti-Tax-1, anti-Flag, anti-RelA antibodies, followed by specific secondary antibodies labeled with AlexaFluor488, AlexaFluor633, AlexaFluor546, respectively, as described in Materials and methods) and analyzed by confocal microscopy.



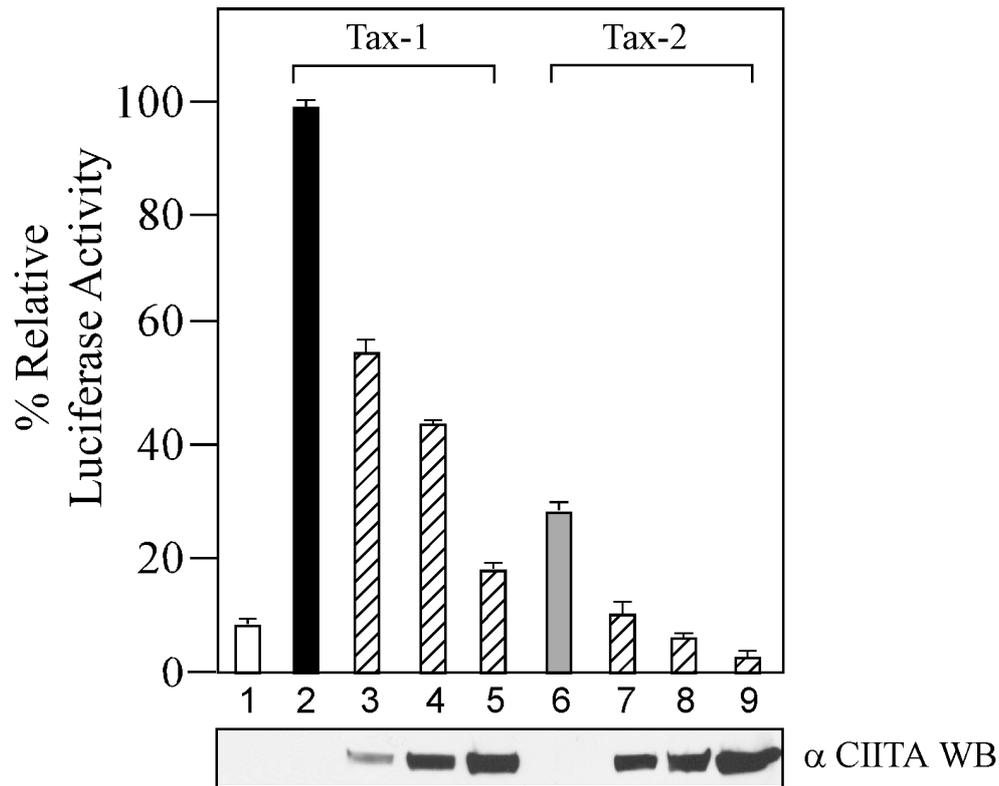
**Figure 13. The interaction of Tax-1 with RelA and IKK $\gamma$  is not affected by CIITA.**

(A) 293T cells were transfected with vectors expressing flag-CIITA or Tax-1 alone or both proteins. Cell extracts were immunoprecipitated with anti-RelA antibody ( $\alpha$  RelA IP) and the immunoprecipitated complexes were analyzed with anti-Tax-1, anti-CIITA and anti-RelA, antibodies. The input represents ten percent of the whole cell extract. The results obtained with the lysate of 293T cells transfected with the mock vector are shown in column 1.

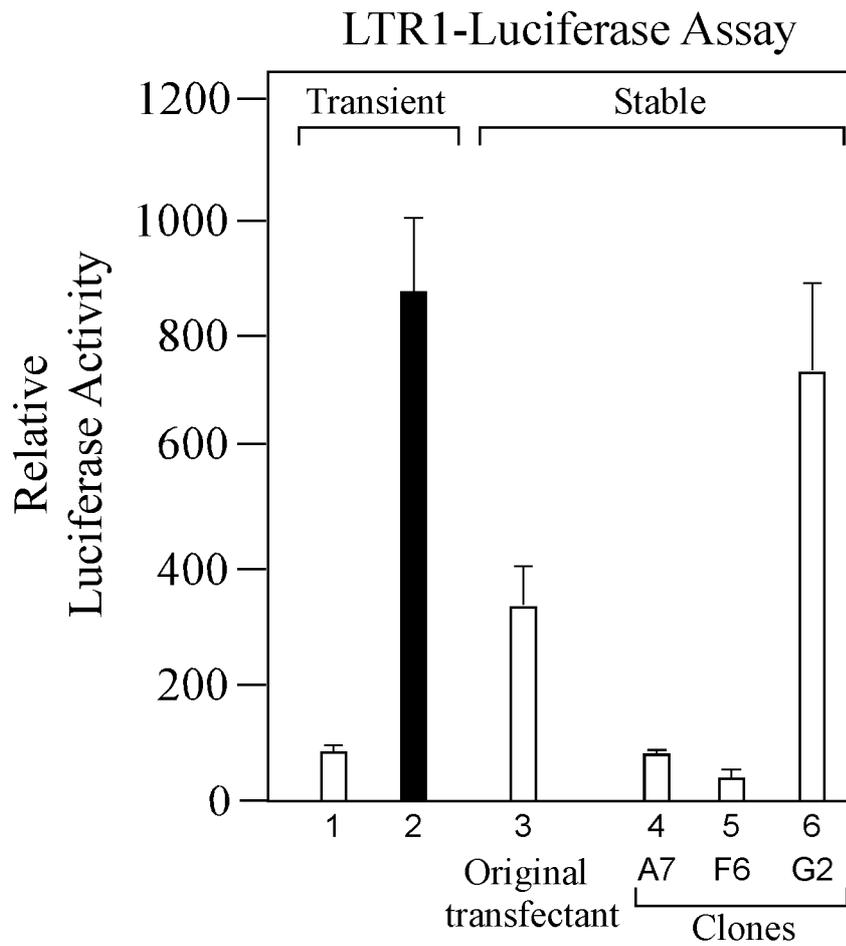
(B) 293T cells were transfected with vectors expressing flag-CIITA or Tax-1 alone or both proteins. Cell extracts were immunoprecipitated with anti-IKK $\gamma$  antibody ( $\alpha$  IKK $\gamma$  IP) and the immunoprecipitated complexes were analyzed with anti-Tax-1, anti-CIITA and anti-IKK $\gamma$ , antibodies. The input represents ten percent of the whole cell extract. The results obtained with the lysate of 293T cells transfected with the mock vector are shown in column 1.



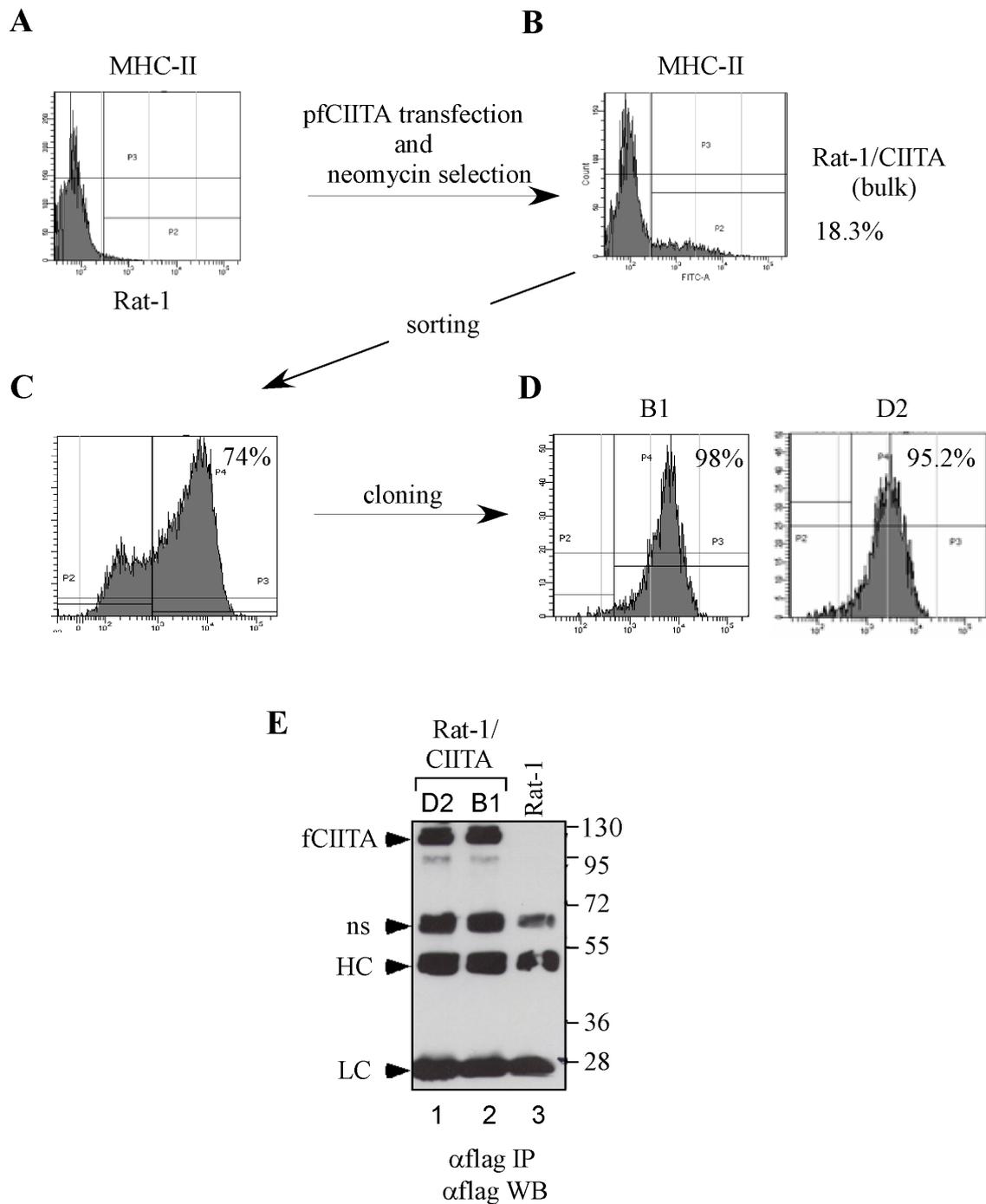
**Figure 14. IκB phosphorylation induced by Tax-1 is inhibited by CIITA.** 293T cells were transfected with pCDNA3 empty vector (pC) or with Tax-1 in the presence or in the absence of flagCIITA. Cells were treated with the proteasome inhibitor MG132 or with its vehicle (DMSO). The expression of total IκB, P-IκB and flagCIITA was assessed by western blotting.



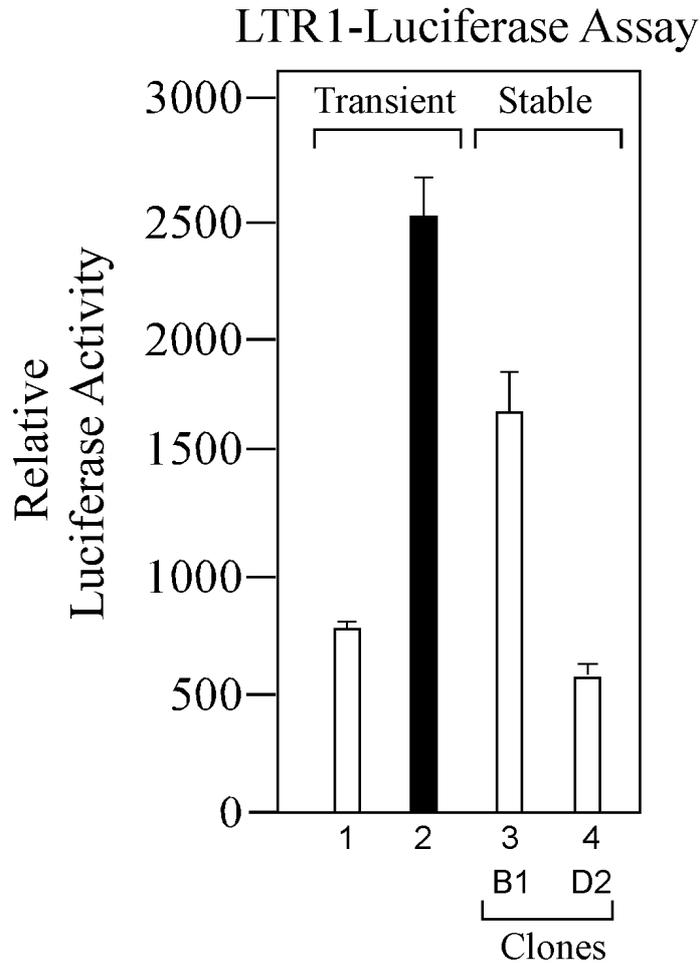
**Fig 15. Tax-dependent activation of canonical NF- $\kappa$ B pathway is inhibited by CIITA.** 293T cells were co-transfected with fixed amounts of pNF- $\kappa$ B-Luc, phRL-CMV, and pTax-1 (black column 2) or pTax-2 (grey column 6) plus increasing amounts (0.2, 0.4, and 0.8  $\mu$ g) of the plasmid coding for flag-tagged CIITA (hatched columns). The results of a representative experiment are shown. Mean luciferase activities, normalized to *Renilla* activity (ordinate axis), are presented as percentages relative to activation by Tax-1 set to 100% (black column 2). Column 1 represents the activity of 293T cells transfected with the pcDNA3 vector. Error bars represent the SD. The expression of fCIITA protein in cell extracts was evaluated by anti- CIITA western blotting.



**Figure 16. Functional characterization of Rat-1/Tax-1 cells.** Rat-1 cells stably expressing Tax-1 (bulk and derived A7, F6, G2 clones, columns 3-6) were transfected with pLTR1-Luc and pRL-CMV plasmids. At 48 hours post-transfection the luciferase activity was analyzed and compared with that of 293T cells transiently transfected with Tax-1 expression vector (black column 2). Column 1 represents the activity of 293T cells transfected with the pcDNA3 vector. Luciferase values normalized to Renilla are shown.

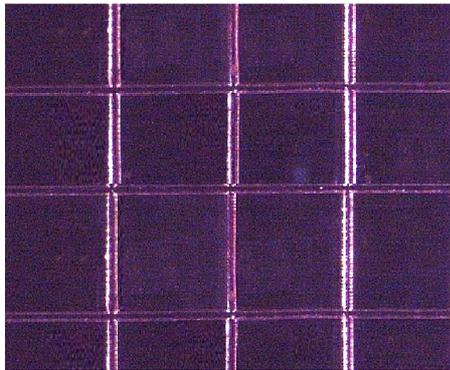


**Figure 17. Generation of Rat-1 cells expressing CIITA and MHC-II.** MHC-II negative Rat-1 cells (A) were stably transfected with pfCIITA plasmid, selected with Neomycin and analyzed by IF and cytofluorometry for the cell surface expression of MHC-II molecules. Eighteen percent of bulk transfected cells express MHC-II (B). This population was enriched to 74% by FACS sorting (C). By limiting dilution cloning two clones > 95% positive for MHC-II were isolated (B1 and D2) (D). CIITA expression in the two clones was assessed by IP with anti-Flag agarose beads followed by anti-Flag Western blotting (lanes 1 and 2). Rat-1 parental cells were also analyzed as negative control for CIITA expression (lane 3). Ns: not specific; HC: Ig heavy chain; LC: Ig light chain.

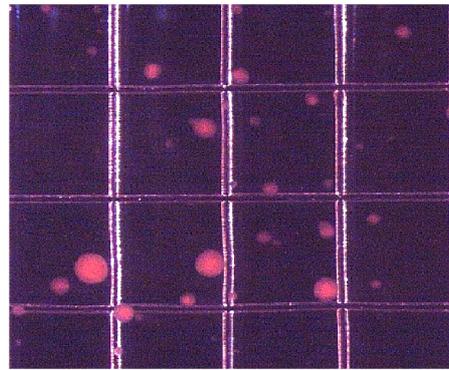


**Figure 18. Tax-1-mediated LTR1 transactivation is inhibited in Rat1/CIITA clones, expressing functional CIITA.** Rat-1/CIITA clones were transiently transfected with pLTR1-Luc gene reporter vector (0.3  $\mu$ g) driving the expression of luciferase in Tax-1-dependent manner, the phRL-CMV (20 ng) and Tax-1 (12.5 ng) plasmids (columns 3 and 4). At 48 hours post-transfection the luciferase activity was analyzed and compared with that of Rat-1 cells transiently transfected with Tax-1 expression vector (black column 2). Column 1 represents the activity of Rat-1 cells transfected with the pcDNA3 vector (32.5 ng). Luciferase values normalized to renilla are shown.

Rat-1/Tax-1 G2 clone



Rat-1/pC



**Figure 19. Rat-1/Tax-1 transfectant fails to form foci in soft agar.** Rat-1/Tax-1 G2 clone or Rat-1 cells transfected with pCDNA3 were plated in soft agar for colony formation assay. Images shown were taken with light microscope at day 14 after plating.

## VI. CONCLUSIONS AND OPEN QUESTIONS

Adaptive and innate immune responses represent the most powerful tool used by the host to counteract infectious agents. Additional intrinsic defense systems against viral infections have been recently identified. They include host-encoded restriction factors, such as APOBEC, TRIM family members, tetherin and SAMHD1, initially described for their inhibitory effect on immunodeficiency virus type 1 (HIV-1) infection. Besides HIV-1, the phenomenon of viral restriction has been investigated for other viruses including HTLV-1 infection, but the role of APOBEC and SAMHD1 in HTLV-1 infection is controversial (Forlani et al. 2013 and references therein).

In our laboratory, we have described the MHC-II transactivator CIITA as a new cellular factor restricting HIV-1, HTLV-1 and HTLV-2 infections. CIITA targets the viral transactivators Tat, Tax-1 and Tax-2, respectively, to inhibit the viral replication, by exploiting different molecular mechanisms (Casoli et al., 2004; Tosi et al., 2006, Tosi et al., 2011). Interestingly, whereas for other restriction factors, the viruses have evolved molecular countermeasures, so far, no viral products are known to counteract CIITA-mediated restriction.

Besides its role as transcriptional activator of HTLV-1 genome transcription, Tax-1 plays a major role in viral pathogenesis and T cell immortalization by deregulating the expression of cellular genes mostly by the constitutive activation of NF- $\kappa$ B pathway and the inhibition of p53 tumor suppressor. Thus, we asked whether CIITA might exert a broader effect on HTLV-1 infection by counteracting Tax-1 oncogenic potential.

In particular, my PhD project was aimed at revealing a potential role of CIITA in the inhibition of Tax-1-mediated de-regulation of NF- $\kappa$ B pathway and, if so, at identifying the fine mechanisms responsible of this inhibitory function.

The unprecedented results reported in this work clearly demonstrate that CIITA suppresses NF- $\kappa$ B activation by Tax-1 and that the N-terminal region of CIITA mediates this effect. This region contains the minimal domain that inhibits Tax-1-dependent LTR transactivation by squelching crucial cellular co-factors (CREB/ATF1 and P/CAF) used by Tax-1 to exert its transcriptional function.

Importantly, our localization studies (IF and fractionation) demonstrate that Tax-1 is retained in the cytoplasm of the majority of the cells when expressed with full-length CIITA. This

suggest that the cytoplasmic fraction of CIITA could be responsible for the sequestration of Tax-1 and its inability to activate NF- $\kappa$ B.

Consistent with this interpretation was the finding that the crucial component of the NF- $\kappa$ B complex RelA remains cytoplasmic in cells co-expressing Tax-1 and full-length CIITA, but not the non-inhibiting CIITA mutant 253-410. The impaired nuclear import of RelA explains the failure of Tax-1 to activate NF- $\kappa$ B in the presence of CIITA. Importantly, CIITA binds to, but does not disturb the interaction of RelA with Tax-1, a crucial step for the recruitment of RelA in nuclear bodies. Because RelA migration into the nucleus depends on the activation of IKK through the binding of Tax-1 to IKK $\gamma$ /NEMO, we also verified whether CIITA could affect this crucial interaction. Our results show that although the interaction of Tax-1 to IKK $\gamma$  is not affected by CIITA, the enzymatic activity of IKK kinase, promoted by Tax-1, is impaired in the presence of CIITA. Taken together, these findings strongly suggest that most of the inhibitory action of CIITA on the Tax-1-dependent NF- $\kappa$ B activation takes place in the cytoplasm. Nevertheless, several considerations based also on findings reported here may additionally corroborate the idea that nuclear CIITA can also play a role in the above inhibition. First of all we found that the N-terminal region of CIITA mediates the inhibition of Tax-1-dependent NF- $\kappa$ B activation. This region contains the minimal domain that inhibits Tax-1-dependent LTR transactivation by squelching crucial nuclear co-factors (CREB/ATF1 and P/CAF) used by Tax-1 to exert its transcriptional function. Moreover it is known that one of the two regions of CIITA that bind the nuclear p300, corresponds to that inhibiting Tax-1-dependent activation of NF- $\kappa$ B. Thus, an alternative mechanism for the negative effect of CIITA on the activation of NF- $\kappa$ B pathway by Tax-1 could be due to the squelching of p300. This HAT protein acetylates Tax-1 in the nucleus triggering Tax-1 activation of NF- $\kappa$ B. It will be interesting to verify whether the over-expression of p300 could revert CIITA-mediated inhibition of Tax-1-dependent activation of NF- $\kappa$ B and/or restore Tax-1 localization in nuclear bodies. Similarly, we could counteract the inhibitory effect of CIITA on Tax-1-dependent LTR transactivation by over-expressing P/CAF or CREB/ATF1 (Tosi et al., 2011). Several post-translational modifications (phosphorylation, ubiquitination, sumoylation and acetylation) of Tax-1 regulate its ability to activate NF- $\kappa$ B. Thus it is possible that the binding of CIITA to Tax-1 might also affect these modifications resulting in the impaired activation of NF- $\kappa$ B.

Overall, our findings demonstrate that CIITA inhibits the activation of the canonical NF- $\kappa$ B pathway. This is supported by the fact that CIITA inhibits also the activation of NF- $\kappa$ B by Tax-2, which is known to trigger the canonical, but not the non-canonical NF- $\kappa$ B pathway. At the present, we cannot exclude that CIITA might exerts its inhibitory function also on the non-canonical route.

Despite these important findings, many questions remain to be addressed.

In order to prove the relevance of our results in HTLV-1 mediated oncogenesis, we need to verify whether CIITA-mediated inhibition of NF- $\kappa$ B activation by Tax-1 correlates with the suppression of Tax-1-driven cellular transformation. To this end, several transformation assays are available, such as the CFSA assay previously established with the Rat-1 fibroblasts cellular model. We have generated Rat-1 cells stably expressing Tax-1 or CIITA but, surprisingly, no colony formation was observed with Rat-1/Tax-1 cells. We attributed this failure to an excessive expression of Tax-1, which might be toxic to the cells. Other Rat-1/Tax-1 clones with a lower Tax-1 expression need to be selected in order to obtain reliable results and establish the double Rat-1/Tax-1/CIITA transfectant.

Finally, it remains to be determined whether CIITA might counteract the inhibition of p53, which is also targeted by Tax-1 to promote malignant transformation.

Further investigation in these area will no doubt provide novel insights into the possible involvement of CIITA against Tax-1-induced T-cell transformation and contribute to establish a rational to develop new therapeutic approaches against HTLV-1 infection based on biological and/or pharmacological strategies aimed at up-regulating, in a controlled manner, the expression of CIITA in cells that are targeted by the virus.

## VII. References

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