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**HTLV Tax, HBZ and APH-2 regulatory protein interaction with host cell factors: implications for NF-κB pathway deregulation and tumorigenesis.**

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*HTLV Tax, HBZ and APH-2 regulatory protein interaction with host cell factors:*
*implications for NF-κB pathway deregulation and tumorigenesis.*

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**ABSTRACT**

Human T-cell leukemia virus type 1 (HTLV-1) is a retrovirus that infects approximately 20 million people worldwide and 5% of them develop adult T-cell leukemia (ATL), a fatal T-cell malignancy with a poor prognosis. The HTLV-1 genome encodes two proteins that play a pivotal role in the oncogenic process, the regulatory protein Tax and the accessory protein HBZ. The expression of HTLV-1 oncoprotein Tax plays a key role in $CD4^+$ T-cell transformation, interacting with host factors and deregulating several cell pathways implicated in the regulation of cell cycle and cell survival. Among them, Tax activates contitutively the NF-κB pathway, which play a primary role in inflammation, immunity, cell proliferation, apoptosis and cancer. HTLV-1 basic zipper protein (HBZ), encoded by the antisense viral genome strand, is essential for viral persistence and promotion of T-cell proliferation, acting in concert with Tax and contributing to ATL development.

The purpose of my PhD research is to expand the knowledge of the molecular mechanism of NF-κB pathway deregulation mediated by the interactions of Tax and HBZ regulatory proteins with selected host factors. We studied the effect of Tax-1 and HBZ on NF-κB promoter activation by comparative analyses with the homologous regulatory proteins expressed by the genetically related HTLV type 2, which is not associated with ATL disease. We focused the experimental analyses on two relevant aspects of the NF-κB deregulation: the interactions of Tax and the antisense proteins with key factors of the NF-κB pathway mediating p65 activation, and their role on the alternative NF-κB pathway modulation. Our data demonstrated for the first time that HBZ and APH-2 differ in the inhibitory mechanism of Tax-dependent NF-κB activation. By confocal microscopy, we observed that APH-2, unlike HBZ, was recruited in Tax-2-cytoplasmic structures containing the NF-κB factors that are essential for the activation of the pathway, the adaptor protein TAB2 and the NF-κB modulator NEMO. The formation of these complexes results in the impairment of p65 transcription factor translocation into the nucleus. The analyses of these complexes showed that TRAF3, a key

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factor of the alternative NF-κB pathway, interacts with Tax and APH-2. Applying the CRISPR/Cas9 system, we generated a cell model that allowed us to define the contribution of TRAF3 in the inhibition of NF-κB. The results obtained revealed for the first time that the absence of TRAF3 dramatically reduced the Tax-1 transactivating activity of NF-κB.

In conclusion, the results of my PhD thesis identify a new cellular factor essential for the action of HTLV Tax protein in the deregulation of cellular pathways and support the hypothesis that the different molecular mechanism of HBZ and APH-2 in the NF-κB inhibition may reflect divergent effects on HTLV-infected cells survival and probably on leukemogenesis induced by HTLV.

**SUMMARY/RIASSUNTO**

Il virus T-linfotropico umano di tipo 1 (HTLV-1) è stato il primo retrovirus ad essere scoperto nell'uomo. La sua infezione è la causa dello sviluppo, dopo anni d'infezione asintomatica, di un'aggressiva neoplasia conosciuta come leucemia delle cellule T dell'adulto (ATL), per la quale non esiste attualmente una terapia efficace. A oggi rimangono sconosciuti gli eventi che portano alla malattia nel 5% dei soggetti infettati, ma è dimostrato che i primi eventi dell'infezione possono essere determinanti per la persistenza del virus e la conseguente patogenicità. Due proteine codificate dal genoma di HTLV-1, le proteine Tax e HBZ, svolgono un ruolo fondamentale nel processo oncogenico, e sono richieste dal virus per completare il ciclo infettivo (Tax) e rendere persistente l'infezione (HBZ). L'espressione dell'oncoproteina Tax svolge un ruolo chiave nella trasformazione delle cellule T CD4$^+$, interagendo con fattori della cellula ospite e deregolando diverse vie di segnalazione cellulare che controllano il ciclo cellulare, la risposta immunitaria e la proliferazione. In particolare, la proteina Tax attiva il fattore di trascrizione NF-κB che svolge un ruolo primario nella regolazione della risposta immunitaria, nell'infiammazione, nella proliferazione cellulare, nell'apoptosi e nel cancro. La proteina HBZ è codificata dal filamento antisenso del genoma virale e svolge un ruolo essenziale nella persistenza del virus e nella promozione della proliferazione delle cellule T in concerto con Tax, contribuendo allo sviluppo della patologia ATL.

Lo studio condotto durante il dottorato di ricerca ha avuto come obiettivo principale migliorare le nostre conoscenze sulle interazioni delle proteine virali Tax e HBZ con fattori cellulari ed i loro effetti sul meccanismo molecolare che porta ad attivazione di NF-κB. A questo scopo abbiamo utilizzato l'analisi comparativa degli effetti dell'espressione delle proteine Tax e HBZ con le proteine omologhe prodotte da HTLV di tipo 2 (HTLV-2), un retrovirus geneticamente correlato ad HTLV-1, ma meno patogeno e soprattutto non associato allo sviluppo della malattia ATL. Gli studi comparativi sull'attività funzionale di Tax-1 e HBZ e gli omologhi di HTLV-2, Tax-2 e APH-2,

contribuiscono all'identificazione dei processi necessari alla tumorigenesi di HTLV. Nello studio abbiamo focalizzato l'attenzione su due aspetti rilevanti della deregolazione NF-κB: le interazioni di Tax e le proteine antisenso con i fattori chiave della via di segnalazione NF-κB, e il loro ruolo nella modulazione del pathway alternativo di NF-κB. I risultati dello studio hanno dimostrato per la prima volta che HBZ e APH-2 differiscono nel meccanismo molecolare che porta all'inibizione di NF-κB mediata da Tax. Attraverso studi di microscopia confocale, abbiamo osservato che APH-2, diversamente da HBZ, è reclutata in strutture citoplasmatiche in cui è presente Tax-2 e proteine essenziali per l'attivazione della via NF-κB, la proteina adattatrice TAB2 e il modulatore NEMO. La formazione di questi complessi rende inefficace il trasferimento nel nucleo del fattore di trascrizione p65. Dall'analisi dei complessi è derivata l'evidenza che un fattore chiave della via non canonica NF-κB, il fattore TRAF3, interagisce con le proteine Tax e APH-2. Applicando il sistema CRISPR/Cas9, abbiamo generato un modello cellulare che ha permesso di definire il contributo di TRAF3 nell'inibizione del pathway alternativo di NF-κB. I risultati ottenuti hanno dimostrato per la prima volta che l'assenza del fattore TRAF3 riduce drasticamente l'attivazione di NF-κB mediata da Tax-1.

In conclusione, i risultati di questa tesi identificano un nuovo fattore cellulare necessario all'azione di deregolazione dei pathway cellulari mediata dalla proteina Tax di HTLV e supportano l'ipotesi che il diverso meccanismo molecolare d'inibizione di NF-κB mediato da HBZ e APH-2 possa riflettere effetti divergenti sulla sopravvivenza delle cellule infette da HTLV e probabilmente sulla leucemogenesi indotta dal virus.

Dedicated to my parents.

**PUBLICATIONS**

1. **Stefania Fochi**, Simona Mutascio, Umberto Bertazzoni, Donato Zipeto, Maria Grazia Romanelli (2018). *HTLV deregulation of the NF-κB pathway: an update on Tax and antisense proteins role.* Front. Microbiol. 9:285 doi: 10.3389/fmicb.2018.00285

2. Louise Dubuisson, Florence Lormières, **Stefania Fochi**, Jocelyn Turpin, Amandine Pasquier, Estelle Douceron, Anaïs Oliva, Ali Bazarbachi, Valérie Lallemand-Breitenbach, Hugues De Thé, Chloé Journo, Renaud Mahieux (2018). *Stability of HTLV-2 Antisense Protein is Controlled by PML Nuclear Bodies in a SUMO-Dependent Manner.* Oncogene. doi: 10.1038/s41388-018-0163-x.

3. **Stefania Fochi**, Simona Mutascio, Francesca Parolini, Donato Zipeto, Maria Grazia Romanelli (2017). *HTLV antisense proteins role in the NF-κB modulation. Virol Res J. Volume 1 Issue 3.*

**CONGRESSES ATTENDED AND ABSTRACTS**

ORAL PRESENTATIONS

1. **Stefania Fochi**, Elisa Bergamo, Michela Serena, Donato Zipeto, Maria Grazia Romanelli. *HTLV-1 and HTLV-2 Tax, HBZ and APH-2 interaction with host factors: their involvement in cellular pathways regulation.* HERN, HTLV European Research Network 2016, Bucarest, Romania. 20-22.05.2016 (**oral presentation**).

2. **Stefania Fochi**, Simona Mutascio, Francesca Parolini, Donato Zipeto, Maria Grazia Romanelli. *HTLV-1 basic leucin zipper factor and its homologous APH-2 impair NF-κB activation mediated by the viral oncoprotein Tax.* Associazione Italiana di Biologia e Genetica, PhD Meeting Santa Margherita Ligure, Italy. 11-13.05.2017 (**oral presentation**).

CONFERENCES CONTRIBUTIONS

1. Elisa Bergamo, Erica Diani, **Stefania Fochi**, Pamela Lorenzi, Michela Serena, Donato Zipeto, Maria Grazia Romanelli. *The human T-cell leukemia virus -2 (HTLV-2) antisense protein APH-2 interacts with p65 and inhibits NF-κB Tax-2 activation.* Società Italiana di Biofisica e Biologia Molecolare, SIBBM Meeting, Turin, Italy. 01-03.07.2015.

2. Oncogenic Viruses Workshop, Padova, Italy. 25-26.09.2015.

3. **Stefania Fochi**, Elisa Bergamo, Michela Serena, Pamela Lorenzi, Donato Zipeto, Maria Grazia Romanelli. *Functional role of HTLV Tax, HBZ and APH-2 regulatory proteins on cellular autophagy pathway.* PhD-Day Meeting, University of Verona, Italy. 01.2016.

4. **Stefania Fochi**, Elisa Bergamo, Michela Serena, Donato Zipeto, Maria Grazia Romanelli. *Human T-cell leukemia virus HBZ and APH-2 antisense proteins interaction with host factors and their involvement in NF-κB activation.* Società Italiana di Biofisica e Biologia Molecolare,

SIBBM Meeting Naples, Italy. From Genomes to Functions. 16-18.06.2016.

5. **Stefania Fochi**, Elisa Bergamo, Michela Serena, Francesca Parolini, Simona Mutascio, Donato Zipeto, Maria Grazia Romanelli. *The interplay between HTLV proteins and cellular factors: impact on the NF-κB cell signaling.* PhD-Day, University of Verona, Italy. 12.2017.

6. Francesca Parolini, Simona Mutascio, Michela Serena, **Stefania Fochi**, Maria Grazia Romanelli, Donato Zipeto. *CRISPR/Cas9 as a powerful tool for genome editing.* Convegno primavera dermatologica, Bucarest, Romania 29.03-02.04.2017.

7. **Stefania Fochi**, Simona Mutascio, Francesca Parolini, Donato Zipeto, Maria Grazia Romanelli. *A CRISPR/Cas9 based approach to study the implication of HTLV regulatory proteins in the NF-κB modulation.* Association of Cell Cultures meeting: The future of cancer therapy: the genome editng era, Catanzaro, Italy. 07-09.06.2017.

8. Francesca Parolini, Simona Mutascio, Michela Serena, **Stefania Fochi**, Maria Grazia Romanelli, Donato Zipeto. *CRISPR/Cas9 for the Study of the Interactions between Viruses and Host.* Association of Cell Cultures meeting: The future of cancer therapy: the genome editng era, Catanzaro, Italy. 07-09.06.2017.

9. **Stefania Fochi**, Simona Mutascio, Francesca Parolini, Donato Zipeto, Maria Grazia Romanelli. *Host-virus interactions: HTLV antisense regulatory proteins play a role in the dysregulation of NF-κB pathway.* Società Italiana di Biofisica e Biologia Molecolare, SIBBM Meeting, Milan, Italy. From Single Cells to 3D-Cell Culture. 14-16.06.2017.

10. Francesca Parolini, Simona Mutascio, Michela Serena, **Stefania Fochi**, Maria Grazia Romanelli, Donato Zipeto. *CRISPR/Cas9 to study virus-host interactions.* Società Italiana di Biofisica e Biologia Molecolare, SIBBM Meeting, Milan, Italy. From Single Cells to 3D-Cell Culture. 14-16.06.2017.

11. Federica Ferrarini, Francesca Martinetto, Roberta Galavotti, **Stefania Fochi**, Pamela Lorenzi, C., Di Gaetano, B., Pardini, A., Naccarati, D. De Pietri Tonelli, Maria Grazia Romanelli, Patricia M.-J. Lievens. *Characterization of FOXP2-Δ11, a novel alternatively spliced product of FOXP2 gene*. Società Italiana di Biofisica e Biologia Molecolare, SIBBM Meeting, Milan, Italy. From Single Cells to 3D-Cell Culture. 14-16.06.2017.

12. **Stefania Fochi**, Simona Mutascio, Francesca Parolini, Donato Zipeto, Maria Grazia Romanelli. *HTLV antisense proteins role in the NF-κB modulation*. International Virology Conference, Toronto, Canada. 30-31.10.2017.


AWARDS

1. **Poster award: Stefania Fochi**, Elisa Bergamo, Michela Serena, Donato Zipeto, Maria Grazia Romanelli. *Human Tcell leukemia virus HBZ and APH-2 antisense proteins interaction with host factors and their involvement in NF-κB activation.* Società Italiana di Biofisica e Biologia Molecolare, SIBBM Meeting Naples, Italy. From Genomes to Functions. 16-18.06.2016.

2. **Poster award: Stefania Fochi**, Elisa Bergamo, Michela Serena, Francesca Parolini, Simona Mutascio, Donato Zipeto, Maria Grazia Romanelli. *The interplay between HTLV proteins and cellular factors: impact on the NF-κB cell signaling.* PhD-Day, University of Verona, Italy. 12.2017.

3. **Poster award: Stefania Fochi**, Simona Mutascio, Francesca Parolini, Donato Zipeto, Maria Grazia Romanelli. *A CRISPR/Cas9 based approach to study the implication of HTLV regulatory proteins in the NF-κB modulation.* Association of Cell Cultures meeting: The future of cancer therapy: the genome editing era, Catanzaro, Italy. 07-09.06.2017.

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**ABBREVIATIONS**

**aa**: amino acid

**AP-1**: Activator Protein 1

**ATL**: Adult T-cell Leukemia

**ATLL**: Adult T-cell Leukemia/ Lymphoma

**AZT:** Azidothymidine

**BAF**: B-cell Activating Factor

**bp:** base pairs

**bZIP**: basic leucine zipper

**CBP**: CREB Binding Protein

**CD4, CD8**: T-cell mature phenotype

**CNS**: Central Nerve System

**CRE**: Cyclic-AMP Response Element

**CREB**: Cyclic AMP Response Element Binding protein

**DMEM:** Dulbecco's Modified Essential Medium

**dsDNA:** double stranded DNA

**EBV**: Epstein-Barr Virus

**EGFR**: Epidermal Growth Factor Receptor

**Env**: envelope

**FBS**: Fetal Bovine Serum

**GAG**: Group-Associated Gene

**GFP**: Green Fluorescent Protein

**GLUT1**: Glucose Transporter 1

**HAM/TSP**: HTLV-1-associated myelopathy/tropical spastic paraparesis

**HBV**: hepatitis B virus

**HBZ**: HTLV-1 bZIP factor

**HCC**: Hepatocellular Carcinoma

**HCV**: hepatitis C virus

**HHV-8**: Human Herpesvirus-8

**HIV**: Human Immunodeficiency Virus

**HPV**: Human Papilloma Virus

**HRP**: Horseradish Peroxidase

**HSPG:** heparin sulfate proteoglycan

**hTERT**: human Telomerase Reverse Transcriptase

**HTLV**: Human T-cell Leukemia Virus

**IARC**: Internation Agency of Cancer Research

**IKK**: IκB kinase

**ICAM-1**: the intercellular adhesion molecule-1

**IL-2**: Interleukin-2

**IL-6:** Interleukin-6

**IL-15**: Interleukin-15

**IFN-α**: Interferon-α

**LC3**: Microtubule-associated protein 1A/1B-Light Chain 3

**LMP1**: Latent infection Membrane Protein 1

**LT-β**: LymphoToxin B

**LTR**: Long Terminal Repeat

**MAPK**: Mitogen-Activated Protein Kinase

**mTOR**: mammalian Target Of Rapamycin

**NB**: Nuclear Body

**NES**: Nuclear Export Signal

**NF-κB**: Nuclear Factor kappa-light-chain-enhancer of activated B cells

**NIK**: NF-κB-Inducing Kinase

**NLD**: Nuclear Localization Determinant

**NLS**: Nuclear Import Signal

**NRP-1**: VEGF-165 receptor neuropilin-1

**nt**: nucleotide

**ORF:** Open Reading Frame

**PBM**: PDZ-binding domain motif

**PML**: Promyelocytic leukemia protein–containing nuclear bodies

**PTM**: Post-Translational Modification

**RANKL**: receptor activator of nuclear factor kappa-B ligand

**Rex:** Regulator of viral protein expression

**RxRE**: Rex response element

**SDS-PAGE**: Sodium Dodecyl Sulphate Polyacrilamide Gel Electrophoresis

**ssRNA+**: positive single-stranded RNA

**STAT3**: Signal Transducer and Activator of Transcription 3

**STLV**: Simian T cell Leukemia Virus

**SUMO**: Small Ubiquitin-like Modifier

**TAB2**: TAK1-Binding protein 2

**TAK1**: Transforming growth factor-β–Activated Kinase 1

**Tax:** Transcriptional activator of pX region

**TCR**: T Cell Receptor

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**TGFβ:** transforming growth factor beta

**TNFR1**: Tumor Necrosis Factor Receptor 1

**TRADD**: Tumor necrosis factor Receptor type 1-Associated DEATH Domain protein

**TRAF**: Tumor necrosis factor Receptor-Associated Factor

**TRE1**: Tax-responsive element 1

**WB**: Western Blot

# 1. INTRODUCTION

## 1.1 Human viral oncogenesis

In the early 1960s, the concept of human viral oncogenesis was an implausible idea that the medical community accepted at the time of Epstein-Barr Virus identification in Burkitt lymphoma cells before, and of Human T-cell leukemia viruses (HTLV-1) discovery later (Tagaya et al., 2017). Cancers caused by pathogens, including bacteria and viruses, are estimated to be >20%, and human cancer properly related to oncovirus infection is approximately 12/15% (Mesri et al., 2014). Seven human viruses (**Table 1**) have been included by the International Agency of Cancer Research (IARC) in the Group 1 of biological carcinogenic agents: Epstein-Barr virus (EBV), Human Herpesvirus-8 (HHV-8), high-risk Human Papilloma Viruses (HPV), Hepatitis C Virus (HCV), Hepatitis B Virus (HBV), Human T-Cell Lymphotropic/Leukemia Virus-1 (HTLV-1) and Merkel Cell Polyomavirus (MCV) (Bouvard et al., 2009).

**Table 1.** The human onco-viruses.

| Family and Genome | Virus | Tumors Related |
| --- | --- | --- |
| Herpesviridae (DNA) | Epstein-Barr virus (EBV) | Burkitt lymphoma, nasopharyngeal carcinoma, Hodgkin lymphoma |
| | Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) | Kaposi Sarcoma |
| Papovaviridae (DNA) | Human Papilloma virus (HPV-16,-18) | Cervical cancer |
| Hepadnaviridae (DNA) | Hepatitis B virus (HBV) | Hepato cellular carcinoma |
| Flaviviridae (RNA) | Hepatitis C virus (HCV) | Hepato cellular carcinoma |
| Retroviridae (RNA) | Human T-lymphotropic virus (HTLV-1) | Adult T-cell leukaemia |
| Poliomaviridae (DNA) | Merkel Cell Polyomavirus (MCV) | Merkel cell carcinoma |

Neoplasms arise as pathobiological consequences of viral infection that evolve specific mechanisms to replicate, persist and evade the immune system through the deregulation of host oncogenic signaling (Mesri et al., 2014). HPV type 16 and 18 are responsible for 60/70% of cervical cancer worldwide, but also vaginal, vulvar, penis, anus cancers, and some head and neck cancers (Khan et al., 2005). HBV and HCV infections associated with hepatocellular carcinoma (HCC) cancer in approximately 80% of cases (Rosa et al., 2017). Some oncoviruses required co-factors and/or micro-environmental alterations to facilitate their oncogenic ability. For instance, Burkitt lymphoma EBV-infection associated is coincident with *Plasmodium falciparum* infection, the etiological agent of malaria (Rochford et al., 2005). HHV-8 also contributes to the development of Kaposi's sarcoma and lymphoproliferative disorders in collaboration with the Human Immunodeficiency Virus (HIV) (Bhutani et al., 2015). MCV was recently discovered to cause a rare, aggressive skin tumor, Merkel cell carcinoma, and the induced oncogenic mechanism is still under investigation (Feng et al., 2008).

In 1980s, the retrovirus HTLV-1 was isolated from leukemic cells from a patient with Adult T-cell leukemia/lymphoma (ATLL) (Yoshida et al., 1982). Several evidences indicate that the oncogenesis induced by HTLV-1 may involve many mechanisms: a) chronic inflammation as a consequence of viral persistence; b) the insertion of oncogenes into the genome of the host cell and the expression of tumor suppressor genes; c) host immune response affection to permit the pre-cancerous cells to evade the immune system surveillance (Zhang et al., 2017). For all these properties HTLV-1 represent a useful model to investigate virus-host cell interactions and discover the critical steps that ultimately can lead to cancer (Tagaya et al., 2017).

**1.2 Human T-cell leukemia virus (HTLV) and Epidemiology**

The discovery of the first human retrovirus, Human T-cell lymphotropic/leukemia virus (HTLV), proceeded quite independently in the United States and Japan. HTLV was first isolated in T-cells from a patient affected by cutaneous T-cell lymphoma, and then in fresh peripheral lymphocytes from a patient with ATL, at

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the beginning of the 80s (Poiesz et al., 1980a, b; Yoshida et al., 1982). HTLVs originate from the gradual evolution of the simian T-lymphotropic viruses (STLVs) transmitted from monkeys to humans in Africa, approximately 35.000 years ago (Watanabe et al., 1984). To date four types of HTLVs, HTLV1/2/3/4, have been identified. HTLV-1 predominantly infects T lymphocytes and is estimated to infect from 10 to 20 million people worldwide (Gessain and Cassar, 2012). HTLV-1 has spread to different geographic regions with human migration and now is endemic in the Southwestern part of Japan, Saharan Africa and South America and, the Caribbean area. In the Middle East Europe, Romania seems to represent a HTLV-1 endemic region (**Figure 1**) (Gessain and Cassar, 2012). Human T-cell lymphotropic virus subtype two (HTLV-2) was isolated from a T-cell variant of hairy cell leukemia in 1982, and differently to HTLV-1, a direct link between HTLV-2 infection and neoplasm development has not been identified (Kalyanaraman et al., 1982). HTLV-2, is prevalent among the native Amerindian population in North America as well as South America, and among intravenous drug users in the United States and Europe (Vrielink & Reesink, 2004). HTLV-3 and HTLV-4, have been isolated and characterized in 2005, and currently, their infection has not been associated with any disease (Mahieux and Gessain, 2009).
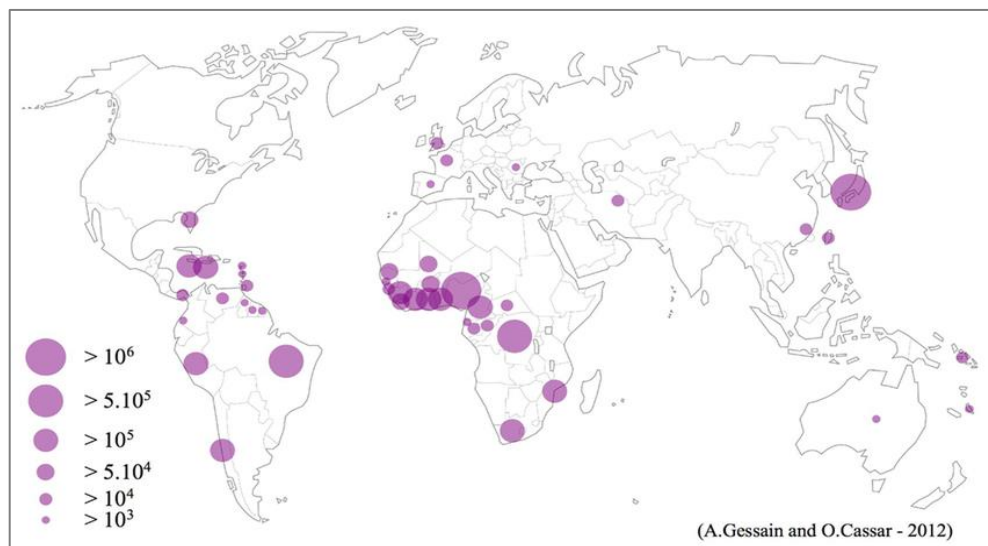


**Figure 1**. **Geographical distribution of the main foci of HTLV-1 infection.** Epidemiological data of HTLV-1 infected carriers obtained from approximately 1.5 billion of individuals (Gessain and Cassar 2012).

## 1.3 HTLV transmission and disease association

In HTLV-1 infected subject free virions are not detected in the serum. The HTLV-1 spread occurs by cell-to-cell transmission (Bangham et al., 2017). HTLV-1 is generally transmitted via three routes *in vivo*: sexual intercourse, parenteral transmission (blood transfusion or intravenous drug use) and mother-to-infant transmission (mainly by breast feeding) (Matsuoka et al., 2005). HTLV-1 infection has been associated with two major diseases, Adult T-cell leukemia/lymphoma (ATLL) and HTLV-1 associated myelopathy/tropical paraparesis (HAM/TSP) (Hinuma et al., 1981; Gessain et al., 1985). In addition, HTLV-1 infection has been linked to a number of inflammatory diseases including alveolitis, dermatitis, polymyositis, uveitis, arthritis, and peripheral myopathy (Gonçalves et al., 2010).

## 1.3.1 Adult T-cell Leukemia/lymphoma (ATLL)

Adult T-cell leukemia/lymphoma (ATLL) is an aggressive peripheral T-cell malignancy with a poor prognosis and no cure currently available. Approximately 6–7% of male and 2–3% of female HTLV-1 carriers develop ATL after a long period of latency, 20–40 years from initial infection (Kogure et al., 2017). Based on the diversity in clinical features and prognosis, ATL has been classified into four clinical type acute, lymphomatous, chronic, and smoldering subtypes; the majority of infected people are asymptomatic throughout their lives. The overexpression of various cytokines and chemokines in ATL patients provides a lymphocytic infiltration in some organs such as the skin, liver, spleen, the gastrointestinal tract, and lungs. ATL patients show numerous erythematous, nodules, and plaques with ulceration (**Figure 2, A**). The acute stage of ATL is characterized by lymphocytes with multilobulated nuclei (flower cells) (**Figure 2, B**), whereas the chronic stage results in leukocytosis.
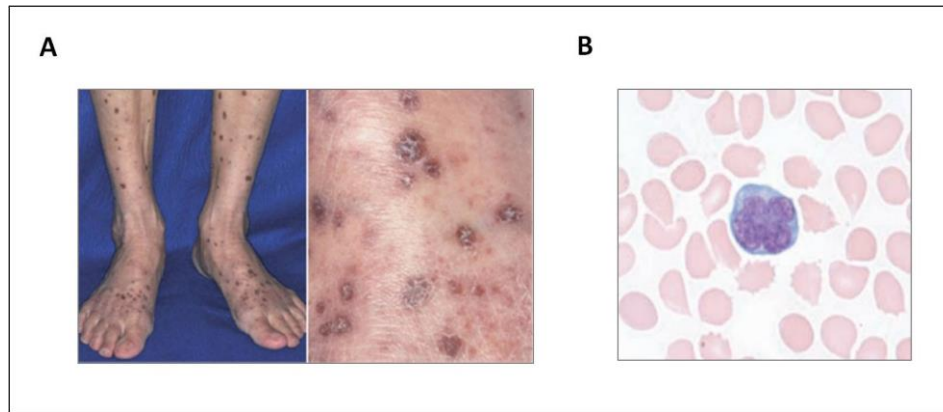
**Figure 2**. **(A)** Clinical features of an ATL patient (www.medicaljournals.se); **(B)** Typical "flower cell" in the peripheral blood of an acute ATL patient (Graham et al., 2014).

The median survival time for acute ATL patients is 6 to 10 months even with intense chemotherapy. Chemotherapy, monoclonal antibodies, allogeneic bone marrow transplants in combination with interferon-α (IFN-α) and azidothymidine (AZT) are the current recommended therapies for ATL (Martin et al., 2016; Macchi et al., 2017). Very recent studies proposed mogamulizumab and lenalidomide as promise in the treatment of ATL (Mehta-Shah et al., 2017).

**1.3.2 HTLV-1 Associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP)**

HTLV-1 is also associated with a chronic neuromyelopathy, known as HTLV-1 Associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP). HAM/TSP consists in a slowly progressive inflammatory demyelinating disease of the central nervous system (CNS) with gradual spastic paraparesis. Clinical analyses of HAM/TSP patients reveal multiple white matter lesions in both the spinal cord and the brain, involving perivascular demyelination and axonal degeneration. Approximately 0.25 to 5% of HTLV-1 carries develop HAM/TSP and it mainly develops in adults with a mean age at onset of 40–50 years. Contrary to ATL, HAM/TSP is more common in women than in men (male/female ratio = 1.4 VS male/female ratio = 0.4) (Gessain and Mahieux, 2012). Antispasmodic and anti-inflammatory medications are included as treatment for symptoms HAM/TSP-associated (Martin et al., 2016).

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### 1.3.3 HTLV-2 Associated Disease

HTLV-2 was isolated from cases of T-lymphocyte variant of hairy cell leukemia, CD8$^+$ T cell leukemia and granular lymphocytic leukemia, but there is no link between infection and development of malignancy (Kalyanaraman et al., 1982). Thus, the etiological role of HTLV-2 in hematological neoplasms remains unresolved. HTLV-2 infection was associated with a few cases of chronic neurodegenerative disease, similar to HAM/TSP (Jacobson et al., 1993) and to an increased incidence of respiratory infections, and inflammatory conditions, such as arthritis (Araujo et al., 2004; Roucoux et al., 2004; Biswas et al., 2010).

### 1.4 HTLV Cell to Cell Transmission and Infection

HTLVs are members of the delta-retrovirus family organized in enveloped virions of 80–100 nm in diameter, with two copies of a single stranded positive sense RNA genome (**Figure 3**). HTLV preferably infects CD4$^+$ and CD8$^+$ T cells, but presents a tropism for other cell types *in vivo*, including CD25$^+$ lymphocytes, monocytes and B-lymphocytes, macrophages, dendritic cells, megakaryocytes as well as glial cells (astrocytes and microglial cells) (Koyanagi et al., 1993).
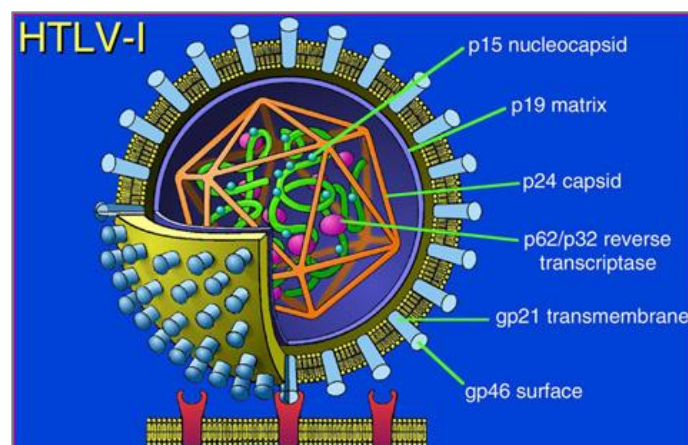


**Figure 3.** Schematic representation of the human T-cell leukemia virus (http://researchnews.osu.edu/archive/htlvil2pics.htm).

HTLV-1 spreads efficiently between T-cells via a tight organized cell-cell contact known as the virological synapse. Two types of cell-cell contacts have been identified to be critical for HTLV-1 transmission: tight cell-cell contacts and cellular conduits (Gross et al., 2016). In both cases, viral particles are transmitted in confined areas protected from the immune response of the host. The HTLV infection begins with a mature virion expressing the surface unit of the viral protein envelope (Env) and binding to a specific cellular receptor (**Figure 4**). HTLV-1 and HTLV-2 use the cellular receptors glucose transporter-1 (GLUT1) and neuropilin-1 (NRP-1). HTLV-1, differently to HTLV-2, also utilizes heparin sulfate proteoglycan (HSPG). When an infected cell comes into contact with an uninfected cell, a microtubule-organizing center (MTOC) is polarized at cell–cell junctions, termed virological synapses, impacting on the localization of HTLV Gag, Env and the genomic RNA. The formation of the virological synapses occurs when the intracellular adhesion molecule-1 (ICAM-1) is engaged by its ligand lymphocyte function-associated antigen 1 (LFA1) (Nejmeddine et al., 2009). When the viral RNA genome is delivered into the cytoplasm, it undergoes reverse transcription to convert the ssRNA molecule into dsDNA. The dsDNA translocates into the nucleus and integrates into the host cell genome, where the provirus is then transcribed by host RNA polymerase II. Once viral mRNA is transported into the cytoplasm the viral proteins are translated by the cell translation machinery. The structural proteins Gag, Pol and Env, together with the viral genomic RNA, assemble at a virus budding site along the plasma membrane forming an immature virus particle. When the budding particles are released from the plasma membrane the viral protease cleaves the viral polyproteins inducing a maturation of the virus particle (Martin et al., 2016). During primary infection, HTLV-1 has a period of active replication. The subsequent proliferation occurs mainly through clonal expansion of infected cells or by viral synapses. HTLV-1 integration into the host genome shows associations with specific transcription factor binding sites (Melamed et al., 2013), especially STAT1 and p53, and specific sites upstream of certain proto-oncogenes that are associated with ATL (Cook et al., 2014).
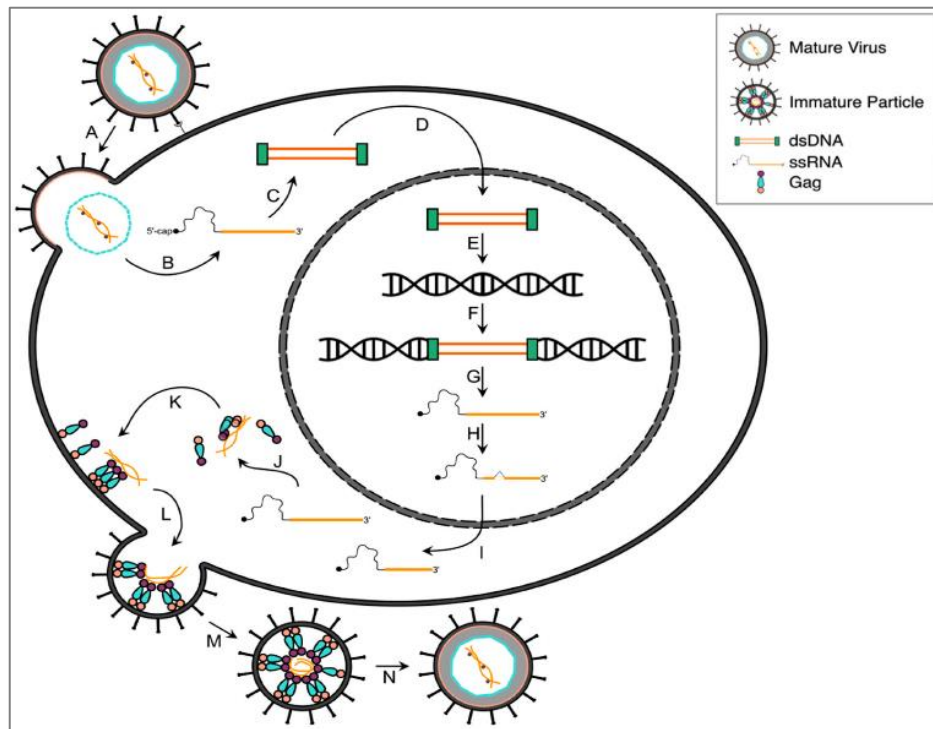
**Figure 4.** HTLV life cycle. Infectious HTLV-1 virion interacts with the target cell surface receptors GLUT1/HSPG/NRP-1 and fuses to the cell membrane (**A**). Following fusion, the viral genomic RNA is delivered into the cytoplasm (**B**), and undergoes reverse transcription to convert the gRNA into dsDNA (**C**). The dsDNA is then integrated into the host genome (**D**, **E, F**). The provirus is then transcribed by cellular RNA polymerase II (**G**), as well as post-transcriptionally modified (**H**). Both full-length and spliced viral mRNAs are exported from the nucleus to the cytoplasm (**I**). The viral proteins are then translated (**J**), and the structural proteins transported to the plasma membrane along with of the gRNA genome (**K**) where they assemble to form an immature virus particle (**L**). The budding particle releases from the cell surface (**M**), and undergoes a maturation process (**N**) (Martin et al., 2017).

## 1.5 HTLV-1 and HTLV-2 Genomic Organization

HTLV-1 and HTLV-2 have a similar genomic structure (**Figure 5**) and share approximately 70% nucleotide sequence homology (Romanelli et al., 2013). HTLV proviral genome, like other retroviruses, encodes the *gag* (structural and core proteins)*, pro* (protease)*, pol* (reverse transcriptase)*,* and *env* (envelope

glycoproteins) genes flanked by the long terminal repeat (LTR). The LTRs contain the U3, R and U5 regions at both the 5'- and 3'-ends, serving as promoters and regulators of viral gene expression. The translation of the polypeptide Gag is followed by cleavage into the 19 kDa matrix protein (p19), 24 kDa capsid protein (p24) and 15 kDa nucleocapsid protein (p15). The poly-protein precursor Env is cleaved into the 46 kDa surface glycoprotein (gp46) and 21 kDa transmembrane protein (p21). In addition, the HTLVs genome produces accessory and regulatory genes corresponding to the open reading frame (ORF) pX, which is located between env and the 3'-LTR.

### 1.5.1 The pX region

The HTLV positive strand of the pX region encodes the regulatory proteins Tax and Rex by the same doubly-spliced mRNA in two separate but overlapping reading frames, and the accessory proteins.
The HTLV negative strand encodes HTLV-1 basic leucine zipper factor (HBZ) protein, and HTLV-2 antisense protein (APH-2), for HTLV-1 and HTLV-2, respectively.

### 1.5.1.1 The accessory proteins

The HTLV accessory genes contribute to the regulation of viral gene expression and the evasion of the host's immune response. The p30Tof/p28, p12/p10, and p21Rex/tRex proteins, in HTLV-1 and HTLV-2, respectively, are considered to be homologous based on their structure and functional properties. Among the other accessory proteins p13 and p8 are unique to HTLV-1, and p11 is peculiar to HTLV-2 (Ciminale et al., 2013). HTLV-1 p30Tof and HTLV-2 p28 play an important role in viral latency, sequestering the tax/rex mRNA in the nucleus. p12 also contributes to hinder lysis of HTLV-1-infected cells by CTL, reducing the expression of the $\beta$ and $\gamma_c$ chains of the interleukin-2 receptor (IL-2R), and of MHC-I (Mulloy et al., 1996, Johnson et al., 2001). The p12 protein is cleaved in p8, which increases cell-to-cell viral transmission through the formation of

intercellular conduits (Prooyen et al., 2010a, b). In analogy to p12, HTLV-2 p10 binds the MHC heavy chain; however, p10 does not bind the IL2R β chain. HTLV-1 p13 influences both the turnover of infected cells and the balance between viral latency and productive infection (Silic-Benussi et al., 2010). The function of HTLV-2 p11 is still unclear; however, it has been demonstrated to bind the MHC heavy chain (Johnson et al., 2000).

### 1.5.1.2 Rex

Rex is the major factor involved in the post-transcriptional regulation; it binds viral mRNAs that contain cis acting sequences termed Rex response element (RxRE) at the R region of the viral LTR. The low expression of Rex during the early stages of the viral gene transcription results in the production of *tax, rex, p30, p12, p13* and *hbz* mRNAs. In the late stages of transcription, the expression levels of Rex become higher and its splicing activity is reduced encoding the singly spliced (*env*) and unspliced (*gag-pro-pol*) mRNAs, which are then transported to the cytoplasm and translated into enzymes and structural proteins. Recent evidences demonstrate the presence of three alternatively spliced transcripts coding for novel Rex isoforms in primary samples from infected patients (Rende et al., 2015). These isoforms exhibit activities comparable to canonic Rex in terms of HTLV-1 protein expression regulation. However, from the analysis of kinetic expression of Rex, it has been observed that the early expression of dicistronic *tax/rex* mRNAs is followed by the expression of the monocistronic *rex* mRNAs, resulting in a prolonged duration of Rex function. The resulting pattern of viral gene expression is important to temporally restrain the expression of highly immunogenic viral epitopes (e.g. Tax, Gag, Env), thus favoring viral persistence and immune response escape (Rende et al., 2015).
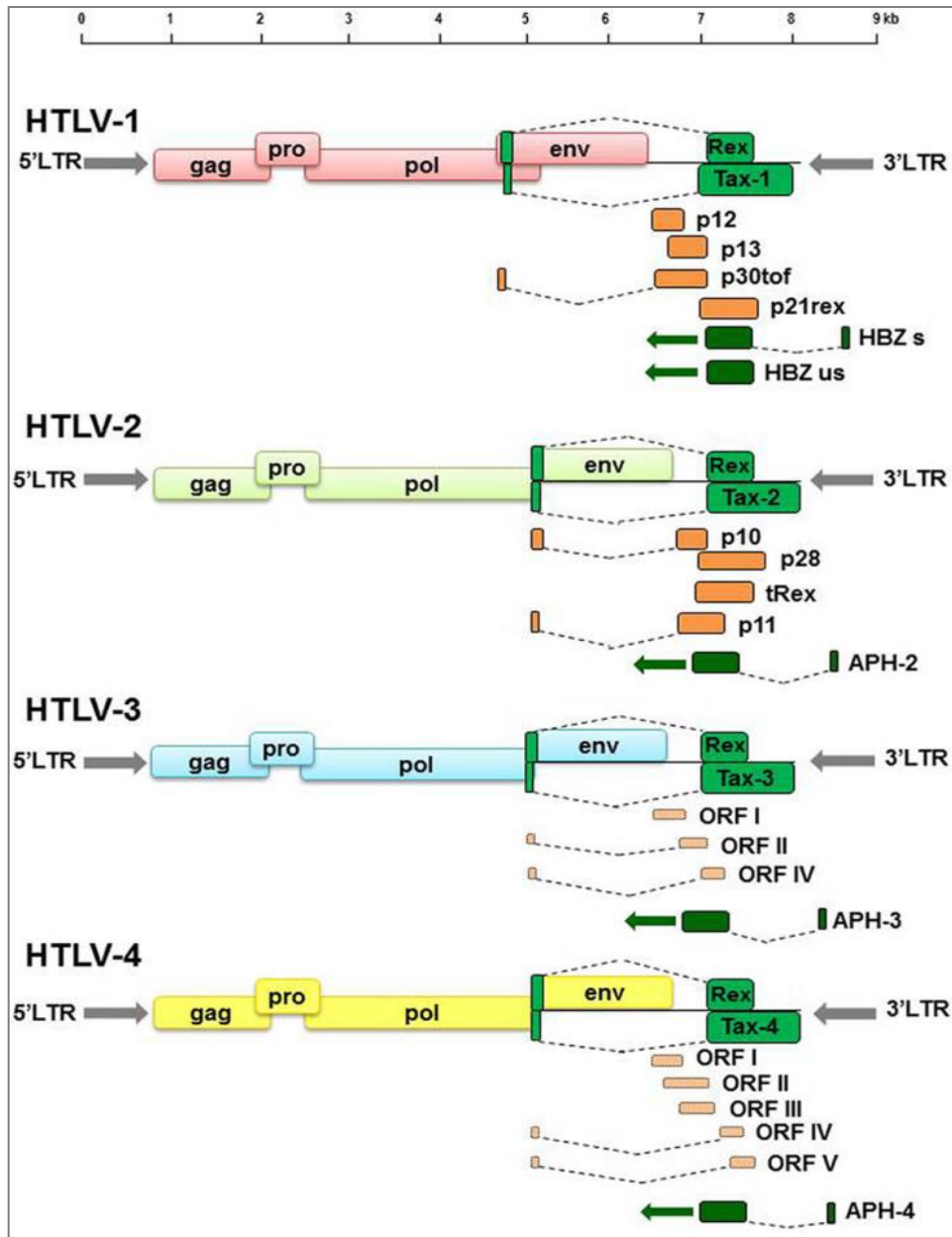
**Figure 5.** Schematic representation of HTLV-1, HTLV-2, HTLV-3, and HTLV-4 genomic organization. Green colored boxes indicate ORF encoding regulatory proteins. Dark orange colored boxes indicate ORF encoding auxiliary proteins. Light orange colored boxes indicate putative ORF deduced by genomic sequence analyses (Romanelli et al., 2013).

**1.6 HTLV Tax oncoproteins**

**1.6.1 Protein Structure of Tax-1 and Tax-2**

The principal role of Tax during viral replication is to activate the LTR promoter transcription through a process that involves the recruitment of CREB/ATF complexes in the U3 region. Tax is recognized as the main viral oncoproteins necessary for the initial steps of T-cell transformation by HTLV. Tax-1 controls many cellular pathways implicated in cell survival and proliferation, directly interacting with components of the PI3K, AKT, MAPK, TGFβ, SRF and NF-κB pathways (Romanelli et al., 2013).

The *tax* gene is highly conserved between all four genotypes and different subtypes of the HTLV (Romanelli et al., 2013). The structural and functional domains of Tax-1, Tax-2, Tax-3, and Tax-4 are shown in **Figure 6**. The most characterized Tax-2 protein is the Tax-2B subtype that shares 75% nucleotide sequence homology and 85% amino acid sequence similarity with Tax-1. The N-terminal region of all Tax proteins contains an ATF/CREB-activating domain and two functional regions involved in CREB-binding protein (CBP)/p300 binding in the C-terminal region, which are required for HTLV transcription regulation, interaction with proteins involved in transcription, cell cycle progression, and cell signaling regulation (Rende et al., 2012). A nuclear localization signal (NLS) is present within the first 60 aa in Tax-1, Tax-3 and Tax-4, whereas Tax-2 possesses a nuclear localization determinant (NLD) within the first 42 aa (Romanelli et al., 2013). All Tax proteins contain a nuclear export sequence (NES) that is located at aa position 189–202 in Tax-1 and Tax-2 (Alefantis et al., 2003; Chevalier et al., 2005). The main characteristic that distinguishes the Tax-1 and Tax-2 is that only Tax-1 presents two leucine zipper-like motif regions (LZRs) at the 116-145 aa and 213-248 aa positions, whereas Tax-2 does not. These regions are required for Tax-1 to activate the non-canonical NF-κB pathway through interaction with the p100 factor (Shoji et al., 2009). Tax-1 and Tax-3, in contrast to Tax-2 and Tax-4 are characterized by the presence of a PDZ-binding motif (PBM) at the C-terminal region (Chevalier et al., 2006).
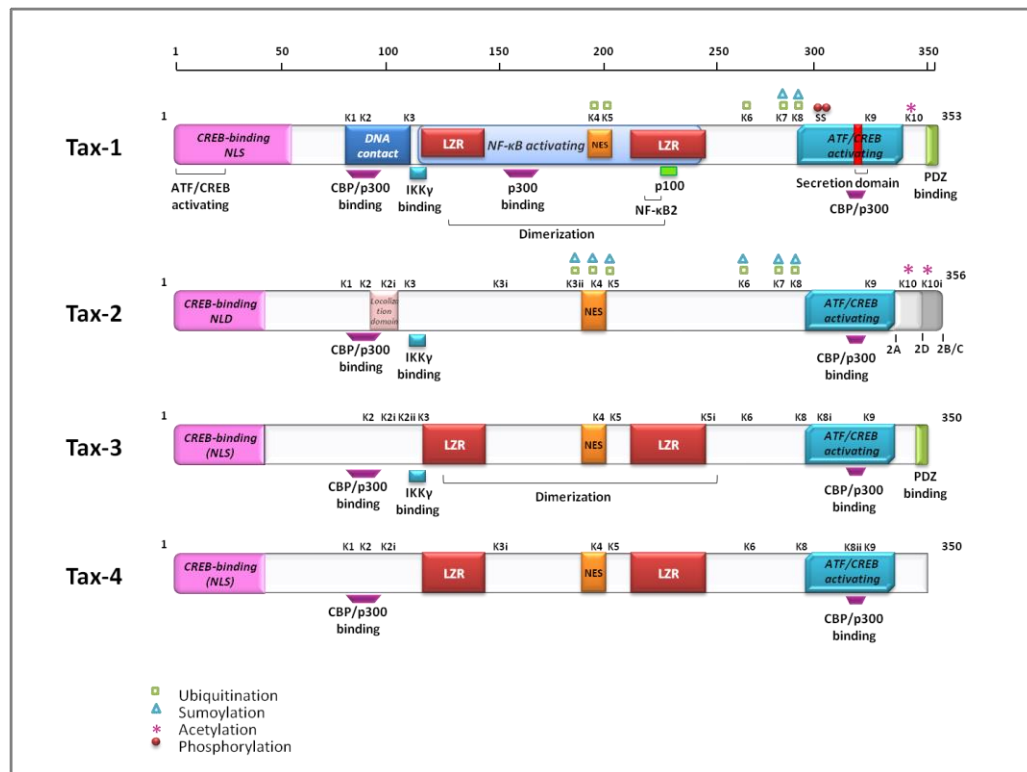
**Figure 6**. Structural and functional domains of the Tax proteins (Romanelli et al., 2013).

## 1.6.2 Cellular Localization of Tax-1 and Tax-2 Proteins

Tax-1 prevalently localizes into the nucleus in speckled structure or nuclear bodies (NBs) that are site of transcriptional activity (**Figure 7**). Promyelocytic leukemia (PML) protein–containing nuclear bodies also have been functionally linked to transcription, of viral genes and other nuclear processes such as RNA processing, transport, and RNP assembly (Mikecz et al., 2000). These unique nuclear structures containing Tax, also include NF-κB p50 and p65 and participate in the Tax-mediated activation of gene expression via the NF-κB pathway (Bertazzoni et al., 2011). In the cytoplasm Tax colocalizes with several host factors, among them we have observed that, Tax relocalizes p65 to cytoplasmic dotted structures which include the NF-κB factors TAB2, NEMO and the calreticulin (Avesani et al., 2010; Turci et al., 2012).

Differently from Tax-1, Tax-2 localizes predominantly in the cytoplasm of the HTLV-2 immortalized or transformed infected T-cells (**Figure 7**) (Marteens et al.,

2004) and in nuclear bodies (Turci et al., 2009). The domain between 89-113 aa confers to Tax-2 the property to accumulate in the cytoplasm.

Comparative analyses of Tax-1 and Tax-2 post-translational modifications highlighted the functional contribution of ubiquitination and sumoylation to the intracellular localization of Tax and its ability to activate NF-κB (Romanelli et al., 2013). Specific lysines are targeted of sumoylation and ubiquitination in Tax-1 and Tax-2 (lysines from K1 to K10 - **Figure 6**). Lysines K6 and K8, which are highly conserved in all Tax proteins, are critical for NF-κB activation.
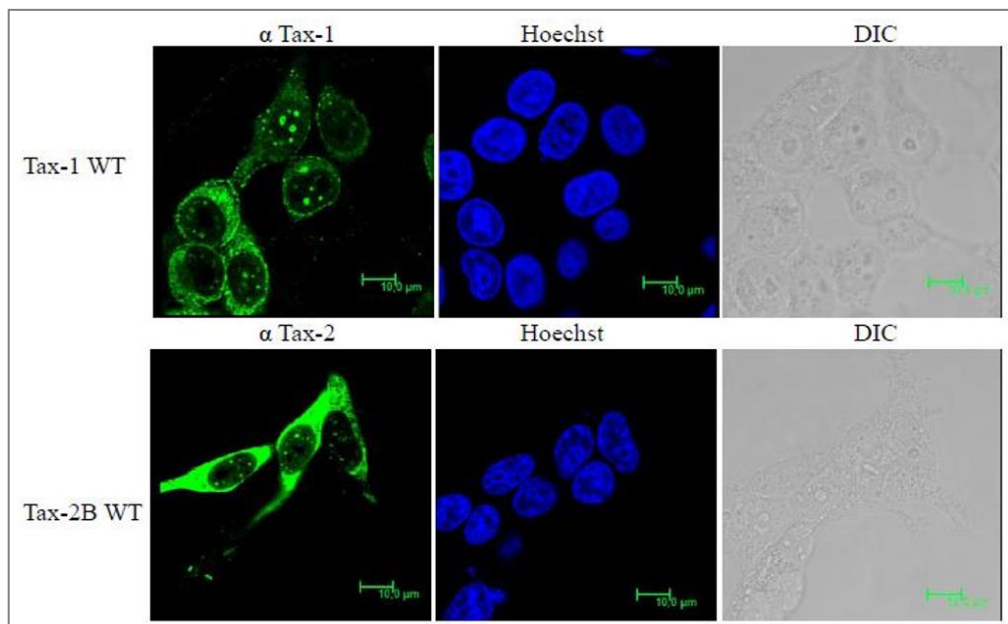


**Figure 7**. Comparison of Tax-1 and Tax-2 subcellular localization (Bertazzoni et al., 2011).

The main structural differences between Tax-1 and Tax-2 reflect different protein interactions, intracellular localization, but also transformation abilities, as described in **Table 2**. Tax-2, compared to Tax-1, is less efficient in the induction of micronuclei formation (Semmes et al., 1996b) and do not alter the expression of the cdk inhibitors p21 and p27 involved in the control of the cell cycle (Tripp et al., 2005).

**Table 2.** Summary of main functional and structural differences between Tax-1 and Tax-2 (Romanelli et al., 2013).

| | Tax-1 | Tax-2[a] | References |
|---|---|---|---|
| Transactivating activity | Higher[b] | Lower[b] | [Semmes et al. 1996a] |
| Transformation capacity | Higher | Lower | [Endo et al. 2002] |
| Micronuclei formation | + | − | [Semmes et al. 1996b] |
| Cell cycle arrest | + | − | [Tripp et al. 2005] |
| Hematopoiesis suppression | + | − | [Kubota et al. 1994] |
| Reduction of histone gene expression | + | − | [Ego et al. 2002; Harrod et al. 2000] |
| Inhibition of p53 functions | Higher | Lower | [Mahieux et al. 2000; Meertens et al. 2004; Jeong et al. 2005; Calattini et al. 2006] |
| Total viral mRNA expression | Higher | Lower | [Li and Green 2007] |
| Proinflammatory cytokine expression | Higher | Lower | [Banerjee et al. 2007] |
| Presence of PDZ motif | + | − | [Feuer and Green 2005] |
| Interaction with PDZ binding proteins | + | − | [Higuchi and Fujii 2009] |
| Interaction with p100 | + | − | [Shoji et al. 2009] |
| Preferential cellular localization | Nucleus | Cytoplasm | [Turci et al. 2009] |
| NF-κB transactivation | + | + | [Chevalier et al. 2012] |
| NF-κB transactivation (lipid raft translocation of IKK) | + | − | [Huang et al. 2009] |
| *In vitro* CK2 phosphorylation | + | − | [Bidoia et al. 2010] |
| Oligo-sumoylation | + | − | [Turci et al. 2009] |
| Nuclear bodies | Larger | Smaller | [Turci et al. 2009] |
| Ubiquitination and sumoylation | + | + | [Zane et al. 2012] |
| Nuclear localization | + | + | [Calattini et al. 2006] |
| T-cell immortalization | + | + | [Imai et al. 2013; Chevalier et al. 2006] |

[a] The properties of Tax-2 include both the results of Tax-2A and Tax-2B reported in the literature.

[b] Higher and lower are relative to Tax-1 and Tax-2 comparison.

### 1.6.3 The Transforming Activity of Tax

The expression of Tax is crucial for promoting human T lymphocyte survival, proliferation and initiating HTLV-1-mediated oncogenesis (Ren et al., 2013). The transforming activity of Tax was first demonstrated using HTLV-1 infectious molecular clone in which the *tax* gene was disrupted. The wild type molecular clone easily transformed human primary T cells, while the tax mutant molecular clone failed to do so (Akagi et al., 1997). In Tax transgenic mice model, Tax-1

induces ATL-like leukemia (Grossman et al., 1995). The transforming activity of Tax is dependent on its ability to activate NF-κB, in fact Tax mutant defective in activating NF-κB fails to transform cells *in vitro*. In addition, Tax activates the transcription of cellular genes by activating the activating protein-1 (AP-1) transcription. This action is thought to contribute to the deregulated phenotypes and leukemogenesis of T cells infected with HTLV-1 (Gazon et al., 2017). AP-1 is composed of 18 dimeric complexes which included members of four families of DNA-binding proteins. Among these factors, c-Fos, Fra-1, c-Jun, JunB, and JunD genes have been shown to be activated by Tax at the transcriptional level (Fujii et al., 2000; Iwai et al., 2001). Studies conducted on Tax transgenic mouse model highlighted that, functional inactivation of p53 by HTLV-1 Tax is not critical for the initial tumor formation, but contributes to late-stage tumor progression (Portis et al., 2001).

Recent studies have demonstrated that, in addition to Tax, the antisense protein HBZ plays an essential role in oncogenesis by regulating viral transcription and modulating cellular signaling pathways (Satou and Matsuoka, 2012).

**1.7 HBZ and APH-2 antisense proteins from HTLV-1 and HTLV-2**

**1.7.1 Protein Structure of HBZ and APH-2**

HBZ and APH-2 are encoded by the minus strand from the 3' LTR of HTLV-1 and HTLV-2 proviral genome, respectively. HBZ was first characterized for its ability to inhibit Tax-mediated viral transcription (Zhao et al., 2016). The inhibition is carried out by: the interaction between HBZ and CREB-2/CREB, and/or the interaction with CBP/p300, resulting in the inhibition of Tax-mediated recruitment of these transcription factors to the HTLV-1 promoter (Zhao et al., 2016). APH-2 can suppress Tax2-mediated viral transcription by a similar mechanism (Halin et al., 2009).

Two transcripts have been reported to be encoded by *HBZ* gene: a 206 amino acid protein spliced (sHBZ) and a 209 aa protein unspliced (usHBZ). Evidences demonstrated that the spliced form of *HBZ* gene is expressed in all ATL cells (Satou et al., 2006). HBZ protein is characterized by three distinct domains: 1) an

activation domain which contains two LXXLL-like motifs important for protein-protein interactions at the N-terminus and with transactivating potential; 2) a central domain which contains NLSs; 3) a basic leucine zipper (bZIP) domain for DNA binding. The bZIP domain enables HBZ to hetero-dimerize with cellular bZIP proteins such as CREB2, c-Jun, JunB, JunD, CREB, and ATF3 (Barbeau et al., 2013) (**Figure 8**). Concerning APH-2, only a spliced mRNA has been identified (Halin et al., 2009). APH-2 is a 183 aa protein, which exhibits less than 30% homology to HBZ. APH-2 contains a non-canonical bZIP region responsible for its interactions with c-Jun and JunB and a C-terminal CREB-binding motif responsible for its interaction with CREB (Halin et al., 2009; Marban et al., 2012). Unlike HBZ, APH-2 is not able to bind p300. The region from aa 102 to 183 is required to form complex between Tax-2B and APH-2 (Marban et al., 2012).
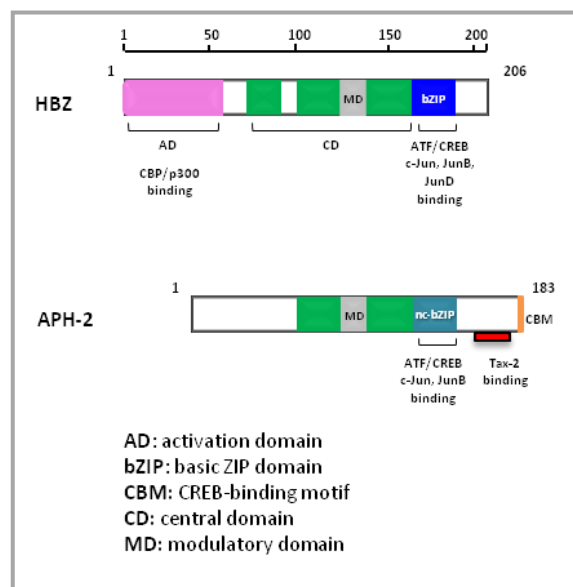


**Figure 8**. Schematic representations of HBZ and APH-2 functional domains (modified from Fochi et al., 2018).

### 1.7.2 Cellular Localization of Antisense Proteins

HBZ exhibits a speckled distribution into the nucleus in infected cells and ATL cells, colocalizing with nucleolar structures (Raval et al., 2015). Three regions distributed in the central domain of the protein are associated with nuclear localization, including a DNA binding domain. HBZ contains a functional NES

site in its N-terminal region and it is exported from the nucleus via a CRM1-dependent pathway. Nuclear export of HBZ is essential for the induction mTOR pathway, resulting in the inhibition of the autophagic machinery (Mukai et al., 2014). In a recent study, it has been demonstrated that, in contrast with the HBZ nuclear localization in ATL, HAM/TSP patients show a HBZ localization confined to the cytoplasm, proposing this feature as a possible biomarker for HTLV-1-associated HAM/TSP (Baratella et al., 2017). The HTLV type 2 counterpart, APH-2, localizes in the nucleus, and its distribution in the cytoplasm is more evident compared to HBZ (Halin et al., 2009).

### 1.7.3 The Oncogenic Potential of HBZ

Approximately 60% of ATL cases lack Tax expression because of genetic and epigenetic changes in the proviral genome of HTLV-1, whereas HBZ is consistently expressed in all ATL cases (Zhao, 2016). The constitutive expression of HBZ promotes the spread of infection (Matsuoka and Green, 2009; Zhao and Matsuoka, 2012). HBZ is not essential for *in vitro* immortalization and but it is required for efficient infection, T-cell survival, and persistence of the virus *in vivo* (Arnold et al., 2006; Arnold et al., 2008). Several studies have provided evidences for HBZ role in HTLV-1 oncogenesis, which may be summarized in five actions: a) suppression of viral transcription mediated by Tax; b) promotion of T-cell proliferation; c) suppression of cellular apoptosis and senescence; d) induction of regulatory T-cell differentiation; e) impairment of cell-mediated immunity (Zhao et al., 2016).

A limited number of studies have compared the effects of APH-2 and HBZ on cellular pathways. The main results are summarized in **Table 3.** The comparative studies between HBZ and APH-2, have indicated that APH-2, unlike HBZ, does not promote T cell proliferation and lymphocytosis (Douceron et al., 2012). These different functions represent interesting aspect to investigate in order to clarify the distinct pathobiologies belonging to HTLV-1 and HTLV-2. Like HBZ, the APH-2 expression has been found to positively correlate with the proviral load in HTLV-2-infected carriers (Douceron et al., 2012). Interestingly, studies conducted in rabbits infected with HTLV-2 have demonstrated that the absence of APH-2

results in a high antibody response to the viral antigens and in an increased proviral load (Yin et al., 2012), suggesting that HTLV-2 replicates better when APH-2 is not expressed. HBZ and APH-2 show opposite effect on the modulation of cellular pathways, such as TGF-β and IRF-1 cell signaling and in the activation of AP-1 transcription.

**Table 3**. Overview of HBZ and APH-2 regulatory functions (Panfil et al., 2016).

| Function | Finding for: | |
|---|---|---|
| | HBZ | APH-2 |
| Expression in HTLV-infected cells | Yes (100%) | Yes (most but not all cases) |
| Required for in vitro immortalization | No | No |
| Required for efficient in vivo infection and persistence | Yes | No |
| HTLV 5' LTR[a] transcription | Inhibits | Inhibits |
| Promotion of T-cell proliferation | Yes | No |
| AP-1 transcription | Inhibits | Activates |
| JunD transcription | Activates | Activates |
| TGF-β signaling | Activates | Inhibits (if overexpressed) |
| Modulation of classical NF-κB pathway | Inhibits | Inhibits |
| Modulation of IRF-1 pathway | Inhibits | Activates |

[a]LTR, long terminal repeat.

**1.8 Effect of Tax, HBZ and APH-2 on NF-κB Signaling Transduction**

As stated above, one of the mayor effects derived by of Tax and antisense protein expression is the deregulation of cellular pathways. The deregulation of the NF-κB pathway has been investigated during the study presented in this PhD thesis. In the following paragraphs, it will be summarized the current knowledge of the involvement of Tax, HBZ and APH-2 on NF-κB signaling.

**1.8.1 NF-κB Pathways**

NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells, is a family of transcriptional factors that control a large number of cellular processes, such as immune and inflammatory responses, developmental processes, cell proliferation, apoptosis and oncogenesis (Masoumi et al., 2011). It has been demonstrated that aberrant NF-κB plays a crucial role in the initiation and progression of cancer. Many viruses have developed strategies to manipulate NF-κB signaling through the use of viral proteins. NF-κB family proteins consist of five transcriptional factors, p65 (termed also RelA), RelB, c-Rel, NF-κB1 (known also p50) and NF-κB2 (alias p52). The different NF-κB members form various homodimers and heterodimers and once located in the nucleus, transactivate target genes bearing a κB enhancer sequence.

The activation of the NF-κB pathway occurs through two distinct pathways, known as the canonical/classical pathway and the non-canonical/alternative pathway, which involve different upstream, intermediated and effector factors (**Figure 9**). The classical NF-κB pathway is triggered by pro-inflammatory cytokines, which bind relevant cell surface receptor such as tumor necrosis factor receptor 1 (TNFR1) or Toll-like receptor. The ligand-receptor binding leads to the recruitment of the adaptor proteins as TNF receptor associated factors (TRAFs), tumor necrosis factor receptor type 1-associated DEATH domain protein (TRADD), or receptor interacting protein (RIP) to the cytoplasmic domain of cell membrane receptors. These adaptor proteins assemble a platform to recruit and activate the IKK complex (IκB kinase), composed of two catalytic kinase subunits, IKKα and IKKβ, and the regulatory non-enzymatic scaffold protein NEMO (termed also IKKγ). The activation of the IKK complex lead to the phosphorylation and degradation of the IκB inhibitor, that in physiological conditions, sequester the NF-κB proteins in the cytoplasm. This mechanism results in the nuclear translocation of the p50/RelA transcriptional factors.

In contrast, the non-canonical NF-κB pathway is induced by lymphotoxin B (LT-β), B-cell activating factor (BAF) or CD40 and involves an IKK complex formed by two subunits of IKKα kinases activated by the NF-κB-inducing kinase (NIK). The upstream ubiquitin ligase TRAF3, together with TRAF2 and cIAPs proteins,

_____

acts as a negative regulator of the alternative pathway targeting NIK for constant ubiquitination and proteasomal degradation, in unstimulated cells. Upon stimulation, TRAF3 is degraded, allowing NIK to activate the IKKα complex, resulting in the processement of the precursor p100 to yield p52 and the final release of p52/RelB active heterodimer in the nucleus (Durand et al., 2017).
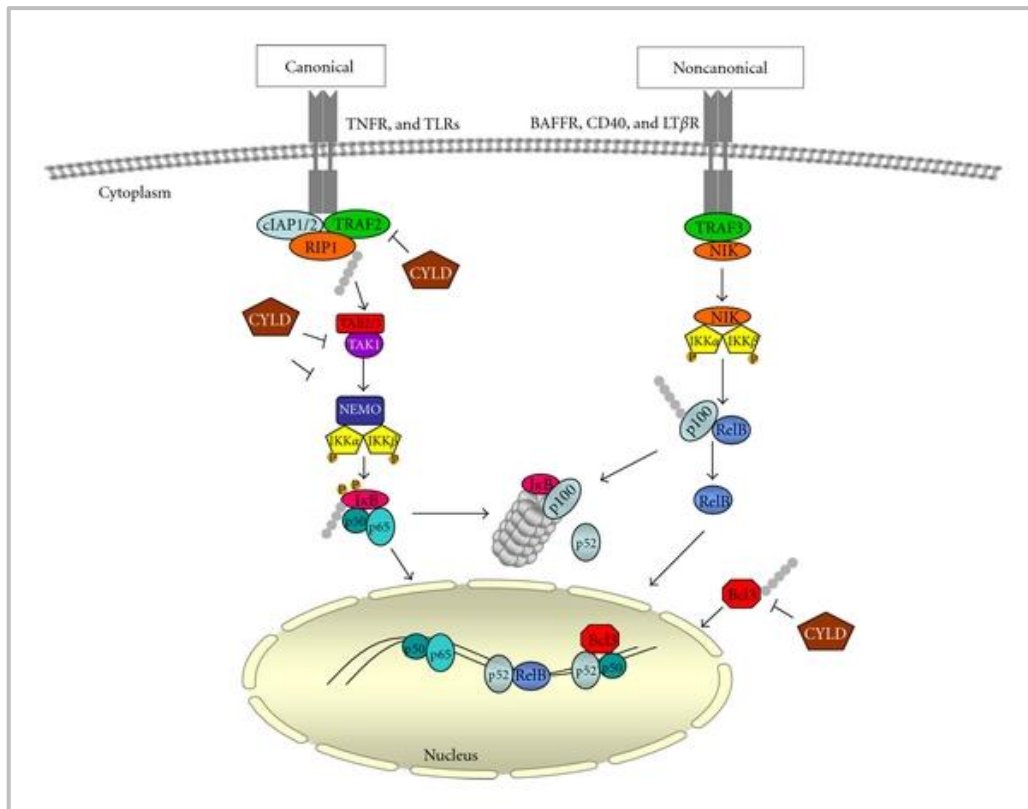


**Figure 9.** The NF-κB pathway (Masoumi et al., 2011).

## 1.8.2 Tax and NF-κB Signaling

It is well established that Tax-1 is a potent transactivator of the NF-κB cell signaling and its activity is critical for immortalization and transformation of HTLV-1-infected T cells (Giam et al., 2016). The activation of NF-κB, results in the transcriptional activation of many cellular genes that govern normal growth-signal transduction, such as cytokines and growth factors, including IL-2, IL-6, IL-15, TNF, GM-CSF, proto-oncogenes (c-Myc), and antiapoptotic proteins (bcl-xl) (Hiscott et al., 2001). Tax-mediated activation of the NF-κB transcription

factors, involves two principal molecular mechanisms: the recruitment of Tax in cellular protein complexes (Qu and Xiao, 2011; Bertazzoni et al., 2011) and post-translational protein modifications (Lavorgna and Harhaj, 2014). Comparative studies between Tax-1 and Tax-2 highlighted relevant differences in the activation of NF-κB because of their divergent ability to interact with NF-κB members (**Figure 10**). Tax-1, unlike Tax-2, triggers the activation of the non-canonical pathway, directly interacting with p100 and promoting its processment to yield p52 (Higuchi et al., 2007; Shoji et al., 2009). Both Tax proteins interact with TAB2 and NEMO/IKKγ stimulating the translocation of the p50/p65 heterodimers into the nucleus, but only Tax-1 interacts with TRAF6, an E3 ligase that triggers the ubiquitination and activation of the downstream NF-κB signaling cascade (Avesani et al., 2010; Journo et al., 2013). We also contributed in a recent study to the identification of complex formation between Tax and two non-canonical IκB kinases, IKKε and TBK1, which act as regulatory factors in IFNα, STAT3 and NF-κB signaling (Shen and Hahn, 2011; Diani et al., 2015). The presence of Tax and TBK1 in lipid raft microdomains along with canonical IκB, supports the role of Tax as molecular crosstalker between the canonical IKKs and TBK1/IKKε, through NF-κB activity (Zhang et al., 2016). It is well known that Tax-1 forms complexes with the ubiquitin-conjugating enzyme *Ubc13,* NEMO, Tax1 binding protein1 (TAX1BP) and NRP/Optineurin in the membrane lipid rafts microdomain. Interestingly, in these complexes, the cell adhesion molecule 1 (CADM1) acts as a molecular scaffold recruiting Tax-1 and this interaction results in the activation of IKK complex and inactivation of the NF-κB negative regulator A20 enzyme (Pujari et al., 2015). An additional consequence of the Tax reorganization of the component of the lipid raft is the autophagy deregulation. Tax, in fact, acts as molecular crosstalker connecting the IKK complex to the autophagic complexes by interacting directly with Beclin1 and PI3KC3, participating in the assembly of authophagosomes (Ren et al., 2015; Chen et al. 2015). Tax induction of NF-κB also increases the expression of inhibitors of apoptosis, such as the anti-apoptotic c-Flip gene, and genes involved in cell cycle progression (Bangham and Matsuoka, 2017).
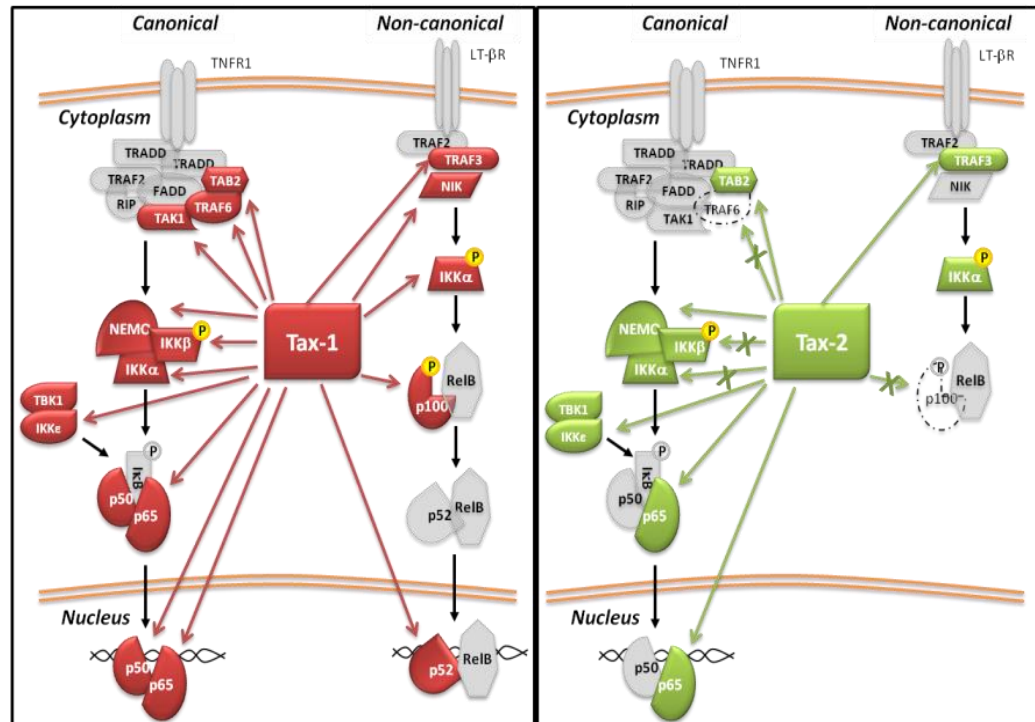
**Figure 10.** Schematic representation of Tax-1 (in red) and Tax-2 (in green) interactions with factors of NF-κB pathway (Romanelli et al., 2013).

Currently, few host factors have been defined to exert a suppressive role on Tax–activation of NF-κB. Among them, the transcriptional regulator of the major histocompatibility complex class II (CIITA), which inhibits the persistent activation of NF-κB in two different manners. In the cytoplasm CIITA retains Tax impairing the nuclear translocation of p65, while in the nucleus, CIITA directly interacts with Tax-1/p65 in nuclear bodies, preventing Tax-1 mediated activation of NF-κB-responsive promoters (Forlani et al., 2013; Forlani et al., 2016). In addition to CIITA, the apoptotic regulator Bcl-3 inhibits p65 nuclear translocation and its DNA binding activity, resulting again in a downregulation of Tax-induced NF-κB activation (Wang et al., 2013). The reduction of Tax-NF-κB activation may also derive by Tax proteosomal degradation induced by host factor interaction (Lavorgna and Harhaj, 2014).

A second mechanism required for Tax-1 and Tax-2 mediated NF-κB activation occurs by post-translational modification, which includes ubiquitination, SUMOylation and phosphorylation. It is well known that Tax phosphorylation is

necessary for Tax nuclear translocation in the nuclear bodies where co-localize with p65 (Bex et al., 1997; Turci et al., 2006). The requirement of ubiquitination and SUMOylation is intensively investigated, but not fully clarified. A recent study proposed Tax as a ubiquitin E3 ligase protein, which catalyzes the assembly of mixed polyUb chains, in association with ubiquitin-conjugating enzyme (E2) (Wang et al., 2016). Afterwards, Shibata *et al.* demonstrated that Tax does not possess E3 ligase activity and they proposed a model in which Tax acts as a scaffold protein recruiting ubiquitin ligases, generating K63- and M1-linked hybrid polyubiquitin chains. In this interacting platform termed by the authors Taxisome, NEMO proteins and Ub-chains lead to the activation of the IKK complex (Shibata et al., 2017).

An additional mechanism that operates in the cells to maintain the NF-κB activation induced by Tax is the positive feedback loop derived by NF-κB target genes. Recently, it has been described that both NF-κB activation and Tax-induced the early growth response protein 1 (EGR1) stability upregulated EGR1, which in turn enhanced constitutive NF-κB activation (Huang et al., 2017). A similar positive loop has been defined by the overexpression of the interleukin receptor IL-17RB. Tax-1 promotes the expression of IL-17RB by NF-κB activation and establishes an IL-17RB-NF-κB feed-forward autocrine loop that maintains persistent NF-κB activation (Lavorgna et al., 2014).

### 1.8.3 HBZ, APH-2 and NF-κB Signaling

HBZ suppresses the classical NF-κB pathway activation, mediated by both the viral protein Tax and the host transcription factor p65 (Zhao et al., 2009). This inhibition derives by four properties of HBZ: a) the interaction with p65; b) the inhibition of p65 DNA binding; c) the enhanced degradation of p65 through PDLIM2 E3 ubiquitin ligase; d) the reduction of p65 acetylation (**Figure 11**). All these processes consequently result in the reduction of the expression of several NF-κB target genes (Zhao et al., 2009; Wurm et al., 2012). In this vein, Ma *et al.* demonstrated in a recent study that HBZ-mediated NF-κB inhibition contributes to the suppression of *cyclin D1* gene, an essential regulator of the G1/S phase

transition of the cell cycle (Ma et al., 2017). Many evidences supported the hypothesis that HTLV-1 may benefit from the antagonistic functions of Tax and HBZ in the deregulation of cellular signaling pathways, resulting in the loss of control of many biological processes such as cell proliferation and survival of HTLV-1-infected cells (Karimi et al., 2017).
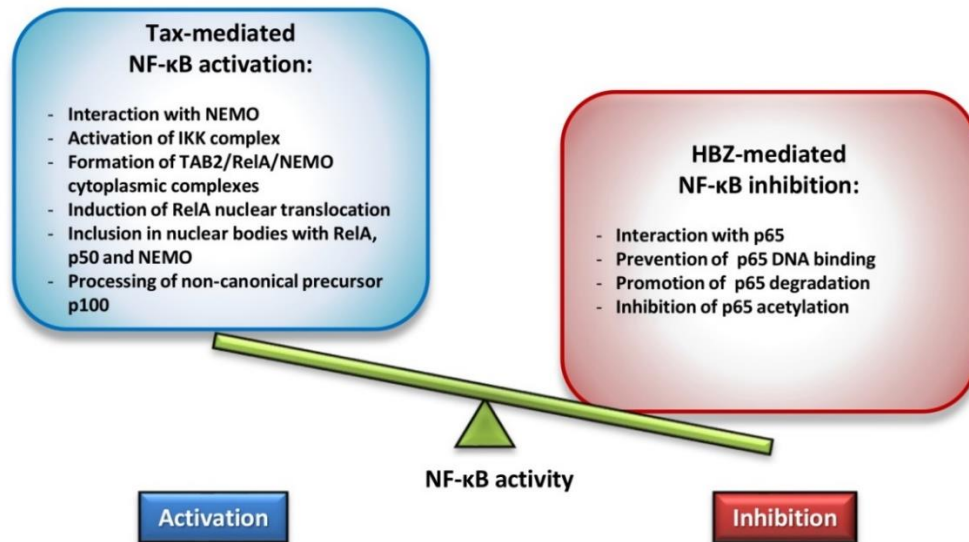


**Figure 11.** Tax and HBZ effect on NF-κB. Schematic representation of the opposite effects in regulating NF-κB activity mediated by Tax-1 and HBZ (Fochi et al., 2018).

The hyper-activation of NF-κB induced by Tax results in an arrest of cell proliferation due to its induction of the cyclin-dependent kinase inhibitors p21 and p27 over-expression (Kuo et al., 2006; Zhang et al., 2009; Zhi et al., 2011). In contrast, the HBZ downregulation of NF-κB mitigates this phenomenon termed Tax-induced cellular senescence (Zhi et al., 2011). It has been proposed that the p21 and p27 function impairment, together with the HBZ downregulation of NF-κB, may contrast senescence induced by Tax, and may promote expansion of HTLV-1-infected cells (Giam and Semmes, 2016). **Table 4** summarizes the results of the comparative studies of Tax-1 and Tax-2, HBZ and APH-2 on the NF-κB cell signalling pathway (Fochi et al., 2018).

A recent study revealed that APH-2, like HBZ, inhibits NF-κB activation, but they differ in their molecular action (Panfil et al 2016). They may act differently on the

non-canonical NF-κB pathway, but the molecular mechanism is unknown and need to be investigated.

**Table 4.** Comparative effect of the HTLV regulatory proteins on the NF-κB pathway (Fochi et al., 2018).

| | Tax-1 | Tax-2 | References |
|---|---|---|---|
| Canonical NF-κB transactivation | + | + | Sun et al., 1994 |
| Non-canonical NF-κB transactivation | + | - | Higuchi et al., 2007 |
| NF-κB transactivation (lipid raft translocation of IKK) | + | - | Huang et al., 2009 |
| Interaction with p100/p52 | + | - | Shoji et al., 2009 |
| Interaction with p65 | + | + | Zhao et al., 2009; Panfil et al., 2016 |
| | **HBZ** | **APH-2** | **References** |
| Canonical NF-κB transactivation | + | **+** | Zhao et al., 2009; Panfil et al., 2016 |
| Non-canonical NF-κB transactivation | - | nd | Zhao et al., 2009 |
| Interaction with p65 | + | + | Zhao et al., 2009; Panfil et al., 2016 |
| Inhibition of Tax-mediated transactivation of NF-κB | + | nd | Zhao et al., 2009 |
| Binding to Tax | - | + | Marban et al., 2012 |
| Inhibition of p65 DNA binding capacity | + | nd | Zhao et al., 2009 |
| p65 degradation | + | - | Panfil et al., 2016 |
| Inhibition of p65 acetylation | + | nd | Wurm et al., 2012 |

nd, Not determined

## 1.8.4 HTLV proteins deregulation of NF-κB in pathogenesis

The HTLV-1 infection may lead to two alternative outcomes as shown in **Figure 12**. The levels of Tax expression in infected cells are determined by the proviral integration sites (transcriptionally active loci or transcriptionally less active loci).

_____

High levels of Tax override HBZ-dependent inhibition of viral replication, NF-κB hyperactivation, and cellular senescence. When the levels of Tax are low, HBZ inhibits LTR trans-activation, NF-κB activation, and senescence induction by Tax and viral mRNA nuclear export by Rex. Moderated NF-κB activation and other mitogenic activities of Tax, in turn, promote survival and oligoclonal expansion of HTLV-1-infected T cells due to the presence of HBZ and complemented by the immunogenic Tax that is expressed but at low levels. The latently cells can be selected by cytotoxic T lymphocyte resulting in a control of virus replication in asymptomatic carriers (Zhi et al., 2011; Giam and Semmes, 2016). A fine regulation of the balance between Tax and HBZ expression, is required for HTLV-1 positive cell transformation and expansion.
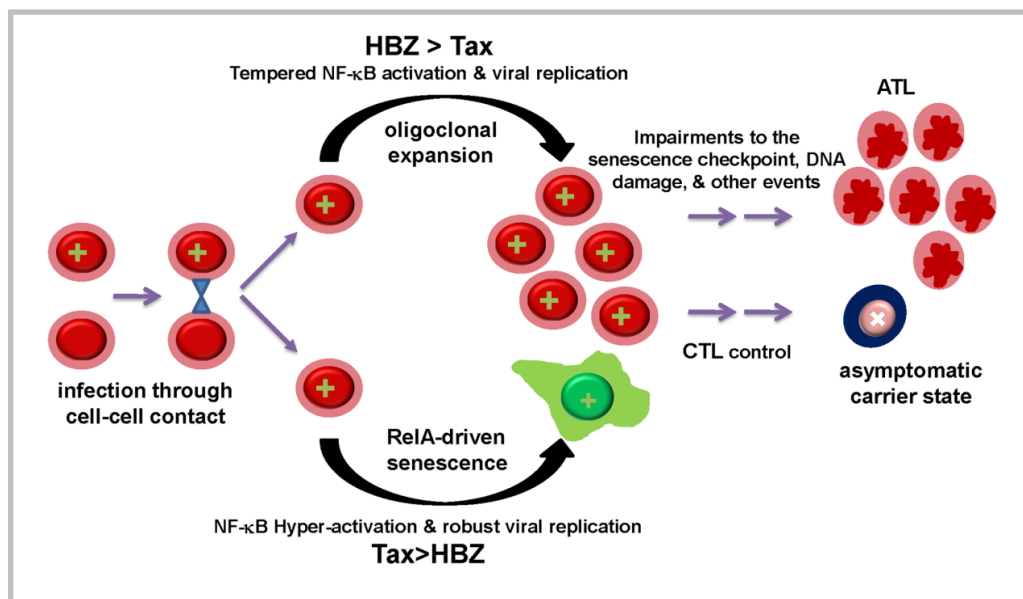


**Figure 12.** The balance between Tax and HBZ expression regulates the outcome of HTLV-1 infection (Zhi et al., 2011).

### 1.8.5 The host factor TRAF3 and the alternative NF-κB pathway

One of the key factors acting on the non-canonical NF-κB pathway is represented by the adaptor protein TRAF3 that we have previously demonstrated to interact with both Tax-1 and Tax-2. TRAF3 belongs to the tumor necrosis factor receptor (TNFR) -associated factor (TRAF) proteins, which are essential components of

signaling pathways activated by TNFR or Toll-like receptor (TLR) family members. TRAFs control many biological processes, including cell survival and the production of cytokines. TRAFs has the ability to bind receptors on the cellular membrane and to recruit the cellular inhibitor of apoptosis (cIAP). TRAF proteins also act as E3 ubiquitin ligases, a function required for the activation of downstream signaling events (Häcker et al., 2011). Emerging evidence indicate that TRAF3 play an essential role in tumorigenesis. Loss-of function mutations of TRAF3 are in fact associated with Hodgkin's lymphoma and multiple myeloma. TRAF3 is also unique because of its different action on several signaling pathways, for example, it positively controls type I interferon (IFN-I) production, but negatively regulates mitogen-activated protein kinase (MAPK) activation and alternative NF-κB. In particular, TRAF3 interacts with TLRs, such as TRIF and MyD88 promoting the recruitment of the kinases TBK1 and IKKε, and the formation of this complexes results in the phosphorylation of IRF-3 (Yang et al., 2015). Alternative NF-κB stimuli induce TRAF3 degradation by the proteasome leading to NIK protein accumulation (Sun, 2017). The accumulation of NIK results in IKKα activation and p100 processing to yield p52, suggesting that TRAF3 degradation is a mechanism sufficient to mediate alternative NF-κB activation (Yang et al., 2015). The degradation of TRAF3 is tightly controlled by the deubiquitinase Otub7b, which is also implicated in the regulation of canonical NF-κB activation via deubiquitination of TRAF6. Another study revealed that TRAF3 could be degraded also in autophagic/lysosomal dependent manner (Xiu et al., 2015). Our research group has recently demonstrated that in transfected cells the IFN-β promoter activation is increased when Tax-1 and TRAF3 were co-expressed with IKKε or TBK1 (Diani et al., 2015). The contribution of TRAF3 in the Tax-modulation of NF-κB is unknown, for this reason we focused our attention on developing studies to assess the functional role of Tax-TRAF3 interaction in the alternative NF-κB pathway.

## 2. AIM OF THE RESEARCH

The retroviruses HTLV-1 and HTLV-2 show strong genomic similarities. HTLV-1 causes adult T-cell leukemia, an aggressive form of leukemia, whereas HTLV-2 has not been associated with cancer. The HTLV-1 viral genome encodes, among others, two oncogenic proteins: the regulatory protein Tax, and the accessory protein HBZ. Tax is a potent transcriptional activator of viral and host genes, that induces cellular transformation. HBZ is required for efficient HTLV-1 infection and persistence. The oncogenic capacity of HTLV-1 is associated, among other effects, to Tax-mediated constitutive activation of the NF-κB pathway. However, Tax-1 is frequently silenced during the late stages of leukemogenesis, in contrast to HBZ, that is always expressed in ATL cells. The functional comparison of HTLV-1 Tax and HBZ proteins and the related HTLV-2 Tax and APH-2 proteins highlights differences that may contribute to clarify their distinct pathobiologies. APH-2 shares with HBZ the ability to repress HTLV transactivation mediated by Tax, but differs in the modulation of several intracellular pathways. Because of their recent discovery, a limited number of studies have compared the effects of HBZ and APH-2 on Tax-modulated pathways, and differences in their action may contribute to define critical steps of oncogenesis.

My thesis project aimed to expand the knowledge of the molecular mechanisms induced by HBZ, APH-2 and Tax in the modulation of the NF-κB signaling. The NF-κB signaling pathway is known to be important for the promotion of T lymphocyte survival and proliferation, and aberrant NF-κB is associated with initiation and progression of cancer. Tax has been reported to constitutively activate NF-κB by several mechanisms. Recent studies have revealed that HBZ antagonizes NF-κB activation and that APH-2 differs from HBZ in the mechanism of NF-κB inhibition; however, the inhibitory mechanism has not yet been clarified. In order to deepen the knowledge of the molecular mechanisms undertaken by the HTLV antisense proteins to deregulate the NF-κB pathway, we focused our attention on two relevant aspects of the NF-κB deregulation mediated by HTLVs oncoproteins: a) their interactions with key factors of the NF-κB

pathway and p65 activation b) their role on alternative NF-κB pathway deregulation.

The experimental approach was based on cell line models transiently transfected with recombinant vectors, expressing Tax and antisense proteins, analyzed for: a) NF-κB promoter activation by luciferase reporter gene assays; b) protein intracellular distribution and co-localization by confocal microscopy analyses; c) protein-protein interaction, by co-immunoprecipitation analyses; d) protein expression levels, by western blot analyses, after cell treatment with specific inhibitors of cellular degradation systems; e) TRAF3 knock-out and its contribution on the alternative NF-κB pathway, applying the CRISPR/Cas9 technique.

The results of this study will contribute to unveiling the involvement of relevant host factors in the mechanisms of HTLV viral proteins deregulation of NF-κB pathway in order to elucidate the HTLV-1 pathogenesis.

## 3. MATERIALS AND METHODS

### 3.1 Cell culture and transfection

Adherent Human embryonic kidney 293T (HEK 293T), HeLa and Human Bone Osteosarcoma Epithelial U2OS cells were maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM), Penicillin G (100 U/L)/Streptomycin (100 mcg/L). Suspension T-lymphocyte cells Jurkat clone E6-1 were grown to a density of $5x10^5$ cells/mL in 1640 RPMI medium supplemented with 10% FCS, L-glutamine (2 mM) and Penicillin G (100 U/L)/Streptomycin (100 mcg/L). All the cell lines were grown at 37°C in a humidified atmosphere with 5% $CO_2$.

For immunoprecipitation and confocal analysis, $4x10^5$ 293T and HeLa cells were plated in 6-well plates. For transactivation studies, $2x10^5$ 293T cells were seeded in 12-well plates. At 70-80% of confluence cells were transfected using TransIT®-LT1 transfection reagent (MIR2300, Mirus Bio), following the manufacturer's protocol. For confocal and transactivation analysis of Jurkat cells, $2x10^6$ cells were transfected by electroporation using the Neon Transfection System (Thermo Fisher Scientific) applying three pulsations of 10 ms at 1,325 V.

### 3.2 Plasmids, antibodies and reagents

pJFE-Tax-1, pJFE-Tax-2B, Tax-M22 and Tax-1 K1-10R full length expression vectors were previously described (Turci et al., 2009). pFlag-APH-2, pGFP-APH-2, pFlag-HBZ, expression plasmids were kindly provided by Dr. Sheehy (Marban et al., 2012). pRSV-RelA/p65, pCMVF-TAB2 and Flag-TRAF3, HA-TRAF3 expression vectors were above described (Avesani et al., 2010; Diani et al., 2015). pEF-p52 was kindly provided by Dr. Fujii and Dr. Matsuoka (Zhao et al., 2009). pCDNA3-VSV-APH-2, pSG5M-Tax2-His, His-HBZ were kindly provided by Dr. Mahieux (Journo et al., 2013). pSpCas9(BB)-2A-Puro (PX459) V2.0 vector was purchased from Addgene. NF-κB-Luc and phRG-TK plasmids were previously described (Bergamo et al., 2017).

The primary antibodies used in western blot, co-immunoprecipitation and immunofluorescence staining experiments were anti-Flag (Sigma-Aldrich), anti-

VSV (Sigma-Aldrich), anti-His (Abcam), anti-HA (Sigma-Aldrich or Covance), anti-V5 (Invitrogen), anti IκB (Cell Signaling), anti-TRAF3 (ProteinTech), anti-p65 (Santa Cruz, Abcam or Merck Millipore), anti-IKKγ (BD Bioscience), anti-NF-κB2 p100/p52 (Cell Signaling Technology) and anti-β-tubulin (H-235). The anti-Tax-1 monoclonal antibody derives from hybridoma 168-A51 (AIDS research and Reagent Program, National Institutes of Health). The anti-Tax-2 rabbit polyclonal specific antibody was obtained as previously described (Turci et al., 2012). The secondary antibodies used in western blot analysis were the goat anti-mouse HRP-conjugated and goat anti-rabbit HRP-conjugated (Thermo Scientific). The secondary antibodies used in immunofluorescence staining analysis were anti-rabbit488 (Abcam), anti-goat488 (Abcam), anti-mouse549 (Vector Lab), anti-goat TexasRed (Jackson Imm.Res.), anti-mouse 647 (Abcam), anti-rabbit 649 (AbD serotec). Bafilomycin A (BFA) and MG132 were from Santa Cruz and Sigma-Aldrich.

### 3.3 Co-immunoprecipitation

293T cells were transfected with 1μg of p65, p52, HA-TRAF3, Flag-APH-2, Flag-HBZ, Tax-1 or Tax-2B expressing vectors. 24h after transfection 293T were washed with PBS (phosphate-buffered saline) and lysed in non-denaturing buffer containing 10mM Tris–HCl pH 7.5, 5mM EDTA, 150mM NaCl, 1% TritonX-100, and supplemented with protease inhibitors Complete Protease Inhibitor Cocktail EDTA-free (Roche). Lysates were sonicated twice for 5s and frozen at -80°C for 1h and centrifuged for 30min at 14000 rpm at 4°C. 1/10 of cell lysate was stored at -20°C and then analyzed by western blot to verify the expression of interest proteins (input). Cellular lysates were subjected to co-immunoprecipitation with the appropriate primary antibodies overnight at 4°C. The immunocomplexes were linked to magnetic beads of either Dynabeads Protein G or A (Life Technologies) for 30min at 4°C. The beads were then washed 3 times and resuspended in elution buffer containing Tris-buffered saline and Tween-20 (TBS-T), NuPAGE loading buffer (Life Technologies) and 0.25 mM DTT.

### 3.4 Western blot analysis

293T cells were transfected with Flag-TRAF3, Flag-APH-2, Flag-HBZ, Tax-1 or Tax-2B expressing vectors in a range from 0.5 to 3μg. The concentration of the total proteins in cell lysates was determined by Bradford Comassie brilliant blue assay (Sigma-Aldrich). Equal amounts of cellular proteins were resolved in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Polyvinylidene fluoride membrane (PVDF) (GEHealthcare). The membranes were first blocked in TBS solution containing 5% nonfat-milk and 0.1% Tween-20 for 1h at room temperature, incubated overnight at 4°C with specific primary antibodies and 1h at room temperature with secondary antibodies. Anti-β-tubulin blot was used for the protein loading control. The bound antibodies were visualized with appropriate HRP-linked secondary antibodies using ECL Western blotting Substrate (Promega), according to the manufacturer's instructions. Densitometry analysis of western blot protein bands was performed using GelQuantNet software.

### 3.5 NF-κB luciferase reporter assay

$2x10^5$ 293T cells or $2x10^6$ Jurkat cells were transfected with 500ng of NF-κB-Luc reporter plasmid together with empty vector or p65, Tax-1, Tax-2, Flag-APH2, Flag-HBZ-encoding plasmids, in a range from 50ng to200 ng. The transfection efficiency was normalized using *Renilla* luciferase vector. 24h after transfection, the cells were washed with PBS and lysed with Passive Lysis Buffer (Promega). Luciferase activity was assayed using the Dual-Luciferase reporter assay system (Promega). The level of firefly and *Renilla* luciferases were measured with a 20/20n Single Tube Luminometer (Promega). The experiments were repeated at least three times and luciferase values were calculated relative to the promoter alone and expressed as the mean plus or minus standard deviation. Results were analyzed using the Student's t-Test. *, $P < 0.05$ was considered to be significant; **, $P < 0.01$ and ***, $P < 0.001$ was considered to be strongly significant.

**3.6 Immunofluorescence and confocal microscopy analysis**

HeLa and Jurkat cells were seeded on coverglasses pre-treated with poly-L-Lysine (Sigma-Aldrich). At 70-80% of confluence, the cells were transfected with specific plasmids and 24h post-transfection were fixed with 4% paraformaldehyde/PBS or formalin at room temperature for 30 min. The cells were then permeabilized with 0.5% Triton X-100/PBS for 20 min, blocked with a 5% milk/PBS solution and incubated with appropriate antibodies for 45' at room temperature. Coverglasses were washed, mounted in DAPI-containing Fluoromount-G (Southern Biotech). The samples were stored at 4°C and examined after 24h under a ZEISS LSM800 confocal microscope. The images were acquired using a 63x objective. The degree of co-localization between fluorophores was calculated using Mander's coefficient and represented as mean ± standard deviation. To quantify the p65 nuclear translocation, the ratios of the nuclear/total p65 mean brightness staining were calculated using ImageJ software, analyzing ten cells for each field. Results were analyzed using the Student's t-Test, considered significant at $p < 0.05$ and presented as a histogram. Results were presented also as box-plot, a method for graphically depicting groups of numerical data through their quartiles.

**3.7 Development of TRAF3 knock-out cell lines by CRISPR/Cas9**

TRAF3 knockout was generated by the CRISPR/Cas9 system according to the protocols described in Ran *et al*. (2013).

The CRISPR (clustered regularly interspaced palindromic repeats)-Cas (CRISPR-associated proteins) system is an adaptive bacterial and archeal mechanism to protect themselves from subsequent infection, recognizing and cleaving incoming foreign nucleic acids. The processes and key components of the *Streptococcus pyogenes* CRISPR-Cas9 system have been well studied and adapted for genome engineering in mammalian cells. The system is based on the interaction between a specific target sites of the gene of interest and the endonuclease Cas9, guided by synthetic guides RNA (gRNAs). Only the genome DNA that contains protospacer-adjacent motif PAMs can be identified and bound by the gRNA/Cas9 complex to generate double-strand breaks (DSBs). These breaks can be repaired

two major endogenous DNA repair mechanisms: the non-homologous end joining (NHEJ) pathway that is error-prone, will directly ligate the broken DNA and result in the chance of introducing wrong base-pair deletions and insertions for a gene knock-out; the homology directed repair (HDR) is another mechanism by which exogenous nucleotide sequences can be introduced into the genome through homologous recombination for precise gene editing (Ran et al., 2013).

The knock-out of TRAF3 gene was performed as summarized in the **Figure 13**. At first, *in silico* analyses were performed in order to define the optimal target sites to induce TRAF3-KO. Based on the CHOP-CHOP tool results, three different gRNAs were designed (**Table 5**).

**Table 5.** Primer sequences for gRNAs cloned into pSpCas9 vector.

| gRNA | Sequence |
|------|----------|
| **gRNA 1** | CCAGTTTTTGTCCCTGAACA |
| **gRNA 2** | AGCCCGAAGCAGACCGAGTG |
| **gRNA 3** | TCTTGACACGCTGTACATTT |

These gRNAs were selected based on the minimal off-target genomic activity that may derived by each gRNA. Each selected gRNA was cloned independently, using the T4 DNA ligase (Promega), into BbS1 restriction sites of Cas9 expressing vectors (pSpCas9(BB)-2A-Puro (PX459) V2.0 vector, Addgene – **Figure 14**), which contains the puromycin resistance.
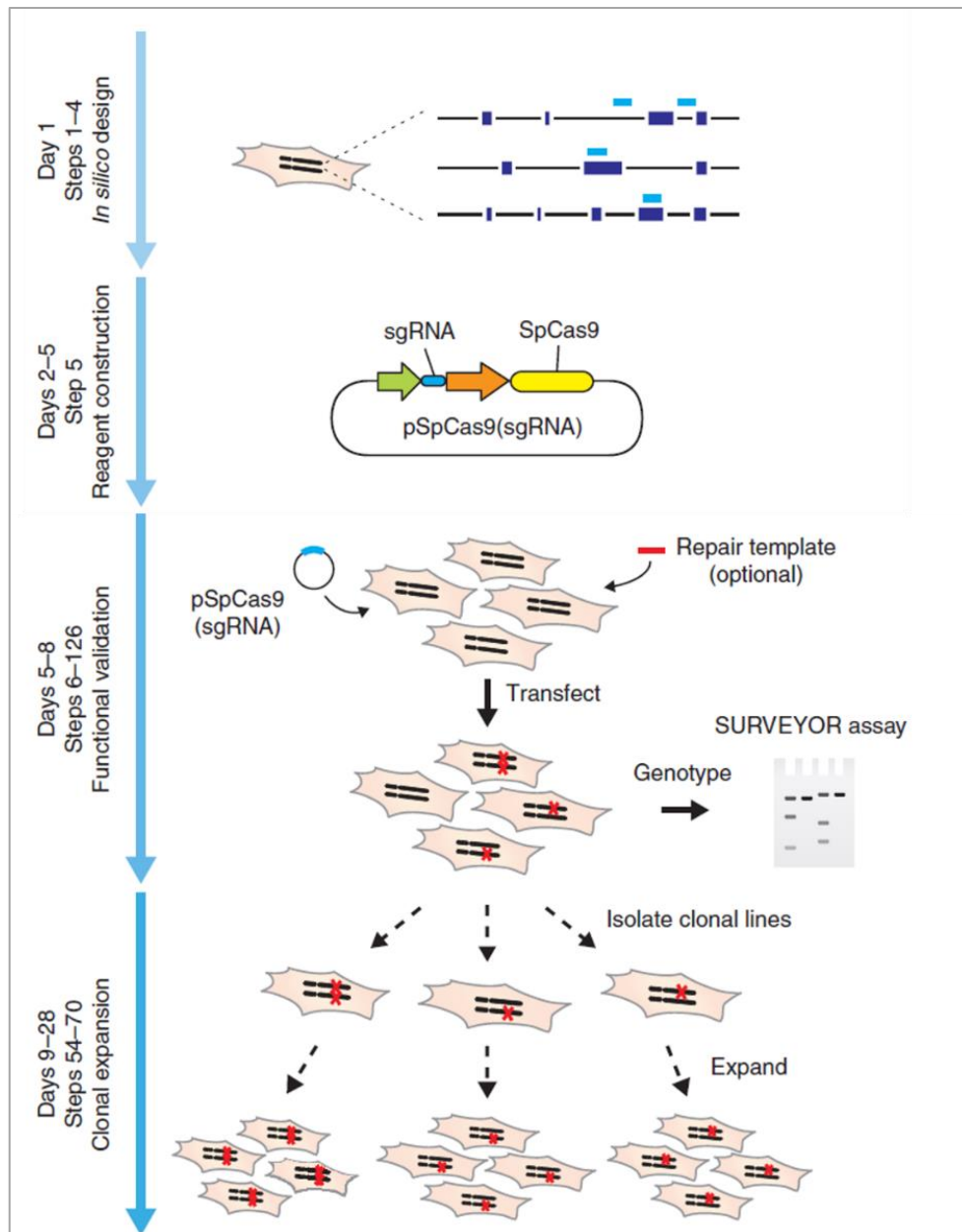
**Figure 13.** CRISPR/Cas9 workflow. gRNAs (light blue bars) for each target are designed *in silico* via the CRISPR Design Tool. gRNAs are then cloned into an expression plasmid containing both sgRNA scaffold backbone and Cas9, obtaining the pSpCas9(sgRNA) plasmid that must be sequence-verified. pSpCas9(sgRNA) plasmid is then transfected into cells and the targeted cleavage is verified. Finally, transfected cells can be clonally isolated and expanded (Ran et al., 2013).
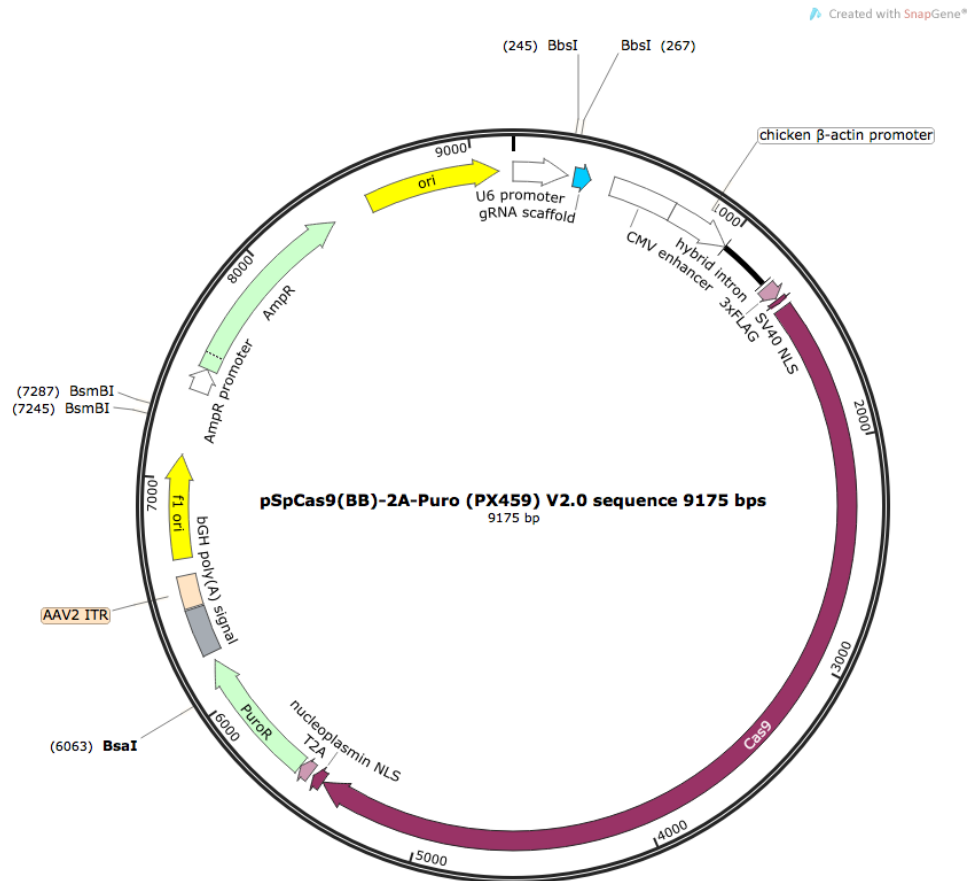
**Figure 14.** pSpCas9(BB)-2A-Puro (PX459) V2.0 (Addgene – Plasmid #62988).

Each plasmid was used to transform TOP10 chemically competent *E. coli*. To determine the presence or absence of insert DNA in the plasmid constructs, a colony PCR was performed, applying the following conditions:

| Cycle step | Temp. | Time |
|---|---|---|
| Initial denaturation | 94°C | 5' |
| Denaturation | 94°C | 30'' |
| Annealing | 56°C | 30'' |
| Elongation | 72°C | 30'' |
| Final elongation | 72°C | 7' |

Each construct was verified by restriction analysis and sequencing.

gRNAs expressing plasmid were transfected in HEK293T cells and the cell pools were selected with 0.5 µg/mL of puromycin for 3 days. The isolation of clonal gene-knockout cell lines was performed by serial dilution according to Poisson's distribution and the selected cell pools were tested by western blot using TRAF3 specific antibody.

## 4. RESULTS

## 4.1 HBZ and APH-2 interact with p65 and suppress the NF-κB pathway

The molecular mechanism carried out by the HTLV antisense protein in the modulation of NF-κB is not completely understood. Previous results indicate that HBZ acts as a negative regulator of NF-κB activation altering p65 function. Recent studies indicate that, also APH-2 may inhibit NF-κB, but though a different mechanism which has not been investigated yet. In order to explore the molecular mechanisms of NF-κB inhibition induced by the antisense viral proteins, we compared the effect of both HBZ and APH-2 on NF-κB promoter activation, by luciferase reporter assay. HEK293T were transfected with NF-κB-responsive luciferase reporter plasmid (pNFκB-Luc), p65 expression vector, and increasing amounts of Flag-HBZ, Flag–APH-2 expressing vectors, or an empty vector. Transfection efficiency was normalized with equal amount of phRG-TK (Renilla) reporter plasmid. Luciferase activity was measured 24h after transfection and the results were represented as fold increase of luciferase activity relative to the NF-κB promoter alone. The results showed that in the presence of increasing amounts of HBZ protein, the NF-κB promoter activity induced by p65 was reduced in a dose-dependent manner (**Figure 15** - panel A). A similar result was observed in the presence of APH-2 (**Figure 15** - panel B) and confirmed in lymphocyte T Jurkat cell line (**Figure 15** – panel C).

It has been demonstrated that HBZ interacts directly with p65 through the bZIP domains in immunoprecipitated cell lysates (Zhao et al., 2009). APH-2 protein contains a non-canonical bZIP domain and it might act differently from HBZ in the interaction with p65. In order to analyze the presence of APH-2 or HBZ in complexes containing p65, we transfected HEK293T cells with p65 and Flag-APH-2 or Flag-HBZ expression vectors and performed immunoprecipitation assay using anti-Flag antibody. The presence of p65 was detected by western blot. The results showed that, both HBZ and APH-2 were present in complexes containing p65 (**Figure 15** – panel D – lines 4 and 6). The interaction was also detected in the presence of HBZ and the endogenous p65 (**Figure 15** - panel D – line 5).

Taken together, these results indicate that HBZ and APH-2 inhibit NF-κB activity in the presence of the active nuclear transcription factor p65 and both HBZ and APH-2 interact with p65.
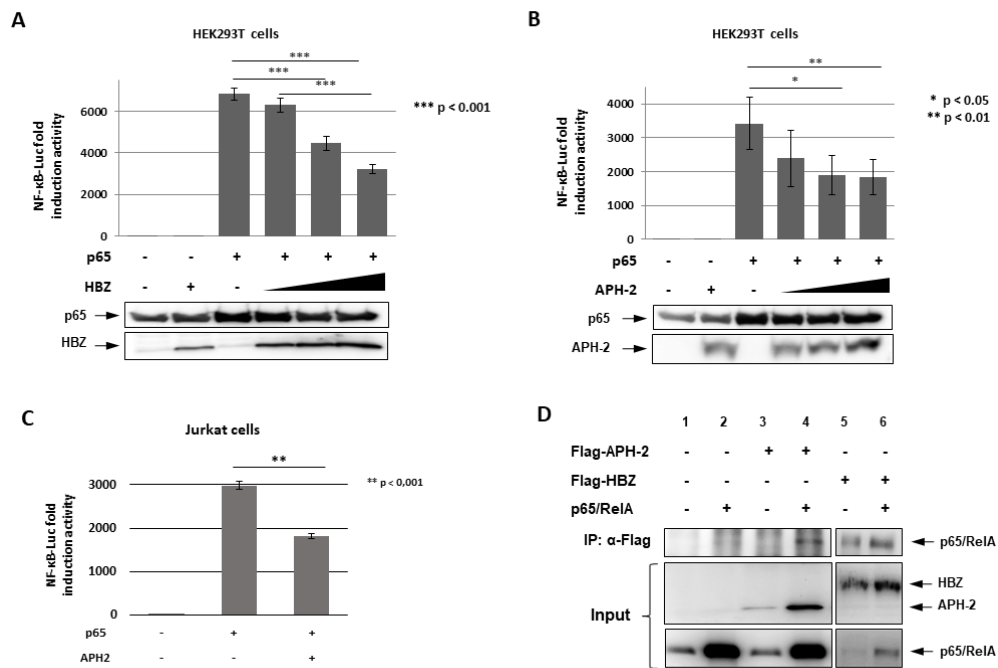


**Figure 15. HBZ and APH-2 interact with p65 and suppress the NF-κB pathway.**
**(A), (B)** HEK293T cells were transfected with 200 ng TK-renilla control, 500 ng κB-luciferase reporter, 50 ng p65 expression plasmid, and increasing amounts of Flag-HBZ, Flag-APH-2 (from 100 ng to 200 ng), or an empty vector. 24 h after transfection, cell lysates were collected and luciferase levels were measured. Asterisk (*), (**) or (***) indicate statistical significance of $p = <0.05, p = <0.01$ or $p = <0.001$ respectively obtained by Student's $t$-test (Top). Immunoblot analysis was performed to detect the expression levels of Flag-HBZ and Flag-APH-2 (Bottom). **(C)** Jurkat cells were transfected with 200 ng TK-renilla, 500 ng κB-luciferase reporter, 50 ng p65 expression plasmid, and 200 ng of Flag-APH-2, or an empty vector. 24 h after transfection, cell lysates were collected and luciferase levels were measured. **(D)** HEK293T cells were transfected with p65, Flag-APH-2, or Flag-HBZ vectors. 24 h after transfection, tagged proteins were immunoprecipitated with anti-Flag antibody and the presence of p65 protein was examined by western blot analysis.

## 4.2 HBZ and APH-2 inhibit the Tax-mediated NF-κB activation

HBZ antagonizes many of the activities mediated by Tax, including the transcription of the 5'LTR (Barbeau et al., 2013). In order to elucidate the contribution of HBZ expression in counteracting the activation of NF-κB mediated by Tax-1, we performed NF-κB luciferase reporter assay comparing NF-κB activation mediated by Tax proteins in the presence of APH-2 or HBZ. HEK293T were transfected with pNF-κB-Luc, increasing amounts of Tax-1 or Tax-2, Flag-HBZ or Flag–APH-2 expressing vectors, or an empty vector. The results showed that, as expected, the NF-κB promoter is activated in the presence of increasing amounts of expressing Tax-1, whereas the presence of HBZ reduced the NF-κB activation in the presence of Tax-1. The inhibitory effect of HBZ was not revealed when Tax-1 was expressed at high levels (**Figure 16** – panel A). Interestingly, when we compared the APH-2 effect on Tax-mediated NF-κB activation, we observed that the overexpression of Tax-2, differently from HBZ, did not restore the activation of NF-κB (**Figure 16** – panel B). These results indicate that HBZ and APH-2 differ in the inhibition of Tax-mediated NF-κB activation. On the basis of these results we proceeded to investigate the role of HBZ and APH-2 interactions with Tax and host factors in the inhibition of the NF-κB pathway.
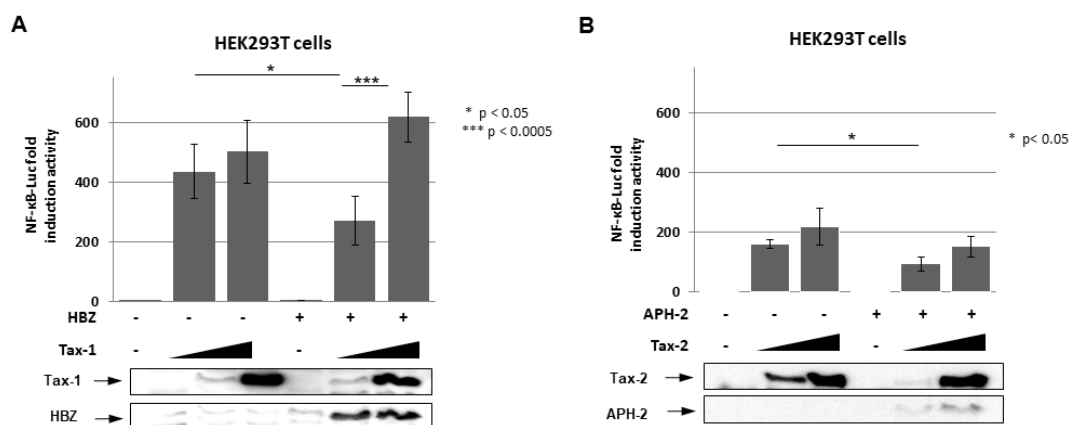


**Figure 16. HBZ and APH-2 inhibit the Tax-mediated NF-κB activation.**
**(A), (B)** HEK293T cells were transfected with 200 ng TK-renilla control, 500 ng κB-luciferase reporter, and increasing amounts of Tax-1 or Tax-2 (from 100 ng to 200ng), and Flag-HBZ, Flag-APH-2, or an empty vector. 24 h after transfection, cell

lysates were collected and luciferase levels were measured. Asterisk (*), (***) indicate statistical significance of $p = <0.05$, or $p = <0.0005$ respectively obtained by Student's *t*-test (Top). Immunoblot analysis was performed to detect the expression levels of Tax, Flag-HBZ and Flag-APH-2 (Bottom).

**4.3 APH-2 is recruited in cytoplasmic structures in the presence of Tax-2**

In previous research, it has been demonstrated that Tax-1 does not interact with HBZ, whereas Tax-2 interact with APH-2. Based on the property of Tax-1 and Tax-2 to localize differently in cellular compartments (Bertazzoni et al., 2011), we formulated the hypothesis that HBZ and APH-2 may inhibit NF-κB through a divergent molecular mechanism that involves the recruitment of selected host factors in the nucleus and in the cytoplasm. In order to verify this hypothesis, we performed co-immunoprecipitation analyses to demonstrate the interaction in a cellular system and we investigated the subcellular co-localization of the complexes formed by the Tax proteins and the antisense proteins, by confocal microscopy. Cell lysates of HEK293T cells, transfected with Tax-1 or Tax-2, and Flag-HBZ or Flag-APH-2 expressing vectors (**Figure 17** – panel A), were immunoprecipitated using anti-Flag antibody. The presence of Tax-2 protein, detected by western blot analysis, was confirmed in complexes containing APH-2 (**Figure 17** – panel A – line 6), whereas Tax-1 was not detectable in complexes containing HBZ. By confocal microscopy analyses, we found that in HeLa cells, Flag-HBZ localized into the nucleus (red signal), but when it was co-expressed with Tax-1 they did not co-localize (**Figure 17** – panel B). When we analyzed the APH-2 distribution, we observed that differently to HBZ, the intracellular localization of GFP-APH-2 was not exclusively nuclear (green signal), whereas Tax-2, as expected, was mainly distributed in the cytoplasm (red signal). Interestingly, the co-expression of APH-2 and Tax-2 resulted in APH-2 redistribution, which co-localized with Tax-2 in cytoplasmic structures (yellow signal) (**Figure 17** – panel C). These analyses were performed in Jurkat T cells transiently transfected with a VSV-APH-2 vector. The results showed that APH-2 is mainly distributed in the nucleus, and re-localized in the cytoplasm (yellow signal) in the presence of Tax-2 (red signal) (**Figure 17** – panel D). The co-localization degree of APH-2 signal with Tax-2 signal measured using Mander's

overlap coefficient was 0,83±0,16. Altogether, these results suggest that HBZ differs from APH-2 in the subcellular distribution when Tax proteins are expressed.
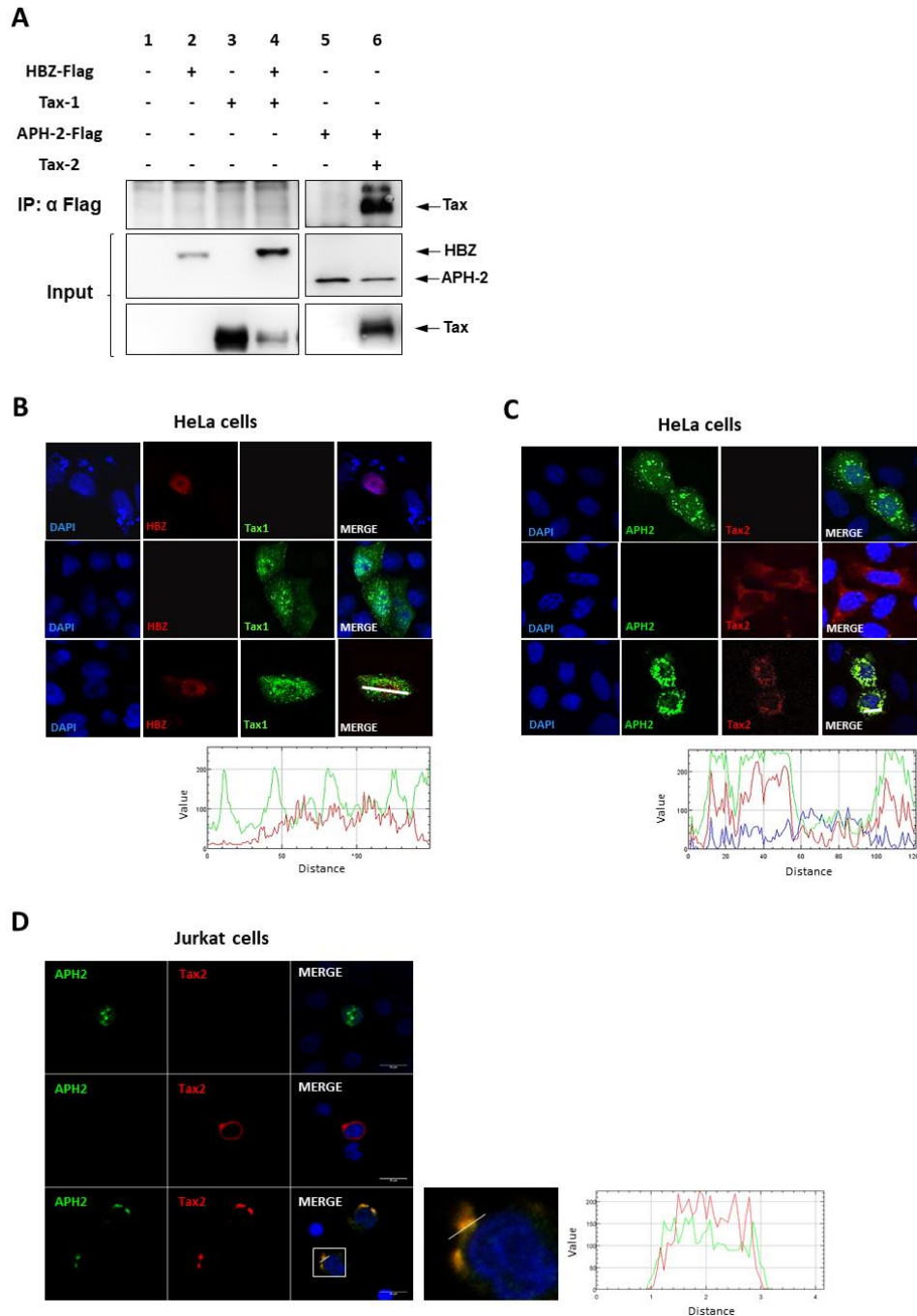


**Figure 17. APH-2 interacts with Tax-2 and it is recruited in cytoplasmic structures in the presence of Tax-2.**
(**A**) HEK293T cells were transfected with Tax-1 or Tax-2 and Flag-APH-2 or Flag-HBZ vectors. 24 h after transfection, tagged proteins were immunoprecipitated with

anti-Flag antibody and the presence of Tax protein was examined by western blot analysis. **(B), (C)** HeLa cells were transfected with Tax-1 or Tax-2 and Flag-APH-2 or Flag-HBZ vectors. 24 h after transfection, cells were fixed with 4% PFA, and stained with anti-Flag, anti-Tax-1 primary antibodies, Alexa 594, Alexa 488 secondary antibodies to detect HBZ (red signal) and Tax-1 (green signal), respectively; anti-Tax-2 primary antibody and Alexa 594 secondary antibody, to detect Tax-2 (red signal) whereas APH-2 is fused to a GFP protein (green signal). Nuclei are stained with DAPI (blue signal). The intensity of fluorescence overlapping the white line drawn on the merged images is plotted in the diagrams on the right. **(D)** Jurkat cells were transfected with VSV-APH-2 and Tax-2 vectors. 24 h after transfection, cells were fixed with formalin, and stained with anti-VSV, anti-Tax-2 primary antibodies and Alexa 488, TexasRed secondary antibodies to detect APH-2 (green signal) and Tax-2 (red signal), respectively. Nuclei are stained with DAPI (blue signal). Enlargements are shown next the "Merge" panel. The intensity of fluorescence along the white line drawn on the merged images is plotted in the diagrams on the right. Scalebar, 10μm.

## 4.4 APH-2 is recruited in cytoplasmic complexes with TAB2 and NEMO in the presence of Tax-2

Given that APH-2 interacts with Tax-2, and Tax-2 forms complexes in the cytoplasm with the NF-κB pathway factors TAB2 and NEMO, we speculated that APH-2 can be recruited in these protein complexes. In order to verify this hypothesis, we investigated the co-localization of APH-2 with the Tax-2-interacting factors TAB2 and NEMO in Jurkat cells by confocal microscopy. The results showed that TAB2 was distributed in the cytoplasm in punctuated structures (magenta signal), and when APH-2 was co-expressed, they partially co-localized in the cytoplasm (**Figure 18** – panel A). Interestingly, the co-expression of Tax-2, APH-2 and TAB2 resulted in the redistribution of the three proteins in cytoplasmic structures where they co-localized. The co-localization degree measured using Mander's overlap coefficient was 0,81±0,08. The same results were reproduced in two different cell lines that are useful for the analyses of cytoplasmic structures, HeLa and U2OS cells (**Figure 18** – panel B, C).
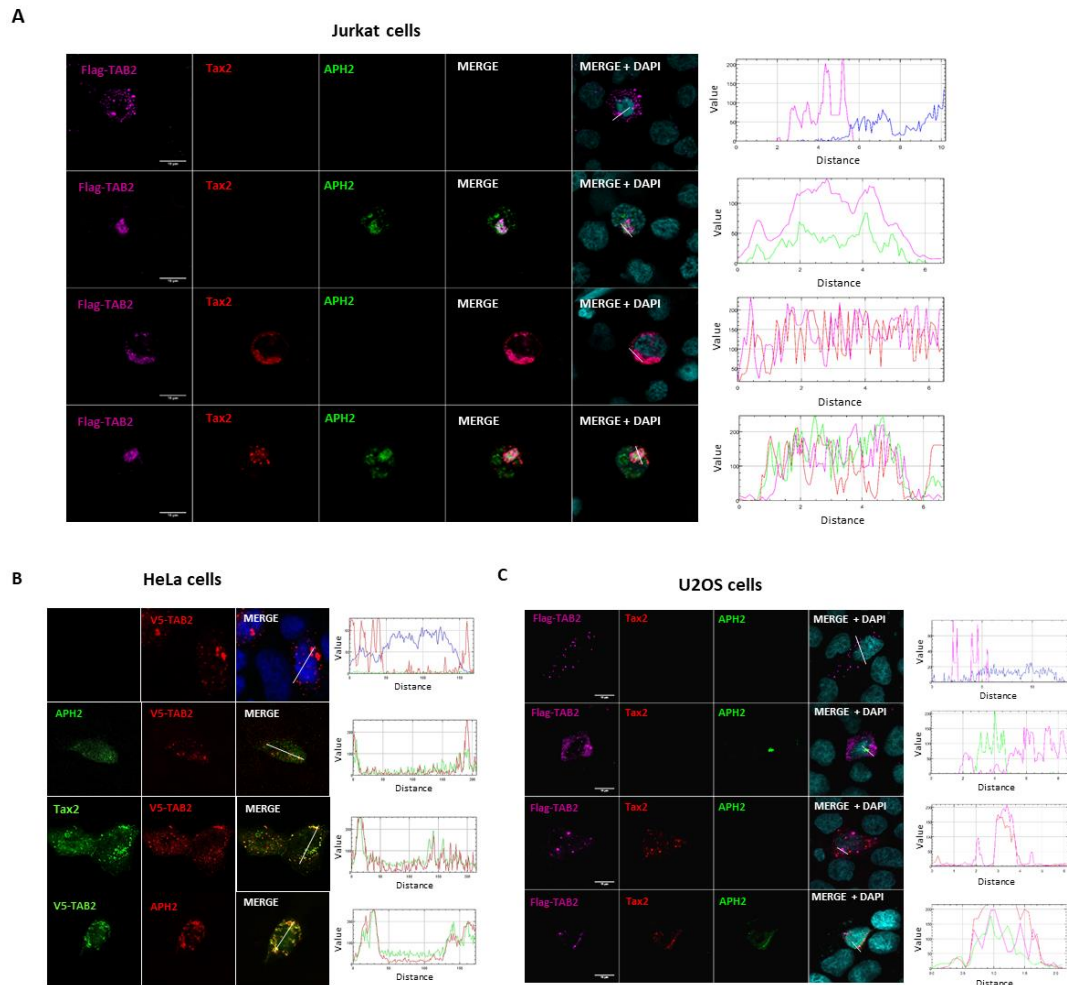
**Figure 18. APH-2 is recruited in cytoplasmic complexes with TAB2 in the presence of Tax-2.**
(**A**) Jurkat cells were transfected with VSV-APH-2, Tax-2 and Flag-TAB2 vectors. 24 h after transfection, cells were fixed with formalin, and stained with anti-VSV, anti-Tax-2, anti-Flag primary antibodies, Alexa 488, TexasRed, Alexa 649 secondary antibodies to detect APH-2 (green signal), Tax-2 (red signal), and TAB2 (magenta signal), respectively. Nuclei are stained with DAPI (blue signal). Scalebar, 10µm. The intensity of fluorescence overlapping the white line drawn on the merged images is plotted in the diagrams on the right. (**B**) The same analysis was performed in HeLa cells using anti-Flag, anti-Tax-2 and anti-V5 primary antibodies and Alexa 488, Alexa 594 secondary antibodies to detect respectively APH-2, Tax-2 (green signal) and TAB2 (red signal), and (**C**) in U2OS cells with the same staining condition used in panel A.

A partial cytoplasmic co-localization of NEMO with Tax-2 and APH-2 was detected when both APH-2 and Tax-2 were co-expressed (**Figure 19** – panel A).

The co-localization degree measured using Mander's overlap coefficient was 0,69±0,13. The same results were obtained in U2OS cells (**Figure 19** – panel B). Taken together, these results demonstrate that APH-2 is recruited in the cytoplasm in Tax-2-complexes containing the NF-κB factors TAB2 and NEMO.
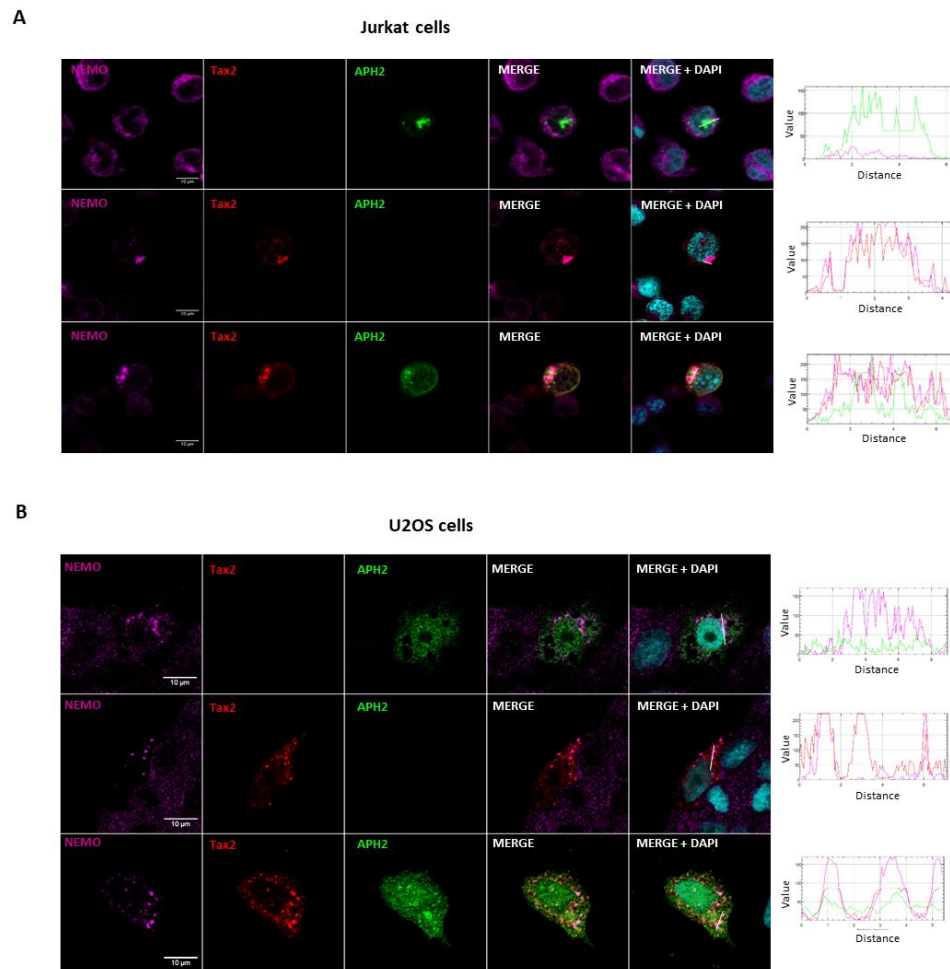


**Figure 19. APH-2 is recruited in cytoplasmic complexes with NEMO in the presence of Tax-2.**
**(A)** Jurkat cells were transfected with VSV-APH-2 and Tax-2 vectors. 24 h after transfection, cells were fixed with formalin, and stained with anti-VSV, anti-Tax-2 and anti-NEMO primary antibodies, Alexa 488, TexasRed and Alexa 647 secondary antibodies to detect APH-2 (green signal), Tax-2 (red signal), and endogenous NEMO (magenta signal), respectively. Nuclei are stained with DAPI (blue signal). The intensity of fluorescence overlapping the white line drawn on the merged images is plotted in the diagrams on the right. **(B)** The same analysis was performed in U2OS cells. Scalebar, 10µm.

**4.5 p65 nuclear translocation is impaired in the presence of APH-2**

In order to analyze if the APH-2 inhibition of Tax-dependent NF-κB activation may derive by p65 impairment in the translocation to the nucleus, we analyzed the intracellular distribution of p65 in the presence of APH-2 and Tax-2 in Jurkat cells (**Figure 20** – panel A). The results obtained by confocal microscopy analyses revealed that endogenous p65 localized in the cytoplasm (magenta signal) in the presence of APH-2 (green signal). When Tax-2 was expressed (red signal) p65 was detected also in the nucleus (magenta signal), as expected. Surprisingly, the co-expression of APH-2 and Tax-2 resulted in their cytoplasmic co-localization and in the impairment of p65 nuclear translocation. Quantification of the nuclear p65 signal indicated that when APH-2 and Tax-2 were co-expressed, p65 nuclear dye decreased of almost five times relative to the presence of Tax-2 alone (**Figure 20** – panel B). The same results were observed in U2OS cells (**Figure 20** – panel C).
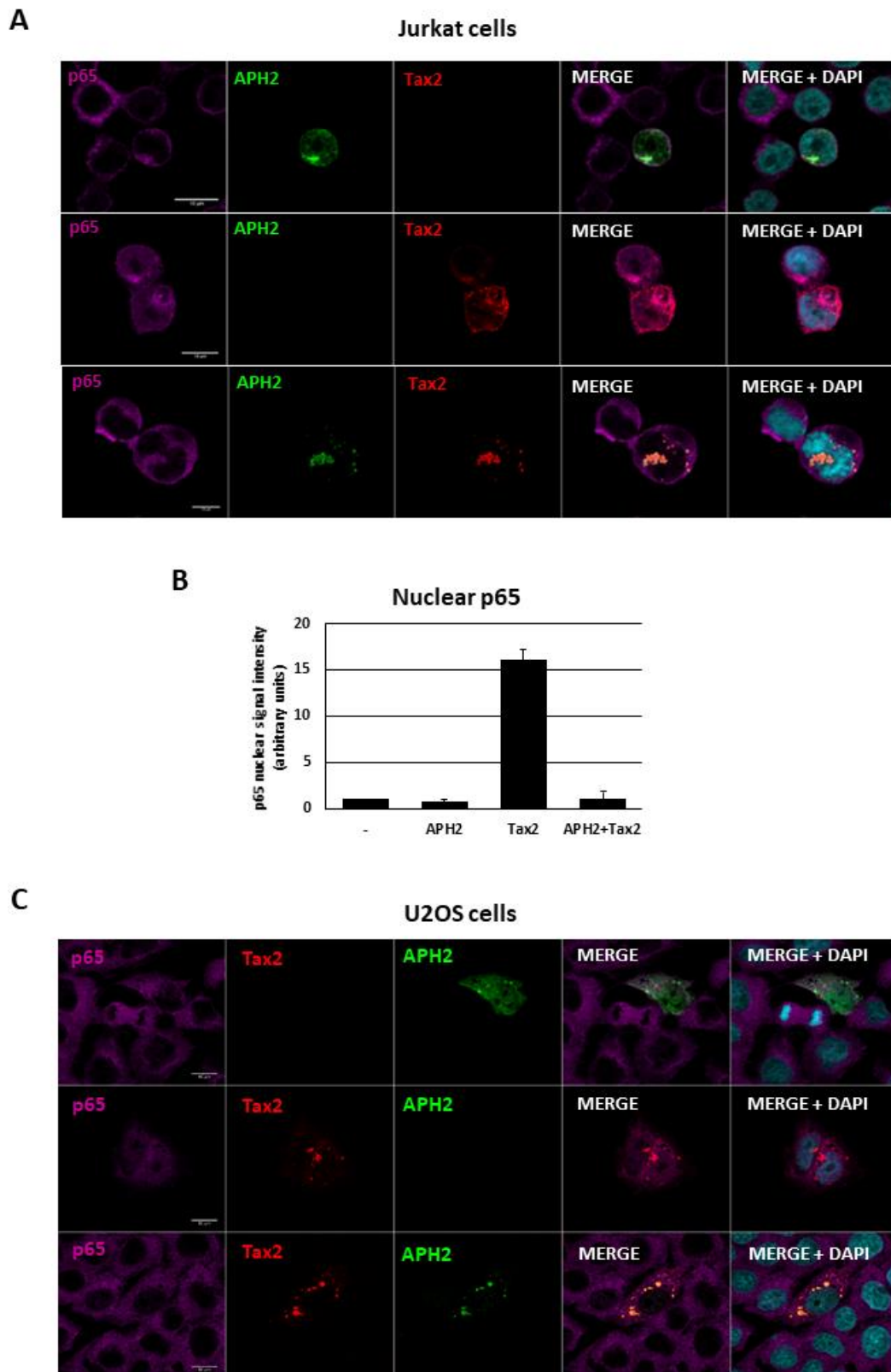
**Figure 20. p65 nuclear translocation is impaired in the presence of APH-2.**
(**A**) Jurkat cells were transfected with VSV-APH-2 and Tax-2 vectors. 24 h after transfection, cells were fixed with formalin, and stained with anti-VSV, anti-Tax-2

and anti-p65 primary antibodies, Alexa 488, Alexa 594 and Alexa 647 secondary antibodies to detect APH-2 (green signal), Tax-2 (red signal), and endogenous p65 (magenta signal), respectively. Nuclei are stained with DAPI (blue signal). **(B)** Total cells and nuclei were delineated using Image J software, and the mean brightness ratio of the nuclear p65 staining was calculated. Values obtained in non-transfected cells were then subtracted from those for APH-2, Tax or APH-2+Tax-expressing cells. p65 nuclear signal intensity in the presence of the viral proteins is plotted. **(C)** The same analysis was performed in U2OS cells. Scalebar, 10μm.

Based on these observations and given that HBZ co-localizes with p65 in the nucleus (Zhao et al., 2009), we hypothesized that HBZ may diverge from APH-2 in the control of p65 nuclear translocation, by affecting the degradation of the IκB inhibitory molecules. We verified the expression levels of endogenous IκB in the presence of HBZ or APH-2 and Tax, by western blot analyses (**Figure 21**). We found that, as expected, the expression of IκB was reduced of about 50% in the presence of both Tax-1 (**Figure 21** - panel A) and Tax-2 proteins (panel B). The reduced expression of IκB was also detected when HBZ was co-expressed with increasing amount of Tax-1 (**Figure 21** - panel A). Surprisingly, we found that, differently from HBZ and Tax-1, when APH-2 and Tax-2 were co-expressed, the IκB intensity band was not reduced. A similar result was obtained in Jurkat cells (**Figure 21** - panel C).

Taken together, these results suggest that APH-2 exerts its inhibitory effect on NF-κB in the cytoplasm. In particular, APH-2, differently to HBZ, might counteract the IκB degradation mediated by Tax-2, resulting in the reduction of the p65 nuclear translocation.
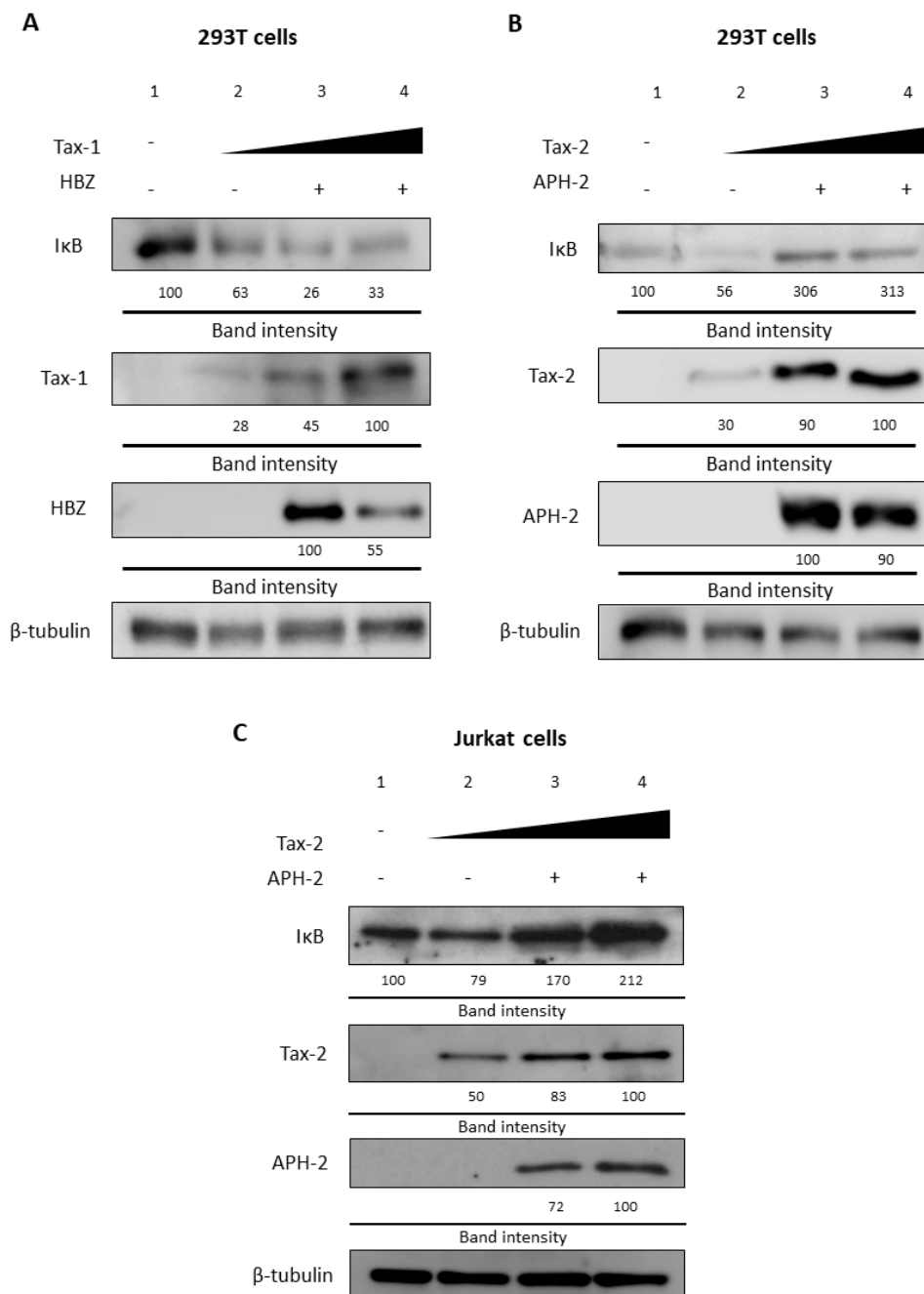
**Figure 21. APH-2 suppresses the IκB degradation mediated by Tax-2.**
**(A), (B)** HEK293T were co-transfected with Flag-HBZ and increasing amount of Tax-1 or with Flag-APH-2 and increasing amount of Tax-2. Immunoblot analysis was performed 24 h after transfection to compare the levels of endogenous IκB in the presence of the viral regulatory proteins. **(C)** Jurkat cells were cotransfected with VSV–APH-2 and increasing amount of Tax-2 or the empty vector. Immunoblot analysis was performed 24 h after transfection to compare the levels of endogenous IκB in the presence of the viral regulatory proteins. The amount of IκB was measured relative to the amount of β-tubulin.

## 4.6 APH-2 and Tax-2 form complexes with TRAF3

In previous studies it has been proposed that HBZ selectively inhibits the classical NF-κB pathway, whereas its role in the regulation of the alternative pathway is not completely clear. ATL cells exhibit high levels of NF-κB inducing kinase (NIK), a positive regulator of the alternative NF-κB pathway, which turnover is regulated by the upstream E3 ubiquitin ligase TRAF3 (Chan et al., 2012). In order to study the role of HBZ in the alternative NF-κB pathway, we first investigated the presence of HBZ in complex with the upstream regulator TRAF3, compared to APH-2, performing co-immunoprecipitation analyses (**Figure 22**). The results showed that in transfected cells, APH-2 interacted with TRAF3 and Tax-2, whereas TRAF3 signal was less represented when HBZ and Tax-1 were co-expressed (**Figure 22** - line 8 and 9).
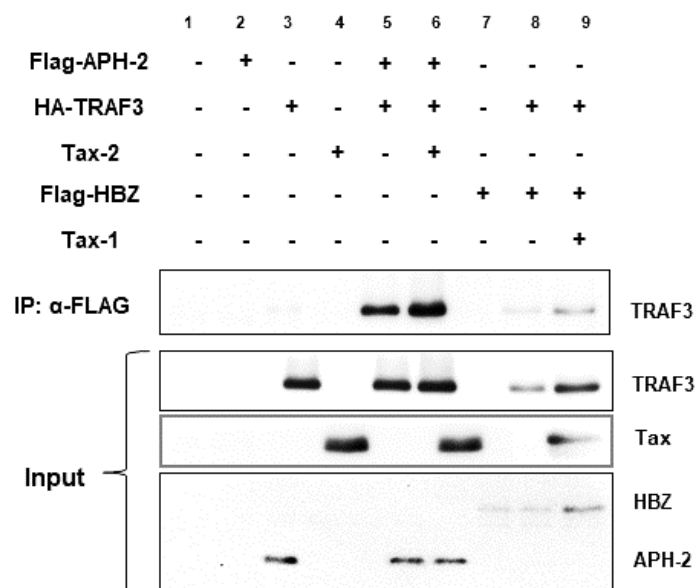


**Figure 22. APH-2 and Tax-2 interact with TRAF3.**
HEK293T cells were transfected with Tax-1 or Tax-2, Flag-APH-2 or Flag-HBZ and HA-TRAF3 vectors. 24 h after transfection, tagged proteins were immunoprecipitated with anti-Flag antibody and the presence of TRAF3 protein was examined by anti-HA primary antibody and an appropriate HRP-conjugated secondary antibody, by western blot.

We have previously shown that TRAF3 interacts with Tax-1 (Diani et al., 2015). In order to verify if post-translational modifications or selected mutations of Tax proteins might affect its interaction with TRAF3, we performed co-immunoprecipitation analyses using the Tax-1 K1-10R or Tax-M22 mutants (**Figure 23**). Tax-1 K1-10R mutant, is a construct in which the lysines targets of sumoylation and ubiquitination post-translational modification have been substituted by arginines. Tax-M22 is a functional mutant defective of NF-κB activation. The results showed that both Tax-M22 and Tax-1 K1-10R form complexes with TRAF3.
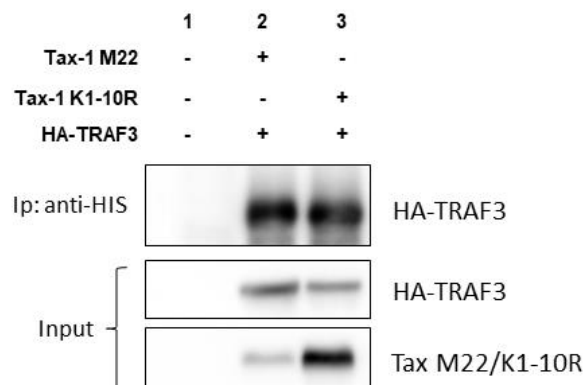


**Figure 23. Tax-M22 and Tax-1 K1-10R interact with TRAF3.**
HEK293T cells were transfected with His-Tax-M22 or His-Tax-1 K1-10R and HA-TRAF3 vectors. 24h after transfection, His-tagged proteins were immunoprecipitated with anti-His antibody and the presence of TRAF3 protein was examined by anti-HA primary antibody and an appropriate HRP-conjugated secondary antibody, by western blot.

Confocal microscopy analyses were performed in order to investigate the APH-2 co-localization with TRAF3 compared to HBZ. HBZ (red signal) was distributed into the nucleus, Tax-1 (green signal) showed a speckled pattern, and TRAF3 was exclusively present in the cytoplasm (green signal) (**Figure 24** – panel A). When HBZ was co-expressed with Tax-1 or TRAF3 no co-localizations were detected. In contrast, the co-expression of Tax-1 and TRAF3 presented some points of co-

localization (yellow signal) in the cytoplasm. HBZ (red signal) and endogenous TRAF3 (green signal) did not co-localize in Jurkat cells (**Figure 24** - panel B). We proceeded to compare the intracellular distribution of APH-2 and Tax-2 proteins in the presence of ectopic or endogenous TRAF3.
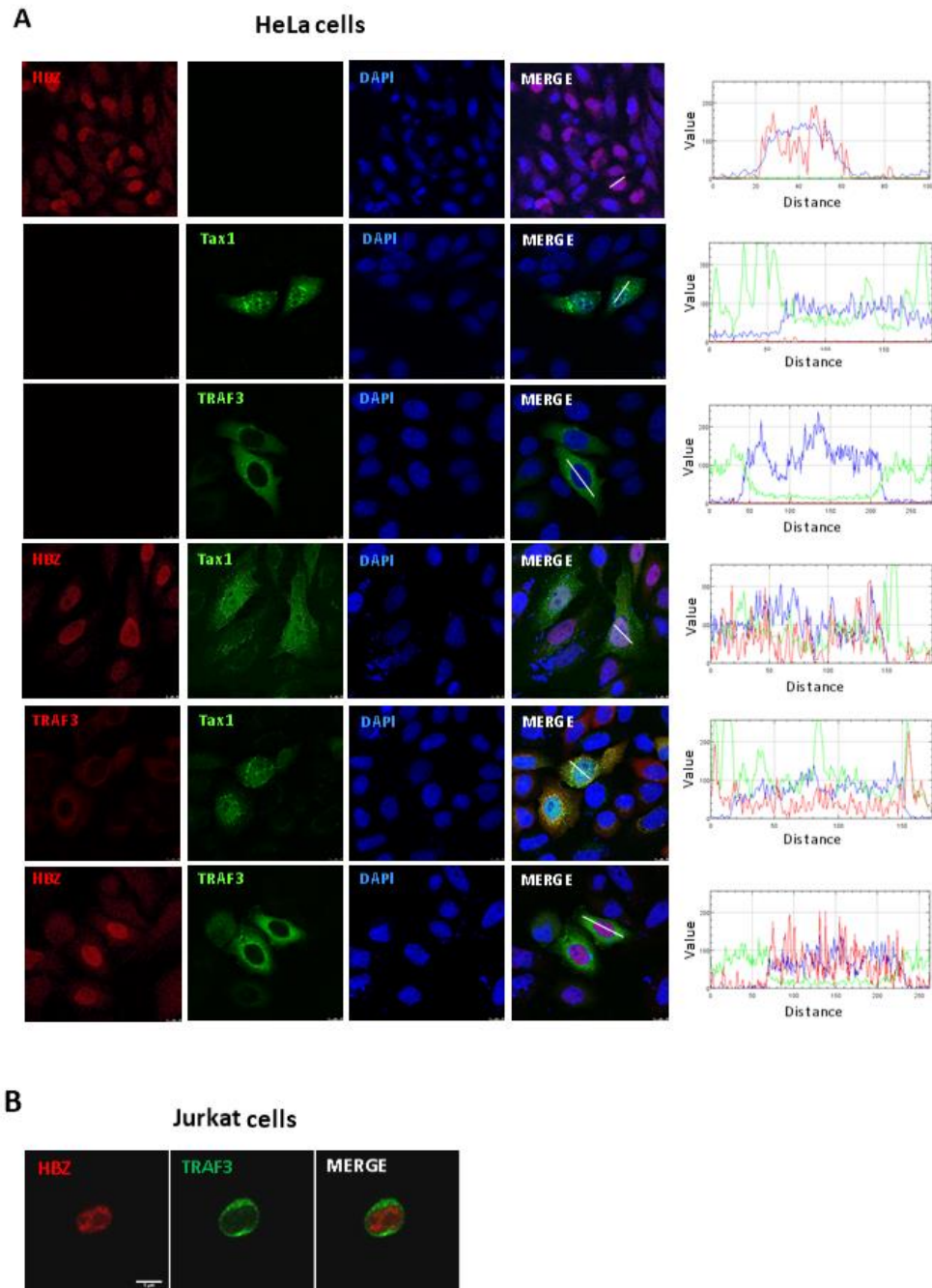


**Figure 24. HBZ did not form complexes with TRAF3 and Tax.**
(**A**) HeLa cells were transfected with Flag-HBZ, Tax-1and HA-TRAF3 vectors. 24 h after transfection, cells were fixed with 4% PFA, and stained with anti-Flag, anti-

Tax-1 and anti-HA primary antibodies, Alexa 594 and Alexa 488 secondary antibodies to detect HBZ (red signal), Tax-1 and TRAF3 (green signal), respectively. The intensity of fluorescence overlapping the white line drawn on the merged images is plotted in the diagrams on the right. **(B)** Jurkat cells were transfected with Flag-HBZ. 24 h after transfection, cells were fixed with formalin, and stained with anti-FLAG and anti-TRAF3 primary antibodies, Alexa 594 and Alexa 488 secondary antibodies to detect HBZ (red signal) and endogenous TRAF3 (green signal), respectively. Nuclei are stained with DAPI (blue signal). Scalebar, 10µm.

Confocal microscopy analyses revealed that TRAF3 localized in the cytoplasm (magenta signal) in Jurkat cells, and when it was co-expressed with APH-2 (green signal), they partially co-localized in the cytoplasm (**Figure 25** – panel A). In the presence of Tax-2 (red signal) some points of co-localization with TRAF3 were visible (pink signal). Finally, the co-expression of TRAF3, Tax-2 and APH-2 resulted in their co-localization in the cytoplasm (white and yellow signal). The same results were reproduced analyzing endogenous TRAF3 in Jurkat cells (**Figure 25** – panel B), and in HeLa cells (**Figure 25** – panel C). The co-localization degree measured using Mander's overlap coefficient between Tax-2, APH-2 and transfected TRAF3 was 0,73±0,10, and 0,70±0,08 between the Tax-2, APH-2 and endogenous TRAF3.

Taken together, these results indicate that APH-2 is recruited in Tax-2-complexes containing TRAF3 differently to HBZ and Tax-1, suggesting that they might differ in the deregulation of the non-canonical NF-κB.

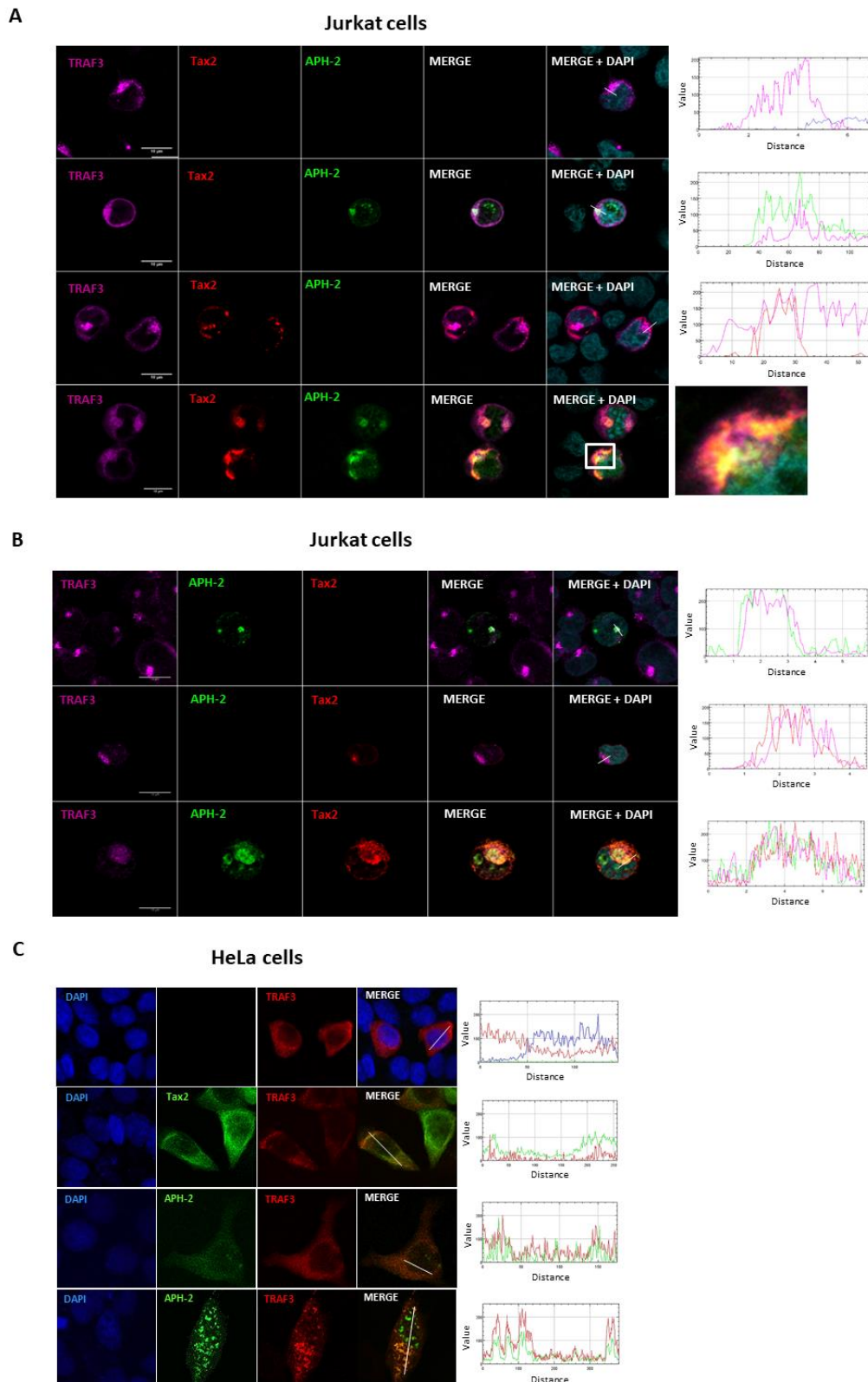**Figure 25. APH-2, unlike HBZ, form complexes with TRAF3 and Tax.**
(**A**) Jurkat cells were transfected with VSV-APH-2, Tax-2 and HA-TRAF3 vectors.
24 h after transfection, cells were fixed with formalin, and stained with anti-VSV,

anti-Tax-2 and anti-HA primary antibodies and Alexa 488, Alexa 594 and Alexa 649 secondary antibodies to detect APH-2 (green signal), Tax-2 (red signal), and TRAF3 (magenta signal), respectively. Nuclei are stained with DAPI (cyan signal). Enlargements are shown next the "Merge" panel. The intensity of fluorescence along the white line drawn on the merged images is plotted in the diagrams on the right. **(B)** Jurkat cells were transfected with VSV-APH-2 and Tax-2 vectors. 24 h after transfection, cells were fixed with formalin, and stained with anti-VSV, anti-Tax-2 and anti-TRAF3 primary antibodies and Alexa 488, Alexa 594 and Alexa 649 secondary antibodies detect APH-2 (green signal), Tax-2 (red signal), and endogenous TRAF3 (magenta signal), respectively. Nuclei are stained with DAPI (blue signal). The intensity of fluorescence along the white line drawn on the merged images is plotted in the diagrams on the right. **(C)** The same analysis was performed in HeLa cells. Scalebar, 10μm.

### 4.7 TRAF3 expression is affected by the presence of HBZ and APH-2

To assess whether HBZ may deregulate the alternative NF-κB pathway affecting the expression of TRAF3, we transfected HEK293T cells with increasing amount of HBZ and evaluated the expression levels of TRAF3 by western blot analysis. The results demonstrated a remarkable reduction of ectopic TRAF3 protein expression in the presence of HBZ (**Figure 26** - panel A), as well as a reduced expression of endogenous TRAF3 (about 50%) (**Figure 26** - panel C). Similar results were obtained when APH-2 was co-expressed with TRAF3 (**Figure 26** - panel B, D).
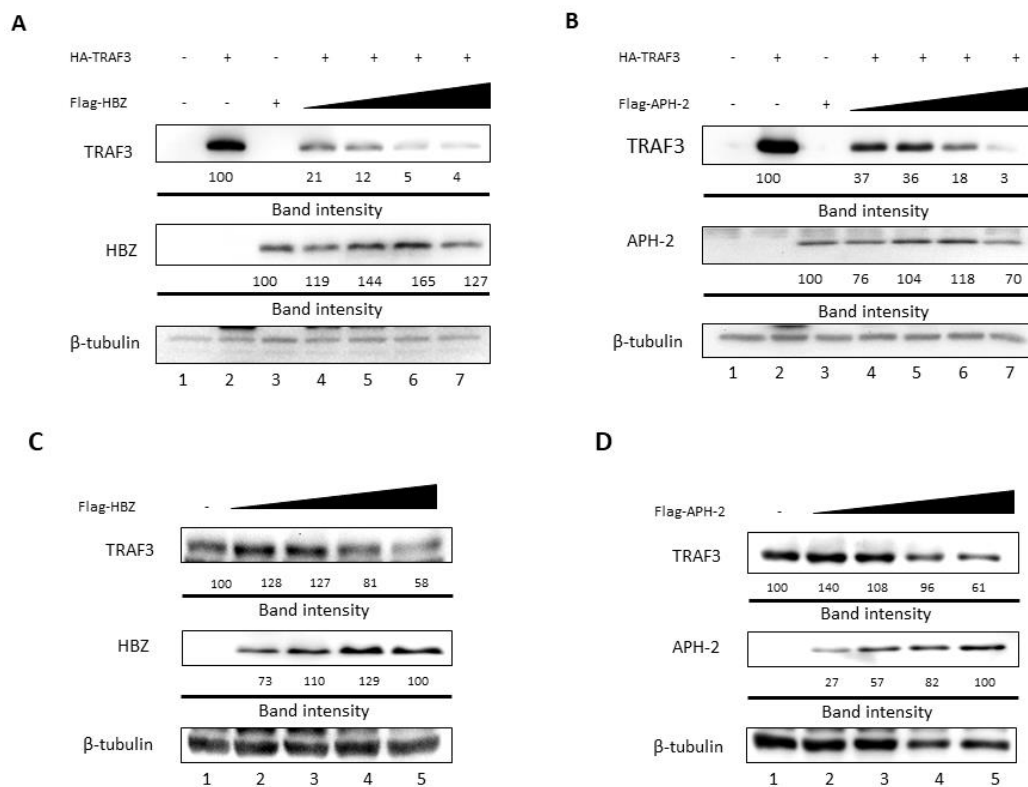
**Figure 26. TRAF3 expression is affected by the presence of HBZ and APH-2.**
HEK293T were cotransfected with HA–TRAF3 and increasing amount of Flag-HBZ
(**A**) or Flag-APH-2 (**B**) or an empty vector. Immunoblot analysis was performed 24 h
after transfection to compare the levels of TRAF3 in the presence of the viral
regulatory proteins. The amount of TRAF3, HBZ and APH-2, were normalized with
β-tubulin. (**C), (D**) The same experiments were performed to analyze the expression
of endogenous TRAF3 in the presence of HBZ and APH-2.

The reduced expression of TRAF3 protein may be the result of two mechanisms
of cellular degradation: via ubiquitin-proteasome pathway and/or lysosomal
autophagic system. We investigated the mechanism induced by HBZ in the
regulation of endogenous TRAF3 protein expression, treating the cells with two
inhibitors, MG132 and Bafilomycin A (BFA) (**Figure 27**). MG132 is a
proteasome inhibitor, which suppresses the degradation of Ub-conjugated
proteins, and BFA is an autophagic inhibitor, which blocks fusion of
autophagosome and lysosome (Tang et al., 2013). The results showed that the
expression level of HBZ was two times higher after treatment with BFA
compared to the control (**Figure 27** - panel A), suggesting that HBZ could be

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degraded by the lysosomal autophagyc system. In contrast, APH-2 expression was six times more represented in correspondence to the treatment with MG132 (**Figure 27** - panel B) compared to the control, suggesting that APH-2 could be degraded via ubiquitin-proteasome pathway.

The panel C in **figure 27** showed a reduction of TRAF3 of about 40% in the presence of HBZ, compared to the control cells. The expression level of endogenous TRAF3 in cell treated with MG132 or BFA was similar, suggesting that HBZ-induced TRAF3 degradation might occur by both proteasome and autophagy system.
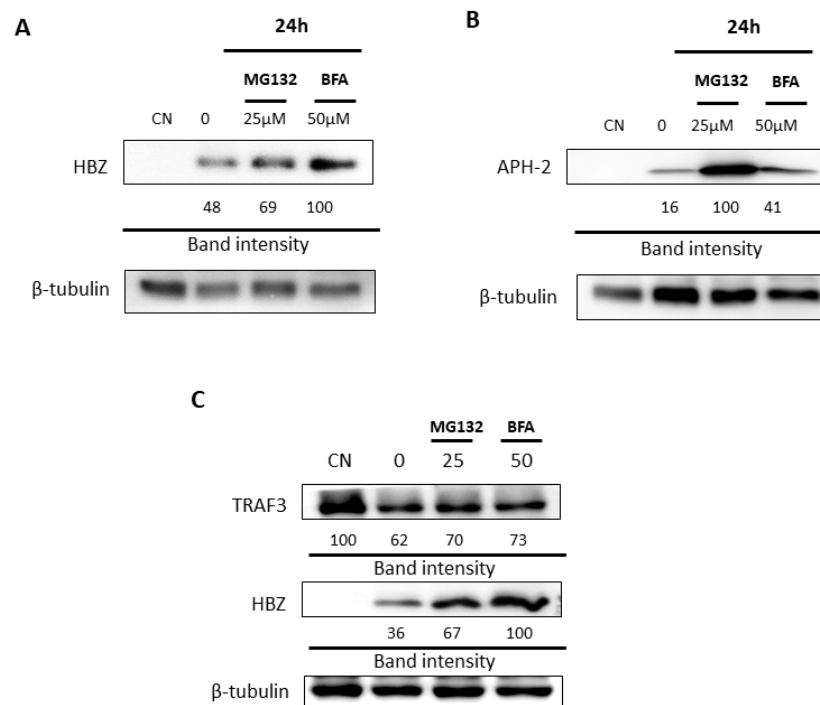


**Figure 27. HBZ reduces TRAF3 expression through both autophagic and proteasomal degradation.**
HEK293T were transfected with Flag-HBZ **(A)**, or Flag-APH-2 **(B)**, or an empty vector. Inhibition of proteosomal and autophagic degradation was induced treating cells with MG132 (25µM) and BFA (50 nM) for 24h, respectively. Immunoblot analysis was performed to evaluate the amount of endogenous TRAF3. **(C)** HBZ and APH-2 and each condition was measured relative to the amount of β-tubulin.

## 4.8 HBZ interacts with p52

The differences emerged analyzing the HBZ and APH-2 binding to the upstream non-canonical regulator TRAF3, let us to assess their interaction with the final effector of the non-canonical NF-κB signaling, the protein p52 (**Figure 28**). Surprisingly, co-immunoprecipitation analysis revealed that HBZ was found in complex with p52 (**Figure 28** - line 6), in contrast to APH-2, which did not interact with p52 (line 4). These results indicate that the antisense proteins interact selectively with the host factors of the non-canonical NF-κB pathway.
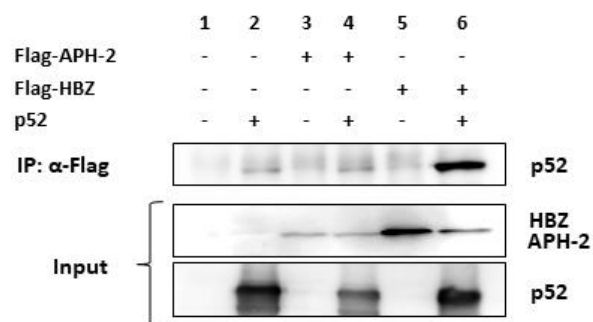


**Figure 28. HBZ interacts with the non-canonical transcriptional factor p52.**
HEK293T cells were transfected with p52, Flag-APH-2, or Flag-HBZ vectors. 24 h after transfection, tagged proteins were immunoprecipitated with anti-Flag antibody and the presence of p52 protein was examined by western blot analysis.

## 4.9 Analysis of the NF-κB activity in TRAF3$^{-/-}$ cell line

Based on the different properties of HBZ and APH-2 in interacting with TRAF3 we sought to investigate the role of TRAF3 in the non-canonical NF-κB pathway deregulation, mediated by the viral proteins. Applying the CRISPR/Cas9 genome editing, we produced stably TRAF3 knock-out cell lines. TRAF3 knock-out in 293T was generated transfecting pSpCas9 (PX459) vector and gRNAs. The knock-out of TRAF3 was verified by western blot (**Figure 29**). Seven of eight selected clones did not express TRAF3, compared to the parental cell line.
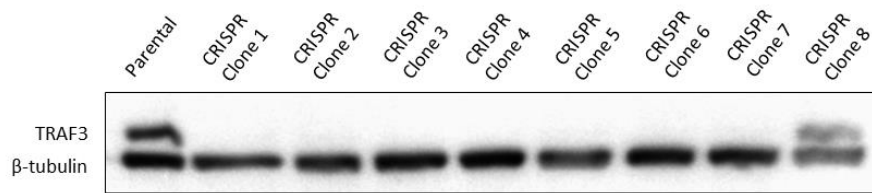
**Figure 29. Generation of TRAF3 knock-out cell lines.**
HEK293T cells were transfected with pSpCas9(sgRNA) vectors. 6 h after transfection, cells were treated with puromycin for three days. TRAF3 knock-out was assessed by western blot.

Using the TRAF3$^{-/-}$ cell line, we analyzed the activity of NF-κB promoter in the absence of cellular stimulation. We transfected TRAF3$^{-/-}$ cell line with pNF-κB-Luc and the NF-κB promoter activity was determined by luciferase reporter assay (**Figure 30** – panel A and B). The results showed that the NF-κB promoter is activated in TRAF3$^{-/-}$ cell line compared to the parental one (50 times compared to the control) (**Figure 30** – panel A). Conversely, as shown in **figure 30** panel B, increasing amount of ectopic TRAF3 expression resulted in the inhibition of the NF-κB promoter activity, in a dose dependent manner. To verify if the activation of NF-κB involved the alternative NF-κB pathway, we analyzed the processing of p100 to generate the p52 transcriptional factor. As shown in **figure 30** panel C, the processing of p100 was detectable only in the TRAF3$^{-/-}$ cell lines, suggesting that the absence of TRAF3 led to a basal activation of the alternative NF-κB pathway. Finally, we verified the intracellular distribution of the transcriptional factor p65, as an indicator of NF-κB transcription activity, by immunofluorescence analyses. Compared to the parental cells in which p65 (green signal) was distributed only in the cytoplasm, in TRAF3 negative cells, we detected a faint green fluorescence also into the nucleus (**Figure 30** - panel D).
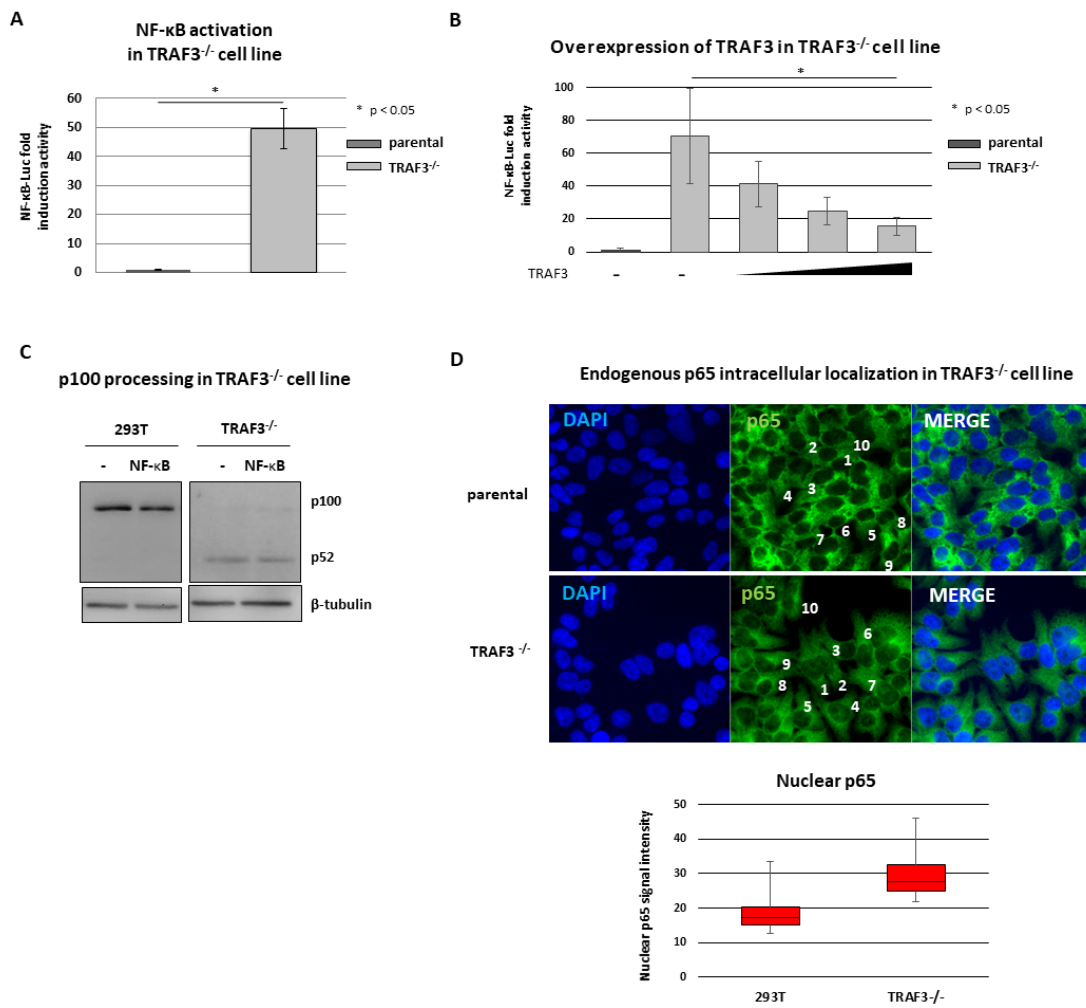
**Figure 30. Analysis of the NF-κB activity in TRAF3$^{-/-}$ cell line.**
(**A**) HEK293T and TRAF3$^{-/-}$ cells were transfected with 200 ng TK-renilla control, 500 ng κB-luciferase reporter. (**B**) HEK293T and TRAF3$^{-/-}$ cells were transfected with 200 ng TK-renilla control, 500 ng κB-luciferase reporter and increasing amount of TRAF3 expressing vector (from 50 to 150 ng). 24 h after transfection, cell lysates were collected and luciferase levels were measured. Asterisk (*) indicates statistical significance of $p = <0.05$, obtained by Student's *t*-test. (**C**) Immunoblot analysis was performed starting from the same cell lysates analyzed in panel A, to detect the expression levels of p100/p52 proteins in HEK293T and TRAF3$^{-/-}$ cells. (**D**) Immunofluorescence analysis revealed the endogenous p65 subcellular distribution in HEK293T and TRAF3$^{-/-}$ cells. Cells were fixed with 4% PFA, and stained with anti-p65 (green signal) primary antibody and Alexa 488 as secondary antibody. Nuclei are stained with DAPI (blue signal). Scalebar, 10µm. Total cells and nuclei of cells (n=10) indicated by a number from 1 to 10 in white were delineated using Image J software, and the mean brightness ratio of the nuclear RelA/p65 staining was calculated and presented as box-plot.

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The generation and characterization of TRAF3 negative cell model give us the opportunity to analyze the contribution of TRAF3 in the deregulation of NF-κB mediated by the Tax-1 protein.


**4.10 Tax-1-mediated NF-κB activation is impaired in the absence of TRAF3**

Previous paper published by our laboratory highlighted that TRAF3 overexpression does not affect Tax-mediated NF-κB activation in transfected cells (Diani et al., 2015). Here, we wondered if the absence of TRAF3 could affect Tax-mediated NF-κB activation. We found that unexpectedly, the activation of NF-κB promoter mediated by Tax-1 is about six times reduced in TRAF3$^{-/-}$ cells (**Figure 31** – panel A).

In order to verify if the reduced activity of NF-κB activation mediated by Tax protein could be due to a different intracellular distribution of Tax-1 and of the endogenous p65, we performed immunofluorescence analyses (**Figure 31** – panel B). As shown in **figure 32** panel B, the amount of nuclear p65 signal (green signal) was higher in TRAF3$^{-/-}$ comparing to HEK293T TRAF3$^{-/-}$ cells. The presence of Tax-1 induced the nuclear translocation of p65 in both the parental cells and the TRAF3$^{-/-}$ cells. The reduced activation of NF-κB mediated by Tax-1 in TRAF3$^{-/-}$ cells can be explained from the analyses of the p65 nuclear translocation in terms of nuclear p65 signal intensity. As shown in the plot (**Figure 31** - panel B) representative of the nuclear p65 staining analyses, we found that in the TRAF3$^{-/-}$ cells in the presence of Tax-1, p65 nuclear staining is reduced of about 1.5 times compared to the parental cell line.

Taken together, these results indicate that the absence of TRAF3 can impair the NF-κB transcription activity mediated by Tax-1.

**A**



**B**



☐ Cell transfected with vector expressing Tax
■ Cell transfected with empty vector



**Figure 31. Tax-1-mediated NF-κB activation is impaired in the absence of TRAF3.**
**(A)** HEK293T and TRAF3$^{-/-}$ cells were transfected with 200 ng TK-renilla control, 500 ng κB-luciferase reporter, and 50 ng Tax-1, or the control expression vector. 24 h after transfection, cell lysates were collected and luciferase levels were measured. Asterisk (*) indicates statistical significance of $p = <0.05$, obtained by Student's *t*-test (Top). Immunoblot analysis was performed to detect the expression of Tax-1 protein (Bottom). **(B)** Immunofluorescence analysis revealed the endogenous p65 subcellular distribution in HEK293T and TRAF3$^{-/-}$ cells in the presence of Tax-1. Cells were fixed with 4% PFA, and stained with anti-p65, anti-Tax-1 primary

antibodies, Alexa 488, Alexa 594 secondary antibodies detect p65 (green signal), and Tax-1 (red signal), respectively. Nuclei are stained with DAPI (blue signal). Scalebar, 10µm. Total cells and nuclei of cells indicated by the white arrow (non-trasfected cells) and red arrow (cells transfected with Tax-1) were delineated using Image J software, and the mean brightness ratio of the nuclear p65 staining was calculated and plotted.
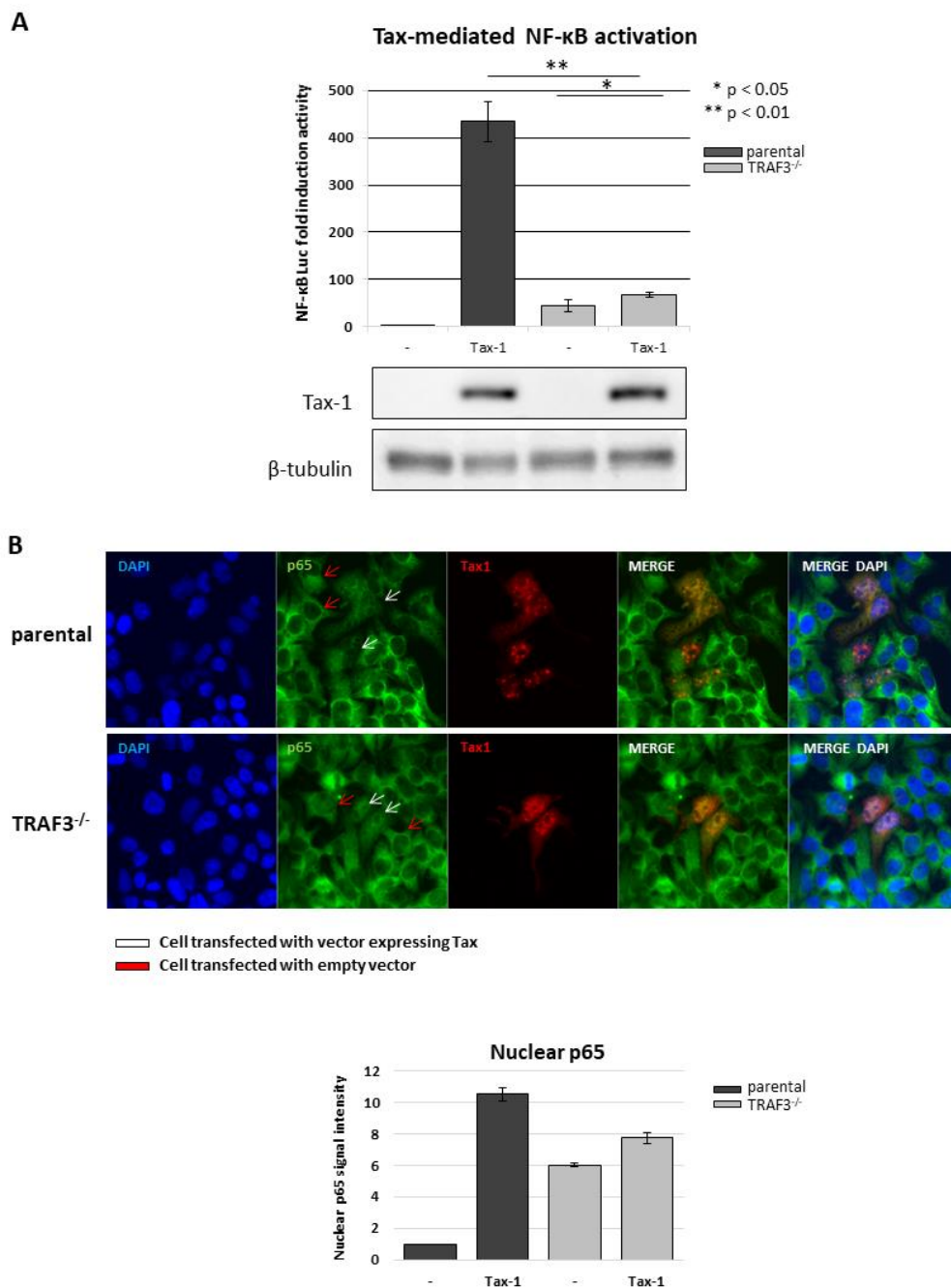
## 4.11 The LXXLL2 domain of APH-2 is required for PML localization

During the PhD program I spent a period abroad participating in research activities at the International Center for Infectiology Research (CIRI) in Lyon collaborating with Renaud Mahieux's research group. This experience gave me the opportunity to apply confocal microscopy analyses, aimed to investigate the differences in the post-translational mechanisms that control the expression of HBZ and APH-2 proteins.

Previous studies demonstrated that APH-2, unlike HBZ, presents at the C-terminal the $LXXLL_2$ domain, which represents a protein-protein interaction motif necessary to form complexes with CREB into the nucleus to contrast the Tax-mediated viral transcription (Yin et al., 2012). In order to examine whether the C-terminal $LXXLL_2$ motif may be involved in the PML-nuclear bodies localization, I investigated the intracellular distribution of a truncated version of the APH-2 protein, $APH-2_{1-178}$, which lacks the C-terminal $LXXLL_2$ motif. Jurkat cells were transfected with full-length or $APH-2_{1-178}$ and analyzed by confocal microscopy imaging after PML staining (**Figure 32**). The results showed that, while full-length APH-2 was distributed in PML-NBs, $APH-2_{1-178}$ mutant was found diffuse in the cytoplasm of cells, indicating that in T-cells, the $LXXLL_2$ domain of APH-2 is required for nuclear localization and more specifically for PML-NBs localization.
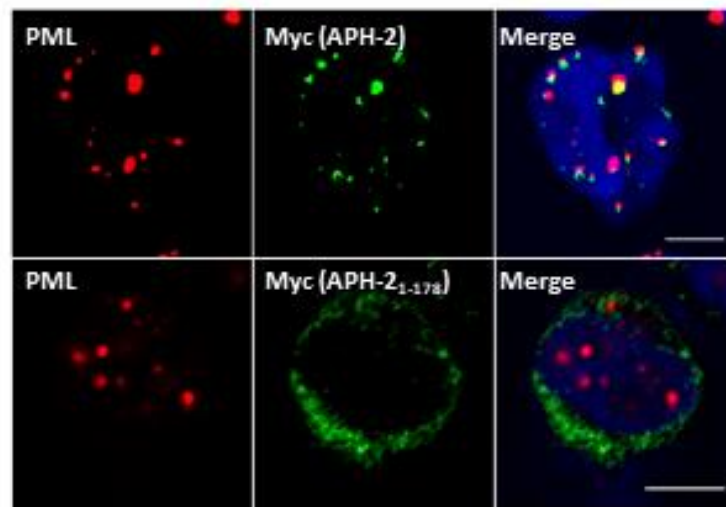
**Figure 32. The LXXLL$_2$ domain of APH-2 is required for PML localization.**
Jurkat T-cells were transfected with full length APH-2-Myc or with APH-2$_{1-178}$ and fixed 24 hours after transfection. Subcellular localization of APH-2 was examined by confocal microscopy after anti-Myc and anti-PML staining. Nuclei were counterstained with DAPI (blue). Scale bar = 10 µm.

## 5. DISCUSSION

NF-κB is a transcription factor family that regulates a large number of genes that are involved in cell survival, inflammation, and immune responses (Sun et al., 2011). Constitutive expression of NF-κB has been associated with several types of cancer (Xia et al., 2014). The activation of the classical NF-κB cell signaling pathway has been extensively studied for its role in cancer, whereas the understanding of the role of the alternative NF-κB pathway regulation is limited. Several evidences demonstrated that a subset of human neoplasms shows constitutive activation of the alternative pathway of NF-κB, modulating the progression of cancer, such as prostate cancer, spontaneous squamous cell-like carcinoma and melanoma cells (Guo et al., 2011; Liu et al., 2006; Dewert et al., 2016). NF-κB is an attractive target also for human oncogenic viruses, including HTLV. These viruses evolved powerful mechanisms to persist and replicate through deregulation of the NF-κB pathways, conveying cancer hallmarks to the infected cell (Mesri et al., 2014). HTLV Tax-1 and Tax-2 are constitutive activators of NF-κB and despite their homology, they differ in the activation of canonical and non-canonical NF-κB pathways. To which extent these differences may contribute to the diversity in the pathogenic potential of the two viruses is not yet understood. The recent characterization of the role of the HTLV antisense proteins that counteracts Tax activities, such as their different impact on NF-κB, has opened a new area of investigation on their interplay in deregulation of cell pathways.

Previous studies from our research group have contributed to the characterization of the Tax intracellular distribution and co-localization with host factors. Recently we identified a new interaction of the Tax proteins with the TRAF3 factor that plays a key role on IFN and non-canonical NF-κB pathway. The distinct functions that HBZ and APH-2 may exercise on the NF-κB pathway based on their host factor interaction are almost unknown. My PhD thesis was focused on the investigation of the role of the antisense HTLV proteins, HBZ and APH-2, in inhibiting the NF-κB pathway.

In this study, we demonstrated for the first time that the antisense protein APH-2 differs in the molecular mechanism of the NF-κB pathway inhibition, compared to HBZ, furthermore, we identified that Tax recruitment of the host regulator TRAF3 is required for an efficient activation of NF-κB.

We demonstrated that both HBZ and APH-2 reduce the NF-κB pathway activation mediated by the host key factor p65 and the viral transactivator Tax. At first, we verified that both HBZ and APH-2 interact with p65 and reduce p65-mediated NF-κB promoter activation in HEK293T cells, validating the previous data shown by Panfil *et al.* (2016). We then confirmed the inhibitory effect induced by APH-2 in Jurkat T cells, demonstrating that APH-2 can inhibit p65 also in a cell model closest to infection. This result is in contrast with that obtained by Panfil *et al*. in which they affirmed that APH-2 does not inhibit NF-κB in T cells. The authors highlighted inherent difficulties in transient transfection of lymphoid cells that may explain the absence of the inhibitory effect. Herein, to bypass the technical difficulties highlighted, we used electroporation as a mechanical transfection method to transiently express viral proteins in Jurkat cells. This may explain the difference in the results.

We showed that HBZ is less efficient in contrasting Tax-mediated NF-κB activity compared to APH-2. The co-expression of HBZ and high levels of Tax, in fact, results in the re-establishment of the NF-κB activation, whereas APH-2 maintains its inhibitory effect in the presence of Tax-2. We verify if the more efficient inhibitory effect induced by APH-2 on Tax-mediated NF-κB activation could be explained by the fact that APH-2 interacts with Tax-2 and may be recruited in the cytoplasmic protein complexes that contain Tax-2. A previous study conducted by our research group describes the presence of Tax in calreticulin-containing cytoplasmic structures together with the final effector p65, the essential NF-κB modulator NEMO and the adaptor protein TAB2 (Avesani et al., 2010). In the present study, we showed that when the antisense proteins are co-expressed with Tax proteins, APH-2, but not HBZ, is recruited in cytoplasmic structures containing Tax-2, TAB2 and NEMO. We demonstrate for the first time by confocal microscopy that in the presence of APH-2 and Tax-2, p65 is mostly localized in the cytoplasm suggesting that the down-regulation of the Tax-

mediated NF-κB activation by APH-2 may be due to the impairment of p65 nuclear translocation. This implies that APH-2 might interfere with the IKK complex activation. This hypothesis is supported by our results showing that in the presence of APH-2, differently to HBZ, NF-κB inhibitor IκB accumulates in the cell lysates. In accord with our results, Marban *et al.*, demonstrated a redistribution of APH-2 in the cytoplasm in the presence of Tax-2. They revealed that Tax-2B expression alters APH-2 localization, which moves from the nucleus to the cytoplasm, a mechanism that could prevent APH-2 from activating AP-1 transcription (Marban et al., 2012).

An additional mechanism that may explain the different action of HBZ and APH-2 in the inhibition of Tax-mediated NF-κB is their effect on the alternative NF-κB pathway. It has been demonstrated that Tax-negative leukemic cell lines as well as freshly isolated ATL cells exhibit abnormally high levels of expression of the IKKα activating the kinase NIK. This suggests that alternative NF-κB factor NIK could play an important role during the late stages of ATL. It has previously suggested that HBZ selectively inhibits the classical NF-κB pathway (Zhao et al., 2009). However, another study has shown that the level of endogenous p100 was lowered in HBZ-expressing cells (Zhi et al., 2011). No information is available relative to the APH-2 selective action on canonical or non-canonical NF-κB pathway. We have previously demonstrated that Tax-2 interacts with an upstream adaptor factor TRAF3, which is a negative regulator of the alternative NF-κB pathway. TRAF3 is an ubiquitin ligase that controls the NIK expression levels (Häcker et al., 2011). In the present study, we demonstrate for the first time that APH-2, in contrast to HBZ, forms cytoplasmic complexes with TRAF3 and Tax-2 and that in the presence of HBZ and APH-2, TRAF3 is less represented in cell lysates. This last phenomenon appears to be more represented in the presence of HBZ. We have not yet clarified how HBZ can negatively regulate the expression of TRAF3. The reduction of TRAF3 abundance is a mechanism induced by other viruses to control the activation of the alternative NF-κB cell signaling. The oncoprotein Tio of the T-lymphotropic virus *Herpesvirus ateles*, acts as a molecular probe to decipher oncogenic signaling cascades in T cells, like HTLV-1 Tax (de Jong et al., 2010). Tio directly interacts with TRAF3 inducing a

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stabilization of NIK that consequently activates the non-canonical NF-κB signaling (de Jong et al., 2013).

TRAF3$^{-/-}$ 293T cell lines displayed moderately enhanced NF-κB promoter activation, in the absence of NF-κB stimulus and a detectable nuclear distribution of the transcriptional factor p65. We have confirmed that NF-κB is activated in the absence of TRAF3, whereas the transient expression of TRAF3 abolished the NF-κB promoter activity. These analyses are in accord with studies conducted *in vivo* in which it has been demonstrated that mouse B cells deficient in TRAF3 correlate with constitutive activation of the alternative NF-κB pathway (Xie et al., 2007). When we analyzed Tax-1 activity in TRAF3 negative cell line, we found unexpectedly that Tax-1 does not induce the activation of NF-κB. This result is in line with another study, which analyses the Epstein-Barr virus-encoded oncoprotein latent membrane protein 1 (LMP1) activity on the NF-κB cell signaling (Xie et al., 2004). LMP1 is a major contributing factor to the development of EBV-associated lymphoproliferative disease and lymphomas. LMP1 is an integral membrane protein that activates NF-κB pathway recruiting TRAFs adaptor proteins, Xie *et al.* demonstrate that LMP1-mediated activation of NF-κB is substantially affected by TRAF3 deficiency, in B cells.

By comparing the NF-κB activation mediated by others human oncogenic viruses, it is of interest to note that the viral vFLIP protein expressed by KSHV activates NF-κB interacting with the IKK complex, and the TRAF2/3 complex. The immediate–early BZLF1 protein encoded by EBV, like HTLV-1 HBZ protein, inhibits the classical NF-κB pathway through the interaction with p65 (Morrison et al., 2004). The role of NF-κB in EBV and KSHV-mediated oncogenesis has been highlighted by studies demonstrating that the IKK inhibitor Bay11-7082 induces apoptosis in infected cell lines and in mice (Keller et al., 2006). Concerning HIV-1, the coded Tat protein enhances NF-κB by hijacking the inhibitor IκB-α and by preventing the repressor binding to the NF-κB complex, whereas Nef protein suppresses this pathway inducing the expression of IκB-α, resulting in the viral evasion of protective T cell responses (Qiao et al., 2006; Fiume et al., 2012).

## 6. CONCLUSION AND PERSPECTIVES

Our data highlight for the first time that the two antisense proteins of HTLV-1 and HTLV-2 exert a different molecular mechanism in the down-modulation of the NF-κB pathway. We propose that the different molecular mechanism induced by the viral protein, can be explained by the recruitment of APH-2 in Tax-2-cytoplasmic structures with NF-κB members that affect p65 nuclear translocation, differently to HBZ which acts mainly in the nucleus at the transcriptional level (**Figure 33**).
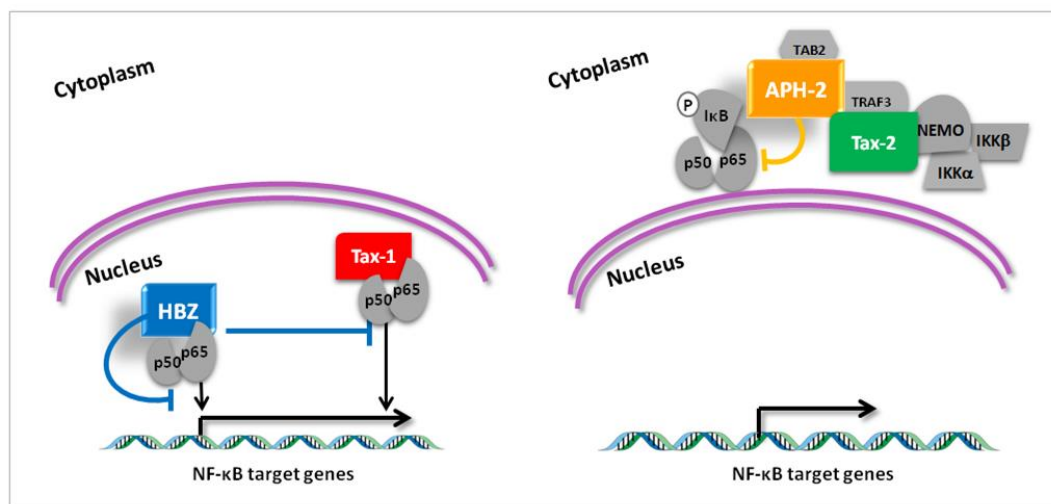


**Figure 33.** Molecular model of APH-2 and HBZ inhibitory mechanism of NF-κB pathway.

In addition, we demonstrate the importance of a novel interaction between the host factor TRAF3 and the oncoprotein Tax-1 in the modulation of the NF-κB pathway. This allows us to highlight the relevance of the non-canonical NF-κB pathway in contributing to the differences of the viral antisense proteins' activities. It is worthy of note that TRAF3 is implicated in several cell signaling pathways, such as IFN and autophagy. TRAF3 is crucial for type I IFN and IL-10 production, in fact, it has been demonstrated that cells lacking TRAF3 are defective in type I IFN responses activated by several different TLRs (Häcker et al., 2011). A recent report demonstrates that TRAF3 degradation is regulated by autophagy leading to NF-κB induction, a phenomenon that culminates in nuclear

translocation of the transcription factor RelB (Newman et al., 2017). Further studies will be devoted to analyze the interplay of TRAF3 and antisense viral proteins in deregulating the IFN response and autophagy. The deep knowledge in the field of the NF-κB activation, as cellular response or mediated by pathogens contributes to drug discovery and paves the way for our current understanding of biological processes of cell proliferation and survival.

# 7. REFERENCES

Akagi, T., Ono, H., Nyunoya, H., Shimotohno, K. (1997). Characterization of peripheral blood T-lymphocytes transduced with HTLV-I Tax mutants with different trans-activating phenotypes. *Oncogene*. 14(17):2071-8.

Alefantis, T., Barmak, K., Harhaj, E.W., Grant, C., Wigdahl, B. (2003). Characterization of a nuclear export signal within the human T cell leukemia virus type I transactivator protein Tax. *J Biol Chem.* 278(24):21814-22.

Andrade, R.G., Gonçalves, P. C., Ribeiro, M.A., Romanelli, L.C., Ribas, J.G., Torres, E.B., et al. (2013). Strong correlation between tax and HBZ mRNA expression in HAM/TSP patients: distinct markers for the neurologic disease. *J Clin Virol.* 56(2):135-40. doi: 10.1016/j.jcv.2012.10.003.

Araujo, A. and Hall., W.W. (2004). Human T-lymphotropic virus type II and neurological disease. *Ann Neurol.* 56:10-9.

Arnold, J., Yamamoto, B., Li, M., Phipps, A.J., Younis, I., Lairmore, M.D. et al. (2006). Enhancement of infectivity and persistence in vivo by HBZ, a natural antisense coded protein of HTLV-1. *Blood.* 107(10):3976-82.

Arnold, J., Zimmerman, B., Li, M., Lairmore, M.D. and Green, P.L. (2008). Human Tcell leukemia virus type-1 antisense-encoded gene, HBZ, promotes T-lymphocyte proliferation. *Blood.* 112(9):3788-97. doi: 10.1182/blood-2008-04-154286.

Avesani, F., Romanelli, M.G., Turci, M., Di Gennaro, G., Sampaio, C., Bidoia, C., et al. (2010). Association of HTLV Tax proteins with TAK1binding protein 2 and RelA in calreticulin-containing cytoplasmic structures participates in Tax-mediated NF-κB activation. *Virology.* 408(1):39-48. doi: 10.1016/j.virol.2010.08.023.

Banerjee, P., Rochford, R., Antel, J., Canute, G., Wrzesinski, S., Sieburg, M. et al. (2007). Proinflammatory cytokine gene induction by human T-cell leukemia virus type 1 (HTLV-1) and HTLV-2 Tax in primary human glial cells. *J. Virol.* 81 1690–170010.1128/JVI.01513-06.

Bangham, C.R.M. and Matsuoka, M. (2017). Human T-cell leukaemia virus type 1: parasitism and pathogenesis. *Philos Trans R Soc Lond B Biol Sci.* 372(1732). pii: 20160272. doi: 10.1098/rstb.2016.0272.

Baratella, M., Forlani, G., Raval, G.U., Tedeschi, A., Gout, O., Gessain, A. et al. (2017). Cytoplasmic Localization of HTLV-1 HBZ Protein: A Biomarker of HTLV-

1-Associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP). *PLoS Negl Trop Dis.* 11(1):e0005285. doi: 10.1371/journal.pntd.0005285.

Barbeau, B. and Mesnard, J.M. (2011). Making sense out of antisense transcription in human T-cell lymphotropic viruses (HTLVs). *Viruses.* 3(5):456-68. doi:10.3390/v3050456.

Barbeau, B., Peloponese, J.M. and Mesnard, J.M. (2013). Functional comparison of antisense proteins of HTLV-1 and HTLV-2 in viral pathogenesis. *Front Microbiol.* 4:226. doi: 10.3389/fmicb.2013.00226.

Baydoun, H.H., Cherian, M.A., Green, P., Ratner, L. (2015). Inducible nitric oxide synthase mediates DNA double strand breaks in Human T-Cell Leukemia Virus Type 1-induced leukemia/lymphoma. *Retrovirology.* 12:71. doi: 10.1186/s12977-015-0196-y.

Becker, J.C. (2010). Merkel cell carcinoma. *Ann. Oncol.* 21(Suppl. 7), vii81–vii85. 10.1093/annonc/mdq366.

Bergamo, E., Diani, E., Bertazzoni, U., Romanelli, M.G. (2017). A Luciferase Functional Quantitative Assay for Measuring NF-kB Promoter Transactivation Mediated by HTLV-1 and HTLV-2 Tax Proteins. *Methods Mol Biol.*1582:79-87. doi: 10.1007/978-1-4939-6872-5_6.

Bertazzoni, U., Turci, M., Avesani, F., Di Gennaro, G., Bidoia, C., Romanelli, M.G. (2011). Intracellular localization and cellular factors interaction of HTLV-1 and HTLV-2 Tax proteins: similarities and functional differences. *Viruses.* 3(5):541-60. doi: 10.3390/v3050541.

Bex, F., McDowall, A., Burny, A., Gaynor, R. (1997). The human T-cell leukemia virus type 1 transactivator protein Tax colocalizes in unique nuclear structures with NF-kappaB proteins. *J Virol.* 71(5):3484-97.

Bhutani, M., Polizzotto, M.N., Uldrick, T.S., Yarchoan, R. (2015). Kaposi sarcoma-associated herpesvirus-associated malignancies: epidemiology, pathogenesis, and advances in treatment. *Semin. Oncol.* 42, 223–246. 10.1053/j.seminoncol.2014.12.027

Bidoia C., Mazzorana M., Pagano M. A., Arrigoni G., Meggio F., Pinna L. A. et al. (2010). The pleiotropic protein kinase CK2 phosphorylates HTLV-1 Tax protein in vitro, targeting its PDZ-binding motif. *Virus Genes* 41 149–15710.1007/s11262-010-0494-3

Biswas, H.H., Kaidarova, Z., Garratty, G., Gibble, J.W., Newman, B.H., Smith, J.W. et al. (2010) Increased all-cause and cancer mortality in HTLV-II

infection. *J Acquir Immune Defic Syndr*. 54(3):290-6. doi: 10.1097/QAI.0b013e3181cc5481.

Bouvard, V., Baan, R., Straif, K., Grosse, Y., Secretan, B., El Ghissassi, F. et al. (2009) A review of human carcinogens-Part B: biological agents. *The lancet oncology*. 10:321–322.

Calattini S., Chevalier S. A., Duprez R., Afonso P., Froment A., Gessain A. et al. (2006). Human T-cell lymphotropic virus type 3: complete nucleotide sequence and characterization of the human tax3 protein. *J. Virol*. 80 9876–988810.1128/JVI.00799-06

Calattini, S., Chevalier, S., Duprez, R., Bassot, S., Froment, A., Mahieux, R. and Gessain, A. (2005). Discovery of a new human T-cell lymphotropic virus (HTLV-3) in Central Africa. *Retrovirology*. 2(1):30.

Chan, J.K. and Greene, W.C. (2012). Dynamic roles for NF-κB in HTLV-I and HIV-1 retroviral pathogenesis. *Immunol Rev*. 246(1):286-310. doi: 10.1111/j.1600-065X.2012.01094.x.

Chen, L., Liu, D., Zhang, Y., Zhang, H., Cheng, H. (2015). The autophagy molecule Beclin 1 maintains persistent activity of NF-κB and Stat3 in HTLV-1-transformed T lymphocytes. *Biochemical and Biophysical Research Communications*. 465(4), 739–745. doi: 10.1016/j.bbrc.2015.08.070

Chevalier, S.A., Durand, S., Dasgupta, A., Radonovich, M., Cimarelli, A., Brady, J.N. et al. (2012). The transcription profile of Tax-3 is more similar to Tax-1 than Tax-2: insights into HTLV-3 potential leukemogenic properties. *PLoS ONE*. 7:e41003 10.1371/journal.pone.0041003

Chevalier, S.A., Meertens, L., Calattini, S., Gessain, A., Kiemer, L., Mahieux, R. (2005). Presence of a functional but dispensable nuclear export signal in the HTLV-2 Tax protein. *Retrovirology*. 14;2:70.

Chevalier, S.A., Meertens, L., Pise-Masison, C., Calattini, S., Park, H., Alhaj, A.A. et al. (2006). The tax protein from the primate T-cell lymphotropic virus type 3 is expressed in vivo and is functionally related to HTLV-1 Tax rather than HTLV-2 Tax. *Oncogene* 25, 4470-82.

Ciminale, V., Rende, F., Bertazzoni, U., Romanelli, M.G. (2014). HTLV-1 and HTLV-2: highly similar viruses with distinct oncogenic properties. *Front Microbiol*. 5:398. doi: 10.3389/fmicb.2014.00398.

Cook, L.B., Melamed, A., Niederer, H., Valganon, M., Laydon, D., Foroni, L. et al. (2014). The role of HTLV-1 clonality, proviral structure, and genomic integration

site in adult T-cell leukemia/lymphoma. *Blood* 123(25):3925-31. doi: 10.1182/blood-2014-02-553602.

de Jong, S.J., Albrecht, J.C., Giehler, F., Kieser, A., Sticht, H., Biesinger, B. (2013). Noncanonical NF-κB activation by the oncoprotein Tio occurs through a nonconserved TRAF3-binding motif. *Sci Signal*. 6(272):ra27. doi: 10.1126/scisignal.2003309.

de Jong, S.J., Albrecht, J.C., Schmidt, M., Müller-Fleckenstein, I., Biesinger, B. (2010). Activation of noncanonical NF-kappaB signaling by the oncoprotein Tio. *J Biol Chem*. 285(22):16495-503. doi: 10.1074/jbc.M110.102848.

Dewert, N., Amschler, K., Lorenz, V., Schön, M.P. (2016). The IKKα-dependent non-canonical pathway of NF-κB activation is constitutively active and modulates progression-related functions in a subset of human melanomas. *Arch Dermatol Res*. 308(10):733-742

Diani, E., Avesani, F., Bergamo, E., Cremonese, G., Bertazzoni, U., Romanelli, M. G. (2015). HTLV-1 Tax protein recruitment into IKKε and TBK1 kinase complexes enhances IFN-I expression. *Virology* 476, 92–99. doi: 10.1016/j.virol.2014.12.005

Doi, K., Wu, X., Taniguchi, Y., Yasunaga, J., Satou, Y., Okayama, A. et al. (2005). Preferential selection of human T-cell leukemia virus type I provirus integration sites in leukemic versus carrier states. *Blood* 106(3):1048-53.

Douceron, E., Kaidarova, Z., Miyazato, P., Matsuoka, M., Murphy, E.L., Mahieux, R. (2012). HTLV-2 APH-2 expression is correlated with proviral load but APH-2 does not promote lymphocytosis. *J Infect Dis*. 205(1):82-6. doi: 10.1093/infdis/jir708.

Dubuisson, L., Lormières, F., Fochi, S., Turpin, J., Pasquier, A., Douceron, E. et al. (2018). Stability of HTLV-2 Antisense Protein is Controlled by PML Nuclear Bodies in a SUMO-Dependent Manner. *Oncogene* doi:10.1038/s41388-018-0163-x

Durand, J.K. and Baldwin, A.S. (2017). Targeting IKK and NF-κB for Therapy. Advances in Protein Chemistry and Structural Biology. *Elsevier Inc.* 1st ed., Vol. 107 doi: 10.1016/bs.apcsb.2016.11.006

Durkin, S.S., Guo, X., Fryrear, K.A., Mihaylova, V.T., Gupta, S.K., Belgnaoui, S.M. et al. (2008). HTLV-1 Tax oncoprotein subverts the cellular DNA damage response via binding to DNA-dependent protein kinase. *J Biol Chem.* 283(52):36311-20. doi: 10.1074/jbc.M804931200.

Ego T., Ariumi Y., Shimotohno K. (2002). The interaction of HTLV-1 Tax with HDAC1 negatively regulates the viral gene expression. *Oncogene* 21 7241–724610.1038/sj.onc.1205701

Endo K., Hirata A., Iwai K., Sakurai M., Fukushi M., Oie M. et al. (2002). Human T-cell leukemia virus type 2 (HTLV-2) Tax protein transforms a rat fibroblast cell line but less efficiently than HTLV-1 Tax. *J. Virol.* 76 2648–265310.1128/JVI.76.6.2648-2653.2002

Feng, H., Shuda, M., Chang, Y., Moore, P.S. (2008). Clonal integration of a polyomavirus in human Merkel cell carcinoma. *Science* 319, 1096–1100. 10.1126/science.1152586

Feuer, G., Green, P.L. (2005). Comparative biology of human T-cell lymphotropic virus type 1 (HTLV-1) and HTLV-2. *Oncogene* 24 5996–600410.1038/sj.onc.1208971

Fiume, G., Vecchio, E., De Laurentiis, A., Trimboli, F., Palmieri, C., Pisano, A. et al. (2012). Human immunodeficiency virus-1 Tat activates NF-κB via physical interaction with IκB-α and p65. *Nucleic Acids Res*. 40(8):3548-62. doi: 10.1093/nar/gkr1224.

Fochi, S., Mutascio, S., Bertazzoni, U., Zipeto, D., Romanelli, M.G. (2018). HTLV deregulation of the NF-κB pathway: an update on Tax and antisense proteins role *Front. Microbiol.* https://doi.org/10.3389/fmicb.2018.00285

Forlani, G., Abdallah, R., Accolla, R. S., Tosi, G. (2016). The Major Histocompatibility Complex Class II Transactivator CIITA Inhibits the Persistent Activation of NF-κB by the Human T Cell Lymphotropic Virus Type 1 Tax-1 Oncoprotein. *Journal of Virology.* 90(7), 3708–3721. doi: 10.1128/JVI.03000-15

Forlani, G., Abdallah, R., Accolla, R.S., Tosi, G. (2013). The MHC-II transactivator CIITA, a restriction factor against oncogenic HTLV-1 and HTLV-2 retroviruses: similarities and differences in the inhibition of Tax-1 and Tax-2 viral transactivators. *Front Microbiol*. 4:234. doi: 10.3389/fmicb.2013.00234.

Fujii, M., Iwai, K., Oie, M., Fukushi, M., Yamamoto, N., Kannagi, M. et al. (2000) Activation of oncogenic transcription factor AP-1 in T cells infected with human T cell leukemia virus type 1. *AIDS Res Hum Retroviruses*. 16(16):1603-6.

Fujikawa, D., Nakagawa, S., Hori, M., Kurokawa, N., Soejima, A., Nakano, K. et al. (2016). Polycomb-dependent epigenetic landscape in adult T-cell leukemia. *Blood* 127(14):1790-802. doi: 10.1182/blood-2015-08-662593.

Furukawa, Y., Kubota, R., Tara, M., Izumo, S., Osame, M. (2001). Existence of escape mutant in HTLV-I tax during the development of adult T-cell leukemia. *Blood* 97(4), 987–993.

Gall, R.C. (2005). History of the discoveries of the first human retroviruses: HTLV-1 and HTLV-2. *Oncogene* 24(39):5926-30.

Gallo, R.C., Willems, L., Tagaya, Y. (2017). Time to Go Back to the Original Name. *Front Microbiol*. 8:1800. doi: 10.3389/fmicb.2017.01800.

Gazon, H., Barbeau, B., Mesnard, J.M., Peloponese, J.M. (2017). Hijacking of the AP-1 Signaling Pathway during Development of ATL. *Front Microbiol*. 8:2686. doi: 10.3389/fmicb.2017.02686.

Gessain, A. and Cassar, O. (2012). Epidemiological Aspects and World Distribution of HTLV-1 Infection. *Front Microbiol*. 3:388. doi: 10.3389/fmicb.2012.00388.

Gessain, A. and Mahieux, R. (2012). Tropical spastic paraparesis and HTLV-1 associated myelopathy: clinical, epidemiological, virological and therapeutic aspects. *Rev Neurol*. 168(3):257-69. doi: 10.1016/j.neurol.2011.12.006.

Gessain, A., Barin, F., Vernant, J.C., Gout, O., Maurs, L., Calender, A. et al. (1985). Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis. *Lancet*. 2(8452):407-10.

Giam, C.Z. and Semmes, O.J. (2016). HTLV-1 infection and adult T-cell leukemia/ lymphoma-A tale of two proteins: Tax and HBZ. *Viruses* 8(6). doi: 10.3390/v8060161

Gonçalves, D.U., Proietti, F.A., Ribas, J.G., Araújo, M.G., Pinheiro, S.R., Guedes, A.C. et al. (2010). Epidemiology, treatment, and prevention of human T-cell leukemia virus type 1-associated diseases. *Clin Microbiol Rev*. 23(3):577-89. doi: 10.1128/CMR.00063-09.

Graham, R.L., Burch, M., Krause, J.R. (2014). Adult T-cell leukemia/lymphoma. *Proc (Bayl Univ Med Cent)*. 27(3): 235–238.

Gross, C., Thoma-Kress, A.K. (2016). Molecular Mechanisms of HTLV-1 Cell-to-Cell Transmission. *Viruses* 8(3):74. doi: 10.3390/v8030074.

Guo, F., Kang, S., Zhou, P., Guo, L., Ma, L., Hou, J. (2011). Maspin expression is regulated by the non-canonical NF-κB subunit in androgen-insensitive prostate cancer cell lines. *Molecular Immunology* 49(1–2), 8–17. http://doi.org/10.1016/j.molimm.2011.07.013

Häcker, H., Tseng, P.H., Karin, M. (2011). Expanding TRAF function: TRAF3 as a tri-faced immune regulator. *Nat Rev Immunol.* 10;11(7):457-68. doi: 10.1038/nri2998.

Häcker, H., Redecke, V., Blagoev, B., Kratchmarova, I., Hsu, L-C., Wang, G.G. et al. (2006) Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. *Nature* 439, 204–7. doi:10.1038/nature04369.

Hagiya, K., Yasunaga, J., Satou, Y., Ohshima, K., Matsuoka, M. (2011). ATF3, an HTLV-1 bZip factor binding protein, promotes proliferation of adult T-cell leukemia cells. *Retrovirology* 8:19. doi: 10.1186/1742-4690-8-19.

Hahn, B., Manzari, V., Colombini, S., Franchini, G., Gallo, R.C., Wong-Staal, F. (1983). Common site of integration of HTLV in cells of three patients with mature T-cell leukaemia-lymphoma: a retraction. *Nature* 305:340. 10.1038/305340a0

Halin, M., Douceron, E., Clerc, I., Journo, C., Ko, N.L., Landry, S. et al. (2009). Human T-cell leukemia virus type 2 produces a spliced antisense transcript encoding a protein that lacks a classic bZIP domain but still inhibits Tax2-mediated transcription. *Blood* 114(12):2427-38. doi: 10.1182/blood-2008-09-179879.

Hariri, S., Unger, E.R., Sternberg, M., Dunne, E.F., Swan, D., Patel, S. et al. (2011). Prevalence of genital human papillomavirus among females in the United States, the National Health And Nutrition Examination Survey, 2003–2006. *J. Infect. Dis*. 204, 566–573. 10.1093/infdis/jir341

Harrod, R., Kuo, Y.L., Tang, Y., Yao, Y., Vassilev, A., Nakatani, Y. et al. (2000). P300 and p300/camp-responsive element-binding protein associated factor interact with human T-cell lymphotropic virus type-1 Tax in a multi-histone acetyltransferase/activator-enhancer complex. *J. Biol. Chem.* 275 11852–1185710.1074/jbc.275.16.11852

Higuchi, M. and Fujii, M. (2009). Distinct functions of HTLV-1 Tax1 from HTLV-2 tax2 contribute key roles to viral pathogenesis. *Retrovirology* 6 11710.1186/1742-4690-6-117

Higuchi, M., Tsubata, C., Kondo, R., Yoshida, S., Takahashi, M., Oie, M. et al. (2007). Cooperation of NF-kappaB2/p100 activation and the PDZ domain binding motif signal in human T-cell leukemia virus type 1 (HTLV-1) Tax1 but not HTLV-2 Tax2 is crucial for interleukin-2-independent growth transformation of a T-cell line. *J Virol.* 81(21):11900-7.

Hinuma, Y., Nagata, K., Hanaoka, M., Nakai, M., Matsumoto, T., Kinoshita, K. et al. (1981). Adult T-cell leukemia: antigen in an ATL cell line and detection of

antibodies to the antigen in human sera. *Proc. Natl. Acad. Sci. U.S.A.* 78, 6476–6480. 10.1073/pnas.78.10.6476

Hiscott, J., Kwon, H., Génin, P. (2001). Hostile takeovers: viral appropriation of the NF-kB pathway. *J Clin Invest*. 107(2): 143–151. doi:10.1172/JCI11918

Hjelle, B., Appenzeller, O., Mills, R., Alexander, S., Torrez-Martinez, N., Jahnke, R. et al. (1992). Chronic neurodegenerative disease associated with HTLV-II infection. *Lancet* 339:645-646.

Huang, J., Ren, T., Guan, H., Jiang, Y., Cheng, H. (2009). HTLV-1 Tax is a critical lipid raft modulator that hijacks IkappaB kinases to the microdomains for persistent activation of NF-kappaB. *J Biol Chem*. 284, 6208-17.

Huang, Q., Niu, Z., Han, J., Liu, X., Lv, Z. (2017). HTLV-1 Tax upregulates early growth response protein 1 through nuclear factor-κB signaling. *Oncotarget*. 8(31): 51123–51133. doi:10.18632/oncotarget.17699

Imai, M., Higuchi, M., Kawamura, H., Yoshita, M., Takahashi, M., Oie, M. (2013) Human T cell leukemia virus type 2 (HTLV-2) Tax2 has a dominant activity over HTLV-1 Tax1 to immortalize human CD4+ T cells. *Virus Genes*. 46, 39-46.

Iwai, K, Mori N, Oie M, Yamamoto N, Fujii M. (2001) Human T-cell leukemia virus type 1 tax protein activates transcription through AP-1 site by inducing DNA binding activity in T cells. *Virology*. 279(1):38-46.

Jacobson, S., Lehky, T., Nishimura, M., Robinson, S., McFarlin, D.E., DhibJalbut, S. (1993). Isolation of HTLV-II from a patient with chronic, progressive neurological disease clinically indistinguishable from HTLV-Iassociated myelopathy/tropical spastic paraparesis. *Ann Neurol*. 33:392-396.

Jeong, S.J., Pise-Masison, C.A., Radonovich, M.F., Park, H.U., Brady, J.N. (2005). Activated AKT regulates NF-κB activation, p53 inhibition and cell survival in HTLV1-transformed cells. *Oncogene* 24, 6719-6728.

Jin, D.Y., Spencer, F., Jeang, K.T. (1998). Human T cell leukemia virus type 1 oncoprotein Tax targets the human mitotic checkpoint protein MAD1. *Cell*. 93(1):81-91.

Jin, J., Hu, H., Li, H.S, Yu, J., Xiao, Y., Brittain, G.C. et al. (2014). Noncanonical NF-κB Pathway Controls the Production of Type I Interferons in Antiviral Innate Immunity. *Immunity* 40(3): 342-354. doi:10.1016/j.immuni.2014.02.006

Johnson, J.M., Mulloy, J.C., Ciminale, V., Fullen, J., Nicot, C., Franchini G. (2000) The MHC class I heavy chain is a common target of the small proteins encoded by the 3' end of HTLV type 1 and HTLV type 2. *AIDS Res Hum Retroviruses*. 16(16):1777-81.

Johnson, J.M., Nicot, C., Fullen, J., Ciminale, V., Casareto, L., Mulloy, J.C. et al. (2001) Free major histocompatibility complex class I heavy chain is preferentially targeted for degradation by human T-cell leukemia/lymphotropic virus type 1 p12(I) protein. J *Virol*. 75(13):6086-94.

Journo, C., Bonnet, A., Favre-Bonvin, A., Turpin, J., Vinera, J., Côté, E. et al. (2013) Human T cell leukemia virus type 2 tax-mediated NF-κB activation involves a mechanism independent of Tax conjugation to ubiquitin and SUMO. *J Virol.* 87(2):1123-36. doi: 10.1128/JVI.01792-12.

Journo, C., Filipe, J., About, F., Chevalier, S.A., Afonso, P.V., Brady, J.N. (2009). NRP/Optineurin Cooperates with TAX1BP1 to potentiate the activation of NF-kappaB by human T-lymphotropic virus type 1 tax protein. *PLoS Pathog.* 5(7):e1000521. doi: 10.1371/journal.ppat.1000521.

Kalyanaraman, V.S., Sarngadharan, M.G., Robert-Guroff, M., Miyoshi, I., Golde, D., Gallo, R.C. (1982). A new subtype of human T-cell leukemia virus (HTLV-II) associated with a T-cell variant of hairy cell leukemia. *Science*. 218(4572):571–3.

Kannian, P., Yin, H., Doueiri, R., Lairmore, M.D., Fernandez, S., Green, P.L. (2012). Distinct Transformation Tropism Exhibited by Human T Lymphotropic Virus Type 1 (HTLV-1) and HTLV-2 Is the Result of Postinfection T Cell Clonal Expansion. *J Virol*. 86(7):3757–66

Karimi, M., Mohammadi, H., Hemmatzadeh, M., Mohammadi, A., Rafatpanah, H., Baradaran, B. (2017). Role of the HTLV-1 viral factors in the induction of apoptosis. *Biomedicine and Pharmacotherapy*. 85, 334–347. doi: 10.1016/j.biopha.2016.11.034

Karimi, M., Mohammadi, H., Hemmatzadeh, M., Mohammadi, A., Rafatpanah, H., Baradaran, B. (2017). Role of the HTLV-1 viral factors in the induction of apoptosis. *Biomedicine and Pharmacotherapy* 85, 334–347. doi: 10.1016/j.biopha.2016.11.034

Keller, S.A, Hernandez-Hopkins, D., Vider, J., Ponomarev, V., Hyjek, E., Schattner, E.J. et al. (2006). NF-kappaB is essential for the progression of KSHV- and EBV-infected lymphomas in vivo. *Blood* 107(8):3295-302.

Khan, M.J., Castle, P.E., Lorincz, A.T., Wacholder, S., Sherman, M., Scott, D.R. et al. (2005). The elevated 10-year risk of cervical precancer and cancer in women with human papillomavirus (HPV) type 16 or 18 and the possible utility of type-specific HPV testing in clinical practice. *J. Natl. Cancer Inst*. 97, 1072–1079. 10.1093/jnci/dji187

Kogure, Y. and Kataoka, K. (2017). Genetic alterations in adult T-cell leukemia/lymphoma. *Cancer Sci*. 108(9):1719-1725. doi: 10.1111/cas.13303.

Koralnik, I.J., Boeri, E., Saxinger, W.C., Monico, A.L., Fullen, J., Gessain, A. et al. (1994). Phylogenetic associations of human and simian T-cell leukemia/lymphotropic virus type I strains: evidence for interspecies transmission. *J Virol*. 68(4):2693–707.

Kubota, R., Umehara, F., Izumo, S., Ijichi, S., Matsumuro, K., Yashiki, S. et al. (1994). HTLV-I proviral DNA amount correlates with infiltrating CD4+ lymphocytes in the spinal cord from patients with HTLV-I-associated myelopathy. *J Neuroimmunol*. 53(1):23-9.

Kuo, Y.L. and Giam, C.Z. (2006). Activation of the anaphase promoting complex by HTLV-1 tax leads to senescence. *The EMBO Journal*. 25(8), 1741–52. doi: 10.1038/sj.emboj.7601054

Kusano, S., Yoshimitsu, M., Hachiman, M., Ikeda, M. (2015). I-mfa domain proteins specifically interact with HTLV-1 Tax and repress its transactivating functions. *Virology* 486:219-27. doi: 10.1016/j.virol.2015.09.020.

Lavorgna, A. and Harhaj, E. W. (2014). Regulation of HTLV-1 tax stability, cellular trafficking and NF-κB activation by the ubiquitin-proteasome pathway. *Viruses* 6(10), 3925–3943. doi: 10.3390/v6103925

Lavorgna, A., Matsuoka, M., Harhaj, E.W. (2014). A critical role for IL-17RB signaling in HTLV-1 tax-induced NF-κB activation and T-cell transformation. *PLoS Pathog*. 10(10):e1004418. doi: 10.1371/journal.ppat.1004418.

Li, M. and Green, P.L. (2007). Detection and quantitation of HTLV-1 and HTLV-2 mRNA species by real-time RT-PCR. *J. Virol. Methods* 142 159–16810.1016/j.jviromet.2007.01.023

Li, Y., Wang, H., Zhou, X., Xie, X., Chen, X., Jie, Z. et al. (2016). Cell intrinsic role of NF-κB-inducing kinase in regulating T cell-mediated immune and autoimmune responses. *Sci Rep*. 6:22115. doi: 10.1038/srep22115.

Liao, G., Zhang, M., Harhaj, E.W., Sun, S.C. (2004). Regulation of the NF-kappaB-inducing kinase by tumor necrosis factor receptor-associated factor 3-induced degradation. *J Biol Chem.* 279(25):26243-50.

Liu, B., Park, E., Zhu, F., Bustos, T., Liu, J., Shen, J. et al. (2006). A critical role for I kappaB kinase alpha in the development of human and mouse squamous cell carcinomas. *Proceedings of the National Academy of Sciences of the United States of America*, 103(46), 17202–7. http://doi.org/10.1073/pnas.0604481103

Ma, Y., Zhang, B., Wang, D., Qian, L., Song, X., Wang, X. et al. (2017). HTLV-1 basic leucine zipper factor downregulates cyclin D1 expression via interactions with NF-κB. *Int J Mol Med.* (22), 764–770. doi: 10.3892/ijmm.2017.2868.

Macchi, B., Balestrieri, E., Frezza, C., Grelli, S., Valletta, E., Marçais, A. (2017). Quantification of HTLV-1 reverse transcriptase activity in ATL patients treated with zidovudine and interferon-α *Blood Adv*. 1(12): 748–752. doi: 10.1182/bloodadvances.2016001370

Mahieux, R. and Gessain, A. (2009). The human HTLV-3 and HTLV-4 retroviruses: new members of the HTLV family. *Pathol Biol*. 57(2):161-6. doi: 10.1016/j.patbio.2008.02.015.

Mahieux, R. and Gessain, A. (2011). HTLV-3/STLV-3 and HTLV-4 viruses: discovery, epidemiology, serology and molecular aspects. *Viruses* 3(7):1074-90. doi: 10.3390/v3071074.

Mahieux, R., Pise-Masison, C.A., Lambert, P.F., Nicot, C., De Marchis, L., Gessain, A. et al. (2000). Differences in the ability of human Tcell lymphotropic virus type 1 (HTLV-1) and HTLV-2 tax to inhibit p53 function. *J. Virol*. 74, 6866-6874.

Majone, F., Semmes, O.J., Jeang, K.T. (1993). Induction of micronuclei by HTLV-I Tax: a cellular assay for function. *Virology* 193(1):456-9.

Marban, C., McCabe, A., Bukong, T.N., Hall, W.W., Sheehy, N. (2012). Interplay between the HTLV-2 Tax and APH-2 proteins in the regulation of the AP-1 pathway. *Retrovirology* 9:98. doi: 10.1186/1742-4690-9-98.

Marriott, S.J. and Semmes, O.J. (2005). Impact of HTLV-I Tax on cell cycle progression and the cellular DNA damage repair response. *Oncogene* 24(39):5986-95.

Martin, J.L., Maldonado, J.O., Mueller, J.D., Zhang, W., Mansky, L.M. (2016). Molecular Studies of HTLV-1 Replication: An Update *Viruses* 8(2): 31. doi: 10.3390/v8020031

Masoumi, K.C., Shaw-Hallgr, G, Massoumi, R. (2011). Tumor Suppressor Function of CYLD in Nonmelanoma Skin Cancer. *J Skin Cancer.* 2011:614097. doi: 10.1155/2011/614097.

Matsuoka, M. (2005) Human T-cell leukemia virus type I (HTLV-I) infection and the onset of adult T-cell leukemia (ATL). *Retrovirology* 2:27.

Matsuoka, M. and Green, P.L. (2009). The HBZ gene, a key player in HTLV-1 pathogenesis. *Retrovirology* 6:71. doi: 10.1186/1742-4690-6-71.

Matsuoka, M. and Yasunaga, J. (2013) Human T-cell leukemia virus type 1: replication, proliferation and propagation by Tax and HTLV-1 bZIP factor. *Curr Opin Virol*. 3(6):684-91. doi: 10.1016/j.coviro.2013.08.010.

Meertens, L., Pise-Masison, C., Quere, N., Brady, J., Gessain, A., Mahieux, R. (2004). Utilization of the CBP but not the p300 co-activator by human T-lymphotropic virus type-2 Tax for p53 inhibition. *Oncogene*. 23, 5447-5458.

Melamed, A., Laydon, D.J., Gillet, N.A., Tanaka, Y., Taylor, G.P., Bangham, C.R. (2013) Genome-wide determinants of proviral targeting, clonal abundance and expression in natural HTLV-1 infection. *PLoS Pathog*. (3):e1003271

Mehta-Shah, N., Ratner, L., Horwitz, S.M. (2017). Adult T-Cell Leukemia/Lymphoma. *J Oncol Pract.* 13(8):487-492. doi: 10.1200/JOP.2017.021907.

Mesri, E.A., Feitelson, M., Munger, K. (2014). Human viral oncogenesis: a cancer hallmarks analysis. *Cell Host Microbe*. 15(3): 266–282. doi: 10.1016/j.chom.2014.02.011

Mikecz, A., Zhang, S., Montminy, M., Tan, E.-M., Hemmerich, P. (2000). Creb-Binding Protein (Cbp/P300) and RNA Polymerase II Colocalize in Transcriptionally Active Domains in the Nucleus. *J Cell Biol*. 150(1): 265–274.

Mitobe, Y., Yasunaga, J., Furuta, R., Matsuoka, M. (2015). HTLV-1 bZIP Factor RNA and Protein Impart Distinct Functions on T-cell Proliferation and Survival. *Cancer Res*. 75(19):4143-52. doi: 10.1158/0008-5472.CAN-15-0942.

Miyazaki, M., Yasunaga, J.I., Taniguchi, Y., Tamiya, S., Nakahata, T., Matsuoka, M. (2007). Preferential Selection of Human T-Cell Leukemia Virus Type 1 Provirus Lacking the 5' Long Terminal Repeat during Oncogenesis. *Journal of Virology*. 81(11), 5714–5723. doi: 10.1128/JVI.02511-06

Mori, N., Sato, H., Hayashibara, T., Senba, M., Hayashi, T., Yamada, Y. et al. (2002). Human T-cell leukemia virus type I Tax transactivates the matrix

metalloproteinase-9 gene: potential role in mediating adult T-cell leukemia invasiveness. *Blood* 99(4):1341-9.

Morrison, T.E. and Kenney, S.C. (2004). BZLF1, an Epstein-Barr virus immediate-early protein, induces p65 nuclear translocation while inhibiting p65 transcriptional function. *Virology* 328(2):219-32.

Motai, Y., Takahashi, M., Takachi, T., Higuchi, M., Hara, T., Mizuguchi, M. et al. (2016). Human T-cell leukemia virus type 1 (HTLV-1) Tax1 oncoprotein but not HTLV-2 Tax2 induces the expression of OX40 ligand by interacting with p52/p100 and RelB. *Virus Genes. 52*(1), 4–13. doi: 10.1007/s11262-015-1277-7

Mukai, R. and Ohshima, T. (2014). HTLV-1 HBZ positively regulates the mTOR signaling pathway via inhibition of GADD34 activity in the cytoplasm. *Oncogene* 33(18):2317-28. doi: 10.1038/onc.2013.181.

Mulloy, J.C., Crownley, R.W., Fullen, J., Leonard, W.J., Franchini, G. (1996). The human T-cell leukemia/lymphotropic virus type 1 p12I proteins bind the interleukin-2 receptor beta and gammac chains and affects their expression on the cell surface. *J Virol.* 70(6):3599-605.

Murphy, E.L., Wilks, R., Hanchard, B., Cranston, B., Figueroa, J.P., Gibbs, W.N. et al. (1996). A case-control study of risk factors for seropositivity to human T-lymphotropic virus type I (HTLV-I) in Jamaica. *Int J Epidemiol.* 25(5):1083-9.

Nejmeddine, M., Barnard, A.L., Tanaka, Y., Taylor, G.P., Bangham, C.R. (2005). Human T-lymphotropic virus, type 1, tax protein triggers microtubule reorientation in the virological synapse. *J Biol Chem.* 280(33):29653-60.

Nejmeddine, M., Negi, V.S., Mukherjee, S., Tanaka, Y., Orth, K., Taylor, G.P. et al. (2009) HTLV-1-Tax and ICAM-1 act on T-cell signal pathways to polarize the microtubule-organizing center at the virological synapse. *Blood* 114(5):1016-25. doi: 10.1182/blood-2008-03-136770.

Newman, A.C., Kemp, A.J., Drabsch, Y., Behrends, C., Wilkinson, S. (2017). Autophagy acts through TRAF3 and RELB to regulate gene expression via antagonism of SMAD proteins. *Nat Commun.* 8(1):1537. doi: 10.1038/s41467-017-00859-z.

Niederer, H.A., Laydon, D.J., Melamed, A., Elemans, M., Asquith, B., Matsuoka, M. et al. (2014) HTLV-1 proviral integration sites differ between asymptomatic carriers and patients with HAM/TSP. *Virol J.* 11:172. doi: 10.1186/1743-422X-11-172.

Oliveira, P.D., Kachimarek, A.C., Bittencourt, A.L. (2017). Early Onset of HTLV-1 Associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP) and Adult T-cell Leukemia/Lymphoma (ATL): Systematic Search and Review. *J Trop Pediatr*. doi: 10.1093/tropej/fmx039.

Osame, M., Usuku, K., Izumo, S., Ijichi, N., Amitani, H., Igata, A. et al. (1986). HTLV-I associated myelopathy, a new clinical entity. *Lancet*. 1(8488):1031–2.

Panfil, A.R., Dissinger, N.J., Howard, C.M., Murphy, B.M., Landes, K., Fernandez, S.A. et al. (2016). Functional Comparison of HBZ and the Related APH-2 Protein Provide Insight into HTLV-1 Pathogenesis. *J Virol*. pii: JVI.03113-15.

Pilotti, E., Bianchi, M.V., De Maria, A., Bozzano, F., Romanelli, M.G., Bertazzoni, U. et al. (2013) HTLV-1/-2 and HIV-1 co-infections: retroviral interference on host immune status. *Front Microbiol*. 4:372. doi: 10.3389/fmicb.2013.00372.

Poiesz, B. J., Ruscetti, F. W., Gazdar, A. F., Bunn, P. A., Minna, J. D., and Gallo, R. C. (1980). Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc. Nat. Acad. Sci. U.S.A.* 77, 7415–7419. doi: 10.1073/pnas.77.12.7415

Portis, T., Grossman, W.J., Harding, J.C., Hess, J.L., Ratner, L. (2001). Analysis of p53 inactivation in a human T-cell leukemia virus type 1 Tax transgenic mouse model. *J Virol.* 75(5):2185-93.

Prooyen, N., Andresen, V., Gold, H., Bialuk, I., Pise-Masison, C., Franchini, G. (2010). Hijacking the T-cell communication network by the human T-cell leukemia/lymphoma virus type 1 (HTLV-1) p12 and p8 proteins. *Mol Aspects Med*. 31(5):333-43. doi:10.1016/j.mam.2010.07.001.

Prooyen, N., Gold, H., Andresen, V., Schwartz, O., Jones, K., Ruscetti, F. et al. (2010). Human T-cell leukemia virus type 1 p8 protein increases cellular conduits and virus transmission. *Proc Natl Acad Sci U S A*. 107(48):20738-43. doi: 10.1073/pnas.1009635107.

Pujari, R., Hunte, R., Thomas, R., van der Weyden, L., Rauch, D., Ratner, L. et al. (2015). Human T-cell leukemia virus type 1 (HTLV-1) tax requires CADM1/TSLC1 for inactivation of the NF-κB inhibitor A20 and constitutive NF-κB signaling. *PLoS Pathog*. 11(3):e1004721. doi: 10.1371/journal.ppat.1004721.

Qiao, X., He, B., Chiu, A., Knowles, D.M., Chadburn, A., Cerutti, A. (2006). Human immunodeficiency virus 1 Nef suppresses CD40-dependent immunoglobulin class switching in bystander B cells. *Nat Immunol.* 7(3):302-10.

Qu, Z. and Xiao, G. (2011). Human T-cell lymphotropic virus: A model of NF-κB-associated tumorigenesis. *Viruses*. 3(6), 714–749. doi: 10.3390/v3060714

Ran, F.A., Hsu, P.D., Wright, J., Agarwala, V., Scott, D.A., Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nat Protoc*. 8(11):2281-2308. doi: 10.1038/nprot.2013.143

Raval, G.U., Bidoia, C., Forlani, G., Tosi, G., Gessain, A., Accolla, R.S. (2015). Localization, quantification and interaction with host factors of endogenous HTLV-1 HBZ protein in infected cells and ATL. *Retrovirology* 12:59. doi: 10.1186/s12977-015-0186-0.

Ren, T. and Cheng, H. (2013). Differential transforming activity of the retroviral Tax oncoproteins in human T lymphocytes. *Front Microbiol*. 4:287. doi: 10.3389/fmicb.2013.00287

Ren, T., Takahashi, Y., Liu, X., Loughran, T.P., Sun, S.C., Wang, H.G. et al. (2015). HTLV-1 Tax deregulates autophagy by recruiting autophagic molecules into lipid raft microdomains. *Oncogene*. 34(3):334-45. doi: 10.1016/j.virol.2008.08.028.

Rende, F., Cavallari, I., Andresen, V., Valeri, V.W., D'Agostino, D.M., Franchini, G. et al. (2015). Identification of novel monocistronic HTLV-1 mRNAs encoding functional Rex isoforms. *Retrovirology* 12:58. doi: 10.1186/s12977-015-0184-2.

Rende, F., Cavallari, I., Romanelli, M.G., Diani, E., Bertazzoni, U. Ciminale, V. (2012). Comparison of the Genetic Organization, Expression Strategies and Oncogenic Potential of HTLV-1 and HTLV-2. *Leuk Res Treatment*. 2012:876153. doi: 10.1155/2012/876153.

Robek, M.D. and Ratner, L. (1999). Immortalization of CD4(+) and CD8(+) T lymphocytes by human T-cell leukemia virus type 1 Tax mutants expressed in a functional molecular clone. *J Virol*. 73(6):4856-65.

Rochford, R., Cannon, M.J., Moormann, A.M. (2005). Endemic Burkitt's lymphoma: a polymicrobial disease? *Nature reviews Microbiology*. 3:182–187.

Román, G.C. and Osame, M. (1988). Identity of HTLV-I-associated tropical spastic paraparesis and HTLV-I-associated myelopathy. *Lancet*. 1(8586):651.

Romanelli, M.G., Diani, E., Bergamo, E., Casoli, C., Ciminale, V., Bex, F. et al. (2013). Highlights on distinctive structural and functional properties of HTLV Tax proteins. *Front Microbiol*. 4:271. doi: 10.3389/fmicb.2013.00271.

Rosa, A.S., Araujo, O.C., Savassi-Ribas, F., Fernandes, C.A., Coelho, H.S., Niel, C. et al. (2017) Prevalence of occult hepatitis B virus infection and Torque teno virus infection and their association with hepatocellular carcinoma in chronic hepatitis C patients. *Virus Res*. 242:166-172. doi: 10.1016/j.virusres.2017.09.022

Roucoux, D. F. and Murphy, E. L. (2004). The epidemiology and disease outcomes of human T-lymphotropic virus type II. *AIDS Rev* 6:144-54.

Rowe, A.M., Murray, S.E., Raué, H.P., Koguchi, Y., Slifka, M.K., Parker, D.C. (2013). A cell-intrinsic requirement for NF-κB-inducing kinase in CD4 and CD8 T cell memory. *J Immunol*. 191(7):3663-72. doi: 10.4049/jimmunol.1301328.

Saito, M., Matsuzaki, T., Satou, Y., Yasunaga, J., Saito, K., Arimura, K. et al. (2009). In vivo expression of the HBZ gene of HTLV-1 correlates with proviral load, inflammatory markers and disease severity in HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP). *Retrovirology* 6:19. doi: 10.1186/1742-4690-6-19.

Satou, Y. and Matsuoka, M. (2011). Molecular and Cellular Mechanism of Leukemogenesis of ATL: Emergent Evidence of a Significant Role for HBZ in HTLV-1-Induced Pathogenesis. *Leuk Res Treatment*. 2012:213653. doi: 10.1155/2012/213653.

Satou, Y., Yasunaga, J., Yoshida, M., Matsuoka, M. (2006). HTLV-I basic leucine zipper factor gene mRNA supports proliferation of adult T cell leukemia cells. *Proc Natl Acad Sci U S A*. 103(3):720-5.

Semmes, O.J., Barrett, J.F., Dang, C.V., Jeang, K.T. (1996)a. Human T-cell leukemia virus type I Tax masks c-myc function through a cAMP-dependent pathway. *J Biol Chem*. 271, 9730-8.

Semmes, O.J., Majone, F., Cantemir, C., Turchetto, L., Hjelle, B., Jeang, K.T. (1996)b. HTLV-I and HTLV-II Tax: Differences in induction of micronuclei in cells and transcriptional activation of viral LTRs. *Virology*. 217, 373-379.

Shen, R.R. and Hahn, W.C. (2011). Emerging roles for the non-canonical IKKs in cancer *Oncogene*. 30(6): 631–641. doi: 10.1038/onc.2010.493

Shibata, Y., Tokunaga, F., Goto, E., Komatsu, G., Gohda, J., Saeki, Y. et al. (2017). HTLV-1 Tax Induces Formation of the Active Macromolecular IKK Complex by Generating Lys63- and Met1-Linked Hybrid Polyubiquitin Chains. *PLoS Pathogens*. 13(1), 1–25. doi: 10.1371/journal.ppat.1006162

Shoji, T., Higuchi, M., Kondo, R., Takahashi, M., Oie, M., Tanaka, Y. et al. (2009). Identification of a novel motif responsible for the distinctive transforming activity of human T-cell leukemia virus (HTLV) type 1 Tax1 protein from HTLV-2 Tax2. *Retrovirology* 6, 83.

Silic-Benussi, M., Biasiotto, R., Andresen, V., Franchini, G., D'Agostino, D.M., Ciminale, V. (2010). HTLV-1 p13, a small protein with a busy agenda. *Mol Aspects Med*. 31(5):350-8. doi: 10.1016/j.mam.2010.03.001.

Simonsen, L., Buffington, J., Shapiro, C.N., Holman, R.C., Strine, T.W., Grossman, B.J. et al. (1995). Multiple false reactions in viral antibody screening assays after influenza vaccination. *Am J Epidemiol*. 141(11):1089-96.

Sun, S.C. (2017). The non-canonical NF-κB pathway in immunity and inflammation. *Nat Rev Immunol*. 17(9):545-558. doi: 10.1038/nri.2017.52.

Sun, S.C., Elwood, J., Béraud, C., Greene, W. C. (1994). Human T-cell leukemia virus type I Tax activation of NF-kappa B/Rel involves phosphorylation and degradation of I kappa B alpha and RelA (p65)-mediated induction of the c-rel gene. *Mol. Cell. Biol*. 14, 7377–7384. doi: 10.1128/MCB.14.11.7377

Tagaya, Y. and Gallo, R.C. (2017). The Exceptional Oncogenicity of HTLV-1. *Front Microbiol*. 8: 1425. doi: 10.3389/fmicb.2017.01425

Takeda, S., Maeda, M., Morikawa, S., Taniguchi, Y., Yasunaga, J., Nosaka, K. et al. (2004). Genetic and epigenetic inactivation of tax gene in adult T-cell leukemia cells. *Int J Cancer*. 109(4):559-67.

Tang, S.W., Chen, C.Y., Klase, Z., Zane, L., Jeang, K.T. (2013). The cellular autophagy pathway modulates human T-cell leukemia virus type 1 replication. *J Virol*. 87(3):1699-707. doi: 10.1128/JVI.02147-12.

Taniguchi, Y., Nosaka, K., Yasunaga, J., Maeda, M., Mueller, N., Okayama, A. et al. (2005). Silencing of human T-cell leukemia virus type I gene transcription by epigenetic mechanisms. *Retrovirology* 2, 64. doi: 10.1186/1742-4690-2-64

Tripp, A., Banerjee, P., Sieburg, M., Planelles, V., Li, F., Feuer, G. (2005). Induction of cell cycle arrest by human T-cell lymphotropic virus type 1 Tax in hematopoietic progenitor (CD34+) cells: modulation of p21cip1/waf1 and p27kip1 expression. *J Virol*. 79(22):14069-78.

Turci, M., Lodewick, J., Di Gennaro, G., Rinaldi, A.S., Marin, O., Diani, E. et al. (2012). Ubiquitination and sumoylation of the HTLV-2 Tax-2B protein regulate its NF-κB activity: a comparative study with the HTLV-1 Tax-1 protein. *Retrovirology* 9:102. doi: 10.1186/1742-46909-102.

Turci, M., Lodewick, J., Righi, P., Polania, A., Romanelli, M.G., Bex, F. et al. (2009). HTLV-2B Tax oncoprotein is modified by ubiquitination and sumoylation and displays intracellular localization similar to its homologue HTLV-1 Tax. *Virology* 386(1):6-11. doi: 10.1016/j.virol.2009.01.003.

Turci, M., Romanelli, M.G., Lorenzi, P., Righi, P., Bertazzoni, U. (2006). Localization of human T-cell lymphotropic virus type II Tax protein is dependent upon a nuclear localization determinant in the N-terminal region. *Gene* 365:119-24.

Usui, T., Yanagihara, K., Tsukasaki, K., Murata, K., Hasegawa, H., Yamada, Y. et al. (2008) Characteristic expression of HTLV-1 basic zipper factor (HBZ) transcripts in HTLV-1 provirus-positive cells. *Retrovirology* 5:34. doi: 10.1186/1742-4690-5-34.

Wang, C., Long, W., Peng, C., Hu, L., Zhang, Q., Wu, A. et al. (2016). HTLV-1 Tax Functions as a Ubiquitin E3 Ligase for Direct IKK Activation via Synthesis of Mixed-Linkage Polyubiquitin Chains. *PLoS Pathogens*. 12(4), 1–25. doi: 10.1371/journal.ppat.1005584

Wang, J., Li, J., Huang, Y., Song, X., Niu, Z., Gao, Z. et al. (2013). Bcl-3 suppresses Tax-induced NF-κB activation through p65 nuclear translocation blockage in HTLV-1-infected cells. *Int J Oncol*. 42(1):269-76. doi: 10.3892/ijo.2012.1685.

Watanabe, T., Seiki, M., Yoshida, M. (1984). HTLV type I (U. S. isolate) and ATLV (Japanese isolate) are the same species of human retrovirus. *Virology* 133(1):238-41.

Wooff, J.C., Trites, J.R., Walsh, N.M., Bullock, M.J. (2010). Complete spontaneous regression of metastatic merkel cell carcinoma: a case report and review of the literature. *Am. J. Dermatopathol*. 32, 614–617.

Wurm, T., Wright, D.G., Polakowski, N., Mesnard, J.M., Lemasson, I. (2012). The HTLV-1-encoded protein HBZ directly inhibits the acetyl transferase activity of p300/CBP. *Nucleic Acids Res*. 40(13):5910-25. doi: 10.1093/nar/gks244.

Xia, Y., Shen, S., Verma, I.M. (2014). NF-κB, an active player in human cancers. *Cancer Immunol Res*. 2(9):823-30. doi: 10.1158/2326-6066.CIR-14-0112.

Xie, L. and Green, P.L. (2005). Envelope Is a Major Viral Determinant of the Distinct In Vitro Cellular Transformation Tropism of Human T-Cell Leukemia Virus Type 1 (HTLV-1) and HTLV-2. *Journal of Virology*. 79(23):14536–45

Xie, P., Hostager, B.S., Bishop, G.A. (2004). Requirement for TRAF3 in signaling by LMP1 but not CD40 in B lymphocytes. *J Exp Med*. 199(5):661-71.

Xie, P., Stunz, L.L., Larison, K.D., Yang, B., Bishop, G.A. (2007). Tumor necrosis factor receptor-associated factor 3 is a critical regulator of B cell homeostasis in secondary lymphoid organs. *Immunity*. 27(2):253-67.

Xiu, Y., Xu, H., Zhao, C., Li, J., Morita, Y., Yao, Z. et al. (2014). Chloroquine reduces osteoclastogenesis in murine osteoporosis by preventing TRAF3 degradation. *J Clin Invest*. 124(1):297-310. doi: 10.1172/JCI66947

Yamagishi, M. and Watanabe, T. (2012). Molecular hallmarks of adult T cell leukemia. *Front Microbiol*. 3:334. doi: 10.3389/fmicb.2012.00334.

Yamagishi, M., Nakano, K., Miyake, A., Yamochi, T., Kagami, Y., Tsutsumi, A. et al. (2012). Polycomb-mediated loss of miR-31 activates NIK-dependent NF-κB pathway in adult T cell leukemia and other cancers. *Cancer Cell*. 21(1):121-35. doi: 10.1016/j.ccr.2011.12.015.

Yang, J., Aittomäki, S., Pesu, M., Carter, K., Saarinen, J., Kalkkinen, N. et al. (2002). Identification of p100 as a coactivator for STAT6 that bridges STAT6 with RNA polymerase II. *EMBO J*. 21(18):4950-8.

Yang, X.D. and Sun, S.C. (2015). Targeting signaling factors for degradation, an emerging mechanism for TRAF functions. *Immunol Rev*. 266(1): 56–71. doi: 10.1111/imr.12311

Yin, H., Kannian, P., Dissinger, N., Haines, R., Niewiesk, S., Green, P. L. (2012). Human T-cell leukemia virus type 2 antisense viral protein 2 is dispensable for in vitro immortalization but functions to repress early virus replication in vivo. *J Virol*. 86(16), 8412–21. doi: 10.1128/JVI.00717-12

Yoshida, M., Miyoshi, I., Hinuma, Y. (1982). Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. *Proc Natl Acad Sci U S A*. 79(6):2031-5.

Yoshida, M., Satou, Y., Yasunaga, J., Fujisawa, J., Matsuoka, M. (2008). Transcriptional control of spliced and unspliced human T-cell leukemia virus type 1 bZIP factor (HBZ) gene. *J Virol*. 82(19):9359-68. doi: 10.1128/JVI.00242-08.

Yu, Q., Minoda, Y., Yoshida, R., Yoshida, H., Iha, H., Kobayashi, T. et al. (2008). HTLV-1 Tax-mediated TAK1 activation involves TAB2 adapter protein. *Biochemical and Biophysical Research Communications*. *365*(1), 189–194. doi: 10.1016/j.bbrc.2007.10.172

Zane L. and Jeang K. T. (2012). The importance of ubiquitination and sumoylation on the transforming activity of HTLV Tax-1 and Tax-2. *Retrovirology* 910310.1186/1742-4690-9-103

Zhang, H., Chen, L., Cai, S.H., Cheng, H. (2016). Identification of TBK1 and IKKε, the non-canonical IκB kinases, as crucial pro-survival factors in HTLV-1-transformed T lymphocytes. *Leuk Res.* 46:37-44. doi: 10.1016/j.leukres.2016.04.012.

Zhang, L., Wei, J., Wang, L., Huang, S., Chen, J.L. (2017). Human T-cell lymphotropic virus type 1 and its oncogenesis. *Acta Pharmacologica Sinica*. 38(8):1093-1103. doi: 10.1038/aps.2017.17.

Zhang, L.L., Wei, J.Y., Wang, L., Huang, S.L., Chen, J.L. (2017). Human T-cell lymphotropic virus type 1 and its oncogenesis. *Acta Pharmacol Sin*. 38(8):1093-1103. doi: 10.1038/aps.2017.17.

Zhao, T. (2016). The Role of HBZ in HTLV-1-Induced Oncogenesis. *Viruses* 8(2). pii: E34. doi: 10.3390/v8020034.

Zhao, T. and Matsuoka, M. (2012). HBZ and its roles in HTLV-1 oncogenesis. *Front Microbiol.* 3:247. doi: 10.3389/fmicb.2012.00247.

Zhao, T., Coutts, A., Xu, L., Yu, J., Ohshima, K., Matsuoka, M. (2013). HTLV-1 bZIP factor supports proliferation of adult T cell leukemia cells through suppression of C/EBPα signaling. *Retrovirology* 10:159. doi:10.1186/1742-4690-10-159.

Zhao, T., Yasunaga, J., Satou, Y., Nakao, M., Takahashi, M., Fujii, M. et al. (2009). Human T-cell leukemia virus type 1 bZIP factor selectively suppresses the classical pathway of NF-kappaB. *Blood* 113(12):2755-64. doi: 10.1182/blood-200806-161729.

Zhi, H., Yang, L., Kuo, YL., Ho, YK., Shih, HM., Giam, CZ. (2011). NF-κB hyperactivation by HTLV-1 tax induces cellular senescence, but can be alleviated by the viral anti-sense protein HBZ. *PLoS Pathog*. 7(4):e1002025. doi: 10.1371/journal.ppat.1002025.

zur Hause, H. (2009). The search for infectious causes of human cancers: where and why (Nobel lecture). *Angew Chem Int Ed Engl*. 48(32):5798-808. doi: 10.1002/anie.200901917.

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## 8. ATTACHMENTS

1. Fochi, S., Mutascio, S., Bertazzoni, U., Zipeto, D., Romanelli, M.G. (2018). HTLV deregulation of the NF-κB pathway: an update on Tax and antisense proteins role *Front. Microbiol.* 9:285 doi: 10.3389/fmicb.2018.00285

2. Dubuisson, L., Lormières, F., Fochi, S., Turpin, J., Pasquier, A., Douceron, E., Oliva, A., Bazarbachi, A., Lallemand-Breitenbach, V., de Thé, H., Journo, C., Mahieux, R. (2018) Stability of HTLV-2 Antisense Protein is Controlled by PML Nuclear Bodies in a SUMO-Dependent Manner. *Oncogene*. 2018. doi:10.1038/s41388-018-0163-x

3. Fochi, S., Mutascio, S., Parolini, F., Zipeto, D., Romanelli, M.G. (2017). HTLV antisense proteins role in the NF-κB modulation. *Virol Res J*. Volume 1 Issue 3.

# HTLV Deregulation of the NF-κB Pathway: An Update on Tax and Antisense Proteins Role

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Human T-cell lymphotropic virus type 1 (HTLV-1) is the causative agent of adult T-cell leukemia (ATL), an aggressive $CD4^+/CD25^+$ T-cell malignancy and of a severe neurodegenerative disease, HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP). The chronic activation or deregulation of the canonical and non-canonical nuclear factor kappa B (NF-κB) pathways play a crucial role in tumorigenesis. The HTLV-1 Tax-1 oncoprotein is a potent activator of the NF-κB transcription factors and the NF-κB response is required for promoting the development of HTLV-1 transformed cell lines. The homologous retrovirus HTLV-2, which also expresses a Tax-2 transforming protein, is not associated with ATL. In this review, we provide an updated synopsis of the role of Tax-1 in the deregulation of the NF-κB pathway, highlighting the differences with the homologous Tax-2. Special emphasis is directed toward the understanding of the molecular mechanisms involved in NF-κB activation resulting from Tax interaction with host factors affecting several cellular processes, such as cell cycle, apoptosis, senescence, cell proliferation, autophagy, and post-translational modifications. We also discuss the current knowledge on the role of the antisense viral protein HBZ in down-regulating the NF-κB activation induced by Tax, and its implication in cellular senescence. In addition, we review the recent studies on the mechanism of HBZ-mediated inhibition of NF-κB activity as compared to that exerted by the HTLV-2 antisense protein, APH-2. Finally, we discuss recent advances aimed at understanding the role exerted in the development of ATL by the perturbation of NF-κB pathway by viral regulatory proteins.

Keywords: HTLV, NF-κB, Tax, HBZ, APH-2, adult T-cell leukemia, cell proliferation, apoptosis

## INTRODUCTION

Human T-cell lymphotropic/leukemia virus type 1 (HTLV-1) is the etiological agent of adult T-cell leukemia (ATL), a malignancy of $CD4^+/CD25^+$ T cells and of a chronic inflammatory disease called HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Poiesz et al., 1980; Hinuma et al., 1981; Gessain et al., 1985; Gallo et al., 2017). It is estimated that at least 20 million people worldwide are infected with HTLV-1 (Gessain and Cassar, 2012; Willems et al., 2017) and approximately 5% of HTLV-1 carriers develop ATL after a latency of 20–50 years from infection (Zhang et al., 2017). HTLV-1 provirus encodes, among others, a regulatory protein, Tax and an accessory antisense strand product HTLV-1 bZip protein (HBZ), which are pivotal factors in

HTLV-1 pathogenesis (Yasuma et al., 2016). Tax is a transcriptional activator of the viral long terminal repeat (LTR) with the capability to unsettle several cellular signal transduction pathways. HBZ is an inhibitor of 5′ LTR Tax-1 transactivation and is required for viral persistence (Barbeau et al., 2013). HBZ is a potent viral oncoprotein which plays an important role in deregulating several cellular processes in concerted action with Tax, affecting cell proliferation, apoptosis, autophagy, and immune escape (Zhao, 2016). Both these viral regulatory proteins promote T-cell proliferation. However, the exact mechanism underlying their role in inducing cell proliferation is still not clearly understood. The genetically related HTLV type 2 virus, although its association with ATL has not been established, encodes a homolog Tax-2 regulatory protein that induces T-cell proliferation *in vitro* and an antisense protein, named antisense protein HTLV-2 (APH-2) that, unlike HBZ, is dispensable for HTLV-2 infection and persistence (Yin et al., 2012). Their structural properties are shown in **Figures 1A,B**. Comparative studies between HTLV-1 and HTLV-2 have contributed to highlight differences in the virus-host interaction that may have key roles in tumorigenesis (Higuchi and Fujii, 2009; Bertazzoni et al., 2011; Romanelli et al., 2013).

Persistent activation of NF-κB by Tax is a key event for the T-cell transformation and development of ATL (Qu and Xiao, 2011; Zhang et al., 2017). Accumulating evidence indicates that the HTLVs have evolved specific strategies mediated by Tax and antisense proteins to deregulate NF-κB signaling pathways. While HBZ is consistently expressed in all ATL cells, Tax is not expressed in approximately 60% of them, even though the HTLV-1 proviral genome is integrated and NF-κB is constitutively activated (Zhao, 2016). This suggests that additional factors contribute to sustain the persistent activation of NF-κB, in the absence of Tax, in ATL cells (Matsuoka and Jeang, 2007). The alteration of the NF-κB signaling pathway could also be involved in the inflammatory state observed in HAM/TSP (Peloponese et al., 2006). An interesting aspect of Tax and HBZ functions is their opposite effect on the regulation of cellular signaling pathways (Zhao and Matsuoka, 2012; Ma et al., 2016) as further discussed here.

In this review, we summarize the recent advances in understanding the molecular mechanisms involved in NF-κB deregulation, mediated by Tax and antisense proteins, through the interaction with host factors and their roles in cell survival and proliferation.

## TAX-MEDIATED NF-κB ACTIVATION

Two distinct pathways lead to NF-κB activation, known as the canonical and the non-canonical pathways that involve different upstream, intermediate, and effector factors. A common step of both pathways is the activation of a complex that contains a serine–specific IκB kinase (IKK) composed by two catalytic kinase subunits, IKKα and IKKβ, and the regulatory non-enzymatic scaffold protein NEMO (known as IKKγ). In the canonical pathway, adaptor proteins (TRAFs) are recruited to the cytoplasmic domain of the cell membrane tumor necrosis

factor receptor (TNF-R) and activate the IKK complex thus inducing the phosphorylation of IκB inhibitor and the seclusion of NF-κB precursors within the cytoplasm (Sun, 2017). This phenomenon leads to IκB degradation and nuclear translocation of the p50/RelA transcriptional effectors. At variance with the canonical pathway, the non-canonical one involves an IKK complex that does not contain NEMO, but two IKKα subunits. The NF-κB-inducing kinase (NIK) activates the IKK complex, leading to p100 processing and the final release in the nucleus of p52/RelB active heterodimer (Durand and Baldwin, 2017).

Based on the study of the molecular mechanisms of NF-κB activation driven by Tax-1, two relevant aspects emerged: the recruitment of Tax in cellular protein complexes (Bertazzoni et al., 2011; Qu and Xiao, 2011) and their post-translational modifications (Lavorgna and Harhaj, 2014). Studies comparing Tax-1 and Tax-2 have highlighted relevant differences in their activation of the NF-κB pathway as a result of protein interaction: both proteins activate the classical pathway, but only Tax-1 activates the non-canonical one; Tax-1, unlike Tax-2, triggers the activation of the non-canonical pathway recruiting NEMO and IKKα to p100, promoting the processing of p100 to p52 (Xiao et al., 2001; Higuchi et al., 2007; Shoji et al., 2009); both Tax proteins interact with TAB2 and NEMO/IKKγ stimulating the translocation of the p50/RelA heterodimers into the nucleus, but only Tax-1 interacts with TRAF6, an E3 ligase that triggers the ubiquitination and activation of the downstream NF-κB signaling cascade (Avesani et al., 2010; Journo et al., 2013). Furthermore, only Tax-1 interacts with the p52/p100 and RelB factors of the non-canonical pathway, inducing the expression of OX40L, a T-cell co-stimulatory molecule of the tumor necrosis factor family implicated in the adaptive immunity (Motai et al., 2016).

We have recently shown that Tax-1 and Tax-2 form complexes with two homologous non-canonical IκB kinases, IKKε and TBK1, which are not component of IKK complexes, but are implicated in the activation of NF-κB, STAT3 and induction of IFNα (Shen and Hahn, 2011; Diani et al., 2015). An additional study demonstrating the presence of Tax and TBK1 in lipid raft microdomains along with canonical IκB supports the role of Tax-1 as a promoter of the molecular crosstalk between the canonical IKKs and additional signaling pathways involved in cell survival and proliferation (Zhang et al., 2016). Interestingly, it has also been reported that Tax-1 forms complexes with the ubiquitin-conjugating enzyme Ubc13, NEMO, Tax1 binding protein1 (TAX1BP) and NRP/Optineurin in the membrane lipid rafts microdomain. In these complexes, the cell adhesion molecule 1 (CADM1) acts as a molecular scaffold recruiting Tax-1 (Pujari et al., 2015). This interaction contributes to the activation of the IKK complex and the inactivation of the NF-κB negative regulator A20 enzyme, thus maintaining a persistent NF-κB activation. An additional consequence of the Tax reorganization of the component of the lipid raft is the deregulation of autophagy. Tax-1, in fact, participates to the connection of the IKK complex to the autophagy molecular complexes by interacting directly with Beclin1 and PI3KC3 and contributing to the assembly of autophagosomes (Ren et al., 2012, 2015; Chen et al., 2015). Tax-1 induction of NF-κB also increases the expression of inhibitors of apoptosis, such as the anti-apoptotic
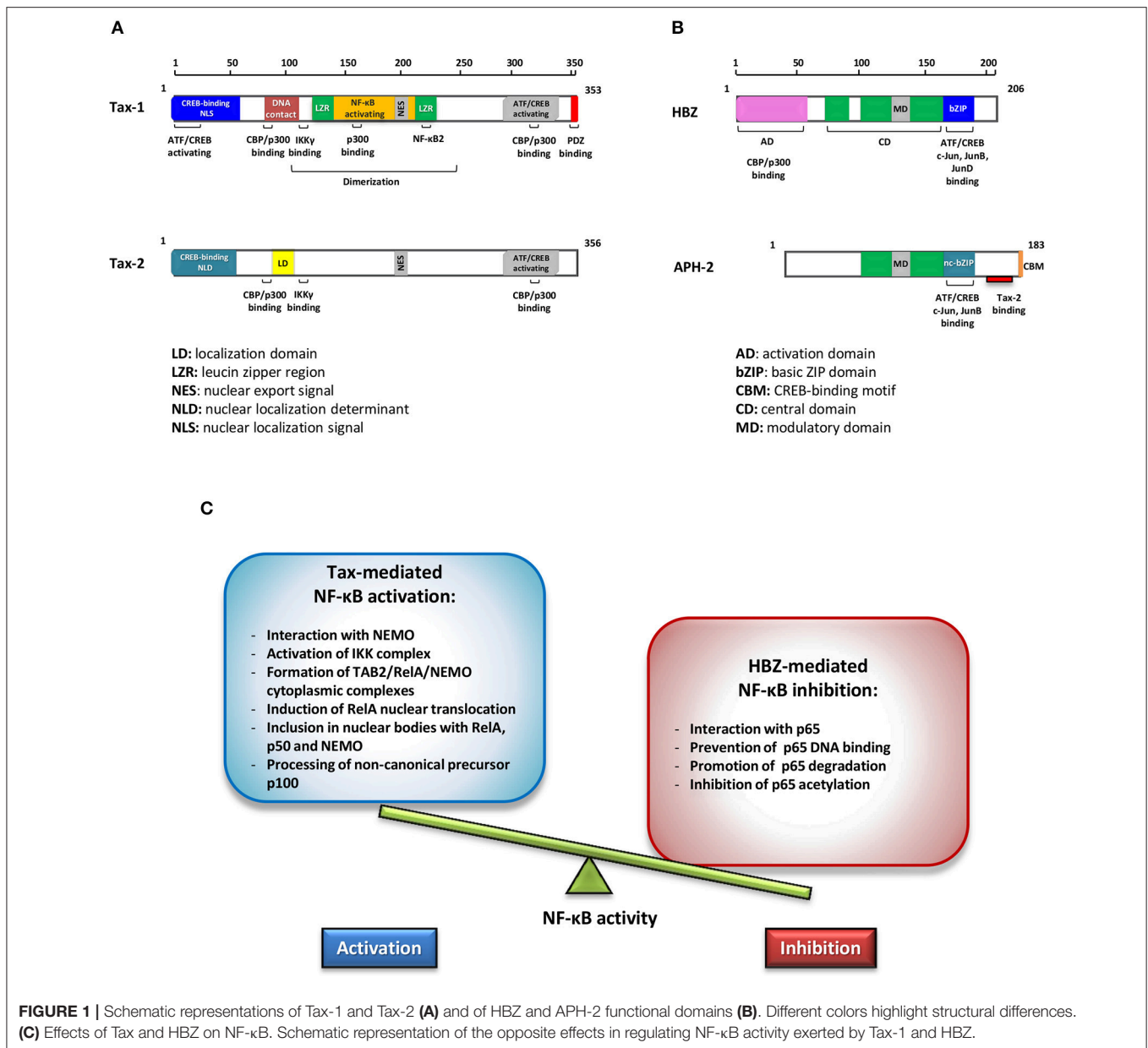
**FIGURE 1 |** Schematic representations of Tax-1 and Tax-2 **(A)** and of HBZ and APH-2 functional domains **(B)**. Different colors highlight structural differences. **(C)** Effects of Tax and HBZ on NF-κB. Schematic representation of the opposite effects in regulating NF-κB activity exerted by Tax-1 and HBZ.

*c-Flip* gene, and of genes involved in cell cycle progression, including cyclin D2, cyclin E, E2F1, CDK2, CDK4, and CDK6 (Wang et al., 2014; Bangham and Matsuoka, 2017; Karimi et al., 2017).

It has been recently reported that Tax-activation of NF-κB can be suppressed by host factors. Among them, the transcriptional regulator of the major histocompatibility complex class II (CIITA) impairs the nuclear translocation of RelA and directly interacts with Tax-1/RelA in nuclear bodies, preventing Tax-1 mediated activation of NF-κB-responsive promoters (Forlani et al., 2013, 2016). In addition, the apoptotic regulator Bcl-3 has been demonstrated to inhibit RelA nuclear translocation and its DNA binding activity, resulting in a downregulation of Tax-induced NF-κB activation (Wang et al., 2013). The decrease

in Tax-NF-κB activation could also be due to Tax proteasomal degradation induced by host factor interaction (Lavorgna and Harhaj, 2014). Tax-1 interaction with the molecular chaperone HSP90 was shown to protect Tax from proteasomal degradation (Gao and Harhaj, 2013), whereas the interaction with PDLIM2 (PDZ-LIM domain-containing protein) within the nuclear matrix induces its polyubiquitination-mediated proteasomal degradation (Yan et al., 2009; Fu et al., 2010). Furthermore, two tumor suppressor genes, MDFIC and MDF, have been recently identified as Tax-1 interactors that alter its subcellular distribution and stability, reducing Tax-dependent activation of NF-κB (Kusano et al., 2015).

The second major mechanism required for Tax-1 and Tax-2 NF-κB activation is the process of post-translational

modification, which includes ubiquitination, SUMOylation and phosphorylation. It is well established that Tax phosphorylation is required for its nuclear translocation and stabilization in the nuclear bodies containing RelA (Bex et al., 1999; Turci et al., 2006). The requirements of ubiquitination and SUMOylation are more complex to define. Both the E2 enzyme Ubc13 and the E3 Ring Finger Protein 8 (RNF8) promote Tax K63-linked polyubiquitination and are essential for the activation of the IKK complex (Shembade et al., 2007; Ho et al., 2015). Other proteins, including E3 ubiquitin ligases, TRAF2, 5, or 6, can potentiate Tax polyubiquitination (Yu et al., 2008). SUMOylated Tax has been demonstrated to bind p300, RelA and NEMO in nuclear bodies (Nasr et al., 2006). In addition, SUMOylation of Tax may be involved in the regulation of Tax stability and NF-κB pathway activation (Kfoury et al., 2011). We have described that SUMOylation and ubiquitination influence Tax proteins intracellular localization, as well as the interaction with NF-κB factors and their transactivating activity (Turci et al., 2012). However, the role of Tax SUMOylation in NF-κB activation remains controversial, given that Tax-induced IKK activation has been shown to correlate with the level of Tax ubiquitination, but not with Tax SUMOylation (Bonnet et al., 2012; Pène et al., 2014). A recent study suggests that Tax itself may function as an ubiquitin E3 ligase that, in association with the ubiquitin-conjugating enzyme E2, catalyzes the assembly of mixed polyUb chains (Wang et al., 2016). However, a more recent study does not attribute to Tax an E3 ligase activity, while suggesting that multivalent interactions between NEMO proteins and Ub-chains can lead to the formation of a macromolecular Taxisome and consequently to the activation of the IKK complex (Shibata et al., 2017).

An additional mechanism that operates within the cells to maintain the NF-κB activation induced by Tax-1 is the positive feedback loop derived by NF-κB target genes. A recent report describes that the over-expression of the early growth response protein 1 (EGR1) induced by Tax-1 activation of NF-κB, results in the stabilization of EGR1 by direct interaction with Tax and nuclear translocation of p65, enhancing NF-κB activation (Huang et al., 2017). A similar positive loop is fostered by the overexpression of the interleukin receptor IL-17RB. Tax-1 promotes the expression of IL-17RB by NF-κB activation and establishes an IL-17RB-NF-κB feed-forward autocrine loop that maintains persistent NF-κB activation (Lavorgna et al., 2014).

## TAX AND HBZ INTERPLAY ON NF-κB DEREGULATION

HBZ can promote viral latency by antagonizing many of the activities mediated by Tax. HBZ inhibits the activation of the HTLV-1 5′ LTR preventing the formation of the Tax transactivation complex (Gaudray et al., 2002; Clerc et al., 2008). The activation of the classical NF-κB pathway by Tax is inhibited selectively by HBZ expression (Zhao et al., 2009; Wurm et al., 2012). This inhibition is connected to the following properties of HBZ as shown in **Figure 1C**: (a) the interaction with p65; (b) the inhibition of p65 DNA binding; (c) the enhanced degradation

of p65 through PDLIM2 E3 ubiquitin ligase; (d) the reduction of p65 acetylation. All these processes result in the reduction of the expression of several NF-κB target genes. A typical example is the *cyclin D1* promoter gene, an essential regulator of the G1/S phase transition of the cell cycle that is overexpressed by Tax-mediated NF-κB activation, while it is downregulated by HBZ interaction with p65 (Ma et al., 2017).

The HBZ inhibition of NF-κB has been proposed to be a critical step in the oligoclonal expansion of HTLV-1-infected cells by downregulating the senescence process (Giam and Semmes, 2016). NF-κB hyper-activation induced by Tax leads to the over-expression of the cyclin-dependent kinase inhibitors, p21 and p27, thus promoting an arrest of cell proliferation that triggers senescence. The proposed model envisages that in HTLV-1 infected cells, in which the p21/p27 functions is impaired, the HBZ downregulation of NF-κB may contrast the senescence induced by Tax hence promoting the expansion of the infected cells (Kuo and Giam, 2006; Zhang et al., 2009; Zhi et al., 2011).

In contrast to HBZ, the HTLV-2 homolog protein APH-2 is dispensable for HTLV infection and persistence and does not promote T-cell proliferation *in vitro* (Yin et al., 2012; Barbeau et al., 2013). In addition, APH-2 expression correlates with the proviral load in HTLV-2 infected subjects and, contrary to HBZ, does not promote lymphocytosis (Saito et al., 2009; Douceron et al., 2012). Of note, HBZ and APH-2 also diverge in the interaction with Tax, since HBZ does not bind Tax-1, whereas Tax-2 interacts with APH-2 (Marban et al., 2012). A recent study has shown that despite HBZ and APH-2 interact with p65/RelA and repress its transactivation activity in transfected cells, they diverge in the induction of p65 degradation since this is not detected in the presence of APH-2 (Panfil et al., 2016). This different effect suggests that the two proteins may adopt different mechanisms to interfere with NF-κB activation. The differences between regulatory proteins of HTLV-1 and HTLV-2 in deregulating NF-κB are outlined in **Table 1**.

## ROLE OF TAX AND HBZ IN ATL DEVELOPMENT

The opposite functions of Tax and HBZ in the regulation of signaling pathways and their effects in survival and proliferation appear as relevant steps during HTLV-1 cellular transformation and tumorigenesis (Giam and Semmes, 2016; Bangham and Matsuoka, 2017; Zhang et al., 2017). The absence of Tax expression in the late stages of the infection is linked to *tax* gene mutations and DNA methylation of the 5′ LTR provirus (Furukawa et al., 2001; Koiwa et al., 2002). On the opposite, the 3′ LTR negative strand remains intact and non-methylated, allowing HBZ to be systematically expressed in ATL cells (Taniguchi et al., 2005; Miyazaki et al., 2007). Unlike HBZ, Tax-1 is highly immunogenic and its inactivation may represent a fundamental strategy to evade the host immune system, a critical step in ATL development (Kogure and Kataoka, 2017). HBZ, like Tax-1, deregulates cell proliferation by targeting key factors implicated in cell survival. HBZ, in fact, binds to ATF3/p53 complexes and inhibits the p53 expression induced by ATF3, thus

| | Tax-1 | Tax-2 | References |
|---|---|---|---|
| Canonical NF-κB transactivation | + | + | Sun et al., 1994 |
| Non-canonical NF-κB transactivation | + | − | Higuchi et al., 2007 |
| NF-κB transactivation (lipid raft translocation of IKK) | + | − | Huang et al., 2009 |
| Interaction with p100/p52 | + | − | Shoji et al., 2009 |
| Interaction with p65 | + | + | Zhao et al., 2009; Panfil et al., 2016 |

| | HBZ | APH-2 | References |
|---|---|---|---|
| Canonical NF-κB inhibition | + | + | Zhao et al., 2009; Panfil et al., 2016 |
| Non-canonical NF-κB inhibition | − | nd | Zhao et al., 2009 |
| Interaction with p65 | + | + | Zhao et al., 2009; Panfil et al., 2016 |
| Inhibition of Tax-mediated transactivation of NF-κB | + | nd | Zhao et al., 2009 |
| Binding to Tax | − | + | Marban et al., 2012 |
| Inhibition of p65 DNA binding capacity | + | nd | Zhao et al., 2009 |
| p65 degradation | + | − | Panfil et al., 2016 |
| Inhibition of p65 acetylation | + | nd | Wurm et al., 2012 |

*nd, Not determined.*

promoting ATL cells proliferation (Hagiya et al., 2011). HBZ also induces the expression of the anti-apoptotic genes *BCL2* and *Flip*, interacting with C/EBPα and deregulating the C/EBP signaling (Zhao et al., 2013). Both Tax-1 and HBZ are involved in the inhibition of the tumor suppressor p53. In particular, Tax inhibits p53 activity through the p65 subunit of NF-κB or by sequestering p300/CBP from p53 (Ariumi et al., 2000; Karimi et al., 2017). Recent studies revealed that HBZ, by binding p300/CBP, inhibits p53 acetylation and decreases the p53 activity (Wright et al., 2016).

The selectivity of HBZ in inhibiting the classical NF-κB pathway opens an interesting area of investigation on the role of the non-canonical NF-κB pathway in tumorigenesis. During ATL development, HBZ might downmodulate the classical NF-κB pathway more efficiently when Tax expression is silenced, leading to predominant activation of the alternative pathway (Zhao et al., 2009). It has also been demonstrated that freshly isolated ATL cells display high expression levels of NIK, persistent phosphorylation of IκBα, aberrant processing of p52, and nuclear translocation of p50, p52, and RelB, despite the absence of Tax-1 expression (Chan and Greene, 2012).

Genetic and epigenetic alterations, including miRNAs expression profile, have been intensively investigated in the genome of ATL patients (Yeung et al., 2008; Bellon et al., 2009; Yamagishi and Watanabe, 2012; Watanabe, 2017). It has been proposed that the genomic instability may derive from Tax inhibition of DNA double-strand break repair and induction of micronuclei formation. ATL cells are characterized by frequent gain-of-function alterations of genes involved in the T-cell receptor/NF-κB signaling pathway, such as PLCG1,

PKCB, and CARD11 or loss-of-function mutations in upstream factors, such as TRAF3 (Cook et al., 2017; Kogure and Kataoka, 2017). Mutations or intragenic deletions of these genes result in NF-κB induction in the absence of Tax-1. A progressive epigenetic downregulation of *miR-31* has been demonstrated in ATL (Fujikawa et al., 2016). Of note, miR-31 negatively regulates the expression of NIK and miR-31 loss in ATL triggers the persistent activation of NF-κB, inducing apoptosis resistance and contributing to the abnormal proliferation of cancer cells (Yamagishi et al., 2012). In addition, Fujikawa et al. (2016) showed that Tax-mediated NF-κB activation induces the over-expression of the histone-lysine methyltransferase, EZH2, leading to host epigenetic machinery deregulation. It has been proposed that EZH2 may contribute to NF-κB activation through miR-31 silencing and consequently NIK induction, in a positive feedback loop (Sasaki et al., 2011; Fujikawa et al., 2016). Genetic mutations have been also suggested to cause IL-17RB overexpression which triggers classical NF-κB activation by an autocrine-loop in a subset of Tax-negative ATL cell lines (Lavorgna et al., 2014).

## CONCLUSIONS AND PERSPECTIVES

HTLV-1 appears to benefit from the antagonistic functions of Tax and HBZ in the deregulation of cellular signaling pathways, resulting in the loss of control of many biological processes such as proliferation and survival of HTLV-1-infected cells. The interplay between Tax and HBZ on NF-κB regulation has a prominent role in viral persistence in ATL cells, thus contributing to leukemic transformation. The intensive studies conducted in recent years aimed at understanding the effect of Tax constitutive activation and HBZ inhibition of NF-κB have contributed to further elucidate the molecular mechanism of NF-κB activation. However, several open questions about its functional role in ATL development still need to be addressed: the exact role of the persistent NF-κB activation in ATL cells; the contribution to tumorigenesis of the alternative pathway activation; the role of the different mechanisms that are adopted by HBZ and APH-2 to interfere with NF-κB activation; the dynamic organization of lipid raft complexes in HTLV-1 infected cell. It is hoped that the application of the CRISPR/Cas9 genome editing new technique will offer a useful tool to investigate the requirement of specific interactions of Tax and HBZ with cell factors that activate the mechanisms driving to tumorigenesis.

## AUTHOR CONTRIBUTIONS

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# REFERENCES

Ariumi, Y., Kaida, A., Lin, J. Y., Hirota, M., Masui, O., Yamaoka, S., et al. (2000). HTLV-1 Tax oncoprotein represses the p53-mediated trans-activation function through coactivator CBP sequestration. *Oncogene* 19, 1491–1499. doi: 10.1038/sj.onc.1203450

Avesani, F., Romanelli, M. G., Turci, M., Di Gennaro, G., Sampaio, C., Bidoia, C., et al. (2010). Association of HTLV Tax proteins with TAK1-binding protein 2 and RelA in calreticulin-containing cytoplasmic structures participates in Tax-mediated NF-κB activation. *Virology* 408, 39–48. doi: 10.1016/j.virol.2010.08.023

Bangham, C. R. M., and Matsuoka, M. (2017). Human T-cell leukaemia virus type 1: parasitism and pathogenesis. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 372:20160272. doi: 10.1098/rstb.2016.0272

Barbeau, B., Peloponese, J. M., and Mesnard, J. M. (2013). Functional comparison of antisense proteins of HTLV-1 and HTLV-2 in viral pathogenesis. *Front. Microbiol.* 4:226. doi: 10.3389/fmicb.2013.00226

Bellon, M., Lepelletier, Y., Hermine, O., and Nicot, C. (2009). Deregulation of microRNA involved in hematopoiesis and the immune response in HTLV-I adult T-cell leukemia. *Blood* 113, 4914–4917. doi: 10.1182/blood-2008-11-189845

Bertazzoni, U., Turci, M., Avesani, F., Di Gennaro, G., Bidoia, C., and Romanelli, M. G. (2011). Intracellular localization and cellular factors interaction of HTLV-1 and HTLV-2 tax proteins: similarities and functional differences. *Viruses* 3, 541–560. doi: 10.3390/v3050541

Bex, F., Murphy, K., Wattiez, R., Burny, A., and Gaynor, R. B. (1999). Phosphorylation of the human T-cell leukemia virus type 1 transactivator tax on adjacent serine residues is critical for tax activation. *J. Virol.* 73, 738–745.

Bonnet, A., Randrianarison-huetz, V., Nzounza, P., Nedelec, M., Chazal, M., Waast, L., et al. (2012). Low nuclear body formation and tax SUMOylation do not prevent NF-kappaB promoter activation, *Retrovirology* 9:77. doi: 10.1186/1742-4690-9-77

Chan, J. K., and Greene, W. C. (2012). Dynamic roles for NF-κB in HTLV-I and HIV-1 retroviral pathogenesis. *Immunol. Rev.* 246, 286–310. doi: 10.1111/j.1600-065X.2012.01094.x

Chen, L., Liu, D., Zhang, Y., Zhang, H., and Cheng, H. (2015). The autophagy molecule Beclin 1 maintains persistent activity of NF-κB and Stat3 in HTLV-1-transformed T lymphocytes. *Biochem. Biophys. Res. Commun.* 465, 739–745. doi: 10.1016/j.bbrc.2015.08.070

Clerc, I., Polakowski, N., André-Arpin, C., Cook, P., Barbeau, B., Mesnard, J. M., et al. (2008). An interaction between the human T cell leukemia virus type 1 basic leucine zipper factor (HBZ) and the KIX domain of p300/CBP contributes to the down-regulation of Tax-dependent viral transcription by HBZ. *J. Biol. Chem.* 283, 23903–23913. doi: 10.1074/jbc.M803116200

Cook, L., Melamed, A., Yaguchi, H., and Bangham, C. R. (2017). The impact of HTLV-1 on the cellular genome. *Curr. Opin. Virol.* 26, 125–131. doi: 10.1016/j.coviro.2017.07.013

Diani, E., Avesani, F., Bergamo, E., Cremonese, G., Bertazzoni, U., and Romanelli, M. G. (2015). HTLV-1 Tax protein recruitment into IKKε and TBK1 kinase complexes enhances IFN-I expression. *Virology* 476, 92–99. doi: 10.1016/j.virol.2014.12.005

Douceron, E., Kaidarova, Z., Miyazato, P., Matsuoka, M., Murphy, E. L., and Mahieux, R. (2012). HTLV-2 APH-2 expression is correlated with proviral load but APH-2 does not promote lymphocytosis. *J. Infect. Dis.* 205, 82–86. doi: 10.1093/infdis/jir708

Durand, J. K., and Baldwin, A. S. (2017). Targeting IKK and NF-κB for therapy. *Adv. Protein Chem. Struct. Biol.* 107, 77–115. doi: 10.1016/bs.apcsb.2016.11.006

Forlani, G., Abdallah, R., Accolla, R. S., and Tosi, G. (2013). The MHC-II transactivator CIITA, a restriction factor against oncogenic HTLV-1 and HTLV-2 retroviruses: similarities and differences in the inhibition of Tax-1 and Tax-2 viral transactivators. *Front. Microbiol.* 4:234. doi: 10.3389/fmicb.2013.00234

Forlani, G., Abdallah, R., Accolla, R. S., and Tosi, G. (2016). The major histocompatibility complex class II transactivator CIITA inhibits the persistent activation of NF-κB by the human T cell lymphotropic virus type 1 Tax-1 oncoprotein. *J. Virol.* 90, 3708–3721. doi: 10.1128/JVI.03000-15

Fu, J., Yan, P., Li, S., Qu, Z., and Xiao, G. (2010). Molecular determinants of PDLIM2 in suppressing HTLV-I Tax-mediated tumorigenesis. *Oncogene* 29, 6499–6507. doi: 10.1038/onc.2010.374

Fujikawa, D., Nakagawa, S., Hori, M., Kurokawa, N., Soejima, A., Nakano, K., et al. (2016). Polycomb-dependent epigenetic landscape in adult T-cell leukemia. *Blood* 127, 1790–1802. doi: 10.1182/blood-2015-08-662593

Furukawa, Y., Kubota, R., Tara, M., Izumo, S., and Osame, M. (2001). Existence of escape mutant in HTLV-I tax during the development of adult T-cell leukemia, *Blood* 97, 987–993. doi: 10.1182/blood.V97.4.987

Gallo, R. G., Willems, L., and Tagaya, Y. (2017). Time to go back to the original name. *Front. Microbiol.* 2017, 8:1800. doi: 10.3389/fmicb.2017.01800

Gao, L., and Harhaj, E. W. (2013). HSP90 protects the human T-cell leukemia virus type 1 (HTLV-1) tax oncoprotein from proteasomal degradation to support NF-κB activation and HTLV-1 replication. *J. Virol.* 87, 13640–13654. doi: 10.1128/JVI.02006-13

Gaudray, G., Gachon, F., Basbous, J., Biard-Piechaczyk, M., Devaux, C., and Mesnard, J. M. (2002). The complementary strand of the human T-cell leukemia virus type 1 RNA genome encodes a bZIP transcription factor that down-regulates viral transcription. *J. Virol.* 76, 12813–12822. doi: 10.1128/JVI.76.24.12813-12822.2002

Gessain, A., Barin, F., Vernant, J. C., Gout, O., Maurs, L., Calender, A., et al. (1985). Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis. *Lancet* 2, 407–410. doi: 10.1016/S0140-6736(85)92734-5

Gessain, A., and Cassar, O. (2012). Epidemiological aspects and world distribution of HTLV-1 infection. *Front. Microbiol.* 3:388. doi: 10.3389/fmicb.2012.00388

Giam, C. Z., and Semmes, O. J. (2016). HTLV-1 infection and adult T-cell leukemia/lymphoma-a tale of two proteins: Tax and HBZ. *Viruses* 8:E161. doi: 10.3390/v8060161

Hagiya, K., Yasunaga, J. I., Satou, Y., Ohshima, K., and Matsuoka, M. (2011). ATF3, an HTLV-1 bZip factor binding protein, promotes proliferation of adult T-cell leukemia cells. *Retrovirology* 8:19. doi: 10.1186/1742-4690-8-19

Higuchi, M., and Fujii, M. (2009). Distinct functions of HTLV-1 Tax1 from HTLV-2 Tax2 contribute key roles to viral pathogenesis. *Retrovirology* 6:117. doi: 10.1186/1742-4690-6-117

Higuchi, M., Tsubata, C., Kondo, R., Yoshida, S., Takahashi, M., Oie, M., et al. (2007). Cooperation of NF-kappaB2/p100 activation and the PDZ domain binding motif signal in human T-cell leukemia virus type 1 (HTLV-1) Tax1 but not HTLV-2 Tax2 is crucial for interleukin-2-independent growth transformation of a T-cell line. *J. Virol.* 81, 11900–11907. doi: 10.1128/JVI.00532-07

Hinuma, Y., Nagata, K., Hanaoka, M., Nakai, M., Matsumoto, T., Kinoshita, K. I., et al. (1981). Adult T-cell leukemia: antigen in an ATL cell line and detection of antibodies to the antigen in human sera. *Proc. Natl. Acad. Sci. U.S.A.* 78, 6476–6480. doi: 10.1073/pnas.78.10.6476

Ho, Y. K., Zhi, H., Bowlin, T., Dorjbal, B., Philip, S., Zahoor, M. A., et al. (2015). HTLV-1 Tax stimulates ubiquitin E3 ligase, ring finger protein 8, to assemble lysine 63-linked polyubiquitin chains for TAK1 and IKK activation. *PLoS Pathog.* 11, 1–19. doi: 10.1371/journal.ppat.1005102

Huang, J., Ren, T., Guan, H., Jiang, Y., and Cheng, H. (2009). HTLV-1 Tax is a critical lipid raft modulator that hijacks IkappaB kinases to the microdomains for persistent activation of NF-kappaB. *J. Biol. Chem.* 284, 6208–6217. doi: 10.1074/jbc.M806390200

Huang, Q., Niu, Z., Han, J., Liu, X., Lv, Z., Yuan, L., et al. (2017). HTLV-1 Tax upregulates early growth response protein 1 through nuclear factor-κB signaling. *Oncotarget* 8, 51123–51133. doi: 10.18632/oncotarget.17699

Journo, C., Bonnet, A., Favre-Bonvin, A., Turpin, J., Vinera, J., Côté, E., et al. (2013). Human T cell leukemia virus type 2 tax-mediated NF-κB activation involves a mechanism independent of Tax conjugation to ubiquitin and SUMO. *J. Virol.* 87, 1123–1136. doi: 10.1128/JVI.01792-12

Karimi, M., Mohammadi, H., Hemmatzadeh, M., Mohammadi, A., Rafatpanah, H., and Baradaran, B. (2017). Role of the HTLV-1 viral factors in the induction of apoptosis. *Biomed. Pharmacother.* 85, 334–347. doi: 10.1016/j.biopha.2016.11.034

Kfoury, Y., Setterblad, N., El-Sabban, M., Zamborlini, A., Dassouki, Z., El Hajj, H., et al. (2011). Tax ubiquitylation and SUMOylation control the dynamic shuttling of Tax and NEMO between Ubc9 nuclear bodies and the centrosome. *Blood* 117, 190–199. doi: 10.1182/blood-2010-05-285742

Kogure, Y., and Kataoka, K. (2017). Genetic alterations in adult T-cell leukemia/lymphoma. *Cancer Sci.* 108, 1719–1725. doi: 10.1111/cas.13303

Koiwa, T., Hamano-Usami, A., Ishida, T., Okayama, A., Yamaguchi, K., Kamihira, S., et al. (2002). 5′-long terminal repeat-selective CpG methylation of latent human T-cell leukemia virus type 1 provirus *in vitro* and *in vivo*. *J. Virol.* 76, 9389–9397. doi: 10.1128/JVI.76.18.9389-9397.2002

Kuo, Y. L., and Giam, C. Z. (2006). Activation of the anaphase promoting complex by HTLV-1 tax leads to senescence. *EMBO J.* 25, 1741–1752. doi: 10.1038/sj.emboj.7601054

Kusano, S., Yoshimitsu, M., Hachiman, M., and Ikeda, M. (2015). I-mfa domain proteins specifically interact with HTLV-1 Tax and repress its transactivating functions. *Virology* 486, 219–227. doi: 10.1016/j.virol.2015.09.020

Lavorgna, A., and Harhaj, E. W. (2014). Regulation of HTLV-1 tax stability, cellular trafficking and NF-κB activation by the ubiquitin-proteasome pathway. *Viruses* 6, 3925–3943. doi: 10.3390/v6103925

Lavorgna, A., Matsuoka, M., and Harhaj, E. W. (2014). A critical role for IL-17RB signaling in HTLV-1 tax-induced NF-κB activation and T-cell transformation. *PLoS Pathog.* 10:e1004418. doi: 10.1371/journal.ppat.1004418

Ma, G., Yasunaga, J., and Matsuoka, M. (2016). Multifaceted functions and roles of HBZ in HTLV-1 pathogenesis. *Retrovirology* 13:16. doi: 10.1186/s12977-016-0249-x

Ma, Y., Zhang, B., Wang, D., Qian, L., Song, X., Wang, X., et al. (2017). HTLV-1 basic leucine zipper factor downregulates cyclin D1 expression via interactions with NF-κB. *Int. J. Mol. Med.* 764–770. doi: 10.3892/ijmm.2017.2868

Marban, C., McCabe, A., Bukong, T. N., Hall, W. W., and Sheehy, N. (2012). Interplay between the HTLV-2 Tax and APH-2 proteins in the regulation of the AP-1 pathway. *Retrovirology* 9:98. doi: 10.1186/1742-4690-9-98

Matsuoka, M., and Jeang, K. T. (2007). Human T-cell leukaemia virus type 1 (HTLV-1) infectivity and cellular transformation. *Nat. Rev. Cancer* 7, 270–280. doi: 10.1038/nrc2111

Miyazaki, M., Yasunaga, J. I., Taniguchi, Y., Tamiya, S., Nakahata, T., and Matsuoka, M. (2007). Preferential selection of human T-cell leukemia virus type 1 provirus lacking the 5′ long terminal repeat during oncogenesis. *J. Virol.* 81, 5714–5723. doi: 10.1128/JVI.02511-06

Motai, Y., Takahashi, M., Takachi, T., Higuchi, M., Hara, T., Mizuguchi, M., et al. (2016). Human T-cell leukemia virus type 1 (HTLV-1) Tax1 oncoprotein but not HTLV-2 Tax2 induces the expression of OX40 ligand by interacting with p52/p100 and RelB. *Virus Genes* 52, 4–13. doi: 10.1007/s11262-015-1277-7

Nasr, R., Chiari, E., El-sabban, M., Mahieux, R., Kfoury, Y., Abdulhay, M., et al. (2006). Tax ubiquitylation and sumoylation control critical cytoplasmic and nuclear steps of NF-κB activation Tax ubiquitylation and sumoylation control critical cytoplasmic and nuclear steps of NF-κB activation. *Blood* 107, 4021–4029. doi: 10.1182/blood-2005-09-3572

Panfil, A. R., Dissinger, N. J., Howard, C. M., Murphy, B. M., Landes, K., Fernandez, S. A., et al. (2016). Functional comparison of HBZ and the related APH-2 protein provides insight into human T-cell leukemia virus type 1 pathogenesis. *J. Virol.* 90, 3760–3772. doi: 10.1128/JVI.03113-15

Peloponese, J. M., Yeung, M. L., and Jeang, K. T. (2006). Modulation of nuclear factor-kappaB by human T cell leukemia virus type 1 Tax protein: implications for oncogenesis and inflammation. *Immunol. Res.* 34, 1–12. doi: 10.1385/IR:34:1:1

Pène, S., Waast, L., Bonnet, A., Bénit, L., and Pique, C. (2014). A non-SUMOylated tax protein is still functional for NF-κB pathway activation. *J. Virol.* 88, 10655–10661. doi: 10.1128/JVI.01827-14

Poiesz, B. J., Ruscetti, F. W., Gazdar, A. F., Bunn, P. A., Minna, J. D., and Gallo, R. C. (1980). Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc. Nat. Acad. Sci. U.S.A.* 77, 7415–7419. doi: 10.1073/pnas.77.12.7415

Pujari, R., Hunte, R., Thomas, R., van der Weyden, L., Rauch, D., Ratner, L., et al. (2015). Human T-cell leukemia virus type 1 (HTLV-1) tax requires CADM1/TSLC1 for inactivation of the NF-κB inhibitor A20 and constitutive NF-κB signaling. *PLoS Pathog.* 11:e1004721. doi: 10.1371/journal.ppat.1004721

Qu, Z., and Xiao, G. (2011). Human T-cell lymphotropic virus: a model of NF-κB-associated tumorigenesis. *Viruses* 3, 714–749. doi: 10.3390/v3060714

Ren, T., Dong, W., Takahashi, Y., Xiang, D., Yuan, Y., Liu, X., et al. (2012). HTLV-2 tax immortalizes human CD4+ memory T lymphocytes by oncogenic activation and dysregulation of autophagy. *J. Biol. Chem.* 287, 34683–34693. doi: 10.1074/jbc.M112.377143

Ren, T., Takahashi, Y., Liu, X., Loughran, T. P., Sun, S. C., Wang, H. G., et al. (2015). HTLV-1 Tax deregulates autophagy by recruiting autophagic molecules into lipid raft microdomains. *Oncogene* 34, 334–345. doi: 10.1038/onc.2013.552

Romanelli, M. G., Diani, E., Bergamo, E., Casoli, C., Ciminale, V., Bex, F., et al. (2013). Highlights on distinctive structural and functional properties of HTLV Tax proteins. *Front. Microbiol.* 4:271. doi: 10.3389/fmicb.2013.00271

Saito, M., Matsuzaki, T., Satou, Y., Yasunaga, J. I., Saito, K., Arimura, K., et al. (2009). *In vivo* expression of the HBZ gene of HTLV-1 correlates with proviral load, inflammatory markers and disease severity in HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP). *Retrovirology* 6:19. doi: 10.1186/1742-4690-6-19

Sasaki, D., Imaizumi, Y., Hasegawa, H., Osaka, A., Tsukasaki, K., Choi, Y. L., et al. (2011). Overexpression of enhancer of zeste homolog 2 with trimethylation of lysine 27 on histone H3 in adult T-cell leukemia/lymphoma as a target for epigenetic therapy. *Haematologica* 96, 712–719. doi: 10.3324/haematol.2010.028605

Shembade, N., Harhaj, N. S., Yamamoto, M., Akira, S., and Harhaj, E. W. (2007). The human T-cell leukemia virus type 1 Tax oncoprotein requires the ubiquitin-conjugating enzyme Ubc13 for NF-kappaB activation. *J. Virol.* 81, 13735–13742. doi: 10.1128/JVI.01790-07

Shen, R. R., and Hahn, W. C. (2011). Emerging roles for the non-canonical IKKs in cancer *Oncogene* 30, 631–641. doi: 10.1038/onc.2010.493

Shibata, Y., Tokunaga, F., Goto, E., Komatsu, G., Gohda, J., Saeki, Y., et al. (2017). HTLV-1 Tax induces formation of the active macromolecular IKK complex by generating Lys63- and Met1-linked hybrid polyubiquitin Chains. *PLoS Pathog.* 13, 1–25. doi: 10.1371/journal.ppat.1006162

Shoji, T., Higuchi, M., Kondo, R., Takahashi, M., Oie, M., Tanaka, Y., et al. (2009). Identification of a novel motif responsible for the distinctive transforming activity of human T-cell leukemia virus (HTLV) type 1 Tax1 protein from HTLV-2 Tax2. *Retrovirology* 6:83. doi: 10.1186/1742-4690-6-83

Sun, S. C. (2017). The non-canonical NF-κB pathway in immunity and inflammation. *Nat. Rev. Immunol.* 17, 545–558. doi: 10.1038/nri.2017.52

Sun, S. C., Elwood, J., Béraud, C., and Greene, W. C. (1994). Human T-cell leukemia virus type I Tax activation of NF-kappa B/Rel involves phosphorylation and degradation of I kappa B alpha and RelA (p65)-mediated induction of the c-rel gene. *Mol. Cell. Biol.* 14, 7377–7384. doi: 10.1128/MCB.14.11.7377

Taniguchi, Y., Nosaka, K., Yasunaga, J., Maeda, M., Mueller, N., Okayama, A., et al. (2005). Silencing of human T-cell leukemia virus type I gene transcription by epigenetic mechanisms. *Retrovirology* 2:64. doi: 10.1186/1742-4690-2-64

Turci, M., Lodewick, J., Di Gennaro, G., Rinaldi, A. S., Marin, O., Diani, E., et al. (2012). Ubiquitination and sumoylation of the HTLV-2 Tax-2B protein regulate its NF-κB activity: a comparative study with the HTLV-1 Tax-1 protein. *Retrovirology* 9:102. doi: 10.1186/1742-4690-9-102

Turci, M., Romanelli, M. G., Lorenzi, P., Righi, P., and Bertazzoni, U. (2006). Localization of human T-cell lymphotropic virus type II Tax protein is dependent upon a nuclear localization determinant in the N-terminal region. *Gene* 365, 119–124. doi: 10.1016/j.gene.2005.09.043

Wang, C., Long, W., Peng, C., Hu, L., Zhang, Q., Wu, A., et al. (2016). HTLV-1 Tax functions as a ubiquitin E3 ligase for direct IKK activation via synthesis of mixed-linkage polyubiquitin Chains. *PLoS Pathog.* 12, 1–25. doi: 10.1371/journal.ppat.1005584

Wang, J., Li, J., Huang, Y., Song, X., Niu, Z., Gao, Z., et al. (2013). Bcl-3 suppresses Tax-induced NF-κB activation through p65 nuclear translocation blockage in HTLV-1-infected cells. *Int. J. Oncol.* 42, 269–276. doi: 10.3892/ijo.2012.1685

Wang, W., Zhou, J., Shi, J., Zhang, Y., Liu, S., Liu, Y., et al. (2014). Human T-cell leukemia virus type 1 Tax-deregulated autophagy pathway and c-FLIP expression contribute to resistance against death receptor-mediated apoptosis. *J. Virol.* 88, 2786–2798. doi: 10.1128/JVI.03025-13

Watanabe, T. (2017). Adult T-cell leukemia: molecular basis for clonal expansion and transformation of HTLV-1-infected T cells. *Blood* 129, 1071–1081. doi: 10.1182/blood-2016-09-692574

Willems, L., Hasegawa, H., Accolla, R., Bangham, C., Bazarbachi, A., Bertazzoni, U., et al. (2017). Reducing the global burden of HTLV-1 infection: an agenda for research and action. *Antiviral Res.* 137, 41–48. doi: 10.1016/j.antiviral.2016.10.015

Wright, D. G., Marchal, C., Hoang, K., Ankney, J. A., Nguyen, S. T., Rushing, A. W., et al. (2016). Human T-cell leukemia virus type-1-encoded protein HBZ

represses p53 function by inhibiting the acetyltransferase activity of p300/CBP and HBO1. *Oncotarget* 7, 1687–1706. doi: 10.18632/oncotarget.6424

Wurm, T., Wright, D. G., Polakowski, N., Mesnard, J. M., and Lemasson, I. (2012). The HTLV-1-encoded protein HBZ directly inhibits the acetyl transferase activity of p300/CBP. *Nucleic Acids Res.* 40, 5910–5925. doi: 10.1093/nar/gks244

Xiao, G., Cvijic, M. E., Fong, A., Harhaj, E. W., Uhlik, M. T., Waterfield, M., et al. (2001). Retroviral oncoprotein Tax induces processing of NF-kappaB2/p100 in T cells: evidence for the involvement of IKKalpha. *EMBO J.* 20, 6805–6815. doi: 10.1093/emboj/20.23.6805

Yamagishi, M., Nakano, K., Miyake, A., Yamochi, T., Kagami, Y., Tsutsumi, A., et al. (2012). Polycomb-mediated loss of miR-31 activates NIK-dependent NF-κB pathway in adult T cell leukemia and other cancers. *Cancer Cell* 21, 121–135. doi: 10.1016/j.ccr.2011.12.015

Yamagishi, M., and Watanabe, T. (2012). Molecular hallmarks of adult T cell leukemia. *Front. Microbiol.* 3:334. doi: 10.3389/fmicb.2012.00334

Yan, P., Fu, J., Qu, Z., Li, S., Tanaka, T., Grusby, M. J., et al. (2009). PDLIM2 suppresses human T-cell leukemia virus type I Tax-mediated tumorigenesis by targeting Tax into the nuclear matrix for proteasomal degradation. *Blood* 113, 4370–4380. doi: 10.1182/blood-2008-10-185660

Yasuma, K., Yasunaga, J. I., Takemoto, K., Sugata, K., Mitobe, Y., Takenouchi, N., et al. (2016). HTLV-1 bZIP factor impairs anti-viral immunity by inducing co-inhibitory molecule, T cell immunoglobulin and ITIM domain (TIGIT). *PLoS Pathog.* 12, 1–22. doi: 10.1371/journal.ppat.1005372

Yeung, M. L., Yasunaga, J., Bennasser, Y., Dusetti, N., Harris, D., Ahmad, N., et al. (2008). Roles for microRNAs, miR-93 and miR-130b, and tumor protein 53-induced nuclear protein 1 tumor suppressor in cell growth dysregulation by human T-cell lymphotrophic virus 1. *Cancer Res.* 68, 8976–8985. doi: 10.1158/0008-5472.CAN-08-0769

Yin, H., Kannian, P., Dissinger, N., Haines, R., Niewiesk, S., and Green, P. L. (2012). Human T-cell leukemia virus type 2 antisense viral protein 2 is dispensable for *in vitro* immortalization but functions to repress early virus replication *in vivo*. *J. Virol.* 86, 8412–8421. doi: 10.1128/JVI.00717-12

Yu, Q., Minoda, Y., Yoshida, R., Yoshida, H., Iha, H., Kobayashi, T., et al. (2008). HTLV-1 Tax-mediated TAK1 activation involves TAB2 adapter protein. *Biochem. Biophys. Res. Commun.* 365, 189–194. doi: 10.1016/j.bbrc.2007.10.172

Zhang, H., Chen, L., Cai, S. H., and Cheng, H. (2016). Identification of TBK1 and IKKε, the non-canonical IκB kinases, as crucial pro-survival factors in HTLV-1-transformed T lymphocytes. *Leuk. Res.* 46, 37–44. doi: 10.1016/j.leukres.2016.04.012

Zhang, L. L., Wei, J., Wang, L., Huang, S., and Chen, J. L. (2017). Human T-cell lymphotropic virus type 1 and its oncogenesis. *Acta Pharmacol. Sin.* 38, 1093–1103. doi: 10.1038/aps.2017.17

Zhang, L., Zhi, H., Liu, M., Kuo, Y. L., and Giam, C. Z. (2009). Induction of p21(CIP1/WAF1) expression by human T-lymphotropic virus type 1 Tax requires transcriptional activation and mRNA stabilization. *Retrovirology* 6:35. doi: 10.1186/1742-4690-6-35

Zhao, T. (2016). The role of HBZ in HTLV-1-induced oncogenesis. *Viruses* 8, 1–12. doi: 10.3390/v8020034

Zhao, T., Coutts, A., Xu, L., Yu, J., Ohshima, K., and Matsuoka, M. (2013). HTLV-1 bZIP factor supports proliferation of adult T cell leukemia cells through suppression of C/EBPα signaling. *Retrovirology* 10:159. doi: 10.1186/1742-4690-10-159

Zhao, T., and Matsuoka, M. (2012). HBZ and its roles in HTLV-1 oncogenesis. *Front. Microbiol.* 3:247. doi: 10.3389/fmicb.2012.00247

Zhao, T., Yasunaga, J., Satou, Y., Nakao, M., Takahashi, M., Fujii, M., et al. (2009). Human T-cell leukemia virus type 1 bZIP factor selectively suppresses the classical pathway of NF-kappaB. *Blood* 113, 2755–2764. doi: 10.1182/blood-2008-06-161729

Zhi, H., Yang, L., Kuo, Y. L., Ho, Y. K., Shih, H. M., and Giam, C. Z. (2011). NF-κB hyper-activation by HTLV-1 tax induces cellular senescence, but can be alleviated by the viral anti-sense protein HBZ. *PLoS Pathog.* 7, 1–12. doi: 10.1371/journal.ppat.1002025

**ARTICLE**

# Stability of HTLV-2 antisense protein is controlled by PML nuclear bodies in a SUMO-dependent manner

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## Abstract

Since the identification of the antisense protein of HTLV-2 (APH-2) and the demonstration that APH-2 mRNA is expressed in vivo in most HTLV-2 carriers, much effort has been dedicated to the elucidation of similarities and/or differences between APH-2 and HBZ, the antisense protein of HTLV-1. Similar to HBZ, APH-2 negatively regulates HTLV-2 transcription. However, it does not promote cell proliferation. In contrast to HBZ, APH-2 half-life is very short. Here, we show that APH-2 is addressed to PML nuclear bodies in T-cells, as well as in different cell types. Covalent SUMOylation of APH-2 is readily detected, indicating that APH-2 might be addressed to the PML nuclear bodies in a SUMO-dependent manner. We further show that silencing of PML increases expression of APH-2, while expression of HBZ is unaffected. On the other hand, SUMO-1 overexpression leads to a specific loss of APH-2 expression that is restored upon proteasome inhibition. Furthermore, the carboxy-terminal LAGLL motif of APH-2 is responsible for both the targeting of the protein to PML nuclear bodies and its short half-life. Taken together, these observations indicate that natural APH-2 targeting to PML nuclear bodies induces proteasomal degradation of the viral protein in a SUMO-dependent manner. Hence, this study deciphers the molecular and cellular bases of APH-2 short half-life in comparison to HBZ and highlights key differences in the post-translational mechanisms that control the expression of both proteins.

## Introduction

In contrast to human T lymphotropic virus type 1 (HTLV-1) that is etiologically linked to severe diseases, such as adult T-cell lymphoma/leukemia (ATLL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), infection by HTLV-2 remains asymptomatic in most infected carriers with rare cases of spinal cord disease (for a review, see [1, 2]). While HTLV-1 infects mostly CD4+ T-cells, HTLV-2 preferentially infects CD8+ T-cells [3, 4].

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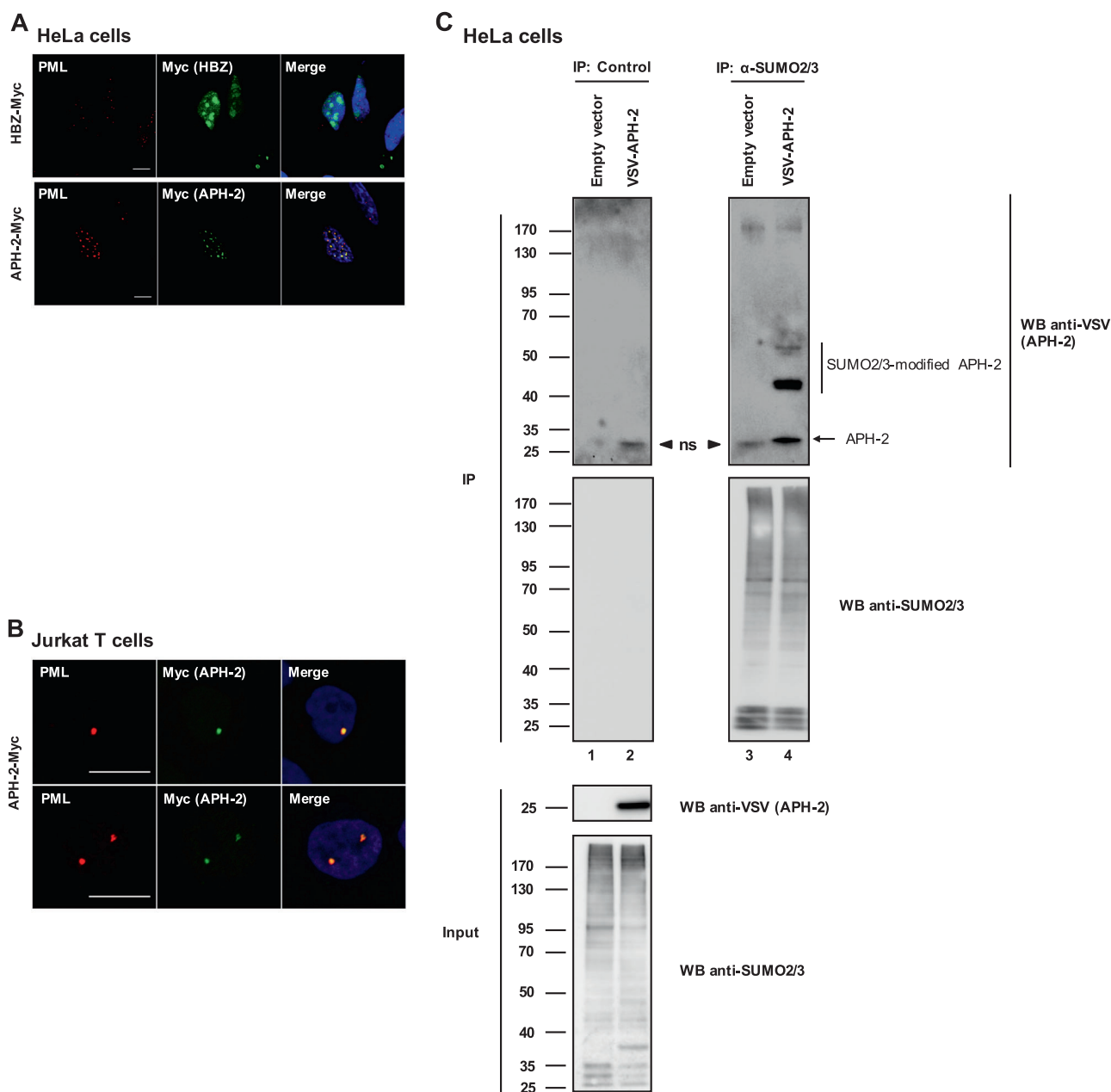
**Fig. 1** APH-2 localizes in PML nuclear bodies and is SUMOylated. **a** HeLa cells and **b** Jurkat T-cells were transfected with HBZ-Myc or APH-2-Myc as indicated, and fixed 24 h after transfection. Subcellular localization of APH-2 and HBZ was examined by confocal microscopy after anti-Myc and anti-PML staining. Nuclei were counterstained with DAPI (blue). Scale bar = 10 μm. Images representative of at least three independent experiments are shown. **c** HeLa cells were transfected with SUMO2/3 and VSV-APH-2 or with empty vector. After treatment with MG132, anti-SUMO2/3 immunoprecipitation was performed on cell lysates in denaturing conditions, allowing specific retention of post-translationally modified APH-2. Precipitates were analyzed by western blot using anti-SUMO2/3 and anti-VSV antibodies. Results are representative of three independent experiments. *ns* non-specific signal

Because HTLV-1 and HTLV-2 share a similar genomic organization, comparative approaches meant to highlight correlations between functional properties of HTLV-1/2 genes and pathogenicity have been undertaken in the last years.

At the molecular level, HTLV-1-induced pathogenicity has been linked to the expression of two regulatory genes encoding the transactivator Tax and the antisense protein HBZ (HTLV-1 basic leucine zipper [bZip] factor), respectively [5–7]. In addition to its viral function in transactivating the viral LTR promoter, Tax interferes with many cellular functions by interacting with cellular partners and transactivating or transrepressing cellular genes [8]. For instance, Tax induces a constitutive activation of the

canonical and non-canonical NF-κB pathways that are involved in both cell transformation (in the course of ATLL development [9, 10]) and inflammation (in the course of HAM/TSP development [11]). As such, Tax is considered as the main viral oncoprotein. HBZ was identified in 2002 as a viral protein encoded by the negative strand of the provirus and controlled by a promoter located in the 3′LTR [12]. Of note, while *tax* expression is severely decreased and difficult to detect in most patients upon ATLL development, *hbz* spliced form (sHBZ/SP1) is consistently expressed in HTLV-1-infected asymptomatic individuals, as well as in ATLL patients. Surprisingly, HBZ-transgenic mice expressing HBZ in CD4+ T-cells, which are the main HTLV-1 target cells in vivo, also develop lymphoma and dermatitis in an IFN-γ-dependent manner [13], indicating that HBZ is intrinsically capable of inducing inflammation and cell transformation. HBZ contributes to the negative regulation of viral expression by inhibiting transcription from the 5′LTR after competition with transcription co-activators required by Tax [12]. It is now well established that HBZ cooperates with Tax for cell transformation, inflammation, and maintenance of transformed state in T-cells (for a recent review, see [14]), although Tax alone is sufficient for transforming T-cells in vitro [15] and in animal models in vivo [16, 17].

We previously reported the existence of the antisense protein of HTLV-2, termed APH-2 (ref. [18]). Similar functions have been attributed to APH-2 and HBZ regarding negative regulation of viral expression, since APH-2 is also able to inhibit transcription from HTLV-2 5′LTR [18]. However, APH-2 is unable to induce cell proliferation in vitro and is dispensable for cell immortalization in an in vivo rabbit model [19, 20]. A recent study also highlighted distinctive features of APH-2 vs. HBZ in vitro in modulating cell signaling pathways [21]. Differences in the functions of APH-2 vs. HBZ might thus contribute to the distinct ability of HTLV-2 vs. HTLV-1 to cause leukemia in vivo.

We previously observed that APH-2 had a nuclear localization that is very different from that of HBZ [18]. Importantly, APH-2 expression was also difficult to detect in vitro even in overexpression assays using expression vectors with strong promoters, indicating that the protein might be very unstable in cells. Recently indeed, Panfil et al. reported a half-life of about 30 min in transfected HEK293T cells, and of about 20 min in transfected Jurkat T-cells [21].

The aim of the present study was to understand the molecular and cellular bases of this instability in comparison to HBZ. We show here that APH-2 is covalently linked to SUMO moieties and addressed to PML nuclear bodies in all cell types tested, including T-cells. We further show that

this natural targeting of APH-2 to PML nuclear bodies induces proteasomal degradation of the viral protein in a SUMO-dependent manner. Hence, APH-2 short half-life is controlled by its post-translational modifications and its subnuclear distribution. This might explain the decreased pathogenicity of HTLV-2 viral infection.
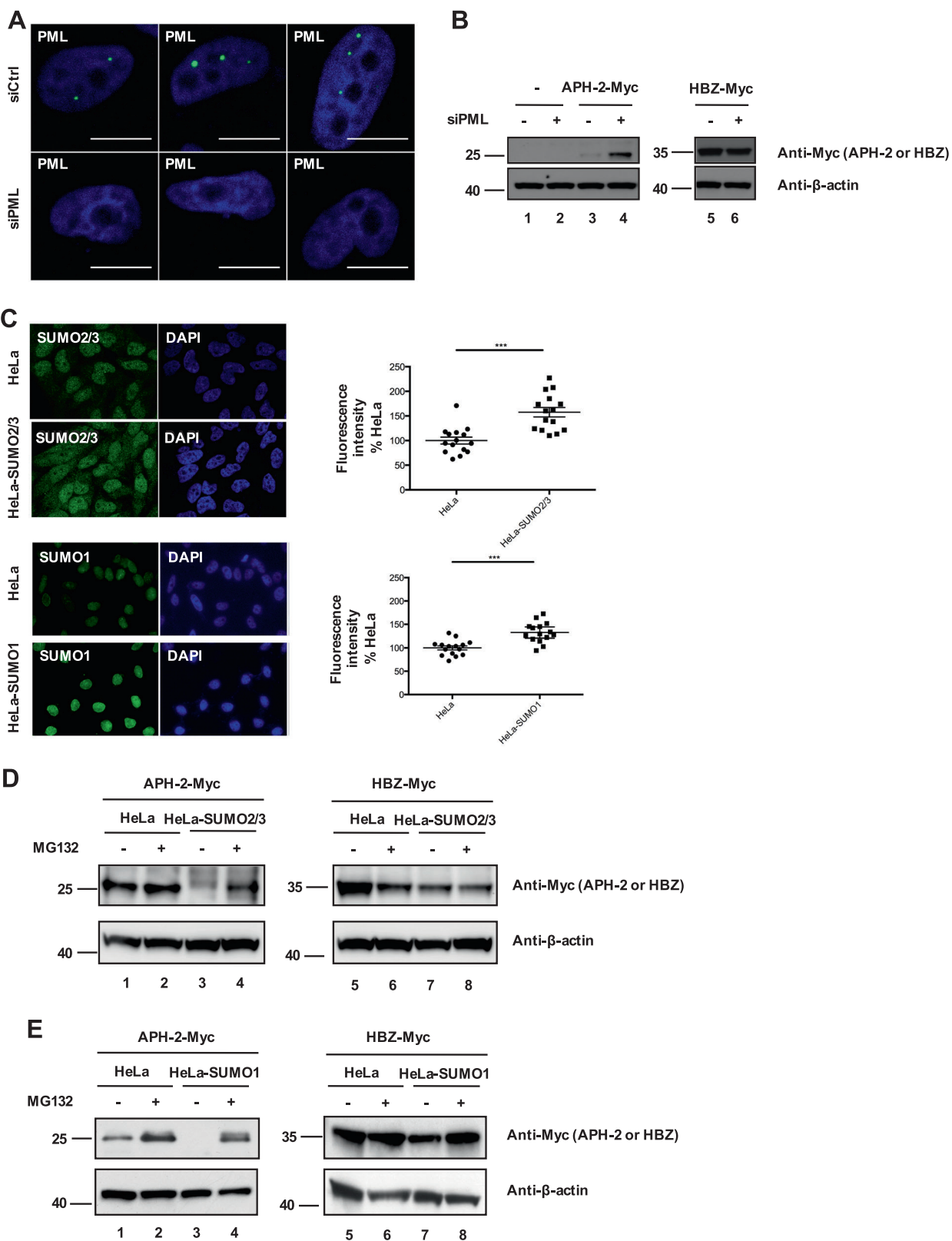
## Results

### APH-2 localizes in PML nuclear bodies

We previously described APH-2 as a nuclear protein that was distributed in nuclear speckles distinct from nucleoli and which stained negative for sc-35 (ref.[18]).To further characterize APH-2 subnuclear distribution, HeLa cells were transfected with expression vectors encoding APH-2 or HBZ and sharing the same tag (Fig. 1a). As previously shown [18], confocal imaging revealed that APH-2 was distributed in nuclear speckles. Co-staining with anti-PML antibodies indicated that these speckles were PML nuclear bodies (PML-NBs) (Fig. 1a), while HBZ did not colocalize with PML, consistent with a previous report indicating that HBZ is a nucleolar protein [22].

To extend these results to T-cells, the main in vivo target of HTLV-2, Jurkat cells were transfected with an APH-2 expression vector and stained for PML (Fig. 1b). APH-2 also co-localized with PML in T-cell nuclei, confirming that this localization is not cell-type specific. To exclude the fact that these results were due to the tag used, experiments were also repeated with expression vectors containing different tags and showed the same pattern (data not shown).

We then hypothesized that APH-2 might be addressed to PML-NBs following covalent poly-SUMOylation, which may be in the form of poly-SUMO2/3 chains terminated by SUMO-1 moieties. Therefore, SUMO2/3 was immunoprecipitated from cells co-transfected with APH-2 and SUMO2/3 vectors. Those cells were treated with the MG132 proteasome inhibitor to facilitate APH-2 detection (Fig. 1c). Immunoprecipitation was performed under denaturing conditions to eliminate non-covalent partners. Purification of SUMO2/3 followed by anti-VSV-APH-2 western blot led to the detection of two SUMO2/3-modified forms of APH-2 that were enriched compared to empty vector-transfected cells (Fig. 1c compare lane 3 vs. 4). These results are typical of SUMO-modified proteins. Complementary experiments also indicated that APH-2 is modified by SUMO1 (Supplemental Fig. 1). Thus, APH-2 is SUMOylated in cells. Taken together, these observations suggest that SUMOylated APH-2 could be addressed to PML-NBs where it accumulates.

## PML nuclear bodies control APH-2 stability in a SUMO-dependent manner

In order to demonstrate the involvement of PML-NBs in the control of APH-2 stability, APH-2 expression was monitored in PML-silenced cells (Fig. 2a, b). Cells were transfected with siRNA targeting PML or control siRNA, together with Myc-tagged APH-2 or HBZ constructs. First, PML silencing was validated by confocal microscopy after PML staining (Fig. 2a, see difference in cells treated with siRNA against PML or control siRNA). In PML-silenced cells, APH-2 expression levels were higher than in control cells (Fig. 2b, compare lane 4 vs. 3), indicating that indeed, PML is required for the low stability of APH-2. In contrast, HBZ expression was unaffected by PML silencing (Fig. 2b, compare lane 6 vs. 5), indicating that PML specifically controls APH-2 stability.

Because PML-NBs are enriched in SUMOylated proteins, and because SUMO modifications are known as a degradation signals for PML-NBs-associated proteins, SUMO-1 and SUMO2/3 signals were first checked by immunofluorescence and quantified in HeLa, HeLa-SUMO1, and HeLa-SUMO2/3 cells (Fig. 2c upper panel for SUMO2/3 and lower panel for SUMO1). Then, APH-2 and HBZ were transfected in HeLa-SUMO2/3 (Fig. 2d) and HeLa-SUMO1 (Fig. 2e), as well as in control HeLa cells. As compared to control HeLa cells, HeLa SUMO2/3 as well as HeLa-SUMO1 cells showed a severe decrease in APH-2 detection (Fig. 2d,e compare lanes 1 vs. 3). Proteasome inhibition by MG132 restored detection of APH-2 in HeLa-SUMO2/3 and HeLa-SUMO1 cells (Fig. 2d, e compare lanes 3 vs. 4), indicating that APH-2 is degraded by the proteasome in a SUMO-dependent manner. Of note, HBZ expression levels were unaffected by SUMO2/3 and

SUMO1 overexpression and only marginally affected by proteasome inhibition (Fig. 2d,e right panel), again indicating that SUMO-dependent degradation is specifically controlling APH-2 stability.

## The LXXLL$_2$ domain of APH-2 is required for PML localization and is responsible for the short half-life of the protein

APH-2 harbors a C-terminal LXXLL motif (aa 179-183, LXXLL$_2$, Fig. 3a left panel) that was previously reported to be required for efficient APH-2 binding to CREB and hence for efficient repression of 5′LTR transcription by APH-2 (ref. [20]). It also harbors a LXXLL-like motif located in the central domain of the protein (aa 64-68, LXXLL$_1$, Fig. 3a left panel) whose function is unclear. In order to investigate whether the C-terminal LXXLL motif is involved in PML-NB localization, we generated a truncated version of the protein, APH-2$_{1-178}$, which lacks the C-terminal LXXLL$_2$ motif. Jurkat cells were transfected with full-length or truncated APH-2 and imaged by confocal microscopy after PML staining (Fig. 3a right panel). While full-length APH-2 was distributed in PML-NBs, APH-2$_{1-178}$ was found diffuse in the cytoplasm of cells, indicating that in T-cells, the LXXLL$_2$ domain of APH-2 is required for nuclear localization and more specifically for PML-NBs localization. This suggests that the 179-183 motif could allow APH-2 to interact with cellular partners accumulated at PML-NBs, and hence induce its association with these nuclear subdomains. However, we could not show any accumulation of CREB in PML-NBs (data not shown), suggesting that the interaction with CREB does not mediate APH-2 association with PML-NBs.

We next used mutant APH-2 constructs to confirm that localization in PML-NBs is responsible for the short half-life of APH-2. We first analyzed the expression levels of wild-type APH-2 compared to mutant proteins that have lost the localization in PML-NBs (APH-2$_{1-178}$, see Fig. 3a, and APH-2$_{LL182-183AA}$ in which the C-terminal LXXLL motif was mutated to abrogate its function). As a control, APH-2$_{LL67-68AA}$ in which the central LXXLL-like motif was mutated, was included, as well as the APH-2$_{LL67-68AA-LL182-183AA}$ double mutant (Fig. 3b). While WT APH-2 expression was low as usual (Fig. 3b, lane 2), expression levels of APH-2$_{1-178}$, APH-2$_{LL182-183AA}$, and APH-2$_{LL67-68AA-LL182-183AA}$ were greatly enhanced (Fig. 3b, lanes 3, 5 and 6, see graph for quantification), demonstrating the importance of the 179–183 amino acid sequence for the control of APH2 stability. Expression levels of APH-2$_{LL67-68AA}$ were similar to WT APH-2 (Fig. 3b, lane 4, and see graph for quantification), indicating that mutation of LXXLL-like motifs per se did not affect APH-2 stability. Thus, deletion or substitutions in the C-terminal LXXLL motif of APH-2

**Fig. 3** The LXXLL$_2$ domain of APH-2 is required for PML localization and is responsible for the short half-life of APH-2. **a** Jurkat T-cells were transfected with full length APH-2-Myc or with APH-2$_{1-178}$ and fixed 24 h after transfection. Subcellular localization of APH-2 was examined by confocal microscopy after anti-Myc and anti-PML stainings. Nuclei were counterstained with DAPI (blue). Scale bar = 10 μm. Images are representative of two independent experiments. **b** HeLa cells were transfected with wild-type (WT) and mutant APH-2-Myc expression vectors. APH-2 expression levels were analyzed by western blot using an anti-Myc antibody. β-actin was used as a loading control. Signals were quantified and normalized to actin, and are shown in the graph (representative of two independent experiments). **c** HeLa cells were transfected with wild-type APH-2 or APH-2$_{LL182-183AA}$. Cells were treated the following day with cycloheximide for the indicated time. Immunoblot analysis was performed to monitor APH-2 expression levels. β-actin was used as a loading control. Signals were quantified and normalized to actin, and are shown on the graph (representative of two independent experiments). **d** HeLa cells were transfected with SUMO2/3 and VSV-APH-2, VSV-APH-2$_{LL182-183AA}$ or with empty vector. After treatment with MG132, anti-SUMO2/3 immunoprecipitation was performed on cell lysates in denaturing conditions, allowing specific retention of post-translationally modified APH-2. Precipitates were analyzed by western blot using anti-SUMO2/3 and anti-VSV antibodies. Results are representative of three independent experiments. *nc bZip* non-conventional bZip domain
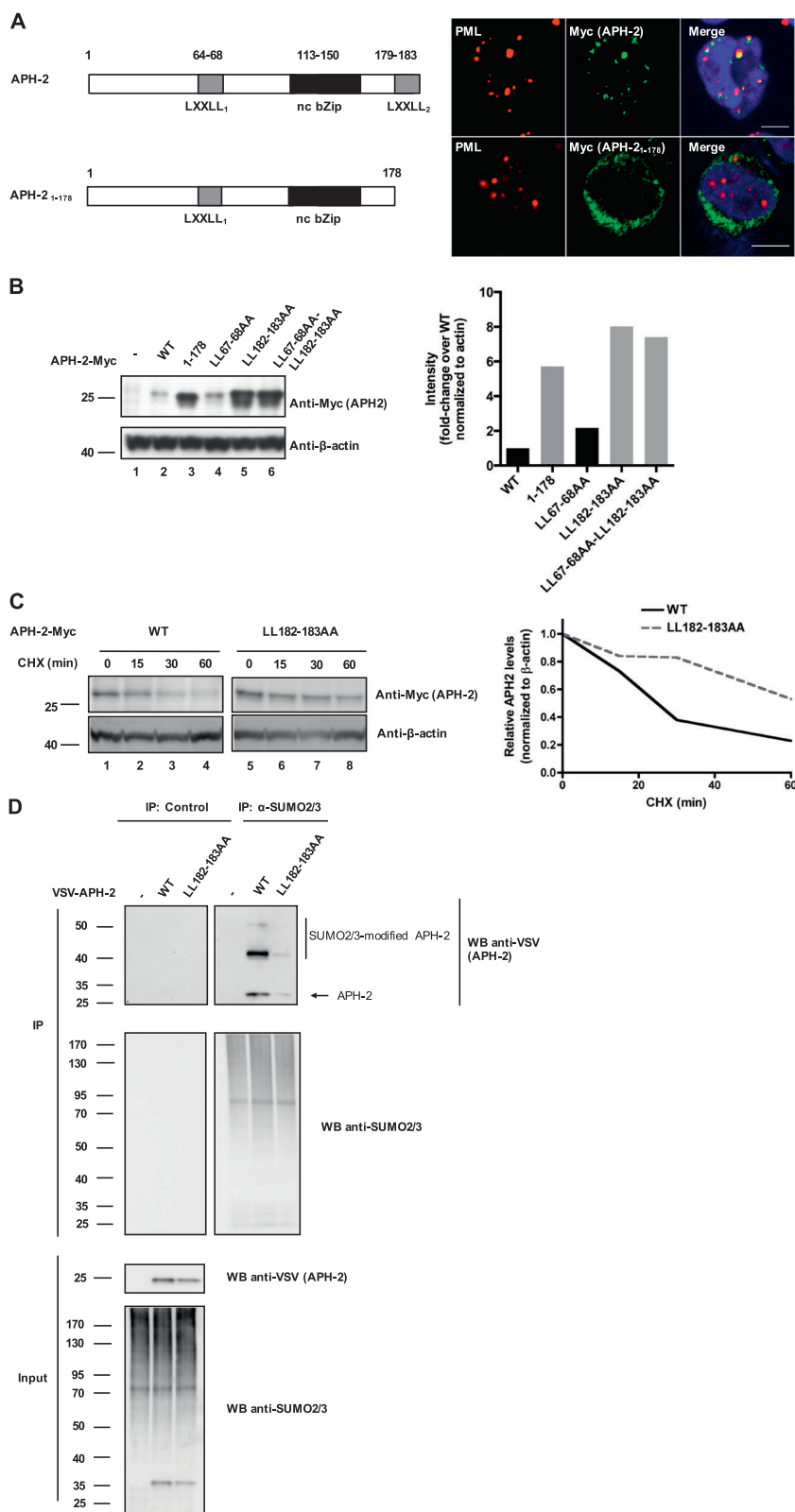


are sufficient to stabilize the protein, indicating that this motif is responsible for the short half-life of the protein. Because this motif is also responsible for targeting APH-2 to PML-NBs, this result is a strong additional indication that localization in PML-NBs is responsible for the short half-life of APH-2.
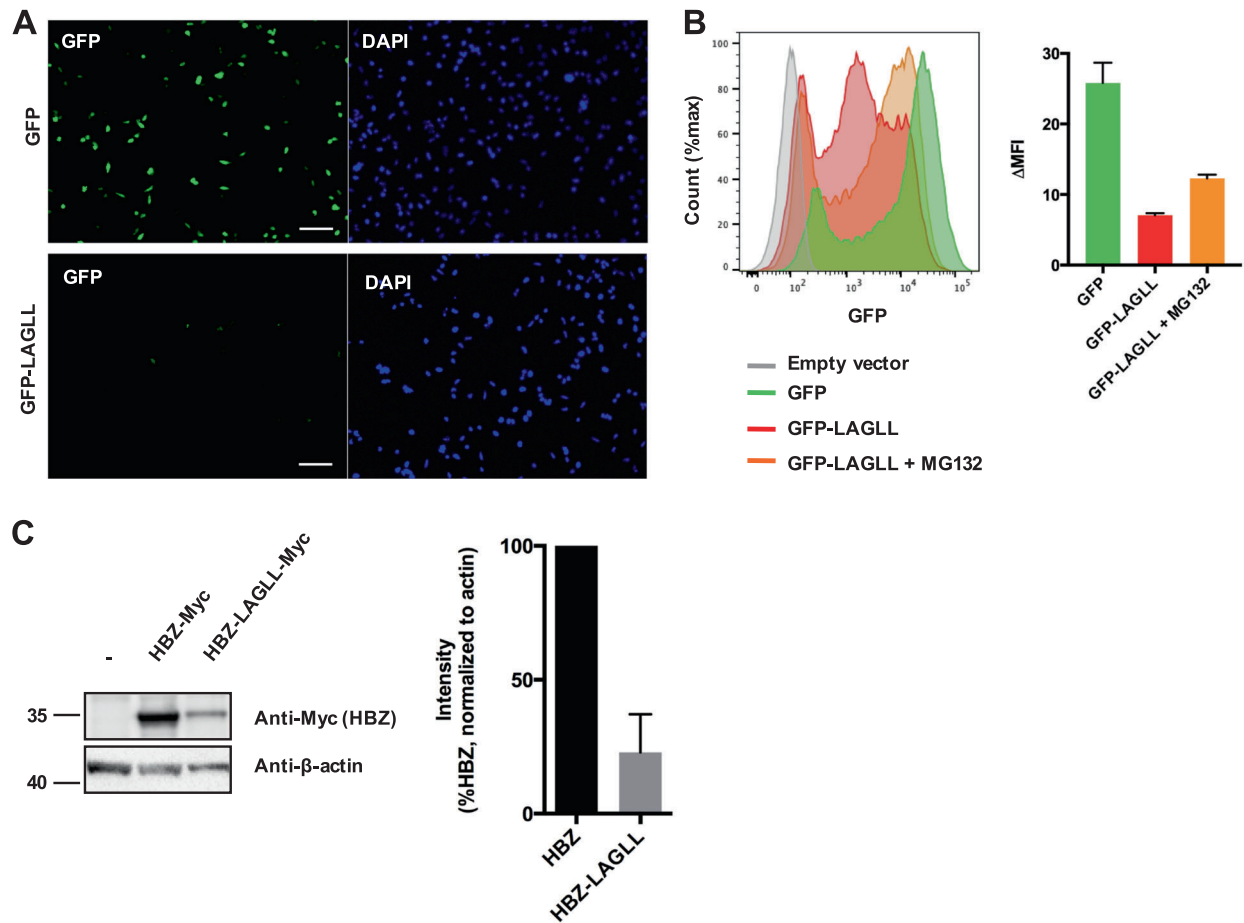
Fig. 4 The LAGLL domain of APH-2 is sufficient to induce proteasomal degradation of stable proteins. **a** HeLa cells were transfected with GFP or GFP-LAGLL constructs and fluorescence was analyzed after 24 h by epifluorescence microscopy. Nuclei were counterstained with DAPI (blue). Scale bar = 50 μm. Images are representative of at least three independent experiments. **b** HeLa cells were transfected with GFP or GFP-LAGLL constructs and treated with MG132, when indicated. Fluorescence was analyzed by flow cytometry after 24 h following live cell gating by FCS/SSC. The gray curve represents mock-transfected cells. Mean fluorescence intensities (MFI) are shown as ΔMFI over mock-transfected cells. The graph shows mean and SEM from three independent experiments. **c** HeLa cells were transfected with HBZ-Myc or HBZ-LAGLL-Myc expression vectors. HBZ and HBZ-LAGLL expression levels were analyzed by western blot using an anti-Myc antibody. β-actin was used as a loading control. Signals were quantified and normalized to actin, and are shown in the graph as mean and SEM from two independent experiments

To confirm that the presence of the $LXXLL_2$ motif of APH-2 is responsible for the short half-life of the protein, we performed cycloheximide (CHX) chase experiments (Fig. 3c). Cells were transfected with WT APH-2 or APH-$2_{LL182-183AA}$ and treated with CHX for varying durations to inhibit protein translation. APH-2 levels were then monitored by western blot analyses. Consistent with previous reports [21], WT APH-2 levels decreased as soon as 15 min after CHX treatment. APH-2 level was reduced to 20% of initial protein amount by 60 min of treatment (Fig. 3c, see right panel for quantification). In contrast, APH-$2_{LL182-183AA}$ levels were maintained at 80% of initial protein amount up to 30 min of treatment and reduced to 50% of initial protein amount by 60 min of treatment (Fig. 3c, right panel). This confirms that mutation of the $LXXLL_2$ motif of APH-2

greatly stabilizes the protein and thus that this motif contributes to the short half-life of APH-2.

Interestingly, analysis of APH-$2_{LL182-183AA}$ SUMOylation status indicated that although SUMO2/3-modified forms remained detectable, they were markedly reduced compared to the WT APH-2 (Fig. 3d), further strengthening the conclusion that a SUMO-dependent mechanism reduces APH-2 stability.

To confirm the importance of the $LXXLL_2$ domain of APH-2 in the control of protein stability, this motif (which exact sequence is LAGLL) was fused at the C-terminus of either GFP or HTLV-1 HBZ (Fig. 4). Expression of GFP or GFP-LAGLL and HBZ or HBZ-LAGLL was evaluated by fluorescence microscopy (Fig. 4a), flow cytometry (Fig. 4b), and western blot (Fig. 4c), respectively. Fusion of the

LAGLL motif at the C-terminus of GFP drastically decreased its detection (Fig. 4a,b). The mean fluorescence intensity of GFP-LAGLL was ~10-fold reduced compared to GFP backbone protein alone (Fig. 4b, right panel). Of note, proteasome inhibition using MG132 partially rescued GFP-LAGLL detection (Fig. 4b), indicating that the LAGLL motif targets GFP to proteasomal degradation. It is worth noting however that GFP-LAGLL was not re-localized to PML-NBs in the presence of MG-132, suggesting that this motif is not sufficient to address GFP to PML-NBs. HBZ-LAGLL levels were also seriously reduced compared to wild-type HBZ (Fig. 4c and graph on the right for quantification of the signal). Thus, the LAGLL motif found at the C-terminus of APH-2 is sufficient to promote decreased protein stability, independently of the protein to which it is fused. Taken together, these results indicate that (i) the LAGLL motif decreases protein abundance when fused to a stable protein such as GFP, (ii) that this decrease in protein abundance is due, at least in part, to proteasomal degradation, (iii) that deletion or mutation of the LAGLL motif slows APH-2 degradation over time. These three characteristics define the LAGLL sequence in APH-2 as a degron, i.e. a sequence controlling protein degradation, in addition to its role as a PML-targeting sequence.

Taken together, these results demonstrate that APH-2 is degraded by the proteasome in a process that depends both on PML and on SUMO-1 modification. Thus, we propose that APH-2 is SUMOylated and addressed to PML-NBs before being targeted to proteasomal degradation.

## Discussion

It is well established that several viruses have developed mechanisms that allow them to manipulate the cellular SUMOylation mechanisms to their profit. In general, this helps their replication and is detrimental to the infected host [23]. A substantial number of viral proteins were previously shown to be SUMOylated with either positive or negative consequence on the function of these proteins (for a review, see ref. [24]). During HTLV-1 infection, it is well established that the HTLV-1 Tax oncoprotein is SUMOylated [25], although SUMOylation does not seem to be critical for NF-κB activation, an important step required during cell transformation [26]. Interestingly, it was demonstrated that arsenic/interferon combination which is used to treat ATLL patients, leads to HTLV-1 Tax poly-SUMOylation followed by degradation in PML nuclear bodies [27]. SUMO also contributes to the anti-HIV-1 effect of interferon [28]. HTLV-2 is barely pathogenic, and on the contrary to HTLV-1 Tax, its Tax protein is barely SUMOylated [29].

As HTLV-1, which encodes HBZ, HTLV-2 infection leads to transcription of the antisense protein of HTLV-2 (aph-2) mRNA that is expressed in vivo in most HTLV-2 carriers. APH-2 protein is barely detectable in vitro with a half-life of 20 min in T-cells, and this short half-life is mainly due to a low protein stability [21]. The lack of ability of APH-2 to enhance TGF-β signaling, or to repress NF-κB p65 or IRF-1 transactivation [21] might be due to the very low availability of the protein in cells.

A number of previously published studies demonstrated that the replication of some DNA viruses is targeted by PML-NBs (for a review see ref. [30]). Here, our results show that APH-2 is very efficiently addressed to PML-NBs. However, the situation is distinct from DNA viruses, since APH-2 is absent from the viral particle, thus its expression requires productive infection. We have demonstrated here that the C-terminal LXXLL domain of APH-2 is required for the efficient localization of the protein to PML-NBs. Interestingly, this motif represents a protein-protein interaction motif that was previously identified as necessary for APH-2 interaction with CREB [20]. However, we excluded the possibility that CREB mediated the recruitment of APH-2 in PML-NBs. The "LAGLL" sequence was also identified as a Sin3a-interacting motif in the Sin3a-associated protein SAP25 (ref. [31]), raising the possibility that APH-2 could also interact with Sin3a. Sin3a is a transcriptional modulator that interacts with multiple partners including transcriptional repressors, enzymes that perform post-translational modifications, DNA binding factors and docking proteins (for a review, see [32]). Sin3a is also able to maintain HIV-1 replication at a latency stage [33]. Because SAP25 was shown to concentrate in PML-NBs, it has been postulated that SAP25 could address the Sin3a complex to PML-NBs [31], a hypothesis consistent with the observation that Sin3a interacts with PML [34]. Interestingly, we observed that APH-2 interacts with Sin3a, although its LAGLL motif is dispensable for the interaction (data not shown). Thus, whether APH-2 interferes with the PML-dependent activities of the Sin3a complex is under current investigation. Altogether, these observations indicate that APH-2 could both be controlled by PML-dependent processes, but also modulate PML-dependent cellular activities, such as transcriptional regulation.

We previously showed that HTLV-2 neo-infection is very sensitive to type I interferon (IFN-I) [35]. Because PML is an IFN-I-induced gene product that was shown to mediate the IFN-I-induced antiviral state against human retroviruses such as human foamy virus [36], it is possible to hypothesize that PML has two functions during HTLV-2 infection: (1) inducing APH-2 degradation and (2) being involved in the IFN-induced antiretroviral defense mechanism.

## Materials and methods

### Cell culture

HeLa and Jurkat T cells were obtained from the American type culture collection (ATCC). HeLa cells stably expressing SUMO-1 (HeLa-SUMO-1) and parental HeLa cells were described elsewhere [37]. HeLa cells and stable HeLa-SUMO1 cells were maintained in high glucose, GlutaMAX Dulbecco's modified Eagle's medium (DMEM, Gibco, Life Technologies) supplemented with 10% fetal bovine serum (FBS, Biosera) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin, Gibco, Life Technologies). Jurkat cells were maintained in GlutaMAX Roswell Park Memorial Institute medium (RPMI, Gibco, Life Technologies) supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin, Gibco, Life Technologies). All cells were maintained at 37 °C in 5% $CO_2$ and tested for mycoplasma contamination at regular intervals.

### Plasmids and siRNA

pcDNA3.1-APH2-Myc-His and pcDNA3.1-HBZ-Myc-His expression vectors [18, 38], as well as HA-SUMO1 and HA-SUMO3 expression vectors were previously described [25]. APH2 truncation mutant ($APH2_{1-178}$) as well as HBZ-LAGLL fusion protein were PCR-amplified and cloned between EcoRI and HindIII sites into the pcDNA3.1-Myc-His vector (Promega). The LAGLL peptide sequence was inserted downstream the GFP coding sequence by primer hybridization and cloning between the EcoRI and BamHI sites of the pEGFP-C3 vector (Clontech). Expression vectors for mutated $APH2\text{-}LXXAA_1$ ($^{67}LL^{68}$ to $^{67}AA^{68}$), $APH2\text{-}LXXAA_2$ ($^{182}LL^{183}$ to $^{182}AA^{183}$), and $APH2\text{-}LXXAA_{1\text{-}2}$ (combining mutations in both LXXLL motifs) were obtained using the QuikChange II site-directed mutagenesis kit (Stratagene) and verified by Sanger sequencing (GATC).

The following siRNAs directed against PML were used: 5′CACCCGCAAGACCAACAACAU3′ and 5′GUGUACCGGCAGAUUGUGGAU3′ (Sigma-Aldrich).

### Antibodies

The following primary antibodies were used: mouse anti-PML (sc-966, Santa Cruz Biotechnology); mouse anti-Myc (clone 4A6, Merck Millipore); FITC-labeled mouse anti-Myc (clone 9E10, Sigma); mouse anti-β-actin (clone AC-74, Sigma-Aldrich); anti-GMP-1/SUMO-1 (clone 21C7, Invitrogen), mouse anti-VSV (clone P5D4, Sigma-Aldrich); rabbit anti-VSV (V4888, Sigma-Aldrich); rabbit anti-SUMO2/3 (ab3742, Abcam), anti-SUMO2/3-HRP (from Signal-Seeker™ SUMOylation 2/3 Detection Kit,

Cytoskeleton), rabbit anti-His (sc-804, Santa Cruz). The following secondary antibodies were used in western blot: horseradish peroxidase-conjugated anti-mouse IgG (NA9310, GE Healthcare or #32430, Thermo Fisher) and anti-rabbit IgG (NA9340, GE Healthcare or #32460, Thermo Fisher).

### Transient transfections

For western blot, immunoprecipitation and immuno-fluorescence assays, HeLa cells were transiently transfected using Effectene or Polyfect transfection reagents (Qiagen), according to the manufacturer's guidelines. For siRNA experiments, $2 \times 10^5$ HeLa cells were seeded in six-well plates and transfected the following day with 50 pmol of siPML using Lipofectamine 2000 Reagent (Life Technology).

Jurkat cells were transfected by electroporation with Neon Transfection kit (MPK5000, Invitrogen). Briefly, $4 \times 10^5$ cells were electroporated with 2 µg of DNA using a 10 µl tip (MPK1096, Invitrogen). Electroporation conditions were: voltage 1325 V, 3 pulsations of 10 ms. Cells were maintained in antibiotic-free RPMI containing 10% FBS for 24 h post-transfection and seeded on poly-lysine-coated coverslips for immunofluorescence assays.

### Cycloheximide analysis

Sixteen hours post-transfection, cells were incubated in the presence of cycloheximide (CHX, 100 µg/ml) for varying durations before harvesting and western blot analysis. Results are representative of two independent experiments.

### Proteasome inhibition

Twenty-four hours post-transfection, HeLa and stable HeLa-SUMO-1 cells were treated with 10 µM of the proteasome inhibitor MG-132 (Calbiochem) for 16 h at 37 °C. DMSO was used as a vehicle.

### Fluorescence microscopy

Cells seeded on coverslips were fixed 24 h after transfection with formalin (HT5011, Sigma-Aldrich) for 20 min. Cells were washed in PBS and permeabilized in PBS–Triton X-100 0.5% for 30 min at room temperature. Cells were then washed, saturated in PBS–Tween 0.2%–milk 5% for 1 h and incubated for 45 min with primary antibodies in saturation buffer. Cells were then washed and incubated for 45 min with the appropriate conjugated secondary antibodies prepared in saturation buffer. Coverslips were mounted in mounting medium containing DAPI (Fluoromount-G, 0100-20, Southern Biotech).

For confocal analyses, slides were examined under a Leica spectral SP5 confocal microscope equipped with a 63× 1.4–0.6 oil-immersion objective using the LAS-AF software, or under an inverted confocal microscope (LSM 800; Carl Zeiss MicroImaging) equipped with a 63× 1.4 plan apochromat oil-immersion objective on the ZEN software and analyzed using the ImageJ software. Images representative of at least two independent experiments are shown.

For epifluorescence imaging, slides were examined under an AxioImager.Z1 microscope (Zeiss) equipped with a 63×/1.4 Plan Apochromat oil-immersion objective. Images were acquired using a Coolsnap HQ monochrome CCD (Zeiss; $1392 \times 1040 - 6.45\,\mu m$ pixel; 12 bit) camera and the Metamorph software. Fluorescence intensity was measured in 15 cells/condition using ImageJ software and Student's $t$-test was used to compare means. GFP epifluorescence was analyzed under an AMG Evos fl digital inverted fluorescence microscope. Images are representative of at least three independent experiments.

## Flow cytometry analyses

Cells were fixed in 4% paraformaldehyde and analyzed on a MACSQuant cytometer (Miltenyi) in three independent experiments. FACS analyses were performed under the FlowJo software.

## Western blots

Cells were lyzed in radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% Na-Doc, 5 mM PMSF, and protease inhibitors (Complete Protease Inhibitor Cocktail EDTA-free, Roche) at 4 °C for 20 min. After centrifugation and protein concentration determination (Bradford, Biorad), lysates were separated by SDS-PAGE on Criterion XT precast gels 10% Bis–Tris (Biorad) or on NuPAGE Novex 4–12% Bis–Tris Gel (Invitrogen), transferred on PVDF membrane and saturated in TBS–Tween 0.1%–milk 5%. After incubation with the appropriate primary and secondary antibodies, ECL prime western blotting detection reagent (GE healthcare) and super signal west femto maximum sensitivity substrate (Thermo Scientific) were used for revelation. Signals were quantified with ImageJ software.

## Denaturing immunoprecipitation

To examine covalent protein–protein interaction between APH2 and SUMO-2/3, HeLa cells were treated with 10 μM MG-132 for 16 h before lysis and immunoprecipitation of SUMO2/3 using the Signal-Seeker™ SUMOylation 2/3 Detection Kit (Cytoskeleton) according to the manufacturer's instructions. Briefly, three 100 mm dishes of HeLa cells were harvested in lysis buffer. After protein quantification, 1.2 mg of proteins were incubated with control beads or SUMO2/3 affinity beads 2 h at 4 °C. After wash, proteins were eluted and analyzed by western blot together with whole cell extracts (input). Results are representative of three independent experiments.

To examine covalent protein–protein interaction between APH-2 and SUMO1, HeLa cells were treated with 10 μM MG-132 for 16 h before lysis in Abis–Guanidine buffer (Guanidine 6 M, $NaH_2PO_4$ 100 mM, imidazole 10 mM). After sonication, proteins were incubated with Ni-NTA beads (His-select HF Agarose Beads, Sigma-Aldrich) overnight at 4 °C. Bound fractions were then washed three times in Abis–Guanidine buffer, twice in 0.25× Abis–Guanidine buffer (diluted in Tris–HCl 25 mM, imidazole 10 mM), and twice in Tris–HCl 25 mM, imidazole 10 mM, before elution in Laemmli buffer followed by western blot analysis. Results are representative of two independent experiments.

## Statistical analyses

No specific test was used to calculate the sample size. When appropriate, unpaired two-sided Student's $t$-test with Welch's correction was used (GraphPad Prism) to compare means of two experimental groups for which data followed a normal distribution.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

## References

1. Bruhn RMR. Human lymphotropic viruses: HTLV-1 and HTLV-2. In: Richman DWR, editor. Clinical virology. 4th ed. Washington, DC: AM Press; 2017. p. 771–94.
2. Ciminale V, Rende F, Bertazzoni U, Romanelli MG. HTLV-1 and HTLV-2: highly similar viruses with distinct oncogenic properties. Front Microbiol. 2014;5:398.
3. Wang TG, Ye J, Lairmore MD, Green PL. In vitro cellular tropism of human T cell leukemia virus type 2. AIDS Res Hum Retrovir. 2000;16:1661–8.
4. Xie L, Green PL. Envelope is a major viral determinant of the distinct in vitro cellular transformation tropism of human T-cell

leukemia virus type 1 (HTLV-1) and HTLV-2. J Virol. 2005;79:14536–45.

5. Currer R, Van Duyne R, Jaworski E, Guendel I, Sampey G, Das R, et al. HTLV tax: a fascinating multifunctional co-regulator of viral and cellular pathways. Front Microbiol. 2012;3:406.

6. Zhao T, Matsuoka M. HBZ and its roles in HTLV-1 oncogenesis. Front Microbiol. 2012;3:247.

7. Mesnard JM, Barbeau B, Cesaire R, Peloponese JM. Roles of HTLV-1 basic Zip factor (HBZ) in viral chronicity and leukemic transformation. potential new therapeutic approaches to prevent and treat HTLV-1-related diseases. Viruses. 2015;7:6490–505.

8. Journo C, Douceron E, Mahieux R. HTLV gene regulation: because size matters, transcription is not enough. Future Microbiol. 2009;4:425–40.

9. Kfoury Y, Nasr R, Journo C, Mahieux R, Pique C, Bazarbachi A. The multifaceted oncoprotein Tax: subcellular localization, post-translational modifications, and NF-kappaB activation. Adv Cancer Res. 2012;113:85–120.

10. Watanabe T. Adult T-cell leukemia: molecular basis for clonal expansion and transformation of HTLV-1-infected T cells. Blood. 2017;129:1071–81.

11. Yamano Y, Sato T. Clinical pathophysiology of human T-lymphotropic virus-type 1-associated myelopathy/tropical spastic paraparesis. Front Microbiol. 2012;3:389.

12. Gaudray G, Gachon F, Basbous J, Biard-Piechaczyk M, Devaux C, Mesnard JM. The complementary strand of the human T-cell leukemia virus type 1 RNA genome encodes a bZIP transcription factor that down-regulates viral transcription. J Virol. 2002;76:12813–22.

13. Satou Y, Yasunaga J, Zhao T, Yoshida M, Miyazato P, Takai K, et al. HTLV-1 bZIP factor induces T-cell lymphoma and systemic inflammation in vivo. PLoS Pathog. 2011;7:e1001274.

14. Giam CZ, Semmes OJ. HTLV-1 infection and adult T-cell leukemia/lymphoma-A tale of two proteins: Tax and HBZ. Viruses. 2016;8:E161. https://doi.org/10.3390/v8060161.

15. Nicot C. HTLV-I Tax-mediated inactivation of cell cycle checkpoints and DNA repair pathways contribute to cellular transformation: 'A Random Mutagenesis Model'. J Cancer Sci 2015; 2. https://doi.org/10.13188/2377-9292.1000009.

16. Shirinian M, Kambris Z, Hamadeh L, Grabbe C, Journo C, Mahieux R, et al. A transgenic Drosophila melanogaster model to study human T-lymphotropic virus oncoprotein Tax-1-driven transformation in vivo. J Virol. 2015;89:8092–5.

17. El Hajj H, El-Sabban M, Hasegawa H, Zaatari G, Ablain J, Saab ST, et al. Therapy-induced selective loss of leukemia-initiating activity in murine adult T cell leukemia. J Exp Med. 2010;207:2785–92.

18. Halin M, Douceron E, Clerc I, Journo C, Ko NL, Landry S, et al. Human T-cell leukemia virus type 2 produces a spliced antisense transcript encoding a protein that lacks a classic bZIP domain but still inhibits Tax2-mediated transcription. Blood. 2009;114:2427–38.

19. Douceron E, Kaidarova Z, Miyazato P, Matsuoka M, Murphy EL, Mahieux R. HTLV-2 APH-2 expression is correlated with proviral load but APH-2 does not promote lymphocytosis. J Infect Dis. 2012;205:82–6.

20. Yin H, Kannian P, Dissinger N, Haines R, Niewiesk S, Green PL. Human T-cell leukemia virus type 2 antisense viral protein 2 is dispensable for in vitro immortalization but functions to repress early virus replication in vivo. J Virol. 2012;86:8412–21.

21. Panfil AR, Dissinger NJ, Howard CM, Murphy BM, Landes K, Fernandez SA, et al. Functional comparison of HBZ and the related APH-2 protein provides insight into human T-cell leukemia virus type 1 pathogenesis. J Virol. 2016;90:3760–72.

22. Hivin P, Basbous J, Raymond F, Henaff D, Arpin-Andre C, Robert-Hebmann V, et al. The HBZ-SP1 isoform of human T-cell leukemia virus type I represses JunB activity by sequestration into nuclear bodies. Retrovirology. 2007;4:14.

23. Lowrey AJ, Cramblet W, Bentz GL. Viral manipulation of the cellular sumoylation machinery. Cell Commun Signal. 2017;15:27.

24. Everett RD, Boutell C, Hale BG. Interplay between viruses and host sumoylation pathways. Nat Rev Microbiol. 2013;11:400–11.

25. Nasr R, Chiari E, El-Sabban M, Mahieux R, Kfoury Y, Abdulhay M, et al. Tax ubiquitylation and sumoylation control critical cytoplasmic and nuclear steps of NF-kappaB activation. Blood. 2006;107:4021–9.

26. Pene S, Waast L, Bonnet A, Benit L, Pique C. A non-SUMOylated tax protein is still functional for NF-kappaB pathway activation. J Virol. 2014;88:10655–61.

27. Dassouki Z, Sahin U, El Hajj H, Jollivet F, Kfoury Y, Lallemand-Breitenbach V, et al. ATL response to arsenic/interferon therapy is triggered by SUMO/PML/RNF4-dependent Tax degradation. Blood. 2015;125:474–82.

28. Sahin U, Ferhi O, Carnec X, Zamborlini A, Peres L, Jollivet F, et al. Interferon controls SUMO availability via the Lin28 and let-7 axis to impede virus replication. Nat Commun. 2014;5:4187.

29. Journo C, Bonnet A, Favre-Bonvin A, Turpin J, Vinera J, Cote E, et al. Human T cell leukemia virus type 2 tax-mediated NF-kappaB activation involves a mechanism independent of Tax conjugation to ubiquitin and SUMO. J Virol. 2013;87:1123–36.

30. Komatsu T, Nagata K, Wodrich H. The role of nuclear antiviral factors against invading DNA viruses: the immediate fate of incoming viral genomes. Viruses. 2016;8:E290. https://doi.org/10.3390/v8100290.

31. Shiio Y, Rose DW, Aur R, Donohoe S, Aebersold R, Eisenman RN. Identification and characterization of SAP25, a novel component of the mSin3 corepressor complex. Mol Cell Biol. 2006;26:1386–97.

32. Kadamb R, Mittal S, Bansal N, Batra H, Saluja D. Sin3: insight into its transcription regulatory functions. Eur J Cell Biol. 2013;92:237–46.

33. Jean MJ, Power D, Kong W, Huang H, Santoso N, Zhu J. Identification of HIV-1 Tat-associated proteins contributing to HIV-1 transcription and latency. Viruses. 2017;9:E67. https://doi.org/10.3390/v9040067.

34. Khan MM, Nomura T, Kim H, Kaul SC, Wadhwa R, Shinagawa T, et al. Role of PML and PML-RARalpha in Mad-mediated transcriptional repression. Mol Cell. 2001;7:1233–43.

35. Cachat A, Chevalier SA, Alais S, Ko NL, Ratner L, Journo C, et al. Alpha interferon restricts human T-lymphotropic virus type 1 and 2 de novo infection through PKR activation. J Virol. 2013;87:13386–96.

36. Regad T, Saib A, Lallemand-Breitenbach V, Pandolfi PP, de The H, Chelbi-Alix MK. PML mediates the interferon-induced antiviral state against a complex retrovirus via its association with the viral transactivator. EMBO J. 2001;20:3495–505.

37. Ivanschitz L, Takahashi Y, Jollivet F, Ayrault O, Le Bras M, de The H. PML IV/ARF interaction enhances p53 SUMO-1 conjugation, activation, and senescence. Proc Natl Acad Sci USA. 2015;112:14278–83.

38. Thebault S, Basbous J, Hivin P, Devaux C, Mesnard JM. HBZ interacts with JunD and stimulates its transcriptional activity. FEBS Lett. 2004;562:165–70.

## HTLV antisense proteins role in the nf-κb modulation

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The retrovirus HTLV-1 is the causative agent of adult T-cell leukemia, whereas the genetically related sierotype HTLV-2 is sporadically associated with neurological diseases. The HTLV-1 genome encodes regulatory proteins, such as the oncoprotein Tax and the antisense proteins HBZ, involved into T-cells proliferation and transformation. Tax-1, HBZ, and the HTLV-2 homologs, Tax-2 and APH-2 interact with many host cell factors imparing cell signaling pathways involved in the mechanisms of survival, and proliferation, including the NF-κB pathway. The aim of this study is to investigate the involvement of the regulatory proteins HBZ and APH-2 in the constitutively Tax-mediated NF-κB activation. We demonstrated that HBZ and APH-2 differ in the NF-κB promoter suppression. The APH-2 protein, differently from HBZ, localizes into the cytoplasm in presence of Tax, where it prevents the degradation of the inhibitor IκB, hindering the nuclear translocation of p65. Unlike HBZ, we found that APH-2 interacts with the E3 ubiquitin ligase TRAF3, an upstream inhibitor of the alternative NF-κB pathway. By generating a TRAF3-KO cell line applying the CRISPR/Cas9 technique, we are investigating the HBZ and APH-2 activity on the alternative NF-κB cell signaling. This study may provide insight into the effect of host-viral interactions in human viral oncogenesis.

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