

**ROLE OF UBE4B IN THE UBIQUITINATION OF THE HTLV-1
TAX ONCOPROTEIN AND NF- κ B ACTIVATION**

by
Teng Han

A thesis submitted to Johns Hopkins University in conformity with the requirements for
the degree of Master of Science

Baltimore, Maryland

April, 2014

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ABSTRACT

Human T-cell leukemia virus type 1 (HTLV-1) is the etiological agent of adult T-cell leukemia and lymphoma (ATLL), an aggressive CD4⁺CD25⁺ malignancy. The HTLV-1 genome encodes the Tax protein that plays essential regulatory roles in oncogenic transformation of T lymphocytes by deregulating different cellular pathways, most notably NF- κ B. Lysine 63 (K63)-linked polyubiquitination of Tax provides an important regulatory mechanism that promotes Tax-mediated interaction with the IKK complex and activation of NF- κ B. However, the E3 ligase(s) and other host proteins regulating Tax ubiquitination are currently unknown. To identify novel Tax interacting proteins that may regulate its ubiquitination we conducted a yeast two-hybrid screen using Tax as bait. This screen yielded the E3/E4 ligase ubiquitin conjugation E4 B (UBE4B) as a novel binding partner for Tax. Here, we confirmed the interaction between Tax and UBE4B in mammalian cells by co-immunoprecipitation assays and demonstrated that they co-localized in the cytoplasm by confocal microscopy. Overexpression of UBE4B specifically enhanced Tax-induced NF- κ B activation, whereas knockdown of UBE4B impaired Tax-induced NF- κ B activation and induction of NF- κ B target genes in Jurkat T cells and ATL cell lines. Although the UBE4B promoter contains putative NF- κ B binding sites, its expression was not upregulated by Tax. Furthermore, depletion of UBE4B with shRNA promoted apoptotic cell death and diminished the proliferation of ATL cell lines. Finally, overexpression of UBE4B enhanced Tax polyubiquitination and knockdown of UBE4B suppressed the K63-linked polyubiquitination of Tax. Collectively, these results implicate UBE4B in Tax-induced NF- κ B activation, cell survival and Tax K63-linked polyubiquitination.

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I. INTRODUCTION

1.1. Ubiquitination and UBE4B

Ubiquitin is a 76 amino acid polypeptide that can be covalently attached via its carboxyl (C)-terminal glycine residue to the ϵ -amino group of a substrate lysine residue (1). This reaction is catalyzed by three enzymes: E1 ubiquitin activating enzyme, E2 ubiquitin conjugating enzyme and E3 ubiquitin ligase (2). E1 activates ubiquitin and transfers it to the active-site cysteine residue of an E2 ubiquitin conjugating enzyme forming an E2-ubiquitin thioester in an ATP-dependent manner. The E3 ligase acts as an adaptor that binds both substrate and charged E2 so as to facilitate isopeptide bond formation between ubiquitin and substrate. The E3 ligase plays a central role in these ubiquitin-conjugating processes since it determines the specificity of ubiquitination by directly interacting with the substrate. E3 ligases can be further subdivided into different groups based on distinct ubiquitin transfer mechanisms. For example, HECT (homologous to E6-associated protein C-terminus) domain ligases (3), such as AIP4/ITCH, form a thioester intermediate with ubiquitin and directly transfer the ubiquitin to a substrate (4). However, RING (really interesting new gene) domains, U-box and F-box catalyze the transfer of ubiquitin from E2s to substrates since they lack catalytic cysteine residues needed to form the thioester intermediate (5). Dysregulation of ubiquitin ligases can give rise to a host of diseases including cancer, immune deficiency, Angelman syndrome and Parkinson's disease. The human genome encodes 2 E1 enzymes, ~40-50 E2 enzymes and over 600 E3 ligases (5), which enhances the complexity and versatility of ubiquitination. A newly discovered ubiquitin chain assembly factor E4 was

shown to be necessary for degradation of select proteins via the ubiquitin fusion degradation (UFD) pathway (6).

Proteins can be modified by ubiquitin in a variety of ways. Some proteins are conjugated with a single ubiquitin moiety on a lysine residue (mono-ubiquitination) or on multiple lysine residues (multi-mono-ubiquitination). Proteins can also be modified with polyubiquitin chains, which can be formed by either linking through the internal lysine residues (K6, K11, K27, K29, K33, K48 and K63) or head-to tail assembly through the α -amino group at the amino (N)-terminus. Different linkages may lead to distinct ubiquitin chain structure- K48-linked polyubiquitin chains have a compact architecture, in contrast, K63-linked and linear polyubiquitin chains adopt open conformations. These structural differences account for the unique functions of linkage-specific polyubiquitination. The K48 ubiquitin linkage was discovered first and for a long time it was thought to be the only relevant linkage type in cells. K48-linked polyubiquitination functions as a molecular tag that targets proteins for proteasomal degradation (7), and mutation of K48, but not other lysine residues, blocks the vegetative growth of *Saccharomyces cerevisiae* (8). In contrast, K63-linked polyubiquitin chains are mainly not associated with protein degradation, but rather this chain type was involved in the signal transduction leading to NF- κ B activation as well as in DNA-damage responses (9).

Compared with our knowledge about K48 and K63-linked polyubiquitination, little is known about the atypical chain types (K6, K11, K27, K29, K33 and linear). Recent data from mass spectrometry (MS)-based proteomics studies indicate a high abundance of these atypical chain types, accounting for more than 50 percent of all linkages in *Saccharomyces cerevisiae* (10). K6, K9 and K33-linked polyubiquitin chains play a role

in protein stability, lysosomal degradation, and regulation of the enzymatic activity of kinases (11, 12). K11-linked polyubiquitin chains may function as a proteasomal degradation signal in cell cycle regulation (13). Upon mitochondrial damage, several mitochondrial proteins are modified by the RBR E3 ligase with K27-linked polyubiquitin chains, which are recognized by the autophagy adaptor p62 to trigger the clearance of damaged mitochondria by mitophagy (14, 15). Since dysfunctional mitochondria play central roles in neurodegenerative disorders, and mutations in parkin cause early-onset Parkinson's disease, it would be intriguing to determine whether K27-linked polyubiquitination plays a critical role in the pathogenesis of neurodegenerative diseases. If so, novel therapeutics could potentially target this specific ubiquitin-linkage. Linear (or Met1)-linked ubiquitin chains, catalyzed by LUBAC (linear ubiquitin chain assembly complex) in a stimulus-dependent manner (e.g. tumor necrosis factor), have been tightly linked to NF- κ B activation (16). The significance of linear ubiquitination mediated by LUBAC in the TNF pathway has been underscored with genetic evidence. Depletion of Sharpin, a key component of the LUBAC complex, abrogates linear ubiquitination and leads to cell death (17). Mice with a mutation in the *Sharpin* gene develop chronic proliferative dermatitis (*cpdm*), an inflammatory disorder that arises from defective NF- κ B activation (18). Surprisingly, the *cpdm* phenotype could be rescued by simultaneous deletion of TNFR, indicating an interplay between TNF signaling and linear polyubiquitin chains in this pathway (19). Undoubtedly, there is still much work to be done to elucidate the physiological significance of atypical polyubiquitin chains.

Akin to phosphorylation, ubiquitination is highly regulated and reversible. Deubiquitinases (DUBs) are proteases that can cleave ubiquitin from target proteins and

therefore counter-regulate the activities of E3 ubiquitin ligases. The human proteome contains approximately 100 DUBs (20), which can be subdivided into five families according to their specific structural domains: ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), ovarian tumor proteases (OTUs), Josephins, and JAB1/MPN/MOV34 metalloenzymes (JAMMs) (21). The USPs comprise the largest subfamily of DUBs with more than 50 members (22). Many DUBs harbor motifs that can interact with ubiquitin, such as the zinc finger ubiquitin-specific protease (ZnF-UBP) domain, the ubiquitin-interacting motif (UIM) and the ubiquitin-associated domain (UBA), to recognize and facilitate recruitment to ubiquitinated substrates. However, a number of DUBs rely on ubiquitin-binding domain containing adaptors to coordinate the recognition and specificity. TNFAIP3 (A20) is a well-known deubiquitinase and potent negative regulator of multiple immune signaling pathways, including TNFR, Toll-like receptor (TLR), NOD-like receptor (NLR), RIG-I-like receptor (RLR) and T-cell receptor (TCR) (23). Among these pathways, the regulation of TNFR and interleukin-1 receptor (IL-1R)/TLR4 pathways by A20 is most studied. After ligand binding, A20 is rapidly induced in a negative feedback loop by NF- κ B and forms a protein complex with TAX1BP1, ITCH, RNF11 and ABIN-1, factors that are thought to be essential for the recruitment and catalytic activity of A20 to terminate the signal transduction, but the physiological roles of these co-factors still need to be clarified in animal models.

UBE4B is a human homolog of the *Saccharomyces cerevisiae* UFD2. Yeast UFD2, encoded by a single-copy gene (24), is the first reported E4 ubiquitination factor and is required for elongation of an oligoubiquitin chain on certain type of substrates that are subsequently recognized by the 26S proteasome for degradation (25). UFD2 is not

essential for the viability of yeast, although it does regulate cell survival under stress conditions (26). UBE4B and its homologs share a 70-amino acid U-box domain that confers ligase activity. Sequence profile alignments revealed that the U-box is a derived version of the RING finger domain, but the signature cysteines in the RING finger domain that are responsible for metal chelating, are not conserved in the U-box (27). However, the predicted structure of the U-box is very similar to that of the RING finger domain (28), which indicates that U-box proteins may also have the capability to function as E3 ligases independently. Indeed, mouse Ubc4b can ubiquitinate substrates *in vitro* together with E1 and E2 in the absence of other E3 ligases (29). Also, it was reported that yeast UFD2 can function as a bona fide E3 ubiquitin ligase to promote ubiquitin conjugation of substrates (30). Thus, UBE4B and its homologs can clearly function as E3 ligases, and their E4 function may represent a specialized type of E3 activity with mono- or oligoubiquitinated proteins as substrates.

E3/E4 ligases, including UBE4B, often regulate the ubiquitination of multiple substrates (31). UBE4B is predominantly expressed in mouse neuronal tissues (32), indicating that it may play a critical role in the nervous system. Indeed, UBE4B was shown to be a master E3/E4 ligase that associated with p53 family members in regulating neuronal survival. p53 is a well-known tumor suppressor gene that can function as a transcription factor by regulating gene expression, such as Bax, Puma and Noxa (33), to influence the intrinsic apoptotic pathway. Conversely, p53 may act directly on the mitochondria to promote apoptosis (34). The gene encoding p53 is inactivated in more than 50% of all human tumors, and p53 mutations are common genetic events in cancer cells. In contrast, p53 overexpression and p53-mediated neuronal cell death have been

reported in many neurodegenerative conditions and diseases, such as Parkinson's, Huntington's, Alzheimer's, etc. (35, 36, 37). Thus, tightly regulated p53 expression and stability is paramount for maintaining normal cell growth (38). The E3 ligase Mdm2 (HDM2 in human) is a critical negative regulator of p53 by either sequestration of p53 in the cytoplasm, blockade of its DNA binding activity, or proteasome-dependent turnover (39). Notably, Mdm2 mediates monoubiquitination or multiple-monoubiquitination of p53 (40), however only polyubiquitin chains are efficiently recognized by the 26S proteasome (41), indicating that additional ubiquitin ligases are required for Mdm2-mediated p53 polyubiquitination and degradation. Recently, UBE4B was identified as an E4 ligase that collaborates with MDM2 to catalyze the polyubiquitination and degradation of p53 (42). Also, *Ube4b*^{-/-} mice are embryonically lethal due to severe apoptosis (43), which partly supports the regulatory role of UBE4B on p53 and diminishes the possibility that other ligases can compensate for UBE4B in p53 regulation.

Two additional members in the p53 protein family harboring pro-apoptotic functions, p63 and p73, are also regulated by UBE4B. Unlike p53, p63 and p73 proteins are expressed as different isoforms in tissues throughout the human body. For p63, these consist of p63 α , p63 β , p63 γ , TAp63 and Δ Np63. For p73, these consist of p73 α , p73 β , TAp73 and Δ Np73 isoforms. TAp63 and TAp73 are transcriptionally active isoforms, however, Δ Np63 and Δ Np73 lack the N-terminal transactivation domain. It has been reported that UBE4B specifically interacts with p73 α , but not p73 β . UBE4B induces the proteasomal degradation of p73 α without triggering its ubiquitination (44), which raises the question regarding the role of UBE4B as an E3/E4 ligase in this process. The regulation of p63 by UBE4B is more complex. The only p63 isoform found to be

regulated by UBE4B was Δ Np63 α , whereas the TA isoform was not subject to regulation by UBE4B (45). Paradoxically, rather than promoting its proteasomal degradation, UBE4B stabilized Δ Np63 α by antagonizing its ubiquitination; however, the degradation of Δ Np63 α occurred after cisplatin treatment. Consistently, when overexpressed, UBE4B significantly extends the half-life of Δ Np63 α . The fact that UBE4B is not linked with either of the β isoforms of either p63 or p73 might also explain the specificity of this ligase. Taken together, UBE4B is a key regulator of nervous system development via control of p53 family proteins, although the underlying mechanisms remain unclear.

In addition to regulating p53 family members, UBE4B has also been implicated in other pathways. UBE4B was shown to polyubiquitinate and degrade ataxin-3, which contains a polyglutamine tract that can cause spinocerebellar ataxia type 3 when aberrantly expanded (46). It was proposed that VCP, an ATPase valosin-containing protein (47), mediates the dissociation of UBE4B from ataxin-3; however this dissociation was impaired for the pathological form of ataxin-3 thus resulting in the abnormal accumulation of ataxin-3. Based on this finding, UBE4B is considered to be a rate-limiting factor in mediating the ubiquitination and polyglutamine aggregation in neurodegenerative diseases. Moreover, UBE4B is thought to be essential for FEZ1 (fasciculation and elongation protein zeta-1)-mediated neurite extension. UBE4B interacted with and induced the polyubiquitination of FEZ1; however, UBE4B did not affect FEZ1 stability, indicating that UBE4B-induced ubiquitination of FEZ1 was not a degradation signal. Rather, the polyubiquitination of FEZ1 induced by UBE4B was mainly K27-linked which regulated the function of FEZ1 (48). Recently, it was also reported that UBE4B regulated the endosomal sorting and downstream signaling of

EGFR (epidermal growth factor receptor) by catalyzing polyubiquitination of EGFR and dispatching it for lysosomal degradation (49).

1.2. Mechanisms of HTLV-1 oncogenesis

1.2.1. HTLV-1 background

Discovered in the early 1980s in the United States and Japan by two independent groups, human T-cell leukemia virus type 1 (HTLV-1) was the first human retrovirus associated with a disease and remains the only one that can cause a malignancy (50, 51). Along with other HTLV family members discovered later (HTLV-2-4), HTLV-1 is a complex retrovirus that is part of the *deltaretrovirus* family (52).

HTLV-1 infects over 20 million people worldwide and is the etiological agent of an aggressive CD4+CD25+ malignancy termed adult T-cell leukemia and lymphoma (ATLL) (53). HTLV-1 is endemic in various regions throughout the world, including southwestern Japan, intertropical Africa, the Caribbean and South America (54). The virus has three major routes of transmission: mother-infant (mainly through breast-feeding), sexual contact and parenteral (e.g. intravenous injection). Cell-free HTLV-1 is poorly infectious and cell-cell contact and formation of a virological synapse is required for transmission between individuals (55). ATLL develops upon mono- or oligoclonal expansion of HTLV-1-infected T-cell clones and all ATLL leukemic cells have an integrated HTLV-1 provirus, which clearly implicates the virus as the causal etiology for leukemogenesis. About 3-5% of HTLV-1 infected individuals develop ATLL after a long latent period lasting 20-30 years. ATLL consists of four clinical subtypes: chronic, smoldering, acute and lymphoma. The latter two represent extremely aggressive forms of

the disease and most patients die within one year of diagnosis. Conversely, chronic and smoldering ATLL are more indolent forms of the disease and patients have better survival rates.

While HTLV-1 can infect several different cell types such as CD8⁺ T cells, B cells and dendritic cells (DCs), it preferentially infects CD4⁺ T lymphocytes *in vivo* (56). Indeed, HTLV-1 infected CD4⁺ T cells constitutes the vast majority of the viral load in infected individuals. However, it was suggested that CD8⁺ T cells and B cells can serve as additional reservoirs for the virus *in vivo* (57). Three types of cell surface proteins and molecules function in concert to mediate HTLV-1 cellular entry: heparin sulfate proteoglycans (HSPGs), Neuropilin-1 (NRP1), and Glucose transporter-1 (GLUT1) (58, 59, 60). DC-Specific adhesion molecule-3 ICAM-3 Grabbing Nonintegrin (DC-SIGN) may also facilitate HTLV-1 entry into DCs (61).

Typical of all retroviruses, the HTLV-1 genome is flanked by 5' and 3' long terminal repeats (LTRs). Each contain cis-acting promoter elements that drive the transcription of viral genes. The *gag*, *pol*, and *env* structural and enzymatic genes are transcribed from the 5' LTR. Also, the HTLV-1 regulatory genes Tax, Rex, p21, p12, p13 and p30 are all encoded by various open reading frames (ORFs) in the pX region located in the 3' end of the genome. In addition, the HTLV-1 basic leucine zipper factor (HBZ) regulatory protein is transcribed in an antisense manner directed by the 3' LTR. Of note, the 5' LTR is frequently deleted and methylated, whereas the 3' LTR retains intact in all cases of ATLL (62). As such, HBZ is expressed in virtually all ATLL tumors and may play key roles in oncogenesis and maintenance of the transformed phenotype.

Tax plays an essential role as a trans-activating protein that regulates viral gene expression at the LTRs together with CREB and CBP. Tax is also a powerful oncoprotein that plays a critical role in the pathogenesis of HTLV-1-associated diseases. The Tax protein consists of 353 amino acids and Tax has multiple structural and functional domains that mediate its trans-activation and transformation functions. It has been reported that Tax is sufficient to transform murine fibroblasts (63), immortalize primary human lymphocytes (64), and to induce considerable pathological effects, including ATLL-like disease, mesenchymal tumors and neurofibromas, in transgenic mice (65, 66). These oncogenic properties of Tax are tightly associated with its ability to deregulate host signaling pathways controlling cell survival and proliferation, DNA damage response, and immune response. Despite other HTLVs (HTLV-2-4) encoding Tax proteins, only Tax1 appears to possess the unique capability of cell transformation and ATLL-like disease when expressed as a transgene in mice (67).

1.2.2. Tax: a powerful activator of the NF- κ B pathway

NF- κ B was first described in B lymphocytes as a nuclear factor that can bind the enhancer of the immunoglobulin kappa light chain gene (68). Later studies revealed NF- κ B to be one of the most pluripotent transcription factor families that played a crucial role in proliferation, apoptosis, oncogenesis, development and the immune response. There are five members of the NF- κ B family that are all ubiquitously expressed: p65 (RelA), c-Rel, RelB, p50/p105, and p52/p100 (69). All of the members share a highly conserved Rel homology domain, which contains a DNA-binding domain, a dimerization domain, a region of interaction with the inhibitory I κ B proteins and a nuclear localization

sequence (NLS) (70, 71). NF- κ B proteins are capable of undergoing homo- or heterodimerization with all possible combinations, with the exception of RelB which dimerizes only with p50 or p52 (72).

Dimeric NF- κ B proteins are sequestered in the cytoplasm as inactive forms bound to a member of the I κ B family. Eight members in the I κ B family have been identified thus far: I κ B- α , I κ B- β , I κ B- δ , I κ B- ϵ , I κ B- γ , Bcl3, and the NF- κ B precursors p100 and p105 (73). They all share multiple ankrin-repeat motifs, which mediate interactions with NF- κ B family members. This interaction masks the NLS of the NF- κ B proteins resulting in their sequestration as latent transcription factors in the cytoplasm (74). In response to a diverse array of stimuli, I κ B is phosphorylated by the IKK (I κ B kinase) complex consisting of the catalytic subunits IKK- α and IKK- β and the regulatory subunit IKK- γ (NEMO). Phosphorylated I κ Bs are conjugated with K48-linked polyubiquitin chains and targeted for proteasomal degradation.

NF- κ B can be activated by a variety of stimuli through two distinct pathways: the canonical and the non-canonical pathway. The canonical pathway is activated by pathogens (e.g. viruses, bacteria, etc.), cytokines (e.g. TNF, IL-1), stress signals (e.g. ultraviolet radiation, DNA damaging drugs) and antigen receptors (e.g. TCR and BCR) and involves the degradation of either I κ B- α , I κ B- β or I κ B- ϵ and the nuclear translocation of NF- κ B heterodimers that typically contain RelA (75). The kinase activity of IKK- β is essential for activating the canonical NF- κ B pathway (76). Conversely, the non-canonical pathway is critical for regulating lymphoid organogenesis, B-cell survival and maturation, DC activation and bone metabolism, and is dependent on the IKK- α catalytic subunit and

the upstream kinase NIK (NF- κ B inducing kinase). This pathway is triggered by members of the TNFR superfamily (e.g. BAFF, CD40 lymphotoxin beta) and results in IKK α -induced phosphorylation of the p100 precursor leading to its processing into the p52 subunit by the proteasome (77). As a consequence, heterodimers containing p52 and RelB translocate into the nucleus and activate transcription of specific target genes.

IKK- γ (NEMO), which is encoded on the X chromosome, is required for canonical NF- κ B activation. NEMO is composed of 419 amino acids and has a molecular weight of 50 kDa. NEMO contains two coiled-coil domains (CC1 and CC2), a NEMO ubiquitin-binding domain (NUB), a leucine zipper (LZ) domain and a zinc finger (ZF) domain. The CC1 domain is responsible for interacting with the effector subunits of the IKK complex, IKK- α and IKK- β . The CC2, NUB and LZ domains together are responsible for oligomerization of NEMO. Notably, the NUB domain participates in the interaction of NEMO with ubiquitinated proteins such as RIP1 in the TNF pathway (78). NEMO functions as a platform for the recruitment of activators and inhibitors of the IKK complex, but the exact mechanism underlying the regulation of IKK activity is still largely unknown. Recently, several reports demonstrated that post-translational modifications, including SUMOylation and ubiquitination, play a crucial role in the activation of the NF- κ B pathway.

ATLL is characterized by aberrant expression of lymphokines and lymphokine receptors that are trans-activated by NF- κ B (79). Normally, NF- κ B is transiently activated in response to an acute stimulus due to negative-feedback inhibitors that terminate NF- κ B signaling. However, in HTLV-1-transformed cell lines, Tax-expressing

cells and in ATLL samples isolated from patients, both canonical and non-canonical NF- κ B pathways are constitutively activated (80, 81). Indeed, Tax acts at multiple levels to induce and maintain pathological NF- κ B activation. Also, post-translational modifications, particularly phosphorylation and ubiquitination, play a critical role in Tax-mediated NF- κ B activation.

Tax triggers the persistent phosphorylation, ubiquitination and subsequent proteasomal degradation of I κ B- α leading to aberrant NF- κ B activation. Tax induces the constitutive phosphorylation and activation of the IKK complex to sustain NF- κ B activation. The role of Tax in the activation of the IKK- α and IKK- β subunits in HTLV-1 infected or Tax-transfected cells was reported just shortly after the discovery of the individual constituents of the IKK complex (82, 83, 84). Tax binds to the IKK complex (85) via a direct interaction with NEMO, which is mediated by the LZ domains within the N- and C-terminal regions of NEMO and the LRRs (leucine rich repeats) in Tax (86). This interaction facilitates the subsequent recruitment of the catalytic subunits of IKK to activate downstream events. Surprisingly, in contrast to TNFR superfamily members which require only IKK- α , Tax requires both IKK- α and NEMO to activate the non-canonical NF- κ B pathway, forming a complex that also contains p100 (87). Tax physically recruits IKK- α to p100, triggering its phosphorylation, ubiquitination and proteasomal processing of p100 to p52 (88). Tax likely requires NEMO for activation of the non-canonical pathway in order to engage the IKK- α subunit.

Not only does Tax activate both the canonical and non-canonical NF- κ B pathways by targeting different IKK complex, but it also facilitates cross-talk and cross-regulation

between these two pathways. It was demonstrated that Tax activation of the non-canonical pathway suppresses the expression of the WWOX tumor suppressor gene, which specifically inhibits Tax activation of the canonical NF- κ B pathway (89). At the level of IKK activation, Tax was reported to inhibit the serine/threonine protein phosphatase 2A (PP2A) that may dephosphorylate and inactivate IKK. Consistently, Tax mutants incapable of binding PP2A were impaired in activating the NF- κ B pathway (90). Tax may also activate the IKK complex indirectly through activation of upstream kinases such as MEKK1 and NIK. Tax was shown to interact with MEKK1, which can phosphorylate IKK- β , albeit these studies were performed under overexpression conditions and the physiological relevance remains unclear (91). Furthermore, the role of NIK in Tax-mediated NF- κ B activation remains controversial. One report showed that dominant negative mutants of NIK inhibited the activation of the NF- κ B pathway suggesting that NIK is downstream of Tax (92); however, another group demonstrated that the activation of the non-canonical pathway by Tax was independent of NIK. Additional studies using shRNAs or gene-targeted knockout cells are warranted to firmly establish or refute a role for these proteins.

There is no doubt that the activation of NF- κ B by Tax is complex and may consist of many host effector proteins. For example, it was shown that Tax interacts with TGF- β -activating kinase 1 (TAK1) through TAK1 binding protein 2 (TAB2) and activates the kinase activity of TAK1. Tax also mediates the recruitment of the IKK complex to TAK1 and TAB2 (93, 94). Also, it has been reported that Tax interacts with the adaptor molecule TAX1BP1 to disrupt its interactions with A20 and Itch, thereby inactivating the A20/TAX1BP1/Ubc13 ubiquitin-editing complex and stabilizing the E2 enzyme Ubc13

involved in Tax ubiquitination (95, 96). Following this discovery, an additional study reported that Tax, TAB2, RelA, calreticulin, TAX1BP1 and NEMO co-localize and form complexes in cytoplasmic punctate structures (97). Similarly, NEMO-related protein (NRP), also known as optineurin, has been demonstrated to interact with ubiquitinated Tax through its ubiquitin-binding domain (UBD), leading to the stabilization of this complex and to potentiate NF- κ B activation by Tax (98). Peptidylproline cis-trans isomerase (PIN1), which is overexpressed in HTLV-1-infected T-cells and Tax transfected cells, may also contribute to NF- κ B activation by Tax (99).

Tax may also promote NF- κ B activation through the regulation of I κ B proteins since Tax can directly interact with I κ B proteins via their ankyrin repeat domains. Indeed, Tax was shown to directly bind to I κ B- α (100) and p105 (101). This interaction promotes NF- κ B activation either by disrupting NF- κ B/I κ B complexes or by recruiting I κ B proteins directly to the proteasome. Tax targets p105 to the proteasome to accelerate its cleavage to the active form p50. The precursor p100 is another target of Tax, as well as I κ B- β (102, 103). In addition to phosphorylation-dependent ubiquitination, Tax also induces I κ B- α degradation by enhancing its interaction with the proteasome (104). Tax interaction with these I κ B proteins may also affect Tax localization. Although Tax localizes in the cytoplasm, it translocates into the nucleus when bound to p52 (105).

Tax can also directly engage the NF- κ B proteins p50, p52, RelA and c-Rel (106). Tax binds to the Rel homology domain and promotes their homo- or hetero-dimerization, resulting in an enhancement of DNA-binding and transcriptional activity. Also, it was reported that Tax, RelA, p50, RNA polymerase II and CBP/p300 co-localize in

transcriptionally active nuclear foci (107, 108). Indeed, the transcriptional co-activators CBP/p300 are recruited to RelA by Tax, which significantly increases the NF- κ B transcriptional activity (109).

Tax ubiquitination, both proteolytic and non-proteolytic, is tightly linked to NF- κ B activation. It was first reported that Tax is mono-ubiquitinated in HTLV-1-transformed lymphocytes and Tax-transfected cells. According to this report, mono-ubiquitination of Tax inhibits the activation of the NF- κ B pathway but does not lead to Tax proteasomal degradation (110). Later studies demonstrated that both transfected and endogenous Tax in ATL cells can be both mono- and polyubiquitinated. Through site-directed mutagenesis, it was demonstrated that the C-terminal 263, 280 and 284 lysines serve as the main targets for Tax ubiquitination and are required for the interaction between Tax and the proteasome (111). Furthermore, it was demonstrated that Tax is conjugated by K48-linked polyubiquitin chains and degraded in the nuclear matrix. PDLIM2 is an E3 ligase that can conjugate Tax with K48-linked polyubiquitin chains. Tax interacts directly with PDLIM2 through its α -helix motif located between amino acids 236-254. It was also demonstrated that PDLIM2 inhibits the NF- κ B transcriptional activity of Tax and that the expression of PDLIM2 is suppressed in HTLV-1-transformed T lymphocytes (112, 113).

It has been reported by our group and others that Tax can be modified by K63-linked polyubiquitin chains. This results in the recruitment of the IKK complex to the cis-Golgi, which serves as the platform for Tax/IKK interaction and IKK activation. Tax K63-linked polyubiquitination on its C-terminal lysines is critical for Tax binding to the IKK complex and for the subsequent activation of NF- κ B (114, 115). Consistent with these findings, Tax/NEMO interaction is abolished upon siRNA-induced silencing of the E2

enzyme Ubc13, indicating that NEMO binds to Tax through the recognition of K63-linked polyubiquitin chains on Tax through its ubiquitin-binding motifs (116).

Taken together, a number of E2s, E3s and deubiquitinating enzymes have been implicated in the regulation of Tax ubiquitination. As described above, PDLIM2 functions as an E3 enzyme to catalyze Tax K48-linked polyubiquitination and degradation in the nuclear matrix. Also, Ubc13 was reported to be the E2 ubiquitin-conjugating enzyme that catalyzes Tax K63-linked polyubiquitination in the cytoplasm. Recently, it was also demonstrated that Tax is a novel substrate for RNF4, which catalyzes ubiquitination on SUMO-modified proteins. This ubiquitination redistributes nuclear Tax to the cytoplasm resulting in increased Tax-induced NF- κ B activation (117). Overexpression of the E3 ligases TRAF2, TRAF5 and TRAF6 strongly enhance Tax polyubiquitination, however it remains unclear whether these enzymes directly catalyze endogenous Tax ubiquitination and whether there are yet to be identified E3 ligases involved in K63-linked polyubiquitination of Tax (94). It was proposed that A20 may deubiquitinate Tax since TAX1BP1, a binding partner of Tax, is a component of the A20 complex. However, this argument was refuted by our group since we showed that Tax interacts with TAX1BP1 and actually disrupts the TAX1BP1, A20 and Itch complex to maintain constitutive NF- κ B activity (96). Tax also interacts with Ubc13 and protects it from TNF-induced ubiquitination and its subsequent proteasomal degradation. Similarly, Tax prevents A20 from interacting with TRAF6 following IL-1 stimulation. Therefore, Tax preserves the E2:E3 complexes that are essential for NF- κ B activation and prevents the degradation of Ubc13 which is required for Tax K63-linked polyubiquitination (97). Recently, it was described that ubiquitin specific peptidase USP20 can deubiquitinate Tax

and terminate Tax-induced NF- κ B activation. It was also found that USP20 expression is down-regulated in HTLV-1-infected T cells, which may ensure high levels of NF- κ B activation by Tax (118). However, there are still many unanswered questions regarding Tax ubiquitination and NF- κ B activation: 1) What is the E3 ligase that conjugates Tax with K63-linked polyubiquitin chains; 2) Does Tax have a ubiquitin binding domain (UBD); and 3) Is Tax also modified by polyubiquitin chains with atypical linkages (e.g. linear polyubiquitination)?

1.2.3. Other cellular pathways deregulated by Tax

HTLV-1 Tax deregulates the cellular machinery at multiple levels to promote the conversion of normal T cells to leukemic cells. For instance, Tax can evade and overcome apoptosis and/or senescence signals induced by virus infection (119). Tax also inactivates key cell cycle checkpoints induced by genetic damage, to promote cell-cycle progression and cell proliferation (120).

In addition to NF- κ B, the phosphatidylinositol 3 kinase (PI3K)-Akt (also known as Protein Kinase B) axis is another key anti-apoptotic pathway targeted by Tax. Akt is a serine/threonine-specific protein kinase that influences cell survival and proliferation. Akt is activated by PI3K through site-specific phosphorylation, primarily on Ser473 (121). Activated Akt transduces signals to downstream transcription factors such as activator protein 1 (AP1), which is overexpressed in many invasive human cancers (122), including ATLL (123). Furthermore, Akt-mediated phosphorylation of Bad, a pro-apoptotic member of the BCL-2 family, inactivates the apoptotic activity of Bad (124). Moreover, Akt regulates cyclin D1, likely via interactions with the p27 and p21 proteins

(125). Akt is also a signaling intermediate upstream of NF- κ B and CREB pathways, and thus regulates the expression of important survival genes. Tax promotes Akt phosphorylation by interacting with the p85 subunit of PI3K (126). Tax-induced activation of the PI3K-Akt axis promotes the survival and proliferation of virus-infected cells. Consistent with this notion, PI3K inhibitors (which prevent Akt phosphorylation) induce apoptotic cell death of HTLV-1 transformed cell lines (127).

Tax also actively suppresses the trans-activation function of p53, a critical tumor-suppressor protein involved in the regulation of the cell cycle, apoptosis and maintenance of genome integrity. Somatic mutations occur in the *p53* gene in up to 60% of all human tumors, however mutant p53 is found in only a small percentage of ATLL tumors. Nevertheless, p53 is inactivated in ATLL by Tax and in Tax- tumors by an unidentified mechanism. By inactivating p53, Tax sets the stage for T-cell immortalization by destabilizing the genome, leading to the accumulation of oncogenic mutations and other types of chromosomal aberrations. Various mechanisms have been proposed for Tax-mediated p53 inactivation. It was shown that p53 is hyperphosphorylated at Ser-15 and Ser-392 in HTLV-1-infected cells, a process dependent on activation of the NF- κ B pathway (128, 129). Although phosphorylation at these sites had no effect on p53 DNA binding activity, it prevented p53 interactions with the basal transcription factor TFIID and the E3 ligase MDM2. Loss of these interactions impaired p53 transcriptional function and stabilized p53. It was also demonstrated that p53 phosphorylation at Ser-15 and Ser-392 was required for Tax-mediated formation of a RelA-p53 complex (130). In addition, the Akt pathway was recently implicated in Tax-induced p53 inactivation, and was associated with increased expression of MDM2 (131). Although Tax strongly suppresses

p53 trans-activation, the precise mechanisms and the role of NF- κ B remain unclear. Finally, Tax also inactivates the Rb tumor suppressor by favoring its hyperphosphorylated/inactive form (132), and by inducing MDM2-mediated Rb proteasomal degradation (133).

Although Tax does not directly elicit DNA damage (134), it can restrict the DNA damage response by various mechanisms to establish genomic instability in HTLV-1 transformed cells. In fact, Tax actively antagonizes DNA damage responses, including base excision repair (BER), nucleotide excision repair (NER), and mismatch repair (MMR), via the inhibition of the kinases CHK1 and CHK2 and DNA β -polymerase (135, 136). Also, it has been reported that Tax inhibits the activity and suppresses the expression of the KU80 protein (137). Chromosome end-to-end fusions and shortened telomeres were also found in ATLL cells (138), which could be explained partly by deregulation of KU80 since it can serve to protect the DNA ends. In addition, it was shown that Tax inhibits the expression of human telomerase reverse transcriptase (TERT) at the early stages of transformation, which reduces the capability of cells to protect new double strand breaks (DSB) as well as extant chromosome ends (139). However, in ATLL cells lacking Tax expression, there is an increased TERT activity (140). How TERT expression is modulated during the course of HTLV-1 transformation is poorly understood, although plausibly it could be regulated by other viral proteins such as HBZ. Tax also induces centrosome overduplication and multipolar mitoses by targeting its binding partner TAX1BP2, a novel cellular inhibitor of centrosome amplification (141). Finally, although Tax does not directly trigger DNA damage, it was reported that Tax

expression induces reactive oxygen species (ROS) that elicits DNA damage and expression of cellular senescence markers (142).

II. MATERIAL AND METHODS

2.1. Reagents, plasmids and antibodies.

Human embryonic kidney cells (HEK 293T) were purchased from ATCC. The HTLV-1-transformed cell lines C8166, MT-2 and HuT-102 were described previously (143). HEK 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM); C8166, MT-2 and HuT-102 cells were cultured in RPMI medium. Medium was supplemented with fetal bovine serum (10%) and penicillin-streptomycin (1%). Expression vectors encoding FLAG-Tax, pCMV4-Tax, Tax M22, Tax M47, HTLV-1 LTR-Luc and NF- κ B-Luc were described previously (116, 144, 145). pDEST51-UBE4B-FLAG was a gift from James A. Mahoney (146). The monoclonal anti-Tax antibody was prepared from a Tax hybridoma (168B17-46-34) received from the AIDS Research and Reference Program, NIAID, National Institutes of Health. Alexa Fluor 555-conjugated donkey anti-mouse IgG and Alexa Fluor 488-conjugated donkey anti-rabbit IgG were purchased from Life Technologies. The FLAG M2 antibody was purchased from Sigma. The monoclonal hemagglutinin (HA) antibody (12CA5) was purchased from Roche Applied Science. The β -Actin antibody was from Abcam. UBE4B antibodies were purchased from Bethyl Laboratories and Santa Cruz Biotechnology. The pRb, p53 and Caspase3 antibodies were from Santa Cruz Biotechnology. DYKDDDDK Tag rabbit polyclonal antibody, PARP, polyubiquitin and Lysine 63 (K63) linkage-specific polyubiquitin antibodies were from

Cell Signaling Technology. DAPI (4', 6-diamidino-2-phenylindole) and MG-132 were purchased from EMD Biosciences.

2.2. Transfections and luciferase reporter assays.

293T cells were transiently transfected with GenJet In Vitro DNA Transfection Reagent (SignaGen Laboratories). Luciferase reporter assays were performed 24 h after DNA transfection, unless otherwise indicated, using the Dual-Glo luciferase assay system (Promega). Firefly luciferase values were normalized based on the *Renilla* luciferase internal control values. Luciferase values are presented as “fold induction” compared to the control transfected with empty vector.

2.3. Immunoblotting, co-immunoprecipitation and ubiquitination assays.

Whole-cell lysates were generated by lysing cells in RIPA buffer (50 mM Tris-Cl [pH 7.4], 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1× Roche complete mini-protease inhibitor cocktail) on ice, followed by centrifugation. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and subjected to immunoblotting. For co-immunoprecipitations (co-IPs), lysates were diluted 1:1 in RIPA buffer and precleared with protein A agarose beads (Roche Applied Science) for 60 min at 4°C. Pre-cleared lysates were further incubated at 4°C overnight with the indicated antibodies (1 to 3 µl) and protein A agarose. Immunoprecipitates were washed three times with RIPA buffer (LSB) to elute bound proteins. An additional wash with RIPA buffer supplemented with 1 M urea was performed for ubiquitination assays.

2.4. Confocal microscopy.

HeLa and MT-2 cells were cultured overnight on glass coverslips coated with poly-L-lysine in 12-well plates. Cells were fixed with 1% paraformaldehyde for 10 min and permeabilized with 0.2% Triton X-100 for 10 min. The fixed cells were then incubated with Super Block buffer (Thermo Scientific) for 45 min followed by staining with mouse anti-Tax and either anti-DYKDDDDK or anti-UBE4B antibodies. Finally, coverslips were incubated with Alexa Fluor 555-conjugated donkey anti-mouse IgG, Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Life Technologies), and DAPI to stain nuclei. Images were obtained using a Nikon C1si confocal microscope.

2.5. qRT-PCR.

RNA was isolated using the RNeasy minikit (Qiagen). RNA was converted to cDNA using the First Strand cDNA synthesis kit for reverse transcription (RT)-PCR (avian myeloblastosis virus [AMV]; Roche Applied Science). Quantitative real-time PCR (qRT-PCR) was performed with an Applied Biosystems 7500 Real-Time PCR system using KiCqStart®SYBR®Green qPCR ReadyMix™ (Sigma). Gene expression was normalized to the internal control 18S rRNA. The following primers were used for qRT-PCR:

Tax forward primer, 5'-ATACCCAGTCTACGTGTTTGGAG-3'

Tax reverse primer, 5'-CCGATAACGCGTCCATCGATG-3'

CD25 forward primer, 5'-ATGCAAGAGAGGTTTCCGCA-3'

CD25 reverse primer, 5'-AGTGGCAGAGCTTGTGCATTG-3'

IRF4 forward primer, 5'-GGATTGTTCTGAGGGAGCC-3'

IRF4 reverse primer, 5'-AGGGTAAGGCGTTGTCATGG-3'

UBE4B forward primer, 5'-GAGAAAAGCGGAGCCTCAGT-3'

UBE4B reverse primer, 5'-GGTCCTTCCAAGAGACACGG-3'

2.6. CHX chase assay.

Cycloheximide (CHX) chase assays were performed as described previously (147). Cells were treated with CHX (100µg/ml) for various times 2 days after transfection. Cells were lysed in RIPA buffer, and immunoblotting (IB) was conducted with anti-Tax.

2.7. Knockdown of UBE4B with lentiviral shRNA.

Three lentiviral Mission short hairpin RNA (shRNA) clones targeting UBE4B were obtained from Sigma. HEK293T cells were transfected with the lentiviral shRNA-targeting vectors pAX packaging plasmid and vesicular stomatitis virus glycoprotein (VSV-G). After 48 h, the supernatants were collected and centrifuged at 25,000 rpm for 2 h at 4°C. The supernatants were removed, and the pellets were re-suspended in ice-cold phosphate-buffered saline (PBS). Viral stocks were used to infect MT-2 and HuT-102 cells.

2.8. Cell viability and proliferation assays.

Cell viability was determined using the CellTiter-Glo luminescent cell viability assay (Promega), which quantitates ATP as a measure of metabolically active cells. A total of 50 µl of suspended cells and 50 µl of CellTiter-Glo solution were mixed and incubated at room temperature for 10 min, and the luminescence was quantified with a GloMax96 microplate luminometer (Promega).

2.9 Yeast Two-Hybrid Analysis

Yeast two-hybrid screening was performed by Hybrigenics Services, S.A.S., Paris, France (<http://www.hybrigenics-services.com>). The coding sequence for the full length Tax protein was PCR-amplified and cloned into pB27 as a C-terminal fusion to LexA (N-LexA-Tax-C). The construct was checked by sequencing the entire insert and used as a bait to screen a random-primed human leukocyte and activated mononuclear cells cDNA library constructed into pP6. pB27 and pP6 derive from the original pBTM116 (Vojtek and Hollenberg, 1995) and pGADGH (Bartel et al., 1993) plasmids, respectively. 46.7 million clones were screened using a mating approach with YHGX13 (Y187 *ade2-101::loxP-kanMX-loxP*, *mata*) and L40-Gal4 (*mata*) yeast strains as previously described (Fromont-Racine et al., 1997). 282 His⁺ colonies were selected on a medium lacking tryptophan, leucine and histidine. The prey fragments of the positive clones were amplified by PCR and sequenced at their 5' and 3' junctions. The resulting sequences were used to identify the corresponding interacting proteins in the GenBank database (NCBI) using a fully automated procedure. A confidence score (PBS, for Predicted Biological Score) was attributed to each interaction as previously described (Formstecher et al., 2005).

2.10. Statistical analysis.

Data are expressed as mean fold increase \pm standard deviation relative to the control from a representative experiment performed 3 times in triplicate. In the Figures, an asterisk (*) indicates a *P* value of <0.05 as determined by Student's *t* test.

III. RESULTS

3.1. Tax interacts with UBE4B.

In order to identify new binding partners of Tax, we conducted a yeast two-hybrid screen using full-length Tax as bait with a cDNA library derived from human leukocytes and activated mononuclear cells. This screen yielded the E3/E4 ligase UBE4B as a putative binding partner for Tax. To confirm the interaction between Tax and UBE4B, a co-IP experiment was performed with lysates from 293T cells transfected with Tax and epitope-tagged UBE4B plasmids. As shown in Fig. 1A, UBE4B interacted with Tax when both proteins were overexpressed. The reciprocal IP in which UBE4B was immunoprecipitated also confirmed Tax and UBE4B interactions (Fig. 1B). The interaction was further examined by co-IPs using Tax and a UBE4B catalytically inactive mutant, in which the highly conserved proline at position 1140 was replaced with alanine (UBE4B P1140A) (48). As shown in Fig. 1C, the dominant-negative mutant (UBE4B P1140A) still interacted with Tax.

3.2. Co-localization of UBE4B and Tax in the cytoplasm.

Tax shuttles between the cytoplasm and nucleus and can be found in both compartments at steady state (147, 148). Similarly, UBE4B distributes in both the cytoplasm and nucleus (149), therefore we aimed to determine where Tax and UBE4B interacted in cells. HeLa cells were transfected with Tax and UBE4B plasmids as indicated (Fig. 2A), followed by immunostaining and confocal microscopy. Tax appeared to co-localize with UBE4B in the cytoplasm (Fig. 2A). Next, to further confirm this result, we examined the localization of endogenous Tax and UBE4B proteins in the HTLV-1-

transformed cell line MT-2. Consistent with the overexpression experiment, Tax strongly co-localized with UBE4B in the cytoplasm (Fig. 2B).

3.3. UBE4B enhances Tax-mediated activation of NF- κ B.

NF- κ B is one of the key signaling pathways that is targeted by Tax and is central for HTLV-1-induced transformation. Tax activates the IKK complex by directly interacting with NEMO, an event critical for activating downstream NF- κ B signaling. Since Tax mainly activates IKK and NF- κ B in the cytoplasmic fraction (150), the cytoplasmic co-localization of Tax and UBE4B led us to hypothesize that UBE4B may regulate Tax-induced NF- κ B activation. To test this hypothesis, 293T cells were transfected with FLAG-Tax in combination with FLAG-UBE4B or FLAG-UBE4B P1140A, and NF- κ B luciferase assays were performed. As expected, Tax expression resulted in potent activation of the NF- κ B luciferase reporter (Fig. 3A). Transfection of wild-type UBE4B, but not catalytically inactive UBE4B P1140A, significantly enhanced Tax-mediated NF- κ B activation (Fig. 3A). Wild-type UBE4B or UBE4B P1140A alone had no effect on NF- κ B activation (Fig. 3A). In contrast, overexpression of UBE4B had no effect on Tax-mediated HTLV-1 LTR activation, therefore UBE4B appears to selectively modulate Tax activation of NF- κ B (Fig. 3B).

Next, UBE4B expression was suppressed with shRNAs in 293T cells (Fig. 3C) and the HTLV-1 transformed cell line MT-2 (Fig. 3E) using recombinant shRNA expressing lentiviruses. Tax-induced NF- κ B activation was impaired in 293T cells expressing UBE4B shRNA (Fig. 3D), and the effects of UBE4B on NF- κ B appeared to be specific for Tax since UBE4B depletion had no effect on TNF-induced NF- κ B activation (Fig.

3D). To determine the role of UBE4B in persistent NF- κ B activation by Tax in an HTLV-1 transformed cell line, we performed an NF- κ B DNA binding assay (EMSA) using nuclear extracts from MT-2 cells. As expected, constitutive NF- κ B DNA binding was observed with MT-2 cells expressing control shRNA; however, shRNA-mediated knockdown of UBE4B in MT-2 cells significantly impaired NF- κ B DNA binding (Fig. 3F).

3.4. Knockdown of UBE4B impairs Tax-induced expression of NF- κ B target genes.

Tax is required for the early steps of HTLV-1 transformation, but is dispensable at later times after development of ATLL. The HTLV-1 transformed cell lines MT-2, HuT-102 and C8166 have high levels of Tax expression and persistent NF- κ B activation. However, since Tax is highly immunogenic and represents a main target of CD8⁺ CTL responses, its expression is silenced by genetic or epigenetic mechanisms in ~60% of ATLL tumors. The ATLL cell lines TL-OM1, ED40515(-) and ATL-43T derived from patients all lack Tax expression. Although Tax is not expressed in these ATLL cell lines, persistent NF- κ B activation is still maintained by unknown mechanisms. Therefore, these ATLL cell lines represent useful models to examine the function of UBE4B. To confirm the effects of UBE4B on Tax-mediated NF- κ B activation, the mRNA expression of several NF- κ B target genes were analyzed in ATLL cell lines by qRT-PCR. CD25 is the high affinity subunit of the IL-2 receptor and is critical for T-cell proliferation. UBE4B knockdown (Fig. 4A) significantly impaired the expression of CD25 in MT-2, HuT-102 and C8166 cell lines (Fig. 4B). Expression of IRF-4, a transcription factor that mediates resistance to AZT/IFN therapy for ATLL patients, was significantly impaired in MT-2, slightly impaired in HuT-102, but not affected in C8166 cells upon UBE4B depletion

(Fig. 4C). Expression of cIAP-2, an anti-apoptotic NF- κ B target gene, was slightly decreased in MT-2, dramatically diminished in HuT-102, but was not affected in C8166 cells when UBE4B was knocked down (Fig. 4D). To reconcile these results, it is plausible that in different HTLV-1 transformed cell lines, the responsiveness may be distinct for different NF- κ B target genes. Importantly, Tax expression was not affected in these ATLL cell lines upon UBE4B knockdown (Fig. 4E).

Next, the mRNA expression of the NF- κ B target genes CD25, IRF-4 and cIAP-2 were examined by qRT-PCR in the Tax- ATLL cell line TL-OM1 after UBE4B knockdown (Fig. 4A). The expression of NF- κ B target genes was not affected in TL-OM1 cells after depletion of UBE4B (Fig. 4F). To further confirm the effect of UBE4B on Tax-mediated NF- κ B activation, UBE4B was knocked down in Jurkat T cells and Tax was introduced into the cells by lentiviral transduction. Cells were lysed two days later and mRNA was extracted for qRT-PCR experiments. The expression of both CD25 and IRF-4 were drastically diminished after UBE4B knockdown in Jurkat T cells expressing Tax (Fig. 4G, H and I). However, Tax expression was comparable between the UBE4B knockdown and control samples (Fig. 4J). Taken together, UBE4B specifically mediates Tax-induced NF- κ B activation and does not play a role in NF- κ B activation in Tax- ATLL cells.

3.5. UBE4B expression is not regulated by NF- κ B.

We found by bioinformatics analysis a putative NF- κ B binding site within the promoter of UBE4B (data not shown), raising the possibility that Tax may upregulate UBE4B expression through NF- κ B to potentiate NF- κ B signaling. To test this hypothesis, Jurkat Tax Tet-on T cells were treated with doxycycline (Dox) to induce Tax expression

and after two days mRNA was extracted for qRT-PCR analysis. As expected, Tax upregulated the expression of NF- κ B target genes including CD25, however UBE4B expression was not affected by Tax (Fig. 5A). Next, UBE4B expression was examined in a panel of HTLV-1 transformed cell lines that all exhibit persistent NF- κ B activation. Compared to Jurkat T cells or peripheral blood mononuclear cells (PBMCs) from a normal donor, there was no significant induction of UBE4B mRNA (Fig. 5B) or protein (Fig. 5C) in ATLL cell lines. Therefore, UBE4B expression does not appear to be regulated through NF- κ B.

3.6. Knockdown of UBE4B promotes apoptotic cell death in Tax positive HTLV-1 transformed cell lines.

Given that NF- κ B is essential for HTLV-1-induced oncogenesis and cell survival, we hypothesized that UBE4B depletion with shRNA would trigger apoptosis of HTLV-1-transformed cells. Indeed, knockdown of UBE4B in MT-2 and HuT-102 cells yielded cleaved forms of PARP and caspase 3 as detected by western blotting (Fig. 6A), indicative of apoptotic cell death. We next examined the effect of UBE4B knockdown on the viability and proliferation of the HTLV-1-transformed cell lines MT-2, HuT-102 and C8166 using CellTiter-Glo luminescent cell viability assay, which measures metabolically active cells by quantifying ATP levels. MT-2, HuT-102 and C8166 cells were infected with recombinant lentiviruses expressing either UBE4B or control shRNA. Metabolically active cells were quantified every 24 h for 5 consecutive days to measure cell proliferation. As expected, cells expressing control scrambled shRNA proliferated vigorously throughout the time course (Fig. 6B-D). However, the proliferation of MT-2, HuT-102 and C8166 cells was significantly impaired upon UBE4B knockdown (Fig. 6B-

6D). Therefore, HTLV-1 transformed cell lines are critically dependent on UBE4B for proliferation and survival.

3.7. UBE4B does not promote pRb and p53 degradation.

The retinoblastoma (Rb) protein regulates a number of key cellular processes, including cell division, differentiation, senescence and apoptosis (151). It was reported that Tax can directly associate with Rb and target it for proteasomal degradation (133). UBE4B may potentially be recruited to Rb by Tax and function as an E3/E4 ligase to catalyze K48-linked polyubiquitination and degradation of Rb. To test this notion, UBE4B was knocked down in Jurkat, MT-2, C8166 and HuT-102 cells, and lysates were subjected to western blotting to examine Rb expression. Consistent with the previous study, low levels of Rb protein were observed in the HTLV-1 transformed cell lines, MT-2, C8166 and HuT-102, compared to control Jurkat T cells (Fig. 7). However, Rb protein expression was not affected upon UBE4B knockdown in MT-2, C8166 and HuT-102 cells, which indicated that UBE4B does not regulate Tax-induced Rb degradation (Fig. 7). Another study demonstrated that UBE4B promoted Hdm2-mediated degradation of p53 in the nervous system (42). To examine whether UBE4B regulated p53 in HTLV-1-transformed cell lines, p53 protein expression was monitored by western blotting after UBE4B knockdown. However, the expression of p53 protein was unchanged in MT-2, C8166 and HuT-102 cells after UBE4B knockdown. Therefore, we conclude that UBE4B does not regulate p53 stability in HTLV-1 transformed cell lines, therefore the regulation of p53 by UBE4B may be cell-type specific.

3.8. Tax K63-linked polyubiquitination is dependent on UBE4B.

Since UBE4B is required for Tax-mediated NF- κ B activation as shown above, we next examined whether UBE4B promotes Tax ubiquitination. Indeed, overexpressed FLAG-UBE4B enhanced Tax polyubiquitination (Fig. 8A). We next performed loss of function studies using 293T cells stably expressing control shRNA or UBE4B shRNA. Both total and K63-linked polyubiquitination of Tax were impaired when UBE4B expression was suppressed (Fig. 8B). Thus, UBE4B positively regulates Tax ubiquitination, particularly K63-linked polyubiquitination.

IV. DISCUSSION

This study was initiated by the identification of UBE4B as a putative interacting protein of HTLV-1 Tax using a yeast two-hybrid screen. We have confirmed using biochemical assays and confocal microscopy that UBE4B is indeed a novel binding partner for Tax. UBE4B and Tax co-localize in the cytoplasm, however additional organelle markers are required to determine their exact co-localization site. Tax partially localizes within the cis-Golgi apparatus within lipid rafts where it directs relocation of the IKK complex to this structure for activation (152, 153). It is tempting to speculate that Tax and UBE4B localize to this sub-structure to trigger IKK activation. Since Tax K63-linked polyubiquitination is necessary to relocate IKK to the cis-Golgi, UBE4B may be critical for Tax to relocate IKK to the cis-Golgi.

Tax is a potent activator of NF- κ B and CREB, which is essential for HTLV-1 LTR activation. Interestingly, UBE4B, but not the U box mutant, specifically enhanced Tax-induced NF- κ B but not HTLV-1 LTR activation. However, UBE4B appears to

specifically mediate Tax-induced NF- κ B activation, but not TNF-triggered NF- κ B suggesting a specific role of UBE4B in the regulation of Tax. Knockdown of UBE4B in the HTLV-1 transformed cell line MT-2 significantly impaired constitutive NF- κ B DNA binding but had no effect on Oct-1 (Fig. 3F). EMSA supershift analysis will be necessary to determine the specific NF- κ B subunits that are dependent on UBE4B for DNA binding in HTLV-1 transformed cells. Furthermore, we will need to examine the roles of UBE4B on upstream steps of NF- κ B activation, including IKK α / β phosphorylation, I κ B phosphorylation and degradation.

UBE4B was also found to be essential for the aberrant expression of NF- κ B target genes, including CD25, IRF-4 and cIAP-2, in the HTLV-1 transformed cell lines MT-2, HuT-102 and C8166. However, knockdown of UBE4B in the Tax negative ATLL cell line TL-OM1 had no effect on NF- κ B target genes (Fig. 4F), indicating that UBE4B specifically regulated Tax-induced NF- κ B activation. Consistently, Tax induction of NF- κ B target genes in Jurkat T cells was critically dependent on UBE4B (Fig. 4H, I). Because NF- κ B regulates key genes that control cell survival and proliferation, it was plausible that UBE4B would mediate the proliferation and survival of HTLV-1 transformed T cells. Indeed, knockdown of UBE4B triggered apoptotic cell death and diminished the proliferation of Tax positive HTLV-1 transformed cell lines (Fig. 6). Since UBE4B promotes Hdm2-mediated p53 proteasomal degradation in the nervous system (42) and Tax induces Hdm2-mediated Rb ubiquitination and subsequent degradation (133) we also conducted experiments to determine if UBE4B regulated p53 and Rb in HTLV-1 transformed T cells. However, knockdown of UBE4B had no influence on p53 and Rb protein expression in Tax expressing cells (Fig. 7). Therefore, it

appears that the effects of UBE4B on the proliferation and survival of HTLV-1 transformed cells are mediated through NF- κ B signaling. Although UBE4B is clearly important for survival of HTLV-1 transformed T cells, it is unknown if UBE4B plays a role in early events in the transformation process. To investigate if UBE4B is required for HTLV-1-induced T cell immortalization, we will conduct a well established co-culture assay using primary human CD4⁺ T cells and lethally irradiated MT-2 cells (as a source of HTLV-1) in the presence of IL-2. Under these conditions, primary T cells are invariably immortalized by HTLV-1 after 6-8 weeks of co-culture. UBE4B expression will be suppressed in the T cells using lentiviral shRNAs and co-cultured for 8-10 weeks to determine if it plays a role in T-cell immortalization by HTLV-1.

UBE4B is an E3/E4 ligase that catalyzes ubiquitination of specific substrates. Although it has not been demonstrated that UBE4B can catalyze K63-linked polyubiquitin chains, this does not rule out the possibility that UBE4B is an E3/E4 for Tax K63-polyubiquitination. The specificity of the polyubiquitin linkage catalyzed by a U-box ligase is determined by the E2 ubiquitin-conjugating enzyme, rather than the E3 itself. Furthermore, it has been demonstrated that one of the E2 enzymes utilized by UBE4B is UbcH5c, which has the potential to catalyze K63-linked polyubiquitination (97, 154). Indeed, our results support a key role of UBE4B in Tax polyubiquitination, especially K63-linked polyubiquitination. However, additional experiments are needed to further investigate the role of UBE4B and determine if it functions as a bona fide E3 ligase for Tax polyubiquitination. First, it is necessary to determine if the catalytic activity of UBE4B is essential for Tax polyubiquitination. Second, *in vitro* ubiquitination assays will be conducted with recombinant E1, E2 (UbcH5c or Ubc13), E3 (wild-type

UBE4B and U box mutant UBE4B), substrate (Tax), ubiquitin and ATP to determine if UBE4B can directly conjugate Tax with K63-linked polyubiquitin chains. Finally, a mass spectrometry screen will be conducted to identify novel host factors that interact with UBE4B in the presence of Tax and vice versa. If UBE4B functions as an E4 for Tax ubiquitination and simply elongates ubiquitin chains primed by a distinct E3 enzyme, this effort may help identify the E3 that collaborates with UBE4B in this regard. Since Tax K63-linked polyubiquitination is required for constitutive NF- κ B activation and subsequent immortalization and transformation of CD4+ T cells, UBE4B may represent a novel therapeutic target for early stage HTLV-1-induced leukemogenesis and also Tax+ ATLL tumors (~40% of all ATLL tumors express Tax).

FIGURES

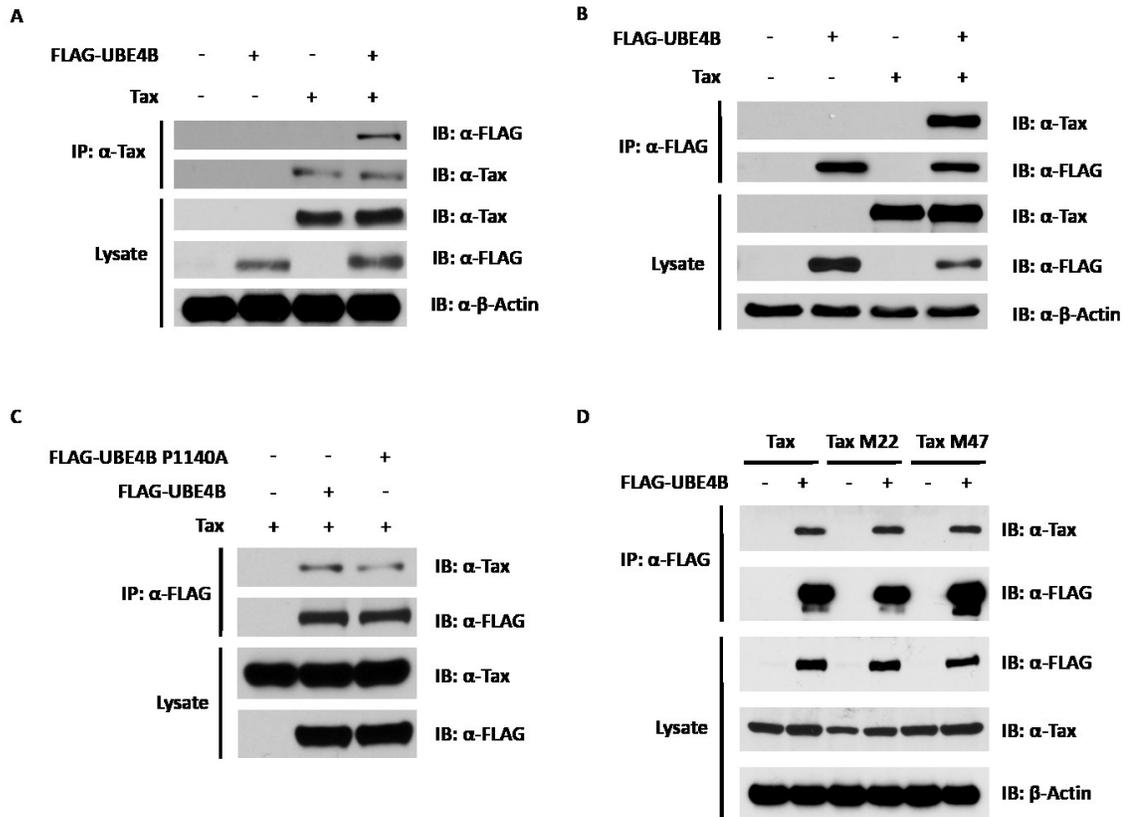


Figure 1. Tax interacts with UBE4B. (A) 293T cells were transfected with vector, FLAG-UBE4B, pCMV-Tax or FLAG-UBE4B together with pCMV-Tax. After 24 h, cells were lysed and immunoprecipitated (IP) with anti-Tax, followed by immunoblotting (IB) with anti-FLAG. Lysates were examined for Tax and FLAG-UBE4B expression. (B) The reciprocal IP was also performed with FLAG antibody and IB with anti-Tax. Lysates were subjected to immunoblotting with anti-Tax, anti-FLAG and anti-Actin. (C) 293T cells were transfected with pCMV-Tax, FLAG-UBE4B and FLAG-UBE4B P1140A as indicated. After 24 h, cell lysates were immunoprecipitated with Flag antibody and IB was performed with anti-FLAG and anti-Tax. Lysates were examined for Tax and

UBE4B expression. (D) 293T cells were transfected with FLAG-UBE4B, Tax, Tax M22 and Tax M47 plasmids. After 24 h, cell lysates were immunoprecipitated with anti-Tax, and IB was performed with anti-FLAG and anti-Tax. Lysates were subjected to IB with anti-FLAG, anti-Tax and anti-Actin.

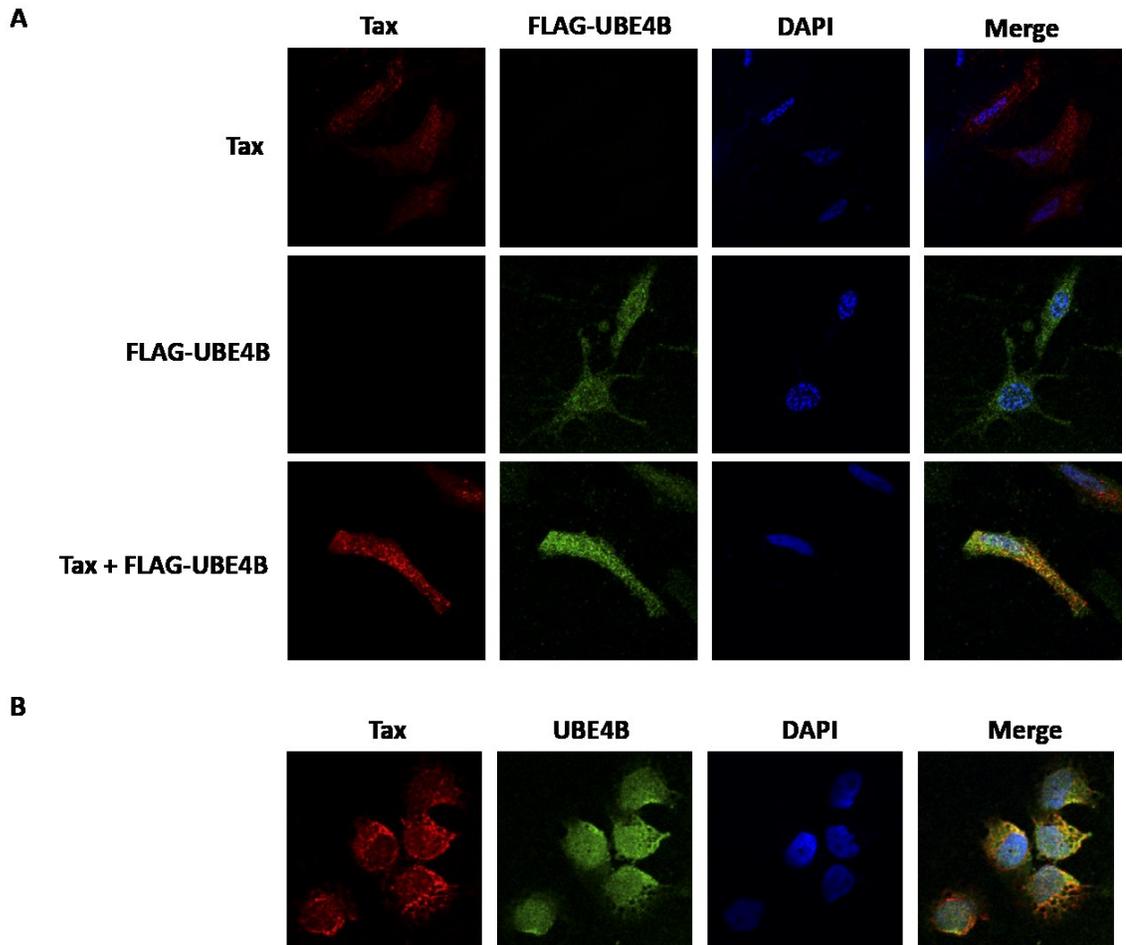


Figure 2. Co-localization of UBE4B and Tax in the cytoplasm. (A) HeLa cells were transfected with Tax and FLAG-UBE4B plasmids. After 24 h, cells were stained with DAPI, anti-Tax and anti-FLAG and subjected to confocal microscopy. (B) MT-2 cells were seeded on poly-L-lysine-coated coverslips and subjected to confocal microscopy to visualize endogenous Tax (red), endogenous UBE4B (green) and nuclei using DAPI (blue).

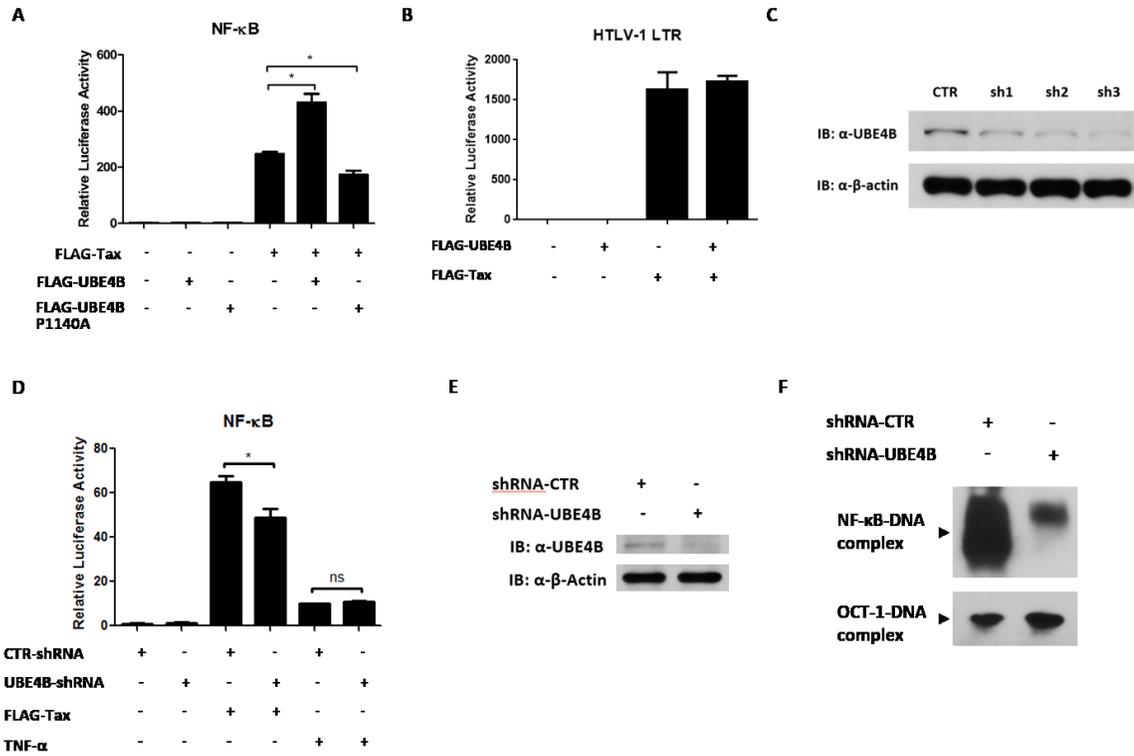


Figure 3. UBE4B enhances Tax-mediated activation of NF- κ B. (A) 293T cells were transfected with κ B-TATA luciferase, pRL-tk, FLAG-UBE4B, FLAG-UBE4B P1140A and FLAG-Tax as indicated. After 24 h, cells were lysed and lysates were subjected to a dual luciferase reporter assay. (B) 293T cells were transfected with HTLV-1 LTR luciferase, pRL-tk, FLAG-UBE4B and FLAG-Tax as indicated. After 24 h, cells were lysed and lysates were subjected to a dual luciferase reporter assay. (C) 293T cells expressing either control shRNA, UBE4B shRNA1, UBE4B shRNA2 or UBE4B shRNA3 were lysed and cell lysates were subjected to IB with anti-UBE4B and anti- β -Actin. (D) 293T cells expressing either control shRNA or UBE4B shRNA were transfected with κ B-TATA luciferase, pRL-tk, FLAG-Tax or stimulated with TNF as indicated. After 24 h, cells were lysed and lysates were subjected to a dual luciferase

reporter assay. (E) MT-2 cells expressing either control shRNA or UBE4B shRNA3 were lysed and lysates were subjected to IB with anti-UBE4B and anti- β -Actin. (F) MT-2 cells expressing either control shRNA or UBE4B shRNA were lysed and subjected to nuclear and cytoplasmic subcellular fractionation. The nuclear extracts were used for NF- κ B and Oct-1 EMSA assays.

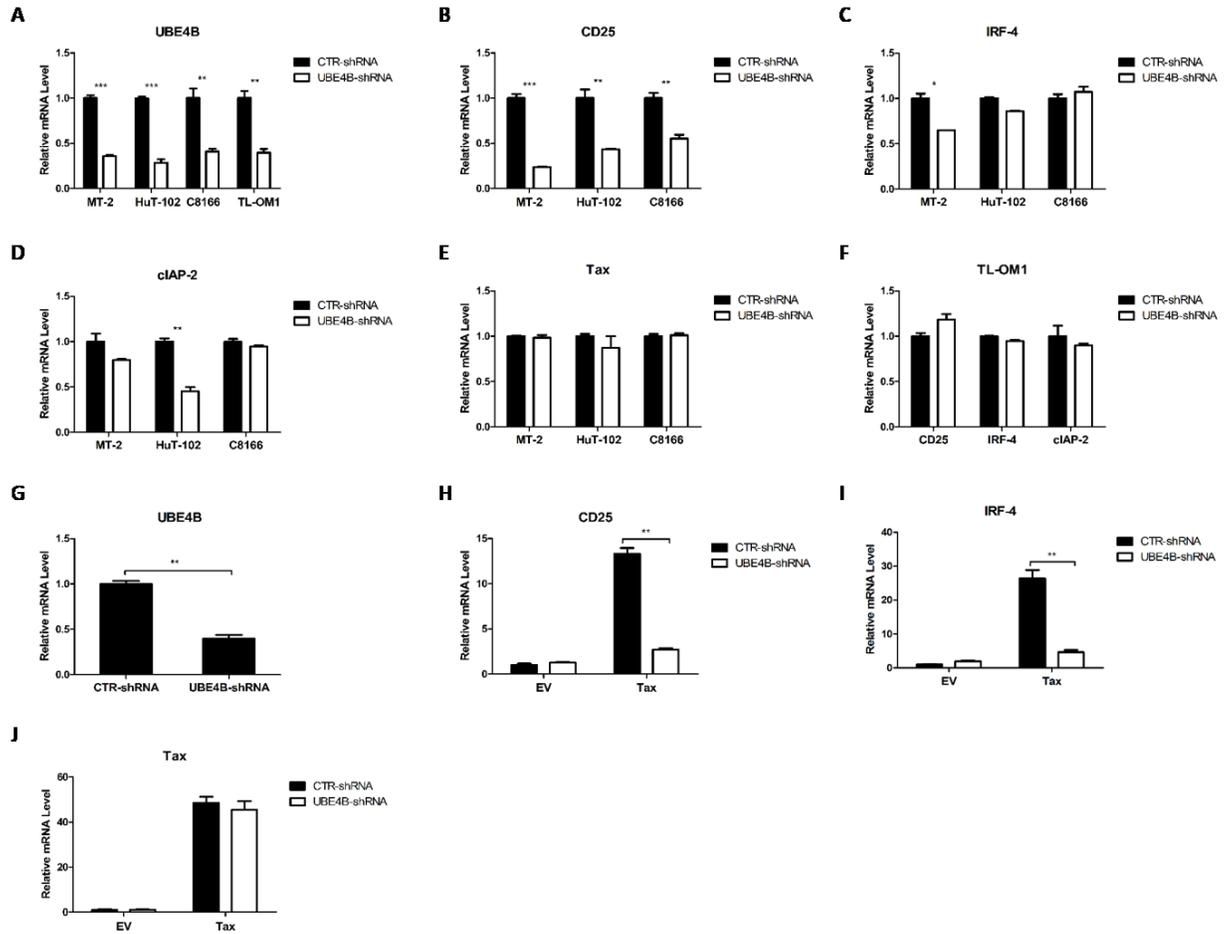


Figure 4. Knockdown of UBE4B impairs Tax-induced NF- κ B target gene expression.

(A-F) The mRNA isolated from MT-2, HuT-102, C8166 and TL-OM1 cells either expressing control shRNA or UBE4B shRNA were used for qRT-PCR to detect UBE4B, CD25, IRF-4, cIAP-2 and Tax, which were normalized to control 18S rRNA. (G-J) Jurkat T cells either expressing control shRNA or UBE4B shRNA were transduced with lentivirus expressing Tax; mRNA was isolated for qRT-PCR to detect UBE4B, CD25, IRF-4 and Tax, which were all normalized to control 18S rRNA.

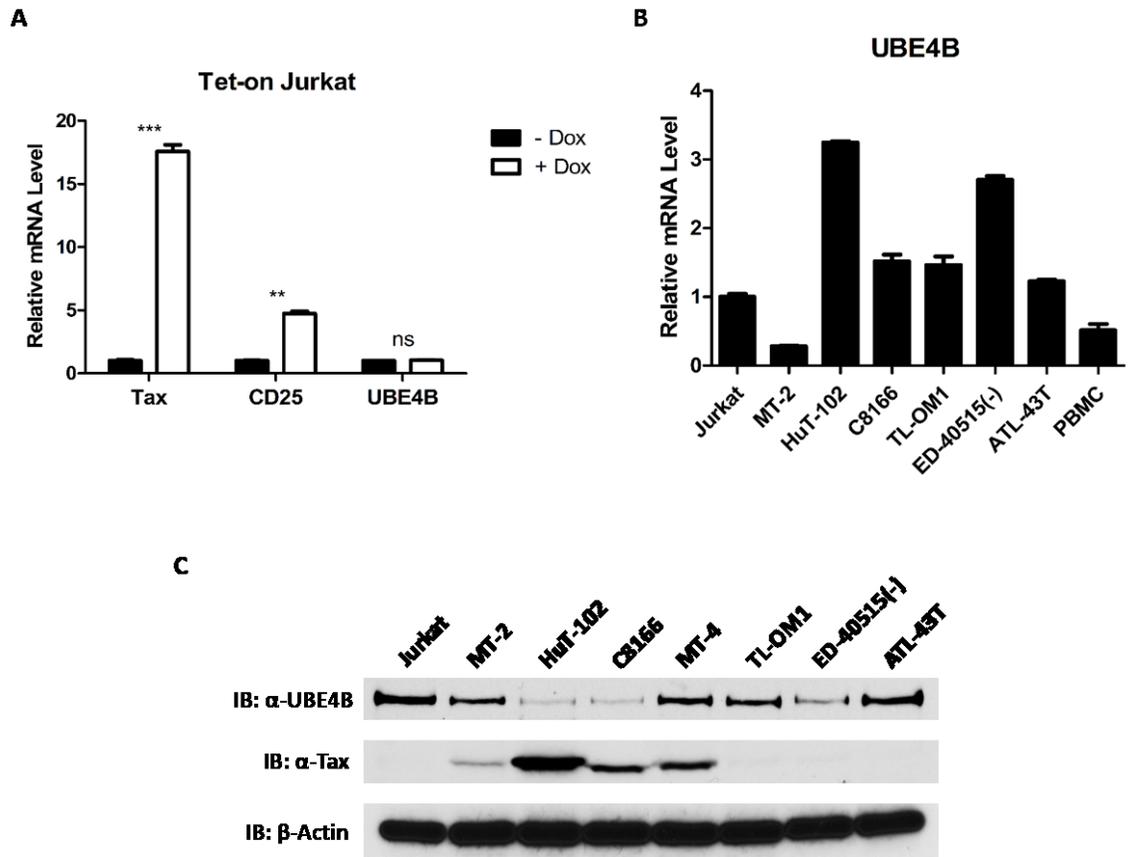


Figure 5. UBE4B expression is not induced by Tax. (A) Jurkat Tax Tet-on cells were treated either with doxycycline (Dox) or DMSO. The mRNA was isolated and used for qRT-PCR to detect Tax, CD25 and UBE4B expression, which were normalized to control 18S rRNA. (B) Jurkat, ATLL cell lines and PBMCs were subjected to qRT-PCR for UBE4B, which was normalized to control 18S rRNA. (C) Whole cell lysates from Jurkat and ATLL cell lines were subjected to IB with anti-UBE4B and anti-Tax.

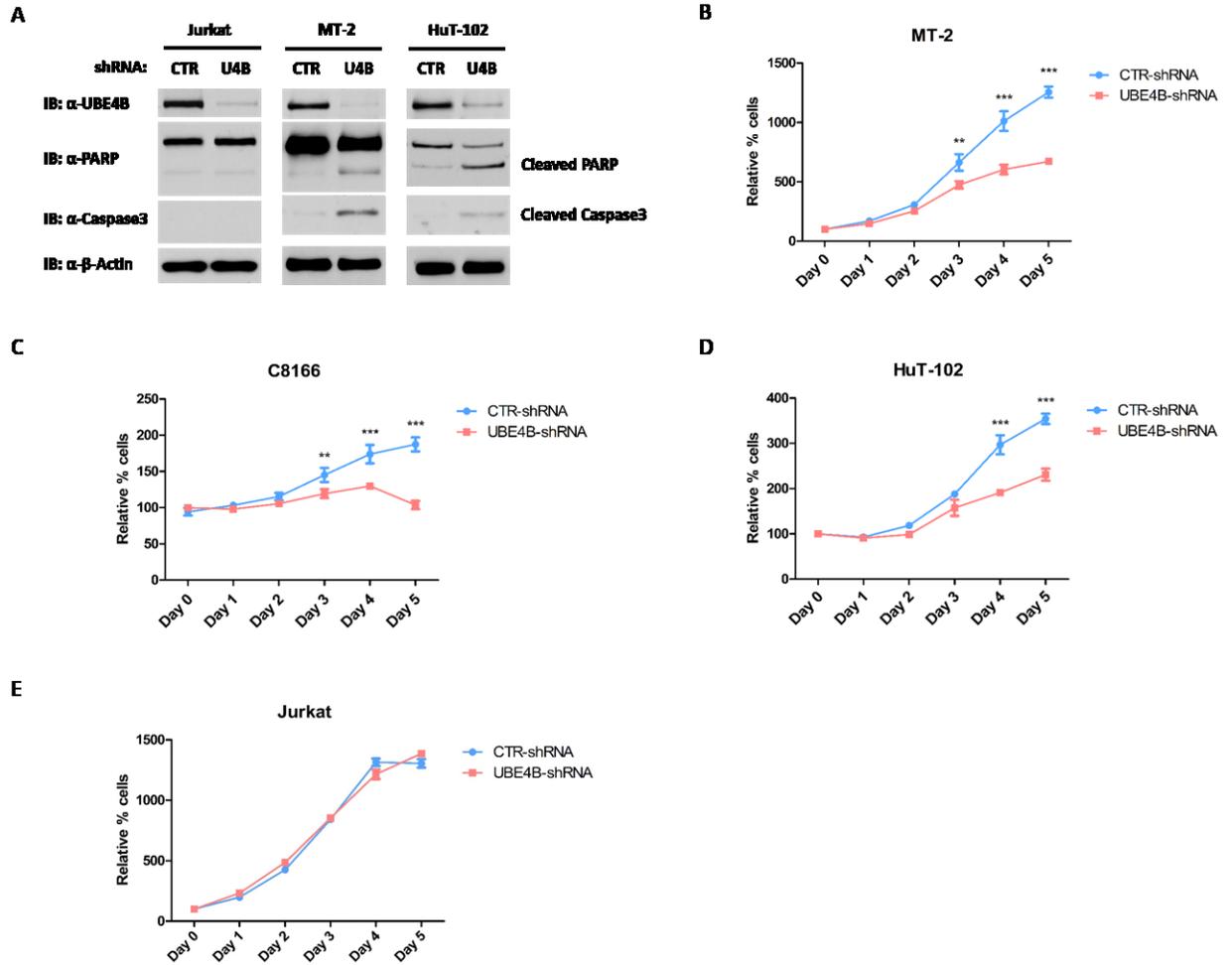


Figure 6. Knockdown of UBE4B promotes apoptotic cell death in Tax positive HTLV-1 transformed cell lines. (A) MT-2 and HuT-102 cells were infected with lentiviral UBE4B shRNA or control shRNA, and the lysates were subjected to IB with anti-UBE4B, anti-PARP, anti-Caspase3 and anti- β -Actin. (B-D) MT-2, HuT-102 and C8166 cells were infected with lentiviral UBE4B shRNA or control shRNA. Cell viability was determined with the CellTiter-Glo viability assay.

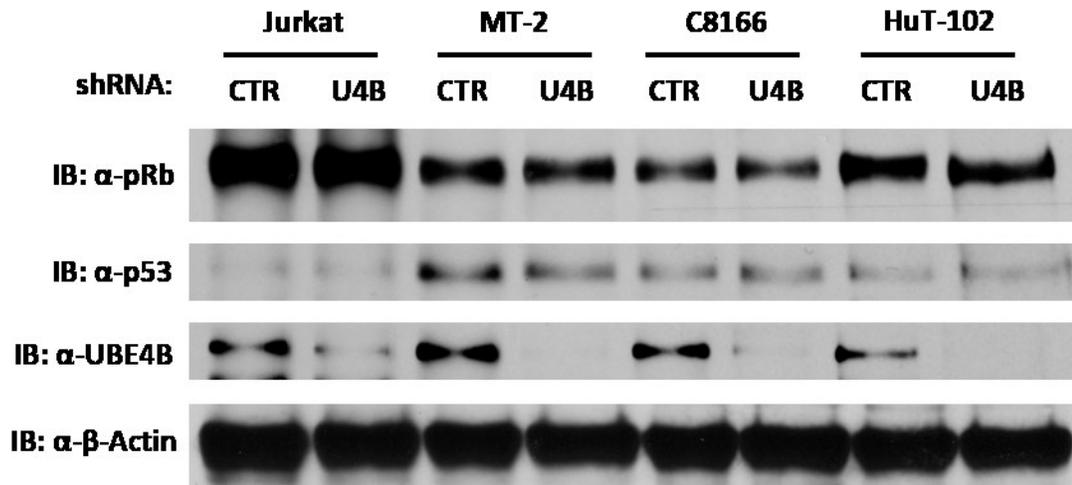


Figure 7. UBE4B does not promote pRb and p53 degradation. Jurkat, MT-2, HuT-102 and C8166 cells were infected with lentiviruses expressing control or UBE4B shRNAs. IB was performed with anti-pRb, anti-p53, anti-UBE4B and anti-Actin.

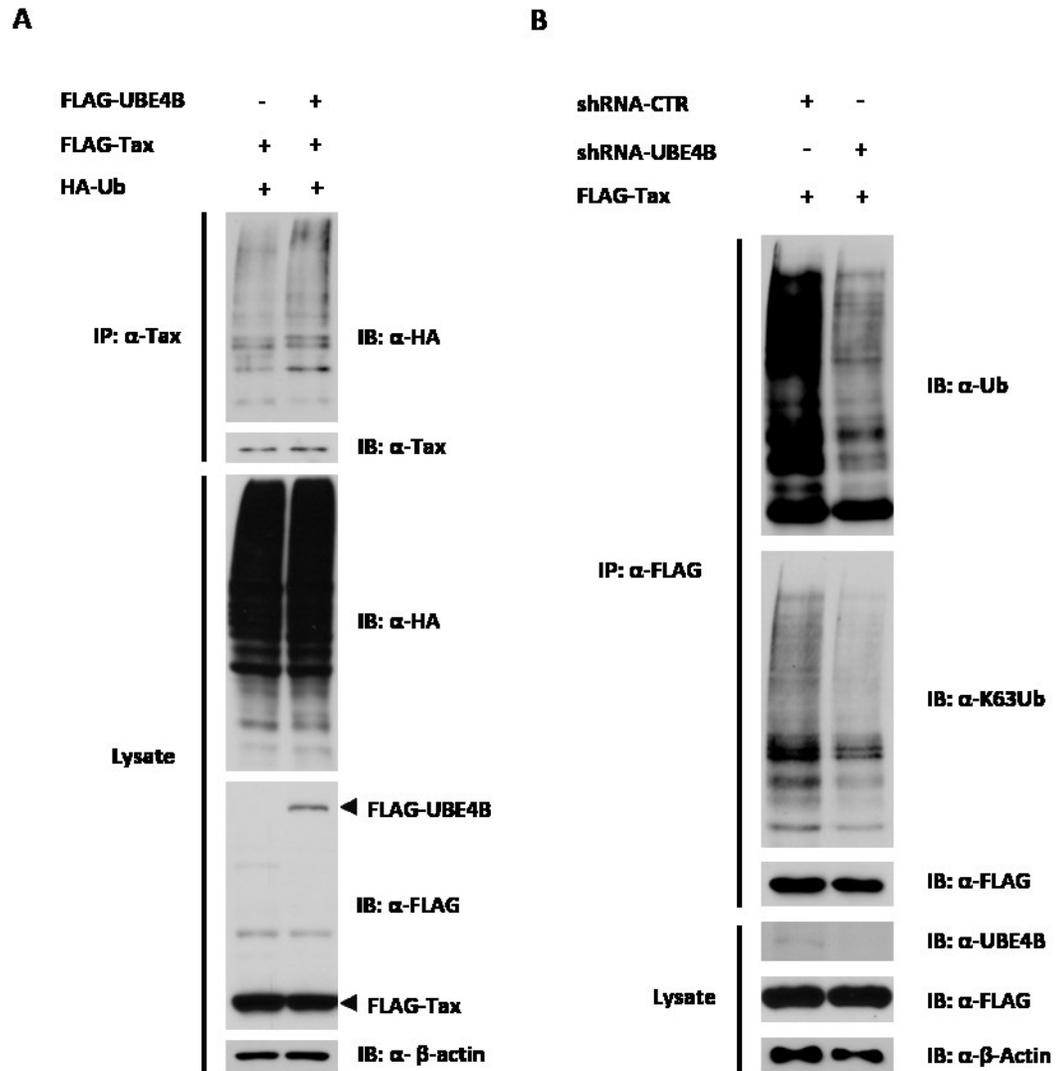


Figure 8. Tax K63-linked polyubiquitination is dependent on UBE4B. (A) 293T cells were transfected with FLAG-Tax and HA-Ub or FLAG-Tax, HA-Ub and FLAG-UBE4B. After 24 h, cells were lysed and immunoprecipitated (IP) with anti-Tax, followed by IB with anti-HA and anti-Tax. Lysates were examined for HA-Ub, FLAG-UBE4B and FLAG-Tax expression. (B) Control 293T cells and 293T cells stably knocked down for UBE4B were transfected with FLAG-Tax. After 24 h, cells were lysed and

immunoprecipitated (IP) with anti-FLAG, followed by IB with anti-Ubiquitin, anti-K63 Ubiquitin and anti-FLAG. Whole cell lysates were subjected to IB with anti-UBE4B, anti-FLAG and anti- β -Actin.

ACKNOWLEDGMENTS

First of all, I thank Dr. Edward Harhaj very much for accepting me into his lab for my rotation and thesis research. He provided me a great chance to learn everything in the lab and do research independently, at the same time he gave me a lot of advises and encouragements for my project by our daily interactions and weekly lab meeting, which helped me make a big progress for my rationale as well as experimental techniques. Second, I also acknowledge that Dr. Alfonso Lavorgna, Dr. Young Choi and other people in Dr. Edward Harhaj's lab for the help provided after I came into this lab. Moreover, I want to thank my departmental advisor: Dr. Xiao-fang Yu for the valued suggestions during my study and the critical reading for my thesis draft. Finally, I highly appreciate the well-organized and flexible structured master degree program in MMI providing me the best opportunities to tailor my interest and to pave the way for my future career.

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CURRICULUM VITAE

TENG HAN

Date of Birth: 01/11/1989

Email: than6@jhu.edu

Telephone: 443-388-1170

Address: 929 N Wolfe ST, Apt208, Baltimore, MD21205

EDUCATION

JOHNS HOPKINS UNIVERSITY (JHU)

School of Public Health

Aug. 2012 - Present

Molecular Microbiology and Immunology (MMI) Department

Master of Science in Molecular Microbiology and Immunology

BEIJING UNIVERSITY OF TECHNOLOGY (BJUT)

College of Life Sciences and Bioengineering

Sep. 2007 - Jul. 2011

Bachelor of Engineering in biotechnology

RESEARCH INTERESTS

Cancer Biology; Immunology; Signal Transduction

PROFESSIONAL SKILLS

Molecular Cloning; Cell Culture; Biochemical Assays

PUBLICATIONS

Shiwei Wang, Wei Tao, Yingting Sit, **Teng Han**, Xiang Gao, Jun Gu. gC1qR mediates C1q suppressed inflammatory response through the inhibition of RIP1 signaling. Submitted.