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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

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Abstract

Background: Retroviruses HTLV-1 and HTLV-2 have homologous genomic structures but differ significantly in pathogenicity. HTLV-1 is associated with Adult T cell Leukemia (ATL), whereas infection by HTLV-2 has no association with neoplasia. Transformation of T lymphocytes by HTLV-1 is linked to the capacity of its oncoprotein Tax-1 to alter cell survival and cell cycle control mechanisms. Among these functions, Tax-1-mediated activation of cellular gene expression via the NF-κB pathway depends on Tax-1 post-translational modifications by ubiquitination and sumoylation. The Tax-2 protein of HTLV-2B (Tax-2B) is also modified by ubiquitination and sumoylation and activates the NF-κB pathway to a level similar to that of Tax-1. The present study aims to understand whether ubiquitination and sumoylation modifications are involved in Tax-2B-mediated activation of the NF-κB pathway.

Results: The comparison of Tax-1 and Tax-2B lysine to arginine substitution mutants revealed conserved patterns and levels of ubiquitination with notable difference in the lysine usage for sumoylation. Neither Tax-1 nor Tax-2B ubiquitination and sumoylation deficient mutants could activate the NF-κB pathway and fusion of ubiquitin or SUMO-1 to the C-terminus of the ubiquitination and sumoylation deficient Tax-2B mutant strikingly restored transcriptional activity. In addition, ubiquitinated forms of Tax-2B colocalized with RelA and IKKγ in prominent cytoplasmic structures associated with the Golgi apparatus, whereas colocalization of Tax-2B with the RelA subunit of NF-κB and the transcriptional coactivator p300 in punctate nuclear structures was dependent on Tax-2B sumoylation, as previously observed for Tax-1.

Conclusions: Both Tax-1 and Tax-2 activate the NF-κB pathway via similar mechanisms involving ubiquitination and sumoylation. Therefore, the different transforming potential of HTLV-1 and HTLV-2 is unlikely to be related to different modes of activation of the canonical NF-κB pathway.

Keywords: HTLV-1, HTLV-2, Retrovirus, Tax, Oncoprotein, Leukemia, Post-translational modification, Ubiquitination, Sumoylation, NF-κB pathway

Background

Human T-cell leukemia viruses type 1 (HTLV-1) and type 2 (HTLV-2) share a common genomic structure but differ significantly in their pathogenic properties [1,2]. This difference is generally attributed to the properties of their transactivating Tax proteins, Tax-1 and Tax-2,

both of which activate gene expression via ATF/CREB and NF-κB pathways [3]. The transforming activity of Tax-1 is linked to its ability to activate the NF-κB pathway, but also to promote cell cycle progression, genome instability and inactivation of the p53 and pRb tumor suppressors resulting in the survival and proliferation of HTLV-1 infected T-cells [4-11].

Because less is known about Tax-2, a comparative analysis between Tax-1 and Tax-2 is important in order to reach a better understanding of the differences in pathogenesis. In a recent review [12], the known features

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and functional differences of Tax-1 and Tax-2 were discussed. Although Tax-1 and Tax-2B share 85 percent of amino acid similarity, two basic structural features differentiate the two proteins. First, a domain outlined by amino acids 225 and 232 of Tax-1 is responsible for p100 processing into p52 leading to activation of the non-canonical NF-κB pathway [13,14]. Second, the C-terminus of Tax-1 contains a domain involved in micronuclei formation [15] and a PDZ binding motif (PBM) encompassing the four C-terminal amino acids responsible for the binding to several PDZ domain-containing proteins [16-18]. In addition, some HTLV-2 subtypes express shorter versions of Tax-2 (namely Tax-2A and Tax-2CG) which, contrary to Tax-2B, do not functionally inactivate p53 [10,19].

Recent studies have demonstrated a hierarchical sequence of post-translational modifications that control Tax-1 intracellular localization and transcriptional activities (reviewed in [20,21]). Phosphorylation-dependent ubiquitination controls both Tax-1 retention in the cytoplasm and subsequent targeting to Golgi-associated structures, in which Tax-1 colocalizes with RelA and IKKγ leading to activation of the IκB kinase (IKK) complexes [22-25]. These steps determine phosphorylation and degradation of the NF-κB inhibitor IκBα and the migration of the active RelA subunit of NF-κB to the nucleus. In the nucleus, Tax-1 is polysumoylated at lysine residues K7 and K8 at amino acid positions 280 and 284. Polysumoylation determines the retention of Tax-1 in the nucleus, the formation of Tax nuclear bodies (NBs) and the recruitment within these NBs of various cellular transcription factors, including the RelA subunit of NF-κB [26] and the transcriptional coactivator p300 [27]. Thus, phosphorylated and polyubiquitinated Tax-1 molecules in the cytoplasm, as well as polysumoylated Tax-1 molecules in the nucleus, cooperate for activation of the NF-κB pathway [22,25,28].

The recent findings that Tax-2B is also modified by ubiquitination and sumoylation [29] and that Tax-1 and Tax-2B form complexes and colocalize with the same cellular factors [30] point to a common intracellular distribution and interactome. To determine whether ubiquitination and sumoylation control Tax-2B transcriptional activity and to outline possible differences between Tax-1 and Tax-2B, we have constructed a series of Tax-2B mutants with substitution of specific lysine residues by arginines as well as C-terminal fusions of these mutants to ubiquitin or SUMO-1. We have compared the ubiquitination and sumoylation status of the Tax-2B mutants, their intracellular distribution and their capacity to activate gene expression via the NF-κB pathway, with the patterns of the corresponding Tax-1 mutants. This study reveals that the transcriptional activity of the Tax-2B lysine to arginine substitution mutants and the ubiquitin and SUMO-1 fusions correlate with their ubiquitination and sumoylation status, suggesting a common mechanism of NF-κB activation for Tax-1 and Tax-2B.

Results

Mutation of multiple lysine residues determines loss of ubiquitination and sumoylation of both Tax-1 and Tax-2B

Previous studies indicated that Tax-1 is polysumoylated at lysine residues K7 and K8 and polyubiquitinated at the five central lysine residues K4 to K8 [22]. These five amino acids are maintained with similar sequence environment in the Tax-2B amino acid sequence. The comparative alignment of the Tax-1 and Tax-2B amino acid sequences is shown in Figure 1. The nomenclature of the 14 lysine residues in Tax-2B was designed to maintain identical numbers for equivalent lysine residues in the two sequences.

To investigate the role of post-translational modifications of Tax-2B in activating the NF-κB pathway, we first constructed Tax-2B lysine to arginine substitution mutants equivalent to Tax-1 mutants K7-8R (sumoylation deficient) as well as K4-8R and K1-10R (sumoylation and ubiquitination deficient) mutants, as described in [22]. The generated mutants Tax-2 K7-8R, K3ii-8R and K1-10iR were fused to a C-terminal 6 histidine tag. Mutants were coexpressed with either HA-Ubiquitin or HA-SUMO-1 and purified by Ni-NTA pulldown. The extent of ubiquitination and sumoylation in comparison with the equivalent Tax-1 mutants is shown in Figure 2.

The ladders of ubiquitinated forms were similar for wild type Tax-1 and Tax-2B, though a lower rate of mono-ubiquitination was observed for Tax-2B (Figure 2A). The abundance of the ubiquitinated forms was progressively reduced with increasing number of substituted lysine residues, ultimately leading to undetectable ubiquitinated forms for the two lysine-less mutants Tax-1 K1-10R and Tax-2 K1-10iR. Similar patterns of sumoylated forms for wild type Tax-1 and Tax-2B were also observed (Figure 2B). Interestingly, although mutations of lysine K7 and K8 completely abolished sumoylation of mutant Tax-1 K7-8R, as previously reported [22], the equivalent mutant Tax-2 K7-8R still maintained 81% of sumoylation as compared to wild type Tax-2B. Moreover, Tax-2 K3ii-8R, with all 6 central lysine residues mutated to arginines, still had a detectable level of sumoylation (7%). As expected, when mutants Tax-1 K1-10R and Tax-2 K1-10iR with substitutions of all lysine residues were analyzed, no sumoylated forms were detected. The presence of comparable levels of wild type and mutant Tax proteins was ascertained by Western blot analysis using an anti-6His antibody (Figure 2C). These results indicate that Tax-2B central lysine residues K3ii to K8 are the major targets for ubiquitination, as observed for the

Figure 1 Alignment of the Tax-1 and Tax-2B amino acid sequences. The 10 lysine residues of Tax-1 and the 10 equivalent lysine residues of Tax-2B are designated K1 to K10. The four additional Tax-2B lysine residues are designated K2i, K3i, K3ii and K10i. The two functional domains involved in Tax-1-mediated processing of NF-κB p100 and binding to PDZ domain containing protein (PBM) that are divergent in Tax-2B, are highlighted.

equivalent K4 to K8 lysines of Tax-1. Interestingly, the sumoylation status of Tax-2B mutants was different from those of corresponding Tax-1 mutants. The fact that Tax-2 K7-8R sumoylation was reduced to 81% as compared to wild type Tax-2B indicates that lysines K7 and K8 are at least partly involved in Tax-2B sumoylation. A further reduction to 7% of wild type Tax-2B sumoylation is observed for mutant K3ii-8R indicating that the 4 lysines K3ii to K6 can also be sumoylated. Thus, contrary to Tax-1, lysine residues other that K7 and K8 can be sumoylated in Tax-2B.

Ubiquitination and sumoylation deficiency alters the intracellular localization of both Tax-1 and Tax-2B

The following series of experiments were designed to shed light on the controversy regarding the specifics of the intracellular distribution for Tax-1 and Tax-2B. First, we developed an anti-Tax-2B rabbit polyclonal antibody (cf Methods, Additional file 1). Second, the Tax-2B and Tax-1 genes were cloned in both pcDNA6.2 and pJFE14 expression vectors. We determined that cells transfected with pJFE-Tax vectors express 20 times more Tax proteins than cells transfected with the pcDNA-Tax vectors (data not shown). Third, we analyzed the intracellular distribution of Tax-2B with respect to Tax-1 by immunofluorescence staining using the newly developed anti-

Tax-2B antibody in HeLa or 293T cells transfected with either the high level (pJFE) or the low level (pcDNA) expression vectors (Figure 3).

The results indicated that Tax-2B had a punctate nuclear distribution reminiscent of the Tax-1 nuclear bodies only in 293T cells transfected with pcDNA-Tax-2B vector. Either high and low level expression of Tax-2B in HeLa cells or high level expression in 293T cells strongly limited detection of Tax-2B in the nucleus and resulted in its accumulation in the cytoplasm. In contrast, Tax-1 NBs were observed in all tested conditions. Note that expression of Tax-1 using the low level expression vector in 293T cells resulted in undetectable Tax-1 in the cytoplasm and its concentration in Tax NBs in the majority of the cells. These results suggest that the balance between the cytoplasmic and nuclear distribution is more dependent upon the level and cellular background of expression for Tax-2B than for Tax-1.

Because of the differences described in the preceding paragraph showing parameters that impact on Tax-2B distribution in the nucleus, we tested the intracellular localization of lysine to arginine substitution Tax-2B mutants in 293T cells transfected with pcDNA vectors expressing wild type or mutant Tax-2B proteins. Colocalization of Tax-1 with the RelA subunit of NF-κB and the transcriptional coactivator p300 in NBs is critical for Tax-1-mediated activation of the NF-κB pathway [27].
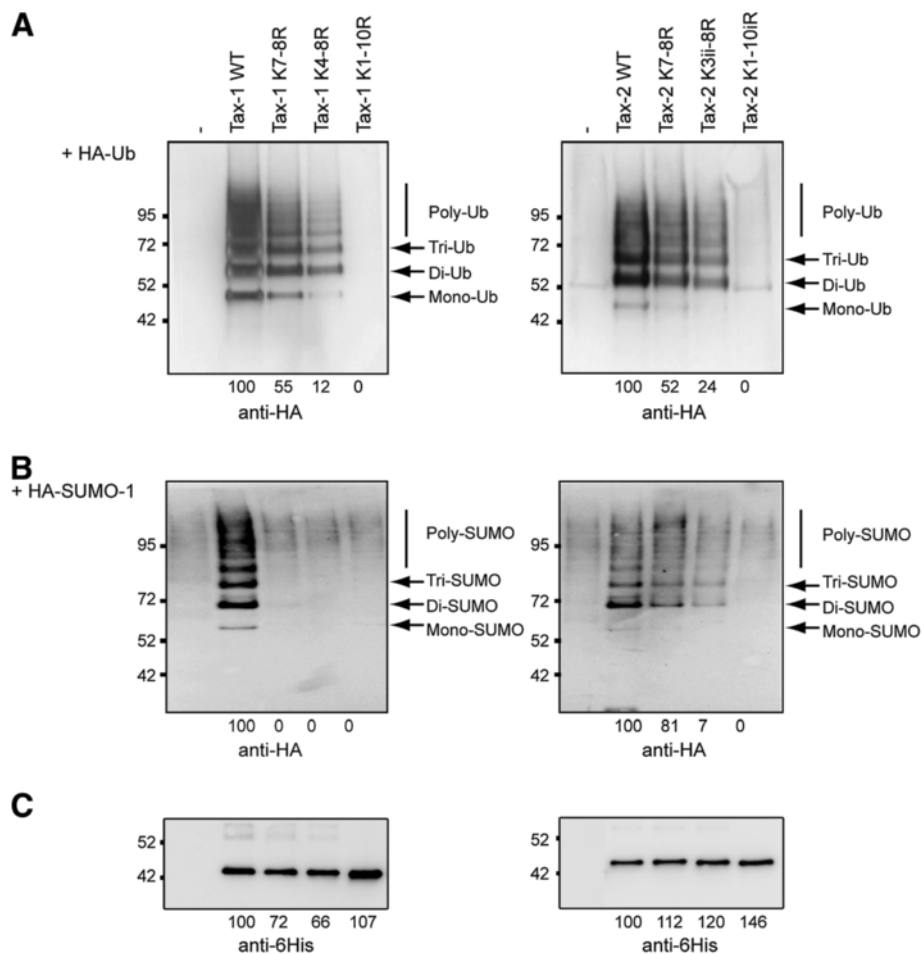
Figure 2 Mutation of multiple Tax-1 or Tax-2B lysine residues leads to ubiquitination and sumoylation deficiency. 293T cells were cotransfected with vectors expressing wild type (WT) or lysine to arginine substitution mutants of Tax-1-6His and Tax-2B-6His, and with vectors expressing (**A**) HA-Ubiquitin or (**B**) HA-SUMO-1. Purification of the ubiquitinated and sumoylated forms was performed by Ni-NTA pulldown and the purified proteins were analyzed by Western Blot using an anti-HA antibody. (**C**) Comparable levels of wild type and mutant Tax proteins in the Ni-NTA pulldown preparations were ascertained by Western blot analysis using an anti-6His antibody. The abundance of the different species on the blots was estimated, and the percentages relatively to wild type species are indicated under each Western blot image.

Therefore, we compared the intracellular localization of Tax proteins, as well as RelA and p300, by triple immunofluorescence staining in 293T cells cotransfected with vectors expressing wild type or mutant Tax proteins and p300-HA (Figure 4).

In cells that did not express Tax proteins, overexpressed p300-HA was detected only in the nucleus and displayed a speckled distribution, whereas endogenous RelA was only detected in the cytoplasm, as previously reported [27]. As indicated by the intensity of the fluorescence staining along a line drawn across the nucleus, wild type Tax-1 or Tax-2B concentrated within punctate nuclear structures in which both p300 and RelA were recruited. The punctate nuclear structures containing Tax-1, RelA and p300 were previously identified as Tax NBs by electron microscopy analysis [26]. It is interesting to note that, although both wild type Tax proteins

were also detected in the cytoplasm, Tax-1, but much less frequently Tax-2B, concentrated with RelA in cytoplasmic structures at the boundary of the nucleus (highlighted by arrows in Figure 4). This observation will be further discussed below.

Remarkably, the Tax-2 K7-8R mutant, which maintained 52% of ubiquitination and 81% of sumoylation with respect to wild type Tax-2B, formed nuclear punctate structures in which p300 and RelA colocalized. This is in marked contrast with the equivalent ubiquitinated (55%), but non-sumoylated Tax-1 K7-8R mutant, which was deficient for formation of nuclear bodies but was able to induce the migration of RelA to the nucleus in a majority of the cells, as previously demonstrated [22]. Furthemore, Tax-2 K3ii-8R was still partly sumoylated and both RelA and p300 concentrated in nuclear punctate structures in a significant fraction of the cells,
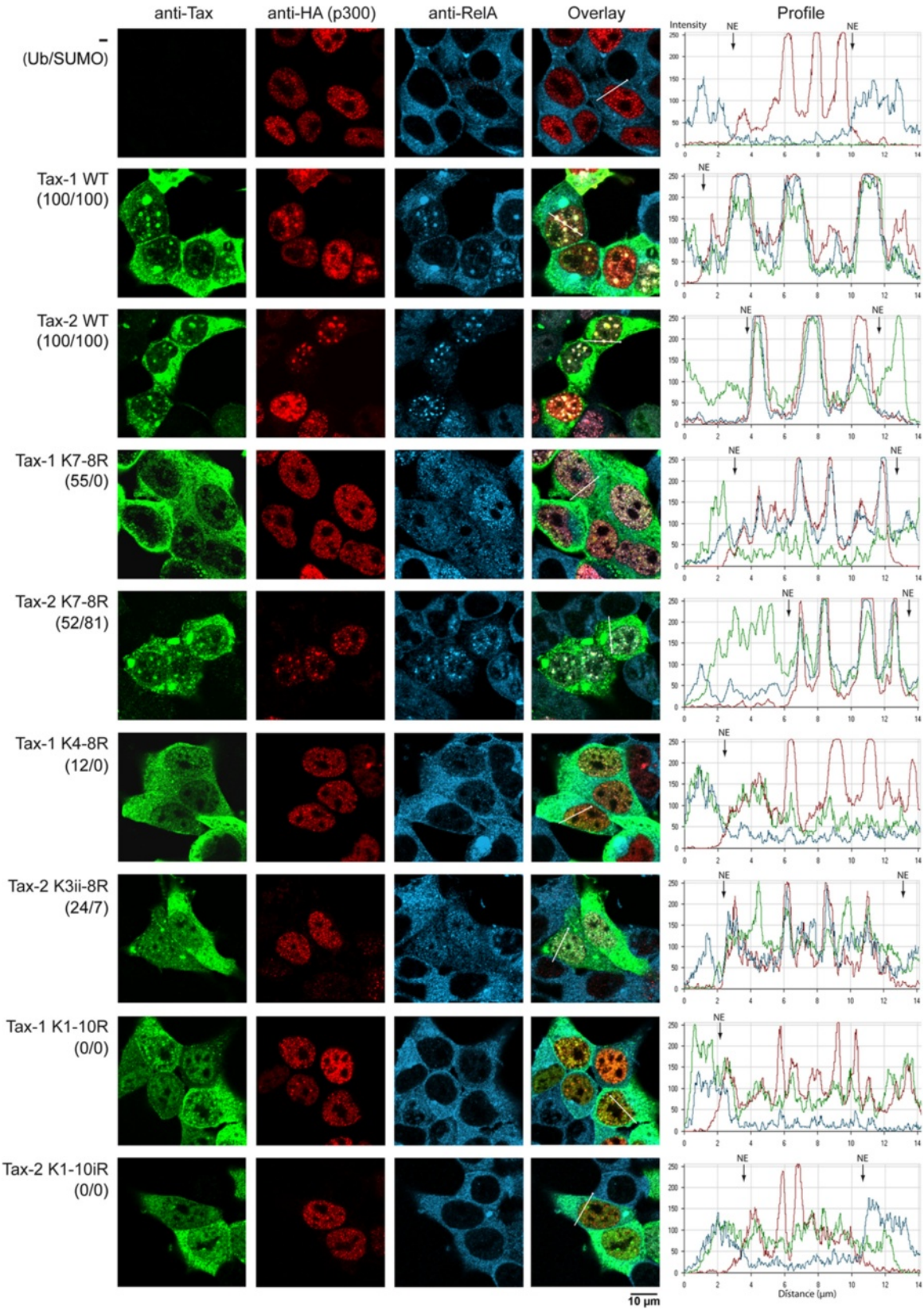
Figure 3 The intracellular distribution of Tax-2B depends on the level and cellular context for expression. HeLa or 293T cells were transfected with high level (pJFE) or low level (pcDNA) expression vectors for either Tax-1 or Tax-2B and analyzed by immunofluorescence staining and confocal microscopy with anti-Tax-1 IgG2a monoclonal antibody or anti-Tax-2B rabbit polyclonal antibody followed by anti-rabbit or anti-mouse IgG antibodies conjugated to Dylight 488. DIC, differential inference contrast.

Figure 4 (See legend on next page.)

(See figure on previous page.)
Figure 4 Ubiquitination and sumoylation deficiency alters the intracellular localization of both Tax-1 and Tax-2B. 293T cells were cotransfected with vectors expressing wild type or mutant Tax-1 or Tax-2B and the transcriptional coactivator p300-HA. The cells were fixed, stained by triple immunofluorescence staining and analyzed by laser scanning confocal microscopy. Staining of Tax-1 expressing cells was with anti-Tax-1 IgG2a monoclonal antibody, anti-HA rabbit polyclonal antibody for the detection of p300-HA and anti-RelA IgG1 monoclonal antibody. For cells expressing Tax-2B, anti-Tax-2B rabbit polyclonal antibody and anti-HA monoclonal IgG2a antibody for the detection of p300-HA and the anti-RelA IgG1 monoclonal antibody were used. The secondary antibodies were goat anti-mouse IgG2a conjugated to Dylight 488, goat anti-rabbit IgG conjugated to Dylight 549 and goat anti-mouse IgG1 conjugated to Dylight 649. The profiles of the intensity of the fluorescence staining along lines crossing the nuclei are shown in the right panels (NE: nuclear envelope). The percentages of ubiquitination and sumoylation obtained in Figure 2 are indicated for each mutant (Ub/SUMO). White arrows point to cytoplasmic structures that contain Tax-1 and RelA at the boundary of the nucleus.

contrary to what was observed for the equivalent ubiquitination and sumoylation deficient Tax-1 K4-8R mutant.

Finally, the ubiquitination and sumoylation deficient Tax-1 K1-10R and Tax-2 K1-10iR mutants had diffuse distributions in the cytoplasm and the nucleus and did not induce the translocation of RelA to the nucleus. We also observed that endogenous p300 colocalized with Tax-2B in punctate nuclear structures, as demonstrated above for overexpressed p300 (Additional file 2). These results established a direct correlation between ubiquitination and translocation of RelA to the nucleus as well as between sumoylation and formation of punctate nuclear structures in which RelA and p300 were recruited for both Tax-1 and Tax-2B.

Activation of gene expression via the NF-κB pathway correlates with the sumoylation and ubiquitination status of both Tax-1 and Tax-2B

We then investigated the relevance of modification by ubiquitination and sumoylation in Tax-2B-mediated

activation of the NF-κB pathway. We analyzed the capacities of wild type and mutant Tax-2B to activate gene expression from a construct expressing the luciferase reporter gene under the control of a promoter containing κB binding sites (NF-κB-luc reporter construct). The results were compared to those obtained for the equivalent wild type and mutant Tax-1 proteins (Figure 5). Notable differences between Tax-1 and Tax-2B emerged. Tax-1 K7-8R and K4-8R mutants were defective for NF-κB activation, thus confirming our previous findings [22], whereas the homologous Tax-2 K7-8R and K3ii-8R mutants maintained 70 and 18% of NF-κB activation, respectively. Both Tax-1 and Tax-2B mutants with all lysine residues substituted by arginines were defective for activation of gene expression via the NF-κB pathway. The expression level of each construct was controlled by detection with anti-Tax-1 and anti-Tax-2B antibodies. The fact that both Tax-2 K7-8R and K3ii-8R mutants were at least partly sumoylated and concentrated with RelA and p300 in nuclear punctate structures contrary
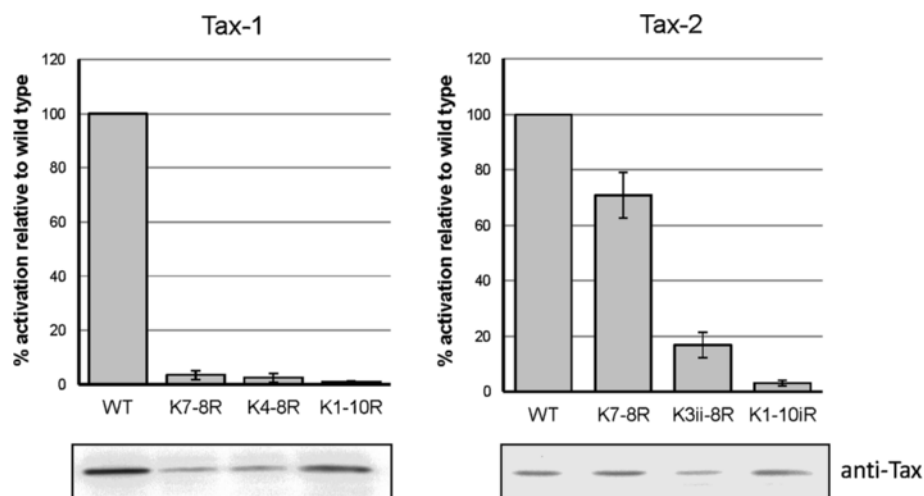

Figure 5 Activation of gene expression via the NF-κB pathway correlates with Tax-1 and Tax-2B ubiquitination and sumoylation status. 293T cells were cotransfected with 50 ng of vectors expressing wild type or mutant Tax-1 or Tax-2B and 250 ng of NF-κB-Luc reporter plasmid. Fifty ng of the phRG-TK Renilla Luciferase vector were added to the reaction to normalize for transfection efficiency. Cells were lysed and the luciferase activity was measured. Mutant Tax-1 and Tax-2B activities were expressed as percentages of WT Tax activities. The values reported are the averages of three independent experiments. Comparable levels of wild type and mutant Tax proteins in the lysates were ascertained by Western blot analysis using Tax-1 and Tax-2B antibodies.
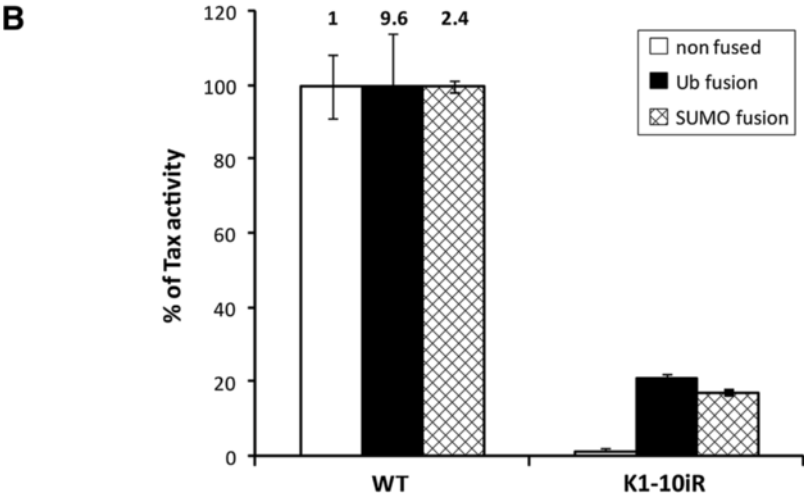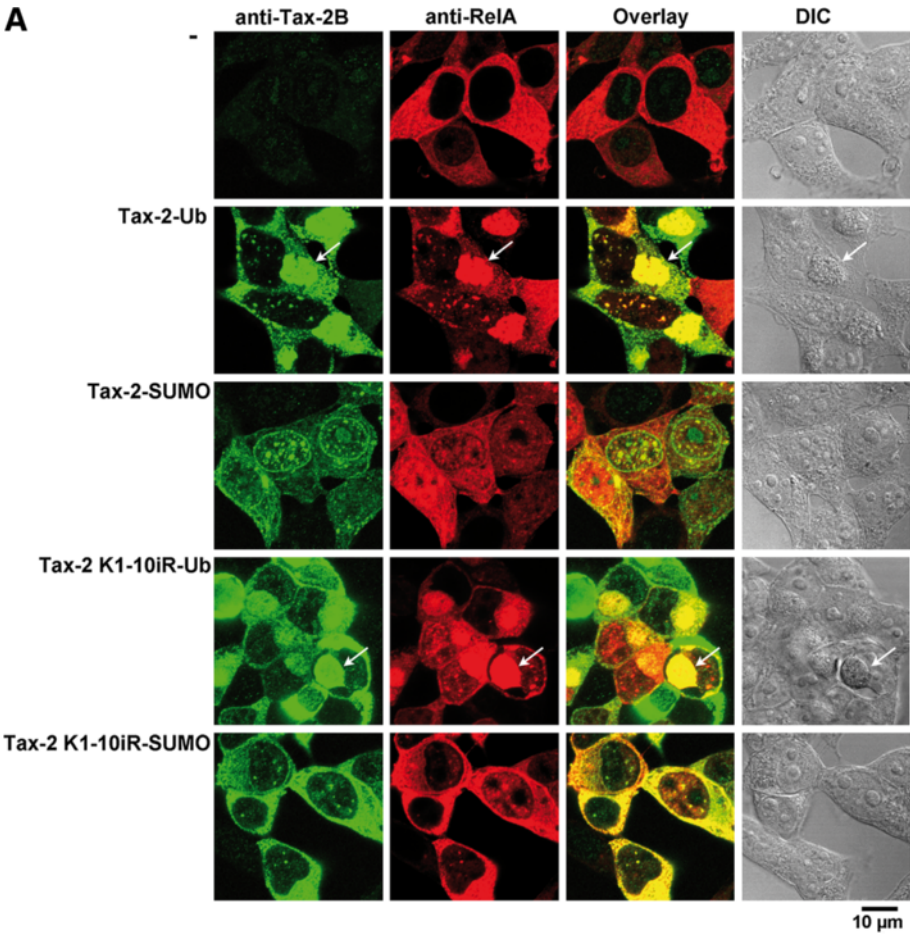
Figure 6 (See legend on next page.)

(See figure on previous page.)
Figure 6 Fusion of ubiquitin or SUMO-1 to the C-terminus of wild type or K1-10iR mutant modifies their intracellular localization and increases NF-κB activity. (A) 293T cells were cotransfected with vectors expressing wild type or K1-10iR Tax-2B mutant, fused to ubiquitin (Tax-2-Ub and Tax-2 K1-10iR-Ub) or SUMO-1 (Tax-2-SUMO and Tax-2 K1-10iR-SUMO). The cells were fixed and stained by dual immunofluorescence staining with anti-Tax-2B rabbit polyclonal antibody and anti-RelA IgG1 monoclonal antibody. The secondary antibodies were goat anti-rabbit IgG conjugated to Dylight 549 and goat anti-mouse IgG1 conjugated to Dylight 649. The images were collected using a laser scanning confocal microscope. White arrows point to cytoplasmic structures at the boundary of the nucleus in which Tax-2-Ub and Tax-2 K1-10iR-Ub fusions colocalize with RelA. DIC, differential inference contrast. **(B)** 293T cells were cotransfected with 50 ng of vectors expressing wild type or K1-10iR mutant Tax-2B fused or not to ubiquitin or SUMO-1 and 250 ng of the NF-κB-Luc reporter construct. 50 ng of the phRG-TK Renilla Luciferase vector was added to normalize for transfection efficiency. Cells were lysed and the luciferase activity was measured in each extracts. The activity of each mutant fusion is expressed as percentages relatively to the equivalent wild type Tax-2-Ub or Tax-2-SUMO fusions. The values were normalized for equal quantity of Tax proteins in each extract. The values reported are the averages of three independent experiments.

to the equivalent Tax-1 mutants, clearly establishes a correlation between the sumoylation status of Tax-1 and Tax-2B mutants, their nuclear punctate distribution and their capacities to activate the NF-κB pathway.

Fusion of ubiquitin or SUMO-1 to the C-terminus of wild type or K1-10iR mutant Tax-2B modifies their intracellular localization

To determine whether ubiquitination and sumoylation had direct consequences on Tax-2B intracellular localization and transcriptional activity, we constructed fusions of ubiquitin or SUMO-1 to the C-terminus of the lysine-less Tax-2 K1-10iR mutant (Tax-2 K1-10iR-Ub and Tax-2 K1-10iR-SUMO). The equivalent fusions of wild type Tax-2B (Tax-2-Ub and Tax-2-SUMO) were constructed as controls. 293T cells transfected with the various fusions were analyzed by dual immunofluorescence staining and confocal microscopy using anti-Tax-2B and anti-RelA antibodies (Figure 6A).

Comparison of the results in Figure 6A with those obtained in Figure 4 indicates that both wild type and K1-10iR mutant Tax-2-Ub fusions colocalized in the majority of the cells with RelA in prominent cytoplasmic structures localized at the boundary of the nucleus (indicated by arrows in Figure 6A), contrary to their unfused versions (see Figure 4). Immunostaining of GM130, a Golgi matrix protein, and IKKγ, the regulatory subunit of the IKK complexes, indicated that the cytoplasmic structures containing the wild type Tax-2-Ub fusion were closely associated with the Golgi apparatus and concentrated IKKγ, in addition to RelA (Additional file 3). These structures are reminiscent of the Tax-1/RelA/IKKγ-containing cytoplasmic "hot spots" previously identified in Tax-1 and Tax-1-Ub expressing cells (see Figure 4 above and [22,23]). Interestingly, the Tax-2 K1-10iR-Ub fusion was able to induce the migration of RelA to the nucleus, contrary to the unfused Tax-2 K1-10iR mutant.

The wild type Tax-2-SUMO fusion was predominantly distributed in prominent nuclear foci that included RelA, and this fusion was less detected in the cytoplasm and did not concentrate in cytoplasmic "hot spots", as compared to the Tax-2-Ub fusion. Furthermore, the Tax-2 K1-10iR-SUMO fusion was distributed in nuclear foci, contrary to the unfused K1-10iR mutant. This fusion was able to induce the migration of RelA to the nucleus, suggesting that fusion of SUMO-1 to the modification deficient K1-10iR mutant can compensate the lack of ubiquitination for activation of the IKK complexes in the cytoplasm. Similar compensation of ubiquitination by SUMO-1 fusion for induction of RelA migration to the nucleus was previously observed for Tax-1 K4-8R-SUMO fusion [22]. Thus, fusion of ubiquitin to the C-terminus of the Tax-2 K1-10iR mutant determined the targeting of the fusion to Golgi-associated structures containing RelA and IKKγ, whereas fusion of SUMO-1 favored the formation of nuclear foci containing RelA. In addition, both fusions were able to induce the translocation of RelA to the nucleus.

Fusion of ubiquitin or SUMO-1 to the C-terminus of the K1-10iR Tax-2B mutant restores NF-κB activity

Finally, we tested whether fusion of ubiquitin or SUMO-1 to wild type or K1-10iR Tax-2B resulted in altered capacities to activate the NF-κB pathway. Figure 6B indicates that the wild type Tax-2-Ub and Tax-2-SUMO fusions had a markedly increased ability to activate the NF-κB pathway as compared to the unfused wild type Tax-2B control (9.6 and 2.4 fold increase, respectively). In addition, although the unfused mutant K1-10iR was deficient for activation of the NF-κB pathway (1% of wild type unfused Tax-2B), ubiquitin or SUMO-1 fusion to this mutant increased its transcriptional activity by 21 and 17 fold as compared to their wild type Tax-2-Ub and Tax-2-SUMO controls, respectively. These results strongly suggest that both ubiquitination and sumoylation are directly involved in Tax-2B-mediated activation of the NF-κB pathway.

Discussion

We have compared the differences between the Tax proteins of HTLV-1 and HTLV-2 that are involved in their capacity to activate the NF-κB pathway. Tax-1-mediated

activation of the NF-κB pathway is regulated by ubiquitination and sumoylation of lysine residues in its central domain and these modifications control Tax-1 intracellular localization. We thus compared the modification patterns of the two proteins by using a panel of lysine to arginine substitution mutants. First, mutations of the lysine residues in the central domain of the two proteins (K4 to K8 for Tax-1 and K3ii to K8 for Tax-2B) resulted in a significant decrease of the ubiquitination level (12 and 24%, respectively), indicating that these central lysine residues are major targets for ubiquitination. Second, sumoylation, which is restricted to lysines K7 and K8 for Tax-1, is targeted to all lysines of the central domains in Tax-2B since only mutation of lysines K3ii to K8 led to a marked reduction of Tax-2 sumoylation level (7%). As expected, substitution of all lysine residues in the two proteins led to ubiquitination and sumoylation deficiency.

Thus, the lysine usage for sumoylation differs between Tax-1 and Tax-2B, which might indicate that the two proteins have different tridimensional folding of their central domain leading to differential interaction with the sumoylation machinery. The differential lysine usage for sumoylation has clear consequences on the intracellular localization and transcriptional activity as indicated by the observations that the sumoylation deficient Tax-1 K7-8R mutant does not assemble nuclear bodies and has no transcriptional activity, contrary to the equivalent Tax-2 K7-8R mutant.

Our results shed light on the differences reported in the literature about Tax-2B distribution in the nucleus. The comparative intracellular localization of Tax-1 and Tax-2B has been thoroughly investigated [29-31], but with controversial results. These studies support the idea that Tax-2B is more predominant in the cytoplasm than Tax-1 and that Tax-1 displays a striking punctate distribution in the nucleus. By using various experimental settings, we find out that overexpression of Tax-2B prevents its detection in the nucleus and leads to its accumulation in the cytoplasm. The level of expression enabling efficient transport to the nucleus appears to depend on the cellular background used for expression.

The comparative analysis of the ubiquitination pattern of Tax-1 and Tax-2B points to a reduced representation of the Tax-2B mono-ubiquitinated forms relatively to Tax-1 (Figure 2), which might explain the differential distribution of Tax-2B and Tax-1 in the cytoplasm and the reduced transport of Tax-2B to the nucleus. This suggestion is supported by the observation that fusion of ubiquitin, which mimics mono-ubiquitination, favors the concentration of Tax-2B (and Tax-1) in Golgi-associated structures at the boundary of the nucleus. The contribution of these structures in Tax-1-mediated activation of IKK complexes has been established [23,24].

However, our previous observations suggest that these cytoplasmic structures might also be involved in the translocation of both Tax-1 and RelA to the nucleus. Whether limited mono-ubiquitination controls the balance between nuclear and cytoplasmic Tax-2B, displacing the equilibrium toward the cytoplasm, and whether the cellular background and the level of expression affect mono-ubiquitination of Tax-2B will require further investigations. In addition, more work is required to determine whether specific differences in the modification patterns of Tax-1 and Tax-2B, including lysine usage for sumoylation and level of mono-ubiquitination, might impact their oncogenic potential.

Besides these differences, our work demonstrates that both ubiquitination and sumoylation control Tax-2B intracellular localization and ability to activate the NF-κB pathway. Polyubiquitination, and specifically K63-branched polyubiquitination, is a critical modifier of several effectors of the NF-κB phosphorylation cascade [32] as well as of Tax-1. Polyubiquitination of Tax-1 was demonstrated to determine the targeting of IKKγ to Golgi-associated structures involved in the activation of the IKK complexes [22-25,33]. Similarly, Tax-2B polyubiquitination could play a role in the release of RelA sequestration by NF-κB inhibitors in the cytoplasm and its subsequent migration to the nucleus. This conclusion is supported by the correlation between the loss of ubiquitination of the lysine-less Tax-2 K1-10iR mutant and its inability to induce the migration of RelA to the nucleus. Furthermore, the striking increase in transcriptional activity resulting from fusion of ubiquitin to the C-terminus of both wild type and the lysine-less Tax-2B mutant correlated with formation of cytoplasmic structures associated with the Golgi apparatus and the recruitment of RelA and IKKγ to these structures.

Polysumoylation is involved in formation of nuclear bodies by many cellular and viral proteins, including Tax-1 [22,25,34]. The direct correlation between the level of sumoylation of Tax-2B lysine mutants and SUMO-1 fusion to the lysine-less mutant, their ability to form punctate nuclear structures that included RelA and p300 and to activate gene expression via the NF-κB pathway support the idea that sumoylation and targeting to punctate nuclear structures play critical roles in Tax-2B transcriptional activity.

The concept that SUMO modification of Tax proteins plays an active role in activation of the NF-κB pathway [22,25,28-30] reviewed in [20,21] is challenged by a new study by Bonnet *et al*. [35]. The study shows that a Tax-1 mutant (P79AQ81A) with sumoylation that is only 23% of wild type Tax-1 and reduced ability to form detectable NBs is still transcriptionally active. Such results might be reconciled with our observations if the Tax-1 mutant P79AQ81A maintains a level of

sumoylation sufficient for threshold activity. A threshold governing the relationship between sumoylation and transcriptional activity is in line with the well known observation that the biological consequences of SUMO conjugation is not proportional to the small fraction of substrate that is modified [36]. In addition, detection of NBs at the resolution level of light microscopy might require a sumoylation threshold higher than that required for transcriptional activity. If we assume that Tax NBs are sites of transcription, it is interesting to note that the estimated size of transcription factories is 70-80 nm [37], which is considerably under the resolution of immunofluorescence staining and confocal microscopy. Thus, detection of Tax NBs might depend on overexpression of Tax or Tax-SUMO fusion, but might be limited in cells expressing Tax-1 mutant P79AQ81A or in HTLV-1 infected T-lymphocytes. These suggestions are supported by the observation that Tax-1 mutant K7R, which has a reduced sumoylation status (30% of wild type Tax-1), gives both NB formation and transcriptional activity similar to the phenotype of mutant P79AQ81A [35]. In a recent paper, Xiao [38] concludes that the studies of Bonnet *et al.* [35] cannot rule out the possibility that Tax sumoylation and/or nuclear bodies may be involved in the transcriptional regulation of some specific NF-κB target genes.

This study supports the idea that both Tax-1 and Tax-2B need to be targeted to specific subcellular domains to achieve NF-κB activation and broadens our model depicting the cytoplasmic and nuclear steps involved in Tax-1-mediated activation of the NF-κB pathway [22]. In addition, Tax-1 and Tax-2B have common partners both in the cytoplasm and nucleus. The results of this study, showing that the punctate nuclear structures assembled by Tax-2B include the RelA subunit of NF-κB and the p300 transcriptional coactivator, indicate a common property with the previously identified Tax-1 NBs. This work is also consistent with our previous studies showing that Tax-2B and Tax-1 colocalize in the cytoplasm with cellular effectors of the NF-κB pathway activation pathway RelA, IKKγ and TAB2 [30].

Conclusions

A huge body of evidence indicates that the transforming activity of Tax-1 depends on its capacity to activate the NF-κB pathway. However, this property is most likely necessary but not sufficient for cellular transformation. Since Tax-2B activates the NF-κB pathway to similar level and via similar mechanisms, the lack of malignancy of HTLV-2 might result from other functional properties that differ between Tax-1 and Tax-2B such as activation of the non-canonical NF-κB pathway, interaction with PDZ-domain proteins and/or other properties yet to be revealed.

Methods
Plasmid constructs

The alignment and nomenclature of the lysine residues in Tax-1 and Tax-2B are shown in Figure 1. The plasmid constructs expressing the wild type or lysine to arginine substitution mutants K7-8R, K4-8R and K1-10R of Tax-1 fused to a C-terminal 6-histidine tag were as previously described [22]. The pcDNA-Tax-1 vector obtained by replacing the sequence coding for Tax-1F in pcDNA6.2 Tax-1F expression vector [29] by the sequence coding for Tax-1. The Tax-2B cDNA was cloned into both the pJFE14 vector [39] and the pcDNA6.2/N expression vector (Invitrogen Corporation; Carlsbad, Ca, USA) using *Eco*RI restriction sites. A similar construct generated pcDNA6.2-Tax-2-6His. This construct was submitted to site-directed mutagenesis to generate the plasmids expressing the Tax-2-6His K7-8R, K3ii-8R and K1-10iR mutants using the Stratagene mutagenesis kit. The vectors for the expression of wild type or K1-10iR mutant Tax-2B fusions to ubiquitin or SUMO-1 were obtained by PCR amplification of the ubiquitin or SUMO-1 coding sequence followed by a 6 histidine tag and the cloning of the resulting fragments at the 3' end of the Tax coding sequence in vectors pcDNA6.2-Tax-2 and pcDNA6.2-Tax-2 K1-10iR. The di-glycine motif involved in the conjugation reaction at the end of the ubiquitin and SUMO-1 sequences were replaced by Gly-Ala to prevent conjugation of the fusions to other substrates. Each mutant or fusion was submitted to nucleotide sequence analysis to verify the correct nucleotide sequence and mutations. The vectors expressing ubiquitin or SUMO-1 tagged with the hemagglutinin epitope of the Influenza virus (HA) at their N-terminus have been previously described [40,41]. The vector for expression of p300-HA has been previously described [42]. The reporter plasmid NF-κB LTR-luc was purchased from Stratagene.

Cell culture and transfection

293T and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-Glutamine, 10% foetal calf serum (FCS), 1% penicillin-streptomycin and 1 mM sodium pyruvate (Gibco) and transfected using the Transit LT1 reagent (Mirus Bio LLC) according to the manufacturer's instructions.

Antibodies

The Tax-2B polyclonal antibody was obtained by immunizing rabbits with a peptide sequence representing the 20 C-terminal amino acids of Tax-2B (AAQGESSTQKVRPSHTNNPK). The antibody was further purified by affinity chromatography against the immobilized peptide. Its binding specificity was determined

by Western Blot and immunoprecipitation analyses (Additional file 1). The anti-Tax-1 monoclonal antibody from hybridoma 168-A51 was obtained from the AIDS research and Reagent Program, National Institutes of Health. The anti-RelA IgG1 mouse monoclonal antibody (F-6), the anti-HA IgG2a monoclonal antibody (F-7), the anti-HA rabbit polyclonal antibody (Y-11) and the anti-IKKγ IgG1 monoclonal antibody (B-3) were purchased from Santa Cruz Biotechnology. The anti-p300 IgG1 mouse monoclonal antibody (RW128) was from Upstate. The anti-pentaHis mouse antibody was from Qiagen. The anti-GM130 was from BD Biosciences. The secondary antibodies used for immunofluorescence staining were Dylight 488-conjugated goat anti-mouse IgG2a, Dylight 549- or 488-conjugated goat anti-rabbit IgG and Dylight 649-conjugated goat anti-mouse IgG1 from Jackson ImmunoResearch.

Luciferase assays

Tax-1- and Tax-2B-mediated activation of gene expression via the NF-κB pathway was assayed by dual luciferase assays using the NF-κB-luc reporter construct. 293T cells (1.25×10^5) were transfected into 12 wells with 250 ng of NF-κB-luc reporter plasmids, 50 ng of each of the different Tax-1 or Tax-2B constructs, and 50 ng of phRG-TK Renilla Luciferase vector (Promega) used to monitor transfection efficiency. Cells were lysed and tested for luciferase activity with a luminometer TD-20/20 (Turner Designs) using the Dual-luciferase reporter assay system (Promega).

Ni-NTA pulldown and western blot analysis

293T cells were lysed 24 h after transfection and Ni-NTA pulldown was done as described previously [22]. The purified proteins were analyzed by electrophoresis on 4-12% Bis-Tris NuPAGE gel (Invitrogen), transferred to Hybond ECL Nitrocellulose membrane (Amersham Pharmacia Biotech), and immunoblotted with primary and corresponding secondary antibodies. Detection was by Lumi-Light Western Blotting Substrate (Roche) or Chemi-luminescent Peroxidase Substrate (Sigma Aldrich). Detection and quantitation of chemiluminescent signals were performed with the Chemi-Smart 5000 and Bio-1D software (Vilber Lourmat) or Autochemi and Gel Pro Analyzer (UVP BioImaging Systems).

Immunofluorescence staining

Cells cultured on glass coverlips were transfected with expression plasmids for 24 h, fixed in Immunohistofix (Gentaur) at room temperature for 10 minutes followed by incubation in methanol for 6 minutes at -20°C and incubated with the primary antibodies overnight at 4°C. After washing, the specimens were incubated for 2 h at room temperature with the secondary antibodies.

Analysis was with a laser scanning confocal microscope (LSM 510, Zeiss) equipped with a 63x objective and light source wavelengths of 488, 543 and 633 nm.

Additional files

Additional file 1: Specificity of the purified anti-Tax-2B rabbit polyclonal antibody. Total cell lysates (30 μg) from 293T cells expressing either Tax-2B or Tax-1 were analyzed (A) by Western blotting with the purified rabbit polyclonal anti-Tax-2B antibody developed in this work (1:2000 dilution). (B) These lysates were also immunoprecipitated with the same anti-Tax-2B antibody. The input represents 1/10 of the immunoprecipitated lysate.

Additional file 2: Tax-1 and Tax-2B colocalize with endogenous p300 in nuclear punctate structures. 293T cells were either transfected or not with the vectors expressing Tax-1 or Tax-2B. The cells were fixed and analyzed by dual immunofluorescence staining with the anti-Tax-1 IgG2a monoclonal antibody or the anti-Tax-2B rabbit polyclonal antibody and an anti-p300 IgG1 monoclonal antibody. The secondary antibodies were goat anti-mouse IgG2a conjugated to Dylight 488, goat anti-mouse IgG1 antibody conjugated to Dylight 649 and goat anti-rabbit IgG antibody conjugated to Dylight 549. The images were collected using a laser scanning confocal microscope. DIC, differential inference contrast.

Additional file 3: The wild type Tax-2B fusion to ubiquitin colocalizes with IKKγ in prominent cytoplasmic structures closely associated with the Golgi apparatus. 293T cells transfected or not with the vector expressing the Tax-2-Ub fusion were fixed and analyzed by dual immunofluorescence staining with anti-Tax-2B rabbit polyclonal antibody and (A) anti-GM130 IgG1 mouse monoclonal antibody or (B) anti-IKKγ IgG1 mouse monoclonal antibody. The secondary antibodies were goat anti-mouse IgG1 antibody conjugated to Dylight 649 and goat anti-rabbit IgG antibody conjugated to Dylight 549. The images were collected using a laser scanning confocal microscopy. DIC, differential inference contrast.

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