The HTLV-1 Tax Protein Cooperates with Phosphorylated CREB, TORC2, and p300 to Activate CRE-Dependent Cyclin D1 Transcription

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Abstract

Adult T-cell leukemia/lymphoma is a fatal malignancy etiologically linked to infection with the human T-cell leukemia virus (HTLV-1). The virally-encoded oncoprotein Tax activates transcription of HTLV-1 and cellular genes by cooperating with cellular transcription factors. Cyclin D1 is a pivotal regulator of cell cycle progression, and increased expression strongly correlates with malignant transformation. Here, we characterize the mechanism of Tax transactivation of cyclin D1. We find that cyclin D1 transcript levels are elevated in HTLV-1 infected cells and that Tax physically associates with the cyclin D1 gene *in vivo*. Tax binds the cyclin D1 promoter-proximal cyclic AMP response element (CRE) in the presence of phosphorylated CREB (pCREB) *in vitro*, and together the Tax/pCREB complex recruits the cellular coactivator p300 to the promoter via this unconventional Tax-responsive element. We further show that Transducer of Regulated CREB 2 (TORC2) cooperates with Tax to further enhance p300 recruitment to the cyclin D1 promoter *in vitro*, consistent with enhanced cyclin D1 expression in the presence of Tax and TORC2. Together, our findings support a model in which Tax-induced accumulation of cyclin D1 shortens the G1 phase of the cell cycle, promotes mitotic replication of the virus, and drives selection and expansion of malignant T-cells.

Keywords

CRE; cell cycle; transducer of regulated CREB; CREB-regulated transcription co-activator; CRTC

Introduction

The human T-cell leukemia virus type 1 (HTLV-1)-encoded oncoprotein, Tax, promotes malignant transformation through disruption of diverse host-cell growth control pathways, resulting in aberrant cell division. Tax adversely influences cellular homeostasis through numerous mechanisms, including the physical interaction with cell cycle regulators and

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Conflict of interest

The authors declare no conflict of interest.

transcriptional activation of cell cycle control genes, that ultimately lead to enhanced cell division and uncontrolled proliferation (Afonso *et al.*, 2007; Jin, 1998; Lemoine and Marriott, 2001; Matsuoka and Jeang, 2007). Of significance, Tax has been shown to activate the cell cycle regulatory genes that encode cyclin D1 and D2 (Huang *et al.*, 2001; Mori *et al.*, 2002). In particular, Tax was shown to stimulate cyclin D1 expression through deregulation of the NF-κB pathway (Mori *et al.*, 2002). Cyclin D1 regulates G1 to S phase progression through formation of active complexes that phosphorylate and inactivate the retinoblastoma (RB) protein. As such, cyclin D1 deregulation is strongly correlated with the development and progression of many cancers. For example, a recent study demonstrated that enforced overexpression of cyclin D1 directly facilitates malignant transformation by promoting genomic instability via centrosome amplification, mitotic spindle abnormalities and aneuploidy (Nelsen *et al.*, 2005).

The oncogenic properties of Tax are primarily exerted through deregulation of two major cellular transcription factor pathways: ATF/CREB and NF-κB. Tax deregulation via NF-κB represents the most prevalent means by which Tax inappropriately alters gene expression levels in the cell. It is well established that Tax activates NF-κB target genes via the sustained release of p50 and p65 from their inhibitors, with subsequent relocation of the transcription factors into the nucleus (Sun and Yamaoka, 2005). Therefore, Tax activation of the NF-κB pathway is indirect, as Tax does not physically associate with the transcription factors bound at their target genes. In contrast, Tax activation through the cellular transcription factor CREB (and possibly other ATF/CREB family members) has only been well characterized at the HTLV-1 promoter. In this case, Tax promotes strong activation of viral transcription through the formation of a promoter-bound complex containing Tax, the Ser^{133}-phosphorylated form of CREB (pCREB) and the cellular coactivators CBP/p300. Within the HTLV-1 promoter, three conserved 21-bp repeat enhancer elements called viral CREs (vCREs) are required for Tax/pCREB complex formation. CREB binds to the central octanucleotide CRE, and Tax binding is stabilized through protein-protein interactions with CREB and protein-DNA interactions with the minor groove of the GC-rich flanks immediately adjacent to the CRE.

The Transducer of Regulated CREB (TORC) proteins have been identified as CREB co-activators that are also targeted by Tax (Bittinger *et al.*, 2004; Conkright *et al.*, 2003; Iourgenko *et al.*, 2003; Koga *et al.*, 2004; Screaton *et al.*, 2004; Siu *et al.*, 2006). This conserved family of proteins includes three members, called TORC1, 2, and 3. TORC1 is predominately expressed in the brain, while TORC2 and TORC3 are highly expressed in B and T cells (Conkright *et al.*, 2003). TORCs associate with CREB and enhance CREB target gene expression (Conkright *et al.*, 2003; Iourgenko *et al.*, 2003). Recently, TORC proteins have been shown to directly interact with Tax and augment Tax activation of HTLV-1 transcription (Koga *et al.*, 2004; Siu *et al.*, 2006). Together, these studies suggest that interactions between Tax, pCREB and TORC proteins cooperate in the strong activation of HTLV-1 transcription.

It is of interest that the cyclin D1 gene is regulated by several transcription factors including NF-κB and pCREB (Castellone *et al.*, 2009; Fox *et al.*, 2008; Guttridge *et al.*, 1999; Hinz *et al.*, 1999; Lee *et al.*, 1999), and that cyclin D1 is upregulated by Tax (Mori *et al.*, 2002). In

this study, we sought to elucidate the mechanism by which Tax activates cyclin D1 gene expression. We show that cyclin D1 transcript levels are elevated in HTLV-1 infected/ transformed cell lines, and that cyclin D1 transcription is activated by Tax through both the NF-κB and CREB pathways. Unexpectedly, chromatin immunoprecipitation assays (ChIP) reveal that Tax physically interacts with the cyclin D1 promoter region *in vivo*. Characterization of transcription factor binding to the cyclin D1 promoter *in vitro* demonstrates that the promoter-proximal CRE, together with pCREB, are required for Tax binding. These data were unexpected, as the cyclin D1 CRE is not flanked by the conserved GC-rich sequences required for Tax/pCREB binding at the vCREs in the HTLV-1 promoter. We find that Tax, in complex with pCREB, significantly increases the recruitment of the cellular coactivator p300 to the cyclin D1 promoter. Moreover, TORC2 also binds the pCREB/cyclin D1 CRE complex, and further increases p300 recruitment in a Tax-dependent manner. This enhanced coactivator binding is likely directly responsible for Tax-dependent activation of cyclin D1 transcription. Together, these findings deepen our understanding of the molecular mechanisms by which Tax activates transcription of cellular genes. Furthermore, our studies suggest that a significant number of CRE-containing cellular genes may be potential targets of deregulation by Tax in the HTLV-1 infected cell. Tax deregulation of the cyclin D1 gene may be of particular significance in HTLV-1-associated disease, as chronically elevated cyclin D1 protein levels may drive cell cycle progression and the potential for malignant transformation.

Materials and Methods

Expression and purification of recombinant proteins

Bacterially expressed Tax-His$_6$ (Zhao and Giam, 1991), CREB$_{327}$ (Lopez *et al.*, 2007), and GST-KIX (CBP aa 588–683) (Giebler *et al.*, 1997) were purified to >98% homogeneity as previously described. CREB was phosphorylated using the catalytic subunit of protein kinase A, as previously described (Giebler *et al.*, 1997). The His$_6$-TORC2 expression plasmid was generated by PCR amplification of pcDNA3.1-V5-TORC2 (Siu *et al.*, 2006) and cloned into the NdeI and HindIII restriction sites of pET3b (Novagen). His$_6$-TORC2 was bacterially expressed and purified by Ni-NTA chromatography. Full-length His$_6$-p300 was expressed from recombinant baculovirus in Sf9 cells and purified as previously described (Geiger *et al.*, 2008; Kraus *et al.*, 1999).

Antibodies

Antibodies against His (H-15), CREB-1 (C-21), p-CREB-1 (Ser-133), CBP (A-22), p300 (N-15), and RNA Pol II (H-224) were purchased from Santa Cruz, Santa Cruz, CA, USA. TORC2 was detected using the anti-His antibody. A Tax monoclonal antibody (hybridoma 168B17-46-92) was obtained from the NIH AIDS Research and Reference Reagent Program. Alexa Fluor IR700 and IR800 goat anti-mouse and goat anti-rabbit secondary antibodies were purchased from Molecular Probes-Invitrogen.

RNA Isolation and RT PCR

Cytoplasmic mRNA was isolated and reverse transcribed (Superscript II, Invitrogen) then used to amplify a cyclin D1 cDNA fragment using upstream primer 5′-

GACCATCCCCCTGACGGCCGAG-3′ and downstream primer 5′-CGCACGTCGGTGGGTGTGC-3′. As an internal control, a thymidine kinase (TK) cDNA fragment was amplified using upstream primer 5′-GAGTACTCGGGTTCGTGAAC-3′ and downstream primer 5′-GGTCATGTGTGCAGAAGCTG-3′.

Transient Transfection Assays

Jurkat cells were grown to a density of 10^6 cells/ml and transfected with a constant amount of DNA (1 ug) using Lipofectamine reagent(Invitrogen, Carlsbad, CA, USA). After 24 hrs, the cells were harvested and lysed, and luciferaseactivity was measured using a dual-luciferase reporter assaysystem (Promega). Reporter plasmids containing the cyclin D1 promoter have been previously described (Albanese *et al.*, 1995; Westerheide *et al.*, 2001). Firefly luciferase activity was normalized to *Renilla* activity (pRL-TK, Promega, Madison, WI, USA). The expression plasmid for TORC2 (pcDNA3.1-V5-TORC2) has been previously described (Siu *et al.*, 2006). The expression plasmids for Tax (pSG-Tax) (Rousset *et al.*, 1996), and Tax mutants M22 (pSG-M22) and M47 (pSG-M47) have been previously described (Smith and Greene, 1990). The transient transfection assays were performed in triplicate and repeated in three independent experiments.

Chromatin Immunoprecipitation Assays

ChIP assays, real-time PCR, and data analysis were performed as previously described (Lemasson *et al.*, 2002; Lemasson *et al.*, 2004). The primers used to detect the cyclin D1 promoter (−83 to +42 from the initiation start site) are as follows: upstream primer 5′-CTGCCGGGCTTTGATCTTTGCTTA-3′ and downstream primer 5′-ACTCTGCTGCTCGCTGCTACT-3′. The amount of immunoprecipitated cyclin D1 promoter DNA relative to that present in the total input was calculated as previously described (Frank *et al.*, 2001; Lemasson *et al.*, 2004). The correlation coefficient was 0.993 and PCR efficiency was in the range of 90–110%.

Immobilized Template Assays

The immobilized cyclin D1 promoter fragments were preincubated for 20 min at 30°C in the presence of Tax and CREB (or pCREB). Recombinant p300 and/or TORC2 were added for an additional 30 min. Protein amounts are indicated in each figure legend, and binding reactions were carried out as previously described (Geiger *et al.*, 2008). Where indicated, CEM nuclear extract (300 ug) was incubated for 1 h at 4°C following preincubation with the above recombinant purified proteins.

The biotinylated double-stranded oligonucleotides were coupled to streptavidin DynabeadsR (Invitrogen) according to the manufacturer's instruction. The top strand of the 5′ biotinylated cyclin D1 promoter sequences of the oligonucleotides are as follows. CD1 wt: 5′-Bio/ GGAATTCCTTTGATCTTTGCTTAACAACAGTAACGTCACACGGACTACAGGGGA GTTTTG TTGAAGTT-3′; CD1 mut1: 5′-Bio/ GGAATTCCTTTGATCTTTGCTTAACAACAGCACGGACTACAGTTGAAGTTGCAA AGTCCT GGAGCCTC-3′; CD1 mut2: 5′-Bio/ GGAATTCCTTTGATCTTTGCTTAACAACAGCACGGACTACAGGGGAGTTTTGTTG

AAGTT GCAAAGTC-3′; CD1 mut3: 5′-Bio/
GGAATTCCTTTGATCTTTGCTTAACAACAGTAACGTCACACGGACTACAGTTGA
AGTTGC AAAGTCCT-3′.

Electrophoretic Mobility Shift Assay (EMSA)

The indicated amounts of purified CREB, Tax, and/or GST-KIX were incubated with either a ^{32}P-end-labeled HTLV-1 vCRE or cyclin D1 CRE probe. Reactions were performed and analyzed as previously described (Kim *et al.*, 2007). The top strand sequence of the oligonucleotide probes are as follows: HTLV-1 vCRE: 5′-TTTCAGGCGTTGACGACAACCCCTCA-3′; cyclin D1 CRE: 5′-TTGACAACAGTAACGTCACACGGACG-3′. The octanucleotide CREs are underlined.

To calculate the apparent binding affinity of Tax for the cyclin D1 CRE (in the presence of pCREB and KIX), we quantified the band intensities of the quaternary complex (Tax/pCREB/KIX/CRE) relative to the ternary complex (pCREB/KIX/CRE) over a range of Tax concentrations. The relative *Kd*, defined as the concentration of Tax at which 50% of the quaternary complex formed, was quantitatively determined by plotting the fraction of Tax-containing quaternary complex versus the total concentration of Tax protein (nM) in each reaction. Reactions with the vCRE probe were performed in parallel to assess the relative binding affinities of the complexes for the respective CREs.

Image Processing

The ImageQuant program (GE, Pittsburg, PA, USA) was used to quantify band intensities. Images were processed in Adobe Photoshop, with minor adjustments made to brightness/contrast as needed (gamma was kept at 1). No bands were obscured or altered. Images were annotated in PowerPoint.

Results and Discussion

Cyclin D1 transcript levels are elevated in HTLV-1-infected T-cell lines

To evaluate cyclin D1 RNA levels in HTLV-1 infected cells, we performed reverse transcriptase-polymerase chain reaction (RT-PCR). We measured cyclin D1 transcript levels in HTLV-1 infected (SLB-1 and C8166) and uninfected (Jurkat and CEM) human T-cell lines. Figure 1a shows elevated cyclin D1 transcript levels in HTLV-1 infected cells, relative to the uninfected cells, consistent with a previous study that reported enhanced cyclin D1 transcription following Tax expression (Mori *et al.*, 2002). These data are not surprising in light of the numerous putative Tax-responsive elements in the cyclin D1 promoter, including NF-κB sites and a promoter proximal CRE (see Fig. 1b).

Tax activates cyclin D1 transcription through the proximal promoter

To determine which promoter elements mediate Tax transactivation of the cyclin D1 gene, we performed transient transfection assays using a collection of cyclin D1 promoter deletion constructs driving luciferase expression (Albanese *et al.*, 1995; Westerheide *et al.*, 2001) (Fig. 1b). Jurkat T-cells were cotransfected with each of the deletion constructs in the absence or presence of an expression plasmid for Tax. Figure 1c shows that deletion of the

upstream cyclin D1 promoter to 66 (66/Luc), relative to the start site, retained Tax responsiveness. This promoter proximal region carries a single NF-κB binding site and a single CREB-binding CRE. Although the mechanism of Tax activation through the NF-κB pathway is well established, Tax activation through cellular CREs has not been explored.

To establish which of these two pathways is utilized by Tax in the activation of cyclin D1, we performed transient transfection assays using several cyclin D1 (−66) promoter/reporter constructs bearing point mutations in the -κB and CRE elements. For reasons that remain unclear, however, the mutants gave highly inconclusive results (data not shown). We therefore performed transient transfection assays using the well-characterized Tax mutants, M22 and M47, which functionally differentiate these two pathways. M22 Tax is primarily defective for activation through NF-κB, whereas M47 Tax is defective for activation of HTLV-1 via the vCREs (Smith and Greene, 1990). As shown in figure 1d, Tax M22 was fully defective for activation of the cyclin D1 −66 reporter plasmid, whereas the Tax M47 mutant was down by approximately half. However, it is important to note that while M22 Tax is >95% defective for NF-κB, it is also 45% defective in the CREB pathway (Smith and Greene, 1990). Based on these observations, it is difficult to quantitatively establish the contribution of each pathway in mediating Tax activation through the transfected cyclin D1 proximal promoter. However, we can easily deduce that both pathways are utilized by Tax to activate cyclin D1 expression in vivo. This conclusion is further supported by our observation that co-transfection of both Tax mutants restores Tax transactivation (Fig. 1d).

Tax associates with the cyclin D1 promoter in HTLV-1 infected cells

Tax activation of NF-κB occurs via phosphorylation and degradation of IκB in the cytoplasm, leading to nuclear translocation of the transcriptionally competent NF-κB proteins (Sun and Ballard, 1999; Sun and Yamaoka, 2005). Although a previous report suggested that Tax physically binds to specific NF-κB proteins at promoter elements (Suzuki *et al.*, 1994), subsequent studies widely support a mechanism in which Tax functions exclusively in the cytoplasm to release the NF-κB proteins, and does not physically accompany the proteins to the promoters of NF-κB-activated cellular genes. Tax activation through CREB (and other ATF/CREB family members) has been well characterized on the HTLV-1 promoter, where Tax strongly activates viral transcription through physical association with CRE-bound CREB and the adjacent minor groove GC-rich DNA (Kimzey and Dynan, 1998; Lenzmeier *et al.*, 1998; Lundblad *et al.*, 1998). *In vitro* studies indicate that cellular CREs, which lack the requisite GC-rich flanking sequences, have significantly reduced capacity for Tax binding (see [Geiger *et al.*, 2008; Lenzmeier *et al.*, 1998; Lundblad *et al.*, 1998]). Consistent with this, there have been relatively few reports of Tax upregulation of cellular genes through CRE sequences (de la Fuente *et al.*, 2006; Hiraiwa *et al.*, 2003; Huang *et al.*, 2001; Iwai *et al.*, 2001; Tanaka *et al.*, 1996; Terme *et al.*, 2008). However, two recent studies reported Tax association with specific CRE-containing cellular gene promoters using the chromatin immunoprecipitation assay (ChIP) (de la Fuente *et al.*, 2006; Wang *et al.*, 2008), suggesting that Tax may physically associate with cellular CRE sequences. In addition, Tax expression further deregulates CREB-dependent cellular transcription via induction of a kinase that leads to constitutively elevated levels of serine 133 phosphorylated CREB (pCREB) (Kim *et al.*,

2007; Trevisan *et al.*, 2004; Trevisan *et al.*, 2006). Together, these data provide evidence for both direct (promoter-binding) and indirect (enhanced CREB phosphorylation) mechanisms of Tax transactivation through cellular CREs.

Since the Tax-responsive cyclin D1 proximal promoter carries a CRE, we investigated whether Tax binds at the cyclin D1 promoter *in vivo* using the chromatin immunoprecipitation assay. We measured Tax binding at the endogenous cyclin D1 promoter in HTLV-1 infected, Tax-expressing SLB-1 T-cells and in uninfected Jurkat T-cells as a negative control. We detected elevated Tax binding at the cyclin D1 promoter only in the SLB-1 cells (Fig. 2). Consistent with this observation and the presence of elevated cyclin D1 transcript levels in these HTLV-1 infected, Tax-expressing cells (see Fig. 1a), we also detected enhanced association of the cellular coactivator CBP and RNA polymerase II at the cyclin D1 promoter. These results reveal that Tax physically associates with the endogenous cyclin D1 promoter and implicate the promoter-proximal CRE as the target site for Tax association. Together, these data support a model in which Tax binding to the CRE facilitates recruitment of CBP/p300 and RNA polymerase II, commensurate with enhanced transcription of the cyclin D1 gene.

Tax binds to the cyclin D1 proximal promoter in vitro

To decipher the molecular basis for the physical interaction between Tax and the cyclin D1 promoter, we performed *in vitro* immobilized template assays. Cyclin D1 proximal promoter constructs carrying either the natural sequence (*wt*) or a deletion of both the CRE and κB sites (ΔCRE/ΔκB) were immobilized on streptavidin-coated magnetic Dynabeads via incorporation of a biotin group on the upstream end of each template, as previously described (Geiger *et al.*, 2008) (see Fig. 3a). The immobilized templates were incubated with nuclear extract derived from CEM cells (an HTLV-1-negative human T-cell line) in the absence or presence of highly purified Tax protein. The templates were extensively washed and promoter-bound proteins were separated by SDS-PAGE and analyzed by western blot. Figure 3B shows that CREB, present in the nuclear extract, bound to the *wild-type* cyclin D1 promoter, but not to the mutant ΔCRE/ΔκB promoter. Notably, we also detected the binding of Tax to the *wild-type* cyclin D1 promoter, but not to the mutant ΔCRE/ΔκB promoter (Fig. 3b, lane 5). These findings corroborate the *in vivo* ChIP assay and further show that Tax specifically associates with the functionally relevant promoter proximal region of cyclin D1.

The CRE is required for Tax binding at the cyclin D1 promoter

We next sought to identify the individual contribution of the CRE and κB sites in mediating Tax association with the cyclin D1 promoter. We designed two additional cyclin D1 promoter constructs carrying targeted deletions of the individual CRE or κB recognition elements. As before, these immobilized templates were incubated with nuclear extract and highly purified Tax. Deletion of the κB site (ΔκB) had no effect on Tax binding, however, deletion of the CRE (ΔCRE) abolished association of Tax with the cyclin D1 promoter (Fig. 3c, lanes 4, 5). Finally, we did not detect Tax binding to the cyclin D1 promoter template in the absence of nuclear extract (data not shown), indicating that the physical association of Tax with the cyclin D1 promoter requires the presence of CREB (or a CREB-like factor) bound at the CRE.

Tax and pCREB together recruit p300 to the cyclin D1 promoter

We next turned our attention to the role of CREB and the cellular coactivators CBP/p300 in facilitating Tax binding to the cyclin D1 promoter. Previous studies examining the binding of Tax at the vCREs in the HTLV-1 promoter established that the Tax/pCREB complex is required for CBP/p300 recruitment, and that this recruitment is essential for strong Tax transactivation (Geiger *et al.*, 2008; Georges *et al.*, 2003; Kim *et al.*, 2007; Kwok *et al.*, 1996; Lemasson *et al.*, 2006; Lu *et al.*, 2002; Ramirez and Nyborg, 2007; Sharma and Nyborg, 2008). Moreover, recent studies demonstrated that the KIX domain of CBP/p300 significantly stabilizes (>60-fold) the association between Tax, pCREB and the DNA (Kim *et al.*, 2007; Ramirez and Nyborg, 2007).

With these observations in mind, we further investigated Tax binding to the cyclin D1 promoter using the immobilized template assay and highly purified CREB, Ser^{133}-phosphorylated CREB (pCREB), and full-length p300. We did not detect Tax binding with DNA-bound CREB or pCREB in the absence of p300 (data not shown), suggesting that Tax has a lower binding affinity for the cyclin D1 CRE than for the vCRE. This observation further supports our model in which Tax and p300 exert mutually stabilizing influences in the assembly of transcriptionally-competent promoter-bound complexes (see below, and [Geiger *et al.*, 2008; Kim *et al.*, 2007]). Based on these results, we proceeded to characterize Tax/CREB complex formation on the cyclin D1 promoter in the presence of full-length p300. We found that Tax binding required the presence of pCREB, as the unphosphorylated form of CREB was insufficient for detectable complex formation (Fig. 4, compare lanes 3 and 5). Together the promoter-bound Tax/pCREB complex supported the association of p300 with the cyclin D1 promoter (Fig. 4, lane 5). Similar results were obtained with purified, full-length CBP (data not shown). It is noteworthy that in the absence of Tax, pCREB alone did not support the binding of full-length p300 (Fig. 4, lane 4), consistent with our previous study (see [Geiger *et al.*, 2008]). This observation is in sharp contrast to numerous publications that have modeled CBP/p300 binding to pCREB based on studies with the isolated KIX domain, and not the full-length coactivators. The significance of Tax-mediated p300 recruitment to the cyclin D1 promoter cannot be overstated, as p300 binding directly correlates with transcriptional output *in vitro* (Geiger *et al.*, 2008), and is a hallmark of transcriptionally competent genes *in vivo* (Heintzman *et al.*, 2007; Visel *et al.*, 2009). Finally, we analyzed, in parallel, Tax, pCREB and p300 binding to the HTLV-1 vCRE (Fig. 4, lane 6). As expected, Tax and p300 binding was greater on the HTLV-1 vCRE than on the cyclin D1 CRE, correlating well with the degree of Tax-mediated transcriptional output from the respective promoters (>100-fold vs. 6–12 fold). While Tax only moderately activates the cyclin D1 gene, the 6–12 fold stimulation observed is significant for a critical cell-cycle regulator and proto-oncogene that must be stringently regulated.

Characterization of Tax binding to the isolated cyclin D1 CRE

Tax association with the HTLV-1 vCREs involves multiple stabilizing interactions that include: Tax binding to GC-rich minor groove sequences adjacent to the octanucleotide core CRE; Tax interaction with CREB; and the synergistic interactions between both Tax and pCREB with the KIX domain of CBP/p300 (Ramirez and Nyborg, 2007). Therefore, the unprecedented nature of Tax binding to a cellular CRE that lacks the adjacent GC-rich DNA

sequences prompted us to more carefully investigate the binding of Tax to this sequence. As shown in figure 4, binding of Tax to the cyclin D1 promoter required the presence of both pCREB and p300. Based on these observations, we performed electrophoretic mobility shift assays (EMSAs) with the cyclin D1 CRE probe and purified Tax, pCREB, and the KIX domain of CBP/p300 (full-length CBP/p300 are not amenable to EMSA due to their large size). As a positive control, we analyzed Tax binding to the HTLV-1 vCRE probe in parallel. As expected based on the data presented in figure 4, we did not detect a Tax-dependent "supershift" of the pCREB/cyclin D1 CRE complex in the absence of KIX, suggesting that Tax did not enter into this complex (Fig. 5a, compare cyclin D1 CRE lanes 12 and 14 with vCRE lanes 2 and 4). However, inclusion of KIX in the binding reactions enabled Tax incorporation into the larger quaternary complex (Tax/pCREB/KIX/CRE), as visualized by the supershifted band. To estimate the relative binding affinity of Tax for the respective CREs, we titrated Tax into binding reactions containing a constant amount of pCREB and KIX, and the Tax-dependent conversion into quaternary complex was measured on both the cyclin D1 CRE and vCRE. Figure 5a shows that higher amounts of Tax were required to produce the quaternary complex on the cyclin D1 CRE, relative to the vCRE, and that the complex formed on the cyclin D1 CRE appeared to be slightly less stable during electrophoresis (Fig. 5a, compare lanes 5–10 with 15–20).

We next plotted the concentration of Tax against the percent conversion into the quaternary complex, and determined the apparent binding affinity based on the midpoint of the binding transition into the quaternary complex (Fig. 5b). The apparent binding affinity of Tax for the cyclin D1 CRE/pCREB/KIX complex was 14-fold lower than that for the vCRE/pCREB/KIX complex (34 nM $\pm$ 5 and 2.4 $\pm$ 0.1 nM, respectively). This 14-fold reduction in the relative binding affinity of Tax for the cyclin D1 CRE is likely due to the absence of GC-rich sequences flanking this cellular CRE. However, the binding data demonstrates that pCREB and KIX (or p300, Fig. 4) partially compensates for the absence of these conserved sequences, as both proteins significantly contribute to the overall stability of Tax on the cellular CRE DNA. These findings, coupled with the functional data shown in figure 1, clearly demonstrate that the cyclin D1 CRE is an authentic and biologically significant Tax-response element that is responsible, in part, for enhanced expression of the cyclin D1 gene.

Tax and TORC2 cooperate in cyclin D1 gene activation and CRE-dependent p300 recruitment

To further characterize the molecular basis for Tax transactivation of the cyclin D1 gene, we investigated whether the TORC proteins, CREB coactivators, also participate in this process. Tax has previously been reported to directly interact with TORC-1, -2, and -3, and transient expression assays have shown that this class of proteins synergize with Tax at the vCREs to activate HTLV-1 transcription (Koga *et al.*, 2004; Siu *et al.*, 2006). Based on these observations, we hypothesized that these proteins facilitate Tax activation of the cyclin D1 gene through the promoter-proximal CRE. To test this hypothesis, we performed transient transfection assays using Tax and TORC expression plasmids and the cyclin D1 (−66/Luc) reporter plasmid. We initially screened all three TORC expression plasmids and found that each protein stimulated cyclin D1 expression and moderately facilitated Tax transactivation (data not shown). Specifically, transfection of TORC2 stimulated cyclin D1 approximately

6-fold, and co-transfection of Tax and TORC2 stimulated cyclin D1 approximately 20-fold (Fig. 6a). These findings indicate that Tax and TORC2 cooperate to activate expression of the cyclin D1 gene. This result is consistent with the previously reported activation of a generic cellular CRE reporter plasmid by Tax and TORC3 (Koga *et al.*, 2004). However, the molecular basis for the observed functional cooperativity between Tax and the TORC proteins is unknown.

To biochemically characterize TORC2 binding at the cyclin D1 promoter, we cloned and expressed the full-length protein, and analyzed its interactions with Tax, pCREB, and p300 (Fig. 6b, c). TORC2 bound the cyclin D1 promoter in the presence of both CREB and pCREB (data not shown), but did not support p300 recruitment (Fig. 6b, lane 3). However, TORC2, in the presence of Tax, enhanced Tax-dependent p300 binding to the promoter template (Fig. 6b, compare lanes 2 and 4). The enhanced binding stability of p300 in the presence of Tax, pCREB, and TORC2 likely derives from the extensive intermolecular protein-protein interactions that take place within this elaborate DNA-bound complex. As such, the increased recruitment of p300 provided by TORC2 binding provides a molecular explanation for the functional synergy observed *in vivo* (Fig. 6a).

We next asked whether the cyclin D1 CRE was required for the formation of the TORC2-containing complex on the cyclin D1 proximal promoter. We compared, in parallel, the binding of these proteins to the wild-type and CRE cyclin D1 promoter templates. Figure 6c shows that the formation of the pCREB/Tax/TORC2/p300 complex on the cyclin D1 promoter is CRE-dependent, as deletion of the CRE prevented complex formation. From these data, we conclude that the CRE serves as the foundation for the assembly of this large (>500 kDa) multiprotein complex on the cyclin D1 promoter.

The data presented herein establish cyclin D1 as a target of Tax transactivation *in vivo*, and provide a molecular basis for the functional actions of Tax. The significance of our findings resides in the molecular dissection of a novel mechanism of Tax binding to a cellular promoter, coupled with the functional outcome of these interactions. First, these data clearly establish the cyclin D1 CRE as the foundation for the assembly of a large, multi-component nucleoprotein complex that facilitates Tax transactivation of this important gene (Fig. 7). The CRE serves as the direct binding site for pCREB, which alone is insufficient for coactivator recruitment (Fig. 4 and 6; also see [Geiger *et al.*, 2008]). Tax incorporation into the CRE-bound pCREB complex promotes p300 recruitment, consistent with the increase in cyclin D1 expression observed in the presence of Tax. The CREB coactivator TORC2 also enters into the pCREB/Tax/CRE complex, and serves to further enhance Tax-dependent p300 recruitment to the cyclin D1 promoter. Again, we find a direct correlation between coactivator binding *in vitro* and transcriptional output *in vivo*, as co-transfection of Tax and TORC2 produce a 20-fold increase in cyclin D1 expression.

Together, these findings establish a role for the promoter-proximal CRE in mediating Tax transactivation of cyclin D1. However, the relative contribution of the CRE and the adjacent κB site in conferring Tax responsiveness to the cyclin D1 gene is difficult to quantitatively discern. Although the Tax mutant M22 is well established for disabling NF-κB activation, it has a significant impact on both the NF-κB and CREB pathways (Smith and Greene, 1990).

Therefore, the marked results obtained with Tax M22 on cyclin D1 reporter gene activation are likely due to defects in Tax transactivation through both the NF-κB and CREB pathways. Our observation that co-transfection of both Tax mutants restores Tax transactivation (likely due to Tax dimerization) further supports our conclusion that both pathways contribute significantly to Tax activation of cyclin D1. Finally, it is notable that the full cyclin D1 promoter carries multiple putative Tax-responsive elements that further contribute to Tax transactivation of the endogenous gene (see Fig. 1b).

These studies illuminate a novel pathway of Tax deregulation of cellular gene expression that may have important implications for HTLV-1 biology and pathogenesis. Cyclin D1 overexpression has been shown to decreases the duration of the G1 phase of the cell cycle (Tashiro *et al.*, 2007), which would enhance viral replication via accelerated mitosis of infected cells. Additionally, this Tax-induced increase in cyclin D1 accumulation likely perturbs cellular homeostasis, creating an environment that favors the creation, selection, and proliferation of malignant T-cells.

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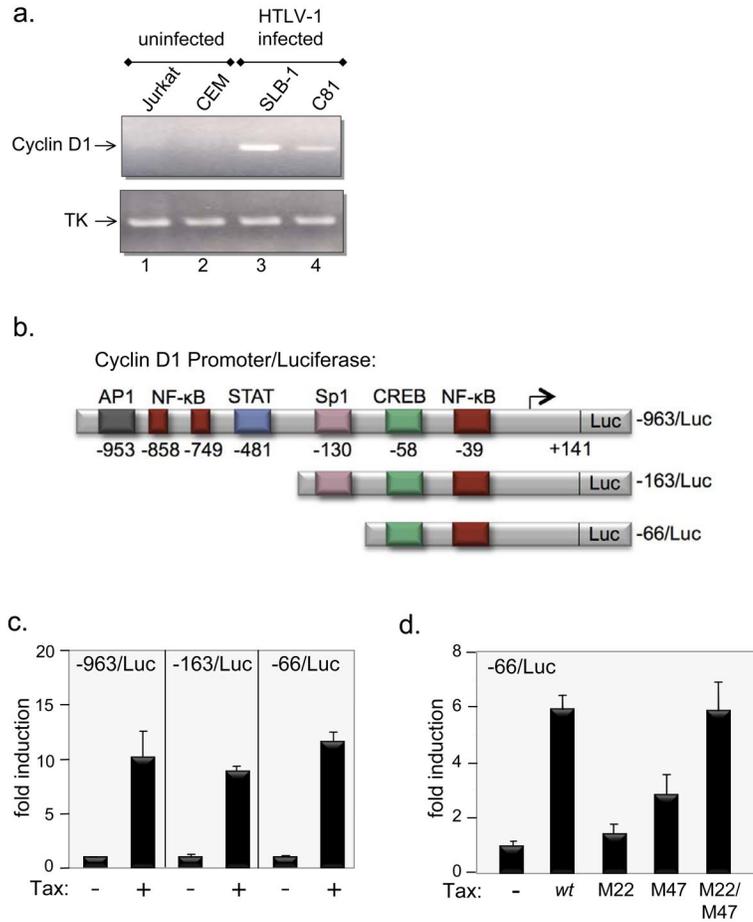
Figure 1.
The human cyclin D1 gene is transcriptionally activated by Tax. (a) *Cyclin D1 transcript levels are elevated in HTLV-1 infected T-cell lines.* Cyclin D1 RNA from uninfected (Jurkat, CEM) and HTLV-1 infected (SLB-1, C81 [C816645]) cells was amplified by RT-PCR and subjected to agarose gel electrophoresis to determine relative transcript levels. Thymidine kinase (TK) levels are shown as an internal control. (b) *Schematic diagram of cyclin D1 promoter.* Relevant cis-acting elements and the deletion constructs used in the assay are shown. (c) *Tax transactivates cyclin D1 through the −66 proximal promoter.* Jurkat cells were cotransfected with the indicated deletion constructs of the human cyclin D1 promoter/Luc (100 ng) (Albanese *et al.*, 1995), in the absence or presence of a Tax expression plasmid (100 ng) (pSG-Tax). Luciferase activity was measured 24 h after transfection and fold-induction was quantified. The graph represents the average of three independent experiments. (d) *The NF-κB and ATF/CREB pathways are utilized in Tax activation of cyclin D1.* Jurkat cells were transiently transfected with the cyclin D1 −66/Luc reporter in the absence or presence of expression plasmids for *wild-type* Tax (pSG-Tax), or the M22 or M47 Tax mutants (pSG-M22, pSG-M47) as indicated. M22 Tax is defective for activation through the NF-κB pathway and M47 Tax is defective for activation through ATF/CREB proteins (Smith and Greene, 1990).
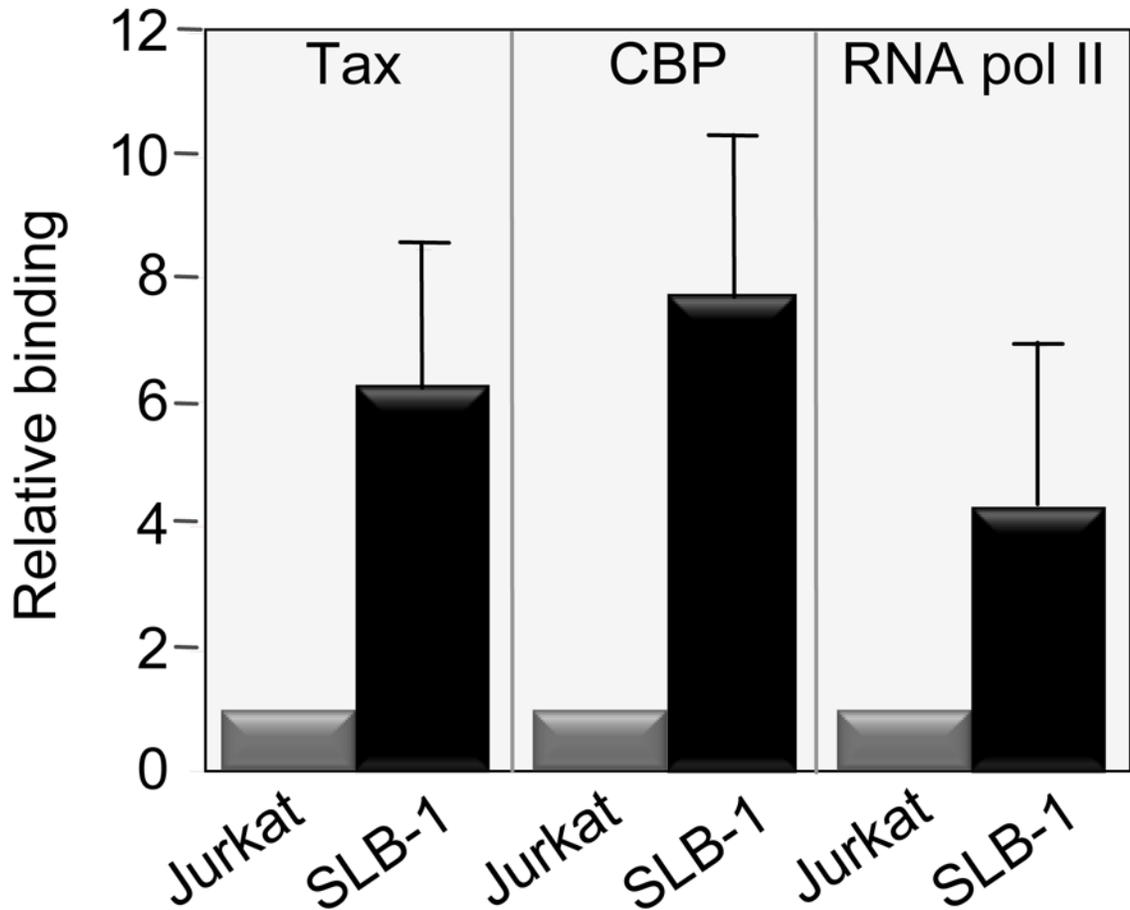
Figure 2. Chromatin immunoprecipitation assays reveal that Tax is associated with the cyclin D1 promoter in HTLV-1 infected cells

Chromatin from Jurkat (uninfected) and SLB-1 (infected) cells was immunoprecipitated using antibodies against the indicated proteins. Graphical representation of the ChIP data following immunoprecipitation and real-time PCR. Tax binding at the IL-2 promoter in SLB-1 cells was used as a negative control. Data shown represents the average of three independent experiments.
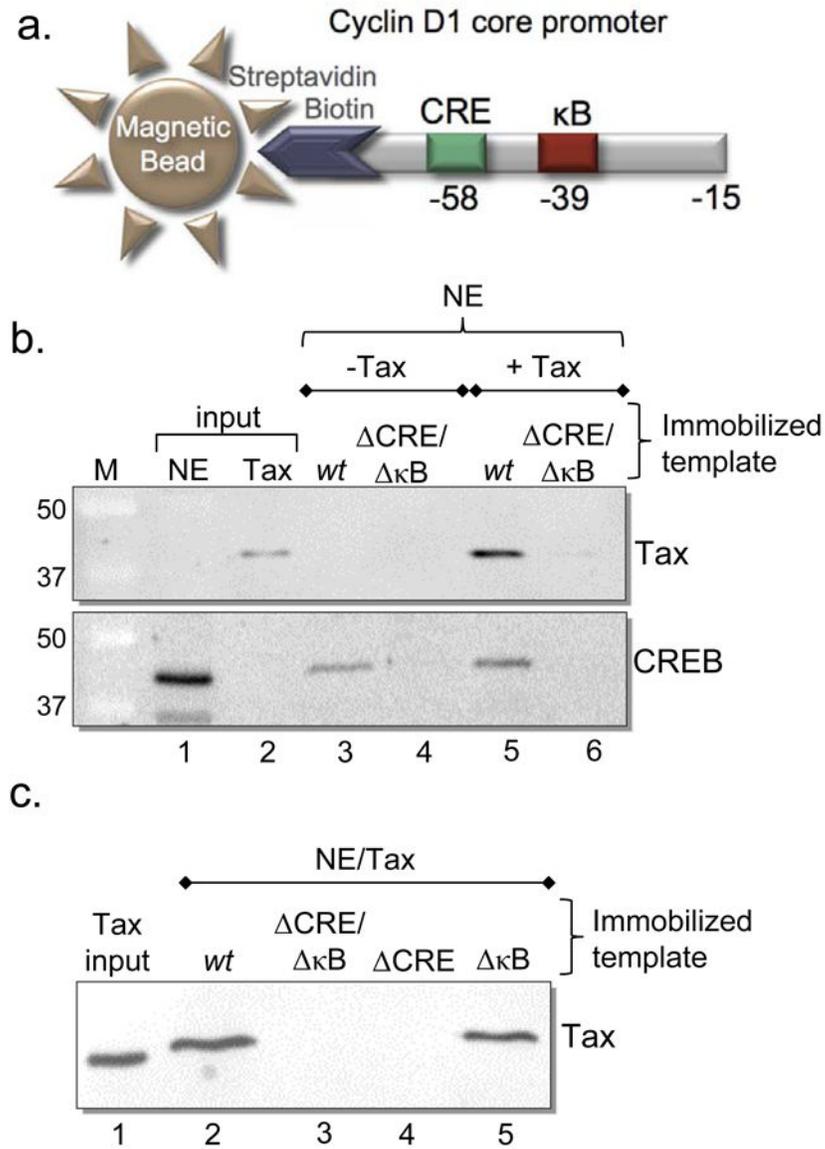
Figure 3. The CRE is required for Tax binding at the cyclin D1 promoter in vitro
(a) *Schematic diagram of the immobilized cyclin D1 core promoter template.* The cyclin D1 core promoter was bound to streptavidin-coated magnetic beads via incorporation of a biotin group at the upstream end of the fragment. (b) *Tax interacts with the cyclin D1 core promoter.* The immobilized template assay was performed with a wild-type (*wt*) cyclin D1 promoter fragment or a mutant fragment carrying substitutions in both the κB and CRE binding sites (ΔCRE/ΔκB) (2 pmol each). Binding reactions were performed with CEM nuclear extract (NE) (300 ug) in the absence or presence of purified Tax (10 pmol). Samples were analyzed by western blot using antibodies against Tax or CREB, as indicated. (c) *The CRE is required for Tax binding to the cyclin D1 promoter.* The immobilized template assay was performed with the wild-type (*wt*) or mutant cyclin D1 promoter fragments (ΔCRE, ΔκB), as indicated. Binding reactions were performed with nuclear extract and purified Tax, as described in panel B, and analyzed by western blot using an anti-Tax antibody.
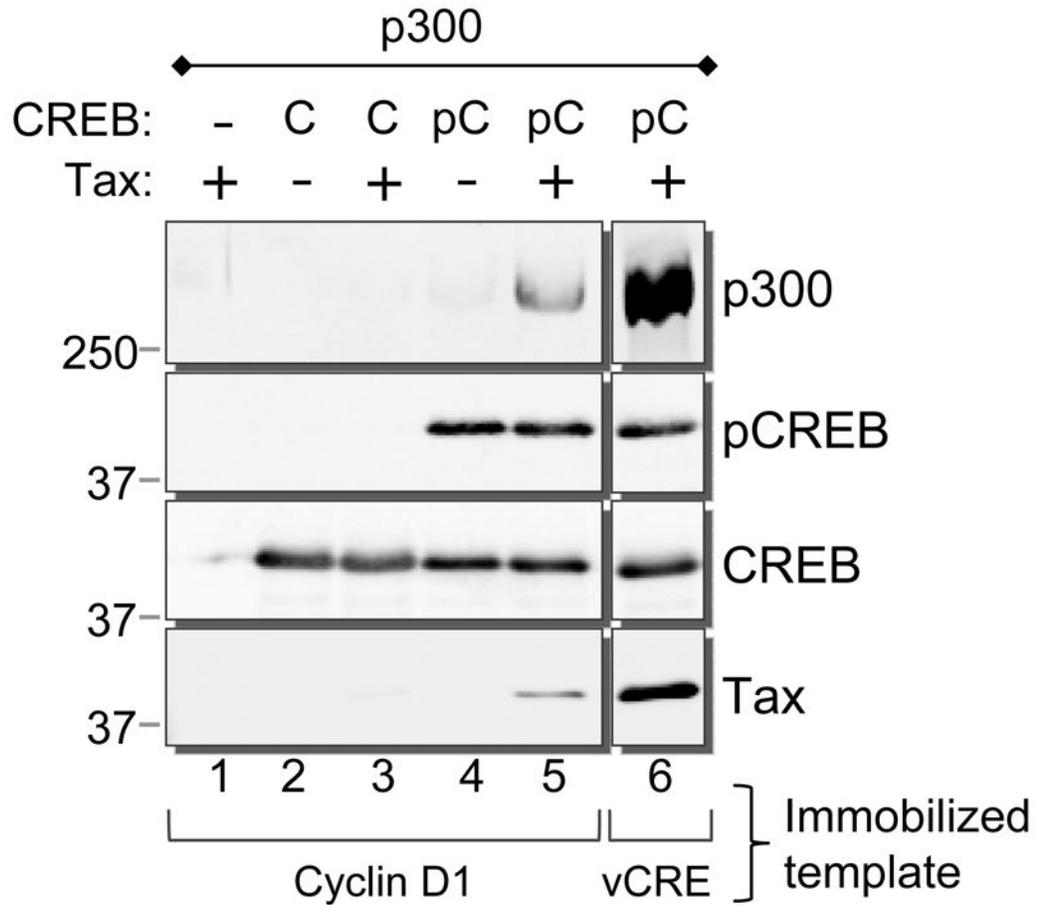
Figure 4. Tax and pCREB together recruit p300 to the cyclin D1 promoter
The immobilized cyclin D1 core promoter (5 pmol) was incubated with purified full-length p300 (4 pmol) in the presence of purified Tax, CREB (C), or Ser[133]-phosphorylated CREB (pC) (10 pmol each), as indicated. Protein binding was detected by western blot using anti-Tax, anti-CREB, anti-Ser[133]CREB, or anti-p300 antibody, as indicated. As a positive control, analysis of protein binding to the immobilized vCRE was performed in parallel with the cyclin D1 core promoter.
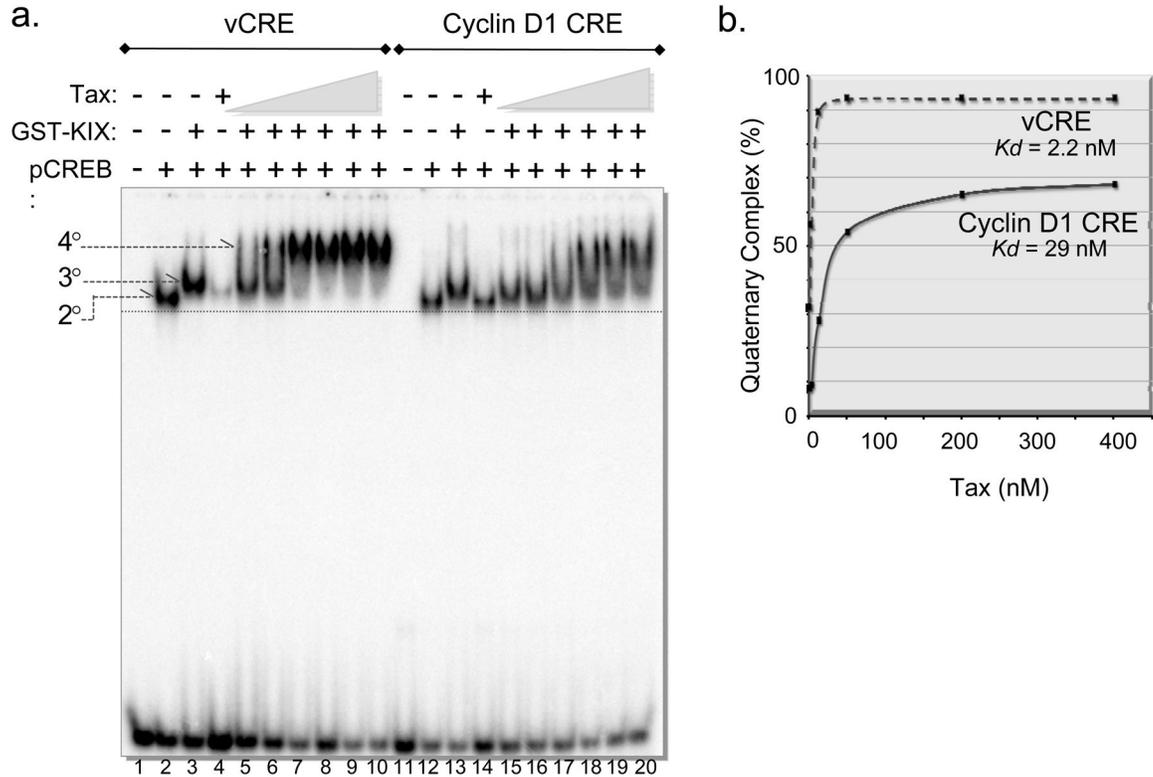
Figure 5. Tax forms a stable complex with pCREB and KIX on the cyclin D1 CRE
(a) *The cyclin D1 CRE supports Tax binding in the presence of pCREB and KIX.*
Electrophoretic mobility shift assays (EMSA) were performed using purified pCREB (24 nM), GST-KIX (5 μM), and increasing concentrations of Tax (0.8, 3.1, 12, 50, 200, 400 nM). Proteins were incubated with the ^{32}P-labeled HTLV-1 vCRE or cyclin D1 CRE (0.15 nM), as indicated. Binding reactions were resolved by 5% nondenaturing PAGE and analyzed by autoradiography. Saturating amounts of GST-KIX were used to visualize the Tax-dependent transition from the ternary complex (pCREB/KIX/CRE) into the slower migrating quaternary complex (Tax/pCREB/KIX/CRE). A dashed line was added to assist in evaluating the migration of the nucleoprotein complexes. The reduced amount of Tax/ pCREB/DNA complex shown in lane 4 is anomalous, and thus not representative of the band typically produced by Tax/pCREB binding to the vCRE (for example, see (Kim *et al.*, 2007). (b) *Relative binding affinities of Tax for the cyclin D1 CRE and vCRE in the presence of pCREB and KIX.* From the data shown in panel A, we quantified and plotted the percent quaternary complex formed with each probe against the total Tax concentration (nM) in each reaction. The concentration of Tax at the mid-point (50%) of the transition from the ternary to the quaternary complex was used to determine the apparent *Kd* of Tax for vCRE/ pCREB/KIX and cyclin D1 CRE/pCREB/KIX, as indicated. The average values from three independent experiments are shown.
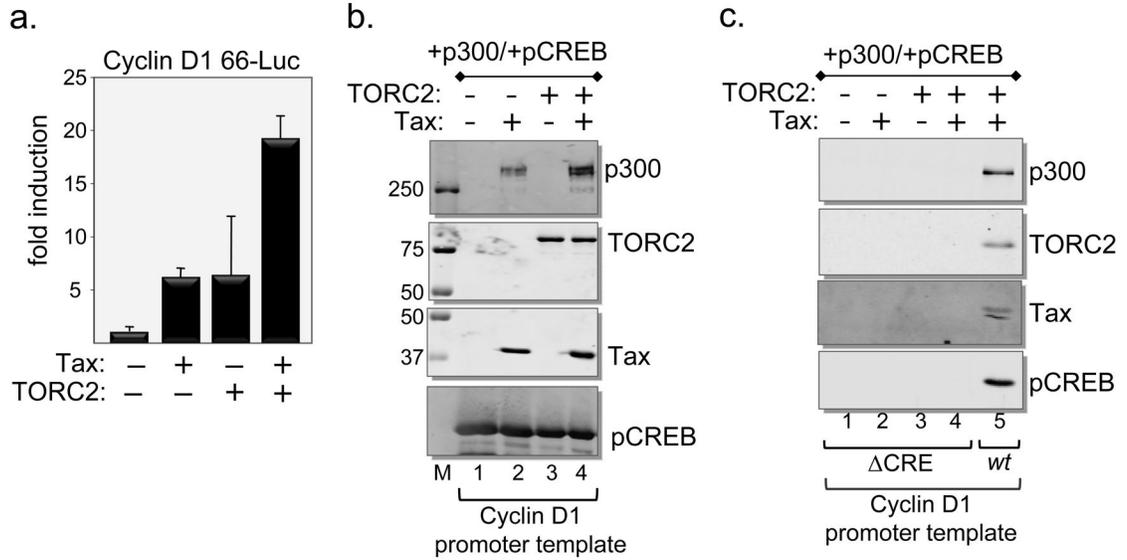
Figure 6. Tax and TORC2 cooperate to activate cyclin D1 via enhanced recruitment of p300
(a) *TORC2 stimulates cyclin D1 expression and facilitates Tax transactivation.* Jurkat cells
were co-transfected with the cyclin D1 −66/Luc reporter construct (100 ng) and Tax and/or
TORC2 expression plasmids (100 ng), as indicated. (b) *Tax and TORC2 cooperate to
enhance p300 recruitment.* Immobilized cyclin D1 core promoter DNA (5 pmol) (see Fig.
3A) was incubated with purified full-length p300 (4 pmol) and pCREB (10 pmol), in the
absence or presence of purified Tax and TORC2 (10 pmol each). Binding reactions were
analyzed by western blot using antibodies against the indicated proteins. (c) *The cyclin D1
CRE is required for Tax-containing complex formation.* The immobilized template assay
was performed using the *wild-type* (wt) and ΔCRE cyclin D1 promoter fragments, as
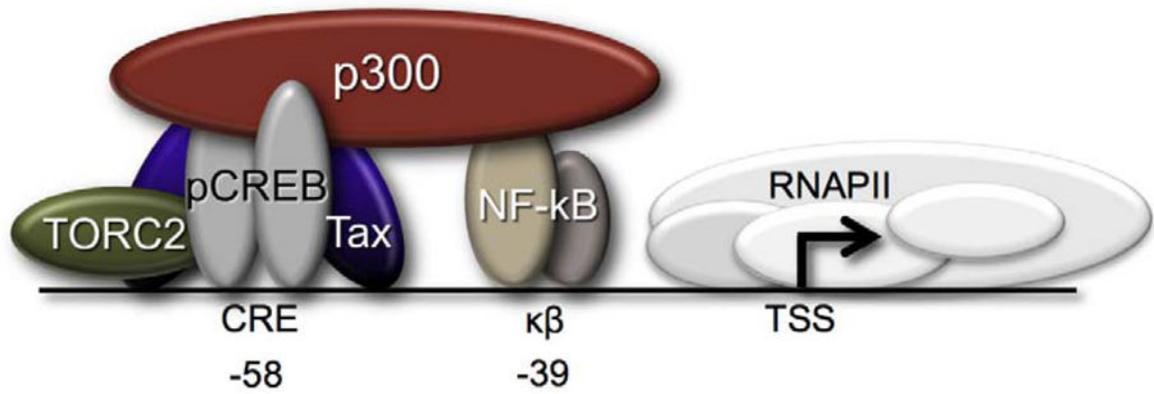described in panel B.

Figure 7. Model depicting the Tax-containing transcription factor complex on the cyclin D1 CRE
Incorporation of Tax into the pCREB/TORC2 complex at the cyclin D1 promoter-proximal CRE promotes enhanced p300 binding, with subsequent association of RNA polymerase II (RNAPII) and activation of the cyclin D1 gene. The schematic shows Tax association with the promoter DNA, however, the extent of Tax-DNA interaction is not known.