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Doctor of Philosophy Degree
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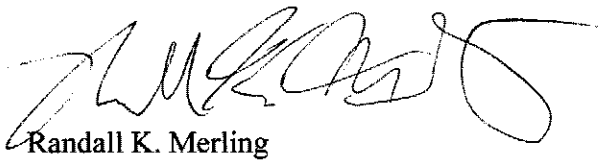

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ABSTRACT

Title of Dissertation: "The Effects of HTLV-1 Tax on Mitotic Regulation"
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The human T-cell leukemia virus 1 (HTLV-1) encodes the *tax* gene (transactivator encoded by the pX region) which is a potent activator of viral transcription and Nuclear Factor kappa B (NF-κB). Recent data indicate that Tax protein activates the anaphase promoting complex/cyclosome (APC) ahead of schedule, thereby causing the premature degradation of cyclin A, cyclin B1, securin, and Skp2. The premature loss of these mitotic regulators is accompanied by mitotic aberrations and leads to rapid senescence and cell cycle arrest in HeLa and *S. cerevisiae* cells. This *tax*-induced rapid senescence (*tax*-IRS) of HeLa cells is mediated primarily through a dramatic stabilization of $p27^{KIP}$, as deficiencies in $p27^{KIP}$ prevent Tax-IRS. Since $p27^{KIP}$ stabilization is accompanied by a surge in the level of $p21^{CIP1/WAF1}$, this cyclin-dependent kinase inhibitor may also play a role in *tax*-IRS.

As part of the research presented in this dissertation, a collection of HTLV-1 *tax* point mutants were isolated that permit normal growth of *S. cerevisiae*. Similar to wild-type *tax*, many mutants (C23W, A108T, L159F, and L235F) transactivated both the HTLV long terminal repeat (LTR) and the NF-κB reporters. The V19M mutant preferentially activated NF-κB, but was attenuated in LTR activation. None of the mutants significantly elevated the levels of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$, indicating that dramatic Tax-induced surges in $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ occurs by mechanisms distinct from NF-κB and LTR activation. Importantly, the ability of these mutants to activate APC was attenuated or abrogated. These data show that Tax-induced rapid senescence is causally associated with APC activation and suggest

that the mitotic abnormalities that are associated with premature APC activation might play an important role in cell transformation by Tax.

In a parallel study, we demonstrate that Tax interacts with both centrosomal Nek2-associated protein 1 (C-Nap1), a large coiled-coil protein that maintains centrosome cohesion, and protein phosphatase 1 (PP1) and that these interactions localize to the centrosome. Furthermore, in cells expressing *tax*, C-Nap1 phosphorylation is greatly diminished. C-Nap1 phosphorylation status plays an important role in centrosome cycle progression; PP1-mediated dephosphorylation of C-Nap1 maintains centriolar cohesion during interphase, and never in mitosis A (NIMA)-related kinase 2 (Nek2A)-mediated phosphorylation of C-Nap1 during G2/M triggers C-Nap1 dissociation from duplicated centrosomes permitting normal centrosome disjunction. Our data suggest that Tax, through recruitment of PP1 to C-Nap1, may permit prolonged C-Nap1 dephosphorylation thereby disrupting normal centrosome disjunction leading to observed Tax-induced centrosome abnormalities and distinct microtubule organizing center (MTOC) loss.

Our studies have revealed two distinct pathways through which Tax could contribute to oncogenesis. First, our Tax mutants that retained LTR and NF-κB activation properties but were impaired in APC activation were also defective in the ability to transform Rat1 fibroblasts. Therefore, premature APC activation by Tax appears to correlate with the ability of Tax to induce cellular transformation. Second, our demonstration of Tax-mediated alterations in C-Nap1 phosphorylation cycle offers an explanation for the centrosome abnormalities and mitotic apparatus defects observed in response to Tax expression. Chromosomal abnormalities resulting from such mitotic apparatus defects may be expected to contribute to the oncogenic potential of Tax.

The Effects of HTLV-1 Tax on Mitotic Regulation

By

Randall K. Merling

Dissertation submitted to the Faculty of the

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My father and mother

Brother and sisters

Jenny Jin

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CHAPTER 1

INTRODUCTION

Overview and Discovery of HTLV

Since their discovery, Human T-lymphotropic viruses (HTLVs) have been extensively studied because of their association with neoplasia and neuropathy. HTLV-1's initial isolation in 1980, from a cutaneous T cell lymphoma patient (159), marked the first identification of a human retrovirus. Furthermore, its demonstrated causative link to adult T-cell leukemia (ATL) marked the first identification of a retroviral-induced human cancer. Takatsuki *et al.* and Uchiyama *et al.* (122, 186) described ATL as a novel clinical entity several years prior to HTLV-1 initial isolation; and cutaneous T cell lymphoma, originally thought to arise from either mycosis fungoides or Sezary T cell leukemia has since been determined to be a form of ATL (46). Approximately, 2-5% of HTLV-1 infected individuals will develop some form of ATL roughly 20-40 years post-infection. The limited susceptibility and long latency period of ATL suggest a multi-step leukemogenic process. Unlike the acute and non-acute transforming animal retroviruses, ATL is neither the result of either oncogene transduction or insertional activation of cellular protooncogenes. HTLV-1 does not acquire exogenous genes, and its proviral integration sites in the chromosomes of ATL patients display a random distribution pattern (19, 39, 123, 146). HTLV-1's leukemogenic potential is attributed to the activities of Tax, the viral transactivating protein; these activities are discussed in detail below.

HTLV is a human retrovirus with subtypes HTLV-1, HTLV-2, HTLV-3 and HTLV-4. The related HTLV-2 virus was initially isolated from a “hairy T cell leukemia” cell line in 1982 (86), but a firm etiologic association with any disease has not been established. HTLV-2 infection is far less prevalent than HTLV-1 and only tentative epidemiological associations between HTLV-2 infection and increased risk of neurological disease development have

been reported (41). The remaining HTLV subtypes, HTLV-3 (16) and HTLV-4 (117), have just been recently discovered near the geographical region of Cameroon and remain to be molecularly and epidemiologically characterized. HTLV-3 is closely related to the central African simian T cell lymphotropic virus (STLV-3) (16). As a side, this newly discovered HTLV-4 (117) is different from a previously named HTLV-4, discovered in 1987, which was later renamed as human immunodeficiency virus (HIV)-2 (94).

HTLV-1 Molecular Virology

The HTLVs belong to the *Deltaretrovirus* genus of the *Orthoretrovirinae* subfamily (7). Like other retroviruses, the HTLV genome consists of two positive strands of RNA and reverse-transcribed cDNA copies of these genomes contain open reading frames (ORFs) corresponding to *gag, pol,* and *env*. HTLV genomes also include two unique genes, *tax* and *rex*; these accessory genes, essential for viral transcription and translation regulation, are located in the 3' "X-region" of the genome. The viral long terminal repeat (LTR) promoter flanks the viral genes at both the 5' and 3' ends of the genome. A detailed depiction of the structure and organization of the HTLV genome can be seen in Figure 1 (81).

For HTLV, the virus life cycle events are not well characterized because infection of cell-free virions is significantly less efficient compared to other retroviruses. Typical retroviral infection begins with attachment of the viral envelope glycoprotein to a cell surface receptor(s) followed by fusion of the virion plus host cell membranes. After entry into the cell, the viral enzymes and structural proteins form a complex in order to convert the viral genomic RNA to DNA catalyzed by viral RNA-dependent DNA polymerase (reverse transcriptase) present in infective virions. HTLV-1 reverse transcription initiation requires a

$tRNA^{Pro}$ primer (173). The synthesized DNA copy of the viral genome relocates to the cell nucleus. HTLV appears to require mitosis-associated nuclear envelope mitosis breakdown to access nuclear components; unlike human immunodeficiency virus (HIV), no mechanism for nuclear transport of an HTLV pre-integration complex has been identified. Viral integrase catalyzes the recombination of the HTLV DNA into the cellular genome. Post-integration, viral DNA is termed provirus (reviewed in Fields Virology, 2001).

LTR

The viral long terminal repeats (LTR) serves as a promoter for HTLV transcription. Full-length proviral transcription provides both mRNA templates for *gag* and *pol* gene product synthesisis and progeny virion genomic RNA. Subgenomic mRNAs are used for expression of the other viral genes. The HTLV-1 LTR is divided into U3, R, and U5 regions. Proviral transcription is controlled from the LTR, specifically its U3 region. In addition to possessing sequences important for termination and polyadenylation of viral mRNAs, U3 contains three imperfect 21-bp repeats, Tax response elements (TREs), necessary for HTLV-1 Tax-mediated transcriptional transactivation (1, 8, 152, 199, 203-205). Along with its essential contribution to viral replication, Tax is responsible for HTLV-1-induced cellular transformation by its ability to alter a number of cellular processes. HTLV-1 Tax effects on cellular processes are discussed in great detail below, and its effects on cellular mitotic regulation are the topic of this dissertation.

LTR and Rex

The LTR's R and U5 regions form the 5' leader sequence of viral mRNAs; and the U5 region also contains the Rex-response element (RRE), required for HTLV Rex-mediated posttranscriptional regulation. Intranuclear phosphorylated Rex, bound to the RRE, plays an important role in nuclear export of incompletely-spliced viral RNA (54, 80, 98) in a manner similar to Rev-mediated nuclear export of unspliced HIV-1 mRNA. Rex is also believed to be necessary for expression of structural and enzymatic proteins and for determination of virion infectivity (53, 54, 80, 98, 200).

Figure 1. Structure and organization of the HTLV-1 genome
The HTLV-1 genes gag, pol, env and the pX region are shown adapted from Jeang et al., 2004, JBC 279:31991-4.

Gag

The *gag* region of HTLV is translated as a polyprotein precursor which is cleaved to form the mature Gag proteins, the core proteins of the virus. These include the 19-kDa matrix, 24-kDa capsid and 15-kDa nucleocapsid. Myristoylated Gag polyprotein is targeted to the inner surface of the cell membrane; Gag matrix domain association with the cell membrane is a critical early step in retroviral assembly and budding (62, 172). During retroviral particle formation Gag associates with the cell membrane via the matrix domain to form viral assembling particles. The late domain is a conserved motif in the Gag polyprotein precursor that, when mutated, leads to virions failing to bud off from the plasma membrane. In HTLV-1, there are two conserved late domain motifs, PPPY and PTAP that are found in the matrix region (161, 188). Specifically, the late domains of HTLV-1 Gag have recently

been found to recruit ubiquitin ligases Nedd4 and Tsg101, a protein of the ESCRT-1 complex, to facilitate the budding process (14). Also, Gag has been reported to associate with tetraspanin enriched microdomains in the cell membrane where it colocalizes with CD82 and other tetraspanins (126). Interestingly, both Gag and CD82 proteins have been found to rapidly segregate to the immune synapse that is formed between Raji B cells and Jurkat T cells in the presence of superantigen (126). HTLV-1 transmission appears to require an intimate association, termed a "virological synapse", between an infected and uninfected cell; this association bears similarities with the immunological synapse associated with T-cell activation. Gag's reported colocalization with cellular tetraspanins, the demonstrated rapid segregation of these proteins to the immune synapse formed between Raji B cells and Jurkat T cells in the presence of superantigen, and the similarity between viral and immunological synapses collectively suggest an important role for Gag and tetraspanins in the formation or function of the viral synapse.

Protease

The HTLV-1 protease is encoded by a region that begins at the 3' end of *gag* and includes 5' *pol*, and protease synthesis is accomplished by ribosomal frameshifting. Maturation of this aspartyl protease into a functional homodimer requires autoproteolysis (85). HTLV protease is responsible for Gag polyprotein processing into its functional products and is, therefore, crucial for virion formation and, hence, viral replication (140). The protease is an important target of pharmacological inhibition in the treatment of both HTLV- and HIV-associated diseases. While the substrate binding region of HTLV-1 protease shares 45% sequence homology with that of HIV-1, their substrate specificity and inhibition profiles

differ substantially (85, 114). One currently-approved therapeutic agent, Indinavir, is an effective inhibitor of both proteases. New protease inhibitors are currently being developed and tested for inhibition of HTLV-1 protease. For instance, one of them is the substrate-based inhibitor containing hydroxymethycarbonyl isostere which showed potent HTLV-1 protease inhibition (116).

Pol

The 5' portion of the HTLV-1 *pol* gene encodes the 896-amino-acid viral reverse transcriptase (RT), and its 3' portion encodes the viral integrase. These two gene products, particularly RT, make retroviruses distinctive. The primary functions of these proteins were mentioned in the replication cycle overview. In addition to its polymerase activity, which is dependent on Mg^{2+} (140, 162), HTLV RT also possesses RNase H activity. This latter activity is essential for processivity of viral DNA production. RT is another important therapeutic target, and 3'-azido-3'-deoxythymidine (AZT), an effective HIV-1 RT inhibitor, has also been shown to inhibit HTLV-1 transmission (115).

Env

The *env* gene encodes a precursor protein of 61 to 69 kDa. The envelope protein is processed through the cellular secretory pathway into a glycoprotein and cleaved into its mature 46 kDa extracellular surface (SU) and 21 kDa transmembrane (TM) protein products (171, 172). The envelope proteins can be detected on the surface of HTLV virions and infected cells. These proteins recognize host cell receptor(s), and possibly co-receptors, critical for initiating virological synapse formation. The envelope proteins' cellular

interaction partners have not been firmly established. Some experimental evidence suggests that the widely-expressed glucose transporter type 1 (GLUT1) functions as an HTLV-1 receptor, but this section will be discussed further in the HTLV-1 Infection section. The envelope proteins' interacting partner(s) are, however, probably ubiquitously expressed since HTLV-1 exhibits a broad tropic spectrum in cell culture.

X-region encoded proteins

HTLV-1's X-region contains four ORFs. The first two encode p12, p13 and p30 proteins, while ORFs III and IV encode Tax and Rex, respectively. The highly hydrophobic p12 protein has been reported to bind to the IL-2 receptor complex. Accordingly, p12 expressing cells displayed a decreased requirement for IL-2 to induce proliferation (143). An ORF II protein, p30, and its truncated form, p13, localize to the nucleus/nucleolus and mitochondria and are believed to function in regulatory roles. The predominantly nucleolar p30 protein is believed to reduce expression of Tax and Rex. ORFs I and II are not required for *in vitro* replication but are indispensable for *in vivo* virus replication in a rabbit model (3, 9, 28, 30, 142, 201). The *tax* and *rex* genes, discussed elsewhere, are indispensable for virus replication whether *in vitro* or *in vivo*.

Diseases Associated with HTLV

Both HTLV-1 and HTLV-2 have been shown to efficiently immortalize and transform T lymphocytes in cell culture, as well as persist in infected humans and experimental animals. However, the clinical manifestations of these two viruses differ significantly (41). HTLV-1 is etiologically associated with adult T-cell leukemia (ATL) and

several chronic inflammatory diseases, most notably HTLV-associated myelopathy tropical spastic paraperesis (HAM-TSP). In contrast, HTLV-2 is less pathogenic and is far less prevalent than HTLV-1 and only a tentative risk of neurological disease development have been reported. As indicated previously, HTLV-3 and HTLV-4 have not yet been demonstrated to cause human disease. However, the particularly high degree of similarity between the HTLV-1 and HTLV-3 Tax proteins suggests the possibility of HTLV-3 pathogenicity.

ATL

The development of ATL has been attributed to the HTLV-1 viral transactivator, Tax though the precise mechanism remains to be fully clarified. ATL occurs in 2-5% of HTLV-1-infected individuals over a latency period of 20 years or more. Individual cases of ATL are typically monoclonal, $CD4^+$ T cell disorders and are generally classified clinically as acute, chronic, lymphomatous, or smoldering. Clinical manifestations of ATL can include hypercalcemia, enlarged liver and spleen, lymphadenopathy, skin involvement, and atypical lymphocytosis (49, 186). Clinical pathologic detection of multilobulated, cerebriform lymphocytes "flower cells" in the peripheral blood of ATL patients is common, particularly those with the acute form of the disease (122).

HAM-TSP

HTLV-1 has also been etiologically associated with several chronic inflammatory diseases, most notably HTLV-associated myelopathy/tropical spastic paraparesis (HAM/TSP). HAM/TSP is a chronic debilitating condition due to degenerative

demyelination within the pyramidal tracts of the spinal cord. The progression of HAM/TSP is characterized by increasing lower limb weakness, hyperreflexia, and loss of function. Other clinical signs result from loss of spinal nerve function such as urinary incontinence and impotence. Unlike ATL, HAM/TSP onset may be rapid. While the pathophysiologic mechanism of HAM/TSP development is not understood, HAM/TSP, unlike ATL, is not a neoplastic disease. However, HTLV-1 Tax may also play an important role in HAM/TSP development as high Tax expression and low $CD8^+$ lymphocyte efficiency have recently been found to correlate with high HTLV-1 proviral load and disease development (49).

HTLV-1 Epidemiology and Transmission

HTLV-1 infection is endemic to central Africa, the Caribbean basin, South America, Japan, Melanesia, and Papua New Guinea (49, 122, 185). Approximately 20 million people are currently infected with HTLV-1. The primary modes of HTLV-1 transmission are via breast milk, sexual intercourse, and blood transfusion. HTLV-1 is inefficiently transmitted. Areas endemic to HTLV tend to maintain a consistent infection rate most likely due to the transmission route of infection which usually occurs early in life.

Transmission of HTLV requires direct cell-cell contact; free virion infectivity is extremely poor in cell culture, and free virions are not detectable in the sera of infected individuals. In support of this claim, both transfusions from frozen plasma from carriers (131) and freeze-thawed mother's breast milk reduced transmission (13, 70, 122). This stringent cell-associated transmission requirement may contribute to viral latency. Furthermore, HTLV tends to remain within families and regions over extended periods of time. They have old-world linkage and related viruses can be found in old-world primates

and their lack of acute pathogenesis suggests the virus has evolved with primates over a large period of time.

HTLV-1 Infection

HTLV-1 infection has been reported to occur through cell-cell association in a variety of cells including non-T cells (27, 63, 195), human endothelial cells (71, 74), monocyte and microglial cells (72), basal mammary epithelial cells (107), B cells, CD8+ T cells and synovial cells (120). HTLV sequences have been detected in post-mortem brains of HAM-TSP patients by *in situ* hybridization (102), demonstrating that astrocytes can be productively infected *in vivo*. HTLV-1 has been more commonly detected in CD4+ T lymphocytes (52) and CD8+ T lymphocytes (37, 56, 134). Like HTLV-1, HTLV-2 has been shown to infect CD4+ and CD8+ T cells, and a variety of other cells which include other peripheral blood mononuclear cells with the cell surface markers CD14, CD16, and CD19 (101). As previously mentioned, the high variability in the tropism of HTLV suggests that its receptor is ubiquitously expressed.

Initial studies suggested the HTLV receptor is glucose transporter type 1 (GLUT1). Although quiescent human T cells do not express GLUT1, its expression is enhanced in T cells after mitogen or TGF-β stimulation. Stimulated T cells which have increased levels of GLUT-1 more readily bind the HTLV virion and its envelope protein, SU (83, 182). Indeed, studies using adherent cell lines have confirmed that GLUT-1 can function as a receptor for HTLV internalization. However, in primary CD4+ T cells, heparan sulfate proteoglycans (HSPGs) are required for the efficient entry of HTLV-1. An analysis of cell surface markers displayed in activated primary T cells, specifically in CD4+ T cells, which are the primary

target of HTLV-1, expressed significantly higher levels of HSPGs than in CD8+ T cells. Conversely, CD8+ T cells, which are the primary target of HTLV-2, expressed GLUT-1 at dramatically higher levels than found on CD4+ T cells (84). Transfection studies have confirmed that the overexpression of the required receptor in a less permissive (denoted as nonpermissive for clarity) cell allows internalization. Specifically, GLUT-1 overexpressed in nonpermissive CD4+ T cells increases entry of HTLV-2 whereas the overexpression of HSPGs in nonpermissive CD8+ T cells increases internalization of HTLV-1 (84). These studies demonstrate the preferences of receptor usage by HTLV-1 and HTLV-2 for their possible role in entry into T cells but the actual demonstration of infection has been eluded.

Because cell-free HTLV-1 virions lack infectivity, it is difficult to analyze the early events of HTLV-1 infection of host cells. To date, many of the available Tax-expressing HTLV-1 transformed T-cell lines have been derived from patients with ATL or HAM-TSP after prolonged passage of their T lymphocytes in culture of long-term cocultivation with X-irradiated HTLV-1 producing cells or with primary cord blood or peripheral T lymphocytes. As a result the initial effects of HTLV infection and Tax expression have not been characterized in cultured cells. The current model for *in vivo* HTLV infection is based on currently available infection systems, in which HTLV-1 infected cells form “virological synapses” with uninfected cells *in vitro*, and this is currently believed how infection occurs. The induced synapses are reminiscent of the T-cell synapse, but they accumulate viral proteins, viral RNA, and microtubules. Importantly, these synapses appear to be important for viral infection (77). The purpose of HTLV's requirement of cell-associated infection may be the provision of mechanisms for immune system evasion and establishment or viral latency *in vivo*; but, in the laboratory, the lack of cell-free HTLV virion infectivity creates

overwhelming difficulties in analyzing all early events in HTLV-1 infection, including viral attachment. Cell-free infection methods, though extremely inefficient and pseudotyped, have been developed to understand entry (34). To further help understand HTLV infection new detection methods have been developed based on detecting Tax expression. One method is based on a luciferase assay (149) while a different method uses green fluorescent reporter (GFP) (203). The Tax-specific GFP reporter (203) which allows for visualization of infected cells should be useful in further understanding the early events of virus transmission.

Overview of HTLV Tax

The 3' end of the HTLV-I genome encodes a 40 kDa transactivator known as Tax (transactivator encoded by the pX region), which can augment HTLV-I viral mRNA transcription and usurp critical cellular regulatory mechanisms to facilitate viral replication. Although *tax* functions like an oncogene and can transform cells in culture, it, unlike retroviral oncogenes such as *v-src* and *v-abl*, is not of cellular origin and is essential for HTLV-1 replication. Examples of the pleiotropic effects of Tax on host cells include potent nuclear factor kappa B (NF-κB) activation (26, 82, 178, 187, 192, 194) and cell cycle perturbation (90, 96, 108, 111, 112, 118, 145). Tax has been shown to activate the expression of interleukin-2 (IL-2), IL-2Rα, IL-3, IL-4, granulocyte-macrophage colony stimulating factor, proliferating cell nuclear antigen (PCNA), transforming growth factor beta (TGF-β), mimentin, proenkephalin, egr-1 (Krox20), egr-2 (Krox24), fra-1, c-fos, c-myc, c-sis, bcl-xL, c-Jun, JunB and JunD (105). Tax expression also leads to micronuclei formation (119, 175), chromosomal instability (108, 112), and centrosome amplification (23, 144, 151).

In HTLV-1 infected cells, Tax expression is negatively regulated by Rex, p30, and a

newly discovered HTLV-1 basic leucine zipper (HBZ) factor encoded by an anti-sense mRNA transcribed from the 3' end of the proviral DNA (5, 18, 103, 168). Despite the role of Tax in cell transformation and leukemogenesis, the leukemic cells of approximately 60% of ATL patients do not express *tax* transcript. The loss of *tax* expression has been shown to correlate with DNA methylation of the 5'LTR or deletion of the 5'LTR, and may be due in part to the fact that Tax is a major target of cytotoxic T lymphocytes (122). It is thought that the oncogenic activities of Tax probably take effect early during the course of ATL development, and sustained *tax* expression is not needed at the late stage of the disease.

HTLV Tax-Induced Transformation and Cancer

The Tax proteins of HTLV-1 (Tax1) and HTLV-2 (Tax2) are both required for viral transcription, but have different effects on cellular metabolism. Although both Tax1 and Tax2 can induce formation of multinucleated cells, the effect of Tax1 is more severe (175). Furthermore, although Tax1 and Tax2 both reduce cell growth, Tax1-expressing cells display more reduced growth kinetics. Tax1 also activates the $p21^{CIP1/WAF1}$ promoter to a much higher level than Tax2 (175). Finally, both Tax1 and Tax2 can transform primary human T lymphocytes (164).

In response to *tax* expression, Rat1 fibroblasts have a loss of contact inhibition, form foci on monolayer, and become transformed by *tax* (38, 69, 121, 197). Several earlier reports have implicated NF-κB activation by Tax as critical for its cell transformation activity (197). Tax1 has been reported to transform Rat1 fibroblasts to a much greater extent than Tax2, though they display 70% amino acid identity (121). Tax1 is still essential for the transformation of human T lymphocytes (164). In addition, only Tax1 has been shown to

induce micronuclei but not Tax2 (175). Tax1-expressing transgenic mice develop neoplastic diseases such as neurofibroma, (141), large granular leukemia-lymphoma (49) and thymus-derived leukemia-lymphoma (49, 61).

LTR Activation

The basic domain-leucine zipper (bZip) transcription factors, CRE-binding proteins (CREB) and activation transcription factors (ATFs), are involved in modulating gene expression in response to intracellular levels of cAMP (153). The full-length CREB protein is 43 kDa in size. CREB and a related protein, ATF-1, form homodimer or heterodimer via their respective carboxyl terminal leucine zipper domain. CREB/ATF-1 homodimers and heterodimer bind to an 8-bp motif known as the cAMP-response element that is present in the promoters of many cAMP-regulated genes. Elevated levels of cAMP lead to the activation of protein kinase A, which, in turn, phosphorylates CREB at Ser-133. The phosphorylated CREB then recruits transcriptional co-activators, CREB-binding protein (CBP) and p300, to the CRE-containing promoters to activate mRNA transcription (25).

The mechanism by which Tax activates viral transcription is well understood, and is a variation on the theme of how CREB activates cellular gene expression. The viral transcriptional enhancer consists of three imperfect Tax-response elements (TREs) in the U3 region of the viral long terminal repeat (LTR). Each of these repeats contains a cAMP response element (CRE) core (TGACGTXX) that is flanked by 5' G-rich and 3' C-rich sequences. By itself, Tax does not bind DNA directly. Rather, it facilitates the dimerization of CREB and ATF1 homodimers and heterodimer, and increases their binding to the 21-bp repeats (1, 8, 152, 199, 204, 205). In the resulting ternary complexes, CREB/ATF-1 binds to

the CRE of the 21-bp repeats, while Tax binds the CREB/ATF-1 bZIP domains and makes contact with the DNA minor groove of the G/C-rich CRE-flanking sequences. In the context of the Tax-CREB-21-bp repeat ternary complex, Tax avidly recruits transcriptional co-activators, CBP/p300, (12, 58, 59, 99, 106) to bring about potent gene activation. Importantly, recruitment of CBP/p300 to the ternary complex by Tax occurs independently of the state of CREB phosphorylation (99), thereby circumventing the cellular signaling process that is required for gene activation. In the presence of Tax, gene transcription driven by the 21-bp repeats increases 100-fold or more. A schematic of Tax-mediated HTLV LTR transactivation is provided in Figure 2 (58).

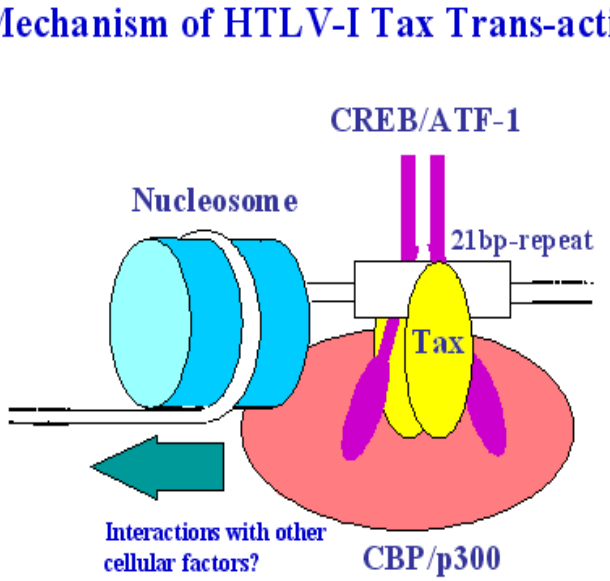

Figure 2. Assembly of a multi-HAT/activator-enhancer complex.
A model of interactions between Tax and CREB/ATF-1 and CBP/p300 on an HTLV-1 21-bp repeat element adapted from Harrod et al., 2000, JBC 275:11852-7.

NF-κB Activation

The transcription factor NF-κB/Rel family of transcription factors plays a role in many biological processes, and its immunological functions are particularly important. The two described NF-κB pathways, known as classical (canonical) and alternative (non-

canonical), are controlled by the inhibitory I-κB proteins, I-κBα, I-κBβ, and by the I-κB-like domains in NF-κB1 (p50) and NF-κB2 (p52). These inhibitors sequester NF-κB/Rel in the cytoplasm as part of multiprotein complexes. When cells are activated by extracellular stimuli such as IL-1 (130), tumor necrosis factor-α (TNF- α), bacterial lipopolysaccharide, or HTLV-1 Tax (26, 44, 48, 73, 78, 192, 194), I-κB kinase (IKK) phosphorylates I-κBα and I-κBβ, marking them for polyubiquitination and proteasome-mediated degradation. The NF-κB1 and NF-κB2 proteins are synthesized as large precursors, p105 and p100, which also undergo proteolytic processing to generate the mature NF-κB subunits, p50 and p52 respectively. While the processing of p105 is constitutive, that of p100 is regulated by the alternative NF-κB pathway. In the mature form, both p50 and p52 participate in target gene transactivation by forming heterodimers with RelA, RelB or c-Rel. The kinetics of I-κB kinase and NF-κB regulatory pathway activation and deactivation are rapid. Tax is able to activate both the classical and alternative pathways (49).

The holo-IκB kinase (IKK) consists of two highly homologous catalytic subunits, 85kDa IKKα and 87kDa IKKβ, and a 48 kDa regulatory subunit, IKKγ/NEMO (NF-κB essential modulator (IKKγ)) (88, 196). Both IKKα and β contain amino-terminal kinase domains followed by leucine zippers and helix-loop-helix domains which mediate protein-protein interactions are important for kinase activity. The activation of IKK requires serine phosphorylation of its activation loop by autophosphorylation and/or by upstream kinases MEKK and TAK1. The polyubiquitination of IKKγ by the ubiquitin ligase, TRAF6, also plays a critical role in initiating IKK activation. TRAF6 acts upstream of TAK1 and might be responsible for the recruitment of TAK1 to IKK (49). Recently, Tax is believed to activate both the classical and alternative NF-κB pathways by recruiting IKKγ and PP2A with either

IKKα or IKKβ. This complex maintains IKK in an active state (44, 49, 73).

Classical NF-κB pathway

In the classical (canonical) NF-κB pathway, IKKβ, one of two IKK catalytic subunits in the holoenzyme complex, is both necessary and sufficient for the phosphorylation and subsequent degradation of IκBα and IκBβ. IKKα, the other catalytic subunit, is dispensable for this pathway. The proteasome-mediated processing of p105, the precursor to p50 (NF-κB1), is constitutive (64). Tax activates IKK in part via a direct interaction with IKKγ by binding to amino acid residues 201-250 in IKKγ (78). While Tax can directly interact with IKKγ, how this interaction leads to IKK activation is not completely understood. Results from this laboratory have indicated that Tax, protein phosphatase 2A (PP2A), and IKKγ (44, 73) form a ternary complex. In the complex, IKK is maintained in an active phosphorylated state and causes the constitutive phosphorylation and degradation of I-κB, the nuclear translocation of NF-κB/Rel, and the potent activation of genes under the control of NF-κB/Rel.

Alternative NF-κB pathway

The alternative (non-canonical) NF-κB pathway is important for B cell proliferation and lymphoid organogenesis and is activated in response to a subset of NF-κB inducers such as lymphotoxin β and B cell activating factor (64). This non-cononical pathway depends on activated NIK-IKKα which phosphorylates p100 and causes its ubiquitination and proteasome-mediated processing to produce active p52 (NF-κB2) and is independent of

IKKγ (64, 193). Tax-mediated activation of the noncanonical pathway, however, requires both IKKα and IKKγ for p100 processing (48, 192, 194). Tax might alter the confirmation of p100 by directly binding to two short amino-terminal helices (αA and αβ) in p100 and at the same time activating IKKγ/IKKα to facilitate p100 phosphorylation and processing. The non-canonical pathway is silent in T-lymphocytes, and its aberrant activation by Tax might play an important role in Tax-mediated T-cell activation and transformation. A diagram of Tax-mediated IKK and NF-κB pathway activation is depicted (49).

Cell Cycle Regulation

An important Tax-mediated effect is the alteration of normal cell cycle progression, a topic of research for this dissertation. The cell cycle is a series of complex and orderly events that a cell undertakes to grow and divide into two daughter cells. The cell cycle consists of four phases: gap1 (G1 phase), DNA synthesis (S phase), gap2 (G2 phase) and mitosis (M phase). The interval between mitosis is referred to as interphase, which covers G1, S, and G2 phases. During mitosis, dramatic changes to the architecture of the cell occur to bring about the faithful segregation of the replicated chromosomes and the division into two identical cells. The progression of the cell cycle is cooperatively controlled by cyclins and cyclin-dependent kinases. Important to this study is the G1/S cyclin-dependent kinase (Cdk), cyclin E/Cdk2, which regulates G1 to S transition; and mitotic cylin-dependent kinase, cyclin B/Cdk1, which controls mitosis and brings about the dramatic cellular changes that occur during mitosis. The activity of cyclinE/Cdk2 is needed for cellular entry into S phase, and is regulated by the level of cyclin E, and the levels of Cdk inhibitors $p21^{CIP1/WAF1}$ and $p27^{KIP1}$. Likewise, the activity of cyclin B/Cdk1 is regulated by activating and inhibitory

phosphorylation, and by the level of cyclin B. Finally, the events of the cell cycle are unidirectional and irreversible. This is largely mediated through targeted polyubiquitination and degradation of cell cycle regulatory proteins by two specific E3 ubiquitin ligases SCF (Skp1/Cullin/F-Box) and APC (anaphase promoting complex) (10, 15, 17, 97, 135-137, 191).

Tax Effects on APC

Entry into mitosis requires both the accumulation of mitotic cyclins (cyclin B) and the activation of Cdk1. Metaphase to anaphase transition and mitotic exit, on the other hand, requires the orderly destruction of mitotic cyclins and other key mitotic regulators such as Pds1p (precocious dissociation of sister chromatids)/securin: the anaphase inhibitor (**Clb2p and Pds1p are *S. cerevisiae* nomenclature**). The levels of Clb2p/cyclin B1 and Pds1p/securin are cell-cycle regulated. They rise in S phase through *de novo* synthesis, peak in G_2/M, and decline rapidly at the end of M phase. The drastic decrease in Clb2p/cyclin B1 and Pds1p/securin is mediated by the multiprotein E3-ubiquitin ligase called the anaphase promoting complex/cyclosome (**APC/C, referred to as APC henceforth**) and its accessory factors, Cdc20 and Cdh1. Activation of APC is mediated through its phosphorylation by Cdk1/cyclin B1 and/or polo-like kinase (Plk1), and sequential association with Cdc20 and Cdh1. Cdc20 and Cdh1 are WD40 repeat-containing proteins that are highly conserved in evolution. Both proteins function as substrate-specific activators of APC and direct APC to ubiquitinate critical mitotic regulators including Clb2p/cyclin B1 and Pds1p/securin, targeting them for degradation by the proteasome in an orderly manner (202).

APC^{Cdc20} is required for the onset of anaphase and APC^{Cdh1} is required for the exit of mitosis. Association of APC with Cdc20 occurs during metaphase to anaphase transition

wherein APC^{Cdc20} targets the destruction of Pds1p/securin, the anaphase inhibitor, and a significant fraction of cyclin B1. The destruction of Pds1p/securin (during metaphase) releases a protease known as Esp1p/separase, which in turn degrades Mcd1p/Scc1p—a protein which constitutes part of a complex called cohesin that holds sister chromatids together. This allows sister chromatids attached to the mitotic spindle and aligned on the metaphase plate to be separated and pulled to the opposite poles of the mitotic spindle. Proper control of Pds1p/securin degradation ensures normal metaphase to anaphase transition and faithful chromosomal transmission during mitosis (29, 183, 202).

The multisubunit E3 ubiquitin ligase, SCF, together with its substrate-recognition subunit, Skp2, is responsible for the ubiquitination and degradation of $p27^{KIP1}$. The level of Skp2 oscillates in a cell cycle-dependent manner like cyclins A and B. Recent studies have shown that Skp2 is a substrate of the Cdh1-associated APC, APC^{Cdh1}, and becomes ubiquitinated and degraded in late M and early G_1 when APC^{Cdh1} is highly active (10, 191). This renders SCF inactive and allows $p27^{KIP1}$ to accumulate in G_1.

The large multisubunit APC complex and its different targeting subunits control cell cycle-related proteins at different phases of the cell cycle (97). The cell cycle progression is regulated by APC through ubiquitin-dependent protein degradation like waves of destruction. The SCF^{Skp2} controls entry into S phase, and APC^{Cdh1} regulates exit from mitosis and controls the duration of G1 by targeting the SCF subunit Skp2 for destruction (10, 15, 17, 97, 135, 136, 191).

Previously, our laboratory has shown that Tax can directly bind and activate APC, which is an E3 ubiquitin ligase that controls metaphase to anaphase transition and mitotic exit by targeting the destruction of critical mitotic regulators. In essence, Tax activates the

cellular program for mitotic exit far in advance of its normal schedule. Tax-induced APC is often accompanied by severe mitotic abnormalities associated with the premature loss of cyclin A, cyclin B1, securin, and Skp2, the substrate targeting subunit of SCF^{Skp2}, another E3 ubiquitin ligase (96, 97). Importantly, the loss of Skp2 renders SCF inactive and dramatically stabilizes its substrate, $p27^{KIP1}$, the major G1/S cyclin-dependent kinases inhibitors (CKI). This results in an immediate and permanent cessation of the cell cycle and arrests cells in a senescence-like state known as Tax-induced rapid senescence (Tax-IRS). In essence, Tax activates the cellular program for mitotic exit far in advance of its normal schedule, thereby driving cells into a state of permanent arrest that is often accompanied by severe mitotic abnormalities in Figure 3 (96).

The expression of $p21^{CIP1/WAF1}$ is increased in Tax-expressing cells via transcriptional activation and mRNA stabilization, both of which might be APC mediated. Tax-IRS induction correlates more closely with Tax's effects on $p27^{KIP1}$. Increased expression of $p27^{KIP1}$ occurs concurrent with initial periods of Tax expression. This is likely due to $p27^{KIP1}$ protein stabilization and loss of Skp2; and Tax-induced increased expression of $p27^{KIP1}$ results in Tax-IRS. Loss of $p27^{KIP1}$ function is a common feature of HTLV-1 transformed human T-cells that constitutively express Tax (96). Interestingly, the mitotic effects of Tax can be faithfully recapitulated in *S. cerevisiae*. Yeast cells expressing *tax* from a galactose inducible promoter also cease proliferating (112). Recent data from our laboratory suggest that a loss of SIC1p, the *S. cerevisiae* G1/S CKI, averts the Tax-IRS.

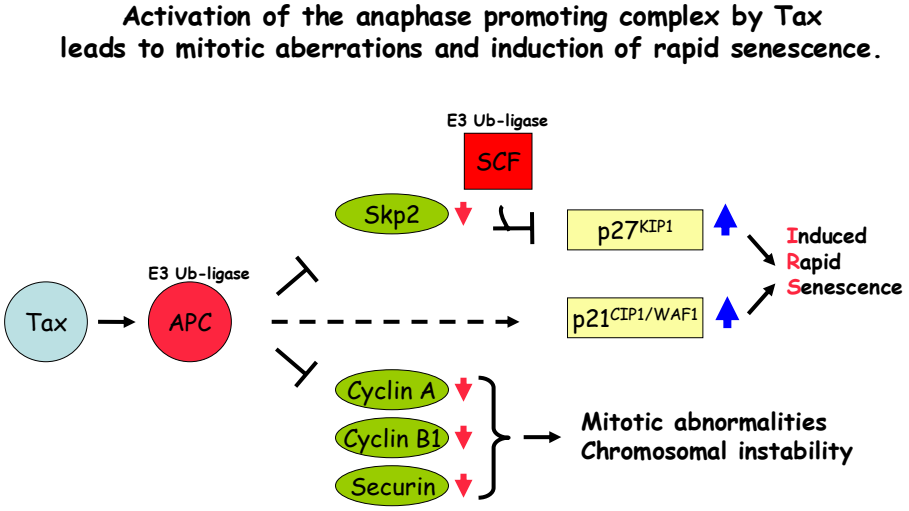

Figure 3. Unscheduled activation of the APC by HTLV-1 Tax and its cell cycle aftermaths.
Unscheduled activation of the APC during S phase by HTLV-1 Tax leads to degradation of cyclin A, cyclin B1, and securin causing mitotic abnormalities/chromosome instability. The degradation of Skp2 results in inhibition/inactivation of SCF^{Skp2}, which leads to the stabilization of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$, committing cells to rapid senescence adapted from Kuo et al., 2006, Embo J., 25:1741-52.

Centrosomes and Tax

Tax has been shown to have a profound effect on cellular mitosis. In addition, Tax-expressing eukaryotic cells are often binucleated or multinucleated. While previous work in this laboratory support the possibility that the premature activation of the APC plays a major role in the mitotic demise of Tax-expressing cells, other cellular mechanisms targeted by Tax might also contribute to this process. ATL cells display centrosome amplification (144), and elucidating the role of Tax in this process is our second research topic.

Centrosome Background

The centrosome functions as the microtubule organizing center (MTOC) and is a cell cycle-regulated organelle that is critical for mitosis. In animal cells, two critical events must

occur in interphase prior to M phase: DNA replication and centrosome duplication. The centrosome duplication is crucial for the the formation of the bipolar mitotic spindle and to impart each daughter cell with a centrosome (113). The centrosome is a small organelle that consists of amorphous material, known as the centrosome matrix or the pericentriolar material (PCM), which surrounds a pair of centrioles. The PCM contains γ-tubulin ring complexes that are essential for nucleating microtubules. The fast-growing plus ends of microtubules project outward and the minus ends remain associated with the centrosome. Although the centrioles and other components of the centrosome are duplicated during interphase, they remain part of a single complex. As such, there is only a single MTOC during interphase. When mitosis begins, this complex divides in two and each centriole pair becomes part of a separate MTOC (128). The significance of centrosomes is further highlighted by their roles in influencing entry into S-phase of the cell cycle (68, 91, 154, 169), cell motility, polarity and shape (169).

Centrosome Disjunction via C-Nap1 Phosphorylation

Located at the centrosome is **c**entrosomal **n**ever-in-mitosis A (NIMA)-related kinase 2A (Nek2A)-**a**ssociated **p**rotein 1 (C-Nap1), a large coiled-coil protein that is critical for centriole attachment and maintenance of duplicated centrosomes (6, 40, 43, 67, 124, 125, 198). In a mechanism not fully understood, C-Nap1 becomes phosphorylated during the G2/M phase of the cell cycle by Nek2A, then dissociates from duplicated centrosomes to allow centrosomal disjunction (known as centrosome splitting). C-Nap1 phosphorylation is believed to be controlled by Nek2A and protein phosphatase 1 (PP1). According to the model depicted in Figure 4 (43), the loss of centriole-centriole cohesion occurs at the onset of

mitosis in order for duplicated centrosomes to separate, in preparation for spindle formation. Several experiments have supported the role that C-Nap1 plays in centrosome cohesion. First, antibody-mediated interference with C-Nap1 function was shown to cause centrosome splitting (125). Second, a dominant negative C-Nap1 mutant (125) and small interfering RNA-mediated depletion of C-Nap1 also caused premature centrosome splitting (6). Third, the overexpression of Nek2A resulted in centrosome splitting (40, 127). Finally, the centrosome normally splits during mitosis due to C-Nap1 phosphorylation (43, 124, 125, 127, 203).

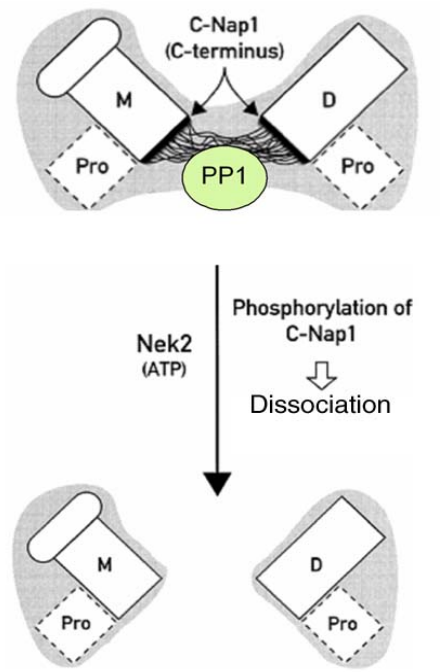

Figure 4. A possible role for C-Nap1 in centriole-centriole cohesion.
In this model, C-Nap1 functions as an important cohesion molecule that connects the proximal ends of centrioles to each other during interphase. PP1 maintains C-Nap1 in the dephosphorylated state throughout interphase. By phosphorylating C-Nap1, Nek2 (or other C-Nap1-specific kinase) may cause dissociation of C-Nap1 during mitosis. This would result in the loss of cohesion and thereby permit centrosome splitting. The symbols are as follows: M denotes the mother centriole (depicted with a distal appendage because it is more complex); D is the daughter centriole; Pro refers to procentrioles; wavy lines indicate the proposed intercentriolar link; gray shading indicate the PCM adapted from Fry et al., 1998, J Cell Biol 141:1563-74.

The kinase that phosphorylates C-Nap1 and leads to centriole splitting is known as Nek2A (43, 127). On the other hand, the PP1 phosphatase counteracts the function of Nek2A by dephosphorylating both Nek2A and C-Nap1 (67, 127, 129). Because a diverse range of cellular functions are regulated by reversible protein phosphorylation through the actions of protein kinases and phosphatases, it is not surprising that PP1 and Nek2A regulate each other and exert opposing influences over C-Nap1 function. Consistent with this notion, the overexpression of NEK2A was found to induce premature centrosome splitting (43). It was also reported that C-Nap1, Nek2A, and PP1 associated in a ternary complex (67). Interestingly, the overexpression of a catalytically inactive Nek2A induces centrosome amplification whereas the wild-type form induces centrosome disjunction but not amplification (40).

PP1 is Important in the Regulation of the Centrosome

PP1 is a serine/threonine specific phosphatase and functions as a ubiquitous eukaryotic enzyme that regulates a variety of cellular processes through the dephosphorylation of its substrates. For example, PP1 enables the storage of energy in the form of glycogen, the relaxation of actomyosin fibers, the return to basal patterns of protein synthesis, the recycling of transcription and splicing factors, the induction of apoptosis when cells are damaged beyond repair and the exit from mitosis (22). Specifically, PP1 dephosphorylates its substrate through the hydrolysis of phosphoric acid monoesters into phosphate ions and molecules with free hydroxyl groups. In direct contrast, kinases attach phosphate groups to their substrates by using energetic molecules such as ATP. Phosphorylation can activate or inactivate enzymes sometimes enabling or disabling protein-

protein interaction. Clearly, kinases and phosphatases are integral members of most signal transduction pathways. Although the PP1 catalytic domain is normally regulated by PP1 regulatory proteins, serine/threonine specific protein phosphatases are regulated by their location within the cell and by specific inhibitor proteins. The three catalytic subunits of PP1, α, β, and γ localize to different regions within cells. Importantly, PP1α localizes to the centrosome (76) whereas PP1β and PP1γ localize in other regions of the cell. Moreover, as indicated earlier, PP1α was demonstrated to interact with C-Nap1 and NEK2A in regulating the centrosome (67, 127).

PP1 is also targeted by certain viruses. The Human immunodeficiency virus (HIV-1) and Herpes simplex virus (HSV) interact with PP1 to enhance viral protein expression. Specifically, the HIV-1 Tat protein interacts with PP1 to increase viral transcription, and HSV expresses the $\gamma_1$34.5 protein, which interacts with PP1 to enhance translation by preventing the termination of protein synthesis (66).

Centrosome Abnormalities

Previously, it was reported that Tax localizes to centrosomes and induces the formation of supernumery centrosomes (151). A recent report demonstrated that Tax can interact with TaxBP1, also known as rootletin, which is closely related to the coiled-coil centrosomal proteins C-Nap1, CEP110, and ninein (23). Both C-Nap1 and rootletin are believed to function in centrosome cohesion. Further support that the HTLV-1 virus, specifically Tax, is involved in centrosome abnormalities was demonstrated in ATL cells, as indicated earlier. ATL cells displayed an average increase of 14% centrosome amplification compared to control cells (144). Other virus-induced examples of centrosome abnormalities

include the human papillomaviruses (HPV), Hepatitis B virus (HBV), and Kaposi's sarcoma-associated herpesvirus (KSHV). The HPV E6 and E7 proteins are associated with centrosome duplication errors (36). Similarly, the KSHV latency associated nuclear antigen is also associated with centrosome amplification (148). Also, HBV X protein induces aberrant centriole replication and abnormal mitotic spindles (42). These examples of centrosome amplification have all been caused by tumor viruses, though the mechanisms are not quite clear.

Centrosome amplification is common in many human cancers. The presence of more than two centrosomes at mitosis is considered abnormal. The possesion of multiple centrosomes is problematic because it can induce the assembly of multipolar spindles which may then lead to unequally distributed chromosomes (176). Centrosome abnormalities and aneuploidy are found in pre-invasive carcinomas and in early events in cellular transformation (50, 157, 176). Invasive breast cancers show a positive, linear correlation between centrosome amplification and aneuploidy (109). Centrosome anomalies are also frequently found in malignant tumors in a variety of other epithelial cancers such as colon, prostate or lung carcinomas (110, 155, 156). In summary, centrosome abnormalities incurred by various insults to the cell have an intimate relationship with cancer.

HYPOTHESIS AND RATIONALE

Research has shown that HTLV-1 Tax expression can transform cells and induce tumors and leukemia in mice. Transformation is a key component of cancer development as it offers cell growth advantages and dysregulates important proteins that are involved in cell cycle regulation. As previously described, the mitotic effects of Tax can be recapitulated in *S.*

cerevisiae. Recent data from our laboratory suggest that a loss of SIC1p, the inhibitor of *S. cerevisiae* G1/S cyclin-dependent kinase, prevents Tax-induced arrest. Importantly, a loss of $p27^{KIP1}$ function is a common feature of HTLV-1 transformed human T-cells that constitutively express Tax (96). In this dissertation, the yeast system is utilized to investigate the mechanisms by which Tax perturbs the cell cycle.

The critical role of centrosomes in maintaining genomic stability through mitosis and cell division, the high frequency of centrosome amplification in malignancy, and the prevalence of centrosome amplification in ATL cells suggest that the mechanism underlying this amplification is an important aspect of ATL molecular pathogenesis. This prompted our interest in elucidating this mechanism. HTLV-1 Tax affects multiple, diverse, cellular processes, any or all of which may be important in ATL molecular pathogenesis. Additionally, Tax expressing eukaryotic cells are often binucleated or multinucleated, and our preliminary data demonstrating direct interaction of Tax with C-Nap1, a protein important for maintaining centrosome cohesion and coordinating centrosome disjunction, suggested a possible role for Tax in HTLV-1-induced centrosome amplification. Subsequent demonstration of direct interaction between Tax and a C-Nap1 regulator, PP1, which shares a high degree of similarity with PP2A, an established biologically relevant Tax-interacting partner (44), suggested a potential mechanism for centrosome amplification.

I propose that the effects of HTLV-1 Tax on mitotic regulation are important in the process leading to aneuploidy. The two specific aims of this dissertation are below.

(1) To determine if HTLV-1 Tax mutants that do not induce G1 arrest are also disabled in activating the anaphase promoting complex and transformation. This hypothesis was tested by determing the ability of Tax mutants generated in yeast to be disabled in inducing cell

cycle arrest; the consistency of the mutants to be disabled in activating the anaphase promoting complex; the ability of the Tax mutants to be disabled in transforming.

(2) To determine if Tax-mediated C-Nap1 dysregulation is an essential component of the mechanism underlying ATL cell centrosome amplification. This hypothesis was tested by determing the ability of Tax expression to induce centrosomal abnormalities; the consistency of the temporal and spatial subcellular localization of Tax, C-Nap1, and PP1 with our hypothesis; the ability of Tax to disrupt both normal C-Nap1 phosphorylation and normal centrosome disjunction.

CHAPTER 2

HTLV-1 Tax Mutants That Do Not Induce G_1 Arrest Are Disabled in Activating the Anaphase Promoting Complex.

Running Title: Tax-Induced Rapid Senescence is Mediated by APC/C

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Key words: HTLV-1 Tax, LTR, NF-κB, anaphase promoting complex/cyclosome, ubiquitination, cell cycle

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ABSTRACT

HTLV-1 Tax is a potent activator of viral transcription and NF-κB. Recent data indicate that Tax activates the anaphase promoting complex/cyclosome (APC/C) ahead of schedule, causing premature degradation of cyclin A, cyclin B1, securin, and Skp2. Premature loss of these mitotic regulators is accompanied by mitotic aberrations and leads to rapid senescence and cell cycle arrest in HeLa and *S. cerevisiae* cells. Tax-induced rapid senescence (*tax*-IRS) of HeLa cells is mediated primarily by a dramatic stabilization of $p27^{KIP}$ and is also accompanied by a great surge in the level of $p21^{CIP1}$ mRNA and protein. Deficiencies in $p27^{KIP}$ prevent Tax-IRS. A collection of *tax* point mutants that permit normal growth of *S. cerevisiae* have been isolated. Like wild-type *tax*, many of them (C23W, A108T, L159F, and L235F) transactivate both the HTLV-LTR and the NF-κB reporters. One of them, V19M, preferentially activates NF-κB, but is attenuated for LTR activation. None of the mutants significantly elevated the levels of $p21^{CIP1}$ and $p27^{KIP1}$, indicating that the dramatic surge in $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ induced by Tax is brought about by a mechanism distinct from NF-κB or LTR activation. Importantly, the ability of these mutants to activate APC/C is attenuated or abrogated. These data indicate that Tax-induced rapid senescence is causally associated with APC/C activation. Finally, the mitotic abnormalities associated with premature APC/C activation may play an important role in cell transformation by Tax.

INTRODUCTION

Human T-lymphotropic virus type I (HTLV-I) is the etiologic agent of adult T-cell leukemia and lymphoma, which occurs in approximately 5% of infected individuals after a long latency period lasting up to 20-40 years. The HTLV-1 viral transactivator/onco-protein Tax is thought to play an important role in T-cell malignancy and HAM/TSP. Tax transactivates the HTLV-1 LTR promoter through its interaction with CREB/ATF-1 (1, 8, 152, 199, 204, 205), CBP/p300 (12, 58, 59, 99, 106), and the Tax-responsive 21-bp repeat element, and activates the NF-κB pathway (26, 82, 178, 187, 192, 194) through the interaction with PP2A/IKKγ (44). In addition to its transactivation functions, Tax also impacts on many aspects of the cell cycle: activating G_1/S transition (90, 118, 145), inactivating p53 functions (158), inducing $p21^{CIP1/WAF1}$ mRNA transcription (2, 24, 33, 89), and inhibiting apoptosis and DNA repair (87, 104). Recent data have indicated that Tax can dramatically perturb mitotic regulation, causing micronuclei formation, cytokinesis failure, and chromosome instability (108, 112, 119). ATL cells are often aneuploid with complex chromosomal abnormalities including trisomy 3, trisomy 7, a partial deletion of 6q, and abnormalities of 14q11 (45). Large lymphocytes with cleaved/cerebriform nuclei are also frequently seen in HTLV-I-positive individuals (93, 166, 174, 180). These pathological findings are likely to be associated with Tax-induced mitotic aberrations.

Indeed, in *tax*-expressing HeLa, MT4, and *S. cerevisiae* cells, the levels of cyclin A, cyclin B and the anaphase inhibitor: securin/Pds1p (precocious dissociation of sister chromatids) were found to be significantly reduced (112). We have found that the loss of cell cycle regulators and the mitotic defects induced by Tax may be causally linked and are associated with premature activation of the anaphase promoting complex/cyclosome

(referred to as APC/C henceforth), a multiprotein E3-ubiquitin ligase that controls the onset of anaphase and mitotic exit by targeting mitotic cyclins and other cell cycle regulators for degradation (111). More recently, we have shown that the cell cycle dysregulation induced by *tax* does not end with mitotic abnormalities. *Tax*-transduced HeLa cells, after passage through a faulty cell division cycle, immediately entered into a senescence-like G_1 arrest termed *tax*-induced rapid senescence, *tax*-IRS (96). These cells expressed high levels of Cdk2 inhibitors: $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ as a consequence of Tax-mediated activation of $p21^{CIP1/WAF1}$ mRNA transcription, and increased stabilization of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ proteins. Consistent with these findings, Tripp et al have also reported that expression of *tax* can cause CD34+ hematopoietic cells to cease proliferation (184).

During normal cell cycle progression, $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ transiently accumulate during G_1, but become degraded in S. The destruction of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ during S phase is regulated by the multisubunit E3 ubiquitin ligase, SCF (Skp-Cullin-F box), together with its substrate-targeting subunit, Skp2 (10, 15, 17, 135, 136, 191) and the cell cycle regulatory protein, Cks1 (10, 47, 191). Recent evidence indicates that Skp2 and Cks1 are both substrates of the Cdh1-associated APC/C (APC^{Cdh1}). They become polyubiquitinated and degraded in late M and early G_1 when APC^{Cdh1} is highly active. This renders SCF^{SKP2} inactive and allows $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ levels to build up in G_1. When *tax* is expressed, APC/C becomes prematurely activated. This causes Skp2 to be polyubiquitinated and degraded starting in S, through G_2/M and in subsequent G_1. The drastic reduction in Skp2 and possibly Cks1, apparently inactivated SCF^{SKP2}, profoundly stabilized $p21^{CIP1/WAF1}$ and $p27^{KIP1}$, thereby committing cells to senescence. The stabilization and surge of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ in *tax*-expressing cells, therefore, is temporally and causally linked to premature

APC/C activation. In essence, Tax activates the cellular program for mitotic exit far ahead of schedule, thereby driving cells into a state of permanent arrest. Interestingly and as might be predicted, we have found that HTLV-1 transformed T-cells invariably express lower levels of $p27^{KIP1}$. Indeed, a loss of $p27^{KIP1}$ function allows cells to evade *tax*-IRS (96). Along this line of reasoning, it is also interesting to note that *tax*-IRS does not occur in Rat1 fibroblasts. Rather, Rat1 cells lose contact inhibition, form foci on monolayer, and become transformed by *tax*. Several earlier reports have implicated NF-κB actication by Tax as critical for its cell transformation activity (38, 69, 89, 121, 197).

Our earlier results have indicated that expression of Tax in *S. cerevisiae* also leads to unscheduled, APC-mediated degradation of Clb2p and Pds1p, G_2/M delay, chromosome aneuploidy, growth arrest, and loss of cell viability (112). Considering the highly conserved nature of the cellular machineries that control mitosis in eukaryotes, this is probably not surprising. The powerful genetics available for *S. cerevisiae* provides an opportunity to dissect the mechanism by which Tax dysregulates APC/C and mitosis, which is otherwise difficult to perform in human cells. Here we report the isolation of a collection of 26 *tax* point mutants whose expression in *S. cerevisiae* did not lead to growth arrest. Five mutants (V19M, C23W, A108T, L159F, and L235F)—with amino acid substitutions that span the majority of Tax protein sequence—were chosen for in-depth analyses. C23W, A108T, L159F, and L235F transactivated both the HTLV-LTR and the NF-κB reporters. One, V19M, preferentially activated NF-κB, but was attenuated in LTR activation. All became impaired or abrogated in their ability (i) to activate APC, (ii) to increase the levels of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$, and (iii) to cause *tax*-IRS. These data strongly suggest that *tax*-IRS, with the associated mitotic aberrations and the accompanying rise in $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ levels, is

coupled to APC/C activation, and is mechanistically unrelated to the CREB/ATF-CBP/p300 or IKK-NF-κB pathway. Finally, although NF-κB activation by Tax plays an important role in cell transformation, the mitotic abnormalities associated with premature APC/C activation may also be required for this process.

MATERIALS AND METHODS

MutagenesisTax mutagenesis and selection for tax mutants. The CEN plasmid, pRS315 Gal10-Tax, that contains *tax* under the control of a galactose-inducible promoter derived from Gal10 gene has been previously described (112). To introduce mutations into *tax* sequence, pRS315 Gal10-Tax plasmid DNA was exposed to hydroxylamine (1 mg/ml) overnight at 37^{o}C (163). The plasmid was then purified using a PCR purification kit (Qiagen), and used (1 μg) to transform *S. cerevisiae*. The transformants were plated on leucine-dropout plates that contained galactose as the sole carbon source. Colonies that appeared on galactose plates were then picked and seeded in grids on galactose plates, transferred to nitrocellulose filters, lysed, and screened for Tax expression using a monoclonal antibody (4C5) against Tax. Only positive clones were chosen for plasmid extraction/isolation. The plasmids extracted from W303-1a were than used to transform competent *E. coli* for DNA preparation and sequence analysis.

Cloning mutant tax alleles into a lentiviral vector. The lentivirus vector, LV-Tax-SV-Puro, which contains the wild-type *tax* gene under the control of the CMV enhancer promoter and **the puromycin-resistance gene** expressed from the SV40 enhancer/promoter (SV-Puro), has been previously reported (96). A mutant *tax* allele, M47, which carries a diagnostic BglII restriction site in the *tax* coding sequence, was cloned into LV-Tax-SV-Puro

via the BamHI (located immediately upstream of the translational initiation codon) and SmaI (downstream of the M47 mutations) restriction sites to generate LV-M47-SV-Puro. Most mutant *tax* alleles were cloned into LV-M47-SV-puro similarly except that an internal MluI site and an XmaI site, located at the aforementioned SmaI site, were used. The recombinants were identified by a loss of the diagnostic BglII site from the recombinant, and confirmed by DNA sequence analysis. For NH2-terminal mutations that lie upstream of the MluI site, DNA fragments harboring the mutations were generated by PCR and cloned into LV-Tax-SV-puro via the BamHI and MluI sites. puro was cloned as described in the submitted paper (96) by first cloning M47 into the HR'CMV vector and then cloning SV40 puro from pBabe puro. Then HTLV-1 Tax from Nehrenburg was subcloned into the NTaxGFP plasmid. Then, Tax was cloned into the HR'CMV M47 SV40 puro at BamH1 to Xma1 to make the HR'CMV Tax puro. Primers used to amplify Tax the NH2 terminal coding N-terminal region of Tax are 5'TaxBamHI 5'-CGCGGATCCGCCACCATGGCCCACTTCCCAGGGTT-3' (with the translational start site underlined) and 3'TaxXmaI 5'-GCTCTAAGCCCCCGGGGGATA-3'.

Construction of the Tax-inducible reporter, 18x21-DsRed and derivation of the 18x21-DsRed indicator cell line. A highly Tax-inducible enhancer/promoter cassette that contains 18 copies of the Tax responsive 21 bp repeat element upstream of a minimal HTLV-1 promoter (18x21) has been reported previously (203). A blunt-ended BamHI fragment containing the 18x21 cassette was inserted upstream of the DsRed reporter gene in the pDsRed2-C1 (Stratagene) plasmid (blunt-ended at AseI and AgeI sites) to make p18x21DsRed-Neo. A HeLa reporter cell line for Tax was derived by transfecting cells with the p18x21-DsRed-Neo plasmid, followed by G418 (1μg/ml, Invitrogen) selection. G418-

resistant clones were expanded and transduced with the HR'CMV-Tax-puro lentiviral vector and observed for DsRed expression. One clone that had low basal expression but high DsRed expression in the presence of Tax was chosen for this study.

DNA transfection and luciferase reporter assay. Approximately 10^5 293T cells/well in a 12-well plate were transfected with the expression construct for each of the *tax* alleles (0.5 μg) together with either HTLV- LTR-Luc (0.1 μg) or E-selectin-Luc reporter plasmid (0.5 μg) using a calcium phosphate transfection kit (Invitrogen). Forty eight hours after transfection, cells were lysed using 250 μl of reporter lysis butter and 20 μl lysate from each transfection was used for the luciferase assay. After injection of 100ul luciferase substrate (Promega), the luciferase activity was measured by a MLX microtiter plate luminometer. Transactivation functions of V19M, C23W, A108T, L159F, and L235F mutants were further confirmed by including in the transfection mixture 0.5 μg of a control plasmid, pRL-TK, that contains the renilla luciferase reporter gene driven by the herpesvirus thymidine kinase promoter.

Lentiviral vector production and gene transduction. Lentiviral vectors (LV) were produced as previously described after transfection of 293T cells. Culture supernatants were harvested at 24, 48 and 72 h after transfection, pooled, filtered, aliquoted, and stored at -80 ˚C. Viral titers were measured by adding serially diluted LV stocks to $2x10^5$ (HeLa 18x21-DsRed-Neo) cells were seeded in a 24-well plate. Polybrene (8 μg/ml, Sigma) was added to the medium together with the vector stocks to facilitate infection. Three days post-transduction, the number of red cells in each well was counted as a measure of viral titer. To transduce *tax* alleles, $2x10^5$ HeLa cells were first plated in a 6-well plate similar as in (96). They were then transduced with LV (m.o.i. = 2) the next day. After 24 h, the medium was

removed and fresh DMEM containing puromycin (1 μg/ml, Sigma) was added. The selection medium and cell debris were removed 48 h after selection by 1x PBS washes, and replaced with fresh puromycin-free DMEM.

Immunoblot analyses of cells transduced with different *tax* alleles. HeLa cells transduced with LV-Tax or LV-mutant Tax were grown to approximately 70% confluency 4-5 days after initial seeding. SDS sample buffer (2x, 60 μl) was added to each well to lyse the cells. Cell lysates were scraped and transferred to an Eppendorf tubes and heated at 100^{o}C for 5 minutes. Total cell proteins were then resolved by SDS/12% PAGE, transferred to nitrocellulose membrane, cut into strips and probed with antibodies (Santa Cruz Biotechnology) against cyclin B1 (sc-752), actin (sc-1616), I-κBα (sc-1643), p52-NFκB2 (sc-7386), Skp2 (sc-7164), p21$^{CIP1/WAF1}$ (sc-397), and p27^{KIP1} (sc-1641). For detection of Tax, a mouse hybridoma antibody, 4C5, which reacts with the COOH terminal region of Tax, was used.

Cell cycle analysis of Tax mutants in mammalian cells. HeLa cells transduced with LV containing either the wild-type or each of the mutants *tax* alleles were selected with puromycin and maintained as described above. Cells were harvested 4-5 days post-transduction for flow cytometry as previously described (111).

Detection of Clb2p in *S. cerevisia*. Detection of Clb2p in *S. cerevisia* was previously described (112) except that yeast cracking buffer (8M urea, 5% SDS, Tris-HCl (pH6.8), EDTA 0.1 mM, Bromophenol blue 0.4 mg/ml, β-mercaptoethanol 10 μl/ml) was used as the lysis buffer. Immunoblots were carried out with anti-HA (Santa Cruz Biotechnology), anti-PP2A-C (Upstate) and 4C5 (Tax) monoclonal antibodies.

Detection of polyubiquitinated cyclin B. HEK 293T cells were co-transfected with HA-tagged ubiquitin and the various *tax* alleles as above. Cells are washed the next day and harvested 48 h later for immunoprecipitation using the cyclin B1 antibody (Santa Cruz Biotechnology) as previously described (111) except that RIPA buffer (Upstate protocol Tris-HCl, NP40 1%, Na-deoxycholate 0.25%, NaCl 150mM, EDTA 1mM, PMSF 1mM, protease inhibitor cocktail (1 μg/ml), Na_3VO_4 1mM, and NaF 1mM) was used instead of the lysis buffer previously described. Immunoblots were carried out with anti-HA (Santa Cruz), anti-cyclin B1 (Santa Cruz Biotechnology) and 4C5 (Tax) monoclonal antibodies.

Cell transformation analysis of Rat1 cells transduced with *tax* alleles. Rat1 cells transduced with LV containing either the wild-type or each of the mutant *tax* alleles were selected with puromycin. The selected colonies were trypsinized and plated in four wells in 6-well plates. One well, after confluency, was used for Tax immunoblot analysis and the other wells were maintained in the presence of puromycin for 2-3 weeks. Media was changed with puromycin containing media every other day with the cells undisturbed. Colony foci on monolayers became visible between 14-21 days after gene transduction and their number was counted as a measure of cell transformation.

RESULTS

Isolation of *tax* mutants that do not cause growth arrest in *S. cerevisiae*.

In the course of a yeast 2-hybrid screen using Tax as bait, we noticed that the yeast strain expressing the *lexA-tax* fusion grew significantly slower than the *lexA* control. This prompted us to examine more closely the effect Tax exerts on the growth and proliferation of *S. cerevisiae*. To this end, W303a, a standard laboratory yeast strain, was transformed with

pRS315-Gal10-Tax, a CEN plasmid carrying the *tax* gene under the control of a galactose inducible promoter (112). As reported previously (112), expression of *tax* after galactose induction lead to a cessation of cell growth and proliferation. Upon Tax expression, the W303a/Gal10-Tax cells initially suffered a delay in $S/G_2/M$ progression (112). They then became arrested at G_1 phase of the cell cycle. The growth-arrested cells became greatly enlarged in size, but were without buds and displayed severe DNA aneuploidy (112). Their viability was also significantly decreased. These results immediately suggest that *tax* mutants that do not cause growth and proliferation arrest may be readily isolated in *S. cerevisiae* and these mutants may have similar or identical properties in human cells. To isolate *tax* mutants impaired in causing growth arrest, we mutated pRS315-Gal10-Tax by hydroxylamine (163). W303a cells were then transformed with the pool of chemically mutated plasmid preparation, and plated to select for galactose-resistant transformants. The colonies on galactose plates were then screened by colony dot blots for *tax* expression using a mouse hybridoma Tax antibody, 4C5. A total of 26 non-growth-arrest *tax* mutants were identified, for which the respective pRS315 vectors were isolated. We next confirmed loss of the growth arrest phenotype by reintroducing plasmids carrying the mutant *tax* alleles into W303a. As expected, all W303a expressing mutant *tax* alleles readily grew on both glucose and galactose plates (Fig. 5B), while W303a expressing the wild-type *tax* arrested failed to grow on galactose plates as previously described (112).

DNA sequence analyses of the *tax* coding sequence revealed that each of the 26 *tax* mutants contained a single amino acid substitution that resulted from a G to A or C to T transition, as might be expected for hydroxylamine mutagenesis. The altered amino acid residues in the Tax protein sequence are listed in Fig. 5A. Many of the amino acid

substitutions are clustered in the NH_2-terminal half of Tax (20/26). Consistent with the notion that the amino acid substitutions had occurred in important regions of Tax, we noticed that the T130I substitution overlap with the dual amino acid substitutions--T130A L131S--in a well characterized *tax* mutant known as M22, which is partially defective in dimerization and is severely impaired in IKKγ/NEMO-binding and NF-κB activation. Two distinct mutations (G61E and G61R) and (A108T and A108V) were isolated for each of the amino acid residues 61 and 108, suggesting the importance of these residues in protein-protein interactions that mediate Tax functions.

Tax mutants selected in W303a are functional in HTLV-1 LTR and NF-κB trans-activation.

Next, we investigated the biological activities of *tax* mutants in mammalian cells. Mutant *tax* alleles were cloned into a lentiviral vector, HR'CMV-SV40-puro. This vector allowed *tax* to be expressed transiently from the CMV immediate early promoter after DNA transfection or stably after lentivirus vector-mediated gene transduction. We first examined the ability of the Tax mutants to transcriptionally activate luciferase reporters driven respectively by the HTLV-1-LTR (LTR-Luc) and the NF-κB-inducible E-selectin-promoter (E-selec-Luc) (170). Twenty one mutants were analyzed by luciferase reporter assays (Fig. 6). The other five mutants (V24E, C36Y, G61R, P92L, and L183F) were excluded from the reporter assays because of either the drastic amino acid alterations caused by the mutations or the existence of alternative amino acid substitution in the same position. Approximately half of the mutants analyzed (S32F, A47T, H52Y, G61E, L75F, T145I, W147L, P169L, A285T, and S300F) were greatly impaired in both transactivation functions of Tax. Many of these

mutations are in the highly conserved NH_2-terminus of Tax. By contrast, some mutants (C23W, P102L, A108T, A108V, H127T, L159F, 235F, G245D, and D264Y) continued to transactivate both LTR and NF-κB reporters to levels (greater than 50%) comparable to those of the wild-type Tax. Of note, V19M was specifically impaired in LTR activation but remained a potent NF-κB activator, while T130I was defective in NF-κB activation, but exhibited significant LTR activation capability, reminiscent of similar properties of the M22 (T130A L131S) mutation mentioned above. These results indicate that mutations that impaired the ability of Tax to arrest growth of W303a cells did not necessarily affect LTR or NF-κB transactivation. We infer from these data that the growth arrest phenotype of Tax most likely involves interactions with a cellular process distinct from the CREB/CBP/p300 and the IKK/NF-κB pathways.

Expression of tax mutants in HeLa cells

We next selected 5 mutants (V19M, C23W, A108T, L159F, and L235F) that retained the ability to transactivate LTR and/or NF-κB for further analysis. Lentivirus vectors (LV) capable of transducing the mutant *tax* alleles were generated by co-transfection of the respective HR'-CMV-*tax*-SV40-puro vectors together with packaging plasmids that encode HIV structural proteins and VSV G protein as previously reported (96, 139). A stable HeLa cell line, HeLa-18x21-DsRed, which expresses DsRed under the control of a Tax-inducible enhancer/promoter cassette containing 18 copies of the 21-bp repeat upstream of a minimal HTLV-1 promoter (203), was used as the cellular background for introducing the *tax* alleles. As the expression of DsRed in HeLa-18x21-DsRed is strictly Tax-dependent, cells that express Tax after gene transduction can be readily detected by fluorescence microscopy.

HeLa-18x21-DsRed cells were infected with LV carrying the wild-type, V19M, C23W, A108T, L159F, L235F *tax* alleles, or the EGFP gene. The LV-transduced cells were then selected in media containing 1 μg/ml puromycin for 2-3 days. Drug-resistant colonies were then grown in puromycin-free medium for 1 day and observed under a fluorescence microscope for DsRed expression. In agreement with the LTR-Luc reporter activities described above (Fig. 6), C23W, A108T, L159F, and L235F, but not V19M activated DsRed expression (Fig. 7B). As expected, HeLa-18x21-DsRed transduced with the LV-EGFP control did not express DsRed. Previously, we have demonstrated that Tax expression in HeLa cells greatly elevated the levels of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ cyclin-dependent kinase inhibitors, thereby causing HeLa cells to enter into a senescence-like G_1 arrest termed Tax-induced rapid senescence (Tax-IRS) (96). The HeLa cells in Tax-IRS are flat, enlarged, vacuolated, often binucleated, and stained positive for the senescence associated β-galactosidase. Indeed, in agreement with previous results, microscopic examination of both the Hela and HeLa-18x21-DsRed cell line transduced with LV-Tax (wild-type) revealed a prevalence of enlarged and binucleated cells (Fig. 7A and Fig. 7B), consistent with the notion that they were in the state of Tax-IRS. By contrast, the morphology of cells transduced with mutant *tax* alleles, with the exception of A108T, resembled those of control cells transduced with the EGFP gene. Finally, we noted that despite some similarity of A018T cells to Tax (wild-type) cells, the extent of arrest and morphological changes in A108T cells appeared to be attenuated.

Tax mutants whose expression is permissible in *S. cerevisiae* do not cause, or are attenuated in inducing cell cycle arrest in HeLa cells, but remain functional in activating I-κB degradation and p100 processing.

To characterize the various *tax* mutants further, we analyzed the LV-mutant-*tax*-transduced cells by flow cytometry. Three days after puromycin selection, asynchronously grown LV-transduced cells were transferred to puromycin-free medium for 24 h and harvested for analyses. As anticipated from the cell morphology in Fig. 8, most cells that expressed the wild-type Tax (75%) appeared in the G_1 phase of the cell cycle. In contrast, G_1 populations for cells transduced with the various *tax* mutant alleles were significantly lower, albeit somewhat higher than that of the EGFP-transduced control. These results support the notion that those *tax* mutants that failed to cause growth arrest in *S. cerevisiae* are also significantly disabled or attenuated in inducing senescence/cell cycle arrest in mammalian cells with varying degrees of attenuation.

The phenotypes of the *tax* mutants were not due to variations in the levels of Tax protein expression as indicated by immunoblotting (Fig. 9). In accordance with the overall cell morphology and flow cytometry analyses, the levels of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ in the various *tax*-transduced cells correlated with their extent of growth arrest or lack thereof, with wild-type *tax* greatly increasing the levels of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$, second by the A108T mutant, and with the remaining mutants having only moderate to no effect (C23W, L159F, L235F, V19M). As might be expected, the levels of cyclin B1 in the respective cell lines inversely correlated with the growth characteristics of the respective cells. Likewise, the levels of Skp2 in the transduced cells also correlated with their respective cyclin B1 levels. Finally, consistent with the ability of the *tax* mutants to transactivate the E-seletin-Luc NF-

κB reporter (Fig. 6), the levels of I-κBα in the *tax*-transduced cells were reduced, while those of p52, the mature NF-κB2, were increased. Here again, in general agreement with the reporter assays, the A108T mutant is equivalent or possibly better than the wild-type *tax* in inducing I-κBα degradation and p52 NF-κB processing, second by V19M and C23W, followed lastly by L235F and L159 mutants. While for some mutants (A108T, L159F, and L235F) there appears to be some correlation between the severity of cell cycle arrest/senescence phenotype and the degree of NF-κB activation, for others such as V19M and C23W, that are strong NF-κB activators, the senescence phenotype was significantly attenuated. These results support the notion that the Tax-induced cell cycle arrest/rapid senescence (Tax-IRS) and increase in $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ levels are causally related and do not involve directly either the CREB/CBP/p300 or the IKK/NF-κB pathway. Whether the IKK/NF-κB pathway may share a common Tax-targeted regulatory factor with the cell cycle/APC pathway remains to be seen.

***S. cerevisiae*-viable *tax* mutants are attenuated in APC/C activation.**

We have shown previously that the mitotic abnormalities and rapid senescence that Tax induces in *S. cerevisiae* and HeLa cells are associated with unscheduled activation of the anaphase promoting complex and the premature degradation of mitotic/cell cycle regulators including cyclin A, Clb2/cyclin B, Pds1/securin, and Skp2 (112). The levels of cyclin B1, Skp2, $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ in the HeLa cells expressing the various *tax* alleles suggest that the *S. cerevisiae*-viable *tax* mutants are impaired in APC/C activation. To determine the effect of the *tax* mutants on APC/C directly, we introduced them into a yeast strain, KY630, which contains a chromosomally integrated HA-CLB2 at the CLB2 locus. As anticipated,

upon induction of *tax* expression for 2 h, a reduction of Clb2p in cells expressing wild-type *tax* was observed compared to the *tax*-null control. By contrast, the *S. cerevisiae*-viable V19M, C23W, A108T, L159F, and L235F *tax* mutants were attenuated in causing Clb2p reduction/degradation (Fig.10A). Similarly, when the wild-type and mutant *tax* alleles were individually co-transfected with HA-tagged ubiquitin into 293T cells, the *tax* mutants were also found to be attenuated in inducing cyclin B1 polyubiquitination when compared to the wild-type control (Fig. 10B). The extents of attenuation of the five *tax* mutants in *S. crevevisiae* versus 293T cells were not exactly identical. This may reflect subtle structural differences between *S. crevevisiae* and human anaphase promoting complex. Finally, the degrees of the mutants to cause cyclin B1 polyubiquitination (A108T ≥ C23W > L159F, L235F, and V19M) correlated largely with the levels of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ increase in the transduced cells (A108T > C23W > L159F, L235F, and V19M). Taken together, these results support the idea that unscheduled activation of the anaphase promoting complex is responsible for the Tax-induced rapid senescence/cessation of cell proliferation in both human and *S. cerevisiae* cells.

NF-κB activation by Tax is not sufficient for the transformation of Rat1 cells.

Tax is known to cause cell transformation in Rat1 cells (38, 69, 89, 121, 197). Rat1 fibroblasts transformed by *tax* lose contact inhibition and form foci on cell monolayer. Several earlier reports have suggested that NF-κB activation by Tax may be important for this process (121, 197). Recent data have also indicated that the PI3K-Akt pathway in the transformed Rat1 cells becomes highly activated. Since the *tax* mutants characterized here are attenuated in inducing mitotic abnormalities and cell cycle arrest, but retain significant to

full NF-κB activating function, we tested their cell transforming function. Lentivirus vectors carrying the respective *tax* alleles were adjusted to similar titers and used to infect Rat1 fibroblasts. The infected cells were then selected and maintained in the presence of puromycin for 2-3 weeks. Colony foci on monolayers became visible between 14-21 days after gene transduction and their number was counted as a measure of cell transformation. As shown in Fig. 14, cells transduced with the wild-type *tax* gene gave rise to multiple foci. By contrast, all but the A108T mutant have completely lost their ability to induce foci formation. Importantly, even though the A108T allele activated NF-κB as well as or better than wild-type *tax*, its ability to induce foci formation is significantly reduced, albeit not completed lost. In aggregate, these results indicate that NF-κB activation by Tax alone is not sufficient for transformation of Rat1 cells. The mitotic/cell cycle abnormalities as a result of APC/C activation may also play an important role in the process.

DISCUSSION

In this study, we have described a collection of 26 *tax* single-point mutants that are disabled in causing cell cycle arrest in *S. cerevisiae*. A total of 21 *tax* alleles were analyzed further by luciferase reporter assays for LTR and NF-κB transactivation. Approximately half of the amino acid substitutions likely have impacted on critical regions of Tax so as to render it severely defective. Special attention was directed to five mutants (V19M, C23W, A108T, L159F, and L235F) that remained strong LTR and NF-κB transactivators. Their phenotypes in HeLa cells were largely consistent with those seen in *S. cerevisiae*—all were attenuated or significantly impaired in causing $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ accumulation, but remained able to induce I-κB degradation and p100 NF-κB2 processing. Whereas the majority of HeLa cells

transduced with wild-type *tax* entered into *tax*-IRS, cells transduced with each of the 5 mutant *tax* alleles continued to proliferate, albeit at rates that varied dependent on the levels of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ expressed. These results validated the utility of *S. cerevisiae* as a model for dissecting the mitotic abnormalities and rapid senescence/cell cycle arrest induced by Tax.

The levels of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ are regulated through transcription, phosphorylation (by cyclinE/Cdk2), subcellular localization, ubiquitination, and proteasome-mediated degradation (15, 57, 179). The E3 ubiquitin ligase, SCF, together with its substrate-recognition subunit, Skp2, mediates the ubiquitination and degradation of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ (10, 47, 191). The level of Skp2 oscillates in a cell cycle-dependent manner. Recent data have indicated that Skp2 and another SCF subunit, Cks1, are substrates of the Cdh1-associated APC/C (APC^{Cdh1}) (10, 191). Both become ubiquitinated and degraded in late M and early G_1 when APC^{Cdh1} is highly active. This inactivates SCF and allows $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ to accumulate transiently in G_1. We have shown recently that in HeLa cells transduced with *tax*, early APC/C activation set in motion premature loss of cyclin A, cyclin B, securin, and Skp2, and cause a dramatic build-up of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ during S phase. After an aberrant mitotic division cycle, the great surge in $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ in *tax*-expressing cells then commits cells into a state of irreversible cell cycle arrest. Results from the present analysis, i.e. mutations in *tax* that abrogated induction/stabilization of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ also disabled APC/C activation, are in agreement with that conclusion.

Activation of $p21^{CIP1/WAF1}$ mRNA transcription by Tax has been reported previously (20, 21, 24, 32). Because a Tax mutant, M47, which is deficient in LTR activation, became

disabled in activating $p21^{CIP1/WAF1}$ promoter, some of these earlier studies have suggested that Tax-induced increase in $p21^{CIP1/WAF1}$ resulted from transactivation via the CREB/ATF-CBP/p300 pathway (24, 32). Since four of the 5 mutants analyzed here activated LTR-luciferase reporter at levels (70-80%) comparable to that of the wild-type *tax*, yet were substantially impaired in causing $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ increase, we conclude that the CREB/ATF-CBP/p300 pathway is most likely not the principal determinant in the accumulation of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$. Likewise, several of the mutants—A108T, V19M and C23W, in particular—are potent activators of IKK-NF-κB as indicated by luciferase reporter assays and the extent of I-κB degradation and p100 processing. These mutants are nevertheless impaired in elevating $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ levels. These results support the notion that the NF-κB pathway is not directly responsible for the Tax-induced increase in $p21^{CIP1/WAF1}$ and $p27^{KIP1}$. Our data indicate that a major factor for Tax-induced $p21^{CIP1/WAF1}$ increase is protein stabilization resulting from APC/C-mediated degradation of Skp2 (96). Therefore, promoter transactivation by Tax may only contribute moderately to the overall build-up of $p21^{CIP1/WAF1}$ protein. This would explain the discrepancy between the data reported here and the earlier studies which relied heavily on $p21^{CIP1/WAF1}$ promoter-luciferase reporter assays. Finally, we have shown that via a tripartite interaction, Tax, PP2A and IKKγ form a stable ternary complex wherein PP2A activity is inhibited or diminished by Tax. In essence, PP2A inhibition by IKKγ-bound Tax maintains IKK in an active, phosphorylated state, causing constitutive phosphorylation and degradation of I-κB, which, in turn, leads to potent activation of genes under NF-κB/Rel control. Since PP2A regulates many critical cellular processes, it is conceivable that APC/C activation by Tax is also mediated through an inhibition of PP2A. In this sense, a *tax* mutant deficient in PP2A interaction will be disabled for both NF-

κB and APC/C activation, but may continue to transactivate LTR. Several tax mutants, H43Q, K85N, and M22 (T130A, L131S) have been shown previously to be disabled for PP2A binding (44). Both H43Q and M22 are deficient in NF-κB but not in LTR transactivation, while K85N is defective for both. Preliminary analyses suggest that these three mutants are also impaired in inducing cell cycle arrest. Mutants like H43Q and M22, with the possible exception of T130I, however, are not highly represented in the current collection. This may be due, in part, to the importance of the PP2A-binding domain of Tax in mediating other critical protein-protein interaction.

Tax has been reported to transform Rat1 fibroblasts previously. The ability of Tax to activate NF-κB appears to play an important role in this process. The present data indicate that NF-κB activation alone is not sufficient for transformation of Rat1 fibroblasts demonstrated by all mutants tested (V19M, C23W, A108T, L159F, and L235F).

The biological/virological relevance for the profound cell cycle arrest induced by Tax remains unclear. It is possible that HTLV-1-infected T-cells that are arrested in a senescence-like state may persist longer *in vivo* and may, in this condition, be co-opted to devote significant cellular resources to virus replication. Alternatively, the dramatic morphological changes associated with the senescence-like arrest may facilitate virus assembly and/or transmission. Many of the *tax* mutants characterized here that remain functional in transactivating viral LTR and NF-κB can be incorporated into an infectious molecular clone of HTLV-1 to address this question.

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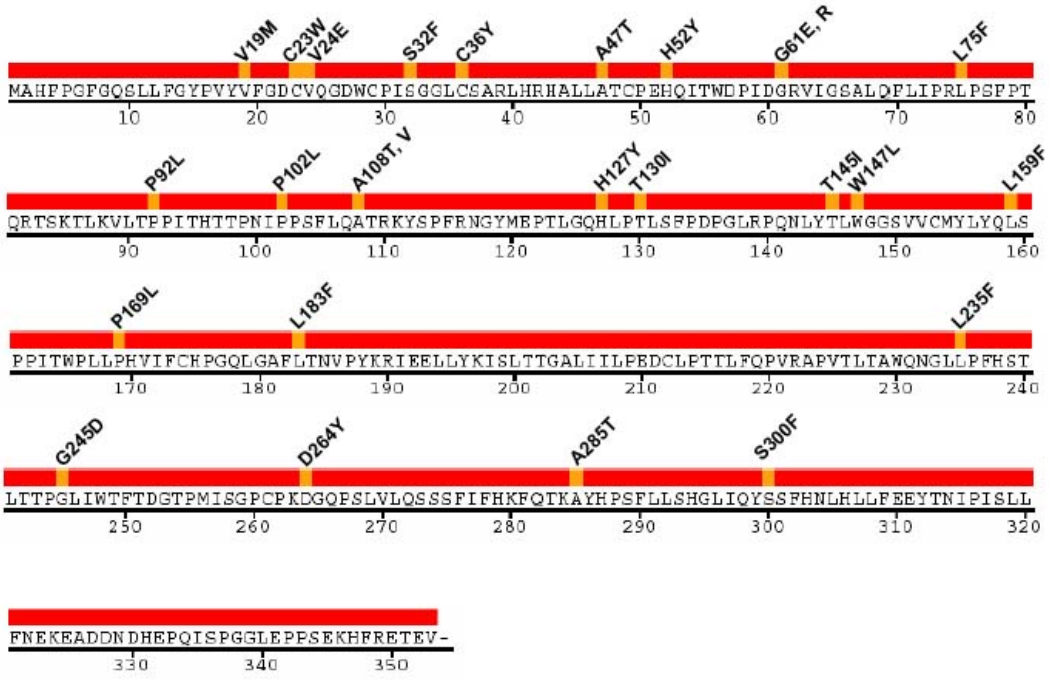
A



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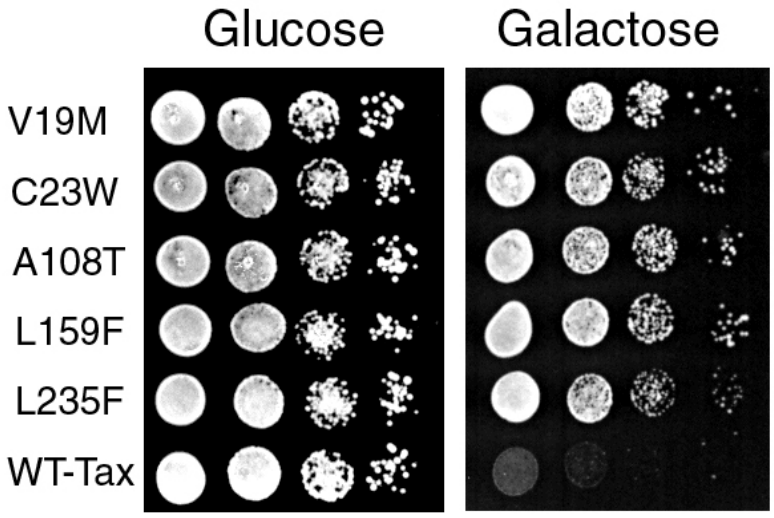


Figure 5. (A) A summary of amino acid substitutions in HTLV-1 *tax* point mutants whose expression did not cause growth arrest in *S. cerevisiae*.
The complete amino acid sequence of HTLV-1 Tax is shown with the amino acid alteration in each point mutant indicated above. **(B) Expression of 5 representative HTLV- *tax* point mutants in *S. cerevisiae*.** W303-1a cells were transformed with the Gal10-Tax (WT-Tax) or its equivalent carrying each of five mutant *tax* alleles and plated on agar plates containing 2% raffinose or 2% raffinose plus 2% galactose. The amino acid alterations in *tax* mutants are as indicated.

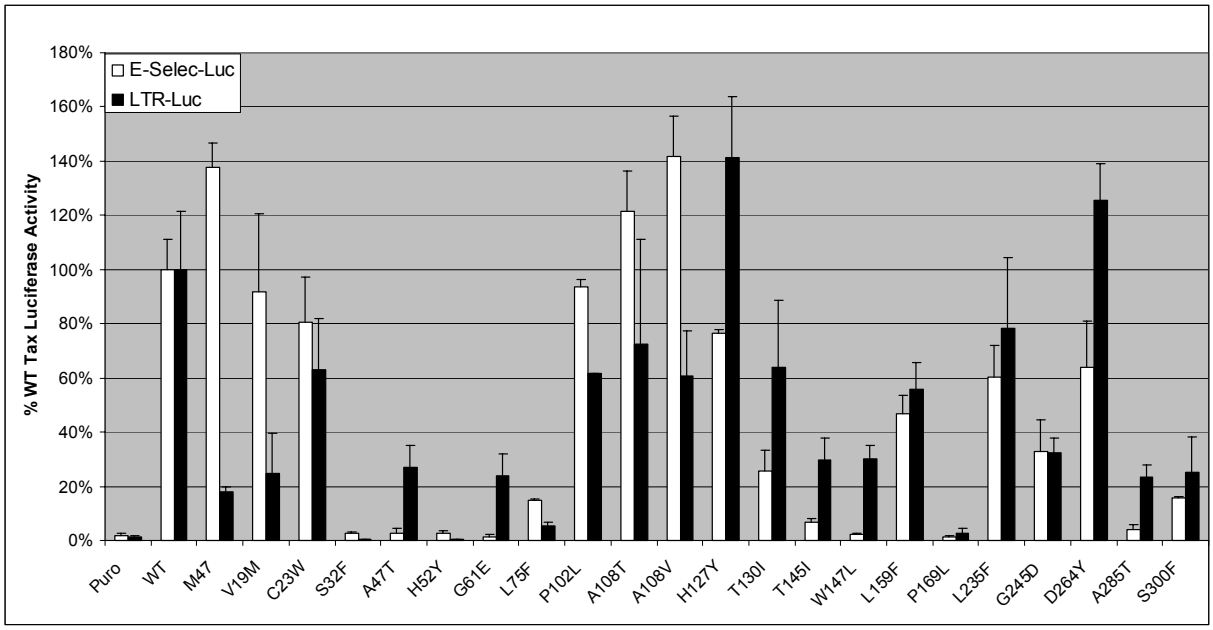
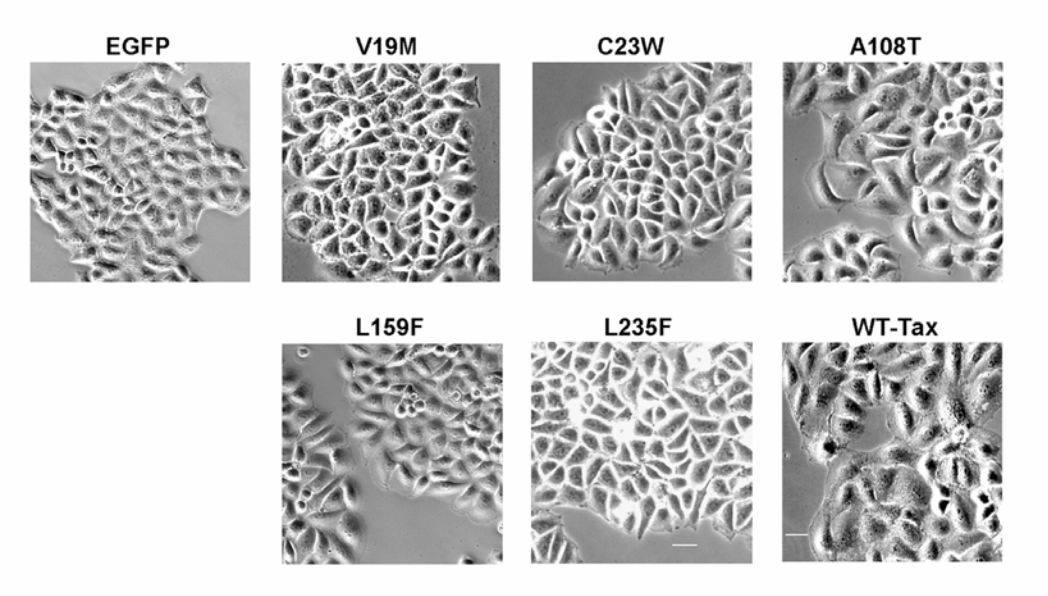


Figure 6. HTLV LTR and NF-κB transactivation functions of *tax* mutants.
The mutant *tax* alleles were subcloned into a lentivirus vector, HR'CMV-SV-puro. The activity of each mutant to transactivate HTLV-1 LTR and NF-κB was determined by cotransfection of an HTLV-1 LTR luciferase construct or an E-selectin luciferase construct with each HR'CMV-*tax* mutant construct into 293T cells. The HTLV LTR (solid bars) and NF-κB (open bars) reporter activities of each mutant were normalized against those of the wild-type *tax* and expressed as % wild-type activity.

A



B

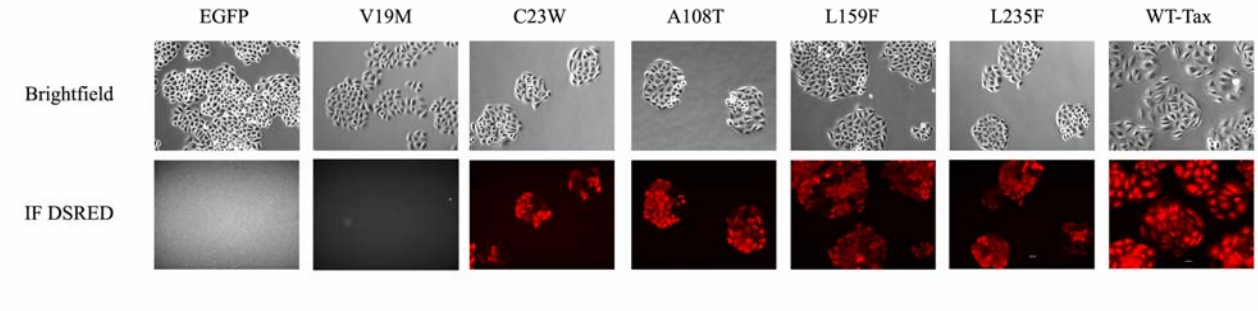


Figure 7. (A) A comparison of the morphologies of HeLa cells transduced with *tax* mutants.
Morphology of Hela cells transduced with a lentivirus vector carrying, respectively, the wild-type, V19M, C23W, A108T, L159F, and L235F mutant *tax* alleles. The *tax*-transduced cells were selected in medium containing puromycin 1 μg/ml for 3 days. The puromycin-resistant colonies were photographed. The scale bar represents 20 μm. **(B) Transactivation of a Tax-specific reporter HeLa cell line transduced with *tax* mutants.** A HeLa cell line containing a stably integrated Tax reporter cassette, 18x21-DsRed, was transduced with a lentivirus vector, HR'CMV-SV-puro, harboring wild-type or each of the mutant tax alleles. The *tax*-transduced cells were then selected in medium as above. Puromycin-resistant colonies were visualized and photographed using an Olympus IX8 inverted fluorescence microscope. The scale bar represents 20 μm.

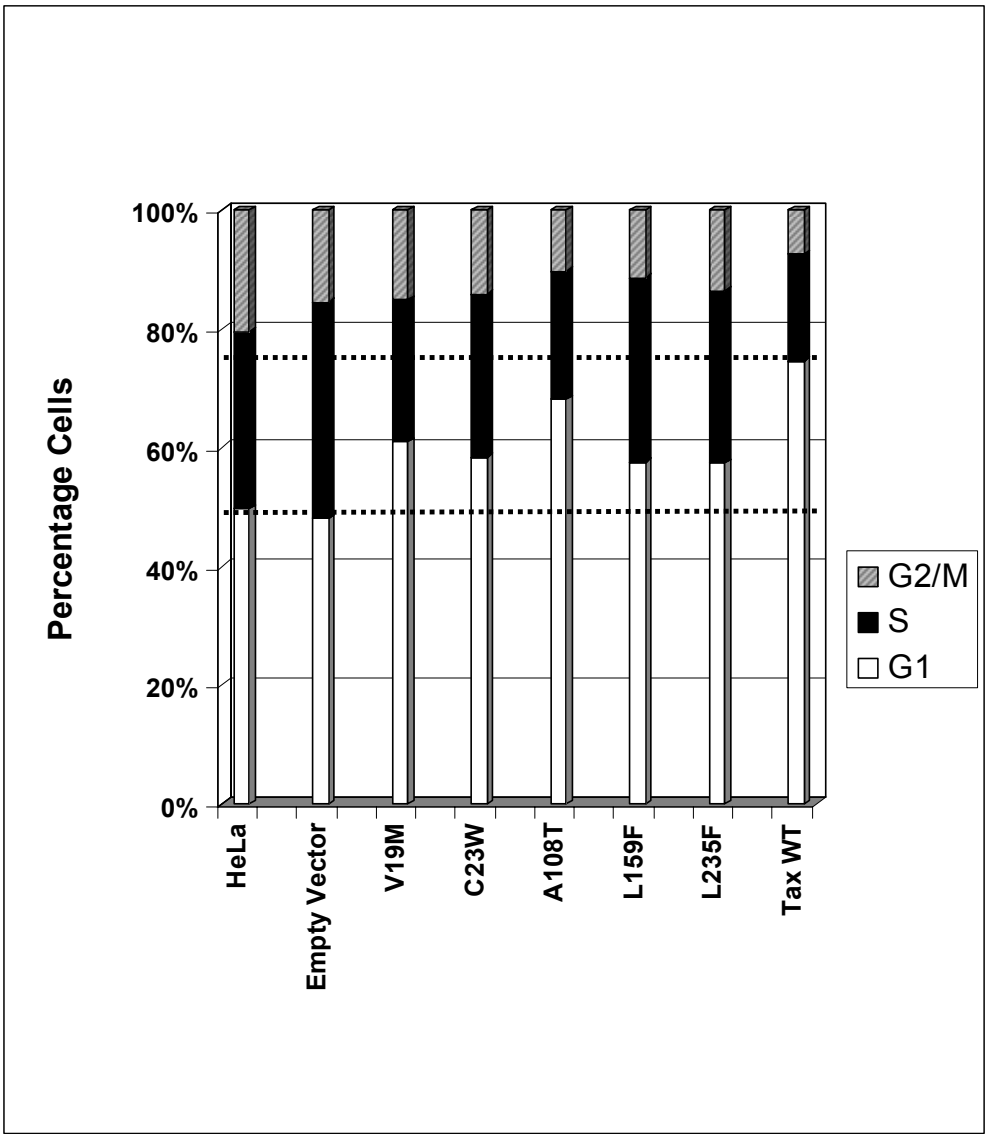


Figure 8. *S. cerevisiae*-viable *tax* mutants are attenuated in inducing cell cycle arrest/senescence in HeLa cells.

HeLa cells transduced with a lentivirus vector carrying wild-type or mutant *tax* were selected in puromycin for 72 h as above and then grown in puromycin-free medium for 24 h. Cells in each group were then fixed in 70% ethanol, stained with propidium iodide, and analyzed by flow cytometry. Based on flow cytometry chromatograms, a bar graph representation of the fraction of cells in G_1, S, G_2/M phases of the cell cycle after transduction with *tax* alleles is shown.

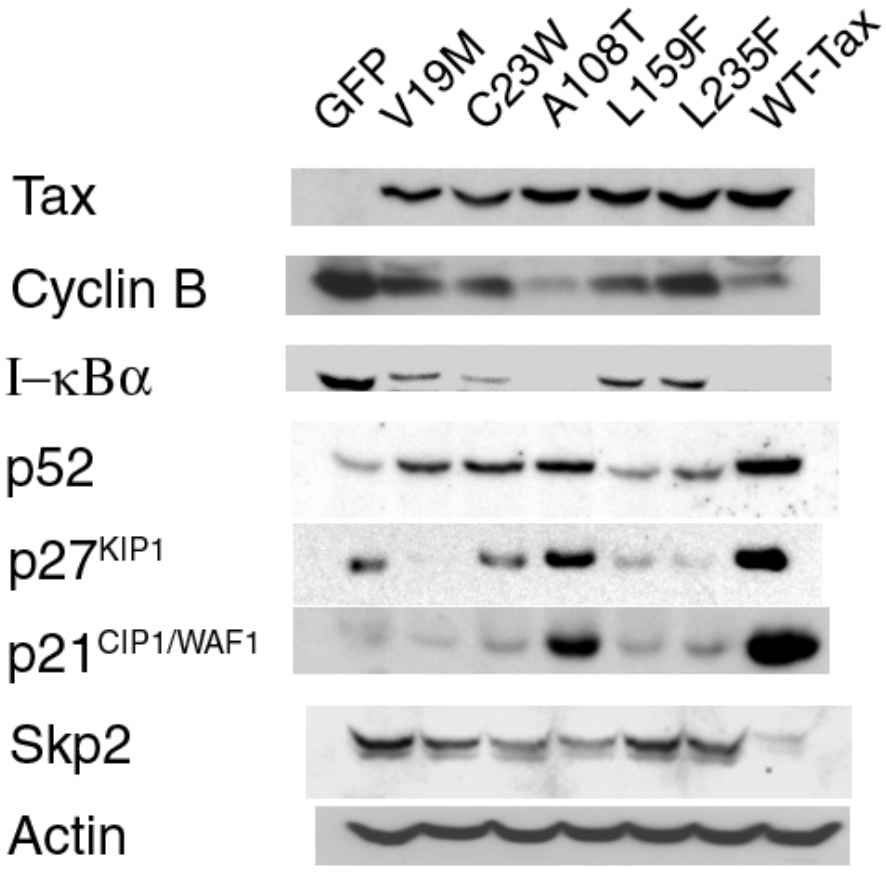


Figure 9. Immunoblot analyses of HeLa cells transduced with wild-type or mutant *tax* alleles.

HeLa cells transduced with the wild-type or the respective mutant *tax* allele were harvested. Cell lysates were prepared, resolved by SDS-12% PAGE, and probed with antibodies against Tax, cyclin B1, I-κBα, NF-κB, p52, $p27^{KIP1}$, $p21^{CIP1/WAF1}$, Skp2, and actin, respectively.

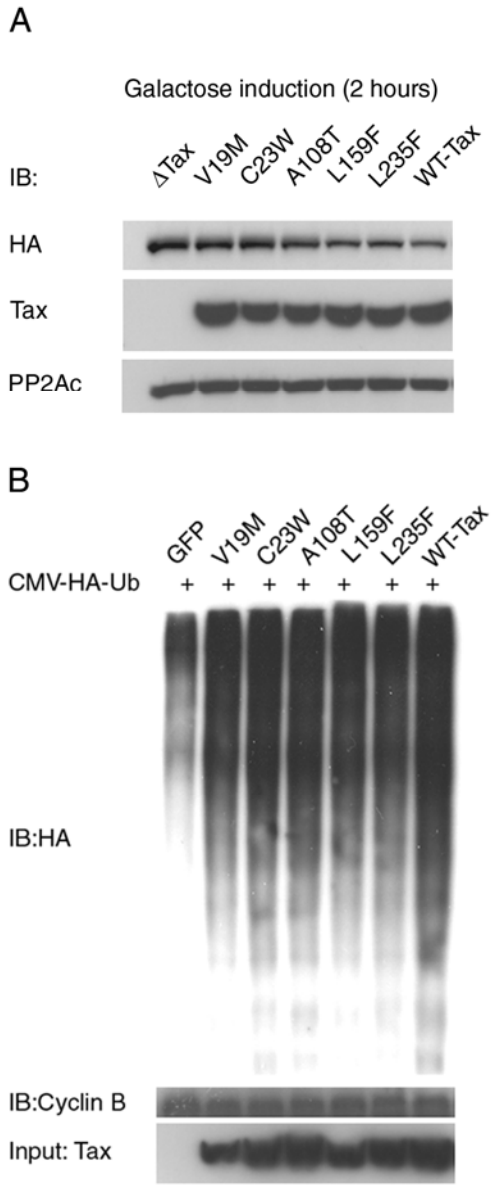


Figure 10. *S. cerevisiae*-viable *tax* mutants are attenuated in APC/C activation.
(A) KY630 cells carrying a gene encoding CLB2-3XHA integrated at the CLB2 locus were transformed with either Gal10-wild-type tax or Gal10-mutant *tax*. KY630/Gal10-*tax* cells were cultured in SC medium containing 2% raffinose at 30°C overnight for 12h, then diluted to 0.3 A_{600}, grown to mid-log phase, and induced for *tax* expression by the addition of 2% galactose. Cell lysates were prepared after 120 minutes. Immunoblots were carried out with anti-HA and 4C5 monoclonal antibodies. (B) 293T cells were transfected with a plasmid encoding HA-tagged human ubiquitin together with an expression plasmid for wild-type tax or mutant *tax*. The cells were arrested at the G_1/S border by a single thymidine treatment, released into complete DMEM containing 10μM MG132 for 5 h, immunoprecipitated with cyclin B1 antibody and immunoblotted with an HA antibody to detect polyubiquitinated cyclin B1.

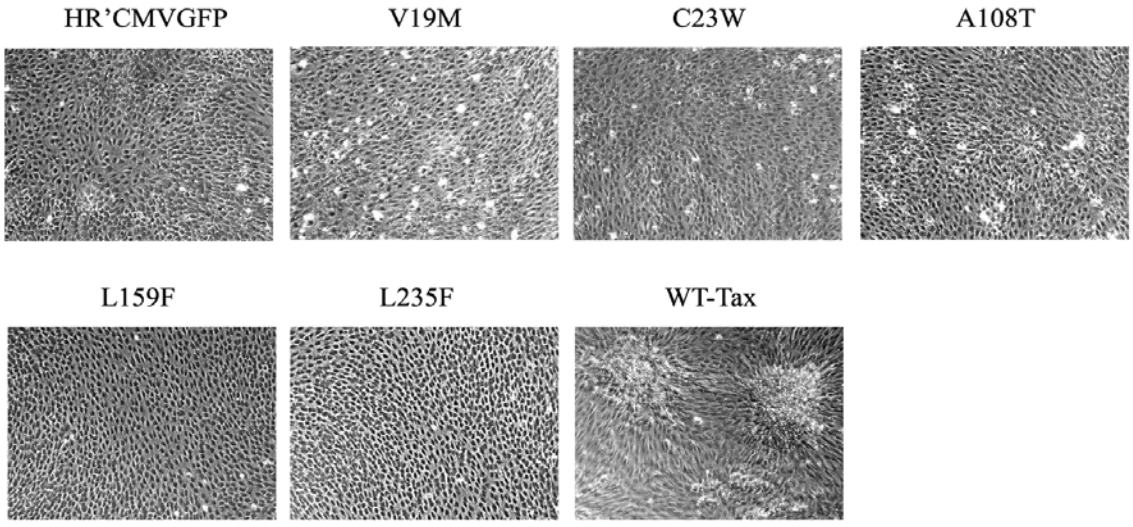


Figure 11. *S. cerevisiae*-viable *tax* mutants do not transform Rat1 fibroblasts.
Rat1 cells were transduced with the various *tax* alleles as above and selected with puromycin (1 μg/ml). Foci formation was observed 21 days after lentiviral vector transduction. Representative foci or lack thereof seen in wild-type or mutant *tax*-transduced Rat-1 fibroblasts.

CHAPTER 3

HTLV-1 Tax Association with C-Nap1: Dysregulated C-Nap1 Phosphorylation and Centrosomal Abnormalities

ABSTRACT

The centrosomal Nek2-associated protein 1 (C-Nap1) is a large coiled-coil protein that plays an important role in centrosome cohesion. C-Nap1 localizes to the ends of centrioles where it links mother and daughter centrosomes together. When C-Nap1 is phosphorylated during G2/M, it releases duplicated centrosomes and allows centrosome disjunction to occur in a process also known as centrosome splitting. C-Nap1 phosphorylation is regulated by never-in-mitosis A (NIMA)-related kinase 2 (Nek2A) and protein phosphatase 1 (PP1). In interphase, C-Nap1 is maintained in the dephosphorylated state by PP1 but becomes phosphorylated by Nek2A during mitosis. Here we show that HTLV-1 Tax can interact directly with C-Nap1 and PP1 by yeast 2-hybrid assay and immunoprecipitation, and Tax co-localizes with both proteins to the centrosome. Importantly, Tax expression leads to a dramatic reduction of C-Nap1 phosphorylation and a failure in centrosome disjunction. Our data suggest that Tax may facilitate the recruitment of PP1 to C-Nap1, thereby prevents C-Nap1 phosphorylation and chromosome disjunction, leading to centrosomal abnormalities and mitotic abnormalities.

INTRODUCTION

The Human T-lymphotropic virus type I (HTLV-I) is the etiologic agent of adult T-cell leukemia and lymphoma, which occurs in approximately 5% of infected individuals after a long latency period of 20-40 years. The HTLV-1 viral transactivator/onco-protein Tax is thought to play an important role in T-cell malignancy and HAM/TSP (49). Tax transactivates the HTLV-1 LTR promoter through its interaction with CREB/ATF-1 (1, 8, 152, 199, 204, 205), CBP/p300 (12, 58, 59, 99, 106), and the Tax-responsive 21-bp repeat element, and activates the NF-κB pathway (26, 44, 82, 178, 187, 194). In addition to its transactivation functions, Tax also impacts on many aspects of the cell cycle (90, 96, 108, 111, 112, 118, 145).

The centrosome functions as the microtubule organizing center (MTOC) and is a cell cycle-regulated organelle critical for mitosis. The centrosomal Nek2-associated protein 1 (C-Nap1) is a large coiled-coil protein of 2,442 amino acid residues in size (43), which, together with another large coiled-coil protein, rootletin, forms the physical linkage between duplicated centrosomes and functions as the cohesive element for centrioles/basal bodies (6, 40, 43, 67, 124, 125, 198). C-Nap1 localization to centrosomes has been demonstrated by immunoelectron microscopy (6, 43, 124). Several lines of evidence support the role of C-Nap1 in centrosome cohesion antibody-mediated interference of C-Nap1 function (125), expression of a dominant negative C-Nap1 (125), and more recently small interfering RNA-mediated depletion of C-Nap1 (6), all lead to premature centrosome splitting. Centrosomes are duplicated in S phase of the cell cycle. It is thought that during that time, the duplicated centrosomes are held together by dephosphorylated C-Nap1, whose state of dephosphorylation is maintained by protein

phosphatase 1. Prior to mitosis, C-Nap1 becomes phosphorylated by the never-in-mitosis A (NIMA)-related kinase 2 (Nek2A) (43, 124, 127, 198). The phosphorylated C-Nap1, in turn, dissociates from centrosomes, thereby allowing centrosome disjunction to occur. Separation of the duplicated centrosomes in mitosis is important for bipolar spindle formation and chromosome segregation.

Nek2A, is the kinase which phosphorylates C-Nap1 leading to centriole splitting (43, 124, 127). By contrast, protein phosphatase 1 α (PP1α) counteracts the function of Nek2A by dephosphorylating both Nek2A and C-Nap1 (67, 127, 129). The reversible and tightly regulated phosphorylation and dephosphorylation of C-Nap1 through the actions of Nek2A and PP1α is therefore critical for proper spindle formation and mitotic progression (67, 127). Consistent with its role in regulating centrosomal functions, PP1α localizes to the centrosome (76). Furthermore, C-Nap1, Nek2A, and PP1 have been shown to form a ternary complex (67).

In a yeast two-hybrid screen, we have found that the extreme COOH-terminal 233 amino acid residues of C-Nap1 interacts strongly with Tax. This region of C-Nap1 is a major target of Nek2A phosphorylation, and is involved in controlling centrosome disjunction and bipolar spindle formation. Because of the critical role of C-Nap1 in centrosome function and multiple recent reports showing that Tax can induce centrosomal abnormalities, we have undertaken a detailed analysis of Tax-C-Nap1 interaction and its impact on centrosome function. Since PP1α plays a critical role in regulating C-Nap1 function, and PP1α catalytic subunit shares significant sequence similarity with the catalytic subunit of PP2A, which we have shown previously (44) to be an important binding partner of Tax, we have included PP1α in our study as well. Here

we show that Tax directly interacts with both C-Nap1 and PP1α, and co-localizes with both proteins to the centrosome. Indeed, upon expression of Tax, the cell-cycle-dependent phosphorylation of C-Nap1 becomes greatly reduced. In agreement with the critical role of C-Nap1 phosphorylation in centrosomal disjunction, in Tax-expressing cells, centrosome splitting is dramatically impaired. Our results are consistent with a model wherein Tax facilitates recruitment of PP1α to C-Nap1, thereby causing dephosphorylation of C-Nap1 and centrosomal non-disjuction. The failure in centrosome disjunction in turn contributes to the severe mitotic abnormalities and centrosomal aberrations seen in Tax-expressing cells.

MATERIALS AND METHODS

Two Hybrid Interaction Screening

Yeast 2-hybrid screen was done in the strain L40 (MATa, his3Δ200, trp1-901, leu2-3112, ade2, LYS2::lexA-HIS3, URA3::lexA-lacZ, gal4, gal80) where LacZ and HIS3 are driven by minimal GAL1 promoters fused to 8 and 4 lexA boxes, respectively. A 2-μ plasmid, BTM116 (TRP), containing the LexA DNA binding domain was used to produce LexA-Tax fusion. The Gal 4 activation domain-based HeLa cDNA library was purchased from Clontech, Inc. All interaction was confirmed in L40 after retransformation of appropriate prey cDNA clones with LexA or LexA-Tax. Stable transformants were selected on dropout plates and assayed for relative β-galactosidase activity using filter assays.

Plasmid Constructions

PP1α cDNA was PCR amplified using 5' PP1 2-hybrid primer CGCGGGATCCCGCCACCatgccgacagcgagaagctc (BamHI site is underlined) and 3' PP1 primer TTGGTACCGTCGACTCTAGAATTCctatttcttggctttggcggaattgcggggtgg (EcoRI is underlined) and an *E. coli* PP1α expression plasmid, pTactac-PPase-1, (kindly provided by Dr. Ernest Lee) as the template. The PCR product was digested with BamHI and EcoRI restriction endonucleases and fused with the coding sequence for VP16 activation domain. Flag-PP1α was similarly derived using the following primer pair: CCGCGGGATCCGCCACCATGGATTACAAGGATGACGACGATAGTCCatgtccgaca gcgagaagctc and TTGGTACCGTCGACTCTAGAATTCctatttcttggctttggcggaattgcggggtgg. The Flag-PP1 PCR product was cloned into the BamHI, SalI site of a lentivirus vector: HR'CMV-SV40-puro.

Likewise, the HA-ΔN1-C-Nap1 coding sequence was generated by PCR using the primer pair: GCGGGATCCGCCACCATGGCGTACCCATACGACGTCCCAGACTACGCT catatgacactgaaggag and TTGGTACCGTCGACTCTAGAATTCctacctggaggcggctt, together with Myc-tagged full-length C-Nap1 (kindly provided by Dr. Erich Nigg) as the template, and cloned into the BamHI and SalI sites of HR'CMV-SV40-puro plasmid. HA-tagged ΔN2-C-Nap1 (HA-ΔN2-C-Nap1) was similarly constructed except the primer pair GCGGGATCCGCCACCATGGCGTACCCATACGACGTCCCAGACTACGCTgagcgg gactcagaacagcaa and TTGGTACCGTCGACTCTAGAATTCctacctggaggcggctt were used.

Cell Culture, Transfection, and Immnochemical Techniques

HEK 293T cells were transfected with 10 μg of each plasmid DNA using a calcium phosphate transfection kit (Invitrogen). Plasmids used were Myc-tagged full-length C-Nap1 (provided by Dr. Erich Nigg), HA-ΔN1-C-Nap1 110 kDa in size, HA-ΔN2-C-Nap1 37 kDa in size, Flag-PP1 and HR'Tax. The transfected cells were washed the next day and harvested 48 h later for cell lysis using a modified RIPA buffer containing Tris-HCl, NP40 1%, Na-deoxycholate 0.25%, NaCl 150mM, EDTA 1mM, PMSF 1mM, protease inhibitor cocktail (1 μg/ml), Na_3VO_4 1mM, and NaF 1mM. Immunoprecipitation and detection was done using monoclonal antibodies to c-Myc (Santa Cruz), HA (Santa Cruz), Tax (4C5), and Flag (Stratagene). Protein G beads (Invitrogen) were added at 4°C overnight to precipitate the immune complex.

Immunofluorescence

Colocalization. HeLa cells were transiently transfected with Myc-tagged full-length C-Nap1, HA-tagged HA-ΔN1-C-Nap1 and Tax expressing plasmids. Cells were fixed with 1% PFA overnight at 4ºC and permeabilized with 0.1% Triton X-100 and double stained with antibodies against (A) cmyc and γ-tubulin, (B) HA and γ-tubulin, (C) HA and Tax, (D) Tax and γ-tubulin, (E) PP1 and γ-tubulin , (F) PP1 and Tax. Monoclonal 4C5 antibody was used against Tax. Antibodies were obtained from the following places as listed monoclonal c-Myc (Santa Cruz), monoclonal HA (Santa Cruz), polyclonal HA (Santa Cruz), monoclonal γ-tubulin (GTU-88, Sigma), polyclonal γ-tubulin (T5192, Sigma), PP1 monoclonal (Santa Cruz), PP1 polyclonal (Upstate) antibodies. Secondary antibodies used were goat anti-mouse-TR (Santa Cruz) and goat anti-rabbit FITC

(Jackson Immuno Research). Images were obtained using an Olympus IX8 inverted fluorescent microscope.

Centrosome observation. HeLa cells were plated on glass coverslips, transduced with a lentiviral vector HR'CMVpuro or HR'Taxpuro at 2 moi, and selected with puromycin for two days. Remaining cell debris was washed off and the cells remaining on the glass cover slips were fixed, permeabilized and stained for γ-tubulin. Localization of γ-tubulin was detected with mouse antibodies to γ-tubulin (GTU-88, Sigma).

Detection of centrosome disjunction. HeLa Tax-reporter (GFP) and Hos Tax-reporter (GFP) cells were plated on glass cover slips and arrested with double Thymidine and infected with Adenovirus Ad-TTA or Ad-Tax as described previously (96) and released for different time points and fixed with 2% PFA; or released for 2 hours, and then arrested with Nocodazole and released for 8 hrs and fixed with 2% PFA. The cells were permeabilized with 0.1% Triton X-100, washed and stained by indirect immunofluorescence with antibodies to γ-tubulin. Tax-induced GFP expression was directly visualized.

Observation of MTOC. LLCPK cells endogenously expressing α-tubulin GFP fusion protein were transduced with a control vector or HR'Taxpuro vector. The next day the transduced cells were selected with 1 μg/ml puromycin for 3 days, washed and visualized for MTOC. For γ-tubulin stain, cells were fixed with 1% PFA, permeabilized with 1x PBS containing 0.1% Triton X-100 and stained with γ-tubulin (GTU-88, Sigma)

Detecting the Phosphorylated Form of C-Nap1

293T cells were cotransfected with HA-ΔN2-C-Nap1 and GFP or Tax using a calcium phosphate transfection kit (Invitrogen). Media was changed the following day.

Cells were arrested with 400 ng/ml nocodazole for 16 hours and harvested. Lysis of the cells was done by using a modified RIPA buffer (Tris-HCl, NP40 1%, Na-deoxycholate 0.25%, NaCl 150mM, EDTA 1mM, PMSF 1mM, protease inhibitor cocktail (1 μg/ml), Na_3VO_4 1mM, and NaF 1mM) and immunoprecipitation was carried out with antibody to HA (Santa Cruz). Immunoblot was performed with antibody to HA (Santa Cruz).

HeLa cells grown to near confluency were cotransfected with HA-ΔN2-C-Nap1 and GFP or Tax using Lipofectamine 2000. The next day, the transfected cells were replated at 1:6 dilution to allow for growth. After 8 hours growth, the cells were arrested with 400 ng/ml nocodazole for 16 hours and then harvested. Detection was performed as above.

In vitro Dephosphorylation of C-Nap1. 293T cells were transfected with HA-ΔN2-C-Nap1 using a calcium phosphate transfection kit (Invitrogen). Media was changed the following day. Cells were arrested with 400 ng/ml nocodazole for 16 hours and were harvested. Lysis of the cells was done by using a modified RIPA buffer and immunoprecipitation was carried out with antibody to HA and protein G beads (Invitrogen) at 4°C. Purified HA CT2 C-Nap1 was incubated at 30°C for 10 minutes with λ phosphatase using 1x reaction buffer plus 2mM $MnCl_2$ (NEB).

^{32}P labeling of C-Nap1. HA-ΔN1-C-Nap1 or Myc-tagged full-length C-Nap1 was co-transfected with Tax into 293T cells. The transfected cells were treated with nocodazole for 16 hours, and then labeled with 1mCi inorganic ^{32}P-phosphate (Amersham) ~$2x10^6$ cells for four hours. Cell lysates were prepared, immunoprecipitated with the HA or Myc antibody, and the immunoprecipates resolved on SDS-PAGE for autoradiography.

RESULTS

C-Nap1 is a Tax-binding protein

In a yeast two-hybrid screen using Tax as bait, we have identified, C-Nap1, as a potential binding partner of Tax (Fig. 12). The C-Nap1 cDNA fragment isolated encodes the COOH terminal amino acid residues 2155-2387 of C-Nap1. C-Nap1 is a large centrosomal coiled-coil protein of approximately 250-270 kDa in size. It is required for centriole-centriole cohesion during interphase of the cell cycle (43, 124, 127, 198). C-Nap1 becomes phosphorylated by Nek2A in a cell cycle-dependent manner during G2/M. The phosphorylated C-Nap1 in turn dissociates from the centrioles. This allows centrosomes to separate at the beginning of mitosis and bipolar spindles to form.

Secondary structure prediction suggests that the Tax-binding region of C-Nap1 is extensively α-helical. This is consistent with previous findings that Tax preferentially interacts with proteins containing α-helical domains including the basic domain-leucine zipper transcription factors, CREB/ATF, and the I-κB kinase (IKK) regulatory subunit, NF-κB essential modulator (NEMO)/IKKγ. Importantly, the COOH terminal region of C-Nap1 has been shown to interact with both Nek2A and PP1α, and is the target of Nek2A phosphorylation and PP1α dephosphorylation.

To verify that Tax-C-Nap1 interaction takes place *in vivo*, expression constructs of Myc-tagged C-Nap1 and Tax were co-transfected into 293T cells and immunoprecipitated with antibodies to Myc and Tax (Fig. 13A). As expected, full-length C-Nap1 and Tax associated with each other. To confirm that the COOH-terminal region of C-Nap1 interacts with Tax, we co-transfected a hemaglutinin-tagged N-terminally deleted C-Nap1 (HA-ΔN1-C-Nap1) that contains 2,442 amino acid residues of C-Nap1

(43) and again performed immunoprecipitation with antibodies to HA and Tax. Consistent with the 2-hybrid results, HA-ΔN1-C-Nap1 and Tax co-immunoprecipitated (Fig. 13B).

Interaction of Tax and PP1α proteins

C-Nap1 function is tightly regulated by Nek2A and PP1α during the cell cycle (67). It is normally kept in the dephosphorylated state throughout interphase by PP1α. Phosphorylation of C-Nap1 by Nek2A (43, 124) is thought to induce a conformational change that causes it to dissociate from the centrosomes. Since C-Nap1 is the physical link between the duplicated centrosomes, dissociation of C-Nap1 leads to centrosome disjunction (43, 124).

We have previously shown that Tax could interact with the catalytic subunit of PP2A (PP2Ac), which shares extensive sequence similarity with PP1α. Because of the association between Tax and C-Nap1, the critical role PP1α plays in C-Nap1 function, and the similarity between PP1α and PP2Ac, we asked if Tax might also interact with PP1α. Indeed, as shown in Fig. 12 above, Tax interacted with PP1α in the yeast 2-hybrid assay. As expected, N-terminal Flag-tagged PP1α co-immunoprecipitated Tax in transfected 293T cells. Furthermore, a PP1α-specific antibody co-immunoprecipitated Tax in MT4 HTLV-1 transformed T cells, which express Tax endogenously; but not in control Jurkat T cells, which lack Tax expression (Fig. 14B). Finally, GST-Tax, but not GST, could readily pull down purified PP1. Taken together, these results strongly suggest that Tax can also directly interact with PP1α.

Tax colocalizes with C-Nap1 and PP1

In keeping with its abilities to activate transcription and I-κB kinase, Tax is localized both in the nucleus and the cytoplasm. Previous studies have shown that Tax localizes to the centrosomes and causes centrosomal abnormalities. To confirm that C-Nap1 and Tax are both localized to the centrosomes, HeLa cells were transiently transfected with Tax and Myc-tagged full-length C-Nap1; stained with the γ-tubulin antibody and 4C5 (Tax) or c-Myc antibody; and visualized by immunofluorescence. Consistent with the centrosomal localization of Tax and C-Nap1, both proteins were found to colocalize with γ-tubulin (Fig. 15). To verify that PP1α and Tax localize to the centrosome, HeLa cells were again transfected with Tax and Flag-tagged PP1α, and stained with the γ-tubulin antibody and 4C5 (Tax) or Flag antibody. In agreement with a previous report (76), we also found PP1α and Tax to colocalize with γ-tubulin to the centrosome (Fig. 16). These results are consistent with the notion that Tax, C-Nap1, and PP1α form a ternary complex at the centrosome; and Tax may facilitate or stabilize the association between C-Nap1 and PP1α.

Tax inhibits C-Nap1 phosphorylation

Previous studies have indicated that Nek2A phosphorylates C-Nap1 during G2/M to activate centrosome disjunction/splitting (43, 127). The activity of Nek2A is countered by PP1α, which dephosphorylates both Nek2A and C-Nap1 (67). Furthermore, phosphorylation of C-Nap1 occurs predominantly at its COOH terminus, where Tax binds. The protein-protein interaction in the ternary Tax-PP1α-C-Nap1 complex could lead to an inhibition of PP1α activity and increased C-Nap1 phosphorylation, or stable

association of PP1α with C-Nap1 and exaggerated dephosphorylation of C-Nap1. With these considerations, we investigated if Tax affected C-Nap1 phosphorylation. An expression construct for a 37 kDa HA-tagged NH_2-terminal truncation of C-Nap1 (HA-ΔN2-C-Nap1) was generated and co-transfected with Tax into 293T cells. The transfected cells were either grown asynchronously, arrested at G1/S phase with thymidine, or arrested at metaphase with nocodazole. The HA-ΔN1-C-Nap1 protein was then immunoprecipitated using the HA antibody, and analyzed by immunoblotting. As shown in Fig. 17B, two HA-ΔN2-C-Nap1 bands were detected in control 293T or HeLa cells treated with nocodazole. Interestingly, the slower-migrating HA-ΔN2-C-Nap1 band disappeared upon treatment of the HA-immunoprecipitate with lambda phosphatase (λPPase), which removes phosphates from phospho-amino acids, suggesting that it most likely represented the hyperphosphorylated form of C-Nap1. Importantly, the hyperphosphorylated HA-ΔN2-C-Nap1 was absent from Tax-transfected HeLa or 293T cells. This suggests that Tax reduced or prevented HA-ΔN2-C-Nap1 phosphorylation. To determine if Tax inhibits C-Nap1 phosphorylation, both HA-ΔN2-C-Nap1 and Myc-tagged full-length C-Nap1 were co-transfected with Tax into 293T cells. The transfected cells were then treated with nocodazole, and labeled with inorganic ^{32}P-phosphate for 4 hours. Cell lysates were then prepared, immunoprecipitated with the HA or Myc antibody, and the immunoprecipates resolved on SDS-PAGE for autoradiography. Consistent with the notion that Tax inhibits C-Nap1 phosphorylation, little ^{32}P-labeled HA-ΔN2-C-Nap1 or Myc-tagged C-Nap1 was detected in cells transfected with Tax, in contrast to the control in Fig. 17D. These data suggest that the association of Tax, PP1α, and C-Nap1 leads to a drastic reduction in C-Nap1 phosphorylation.

Tax induces a failure of centrosome disjunction

Since C-Nap1 phosphorylation is critical for centrosome disjunction, and Tax causes a dramatic decrease in C-Nap1 phosphorylation, we examined if centrosome disjunction/splitting was also affected by Tax expression. To this end, a HeLa cell line, known as HeLa/18x21-EGFP, which contains a reporter cassette wherein the EGFP gene is placed under the transcriptional control of 18 copies of the Tax-responsive 21-bp repeat enhancer was used. This cell line conditionally expresses EGFP in the presence of Tax, but not in its absence, therefore allowing Tax expression to be readily scored. To observe centrosome function, HeLa/18x21-EGFP cells were first synchronized at the G1/S border with a double-thymidine treatment. They were then transduced with Ad-Tax, an adenovirus vector for Tax, or a control vector, Ad-tTA at an MOI of 5 as described previously (96). One hour after transduction, cells were released from the G1/S arrest, and fixed at different time points for immunoflurescence. In a separate experiment, the cells were released for two hours and treated with NOC for 16 hours to synchronize them at metaphase, and then released and fixed at the indicated times for immunofluorescence. As shown in Fig. 18, in the Ad-tTA control, at 4 hours after release from the G1/S block when all cells enter into the S phase, a single centrosome was detected in each cell. At 8 and 12 hours after release, when cells progressed through G2/M, centrosome disjunction could be readily seen. As anticipated, at 14 hours after release when most cells exited mitosis, cells with mono-centrosome were again observed. Finally, at 24 hours post-release when cells enter another G2/M, centrosome separation was again visible. By contrast, in Ad-Tax- transduced cells (indicated by EGFP expression), no discernible

centrosomal disjunction could be observed throughout the course of the experiment. The centrosomes appeared to have remained joined (Fig. 18), and the nuclei of many Tax-expressing cells became highly convoluted at 24 hours post release. As reported previously, the presence of Tax slowed the progression of the cell cycle by approximately 3 hours. This was possibly due to the premature activation of the anaphase promoting complex described earlier (96, 111, 112). The cell cycle delay would have placed Tax-expressing cells in G2/M at 12 and 14 hours post-release. The fact that split centrosomes could not be detected at those time points strongly suggest that centrosomal disjunction was severely impaired by Tax. It should also be pointed out that most Tax-expressing cells eventually exit mitosis and enter into senescence, but a significant fraction (10-15%) remained in G2/M. Finally, Tax-transduced, EGFP-positive cells that had no detectable centrosomes also appeared at high frequency (data not shown).

Evidence of Tax associated centrosome amplification in cells

HeLa cells transduced with Tax virus caused an increase in centrosomes demonstrated by γ–tubulin staining, in Fig. 19, suggesting centrosome amplification or fragmentation. We initially suspected that the Tax and C-Nap1 interaction could be one possible mechanism of causing centrosome abnormalities. This is based on the report that microinjection of anti-C-Nap1 antibodies into cells induced centrosome splitting (125) and further supported by overexpressing dominant negative functioning forms of C-Nap1 (125). A more physiological mechanism possibly involves Tax interaction with PP1. The specific stabilization or recruitment of PP1 to C-Nap1 could keep C-Nap1 and Nek2A in a dephosphorylated state.

Tax interferes with a distinct MTOC

The microtubule organizing center (MTOC) is a centrosome associated structure from which microtubules radiate. Most cells during interphase have one MTOC, usually located near the nucleus and mitotic cells can contain two MTOC after centrosome disjunction. We were able to observe the MTOC in LLCPK cells. These cells express α-tubulin fused with the green fluorescent protein gene (GFP) in which there forms a focal region at the centrosome. The α-tubulin concentrated area allows for direct visualization of the MTOC by fluorescent microscopy. The LLCPK cells were transduced with HR'LacZPuro or HR'TaxPuro and were selected for puromycin resistance for 3 days. The, α-tubulin GFP was visualized directly in which a dramatic loss of the MTOC could be seen in Tax expressing cells in Fig. 20A, which maybe related to the failure of centrosome disjunction caused by Tax. To confirm that the focal region of α-tubulin GFP indeed was located at the centrosome, the puromycin resistant cells were stained by indirect immunofluorescence with antibodies to γ-tubulin shown as red whereas α-tubulin GFP was visualized directly, in Fig. 20B.

DISCUSSION

In this study, we have demonstrated that Tax interacts directly with both C-Nap1 and PP1. Tax-C-Nap1-PP1 interactions correlated with a drastic reduction of C-Nap1 phosphorylation in Tax-expressing cells and a failure in centrosomal disjunction. The impairment in proper centrosome separation appears to be responsible, in large measure, for the centrosome abnormalities induced by Tax. Our current data are consistent with a

model wherein Tax facilitates and stabilizes PP1 association with C-Nap1, thereby causes C-Nap1 to be un- or under-phosphorylated and remain attached to duplicated centrosomes. This, in turn, prevents centrosomal disjunction and bipolar spindle formation. Alternatively, by binding the COOH-terminal region of C-Nap1, Tax may block C-Nap1 phosphorylation directly. In this case, the role of Tax-PP1 interaction is less clear. Finally, we have previously shown that Tax can activate the anaphase promoting complex ahead of schedule. As Nek2A is a substrate of APC (55, 65), the premature APC activation can lower the level of Nek2A, and contributes to the decrease in C-Nap1 phosphorylation.

A previous study has suggested that Tax interaction with Ran and RanBP1 may be important for Tax-induced centrosomal amplification (151). More recently, Tax has been shown to interact with TaxBP2 (23), a large coiled-coil centrosomal protein otherwise known as rootletin. Importantly, rootletin has been found to be a C-Nap1-associated protein, and like C-Nap1, is involved in centrosome cohesion by forming centriole-associated filaments (6, 198). Indeed, small interfering RNA-mediated depletion of either rootletin or C-Nap1 led to centrosome splitting (6). At present, whether rootletin is regulated by Nek2A and PP1 like C-Nap1 is unclear. It would not be surprising, however, if Tax affects both rootletin and C-Nap1 through the same mechanism.

Tax has been shown to cause centrosome supernumeracy previously. How does a failure in centrosome disjunction, as reported here, lead to centrosomal amplification? First, cells that inherited the unsplit centrosome could duplicate them during subsequent cell division cycle to produce daughter cells with multiple centrosomes. Second, a recent

study has found that a cell that inherited no centrosome as a result of laser-ablation of centrioles during mitosis gave rise to multiple centrin aggregates in progeny cells (100). In this regard, those Tax-expressing cells that inherited no centrosome might assemble multiple centrioles *de novo* to compensate for the centrosome loss. As most Tax-expressing HeLa cells enter into senescence, it seems less likely for Tax-expressing cells to undergo additional cell cycle that amplify centrosomes. Whether *de novo* nucleation of centrosomes is involved in the centrosome supernumeracy induced by Tax remains to be fully resolved.

Disruption of centrosome function is known to adversely affect mitotic spindle formation and chromosome segregation. The frequent occurrence of DNA aneuploidy and appearance of bi- and multi-nucleated cells following Tax expression are consistent with the inhibition of C-Nap1 phosphorylation and centrosomal non-disjunction induced by Tax described here. We have shown previously that Tax activates the anaphase promoting complex ahead of normal cell cycle schedule (96, 111, 112), causing the early degradation of multiple mitotic regulators including cyclins A and B, securin, and Skp2, and committing cells to senescence (96). Clearly, both premature APC activation and inhibition of C-Nap1 phosphorylation impact dramatically on the cell cycle progression of Tax-expressing cells. It is not clear, however, if they represent two distinct and unrelated activities of Tax, or alternatively, there may be a causal link yet-to-be resolved between the two. Finally, the role of the Tax-induced mitotic dysfunctions in HTLV-1 life cycle and pathogenesis also remains to be fully elucidated.

ACKNOWLEDGEMENTS

We thank Dr. Erich A. Nigg for the plasmid construct pBKcmycC-Nap1 and Dr. Ernest Lee for the TacTacPP1 construct. We also thank Dr. Xin Xiang and Dr. Kyung Lee for helpful discussions.

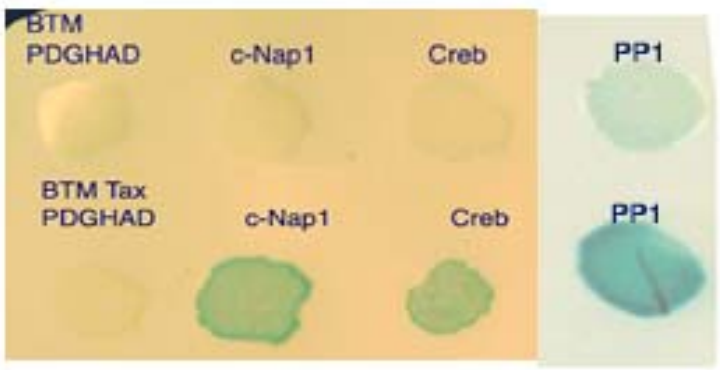


Figure 12. Yeast two-hybrid interaction of Tax with C-Nap1 and PP1
Yeast strain L40 was transformed with the indicated constructs, LexA or LexA DNA-binding domain fused with Tax along with VP16 containing the activation domain fused with C-Nap1, Creb, or PP1. Yeast two-hybrid assay was used to determine protein-protein interaction of the expression constructs that were used. Stable transformants were selected on dropout plates and assayed for relative β-galactosidase activity using liquid filter assays.

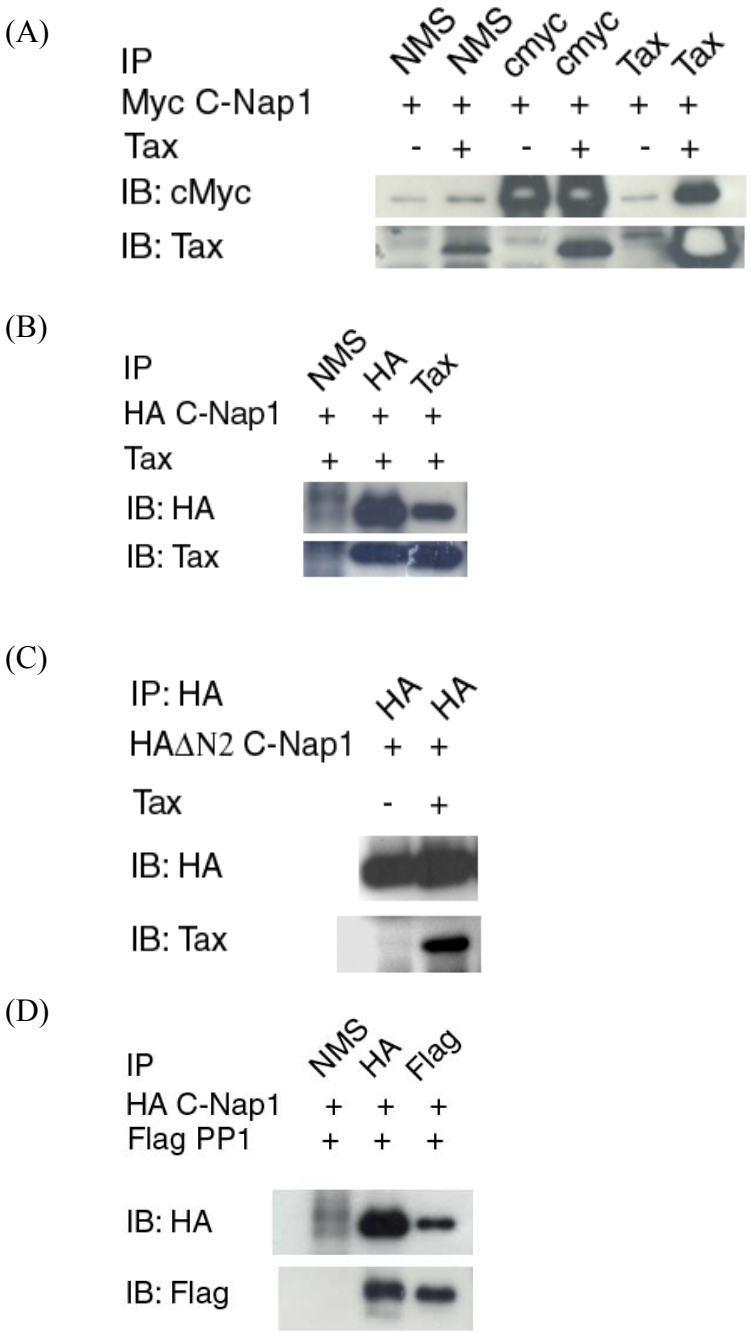
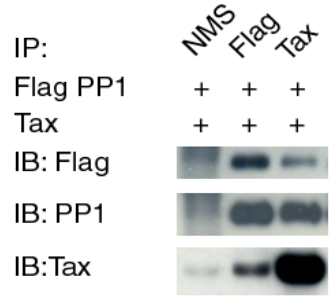
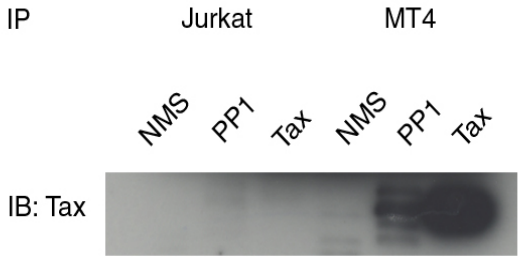


Figure 13. C-Nap1 interacts with Tax and PP1
293T cells were co-transfectioned with C-Nap1 and either Tax or PP1 and coimmunoprecipitations were done to determine interaction. (A) Myc-tagged full-length C-Nap1 and Tax complex was coimmunoprecipitated and observed by c-Myc and Tax (4C5) monoclonal antibodies (B) HA-tagged C-Nap1 (HA-ΔN1-C-Nap1) and Tax complex was detected by HA and Tax monoclonal antibodies. (C) HA-tagged C-Nap1 (HA-ΔN2-C-Nap1) and Tax were detected by HA and Tax monoclonal antibodies. (D) HA-ΔN1-C-Nap1 and Flag-PP1 complex was detected by HA and Flag monoclonal antibodies.

(A)



(B)



(C)

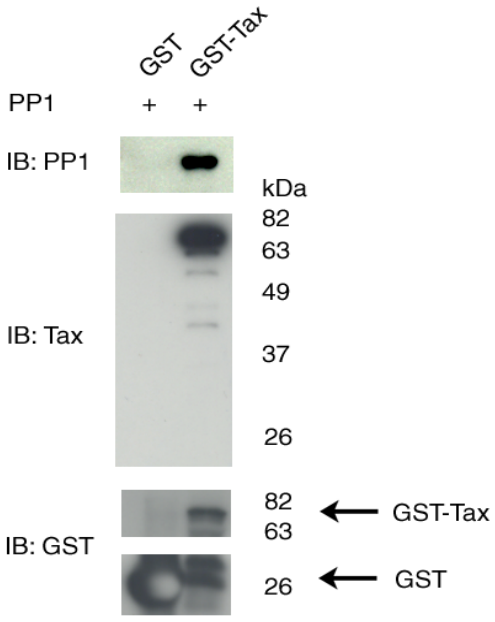


Figure 14. Tax interacts with the catalytic subunit of PP1
Flag epitope-tagged PP1 and Tax were cotransfected into 293T cells and extracts were coimmunoprecipitated to determine interaction. Coimmunoprecipitation was done by anti-Flag and anti-Tax antibodies (B) Jurkat control and MT4 (endogenous Tax expressing cell) cell extracts were immunoprecipitated with mouse IgG, anti-PP1 or anti-Tax antibodies. (C) Purified GST or GST-Tax were incubated with purified PP1α and purified using glutathione-Sepharose. The proteins that bound to the glutathione-Sepharose were resolved in SDS-12% PAGE and Western blotted with anti-Tax 4C5 monoclonal antibody, GST antibody or PP1 monoclonal antibody.

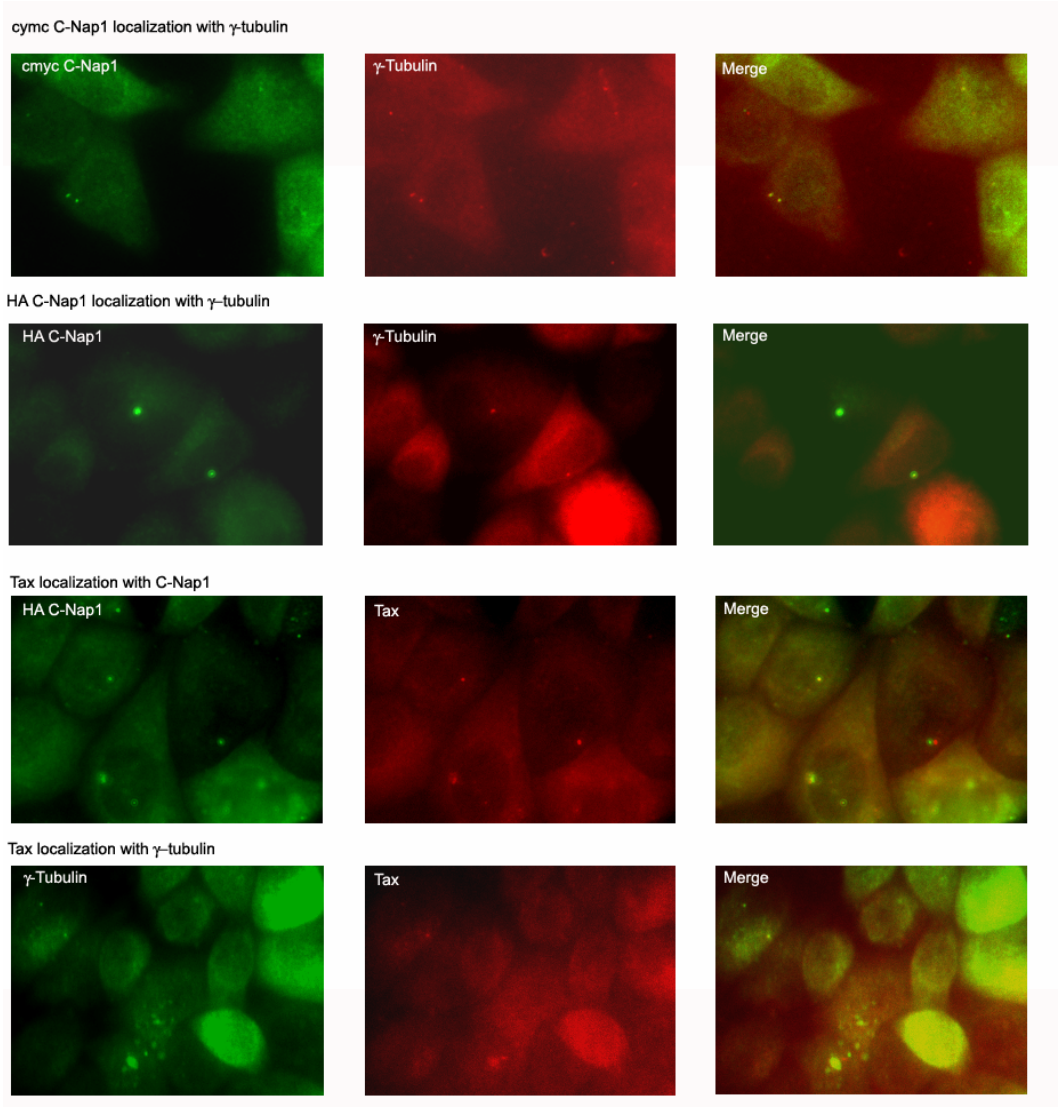


Figure 15. Tax localizes with C-Nap1 to centrosomes in human cells
HeLa cells were transiently co-transfected with Myc-tagged full-length C-Nap1, HA-ΔN1-C-Nap1, or HA-ΔN1-C-Nap1 and Tax and double stained using (1st row) cmyc and γ-tubulin antibodies, (2nd row) HA and γ-tubulin antibodies, (3rd row) HA and 4C5 Tax antibodies, (4th row) anti-Tax 4C5 and γ-tubulin antibodies.

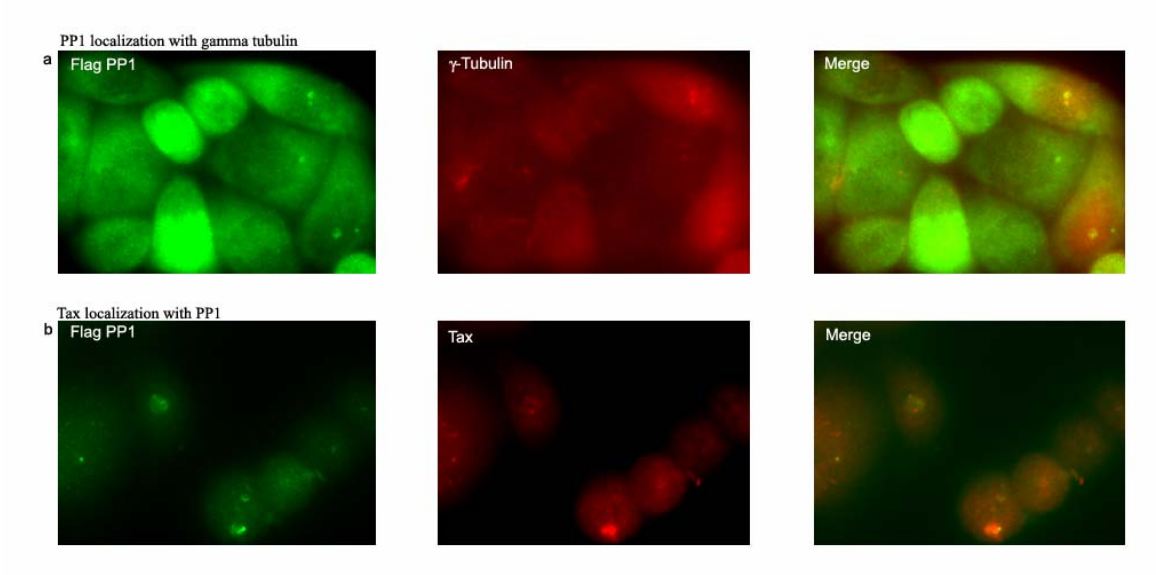


Figure 16. Tax localizes with PP1 in human cells
HeLa cells were transiently co-transfected with Tax and Flag-tagged PP1 then double stained using (A) PP1 and γ-tubulin antibodies, (B) PP1 and 4C5 Tax antibodies, and (C) γ-tubulin and 4C5 Tax antibodies.

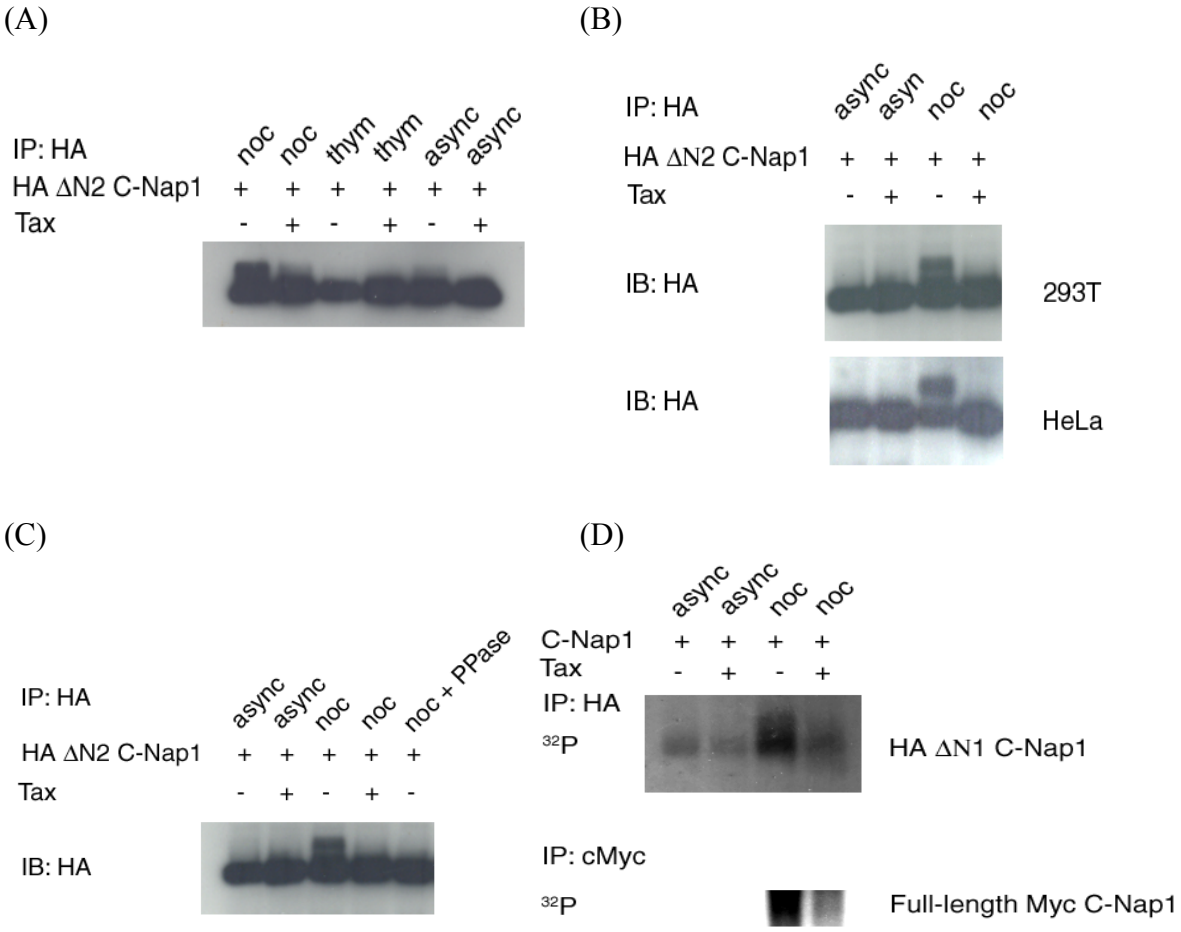
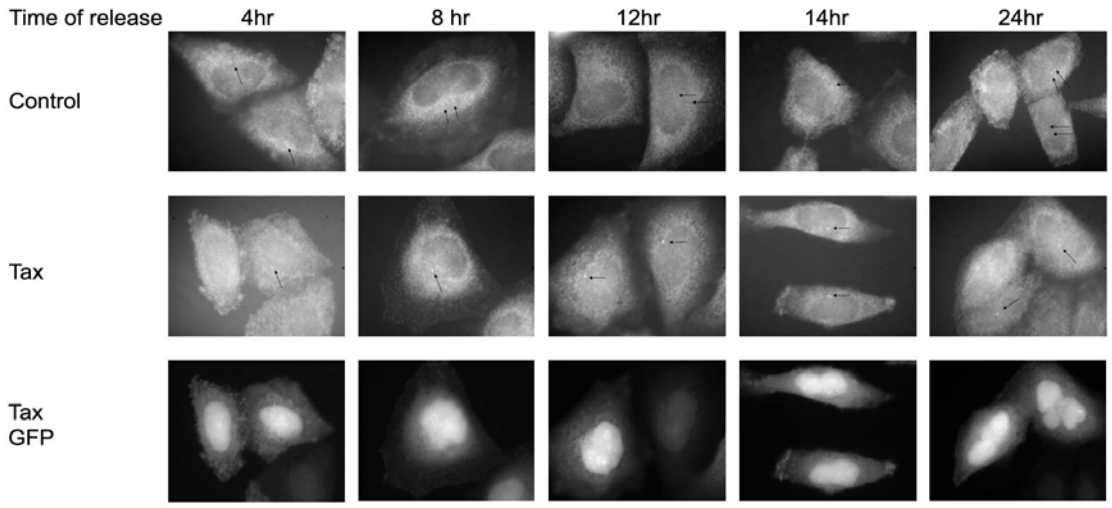
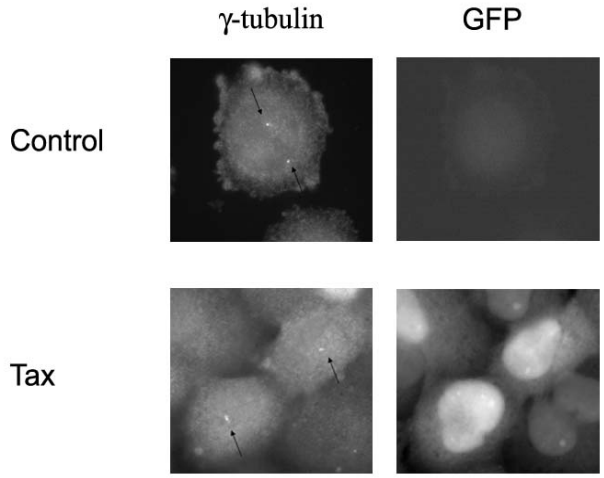


Figure 17. Tax inhibits normal C-Nap1 phosphorylation in mitosis
(A) 293T cells were transfected with a HA-tagged truncated C-Nap1 (HA-ΔN2-C-Nap1) with or without Tax. Then, cells were arrested with 400ng nocodazole, 2.5mM thymidine or left asynchronous. The cells were lysed and immunoprecipitated with HA antibody. The sample was observed by SDS-PAGE for size migration differences. (B) Both 293T cells and HeLa cells were transfected with HA-ΔN2-C-Nap1 with or without Tax. They were left asynchronous or arrested with nocodazole and HA-immunoprecipitated then observed for size migration differences. (C) The upper mobility band of HA-ΔN2-C-Nap1 was treated with λ phosphatase to test for a difference in migration. (D) HA-ΔN1-C-Nap1 or Myc-tagged full-length C-Nap1 were cotransfected with Tax into 293T cells. The transfected cells were treated with nocodazole, and labeled with inorganic p^{32}-phosphate for four hours. Cell lysates were then prepared, immunoprecipitated with the Myc antibody or HA antibody and the immunoprecipates resolved on SDS-PAGE for autoradiography.

(A)



(B)



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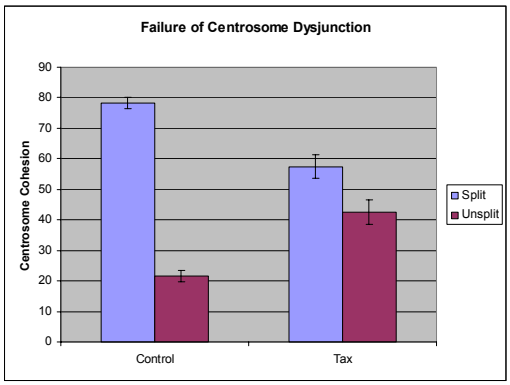


Figure 18. Tax disrupts centrosome disjunction
HeLa Tax-reporter (GFP) cells arrested with Thymidine were infected with Adenovirus Ad-TTA or Ad-Tax as described previously (96). Then, they were released (A) for different time points 4hr, 8hr, 12hr, 14hr, 24hr and fixed or (B) for 2 hours and arrested with Nocodazole and released for an additional 8 hours and fixed. The cells were stained by indirect immunofluorescence with antibodies to γ-tubulin to visualize centrosomes. Tax-induced GFP expression was directly visualized. (C) Centrosomes were quantitated for centrosome disjunction after 12hr thymidine release.

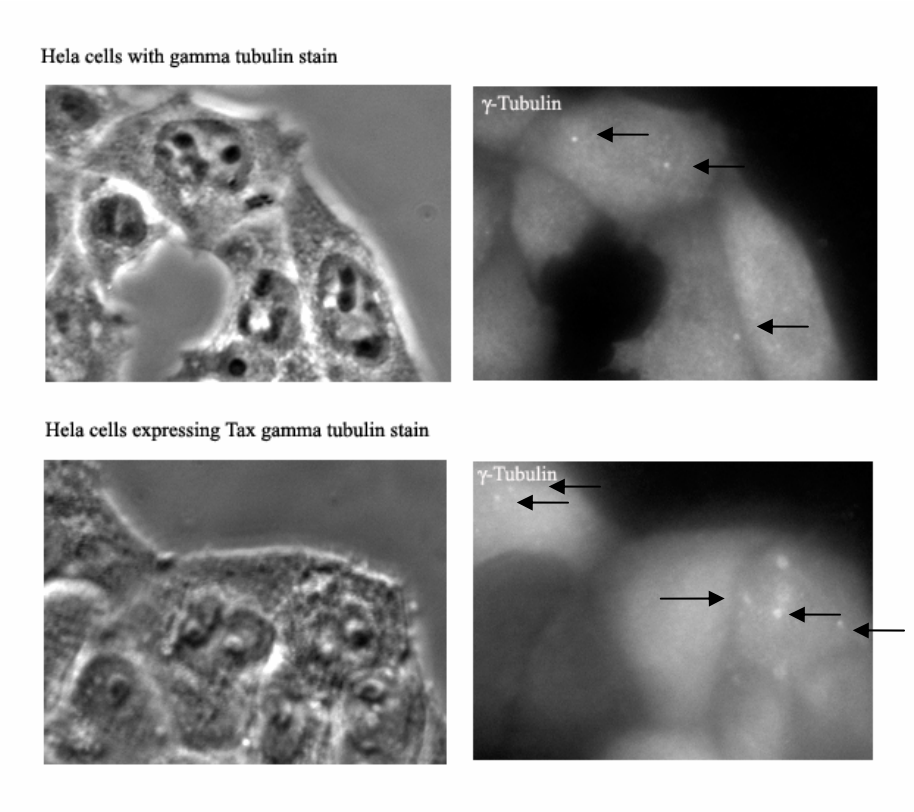
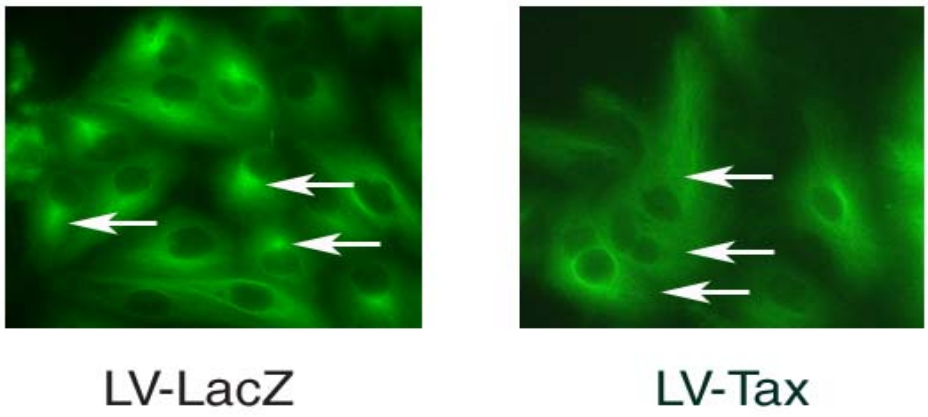


Figure 19. Tax expression causes centrosome abnormalities
HeLa cells were transduced with HR'Puro or HR'TaxPuro. The transduced cells were selected for puromycin resistance for 2-3 days. The puromycin resistant cells were stained by indirect immunofluorescence with antibodies to γ-tubulin.

(A)



(B)

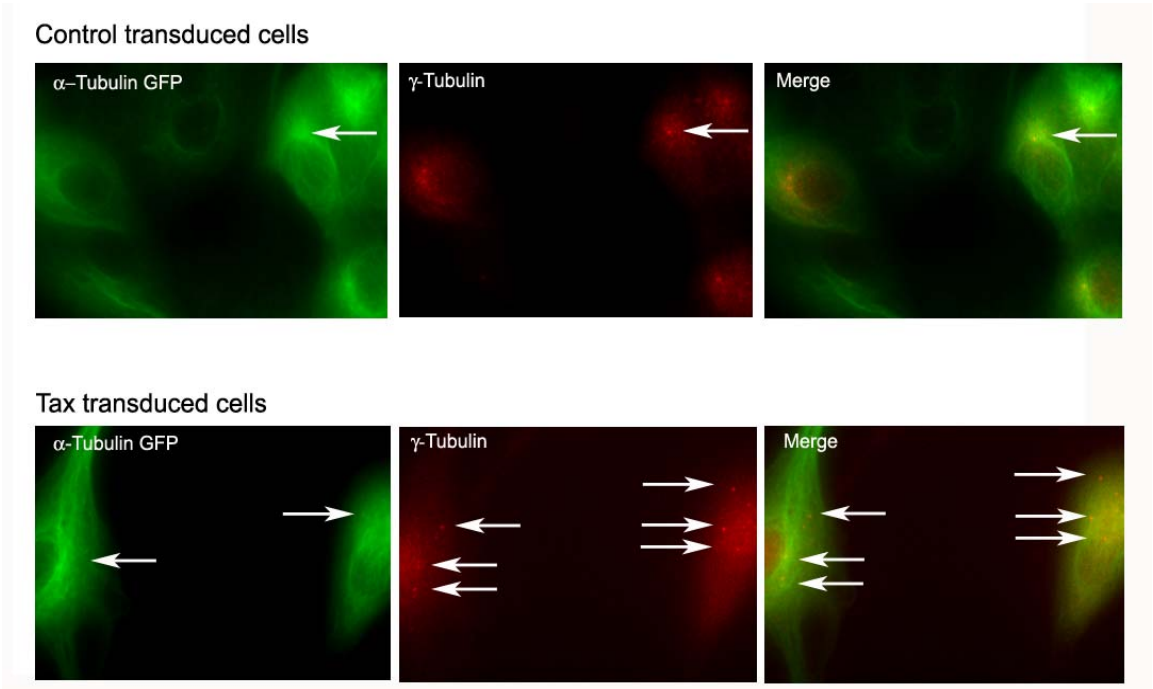


Figure 20. Centrosome abnormalities and loss of a distinct MTOC in Tax expressing cells

LLCPK cells, expressing α-tubulin fused with the green fluorescent protein gene (GFP), were transduced with (A) HR'LacZPuro or HR'TaxPuro. α-tubulin GFP was visualized directly. (B) The transduced cells were selected for puromycin resistance for 3 days. The puromycin resistant cells were stained by indirect immunofluorescence with antibodies to γ-tubulin shown as red. α-tubulin GFP was visualized directly.

CHAPTER 4

DISCUSSION

In this dissertation, I have investigated the effects of HTLV-1 Tax on mitotic regulation. The research was directed towards two specific aims: (1) to determine if HTLV-1 Tax mutants that do not induce G1 arrest are disabled in activating the anaphase promoting complex and transformation; and (2) to determine the biological significance of HTLV-1 Tax association with C-Nap1 in causing centrosomal abnormalities.

Specific Aim 1

HTLV-1 Tax Mutants That Do Not Induce G1 Arrest Are Disabled in Activating the Anaphase Promoting Complex and Transformation

Tax had been shown previously to cause cell cycle arrest in HeLa cells, human diploid fibroblast WI-38 cells, hematopoietic stem cells and in *S. cerevisiae* (96, 108, 111, 112, 184). In human cells, the cell cycle arrest caused by Tax is due to a drastic build-up of $p21^{CIP1/WAF1}$and $p27^{KIP1}$, inhibitors of G1/S cyclin-dependent kinase, Cyclin E/Cdk2. The levels of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ are regulated through transcription, subcellular localization, phosphorylation (by cyclinE/Cdk2), ubiquitination and proteasome-mediated degradation (15, 57, 179). The E3 ubiquitin ligase, SCF, together with its substrate-recognition subunit, Skp2, mediates the ubiquitination and degradation of $p27^{KIP1}$ (10, 47, 191). Recent data have indicated that Skp2 is a substrate of the Cdh1-associated APC (APC^{Cdh1}) (10, 191). Skp2 becomes ubiquitinated and degraded in late M and early G1 phases when APC^{Cdh1} is active. The degradation of Skp2 inactivates SCF and allows $p27^{KIP1}$ to transiently accumulate in G1 phase. Previous work in our laboratory has shown that HeLa cells transduced with *tax*, displayed premature APC activation that resulted in the loss of cyclin A, cyclin B, securin, and Skp2 ahead of normal cell cycle schedule. The *tax*-expressing HeLa cells accumulated

p21$^{CIP1/WAF1}$ and p27^{KIP} starting in S phase. Following an aberrant mitotic division cycle, the dramatic surge in the levels of p21$^{CIP1/WAF1}$ and p27^{KIP1} caused the *tax*-expressing cells to enter into senescence.

Since *tax* expression arrests cell cycle progression in yeast, the *S. cerevisiae* system was used to elucidate the mechanisms by which Tax perturbs the cell cycle. To this end, a collection of *tax* point mutants were isolated that permitted the normal growth of *S. cerevisiae*. Like wild-type *tax*, many of these mutants (C23W, A108T, L159F, and L235F) transactivate both the HTLV-LTR and the NF-κB reporters. One of them, V19M, preferentially activated NF-κB, but was attenuated for LTR activation. Interestingly, none of the *tax* mutants significantly elevated the levels of the cell cycle inhibitors p21$^{CIP1/WAF1}$and p27^{KIP1}, and most could be stably expressed in HeLa cells without causing cell cycle arrest. In essence, those mutations in *tax* that permitted expression in *S. cerevisiae* have abrogated induction/stabilization of p21$^{CIP1/WAF1}$ and p27^{KIP1}. The mutations also attenuated or abrogated the ability of Tax to activate APC. These results are consistent with the notion that premature APC activation by Tax is responsible for the senescence-like cell cycle arrest.

The activation of p21$^{CIP1/WAF1}$ transcription by Tax has been reported previously (20, 21, 24, 32). These earlier studies have suggested that the Tax-induced increase in p21$^{CIP1/WAF1}$ resulted from transactivation via the CREB/ATF-CBP/p300 pathway (24, 32). Since four of the five mutants analyzed in this study activated LTR-luciferase reporter at levels (70-80%) comparable to those of wild-type *tax*, yet were substantially impaired in increasing levels of p21$^{CIP1/WAF1}$ and p27^{KIP1}, I conclude that the CREB/ATF-CBP/p300 pathway is probably not the principal determinant in the accumulation of p21$^{CIP1/WAF1}$ and p27^{KIP1}. Likewise, luciferase reporter assays and the extent of I-κB degradation and p100

processing suggest that the mutants analyzed, particularly A108T, V19M and C23W, are potent activators of IKK-NF-κB. These mutants were nonetheless unable to increase $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ levels when compared to wild-type Tax. This result supports the idea that the NF-κB pathway is not directly responsible for the Tax-induced increase in $p21^{CIP1/WAF1}$ and $p27^{KIP1}$. Studies from others in the laboratory now indicate that protein stabilization resulting from APC-mediated degradation of Skp2 is a major factor for Tax-induced increase in $p27^{KIP}$ (96). Another study conducted by a different laboratory member suggests that the Tax-mediated increase in $p21^{CIP1/WAF1}$ is caused by promoter transactivation and mRNA stabilization. Whether and how these two activities are linked to premature activaton of APC by Tax is currently under investigation. In summary, yeast genetics can be productively used to isolate *tax* mutants which no longer cause cell cycle arrest. Whether this system may also be useful for identifying and studying the cell cycle machineries targeted by Tax remains to be seen.

During the course of this dissertation research, I have used a reporter system (203) developed in this laboratory to derive a stable HeLa cell which harbors a reporter cassette consisting of the DSRed gene under the transcriptional control of 18 copies of the Tax-responsive 21-base pair repeat fused to a minimal HTLV-1 promoter. With this reporter system, DSRed expression is only observed when Tax is expressed. This Tax-specific reporter cell line can be used to monitor Tax expression from *tax*-expression constructs or from HTLV-1 infection. In this study, I used the reporter to analyze the expression of various Tax mutants. The *tax* mutants that remained functional in transactivating viral LTR and NF-κB could be incorporated into an infectious molecular clone of HTLV-1 in order to address the respective role of APC activation and NF-κB activation in T cell infection and

transformation. It is conceivable that an infectious molecular clone of HTLV-1 could be engineered to contain the specific *tax* mutations such that infectious virions with such mutations could be generated and used to infect susceptible cells for testing viral production, viral transmission, viral protein expression, NF-κB activation, or cell cycle arrest and the testing could be done in reporter indicator cells.

The V19M mutant, which activated the NF-κB pathway but did not activate the HTLV LTR or the reporter cell line, could be used to further understand how Tax activates the viral promoter. For instance, the V19M mutant could be tested for an interaction with CREB/ATF or CBP/P300 by yeast-two hybrid assay or co-immunoprecipitation. The electromobility shift assay (EMSA) using the 21-bp repeat of the LTR could also be used to determine if the V19M mutant is part of the CREB/ATF and CBP/P300 complex. Other *tax* mutants could be used similarly to help elucidate the various Tax-induced mitotic effects.

The ability of Tax to cause G1 arrest/senescence appears to contradict with its role in ATL development. Previous works in the laboratory and in other laboratories have found that HTLV-1 transformed, Tax-positive T-cells invariably express significantly lower levels of $p27^{KIP1}$ (96) but produce dramatically high levels of $p21^{CIP1/WAF1}$, which however localizes to the cytoplasm predominantly and, as a result, fails to inhibit cell cycle progression. These results suggest that the loss of $p27^{KIP1}$ expression and the functional inactivation of $p21^{CIP1/WAF1}$ is a pre-requisite for T cell transformation by HTLV-1. Indeed, recent results from this lab indicate a loss of $p27^{KIP1}$ function allows cells to evade *tax*-IRS (96). In aggregate, these results support the notion that deficiency in $p27^{KIP1}$ is a common feature of HTLV-1 transformed cells and possibly of adult T-cell leukemia/lymphoma (133). This deficiency, whether through the loss of $p27^{KIP1}$ gene, the abrogation of mRNA expression,

accelerated protein degradation, or functional inactivation allows virus-infected T cells to overcome the effects of Tax-IRS. In these persistently infected cells, the mitogenic property of Tax could then promote cell proliferation without regard to normal cell cycle regulation (96). The chromosomal instability induced by Tax may also further accelerate cell transformation and ATL development. Finally, at a later time during the cancer development, Tax expression may be negatively selected by cytotoxic lymphocyte killing and because of the inherent proliferative disadvantage of the mitotic aberrations it induces.

To further study the significance of the Tax-induced cell-cycle arrest, I investigated whether the Tax mutants could transform Rat1 fibroblasts, as had been previously reported for WT-Tax (38, 69, 89, 121, 197). In agreement with the notion that a loss of $p27^{KIP1}$ allows cells to evade *tax*-IRS, Rat1 cells are deficient in $p27^{KIP1}$ and *tax*-IRS does not occur in Rat1 fibroblasts. Instead, Rat1 cells lose contact inhibition, form foci on monolayer, and become transformed by *tax*. Several earlier reports have implicated NF-κB activation by Tax as critical for its cell transformation activity. Although the ability of Tax to activate NF-κB appears to play an important role in the transformation process, it is unclear whether NF-κB activation is sufficient for transformation (41). The data here suggest that NF-κB activation alone is insufficient for the transformation of Rat1 fibroblasts because all of the tested mutants (V19M, C23W, A108T, L159F, and L235F) were unable to induce transformation. Although NF-κB activation by Tax plays an important role in cell transformation, the mitotic abnormalities that are associated with premature APC activation might also be required for this process.

The biological relevance of the profound Tax-induced cell-cycle arrest remains unclear. It is possible that HTLV-1-infected T-cells that are arrested in a Tax-induced

senescence-like state might persist longer *in vivo* and devote significant cellular resources to virus replication. Furthermore, the dramatic morphological changes associated with the senescence-like arrest might facilitate virus assembly and/or transmission. At this point, I do not know if Tax expression and/or HTLV-1 infection will induce a senescence-like arrest in human T cells. It should be pointed out that previous studies have suggested that Tax activates T cells and drives cell cycle progression. The possibility that T cells, which are the natural reservoir for the virus, may behave differently in response to Tax expression or HTLV-1 infection because of the presence of T-cell-specific pathways cannot be ruled out at present. It is conceivable that Tax may activate an autocrine mechanism in T cells that allows them to overcome the senescence effects of Tax. In this vein, it should be noted that Tax is known to activate the lymphokines (31, 51) and lymphokine receptors in T cells.

As described, the APC has many roles in cell cycle regulation. It is crucial for the metaphase to anaphase transition and mitotic exit. Indeed, the dysregulation of the APC might have an impact in cell cycle progression, and could lead to mitotic defects, and possibly cancer (11, 138, 190). The APC can be deregulated when one of its activating subunits is altered (190) which is found in squamous cell carcinoma cell lines (132), human colon cancer cells (189) and from human papilloma virus E2 protein expressing cells. Through unscheduled APC activation, Tax exerts a dramatic effect on mitosis. While most Tax-expressing eukaryotic cells enter senescence, many are also found to be binucleated or multinucleated. In future experiments, the mutants generated in this study might be used to help elucidate the mechanisms by which Tax brings about these mitotic abnomalities. While our current data support the hypothesis that the premature activation of the anaphase

promoting complex plays a major role in the mitotic demise of Tax-expression on cells, other cellular mechanisms targeted by Tax may also contribute to this process.

Specific Aim 2

Determine if HTLV-1 Tax Association with C-Nap1 and PP1 is the Cause of Dysregulated C-Nap1 Phosphorylation and Centrosomal Abnormalities

In a parallel study, I investigated how Tax might affect mitotic regulation through its interaction with the centrosomal Nek2A-associated protein, C-Nap1. This analysis was prompted by a yeast 2-hybrid screen (unpublished results of the laboratory) wherein C-Nap1 was identified as a potential protein binding partner of Tax. As outlined above, C-Nap1 is a large coiled-coil protein responsible for centrosome cohesion (6, 43, 124, 125). The cell-cycle-regulated phosphorylation and dephosphorylation of C-Nap1 is essential for centrosome function (6, 43, 198). C-Nap1 becomes phosphorylated by Nek2A in the G2/M phase of the cell cycle. The phosphorylated C-Nap1 dissociates from centrioles. This allows centrosome splitting and formation of bipolar spindle to occur. During interphase, C-Nap1 is kept un- or under-phosphorylated by phosphor-Ser/Thr protein phosphatase 1 α, PP1 α. The unphosphorylated C-Nap1 remains attached to the centrioles and prevents centrosome disjunction (67, 127, 129). Importantly, PP1α also dephosphorylates and inactivates Nek2A (129) to ensure that premature centrosome splitting is prevented.

In this study, I demonstrated that Tax can interact with C-Nap1 and PP1α by yeast two-hybrid assay and coimmunoprecipitation. Furthermore, Tax localizes with both C-Nap1 and PP1α to the centrosome. Although it remains unclear whether the interaction of Tax with C-Nap1 is sufficient to induce centrosome abnormalities, the localization of Tax to the

C-terminal region of C-Nap1 is likely critical. This region is targeted by both PP1α and Nek2A. During the majority of the cell cycle the effect of PP1α over C-Nap1 predominates. The cellular changes needed at the onset of mitosis to offset the dynamics in favor of Nek2A remain unclear. The observation that Tax dramatically decreases C-Nap1 phosphorylation suggests that Tax may recruit PP1α to C-Nap1 and/or preclude Nek2A from interacting with C-Nap1. The Tax-recruited PP1α can then dephosphorylate C-Nap1 and/or Nek2A, thereby prevents C-Nap1 phosphorylation, centrosome disjunction and bipolar spindle formation.

PP2A, an established biologically relevant partner of Tax, is recruited by Tax to the IKK-γ/ IKK complex. A previous report suggests that PP2A is a positive regulator of IκB kinase activity and that Tax expression leads to increased IκB activity (95). This information merely suggests that PP1 might function similarly though the relevance must be substantiated. In terms of function, protein phosphatase activity must be tightly regulated as the ability of analogues to inhibit PP1 and PP2A have been correlated with anti-cancer activity in human cancer cell lines (60, 167). Increased PP1 stability, expression, and nuclear localization has been reported in different cancers (181): oral squamous cell carcinoma cells (75), human hepatomas cells (79, 92), and rat hepatomas cells (79, 165). There are far less phosphatases in comparison to kinases for the same regulatory phosphorylation mechanisms. Therefore, inhibition of a single phosphatase could lead to a breakdown in multiple pathways. Some examples of other viruses interacting with PP1 include HIV-1 (4), a close relative of HTLV-1, and herpes simplex virus (66).

The dramatic decrease in C-Nap1 phosphorylation in mitosis led us to investigate the possible role of Tax on centrosome disjunction, also known as centrosome splitting. C-Nap1 is critical for the cohesion of duplicated centrosomes while C-Nap1 phosphorylation, during

mitosis, leads to centrosome disjunction and bipolar spindle formation. By using synchronized Tax-specific reporter cells, I was able to visualize centrosome disjunction in normal HeLa cells but observed a dramatic reduction of centrosome disjunction in Tax expressing cells. Therefore, the failure of C-Nap1 phosphorylation correlated directly with the failure of centrosome disjunction in Tax-expressing cells. Furthermore, quantitative relationships between centrosome non-disjunction and chromosome loss in the cause of aneuploidy have been demonstrated (150).

Tax has been shown to cause centrosome supernumeracy previously (23, 144, 151). I also observed that prolong periods of Tax expression led to centrosome amplification in HeLa cells. How does a failure in centrosome disjunction, as reported here, lead to centrosomal amplification? First, cells that inherited the unsplit centrosome could duplicate them during subsequent cell division cycle to produce daughter cells with multiple centrosomes. Second, a recent study has found that a cell that inherited no centrosome as a result of laser-ablation of centrioles during mitosis gave rise to multiple centrin aggregates (100). In this regard, those Tax-expressing cells that inherited no centrosome might assemble multiple centrioles *de novo* to compensate for the centrosome loss. As most Tax-expressing HeLa cells enter into senescence, it seems less likely for Tax-expressing cells to undergo additional cell cycle that amplify centrosomes. Whether *de novo* nucleation of centrosomes is involved in the centrosome supernumeracy induced by Tax remains to be fully resolved.

Further support of Tax-induced centrosome abnormalities was demonstrated in LLCPK cells which express α-tubulin-GFP fusion protein. The α-tubulin proteins nucleated at the centrosome or also known as the MTOC. This focal region of α-tubulin-GFP was shown to colocalize with γ-tubulin, a common marker used to indicate the centrosome. I

observed that Tax-induced an increased number of centrosomes in LLCPK cells but, unexpectedly, resulted in the frequent loss of a distinct MTOC, as shown by the loss of the α-tubulin-GFP focal region. Cells that develop abnormal centrosomes might also have abnormal nucleation of microtubules. This is supported by the finding that microinjection of antibodies against centrosomal proteins CEP110 and ninein, which co-localize with C-Nap1, disrupted the centrosome affecting the centrosome's ability to function as a MTOC (147). The disruption of a MTOC could also affect chromosome segregation though the relevance of this data still needs to be substantiated.

Whether centrosome amplification in Tax-expressing cells can cause overt spindle multipolarity remains to be determined. While abnormal centrosomes and spindle multipolarity are commonly linked with cancer, it is only beginning to be understood how cancer cells remain viable in the presence of such mitotic abnormalities. Somatic cells possess abilities to mute the effects of centrosome amplification. Spindle multipolarity is avoided by microtubule dynein which supports centrosomal clustering to form bipolar spindles (160, 177) and whether this is important in Tax-expressing cells is not known at present. While spindle-pole bundling leads to bipolar division, chromosome segregation in this instance may not always be exactly equal (177). Another means by which cells employ to mute the effects of centrosome amplification is to attempt a multipolar division where only one cleavage furrow is allowed to persist, yielding two daughter cells containing possibly different chromosome complements. Furthermore, in the absence of a centrosome, cells possess an alternative pathway that assembles bipolar spindles (176) which may be important in Tax-expressing cells which do not inherit any centrosomes. Therefore, although Tax-

expressing cells develop centrosome abnormalities, various mechanisms may be engaged by the cells to overcome such mitotic challenges.

Centrosome amplification is frequently found in malignant epithelial cancers such as breast, colon, prostate and lung carcinomas (35, 110, 155, 156). Cells infected by tumor viruses such as hepatitis B virus (42), human papillomaviruses (36), and Kaposi's sarcoma-associated herpesvirus (148) have also been demonstrated to contain supernumerous centrosomes. These examples of centrosome amplification have been suggested to play an important role in tumorigenesis. Disruption of centrosome function is known to adversely affect mitotic spindle formation and chromosome segregation. The frequent occurrence of DNA aneuploidy and appearance of bi- and multi-nucleated cells following Tax expression are consistent with the inhibition of C-Nap1 phosphorylation and centrosomal non-disjunction induced by Tax described here. Recently, centrosome amplification has also been demonstrated in ATL cells from HTLV-infected patients, lending further support to the biological relevance of our studies. Lymphocytes isolated from ATL patients displayed increased numbers of centrosomes per cell when compared to normal patients, and the same phenomenon was observed in Tax-expressing T cells (144). These results suggest that HTLV-1 infection causes centrosomal abnormalities; and the centrosome abnormalities/amplification induced by HTLV-1 Tax may be an important aspect to ATL development.

A previous study has suggested that Tax interaction with Ran and RanBP1 may be important for Tax-induced centrosomal amplification. More recently, Tax has been shown to interact with TaxBP2, a large coiled-coil centrosomal protein otherwise known as rootletin. Importantly, rootletin has been found to be a C-Nap1-associated protein, and like C-Nap1, is

involved in centrosome cohesion by forming centriole-associated filaments. Indeed, small interfering RNA-mediated depletion of either rootletin or C-Nap1 led to centrosome splitting (6). At present, whether rootletin is regulated by Nek2A and PP1 like C-Nap1 is unclear. It would not be surprising, however, if Tax affects both rootletin and C-Nap1 through the same mechanism (23).

Finally, the Tax-mediated premature activation of the APC can also contribute to the significant decrease in C-Nap1 phosphorylation. The APC has been shown to target Nek2A for degradation during early mitosis (55, 65). The loss of Nek2A as a result of premature APC activation by Tax could contribute to the decreased phosphorylation of its substrate, C-Nap1. Experiments to test for the levels of Nek2A at different stages of the cell cycle of Tax-expressing cells are in progress. In this vein, over-expression of Nek2A has been shown to cause centrosome splitting while dominant negative Nek2A prevents centrosomal disjunction.

In conclusion, research in this dissertation has indicated that both premature APC activation and inhibition of C-Nap1 phosphorylation impact dramatically on the cell cycle progression of Tax-expressing cells. It is not clear, however, if they represent two distinct and unrelated activities of Tax, or alternatively, there may be a causal link yet-to-be resolved between the two. Finally, the role of the Tax-induced mitotic dysfunctions in HTLV-1 life cycle and pathogenesis also remains to be fully elucidated. With better understanding, new therapeutic agents could be developed for HTLV infection, HTLV-associated diseases, and cancers with similar mitotic dysfunctions.

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