Mechanisms controlling CDK9 activity

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1. ABSTRACT

This review primarily focuses on the mechanisms that modulate CDK9 activity and its recruitment to cellular genes, where it phosphorylates the C-terminal domain of RNA polymerase II (RNAPII) as well as negative elongation factors. CDK9 associates with each of four cyclins (T1, T2a, T2b and K), forming distinct positive transcription elongation factors (P-TEFb). Research done during the past decade has demonstrated a role for P-TEFb in stimulating elongation of otherwise paused RNAPII transcripts. Recent work suggests that P-TEFb also positively modulates other steps during transcription. In addition, "abnormal" CDK9 function is associated with certain diseases. Specifically, the activity of the cyclin T1/CDK9 complex is essential for HIV-1 replication and CDK9 upregulation is associated with cardiac hypertrophy. Thus, the role of CDK9 in these processes, and the possibility of therapeutically targeting CDK9, will also be briefly discussed.

2. INTRODUCTION: IDENTIFICATION AND CHARACTERIZATION OF T-TYPE CYCLIN/CDK9 COMPLEXES

CDK9 (cyclin dependent kinase 9) is a Ser/Thr Pro-directed protein kinase that was identified in a human cDNA library screen aimed at discovering novel members of the Cdc2 family of protein kinases (1, 2). CDK9 was originally named PITALRE (1), which designated the single letter amino acid sequence of its putative PSTAIRE-like cyclin binding motif (Figure 1B). This motif is present in CDKs and related kinases, and it is used to name CDK-like kinases without known cyclin partners (reviewed in 3). Accordingly, PITALRE was renamed CDK9 when its cyclin partners were identified (4-6). The region of CDK9 that is conserved with other CDKs spans residues 19-315, sharing 33-47 % amino acid identity. Originally, CDK9 was identified as a protein with a molecular mass of approximately 42 kD ($CDK9_{42}$) (1), which is expressed in all cell types tested to date. Accordingly, CDK9 expression is regulated by a TATA-less housekeeping promoter (7) (Figure 1A). A second CDK9 isoform, $CDK9_{55}$, has recently been identified, which exhibits a 13 kD Pro and Gly-rich N-terminal extension (8). $CDK9_{55}$ has an approximate molecular mass of 55 kD and, like $CDK9_{42}$, localizes to the nucleus (1, 8, 9). Interestingly, $CDK9_{55}$ expression is apparently directed by a TATA-box containing promoter upstream of the $CDK9_{42}$ promoter (8, 10) (Figure 1A). Of note, the ratio of $CDK9_{42}$/$CDK9_{55}$ expression changes with cell type and in certain cell types with the differentiation/activation stage (8, 10, 11). The presence of two independent promoters (7, 8, 10) and the differential expression of CDK9 mRNA species in tissues
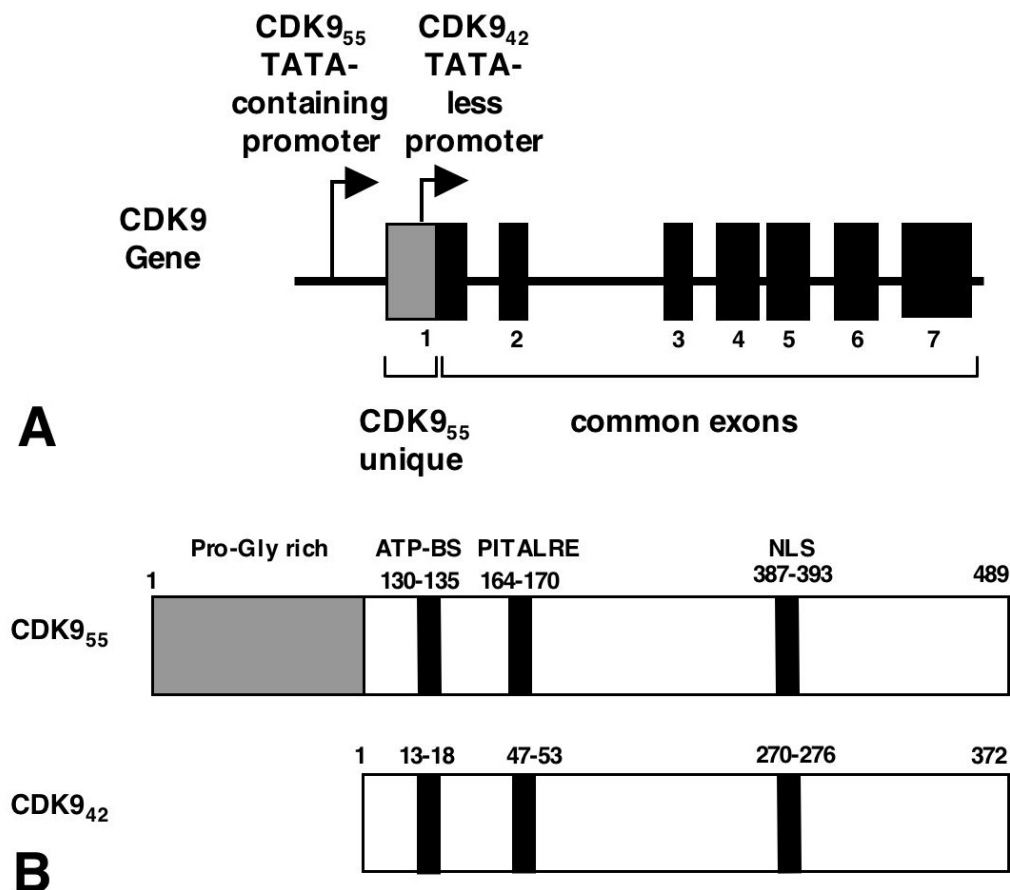
Figure 1. Diagram of the human CDK9 isoforms. (A) The CDK9 gene has a TATA-less promoter that directs expression of CDK9$_{42}$, whereas, CDK9$_{55}$ is thought to be dependent on an upstream TATA-containing promoter. (B) CDK9$_{42}$ consists of 372 amino acids. CDK9$_{55}$ exhibits a unique 117 amino acid N-terminal domain rich in proline and glycine residues (Pro-Gly rich). This extension is predicted to be followed by 372 residues identical to CDK9$_{42}$. Both CDK9 isoforms contain the ATP-binding site (ATP-BS) as well as the 11 subdomains conserved in the catalytic domain of protein kinase (not shown), the PSTAIRE-like motif PITALRE, and a putative nuclear localization signal (NLS).

(1) suggest that transcription of these two CDK9 isoforms is regulated in a tissue specific manner.

Early experiments showed that the majority of cellular CDK9 activity was associated with protein complexes, but not monomeric CDK9, suggesting the existence of cyclin regulatory subunits (12). This suggestion was confirmed with the identification of T-type cyclins as required partners for CDK9 activation (5, 6, 13). Purification of the *Drosophila* Positive Transcription Elongation Factor b (P-TEFb) led to the identification of CDK9 and cyclin T1 as its two subunits (5, 14). This was the first hint of CDK9 cellular function, as P-TEFb had previously been shown to exhibit a DRB-sensitive C-terminal RNA polymerase II (RNAPII) kinase activity required for the transition into transcriptional productive elongation (15, 16). CDK9 activity was also found to be the HIV-1 Tat associated kinase (TAK) (14, 17), a DRB-sensitive kinase activity previously shown to be associated with the domains in Tat required to stimulate HIV

transcription (18, 19). Human cyclin T1 was subsequently identified as a Tat associated protein (13) and as a component of mammalian P-TEFb (6), independently. In mammals, there are several distinct complexes that result from the combination of the two CDK9 isoforms with four activating cyclin subunits, named cyclin T1, cyclin T2a, cyclin T2b and cyclin K (6, 8, 20). Each T-type cyclin/CDK9 complex forms a distinct P-TEFb unit, which phosphorylates the CTD of RNAPII and positively regulates transcriptional elongation (6, 8). Cyclin K/CDK9 also phosphorylates the CTD of RNAPII (20), but can only stimulate transcription when tethered to promoters via RNA, not DNA (21).

T-type cyclins and cyclin K are more closely related to cyclins C, H and L than to cyclins with primary functions in cell cycle control. Indeed, similarly to T-type and K cyclins, cyclins C, H and L are all known to activate CDKs, which play distinct roles in transcription (reviewed in 3). The Cyclin T1 gene encodes a 726 amino acid
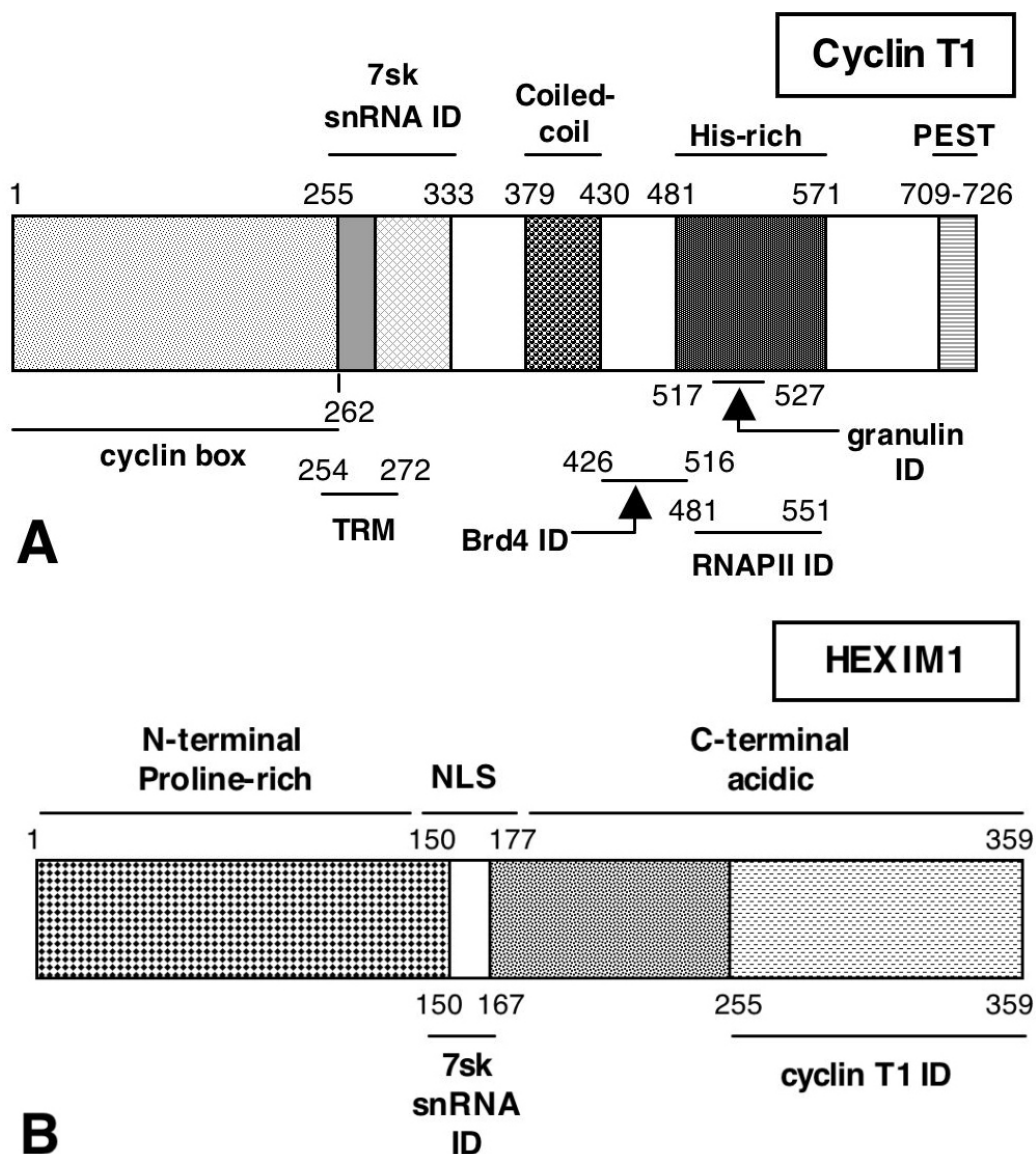
Figure 2. Diagram of the human cyclin T1 and HEXIM1/MAQ1 proteins. (A) Cyclin T1 is a 726 amino acid protein with a cyclin box, Tat recognition motif (TRM), 7sk snRNA-interacting domain (ID), coiled-coil motif, histidine-rich region (His-rich) essential for binding RNAPII and granulin, and a putative PEST sequence. (B) HEXIM1 is a 359 amino acid protein containing a N-terminal proline-rich region, a nuclear localization signal (NLS), 7sk snRNA-interacting domain, C-terminal acidic region and a cyclin T1 interacting domain.

protein, while cyclins T2a and T2b are derived from a single gene as a result of alternative splicing (6). Consequently, cyclin T2a consists of 663 amino acids, of which cyclin T2b shares the first 642 residues. In addition, cyclin T2b exhibits an additional C-terminal extension for a total of 730 amino acids (6). Cyclin T1 is the major CDK9-associated cyclin in HeLa cells (6) and likely other cell types. However, the relative ratio of distinct P-TEFb complexes is likely to be cell type, differentiation, and/or activation-dependent, as differences in the expression of CDK9 isoforms and T-type cyclins have been reported (1, 6, 8, 10, 11, 13)(Figure 2A). T-type cyclins share 81 % identity within the N-terminal cyclin box, which contains

two 5-α-helix predicted folds characteristic of cyclins (5, 6). However, cyclin T1 and cyclin T2 share only 46 % identity in the C-terminus (6). Both cyclin T1 and T2 contain a His-rich motif and cyclin T1 exhibits a putative PEST sequence (Figure 2A). The CTD of RNAPII binds the cyclin T1 His-rich motif at amino acids 481 to 551 (22). In contrast to other PEST-motif containing proteins (reviewed in 23), the PEST sequence of cyclin T1 does not appear to be involved in regulation of its protein stability, but might be important for recruitment of a ubiquitin ligase that mediates CDK9 ubiquitination (see below). Also, cyclin T1 contains a putative coiled-coil motif and a Tat-recognition motif (TRM) (13)(Figure 2A). This TRM

contains a critical Cys residue (Cys[261]) required for binding to Tat to form the TAK complex (24-26). Importantly, cyclins T2a and T2b lack the TRM motif, which prevents their association with Tat (26, 27). Of note, murine cyclin T1 also lacks the Cys[261] residue explaining why murine cells do not support LTR-Tat-dependent transactivation, and reveling one of multiple blocks to HIV-1 replication in murine cells (24-26, 28). Therefore, although all T-type cyclins are capable of forming distinct P-TEFb complexes, only cyclin T1/CDK9 can form TAK.

3. ROLE OF P-TEFb IN TRANSCRIPTION

As mentioned above P-TEFb was first discovered as a cellular factor with the ability to stimulate transcriptional elongation. However, more recent work suggests roles for P-TEFb at other steps in transcription and RNA processing. In this section, we will briefly review the roles of P-TEFb in transcriptional initiation, elongation and RNA processing (the following subsections are ordered according to chronological discovery rather than by the order of each phase in the transcriptional process).

3.1. Elongation

P-TEFb was originally identified as a 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB)-sensitive factor strictly required for production of mature mRNA transcripts *in vitro* by RNAPII at initiated promoters (29). Previously, it had been shown that DRB dramatically reduces the production of mRNA when added to mammalian cells in culture (30) and inhibits transcriptional elongation by RNAPII *in vitro* (31). Subsequently, it was shown that elongation by RNAPII required its C-terminal domain (CTD) and that P-TEFb exhibited a DRB-sensitive kinase activity that phosphorylated the CTD in elongating complexes. This lead to the proposal that P-TEFb promotes productive elongation by phosphorylating the CTD of RNAPII (15). The mammalian CTD of RNAPII is composed of 52, mostly perfect, tandem heptad peptide repeats with the following consensus sequence Tyr-Ser[2]-Pro-Thr-Ser[5]-Pro-Ser, which becomes mostly phosphorylated on Ser residues during transcription (reviewed in 32). CTD phosphorylation is thought to facilitate recruitment of elongation and RNA processing factors (reviewed in 33). Phosphorylation of the CTD on Ser[5] is detected at gene promoters, while phosphorylation on Ser[2] is found in coding regions (34, 35). It has been proposed that Ser[5] is phosphorylated by CDK7, the kinase catalytic subunit of TFIIH, while P-TEFb phosphorylates Ser[2]. Interestingly, in the presence of Tat, P-TEFb also phosphorylates Ser[5] (36). A model has arisen in which hypophosphorylated RNAPII is recruited to promoters and is then phosphorylated by CDK7 on Ser[5], coinciding with promoter clearance, initiation of transcription, and recruitment of RNA capping enzymes. Pausing near the promoter is subsequently abrogated concomitantly with phosphorylation of Ser[2] by P-TEFb (reviewed in 32)(Figure 3A and B). P-TEFb also phosphorylates DSIF and NELF, two N-TEF complexes, which cooperate to repress elongation by RNAPII and confer a requirement for P-TEFb during elongation in extracts (reviewed in 33, 37, 38). NELF negatively

regulates RNAPII by binding to RNAPII/DSIF complexes and nascent transcripts, as one of the NELF subunits binds RNA (39). P-TEFb phosphorylates the SPT5 subunit of DSIF and the RNA-binding RD subunit of NELF, releasing their repressive action on RNAPII following initiation (14, 40-45). Phosphorylated SPT5 remains associated with the polymerase and becomes a positive elongation factor (reviewed in 38). RD phosphorylation apparently inhibits its RNA binding activity, at least in the context of the HIV-1 nascent transcript, where RD binds TAR, but NELF may remain associated with the elongating RNAPII (45, 46)(Figure 3A and B).

3.2. RNA processing

Work utilizing model organisms suggests that CDK9, may play additional roles in RNA processing in a manner dependent on its ability to phosphorylate the CTD of RNAPII. Ctk1, the catalytic subunit of the *Saccharomyces cerevisiae* CTD Kinase 1, is a CTD kinase with structural and functional similarities to P-TEFb, which phosphorylates Ser[2] during elongation (47). However, Ctk1 is not required for recruitment of elongation factors, as RNAPII elongates transcripts from multiple genes in the absence of Ctk1 (48). Instead, Ctk1 is necessary for recruitment of polyadenylation factors and processing of 3' end RNA (48, 49). Likewise, in *Drosophila melanogaster* Kc cells inhibition of P-TEFb activity with flavopiridol prevented accumulation of hsp70 and hsp26 RNAs with concomitant inhibition of Ser[2] phosphorylation but without altering the distribution of RNAPII and other transcription factors (50). Interestingly, this phenomenon appears to result from inadequate recruitment of 3' end processing factors in the absence of P-TEFb activity. Similarly, DRB blocked transcription-coupled splicing and poly(A) site cleavage in *Xenopus* oocytes (51). Finally, although it is not known whether P-TEFb plays similar roles in mammalian cells, flavopiridol had little effect on RNA cleavage in human RNAPII elongation complexes *in vitro* (52).

3.3. Initiation

P-TEFb has been detected at the HIV preinitiation complex (PIC), where it is thought to be inactive, and subsequently become activated concomitantly with TFIIH release during early elongation (53). However, a recent report suggests that P-TEFb also plays a key role in transcription initiation (54). Previous work has shown that the PIC is formed at the promoter by the assembly of RNAPII and at least six basal transcription factors including TFIID. TFIID consists of the TATA-box-binding protein (TBP) and TBP-associated factors designated TAFs (reviewed in 55). Using chromatin immunoprecipitation combined with DNA/RNA tethering of various potent transactivators, it has been observed that Tat and P-TEFb recruit TBP to various promoters in the absence of TAFs. Recruitment of TBP by Tat requires HIV-1 TAR and Tat transactivation is not inhibited in the absence of TAFs. A model arises in which P-TEFb is recruited by Tat or cellular factors, which in turn results in recruitment of TBP without TAFs to form PICs. Thus, P-TEFb induces transcription complex assembly (54). These results also suggest the existence of two major distinct classes of

Figure 3. Recruitment of P-TEFb to gene promoters and downstream regions and dynamic regulation of cellular P-TEFb pools. (A) P-TEFb is recruited near paused RNAPII via specific transcription factors and/or activators (TF), such as myc or NF-kappaB. P-TEFb then phosphorylates negative elongation factors, NELF and DSIF, as well as the C-terminal domain of RNAPII on Ser^2 (red *P*), which allows for productive elongation (yellow *P* represent Ser^5 phosphorylation by TFIIH, see text for details). Both DSIF and NELF remain bound to RNAPII and may play other roles during elongation. (B) Tat recruits P-TEFb to the TAR RNA structure that forms at the 5' end of the nascent HIV RNA. Then, CDK9 phosphorylates RNAPII as well as NELF and DSIF to allow for productive elongation. Note that NELF no longer contacts TAR upon phosphorylation. (C) 7sk/HEXIM binds and sequesters P-TEFb in an inactive form in the nucleus. Stress signals, such as actinomycin D, UV or hypertrophic signals, induce release of P-TEFb from it's inhibitory complex. Release of active P-TEFb allows for its association with Brd4 and perhaps other transcription factors which target P-TEFb to specific gene targets. (D) Brd4 recruits active P-TEFb to acetylated DNA during transcription initiation. The Mediator may also facilitate recruitment of P-TEFb via its association with Brd4 (see text).

promoters in human cells, TAF-dependent and TAF-independent, a notion already demonstrated in yeast (56, 57). Nevertheless, these results do not question an additional role for P-TEFb during elongation and/or RNA processing.

4. MECHANISMS CONTROLLING CDK9 ACTIVITY

4.1. Assembly

CDK9 is part of multiprotein complexes of variable molecular mass (1, 12, 58). Cellular CDK9 activity was found to be mainly associated with multiprotein complexes rather than monomeric CDK9, suggesting that other regulatory subunits were required for efficient kinase activation (12). With the identification of T-type cyclins, it was possible to reconstitute active CDK9 complexes *in vitro*, showing that recombinant CDK9 had no activity in the absence of associated cyclins (6). In 293 cells ectopically expressing CDK9 (B4 cells), additional proteins were identified bound to CDK9, including Hsp90, Hsp70 and Cdc37 (58). Identification of these proteins led to the elucidation of a multi-step, chaperone-dependent, pathway responsible for proper CDK9/cyclin T1 complex assembly (58). Free CDK9, which accounts for approximately 80% of CDK9 in B4 cells, is rapidly degraded. Newly synthesized CDK9 sequentially interacts with the molecular chaperone Hsp70, the kinase-specific chaperone complex Hsp90/cdc37, and finally cyclin T1, leading to production of the mature cyclin T1/CDK9 complex which, as opposed to free CDK9, is very stable (58).

4.2. Upregulation of cyclin T1 and CDK9 during T-cell Activation

T cells and macrophages are primary targets for HIV-1 infection and pathogenesis. HIV-1 replicates poorly in resting lymphocytes and CD4$^+$ memory T cells, where it becomes latent. Thus, CD4$^+$ memory T cells are reservoirs of latent HIV-1 viruses and T cell reactivation triggers HIV-1 replication (reviewed in 59). While searching for factors limiting for HIV-1 replication in resting T cells, it was found that TAK activity was low in quiescent Peripheral Blood Lymphocytes (PBLs) and increased following mitogenic stimulation (17). The discovery of the subunits of P-TEFb/TAK, allowed analysis of their expression in quiescent and mitogen stimulated PBLs and CD4$^+$ T cells. Stimulation of PBLs with Phorbol 12-myristate 13-acetate (PMA), and/or phytohemagglutinin (PHA), as well as with combinations of αCD3 and αCD28 antibodies, the cytokine IL-2, and ionomycin (a calcium ionophore) leads to upregulation of both cyclin T1 and CDK9 (60, 61). Upregulation of cyclin T1 correlates with RNAPII phosphorylation and HIV-1 replication (60, 62). Interestingly, the mechanisms controlling cyclin T1 upregulation during T cell activation are complex and are regulated by independent mitogenic pathways (62). PHA induces cyclin T1 mRNA and protein accumulation via cyclin T1 mRNA stabilization. This process requires both calcineurin and JNK activation. In contrast, PMA induces PKC-dependent stabilization of the cyclin T1 protein, without major changes in mRNA expression (62). Of note,

upregulation of cyclin T1 during T cell activation is not a unique event, but rather appears to be coordinated with the upregulation of other components of the RNAPII transcription machinery, including CDK9, the CDK7 and cyclin H subunits of TFIIH, RNAPII and likely other basal transcription factors (62). While the particular mechanism involved in upregulation of each of these other factors is unknown, in the case of CDK9 it might involve protein stabilization, as expression of the cyclin T1 subunit appears to be limiting for stabilization of CDK9 in the chaperone pathway leading to complex assembly in various cell types (58, 63, 64). Nevertheless, the upregulation of these basal transcription factors is likely in place to fulfill the increased transcriptional needs associated with the acquisition of functional competence and proliferation during T cell activation.

Of note, while cyclin T1 and CDK9 expression are upregulated during T cell activation, a process that involves cell cycle entry and progression through the cell cycle, their expression and associated kinase activity is not upregulated during cell cycle entry and progression in a variety of human cells (1, 60, 63). In agreement with this notion, cyclin T1 and CDK9 expression appears to be upregulated by a combination of cytokines sufficient to reactivate transcription and replication of latent HIV-1 in CD4$^+$ cells from infected individuals, apparently without inducing proliferation (65).

That said, it is also important to mention that others subsequently reported that they observed little change in cyclin T1 expression during activation of Peripheral Blood Mononuclear Cells (PBMCs), and suggested that the discrepancies between theirs and previous studies could be due to differences in the preparation of cell lysates (66). Additionally, this report suggested that the level of cyclin T1 expression detected in proliferating U937 promonocytic cells was also dependent on cell lysis conditions (66). Thus, we reexamined upregulation of cyclin T1 in both PBLs and monocyte/macrophages. Using whole lysates of PBLs (devoid of macrophages) prepared under denaturing conditions we confirmed that cyclin T1 is upregulated by various mitogens (62). In agreement with these results, upregulation of cyclin T1 and CDK9 during PBL activation has also been confirmed by indirect immunofluorescence following fixation of intact PBLs (67). However, we observed that quiescent PBLs appear to contain a protease with the ability to degrade a small fraction of cyclin T1 during non-denaturing cell lysis even in the presence of complex mixtures of protease inhibitors (62). Unfortunately, this prevents direct accurate measurement of CDK9 activity, which requires preparation of cell lysates under non-denaturing conditions. Thus, it is important to clarify that while cyclin T1 expression is clearly upregulated during T cell activation, previous studies may have slightly overestimated the fold increase in cyclin T1 expression.

On the other hand, similar analysis performed in proliferating HL-60 promyelocytic cells and in primary macrophages revealed the presence of a potent protease,
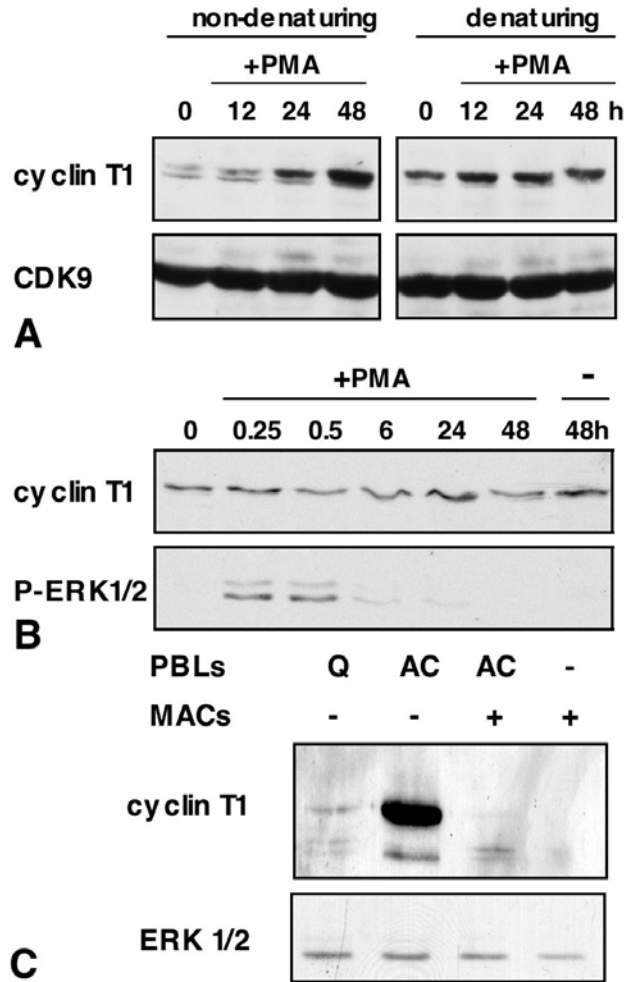
Figure 4. Cyclin T1 expression is not upregulated during differentiation along the monocyte/macrophage lineage. (A) Cyclin T1 expression is not upregulated in PMA-treated HL60 cells. HL60 cells were plated at a concentration of 2×10^5 cells/mL and treated with vehicle or 3 ng/mL PMA for the indicated times. Whole cell lysates were prepared under denaturing (125 mM Tris (pH 6.8), 2.0% SDS, 0.1 M DTT, 10% glycerol and Complete Mini Protease Inhibitor Cocktail Tablet, Roche) as described previously (62) and non-denaturing conditions (50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.1% Triton X-100, 0.1 mM sodium vanadate, 1 mM PMSF, 10 μg/mL leupeptin, 4 μg/mL aprotinin, and 40 μg/mL pepstatin). 20 μg of protein was resolved by 8 % SDS-PAGE. Western blot analysis was performed with anti-cyclin T1 and anti-CDK9 antibodies. (B) Cyclin T1 is not upregulated during PHA-induced differentiation of U937 cells. U937 cells were plated at 2×10^5 cells/mL and treated with vehicle or 3 ng/mL PMA for the indicated times. Cells were collected and lysates prepared under non-denaturing lysis conditions (see A). 10 μg of total protein was resolved by 8 % SDS-PAGE and Western blot analysis performed using anti-cyclin T1 and anti-phospho-ERK1/2 antibodies. (C) Macrophages appear to contain a potent protease that degrades cyclin T1 in whole cell extracts of mixed activated PBLs and macrophages. Macrophages were depleted from the PBMCs by magnetic affinity column separation using anti-CD14 positive selection. PBLs were treated with vehicle (Q) or stimulated with 1 ng/mL PMA and 1 μg/mL PHA for 48 h (AC). Activated PBLs (AC) and macrophages (MACs) were mixed, pelleted and whole cell lysates were prepared immediately under non-denaturing conditions as previously described (62). 20 μg of whole cell lysate was resolved by 8 % SDS-PAGE. Western blot analysis was performed with anti-cyclin T1 and anti-ERK1/2 (loading control) antibodies.

which degrades cyclin T1 in non-denaturing lysates. Figure 4A shows that the apparent upregulation of cyclin T1 seen following PMA-induced differentiation of HL60 promyelocytic leukemic cells along the monocyte/macrophage lineage is misleading, as it is not observed if the cell lysates are prepared under denaturing conditions. Also, we observed little to no upregulation of cyclin T1 following treatment of U937 cells with PMA (Figure 4B). More direct evidence is provided in Figure 4B, where non-denaturing cell lysis of a mixture of primary macrophages and mitogen-stimulated PBLs leads to complete disappearance of the cyclin T1 present in PBLs, which is otherwise stable. Thus, it appears that cyclin T1 expression is not upregulated during differentiation along the monocyte/macrophage lineage. Also, these data warn that caution should be taken when analyzing cyclin T1 expression in hematopoietic cells, especially in cells of the monocyte/macrophage lineage.

4.3. Autophosphorylation

Initial studies assaying CDK9 associated kinase activity revealed that most proteins specifically co-immunoprecipitated with CDK9 antibodies, including CDK9 itself, were phosphorylated *in vitro* (1). Subsequent studies showed that the kinase activity detected in CDK9 complexes was solely due to the CDK9 subunit, and thus demonstrated that CDK9 autophosphorylates *in vitro* (12). The functional relevance of CDK9 autophosphorylation was revealed later, when it was demonstrated that CDK9 autophosphorylation in several Ser/Thr C-terminal residues increased the affinity of the Tat/cyclin T1/CDK9 complex for TAR (68, 69). Interestingly, it has been shown that CDK9 autophosphorylation is negatively regulated by TFIIH in HIV-1 promoter PICs, and that release of TFIIH during elongation results in CDK9 autophosphorylation facilitating binding of the Tat/cyclin T1/CDK9 complex to TAR (53). It is conceivable that CDK9 autophosphorylation is also important for the interaction of P-TEFb with other macromolecules.

4.4. 7sk snRNA/HEXIM

Several of the well characterized CDKs are known to be negatively regulated by proteins that stoichiometrically bind to either the cyclin/CDK dimer or the monomeric catalytic subunit, thereby preventing binding of the regulatory cyclin (reviewed in 70). Of note, CDK9 is negatively regulated by an unprecedented mechanism involving both a RNA molecule and a protein factor (Figure 3C). The 7sk small nuclear RNA (7sk snRNA) was found associated with the inactive pool of P-TEFb, which in HeLa cells is about 50% (71, 72). Importantly, the 7sk snRNA-containing complex (400-500 kD) could be converted to a smaller, kinase active complex (130 kD) following salt or RNase-treatment *in vitro* as well as stress inducing and transcriptional inhibiting agents, such as UV irradiation and actinomycin D, *in vivo* (71, 72). Paradoxically, while UV irradiation and actinomycin D treatment inhibit cellular transcription, HIV-1 transcription is upregulated (73-75). Thus, it is conceivable that under certain stress conditions that globally shut down cellular transcription, increased P-TEFb activity may facilitate transcription of a subset of cellular genes, perhaps stress response genes.

Further characterization of these complexes led to the identification of HEXIM1 (hexamethylene bisacetamide-induced protein 1)/MAQ1 (ménage à quatre) as a cellular protein required for 7sk snRNA inhibition of P-TEFb (76, 77). HEXIM1 was also independently identified as a nuclear protein induced by a potent differentiation inducer, hexamethylene bisacetamide (HMBA), in vascular smooth muscle cells (78). A related HEXIM protein (HEXIM2) has been identified and shown to exhibit activity similar to that of HEXIM1 (79, 80). Interestingly, neither 7sk snRNA nor HEXIM proteins are sufficient to inhibit P-TEFb activity. A model describing the multisubunit structure of inactive P-TEFb complexes containing 7sk snRNA and HEXIM has been proposed based on several recent reports. According to this model, a single molecule of 7sk snRNA binds a HEXIM dimer, inducing a conformational change in the HEXIM proteins

exposing a binding domain for P-TEFb in each HEXIM protein (81)(Figure 3C). Two P-TEFb complexes, previously phosphorylated on T186 of the T loop domain of CDK9 each bind to the 7sk snRNA activated HEXIM proteins, leading to P-TEFb inactivation. Interestingly, this report also demonstrated that CDK9 activity requires phosphorylation on T186 (81). Phosphorylation of this conserved residue in other CDKs is known to be required for activation (reviewed in 82). The enzyme phosphorylating this residue in CDKs, such as CDK1, CDK2 and CDK4, is called CAK (CDK Activating Kinase) and two unrelated kinases have been identified in mammalian cells with this activity; the cyclin H/CDK7 kinase that is also a component of TFIIH (83) and p42 (84). In any case, the nature of the kinase phosphorylating T186 on CDK9 is unknown. While the actual components of the inactive P-TEFb pool have been well characterized, the dynamics of formation/abrogation of this complex in the cell are less well understood. In this regard, it has been shown that physiological stresses in cardiac muscle cells lead to release of P-TEFb from 7sk snRNA complexes, which correlates with hyperphosphorylation of RNAPII and a global increase in RNA and protein synthesis (85), a cellular response typically associated with muscle cell hypertrophy (reviewed in 86). Interestingly, while HEXIM1 and HEXIM2 are expressed in all tissues tested, their relative expression varies in different tissues (79). Of note, HEXIM1 expression is induced in a variety of cells treated with HMBA, a potent inducer of cell differentiation (79). In contrast, HEXIM1 is downregulated by estrogen in breast cancer cells (87). Thus, HEXIM upregulation might positively regulate differentiation and inhibit growth, while its downregulation might favor proliferation and growth. Hence, it is clearly important to study how the expression of HEXIM proteins is regulated in response to signals that induce cell proliferation/differentiation and growth and how this affects P-TEFb activity as well as both global and specific transcription. Alternatively, cells may utilize more dynamic mechanisms to regulate the interaction between 7sk snRNA, HEXIM and P-TEFb in response to cellular signaling.

4.5. CDK9 ubiquitination

Recent work has shown that protein ubiquitination plays roles other than marking proteins for proteasomal degradation (reviewed in 88, 89). The first hint of a potential role for ubiquitination in modulation of the cyclin T1/CDK9 complex was provided by the demonstration that the SCF^{skp2} ubiquitin ligase forms a complex with cyclin T1/CDK9 and ubiquitinates the CDK9 subunit (90). This interaction depends on a PEST motif present at the C-terminal domain of cyclin T1. It was also proposed that ubiquitination regulates CDK9 expression during the cell cycle (90). However, re-examination of these findings confirmed the interaction between cyclin T1/CDK9 and the SCF^{SKP2} ubiquitin ligase, but challenged the notion that ubiquitination affects CDK9 steady-state levels or associated kinase activity during the cell cycle (reviewed in 37). Interestingly, a recent report suggests that CDK9 ubiquitination by the SCF^{SKP2} ubiquitin ligase enhances the affinity of the Tat/cyclin T1/CDK9 complex for TAR, thereby increasing Tat mediated transactivation

Table 1. Factors that affect P-TEFb complex recruitment to genes

P-TEFb Complex	Binding Partner	Gene Target	Negative Transcription Regulator	Positive Transcription Regulator	Reference
CycT1/CDK9	Androgen receptor	PSA	-	+	127
	CIITA	MHC class II	-	+	98
	NF-kappaB	IL-8	-	+	99
	Aryl hydrocarbon receptor	cytochrome P450 1A1	-	+	128
	Myc	CAD, cyclin D2, TERT, NUC, ODC	-	+	100-102
	Tat	HIV-1	-	+	13-14
	BRD4	HIV-1	-	+	105-106
	Runx1	CD4?	+	-	109
CycT/CDK9	HEXIM/7sk snRNA	General sequestration	+	-	71-72, 76-77
CycT2a/CDK9	MyoD	Muscle specific genes	-	+	103-104
CycT/CDK9 (C. Elegans)	PIE-1	?	+	-	107

(91). In agreement with this, Tat dependent transactivation of the HIV-1 promoter is reduced in cells lacking SKP2. Moreover, it has also been shown that ubiquitination of a prototypic transcriptional activator facilitates its recruitment of P-TEFb, in a manner in which ubiquitin interacts directly with the C-terminus of cyclin T1 creating a second contact between the transactivator and cyclin T1 (92). Thus, ubiquitin ligases appear to positively regulate P-TEFb activities by acting at different steps thereby facilitating more robust protein-protein interactions.

4.6. Transcription Factors and Brd4 recruit P-TEFb to genes.
        The expression of many genes is regulated at the level of transcriptional elongation (reviewed in 37, 93). The notion that P-TEFb may be required for transcription of most RNAPII dependent genes is supported by experiments using flavopiridol, a potent CDK inhibitor, which exhibits significant selectivity for CDK9 (94). Flavopiridol potently inhibits transcription by initiated polymerases in run-on assays performed with nuclei isolated from treated HeLa cells (95). In agreement with these results, flavopiridol also decreases the expression of many genes as determined by DNA microarray analysis in a manner highly similar to other transcriptional inhibitors such as actinomycin D and DRB (96). Moreover, targeting CDK9 with siRNA in *C. elegans* embryos demonstrated that CDK9 is essential for expression of many early embryonic genes and phosphorylation of Ser^2 of the RNAPII CTD (97). These data have led to the proposition that P-TEFb serves as a general cellular transcription elongation factor (95). A number of studies have investigated the means by which P-TEFb is recruited to promoters. Taube *et al*. demonstrated that P-TEFb stimulated transcription when tethered to either upstream or downstream of promoters via DNA, and suggested that P-TEFb could mediate the effects of enhancers by stimulating elongation in a manner that required an interaction with the CTD of RNAPII as well as core promoter elements and promoter proximal regions (22). In agreement with this notion, several transcription factors and transcriptional activators have been identified that interact with P-TEFb. A compilation of them is shown in Table 1. The first of these P-TEFb recruiting factors to be identified was CIITA, a class II transactivator that regulates expression of MHC genes (98). CIITA interacts with the same domain in cyclin T1 as Tat. Thus, CIITA and Tat compete for binding when coexpressed, suggesting a mechanism by

which Tat may contribute to the immunopathology associated with AIDS (98). Subsequent studies led to the identification of other transcription factors including NF-kappaB, c-myc, the androgen receptor and Myo D, which were shown to bind P-TEFb and act as coactivators (Table 1, Figure 3A). In particular, RelA was shown to stimulate elongation in a manner dependent on CDK9 activity and cyclin T1 (99). Accordingly, CDK9 and RelA are co-recruited to the IL-8 promoter in response to TNF-alpha stimulation coinciding with phosphorylation of RNAPII on Ser^2 and Ser^5 (99). c-Myc was also shown to associate with cyclin T1 and c-Myc activation induces CDK9 recruitment to a variety of c-Myc inducible promoters (100-102). On the other hand, cyclin T2/CDK9 associates with MyoD and both cyclin T2 and CDK9, but not cyclin T1, are recruited to MyoD target promoters during muscle differentiation (103, 104). All together, these observations support a model in which P-TEFb could be recruited to multiple promoters by particular transcription factors or activators, functioning as coactivators (reviewed in 37). However, recent work suggests that the active cellular pool of P-TEFb is associated with Brd4, a bromodomain motif containing protein, which binds acetylated histones (105, 106). In HeLa cells, modulation of Brd4 expression by overexpression or siRNA knockdown leads to increased or decreased RNAPII phosphorylation and transactivation of various cellular promoters, respectively. Of note, promoter recruitment of P-TEFb is dependent on Brd4 and enhanced by an increase in chromatin acetylation (106). It has also been suggested that the Mediator complex plays a role in P-TEFb recruitment (105, 106). Interestingly, the two pools of cellular P-TEFb, inactive P-TEFb/7sk snRNA/HEXIM1 and transcriptionally active P-TEFb/Brd4 are kept in a dynamic equilibrium and convert into each other in response to stress signals (105)(Figure 3C and 3D). Thus, the requirement of P-TEFb activity for the transcription of most cellular RNAPII genes mentioned above is compatible with a model in which Brd4 would recruit P-TEFb to promoters by mechanisms dependent on the acetylation state of histones and/or other general mechanisms (Figure 3D), rather than promoter and/or enhancer specific sequences. Thus, the contribution of transcription factors/activators to P-TEFb promoter recruitment remains an open question. It is possible that at least for certain genes, P-TEFb recruitment to promoters, enhancers and/or coding regions is modulated by particular transcription factors/activators (Figure 3A), perhaps cooperating with Brd4. Analysis of P-TEFb

recruitment/co-recruitment to distinct cellular genes will be necessary to answer this question.

4.7. Repressors of P-TEFb activity

Recent studies have also revealed that P-TEFb is targeted by repressors in a manner that leads to inhibition of transcriptional elongation by RNAPII. *Caenorhabditis elegans* PIE-1, a global transcription repressor in early embryonic germ cells, inhibits transcriptional elongation in a cyclin T1-dependent binding manner (107). PIE-1 associates with the C-terminal domain of cyclin T1 that contains the his-rich region, preventing its interaction with RNAPII. Interestingly, PIE-1 exhibits a "decoy" repeat similar to the heptapeptide repeats of the RNAPII CTD, but with Ala residues at the Ser positions 2 and 5 (107). This domain is important for the transcriptional silencing function of PIE-1. Another example is Runx1, which functions as a potent transcriptional repressor of the CD4 gene in immature thymocytes (108). Runx1 contains an inhibitory domain that binds to cyclin T1, which may mediate its inhibitory effects on elongation by RNAPII (109). Altogether, it seems that both transcriptional activators (section 4.6.) and repressors (section 4.4 and this section) can target P-TEFb through multiple domains and via distinct mechanisms, positively or negatively modulating elongation by RNAPII and perhaps other aspects of transcription. The complexity of P-TEFb regulation is in accordance with its potential involvement at multiple steps during the transcription process as well as its involvement in the transcription of many genes.

4.8. Interaction with other proteins

A number of proteins have been identified in yeast two-hybrid screens using cyclin T1 as a bait. Among these are granulins and the human I-mfa domain containing protein (HIC) (110, 111). Granulins are growth factors that have been found to regulate many cellular processes, such as cell growth, development, wound repair, and transcription. Granulins are expressed in many different human cell types and elevated levels of granulin have been found in several different human cancers (reviewed in 112). Both granulins and HIC interact with cyclin T1, but not with cyclin T2, as this interaction involves the His-rich region of cyclin T1, which also mediates a interaction with RNAPII (110, 111). Of note, both proteins also interact with Tat although through distinct domains (113), and modulate Tat dependent transactivation, with granulins being inhibitory (110) and HIC exhibiting positive effects (111). However, whether the expression of these proteins is rate-limiting for P-TEFb transcriptional activities in cells is not known.

5. CDK9 AND HUMAN DISEASE

As discussed in the preceding sections, mammalian T type cyclin/CDK9 complexes and their orthologues in eukaryotic cells play critical roles at distinct steps during transcription. Work over the last few years has shown that abnormal CDK9 function is associated with human diseases such as AIDS, cardiac hypertrophy and perhaps even cancer.

As mentioned above, the cyclin T1/CDK9 complex is a target of HIV-1 Tat. Cyclin T1 and Tat contact the nascent HIV-1 TAR recruiting CDK9 in close proximity to RNAPII and N-TEF, which become hyperphosphorylated allowing productive elongation (Figure 3B)(reviewed in 33, 37) and, as recently reported, also stimulating subsequent rounds of transcription complex assembly at the HIV-1 promoter and re-initiation (54). Interestingly, HIV-1 transcription is highly sensitive to inhibition of CDK9 activity. In this regard, a variety of pharmacological kinase inhibitors that exhibit selectivity for CDK9 are potent inhibitors of HIV-1 transcription and replication in various cellular assays (114-116). These assays have mostly been performed using transformed cells, but it has also been shown that some of these compounds inhibit HIV-1 replication in PBMCs at concentrations that are apparently non-toxic (116). In agreement with these results, direct inhibition of cyclin T1/CDK9 activity or expression using dominant-negative CDK9 or siRNA, respectively, also inhibits HIV-1 replication without causing toxicity in transformed cells (64, 116). However, these results do not mean that CDK9 activity is not essential for cellular transcription and/or viability. Indeed, at significantly higher concentrations, pharmacological inhibitors of CDK9 are toxic, presumably due to inhibition of cellular transcription. Given the observation that cyclin T1 and CDK9 expression is low in quiescent PBLs and that T cell activation correlates with accumulation of these subunits and an increase in global RNA synthesis, it is important to note that these cells might be particularly sensitive to CDK9 inhibition. Thus, further work is needed to establish whether CDK9 or, in particular, the cyclin T1/CDK9 complex can be safely inhibited in primary cells with no toxicity and without affecting cellular function.

Mechanical stress of the ventricular wall as a result of hypertension, valvular disease, congenital defects, inherited cardiomyopathies, or myocardial infarction causes cardiac hypertrophy, a disease state characterized by a global increase in cellular RNA and protein content resulting in increased myocyte size (reviewed in 86, 117, 118). The increase in ventricular wall thickness is an increased risk factor for cardiac mortality in humans (119, 120). Interestingly, RNA polymerase II activity is limiting in cardiac myocytes in the absence of trophic signals and hypertrophic signals stimulate transcriptional elongation by RNAPII in a manner dependent on CDK9 activity (85). In cultured cardiomyocytes, hypertrophic agonists induce rapid RNAPII hyperphosphorylation, apparently via release of cyclin T1/CDK9 from the 7sk snRNA inhibitory complex. Importantly, this situation is also observed in transgenic mice expressing Gq proteins and calcineurin under the control of alpha-MHC promoters, as well as in a model of mechanical load (85, 86). In agreement with these results, ablation of the mouse HEXIM1 (also designated CLP-1) gene leads to cardiac hypertrophy and late embryonic lethality (121). Surprisingly, subsequent studies have also shown that genes associated with mitochondrial function are downregulated in the mouse myocardium of alpha-MHC-cyclin T1 transgenic mice and by cyclin T1/Cdk9 in cultured cardiomyocytes (122). In

this context, cyclin T1/CDK9 activation inhibits the transcription of a master regulator of mitochondrial function, peroxisome proliferator-activated receptor gamma coactivator 1 (PGC-1), which apparently leads to decreased mitochondrial membrane potential, and sensitizes cardiomyocytes to apoptosis. This suggests that deregulation of cyclin T1/CDK9 function in cardiomyocytes triggers both an increase in cell mass as well as mitochondrial dysfunction, which may contribute to heart failure (122). Cumulatively, these results have lead to the proposal that inhibitors of CDK9 could be used therapeutically to decrease the mass of the ventricular wall in pathological conditions and perhaps prevent other deleterious consequences (reviewed in 123).

A potential link between cyclin T1/CDK9 activity and cancer is suggested in ER positive breast cancer cells, where estrogens have been shown to downregulate the expression of HEXIM1, also known as Estrogen Downregulated Gene (EDG1) (87). HEXIM1/EDG1 interact with the ER receptor and, as mentioned above, with cyclin T1, apparently in a mutually exclusive manner (124). When overexpressed in breast cells, HEXIM1/EDG1 inhibits cell proliferation and anchorage-independent growth (87). Also, considering the role of HEXIM1 in limiting P-TEFb activity and the effects of hexamethylene bisacetamide (HMBA), which potently induces HEXIM-1 expression and differentiation in a variety of neoplastic cells (78), it is tempting to speculate that a fine balance in cellular T-type cyclin/CDK9 activity may influence the neoplastic capacity of certain tumor cells. Another possible link between potentially deregulated cyclin T1/ CDK9 complexes and cancer is suggested by a study describing upregulation of CDK9/cyclin T1 expression in lymphomas derived from precursor B and T cells (125). However, considering that cyclin T1 and CDK9 are upregulated during T cell activation (60, 61), it is conceivable that expression of these subunits in B/T cell lymphomas reflects the activated and/or differentiation stage of these cells. In any case, while deregulation of CDK9 activity may occur in certain tumor types, the therapeutic value of inhibiting P-TEFb to treat cancer patients is questionable, as flavopiridol has failed as a single agent in most clinical trials, showing little anti-tumoral activity at non-toxic concentrations (reviewed in 126). Nevertheless, it might still prove to be a potentially effective agent when used against tumors highly dependent on transcription for survival, or perhaps in combination with other drugs.

In summary, it is conceivable that potent selective inhibitors of CDK9 activity may have useful therapeutic applications. Highly selective inhibition of cyclin T1/CDK9 complexes, without affecting other P-TEFb complexes, could potentially block HIV-1 replication, if normal cells can function with low levels of cyclin T1/CDK9 activity and/or if other T-type cyclins can substitute for cyclin T1 to maintain normal cellular transcription. Alternatively, an inhibitor interfering with the cyclin T1/CDK9/Tat complex, which does not interfere with the ability of cyclin T1/CDK9 to be recruited to cellular promoters, could also potentially work.

Pharmacological agents targeting cellular proteins have the presumed advantage of limiting the selection of drug-resistant viral variants, which is a current problem in patients under Highly Active Anti-Retroviral Therapy (HAART). This makes the cyclin T1/CDK9 complex an attractive target. A number of CDK9 pharmacological agents antagonize HIV-1 replication at non-toxic concentrations in a variety of cells, and it is conceivable that more selective analogs could be generated with even lower HIV-1 replication inhibition/cellular toxicity IC_{50} ratios. In this case, as these drugs do not kill the virus and only prevent its replication, it is critical to note that patients would have to be treated chronically, perhaps in combination with other HAART drugs to prevent reactivation of latent proviruses. On the other hand, using CDK9 inhibitors to treat cardiac hypertrophy is completely different, as the objective now becomes to reduce cellular transcription. Thus, the magnitude of CDK9 inhibition required will likely be significantly higher. While chronic treatments may not be necessary in this case, the challenge would reside on finding drug concentrations that effectively reduce hypertrophy without affecting cell viability. Perhaps, however, a better understanding of the signaling pathways that result in activation of CDK9 in cardiomyocytes following stresses could unveil upstream targets, whose inhibition would be less detrimental to normal cell function.

6. PERSPECTIVES

Although we have witnessed major progress in delineating the mechanisms regulating CDK9 activity and P-TEFb function over the last decade, numerous important questions remain unanswered. Among these are the following: (i) Do Brd4 and transcriptional activators cooperate to recruit P-TEFb to common genes or alternatively target separate genes? Does P-TEFb play the same role in transcription when recruited by Brd4 as when recruited via transcriptional activators? For instance, Brd4 recruitment could facilitate a potential role for P-TEFb during initiation and/or early elongation, while transcriptional activators may facilitate P-TEFb's role during elongation and/or RNA processivity for inducible genes. Do other recruitment mechanisms exist? (ii) Is the transcription of most genes modulated by P-TEFb in the same manner? Or is P-TEFb an integrator of multiple cellular signals? Are different P-TEFb complexes redundant? Do they target different genes? Do they target the same genes but respond to different cellular cues? (iii) It is also important to learn how HEXIM proteins and Brd4 are regulated in response to cellular cues. What are the effects of altering the ratios among these proteins in cellular processes such as proliferation, growth, differentiation, and apoptosis using both primary cells and animal models? (iv) What posttranslational modifications on CDK9, T-type cyclins as well as their main regulatory partners are critical and how do they work? (v) Finally, it is important to establish the extent to which inhibition of CDK9 activity, as well as the activity of particular T-type cyclin/CDK9 complexes, is compatible with cell viability and normal cell function in both primary cells and animal models, in order to establish whether CDK9 or a subset of

T-type cyclin/CDK9 complexes can be therapeutically targeted.

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