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Developmental regulators containing the I-mfa domain interact with T cyclins and Tat and modulate transcription

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Summary

Positive transcription elongation factor b (P-TEFb) complexes, composed of cyclin-dependent kinase 9 (CDK9) and cyclin T1 or T2, are engaged by many cellular transcription regulators that activate or inhibit transcription from specific promoters. The related I-mfa (inhibitor of MyoD family a) and HIC (human I-mfa-domain-containing) proteins function in myogenic differentiation and embryonic development by participating in the Wnt signaling pathway. We report that I-mfa is a novel regulator of P-TEFb. Both HIC and I-mfa interact through their homologous I-mfa domains with cyclin T1 and T2 at two binding sites. One site is the regulatory histidine-rich domain that interacts with CDK9 substrates including RNA polymerase II. The second site contains a lysine- and arginine-rich motif that is highly conserved between the two T cyclins. This site overlaps and includes the previously identified Tat/TAR recognition motif of cyclin T1 required for activation of human immunodeficiency virus type 1 (HIV-1) transcription. HIC and I-mfa can serve as substrates for P-TEFb. Their I-mfa domains also bind the activation domain of HIV-1 Tat and inhibit Tat- and P-TEFb-dependent transcription from the HIV-1 promoter. This transcriptional repression is cell-type specific and can operate via Tat and cyclin T1. Genomic and sequence comparisons indicate that the I-mf and HIC genes, as well as flanking genes, diverged from a duplicated chromosomal region. Our findings link I-mfa and HIC to viral replication and suggest that P-TEFb is modulated in the Wnt signaling pathway.

Keywords

I-mfa; HIC; HIV transcription; cyclin T1/T2; P-TEFb

Introduction

The inhibitor of MyoD family protein a (I-mfa) and the human I-mfa domain containing protein (HIC) share a highly homologous cysteine-rich C-terminal region named the I-mfa domain.¹ I-mfa functions as a repressor of MyoD family basic helix-loop-helix (bHLH) transcription factors that are essential for myogenesis.² It also plays a pivotal role in differentiation of placental trophoblast giant cells via its interaction with Mash2 and its induction by PPAR β .³

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⁴ I-mfa and XIC (the *Xenopus* ortholog of HIC) play critical roles in mouse and frog development and both are essential players in the Wnt signaling pathway in *Xenopus*.^{3,5,6} Signaling through the Wnt cascade involves the coactivator β -catenin and the transcription factors TCF/LEF (T cell factor/lymphoid enhancer-binding factor). Both XIC and I-mfa bind TCF/LEF via their I-mfa domain and this domain is sufficient to inhibit the binding of *Xenopus* Tcf3 to its target DNA.⁶ I-mfa and HIC also bind to Axin and modulate Axin-mediated signaling in the Wnt and c-Jun N-terminal kinase (JNK) pathway.⁷ More recently, it was shown that I-mfa binds directly to β -catenin and that this interaction relieves I-mfa suppression of myogenesis in a myogenic model system.⁸ In addition to the crucial roles attributed to the I-mfa family proteins in differentiation and development, HIC modulates transcription from the promoters of two human retroviruses, HTLV-I and HIV-1.^{1,9,10}

We identified HIC as a novel regulator of the positive transcription elongation factor P-TEFb via interactions with the cyclin T1 subunit of P-TEFb and its viral ligand HIV-1 Tat (trans-activator of transcription).⁹ P-TEFb is a general transcription elongation factor involved in transcription of more than 90% of RNA polymerase II (Pol II) genes.¹¹ It phosphorylates the carboxy-terminal domain (CTD) of Pol II and additional substrates involved in general and gene specific transcription.^{12–17} CTD hyperphosphorylation overcomes abortive elongation and greatly increases the production of RNA.^{18,19} P-TEFb itself is found in active and inactive forms. A large fraction of the active form interacts with the bromodomain 4 (Brd4) recruiting factor which is necessary for its general transcription activity,^{20,21} and ~ 50% of P-TEFb in the cell is present in inhibitory complexes containing HEXIM proteins (HEXIM1 or 2) bound to 7SK, an abundant small nuclear RNA.^{22–29}

P-TEFb contains the cyclin-dependent kinase CDK9 and a cyclin partner, either cyclin T1, T2a, T2b, or K.^{30–32} The T cyclins (T1, T2a,b) are exceptionally long cyclins and share a highly homologous cyclin domain at their N termini which binds CDK9 and various cellular transcription modulators that recruit P-TEFb to specific promoters.^{31,33–44} Their long C termini are divergent but contain a histidine-rich regulatory domain essential for binding RNA polymerase II and other CDK9 substrates and transcription modulators.^{13,45,46} Since its discovery, P-TEFb has emerged as an essential factor in diverse cellular systems including cell growth, differentiation and apoptosis.^{13,37,39,44,47–49}

Another conspicuous role for P-TEFb is in HIV-1 replication^{19,30,50} where the viral transcription elongation factor Tat and the viral RNA element TAR (trans-activation response element) associate with P-TEFb complexes that contain cyclin T1. Tat and TAR bind to the cyclin box of cyclin T1 and to an adjacent basic sequence named the Tat-TAR recognition motif (TRM).⁵¹ Tat is a small protein (101 aa) composed of several versatile domains. Its N-terminal activation domain, which is necessary and sufficient for binding to cyclin T1, contains six conserved cysteines in a short cysteine-rich domain as well as a conserved core domain. Tat's interaction with TAR RNA is mediated via its arginine/lysine-rich basic domain. In addition to its major role in recruiting P-TEFb, Tat interacts with numerous cellular proteins to facilitate HIV-1 infection (for reviews see^{52–55}). We and others have previously shown that HIC interacts with first exon Tat (aa 1–72) in vivo.^{9,10} HIC cDNA activates transcription from the HIV-1 promoter,⁹ an action that we have recently ascribed primarily to the extraordinarily long (2.8kb) 3' untranslated region of the HIC mRNA which can displace the inhibitory 7SK RNA from P-TEFb (Young, Mathews and Pe'ery, submitted for publication).

We demonstrate that I-mfa is a novel P-TEFb binding partner that modulates expression from the HIV-1 promoter in a cell-type specific manner. Like HIC, I-mfa interacts with the cyclin T1 subunit of P-TEFb and with HIV-1 Tat. The homologous I-mfa domains of HIC and I-mfa are responsible for binding to cyclin T1 and Tat. Both I-mfa domains inhibit P-TEFb- and Tat-dependent transcription, with the domain in the I-mfa protein being more potent than that of

HIC. The I-mfa domains can also serve as P-TEFb substrates. Notably, HIC and I-mfa interact with two prominent regulatory domains present in both cyclin T1 and cyclin T2. One of these, the histidine-rich domain that is a site for binding Pol II and other substrates, interacts in a zinc-dependent manner. The other site, which includes the Tat/TAR binding motif of cyclin T1, is therefore identified as a region that engages cellular as well as viral transcription regulators.

Results

I-mfa and HIC are differentially expressed

The I-mfa protein shares sequence homology with HIC in two main regions (Figure 1(a)). The signature I-mfa domain is present at their C-termini and, based on the complete human genome sequence, these are the only two proteins that contain this unique cysteine-rich domain. In addition, a short region (~20aa) near the center of the protein sequence (Figure 1(a), dashed line) contains 7 conserved basic amino acids and has nucleic acid binding properties (Wang et al., in preparation). In a survey of cell lines, HIC mRNA was readily detected in 8 of 12 cell lines examined (Figure 1(b)). I-mfa mRNA was found at a high level in one line (COLO 205) and in lesser abundance in 6 others. No cell line contained high levels of both mRNAs. Thus, most cell lines had one or the other mRNA and only MCF-7 cells appeared to lack both.

I-mfa is a novel P-TEFb binding partner

HIC and I-mfa have been reported to exhibit similar but distinct functions in transcription and differentiation. Our finding that HIC interacts with P-TEFb via its I-mfa domain⁹, prompted us to ask whether the I-mfa protein is also a cellular partner of this transcription elongation factor. COS cells were transfected with plasmids expressing FLAG-tagged versions of I-mfa and HIC and their various truncations (Figure 2(a)). Whole cell extract was subjected to immunoprecipitation with anti-FLAG antibody followed by immunoblotting with antibodies directed against cyclin T1 or CDK9 (Figure 2(b)). Endogenous cyclin T1 and CDK9 coimmunoprecipitated with full-length I-mfa and HIC and with their C-terminal fragments (I-mfa-I and HIC-I) but not with their N-terminal fragments (HIC-N and I-mfa-N). We conclude that I-mfa is a novel P-TEFb interacting protein and that its I-mfa domain is necessary and sufficient for this interaction in cells. The presence of the conserved basic region in HIC-C and I-mfa-C did not alter the interaction with P-TEFb.

The I-mfa domains of I-mfa and HIC interact with cyclin T1 at two sites

To map the I-mfa domain binding site on cyclin T1 we performed GST pull-down assays using various GST-cyclin T1 truncated proteins (Figure 3(a), (b)) and in vitro synthesized ³⁵S-labeled I-mfa or HIC (Figure 3(c)). HIC interacted with full-length cyclin T1 and with its His-rich regulatory domain, as expected from results obtained with yeast two-hybrid system.⁹ In addition, it interacted with the N-terminal region of cyclin T1. Cyclin T1 (1–300), containing the cyclin domain, was sufficient for this interaction. Cyclin T1 truncations T1 (1–300) and T1 (1–479) bound HIC to a lesser extent compared with the full-length protein but the binding efficiency was restored when the His-rich domain was included in cyclin T1 (1–551). The C-terminal region of cyclin T1, T1 (402–726), displayed binding activity comparable to the N-terminal truncations T1 (1–300) and T1 (1–479). While the His-rich domain alone, T1 (501–530), bound HIC weakly, the longer C-terminal fragment, T1 (480 to 570), interacted more avidly with HIC, suggesting that regions flanking the His-rich region also participate in the interaction. Similar results were obtained with the I-mfa protein (Figure 3(c)). Hence, both HIC and I-mfa interact with cyclin T1 at two binding sites.

Since the I-mfa domain is cysteine-rich and binds to the His-rich region of cyclin T1, we investigated the dependence of this interaction on divalent metal ions. In the presence of zinc ions, the binding of I-mfa or HIC to full-length cyclin T1 and to all cyclin T1 C-terminal

fragments that contain the His-rich sequence was dramatically increased (Figure 3(c)). Other divalent metal ions such as Mg^{2+} , had a weaker effect (data not shown). In contrast, the binding of I-mfa or HIC to the two N-terminal fragments of cyclin T1 was not affected by the addition of $ZnCl_2$. The addition of 10mM EDTA further reduced the binding activity of the C-terminal fragments while the N-terminal fragments retained the same ability to bind I-mfa or HIC (Figure 3(c)).

Similar pull-down assays were used to map the regions of I-mfa and HIC that are necessary for interaction with cyclin T1 in vitro (Figure 3(d)). The I-mfa domain alone (I-mfa-I and HIC-I) is sufficient to confer binding to GST-cyclin T1, while the first 163 amino acids (I-mfa-N and HIC-N) are dispensable for this interaction. As expected from the data of (Figure 3(c)), C-terminal fragments of cyclin T1 containing the His-rich domain, displayed zinc dependency when binding to the I-mfa domain but the N-terminal fragment did not (data not shown). Thus the I-mfa domains of I-mfa and HIC are necessary and sufficient for binding to both sites on cyclin T1, and the interactions of the I-mfa domains with the His-rich containing region are zinc dependent.

I-mfa and HIC are P-TEFb substrates and their I-mfa domains contain CDK9 phosphorylation sites

P-TEFb is a CTD kinase and it also phosphorylates a number of proteins that interact with one or both of its subunits.^{12,44,56–61} To investigate whether HIC and I-mfa can serve as P-TEFb substrates in vitro, we first used GST-HIC and GST-I-mfa to pull down P-TEFb from HeLa cell extracts (Figure 4(a)). Immunoblotting for cyclin T1 and CDK9 showed that, as expected, full-length HIC and I-mfa and the I-mfa domains of both proteins pulled down P-TEFb from HeLa cell extract whereas HIC-N, I-mfa-N and GST did not. Approximately the same amounts of GST fusion proteins were used (Figure 4(b)). The proteins that had been pulled down were subjected to kinase assays (Figure 4(c)). GST-HIC, GST-I-mfa and the I-mfa domains of both proteins (HIC-I and I-mfa-I) were found to be phosphorylated.

Since additional kinases may be present in the GST pull-down samples, we examined the ability of P-TEFb immunocomplexes to phosphorylate HIC and I-mfa. P-TEFb immunoprecipitated from HeLa cell extract with anti-CDK9 antibody was shown to phosphorylate a synthetic substrate, CTD4 (Figure 4(d)). Purified GST fusion proteins (Figure 4(e)) were tested as substrates for the immunopurified kinase (Figure 4(f)). Only the proteins that bind P-TEFb, i.e., full-length HIC and I-mfa and their I-mfa domains (GST-HIC-I and GST-I-mfa-I), became phosphorylated. Several additional bands were also radiolabeled, including cyclin T1 and CDK9 as observed previously.^{19,56,57} As expected, the phosphorylation of GST-HIC was sensitive to the P-TEFb inhibitor 5,6-dichloro-1- β -D-ribofuranosyl benzimidazole (DRB; Figure 4 (d)). Taken together, these results support the conclusion that HIC and I-mfa engage in intimate interactions with P-TEFb, and that their I-mfa domains contain one or more CDK9 phosphorylation sites.

Differential binding of Tat to cyclin T1 and the I-mfa domain

HIC interacts with HIV-1 Tat, both in the yeast two-hybrid system and in vivo, and this interaction is dependent on the I-mfa domain.⁹ To determine whether the I-mfa protein behaves similarly, we compared the ability of full length I-mfa and HIC and their truncations to bind to GST-Tat72 (Figure 5(a)). Both proteins bound, as did the truncations that contain the I-mfa domains (I-mfa-I, I-mfa-C, HIC-I and HIC-C) but not the N-terminal truncations (I-mfa-N and HIC-N). Thus, the I-mfa domains of I-mfa and HIC are necessary and sufficient for the interaction with Tat. To verify that they also form complexes with Tat inside cells, we expressed FLAG-HIC, FLAG-I-mfa and their FLAG-tagged truncations, alone or together with HA-Tat72 in COS cells. Whole cell extracts were prepared and subjected to immunoprecipitation

with anti-FLAG antibody followed by immunoblotting with anti-HA antibody. HA-Tat72 coimmunoprecipitated with HIC as expected,⁹ and also with I-mfa (Figure 5(b)). Similar to their interactions with cyclin T1, the I-mfa domains of both proteins are sufficient for interaction with Tat inside cells (Figure 5(b)).

To map the sites on Tat that are necessary for this interaction we used full-length Tat and its truncations in the form of GST-fusion proteins (Figure 5(c)) and tested their binding to HIC and I-mfa in GST pull-down assays. (Figure 5(d)) (top and bottom panels) shows that HIC and I-mfa bind to the Tat activation domain (aa 1–48). The acidic region (aa 1–21) within the activation domain is dispensable for binding to HIC and I-mfa whereas the adjacent cysteine-rich region of Tat (aa 21–37) is essential for direct interaction with HIC or I-mfa but is not sufficient on its own. The minimal requirement, aa 21–45, is therefore a subset of the requirement for the Tat:cyclin T1 interaction (aa 1–48).¹⁹ However, the Tat:HIC interaction was not disrupted by point mutations in the cysteine-rich or core domains of Tat (C30G and K41A) that abrogate the binding of Tat to cyclin T1 (Figure 5(e)). Furthermore, in contrast to the interactions of cyclin T1 with Tat^{51,62} and HIC, neither zinc nor EDTA affected Tat:HIC binding (Figure 5(d), middle panels). Thus, HIC and cyclin T1 bind overlapping but distinct sites on Tat.

The I-mfa domain inhibits Tat- and P-TEFb-dependent transcription

I-mfa and HIC bind Axin but have opposite effects on β -catenin regulated transcription.⁷ As shown above, I-mfa and HIC display similar binding to P-TEFb and Tat and they are both P-TEFb substrates. We therefore compared the effect of the I-mfa and HIC proteins on expression driven by the HIV-1 promoter which is dependent on P-TEFb and Tat. Whereas transfection of the full-length HIC cDNA was stimulatory,⁹ the HIC open reading frame alone gave no effect in HeLa cells (Young, Mathews and Pe'ery, submitted for publication). In HeLa cells the expression of full-length HIC had no significant effect on Tat transactivation but HIC-I inhibited firefly luciferase expression by 2.5 fold while HIC-N activated transcription by about 50% (Figure 6(a)). The intact I-mfa protein inhibited Tat transactivation and its I-mfa domain alone exerted a 5-fold inhibition on Tat mediated transcription. Deletion of the I-mfa domain from the I-mfa protein (I-mfa-N) completely abolished the repression activity indicating that this domain is solely responsible for the inhibitory effect (Figure 6(a)). Thus, the I-mfa protein acts as an inhibitor of Tat transactivation in HeLa cells and its I-mfa domain is about twice as strong an inhibitor as that of HIC. Similar results were obtained in HT-1080 and A549 cells which express little or no detectable HIC protein (data not shown), but 293 cells were more responsive as observed previously with HIC-I.⁹ In 293 cells, full-length HIC repressed expression by almost two fold and I-mfa inhibition was two and a half fold (Figure 6(b)). HIV-driven transcription was reduced by about 5-fold by either of the two I-mfa domains (HIC-I and I-mfa-I) whereas HIC-N and I-mfa-N activated gene expression 50% and two fold respectively. Thus, in 293 cells HIC and I-mfa exert similar actions on gene expression from the HIV promoter, but I-mfa is somewhat more potent.

Because HIC and I-mfa bind to Tat as well as P-TEFb, their effects may involve I-mfa domain interactions with either or both of these transcription factors. To evaluate their individual contributions, we examined expression from Tat-independent promoters. Transcription driven by the PCNA promoter is also largely independent of P-TEFb.¹³ Correspondingly, expression of firefly luciferase from the PCNA promoter was not significantly modulated in HeLa cells by HIC, I-mfa or their truncations (Figure 6(c)). Similar results were obtained with the c-Myc promoter (Figure 6(d)), which is sensitive to P-TEFb inhibitors,⁶³ suggesting that inhibition exerted by the I-mfa domain in HeLa cells is mediated via Tat rather than P-TEFb. To test this idea, we used the Gal4-cyclin T1 tethering assay, in which P-TEFb activity is recruited to a modified HIV-1 promoter containing Gal4 binding sites without a requirement for Tat.⁶⁴ This

system allows the effects of the interactions of HIC and I-mfa with cyclin T1 on expression from the HIV promoter to be evaluated in the absence of Tat. Inhibition by the I-mfa domains was greatly attenuated in HeLa cells (Figure 6(e)), but it remained strong in 293 cells (Figure 6(f)). We conclude that the I-mfa domain inhibits predominantly via P-TEFb in 293 cells and predominantly via Tat or the Tat:P-TEFb complex in HeLa cells.

An expanded regulatory region in cyclins T1 and T2

P-TEFb complexes containing cyclin T2 account for about 20% of CDK9 in nuclear extracts (compared to 65% for cyclin T1) and are potent regulators of transcription.^{31,65} Cyclins T2a and T2b are splice variants that share their first 642 amino acids but diverge at their C-termini. The cyclin boxes of cyclins T1 and T2 (aa 1–250 and 1–249 respectively) are 81% identical³¹ and their regulatory histidine-rich domains are ~60% identical (Figure 7(a)). Since both I-mfa and HIC bind to the N-terminal region (aa 1–300) and to the His-rich domain of cyclin T1, we examined whether they also bind to cyclin T2 in GST pull-down assays. ³⁵S-labeled full-length I-mfa and HIC were pulled down by GST-cyclin T2b at about the same efficiency as by GST-cyclin T1 (Figure 7(b), upper panels of I-mfa and HIC, lanes 3 and 6). I-mfa and HIC also bound to cyclin T2 (1–300) which contains its cyclin domain (Figure 7(b), upper panels of I-mfa and HIC, lanes 7). The histidine-rich domain of cyclin T2a exhibited a similar weak binding activity to I-mfa and HIC as the histidine-rich domain of cyclin T1 (Figure 7(b), upper panels of I-mfa and HIC, lanes 8) and this binding was greatly increased upon addition of Zn²⁺ (Figure 7(b), lower panels of I-mfa and HIC, lanes 8). Thus, as with cyclin T1, I-mfa and HIC interact with cyclin T2 at two sites and the binding to the histidine-rich region of is zinc dependent.

Tat and TAR interact with cyclin T1 in its cyclin domain and a sequence known as the TRM located immediately downstream at aa 250–262.⁵¹ We therefore considered the possibility that the TRM might be part of a more general cyclin T regulatory region. To test this idea we first determined whether the cyclin domains of cyclins T1 and T2 are sufficient for binding to I-mfa and HIC. The binding of in vitro synthesized I-mfa and HIC to GST-T1 (1–250) and GST-T2 (1–250) was drastically reduced in comparison to their counterparts GST-T1 (1–300) and GST-T2 (1–300) (Figure 7(c), compare lanes 4, 5 and 7, 8). Hence, the cyclin boxes of T1 and T2 alone interact relatively weakly with I-mfa and HIC, and this binding is greatly stimulated by the adjacent 50 amino acids.

Examination of this region of human cyclins T1 and T2 shows that there is 65% sequence identity and 80% similarity between them in their proximal part (aa 251–270 and 250–269, respectively) (Figure 7(d)); residues in the distal part are only 7% identical and 33% similar (Figure 7(e)). Basic amino acids, which are scarce in the distal part, account for 7 of the 20 residues (35%) in the proximal part, and accordingly we refer to it as the K/R-rich motif (KRM) (Figure 7(d) boxed residues). A multiple alignment of the cyclin T species from different organisms revealed that 16 of the 20 residues of the KRM are highly conserved in vertebrates (Figure 7(e)). The vertebrate consensus sequence over the region of the KRM, corresponding to the 20 residues adjacent to the cyclin domain is R-L-K-R/k-I-R/w-N-W-R/k-A-x-Q/e-A-A-x-K-t/p-K-x-D (upper case denotes complete or nearly complete conservation; lower case, alternates occurring at least 3 times; underlines, specific for cyclin T1 or T2). In contrast, the next 30 residues exhibit little conservation between the cyclin T1 and T2 families, but high conservation within each family (Figure 7(e)), compare black highlighting with the two shades of gray highlighting). Presumably these sequences diverged after gene duplication and became specialized for different functions. In the case of cyclin T2, one such function could be to provide an additional site for interaction with RNA pol II.⁶⁵

The high degree of conservation in the KRM prompted us to examine the binding of I-mfa and HIC to a series of cyclin T1 mutants that carry a cluster of alanine substitutions spanning the

region.⁵¹ All of the mutants, in the form of GST fusion proteins, were defective for these interactions (Figure 7(d), upper panels). The substitution closest to the cyclin domain (aa 251–254) was the most deleterious and the most distal substitution (aa 269–272) was the least affected. In contrast, only the aa 256–259 substitution was detrimental for Tat binding (Figure 7(d), third panel) consistent with the findings of Garber et al.⁵¹ The cyclin domain of cyclin T1 (aa 1–250) is sufficient for its interaction with CDK9;^{51,65} accordingly, none of the mutants displayed reduced CDK9 binding (Figure 7(d), fourth panel). We propose that the KRM constitutes a regulatory region that includes the TRM and binds both cellular and viral ligands, including I-mfa, HIC, 7SK,⁶⁶ Tat and TAR.

Discussion

P-TEFb is a pivotal transcription elongation factor involved in multiple cellular systems including cell growth, differentiation and apoptosis and in viral infection. We show that I-mfa, the founding member of the I-mfa/HIC family, binds to cyclin T1 and Tat via its signature I-mfa domain and regulates HIV-1 transcription. Characterization of the interaction sites led to the identification of a regulatory site for I-mfa and HIC, as well as Tat and TAR, on cyclins T1 and T2. This study establishes links between P-TEFb and the Wnt/ β -catenin signaling pathway in development and differentiation.

I-mfa and HIC originated by gene duplication

The I-mfa and HIC proteins share a common domain, the I-mfa domain, and many binding activities and biological functions as shown here and by others.^{6–9} They also both have a conserved basic region that can participate in nucleic acid binding (Wang et al., in prep.). The I-mf (MDFI) and HIC (MDFIC) genes are located on separate chromosomes, at 6(p21) and 7 (q31.2) respectively, but have significant features in common. First, the two genes flanking I-mf, namely FOXP4 (forkhead box P4) and TFEB (transcription factor EB), are homologous to the genes flanking HIC, namely FOXP2 and TFEC, and corresponding genes are transcribed in the same direction (Figure 8(a)). Second, the exon-intron structures of I-mfa and HIC are similar (Figure 8(b)). Although the HIC gene is about 6 times longer than I-mf due to longer introns and UTRs, both have 5 exons and the coding region starts in the second exon. Corresponding coding exons are similar in size, giving rise to proteins of the same length. The I-mfa domains begin at the exon 4/5 junction and are included in the last exons of I-mfa and HIC (Figures 1(a) and (8b)). Third, the intron-exon junctions fall between the first and second nucleotides of codons in both genes. Moreover, aspartate is the first amino acid of the I-mfa domain and is conserved in all organisms sequenced (Figures 8(c) and (d)). Taken together these observations strongly support the idea that I-mfa and HIC and their two flanking genes originated from a single ancestral sequence through duplication of a three-gene block.

The I-mfa domains of human I-mfa and HIC are 74% identical and contain 23 identical cysteines¹, including 5 consecutive cysteines that are essential for HIC's binding to Tat.⁹ Homologous I-mfa domain sequences are found in many vertebrates from bony fish to mammals (Figure 8(d)). Sequence conservation between I-mfa and HIC is found throughout the domain and is especially prominent in its N- and C- terminal regions (Figure 8(d), highlighted on black background). Nearly all of the cysteine residues (22 out of 23 or 24) are conserved, implying their involvement in the structure and/or function of the domain, and several potential phosphorylation sites are conserved. Sequences characteristic of the I-mfa and HIC proteins, which presumably account for their differential activities, are especially frequent in the central part of the I-mfa domain (Figure 8(d), highlighted on dark and light gray backgrounds, respectively). Evidently the two genes are very ancient since they are present in bony fish which evolved about 500 million years ago. Since the divergence of I-mfa and HIC, their distinct I-mfa domains have been well conserved, suggestive of distinctive biological

functions of the domain in the two proteins. Consistent with a functional differentiation, the two genes are frequently differentially expressed in the cell types examined (Figure 1(b)).

No sequenced genome was found that contains only a single I-mfa domain containing protein. Furthermore, no corresponding protein sequences were found in the invertebrate databases scanned. However, a search conducted with the nucleotide sequence of the I-mfa domain revealed that the I-mfa domain of I-mfa, but not of HIC, has similarity to sequences present in the fruitfly, *Caenorhabditis elegans* and *Ciona intestinalis* genomes. Hence it is possible that I-mfa-I is ancestral to HIC-I and gave rise to it by gene duplication.

I-mfa domains interact with T cyclins and identify the KRM motif

P-TEFb is subject to regulation by a growing list of cellular and viral factors. Many such factors, e.g., CIITA,³⁵ NF- κ B,³⁸ c-Myc^{39,43} and estrogen receptor α ,⁶⁷ bind to cyclin T1 via its cyclin boxes and activate transcription. On the other hand, PIE-1 and granulin inhibit P-TEFb transcription by interacting with the histidine-rich region of cyclin T1.^{13,46} Interactions with P-TEFb complexes containing cyclin T2 have been reported for pRB³⁷ and MyoD.⁶¹ MyoD recruits cyclin T2 containing P-TEFb to promote MyoD-dependent differentiation in myoblasts.³³ Runx1, a transcription modulator in the hematopoietic system, was recently reported to repress P-TEFb dependent transcription via complexes containing either cyclin T1 or cyclin T2.⁶⁸ Like Runx1, I-mfa and HIC bind to cyclins T1 and T2. This interaction inhibits the activity of P-TEFb complexes containing cyclin T1 (Figure 6) and presumably also complexes containing cyclin T2. Hence I-mfa and HIC may be able to modulate P-TEFb complexes that associate with a variety of promoters and regulate systems in which cyclin T1 and/or cyclin T2 is present.⁴⁸

I-mfa and HIC are the only cellular proteins identified to date that bind to two sites on T cyclins: the KRM adjacent to the cyclin domain, and the histidine-rich region nearer to the C terminus (Figure 9). The histidine-rich regulatory region is involved in binding Pol II and other CDK9 substrates⁴⁵ and regulators.^{13,46} The binding of I-mfa and HIC to this region is zinc dependent. This represents another example of zinc ions coordinating interactions between histidine-rich and cysteine-rich domains in separate proteins, as previously shown for granulin, also a cysteine-rich protein that interacts with cyclin T1.¹³ The KRM site of cyclins T1 and T2 (Figure 7(d)) includes and extends beyond the TRM, a sequence that is present in human and some other cyclins T1 but not cyclin T2. I-mfa and HIC are the first cellular proteins shown to interact with the TRM, a region that is necessary, but not sufficient, for the binding of HIV-1 Tat and TAR. It contains an essential, species-specific, cysteine residue (C261) that coordinates the zinc-dependent binding of Tat to cyclin T1 but not T2.⁵¹ In contrast, the KRM extends further downstream than the TRM and is conserved among the T cyclins of vertebrates (Figure 7(e)). While no other cellular protein has yet been reported to engage this region, we argue that the KRM is likely to constitute a site for binding regulatory RNA and protein ligands to P-TEFb complexes containing cyclin T1 or T2. A corollary of this idea is that the KRM sequence has been retained through evolution because it is a binding site for conserved ligands that are common to the T cyclins. As with Tat and TAR binding to the TRM, sequences in the cyclin domain are also involved in HIC and I-mfa binding to the KRM. It is notable that a predicted cyclin box α helix extends into the N-terminal part of the TRM/KRM.⁵¹

Effect of the I-mfa domains on P-TEFb-dependent transcription

The isolated I-mfa domain of the I-mfa protein, like that of HIC⁹, interacts with P-TEFb and inhibits its transcription function (Figure 6). Deletion studies have shown that the I-mfa domains of both proteins are also essential for their inhibitory functions in transcription pathways mediated by regulators such as Axin, TCF/LEF, β -catenin, Zic, and myogenic regulatory factors such as MyoD.^{2,6-8,69} Consistent with these findings in cell culture

systems, the I-mfa domain of mouse I-mfa was sufficient to inhibit the formation of the *Xenopus* dorsal axis.⁶ MyoD was recently shown to bind P-TEFb,⁶¹ raising the possibility that I-mfa and HIC exert their transcription inhibitory function during development and differentiation by interacting with P-TEFb. Comparing the magnitude of the inhibition of Tat transactivation in several cell lines, we noted that I-mfa-I is more inhibitory than HIC-I. Since the first 40 residues of HIC-I are sufficient for binding to Tat,⁹ it is likely that the divergent sequences in the central part of the I-mfa domains play a role in determining their potency.

The inhibitory effect of the I-mfa domain is cell-type specific in magnitude and mechanistically (Figure 6). In 293 cells, inhibition was primarily due to its interaction with P-TEFb, whereas interaction with Tat or the Tat:P-TEFb complex was required in HeLa cells. The degree of inhibition was greater in 293 cells than in HeLa cells, and full-length I-mfa and HIC were inhibitory in 293 cells but not in HeLa cells. Furthermore, the isolated N-terminal regions of I-mfa and HIC displayed an activating function that was significant in 293 cells but modest in HeLa cells. In the intact proteins, the N-terminal regions moderated the inhibitory action of the I-mfa domain, possibly because of interactions between these two parts of the protein (Wang et al., manuscript in preparation). These observations imply that 293 cells express factor(s) that cooperate with I-mfa and HIC to modulate P-TEFb activity. In light of the derivation of 293 cells from human embryonic kidney, it is interesting to note that HIC has been isolated frequently from adult cDNA libraries using yeast two-hybrid screens,^{1,9,70} whereas I-mfa has most often been isolated from embryonic libraries.^{2,8,69,71} Correspondingly, we detected HIC RNA but not I-mfa RNA in 19 human tissues (data not shown) and I-mfa RNA was undetectable in most adult mouse tissues.² Although this correlation is not absolute as XIC is involved in embryonic axis formation in *Xenopus*,^{5,6} it may be significant that I-mfa and HIC mRNAs are often expressed in a reciprocal fashion in cell lines (Figure 1(b)). Strikingly, the highest level of I-mfa mRNA was detected in COLO 205 cells which are derived from a human colorectal adenocarcinoma, a cancer that is frequently associated with lesions in the Wnt signaling pathway.⁷²

P-TEFb can phosphorylate I-mfa and HIC (Figure 4) as well as the CTD (Q. Wang, unpublished data) even when bound to the I-mfa domain. Therefore the mechanism by which I-mfa and HIC inhibit transcription may involve recruitment of P-TEFb to the transcription complex rather than a direct effect on its kinase activity. Our studies with Tat truncations (Figure 5(d)) do not support the conclusion¹⁰ that HIC binds to the Tat nuclear localization signal in its basic domain (aa 49–57). Moreover, we did not observe relocalization of Tat to the cytoplasm in the presence of HIC.⁹ Our data indicate that the I-mfa domain of HIC interacts with Tat in the activation domain that is required for it to bind to cyclin T1 (Figure 9). Although the HIC and cyclin T1 sites are distinguishable on the basis of their zinc dependence and precise sequence requirements, we could not detect simultaneous binding of the two proteins to Tat (data not shown). Thus we propose that the I-mfa domain can prevent HIV-1 transactivation by interfering with the Tat:cyclin T1 interaction. Because cyclin T1 has two binding sites for HIC or I-mfa, it is possible that they can bind together with Tat to P-TEFb. Which of these interactions is responsible for transcriptional inhibition in a particular cell type or set of conditions may depend on the balance of transcription factor activities in the cell.

Materials and Methods

Cell culture

Cell lines used in this study were obtained from the American Type Culture Collection. Suspension cells (CEM, Jurkat and U937) were maintained in RPMI 1640 medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% heat-inactivated (at 50°C for 30 min) fetal bovine serum (Sigma-Aldrich, St. Louis, MO). Adherent cells (COS, 3T3, 293, A549, COLO 205, HeLa, HT-1080, HT-29, MCF-7, SiHa, U2-OS) were maintained in

Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum.

Plasmids and plasmid construction

Plasmids for the expression of glutathione S-transferase (GST) fusion proteins were as follows: cyclin T1 [T1 (1–726)] and alanine substitution mutants T1 (1–303)-(251–254)A, T1 (1–303)-(256–259)A, T1 (1–303)-(265–268)A and T1 (1–303)-(269–272)A, from K. A. Jones;⁵¹ cyclin T1 truncations, T1 (1–250), T1 (1–300), T1 (1–479), T1 (1–551) and cyclin T2b full-length, T2 (1–420), T2 (1–300), and T2 (1–250) from B. M. Peterlin^{65,73} Cyclin T1 (402–726) and cyclin T2a (440–663) from D. H. Price;³¹ Tat (1–101), Tat (1–45), Tat (30–72), Tat (40–72) and Tat (50–72) from K. T. Jeang;⁷⁴ and Tat (1–48) and Tat (1–72) from the AIDS Research and Reference Reagent Program, National Institutes of Health. The GST-fusion protein deletion mutants T1 (480–570), T1 (480–530), T1 (501–530) and T1 (501–570) were constructed by M. Hoque in our laboratory,¹³ as were Tat (21–37) and Tat (21–72).⁷⁵

Plasmid vector expressing Tat (1–72) under the control of the Rous sarcoma virus (RSV) promoter and the reporter plasmids pHIV1-LTR-firefly luciferase and pRSV-Renilla luciferase were constructed by S. M. Reza in our laboratory.⁷⁶ The PCNA-firefly luciferase plasmid was provided by T. W. Reichman in our laboratory.¹³ Gal4BD, GAL4BD-cyclin T1 and G5-HIV-firefly luciferase reporter were from L. Lania.⁶⁴ The plasmid pcDNA3-HA-Tat (1–72) was from B. M. Peterlin.⁷³ The c-Myc-firefly luciferase plasmid (pSNLuc), containing the promoter (–424 to +334) of c-Myc gene was from K. Calame.⁷⁷

An empty vector expressing the FLAG tag, pcDNA3.1-Flag, was constructed by chemically synthesizing complementary oligonucleotides corresponding to the FLAG epitope, annealing them, and cloning the duplex into pcDNA3.1 (Invitrogen). The plasmid pHICp32, containing the full-length HIC open reading frame, was constructed as previously described.⁹ Plasmids containing FLAG -HIC, FLAG -HIC-N, FLAG -HIC-C and FLAG -HIC-I were constructed by subcloning the corresponding PCR amplified sequences from the pHICp32 vector into pcDNA3.1-Flag. The full-length I-mfa open reading frame was cloned from COLO 205 total RNA by a reverse transcription-PCR (RT-PCR) procedure using a TITANIUM one-step RT-PCR kit (BD Biosciences, Palo Alto, CA) with the specific primer set (5'-ATA CCG AAG CTT ATG TAC CAG GTG AGC GGC CAG-3' 5'-ATA CCG CTC GAG TCA TCA T CA GGA GGA GAA GCA GAG CCC-3'). The product was cloned into pcDNA3.1-Flag. Plasmids containing FLAG-I-mfa-N, FLAG-I-mfa-C and FLAG-I-mfa-I were constructed by subcloning the corresponding PCR amplified sequences from the pcDNA3.1-Flag-I-mfa vector into pcDNA3.1-Flag.

Antibodies

Goat anti-cyclin T1 antibody (T-18), rabbit anti-cyclin T1 antibody (H-245), rabbit anti-CDK9 antibody (C-20), and rabbit anti-HA (antihemagglutinin) antibody (Y-11) were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-FLAG monoclonal antibody (M2) was from Sigma-Aldrich.

GST pull-down assays

GST fusion proteins were expressed and purified as previously described.¹³ HIC and I-mfa proteins were synthesized with the TNT Quick coupled transcription/translation system (Promega, Madison, WI) in the presence of [³⁵S] Trans-Label (ICN Pharmaceuticals Inc. Costa Mesa, CA). GST or GST-fusion proteins bound to glutathione-Sepharose 4B (Amersham) were mixed with 10 µl of in vitro synthesized labeled proteins in EBCD buffer (50 mM Tris-Cl [pH 8.0], 120 mM NaCl, 0.5% NP-40, 1 mM dithiothreitol [DTT]) and incubated overnight at 4°

C with rocking. The protein complexes bound to the beads were washed and analyzed as previously described.¹³

Detection of mRNA

Cells were grown to 100% confluency in 60-mm tissue culture dishes or in suspension to a density of 10^6 cells/ml. Total RNA was isolated with Trizol (Invitrogen) according to the manufacturer's instructions. RT-PCR was performed as above with specific primers for HIC (5'-ATA CCG AAG CTT ATG TCC GGC GCG GGC GAA G-3' 5'-ATA CCG CTC GAG TTA TTA TTA TGA AGG AAA ACA AAT TCC ACA G-3') or I-mfa (primer set as described above) mRNA. RT-PCR products were analyzed in 0.7% agarose gels.

Coimmunoprecipitation

COS cells were seeded at 1×10^6 cells in 60 mm dishes, transfected 24 h later with 4 μ g of each plasmid (pFlag, pFlag-HIC, pFlag-HIC-N, pFlag-HIC-C, pFlag-HIC-I, pFlag-I-mfa, pFlag-I-mfa-N, pFlag-I-mfa-C, and pFlag-I-mfa-I) and harvested at 48 h posttransfection. Alternatively, 4 μ g of each plasmid and 3 μ g of pcDNA3.1-HA-Tat (1–72) were used for transfection. Transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were lysed in 700 μ l of IP buffer as previously described.¹³ Cell extracts (700 μ g protein) were incubated with anti-FLAG M2 affinity gel (Sigma) overnight at 4° C with rocking. Immunocomplexes were washed and analyzed as previously described.¹³

Kinase assays

Expression and purification of GST fusion proteins and preparation of whole cell extract (WCE) from HeLa cells were described previously.¹³ GST or GST fusion proteins bound to glutathione-Sepharose 4B beads were mixed with 500 μ l (500 μ g protein) of cell extract in EBCD buffer and incubated overnight at 4° C. Then the beads were washed extensively with EBCD buffer, followed by washing with TKB buffer (50 mM Tris-Cl [pH 7.6], 5 mM DTT, 4 mM $MgCl_2$, and 5 mM $MnCl_2$). Kinase reaction was performed in the presence of 2 μ M ATP and 10 μ Ci [γ -³²P] ATP (ICN) at 25° C for 40 min. The proteins were resolved in denaturing 10% polyacrylamide gels. ³²P-labeled proteins were detected by autoradiography. Kinase assays with immunopurified P-TEFb were performed on protein A-Sepharose beads. The beads (10 μ l) were prewashed in IP buffer then mixed with 0.3 μ g of anti-CDK9 antibody. After 1 h of incubation, the beads were washed three times in IP buffer, mixed with 500 μ l of cell extract (500 μ g protein) in IP buffer and incubated for 2 h at 4° C with rocking. The beads were washed extensively in IP buffer, followed by equilibration in TKB buffer. Purified GST or GST fusion proteins (2 μ g) were added to the IP samples and kinase reactions were performed as described above.

Dual luciferase assay

HeLa and 293 cells were seeded at 1.6×10^5 cells in six-well dishes and transfected 24 h later by using Lipofectamine 2000 (Invitrogen). Cells were harvested at 24 h posttransfection and lysed in 300 μ l of passive lysis buffer (Promega Corporation, Madison, WI). Luciferase assays were performed with the Promega dual luciferase reporter system according to the manufacturer's instruction. Data are normalized to internal controls as specified in the Figure legends.

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References

1. Thébaud S, Gachon F, Lemasson I, Devaux C, Mesnard JM. Molecular cloning of a novel human I-mfa domain-containing protein that differently regulates human T-cell leukemia virus type I and HIV-1 expression. *J Biol Chem* 2000;275:4848–57. [PubMed: 10671520]
2. Chen CM, Kraut N, Groudine M, Weintraub H. I-mf, a novel myogenic repressor, interacts with members of the MyoD family. *Cell* 1996;86:731–41. [PubMed: 8797820]
3. Kraut N, Snider L, Chen CM, Tapscott SJ, Groudine M. Requirement of the mouse I-mfa gene for placental development and skeletal patterning. *EMBO J* 1998;17:6276–88. [PubMed: 9799236]
4. Nadra K, Anghel SI, Joye E, Tan NS, Basu-Modak S, Trono D, Wahli W, Desvergne B. Differentiation of trophoblast giant cells and their metabolic functions are dependent on peroxisome proliferator-activated receptor beta/delta. *Mol Cell Biol* 2006;26:3266–81. [PubMed: 16581799]
5. Snider L, Tapscott SJ. XIC is required for Siamois activity and dorsoanterior development. *Mol Cell Biol* 2005;25:5061–72. [PubMed: 15923623]
6. Snider L, Thirlwell H, Miller JR, Moon RT, Groudine M, Tapscott SJ. Inhibition of Tcf3 binding by I-mfa domain proteins. *Mol Cell Biol* 2001;21:1866–73. [PubMed: 11238923]
7. Kusano S, Raab-Traub N. I-mfa Domain Proteins Interact with Axin and Affect Its Regulation of the Wnt and c-Jun N-Terminal Kinase Signaling Pathways. *Mol Cell Biol* 2002;22:6303–405.
8. Pan W, Jia Y, Wang J, Tao D, Gan X, Tsiokas L, Jing N, Wu D, Li L. Beta-catenin regulates myogenesis by relieving I-mfa-mediated suppression of myogenic regulatory factors in P19 cells. *Proc Natl Acad Sci U S A* 2005;102:17378–83. [PubMed: 16301527]
9. Young TM, Wang Q, Pe'ery T, Mathews MB. The Human I-mfa Domain-Containing Protein, HIC, Interacts with Cyclin T1 and Modulates P-TEFb-Dependent Transcription. *Mol Cell Biol* 2003;23:6373–84. [PubMed: 12944466]
10. Gautier VW, Sheehy N, Duffy M, Hashimoto K, Hall WW. Direct interaction of the human I-mfa domain-containing protein, HIC, with HIV-1 Tat results in cytoplasmic sequestration and control of Tat activity. *Proc Natl Acad Sci U S A* 2005;102:16362–7. [PubMed: 16260749]
11. Chao SH, Price DH. Flavopiridol inactivates P-TEFb and blocks most RNA polymerase II transcription in vivo. *J Biol Chem* 2001;276:31793–9. [PubMed: 11431468]
12. Ramanathan Y, Rajpara SM, Reza SM, Lees E, Shuman S, Mathews MB, Pe'ery T. Three RNA polymerase II carboxyl-terminal domain kinases display distinct substrate preferences. *J Biol Chem* 2001;276:10913–20. [PubMed: 11278802]
13. Hoque M, Young TM, Lee CG, Serrero G, Mathews MB, Pe'ery T. The Growth Factor Granulin Interacts with Cyclin T1 and Modulates P-TEFb-Defendent Transcription. *Mol Cell Biol* 2003;23:1688–702. [PubMed: 12588988]
14. Marshall NF, Peng J, Xie Z, Price DH. Control of RNA polymerase II elongation potential by a novel carboxyl-terminal domain kinase. *J Biol Chem* 1996;271:27176–27183. [PubMed: 8900211]
15. Isel C, Karn J. Direct evidence that HIV-1 Tat stimulates RNA polymerase II carboxyl-terminal domain hyperphosphorylation during transcriptional elongation. *J Mol Biol* 1999;290:929–41. [PubMed: 10438593]
16. Ivanov D, Kwak YT, Guo J, Gaynor RB. Domains in the SPT5 protein that modulate its transcriptional regulatory properties. *Mol Cell Biol* 2000;20:2970–83. [PubMed: 10757782]
17. Fujinaga K, Irwin D, Huang Y, Taube R, Kurosu T, Peterlin BM. Dynamics of human immunodeficiency virus transcription: P-TEFb phosphorylates RD and dissociates negative effectors from the transactivation response element. *Mol Cell Biol* 2004;24:787–95. [PubMed: 14701750]
18. Marshall NF, Price DH. Purification of P-TEFb, a transcription factor required for the transition into productive elongation. *J Biol Chem* 1995;270:12335–12338. [PubMed: 7759473]
19. Zhu Y, Pe'ery T, Peng J, Ramanathan Y, Marshall N, Marshall T, Amendt B, Mathews MB, Price DH. Transcription elongation factor P-TEFb is required for HIV-1 tat transactivation in vitro. *Genes Dev* 1997;11:2622–2632. [PubMed: 9334325]
20. Jang MK, Mochizuki K, Zhou M, Jeong HS, Brady JN, Ozato K. The bromodomain protein Brd4 is a positive regulatory component of P-TEFb and stimulates RNA polymerase II-dependent transcription. *Mol Cell* 2005;19:523–34. [PubMed: 16109376]

21. Yang Z, Yik JH, Chen R, He N, Jang MK, Ozato K, Zhou Q. Recruitment of P-TEFb for stimulation of transcriptional elongation by the bromodomain protein Brd4. *Mol Cell* 2005;19:535–45. [PubMed: 16109377]
22. Yang Z, Zhu Q, Luo K, Zhou Q. The 7SK small nuclear RNA inhibits the CDK9/cyclin T1 kinase to control transcription. *Nature* 2001;414:317–22. [PubMed: 11713532]
23. Nguyen VT, Kiss T, Michels AA, Bensaude O. 7SK small nuclear RNA binds to and inhibits the activity of CDK9/cyclin T complexes. *Nature* 2001;414:322–5. [PubMed: 11713533]
24. Michels AA, Fraldi A, Li Q, Adamson TE, Bonnet F, Nguyen VT, Sedore SC, Price JP, Price DH, Lania L, Bensaude O. Binding of the 7SK snRNA turns the HEXIM1 protein into a P-TEFb (CDK9/cyclin T) inhibitor. *Embo J* 2004;23:2608–19. [PubMed: 15201869]
25. Yik JH, Chen R, Nishimura R, Jennings JL, Link AJ, Zhou Q. Inhibition of P-TEFb (CDK9/Cyclin T) kinase and RNA polymerase II transcription by the coordinated actions of HEXIM1 and 7SK snRNA. *Mol Cell* 2003;12:971–82. [PubMed: 14580347]
26. Yik JH, Chen R, Pezda AC, Zhou Q. Compensatory Contributions of HEXIM1 and HEXIM2 in Maintaining the Balance of Active and Inactive Positive Transcription Elongation Factor b Complexes for Control of Transcription. *J Biol Chem* 2005;280:16368–76. [PubMed: 15713661]
27. Byers SA, Price JP, Cooper JJ, Li Q, Price DH. HEXIM2, a HEXIM1-related Protein, Regulates Positive Transcription Elongation Factor b through Association with 7SK. *J Biol Chem* 2005;280:16360–7. [PubMed: 15713662]
28. Barboric M, Kohoutek J, Price JP, Blazek D, Price DH, Peterlin BM. Interplay between 7SK snRNA and oppositely charged regions in HEXIM1 direct the inhibition of P-TEFb. *Embo J* 2005;24:4291–303. [PubMed: 16362050]
29. Egloff S, Van Herreweghe E, Kiss T. Regulation of polymerase II transcription by 7SK snRNA: two distinct RNA elements direct P-TEFb and HEXIM1 binding. *Mol Cell Biol* 2006;26:630–42. [PubMed: 16382153]
30. Wei P, Garber ME, Fang SM, Fischer WH, Jones KA. A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA. *Cell* 1998;92:451–62. [PubMed: 9491887]
31. Peng J, Zhu Y, Milton JT, Price DH. Identification of multiple cyclin subunits of human P-TEFb. *Genes Dev* 1998;12:755–62. [PubMed: 9499409]
32. Fu TJ, Peng J, Lee G, Price DH, Flores O. Cyclin K functions as a CDK9 regulatory subunit and participates in RNA polymerase II transcription. *J Biol Chem* 1999;274:34527–30. [PubMed: 10574912]
33. Giacinti C, Bagella L, Puri PL, Giordano A, Simone C. MyoD recruits the cdk9/cyclin T2 complex on myogenic-genes regulatory regions. *J Cell Physiol* 2006;206:807–13. [PubMed: 16245309]
34. Lis JT, Mason P, Peng J, Price DH, Werner J. P-TEFb kinase recruitment and function at heat shock loci. *Genes Dev* 2000;14:792–803. [PubMed: 10766736]
35. Kanazawa S, Okamoto T, Peterlin BM. Tat competes with CIITA for the binding to P-TEFb and blocks the expression of MHC class II genes in HIV infection. *Immunity* 2000;12:61–70. [PubMed: 10661406]
36. Lee DK, Duan HO, Chang C. Androgen receptor interacts with the positive elongation factor P-TEFb and enhances the efficiency of transcriptional elongation. *J Biol Chem* 2001;276:9978–84. [PubMed: 11266437]
37. Simone C, Bagella L, Bellan C, Giordano A. Physical interaction between pRb and cdk9/cyclinT2 complex. *Oncogene* 2002;21:4158–65. [PubMed: 12037672]
38. Barboric M, Nissen RM, Kanazawa S, Jabrane-Ferrat N, Peterlin BM. NF-kappaB binds P-TEFb to stimulate transcriptional elongation by RNA polymerase II. *Mol Cell* 2001;8:327–37. [PubMed: 11545735]
39. Kanazawa S, Soucek L, Evan G, Okamoto T, Peterlin BM. c-Myc recruits P-TEFb for transcription, cellular proliferation and apoptosis. *Oncogene* 2003;22:5707–11. [PubMed: 12944920]
40. Tian Y, Ke S, Chen M, Sheng T. Interactions between the aryl hydrocarbon receptor and P-TEFb. Sequential recruitment of transcription factors and differential phosphorylation of C-terminal domain of RNA polymerase II at cyp1a1 promoter. *J Biol Chem* 2003;278:44041–8. [PubMed: 12917420]

41. Giraud S, Hurlstone A, Avril S, Coqueret O. Implication of BRG1 and cdk9 in the STAT3-mediated activation of the p21waf1 gene. *Oncogene* 2004;23:7391–8. [PubMed: 15286705]
42. Gomes NP, Bjerke G, Llorente B, Szostek SA, Emerson BM, Espinosa JM. Gene-specific requirement for P-TEFb activity and RNA polymerase II phosphorylation within the p53 transcriptional program. *Genes Dev* 2006;20:601–12. [PubMed: 16510875]
43. Eberhardy SR, Farnham PJ. Myc recruits P-TEFb to mediate the final step in the transcriptional activation of the cad promoter. *J Biol Chem* 2002;277:40156–62. [PubMed: 12177005]
44. Iankova I, Petersen RK, Annicotte JS, Chavey C, Hansen JB, Kratchmarova I, Sarruf D, Benkirane M, Kristiansen K, Fajas L. Peroxisome proliferator-activated receptor gamma recruits the positive transcription elongation factor b complex to activate transcription and promote adipogenesis. *Mol Endocrinol* 2006;20:1494–505. [PubMed: 16484339]
45. Taube R, Lin X, Irwin D, Fujinaga K, Peterlin BM. Interaction between P-TEFb and the C-terminal domain of RNA polymerase II activates transcriptional elongation from sites upstream or downstream of target genes. *Mol Cell Biol* 2002;22:321–31. [PubMed: 11739744]
46. Zhang F, Barboric M, Blackwell TK, Peterlin BM. A model of repression: CTD analogs and PIE-1 inhibit transcriptional elongation by P-TEFb. *Genes Dev* 2003;17:748–58. [PubMed: 12651893]
47. Sano M, Schneider MD. Cyclin-dependent kinase-9: an RNAPII kinase at the nexus of cardiac growth and death cascades. *Circ Res* 2004;95:867–76. [PubMed: 15514168]
48. Liou LY, Haaland RE, Herrmann CH, Rice AP. Cyclin T1 but not cyclin T2a is induced by a post-transcriptional mechanism in PAMP-activated monocyte-derived macrophages. *J Leukoc Biol* 2006;79:388–96. [PubMed: 16330531]
49. Foscett SM, Ghose R, Tang DN, Lewis DE, Rice AP. Antiapoptotic function of Cdk9 (TAK/P-TEFb) in U937 promonocytic cells. *J Virol* 2001;75:1220–8. [PubMed: 11152495]
50. Flores O, Lee G, Kessler J, Miller M, Schlieff W, Tomassini J, Hazuda D. Host-cell positive transcription elongation factor b kinase activity is essential and limiting for HIV type 1 replication. *Proc Natl Acad Sci USA* 1999;96:7208–13. [PubMed: 10377393]
51. Garber ME, Wei P, KewalRamani VN, Mayall TP, Herrmann CH, Rice AP, Littman DR, Jones KA. The interaction between HIV-1 Tat and human cyclin T1 requires zinc and a critical cysteine residue that is not conserved in the murine CycT1 protein. *Genes Dev* 1998;12:3512–27. [PubMed: 9832504]
52. Karn J. Tackling Tat. *J Mol Biol* 1999;293:235–54. [PubMed: 10550206]
53. Gibellini D, Vitone F, Schiavone P, Re MC. HIV-1 tat protein and cell proliferation and survival: a brief review. *New Microbiol* 2005;28:95–109. [PubMed: 16035254]
54. Hetzer C, Dormeyer W, Schnolzer M, Ott M. Decoding Tat: the biology of HIV Tat posttranslational modifications. *Microbes Infect* 2005;7:1364–9. [PubMed: 16046164]
55. Giacca M. The HIV-1 Tat protein: a multifaceted target for novel therapeutic opportunities. *Curr Drug Targets Immune Endocr Metabol Disord* 2004;4:277–85. [PubMed: 15578978]
56. Graña X, DeLuca A, Sang N, Fu Y, Claudio PP, Rosenblatt J, Morgan DO, Giordano A. PITALRE, a nuclear CDC2-related protein kinase that phosphorylates the retinoblastoma protein in vitro. *Proc Natl Acad Sci USA* 1994;91:3834–3838. [PubMed: 8170997]
57. Herrmann CH, Rice AP. Lentivirus Tat proteins specifically associate with a cellular protein kinase, TAK, that hyperphosphorylates the carboxyl-terminal domain of the large subunit of RNA polymerase II: candidate for a Tat cofactor. *J Virol* 1995;69:1612–20. [PubMed: 7853496]
58. Kim JB, Sharp PA. Positive transcription elongation factor B phosphorylates hSPT5 and RNA polymerase II carboxyl-terminal domain independently of cyclin-dependent kinase-activating kinase. *J Biol Chem* 2001;276:12317–23. [PubMed: 11145967]
59. Yamada T, Yamaguchi Y, Inukai N, Okamoto S, Mura T, Handa H. P-TEFb-mediated phosphorylation of hSpt5 C-terminal repeats is critical for processive transcription elongation. *Mol Cell* 2006;21:227–37. [PubMed: 16427012]
60. Garber ME, Mayall TP, Suess EM, Meisenhelder J, Thompson NE, Jones KA. CDK9 autophosphorylation regulates high-affinity binding of the human immunodeficiency virus type 1 tat-P-TEFb complex to TAR RNA. *Mol Cell Biol* 2000;20:6958–69. [PubMed: 10958691]
61. Simone C, Stiegler P, Bagella L, Pucci B, Bellan C, De Falco G, De Luca A, Guanti G, Puri PL, Giordano A. Activation of MyoD-dependent transcription by cdk9/cyclin T2. *Oncogene* 2002;21:4137–48. [PubMed: 12037670]

62. Ramanathan Y, Reza SM, Young TM, Mathews MB, Pe'ery T. Human and rodent transcription elongation factor P-TEFb: interactions with human immunodeficiency virus type 1 tat and carboxy-terminal domain substrate. *J Virol* 1999;73:5448–58. [PubMed: 10364292]
63. Lam LT, Pickeral OK, Peng AC, Rosenwald A, Hurt EM, Giltneane JM, Averett LM, Zhao H, Davis RE, Sathymoorthy M, Wahl LM, Harris ED, Mikovits JA, Monks AP, Hollingshead MG, Sausville EA, Staudt LM. Genomic-scale measurement of mRNA turnover and the mechanisms of action of the anti-cancer drug flavopiridol. *Genome Biol* 2001;2:RESEARCH0041. [PubMed: 11597333]
64. Majello B, Napolitano G, Giordano A, Lania L. Transcriptional regulation by targeted recruitment of cyclin-dependent CDK9 kinase in vivo. *Oncogene* 1999;18:4598–605. [PubMed: 10467404]
65. Kurosu T, Zhang F, Peterlin BM. Transcriptional activity and substrate recognition of cyclin T2 from P-TEFb. *Gene* 2004;343:173–9. [PubMed: 15563843]
66. Chen R, Yang Z, Zhou Q. Phosphorylated positive transcription elongation factor b (P-TEFb) is tagged for inhibition through association with 7SK snRNA. *J Biol Chem* 2004;279:4153–60. [PubMed: 14627702]
67. Wittmann BM, Fujinaga K, Deng H, Ogba N, Montano MM. The breast cell growth inhibitor, estrogen down regulated gene 1, modulates a novel functional interaction between estrogen receptor alpha and transcriptional elongation factor cyclin T1. *Oncogene* 2005;24:5576–88. [PubMed: 15940264]
68. Jiang H, Zhang F, Kurosu T, Peterlin BM. Runx1 binds positive transcription elongation factor b and represses transcriptional elongation by RNA polymerase II: possible mechanism of CD4 silencing. *Mol Cell Biol* 2005;25:10675–83. [PubMed: 16314494]
69. Mizugishi K, Hatayama M, Tohmonda T, Ogawa M, Inoue T, Mikoshiba K, Aruga J. Myogenic repressor I-mfa interferes with the function of Zic family proteins. *Biochem Biophys Res Commun* 2004;320:233–40. [PubMed: 15207726]
70. Mizutani T, Yamada K, Yazawa T, Okada T, Minegishi T, Miyamoto K. Cloning and characterization of gonadotropin-inducible ovarian transcription factors (GIOT1 and -2) that are novel members of the (Cys)(2)-(His)(2)-type zinc finger protein family. *Mol Endocrinol* 2001;15:1693–705. [PubMed: 11579202]
71. Ma R, Rundle D, Jacks J, Koch M, Downs T, Tsiokas L. Inhibitor of myogenic family, a novel suppressor of store-operated currents through an interaction with TRPC1. *J Biol Chem* 2003;278:52763–72. [PubMed: 14530267]
72. Gregorieff A, Clevers H. Wnt signaling in the intestinal epithelium: from endoderm to cancer. *Genes Dev* 2005;19:877–90. [PubMed: 15833914]
73. Fujinaga K, Taube R, Wimmer J, Cujec TP, Peterlin BM. Interactions between human cyclin T, Tat, and the transactivation response element (TAR) are disrupted by a cysteine to tyrosine substitution found in mouse cyclin T. *Proc Natl Acad Sci U S A* 1999;96:1285–90. [PubMed: 9990016]
74. Chun RF, Semmes OJ, Neuveut C, Jeang KT. Modulation of Sp1 phosphorylation by human immunodeficiency virus type 1 Tat. *J Virol* 1998;72:2615–29. [PubMed: 9525578]
75. Hoque M, Tian B, Mathews MB, Pe'ery T. Granulin and granulin repeats interact with the Tat:P-TEFb complex and inhibit tat transactivation. *J Biol Chem*. 2005
76. Reza SM, Rosetti M, Mathews MB, Pe'ery T. Differential activation of Tat variants in mitogen-stimulated cells: implications for HIV-1 postintegration latency. *Virology* 2003;310:141–56. [PubMed: 12788638]
77. Riggs KJ, Saleque S, Wong KK, Merrell KT, Lee JS, Shi Y, Calame K. Yin-yang 1 activates the c-myc promoter. *Mol Cell Biol* 1993;13:7487–95. [PubMed: 8246966]

Abbreviations used

P-TEFb

positive transcription elongation factor b

CDK9

cyclin-dependent kinase 9

I-mfa

inhibitor of MyoD family a

HIC	human I-mfa-domain-containing
HIV-1	human immunodeficiency virus type 1
XIC	the <i>Xenopus</i> ortholog of HIC
TCF/LEF	T cell factor/lymphoid enhancer-binding factor
JNK	c-Jun N-terminal kinase
Tat	trans-activator of transcription
Pol II	RNA polymerase II
CTD	carboxy-terminal domain
TAR	trans-activation response element
TRM	Tat-TAR recognition motif
PCNA	proliferating cell nuclear antigen
KRM	K/R-rich motif

B.

Comparison of HIC and I-mfa protein sequence and mRNA expression. (a) Alignment of human HIC and I-mfa proteins. The solid line indicates the I-mfa domains of both proteins and the dotted line indicates the conserved basic region. Identical, strongly similar, and weakly similar amino acids are marked with stars, double dots, and single dots, respectively. The alignment was processed with the Clustal W (1.82) multiple sequence alignment program (<http://www.ebi.ac.uk/clustalw/>). Bold and underlined residues lie at splice sites. (b) Expression of HIC and I-mfa mRNA in cell lines. Total RNA was prepared and subjected to RT-PCR with primers specific for HIC, I-mfa, or β -actin. The resultant DNA was resolved in agarose gels and detected by staining with ethidium bromide.

Figure 2.

The I-mfa protein is a novel partner of P-TEFb. (a) Schematic representation of the HIC and I-mfa proteins and their truncations used in this study. (b) I-mfa and HIC interact with P-TEFb in vivo. COS cells were transfected with plasmids expressing the FLAG-tagged proteins shown in panel B, or plasmid expressing FLAG tag alone as control. Cell extracts were subjected to immunoprecipitation with anti-FLAG antibody followed by Western blots probed with antibody against cyclin T1 (top panel) or CDK9 (third panel). Protein expression levels were monitored by probing western blots containing 2% of the input cell extract with antibody against cyclin T1 (second panel), CDK9 (fourth panel) and the FLAG epitope (bottom panel).

Figure 3.

Cyclin T1 exhibits two binding sites for I-mfa and HIC, one of which is zinc dependent. (a) Schematic representation of cyclin T1 and its truncations expressed as GST fusion proteins. (b) Protein staining of the GST-cyclin T1 fusion proteins used in the pull-down assays. (c) GST-cyclin T1 (GST-T1) and the truncations indicated were used to pull down *in vitro* synthesized ³⁵S-labeled I-mfa (upper panels) or HIC (lower panels) in the absence or presence of 50 μ M ZnCl₂ or 10 mM EDTA. GST was used as control. (d) The I-mfa domain of I-mfa and HIC is sufficient for direct binding to cyclin T1 binding sites. GST-T1 was used to pull down *in vitro* synthesized ³⁵S-labeled full-length or truncated I-mfa or HIC. Lanes on the left were loaded with 10% of the labeled proteins used as input for the pull-down reactions.

Figure 4.

I-mfa and HIC are substrates of P-TEFb. (a) P-TEFb is pulled down from HeLa cell extracts by GST-HIC and GST-I-mfa. HeLa cell extracts were incubated with the indicated GST fusion proteins bound to glutathione beads and P-TEFb binding was detected using Western blots probed with antibody against cyclin T1 or CDK9. GST was used as control. (b) GST fusion proteins used in the kinase assay shown in panel C. Proteins were stained with Coomassie Brilliant Blue. (c) GST pull-down and kinase assay. Complexes bound to GST-HIC or GST-I-mfa and to their truncated GST fusion proteins (panels (a) and (b)) were tested as substrates in kinase assays. (d) Immunopurified P-TEFb complexes used to phosphorylate purified GST fusion proteins as shown in panel (f). Complexes were immunoprecipitated (IP) from HeLa cell extract with anti-CDK9 antibody, examined by Western blotting with antibody against cyclin T1 or CDK9 (left panel), and used in kinase assays with the CTD4 peptide¹³ (middle panel) or GST-HIC (right panel) as substrate. DRB was present at 50 μ M where indicated. (e) Staining of the affinity purified GST fusion proteins that were used as substrates in the kinase

assay shown in panel (f). (f) Immunoprecipitated P-TEFb complexes and affinity-purified GST or GST fusion proteins (panels (d) and (e)) were subjected to kinase assays. The Control lane contained kinase but no substrate.

Figure 5.

Specific interactions of I-mfa and HIC with HIV-1 Tat. (a) The I-mfa domain of I-mfa and HIC is sufficient for binding to Tat. GST-Tat72 was used to pull down ^{35}S -labeled I-mfa or its truncations (upper panel), and ^{35}S -labeled HIC or its truncations (lower panel). (b) The I-mfa protein interacts with Tat in vivo via its I-mfa domain. COS cells were cotransfected with HA-Tat and FLAG-I-mfa or FLAG-HIC or with their truncations as indicated. Anti-FLAG antibody was used to immunoprecipitate FLAG containing complexes. HA-Tat was detected in the immunocomplexes by Western blot with anti-HA antibody (top panel). Protein expression levels were monitored by probing western blots containing 2% of the input cell extract with antibody against HA-Tat and FLAG-tagged proteins (middle and bottom panels,

respectively). Cells transfected with the FLAG-containing plasmid were used as control. (c) Schematic representation of full-length HIV-1 Tat, its regions and the truncations used in this study. (d) The Cys-rich region of Tat is necessary for the interaction with HIC and I-mfa. ³⁵S-labeled HIC (top panel) or I-mfa (bottom panel) was used in pull-down assays with the GST fusion proteins indicated. GST pull-down experiments were also performed in the presence of 50 μ M ZnCl₂, or 10mM EDTA as indicated. GST was used as control. (e) HIC binds to mutant Tat proteins that cannot bind cyclin T1. COS cells were cotransfected with plasmids expressing wild-type or mutant HA-Tat and EGFP-HIC. Top left panel: HA-Tat was detected in complexes immunoprecipitated with anti-EGFP antibody. Top right panel: endogenous cyclin T1 was detected in complexes immunoprecipitated with anti-HA antibody. Lower panels: control immunoblots containing the corresponding cell extracts before immunoprecipitation.

Figure 6.

Effects of HIC and I-mfa on Tat- and P-TEFb-dependent transactivation in human cells. (a, b) HeLa cells or 293 cells were transfected with plasmids expressing FLAG-HIC, FLAG-I-mfa or their truncations together with RSV-Tat, HIV-LTR-firefly luciferase reporter plasmid and RSV-Renilla luciferase reporter plasmid as control. The production of firefly luciferase was detected with the dual luciferase reporter assay. Transactivation was expressed as relative firefly/Renilla luciferase activity, normalized to the value obtained with control FLAG plasmid. (c, d) HeLa cells were transfected as in (a) except that the Tat vector was omitted and a reporter plasmid containing the PCNA promoter or c-Myc promoter driving firefly luciferase replaced the HIV-LTR-firefly luciferase plasmid. (e, f) As (a,b) except that the Gal4-cyclin T1 tether system was used. Data represent mean of two experiments with standard errors.

Figure 7.

I-mfa and HIC bind to cyclin T2 at two sites, identifying the conserved K/R-rich motif (KRM) adjacent to the cyclin domain. (a) Schematic representation of cyclin T1, T2a and T2b. (b) Cyclin T2 bind I-mfa and HIC. GST-cyclin T1 (GST-T1), GST-cyclin T2 (GST-T2b) and the truncations indicated were used to pull down in vitro synthesized ³⁵S-labeled I-mfa in the absence or presence of 50 μM ZnCl₂ (top and second panels, respectively) or ³⁵S-labeled HIC in the absence or presence of 50 μM ZnCl₂ (third and bottom panels, respectively). (c) Residues 250–300 adjacent to the cyclin domains are required for HIC and I-mfa binding. GST-T1, GST-T2 and their C-terminal truncations were used to pull down in vitro synthesized ³⁵S-labeled I-mfa (top panel) or HIC (bottom panel) as indicated. (d) The K/R-rich motif (KRM) is required

for I-mfa and HIC binding. The amino acid sequences of the KRM (aa 251–270 for T1 and aa 250–269 for T2) are shown for cyclins T1 and T2 (boxed). Basic residues are shown in bold and sequence similarities are marked as in Figure 1(a). The dotted line marks the TRM in cyclin T1. Lines denote the alanine substitution mutants used in the pull-down experiment below. In vitro synthesized ³⁵S-labeled I-mfa, HIC, Tat, or CDK9 were pulled down by the indicated wild type GST-T1 C-terminal truncations and GST-T1 (1-303) alanine substitution mutants. A stained gel containing the GST fusion proteins used in this assay is shown. (e) Multiple alignment of cyclin T from different species. The three invertebrates have a single cyclin T gene. Amino acid residues that are identical in the alignment are highlighted in black. Those that are highly conserved within the cyclin T1 family or cyclin T2 family are highlighted in dark grey or light grey, respectively. The data were obtained from Ensembl Genome Browser (<http://www.ensembl.org/>). The alignment was processed with Clustal W (1.82) multiple sequence alignment program (<http://www.ebi.ac.uk/clustalw/>).

HIC and I-mfa originated from gene duplication. (a) Genomic context of human HIC and I-mfa proteins. Arrows show the direction of gene transcription. Chromosomes are represented by a dotted line (not to scale). (b) Schematic representation of exon-intron structures of human HIC and I-mfa (not to scale). Lines, introns; blocks, exons; hatched area, open reading frame. (c) Schematic representation of exon junctions in HIC and I-mfa mRNAs and proteins. Dotted lines show the positions of intron-exon junctions within codons. The data were obtained from <http://www.ncbi.nlm.nih.gov/entrez/>. (d) Multiple alignment of the C-terminal I-mfa domains of HIC and I-mfa orthologs. Amino acid residues that are identical in the alignment are highlighted in black. Residues that are highly conserved within the HIC family or I-mfa family

are highlighted in dark grey or light grey, respectively. The data were obtained from Ensembl Genome Browser (<http://www.ensembl.org/>) using human HIC mRNAs as query. The existence of the genes was further confirmed by EST (expressed sequence tags) evidence. The alignment was processed with Clustal W (1.82) multiple sequence alignment program (<http://www.ebi.ac.uk/clustalw/>).

Figure 9.

Interaction of HIC or I-mfa with P-TEFb and Tat . (a) The histidine-rich (His) domain and the newly designated K/R motif (KRM) of cyclin T1 are shown as rectangles. HIC or I-mfa are able to dimerize via their I-mfa domains (I) (Wang et al., in preparation) and to bind to sequences on the T cyclins that contain the His and KRM. Other partners of cyclin T1 are shown together with their specific binding sequences which are depicted in black lines. (b) I-mfa or HIC bind to the activation domain of Tat, to sequences containing the cysteine-rich (Cys) and core domains. Tat sequences that are necessary for binding to cyclin T1 or TAR are shown in black lines.