PP2B and PP1 α cooperatively disrupt 7SK snRNP to release P-TEFb for transcription in response to Ca²⁺ signaling

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The positive transcription elongation factor b (P-TEFb), consisting of Cdk9 and cyclin T, stimulates RNA polymerase II elongation and cotranscriptional pre-mRNA processing. To accommodate different growth conditions and transcriptional demands, a reservoir of P-TEFb is kept in an inactive state in the multisubunit 7SK snRNP. Under certain stress or disease conditions, P-TEFb is released to activate transcription, although the signaling pathway(s) that controls this is largely unknown. Here, through analyzing the UV- or hexamethylene bisacetamide (HMBA)-induced release of P-TEFb from 7SK snRNP, an essential role for the calcium ion (Ca²⁺)-calmodulin-protein phosphatase 2B (PP2B) signaling pathway is revealed. However, Ca²⁺ signaling alone is insufficient, and PP2B must act sequentially and cooperatively with protein phosphatase 1 α (PP1 α) to disrupt 7SK snRNP. Activated by UV/HMBA and facilitated by a PP2B-induced conformational change in 7SK snRNP, PP1 α releases P-TEFb through dephosphorylating phospho-Thr186 in the Cdk9 T-loop. This event is also necessary for the subsequent recruitment of P-TEFb by the bromodomain protein Brd4 to the preinitiation complex, where Cdk9 remains unphosphorylated and inactive until after the synthesis of a short RNA. Thus, through cooperatively dephosphorylating Cdk9 in response to Ca²⁺ signaling, PP2B and PP1 α alter the P-TEFb functional equilibrium through releasing P-TEFb from 7SK snRNP for transcription.

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In eukaryotes, the transcription of protein-coding genes is performed by RNA polymerase (Pol) II in a cyclic process consisting of several tightly regulated stages (Sims et al. 2004). During the elongation stage, the C-terminal domain of the largest subunit of Pol II is phosphorylated by the positive transcription elongation factor b (P-TEFb) (Peterlin and Price 2006; Zhou and Yik 2006). This modification is crucial for Pol II to change from abortive to productive elongation and produce full-length RNA transcripts. Consisting of Cdk9 and cyclin T1 (or the minor forms T2 and K), P-TEFb is considered a general transcription factor required for the expression of a vast array of protein-coding genes (Chao and Price 2001; Shim et al. 2002). Not only is P-TEFb critical for cellular gene tran-

scription, it is also a specific host cofactor for the HIV-1 Tat protein. Tat recruits P-TEFb to the TAR RNA element located at the 5' end of nascent viral transcripts, allowing P-TEFb to phosphorylate stalled Pol II and enhance HIV-1 elongation (Peterlin and Price 2006).

However, not every P-TEFb in the nucleus is in a transcriptionally active state. A major reservoir of P-TEFb (~50% of total P-TEFb in HeLa cells) actually exists in an inactive complex termed 7SK snRNP that also contains the 7SK snRNA (Nguyen et al. 2001; Yang et al. 2001) and three nuclear proteins, HEXIM1 (Michels et al. 2003; Yik et al. 2003), BCDIN3 (Jeronimo et al. 2007), and PIP7S/LARP7 (He et al. 2008; Krueger et al. 2008). Within this complex, HEXIM1 inhibits the Cdk9 kinase in a 7SK-dependent manner (Yik et al. 2003). Underscoring the importance of 7SK as a molecular scaffold to maintain the integrity of 7SK snRNP, this RNA is protected from both 5'-3' and 3'-5' exonucleases by the respective actions of BCDIN3, a methylphosphate capping enzyme specific for 7SK (Jeronimo et al. 2007), and

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PIP7S/LARP7, a La-related protein bound to the 3' UUU-OH sequence of 7SK (He et al. 2008).

Besides P-TEFb sequestered in 7SK snRNP, a separate population of P-TEFb exists in a complex together with the bromodomain protein Brd4, which recruits P-TEFb to chromatin templates through interacting with acetylated histones and the mediator complex (Jang et al. 2005; Yang et al. 2005). Recent evidence shows that this recruitment occurs mostly at late mitosis and is essential to promote G1 gene expression and cell cycle progression (Yang et al. 2008). Importantly, the two populations of P-TEFb are kept in a functional equilibrium that can be perturbed by conditions that impact cell growth. For example, treating cells with global transcription inhibitors DRB and actinomycin D or the DNA-damaging agent UV disrupts 7SK snRNP and converts P-TEFb into the Brd4-bound form for stress-induced gene expression (Nguyen et al. 2001; Yang et al. 2001, 2005). Similarly, in cardiac myocytes, hypertrophic signals release P-TEFb from 7SK snRNP, leading to an overall increase in cellular protein and RNA contents, enlarged cells, and hypertrophic growth (Sano et al. 2002). Finally, RNAi-mediated depletion of PIP7S/LARP7 compromises the integrity of 7SK snRNP, resulting in P-TEFb-dependent transformation of mammary epithelial cells (He et al. 2008).

Recent data indicate that the P-TEFb functional equilibrium can also be affected by HMBA (hexamethylene bisacetamide), which is known to inhibit growth and induce differentiation of many cell types (Marks et al. 1994). Interestingly, the response to HMBA displays a biphasic nature (He et al. 2006; Contreras et al. 2007). Shortly after the treatment begins, a disruption of 7SK snRNP and enhanced formation of the Brd4–P-TEFb complex occur. However, when the P-TEFb-dependent HEXIM1 expression markedly increases as the treatment continues, the elevated HEXIM1 levels eventually push the P-TEFb equilibrium back toward the 7SK snRNP side to accommodate an overall reduced transcriptional demand in terminally differentiated cells (He et al. 2006).

Accumulating evidence indicates that 7SK snRNP represents a major reservoir of activity where P-TEFb can be recruited to activate transcription (Zhou and Yik 2006). However, the signaling pathway(s) that controls this process is mostly unknown. Of note, in the course of our studies, it was reported that the activity of PI3K/Akt is required for HMBA to release P-TEFb from 7SK snRNP (Contreras et al. 2007). However, since neither the treatment with various pharmacological activators of the PI3K/Akt pathway nor the expression of a CA form of Akt induces 7SK snRNP disruption (Supplemental Fig. S1), it is unlikely that PI3K/Akt alone is sufficient to accomplish this task.

Here, through analyzing the disruption of 7SK snRNP by UV or short-term HMBA treatment, we show that both agents cause calcium ion (Ca²⁺) influx and activation of a Ca²⁺–calmodulin–PP2B (protein phosphatase 2B, also known as calcineurin) signaling pathway that is necessary although insufficient to cause the disruption.

To dissociate HEXIM1 from P-TEFb, PP2B must act sequentially and cooperatively with PP1 α (protein phosphatase 1 α). Facilitated by a PP2B-induced conformational change in 7SK snRNP, PP1 α releases P-TEFb from 7SK snRNP through dephosphorylating phospho-Thr186 located in the Cdk9 T-loop. This event is also necessary for the subsequent recruitment of P-TEFb by Brd4 to the preinitiation complex (PIC), where Cdk9 has been reported to remain unphosphorylated and inactive until after the synthesis of a short RNA transcript (Zhou et al. 2001). Together, our data are consistent with a model that PP2B and PP1 α act cooperatively and in response to Ca²⁺ signaling to dephosphorylate Cdk9 T-loop, disrupt 7SK snRNP, and generate a pool of P-TEFb that can be recruited to the PIC.

Results

HMBA treatment and UV irradiation trigger Ca²⁺ influx

Abundant evidence points to the importance of Ca²⁺, one of nature's most versatile second messengers, in modulating gene transcription through inducing the phosphorylation or dephosphorylation of specific transcription factors (Crabtree 2001). Given that P-TEFb can be released from 7SK snRNP in cells treated with UV or HMBA (Yik et al. 2003; He et al. 2006), we asked whether Ca²⁺ homeostasis might play a role in this process. First, we examined whether UV and HMBA could trigger Ca²⁺ influx in HeLa cells under the conditions that cause the disruption of 7SK snRNP. Using Fluo-3/AM as an intracellular Ca²⁺ indicator, both agents were shown to significantly increase intracellular Ca²⁺ levels (Fig. 1A). Importantly, pretreating cells with nifedipine, which blocks Ca²⁺ entry by inhibiting the L-type Ca²⁺ channel in plasma membrane (Reid et al. 1997), completely abolished this effect (Fig. 1A).

Ca²⁺ entry is key for UV/HMBA-induced dissociation of HEXIM1 from and activation of P-TEFb

Correlating with its inhibition of Ca²⁺ entry, nifedipine also prevented the UV- or HMBA-induced dissociation of HEXIM1 from P-TEFb in a dose-dependent manner in the HeLa-based F1C2 cells that stably express Cdk9-f (Yang et al. 2001). This is illustrated by Western blotting followed by quantification of the amounts of HEXIM1 bound to Cdk9-f, which was isolated by anti-Flag immunoprecipitation (IP) from nuclear extract (NE) of treated cells (Fig. 1B) (the amount of Cdk9-f-bound HEXIM1 before the treatment was set to 100%). Analysis of 7SK RNA bound to Cdk9-f came to the same conclusion (data not shown). The expression levels of HEXIM1 and the other components of 7SK snRNP were completely unaffected by the highest amount of nifedipine used in this experiment (Supplemental Fig. S2A).

In contrast to nifedipine's inhibitory effect, treating cells with ionomycin, a Ca²⁺-specific ionophore that

Figure 1. Ca^{2+} influx is key for UV/HMBA to dissociate HEXIM1 from P-TEFb and activate P-TEFb-dependent transcription. (A) HeLa cells loaded with Fluo-3/AM, a Ca^{2+} -specific fluorescent indicator, were preincubated with either buffer or Nifedipine (Nife), treated with HMBA or UV, and then analyzed by fluorescence microscopy with untreated cells as a control. (B) F1C2 cells were pretreated with the indicated amounts of nifedipine and then exposed to UV (left) or HMBA (right). Anti-Flag immunoprecipitates derived from NE were analyzed by Western blotting (WB) for the indicated proteins. The levels of HEXIM1 bound to P-TEFb were quantified, with those in untreated cells set to 100%. (C) F1C2 cells were treated with the indicated amounts of HMBA and/or ionomycin. Anti-Flag immunoprecipitates from NE were subjected to WB and quantification to determine the amounts of HEXIM1 bound to P-TEFb. (D) HeLa cells with an integrated HIV-1 LTR-luciferase gene were treated with the indicated amounts of HMBA and/or ionomycin. Cell lysates were obtained to measure luciferase activity. (E) F1C2 cells grown in either DMEM (+ Ca^{2+}) or S-MEM (- Ca^{2+}) were treated with UV or HMBA. Anti-Flag immunoprecipitates from NE were analyzed by WB. The amounts of P-TEFb-bound HEXIM1 were quantified as in B. (F) F1C2 cells cultured in Ca^{2+} /phosphate-free modified DMEM supplemented with the indicated compounds were treated with UV or HMBA. Anti-flag immunoprecipitates were analyzed and quantified as in B. (G,H) HeLa cells with an integrated HIV-1 LTR-luciferase gene were incubated with the indicated compounds. Luciferase activities were analyzed as in D.

triggers Ca^{2+} influx, enabled the dissociation of HEXIM1 from Cdk9-f by a suboptimal dosage (2 mM) of HMBA (optimal dosage is 10 mM), which was otherwise unable to achieve this effect by itself (Fig. 1C, cf. lanes 1 and 4). It is important to note that ionomycin (i.e., Ca^{2+} influx) alone was insufficient to dissociate HEXIM1 (Fig. 1C, lanes 1–3), a point we will come back to later. Finally, like nifedipine, ionomycin did not affect the nuclear levels of HEXIM1 and other 7SK snRNP components (Supplemental Fig. S2A).

The HMBA-induced dissociation of HEXIM1 from P-TEFb has previously been shown to stimulate the HIV-1 LTR-driven luciferase gene expression (He et al. 2006), which is known to be strongly P-TEFb-dependent (Chao and Price 2001; Yang et al. 2001). However, when HMBA

was used at a suboptimal concentration (1 mM), no stimulation of HIV-1 LTR activity was observed (Fig. 1D). This situation was markedly improved when ionomycin was also added into the medium, which enhanced the HMBA-induced HIV-1 transcription in a dosage-dependent manner despite the fact that it alone had only a minimal effect (Fig. 1D). In contrast to ionomycin's stimulatory effect, nifedipine strongly inhibited HIV-1 transcription activated by HMBA (data not shown). Thus, by employing pharmacological compounds with opposite effects on Ca^{2+} homeostasis, our data suggest that modulating the UV/HMBA-induced Ca^{2+} influx can directly affect the abilities of these two agents to disrupt 7SK snRNP and activate P-TEFb-dependent transcription.

Depletion of intra- or extracellular Ca²⁺ abolishes UV/HMBA-induced disruption of 7SK snRNP and activation of P-TEFb

To confirm the reliance on Ca²⁺ for UV/HMBA/-induced HEXIM1 dissociation from P-TEFb, we tested whether the presence or absence of Ca²⁺ in culture media would affect this process. Whereas the dissociation was highly efficient in cells cultured in serum-free DMEM (Dulbecco's modified Eagle's medium) containing Ca²⁺, it was significantly inhibited when S-MEM (minimum essential medium spinner modification) (Fig. 1E), a Ca²⁺-free medium commonly used for studying Ca²⁺ signaling, was used. Similar results were also obtained with F1C2 cells cultured in modified, Ca²⁺-free, and phosphate-free DMEM, which was supplemented with CaCl₂, EGTA, or BAPTA-AM. Again, HEXIM1 was efficiently dissociated from P-TEFb by UV/HMBA in the presence of 2 mM CaCl₂, which mimics the Ca²⁺ concentration in normal DMEM, but remained largely intact when either EGTA, a chelator of divalent cations, or BAPTA-AM, a highly specific intracellular Ca²⁺ chelator, was in the medium (Fig. 1F).

Consistent with these data, treatment of HeLa cells containing an integrated HIV-1 LTR-luciferase reporter gene with either EGTA (Fig. 1G) or BAPTA-AM (Fig. 1H) strongly inhibited HMBA's ability to activate HIV-1 transcription. Notably, the two chelators had only a minor effect in the absence of HMBA. These data collectively implicate Ca²⁺ as a key factor required to transmit the signals initiated by UV/HMBA to dissociate HEXIM1 from P-TEFb.

PP2B and calmodulin are required for UV/HMBA-induced release of P-TEFb from 7SK snRNP

Given the demonstrations that Ca²⁺ signaling is key for UV/HMBA to dissociate HEXIM1 from P-TEFb, we

asked how the signal might be transduced. It has been reported previously that in transgenic mice expressing the CA PP2B, P-TEFb is activated in the myocardium (Sano et al. 2002). This result prompted us to test whether PP2B, a Ca²⁺-activated serine/threonine phosphatase, is required for UV/HMBA to induce the dissociation of HEXIM1 from and activation of P-TEFb.

We first took a pharmacological approach to pretreat F1C2 cells with increasing amounts of cyclosporine A (CsA) or FK506, two well-studied, highly specific PP2B inhibitors (Crabtree 2001). Consistent with a strong dependence on PP2B, the abilities of UV and HMBA to dissociate HEXIM1 were severely inhibited in a CsA (Fig. 2A) or FK506 (Fig. 2B) dosage-dependent manner. Again, the expression of HEXIM1 and the other components of 7SK snRNP was unaffected by the highest concentrations of CsA and FK506 used here (Supplemental Fig. S2A).

Next, the dependence on PP2B for HEXIM1 dissociation was also tested in an experiment that involved ectopic expression of either a constitutively active (CA) form of the PP2B catalytic subunit A α (Chin et al. 1998) or a phosphatase-inactive (IN) mutant derivative (Rusnak and Mertz 2000). Both proteins were expressed to a similar level and did not affect the levels of 7SK snRNP components in transfected HeLa cells (Supplemental Fig. S3A). Compared with cells with an empty vector (Fig. 2C, lanes 1,4), the dissociation of HEXIM1 from P-TEFb in cells treated with less than the optimal dosages of UV (20 J/m²; optimal dosage is 80 J/m²) or HMBA (5 mM) was markedly enhanced by the expression of the CA but not IN form of PP2B (Fig. 2C). In fact, the latter had an effect indistinguishable from that of the vector alone (Fig. 2C, cf. lanes 4 and 6).

Further evidence supporting a key role of PP2B in dissociating HEXIM1 from P-TEFb came from the demonstration that UV and HMBA failed to dissociate HEXIM1

Figure 2. PP2B and calmodulin are required for UV/HMBA-induced dissociation of HEXIM1 from P-TEFb. (A,B) F1C2 cells were preincubated with the indicated amounts of CsA(A) or FK506 (B) and then treated with UV or HMBA. Anti-Flag immunoprecipitates from NE were subjected to Western blotting (WB) and quantification to determine the levels of P-TEFb-bound HEXIM1. (C) HeLa cells cotransfected with the Cdk9-f cDNA and the indicated HA-PP2B-expressing plasmids (CA or IN) or an empty vector (V) were treated with UV or HMBA. Anti-Flag immunoprecipitates from NE were analyzed by WB to determine the levels of P-TEFb-bound HEXIM1. (D) F1C2 cells were preincubated with the indicated amounts of W-7 and then treated with UV or HMBA. Anti-Flag immunoprecipitates were analyzed and quantified to determine the levels of P-TEFb-bound HEXIM1.

in cells pretreated with W-7 [N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide] (Fig. 2D), a specific inhibitor of calmodulin, which is known to bind and activate PP2B together with Ca^{2+} . Consistently, HMBA activation of HIV-1 transcription was also blocked by W-7 in a dose-dependent manner (data not shown). Together, these data indicate the Ca^{2+} -calmodulin-PP2B pathway as key for transducing the UV/HMBA-induced signal to disrupt the HEXIM1-P-TEFb interaction and activate P-TEFb.

PP1 α plays a key role in UV/HMBA-induced dissociation of HEXIM1 from P-TEFb

It is important to point out that ectopic expression of PP2B CA only facilitated the UV/HMBA-induced HEXIM1 dissociation but did not cause the dissociation by itself (Fig. 2C, lanes 1,2). Similarly, activation of PP2B by ionomycin was insufficient to dissociate HEXIM1 in the absence of HMBA or UV treatment (Fig. 1C). Finally, when purified 7SK snRNP was treated in vitro with recombinant PP2B (Calbiochem), no dissociation of HEXIM1 from P-TEFb was detected (see Fig. 4D, below). These observations raise the possibility that PP2B is necessary but not sufficient to induce HEXIM1 dissociation. Another factor(s) activated by the UV- or HMBA-induced signaling is probably required to cooperate with PP2B for this effect.

Previously, we have shown that P-TEFb treated with PP1 cannot be sequestered into 7SK snRNP in vitro (Chen et al. 2004). Consistently, a critical role for PP1 to dephosphorylate Cdk9 and contribute to the control of HIV-1 transcription has also been revealed in vivo (Amosova et al. 2005). To test the hypothesis that PP1 plays a key role in causing the dissociation of HEXIM1 from P-TEFb, we first used a pharmacological approach

to pretreat F1C2 cells with 20 nM calyculin A (CalyA; Calbiochem), a PP1- and PP2A-specific inhibitor. Although CalyA alone did not dissociate HEXIM1 (Fig. 3A, cf. lanes 1 and 4), it markedly blocked the UV/HMBA-induced dissociation (Fig. 3A, lanes 2,3). Similarly, pre-treating cells with microcystin LR (MCLR; Calbiochem), an inhibitor of both PP1 and PP2A, also prevented the UV/HMBA-induced HEXIM1 dissociation (Fig. 3B).

Judging from the respective IC₅₀ of MCLR for PP1 and PP2A (IC₅₀ for PP2A = 40 pM and for PP1 = 1.7 nM) as well as the amounts of MCLR (~5–10 nM) required to achieve the inhibition, we postulated that it is the activity of PP1 that is required for UV/HMBA to dissociate HEXIM1 from P-TEFb. In support of this notion, overexpression of PP1 α but not the other two isoforms of PP1 (PP1 β/δ and PP1 γ) as EGFP fusion proteins in HeLa cells was able to partially induce HEXIM1 dissociation even in the absence of any UV or HMBA treatment (Fig. 3C). The effect of PP1 α was relatively small albeit highly reproducible, and the reason for this will become clear later.

Further evidence indicating an indispensable role of PP1 α in promoting UV/HMBA-induced disruption of 7SK snRNP was obtained in PP1 α knockdown cells. As discussed in detail below (Fig. 6E, below), shRNA depletion of PP1 α in HeLa cells effectively blocked this process.

Finally, a direct role for PP1 α in releasing P-TEFb from 7SK snRNP was demonstrated in vitro through treating immobilized 7SK snRNP (via Cdk9-f and anti-Flag beads) with recombinant PP1 α (15 U; New England Biolabs). After the incubation and then washing, both HEXIM1 and 7SK were removed from Cdk9-f, which remained attached to the beads (Fig. 3D, lanes 1,2). Meanwhile, the dissociated HEXIM1 and 7SK were in the supernatant

Figure 3. PP1 α plays a key role in UV/HMBA-induced disruption of 7SK snRNP. (A,B) F1C2 cells preincubated with the indicated amounts of CalyA(A) or MCLR(B) were treated with UV or HMBA. Anti-Flag immunoprecipitates from NE were analyzed by Western blotting (WB), and the levels of P-TEFb-bound HEXIM1 are quantified below. (C) HeLa cells were cotransfected with cDNA constructs expressing Cdk9-f and the indicated isoforms of PP1 as EGFP fusions. Anti-Flag immunoprecipitates from NE were analyzed by WB and quantification as in A. The levels of the various EGFP-PP1 isoforms in NE were determined by anti-GFP WB. (D) 7SK snRNP immobilized on anti-Flag beads via Cdk9-f was incubated with either buffer alone (–) or PP1 α (+). The reaction supernatant (right) and the materials bound to the beads after extensive washes (left) were analyzed by WB and Northern blotting (NB) for the indicated components.

(Fig. 3D, lanes 3,4). Together, these data are consistent with a direct role for PP1 α in disrupting the HEXIM1–P-TEFb interaction in vitro and in vivo.

PP2B and PP1 α cooperate to dissociate HEXIM1 from P-TEFb and stimulate P-TEFb-dependent HIV-1 transcription

In the above PP1 α experiments, the removal of HEXIM1 from P-TEFb was incomplete even when a large amount of DNA plasmid (30 μ g/150-mm dish) was transfected into cells (Fig. 3C). Moreover, efficient dissociation of HEXIM1/7SK from P-TEFb in vitro was achieved only when a large dose of recombinant PP1 (15 U) was used in the reaction (Fig. 3D; data not shown). These data, coupled with the demonstration that the expression of PP2B CA was necessary but not sufficient to dissociate HEXIM1 from P-TEFb (Fig. 2C), prompted us to investigate whether PP2B and PP1 α must cooperate to produce this effect.

Indeed, while the HEXIM1–P-TEFb interaction was not affected by the separate expression of either wild-type PP1 α or PP2B CA from a moderate level of transfected plasmid (10 μ g/150-mm dish), expression of the two together efficiently disrupted the interaction (Fig. 4A). This cooperativity requires that both phosphatases are catalytically active, as mutations that destroy the activity of either one failed to exert any effect (Fig. 4A, cf. lanes 3–5 and 2). It is also important to note that the cooperation by PP1 α and PP2B completely bypassed the requirement of UV or HMBA to dissociate HEXIM1 from P-TEFb.

To complement the above experiments involving transfected Cdk9-f, we also performed glycerol gradient sedimentation analysis to test the effect of PP1 α and PP2B on endogenous P-TEFb. As shown in Figure 4B, coexpression of the two phosphatases markedly reduced the amounts of HEXIM1 and Cdk9 in the large form P-TEFb (i.e., 7SK snRNP). Meanwhile, a major increase in the amounts of free Cdk9 and HEXIM1 was detected (note that the current conditions disrupted the salt-sensitive Brd4–P-TEFb binding).

Consistent with the cooperative nature of the PP2B/PP1 α -mediated release of P-TEFb from inactive 7SK snRNP, coexpression of wild-type PP1 α and PP2B CA but not their inactive derivatives (PP1 α H66N and PP2B IN) also markedly enhanced Tat-activation of HIV-1 transcription, a process known to be exquisitely P-TEFb-dependent (Fig. 4C; Peterlin and Price 2006).

To determine whether PP2B and PP1 α could also cooperatively dissociate HEXIM1 from P-TEFb in vitro, we treated the immobilized 7SK snRNP (on anti-Flag beads via Cdk9-f) with moderate levels of recombinant PP2B (5.0 U; Calbiochem) and/or PP1 α (2.5 U; New England Biolabs). Consistent with the in vivo results above, HEXIM1 was efficiently dissociated only when the two phosphatases were used together but not separately in the reaction (Fig. 4D).

PP2B facilitates PP1 α 's dissociation of HEXIM1 from P-TEFb

In order to dissociate HEXIM1 from P-TEFb, PP2B and PP1 α not only had to cooperate but also in a particular

Figure 4. PP2B and PP1 α sequentially and cooperatively dissociate HEXIM1 from P-TEFb and stimulate P-TEFb-dependent transcription. (A) HeLa cells were cotransfected with plasmids expressing Cdk9-f and the indicated HA-tagged PP1 α and/or PP2B. Anti-Flag immunoprecipitates from NE were analyzed by Western blotting (WB), and the amounts of HEXIM1 bound to P-TEFb were quantified. The levels of HA-PP1 α and HA-PP2B in NE were examined by anti-HA WB. (B) Lysates of HeLa cells transfected with the indicated cDNA constructs or an empty vector were analyzed by glycerol gradient (10%–30%) sedimentation. The distributions of Cdk9 and HEXIM1 in gradient fractions were examined by WB. (C) HeLa cells containing the HIV-1 LTR-luciferase reporter gene were cotransfected with the indicated expression plasmids. Luciferase activities were measured 48 h later. The levels of HA-PP1 α and HA-PP2B in transfected cells were examined by anti-HA WB. (D) 7SK snRNP attached to anti-Flag beads via Cdk9-f was incubated with the indicated amounts of recombinant PP1 α and/or PP2B. After washing, the levels of HEXIM1 bound to Cdk9-f were determined by WB and quantification. (E) Immobilized 7SK snRNP from F1C2 NE was incubated sequentially with the indicated phosphatases. Extensive washes separated the two incubations. The amounts of HEXIM1 bound to P-TEFb were determined by WB and quantification.

order. In fact, HEXIM1 dissociation was observed only when immobilized 7SK snRNP was subjected to the incubation with PP2B first, followed by extensive washes to remove PP2B and then a second incubation with PP1 α (Fig. 4E, lane 2). No effect was seen if PP2B and PP1 α were added in a reverse order (Fig. 4E, lane 3). We postulate that the target of PP2B in this reaction was 7SK snRNP but not PP1 α . This is because PP1 α , supplied as an active recombinant protein (New England Biolabs), is not known to be a substrate of PP2B. Moreover, no PP2B could be detected on immobilized 7SK snRNP after extensive washes (data not shown). Thus, the stimulatory effect of PP2B on PP1 α -induced dissociation of HEXIM1 from P-TEFb is likely a result of the two enzymes acting sequentially to dephosphorylate 7SK snRNP, which in turn causes the dissociation.

Direct dephosphorylation of Cdk9 at Thr186 by PP1 α

Next, we wanted to locate the precise position in 7SK snRNP where the dephosphorylation coordinated by

PP1 α and PP2B triggers the disruption of 7SK snRNP. Previously, we showed that the dephosphorylation of Cdk9 blocks the formation of 7SK snRNP in vitro (Chen et al. 2004). Moreover, mutation of Thr186 (T186) at the tip of the T-loop in Cdk9 into either Ala or Glu completely abolishes the sequestration of P-TEFb into 7SK snRNP (Chen et al. 2004; see also Fig. 5A). To determine whether T186 is phosphorylated in vivo, and if yes, how a change in its phosphorylation state may control the disruption of 7SK snRNP induced by PP2B and PP1 α , we raised an antibody (termed α -pT186) against a Cdk9 peptide (QPNRYpTNRVVTLC) containing the phosphorylated T186 (pT186). Western analysis indicates that except for two Cdk9 mutants T186A and T186E, which cannot be phosphorylated at T186 and do not interact with HEXIM1 (Chen et al. 2004), α -pT186 was able to recognize all the other Cdk9 mutants (Fig. 5A), some of which (e.g., 4A, 8A, S175A, and S175D) lack phosphorylation at positions other than T186 (Fong and Zhou 2000; Chen et al. 2004).

To further test the specificity of α -pT186 and the abil-

Figure 5. PP2B induces a more relaxed conformation in 7SK snRNP to facilitate PP1 α dephosphorylation of Cdk9 at T186. (A) Anti-Flag immunoprecipitates from NE of HeLa cells expressing wild-type or various mutant Cdk9-f proteins were analyzed by Western blotting (WB) with the indicated antibodies. The levels of HEXIM1 in NE serve as loading controls. (B) Affinity-purified 7SK snRNP was incubated first with RNase A or RNasin and then the indicated amounts of PP1 α . The reaction mixtures were analyzed by WB and quantification to determine the levels of bulk and pT186 form of Cdk9. (C) 7SK snRNP was incubated first with RNase A and then buffer or the indicated amounts of PP1 α or PP2B. The reaction mixtures were analyzed as in B. (D) 7SK snRNP was incubated with the indicated amounts of PP1 α and PP2B in the presence of RNasin. The reaction mixtures were analyzed as in B. (E) HeLa cells were cotransfected with plasmids expressing Cdk9-f and the indicated HA-tagged phosphatases. Anti-Flag immunoprecipitates from NE were analyzed by WB to determine the levels of pT186 and bulk Cdk9. Anti-HA WB was used to monitor the levels of transfected phosphatases in NE. (F) 7SK snRNP was pretreated with (lanes 5,6) or without (lanes 1–4) RNase A and then incubated in reactions containing the indicated components. The reaction mixtures were analyzed by WB with anti-CycT1 antibody. (FL) Full-length; (*) partially degraded CycT1 in untreated 7SK snRNP preparation.

ity of PP1 α to dephosphorylate pT186 in native Cdk9, we incubated 7SK snRNP with increasing amounts of recombinant PP1 α (NEB). This RNP was isolated via anti-Flag IP from NE of HH8, a HeLa-based cell line stably expressing Flag-HEXIM1 (Yik et al. 2003), to ensure that only the 7SK/HEXIM1-bound Cdk9 was analyzed. In contrast to P-TEFb sequestered in 7SK snRNP (7SK RNA protected by RNasin), which turned out to be a poor substrate for PP1 α , the released P-TEFb through RNase A degradation of 7SK was much more responsive to PP1 α , which caused dose-dependent dephosphorylation of pT186 (Fig. 5B). These data confirm the presence of pT186 in 7SK snRNP and also the specificity of α -pT186 toward this epitope. More importantly, they also show that the sequestration of P-TEFb in 7SK snRNP interferes with PP1 α 's dephosphorylation of pT186.

PP2B enhances PP1 α 's dephosphorylation of pT186

Although pT186 in 7SK/HEXIM1-free P-TEFb was efficiently dephosphorylated by 1 U of PP1 α , no dephosphorylation was seen when the same amount or even five times more PP2B was in the reaction (Fig. 5C), suggesting that pT186 is not a natural substrate of PP2B. Given the above demonstration that pretreating 7SK snRNP with PP2B facilitated PP1 α 's removal of HEXIM1 from P-TEFb (Fig. 4E), we asked whether PP2B could also enhance PP1 α 's dephosphorylation of pT186. Indeed, the combination of just 0.5 U of PP2B and 0.25 U of PP1 α almost completely dephosphorylated pT186 even when P-TEFb was still sequestered in 7SK snRNP (Fig. 5D, lane 5). Again, the same units of PP2B and PP1 α did not produce any effect when used separately. Considering that it normally takes >10 U of PP1 α to dephosphorylate pT186 when P-TEFb is within 7SK snRNP (data not shown), the ability of PP2B to allow just 0.25 U of PP1 α to achieve the same effect is remarkable and underscores the efficiency with which the two phosphatases cooperate to dephosphorylate pT186.

Consistent with these in vitro observations and also the cooperation displayed in vivo by PP2B and PP1 α to dissociate HEXIM1 from P-TEFb (Fig. 4A), coexpression of wild-type PP1 α and PP2B CA but not their mutants also dephosphorylated pT186 in vivo (Fig. 5E). Together, our data reveal an excellent correlation between PP2B's abilities to promote the dissociation of HEXIM1 from P-TEFb and dephosphorylation of pT186 by PP1 α .

PP2B induces a more relaxed conformation in 7SK snRNP

Since the ability of PP2B to assist PP1 α 's dephosphorylation of pT186 can be bypassed by RNase degradation of 7SK (Fig. 5B), we hypothesized that a PP2B-induced conformational change in 7SK snRNP similar to that caused by 7SK degradation is required for PP1 α 's action. To test this idea, we examined whether treating 7SK snRNP with recombinant PP2B would alter the sensitivity of CycT1, a subunit of the complex, to partial trypsin di-

gestion. Indeed, PP2B enabled a more efficient cleavage of CycT1 into smaller fragments, which was inhibited by EGTA, a Ca²⁺ chelator and inhibitor of PP2B (Fig. 5F). Moreover, consistent with the data in Figure 5B, the RNase-treated 7SK snRNP showed a similarly enhanced sensitivity to trypsin digestion as the PP2B-treated complex (cf. lanes 2 and 5). Thus, the dephosphorylation of 7SK snRNP at a position other than T186 (Fig. 5C) by PP2B induces a more relaxed conformation, which allows PP1 α to gain better access to the Cdk9 T-loop and dephosphorylate pT186.

UV/HMBA-induced dissociation of HEXIM1 from P-TEFb involves dephosphorylation of pT186

Our data so far indicate that UV and HMBA activate a Ca²⁺-calmodulin-PP2B signaling pathway to dissociate HEXIM1 from P-TEFb. Meanwhile, PP2B and PP1 α sequentially and cooperatively dephosphorylate Cdk9 at T186 and disrupt the HEXIM1-P-TEFb interaction. To establish a direct link between these two sets of findings, it is important to confirm that treating cells with UV/HMBA can lead to Cdk9 dephosphorylation at T186. Indeed, HMBA or UV not only decreased the levels of HEXIM1 bound to the immunoprecipitated Cdk9-f but also led to a major reduction in Cdk9 pT186 levels (e.g., Fig. 6 [lane3], B [lane 5]).

Rephosphorylation of T186 soon after the release of P-TEFb from 7SK snRNP

For UV-treated cells, massive apoptosis detected at ~4 h post-treatment prevented the examination of the pT186 status beyond this time point. In contrast, HMBA only slightly affected F1C2 growth (He et al. 2006) and caused the pT186 levels to show a transient reduction peaking ~4 h after the treatment began (Fig. 6A). During a prolonged treatment (>6 h), pT186 levels began to increase and HEXIM1 also became reassociated with P-TEFb. Notably, the total pT186 levels detected at the 2- to 4-h time points were greater than the levels of HEXIM1-bound P-TEFb (Fig. 6A). It is possible that besides its presence in 7SK snRNP, extra pT186 also existed in the released P-TEFb, which were subsequently rephosphorylated and active in elongation.

The rephosphorylation of T186 soon after the release of P-TEFb from 7SK snRNP has solved an apparent paradox created by the unphosphorylated state of the Cdk9 T-loop. This state, although critical for releasing P-TEFb, is likely to render the released P-TEFb inactive due to restricted access to the catalytic pocket of Cdk9 (Russo et al. 1996). In order for released P-TEFb to become active in transcription, the T-loop must be quickly rephosphorylated. This is precisely what was observed during a prolonged HMBA treatment (Fig. 6A) and also explains why the P-TEFb-dependent HIV-1 transcription is activated under these conditions (He et al. 2006; Contreras et al. 2007). Once the phosphorylated P-TEFb finishes its job in transcription, the excess amount must be con-

verted back into 7SK snRNP, which may explain the restored HEXIM1–P-TEFb interaction detected during a prolonged HMBA treatment (Fig. 6A).

Disruption of Ca^{2+} signaling or PP1 α activity interferes with UV/HMBA-induced dephosphorylation of pT186

Having demonstrated that the UV/HMBA-induced dissociation of HEXIM1 from P-TEFb involves dephosphorylation of pT186, we wanted to confirm that the activities of PP1 α and PP2B, shown above as key for UV/HMBA to dissociate HEXIM1, were also required for the signal-induced pT186 dephosphorylation. Indeed, pre-treating cells with inhibitors of either PP1 α (MCLR and CalyA) (Fig. 6C) or the various steps of the PP2B activation pathway (CsA, FK506, nifedipine, and W-7) (Fig. 6D) effectively blocked the UV-induced dephosphorylation. Notably, these inhibitors did not affect pT186 levels when used alone (Supplemental Fig. S2C). To comple-

ment these pharmacological studies, we also expressed two shRNAs (shPP1 α #1 and #2) to specifically depleted PP1 α in HeLa cells and showed that this severely compromised the UV/HMBA-induced dephosphorylation of pT186 and dissociation of HEXIM1 from P-TEFb (Fig. 6E).

The P-TEFb recruitment factor Brd4 prefers P-TEFb with unphosphorylated Cdk9 T-loop

Given the general belief that T-loop phosphorylation is required for Cdk activation (Russo et al. 1996), it is seemingly counterintuitive for cells to use PP1 α dephosphorylation of pT186 to release P-TEFb from 7SK snRNP for subsequent transcription. However, published data suggest that this scenario may not be so strange after all. Using HIV-1 LTR as a model system, previous studies have shown that Cdk9 is present, albeit inactive, in the PIC (Isel and Karn 1999; Ping and Rana 1999). Subsequently, it was shown that unphosphorylated Cdk9 as-

sociates with the PIC and its phosphorylation is inhibited by TFIIF (Zhou et al. 2001). As TFIIF is released from the PIC between +14 and +36, Cdk9 becomes rephosphorylated and active.

In light of these results, we examined the phosphorylation status of T186 in Cdk9 that is bound to Brd4, the factor known to recruit P-TEFb to the PIC for general elongation (Jang et al. 2005; Yang et al. 2005). To obtain P-TEFb either associated with Brd4 or present in 7SK snRNP, NEs from two HeLa-based cell lines expressing either Brd4-Flag (MCAP) (Jang et al. 2005) or Flag-HEXIM1 (HH8) (Yik et al. 2003) were subjected to anti-Flag IP. When total Cdk9 levels were normalized between the two complexes, a significantly reduced pT186 level was detected in the Brd4-bound P-TEFb (Fig. 6F). Thus, whereas the pT186-containing Cdk9 is sequestered in 7SK snRNP, it is mostly the unphosphorylated form that is recruited by Brd4 to the PIC. Finally, consistent with the notion that P-TEFb is maintained in a dynamic equilibrium between 7SK snRNP and the Brd4-bound complex (Zhou and Yik 2006), coexpression of active PP1 α and PP2B decreased the association with HEXIM1 while increasing the binding to Brd4 by P-TEFb (Fig. 6G). These data agree well with the published findings and indicate that PP1 α dephosphorylation of pT186 is necessary for not only the release of P-TEFb from 7SK snRNP but also the subsequent recruitment of P-TEFb to the PIC by Brd4.

Discussion

The data presented above are consistent with a model (Fig. 7) in which UV and HMBA initiate a Ca^{2+} /calmodulin-dependent signaling pathway to activate PP2B, which in turn acts on an as-yet-unidentified target in 7SK snRNP to induce a more relaxed conformation in the complex. This increases the accessibility to the Cdk9 T-loop and enables PP1 α to dephosphorylate pT186, leading to the release of P-TEFb. Once released, P-TEFb with the unphosphorylated T-loop is recruited by Brd4 to the PIC. Upon the synthesis of a short RNA, Cdk9 is rephosphorylated by a yet-to-be-identified kinase to gain full transcriptional activity. After transcription is completed, the excess amount of phosphorylated P-TEFb can be converted back into inactive 7SK snRNP to maintain a functional equilibrium for optimal cell growth (Zhou and Yik 2006).

During the past decade, several signaling pathways leading to cardiac hypertrophy have been deciphered (Heineke and Molkentin 2006). Among them, the Ca^{2+} -dependent pathway is recognized as one of supreme importance, not only because of its crucial role in governing cardiac functions, but also due to its ability to cross-talk with other hypertrophic signaling pathways (Wilkins and Molkentin 2004). Parallel to these findings, a key regulatory mechanism involving P-TEFb has also been revealed in heart cells. Upon the treatment with various hypertrophy-inducing agents, P-TEFb is released from 7SK snRNP, leading to elevated cellular protein/RNA contents and enlargement of cells (Sano et al. 2002). Con-

Figure 7. A model depicting the cooperation between PP2B and PP1 α to dephosphorylate Cdk9 T-loop and release P-TEFb from 7SK snRNP in response to Ca^{2+} signaling. UV/HMBA triggers Ca^{2+} influx through the L-type Ca^{2+} channels (LTCC) in the plasma membrane. Coupled to Ca^{2+} entry, activation of calmodulin (CaM) results in the nuclear import of activated PP2B, which targets a yet-to-be identified component of 7SK snRNP (indicated by a question mark [?]; the depiction of HEXIM1 as a target is purely hypothetical) and alters the conformation of the complex. This facilitates the dephosphorylation of Cdk9 at T186 by PP1 α , which is activated by UV/HMBA via a separate, as-yet-undefined signaling pathway. As a result of the sequential and cooperative actions of PP2B and PP1 α , P-TEFb is released from 7SK snRNP. This process is blocked at various stages by the indicated pharmacological inhibitors. The released, unphosphorylated P-TEFb is transferred to Brd4, which recruits it to the PIC. After the synthesis of a short RNA, Cdk9 is rephosphorylated at T186 by an unknown kinase to become transcriptionally active. Upon completion of transcription, the excess amount of active, pT186-containing P-TEFb is reassembled into 7SK snRNP to maintain a functional equilibrium for optimal growth.

sistently, ablation of the mouse HEXIM1 (CLP-1) gene also leads to cardiac hypertrophy and embryonic lethality (Huang et al. 2004). Given that Ca^{2+} signaling and induced release of P-TEFb from 7SK snRNP have been separately shown as important for hypertrophic growth, it is gratifying to see that these findings are finally joined together in the present study, which indicates the Ca^{2+} -calmodulin-PP2B pathway as key for UV/HMBA to release P-TEFb from 7SK snRNP.

This conclusion, although based on studies in HeLa cells, a noncardiac cell line, is highly consistent with an earlier demonstration that P-TEFb is activated in the myocardium of transgenic mice expressing the activated form of PP2B (Sano et al. 2002). In addition, several aspects of this conclusion have also been confirmed by experiments conducted for other unrelated studies and/or in different cell types. For example, low dosages of UV, which disrupt 7SK snRNP, can trigger Ca^{2+} influx in HeLa cells (Pu and Chang 2001) and activate PP2B in mononuclear cells (Herman et al. 2002). Similarly, HMBA treatment of erythroleukemia cells has been shown to activate Ca^{2+} signaling (Gillo et al. 1993).

Thus, the disruption of 7SK snRNP via the Ca^{2+} -calmodulin-PP2B pathway by UV/HMBA could be a phenomenon common to many different cell types.

It is important to note that in HeLa cells, Ca^{2+} signaling is necessary but not sufficient for UV/HMBA-induced disruption of 7SK snRNP. The PP1 α dephosphorylation of the Cdk9 T-loop, a process that is greatly aided by PP2B, is additionally required for this effect. Although the exact target of PP2B in 7SK snRNP is still unknown, we show that PP2B induces a more relaxed conformation in this RNP (Fig. 5F), which increases the access to the T-loop by PP1 α . Previously, the phosphorylation state of the Cdk9 C terminus has been shown to influence the conformation of P-TEFb, which in turn affects P-TEFb's ability to interact with the HIV-1 Tat protein and TAR RNA (Fong and Zhou 2000; Garber et al. 2000). It will be interesting to determine whether PP2B can target the same phosphorylated residues at the C terminus, and if yes, whether this may relax 7SK snRNP and help PP1 α 's dephosphorylation of pT186.

Since neither the ionomycin treatment alone (Fig. 1C) nor the sole expression of PP2B CA (Fig. 2C) was able to dissociate HEXIM1 from P-TEFb, the Ca^{2+} -dependent activation of PP2B is obviously not the only rate-limiting step in this process. Thus, despite the fact that PP2B can promote PP1 α 's dephosphorylation of pT186, PP1 α must be activated first via a separate, UV/HMBA-induced signaling pathway before it can work with PP2B (Fig. 7). Since PP1 is controlled by a diverse array of associated regulators, it remains to be determined how UV/HMBA may alter PP1 α 's interaction with a specialized regulator(s), leading to the specific dephosphorylation of pT186.

Of note, in the course of our studies, it was reported that the HMBA-induced disruption of 7SK snRNP involves PI3K/Akt and the phosphorylation of HEXIM1 (Contreras et al. 2007). However, since the effect of PI3K/Akt on purified 7SK snRNP has not been shown, it remains to be seen whether PI3K/Akt can directly phosphorylate and dissociate HEXIM1 from P-TEFb. In fact, there exist several lines of evidence that argue against a central role of PI3K/Akt in this process. For example, neither the treatment with pharmacological activators of PI3K/Akt (e.g., pervanadate, H_2O_2 , phorbol ester TPA, ionomycin, etc.) nor the expression of a CA form of Akt (Myr-Akt) caused HEXIM1 dissociation (Fig. 1C; Supplemental Fig. S1). Furthermore, chemical inhibition of PI3K/Akt or shRNA depletion of Akt had little effect on the induced HEXIM1 dissociation either (Supplemental Fig. S1).

In contrast to PI3K/Akt, the cooperation between PP2B and PP1 α has been indicated here as not only necessary but also sufficient to dissociate HEXIM1 from P-TEFb in vivo and in vitro. However, since UV/HMBA can activate multiple signaling pathways, it is still possible that some of them—e.g., the PI3K/Akt pathway—may cross-talk with PP2B and PP1 α to further promote the dissociation. For example, like PP2B, the PI3K/Akt-dependent phosphorylation of HEXIM1 may provide an alternative way to introduce conformational changes in

7SK snRNP to promote PP1 α dephosphorylation of pT186. Alternatively, PI3K/Akt could be involved in HMBA activation of PP1 α .

Another place where PI3K/Akt or some other signaling pathways may play a more prominent role is the disruption of 7SK snRNP induced by P-TEFb inhibitors such as actinomycin D and DRB (Nguyen et al. 2001; Yang et al. 2001). Our preliminary data indicate that unlike UV/HMBA, these two compounds did not induce Ca^{2+} influx. Moreover, pretreating cells with PP2B inhibitors FK506 and CsA did not prevent actinomycin D or DRB from disrupting 7SK snRNP. Thus, Ca^{2+} signaling is unlikely to be involved. It remains to be seen whether pT186 dephosphorylation still occurs in this process, and if yes, what signaling molecules are required to cause the dephosphorylation.

Although a few details are still missing, our current model (Fig. 7) has linked together several key observations made in the past. For example, P-TEFb has been found to exist in an unphosphorylated and inactive state in the PIC (Isel and Karn 1999; Ping and Rana 1999; Zhou et al. 2001). Meanwhile, PP1 α is probably responsible for Cdk9 dephosphorylation (Amosova et al. 2005). However, it was unclear what triggers PP1 α to dephosphorylate Cdk9, where PP1 α obtains its source of P-TEFb, and how the unphosphorylated P-TEFb enters the PIC. The demonstration that pT186 dephosphorylation by the joint actions of PP1 α and PP2B is important for not only releasing P-TEFb from 7SK snRNP but also the subsequent recruitment of P-TEFb to the PIC by Brd4 provides critical connections among these findings.

What could be the benefit of having inactive Cdk9 in the PIC and also during initial RNA synthesis? It is possible that this helps establish a noninterfering environment for TFIIF to phosphorylate Ser5 in the Pol II CTD that is key for promoter clearance and pre-mRNA capping (Sims et al. 2004). Since the phosphorylated T-loop is generally required for Cdk activity, it is expected that upon the release of TFIIF, Cdk9 is rephosphorylated by a yet-to-be-identified kinase to become active. Indeed, the detection of pT186 soon after HMBA disrupted 7SK snRNP (Fig. 6A) agrees with this prediction. After transcription is completed, the excessive amount of phosphorylated P-TEFb could be repackaged into 7SK snRNP, allowing active P-TEFb levels to be finely controlled for optimal cell growth. Thus, modified successively by PP2B/PP1 α and then a CAK-like kinase (Chen et al. 2004) in a tightly controlled fashion, the phosphorylation state of Cdk9 T-loop undergoes dynamic changes in the transcription cycle in a way similar to those of the Pol II CTD. To fully understand the control of these changes and their biological implications, the signaling pathway identified in this study represents an important step forward.

Materials and methods

Materials

All chemical compounds for studying Ca^{2+} signaling are from Calbiochem except for cyclosporin A and CalyA, which are

from LC Laboratories. Recombinant PP1 α and PP2B are from New England Biolabs and Calbiochem, respectively. Rabbit anti-PP1, anti-PP2B, and anti-EGFP antibodies are from Santa Cruz Biotechnologies. Rabbit polyclonal antibody against a pT186-containing Cdk9 peptide (QPNRYpTNRVVTLCW) was raised in Bethyl, Inc. DMEM free of Ca²⁺ and phosphate was prepared according to the description of Invitrogen by removing CaCl₂ and phosphate from the recipe.

To generate the plasmid expressing HA-PP2B CA, the PCR fragment amplified from pCI-neo-CnA* (provided by Dr. R. Sanders Williams, Duke Medicine), which contains the calcineurin A α isoform missing the autoinhibitory domain and a portion of the calmodulin-binding domain, was cloned into pcDNA3 (Invitrogen). HA-PP2B IN was created by introducing a point mutation (D90A) into HA-PP2B CA. The PP1 α catalytic subunit was PCR-amplified from pRB4891 containing the human PP1 α cDNA (provided by Dr. Bin He, University of Minnesota) and subcloned as a HA-tagged fusion into pCMV5 (Sigma). To generate the inactive form of PP1 α , a point mutation (H66N) was introduced into wild-type PP1 α . All constructs were verified by sequencing. The EGFP-PP1 α , EGFP-PP1 β/δ , and EGFP-PP1 γ constructs were provided by Dr. Laura Trinkle-Mulcahy (University of Dundee). A shRNA that specifically targets PP1 α mRNA was expressed from a DNA oligonucleotide (5'-GATCAAGATCAAGTACCCCGAGAACTTCAAGAGAGTTCTCGGGGTACTTGATCTTTTAA-3') inserted into pSuper-Retro-puro vector (OligoEngine).

Treatment of cells with UV or various pharmacological compounds

Cells at ~50%–70% confluence were preincubated with the various compounds at the concentrations indicated in the figures for 1 h. For UV irradiation, cells were exposed to the indicated dosages of UV in Spectrolinker XL-1000 (Spectronics) without medium and then put back in the original medium for 1 h unless indicated otherwise.

Immuno-affinity purification of P-TEFb and its associated factors for Western and Northern analyses

Flag-tagged Cdk9 and its associated factors were isolated by anti-Flag IP from NE of FIC2 or transfected HeLa cells as described previously (Chen et al. 2004). The levels of the P-TEFb-bound HEXIM1 or the pT186 form of Cdk9 were determined by Western blotting, normalized against the bulk Cdk9 levels, and quantified with the QuantityOne software. The levels of pT186 and HEXIM1 in untreated cells were set to 100%. All experiments were repeated multiple times to ensure reproducibility.

Phosphatase treatment and trypsin digestion of affinity-purified 7SK snRNP

7SK snRNP was affinity-purified from ~250 μ g of FIC2 NE. After washing with buffer D (20 mM HEPES-KOH at pH 7.9, 15% glycerol, 0.2 mM EDTA, 0.1% NP-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) containing 0.3 M KCl (D0.3) and then D0.1, the complex attached to anti-Flag beads was incubated for 1 h at 30°C with either 10 μ L of buffer D0.1 plus 1 mM CaCl₂ and 1 mM MnCl₂ or the indicated amounts of phosphatases in the same buffer. The beads were then washed with buffer D0.3 and eluted with the Flag peptide.

For phosphatase dephosphorylation of pT186, 7SK snRNP was isolated via anti-Flag IP from HH8 cells (Yik et al. 2003) and eluted with Flag peptide in buffer D0.1. The purified complex

was treated with RNase A (100 U) or RNasin (40 U) for 30 min at 30°C and then incubated with the indicated amounts of phosphatases in 10–15 μ L of buffer D0.1 plus 1 mM CaCl₂ and 1 mM MnCl₂. The complex was analyzed by Western blotting to determine the levels of bulk and pT186 form of Cdk9. For trypsin digestion, 7SK snRNP was incubated for 1 h at 30°C with PP2B/calmodulin in buffer D0.1 plus either 1 mM CaCl₂ or 1 mM EGTA. The mixtures were then digested with trypsin as described (Fong and Zhou 2000).

Luciferase assay

HeLa cells with an integrated HIV-1 LTR-luciferase reporter gene (He et al. 2006) were preincubated in six-well plates for 1 h with the various compounds. HMBA (5 mM) was then added into the medium and continuously present for 6 h. To determine the effects of transfected PP1 α and/or PP2B on Tat-activated HIV-1 transcription, the reporter cells were cotransfected with 50 ng per well Flag-Tat plasmid and 1 μ g per well of the desired cDNA constructs as indicated. The total amount of DNA transfected per well was kept constant by the inclusion of empty vectors. After 48 h of transfection, cell lysates were prepared to measure luciferase activity.

Visualization of intracellular free Ca²⁺

Fluorescent probe Fluo-3/AM (Molecular Probes) was used to visualize drug-induced Ca²⁺ influx in HeLa cells as described (Thomas et al. 2000). For Nifedipine treatment, cells were incubated in the HEPES buffer containing 10 μ M Nifedipine during de-esterification. HMBA (10 mM) was then added for 10 min before pictures were taken. For UV, cells were treated with 80 J/m² UV without the HEPES buffer, which was then added back and pictures were taken 1 min later.

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