Acetylation and subnuclear localization regulate P-TEFb activity

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Chapter 1

Introduction

INTRODUCTION

Transcription Elongation

The regulation of gene expression is one of the most intensely investigated areas in all of life sciences. Differential gene expression in multicellular organisms forms the foundation of cell-type specificity. Deregulation of the appropriate pattern of gene expression has profound effects on cellular function and underlies many diseases. Although there are many cellular processes that control gene expression, the most direct regulation occurs during transcription. The transcription of protein-coding genes in eukaryotes is carried out by RNA polymerase II (RNAPII). Until recently, the vast majority of studies aimed at elucidating the molecular mechanisms of transcription regulation have focused on early stages, such as the formation of a transcription initiation complex (pre-initiation) or initiation. For several years, transcript elongation has been thought of as the trivial addition of ribonucleoside triphosphates to the growing mRNA chain, but this process is actually a dynamic and highly regulated stage of the transcription cycle, capable of coordinating downstream events. In particular, recent studies have challenged the once commonly held view that transcription is predominantly regulated at the level of RNA polymerase II (RNAPII) recruitment to the promoter and it is now clear that many genes in organisms from flies to men are regulated by reversing early blocks to RNAPII elongation (Chao and Price, 2001; Price, 2000).

Promoter escape (or promoter clearance)

Transcription starts with pre-initiation complex (PIC) assembly at the promoter. The PIC includes RNAPII and several general transcription factors (GTFs) (Orphanides et al., 1996). After melting of the double stranded DNA, transcription initiation occurs upon addition of two initiating nucleotides triphosphates and formation of the first phosphodiester bond (Goodrich and Tjian, 1994).

The first block to elongation occurs after this step: before RNAPII becomes engaged into productive transcript elongation, it needs to escape the ties that bind it to the promoter (promoter clearance). An important but poorly understood aspect of promoter clearance is that RNAPII now needs to start moving through chromatin,

rather than merely be embedded in it at the promoter (Thoma, 1991). Therefore the PIC must eventually be disassembled: a subset of GTFs remains at the promoter, serving as a scaffold for the formation of the next transcription initiation complex (Yudkovsky et al., 2000; Zawel et al., 1995). During promoter clearance, RNAPII itself must undergo structural and functional maturation in order to break its contacts with promoter-sequence elements and to tighten its grip on the nascent RNA.

The main structural change that occurs to RNAPII during early stages of transcription is the hyper-phosphorylation of the C-terminal domain (CTD) of its largest subunit, Rpb1. RNAPII CTD contains multiple repeats of the heptapeptide sequence YSPTSPS, each of which can be phosphorylated. The number of repeats increases with genomic complexity: 26 in yeast (Allison et al., 1985), 45 in *Drosophila* (Allison et al., 1988; Zehring et al., 1988), and 52 in mammals (Corden et al., 1985). The existence of the hypo- and hyper-phosphorylated forms of RNAPII (IIA and IIO, respectively) was first described in the early 1980s (Dahmus, 1981). Studies using functional assays together with antibodies specific to one or the other form of RNAPII demonstrated that RNAPII in the pre-initiation complex (PIC) was unphosphorylated (Laybourn and Dahmus, 1989; Lu et al., 1991), whereas transcription-competent RNAPII was heavily phosphorylated on its CTD (Christmann and Dahmus, 1981); subsequently, Ser2 and Ser5 residues were identified as the major modification sites.

The three kinases that target the RNAPII CTD are the transcriptional cyclin-dependent kinases Cdk7, Cdk8, and Cdk9 (Prelich, 2002). These enzymes are evolutionarily conserved from yeast to mammals, and all are components of protein complexes.

Cdk8 associates with the Srb/Mediator complex and functions in transcriptional events prior to elongation (Cho et al., 1998; Maldonado et al., 1996). Cdk7 is a subunit of the general transcription factor TFIIH (Orphanides et al., 1996) and phosphorylates RNAPII CTD on Ser5 immediately after the formation of the first phosphodiester bond of the nascent transcript, thus regulating RNAPII promoter clearance (Akoulitchev et al., 1995; Orphanides and Reinberg, 2002; Rodriguez et al., 2000). Cdk9, is part of the P-TEFb (positive transcription elongation factor b)

complex (Zhu et al., 1997) and phosphorylates RNAPII CTD on Ser2 after the positioning of the 5' cap to the mRNA (Cho et al., 2001).
There are also CTD phosphatase that reverse the status of the CTD. Among them, the FCP1 phosphatase targets the CTD of RNAPII (Archambault et al., 1997; Archambault et al., 1998; Chambers and Dahmus, 1994) and participates in RNAPII recycling (Cho et al., 1999; Mandal et al., 2002).

Promoter-proximal pausing

Before becoming a fully productive elongation complex, the early elongation complex undergoes continued adjustments and this process is often accompanied by transcriptional pausing (or stalling) near the promoter. Promoter-proximal pausing is a phenomenon whereby RNAPII pauses in the 5' region of the transcription unit and only progresses efficiently into productive elongation upon stimulation by appropriate signals. It constitutes the second block to transcription elongation and functions as a checkpoint before committing to productive elongation.
The most notable examples of genes that harbor a paused polymerase include the heat-shock-inducible genes and the mammalian proto-oncogenes *MYC* and *FOS* (Lis, 1998); this mechanism, however, is more widely exploited, since it that takes place at many genes in eukaryotes (Raschke et al., 1999) and during viral transcription (Barboric and Peterlin, 2005).
Candidate pausing factors were discovered during attempts to reconstitute *in vitro* transcription that displays the same sensitivity to the inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) as seen *in vivo*. DRB sensitivity inducing factor (DSIF) and negative elongation factor (NELF) (Wada et al., 1998; Yamaguchi et al., 1999) were both required for the inhibition of transcription by DRB *in vitro* and their negative effect could be overcome by the action of the protein that was actually inhibited by DRB, namely P-TEFb.
DSIF and NELF, bind to RNAPIIA, the hypo-phosphorylated form of the polymerase, that contains Ser5P but not Ser2P (Wada et al., 1998; Yamaguchi et al., 1999) and they cooperate to inhibit the elongation rate of RNAPII by increasing the duration of time spent at paused sites (Renner et al., 2001).

The paused RNAPII is then joined by the capping enzyme that interacts with the Ser5P CTD and DSIF (Kim et al., 2002; Wada et al., 1998; Wen and Shatkin, 1999) and the nascent RNA becomes capped. Following the addition of a cap to the 5' end of the nascent RNA, the negative effect of DSIF and NELF are relieved by the action of P-TEFb, which phosphorylates DSIF, NELF and RNAPII CTD on Ser2 (Cho et al., 2001). Once the transition to productive elongation has taken place, NELF leaves the elongation complex while DSIF remains associated and becomes a positive factor.

It is not clear how P-TEFb is recruited to the transcription complexes. P-TEFb may be recruited to RNAPII at the promoter, and its kinase activity may be inactive until the cap has been added. Indeed, the observation that transcriptional activators can interact with P-TEFb suggests that this enzyme may be recruited to the initiation complex at the promoter (Barboric et al., 2001; Kanazawa et al., 2000). Alternatively, P-TEFb may join the paused RNAPII complex after capping: the capping enzyme may assist the recruitment of Cdk9 as in *S. pombe*, in which the Cdk9 ortholog directly binds a subunit of the capping apparatus (Pei et al., 2003).

In all likelihood, efficient elongation requires that the CTD is kept hyper-phosphorylated during the entire length of the run, but the precise mechanism of action of the kinases responsible, and indeed, the processes leading to CTD de-phosphorylation are not yet well defined. CTD de-phosphorylation is likely to be a continuing occurrence during elongation, as kinase inhibitors such as DRB and H8, inhibit elongation both *in vivo* and *in vitro* (Chodosh et al., 1989; Marshall and Price, 1995; Yankulov et al., 1995; Yankulov et al., 1996), and CTD kinases, such as Ctk-1 and P-TEFb, stimulate transcript elongation (Chodosh et al., 1989; Lee and Greenleaf, 1997; Marshall and Price, 1995; Yankulov et al., 1995; Yankulov et al., 1996). This indicates that the hyper-phosphorylated state of the CTD, acquired at the initiation–elongation transition, needs to be maintained, perhaps in order to keep the integrity of elongating RNAPII holoenzyme.

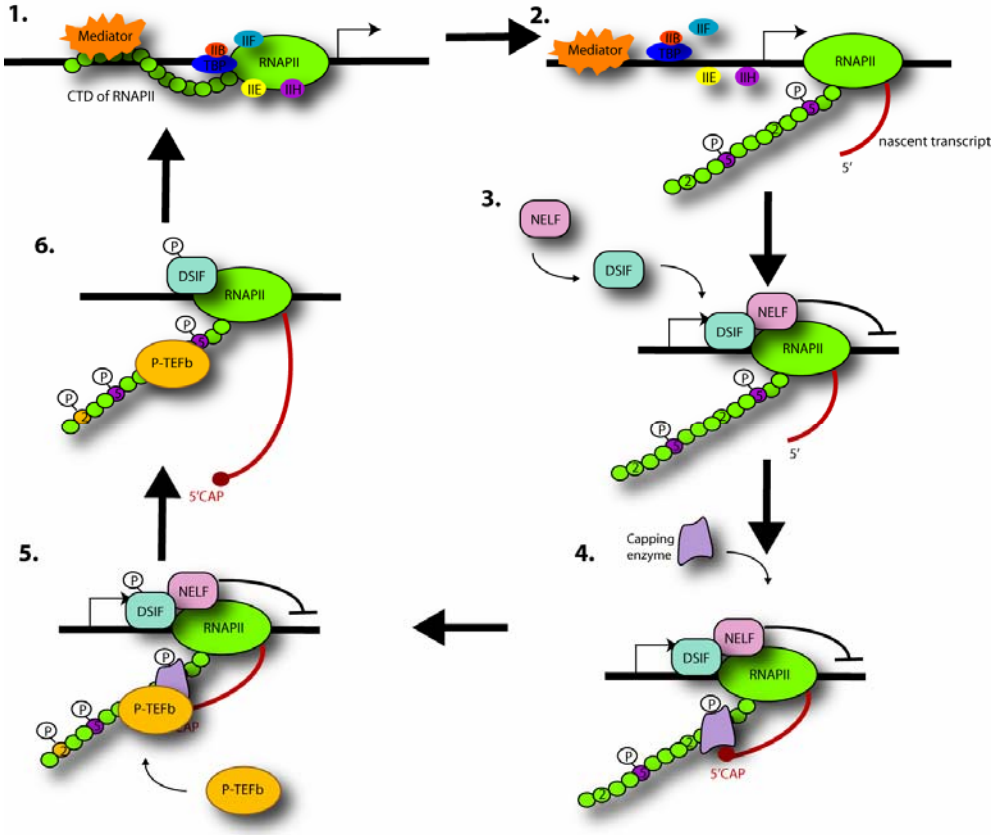

Figure 1. A "Checkpoint" model for the coupling of 5' pre-mRNA capping and early transcription initiation. Adapted from (Orphanides and Reinberg, 2002).

The CTD code

As transcription elongation proceeds, the RNA transcript is matured by capping and splicing, and these events, as well as the termination-coupled processes leading to mRNA polyadenylation, all happen co-transcriptionally, that is, coupled to the progress of RNAPII along the gene (Kornberg, 2001; Proudfoot et al., 2002).

It is now established that the RNAPII CTD plays a central role in the ordered recruitment of protein factors involved in elongation, as well as in mRNA maturation, surveillance, and export (Hirose et al., 1999; Orphanides and Reinberg, 2002; Proudfoot et al., 2002).

The Mediator complex binds RNAPII at least partly via the CTD and enhances TFIIH-mediated CTD phosphorylation up to several hundred-fold (Kim et al., 1994).

Mediator is absent from elongation complexes containing hyper-phosphorylated RNAPII (Pokholok et al., 2002; Svejstrup et al., 1997), thus CTD phosphorylation probably induces the disruption of CTD-Mediator interactions, enabling recycling of Mediator to a new initiating polymerase (Svejstrup et al., 1997); in all likelihood, other RNAPII-GTF interactions are also broken by CTD phosphorylation (Usheva et al., 1992).

While leading to disruption of one set of interactions, CTD phosphorylation might concomitantly establish another set. The phosphorylated CTD recruits capping enzymes (Cho et al., 1997; Rodriguez et al., 2000; Yue et al., 1997) and the SR proteins involved in splicing (Greenleaf, 1993; Kim et al., 1997; Patturajan et al., 1998).

Similar to capping and splicing, transcription termination and 3'-end processing require factors that associate with the RNAPII CTD. Several components of the 3'-end processing machinery interact with the Ser2P CTD *in vitro* (Barilla et al., 2001; Licatalosi et al., 2002), and RNAPII has been shown to stimulate 3'-end processing (Hirose and Manley, 1998; Proudfoot et al., 2002). Moreover 3'-end processing defects were the major alterations resulting from a loss of Ser2 phosphorylation (Ahn et al., 2004; Ni et al., 2004). In addition, the Ser2 kinases Ctk1 and Cdk9 have been shown to be essential for the 3'-end processing in both *S.cerevisiae* (Skaar and Greenleaf, 2002) and mammals (Medlin et al., 2005; Medlin et al., 2003).

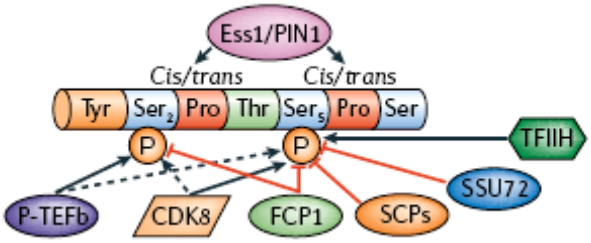

Figure 2. The CTD can be modified by phosphorylation, glycosylation, and *cis/trans* isomerization of prolines (Saunders et al., 2006).

The CTD is a simple repetition of an heptapeptide sequence, so how does such a simple sequence interact with so many targets? A series of different phosphorylation and conformation changes generates configurations specific for binding of particular factors. In essence, the existence of a “CTD code” has been inferred, that specifies the position of RNAPII in the transcription cycle (Buratowski, 2003).

The two phosphorylations on Ser5 and Ser2 help distinguish early and late phases of transcription. Chromatin Immunoprecipitation (ChIP) data on the fate of Ser5 and Ser2 phosphorylation during transcription have indicated that Ser5 phosphorylation is high at the promoter, and then decreases towards the 3’-end of the gene, while Ser2 phosphorylation increases towards the 3’-end of the gene (Cho et al., 2001; Komarnitsky et al., 2000; O'Brien et al., 1994; Schroeder et al., 2000). Thus, it appears that CTD phosphorylation at Ser5 correlates with transcription initiation and early elongation (promoter clearance), whereas Ser2 phosphorylation is associated with RNAP II farther away from the promoter.

It is important to note that a recent report characterizing the antibodies frequently used to distinguish between Ser5 and Ser2 CTD phosphorylation revealed that the Ser2-specific antibody can recognize in some circumstances both the Ser5 and Ser2 phosphorylated forms (Jones et al., 2004). Hence it may be necessary to re-examine some previously published information, although the general trends are likely to remain valid.

In addition to phosphorylation, the CTD code probably also includes cis-trans isomerization at two prolines that follow the phosphorylated serines. The proline isomerase Pin1/Ess1 acts at prolines preceded by a phosphorylated residue and has been involved in mRNA 3’-end formation. It remains to be determined whether phosphorylation changes the equilibrium between the *cis* and *trans* form of the CTD prolines.

Just considering the possible patterns of phosphorylation and proline configurations, sixteen distinct states can be specified within a single CTD repeat. Each state is potentially a specific recognition site for an interacting factor (Buratowski, 2003).

P-TEFb

P-TEFb (positive transcription elongation factor b) was originally identified based on its ability to stimulate DRB-sensitive transcription of long transcripts *in vitro* (Marshall et al., 1996; Marshall and Price, 1992). The heterodimeric P-TEFb complex consists of the Cdk9 kinase that associates either with Cyclin T1, Cyclin T2a, Cyclin T2b, or Cyclin K (Peng et al., 1998; Peng et al., 1998). The elongation activity of P-TEFb is dependent on its kinase activity on RNAPII CTD and DSIF/NELF during promoter clearance (Price, 2000). P-TEFb targets specifically Ser2 of the CTD, as demonstrated by RNA interference studies in *C. elegans* (Shim et al., 2002) and by exploiting the highly specific P-TEFb inhibitor flavopiridol (Ni et al., 2004).

Upon heat shock, P-TEFb is rapidly recruited to transcriptionally active loci on *Drosophila* polytene chromosomes and frequently co-localizes with the promoter-paused hypo-phosphorylated form of RNAPII (Lis et al., 2000). These results suggest that P-TEFb is recruited to facilitate productive elongation upon RNAPII pausing. Additional experiments revealed that P-TEFb appeared to track along with RNAPII during elongation with similar kinetics (Andrulis et al., 2000; Boehm et al., 2003). Therefore P-TEFb seems to act not only during promoter escape but also during transcriptional elongation.

The P-TEFb core complex

Cdk9 is a 43 kDa protein that has been originally identified during a cDNA screening aimed at isolating novel regulators of the mammalian cell cycle (Grana et al., 1994). As no cyclin partner or cell cycle function was demonstrated at that time, Cdk9 was temporarily designated PITALRE for its PSTAIRE-like sequence, a conserved motif found in CDC2 and related kinases (Pines, 1994). The cellular function of Cdk9 remained unknown until the *Drosophila* homologue of human PITALRE, Cdk9, was identified as the small subunit of the P-TEFb factor by Marshall and Price (Marshall and Price, 1995).

Recently, a novel isoform of Cdk9 has been identified, which is transcribed from an alternative upstream promoter (Shore et al., 2005; Shore et al., 2003). The

resulting 55 kDa Cdk9 isoform interacts with Cyclin T1 and 7SK and is able to phosphorylate RNAPII CTD like the 43 kDa isoform.

The two isoforms of Cdk9 have a differential expression. $Cdk9^{55}$ is the minor form of Cdk9 in HeLa and NIH/3T3 cells, comprising less than 20% of total Cdk9. However, the relative amount of the two forms is altered in cultured human macrophages, with $Cdk9^{55}$ predominating. Interestingly, this ratio is altered upon LPS (lipopolysaccharide) treatment or HIV-1 infection of the macrophages, leading to a change in the relative amounts of the two forms with $Cdk9^{42}$ becoming the major form (Shore et al., 2003).

Western analysis of murine tissues has shown that the relative abundance of the two forms of Cdk9 varies across different tissues, with liver having more $Cdk9^{55}$ than $Cdk9^{42}$. During adaptation of primary rat hepatocytes to culture, the ratio of the two forms of Cdk9 changed. Initially, $Cdk9^{55}$ was the predominant form, but as the cells began to enter the cell cycle $Cdk9^{42}$ became the major form. This suggests that $Cdk9^{42}$ and $Cdk9^{55}$ may have specific functions in dividing and nondividing cells respectively, or that dividing cells have a higher need for P-TEFb and that induction of the $Cdk9^{42}$ promoter provides the extra Cdk9 required (Shore et al., 2005).Moreover the two isoforms have different localization: epitope tagged transiently expressed $Cdk9^{42}$ localized diffusely in the nucleoplasm, while $Cdk9^{55}$ accumulated in nucleolus (Liu and Herrmann, 2005).

Cdk9 has two homologs in yeast, Ctk1 and Bur1 (Yao and Prelich, 2002). The two yeast kinases likely contain distinct functional activities and may have different targets *in vivo*. Whereas Bur1 is essential for cell viability, Ctk1 is not (Lee and Greenleaf, 1991; Patturajan et al., 1999; Prelich and Winston, 1993; Yao et al., 2000). Even though Bur1 phosphorylates the RNAPII CTD, genetically interacts with CTD truncations, and co-localizes with elongating RNAPII, mutations in the Bur1 gene do not appear to affect either Ser2 or Ser5 phosphorylation in the cells (Keogh et al., 2003; Murray et al., 2001).

Ctk1 is responsible for elongation-associated Ser2 phosphorylation of the CTD and is localized to the coding regions of genes (Cho et al., 2001; Patturajan et al., 1999). Deletion of Ctk1 results in loss of histone H3-K36 methylation and Set2 recruitment (Krogan et al., 2003; Xiao et al., 2003), further highlighting the role of

Ctk1 in the regulation of transcript elongation. However, the fact that Ctk1 is not essential suggests that other kinases, likely Bur1, can compensate for its deficiency.

The Cyclin T1 regulatory subunit of Cdk9 was independently identified by the groups of Katherine Jones and David Price using different strategies (Peng et al., 1998; Wei et al., 1998). Subsequently, three T-type cyclins were identified in human cells and were named Cyclins T1, T2a and T2b (T-type cyclins; (Peng et al., 1998). Cyclins T2a and T2b are splice variants of a primary transcript. The two cyclins share the first 642 amino acids, but Cyclin T2b contains a larger C-terminal domain. The three T-type cyclins share a highly conserved N-terminus containing an 81% identical cyclin box. However, the C-terminus is much less conserved (46% identity) (Peng et al., 1998). In addition to the N-terminal cyclin domain, Cyclin T1 contains a putative coiled-coil motif, a His-rich motif and a carboxy-terminal PEST sequence (Wei et al., 1998). PEST sequences have been previously identified in proteins with high turnover rates, including G1 cyclins, and appear to regulate protein turnover by ubiquitin-dependent proteolysis (Rechsteiner and Rogers, 1996). However, the role of the PEST sequence present in Cyclin T1 is not well understood.

A more distantly related cyclin, Cyclin K, has also been shown to both interact and form an active complex with Cdk9 (Fu et al., 1999). Interestingly, in contrast to T-type cyclin/Cdk9 complexes, Cyclin K/Cdk9 can only activate transcription when tethered to RNA but not DNA. Cyclin K lacks an essential His-rich region in its carboxy terminus that, in Cyclin T1, helps recognizing the RNAPII CTD (Lin et al., 2002).

P-TEFb regulatory partners

In human HeLa cells, more than half of the P-TEFb heterodimer is associated with large ribonucleoprotein (RNP) complexes which also contain the 7SK small nuclear RNA (Nguyen et al., 2001; Yang et al., 2001) and the HEXIM1 or HEXIM2 protein (Byers et al., 2005; Michels et al., 2003; Yik et al., 2003; Yik et al., 2005). Actually the large complex seems to be formed by one 7SK molecule, multimers of HEXIM1 and 2 and multiple P-TEFb complexes (Dulac et al., 2005; Li et al., 2005).

Association of P-TEFb with 7SK/HEXIM1 is specific and reversible. Inhibition of cellular transcription by chemical agents or ultraviolet irradiation triggers the disruption of the P-TEFb/7SK complex (Nguyen et al., 2001; Yang et al., 2001).
In contrast to its free form, the 7SK/HEXIM-associated fraction of P-TEFb shows little kinase activity, indicating that the 7SK snRNA, in collaboration with HEXIM1, functions as an inhibitory factor of P-TEFb. In HeLa cells, the overexpression of HEXIM1 inhibits the activity of a variety of reporter promoters whereas the siRNA-mediated knockdown of HEXIM1 expression results in transcriptional activation (Fraldi et al., 2005; Yik et al., 2003).
Likewise, the depletion of 7SK snRNA increases the CTD kinase activity of P-TEFb and stimulates transcription from RNAPII specific promoters, including the HIV-1 long terminal repeat (Nguyen et al., 2001; Yang et al., 2001). Thus, it is conceivable that certain cellular processes marked by an increase in global transcription are modulated by changes in the availability of free P-TEFb. An example of such a global process leading to an increase in the synthesis of total RNA is illustrated by cardiac muscle hypertrophy, which is due to increased cardiac workload or defective mechanical performance (Sano et al., 2002). Hypertrophic signals dissociate the 7SK snRNA from T-type cyclins/Cdk9 complexes, leading to an increase in Cdk9 activity that results in hyper-phosphorylation of the CTD of RNAPII (Sano et al., 2002).
Recently two groups have identified a new component of the P-TEFb active complex, the Brd4 protein. Brd4 is a mammalian bromodomain protein that binds to acetylated chromatin and interacts with P-TEFb (specifically with Cyclin T1) through its bromodomain (Jang et al., 2005). It interacts with active P-TEFb free of 7SK/HEXIM and stimulates its kinase and transcriptional activity (Jang et al., 2005; Yang et al., 2005). Moreover chromatin immunoprecipitation (ChIP) assays revealed that the recruitment of P-TEFb to promoters was dependent on Brd4, which contacts DNA through acetylated histones as well as through the Mediator complex (Jang et al., 2005; Yang et al., 2005).

P-TEFb and HIV-1 transcription

In mammalian cells, the role of P-TEFb has been initially highlighted by the association of Cyclin T1 with the human immunodeficiency virus type 1 (HIV-1) Tat transactivator (Mancebo et al., 1997; Wei et al., 1998; Zhu et al., 1997).

The HIV-1 long terminal repeat (LTR) promoter depends on P-TEFb activity more than normal cellular promoters, since activation of that promoter by the viral transactivator Tat is blocked by concentrations of P-TEFb inhibitors that do not affect regular cellular transcription (Chao et al., 2000; Chao and Price, 2001; Flores et al., 1999).

Tat is unique among transcriptional activators in eukaryotic cells in that it functions via RNA rather than DNA promoter elements. It binds the transactivation response element (TAR) that forms a stable RNA stem loop at the 5′ end of all viral transcripts. Thus, Tat requires minimally the transcription of TAR before it can stimulate HIV transcription from the long terminal repeat (LTR). Indeed, in the absence of Tat, RNAPII clears the HIV LTR successfully but soon arrests, yielding predominantly short viral transcripts (Kao et al., 1987). Tat binds the 5′ bulge in TAR via its arginine-rich motif from positions 49 to 57. However, this binding is not sufficient for Tat's function *in vivo*. The N-terminal core and cysteine-rich regions, which form the activation domain of the protein, lie adjacent to the arginine-rich motif. This activation domain binds Cyclin T1 (Wei et al., 1998). As a consequence, a tripartite complex is formed between Tat, Cyclin T1 and TAR at the 5’ end of each viral mRNA. The formation of the P-TEFb-TAR-Tat complex is an essential step towards the assembly of the processive RNAPII machinery at the LTR promoter (Bieniasz et al., 1998; Fujinaga et al., 1998; Garber et al., 1998; Zhou et al., 1998).

The assembly and disassembly of the complex between P-TEFb, Tat, and TAR is a regulated process *in vivo*. Whereas the phosphorylation of Cdk9 (Garber et al., 2000) and the P/CAF-mediated acetylation of the lysine 28 of Tat strengthens this complex, the p300-mediated acetylation of the lysine 50 of Tat weakens it (Kiernan et al., 1999).

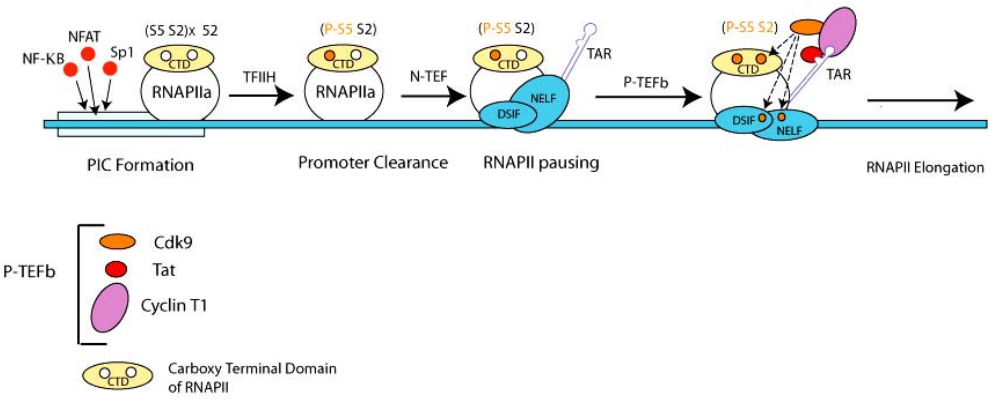

Figure 3. Early phases of HIV-1 transcription. Adapted from (Barboric and Peterlin, 2005).

Interestingly, the HEXIM1 protein binds the same region at the N-terminus of Cyclin T1 that is responsible for Tat binding (Michels et al., 2003). As a matter of fact, GST pull-down experiments and size exclusion chromatography reveal a mutually exclusive binding of the two effectors to Cyclin T1, suggesting a model where HIV-1 Tat competes with HEXIM1 for CyclinT1 binding (Schulte et al., 2005). Furthermore, the 7SK-binding motif in HEXIM1 contains clusters of positively charged residues reminiscent of the arginine-rich RNA-binding motif found in a wide variety of proteins. Part of it is highly homologous to the TAR RNA-binding motif of Tat. A similar RNA-protein recognition mechanism may regulate the formation of both the Tat-TAR-P-TEFb and the HEXIM1-7SK-P-TEFb ternary complexes, which may help convert the inactive HEXIM1/7SK-bound P-TEFb into an active complex for Tat-activated and TAR-dependent HIV-1 transcription (Michels et al., 2004; Yik et al., 2004). Therefore it has been speculated that the TAR RNA/Tat lentiviral system has evolved to subvert the cellular 7SK RNA /HEXIM1 system.

P-TEFb and transcription

Although Tat was the first activator known to recruit P-TEFb to initiating RNAPII, additional members of this group were soon identified. They include another viral

transactivator, HTLV Tax (Zhou et al., 2006), and mammalian activators such as the androgen receptor (Lee et al., 2001), c-Myc (Eberhardy and Farnham, 2001), the class II transactivator (CIITA) (Kanazawa et al., 2000), myoblast determination protein (MyoD) (Giacinti et al., 2006), and nuclear factor κB (NF-κB) (Barboric et al., 2001). These forms of recruitment of P-TEFb are specific and tailored to individual transcription units. Nevertheless P-TEFb involvement in transcription appears to be a more general feature. In fact, in *C. elegans*, genetic inactivation of Cdk9 or Cyclin T1 and Cyclin T2 resulted in the inhibition of all RNAPII transcription (Shim et al., 2002). Although no murine knockouts of subunits of P-TEFb have been reported, DRB and flavopiridol, two ATP analogs that inhibit the kinase activity of Cdk9, can inhibit nearly all transcription by RNAPII in human cells (Chao and Price, 2001). Therefore there should also be a general mechanism for bringing P-TEFb to RNAPII. The association of P-TEFb with the Brd4 protein, which associates with the Mediator and binds acetylated histones, (Jang et al., 2005; Yang et al., 2005), might provide one possible general tethering system.

In any case, these observations do not rule out the possibility that specific P-TEFb complexes could be rate-limiting for the expression of a subset of genes under certain cellular instances.

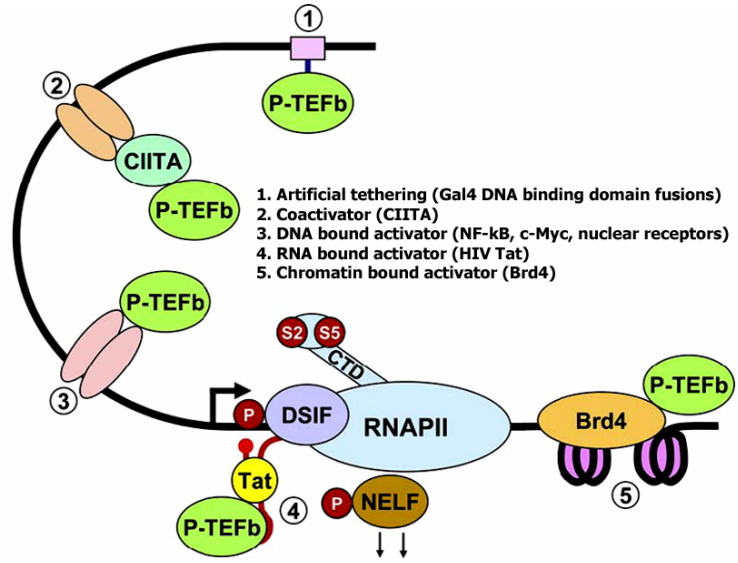

Figure 4. Mechanisms of recruitment of P-TEFb to promoters (Peterlin and Price, 2006).

P-TEFb and cellular differentiation

In contrast to Cdks with known cell cycle regulatory functions, the Cdk9-associated kinase activity is fairly constant throughout both the cell cycle and during cell cycle entry from a quiescent state (Garriga et al., 2003; Grana et al., 1994), but it changes during cell differentiation (De Falco et al., 2005; Foskett et al., 2001; Sano et al., 2002; Simone et al., 2002) and viral infection (Bark-Jones et al., 2005; Liou et al., 2004; Tamrakar et al., 2005; Zhou et al., 2000).

The discovery of Cdk9 as the catalytic subunit of the Tat-Associated Kinase (Herrmann and Rice, 1993; Herrmann and Rice, 1995), the activity of which appeared to be dramatically upregulated during T-cell activation (Yang et al., 1997), prompted the examination of the regulation of P-TEFb expression in cells that are target for HIV-1 infection.

Cyclin T1 protein levels have been shown to increase following activation of peripheral blood lymphocytes (PBLs) and the upregulation of Cyclin T1 to correlate with hyper-phosphorylation of RNAPII and increased HIV replication in these cells (Garriga et al., 1998; Ghose et al., 2001; Herrmann et al., 1998; Marshall et al., 2005).

$Cdk9^{55}$ is expressed at relatively high levels in resting lymphocytes and is not regulated by activation, while $Cdk9^{42}$ is expressed at low levels in resting lymphocytes and seems to be upregulated by activation (Liu and Herrmann, 2005)

In human monocytes and macrophages, Rice and colleagues have observed complex patterns of P-TEFb regulation. CyclinT1 mRNA levels are high but little protein expression can be observed in monocytes freshly isolated from healthy blood donors (Liou et al., 2002). When monocytes are instead cultured under conditions that induce macrophage differentiation, CyclinT1 protein expression is induced to high levels within one or two days. In contrast, Cdk9 protein levels are generally high in freshly isolated monocytes and are not strongly upregulated during differentiation. However, after approximately seven to ten days of macrophage differentiation in culture, CyclinT1 protein expression is shut-off by proteasome-mediated proteolysis (Liou et al., 2004). Macrophages activators such as lipopolysaccharide or other pathogen-associated molecular patterns (PAMPs) can reinduce expression of CyclinT1 after the shut-off, suggesting that induction of

CyclinT1 is a component of the innate immune response (Liou et al., 2004). Interestingly, HIV infection can also induce CyclinT1 expression in the late differentiated-macrophages (Liou et al., 2004). In this respect, however, it should be mentioned that these findings have been challenged by a study that reported no significative differences in CyclinT1 expression levels between unstimulated and stimulated primary lymphocytes (Martin-Serrano et al., 2002).

Besides cells that are target for HIV infection, there are other examples of differentiating cells in which P-TEFb activity appears to be regulated.

In the mouse, the kinase activity and protein expression of Cdk9 are highest in terminally differentiated tissues such as the muscle and brain (Bagella et al., 1998). In keeping with this notion, C2C12 cells induced to differentiate along muscle lineages peaked in Cdk9 kinase activity during differentiation. Overproduction of Cdk9 and of its associated cyclin (Cyclin T2a) stimulates myogenic differentiation in both MyoD-converted fibroblasts and C2C12 muscle cells. Conversely, inhibition of Cdk9 activity by a dominant negative form (Cdk9-dn) represses the myogenic program (Simone et al., 2002). Furthermore, Cdk9, Cyclin T2a and MyoD can be detected in a multimeric complex on promoters where Cdk9 strengthens MyoD-dependent transcription (Giacinti et al., 2006; Simone et al., 2002)

Cardiac Cdk9 kinase activity declines during normal cardiac maturation but is reactivated by hypertrophic signals. In these cases little or no change occurs in the levels of the kinase or of its activator, CyclinT. Instead, Cdk9 activation involves the dissociation of the inhibitor 7SK small nuclear RNA (Sano et al., 2002).

In contrast to this system, the Rice's group has observed that 7SK RNA and HEXIM1 protein expression, and the association of 7SK with P-TEFb, are induced upon activation of quiescent lymphocytes, parallel, however, to the increase of Cdk9 kinase activity (Haaland et al., 2003; Haaland et al., 2005). On the other hand, increased HEXIM1 expression has been shown to correlate with inhibition of P-TEFb activity during erythroleukemia cell differentiation (Turano et al., 2006).

The Cdk9/CyclinT1 complex seems to be also required for neuron differentiation induced by retinoic acid, since the expression level of the complex increases during differentiation. In addition, in samples of neuroblastoma and PNET (Primary Neuroectodermal Tumor), the levels of Cdk9 expression parallel the differentiation

state of the tumor (De Falco et al., 2005). HEXIM1 expression appears to be regulated during neuroblastoma cell differentiation as well (Turano et al., 2006).
Finally P-TEFb has been found to participate in the process of adipogenesis**.** Cdk9, as well as CyclinT1 and CyclinT2, show differences in nuclear localization at distinct stages of adipogenesis. Overexpression of Cdk9 increases the adipogenic potential of 3T3-L1 cells, whereas the inhibition of Cdk9 by specific Cdk inhibitors and by a dominant-negative Cdk9 mutant impairs adipogenesis. The positive effects of Cdk9 on the differentiation of 3T3-L1 cells are mediated by a direct interaction with, and by the phosphorylation of, peroxisome proliferator-activated receptor gamma (PPARgamma), which is the master regulator of this process, on the promoter of PPARgamma target genes. PPARgamma-Cdk9 interaction results in increased transcriptional activity of PPARgamma and therefore increased adipogenesis (Iankova et al., 2006).
Not only cellular differentiation but also viral infection can modify P-TEFb activity. HIV-1 infection induces CyclinT1 expression (Liou et al., 2004), while HCMV infection increases Cdk7 and Cdk9 protein levels and kinase activity (Tamrakar et al., 2005). Besides regulating the overall level of kinase activity, cell infection by different viruses may also change the substrate specificity of Cdk9: in presence of HIV Tat or EBV EBNA2, Cdk9 phosphorylates RNAPII CTD on both Ser2 and Ser5 (Bark-Jones et al., 2005; Zhou et al., 2000).

Cdk9 post-translational modifications

The stability and activity of P-TEFb are regulated by multiple mechanisms.
The maturation of active CyclinT1/Cdk9 complexes is mediated by a chaperone pathway that sequentially transfers newly synthesized Cdk9 from HSP70 to HSP90/Cdc37 and finally to Cyclin T1 (O'Keeffe et al., 2000). A factor in this pathway appears to be rate-limiting for the stabilization of Cdk9, since ectopic expression of an HA-tagged version of Cdk9 leads to downregulation of endogenous Cdk9 (Garriga et al., 2003; Garriga et al., 1996). This downregulation of endogenous Cdk9 when Cdk9 is ectopically overexpressed is due to an increased rate of Cdk9 protein turnover, both endogenous and exogenous (Garriga

et al., 2003; O'Keeffe et al., 2000), suggesting that saturation of the pathway mediating stabilization of Cdk9 induces the degradation of free Cdk9.

Kiernan et al. hypothesized that Cdk9 stability could be regulated by an ubiquitin-proteasome system. In fact, they found that Cdk9 is modified by ubiquitin in an unusual way: CyclinT1 recruits the ubiquitin ligase through its PEST domain, but then it is Cdk9 that is ubiquitinated and degraded by the proteasome (Kiernan et al., 2001). While subsequent work has questioned this conclusion (Garriga et al., 2003), it is commonly accepted that Cdk9 activity can be modulated by ubiquitination. As a matter of fact, Barboric et al. have demonstrated that ubiquitination of Cdk9 by Skp2 facilitates optimal Tat transactivation because it strengthens the formation of the ternary complex between P-TEFb, Tat and TAR (Barboric et al., 2005).

Another important post-translational modification of P-TEFb is phosphorylation. Cdk9 can be phosphorylated on both serine and threonine residues. In particular, like all the other Cdks, it must be phosphorylated on Thr 186 of the T-loop for the catalytic pocket to become correctly folded (Ramanathan et al., 1999). Other sites of Cdk9 phosphorylation lie within the C-terminus of the protein and can be modified by Cdk9 itself or by other protein kinases such as PKA (Garber et al., 2000).

Evidence has been provided that Cdk9 autophosphorylation represents an important step in binding its cyclin partner and forming a stable interaction with the HIV-1 Tat/TAR complex (Fong and Zhou, 2000; Garber et al., 2000). Cdk9 phosphorylation is regulated during HIV transcription: in fact TFIIH in the pre-initiation complex inhibits Cdk9 phosphorylation, whereas Cdk9 is phosphorylated and active during transcription elongation when TFIIH is absent from the transcription complex (Zhou et al., 2001).

Subcellular localization of P-TEFb

Transcriptional regulation in mammalian cells is a highly dynamic process, requiring temporal and spatial coordination of functional protein complex assembly (Carmo-Fonseca, 2002; Stein et al., 2000). In addition, transcription overlaps extensively with downstream processes of mRNA maturation (Hirose and Manley,

2000). For these reasons, several of the factors participating in these events are found localized in specific subnuclear compartments; among them, CyclinT1 and Cdk9 co-localize in the non-nucleolar nucleoplasm with an evident speckled pattern indicative of distribution within compartments (Herrmann and Mancini, 2001).

In particular, CyclinT1 is exclusively present in nuclear speckled structures while Cdk9, albeit mainly nuclear, can also be visualized in the cytoplasm where it is actively exported from the nucleus through a leptomycin B-sensitive pathway. Interestingly, enforced expression of CyclinT1 enhances nuclear localization of Cdk9 and this effect requires the catalytic activity of the kinase (Napolitano et al., 2002; Napolitano et al., 2003). Cdk9 localization also depends on the differentiation status of the cell: in fact, Cdk9 undergoes a change in subcellular localization from nucleus to cytoplasm during *in vitro*-induced myogenesis (MacLachlan et al., 1998).

The subnuclear foci in which CyclinT1 resides appear juxtaposed, although not exactly coincident, with nuclear speckles, while they co-localize with promyelocytic leukaemia (PML) bodies, either upon overexpression of PML or, at least for a subset of them, at the levels of expression of the endogenous proteins. Localization of CyclinT1 into PML nuclear bodies depends on the physical interaction between the two proteins. In fact, CyclinT1 specifically binds PML not only in *in vitro* binding and co-immunoprecipitations experiments, but also in fluorescence resonance energy transfer (FRET) experiments that (as explained in chapter 5) indicate a direct interaction between the two proteins. Accordingly, CyclinT1 deletion mutants unable to associate with PML are also less prone than the wild-type protein to form nuclear foci *in vivo*. Most notably, human CyclinT1 has a diffuse localization in the nucleoplasm of fibroblasts derived from PML-knockout mice (Marcello et al., 2003).

The overexpression of Tat, which specifically binds CyclinT1 in FRET experiments, determines the recruitment of CyclinT1 outside the Cyclin T1 bodies and promotes HIV-1 transcriptional activation (Marcello et al., 2001), whereas CyclinT1 or PML overexpression forces CyclinT1 in nuclear bodies and correlates with an inhibition of HIV transcription (Marcello et al., 2003).

Together, these observations favor a model by which nuclear bodies modulate the activity of the HIV promoter by coordinating the availability of several factors that act in concert and are transiently part of the same complex assembled onto the LTR, including P-TEFb, p300/CBP, RNAPII and PML itself. Forced expression of PML or of other factors regulated by nuclear bodies such as CyclinT1 or PML might shift this dynamic equilibrium toward the formation of larger bodies that do not participate in transcription. In contrast, the expression of Tat might recruit these factors outside nuclear bodies in a region of nucleoplasm favorable for transcription. This model, which is schematically depicted in Figure 5, implies that nuclear body proteins shuttle in and out of these domains (Boisvert et al., 2001; Phair and Misteli, 2000), and is in agreement with the notion that transcription itself might occur at the periphery of the nuclear bodies (Boisvert et al., 2000).

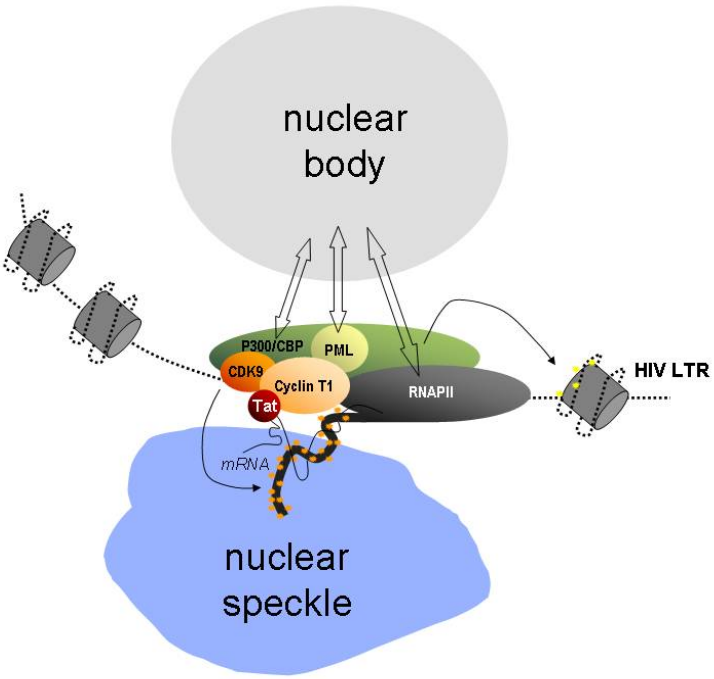

Figure 5. HIV-1 transcription and nuclear bodies.

PML bodies

The mammalian nucleus is a complex organelle organized into chromatin territories and discrete nuclear compartments or bodies. One of these is the promyelocytic leukemia (PML) body, also known as the PML oncogenic domain (POD), nuclear domain 10 (ND 10) or Kremer (Kr) body. There are approximately 5-30 bodies observed per nucleus, ranging in size from ~0.2 to 1 µm (Melnick and Licht, 1999). The major structural component of the PML bodies is the PML protein (Dyck et al., 1994; Koken et al., 1994; Weis et al., 1994).

The importance of PML bodies in cell differentiation and cell growth was first indicated in studies of promyelocytes from patients suffering from acute promyelocytic leukemia (APL). APL is a common form of acute myeloid leukemia (AML) that can be morphologically characterized by a distinct blockage of myeloid differentiation and accumulation of immature promyelocytes in patient bone marrow and peripheral blood (Warrell et al., 1993). In 99% of APL cases, a fusion of the PML protein and the retinoic acid receptor α (RARα) occurs as a result of the chromosomal translocation t(15:17) leading to the production of a PML-RARα chimeric protein (de The et al., 1991; Goddard et al., 1991; Kakizuka et al., 1991). Through its ability to heterodimerize with PML and RXR, PML-RARα interferes with both the PML and RAR/RXR-RA pathways, thus acting as a double dominant negative oncogenic product (Dyck et al., 1994; Kastner et al., 1992).

The role of PML in cell growth and tumorigenesis has been further characterized by overexpression and knock out of the PML protein. Stable overexpression of PML significantly reduced the growth rate of HeLa cells lengthening the G1 phase of cell cycle (Mu et al., 1997); in contrast, $PML^{-/-}$ MEFs grew faster than $PML^{+/+}$ MEFs (Wang et al., 1998). These findings demonstrate that PML can function *in vivo* as a negative growth regulator. Furthermore, $PML^{-/-}$ mice are highly susceptible to develop tumors in several *in vivo* models of physical or chemical induced carcinogenesis (Wang et al., 1998), thus indicating that PML can act as a tumor suppressor in vivo and that PML functional inactivation may be critical in APL leukemogenesis.

Notwithstanding these observations, the following considerations should also be taken into account in an assessment of PML nuclear bodies function. First, the expression of the PML gene is not required for viability, since $PML^{-/-}$ mice develop, in essence, normally (Wang et al., 1998). Second, the PML gene is not evolutionarily conserved among eukaryotes, being absent in *Drosophila melanogaster*, *Saccharomyces cerevisiae*, and *Arabidopsis thaliana* (Borden, 2002). Third, unlike other nuclear organelles, there appears to be no PML bodies in *Xenopus laevis*.

PML protein

The PML protein contains at its N-terminus several important functional domains which collectively form the RBCC or TRIM motif (Jensen et al., 2001). This motif is characterized by the presence of a zinc-binding domain that includes the RING finger motif followed by two additional zinc fingers (B-boxes) and an α-helical coiled-coil motif. The RING finger is a specialized type of zinc finger that is found in factors involved in transcription, tumor suppression and genomic stability and that may confer E3 ubiquitin-protein ligase activity (Jackson et al., 2000). RING and B-box motifs are thought to be involved in protein interactions and do not appear to bind nucleic acid directly (Borden, 2000). The helical coiled-coil region consists of eight heptad repeats (Kastner et al., 1992) and is responsible for multimerization of PML (Kastner et al., 1992; Le et al., 1996) as well as its heterodimerization with PML-RARα (Grignani et al., 1996; Perez et al., 1993).

Alternative splicing generates seven major isoforms of PML, which all share the N-terminal region motif but differ in their C-terminal portions and subcellular distribution (Jensen et al., 2001). The majority of PML protein forms nuclear bodies (Melnick and Licht, 1999); however, there is some PML located in cytoplasmic bodies and a soluble component in the nucleus (Flenghi et al., 1995; Melnick and Licht, 1999). A partial cytoplasmic localization is a feature shared even by the PML isoforms that are predominantly nuclear (Flenghi et al., 1995; Melnick and Licht, 1999).

Post translational modifications of PML regulate PML bodies formation. In particular, SUMO-1 modification of PML appears to regulate the dynamics of

protein localization within PML nuclear bodies (Eskiw et al., 2003; Maul et al., 2000; Zhong et al., 2000). For example, although SUMO-1 modification of the PML protein is not required for the formation of PML aggregates, the accumulation of many other PML nuclear body associated proteins within these structures appears to require the sumoylation of PML (Zhong et al., 2000).

Although the PML protein is the only essential protein for PML body formation (Dyck et al., 1994), many other proteins localize to these structures. Up to date over 77 proteins are known to localize to this compartment (see the Nuclear Protein Database http://npd.hgu.mrc.ac.uk/) (Dellaire et al., 2003), implicating these structures in virtually every nuclear activity, including transcription (reviewed by (Zhong et al., 2000)), DNA repair (reviewed by (Dellaire and Bazett-Jones, 2004)), apoptosis (reviewed by (Takahashi et al., 2004)), tumor suppression (reviewed by (Salomoni and Pandolfi, 2002)), proteolysis (Lallemand-Breitenbach et al., 2001), and the antiviral response (reviewed by (Everett, 2006; Regad and Chelbi-Alix, 2001)).

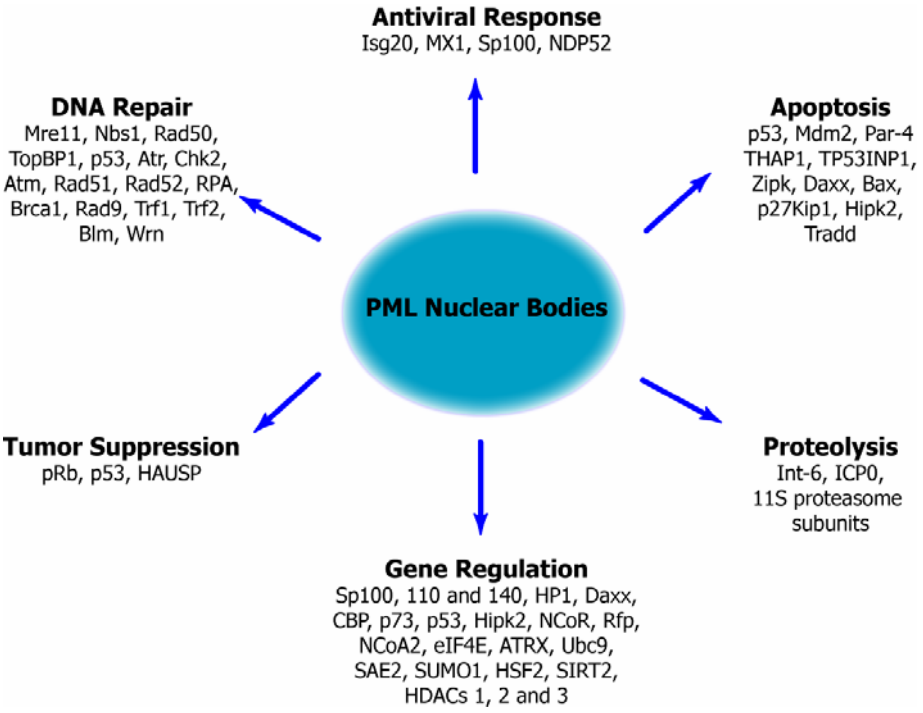

Figure 6. Functions of proteins that localize at PML NBs or associate with PML directly (Dellaire and Bazett-Jones, 2004).

PML bodies and transcription

PML has been touted as both a transcriptional activator and repressor. Although PML itself does not exhibit DNA binding activity, it can regulate transcription through interaction with various transcription factors/cofactors (reviewed in (Zhong et al., 2000)).

PML shows intrinsic repression activity when tethered to a promoter through fusion with the Gal4 DNA binding domain (Ahn et al., 1998; Vallian et al., 1997). Consistent with this repression activity, overexpression of PML led to inhibition of various promoters, including the MDR and EGF-receptor (EGF-R) promoters (Mu et al., 1994). In particular for the EGF-R promoter, the mechanism of repression was attributed to the PML-dependent disruption of the Sp1-DNA interaction (Vallian et al., 1998). PML was also reported to directly interact with the non-phosphorylated form of Rb and to abrogate the enhancing effect of Rb on glucocorticoid receptor-mediated transactivation (Alcalay et al., 1998). Moreover, PML has been shown to interact with histone deacetylases (Wu et al., 2001) and other co-repressors (c-Ski, N-CoR, and mSin3A) (Khan et al., 2001).

On the other hand, several lines of evidence suggest that PML can also act as a transcription co-activator. PML has been shown to upregulate the transcription of genes related to the major histocompatibility complex (Zheng et al., 1998), GATA-2 (Tsuzuki et al., 2000), AP-1 (Vallian et al., 1998). The positive effect of PML on transcription can be partially explained by the fact that it can recruit transcriptional co-activators such as CBP and p300 histone acetyltransferases (Doucas et al., 1999; LaMorte et al., 1998; von Mikecz et al., 2000). In particular for p53 mediated transcription, PML is required for the appropriate formation of certain p53 post-translational modifications (phosphorylation, acetylation, sumoylation) that potentiate its activity (Fogal et al., 2000; Guo et al., 2000; Pearson et al., 2000). At the same time, it can bind and inactivate the negative p53 regulator, Mdm2, thus protecting p53 from Mdm2-mediated degradation (Kurki et al., 2003; Louria-Hayon et al., 2003; Wei et al., 2003).

Recently, the contribution to transcription of the PML nuclear body itself (and not only of the PML protein) has been addressed by artificially tethering reporter plasmids to PML bodies (Block et al., 2006). In these conditions, the PML nuclear

body environment has been shown to modulate the expression of the reporter gene in a promoter-dependent manner (the SV40 promoter was repressed, the CMV promoter was activated and a minimal eukaryotic promoter was not affected).

PML bodies and chromatin

The structure of PML nuclear bodies has been extensively studied using both the light and electron microscope to examine their protein composition and ultrastructure (Boisvert et al., 2000; Eskiw et al., 2003; Grande et al., 1996; Ishov et al., 1999). The bodies are spherical or ring-shaped (Boisvert et al., 2000; Eskiw et al., 2003) and often appear as spheroid shells of proteins, with little protein mass at their core (Boisvert et al., 2000). Like many nuclear bodies, PML nuclear bodies reside in the interchromatin domain (ICD) space (Boisvert et al., 2000).

While LaMorte et al. reported the existence of nascent RNAPII transcripts within this compartment (LaMorte et al., 1998), subsequent analysis demonstrated that the core of PML nuclear body is a dense protein-based structure which does not contain detectable nucleic acid (Boisvert et al., 2000). Although neither chromatin nor RNA are found within the central core of these bodies, newly synthesized RNA is associated with their periphery (Boisvert et al., 2000) and both proteins and chromatin-like fibers appear to connect the bodies via multiple contacts to the surrounding chromatin (Eskiw et al., 2004; Eskiw et al., 2003). These contacts with chromatin at the periphery of the PML nuclear bodies seem to be partly responsible for the positional stability over long time intervals during interphase (Eskiw et al., 2004; Eskiw et al., 2003).

The integrity and stability of PML bodies are lost when chromatin is disrupted by stress, transcriptional repression or early apoptotic events. Under such conditions, PML bodies become mobile (Eskiw et al., 2004; Eskiw et al., 2003; Maul et al., 1995; Nefkens et al., 2003). Contacts between chromatin and PML bodies could have a more profound role than just the maintenance of their positional stability. One hypothesis is that the disruption of PML bodies might directly affect the availability of PML body-associated proteins within the nucleus leading to the release of these proteins. It is equally possible that the association of PML bodies

with chromatin also brings together specific gene loci regulated by PML body-associated proteins.

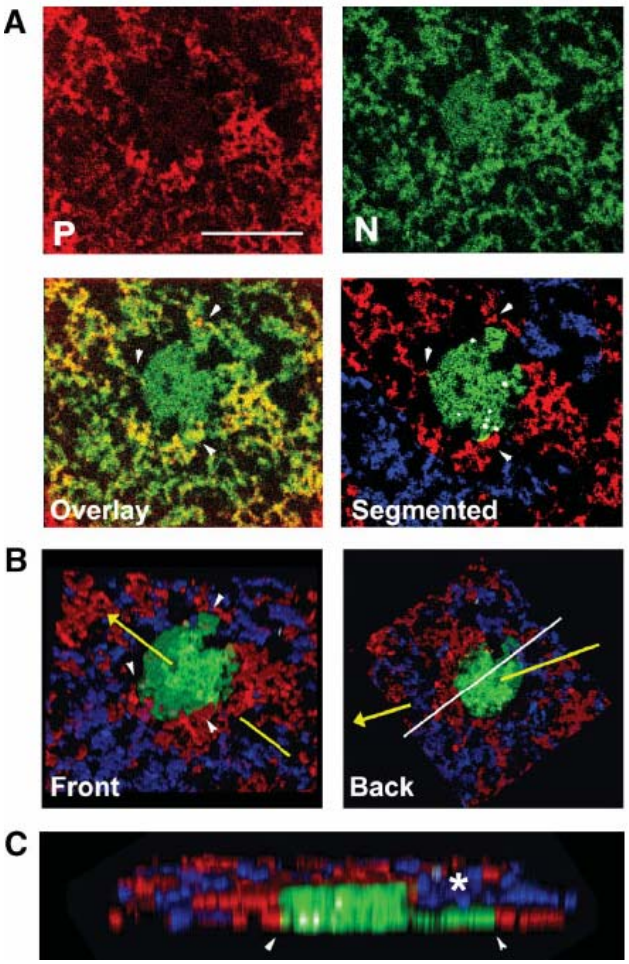

Figure 7. Ultrastructure of PML nuclear bodies (Dellaire and Bazett-Jones, 2004). (**A**) An electron micrograph of a PML NB is shown by electron spectroscopic imaging (ESI). Nucleic acid is visualized in the net phosphorous image (P) and both nucleic acid and protein in the net nitrogen image (N). Overlay of the two images produces an image where protein-rich structures, such as the PML NB, appear green and structures with intermediate protein and nitrogen content, such as chromatin and ribonucleoprotein (RNP) complexes, appear yellow. In the segmented panel, the ratio of nitrogen and phosphorous is used to segment the image into chromatin (red), RNP (blue) and the PML body (green). The PML body has a ring-like appearance with a hole at the centre of the PML NB. In addition, a finger-like projection from the top of the body can be seen. The PML NB is in contact with the surrounding chromatin at several locations (small arrows). (**B**) A three-dimensional volume representation of the PML NB in A. (**C**) A cross-section

through the three-dimensional volume of the PML NB, along the plane shown in the back panel of B (white line), is shown.

Many examples of the non-random association of PML nuclear bodies in mammalian cells with specific chromosomal loci or chromatin have been reported, which include *TP53* (Sun et al., 2003) and the MHC gene cluster (Shiels et al., 2001). Wang et al. have extended the analysis to multiple gene-rich and gene-poor regions on other chromosomes in order to determine whether there is a spatial organization of PML bodies relative to particular regions of the genome. These authors found that PML bodies associate with genomic regions of high transcriptional activity, which is a function of both the gene density and the proportion of genes that are active. Single genes that are highly expressed do not show significant associations with PML bodies alone. The organization of PML bodies in relation to transcriptionally active regions was further supported by the association of PML bodies with many genes on the active X chromosome compared with their homologues on the inactive X chromosome, and with replication-dependent histone genes on chromosome 6 in S-phase cells compared with those in G0/G1 phase cells (Wang et al., 2004).

PML bodies thus seem to associate with chromosomal regions of high transcriptional activity and/or gene density, and, in some cases, the association may be cell cycle-dependent. Yet, not all transcriptionally active regions of genome associate with PML bodies and PML bodies do not serve as obligate transcription sites for the associated genes that were tested, nor are basal transcription levels of these genes altered by knockdown of PML protein. Collectively, these observations suggest that the specificity of PML body association is determined by locus-specific factors rather then by the transcriptional level (Ching et al., 2005; Wang et al., 2004).

The genes brought into the vicinity of the PML body might then be jointly regulated through clustered co-activators such as CBP/p300, and co-repressors, such the histone deacetylases (HDACs). Such a role for PML bodies is reminiscent of that proposed for matrix attachment regions (MARs) in DNA (Cockerill and Garrard, 1986). Furthermore, Kiesslich et al. (Kiesslich et al., 2002) found that, in unsynchronized mammalian cells, approximately 30% of PML bodies were spatially

associated with transcription foci, marked by the presence of hyper-phosphorylated RNA polymerase II and fluorouridine-labeled newly synthesized RNAs; this percentage rose to 70% when cells were synchronized in G1.

These findings may suggest, for the PML bodies, a role analogous or coincident with the so-called "transcription factories". These factories are discrete sites within the nucleus where RNA transcription occurs; growing evidence indicates that when a gene becomes active, its DNA moves to one of those factories. Genes on different chromosome whose proteins work together travel long distances within the nucleus to meet up in the same factories (Pennisi, 2006).

On the other hand, several observations are also in sharp contrast with this conclusion. Knockdown experiments of PML bodies by RNA interference showed no effect on the expression of genes non-randomly associated with PML bodies (Wang et al., 2004). Experiments in the Ana Pombo laboratory have demonstrated by high-resolution imaging of ultrathin cryosections that PML bodies contain no detectable RNAPII or nascent RNA in HeLa cells, but are often surrounded by these markers at a distance >25 nm (Xie and Pombo, 2006). This observation supports the view that, although PML bodies are present in transcriptionally active areas of the nucleus, they are not themselves sites of polymerase assembly, transport or activity. One possible explanation for the difference between these results and those of Kiesslich et al. (Kiesslich et al., 2002) is that *in vivo* labeling of newly made RNA with fluorouridine allows for some transcripts to be completed and move away from the sites of synthesis towards PML bodies. Alternatively, the insufficient resolution provided by confocal microscopy of whole cells may give the false impression that PML bodies are transcriptionally active. Thus, the association of active genes with PML bodies, although nonrandom, can be explained by the coincidental positioning of PML bodies, active genes and polymerases in regions of the nucleus that are transcriptionally active (Wang et al., 2004). Proposed functions of PML bodies would agree with such preferential localization as they include storage of proteins, such as many transcription factors and chromatin modifiers, post-translational modification, like acetylation, phosphorylation and SUMOylation, and proteosomal-dependent degradation.

These results do not exclude the possibility that PML proteins that are freely available throughout the nucleoplasm (i.e. not in PML bodies) interact with RNAPII or regulate gene expression.

Not only human genes, but also viral genomes associate with PML bodies. The genomes and/or replication complexes of HSV-1 and adenovirus were preferentially located in close association with PML bodies (Ishov and Maul, 1996; Maul et al., 1996). This observation has been extended to include the papovaviruses SV40 and polyomavirus, and members of all subfamilies of the *herpesviridae* (for reviews see (Everett, 2001; Maul, 1998)). More recently, similar observations were made in cells infected with the parvovirus AAV (Fraefel et al., 2004).

PML bodies not only are often associated with viral genomes but also facilitate their transcription and replication (Ching et al., 2005), again supporting the hypothesis that these bodies are sites of gene activity. Regarding HIV-1, the group of G. Maul approached the localization of proviral HIV-1 DNA and transcribed HIV-1 mRNAs with respect to speckles and PML bodies in the nucleus of infected cells (Bell et al., 2001). To distinguish between unintegrated and integrated viral DNA, these investigators employed a technique called stress-induced chromosome condensation (SICC), which allows the visualization of the interchromatin space. Surprisingly, unintegrated HIV-1 DNA was found to accumulate within nuclear speckles, while no specific association of either unintegrated viral DNA, or transcription foci, or integrated proviral DNA could be detected with PML nuclear bodies. However, given the fact that these experiments were conducted with a FISH probe covering the whole HIV genome, it should be considered that multiply spliced subgenomic mRNAs might have escaped detection by this method. These considerations still leave the question of what are the sites in the nucleus at which HIV transcription occurs with respect to the known nuclear territories, largely unanswered.

Finally, it is of interest to note that, since PML bodies could also play a role in the mechanism of antiviral action of interferon, viruses have also evolved different ways to alter PML expression and/or localization (for review see (Everett, 2006; Regad and Chelbi-Alix, 2001)).

Function of PML bodies

All the above reported observations on the properties of PML bodies may collectively lead to three possible models to explain their actual function.

In the first model, the bodies are proposed to be aggregations of excess nucleoplasmic protein (Negorev and Maul, 2001): for example, they can sequester and inactivate co-activators, co-repressors or transcription factors (Li and Chen, 2000; Zhong et al., 2000). By titrating these factors from the active pool in the nucleoplasm, the PODs could interfere with transcription, resulting in either activation or repression.

In the second model, PML bodies are proposed to be sites of post-translational modification and degradation of proteins. Observations supporting this model include the acetylation (Pearson et al., 2000), phosphorylation (D'Orazi et al., 2002) and sumoylation (Fogal et al., 2000; Kwek et al., 2001) of p53 at PML bodies and the localization of ubiquitin/proteasome associated proteins at some PML bodies (Anton et al., 1999; Everett et al., 1997; Lafarga et al., 2002; Lallemand-Breitenbach et al., 2001).

In the third model, PML bodies are proposed to be sites of specific nuclear activities, such as transcriptional regulation and DNA replication. Evidence for this model includes the detection of nascent RNA around PML bodies (Boisvert et al., 2000), the association of PML bodies with regions of high transcriptional activity (Wang et al., 2004), and the non-random nature of PML body assembly (based on the conservation of their size and position) following dissociation and re-formation as a result of cellular stress (Eskiw et al., 2003).

These three models of PML body function are not mutually exclusive.

Acetylation

Post-translational modification represents an important mechanism for regulating protein function. Lysine acetylation, or the transfer of an acetyl group from acetyl coenzyme A to the ε-amino group of a lysine residue, was initially discovered on histone proteins about four decades ago (Vidali et al., 1968). Intensive research in the past decade has shown that this modification is quite common and plays important roles in regulating the functioning of eukaryotic, viral and bacterial proteins (Kouzarides, 2000; Sterner and Berger, 2000). Importantly, a protein module termed bromodomain has been shown to possess specific acetyllysine-recognizing ability (Zeng and Zhou, 2002).

Types of acetylated proteins	**Examples**
Core histones	H2A, H2B, H3, H4
Non-histone chromatin proteins	HMGB
HAT autoacetylation	PCAF, p300
Chromatin remodeler	Brm
Sequence specific transcription factors	p53, STAT3, c-MYC, MyoD, E2F, Rb, NF-kB
Transcriptional co-regulators	ACTR, CIITA, Rb
General transcription factors	TFIIE, TFIIF, PC4
DNA replication factors	MCM3, PCNA
DNA metabolic enzyme	Flap-endonuclease I, Werner DNA helicase
Chromatin cohesion proteins	Cohesin subunits, San
Cytoskeletal proteins	α-tubulin
Apoptosis regulator	Ku70
Nucleocytoplasmic trafficking protein	Importinα
Viral protein	Adenoviral E1A, HIV Tat, HIV Integrase
Bacterial protein	Alba, CheY

Table 1. Adapted from (Yang, 2004).

HATs

Lysine acetylation is a reversible post-translational modification that is governed by the opposing action of acetyltransferases and deacetylases (Grozinger and Schreiber, 2002; Yang, 2004).

Since 1995 when the first acetyltransferase was discovered in yeast (Kleff et al., 1995), dozen of proteins have been discovered to possess intrinsic lysine acetyltransferase activity. Although most of these enzymes were first identified as histone acetyltransferases (HAT) and then tested for activities towards other proteins, acetyltransferases only modifying non-histone proteins have also been identified. According to sequence similarity, lysine acetyltransferases can be organized into different groups.

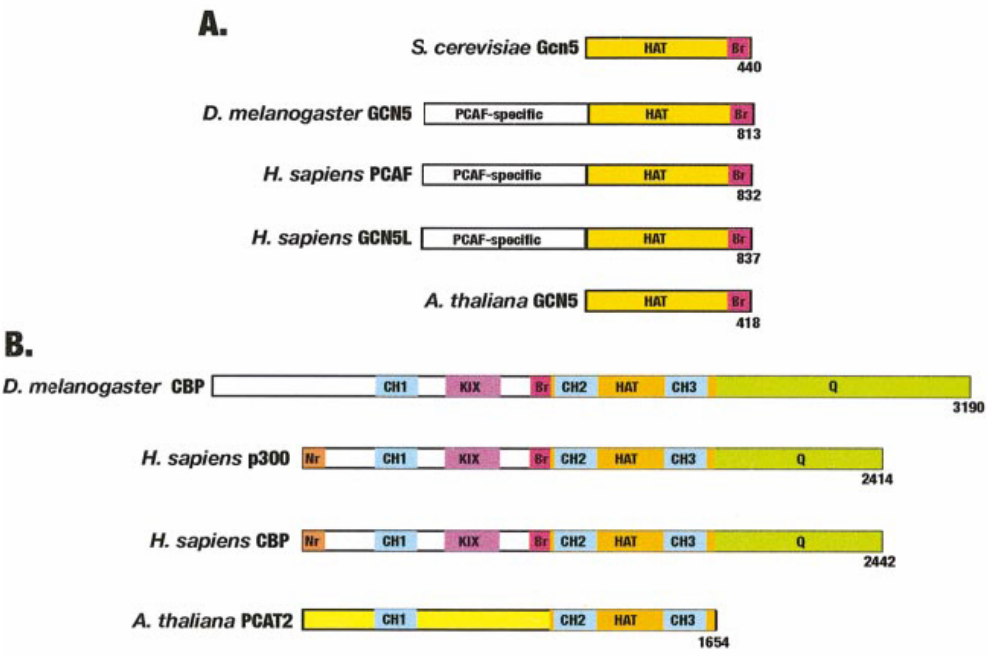

Figure 8. Schematic illustration of the Gcn5/PCAF (A) and p300/CBP (B) families of HATs (Yang, 2004).

The **Gcn5/PCAF** family is composed of GCN5, PCAF and related proteins. Yeast Gcn5 possesses a HAT domain and a bromodomain and is highly homologous to the C-terminal halves of human PCAF and GCN5L (mammalian GCN5 long form)

(Georgakopoulos and Thireos, 1992; Smith et al., 1998; Wang et al., 1997; Xu et al., 1998). Numerous studies indicate that these HATs function as histone acetylating transcriptional co-activators but also that they can acetylate non-histone proteins as well (Yang, 2004).

The **p300/CBP** family is another major group of nuclear HATs that has been extensively characterized (Chan and La Thangue, 2001; Goodman and Smolik, 2000). Like PCAF, both p300 and CBP are transcriptional co-activators able to acetylate histones and non-histone proteins. Reminiscent of PCAF and GCN5L, p300 and CBP form a pair of homologous HATs in mammals.

The **MYST** family of proteins constitutes a third major group of nuclear HATs. The acronym MYST is from its four founding members: human MOZ (monocytic leukemia zinc finger protein), yeast Ybf2, yeast Sas2 and the mammalian Tip60. Compared with the GCN5/PCAF and p300/CBP groups, the MYST family is larger, more diverse and not so well characterized. Despite their similar HAT domains, MYST proteins play different roles in various cellular processes (Yang, 2004).

In addition to these three major groups of HATs, more than a dozen other proteins have been shown to possess acetyltransferase activity.

Most HATs exist as stoichiometric multisubunit complexes *in vivo*. The complexes are typically more active than their respective catalytic subunits and display distinct substrate specificities, suggesting that associated subunits regulate the activities of the respective catalytic subunits. In addition, non-catalytic subunits are also involved in recruiting substrates for targeted action to ensure the specificity. Amazingly, one HAT can be the catalytic subunit of multiple complexes. GCN5L forms at least two distinct multisubunit complexes, and yeast GCN5 is the catalytic subunit of four complexes (reviewed in (Carrozza et al., 2003)).

Regulation of HAT activity

Multiple mechanisms are involved in the control of HAT activity (see (Yang, 2004) and references therein).

First, as an essential cofactor for different acetyltransferases, acetyl-CoA also stabilizes GCN5 and PCAF.

Second, as described above, formation of stoichiometric multisubunit complexes modulates the specific activities and substrate specificities of different HATs.
Third, the enzymatic activities of PCAF, p300 and CBP are regulated by interaction with transcription factors and viral proteins.
Fourth, HATs are subject to covalent modifications such as phosphorylation, acetylation, ubiquitination and sumoylation.
Fifth, HATs are degradated by caspases, calpains and ubiquitin-dependent proteasomes.
Sixth, subcellular compartmentalization is an important regulatory mechanism for HATs. For example, HAT1 binds to 14-3-3 proteins and TIP60 is sequestered to the cytoplasm in a signal-dependent manner.
Finally, while p300, CBP, MOZ and MORF possess PHD fingers, yeast Esa1 and Sas3 associate with PHD finger-containing subunits. PHD fingers are implicated in phosphoinositide binding and may thus provide structural modules for integrating nuclear lipid signals, so that activities of these acetyltransferases may be regulated by nuclear signaling events.

HDACs

Two families of deacetylating enzymes have been identified in eukaryotes: the histone deacetylases, or HDACs, and the Sir2 (silent information regulator-)-like family of NAD-dependent deacetylases, or sirtuins. Both families have been evolutionary conserved from prokaryotes to humans, and both consist of several different proteins with non-redundant cellular functions, many of which involve transcriptional regulation.
The **HDAC** family members can be divided into two classes based on their similarity to yeast histone deacetylase Rpd3 (class I) or Hda1 (class II) (Grozinger et al., 1999). Four class I (HDAC 1, 2, 3 and 8) and five class II (HDAC 4, 5, 6, 7, 9 and 10) HDACs have been identified and partially characterized in humans (Gray and Ekstrom, 2001; Guardiola and Yao, 2002; Zhou et al., 2001). A third group of HDACs has been formed after the discovery of HDAC 11, which contains conserved residues in the catalytic core shared by both class I and class II HDACs

but seems to have distinct physiological roles from those of the known HDACs (Gao et al., 2002).

HDAC1 and HDAC2 are the best characterized HDAC proteins and are generally found in stable, multicomponent complexes, which are recruited to their site of action by DNA binding proteins (Sin3, NuRD and CoREST complexes).

The group of **sirtuins** is related to the yeast transcriptional repressor Sir2, and its members can be divided into five classes based on their primary structure (Frye, 2000). Yeast Sir-2 is the best characterized sirtuin protein: it is found in two major complexes, one of which is responsible for silencing expression from the telomeric and HM loci, while the second one suppresses genetic recombination by forming a closed chromatin structure at the rDNA locus (Grozinger et al., 1999).

Out of seven human sirtuins, only two have been characterized so far, but all seem to have weak or inexistent histone deacetylating activity *in vivo*. Sirt1 regulates processes such as apoptosis and muscle differentiation by deacetylating key proteins (p53, FOXO, PCAF, MyoD) (Hisahara et al., 2005; Vaziri et al., 2001; Yang et al., 2005). Sirt2 deacetylates alpha-tubulin (North et al., 2003) and is involved in the control of mitotic exit in the cell cycle, probably via its role in the regulation of cytoskeleton.

Effects of acetylation on protein function

The consequences of acetylation on protein function vary from one protein to another depending on the primary function of the protein and on where acetylation takes place.

Acetylation has been reported to modulate DNA binding (Gu and Roeder, 1997; Marzio et al., 2000; Yao et al., 2001), protein-protein interactions (Bannister et al., 2000; Cohen et al., 2004; Kovacs et al., 2005; Yuan et al., 2005), protein stability (Bernassola et al., 2004; Jeong et al., 2002; Li et al., 2002) and subcellular localization (Blander et al., 2002; Kawaguchi et al., 2006; Kitamura et al., 2005; Thevenet et al., 2004).

Both `loss-of-function' and `gain-of-function' effects have been reported. Regarding the former, acetylation of the ε-amino group of a lysine residue neutralizes the positive charge, so the modification may affect interaction of the lysine residue with

DNA, RNA and proteins. Such a mechanism may operate with chromatin since the DNA backbone is negatively charged. Indeed, histone acetylation has been shown to affect the nucleosomal structure and the stability of nucleosomal arrays (Garcia-Ramirez et al., 1995). Alternatively, acetylation may render the ε-amino group unable to form hydrogen bonds. For example, Lys11 of Alba is involved in forming a hydrogen bond important for oligomerization, so acetylation of this residue inhibits oligomerization (Bell et al., 2002; Zhao et al., 2003). Acetyl-CoA synthetase from *S. enterica* provides another example of a `loss-of-function' effect (Starai et al., 2002). Lys609 of this enzyme is part of the catalytic center, so acetylation of this residue inhibits enzymatic activity. In eukaryotic cells, the ε-amino group of a lysine residue is also subject to methylation and modification by ubiquitin and ubiquitin-like proteins such as NEDD8 and SUMO. Different modifications are mutually exclusive, thus leading to their potential competition (Caron et al., 2005).

As for `gain-of-function' mechanisms, the addition of an acetyl group to a lysine residue creates a new surface for protein association. Reminiscent of domains that recognize phosphoproteins, bromodomains function as structural modules specific for acetyllysine-containing motifs (Zeng and Zhou, 2002). It has been demonstrated that several chromatin regulators use bromodomains to recognize acetyllysine. Bromodomains are found in many proteins and sequence variations may dictate their binding specificity. Some proteins contain multiple bromodomains, which may cooperate with each other to increase the affinity for binding partners with multiple acetylated lysine residues. Acetylation is also known to stimulate the association with proteins that do not contain bromodomains (Soutoglou et al., 2000).

Although the exact number and variety of proteins that are post-translationally lysine-acetylated in the cell is still unknown, it is clear that far more proteins are modified by this mechanism than initially appreciated. It has, thus been proposed that acetylation is a regulatory modification that rivals phosphorylation in spread (Kouzarides, 2000).

Dynamic acetylation of non-histone proteins has pleiotropic effects on cellular function (see Table 2). Given this wide array of possible outcomes, it is not possible to determine the effect of the acetylation status of any site without empirical testing.

Effect of acetylation	Examples
Increases DNA binding affinity	p53, SRY, STAT3, GATA1, GATA2, E2F1
Decreases DNA binding affinity	YY1, HMG-A1, HMG-N2, p65
Increases transcriptional activation	p53, HMG-A1, STAT3, AR, MyoD, E2F1, GATA1
Decreases transcriptional activation	ERα, HIF1α
Increases protein stability	P53, c-Myc, AR, E2F1, ERα, Smad7
Decreases protein stability	HIF1α
Promotes protein-protein interaction	STAT3, AR, EKLF, Importinα
Disrupt protein-protein interaction	NF-kB, Ku70, Hsp90

Table 2. Adapted from (Glozak et al., 2005)

Acetylation and transcription

The N-terminal tails of histones were the first acetyltransferase substrates to be discovered (Allfrey et al., 1964; DeLange et al., 1969; Vidali et al., 1968). Together with other modifications, such as methylation and ubiquitination, histone acetylation takes part in the determination of the histone code, a complex modification language that is fundamental to the regulation of chromatin structure and function (Strahl and Allis, 2000; Turner, 2000). Chromatin is typically repressive for nuclear processes like transcription (Horn and Peterson, 2002). Acetylation neutralizes the positive charge of the histone tails decreasing their affinity for DNA. As a consequence, histone acetylation loosens chromatin structure, an event that correlates with gene activation. Consistent with this notion, transcriptional co-activators and co-repressors have been found to possess intrinsic HAT and HDAC activities, respectively (Carrozza et al., 2003; Grozinger and Schreiber, 2002). Except for a few cases (De Nadal et al., 2004; Wang et al., 2002), DNA-binding transcriptional activators recruit HATs to execute gene-specific chromatin acetylation and transcriptional upregulation, whereas repressors enlist HDACs to deacetylate specific nucleosomes and inhibit transcription. Histone acetylation also

regulates other nuclear processes such as DNA replication, recombination and repair. Related to this, acetylation of histone H4 at Lys5 and Lys12 is required for deposition of newly synthesized core histones and chromatin assembly (Roth et al., 2001). In addition to histones, other chromatin proteins like high-mobility group (HMG) proteins are also modified by acetylation (Bergel et al., 2000; Sterner et al., 1979).

As far as the other proteins involved in transcription are concerned, lysine acetylation is known to occur in over 40 sequence-specific transcription factors, in transcriptional co-regulators, general transcription factors and also chromatin remodellers (see Table 1). In several cases, this modification potentiates transcription. However, acetylation of NF-kB RelA, HMGI(Y), C/EBPb, IRF2, IRF7, Era, AFX, ACTR, TAFI68 and Brm inhibits transcription, which may serve as a feedback mechanism to control the duration of transcription.

GCN5

Gcn5 (general control of nonderepressible-5) has been the first link between histone acetylation and gene activation: as a matter of fact, the *Tetrahymena* histone acetyltransferase A was found to be homolog of *S. cereviasie* Gcn5, previously identified as a transcriptional co-activator (Brownell et al., 1996). Homologs of Gcn5 have been cloned and sequenced from numerous divergent organisms suggesting that their function is highly conserved throughout the eukaryotes (Brownell et al., 1996; Candau and Berger, 1996; Smith et al., 1998; Xu et al., 1998).

To date, yeast Gcn5 is the best characterized of the HATs, both structurally and functionally and both *in vivo* and *in vitro*. Various studies have mapped and characterized the functional domains of this protein (Candau and Berger, 1996; Candau et al., 1997). These include a C-terminal bromodomain, an Ada2 interaction domain, and the HAT domain, which was found to be required for transcriptional activation *in vivo* (Candau et al., 1997). Alanine scan mutagenesis identified conserved residues critical to HAT activity and demonstrated the direct correlation of Gcn5 HAT function with cell growth, *in vivo* transcription, and

promoter histone acetylation *in vivo* (Kuo et al., 1998; Wang et al., 1998). In addition to affecting the final steady state level of expression of a gene, Gcn5 can also significantly increase the rate of chromatin remodeling and gene activation, as it does for the *PHO5* gene (Barbaric et al., 2001).

The substrate specificity of Gcn5 has also been investigated. *In vitro*, recombinant Gcn5 was found to acetylate histone H3 strongly and H4 weakly in a free histone mixture. Protein sequence analysis of these reaction products revealed that the primary sites of acetylation were Lys 14 on histone H3 and Lys 8 and 16 on histone H4 (Kuo et al., 1996). Although recombinant Gcn5 can acetylate free histones efficiently, it is unable to acetylate nucleosomal histones, the more physiological substrate, except under special conditions and at high enzyme concentrations. Only in the context of multisubunit native complexes such as SAGA and ADA (described later) Gcn5 is able to acetylate nucleosomes effectively, indicating that the influence of other proteins is required to confer this activity.

The human *PCAF* and *GCN5* cDNAs were both identified based on their sequence similarity to yeast *GCN5* (Candau and Berger, 1996; Yang et al., 1996). Two isoforms of the human GCN5 protein have been detected and are proposed to be the result of an alternative splicing event (Smith et al., 1998; Xu et al., 1998). The less abundant short form (GCN5S) is similar in length to yeast Gcn5p, whereas the predominantly expressed long form (GCN5L) contains an extended N-terminal domain akin to PCAF, mouse GCN5 and *Drosophila* GCN5 (Smith et al., 1998; Xu et al., 1998).

The function of human GCN5 has also been investigated *in vitro* and *in vivo*, and it was found to carry out transcriptional adaptor roles analogous to those of yeast Gcn5. Further studies showed that human GCN5 had acetyltransferase activity and that its HAT domain could successfully substitute for that of yeast Gcn5 *in vivo*, indicating the evolutionary conservation of this HAT function (Wang et al., 1997).

The HAT domain of human GCN5 is of course indispensable to its acetylation function, but interestingly, another domain appears to have an influence on its HAT activity and substrate use. While recombinant short-form human GCN5 could acetylate histone H3 (and to a lesser extent H4) only as free histones (Wang et al., 1997), the full-length forms of human and mouse GCN5 were shown to be

competent for the acetylation of nucleosomal histones, implicating the N-terminal region in chromatin substrate recognition (Xu et al., 1998).
GCN5 activity can be regulated by post-translational modifications. hGCN5 can be phosphorylated and inactivated by DNA-dependent protein kinase (Ku-DNA-PK) holoenzyme (Barlev et al., 1998); while yeast Gcn5 has recently been shown to be sumoylated (Sterner et al., 2006)

Yeast GCN5-containing complexes

As previously described, purified yeast Gcn5 is unable to acetylate nucleosomal histones under standard conditions *in vitro*. Because of this, a study was performed which sought to identify native yeast complexes capable of acetylating nucleosomal substrates. Two Gcn5-containing complexes were identified: SAGA and ADA (Grant et al., 1997).
Of the known HAT complexes, yeast SAGA (Spt-Ada-Gcn5) is the best characterized (reviewed by (Sterner and Berger, 2000)). This 1.8 MDa complex is composed of more than 15 subunits and acetylates lysine residues on the histone H3 and H2B N-terminal tails. It comprises products of distinct classes of genes: the transcriptional adaptors (Ada proteins), the TATA-binding protein related set of Spt proteins; a subset of TBP-associated factors (TAFs) and Tra1, the yeast homolog of the human transcription regulatory protein TRAPP.
Gcn5 is the catalytic HAT subunit of this complex (Grant et al., 1997) and is regulated by Ada2 and Ada3: Ada2 potentiates Gcn5 catalytic activity and interacts with acidic activators and Ada3 facilitates nucleosomal acetylation and an expanded lysine specificity (Balasubramanian et al., 2002; Grant et al., 1999; Sterner et al., 1999). It has been shown that SAGA also contains the de-ubiquitylation enzyme Ubp8 which controls the levels of histone H2B ubiquitylation (Daniel et al., 2004; Henry et al., 2003) that are related to transcriptional activity (Bernstein et al., 2002; Daniel et al., 2004; Henry et al., 2003).
The SAGA complex directly participates in the transcription process by being specifically recruited by activators to the promoter of many genes. For instance, activators such as Gal4 and Gcn4 interact directly with the SAGA complex *in vitro* (Brown et al., 2001; Drysdale et al., 1998) and *in vivo* (Bhaumik et al., 2004; Ikeda

et al., 1999; Sterner et al., 1999; Utley et al., 1998), and they recruit SAGA to target gene promoters *in vivo* (Kuo et al., 2000; Larschan and Winston, 2001), an event that can occur independently of transcription (Bhaumik and Green, 2001; Kuo et al., 2000; Topalidou and Thireos, 2003) and that precedes and is required for the recruitment of the RNAPII machinery to certain promoters (Bhaumik and Green, 2001; Bryant and Ptashne, 2003).

In some cases, SAGA recruitment seems to depend on the presence of other chromatin remodeling activities such as the SWI/SNF ATP-dependent nucleosome remodeling complex. The recruitment of one co-activator may stimulate the recruitment of another one, and this interdependence can be reflected in a sequential order of co-activators recruitment to the promoter. Such staged recruitment was shown for the *HO* gene where SWI/SNF recruitment is necessary for, and temporally precedes, recruitment of SAGA by the activator Swi5p to the URS2 element (Cosma et al., 1999). The requirement for SWI/SNF function as a prerequisite for SAGA recruitment by Swi5p at *HO* appears to be restricted to late mitosis and applies even to Gal4p- and Gcn4p-regulated promoters in this phase of the cell cycle, most likely reflecting a highly condensed state of promoter chromatin (Krebs et al., 2000). In fact, SAGA functions are required during interphase for wild-type steady-state recruitment of SWI/SNF by Gcn4p at the target genes *ARG1* and *SNZ1* (Yoon et al., 2003). Thus, the degree of interdependency among co-activators in recruitment can vary from one activator to the next, and even for the same activator depending on the chromatin structure of the promoter.

Once recruited, the Gcn5 HAT subunit of SAGA locally acetylates histones, an event that facilitates transcription by loosening the chromatin structure or by generating specific binding sites for the recruitment of transcription factors (Kuo et al., 1998).

SAGA can also serve an adaptor to recruit the TATA-binding protein (TBP) through its Spt3 subunit in a HAT-independent manner (Bhaumik and Green, 2001; Larschan and Winston, 2001). SAGA can thus substitute for TFIID at certain RNAPII genes. TFIID function seems to regulate the transcription of 90% of the yeast genome, whereas SAGA might have an important role in the transcription of

10% of the genes, most of which seem to be stress induced (Huisinga and Pugh, 2004).

Although the exact mechanism remains to be determined, comparison of the low resolution structures of SAGA and yeast TFIID reveals important similarities and differences, which can be relevant for their function (Timmers and Tora, 2005; Wu et al., 2004).

In addition to its targeted co-activator function, Gcn5 also acetylates histones genome-wide, a phenomenon affecting most nucleosomes in yeast (Waterborg, 2000) and referred to as "global acetylation" (reviewed in (Kurdistani and Grunstein, 2003)). This global activity results in a basal state of histone acetylation throughout the genome that varies among *loci* and over which targeted acetylation superimposes. While the importance of activator-targeted histone acetylation in transcriptional regulation has been extensively documented, the role of global acetylation remains largely elusive. A major complication in addressing this question stems from the ability of Gcn5 and other HATs to function in both a global and a targeted manner, thereby making it difficult to distinguish between the two activities.

Global acetylation seems to affect the activation process by facilitating PIC formation, either by increasing the affinity of one or more general transcription factors for the core promoter or by creating a more accessible chromatin environment. The effects of this mechanism depend on the strength of interaction used to recruit the transcription machinery and on the affinity of the latter for the core promoter (Imoberdorf et al., 2006).

The other known Gcn5-containing complex is ADA**,** which has a size of about 0.8 MDa. Like SAGA, the ADA complex acetylates nucleosomes primarily on histone H3 and H2B *in vitro* but at fewer residues (Grant et al., 1999). It contains Ada2 and Ada3 but none of the other known subunits of SAGA (Grant et al., 1997). Peptide analysis revealed a novel subunit unique to ADA, demonstrating that it is a distinct complex and not a subcomplex or artifactual fragment of SAGA. This subunit, named Ach1 (ADA histone-acetyltransferase component 1), is specifically required for the integrity of the ADA complex, as a knockout mutation disrupted the complex

(Eberharter et al., 1999). Although ADA does contain Gcn5 and two other adaptors, unlike SAGA it does not seem to participate directly in transcription.

Human GCN5-containing complexes

Human homologues of yeast Gcn5, PCAF and GCN5, were found in several multiprotein complexes, such as TBP-free TAF complex (TFTC) (Brand et al., 1999; Wieczorek et al., 1998), SPT3/TAF9/GCN5 acetyltransferase complex (STAGA) (Martinez et al., 1998) and PCAF/GCN5 complexes (Ogryzko et al., 1998). While still incompletely characterized, these multisubunit complexes show related, but not identical, compositions and presumably overlapping but also distinct functions. All contain Gcn5 or PCAF, Ada proteins, Spts, TAFs and TAF-like proteins, in addition to the Tra1 orthologue, TRAPP (transformation/transcription domain-associated protein) (Martinez, 2002). However, TFTC specifically contained TAF5 and TAF6, whose yeast homologues are also found in SAGA, whereas these subunits were not found in STAGA or in PCAF/GCN5 complexes (Carrozza et al., 2003). Recently ataxin-7, the protein that harbors polyglutamine expansion in Spinocerebellar ataxia type 7, was shown to be a TFTC/STAGA subunit (Helmlinger et al., 2004).

These complexes contain a GCN5 HAT activity and were shown to acetylate preferentially histone H3 in both free and nucleosomal contexts and to activate transcription on chromatin templates (Brand et al., 1999; Hardy et al., 2002; Martinez et al., 2001). Furthermore, TFTC has a TFIID-like function like yeast SAGA: it was shown to direct pre-initiation complex assembly in the absence TFIID on naked DNA templates (Wieczorek et al., 1998). In addiction TFTC and STAGA complexes can associate with UV-damaged DNA-binding proteins suggesting a role for the complexes also in DNA repair mechanisms (Brand et al., 2001; Martinez et al., 2001).

Chapter 2

Materials and Methods

MATERIALS AND METHODS

Plasmids and siRNAs

pcDNA3-Cdk9-his was kindly provided by D.H. Price (Iowa City). From this template we subcloned Cdk9 in different expression vectors: pcDNA3 (Invitrogen) for *in vitro* translation, pGEX-2T (Amersham) for recombinant protein purification, pFlag-CMV-2 (Sigma) and pEGFP-C1 (Clontech) for expression in eukaryotic cells.
pGEX-2T-Cdk9 deletion mutants were prepared by PCR amplifcation of Cdk9 with primers specific for the deleted versions. pGEX-2T and pFlag single and double mutants of Cdk9 were constructed using recombinant PCR starting from each original vector.
Gal4-Cdk9 was generously provided by L. Lania (Napoli) (Majello et al., 1999).
pG6(5'Pro) was obtained by B.M.Peterlin (San Francisco).
GST-CTD expressing bacteria were obtained from R. Young (Cambridge) (Hengartner et al., 1998).
pGEX-GCN5 was a kind gift of M. Benkirane (Montpellier).
pGEX-2T-GCN5 deletion mutants were obtained by PCR amplifcation of GCN5 with primers specific for the deleted versions.
pcDNA3-HA-GCN5 was prepared by subcloning of GCN5 in the pCDN3-HA vector.
pcDNA3-HA-GCN5 (Y260A/F261A) (Paulson et al., 2002) was constructed using recombinant PCR starting from original vector.
The pGEX-p300 HAT (aa 1195-1810) was a kind gift of E. Verdin (San Francisco).
pCMVβ-p300 has been previously described (Marzio et al., 1998; Marzio et al., 2000).
The pCMV p300DY-myc plasmid was kindly provided by T.P.Yao (Durham).
The pcDNA3-PML-IV plasmid was obtained by S. Minucci (Milan) while the pFlag-PML-IV by L. Banks (Trieste) (Guccione et al., 2004)

siRNAs against GCN5 (Dharmacon-SMARTpool selected, 5'-AACCAUGGAGCUGGUCAAUGA-3') and Luciferase (5'-

NNAUGAACGUGAAUUGCUCAA-3') (Palhan et al., 2005) were purchased from Dharmacon.

Cell culture and transfection

HEK 293T, U2OS and HeLa cells were maintained in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (1 mg/ml). Transfection were performed by the standard calcium phosphate co-precipitation procedure or by Polyfect or Effectene (Qiagen) according to the manufacturer's instructions.

Deacetylation inhibition was achieved by treatment of cells with trichostatin A (TSA) 200 ng/ml over night.

Recombinant proteins

Recombinant GST fusion proteins were produced and purified from BL21 bacteria transformed with the respective plasmids. Bacterial culture were growth in terrific broth + ampicillin and protein production was induced with IPTG 0.5 mM for 4 hours at 30 °C with OD_{600} between 0.6 and 0.8. Bacteria where then resuspended in lysis buffer (50 mM Tris-HCl pH 8, 100 mM NaCl, 5% glycerol, 2 mM dithiothreitol) and sonicated by 4 pulses of 30 sec each. Bacterial lysates were mixed with a 50% (vol/vol) slurry of glutathione cross-linked agarose beads and the GST-fusion proteins were allowed to bind the beads at 4 °C on a rotating wheel for 1 hour. The suspension was then loaded on an empty plastic column (Bio-Rad, Richmond, CA), letting the unbound proteins pass through, and the beads were washed in 400 beds volumes of lysis buffer. The fusions proteins were eluted in the elution buffer (100 mM Tris-HCl pH 8, 100 mM NaCl, 5% glycerol, 2 mM dithiothreitol) containing 20 mM free glutathione. The purity and integrity of the proteins were routinely checked by SDS-PAGE and Coomassie blue staining.

***In vitro* acetylation assay**

HAT assays were performed as previously reported with minor modifications (Marzio et al., 1998; Marzio et al., 2000). GST fusion proteins used as substrates were incubated with the GST-GCN5 or GST-p300 HAT and [^{14}C]-acetyl-CoA in

HAT buffer (50 mM Tris pH 7.5, 5% glycerol, 0.1 M EDTA, 50 mM KCl, and 2 mM sodium butyrate) in a final volume of 20 µl for 45 min at 30°C. Acetylated proteins were visualized by phosphoimaging (Cyclone) after separation by SDS-PAGE.

***In vitro* binding assays**

[^{35}S]-labelled Cdk9 proteins used for *in vitro* binding assays were produced by using the TNT Reticulocyte Lysate System (Promega) and the corresponding pcDNA3 vectors as templates.

Binding of GST-GCN5 and its truncated variants to [^{35}S]-Cdk9 was performed as previously described (Marzio et al., 1998; Marzio et al., 2000). Briefly, 1 µg of recombinant proteins immobilized on agarose beds, after pretreatment in a solution containing DNaseI 0.25U/µl and RNase 0.2 µg/µl to remove contaminant bacterial nucleic acids, were washed and resuspended in NETN buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 1mM EDTA, 0.5% NP-40, 1mM DTT, 1mM PMSF) supplemented with 0.2 mg/ml of ethidium bromide to inhibit aspecific interactions between residual DNA and proteins. *In vitro* translated [^{35}S]-labeled proteins were added and incubated at 4 °C on a rotating wheel. After 1 hour, bound proteins were washed 5 times with 1 ml of NETN buffer and then resolved by SDS-PAGE electrophoresis. Radioactive proteins were visualized by phosphoimaging (Cyclone).

Antibodies

For the production of a polyclonal, anti-acetylated Cdk9 antibody three rabbits were immunized with a 15-mer Cdk9 peptide (residues 39-53) acetylated at positions 44 and 48 after conjugation with keyhole limpet hemocyanin (Sigma). The IgG fraction was obtained from serum with ImmunoPure (A) IgG purification kit (Pierce). The antibody was tested by dot blot and western blot analysis.

The following antibodies were used: anti-acetylated lysine (Cell Signaling); anti Flag (M2 Sigma), either free or bound to agarose beads; anti HA (Y-11), anti Cyclin T1 (T-18) anti Cdk9 (C-20 and D-7), anti GCN5 (H-75), anti p300 (N-15), anti PML (PG-M3) all purchased from Santa Cruz; anti HEXIM1 kindly provided by O. Bensaude (ENS, Paris); anti Ac-Cdk9 (see above).

Immunoprecipitation and *in vivo* acetylation assay

For immunoprecipitation cell pellets were lysed 36 hours after transfection in a NHEN buffer (20 mM Hepes pH 7.5, 300 mM NaCl, 0.5% NP-40 20% glycerol, 1 mM EDTA) or RIPA buffer (50mM Tris-HCl pH 7.5, 150 mM NaCl, 1% TRITON X-100, 0.1% SDS, 0.5% deoxycolic acid) containing 10 mM of sodium butyrate (Sigma) and protease inhibitors (Roche). The protein concentration of the extracts was determined by the Bradford assay (BioRad). Specific antibodies were incubated overnight at 4°C with cell extracts (2 mg). Next, 30 µl of a 50% suspension of protein-A-trisacryl beads (Pierce) was added. After 2 hours of incubation the immuno-complexes were extensively washed in lysis buffer and then analyzed by western blotting.

Immunoprecipitation-kinase assay and FSBA labeling

For kinase assays phosphatase inhibitors were added to the NHEN lysis buffer (20 mM NaF, 1mM Na_3VO_4). Anti-FLAG M2 agarose beads were incubated overnight at 4°C with 500 µg of cell extract and then extensively washed with NHEN + 20 mM NaF. IP beads were then washed twice with kinase buffer (50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 20 mM NaF) and finally incubated in 20 µl of reaction buffer (kinase buffer + 10 µM ATP + 2.5 mM DTT) with 5 µCi of [^{32}P] ATP and GST-CTD beads (containing 1 µg of proteins) for 30 min at 30 °C. Reaction was stopped by adding SDS sample buffer and boiling. Phosphorylated proteins were visualized by phosphoimaging (Cyclone) after separation by SDS-PAGE.

For FSBA labeling Flag immunoprecipitation was conducted in the same way. After extensive washing with NHEN buffer, Flag-beads were incubated with FSBA 50 µM (alone or in presence of ATP 0.5 or 5 mM) in PBS+0.1% TritonX-100 for 15 min at 30 °C, then washed 4 times with PBS+0.5% TX-100. Proteins were resolved by SDS-PAGE and then subject to western blot with an anti-FSBA antibody kindly provided by M. Wymann (Basel) (Wymann et al., 1996).

All quantifications experiments were performed at least three times.

Transcription assays

U2OS cells were seeded in 6-well plates and transfected with Polyfect (Qiagen) with the indicated plasmids and siRNAs. In each transfection 200 ng of pEGFP-C1 (Clontech) were co-transfected as an internal control. After 48 hours cells were harvested and part of them analyzed by flow cytometry for transfection efficiency. The remaining part was resuspended in 200 µl of Tris-HCl 0.25M pH 8 and lysed by repeated freeze/thaw cycles. The protein concentration of the extracts was determined by the Bradford assay (BioRad) and then 2.5 µg of lysate were used to measure CAT expression with a CAT ELISA kit (Roche) according to the manufacturer's instructions.

The results were reported as percentage of CAT production with respect to the values obtained by transfecting only the reporter pG6(5'Pro) and the activator Gal4-Cdk9.

Each experiment was repeated at least three times.

Immunofuorescence

Following paraformaldehyde fixation, cells were washed with 100 mM glycine and permeabilized with 0.1% Triton X-100 for 5 min. Primary and secondary antibodies were incubated at 37°C for 1 h in a humidified chamber in phosphate-buffered saline with the addition of 1% bovine serum albumin and 0.1% Tween-20. Images were acquired using a TCS-SL Leica confocal microscopy.

Biochemical fractionation

Biochemical fractionation assays were conducted as described by Fogal et al. (Fogal et al., 2000). Briefly 36 hours post transfection cells were collected in PBS and nuclei were separated by lysis in buffer 1 (50 mM Tris-HCl pH 7.9, 10 mM KCl, 1 mM EDTA, 0.2% NP-40, 10% glycerol) and centrifugation at 6000 r.p.m. for 3 min at 4 °C. Pellets were washed in buffer 1 without detergent and lysed in buffer 2 (400 mM NaCl, 1% NP-40, 20% glycerol, 20 mM HEPES pH 7.9, 10 mM KCl, 1mM EDTA) for 20 min at 4 °C. The insoluble and soluble nuclear fractions were separated by centrifugation at 14000 r.p.m. for 10 min.

Chromatin immunoprecipitation

After treatment with TPA for the indicated time periods, cells were fixed by adding a fixing solution (11% formaldehyde, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA pH 8.0, 50 mM Tris–HCl pH 8.0) directly to the cell culture medium at 1% final dilution. Cross-linking was allowed to proceed for 10 min at 37°C and was stopped by the addition of glycine at a final concentration of 0.125 M. Fixed cells were washed once in ice-cold PBS, once in buffer B1 (0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, 10 mM Tris–HCl pH 8.0) and once in buffer B2 (1 mM EDTA, 0.5 mM EGTA, 200 mM NaCl, 10 mM Tris–HCl pH 8.0). Cells were pelletted by centrifugation and resuspended in RIPA 50 buffer (50 mM NaCl, 20 mM Tris–HCl pH 7.4, 1 mM EDTA, 0.5% NP-40, 0.5% deoxycholic acid, 0.1% SDS, protease inhibitors). Chromatin was sheared by sonication (20 pulses, 30 s each) on ice and centrifuged to pellet debris. Immunoprecipitations were carried out using the antibodies indicated (4 µl of each antibody) [Pol II (H224) SC-9001; CDK9(H-169) SC-8338 (all from Santa Cruz); Pol II phosphorylated at Ser 2 in CTD (H5) from Covance; Ac-CDK9 previously described] at 4°C overnight. Immune complexes were collected with protein A–Sepharose CL-4B (Pharmacia), and beads were washed three times with 1 ml RIPA 150 buffer (same as RIPA 50 but with 150 mM NaCl). Protein–DNA complexes were resuspended in 200 µl of TE buffer and digested with 5 U of DNase-free RNase (Roche) for 30 min at 37°C. The samples were treated for 3 h at 56°C with 300 µg/ml Proteinase K (Sigma) in 0.5% SDS, 100 mM NaCl, and for 6 h at 65°C to revert crosslinks. DNA was extracted with phenol/chloroform/isoamyl alcohol, ethanol precipitated and resuspended in water for PCR quantification.

Diluted concentrations of the input (1/10 and 1/100) were used as standards for each primer and TaqMan probe set. Real Time PCR amplifications were performed on an AbiPrism 7000 machine, using the TaqMan technology (Applied Biosystems) with ChIP products standardized for primer efficiency. Fold enrichments (occupancy) for each of the antibodies were calculated as a percent of the input material.

Details of primers used in ChIP analysis:

Primer Name		Sequence	Position
PPR1	For	GCCTCCTAGCATTTCGTCAC	271
PPR1	Rev	CTCGATGTCAGCAGTTCTTTGTAGT	298
PPR1	Probe	AGAGCTGCATCCGGA	316
NUC1A	For	GCTAGCTAGGGAACCCACTGCTTA	497
NUC1A	Rev	CTACAAGTAGTGTGTGCCCGTCT	556
NUC1A	Probe	CCTCAATAAAGCTTGCC	532
U1A	For	ACATCAAGCAGCCATGCAAAA	1368
U1A	Rev	CAGAATGGGATAGATTGCATCCA	1418
U1A	Probe	AAGAGACCATCAATGAGGAA	1394
U1B	For	TCAGAAGCAGGACCCGATAGA	2208
U1B	Rev	TCACTCTTTGGCAGCGACC	2250
U1B	Probe	ACTGTATCCTTTAGCTTCCCT	2225
U1C	For	ATGAAGGGTGCCCACACTAATG	3618
U1C	Rev	CAAAAAATAGCCACAGAAAGCATAGTAA	3667
U1C	Probe	TGAAACAATTAACAGAGGCAGT	3643

Chapter 3

Results

RESULTS

Cdk9 interacts with and is acetylated by GCN5

Cdk9 is acetylated by GCN5 acetyltransferase

It has been shown that Cdk9 is regulated by post-translational modifications such as phosphorylation (Fong and Zhou, 2000; Garber et al., 2000; Zhou et al., 2001) and ubiquitination (Barboric et al., 2005; Garriga et al., 2003; Kiernan et al., 2001). Because a growing number of nonhistone regulatory proteins are covalently modified by acetylation (reviewed in (Glozak et al., 2005)) we decided to investigate whether Cdk9 is also acetylated.

First we wondered whether Cdk9 was acetylated *in vivo*. To this end we immunoprecipitated Flag-Cdk9 or Flag-Luciferase (as a negative control) from 293T transfected cells and probed the immunoprecipitated protein with an antibody that specifically recognizes acetylated lysines (Figure 9A left panel). This experiment revealed that Flag-Cdk9, but not Flag-Luciferase, was acetylated *in vivo* by endogenous acetyltransferases even if the two proteins were equally expressed. To confirm this result we proved that treatment of cells with trichostatin A (TSA), a potent inhibitor of different HDAC activities (Yoshida et al., 1995), increased the Flag-Cdk9 acetylation signal (Figure 9B).

In an attempt to identify which histone acetyltransferase (HAT) is responsible for Cdk9 acetylation, we tested the activity of the GCN5 and p300 enzymes, chosen as prototypes of the two best characterized families of HATs which are involved in transcriptional co-activation (Yang, 2004). We repeated the *in vivo* acetylation assay by transfecting 293T cells with Flag-Cdk9 and either p300 or HA-GCN5 wild type or catalytically inactive mutants. Expression of wild type GCN5, but not of the inactive mutant (Y260A/F261A) was found to remarkably increase Cdk9 acetylation, while p300 had only a modest effect, which was lost completely by using its catalytically inactive variant (Figure 9C). Western blot analysis with the anti-Flag antibody revealed that the same amount of proteins were expressed and immunoprecipitated even in case of HATs co-transfection. Note that the basal level of Cdk9 acetylation shown in Figure 9A and B was not appreciable in these blots

since their exposure time was significantly shorter, in order to visualize the high level of Cdk9 acetylation that follows GCN5 transfection.

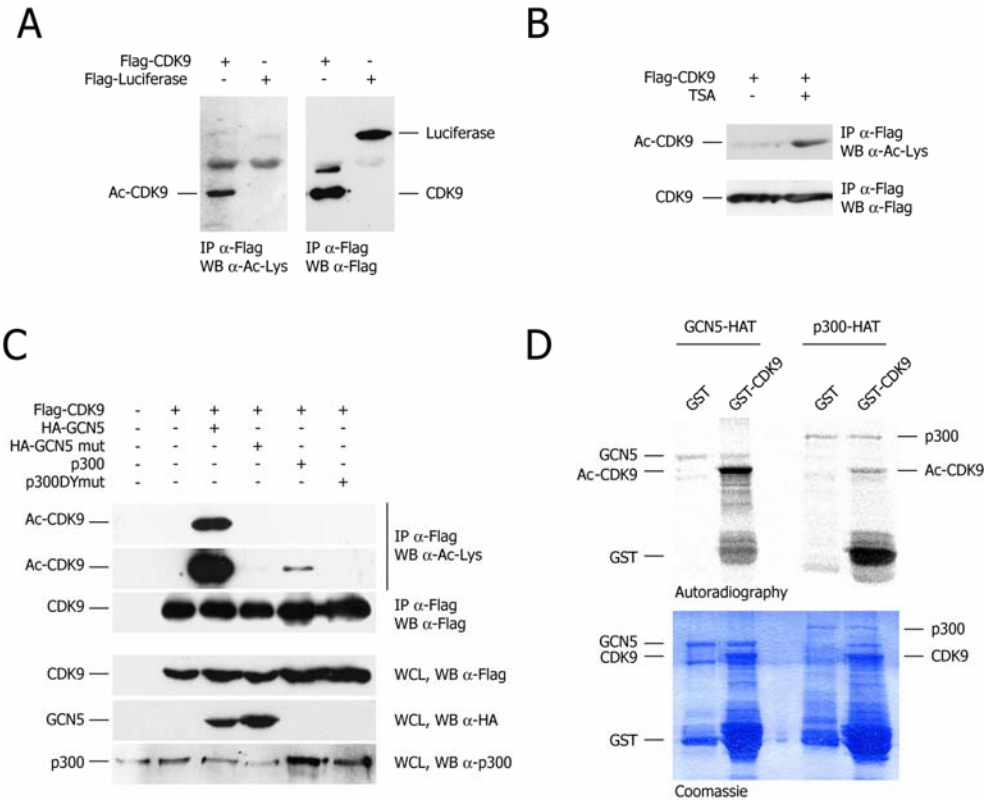

Figure 9. Cdk9 is acetylated by GCN5 *in vivo* and *in vitro*. (**A** and **B**) *In vivo* CDK9 acetylation. Extracts from HEK 293T cells transfected or treated as indicated on top of each lane were immunoprecipitated with an anti-Flag antibody and then immunoblotted with an anti-Ac-Lys antibody. The same filters were then incubated with an anti-Flag antibody. (**C**) Same as in (A) and (B) for the upper three panels. The bottom three panels show transfected protein levels. WCL: whole cell lysate (**D**) *In vitro* CDK9 acetylation. Recombinant purified GST-CDK9 was incubated with GST-GCN5 or GST-p300 HAT in the presence of radioactive labeled [^{14}C]-acetyl-CoA and resolved by SDS-PAGE followed by autoradiography (upper panel). Lower panel shows Coomassie blue staining of the same gel.

These findings suggested that GCN5 was the major responsible for the observed acetylation of Cdk9. To further confirm this finding, we performed an *in vitro* acetylation assay. Recombinant full length GST-Cdk9 was incubated with recombinant GST-p300 HAT or recombinant GST-GCN5 in the presence of

radioactive labeled acetyl-CoA and resolved by SDS-PAGE followed by autoradiography (Figure 9D). While the GST protein, used as a negative control, gave no autoradiographic signal, GST-Cdk9 gave an acetylation signal with both acetyltransferases, but stronger with GCN5. In addition to Cdk9, p300 and GCN5 were also positive for acetylation due to the autocatalytic activity of the enzymes (Creaven et al., 1999; Thompson et al., 2004), thus demonstrating that both HAT proteins were enzymatically active.

This experiment confirmed that GCN5 is a specific acetyltransferase for Cdk9 both *in vivo* and *in vitro*.

Cdk9 interacts with GCN5

Unlike classical enzyme substrates, most known acetylated proteins associate with their respective acetyltransferases, thus we decided to explore whether GCN5 binds Cdk9 and which are the regions that mediate this interaction.

For this experiment we performed immunoprecipitation of GCN5 from HeLa total and nuclear extracts and analyzed bound proteins by Western blot using a specific anti-Cdk9 antibody. Cdk9 could be detected together with GCN5 in the bound fraction (Figure 10A).

To confirm this interaction, a Flag tagged Cdk9 was expressed in 293T cells together with HA-GCN5 wild type or mutated in the catalytic domain (Y260A/F261A), followed by immunoprecipitation using an anti-Flag antibody and immunoblotting with an anti-HA antibody. As shown in the upper panel of Figure 10B, HA-GCN5 wild type and mutated specifically co-precipitated with Flag-Cdk9, while they did not with the pFlag empty vector. In a consistent manner, Flag-Cdk9 was found to co-immunoprecipitate with HA-GCN5 when the immunoprecipitation was performed with HA antibodies. It is interesting to note that both GCN5 wild type and the catalytically inactive mutant (Y260A/F261A) were able to co-immunoprecipitate with Cdk9 suggesting that the interaction between the two proteins did not depend on the HAT activity of GCN5. The same extracts were run on an SDS-PAGE gel and immunoblotted with anti-Flag and anti-HA antibodies to verify protein expression levels.

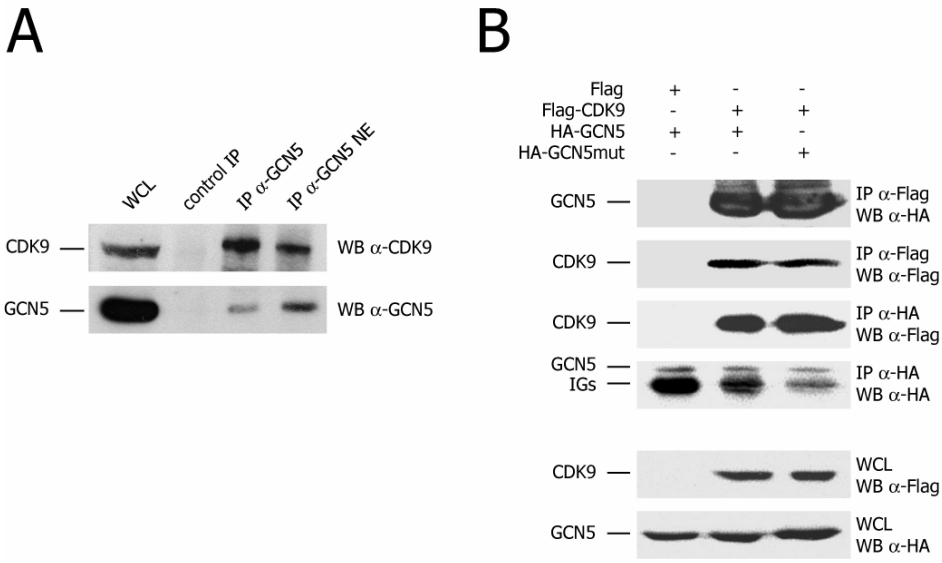

Figure 10. Cdk9 binds GCN5 *in vivo*. (**A**) HeLa whole cell lysate and nuclear extract (NE) were subject to immunoprecipitation with an anti-GCN5 antibody and then analyzed by western blotting using a specific anti-CDK9 antibody. CDK9 could be detected together with GCN5 in the bound fraction. (**B**) Extracts prepared from cells co-transfected with Flag-CDK9 and wt HA-GCN5 or HA-GCN5(Y260A/F261A) were immunoprecipitated with an anti-Flag antibody and immunoblotted with anti-HA antibody and vice versa (upper four panels). The same extracts were run on an SDS-PAGE gel and immunoblotted with anti-Flag and anti-HA antibodies to verify protein expression levels (lower two panels).

Having established that GCN5 and Cdk9 interact *in vivo*, we set out to dissect the regions that mediate this interaction.

To map the Cdk9 interacting domain in GCN5, the latter was divided into four fragments which were subsequently cloned and expressed as GST fusion proteins. Therefore we produced a GST-GCN5 full length (aa 1-476), and four deletions mutant that we called N-terminal domain (aa 1-189), HAT domain (aa 190-270), Intermediate domain (aa 270-383) and Bromo domain (aa 384-476); Figure 11A.

GST pull-down experiments with these fragments were performed using *in vitro* translated [^{35}S]-labeled Cdk9. GST-GCN5 fragments immobilized on beads were incubated with [^{35}S]-Cdk9; the beads were then extensively washed and the proteins resolved by SDS-PAGE.

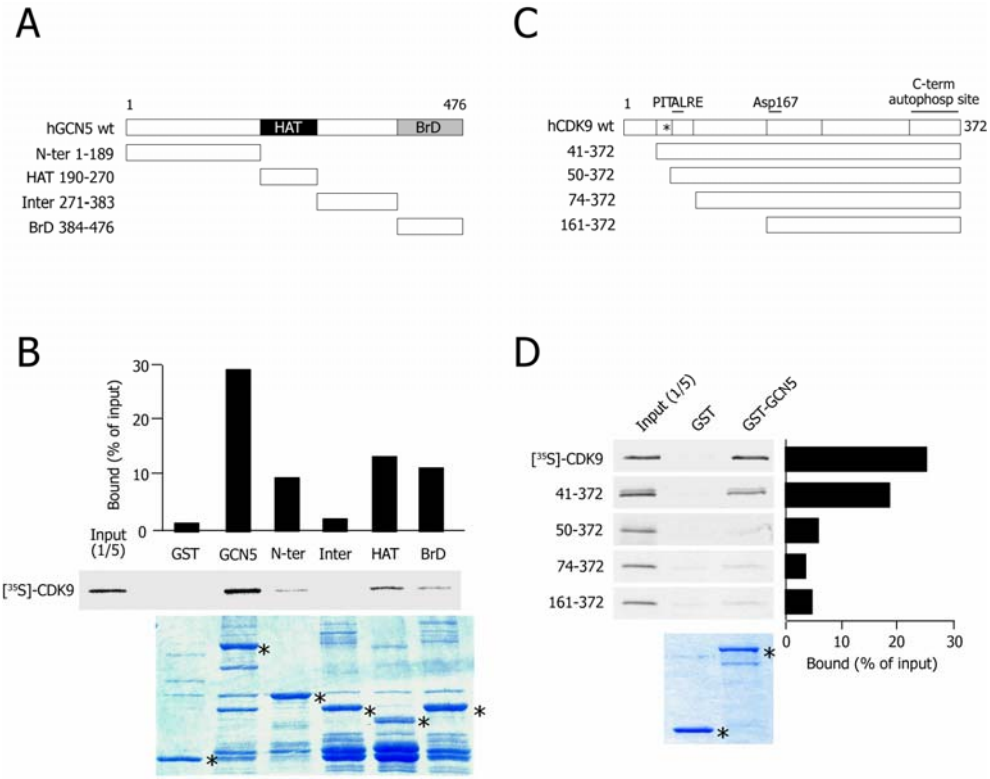

Figure 11. The N-terminus of Cdk9 binds GCN5 Bromo and HAT domains *in vitro*. (**A**) CDK9 binds the bromo- and HAT-domains of GCN5 *in vitro*. Schematic representation of the GST-GCN5 fragments used for pull down assays. (**B**) GST-GCN5 fragments were incubated with [35S]-CDK9, extensively washed and then analyzed by SDS-PAGE. (**C**) GCN5 binds the N-terminus of CDK9 *in vitro*. Schematic representation of the [35S]-CDK9 deletion mutants used for pull down assays. The position of the lysines that are positive for acetylation is indicated by an asterisk. (**D**) GST-GCN5 or GST alone as a control were incubated with [^{35}S]-CDK9 deletion mutant proteins, extensively washed and then analyzed by SDS-PAGE. The upper panels in (B) and (D) show the gels exposed to a phosphoimager screen, while the lower panels the Coomassie stainings of the same gels. The bands corresponding to the relevant intact GST proteins are indicated by asterisks. The graphs show the amounts of bound proteins as percentages of radiolabeled input.

Figure 11B shows the gel stained with Coomassie blue (upper panel) and after autoradiography (lower panel). Labeled Cdk9 was found to bind to GST-GCN5 full length, but not to GST alone. Among the four GCN5 fragments, Cdk9 bound stronger the Bromo and HAT domain, while the binding with the others was

comparable to background signal. Thus the region of GCN5 responsible for the interaction with Cdk9 includes the Bromo and HAT domains. This is not surprising since the bromodomain has a specific affinity for acetyllysine containing motifs (Dhalluin et al., 1999; Hudson et al., 2000; Owen et al., 2000) and the HAT domain is the catalytic domain that has to contact the substrate in order to transfer the acetyl group.

To map the GCN5 interacting domain on Cdk9, a series of [^{35}S]-labeled Cdk9 N-terminal deletion mutants were produced and tested for GST-GCN5 binding. Based on the homology between Cdk9 and the best characterized kinase, Cdk2 (Grana et al., 1994), we produced four Cdk9 mutants. Cdk9 (41-372) lacks subdomain I containing the glycine-rich loop of the ATP-binding site; Cdk9 (50-372) includes half of the subdomain II but lacks the lysine 48 that is homolog to the conserved Cdk2 K33 needed for maximum enzyme function (De Bondt et al., 1993); Cdk9 (74-372) includes domains IV-XI and lacks the PITALRE region that is responsible for the interaction with the cyclin partner; Cdk9 (161-372) contains domains VII-XI that include the T-loop which is the activation region of the molecule (Figure 11C).

As shown in Figure 11D, only wild type Cdk9 and Cdk9 (41-372) were able to bind GCN5 above background level. These results suggest that Cdk9 can specifically interact with GCN5 through the region comprised between its amino acids 41 and 50.

Given these data, we concluded that the N-terminus of Cdk9 interacts with the Bromo and HAT domains of GCN5.

Acetylation modifies crucial residues of Cdk9

Cdk9 is acetylated at lysine 44 and 48 by GCN5

In order to determine which portion of Cdk9 was acetylated by GCN5 we produced N- and C-terminal Cdk9 deletion mutants fused to GST, as schematized in Figure 12A, and then analyzed them by the *in vitro* HAT assay.

As shown in Figure 12B lanes 3 and 4, the deletion of the first 41 aa of the protein had no effect on the acetylation levels. In contrast, when the deletion was extended

to include the first 74 aa (thus also removing the PITALRE region), acetylation of the protein became undetectable.

Between amino acid 41 and 74 there are two groups of lysines: the first one between aa 41-50 (K44, 48 and 49) and the second one in between aa 50-74 (K56 and 68). Therefore we tested a new GST-Cdk9 deletion mutant spanning amino acids 50-372 that only contained the second group of lysines and that was found to be negative for acetylation (Figure 12B lane 11). Thus the region that is target for GCN5 acetylation lies between amino acids 40 and 50. Noteworthy, this is the same region that was found to interact with GCN5.

Since this region contains three lysines at positions 44, 48 and 49, they were changed, either one at a time or in combination, into arginines (this amino acid is characterized by the same positive charge as lysine, but cannot be modified by acetylation).

None of the single mutants was negative for acetylation (Figure 12B lanes 12,13,14), while the double mutant K44,48R was negative (lane 15). From this set of experiments, we concluded that the lysines at positions 44 and 48 are substrates for GCN5-mediated acetylation.

To confirm that Cdk9 is acetylated by GCN5 at positions 44 and 48 also *in vivo* we cloned Cdk9 K44,48R in a Flag-tagged expression vector. Using this mutant and a wild type Cdk9 expression vector, we performed an *in vivo* acetylation assay. Upon GCN5 overexpression, Flag-Cdk9 was found to be markedly acetylated, while the mutant Flag-Cdk9 K44,48R was negative for acetylation even if the two Flag proteins were expressed and immunoprecipitated to the same extent (Figure 12C).

To exclude the possibility that the substitution of Lys 44 and Lys 48 (that lie in the region of Cdk9 that interacts with GCN5) might disrupt the interaction between Cdk9 and GCN5, we also immunoblotted the same filter with a GCN5 antibody and found that both Flag-Cdk9 and Flag-Cdk9 K44,48R were able to co-immunoprecipitate GCN5 at the same efficacy (Figure 12C, bottom panel). This result implies that loss of acetylation in the K44,48R mutant does not result from its inability to associate with GCN5, but from the fact that K44 and 48 are authentic targets for GCN5-mediated acetylation.

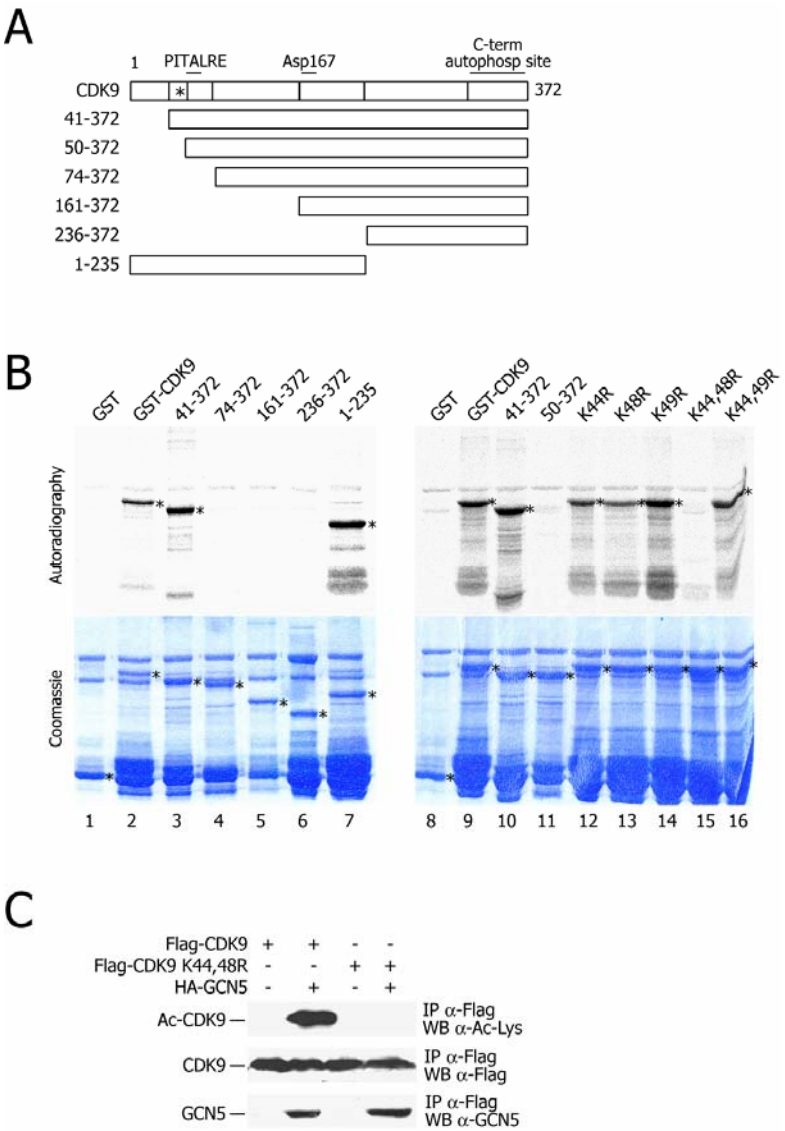

Figure 12. Mapping of Cdk9 acetylation site *in vitro* and *in vivo*. (**A**) Schematic representation of GST-CDK9 fragments used for *in vitro* acetylation assays. Position of lysines found to be positive for acetylation is indicated by an asterisk. (**B**) *In vitro* mapping of CDK9 acetylated lysines. Full length CDK9 (lane 2); a series of CDK9 mutants with N- and C-terminal truncations (lanes 3-7 and 10-11); and point mutants in the lysines in the N-terminal domain (lanes 12-16) were assayed for *in vitro* acetylation by GCN5. Proteins were resolved by SDS-PAGE followed by autoradiography. (**C**) *In vivo* mapping of CDK9 acetylated lysines. Extracts of 293T cells transfected with the indicated plasmids were immunoprecipitated with an anti-Flag antibody and immunoblotted using an anti-Ac-Lys antibody (upper panel). The

same filter was incubated with an anti-Flag antibody (medium panel) and an anti-GCN5 antibody (lowest panel) to verify protein expression levels. Of notice the CDK9 (K44,48R) mutant, which is not acetylated, is still able to bind GCN5.

Cdk9 K44,48R has impaired catalytic activity

Interestingly, the acetylated lysines of Cdk9 lay in close proximity to the PITALRE amino acid sequence (position 60-66). This region is essentially involved in the formation of the catalytic pocket of Cdk9, and its sequence is highly conserved in all members of the Cdk family (Figure 13A). In particular, lysine 48 of Cdk9 corresponds to lysine 33 of Cdk2; structural data indicate that this residue is essentially involved in orienting the ATP phosphate residues as well as in magnesium binding within the catalytic pocket of the enzyme (De Bondt et al., 1993).

This prompted us to investigate whether mutations in this lysine could modify the enzymatic properties of Cdk9 .

First, we transfected U2OS cells with the Flag-Cdk9 K44,48R expression vector and analyzed the subcellular localization of the mutated protein by immunofluorescence with an anti-Flag antibody: Cdk9 K44,48R had a nucleo-cytoplasmic localization analogous to that of wild type Cdk9 (Figure 13B).

Then in order to assess the binding between Flag-Cdk9 K44,48R and the regulatory partners CyclinT1 and HEXIM1, we transfected 293T cells with Flag-Cdk9 wild type or K44,48R and performed immunoprecipitations of the two proteins with an anti-Flag antibody. Western blot analysis revealed that endogenous CyclinT1 co-precipitated with the same efficacy with both proteins, while HEXIM1 showed slightly decreased affinity for the mutated form of Cdk9 (Figure 13C).

Nevertheless the major expected effect of lysines mutation was an impairment of Cdk9 catalytic activity. Therefore we set out to measure Cdk9 kinase activity by immunoprecipitation-kinase assay. Flag-Cdk9 wild type or K44,48R mutant were immunoprecipitated from 293T transfected cells with an anti-Flag antibody and then after extensive washing incubated with recombinant GST-CTD purified from bacteria and [^{32}P]-radiolabeled ATP. The complexes were then resolved by SDS-PAGE and the amount of labeled proteins was quantified after autoradiography.

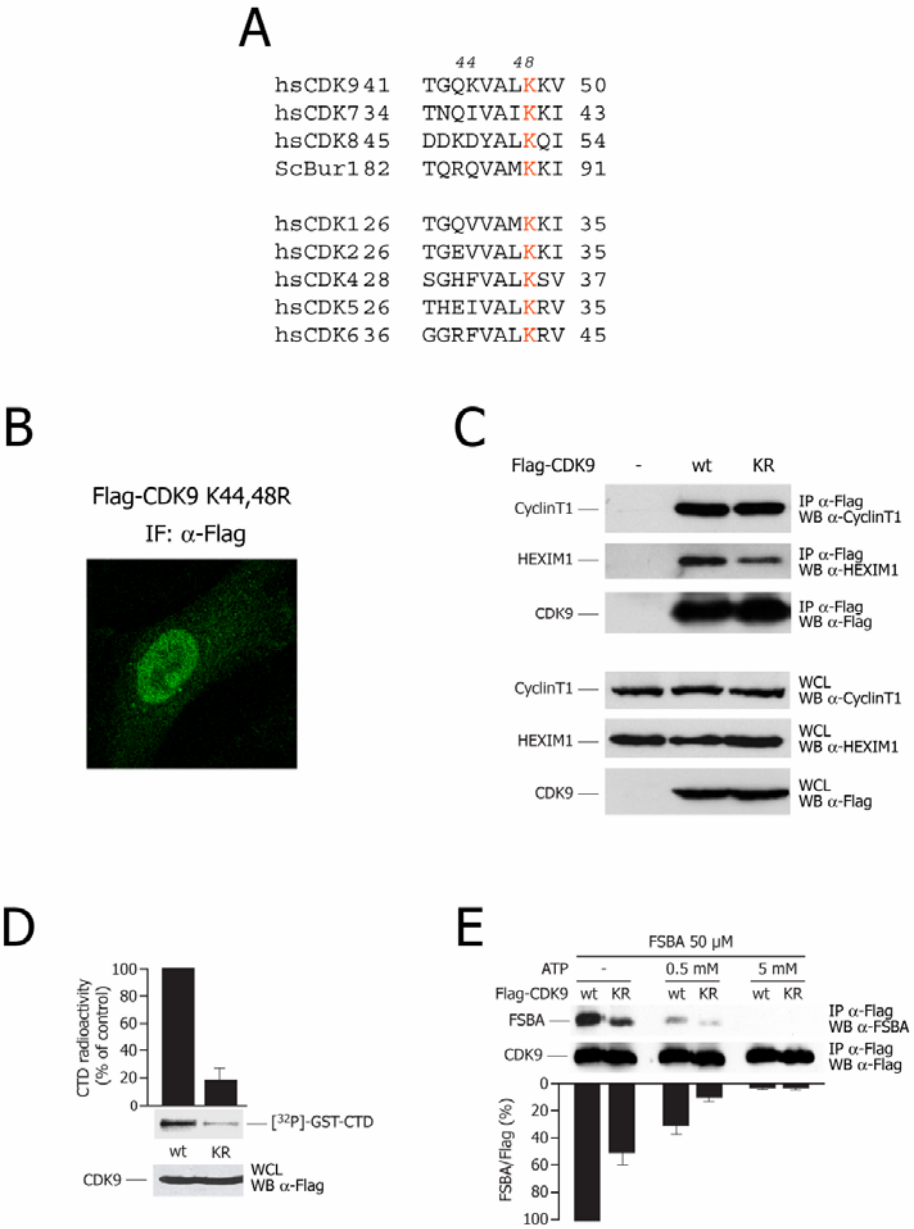

Figure 13. Cdk9 K44,48R characterization. (**A**) Conservation of one of the lysines that are substrates for acetylation (K48) in other Cdks controlling transcription or cell cycle. (**B**) U2OS cells were transfected with Flag-Cdk9 K44,48R and then subject to immunofluorescence with an anti-Flag antibody. (**C**) 293T cells were transfected with Flag-CDK9, either wild type or K44,48R and then subject to immunoprecipitation with an anti-Flag antibody. CyclinT1 and HEXIM1 presence in the bound fraction was analyzed by western blot. The bottom three

panels show protein expression levels in whole cell lysates. (**D**) Mutations of lysines 44 and 48 to arginines inhibits CDK9 kinase activity. Flag-CDK9, either wild type or K44,48R, immunoprecipitated from 293T cells, and recombinant GST-CTD, purified from bacteria, were incubated with [32P]-radiolabeled ATP and then resolved by SDS-PAGE followed by autoradiography. The graph shows the quantification of labeled GST-CTD in three independent experiments (mean±s.d.). The bottom panel shows protein expression levels. (**E**) CDK9 K44,48R mutant has reduced ATP binding activity. Wild type Flag-CDK9 or its K44,48R mutant were transfected into 293T cells, immunoprecipitated with an anti-Flag antibody and labeled with the ATP analog FSBA. Immunoprecipitated proteins were then resolved by SDS-PAGE and analyzed by western blot with an anti-FSBA antibody (upper panel) or anti Flag antibody (lower panel). Incorporation of FSBA was then quantified by densitometric analysis. The graph shows the mean±s.d. of three independent experiments.

The Cdk9 K44,48R mutant had a significantly reduced kinase activity in comparison to wild type Cdk9 even if it was expressed to the same level (Figure 13D).

To examine whether the loss of kinase activity was due to the failure to bind ATP, we utilized the ATP analog FSBA to measure the ATP binding capacity of the two proteins. In fact FSBA contains a crosslinking group in the position analogous to the γ-phosphate of ATP (Kamps et al., 1984) whose transfer and correct positioning are probably regulated by the conserved lysine positioned in the catalytic pocket of protein kinases.

Immunoprecipitated wt or K44,48R mutant Flag-Cdk9 were extensively washed and then incubated with 50 μM FSBA in the presence or absence of competitor ATP for 15 min at 30 °C. Then the proteins were resolved by SDS-PAGE and FSBA incorporation was revealed by western blot with an anti-FSBA antibody (Wymann et al., 1996), while the amount of immunoprecipitated proteins was assessed with anti-Flag antibody (Figure 13E). In keeping with previous results, quantification of the ratio between (FSBA labeled protein) and (total immunoprecipitated Flag protein) by densitometric analysis revealed that Flag-Cdk9 K44,48R mutant had reduced capacity to bind FSBA in comparison to wild type Flag-Cdk9. We proved that the FSBA labeling was specific for the nucleotide binding-site by adding molar excess of ATP in the reaction mix: ATP competed with

FSBA for the binding to the kinase and at higher doses completely abolished the FSBA labeling.

Acetylation inhibits Cdk9 kinase and transcriptional activity

GCN5 mediated acetylation inhibits Cdk9 kinase activity.

Since we found that the lysines that are acetylated by GCN5 are crucial for Cdk9 kinase activity, we wondered what effect could have the addition of an acetyl group on these residues in the context of the wild type Cdk9 protein. As a matter of fact acetylation of residues in the catalytic site of other enzymes has been shown to inhibit their function (Hallows et al., 2006; Mukherjee et al., 2006; Okumura et al., 2006; Schwer et al., 2006; Starai et al., 2002).

For this reason we decided to perform immunoprecipitation-kinase assays from cells transfected with Flag-Cdk9 and increasing amounts of HA-GCN5 in order to induce Cdk9 acetylation. We found that GCN5 transfection impaired Cdk9 kinase activity on the GST-CTD substrate in a dose dependent manner (Figure 14A). This effect was clearly due to the acetyltransferase activity of GCN5 since the mutant HA-GCN5 (Y260A/F261A) that is HAT defective but still able to bind Cdk9 had no effect (Figure 14B). The same extracts used for the kinase assay were run on an SDS-PAGE gel and immunoblotted with an anti-Flag and anti-HA antibody to verify that GCN5 transfection did not modify Flag-Cdk9 expression. These results suggest that Cdk9 acetylation by GCN5 is inhibitory for its kinase activity.

Since Cdk9 kinase activity is regulated by the interaction of the kinase with its cellular cofactors CyclinT1 (Peng et al., 1998; Peng et al., 1998) and HEXIM1 (Michels et al., 2003; Yik et al., 2003), we wondered whether GCN5 transfection could modify these interactions. Therefore the same lysates used for the kinase assay were subject to immunoprecipitation with an anti-Flag antibody and then analyzed for the presence of co-precipitated endogenous CyclinT1 and HEXIM1. As reported in Figure 14C the amount of CyclinT1 and HEXIM1 co-precipitated with Flag-Cdk9 was the same in all lanes supporting the notion that acetylation affects Cdk9 catalytic activity rather than modulating interaction with its known regulatory partners.

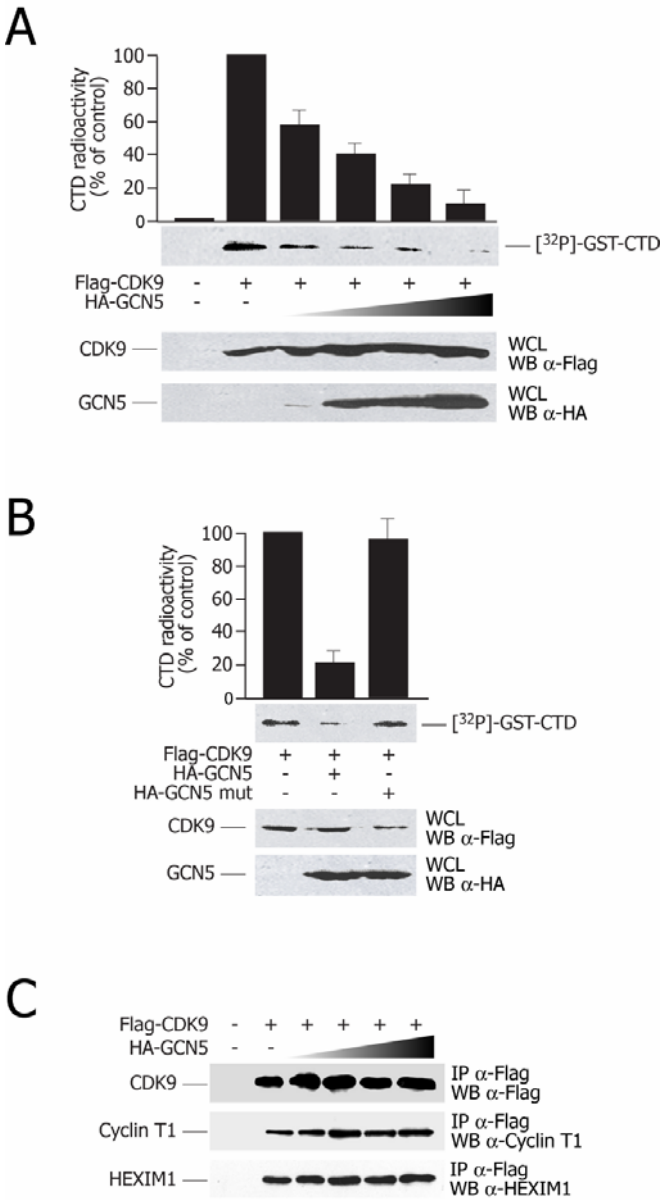

Figure 14. GCN5 inhibits Cdk9 kinase activity. (**A**) Acetylation inhibits CDK9 kinase activity. Flag-CDK9, immunoprecipitated from 293T cells transfected with the indicated plasmids, and recombinant GST-CTD, purified from bacteria, were incubated with [32P]-radiolabeled ATP and then resolved by SDS-PAGE followed by autoradiography. The graph shows the quantification of labeled GST-CTD in three independent experiments (mean±s.d.). The bottom two panels show protein expression levels. (**B**) Immunoprecipitation-kinase assay performed as in (A) from cells co-transfected with Flag-CDK9 and either wt GCN5 or its catalytically

inactive (Y260A/F261A) mutant. (**C**) The same extracts as in (A) were subject to immunoprecipitation with an anti-Flag antibody and then analyzed by western blot for the presence of co-precipitated endogenous CyclinT1 and HEXIM1 proteins.

Inhibition of Cdk9 kinase activity by GCN5 impairs Cdk9 function in transcription.

Since acetylation inhibited the kinase activity of Cdk9 on RNAPII CTD *in vitro*, we analyzed whether this modification might affect the transcriptional regulation of Cdk9 responsive promoters inside the cells. For this purpose, we exploited the pG6(5'Pro) reporter system that contained the HIV LTR linked to the CAT reporter gene. Six repeats of the synthetic Gal4-binding sites (UAS) were introduced upstream of the three Sp1 sites. These UAS repeats replaced the enhancer (EN) in the HIV LTR (Figure 15A). Cdk9 alone, when tethered on this promoter by fusion to the Gal4 DNA binding domain, is able to induce transcription of the reporter gene (Taube et al., 2002). This allowed us to appreciate the effects of Cdk9 acetylation in an *in vivo* system in a simplified, CyclinT1-independent manner.

The pG6(5'Pro) reporter was transfected in U2OS cells together with Gal4-Cdk9 and increasing amounts of HA-GCN5, followed by the analysis of CAT production using an ELISA assay. We observed that promoter activation by Cdk9 was inhibited in a dose-dependent manner by the expression of enzymatically active GCN5, but not by its catalytically inactive variant (Figure 15B)

In keeping with this result, when we used a siRNA against GCN5 to specifically inhibit endogenous GCN5 expression (as shown in the western blot of Figure 15C), we found that co-transfection of this siRNA (and not of an anti-Luciferase siRNA that we used as a negative control) together with the pG6(5'Pro) reporter and the Gal4-Cdk9 plasmid determined a remarkable increase of Cdk9-driven transcriptional activation (Figure 15C).

In addiction, we verified that the non acetylable Cdk9 K44,48R mutant was insensible to GCN5 transfection. To perform this experiment, we exploited the capacity of Cdk9 to increase CyclinT1 transcriptional activity and co-transfected Gal4-CycT1 and the pG6(5'Pro) reporter with Flag-Cdk9 either wild type or mutated. As expected, Flag-Cdk9 K44,48R mutant had a very low transcriptional

activity but sufficient to indicate that it was insensible to GCN5 overexpression, while wild type Flag-Cdk9 was inhibited by GCN5 (Figure 15D).

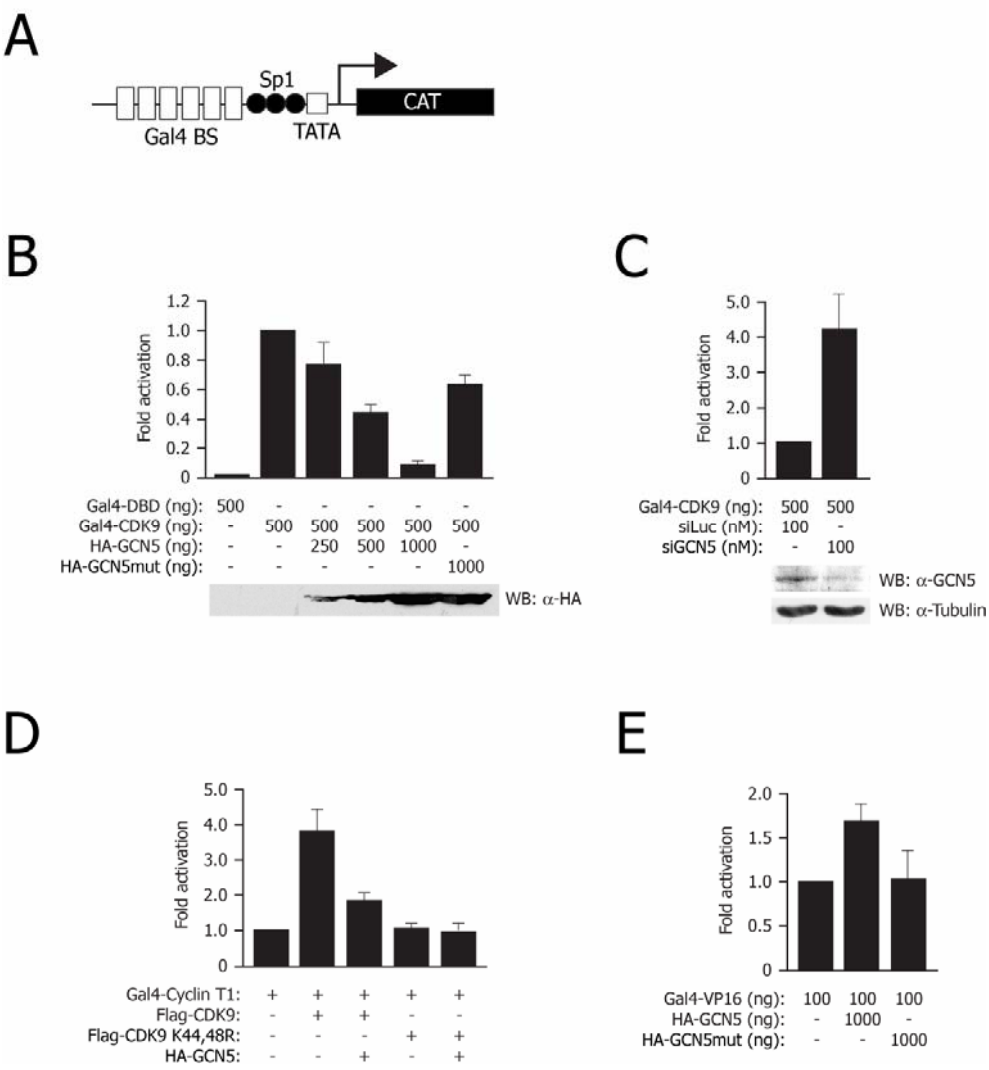

Figure 15. GCN5 inhibits Cdk9-driven transcription. (**A**) Schematic representation of the pG6(5'Pro) reporter vector used for the transcription assays. (**B**) Acetylation inhibits CDK9 transcription activity. Extracts of U2OS cells transfected with the pG6(5'Pro) reporter together with the indicated plasmids were analyzed for CAT protein production by ELISA. The results are reported as percentage of CAT activity with respect to cells transfected with pG6(5'Pro) and Gal4-CDK9 alone; the graph summarizes the results obtained from three independent experiments (mean±s.d.). Expression of wt HA-GCN5 or its catalytically inactive mutant was verified by western blot (lower panel). (**C**) The

same experiment performed as in (B) was carried out by co-transfecting Gal4-CDK9 together with 100 nM siRNAs against GCN5 or Luciferase; the bottom part of the panel reports western blottings for GCN5 and α-tubulin. (**D**) Extracts of U2OS cells transfected with the pG6(5'Pro) reporter together with the indicated plasmids were analyzed for CAT protein production by ELISA. The results are reported as percentage of CAT activity with respect to cells transfected with pG6(5'Pro) and Gal4-CyclinT1 alone; the graph summarizes the results obtained from three independent experiments (mean±s.d.). (**E**) The same experiment as in (B) was carried out by using Gal4-VP16 instead of Gal4-CDK9.

In sharp contrast, expression of GCN5 had an opposite, activation effect when transcription was directed by the Gal4-VP16 transactivator (Figure 15E), as already described in the literature (Candau and Berger, 1996).
Thus, the inhibition of Cdk9 transcriptional activity by enzymatically active GCN5 is consistent with the direct inhibition of Cdk9 kinase activity by acetylation.

Acetylated Cdk9 accumulates at PML bodies

Anti Ac-Cdk9 specific antibody detects endogenous acetylated Cdk9

To better characterize acetylated Cdk9, we raised a specific antibody that recognizes this post-translationally modified form of Cdk9. We obtained a 15 amino acid peptide corresponding to Cdk9 (39-53) acetylated at positions 44 and 48. This peptide was coupled to KHL (Keyhole Limpet Hemocyanin) and used to immunize three rabbits. After some months sera were collected and the specificity of the antibody was tested in dot-blot experiments with increasing amounts of acetylated and not acetylated synthetic peptides as substrates. As shown in Figure 16A the serum specifically recognized the acetylated peptide and not the non acetylated one. Subsequently the IgG fraction was purified from the antiserum on columns bearing protein A. Purified anti-acetylated Cdk9 antibody was then tested by western blotting on immunoprecipitated wild type or K44,48R mutant Cdk9. Cdk9 wild type or mutant were co-transfected with GCN5 (wild type or catalytically inactive mutant) in 293T cells and then immunoprecipitated with anti-Flag antibodies.

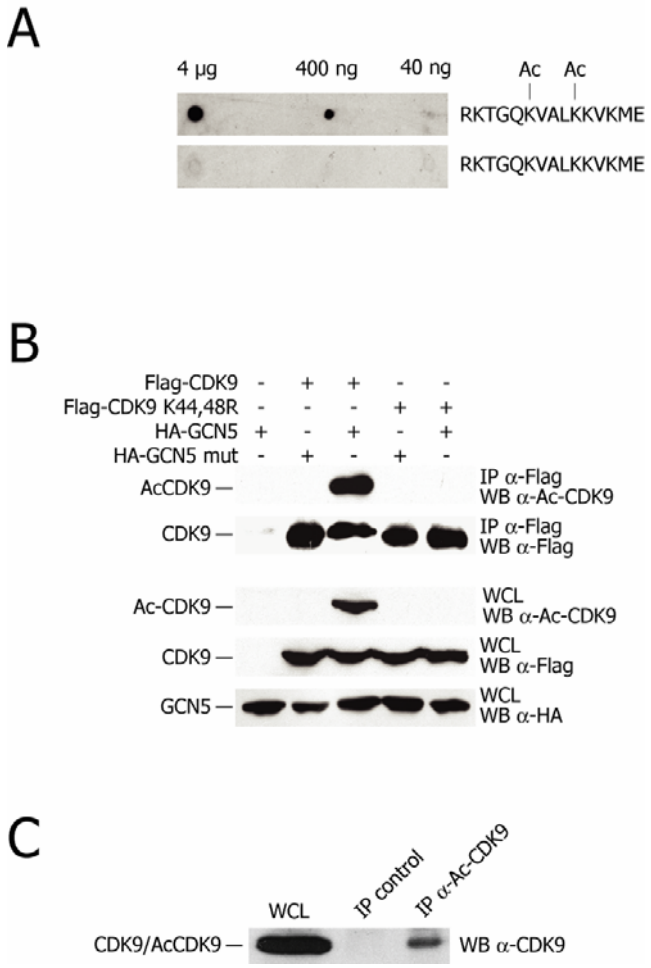

Figure 16. Characterization of anti Ac-CDK9 antibody. (**A**) Dot blot experiment showing specificity of the anti-acetylated CDK9 antibody. Fifteen-mer peptides corresponding to human CDK9 amino acids 39-53, either acetylated or not acetylated on lysines 44 and 48, were blotted onto nitrocellulose filters in the indicated amounts and challenged with IgGs (1:500) from an animal immunized with the acetylated peptide. The antibody clearly only recognizes the acetylated peptide. (**B**) Anti-Ac-CDK9 antibody specifically reacts with wild type CDK9 and not with the not acetylable CDK9 K44,48R mutant. Extracts of 293T cells transfected with the indicated plasmids were immunoprecipitated with an anti-Flag antibody and subject to western blot (WB) with the anti-Ac-CDK9 or anti-Flag antibodies (upper two panels). Protein expression levels were verified by western blot on total cell lysates (lower three panels). WCL: whole cell lysate. (**C**) Anti-Ac-CDK9 antibody is able to detect endogenous acetylated CDK9. TSA treated HeLa whole cell lysates (WCL) were subject to immunoprecipitation with the anti-Ac-CDK9 antibody and then to immunoblotting with an anti-CDK9 antibody. Control IP was performed with the preimmune serum.

After SDS-PAGE and western blot the anti-AcCdk9 antibody recognized only wild type Flag-Cdk9 and not the non acetylable Flag-Cdk9 K44,48R even if the two proteins were immunoprecipitated at the same level (Figure 16B). The same extracts were run on an SDS-PAGE gel and immunoblotted with anti-Ac-Cdk9, anti-Flag and anti-HA antibody to verify protein expression levels.

By using this antibody we were able to detect endogenous acetylated Cdk9 inside the cells thus bypassing the sensitivity limits of standard anti-acetyllysine antibodies. As a matter of fact we could use this antibody to immunoprecipitate endogenous acetylated Cdk9 from HeLa cells treated with TSA and then we could detect it by western blot with an anti-Cdk9 monoclonal antibody (Figure 16C).

Acetylated Cdk9 localizes in speckled structures inside the nucleus.

The anti-Ac-Cdk9 antibody could also detect endogenous Cdk9 in immunofluorescence experiments.

Total Cdk9 as well as transfected GFP-Cdk9 were found distributed throughout the nucleoplasm in both HeLa and U2OS cells, with lower levels of fluorescence also detectable in the cytoplasm, in accordance to published data (Napolitano et al., 2002); Figure 17A. In sharp contrast, acetylated Cdk9 was exclusively found in the nucleus, where it gave rise to a speckled pattern, indicative of distribution within specific subnuclear compartments (Figure 17B). The levels of acetylated protein within the Ac-Cdk9 foci increased upon cell treatment with the histone deacetylase inhibitor TSA. Of notice is the fact that the same treatment also determined the redistribution of transfected GFP-Cdk9 from its diffuse nucleoplasmic localization to form a punctuated pattern (Figure 17B).

Since endogenous, transfected, and CFP-tagged GCN5 also showed a similar subnuclear distribution in a speckled pattern (Figure 17C), we tested whether GCN5 and acetylated Cdk9 co-localized within the same subnuclear structures. Figure 18 shows that a large fraction of GCN5 actually did co-localize with acetylated Cdk9 in the same speckled structures, and that the overall levels of acetylated Cdk9 were remarkably increased upon overexpression of wild type GCN5, but not of its catalytically inactive (Y260A/F261A) mutant.

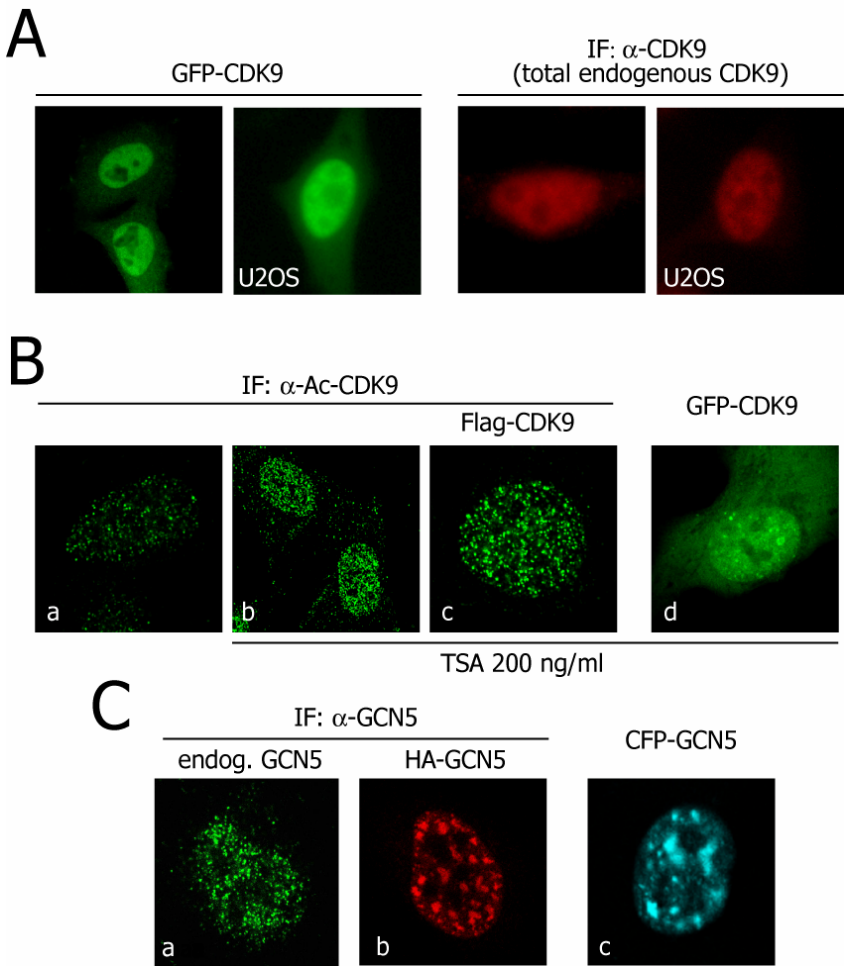

Figure 17. Acetylated Cdk9 and GCN5 have a speckled localization. (**A**) CDK9 shows a diffuse localization inside the cells. Localization of transfected GFP-CDK9 (left panels) and endogenous CDK9 (right panels) in HeLa and U2OS cells (where indicated). (**B**) Ac-CDK9 has a speckled nuclear localization. Localization of endogenous Ac-CDK9 by indirect immunofluorescence with the anti-Ac-CDK9 antibody in HeLa cells mock treated (a) or treated with TSA (b); Localization of Ac-CDK9 in cells transfected with Flag-CDK9 and treated with TSA (c); Localization of total GFP-CDK9 after cells treatment with TSA (d). (**C**) GCN5 accumulates in foci inside the nucleus. Immunofluorescence with an anti-GCN5 antibody to visualize endogenous (a) or transfected (b) GCN5; localization of overexpressed CFP-GCN5 (c).

The speckled distribution of acetylated Cdk9 and GCN5 is reminiscent of the distribution of the main Cdk9 cyclin partner, CyclinT1, which we have shown to

correspond to the sites of accumulation of the promyelocytic leukemia protein (PML) (Marcello et al., 2003). These PML bodies represent subnuclear compartments in which several proteins variously involved in different cellular activities, including transcription, accumulate; most notably, these are sites in which post-translational modification of many proteins occurs (for reviews, see: (Zhong et al., 2000)), as detailed in the Introduction. Thus we decided to further investigate the relationship between acetylation of Cdk9 and PML.

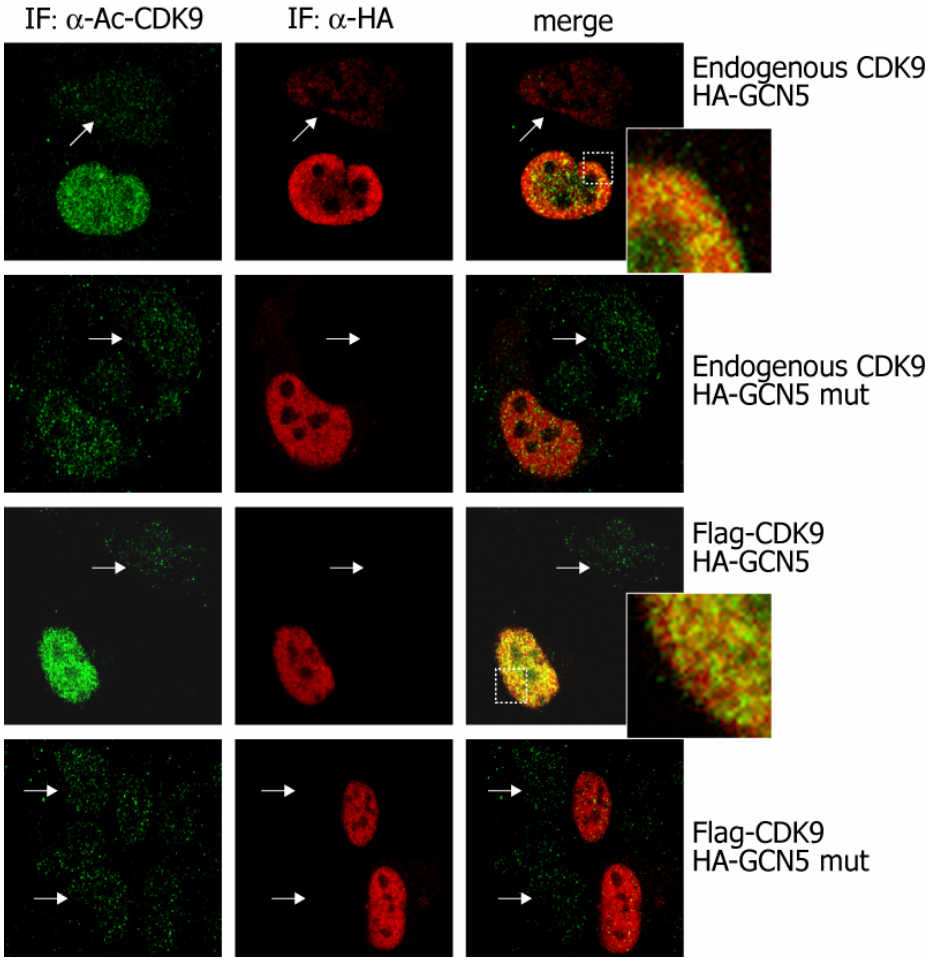

Figure 18. Co-localization of Ac-CDK9 and GCN5 in speckled structures. Transfection of enzymatically active GCN5, but not of its catalytically inactive variant, increases the amount of Ac-CDK9 inside the cells. Double immunofluorescence with anti-Ac-CDK9 and anti-HA antibodies (the latter reacting

with wt or catalytically inactive HA-GCN5) in cells transfected with the indicated plasmids. Expression of enzymatically active GCN5 dramatically increases the levels of Ac-CDK9 in nuclear speckles, which are lower in untransfected cells (arrows) or cells expressing the catalytically inactive GCN5 mutant.

Cdk9 interacts with PML

First we wanted to asses whether total Cdk9 localizes inside PML bodies like its cyclin partner. For this reason we transfected cells with Cdk9 and/or PML-IV expressing vectors and then analyzed the localization of these proteins by immunofluorescence experiments.

We found that at the endogenous level of PML, Cdk9 showed a diffuse localization even when it was overexpressed as GFP-Cdk9 (Figure 19A first row) or Flag-Cdk9 (Figure 19B first and second rows). Nevertheless, after transfection of PML-IV (but not of PMLRARα), Cdk9 (both endogenous and transfected) assumed a speckled distribution that co-localized with PML bodies, thus demonstrating an affinity towards this subnuclear compartment (Figure 19A second and third row, Figure 19B third and fourth rows).

In order to understand whether the observed re-localization of Cdk9 was mediated by an interaction between Cdk9 and the PML protein we performed an *in vitro* binding assay between GST-PML-IV and [^{35}S]-labeled Cdk9. GST-PML-IV immobilized on beads was incubated with [^{35}S]-Cdk9; the beads were then extensively washed and the proteins resolved by SDS-PAGE. Figure 20B shows the gel stained with Coomassie blue (lower panel) and after autoradiography (upper panel). Labeled Cdk9 was found to bind to GST-PML-IV but not to GST alone.

This interaction was confirmed also *in vivo* by co-immunoprecipitations experiments. Flag-Cdk9 was transfected in 293T cells alone or together with PML-IV, then Cdk9 was immunoprecipitated with an anti-Flag antibody and the complexes analyzed by western blot with an anti-PML antibody. PML was found to specifically co-precipitate with Cdk9 when overexpressed, but not at the endogenous level (Figure 20C).

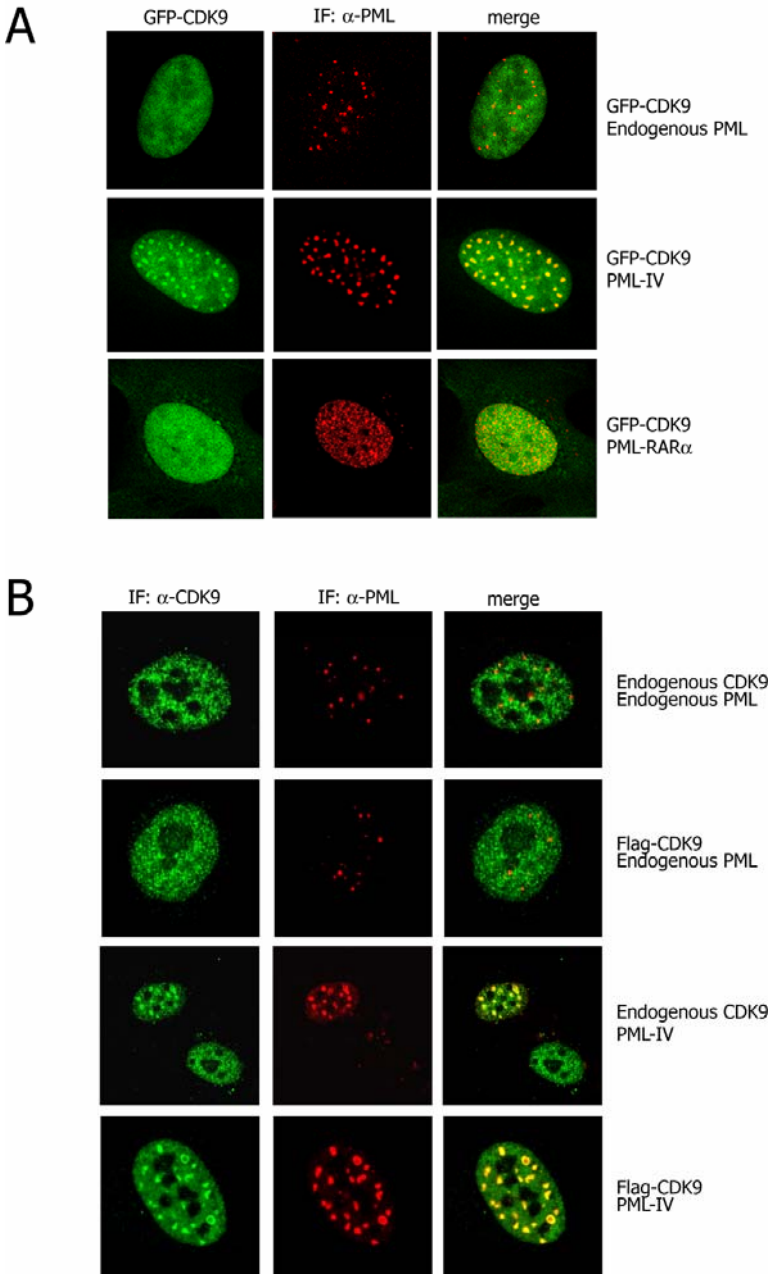

Figure 19. Cdk9 and PML co-localize inside PML bodies. (**A**) GFP-CDK9 co-localizes with PML when PML-IV is overexpressed. (**B**) Endogenous and Flag-CDK9 co-localize with PML when PML-IV is overexpressed. U2OS cells were transfected with indicated plasmids and subject to immunofluorescence with anti-PML antibody (A) or to double immunofluorescence with antibodies against PML and CDK9 (B).

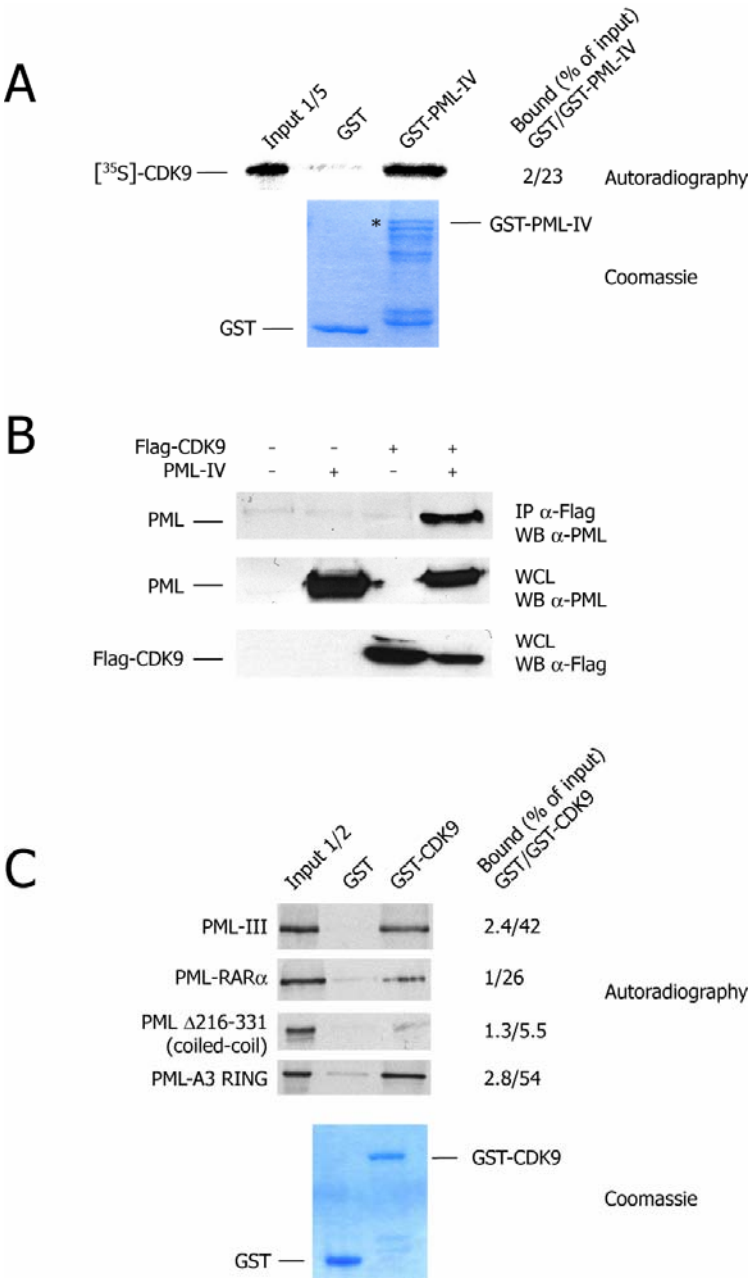

Figure 20. Cdk9 interacts with PML *in vivo* and *in vitro*. (**A**) CDK9 binds PML-IV *in vitro*. GST-PML-IV or GST alone as a control were incubated with [35S]-CDK9, extensively washed and then analyzed by SDS-PAGE. The upper panel shows the gel exposed to a phosphoimager screen, while the lower panel the Coomassie stainings of the same gels. (**B**) CDK9 binds PML-IV *in vivo*. Extracts prepared from cells transfected with indicated plasmids were immunoprecipitated with an anti-Flag

antibody and immunoblotted with anti-PML antibody. The same extracts were run on an SDS-PAGE gel and immunoblotted with anti-PML and anti-Flag antibodies to verify protein expression levels (lower two panels). (**C**) CDK9 binds the coiled coil domain of PML *in vitro*. GST-CDK9 or GST alone as a control were incubated with [^{35}S]-labeled PML isoforms or mutants, extensively washed and then analyzed by SDS-PAGE. The upper panels show the gel exposed to a phosphoimager screen, while the lower panel the Coomassie stainings of one representative gel.

In order to investigate whether the interaction between Cdk9 and PML is isoform specific or common to all PML proteins, besides PML-IV, we analyzed the interaction of Cdk9 also with the PML-III isoform and with the oncogenic form PMLRARα. These three PML variants, which differ in their C-termini but maintain an intact RBCC motif, were all capable of binding Cdk9 *in vitro* (Figure 20D), suggesting that the interaction domain lies within the RBCC region. We also assayed a deletion mutant of PML-III, PMLΔ(216-333), lacking the coiled-coil domain, and another mutant, PML A3 RING, with a non-functional RING domain. While the latter behaved like the wild-type protein, the mutant in the coiled-coil domain lost its ability to associate with Cdk9 (Figure 20D). This experiment demonstrated that the association of Cdk9 with PML is not limited to a subset of PML isoforms and requires an intact coiled-coil domain.

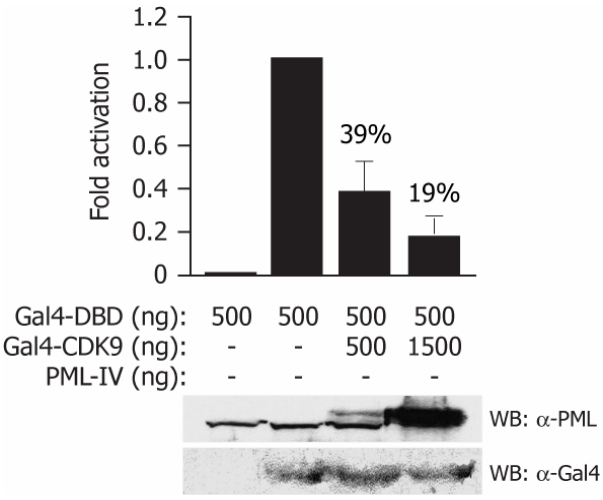

Figure 21. PML inhibits Cdk9-mediated transcription. Extracts of U2OS cells transfected with the pG6(5'Pro) reporter together with the indicated plasmids were analyzed for CAT protein production by ELISA. The results are reported as

percentage of CAT activity with respect to cells transfected with pG6(5'Pro) and Gal4-CDK9 alone; the graph summarizes the results obtained from three independent experiments (mean±s.d.). Expression of PML and Gal4-CDK9 was verified by western blot (lower panels).

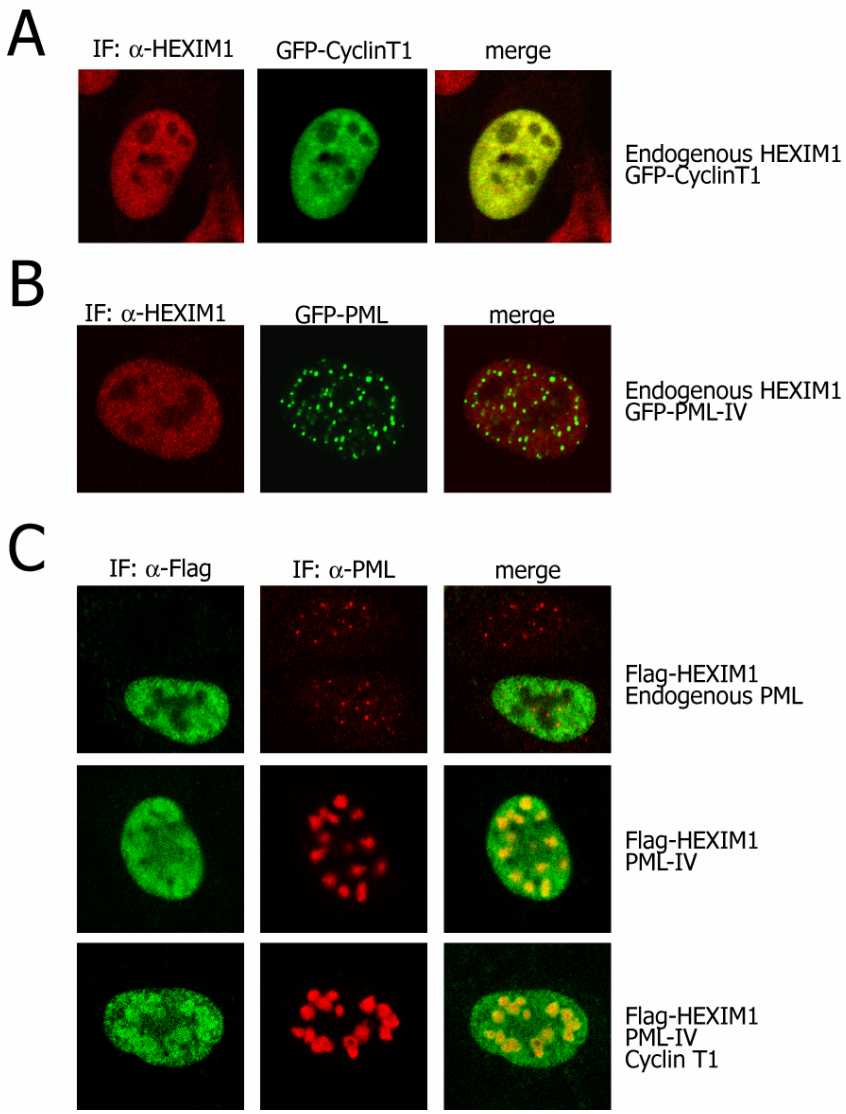

Figure 22. HEXIM1 co-localizes with PML when PML is overexpressed. (**A**) Immunofluorescence of endogenous HEXIM1 in U2OS cells transfected with GFP-CyclinT1. (**B**) Immunofluorescence of endogenous HEXIM1 in U2OS cells transfected with GFP-PML-IV. (**C**) Double immunofluorescence with antibodies against HEXIM1 and PML in U2OS cells transfected with indicated plasmids.

Accumulation of CyclinT1 in PML bodies correlated with inhibition of P-TEFb transcription at the HIV-1 promoter, thus we wondered which was the effect of PML on Cdk9-dependent transcription. For this purpose, we exploited the pG6(5'Pro) reporter system that, as previously described, could be activated by Gal-Cdk9 in a CyclinT1 independent manner. We found that PML-IV transfection inhibited Gal4-Cdk9 transcription activity in a dose dependent manner with an efficacy similar to that observed for GCN5 (Figure 21).

Since one of the best characterized inhibitors of P-TEFb is the HEXIM1 protein (Michels et al., 2003; Yik et al., 2003), we wondered whether accumulation of Cdk9 within PML bodies increased its association with HEXIM1.

First we analyzed the subcellular localization of HEXIM1 in order to see whether it can be found at PML bodies. Endogenous HEXIM1 showed a diffuse localization inside the nucleus even in case of GFP-CyclinT1 (Figure 22A) or GFP-PML-IV (Figure 22B) transfection. Nevertheless when overexpressed as Flag-HEXIM1, it re-localized into PML bodies upon PML overexpression (Figure 22C second row) and more efficiently upon PML plus CyclinT1 co-transfection (Figure 22C third row), thus supporting the notion that PML might facilitate the binding between Cdk9 and HEXIM1 by recruiting both proteins in the same compartment. Anyway, HEXIM1 localization at PML bodies was not observed at endogenous levels of HEXIM (even in case of PML-IV transfection), and was only partial also in case of Flag-HEXIM1 transfection if CyclinT1 was not co-transfected together with PML-IV.

Furthermore we found that PML transfection did not change the interaction between Cdk9 and HEXIM1 or CyclinT1. 293T cells were transfected with Flag-Cdk9 and increasing amounts of pcDNA3-PML-IV, then Flag-Cdk9 was immunoprecipitated with an anti-Flag antibody and bound proteins were analyzed by SDS-PAGE and western blot with CyclinT1 (Figure 23A) or HEXIM1 (Figure 23B) antibodies. These experiments revealed that Flag-Cdk9 co-immunoprecipitated the same amount of CyclinT1 or HEXIM1 protein in presence or in absence of PML-IV overexpression.

These results open the possibility that another mechanism, possibly based on Cdk9 acetylation, might explain the observed inhibitory effect of PML on P-TEFb mediated transcription.

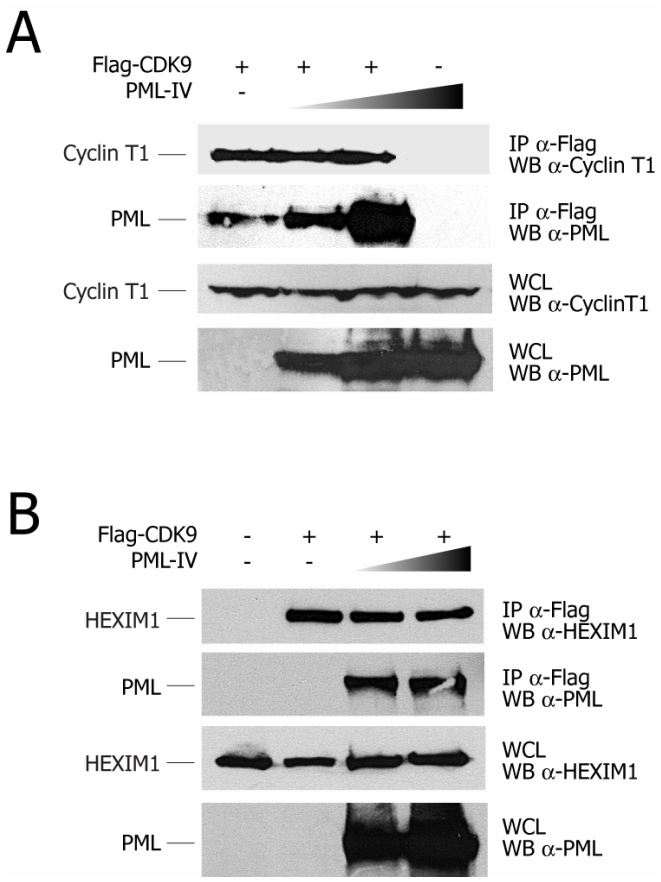

Figure 23. PML does not modify the interaction between CDK9 and its regulatory partners. 293T cells transfected with Flag-CDK9 and increasing amounts of PML-IV were subject to immunoprecipitation with an anti-Flag antibody and then analyzed by western blot for the presence of co-precipitated endogenous CyclinT1 (**A**) and HEXIM1 proteins (**B**). Protein expression was verified by western blot on total cell lysates.

Acetylation increases Cdk9 affinity for PML bodies

The localization of endogenous PML was remarkably different from that of total Cdk9 (which is mainly not acetylated), unless PML was overexpressed (Figure 24A). In contrast, a remarkable proportion of endogenous acetylated Cdk9 localized within endogenous PML bodies, and overexpression of PML or PML plus GCN5 markedly enhanced this localization (Figure 24B), suggesting that acetylation may increase Cdk9 affinity for PML bodies.

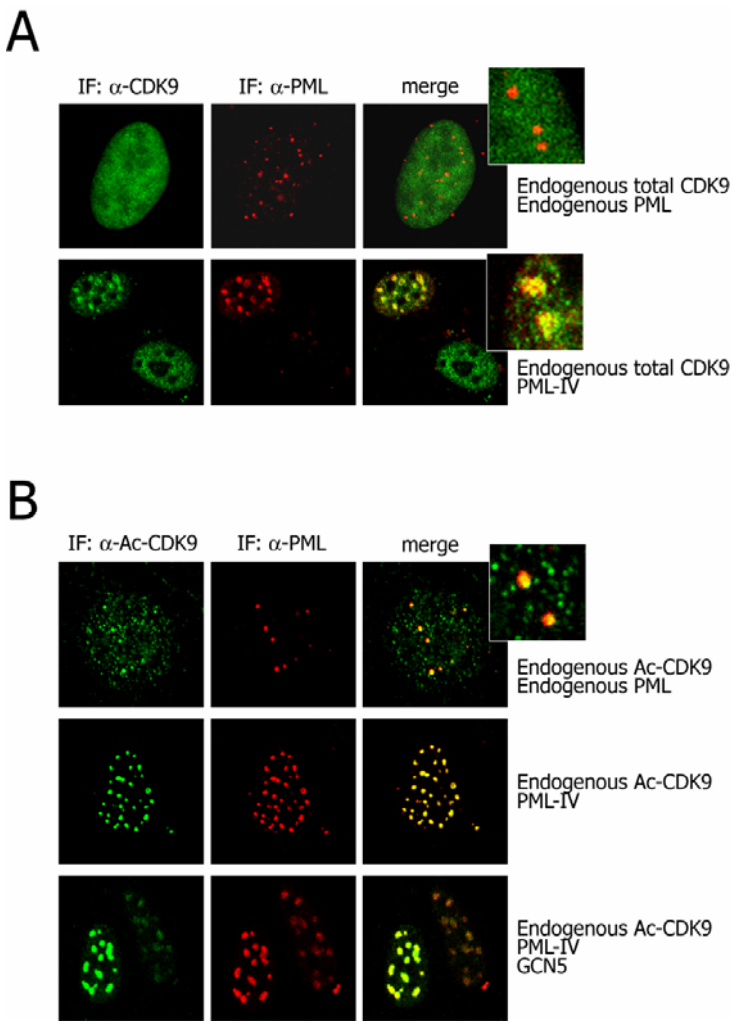

Figure 24. Acetylated CDK9 co-localizes with PML in PML bodies. (**A**) Most endogenous CDK9 does not co-localize with endogenous PML (upper panel); however, overexpression of PML-IV forces redistribution of endogenous CDK9 to nuclear bodies (lower panels). (**B**) Ac-CDK9 co-localizes with both endogenous or transfected PML in nuclear bodies. In (A) and (B) U2OS cells were transfected with indicated plasmids and subjected to double immunofluorescence with antibodies against PML and CDK9 or Ac-CDK9 respectively.

PML bodies are known to associate with the insoluble nuclear matrix fraction after biochemical fractionation (Fogal et al., 2000). We therefore fractionated total extracts from cells that were transfected with Cdk9 and GCN5 in order to enhance acetylation of the former protein. After nuclei isolation, the nuclear soluble fraction

was extracted by lysis in high salt buffer containing NP-40. The same amounts of proteins for each fraction were separated by SDS-PAGE and analyzed by western blot with specific antibodies. Under this conditions, PML-IV was found within the insoluble matrix fraction, while total Cdk9 partitioned in the soluble and insoluble nuclear fractions, as well as in the cytoplasmic fraction. In contrast, acetylated Cdk9 was almost exclusively present in the insoluble nuclear matrix fraction, together with transfected GCN5 and PML (Figure 25A).

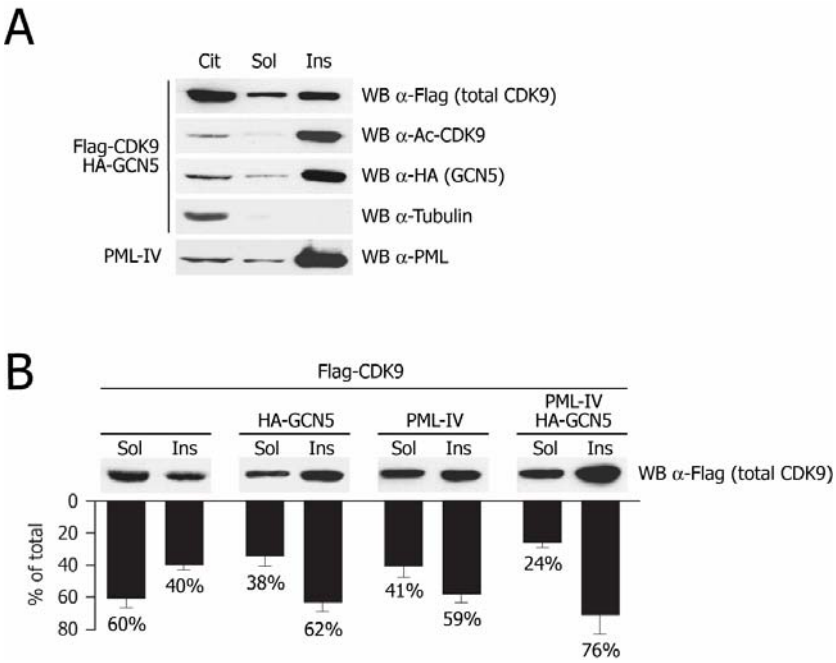

Figure 25. Ac-CDK9 localizes in the nuclear matrix fraction together with PML and GCN5.(**A**) Ac-CDK9 fractionates in the insoluble nuclear fraction together with PML. Extracts of 293T cells transfected with Flag-CDK9 and HA-GCN5 were separated into cytosolic, nuclear soluble and nuclear insoluble fractions as described in Materials and Methods. The same amounts of proteins for each fraction were separated by SDS-PAGE and analyzed by western blot with specific antibodies. As a control of fractionation, PML localization in the insoluble fraction was verified. (**B**) Both GCN5 and PML force re-localization of total CDK9 into the insoluble nuclear fraction. Nuclear extracts of 293T cells transfected with the indicated plasmids were separated into a soluble and insoluble fraction. Comparable amounts of proteins for each fraction were separated by SDS-PAGE and analyzed by western blot with an anti-Flag antibody. The amount of Flag-protein in each fraction was then quantified by densitometric analysis. The graph represents results obtained from three independent experiments (mean±s.d.).

Nevertheless the insoluble fraction of Flag-Cdk9 was more abundant of what we could expect from the diffuse localization that Cdk9 showed in absence of PML transfection. This could be explained by the fact that in the biochemical fractionation assay the cells were co-transfected with HA-GCN5 and, as we can see in Figure 25A, also this protein partitioned in the insoluble nuclear matrix fraction like PML. Therefore GCN5 transfection could have partially re-localized Flag-Cdk9 in the insoluble fraction. In fact, we observed that, upon transfection of GCN5, PML or both these proteins, the fraction of insoluble Cdk9 raised from 40% to 62, 59 and 76% respectively, clearly indicating that the expression of both PML and GCN5 causes significant re-localization of Cdk9 toward matrix-associated structures (Figure 25B).

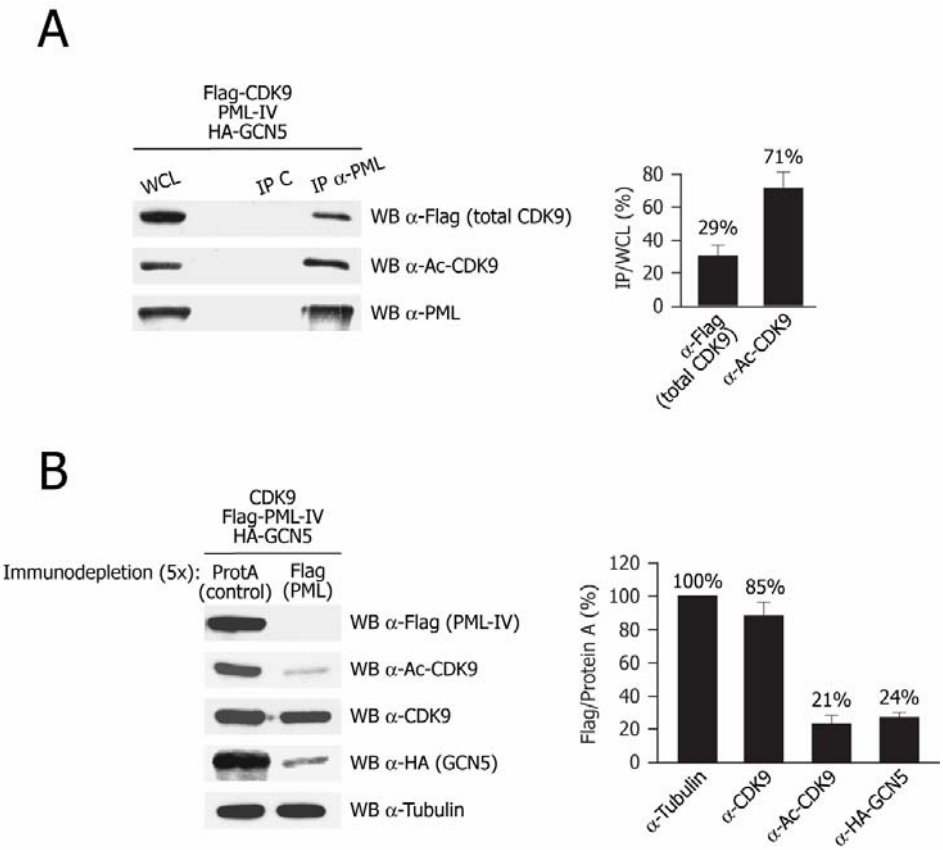

Figure 26. Ac-Cdk9 preferentially binds PML. (**A**) 293T cells transfected with Flag-CDK9, PML-IV and HA-GCN5 were subject to immunoprecipitation with an anti-PML antibody or an irrelevant antibody. Bound proteins were analyzed by

western blot with anti-Flag, anti-Ac-CDK9 and anti-PML antibodies. The amount of bound proteins respect to input was quantified by densitometric analysis. The graph shows the results obtained in three independent experiments (mean±s.d.). (**B**) PML immuno-depletion preferentially removes Ac-CDK9 from cell extracts. Extracts of 293T cells transfected with indicated plasmids were passed through anti-Flag antibody beads or control protein A beads five times. Flag-PML-IV, Ac-CDK9, CDK9, HA-GCN5 and Tubulin protein expression after depletion was verified by western blot using specific antibodies and then quantified by densitometric analysis. The graph shows the results obtained in three independent experiments (mean±s.d.).

The specific interaction between Ac-Cdk9 and PML was further demonstrated by co-immunoprecipitation assays. Cells were transfected with PML-IV, Flag-Cdk9 and HA-GCN5 and then subject to immunoprecipitation with an anti-PML antibody or an irrelevant antibody as a control. After SDS-PAGE and western blot with anti-Cdk9 and anti-Ac-Cdk9 antibodies, the amount of bound proteins respect to input was assessed by densitometric analysis and we found that Ac-Cdk9 co-precipitated with PML more efficiently than total Cdk9 (Figure 26A).

The relative amount of acetylated Cdk9 which was bound to PML was assessed by immunodepletion experiments. After transfection with Flag-PML-IV, Cdk9 and GCN5, cell extracts were passed through anti-Flag antibody beads 5 times, which almost completely removed Flag-PML from the extracts. Strikingly, depletion of PML led to the selective removal of more than 80% of acetylated Cdk9, compared to about 15% of total Cdk9 (which also includes the acetylated portion); Figure 26B. These results further strengthen the notion that acetylated Cdk9 selectively accumulates at PML bodies.

Cdk9 localization at PML bodies is independent from acetylation

Whether acetylation of Cdk9 might be the consequence or the cause of the re-localization in PML bodies still remains to be completely determined. However, we have clues that Cdk9 K44,48R mutant still re-localizes at PML bodies upon PML-IV overexpression, favoring a model by which acetylation is not requires for Cdk9 localization in PML bodies.

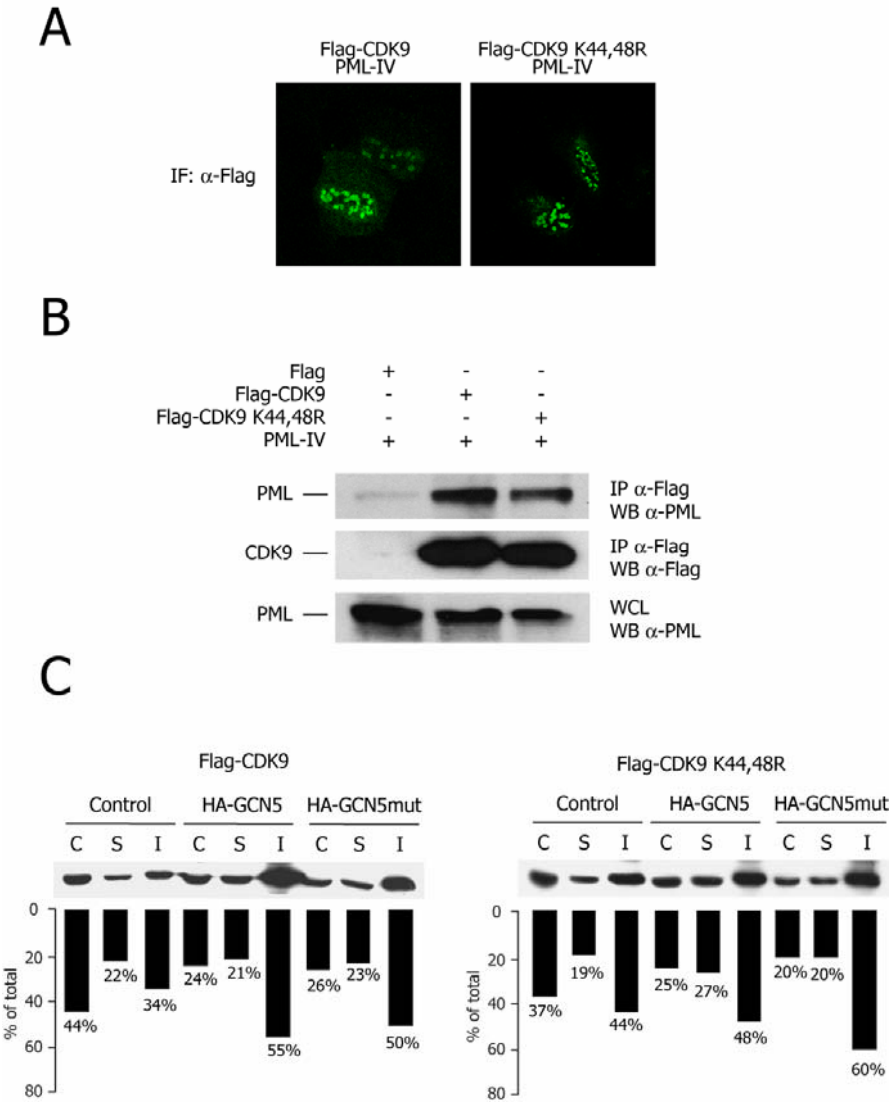

Figure 27. CDK9 K44,48R interacts with PML. (**A**) U2OS cells were transfected with Flag-CDK9 wild type or K44,48R together with PML-IV and subject to immunofluorescence with anti-Flag antibodies. (**B**) 293T cells were transfected with indicated plasmids and subject to immunoprecipitation with anti-Flag antibodies. Proteins in the bound fraction were analyzed with anti-PML and anti-Flag antibodies. (**C**) Extracts of 293T cells transfected with indicated plasmids were separated into cytosolic, nuclear soluble and nuclear insoluble fractions as described in Materials and Methods. The same amounts of proteins for each fraction were separated by SDS-PAGE and analyzed by western blot with anti-Flag antibodies.

After co-transfection of Flag-Cdk9 K44,48R and PML-IV in U2OS cells, immunofluorescence analysis with an anti-Flag antibody revealed that mutant Cdk9 had no longer the nucleo-cytoplasmic distribution previously described, but accumulated at PML bodies like wild type Flag-Cdk9 (Figure 27A).

This localization suggested that Cdk9 K44,48R could still bind the PML protein. In order to verify this finding, we transfected 293T cells with Flag-Cdk9, either wild type or mutated, together with PML-IV, then after immunoprecipitation with anti-Flag antibodies bound proteins were analyzed by western blot with anti-PML antibodies. Flag-Cdk9 K44,48R co-immunoprecipitated with transfected PML-IV with an efficacy similar to wild type Cdk9 (Figure 27B).

Finally we found that, besides wild type Cdk9, also K44,48R Cdk9 could be partially re-localized into the insoluble nuclear matrix fraction upon transfection of both wild type GCN5 or its catalytically inactive mutant (Figure 27C).

These results suggest that the interaction between Cdk9 and GCN5 (which, as already shown, is independent from the HAT activity of GCN5), and not Cdk9 acetylation, targets Cdk9 to PML bodies. There, GCN5-mediated acetylation of the catalytic pocket of Cdk9 might ensure inhibition of P-TEFb activity and, at the same, time sequestration in the nuclear bodies compartment.

GCN5 interacts with PML

The idea that GCN5 might target Cdk9 to PML bodies is further supported by the fact that GCN5 itself, like other cellular HATs, such as p300 and CBP (Marcello et al., 2003; von Mikecz et al., 2000), interacts with the PML protein

We found that transfected GCN5 co-fractionated with PML in the insoluble nuclear matrix (Figure 25A) and that PML immunodepletion removed a high percentage of GCN5 from cell extracts (Figure 26B). Having therefore observed that GCN5 had a speckled distribution within the nucleus (Figure 17C), we wondered whether the compartments in which it resides has any relationship with PML bodies. For this purpose we transfected cells with either HA-GCN5 or PML-IV expressing vectors or both and performed double immunofluorescence with anti-GCN5 and anti-PML antibodies.

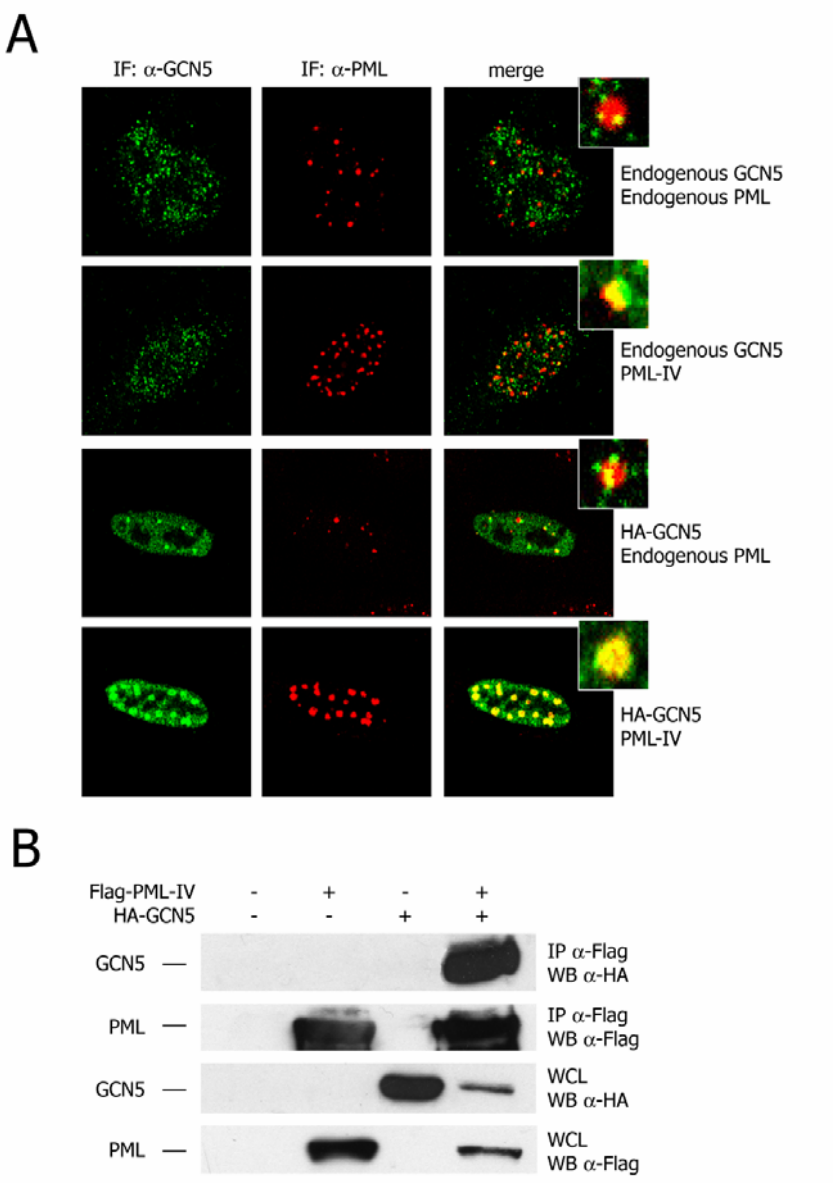

Figure 28. GCN5 interacts with PML. (**A**) GCN5 co-localizes with PML. U2OS cells were transfected with indicated plasmids and subject to double immunofluorescence with antibodies against GCN5 and PML. (**B**) GCN5 binds PML-IV *in vivo*. Extracts prepared from cells transfected with indicated plasmids were immunoprecipitated with an anti-Flag antibody and immunoblotted with anti-HA antibody. The same extracts were run on an SDS-PAGE gel and

immunoblotted with anti-HA and anti-Flag antibodies to verify protein expression levels (lower two panels).

We found that endogenous GCN5 localized in many small foci inside the nucleus, whereas transfected GCN5 became more diffuse in the nucleus but still accumulates in few larger foci. In both situations, GCN5 foci partially co-localized with PML bodies (Figure 28A, first and third rows). This co-localization increased after transfection of PML-IV (Figure 28A second row) and became almost complete when HA-GCN5 and PML-IV were both transfected (Figure 28A fourth row).
The interaction between GCN5 and PML was confirmed by co-immunoprecipitations experiments. Flag tagged PML-IV was expressed in 293T cells together with HA-GCN5, followed by immunoprecipitation using an anti-Flag antibody and immunoblotting with an anti-HA antibody. As shown in the upper panel of Figure 28B, HA-GCN5 specifically co-precipitated with Flag-PML-IV, but not with the control pFlag empty vector. The same extracts were run on an SDS-PAGE gel and immunoblotted with an anti-Flag and anti-HA antibody to verify protein expression levels.
From this set of experiments we concluded that GCN5 interacts with PML *in vivo* inside nuclear bodies.
In conclusion, we have found that Cdk9 can be targeted to PML bodies independently from its acetylation, that GCN5 can localize at PML bodies and that both acetylation and accumulation at PML bodies inhibit Cdk9 transcription.

Selective binding of acetylated Cdk9 to the HIV-1 promoter and HIV-1 genome during transcriptional latency

In order to understand the physiological relevance of Cdk9 acetylation, we exploited HIV-1 transcription as a model system that is highly dependent on Cdk9 kinase activity. In fact, the activation of the HIV-1 long terminal repeat (LTR) promoter by the viral transactivator Tat is blocked by concentrations of P-TEFb inhibitors that do not affect regular cellular transcription (Chao et al., 2000; Chao and Price, 2001; Flores et al., 1999).

In particular we focused on the regulation of HIV-1 latency by exploiting the U1 monocytic cell line, a well defined model of post-integration latency (Lusic et al., 2003). This cell line contains two copies of the integrated provirus, which, under basal conditions, produce almost undetectable levels of HIV-1 mRNA; transcription is markedly induced (over 100 fold) by exposure to different mitogens, including phorbol esters (TPA);Figure 29A.

To determine whether transcriptional activation by TPA induces a modification of Cdk9 acetylation *in vivo* at the HIV-1 genome, aliquots of untreated and TPA-treated U1 cells were analyzed in parallel by chromatin immunoprecipitation (ChIP). Formaldehyde crosslinked, sonicated chromatin fragments from U1 cells were immunoprecipitated using antibodies directed against RNAPII, Ser2P-RNAPII, total Cdk9 and Ac-Cdk9.

Six different genomic sites were investigated. One region (B13) maps on chromosome 19, 5 kb away from the lamin B2 origin in a region not containing any gene and therefore used here as a negative control (Todorovic et al., 2005); while the other five map to contiguous regions in the HIV-1 proviral DNA: two in the promoter (PPR and Nuc-1A), and three in the coding region at the 5' end of the genome (U1A, U1B and U1C); Figure 29B.

In particular the two regions on the HIV-1 promoter have been chosen because they have been extensively studied in relation to the transcriptional state of HIV-1. Experiments performed both *in vivo* (El Kharroubi et al., 1998; Van Lint et al., 1996; Verdin et al., 1993) and *in vitro* (Sheridan et al., 1997; Van Lint et al., 1996) using the HIV-1 promoter reconstituted into chromatin have shown that, independent from the integration site, nucleosomes at the 5' LTR are precisely positioned with respect to cis-acting regulatory elements and define two large nucleosome-free areas. The first one (PPR) is composed of the core promoter, containing three tandem Sp-1 binding sites and the TATA box sequence, and of the LTR enhancer, which is the target for the p50/p65 NF-kB heterodimer; the same region also contains the binding sites for other transcription factors including Ets-1 and USF. The second open area spans the primer-binding site immediately downstream of the 5' LTR. These two open regions are separated by a single nucleosome called nuc-1 (Nuc-1A) that is positioned in the close proximity of the transcription start site

and is specifically and rapidly destabilized during transcriptional activation (El Kharroubi et al., 1998; Van Lint et al., 1996; Verdin et al., 1993). We decided to extend our ChIP analysis also to the three regions inside the HIV-1 genome because, as described in the Introduction, Cdk9 activity is required not only at promoters but also within coding regions.

In uninduced conditions, RNAPII was present on the promoter region but not inside the HIV-1 genome (Figure 29C); most of this polymerase was not phosphorylated on Ser2 (Figure 29D). Following TPA induction, the levels of both total RNAPII and Ser2-phosphorylated RNAPII increased on the promoter region; of interest, Ser2P RNAPII was also found distributed inside the proviral genome (Fig. 21C and D). Phosphorylation of RNAPII Ser2 is known to be essentially catalyzed by Cdk9 (Ni et al., 2004; Shim et al., 2002). Consistent with this notion, the levels of Cdk9 associated with both the promoter and the proviral genome were significantly increased upon TPA induction, thus paralleling those of RNAPII Ser2P (Figure 29E). Most strikingly, by using the specific anti-Ac-Cdk9 antibody, we observed that, in uninduced cells, a large amount of catalytically inactive, acetylated Cdk9 was associated with both the HIV-1 promoter and the HIV-1 genome; acetylated Cdk9 was absent from both regions after TPA stimulation (Figure 29F). Thus, the latent state of HIV-1 is characterized by the recruitment of low levels of RNAPII within the promoter region; this polymerase is not phosphorylated on Ser2 since Cdk9 is kept in an enzymatically inactive state by acetylation. Transcriptional activation is concomitant with an increase in the total levels of recruited Cdk9 and, most notably, in a marked reduction of its acetylated form. These results clearly indicate that acetylation of Cdk9 essentially concurs in maintaining transcriptional latency of the HIV-1 genome.

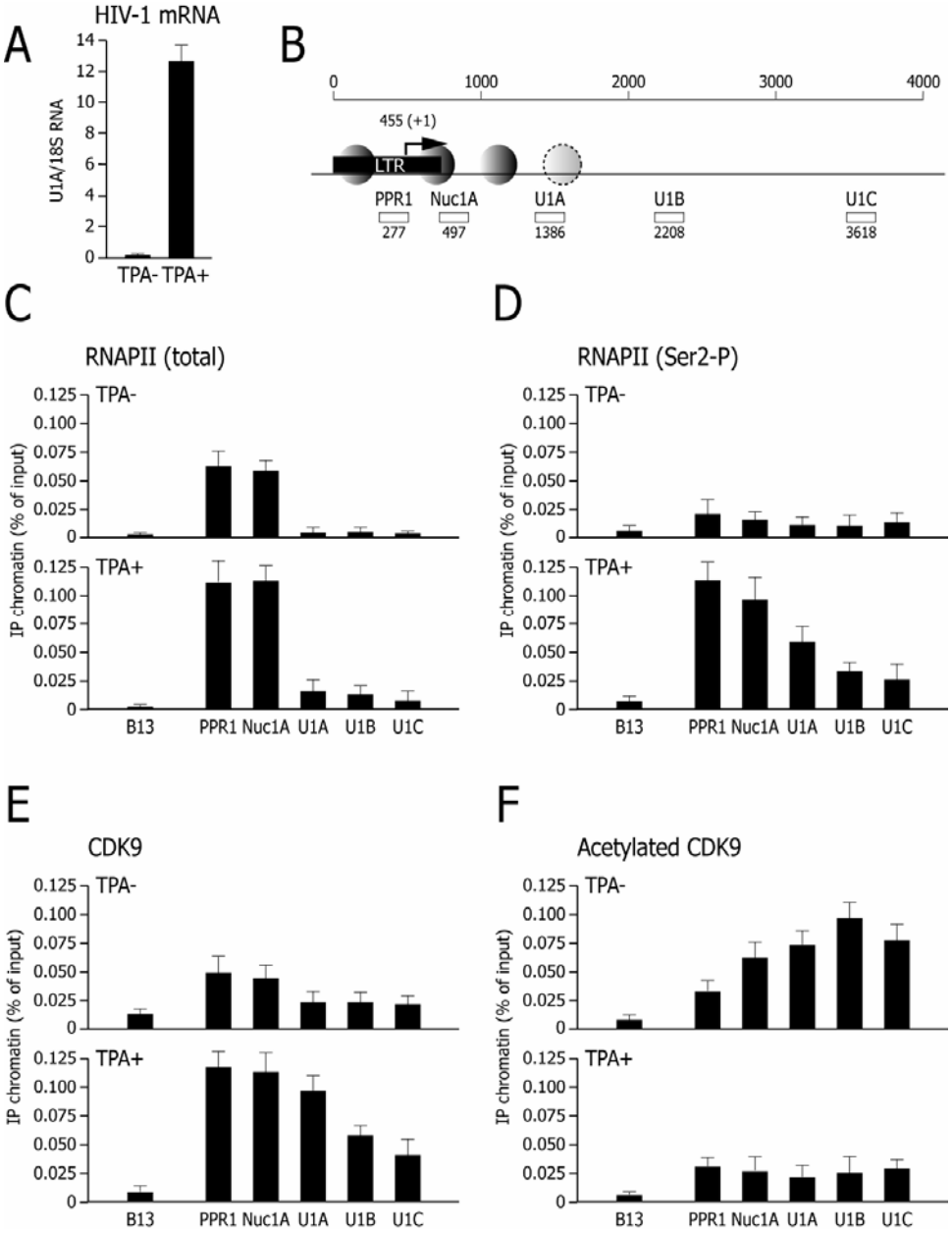

Figure 29. Ac-CDK9 selectively binds HIV-1 genome during transcriptional latency. (**A**) Induction of HIV-1 transcription in U1 cells after TPA stimulation. The levels of HIV-1 mRNA were measured by real time PCR using TaqMan primers and probe corresponding to the Nuc1A region – see panel b; the results are expressed as a ratio between HIV-1 mRNA and the cellular 18S rRNA. (**B**) Positions of primers selected for the amplification of HIV-1 chromatin. The position

of the LTR, including the transcription start site, is indicated, along with the known nucleosomal arrangement at the 5' genome region. **(C-F)** Chromatin immunoprecipitation experiments using antibodies against total RNAPII, RNAPII CTD phosphorylated on serine 2 (RNAPII-Ser2P), total CDK9 and acetylated CDK9 in latent and TPA-activated U1 cells. For each analyzed region, the amount of immunoprecipitated chromatin using the indicated antibodies is shown, expressed as a percentage of input chromatin.

Chapter 4

Discussion

DISCUSSION

One of the essential molecular events that regulate gene expression in eukaryotic cells is the phosphorylation of the carboxy-terminal domain (CTD) of RNA polymerase II (RNAPII), which is required for efficient promoter clearance and transcriptional processivity (Meinhart et al., 2005). Among the different members of the cyclin-dependent kinase family that are known to specifically induce this modification, the P-TEFb complex is mainly responsible for the phosphorylation of Ser2 of CTD, an event representing the hallmark of elongating RNAPII.

Studies performed by using either RNA interference or highly specific inhibitors have indicated that P-TEFb acts as a global co-factor important for most RNAPII transcription (Chao and Price, 2001; Shim et al., 2002). Not surprisingly, therefore, its activity appears to be tightly regulated in different cell types, along differentiation and as part of the cell response to various stimuli (Sano et al., 2002; Yang et al., 1997; Zhou et al., 2000). To achieve this goal, different features of its components, Cdk9 and CyclinT1, are controlled: level of expression, interaction with other proteins, post-translational modifications (i.e. phosphorylation and ubiquitination) and subcellular localization.

Acetylation modifies crucial residues of Cdk9

The work described in this thesis reveals a new mechanism of regulation of P-TEFb that involves both post-translational modification and subcellular localization.

First of all, we demonstrated for the first time that Cdk9 is acetylated *in vitro* and *in vivo* by the GCN5 acetyltransferase. Interestingly, the acetylated lysines of Cdk9 lie in close proximity to the PITALRE amino acid sequence (position 60-66). This region is essentially involved in the formation of the catalytic pocket of Cdk9, and its sequence is highly conserved in all members of the Cdk family (as shown in Figure 13A). Moreover, one of the two acetylated lysines (K48) is conserved in the so-called subdomain II of almost all the eukaryotic protein kinases (conserved in over 95% of 370 sequences), and hence essential for the enzyme function (Hanks and Hunter, 1995). Consistent with this notion, conservative mutations of Cdk9 lysines 44 and 48 into arginines greatly impaired Cdk9 enzymatic activity.

Structural data about Cdk2 indicate that the conserved subdomain II lysine is essentially involved in orienting the ATP phosphate residues as well as in magnesium binding within the catalytic pocket of the enzyme (De Bondt et al., 1993).

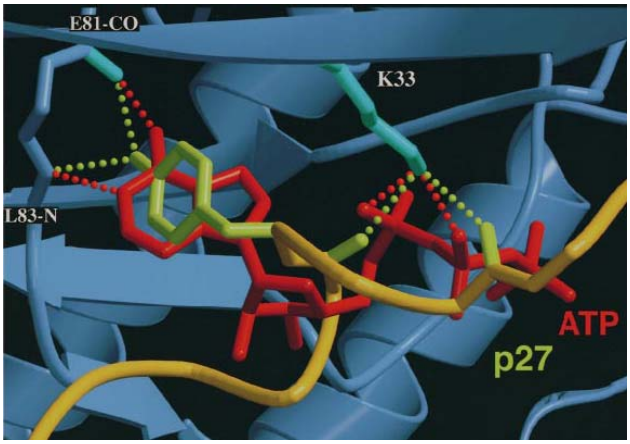

Figure 30. CDK2 catalytic pocket. Adapted from (Pavletich, 1999) .

Not surprisingly, we found that the mutant Cdk9 K44,48R had reduced affinity for the ATP analog, FSBA. FSBA was previously used to map the ATP-binding sites of various proteins, including PKA and smooth muscle myosin light chain kinase (Komatsu and Ikebe, 1993; Zoller et al., 1981). The FSBA reactive residues were subsequently shown to play crucial roles in the phosphotransfer reaction (Madhusudan et al., 1994; Taylor et al., 1992). As a matter of fact FSBA contains a crosslinking group in the position analogous to the γ-phosphate of ATP (Kamps et al., 1984), the transfer and correct positioning of which are probably regulated by the conserved lysine of subdomain II of protein kinases.

In both types of experiments (kinase assay and FSBA binding) the mutant Cdk9 K44,48R maintains a residual activity, while we expected a complete loss of activity. We have still to consider that we introduced a conservative mutation of lysines to arginines while a mutation to alanines would probably have had more profound effects. In keeping with this notion, the K-R mutation in human sphingosine kinase 1 reduced the enzymatic activity to 30%, while the K-A mutation completely abolished it (Pitson et al., 2002). Moreover we have to keep in

mind that even if the subdomain II lysine is very important for the kinase activity, there could be other lysines that might compensate for it. Two examples of situations in which this event occurs are the catalytic subunit of *S. cerevisiae* cAMP dependent protein kinase (Gibbs and Zoller, 1991) and the large subunit of *herpes simplex* virus type 2 of ribonucleotide reductase (ICP10) (Nelson et al., 1996).

Acetylation inhibits Cdk9 kinase activity

As described in the Introduction, acetylation has been reported to modulate DNA binding (Gu and Roeder, 1997; Marzio et al., 2000; Yao et al., 2001), protein-protein interactions (Bannister et al., 2000; Cohen et al., 2004; Kovacs et al., 2005; Yuan et al., 2005), protein stability (Bernassola et al., 2004; Jeong et al., 2002; Li et al., 2002) and subcellular localization (Blander et al., 2002; Kawaguchi et al., 2006; Kitamura et al., 2005; Thevenet et al., 2004) and the consequence of acetylation on protein function may vary from one protein to another depending on where within the protein the acetylation takes place.

Since acetylation neutralizes the positive charge of the ε-amino group of lysines that are positioned in the catalytic pocket of Cdk9 and are involved in ATP binding, it was not surprising to find that overexpression of GCN5 (and the subsequent increased acetylation of Cdk9) inhibited P-TEFb kinase activity on the RNAPII CTD.

This is not the first case of acetylation occurring on residues that lie in the catalytic site of an enzyme: acetylation of a lysine positioned in the catalytic center of Acetyl-CoA synthetase inhibits its enzymatic activity both in *S. enterica* (Starai et al., 2002) and in mammals (Hallows et al., 2006; Schwer et al., 2006), similar to what also happens in the PTEN phosphatase (Okumura et al., 2006). *Yersinia* YopJ acetylates serines and threonines in the activation loop of MAPKK6 thereby blocking their phosphorylation and the activation of the kinase (Mukherjee et al., 2006).

Anyway, Cdk9 is the first example of regulation of a Cdk by acetylation and the observation that one of the lysines that becomes acetylated in Cdk9 is strictly conserved in all members of the family suggests that this mechanism of regulation

might play a broader role in controlling the function of other members of this kinase family. As a matter of fact preliminary observations in our laboratory indicate that some Cdks involved in cell cycle control are indeed acetylated by GCN5. These findings may open a new field of investigation, since GCN5 appears to be involved in the control of many cell-cycle related genes (Kikuchi et al., 2005).

Acetylation does not impair the binding of Cdk9 to its regulatory partners

Even if acetylation of Cdk9 modifies the catalytic site of the enzyme, it does not seem to affect its interaction with CyclinT1 and HEXIM1. As a matter of fact the amount of CyclinT1 and HEXIM1 co-precipitated with Flag-Cdk9 did not change upon GCN5 overexpression, supporting the notion that acetylation affects Cdk9 catalytic activity rather than modulating interaction with its known regulatory partners.

Regarding this point, it is worth mentioning that the catalytic pocket of Cdks, inside which Cdk9 acetylated lysines reside, is completely folded only after the binding to the cyclin partner (Pavletich, 1999). Cdks activation has been understood in structural detail from X-ray crystallographic studies of human Cdk2 in various states of activity.

The active site of Cdk2 is located in a cleft between two lobes of the kinase. ATP binds deep within the cleft, with its phosphates oriented outward. The protein substrate would normally interact with the entrance of the active-site cleft, but this region is obscured in the inactive Cdk2 monomer by the T-loop. Key residues in the ATP-binding site are also misoriented in the Cdk2 monomer, further suppressing its activity (Figure 31A). Cyclin A binding has a major impact on the conformation of the Cdk2 active site (Figure 31B). Several helices in the cyclin box contact both lobes of Cdk2 in the region adjacent to the active-site cleft, resulting in extensive conformational changes in Cdk2.

The most obvious change occurs in the T-loop, in which the L12 helix has been changed into a beta strand, and which no longer occludes the binding site for the protein substrate but lies almost flat at the entrance of the cleft. Major changes also

occur in the ATP-binding site, leading to the correct positioning of the ATP phosphates for the phosphotransfer reaction.

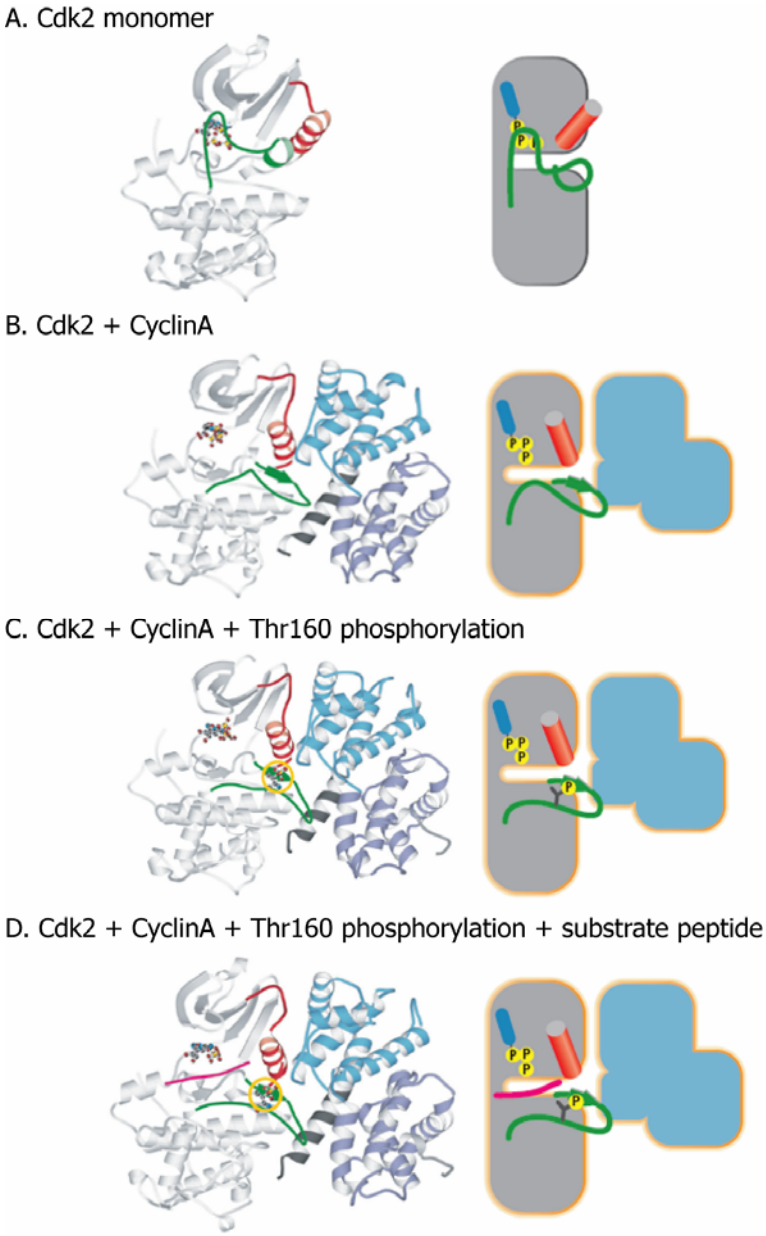

Figure 31. The structural basis of Cdk activation. These diagrams illustrate the structure of human Cdk2 in various states of activity. In each case, the complete

structure is represented in the left column, while the right column provides schematic views that emphasize key substructures, including the ATP in the active site, the T-loop (green) and the PSTAIRE helix (red).

The T-loop of Cdk2 contains Thr 160, the threonine residue whose phosphorylation by the Cdk-activating kinase (CAK) further increases the activity of the Cyclin A–Cdk2 complex. After phosphorylation, the phosphate on Thr 160 is inserted in a cationic pocket and acts as the central node for a network of hydrogen bonds spreading outward to stabilize neighbouring interactions in both the Cdk and Cyclin. The T-loop is flattened and moves closer to Cyclin A (Figure 31C), and this region serves as a key part of the binding site for protein substrates (Figure 31D).
This data indicate that the conserved lysine in the catalytic cleft of Cdks, namely K48 for Cdk9, is partially occluded by the T-loop until the cyclin has bound the kinase, as shown in Figure 32. Therefore, CyclinT1 binding (and maybe also T-loop phosphorylation) might be necessary in order to completely expose the K48 residue of Cdk9 to GCN5-mediated acetylation.

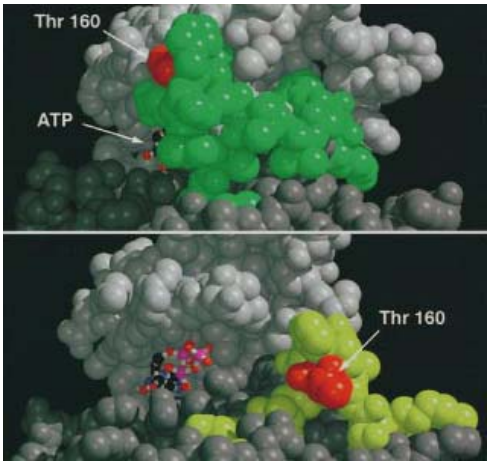

Figure 32. Close-up views of Cdk2 T loop, before and after Cyclin A binding. The T loop of Cyclin A-bound Cdk2 is highlighted in yellow while that of free Cdk2 is green. Thr160, which is phosphorylated by CAK, is colored red (Pavletich, 1999).

The fact that CyclinT1 binds Cdk9 also in case of GCN5 transfection (and thus Cdk9 iper-acetylation), together with these structural data, suggest that acetylation occurs on the already formed CyclinT1/Cdk9 heterodimer and does not disrupt it.

Additional biochemical experiments are required to further characterize the relationship between Cdk9 folding and acetylation.
We have not tested the effect of Cdk9 acetylation on the binding to Brd4 that has a bromodomain and therefore could be a good candidate for increased interaction after acetylation, since *in vitro* binding experiments indicated that actually Brd4 binds CyclinT1 and not Cdk9 (Jang et al., 2005). Interestingly, Cyclin T1 has been recently found in a proteomic search of acetylated proteins (Kim et al., 2006) therefore we can speculate that acetylation of CyclinT1, in contrast to that of Cdk9, has an activating effect on P-TEFb activity increasing the binding to the positive factor Brd4.

Acetylation re-localizes Cdk9 at PML bodies

We demonstrated that Cdk9, like its main cyclin partner Cyclin T1, accumulates at nuclear bodies upon PML overexpression through direct interaction with the PML protein.
Despite the attention paid to these structures, the function of PML bodies is still not fully known. One of the proposed role for PML bodies is to be sites of post-translational modification and degradation of proteins. Observations supporting this model include the acetylation (Pearson et al., 2000), phosphorylation (D'Orazi et al., 2002) and sumoylation (Fogal et al., 2000; Kwek et al., 2001) of p53 at PML bodies and the localization of ubiquitin/proteasome associated proteins at some PML bodies (Anton et al., 1999; Everett et al., 1997; Lafarga et al., 2002; Lallemand-Breitenbach et al., 2001).
Acetylated Cdk9 co-localizes and co-precipitated with PML to a higher extent in comparison with total Cdk9 (mostly not acetylated) indicating that Cdk9 acetylation increases its affinity towards the PML bodies compartment. Whether acetylation of Cdk9 might be the consequence or the cause of the re-localization in PML bodies still remains to be completely determined since we have not rule out whether PML promotes Cdk9 acetylation like it does with p53 (Pearson et al., 2000) and p73 (Bernassola et al., 2004).

However, these data suggests that acetylation actually occurs preferentially inside PML bodies:

- Cdk9 K44,48R mutant re-localizes at PML bodies upon PML-IV overexpression even if it cannot be acetylated;
- both wild type and K44,48R Cdk9 can be re-localized in the nuclear matrix fraction by GCN5 catalytically active or inactive;
- GCN5, like other cellular acetyltransferases, localizes at PML bodies.

Thus we hypothesized that the interaction with either GCN5 or PML protein may target Cdk9 to PML bodies; then if Cdk9 is acetylated by GCN5, this modification strengthens the binding between Cdk9 and PML and entraps Cdk9 in the nuclear matrix compartment probably in a transcriptionally silent context. In keeping with this model, our laboratory has previously described the inhibitory effect of accumulation in PML bodies for CyclinT1 mediated transcription (Marcello et al., 2003). Other examples of proteins that are targeted at PML bodies upon acetylation are the transcription factor FoxO1 (Kitamura et al., 2005) and the DNA helicase Werner (Blander et al., 2002).

Indeed PML bodies have been proposed to be a regulated nuclear depot where proteins such as co-activators, co-repressors or transcription factors are accumulated and released upon specific signals (Li and Chen, 2000; Negorev and Maul, 2001; Zhong et al., 2000). By titrating these factors from the active pool in the nucleoplasm, the PODs could interfere with transcription, resulting in either activation or repression. Both Cdk9 and FoxO1 are transcriptionally inactive upon acetylation and the subsequent accumulation at PML bodies (Kitamura et al., 2005), but in the case of FoxO1, acetylation probably precedes and is required for the localization at PML bodies since the non acetylable mutant of FoxO1 cannot localize at PML bodies.

PML bodies are not the only transcriptionally repressive compartment where acetylated protein can be targeted. The PGC-1α transcription factor is directly acetylated by GCN5 resulting in a transcriptionally inactive protein. This proteins re-localizes to nuclear foci characterized by the presence of the nuclear co-repressor RIP140, but different from PML bodies (Lerin et al., 2006).

Thus even if acetylation (of both histones and transcription factors) generally potentiates transcription, Cdk9 is not the first transcription factor to be inhibited by acetylation. Acetylation of NF-kB p65 (Kiernan et al., 2003), HMGI(Y) (Munshi et al., 2001), IRF7 (Caillaud et al., 2002), AFX (Foxo4) (Fukuoka et al., 2003) Brm (Bourachot et al., 2003) and PGC1α (Lerin et al., 2006) turns off transcription, which may serve as a feedback mechanism to control the duration of transcription.

What is the functional consequence of Cdk9 acetylation?

Several reports have suggested that there should be a post-recruitment regulation of Cdk9 kinase activity on promoter and coding regions and that simple recruitment of P-TEFb does not imply phosphorylation of RNAPII on Ser2.

In vivo chromatin immunoprecipitation assays demonstrated that, during transcription, Cdk9 is present both on promoter and coding regions of different genes, however Ser2P/RNAPII increases towards the 3' of the genes (Ahn et al., 2004; Boehm et al., 2003; Cho et al., 2001). On the human p21 gene (Gomes et al., 2006), low amounts of P-TEFb bind the 5' part of the coding region also in conditions when RNAPII is not phosphorylated on Ser2 (Gomes et al., 2006). Additionally, Cdk9 is present in *in vitro* reconstituted HIV-1 pre-initiation and elongation complexes, but RNAPII CTD Ser2 is phosphorylated only in the last ones (Isel and Karn, 1999; Marshall et al., 1996; Marshall and Price, 1995; Ping and Rana, 1999; Ping and Rana, 2001; Zhou et al., 2001).

In particular, it seems that, on the HIV-1 promoter, a number of mechanisms concentrate high amounts of inactive P-TEFb in close proximity of its substrates, ready to be activated. First, an inhibitory intramolecular interaction between the N- and C-terminal regions of CyclinT1, which prevents the ternary complex assembly, is relieved by the binding of the transcription elongation factor Tat-SF1 to the C-terminal region in CyclinT1 (Fong and Zhou, 2000). Second, the presence of TFIIH in the HIV-1 PIC inhibits Cdk9 phosphorylation and its subsequent kinase activity on RNAPII CTD; as TFIIH is released from the elongation complex, Cdk9 phosphorylation is observed (Zhou et al., 2001). In addition, the ubiquitylation of Cdk9 by Skp2 recruited by Tat increases the TAR-Tat-P-TEFb complex assembly

(Barboric et al., 2005). All these mechanisms contribute to ensure a tight control of the promoter clearance: RNAPII should be phosphorylated by Cdk9 only after the cap is positioned on 5' of the mRNA in order to avoid wasteful rounds of transcription of uncapped mRNAs (Cho et al., 2001).

Here we found that in conditions of HIV-1 latency, when proviral transcription is very low and Ser2P RNAPII is virtually absent from HIV-1 genome, there is a certain amount of catalytically inactive, acetylated Cdk9 already bound to the HIV-1 genome. After TPA-mediated transcription activation, Cdk9 can be found associated with both the promoter and the proviral genome together with RNAPII Ser2P accordingly to literature (Boehm et al., 2003; Bres et al., 2005; Cho et al., 2001; Gomes et al., 2006), whereas Ac-Cdk9 becomes absent from both regions. Taken together, these results suggest that Cdk9 might be recruited onto the genome as an acetylated protein, which is then specifically deacetylated (or exchanged with not acetylated?) when transcription is activated. Several data indicate that the inhibition of Cdk9 kinase activity by flavopiridol or DRB does not impair its recruitment onto DNA (Barboric et al., 2001; Gomes et al., 2006; Ni et al., 2004), so it is conceivable that acetylated Cdk9 can be also recruited to DNA given that it can still bind CyclinT1. Since histone acetylation is continually reversed by deacetylases in order to ensure rapid turnover of chromatin acetylation states (Topalidou et al., 2003; Yamagoe et al., 2003), this mechanism could be active also on non-histone proteins such as Cdk9. Therefore, finding the specific Cdk9 deacetylase and the mechanism that controls its activity will help to understand also the functional role of Cdk9 acetylation.

How all these findings fit with the well established notion of P-TEFb being recruited on HIV-1 promoter by Tat when transcription is activated?

Work from other laboratories has shown that P-TEFb is not present on transcriptionally silent HIV-1 genome (Bres et al., 2005; Kim et al., 2006) nor is recruited when transcription is induced by a mutated Tat protein (C22G) incapable of binding P-TEFb (Kim et al., 2006).

One possible explanation for the differences between these results and ours can be ascribed to the cell lines used: Jurkat cells carrying an integrated lentiviral vector expressing GFP for Kim et al., HeLa P4 containing the lacZ gene under the

control of the integrated HIV-1 LTR for Bres et al. and U1 cells for our experiments. U1 cells contain two integrated HIV proviruses, each with mutant *tat*: one blocked by the deletion of a translational initiation codon and the other encoding a form of Tat containing histidine to leucine mutation at amino acid 13 (Emiliani et al., 1998). This mutation causes a decrease in the transactivation potency of the mutant Tat protein, however, it has been shown that higher levels of Tat can compensate for the defect (Adams et al., 1994; Emiliani et al., 1998) suggesting that it probably retains some ability to bind P-TEFb. In unstimulated conditions, U1 cells are not completely silent, but express low levels of short, incomplete HIV-1 transcripts reflecting the impaired Tat activity (Adams et al., 1994; Adams et al., 1999). Thus we can speculate that this leakiness of transcription regulation can allow the recruitment of small amounts of Cdk9 on HIV-1 genome, but not bypass the inhibitory effect given by acetylation of Cdk9.

HIV-1 Latency

In essence, Cdk9 acetylation contributes to the understanding of the molecular determinants of HIV-1 latency – an issue of major relevance and overall interest.
Indeed, the establishment of a latent infection represents the major obstacle to HIV-1 eradication. The main reservoir is composed of latently infected resting memory $CD4^+$ T cells that carry an integrated provirus that is transcriptionally silent (Chun et al., 1995; Chun et al., 1997). The extremely long half-life of these cells, combined with a tight control of HIV-1 expression, make this reservoir ideally suited to maintain hidden copies of the virus, which are in turn able to trigger a novel systemic infection upon discontinuation of therapy. Given the importance of this reservoir, a lot of effort has been invested to characterize these cells from infected patients and also to understand the molecular determinants that allow the provirus to remain silent. Such mechanisms are mostly related to transcriptional control of viral expression and thus depend on the chromatin environment at the site of viral integration and on the interaction of the viral Tat transactivator with host factors.
In respect to the second point it is worth mentioning that, in resting T-cells, P-TEFb kinase activity is low and that it increases in response to activating stimuli (Ghose

et al., 2001; Herrmann et al., 1998). Moreover we demonstrated that, when the integrated provirus is transcriptionally silent, the low amount of P-TEFb bound to the HIV-1 genome is catalytically inactive because acetylated. On the other hand, in resting T-cell, general transcription activators such as Sp1 are available to bind the HIV-1 LTR, therefore the initiation of transcription is not completely inhibited but, in absence of the Tat protein and of an active P-TEFb complex, transcription is mostly non-processive. As a matter of fact the presence of short, non-polyadenylated promoter-proximal transcripts has been directly demonstrated in highly purified resting $CD4^+$ T-cells from patients on HAART (Lassen et al., 2004).

Taken together these results suggest that, in resting T-cell, there is not an absolute block at the level of transcription initiation. Rather there is a relative block, with transcriptional initiation occurring with reduced efficacy due to the absence in resting cells of crucial host transcription factors and the viral protein Tat. The absence of Tat and active P-TEFb is largely manifest at the subsequent step of transcriptional elongation and the result is the production of abortive rather than full length transcripts.

In this context it will be interesting to address the relationship between Cdk9 acetylation and the kinase activity of P-TEFb in cells that are differently susceptible to HIV-1 infection (monocytes/macrophages and T-lymphocytes).

Chapter 5

Additional findings

DISSECTION OF PROTEIN-PROTEIN INTERACTIONS THAT GOVERN HIV-1 TRANSCRIPTION BY BIOPHYSICAL METHODS.

Introduction

Tat-associated factors and HIV-1 transcription

Understanding the molecular mechanisms controlling silencing and reactivation of the HIV-1 provirus at its integration site within the host cell genome has profound implications for both the elucidation of the HIV disease pathogenesis and for its pharmacological control (Cohen and Fauci, 2001; Finzi et al., 1999). Such mechanisms are mostly related to transcriptional control of viral gene expression and thus depend on the chromatin environment at the site of viral integration and on the interaction of the viral Tat transactivator with host factors. Transcriptional activation occurs essentially through Tat-mediated modulation of chromatin conformation and transcriptional elongation. These processes in fact are regulated by different protein complexes assembled by Tat at the 5' of each viral transcripts.

In particular through direct interaction with the CyclinT1 protein, Tat recruits the P-TEFb complex and therefore promotes hyper-phosphorylation of RNAPII CTD and transcriptional processivity. Interestingly the RelA/p65 subunit of NF-kB (Barboric et al., 2001) as well as the Sp1 transcription factor (Yedavalli et al., 2003), have also been shown to interact with P-TEFb. The recruitment of P-TEFb by these two factors might be responsible for the first rounds of HIV-1 transcription that occurs prior to the synthesis of Tat.

In addition to its functional interaction with P-TEFb, Tat associates with different factors that possess acetyltransferase activity. These factors include the transcriptional co-activators p300 and the highly homologous cyclic adenosine monophosphate (cAMP)-responsive binding protein (CREB)-binding protein (CBP) (Marzio et al., 1998), the p300/CBP-associated factor (P/CAF) (Benkirane et al., 1998), the general control nonderepressible-5 (GCN5) factor (Col et al., 2001), the TIP60 protein (Kamine et al., 1996), and the general transcription factor TAFII250 (Weissman et al., 1998). Chromatin immunoprecipitation experiments at the HIV-1 promoter clearly indicate that transcriptional activation by Tat induces acetylation of

H3 and H4 histones in this region, which precedes the actual onset of transcription. Histone acetylation is paralleled by the recruitment of specific acetyltransferases to the promoter, which is cell-type specific and depends on the stimulus used for transcriptional activation (Lusic et al., 2003). Finally, it is of interest that, besides histones, Tat itself is a substrate for acetylation by p300/CBP, P/CAF, and GCN5 (Col et al., 2001; Deng et al., 2000; Kiernan et al., 1999; Ott et al., 1999). Tat is acetylated by P/CAF on Lys28 and by p300 and hGCN5 on Lys50. The acetylation of Tat has been proposed to regulate two discrete and functionally critical steps in transcription, binding to P-TEFb and release of Tat from TAR RNA (Bres et al., 2002; Dorr et al., 2002; Kaehlcke et al., 2003).

Since cellular factors play a critical role in HIV-1 transcription, it is conceivable that HIV-1 gene expression undergoes the same controls as cellular gene expression. In particular the transient assembly and disassembly of multi-protein complexes involved in nuclear processes, such as transcription, in mammalian cells appear to be highly ordered and spatially defined processes.

For example, it has becoming widely accepted that genes whose proteins work together, and therefore needs to be co-expressed, are transcribed in the same "transcription factory" within the nucleus (Osborne et al., 2004).

An approach to the understanding of the spatial regulation of HIV transcription is the visualization of the subnuclear dynamics of the proteins involved in this process and the study of their reciprocal interactions. These two issues can now be tackled on a submicrometric scale, by the exploitation of high-resolution optical spectroscopy approaches for the visualization of proteins labeled with fluorescent tags. Most of these recent techniques rely on the properties of the green fluorescent protein (GFP) from the jellyfish *A. victoria* (Tsien, 1998). Variants of this autofluorescent protein are continually being engineered with improved optical properties that are exploited in a variety of techniques (Lippincott-Schwartz et al., 2001; Nagai et al., 2002). In particular, protein–protein interactions can be visualized in a single cell by fluorescence resonant energy transfer (FRET) (Day et al., 2001; Selvin, 2000; van Roessel and Brand, 2002).

Fluorescence Resonance Energy Transfer (FRET)

FRET is a physical phenomenon in which energy absorbed by a fluorophore is transferred to another molecule through a nonradiactive pathway. For resonance energy transfer to occur, two specific conditions must be met. (i) The emission spectrum of the fluorophore, also called the donor, must overlap the acceptor molecule's absorbance spectrum. (ii) Donor and acceptor molecules must reside within 10 nm (100 Å) of each other.

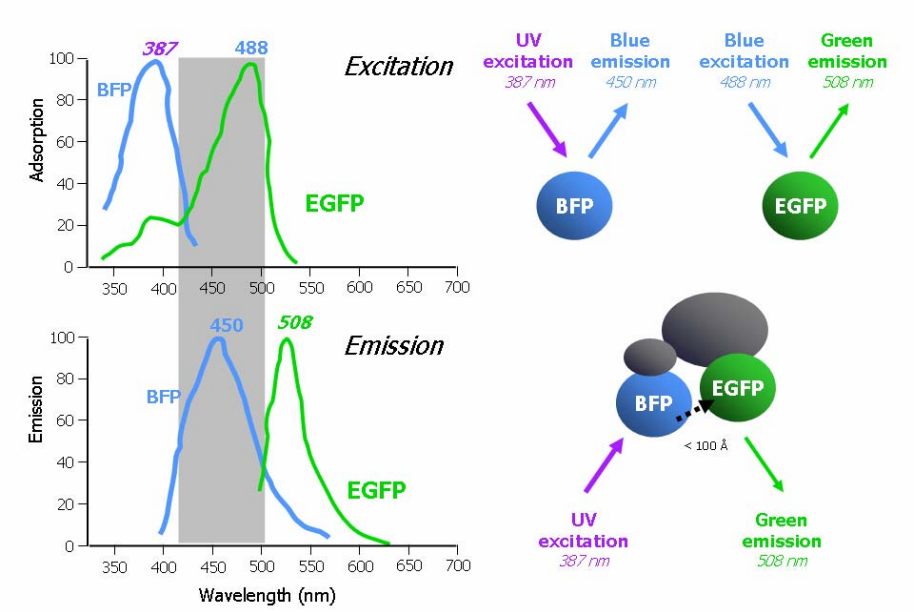

Figure 33. EGFP and EBFP spectra.

Among the fluorophore, pairs with spectral characteristics optimal for FRET are those formed by some variants of the green fluorescent proteins (GFP), namely the CFP (cyan)/YFP (yellow) and the BFP (blue)/EGFP (green) pairs. Technically, the visualization of fluorescence from ECFP/EYFP is easier, due to the higher temporal stability of the photon emission associated to the reduction of photobleaching effects. However, the determination of FRET efficiency between these two proteins requires careful measurement due to the extensive overlapping of their emission spectra (Bastiaens and Pepperkok, 2000; Lippincott-Schwartz et al., 2001). In contrast, the emission of BFP and EGFP overlaps only minimally. Thus, the FRET efficiency can be calculated as a simple ratio of the emission intensities of the

acceptor excited within its absorption band and at resonance with the donor absorption (Marcello et al., 2001). Visualization of BFP-tagged proteins, however, is difficult when compared to other GFP-fusion proteins as the BFP rapidly converts to a dark (non-emissive) state after photoexcitation.

Förster showed that the efficiency of resonance energy transfer, or FRET efficiency (the fraction of the photon energy absorbed by a fluorescent molecule that is transferred to an acceptor through dipole-dipole interactions), has a sixth power dependence on the separation distance between the donor and the acceptor. It is this strong dependence on separation distance that allows FRET imaging to be used to study molecular interactions on a 1- to 10-nm scale. For the green fluorescent protein (GFP):blue fluorescent protein (BFP) pair, the Förster radius (the distances at which FRET efficiency is 50%) is less then 4 nm; this implies that simple co-localization of two proteins is not sufficient to yield energy transfer (Heim and Tsien, 1996; Selvin, 2000; Tsien and Miyawaki, 1998).

FRET presents several advantages in comparison with other techniques employed to detect protein–protein interactions. Firstly, the presence of FRET actually indicates protein–protein interaction at distances of a few nanometers for many pairs of optically matched fluorophores. Secondly, the use of gene-encoded GFP variants allows the visualization of interactions in real time and in living cells (Janetopoulos and Devreotes, 2002; Jiang and Sorkin, 2002; Majoul et al., 2001; Mayr et al., 2001). Thus, the physiological conditions of the experiments can be varied in order to assess whether the formation of a specific protein complex is influenced by external stimuli. Thirdly, FRET actually determines not only ‘‘if’’ but also ‘‘where’’ proteins interact inside the nucleus (Jiang and Sorkin, 2002; Marcello et al., 2001). This is of particular value, especially given the increasing importance ascribed to the compartmentalization of biological processes *in vivo*, as also outlined above.

Intramolecular FRET

Intramolecular FRET can be measured when both the GFP donor and the acceptor are fused to the same host molecule and it allows to detect dynamic conformational changes of proteins with spatiotemporal resolution. FRET based indicators have

been developed to measure several ions, cyclin nucleotides, metabolites, neurotransmitters, the balance between protein kinases and phosphatases activities, and activities of proteases, small G proteins, and histone acetyltransferases.

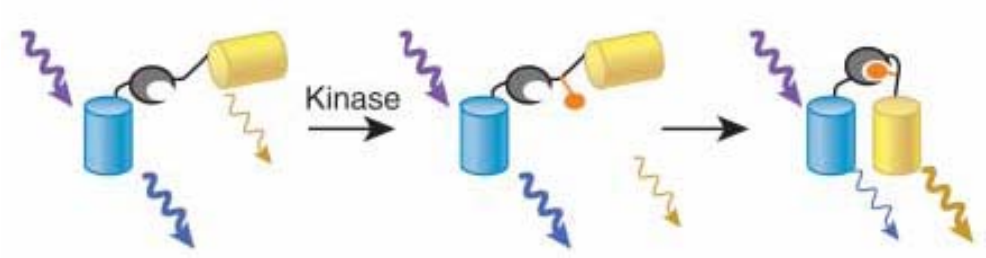

Figure 34. FRET sensor (indirect). A substrate undergoes modification (1) (e.g. phosphorylation, orange dot), causing a conformational change (2) and altering FRET from CFP to YFP.

One of the first demonstration of this technique was performed by Mitra et al. (Mitra et al., 1996) who fused a BFP and GFP in the same molecule, separated by a flexible polypeptide linker containing a factor X_a protease cleavage site. When incubated with factor X_a, cleavage of the linker was followed by a decrease in FRET.

One example of FRET-based calcium biosensor are “camaleons” which consist of a tandem fusion of CFP, Ca^{2+}-calmodulin (CaM), M13 peptide and YFP. Upon an increase in Ca^{2+} concentration, the CaM component of the hybrid protein binds Ca^{2+} and preferentially wraps around the fused M13 peptide. This conformational change results in a decrease in the distance between the two GFP variants, and therefore an increase of FRET (Miyawaki et al., 1997).

Intermolecular FRET

Intermolecular FRET can occur when the GFP donor and the acceptor are on different macromolecules. In these conditions FRET can detect dynamic protein-protein interaction in live cells, provided that the fluorescent proteins get within 6-8 nm of each other. Recently, the possibility of using three fluorescent proteins to study higher order complexes has been addressed by adding a monomeric red fluorescent protein (mRFP) to the CFP/YFP pair. In trimeric complexes, CFP is the

FRET donor for YFP; subsequently, YFP can act as a FRET donor for mRFP. 3-FRET has been shown in multiprotein complexes and in protein trimerization (Galperin et al., 2004; He et al., 2005).

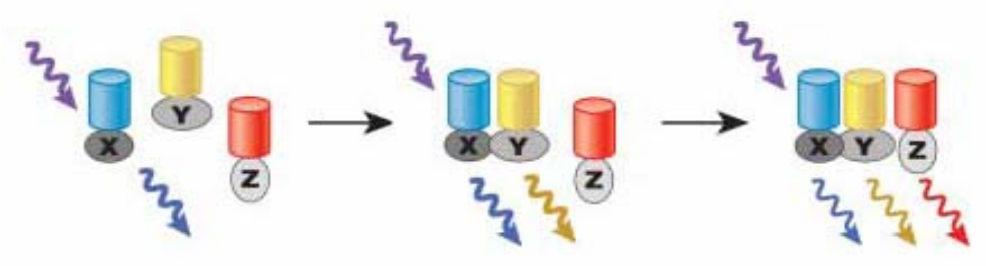

Figure 35. Intermolecular FRET/ 3-FRET. Binding of X to Y (1) leads to CFP-YFP FRET. Additional binding of Z (2) makes RFP an acceptor for X/Y.

An early example of GFP-based intermolecular FRET occurred with Bcl-2 and Bax, which play a crucial role in apoptosis, as well as in the indication and progression of human cancer. A direct interaction in the mitochondria between Bcl-2 and Bax was shown when a GFP fused to the N-terminus of Bcl-2 and a BFP fused at the N-terminus of Bax were co-expressed in the same cell (Rampino et al., 1997; Yin et al., 1997).

FRET has now become one of the preferred methods for studying protein-protein interactions and validate structural details revealed by x-ray crystallography and electron microscopy in living cells. Not surprisingly, the number of PubMed-listed citations using FRET has increased 10-fold over the past 10 years (Vogel et al., 2006).

We have therefore exploited the application of high-resolution confocal microscopy and FRET analysis to study the subcellular localization and structural conformation of different proteins involved in HIV transcription and their reciprocal interactions.

How Is FRET measured?

Numerous methods have been used for measuring FRET (Jares-Erijman and Jovin, 2003; Sekar and Periasamy, 2003). FRET methods can be divided into four fundamental categories: (i) methods that monitor changes in donor fluorescence; (ii) methods that examine changes in acceptor fluorescence; (iii) methods that

simultaneously measure changes in both donor and acceptor fluorescence using spectral imaging; and (iv) methods that monitor changes in the orientation of the fluorophores.

The most popular methods used for measuring FRET involve monitoring acceptor emission as a result of donor excitation (Gordon et al., 1998). This technique is commonly referred to as the three-cube method because it involves acquiring three different images using three fluorescent filter sets. First, a FRET image is generated by using a filter set that excites the donor but measures emission from the acceptor. Images obtained with filter sets that measure emission from donors or acceptors when directly excited are then used to correct the “FRET image.” These corrections are required because the emission of the donor can bleed through into the FRET image and because excitation wavelengths that are used to excite donors can excite acceptors in some cases.

Materials and Methods

Plasmids

EGFP-Cdk9 and Cdk9-EGFP were obtained by PCR amplification of Cdk9 from pcDNA3-Cdk9-his and cloning as an EcoRI/BamHI fragment into pEGFP-C1 and pEGFP-N1 respectively (Clontech).

pcDNA3-Tat-EBFP, pcDNA3-EGFP-CyclinT1 and pEGFP-PML-IV were kindly provided by A. Marcello (Trieste) (Marcello et al., 2001; Marcello et al., 2003).

The plasmid pCycT300-EGFP, carrying EGFP at the 3' end, was obtained by PCR amplification of the N terminus of CyclinT1 and cloning as an EcoRI/BamHI fragment into pEGFP-N1 (Clontech).

The plasmid pBFP-CycT300-EGFP, carrying BFP at the 5'-end and GFP at the 3'-end, was obtained by PCR amplification of the previous construct pCycT300-EGFP and cloning as a HindIII/SalI fragment into pBFPC1 (Clontech).

pGEX-P/CAF(Y760D) and pGEX-P/CAF(Y761D) were obtained by PCR mutagenesis from the template pGEX-P/CAF, kindly provided by M. Benkirane (Montpellier, France). EGFP-tagged derivatives were obtained by subcloning the EcoRI/XhoI fragment from the pGEX constructs into the EcoRI/SalI sites of pEGFP-C2 (Clontech).

FRET

In the experiments described in this thesis, we transfected epithelial cells (HL3T1) by the calcium phosphate method with the various expression plasmids fused to the optically-matched fluorescent tags in four-chambers of glass slides. After 48 h we fixed cells in 4% paraformaldehyde and mounted them in 70% glycerol for FRET analysis.

FRET analysis was performed in two steps. Firstly, enhanced green fluorescent protein (EGFP) emission was collected by integrating the fluorescence signal around 520 nm (bandwidth 40 nm) under direct EGFP excitation at 480 nm (wavelength selection was obtained by 40 nm band-pass filters, excitation power was 5 W/cm2). Secondly, EGFP emission in the same frequency range was measured after excitation of EBFP at 350 nm (power density 2W/cm2 and

bandwidth 60 nm). Background was detected out of the cell under study for each frame and subtracted from the relevant fluorescent signal. Following this procedure, the ratio between the two measured EGFP emissions (data taken following excitation at 350 nm divided by those at 480 nm) provides the FRET signal. Fluorescence was collected by a PentaMax 512-EFT intensified CCD camera with detection times of the order of 0.1 s (in particular, for data taken under excitation at 350 nm they were five times longer than for those relative to 480 nm excitation). Data acquisition and analysis were performed with the Metamorph software from Universal Imaging Corporation, a subsidiary of Molecular Devices. When evaluating FRET ratios, emission intensities were scaled to take into account the different detection times.

Results and Discussion

Cdk9 directly interacts with PML, but not with Tat

CyclinT1, the main Cdk9 partner, has been initially discovered as a cofactor of the human immunodeficiency virus type 1 (HIV-1) Tat transactivator (Wei et al., 1998). Previous studies from the laboratory exploited high resolution fluorescence resonance energy transfer (FRET) to visualize and quantitatively analyze the direct interaction between Tat and CyclinT1 inside the cells (Figure 36). They found that CyclinT1 resides in specific subnuclear foci which are in close contact with nuclear speckles and that Tat determines its redistribution outside of these compartments. Consistent with this observation, strong FRET was observed between the two proteins both in the cytoplasm and in regions of the nucleus outside of CyclinT1 foci and overlapping with Tat localization (Marcello et al., 2001).

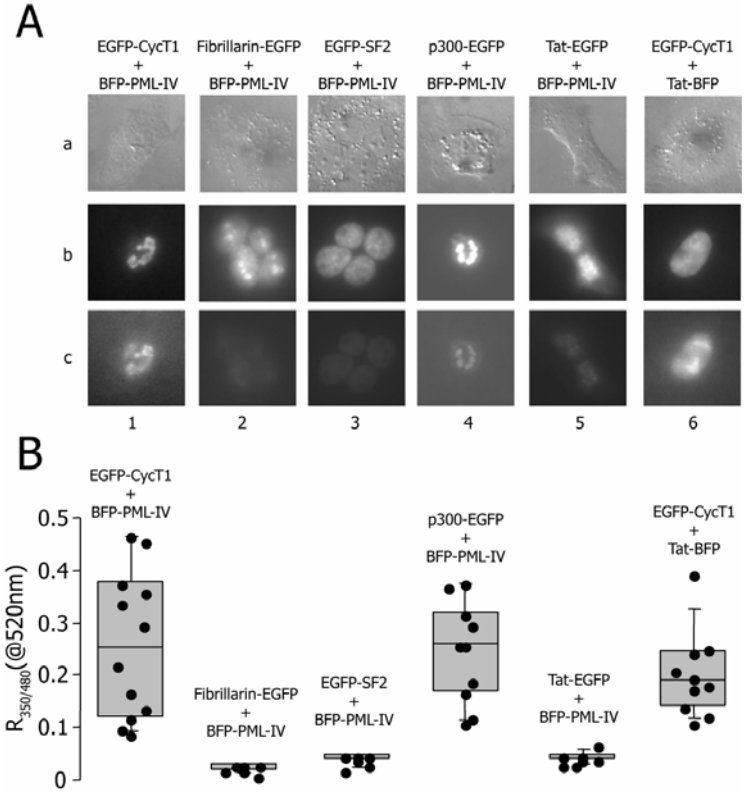

Figure 36. FRET analysis of CyclinT1-Tat-PML interactions (Marcello et al., 2003).

The subnuclear foci in which CyclinT1 resides are coincident with promyelocytic leukaemia (PML) bodies (Marcello et al., 2003) and FRET experiments proved that CyclinT1 physically interacts *in vivo* with the PML protein within these compartments. Whereas Tat expression determines CyclinT1 redistribution outside of these compartments and promotes transcriptional activation (Marcello et al., 2001), CyclinT1 or PML overexpression forces CyclinT1 and also Tat in nuclear bodies and correlates with an inhibition of transcription (Marcello et al., 2003).

In light of our new data on the relationship between Cdk9 and PML, we decided to further extend the FRET analysis at this couple of proteins.

Therefore we co-transfected cells with EGFP-Cdk9 and EBFP-PML-IV. Panels in row b of Figure 37A show the intracellular distribution of fluorescence at 520 nm (the peak wavelength of EGFP emission) under excitation at 480 nm. In these conditions, most cells transfected with EGFP-Cdk9:EBFP-PML-IV showed the characteristic pattern already observed in other experiments and consisting of large nuclear foci of PML-IV and Cdk9. FRET analysis was performed by comparing EGFP emission at 520 nm, following EBFP excitation at 350 nm (Figure 37A, panels in row c), with that following excitation at 480 nm of the same cells (Figure 37A, panels in row b). In these conditions, samples expressing both EGFP-Cdk9 and EBFP-PML-IV scored positive for FRET, indicating direct interaction between the two proteins. The detailed, quantitative analysis of at least 10 cells expressing each of the analyzed protein pairs is presented in Figure 37B, showing the percentile distribution of FRET values. FRET between PML and Cdk9, is not restricted to nuclear bodies but also occurs in the nucleoplasm. This is in agreement with previous data about the interaction between Cyclin T1 and PML, which showed positive FRET values in both compartments (Marcello et al., 2003).

In contrast, no FRET signal was obtained between Cdk9 and Tat even if we tried three different couples of tagged protein: EGFP-Cdk9:Tat-EBFP, Cdk9-EGFP:Tat-EBFP and EBFP-Cdk9:Tat-EGFP (figure A, columns 1, 2, 3). This result is in accord with literature data reporting the interaction between CyclinT1, and not Cdk9, and Tat and provides us a good negative control for the FRET analysis.

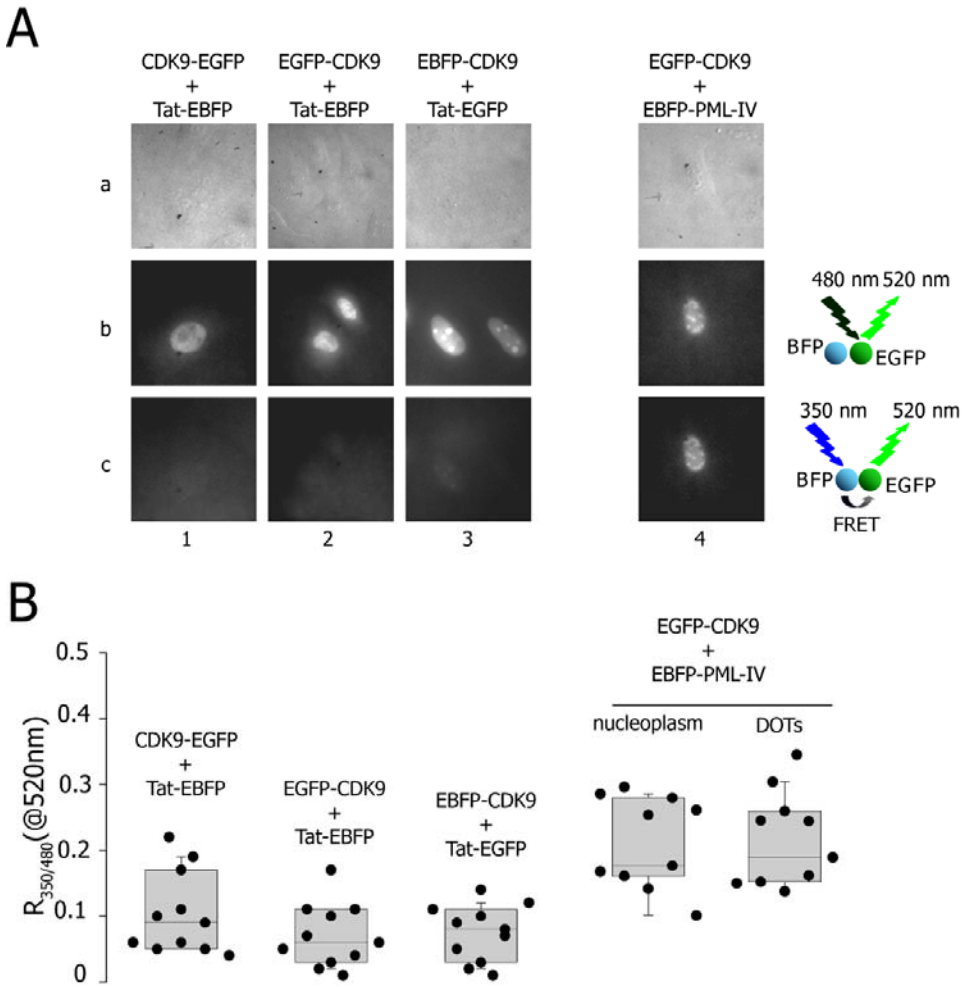

Figure 37. FRET analysis of CDK9-Tat and CDK9-PML interaction.

A structural model of the N-terminus of CyclinT1

Cyclin T1 can be grossly sub-divided into two major domains. The N terminus of the protein (aa 1–300) shares partial homology with other members of the Cyclin T family (cyclin box) (Wei et al., 1998). It contains the Tat recognition motif (TRM) that includes a critical cysteine residue at position 261 present in human CyclinT1, but absent in its rodent homologue (Bieniasz et al., 1998; Garber et al., 1998). The N-terminus domain of CyclinT1 is also important for the protein–protein associations with Cdk9, NF-kB, CIITA and c-Myc (Barboric et al., 2001; Eberhardy and Farnham, 2001; Kanazawa et al., 2000). The C-terminus of CyclinT1 is less characterized and contains a putative coiled-coil region (aa 379–430); a histidine-rich domain (aa 506–530) and a carboxy-terminus PEST sequence (aa 700–726). The C-terminus of CyclinT1 is involved in the association with the RNAPII CTD (Fong and Zhou, 2000; Taube et al., 2002) and the PML protein (Marcello et al., 2003).

At present, no three-dimensional structure of Cyclin T1 is available. Therefore, in collaboration with the Physics Laboratory of Scuola Normale Superiore (F. Beltram, A. Ferrari) and the International School for Advanced Studies (ISAS) and INFM-DEMOCRITOS Modeling Center for Research in Atomistic Simulation in Trieste (P. Carloni, S. Pantano), we have used bioinformatics tools to model the N-terminus (aa 27–263) of CyclinT1, that is the region responsible for binding to Cdk9 and Tat. Since the cyclin box is highly conserved among cyclins, we build a structural model of CyclinT1 based on the X-ray structure of Cyclin H, another member of the cyclin family involved in transcription, whose X-ray structure was available. This model suggested that the N- and C- termini parts of the CyclinT1 cyclin box should be in close proximity. Therefore we took advantage of the complex interactions between the HIV-1 Tat protein and CyclinT1 *in vivo* to validate this prediction. Specifically, we estimated the distance between the N and C termini of the cyclin box by measuring the fluorescence resonance energy transfer (FRET) between fluorescent proteins genetically fused to the N- and C-termini of CyclinT1 cyclin box and to the C-termini of Tat in living cells.

As a first attempt, we tried and provide an estimation of this proximity by intra-molecular FRET in cells transfected with a plasmid expressing CycT1(1-300)

tagged at the N-terminus with the BFP and at the C-terminus with EGFP. FRET image analysis of individually transfected cells are shown in Figure 38A, column 3. FRET analysis was performed by comparing EGFP emission at 520 nm (the peak wavelength of EGFP emission), following BFP excitation at 350 nm (Figure 38A, panels in row c), with that following excitation at 480 nm of the same cells (Figure 38A, panels in row b). As it can be seen from Figure 38, in this case no FRET signal was obtained.

We then proceed to investigate this proximity by intermolecular FRET, taking advantage of the fact the CyclinT1 TRM is located around position 261, immediately after the cyclin box. We therefore co-transfected human HL3T1 cells with plasmids expressing CyclinT1, tagged at the N-termini with EGFP, and Tat tagged at the C-termini with BFP. Most cells transfected with EGFP-CyclinT1:Tat-BFP (Figure 38, panel b1) showed the characteristic nuclear punctuated pattern of CyclinT1 (Herrmann and Mancini, 2001; Marcello et al., 2001). Samples expressing both EGFP-CyclinT1 and Tat-BFP scored positive for FRET. This suggests that the distance between the N-terminus of Cyclin T1 (where is EGFP) and the TRM region (where Tat binds) is small enough for FRET to occur. Based on these findings, we tested whether fusing GFP at the C terminus of CyclinT1 cyclin box provides FRET with Tat-BFP. Cells transfected with CycT300-EGFP:Tat-BFP (Figure 38, column 2) showed a nucleo-cytoplasmic distribution of CycT300-EGFP at 520 nm under excitation at 480 nm. The difference of distribution of the protein with respect to the wild-type CyclinT1 reflected the lack of the carboxy-terminal domain of the protein, which was responsible for the punctuated nuclear localization through binding to the PML protein (Marcello et al., 2003). EGFP emission at 520 nm, following BFP excitation at 350 nm, scored positive for FRET, substantiating the fact that Tat-BFP was within 4 nm to the N and C termini of CycT1(1 – 300). The detailed, quantitative analysis of at least 10 cells expressing for the protein pairs is presented in Figure 38B, showing the percentile distribution of FRET values. We consider the signal from cells transfected with EGFP and BFP (background) as baseline.

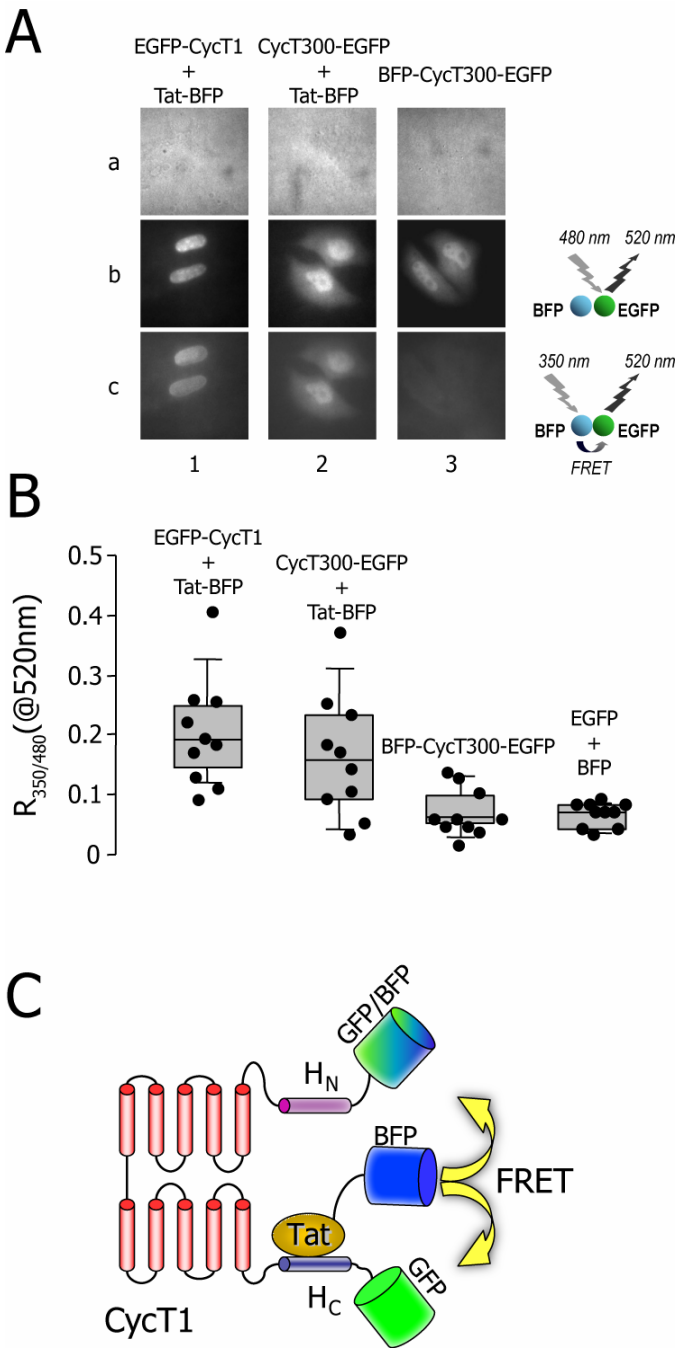

Figure 38. CyclinT1 theoretical structure validation by FRET analysis.

Thus, by measuring FRET between EGFP-CycT1:Tat-BFP and CycT1-EGFP:Tat-BFP complexes we are able to confirm spatial proximity of the N- and C-terminus of cyclin box of CyclinT1 in living cells.

In conclusion, the model presented here is consistent with all experimental data up to the best of our knowledge. However, it must be kept in mind that it suffers the obvious limitations and uncertainties of comparative modeling, particularly at low sequence identity levels. Still, we believe that it may be useful to test working ideas and inspire new biochemical experiments aimed to clarify structural features of protein–protein interactions in which CyclinT1 is involved.

Insights into HIV-1 Tat:P/CAF bromodomain interaction

Besides histones, Tat itself is a substrate for acetylation by p300/CBP, P/CAF, and GCN5 (Col et al., 2001; Deng et al., 2000; Kiernan et al., 1999; Ott et al., 1999).

P/CAF was able to bind to the cysteine-rich region of non-acetylated Tat and acetylated Lys28 within the transcription activation domain of Tat. This post-translational modification abrogated the interaction between P/CAF and Tat, but significantly enhanced the recruitment of P-TEFb by Tat (Bres et al., 2002; Kiernan et al., 1999). The acetylation site of p300/CBP, as well as hGCN5, has been mapped to Lys50 (and, weakly, to Lys51), a highly conserved amino acid within the TAR RNA-binding domain of Tat. This second post-translational modification leads to the dissociation of P-TEFb-Tat from TAR RNA and subsequent binding to the elongating RNAPII (Col et al., 2001; Kaehlcke et al., 2003; Kiernan et al., 1999; Ott et al., 1999). In addition, acetylation of Tat on Lys50 by p300/CBP also served as a signal for the bromodomain of P/CAF to recruit this transcriptional co-activator to the elongating RNAPII. This results in the formation of a P-TEFb-Tat-P/CAF ternary complex associated with the elongation complex during transcriptional elongation (Bres et al., 2002; Dorr et al., 2002; Kiernan et al., 1999; Mujtaba et al., 2002).

Structural insights into the binding mode of P/CAF to Lys50 acetylated Tat were provided by determination of the NMR structure of the peptide SYGRAcK50KRRQRC (that corresponds to the Tat residues 46 to 55, with an acetyl group on lysine 50) in complex with the acetyllysine (AcK)-binding domain of P/CAF (Mujtaba et al., 2002). P/CAF consists essentially of two domains, a catalytic domain with histone acetyltransferase activity, which does not play any role in Tat binding, and an acetyllysine-binding domain, termed the bromodomain (BD). The relatively small association constant estimated for the P/CAF BD:AcK50 Tat peptide complex suggests that important interactions may arise by additional contacts present only in the full-length Tat protein.

Therefore, starting from this BD/peptide structure, we used molecular dynamics simulation techniques to construct a model of the full-length AcK50 Tat:P/CAF BD interaction and then we provide a functional validation of this model inside the cells by using FRET experiments (Pantano et al., 2006).

The model predicted that, in addition to most of the interface contacts found in the NMR structure, a large part of the protein–protein interaction surface is formed by hydrophobic contacts between the core domain of Tat and one loop of P/CAF. In particular, two tyrosine residues of P/CAF are predicted to play a crucial role in Tat binding: Y760, which interacts with AcK50 Tat, and Y761, which forms hydrophobic contacts with the Tat core domain.

To experimentally ascertain the relevance of Tyr760 and Tyr761 in maintaining the interaction between AcK50Tat and P/CAF, we selectively mutated them to aspartate, since the calculations data suggested that the change from a hydrophobic to a hydrophilic amino acid should affect the interaction of the protein with the hydrophobic core of Tat.

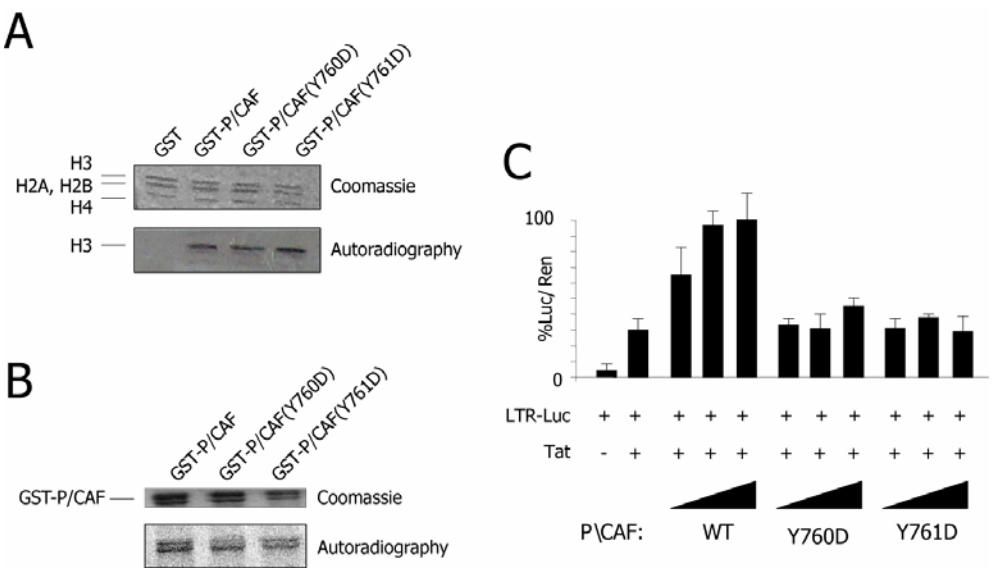

Figure 39. Functional characterization of the P/CAF mutants

Functional characterization of the P/CAF mutants

Wild-type P/CAF and the P/CAF mutants Y760D and Y761D were obtained as recombinant proteins fused to GST, and their enzymatic activity was tested. As shown in Figure 39A, both the wild-type protein and the two mutants, but not unfused GST, were able to acetylate histones, and, in particular, H3. In addition, all three proteins displayed auto-acetylation activity (Figure 39B). These observations

clearly indicate that the Y760D and Y761D mutations do not impair the enzymatic activity of P/CAF. Next, expression vectors were obtained for the two mutants, as well as for the wild-type P/CAF, in which the proteins had EGFP fused at their N-terminus. Wild-type EGFP-P/CAF and the two mutants were co-transfected in HeLa cells together with an expression vector for Tat and an LTR-Luciferase reporter. In the presence of suboptimal concentrations of Tat, the HIV-1 promoter was clearly co-activated by wild type P/CAF but not by either of the two mutants (Figure 39C).

Visualization of *in vivo* interaction between P/CAF and Tat by FRET

The lack of functional co-activation of Tat-mediated transcription by the two P/CAF mutants is consistent with the possibility that the two mutations impair binding of the two proteins, either by disrupting the hydrophobic pocket that homes AcK50 (Y760D) or by hindering contacts between the BD and the Tat core (Y761D). Therefore, we set out to directly visualize the binding between Tat and P/CAF inside the cells by FRET using the two proteins tagged with the BFP:EGFP fluorescent protein pair. FRET experiments between P/CAF and Tat were performed by transfection of HeLa cells with plasmids expressing EGFP fused at the C-terminus of wild-type P/CAF or the P/CAF mutants together with Tat-BFP, followed by the analysis of fluorescence at 520 nm (the peak wavelength of EGFP emission). Representative results of these experiments are shown in Figure 40A. The panels in row b show the intracellular distribution of fluorescence at 520 nm under excitation at 480 nm, characteristic of EGFP. FRET analysis was performed by comparing acceptor EGFP emission at 520 nm, following donor BFP excitation at 350 nm (Figure 40A, panels in row c), with that following excitation at 480 nm of the same cells (Figure 40A, panels in row b). In these conditions, the samples expressing both EGFP-P/CAF wild-type and Tat-BFP scored positive for FRET, indicating direct interaction between the two proteins. In contrast, no FRET was observed between Tat and the nucleolar protein fibrillarin, despite the localization of the latter protein in the same compartments as Tat. We can therefore conclude that P/CAF and Tat directly interact within the nucleus of co-transfected cells. Next we extended this experiment to the P/CAF mutants. Both mutations Y760D and

Y761D completely abolished the FRET signal (Figure 40A), indicating that the two proteins were no longer at a distance favorable for FRET. The detailed, quantitative analysis of at least 10 expressing cells for each of the analyzed protein pairs is presented in Figure 40B, showing the percentile distribution of FRET values.

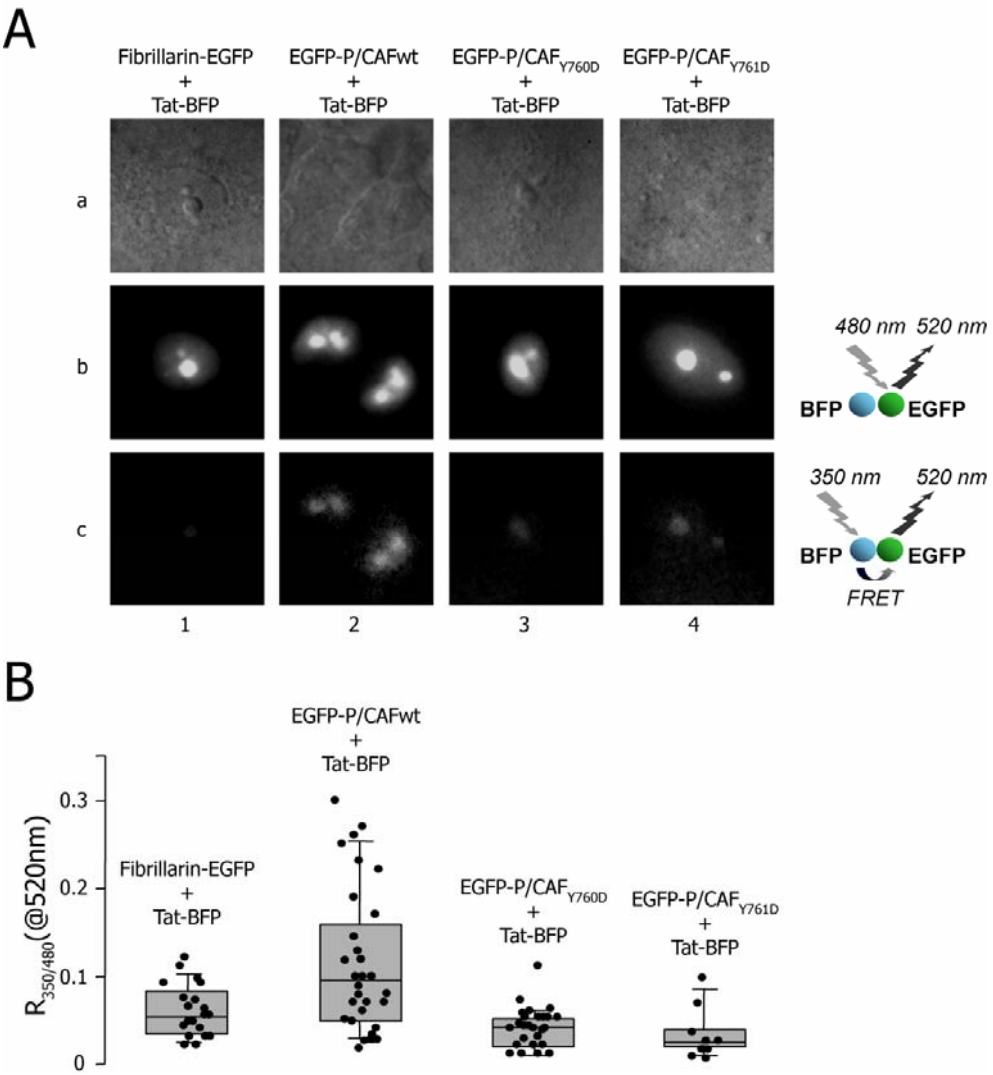

Figure 40. Visualization of *in vivo* interaction between P/CAF and Tat by FRET.

In conclusion the two P/CAF BD mutants at Tyr760 and Tyr761 are both impaired in transactivation and fail to give FRET thus confirming the structural prediction that

these two amino acids are essential to maintain the contacts between Tat acetylated at Lys50 and the P/CAF bromodomain.

Chapter 6

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