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# Cooperative activity of cdk8 and GCN5L within Mediator directs tandem phosphoacetylation of histone H3

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**The human Mediator complex is generally required for expression of protein-coding genes. Here, we show that the GCN5L acetyltransferase stably associates with Mediator together with the TRRAP polypeptide. Yet, contrary to expectations, TRRAP/GCN5L does not associate with the transcriptionally active core Mediator but rather with Mediator that contains the cdk8 subcomplex. Consequently, this derivative 'T/G-Mediator' complex does not directly activate transcription in a reconstituted human transcription system. However, within T/G-Mediator, cdk8 phosphorylates serine-10 on histone H3, which in turn stimulates H3K14 acetylation by GCN5L within the complex. Tandem phosphoacetylation of H3 correlates with transcriptional activation, and ChIP assays demonstrate co-occupancy of T/G-Mediator components at several activated genes *in vivo*. Moreover, cdk8 knockdown causes substantial reduction of global H3 phosphoacetylation, suggesting that T/G-Mediator is a major regulator of this H3 mark. Cooperative H3 modification provides a mechanistic basis for GCN5L association with cdk8-Mediator and also identifies a biochemical means by which cdk8 can indirectly activate gene expression. Indeed our results suggest that T/G-Mediator directs early events—such as modification of chromatin templates—in transcriptional activation.**

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## Introduction

The human Mediator complex is a multisubunit cofactor that regulates RNA polymerase II-dependent transcription (Conaway *et al*, 2005). Although its precise biochemical activity is poorly understood, it is clear that Mediator plays multiple roles in regulating the pre-initiation complex (PIC), which minimally consists of TFIIA, IIB, IID, IIE, IIF, IIH, and RNA polymerase II (pol II) (Hahn, 2004). For example, Mediator interacts directly with pol II and helps recruit this complex to gene promoters (Davis *et al*, 2002; Näär *et al*, 2002); Mediator also stimulates the TFIIH-dependent phosphorylation of the pol II CTD, which is critical for transcription initiation (Kim *et al*, 1994; Akoulitchev *et al*, 2000). Furthermore, Mediator is a general target of DNA-binding transcription factors. Thus, Mediator is believed to function in part by communicating regulatory signals from promoter- and enhancer-bound transcription factors directly to the PIC.

One simple way in which Mediator activity is controlled is through subunit exchange: addition or subtraction of subunits to alter its biochemical function (Taatjes *et al*, 2004; Malik and Roeder, 2005). For instance, a cdk8 subcomplex (consisting of cdk8, cyclin C, Med12 and Med13) can reversibly associate with the 'core' Mediator to inhibit its co-activator function (Taatjes *et al*, 2002; Pavri *et al*, 2005). Notably, core Mediator (~25 subunits and 1.2 MDa in size) has no known enzymatic functions; however, association with the cdk8 subcomplex bestows kinase activity (for example, cdk8) to the complex. Whereas the core Mediator complex strongly interacts with pol II and potentiates activated transcription, the cdk8-Mediator complex does not interact with pol II and is transcriptionally inert (Davis *et al*, 2002; Näär *et al*, 2002; Taatjes *et al*, 2002). Although the mechanism by which cdk8-Mediator inhibits activated transcription is not completely defined, its kinase activity appears to play an important role. In fact, cdk8-dependent phosphorylation of cyclin H (a subunit of TFIIH) can inhibit TFIIH-dependent phosphorylation of the pol II CTD, which facilitates initiation of transcription (Akoulitchev *et al*, 2000). Interestingly, this cdk8-dependent modification does not occur in yeast; rather, an alternate inhibitory mechanism appears to function in yeast whereby cdk8 itself phosphorylates the pol II CTD before PIC assembly (Hengartner *et al*, 1998). Apart from cyclin H and the pol II CTD, however, few cdk8 substrates have been identified in humans.

Although a vast body of literature has demonstrated that the cdk8 subcomplex can negatively regulate transcription, a number of observations suggest that the cdk8 subcomplex may play some roles in activating gene expression (Liu *et al*, 2004; Wang *et al*, 2005; Donner *et al*, 2007). First, activators can interact with either the core Mediator or cdk8-Mediator, suggesting that cdk8-Mediator may play some roles in the activation of transcription (Taatjes *et al*, 2002). Second, although the majority of activators target subunits shared between core- and cdk8-Mediator, a few are known to recruit Mediator directly via the cdk8 subcomplex (Zhou *et al*, 2002; Fryer *et al*, 2004; Kim *et al*, 2006; Wang *et al*, 2006). Third, recent work in human cells suggests that cdk8-Mediator is

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recruited at an early stage in the activation of some genes. For example, at genes regulated by the retinoic acid receptor (RAR) or CEBPβ, recruitment of cdk8-Mediator occurs before activation of transcription; moreover, only upon loss of the cdk8 subcomplex—leaving behind core Mediator—is pol II recruited and transcription activated at these genes (Mo *et al*, 2004; Pavri *et al*, 2005). These results and others (Andrau *et al*, 2006; Zhu *et al*, 2006) suggest that cdk8 recruitment is temporally regulated and can correlate with transcription. However, a biochemical mechanism for cdk8-dependent activation of transcription has remained elusive.

In addition to Mediator and the PIC, histone modifications play major roles in the regulation of transcription (Lee and Workman, 2007; Shahbazian and Grunstein, 2007). Specific histone modifications—such as acetylation and phosphorylation—work to alter chromatin structure and recruit co-activators or co-repressors to specific genomic loci. It is becoming increasingly evident that histone marks work in combinatorial fashion and that modification at one site can promote or antagonize modifications at other sites. One example is tandem S10/K14 phosphoacetylation of histone H3, which correlates strongly with active expression of at least a subset of human genes. Although the precise mechanisms by which tandem H3 phosphoacetylation affect transcription are not completely defined, it is clear that both modifications can stimulate or antagonize recruitment of co-regulatory factors such as 14–3–3 proteins or HP1 (Mateescu *et al*, 2004; Fischle *et al*, 2005; Hirota *et al*, 2005; Winter *et al*, 2008).

Over the years, the identities of many enzymes that post-translationally modify histone substrates have been determined, with most isolated as components of large, macromolecular complexes. These distinct, multi-subunit chromatin-modifying complexes possess many shared subunits. For example, GCN5 is present in multiple chromatin-modifying complexes in yeast and humans (Lee and Workman, 2007). Notably, human GCN5L interacts directly with the 430 kDa TRRAP polypeptide (McMahon *et al*, 2000; Park *et al*, 2001), which is believed to function primarily as a scaffold protein because it has no known enzymatic function but is found in a number of distinct multi-subunit complexes that play varied roles in the cell (Robert *et al*, 2006; Lee and Workman, 2007). Interestingly, the Nut1 subunit of the yeast Mediator complex possesses sequence similarity to GCN5, and yeast Mediator complexes display HAT activity in a Nut1-dependent fashion (Lorch *et al*, 2000). However, the Nut1 subunit is not conserved in higher organisms, and human Mediator is not known to interact with histones in any way.

In this study, we describe the isolation and biochemical activity of a cdk8-Mediator complex that contains the TRRAP and GCN5L polypeptides. Significantly, the cdk8 and GCN5L subunits within this 'T/G-Mediator' complex work cooperatively to catalyze tandem phosphoacetylation of H3 S10/K14, providing a mechanistic basis for GCN5L association with cdk8-Mediator. Because tandem H3 phosphoacetylation is associated with active transcription, these results identify a biochemical role for cdk8 in transcriptional activation. As expected, however, the cdk8-containing T/G-Mediator complex was unable to directly activate transcription in a highly purified, reconstituted human system, suggesting that Mediator itself performs multiple roles in the regulation of gene expression, including potential early events such as post-translational modification of chromatin.

## Results

### *TRRAP/GCN5L stably associate with human Mediator*

This work initiated from an unexpected observation made upon purification of Mediator from partially purified extracts. A typical purification starts with fractionation of HeLa nuclear extract over a phosphocellulose column; Mediator will elute in the P0.5 M and the P1 M fractions. The P1 M fraction is enriched in the core Med26-containing Mediator complex, whereas the P0.5 M fraction is enriched in the larger cdk8-containing Mediator complex (Figure 1A). Further purification using a VP16 activation domain affinity resin indicated that Mediator samples derived from the P0.5 M fraction—but not the P1 M fraction—showed the presence of a polypeptide unique to Mediator that co-migrated with the complex on a glycerol gradient (Figure 1B and data not shown). This polypeptide was of high molecular weight (>300 kDa), enabling its easy detection by gel electrophoresis and silver staining. Notably, we observed similar results using different activation domains for purification from the P0.5 M fraction (data not shown).

To identify this unknown polypeptide, glycerol gradient fractions 15–17 (Figure 1B) were pooled, concentrated by TCA precipitation and separated by SDS–PAGE. A tryptic digest of the unknown band analyzed by mass spectrometry revealed 31 peptide matches to TRRAP/PAF400, a protein found in a number of large, multi-subunit complexes (McMahon *et al*, 1998; Ogryzko *et al*, 1998; Wieczorek *et al*, 1998; Ikura *et al*, 2000). Further MS analysis of other excised bands from the P0.5M-derived Mediator sample identified predicted Mediator subunits, with the exception of one 95 kDa band that was identified as GCN5L (Table I). TRRAP interacts directly with GCN5L and both subunits are found together in other large protein complexes such as TFTC and STAGA (Martinez *et al*, 1998; Wieczorek *et al*, 1998). However, MS analysis did not identify any additional subunits from TFTC/STAGA in these samples. Thus, we suspected that TRRAP/GCN5L might associate with Mediator.

As a first step to investigate a potential TRRAP/GCN5L interaction with Mediator, we generated antibodies against TRRAP and tested whether these antibodies would immunoprecipitate the Mediator complex from HeLa nuclear extracts. As shown in Supplementary Figure 1, Mediator subunits co-immunoprecipitated with the anti-TRRAP antibody. In a reciprocal experiment, TRRAP and GCN5L were observed to co-IP on a cdk8 antibody resin (Supplementary Figure 1). To further test this association, Mediator was affinity-purified from the P0.5 M fraction, eluted and incubated over an anti-TRRAP antibody resin or an anti-Med26 resin. Significantly, the purified Mediator complex specifically bound the anti-TRRAP antibody resin, whereas the same Mediator sample did not bind an anti-Med26 column (compare lanes 2 and 4, Figure 1C). Moreover, Mediator could be eluted from the anti-TRRAP resin with its peptide antigen (Figure 1C, lane 3). Coupled with the MS data (Table I), the results from Figure 1 suggested that TRRAP and GCN5L can stably associate with Mediator.

 

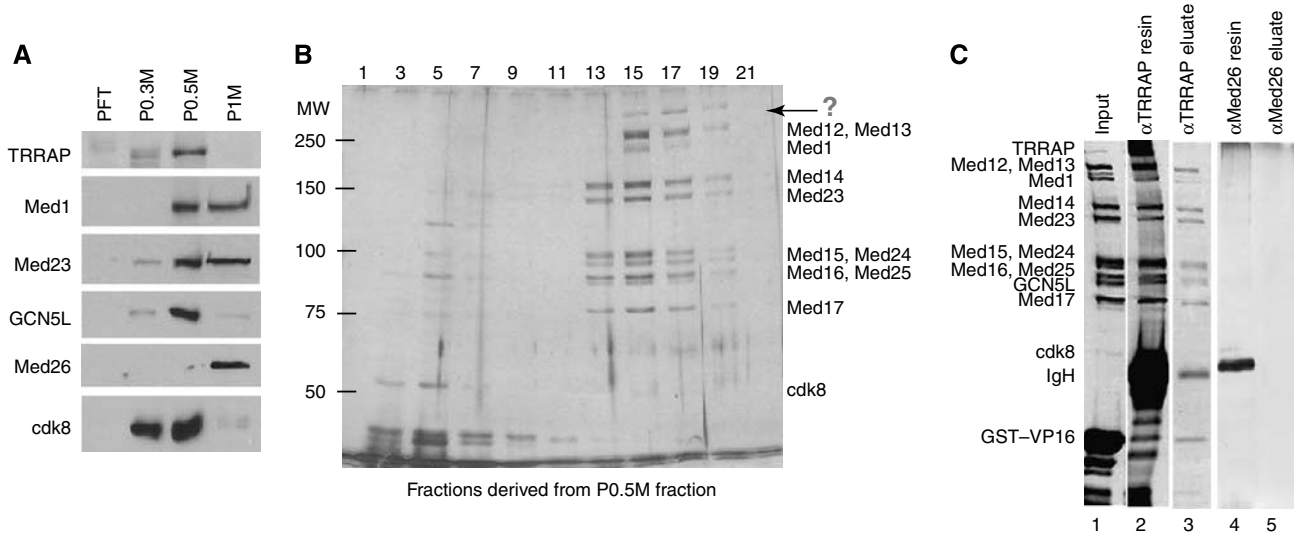


**Figure 1** TRRAP/GCN5L associates with human Mediator. (**A**) TRRAP/GCN5L and cdk8-Mediator are enriched in the P0.5 M fraction; core Mediator is enriched in the P1 M fraction. Each lane (PFT, P0.3, P0.5, P1 M) contained 11 μg total protein. (**B**) Silver-stained gel (7% acrylamide) of glycerol-gradient fractions from VP16 affinity purification. The identities of the subunits are indicated at the right; arrow indicates the obvious 'unknown' co-migrating polypeptide. (**C**) Mediator purified from the P0.5 M fraction (Input, lane 1) was incubated with an anti-TRRAP or anti-Med26 antibody resin. Following extensive washing with 0.5 M KCl HEGN, bound proteins were eluted with TRRAP peptide antigen. Mediator remained bound and was eluted from the anti-TRRAP antibody resin only (lanes 2 and 3). A full-colour version of this figure is available at *The EMBO Journal* Online.

**Table I** MS results from P0.5 M-derived Mediator samples purified using affinity chromatography and glycerol gradient sedimentation

| Identified subunit | Unique peptides |
|---|---|
| TRRAP/PAF400 | 31 |
| Med13 (250) | 15 |
| Med12 (240) | 13 |
| Med1 (220) | 21 |
| Med14 (150) | 18 |
| Med23 (130) | 13 |
| Med24 (100) | 2 |
| Med16 (95) | 5 |
| GCN5L | 8 |
| Med17 (78) | 7 |

Protein bands 70 kDa and larger were excised from a polyacrylamide gel, digested and examined as described in Materials and methods. Data shown are from p53-purified T/G-Mediator; similar results were obtained using VP16 or SREBP-1a activation domains for purification.

### *TRRAP/GCN5L interact with the cdk8-Mediator complex*

Mediator exists in two major forms in human cells: a smaller form that interacts strongly with pol II and activates transcription, and a large form that does not interact strongly with pol II and does not directly activate transcription. Notably, the 'small' and 'large' Mediator complexes differ in their subunit composition: the Med26 subunit preferentially associates with the small, active complex, whereas cdk8, cyclin C, Med12 and Med13 associate with the large Mediator complex (Taatjes *et al*, 2004). Thus, with the exception of Med26, the small Mediator complex represents a core Mediator structure that is present within the large, cdk8-Mediator complex.

To determine whether TRRAP/GCN5L specifically associate with the Med26- or the cdk8-containing Mediator complex (or both), we first examined how TRRAP and GCN5L tracked with Mediator subunits over a series of ion-exchange columns. Although the P0.5 M fraction is enriched in cdk8-Mediator, a small amount of Med26 remains in this fraction (Figure 2A). We can further segregate Med26 from cdk8 on a HiTrap Q column. As shown in Figure 2A, TRRAP, GCN5L, cdk8 and other Mediator subunits co-eluted in the P0.5M/QFT fraction, whereas Med26 was no longer detectable. This suggested that TRRAP/GCN5L associates specifically with the cdk8-Mediator complex. Further purification from the P0.5M/QFT fraction using affinity chromatography followed by glycerol gradient sedimentation indicated that TRRAP and GCN5L co-purified with cdk8-Mediator using this more rigorous purification protocol (Figure 2B–D). As expected, the STAGA subunits SAP130 and SPT3 were not detected in this sample (Figure 2D). Importantly, similar results were obtained using different affinity chromatography resins (for example, SREBP-1a or p53 instead of VP16—see Supplementary Figure 2). In addition, binding assays with anti-Med26 or anti-cdk8 antibody resins indicated specific association of TRRAP/GCN5L with cdk8-Mediator but not core Mediator (Supplementary Figure 3).

As a final means to confirm TRRAP/GCN5L association with the cdk8-Mediator complex, we used electron microscopy (EM). Core Mediator and cdk8-Mediator can be readily distinguished with EM by completing 2D classification of single-particle images, as shown in Figure 3A: the larger cdk8-Mediator complex has a size and shape distinct from core Mediator. As expected, analysis of the purified sample on a gradient (5–15%) polyacrylamide gel indicated the presence of polypeptides specific for cdk8-Mediator (for example, Med12 and Med13), whereas Med26, a subunit specific for core Mediator, was not observed (Figure 3B). This result was substantiated upon EM analysis of the sample: the 2D classes generated from the data revealed complexes of size and shape consistent with cdk8-Mediator, but not core Mediator (representative classes shown in Figure 3C).

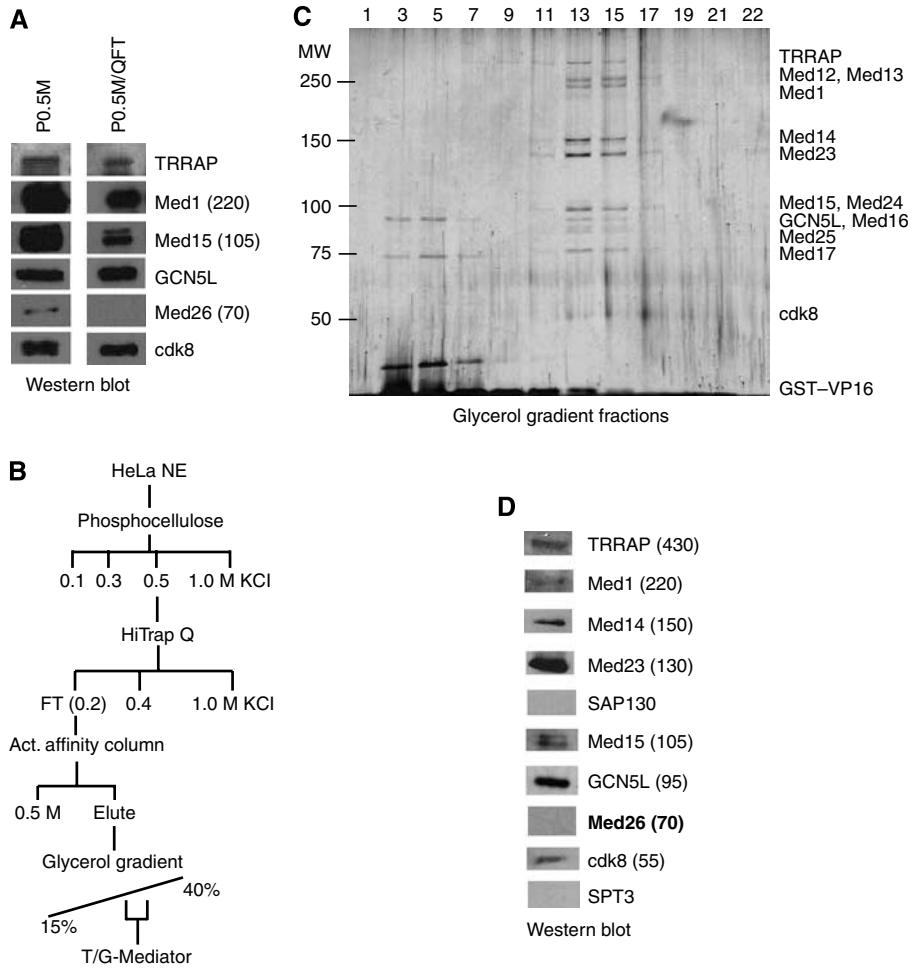


**Figure 2** Purification of T/G-Mediator. (**A**) Western blots against partially purified column fractions. Each lane contained 24 μg total protein. (**B**) Purification protocol used for isolation of T/G-Mediator. (**C**) Silver-stained gel (7% acrylamide) showing glycerol gradient fractions from the purification outlined in (B). Subunit identities are shown at the right. (**D**) Western blots against various subunits from the T/G-Mediator sample. Numbers in parenthesis represent the approximate molecular weight of each protein. Subunits in red are specific to STAGA, whereas Med26 is specific to core Mediator. A full-colour version of this figure is available at *The EMBO Journal* Online.

Taken together, the results summarized in Figures 1–3 indicate that TRRAP/GCN5L stably associate with the cdk8-Mediator complex but not the core Mediator complex. For simplicity, we will refer to this complex as T/G-Mediator.

### *T/G-Mediator cooperatively phosphoacetylates S10/K14 within histone H3*

Having established that TRRAP/GCN5L can stably associate with cdk8-Mediator in human cells, we examined whether T/G-Mediator would display HAT activity. These experiments, which are described further in Supplementary data, demonstrated that T/G-Mediator, but not core Mediator, specifically acetylates lysine 14 on histone H3 (Supplementary Figure 5). Because T/G-Mediator also contains a kinase, cdk8, we tested whether T/G-Mediator might also phosphorylate histones; these experiments were prompted in part by related work in our lab that demonstrated that the cdk8 subcomplex itself was a potent histone kinase (MTK and DJT, unpublished data). As described further in Supplementary data, kinase assays showed that T/G-Mediator, but not core Mediator, specifically phosphorylates serine-10 on histone H3 (Supplementary Figure 6 and 7).

Individually, H3S10 phosphorylation and H3K14 acetylation are histone modifications linked to transcriptional activation. However, mounting evidence suggests that both modifications are important for expression of at least a subset of genes in yeast and mammalian cells (Clayton *et al*, 2000; Lo *et al*, 2000; Thomson *et al*, 2001). Although the data summarized in Supplementary Figures 5 and 7 indicated T/G-Mediator specifically phosphorylates H3S10 and acetylates H3K14, we could not conclude that T/G-Mediator was performing both modifications on the same H3 tail. To demonstrate this, we incubated T/G-Mediator with recombinant core histone octamers in the presence of both acetyl CoA and ATP. Significantly, immunoblotting experiments with antibodies specific for S10/K14 phospho-acetylated H3 confirmed that T/G-Mediator was catalyzing H3S10 phosphorylation and H3K14 acetylation on the same H3 tail (Figure 4A). Because H3S10 phosphorylation is known to stimulate GCN5 acetylation of H3K14 (Cheung *et al*, 2000), we also ran separate acetyltransferase assays with T/G-Mediator in the presence or absence of ATP. As expected, the added ATP had a notable affect on histone acetylation by T/G-Mediator (Figure 4B, compare lanes 2 and 4). In particular, the low levels of H2A, H2B and H4 acetylation by T/G-Mediator

 

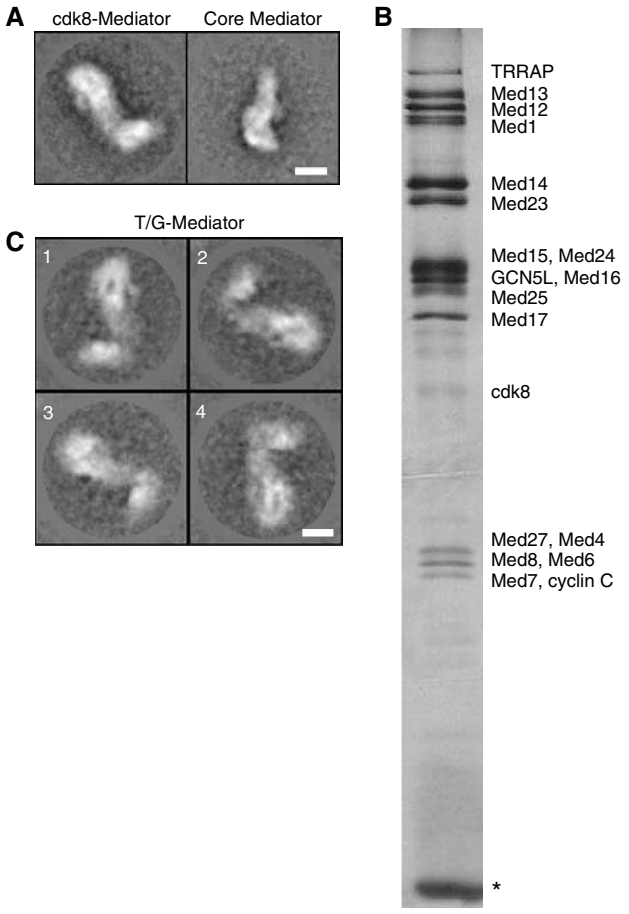


**Figure 3** T/G-Mediator contains the cdk8 submodule. (**A**) Representative 2D classes of cdk8-Mediator and core Mediator. Scale bar: 150 Å. (**B**) Silver-stained gradient (5–15% acrylamide) gel showing the T/G-Mediator sample used for EM analysis. Note that only prominently staining bands are identified and smaller, poorly staining polypeptides representing other consensus Mediator subunits are not labelled (Sato *et al*, 2004). Asterisk: insulin (used for TCA precipitation). (**C**) EM analysis indicates that T/G-Mediator sample has a size and shape consistent with cdk8-Mediator. Representative 2D classes are shown; each class (1–4) was obtained by averaging ~150 aligned single-particle images. Scale bar: 150 Å. A full-colour version of this figure is available at *The EMBO Journal* Online.

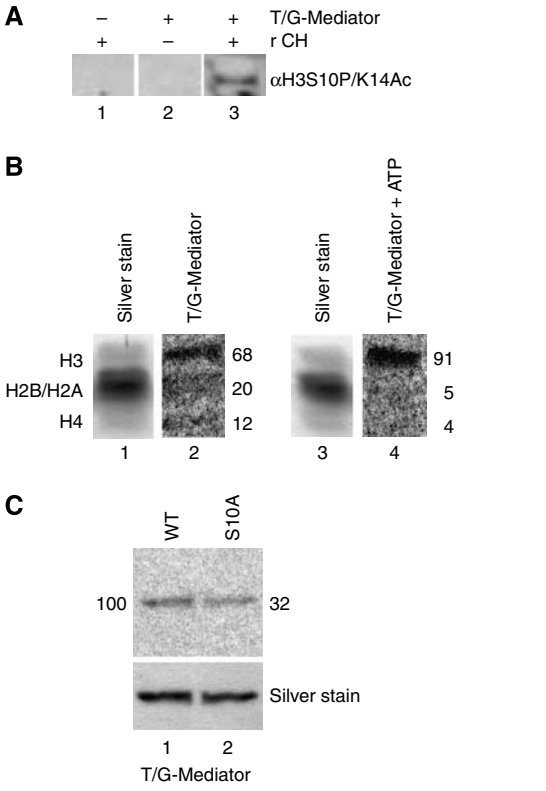


**Figure 4** T/G-Mediator cooperatively phosphoacetylates histone H3. (**A**) Recombinant core histones alone (lane 1), T/G-Mediator alone (lane 2) or T/G-Mediator together with recombinant core histones (lane 3) were incubated with ATP and acetyl CoA. Reactions were then probed with an antibody specific to the doubly modified S10/K14 H3. (**B**) ATP enhances T/G-Mediator acetylation specificity for H3. Acetylation assay with T/G-Mediator and core histones in the absence (lane 2) and presence (lane 4) of ATP. Lane 1 and 3: loading reference for lanes 2 and 4. Quantitation of the [$^{14}$C]-acetyl label is shown to the right of lanes 2 and 4. (**C**) T/G-Mediator-catalysed acetylation of H3K14 is reduced in H3S10A mutant. Equivalent amounts of T/G-Mediator were incubated with wild-type (WT) or mutant (S10A) H3 tails in the presence of ATP and [$^{14}$C]-acetyl CoA. Representative data are shown; quantitation of the [$^{14}$C]-acetyl label is shown beside each radiolabelled band, with WT normalized to 100. The value shown for S10A is the average of four independent experiments ($\sigma = 8.9$). Representative loading controls (silver stain) are shown for each lane. A full-colour version of this figure is available at *The EMBO Journal* Online.

decreased to nearly undetectable levels in the presence of ATP, whereas acetylation of H3 remained high. This data is consistent with previous studies demonstrating cooperativity between H3S10 phosphorylation and H3K14 acetylation (Cheung *et al*, 2000; Clements *et al*, 2003) and offers further evidence that T/G-Mediator can perform both modifications on the same histone H3 tail. Figure 4C also demonstrates cooperativity between H3S10/K14 within T/G-Mediator, as mutation of S10 within the H3 tail significantly reduced acetylation at H3K14. Taken together, the results summarized in Figure 4 indicate that cdk8 and GCN5L work cooperatively within a single complex, T/G-Mediator, to perform tandem phosphoacetylation of histone H3.

### *T/G-Mediator modifies H3 within chromatin templates*

We also performed experiments that demonstrated that T/G-Mediator phosphoacetylates H3 within chromatin templates; these experiments are described further in Supplementary data and are shown in Supplementary Figure 8.

### *Co-occupancy of T/G-Mediator subunits at the CDKN1A and* egr1 *genes*

To investigate the association of TRRAP with a cdk8-containing Mediator complex *in vivo*, we tested for their recruitment to human genes using ChIP assays. We focused on three well-characterized human genes whose activity could be easily manipulated in cell cultures: *CDKN1A*, *egr1* and *c-fos*. *CDKN1A* is a canonical p53 target gene whose transcription is strongly stimulated by Nutlin-3, a pharmacological inhibitor of the p53 repressor MDM2 (Vassilev *et al*, 2004; Donner *et al*, 2007). Nutlin-3 treatment of HCT116 cells (wt p53) leads to sustained accumulation of the *CDKN1A* mRNA (Figure 5A). *egr1* and *c-fos* are genes whose transcription is stimulated in quiescent cells via growth factor-mediated signalling. Consistent with their classification as immediate-early

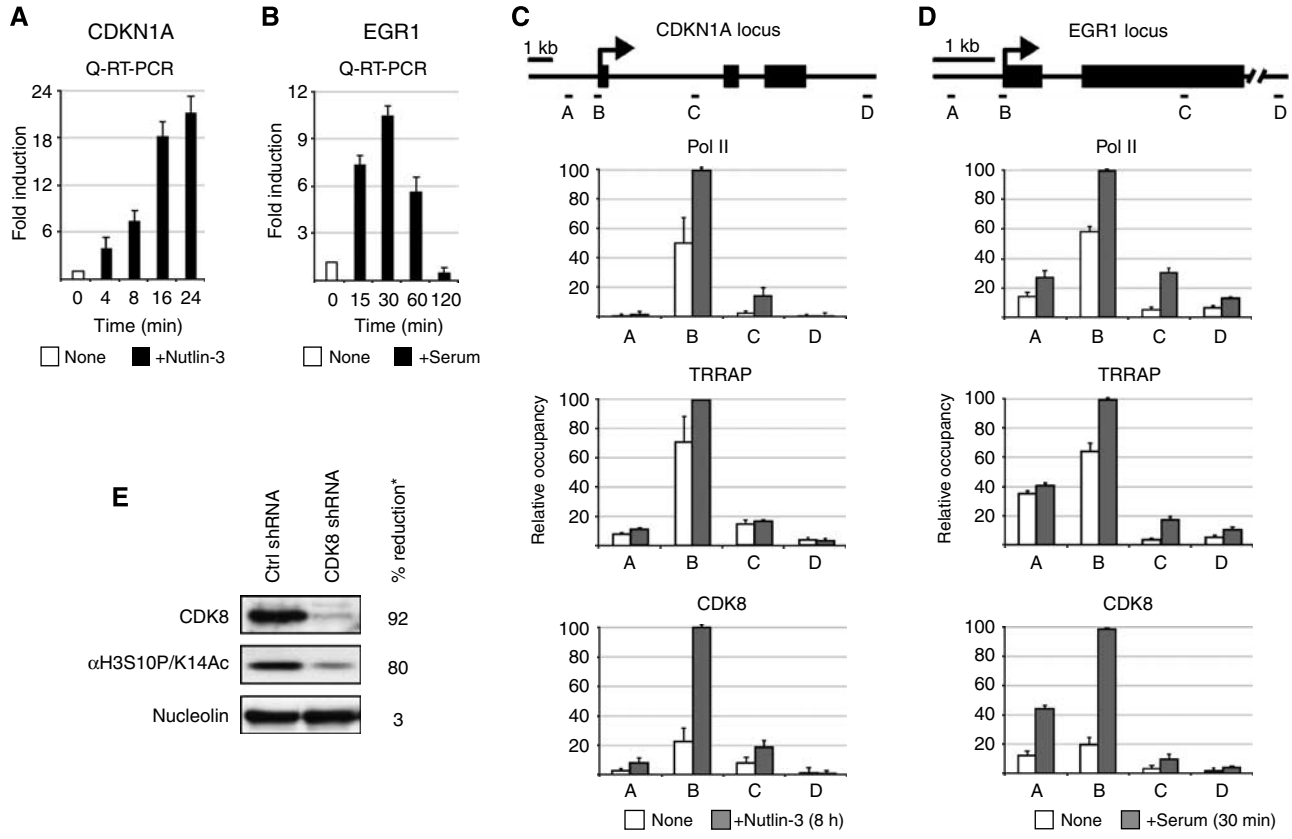


**Figure 5** TRRAP and cdk8 are co-recruited to the promoter regions of active human genes; cdk8 knockdown depletes global levels of phosphoacetylated H3. (**A**) p53-dependent activation of *CDKN1A* in HCT116 cells. cDNA samples were prepared after addition of 10 μM Nutlin-3 to the culture media. Values are expressed as fold induction over untreated cells after normalization to 18S rRNA. (**B**) Serum-induced activation of *egr1* in HCT116 cells. cDNA samples were prepared after serum replenishment, following 24 h of serum withdrawal. (**C**) ChIP analysis of the *CDKN1A* locus. HCT116 cells were treated with vehicle or 10 μM Nutlin-3 for 8 h before harvest and extract preparation. IPs were performed with antibodies against pol II, TRRAP and cdk8. ChIP-enriched DNA was analysed by Q–PCR with four different amplicons (A–D) covering different regions of the *CDKN1A* locus. (**D**) ChIP analysis of the *egr1* locus. Serum-starved HCT116 cells were stimulated with serum for 30 min before harvest. ChIPs were performed as in (C). Error bars represent standard deviations on triplicate experiments. (**E**) HCT116 cells were transduced with control or anti-cdk8 shRNAs and analysed by Western blot. Quantitation of knockdown is reported as % reduction from control cells (*average of two experiments; % reduction measured was 92/92 for cdk8, 86/75 for H3S10P/K14Ac, (−10)/16 for nucleolin (loading control)).

genes, *egr1* and *c-fos* mRNAs rapidly but transiently accumulate upon serum replenishment of serum-starved HCT116 cells (Figure 5B and data not shown). ChIP analysis at the *CDKN1A* promoter reveals that, upon induction, pol II recruitment increases at both the transcription start site (amplicon B) and the intragenic region (amplicon C), thus revealing the presence of elongating pol II. Interestingly, significant amounts of TRRAP can be detected at the *CDKN1A* proximal promoter even before p53 activation, and TRRAP association increases modestly upon induction. Similarly, ChIP assays with cdk8 antibodies reveal that it is recruited to the *CDKN1A* locus upon p53 activation. Importantly, cdk8 is most likely recruited as part of the Mediator complex, as indicated by concomitant association of Med1, Med12 and cyclin C to the active *CDKN1A* locus (Donner *et al*, 2007). These results are consistent with a cdk8-containing Mediator complex coexisting with TRRAP at the active *CDKN1A* locus, suggesting the presence of T/G-Mediator. ChIP analysis of the *egr1* or *c-fos* locus produced almost identical results (Figure 5B and D and data not shown), also consistent with T/G-Mediator occupancy at each promoter. ChIP assays using antibodies recognizing tandem phosphoacetylated H3 at the *CDKN1A*, *egr1* and *c-fos* loci are described in Supplementary data.

### *Knockdown of cdk8 dramatically reduces phosphoacetylated H3 levels in human cells*

Because of the inherent difficulty in identifying and proving that a human gene is specifically dependent upon the chromatin-modifying activity of T/G-Mediator (and not some other recruited kinase or acetyltransferase), we instead examined how shRNA-mediated knockdown of cdk8 affects global levels of phosphoacetylated H3. As shown in Figure 5E, knocking down cdk8 reduced the level of phosphoacetylated H3 by 80%. This corroborates our *in vitro* data and clearly demonstrates that cdk8 acts as a histone kinase in human cells. Furthermore, because cdk8 knockdown impacts the dual H3 S10P/K14Ac state, this suggests that cdk8 activity within T/G-Mediator plays a significant role in the tandem modification of histone H3.

### *T/G-Mediator does not directly activate transcription*

Subunit exchange within the human Mediator complex can dramatically impact its function as a transcriptional

 

co-activator. For example, the core Mediator complex interacts directly with RNA polymerase II and is a potent co-activator of transcription. By contrast, when associated with the 4-subunit cdk8 subcomplex (that is, cdk8, cyclin C, Med12, Med13), Mediator co-activator function is repressed: this distinct 'cdk8-Mediator' complex is unable to activate transcription (Taatjes *et al*, 2002). Notably, T/G-Mediator most closely resembles cdk8-Mediator in that it contains the cdk8 submodule and lacks Med26; however, T/G-Mediator also contains a known co-activator, the GCN5L acetyltransferase.

To determine whether T/G-Mediator possesses direct co-activator function, we tested its activity in our *in vitro* transcription system. This reconstituted human transcription system is similar to one described previously (Taatjes and Tjian, 2004) and uses chromatin templates with highly purified and recombinant general transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH, RNA pol II) together with purified activators and Mediator. Additional details of this reconstituted human system will be provided elsewhere (KDM, MTK and DJT, unpublished data). As shown in Figure 6A, core Mediator displays robust, activator-dependent co-activator function in this assay (compare lanes 1–4), as observed previously (Taatjes and Tjian, 2004). T/G-Mediator, by contrast, was unable to activate transcription in this highly purified system, even when titrated to a concentration threefold higher than core Mediator (Figure 6A, lanes 8–11). In fact, if added together with core Mediator, T/G-Mediator can block transcription in a dose-dependent manner (Figure 6A, lanes 4–7). These results suggest that T/G-Mediator is transcriptionally inert and that repression results from competition between T/G-Mediator and core Mediator for activator (Sp1 in this case) binding/recruitment to the promoter. To ensure the observed activity was not simply a characteristic of the Sp1 promoter template, we also tested T/G-Mediator function on chromatin templates responsive to SREBP-1a/Sp1, VDR/RXR or gal4-VP16. T/G-Mediator displayed similar activity in these assays (Supplementary Figure 9).

Given that T/G-Mediator contains the cdk8 subcomplex, we did not expect it to display co-activator function (Taatjes *et al*, 2002). Yet because T/G-Mediator possesses histone kinase and acetyltransferase activity, we hypothesized that it may possess latent co-activator function. Previous work by the Reinberg lab indicated that, at least at promoters responsive to RARβ, the cdk8-Mediator complex could be 'activated' by PARP-1: PARP-1 was shown to bind RARβ and dissociate the cdk8 subcomplex, leaving behind core Mediator at the promoter, which then worked to activate transcription (Pavri *et al*, 2005). Although clearly distinct from cdk8-Mediator, T/G-Mediator does contain the cdk8 submodule, and therefore we surmised that PARP-1 might dissociate the cdk8 subcomplex from T/G-Mediator (and perhaps TRRAP/GCN5L) to enable transcription activation *in vitro*. However, as shown in Figure 6B, PARP-1 was unable to activate T/G-Mediator in this way, at least at the promoters tested. (Note that similar results were obtained with LDLR-derived promoters or a VDR/RXR promoter, as shown in Supplementary Figure 9.) This supports the conclusions of Pavri *et al*, (2005) that PARP-1 acts in a promoter-specific manner.

## Discussion

Mediator is a primary regulator of PIC function: Mediator physically or functionally interacts with most components of the PIC and also regulates post-recruitment events in transcription initiation (Malik and Roeder, 2005; Wang *et al*, 2005). The results from this study suggest that human Mediator plays an expanded role in transcriptional regulation that involves post-translational modification of histone H3. Given the link between H3 acetylation and activation of transcription, it is somewhat counter-intuitive that TRRAP/GCN5L associates not with core Mediator, which possesses co-activator function, but rather with cdk8-Mediator, which does not directly activate transcription. However, our histone modification assays reveal that within T/G-Mediator, cdk8 phosphorylates H3S10, providing a biochemical rationale for

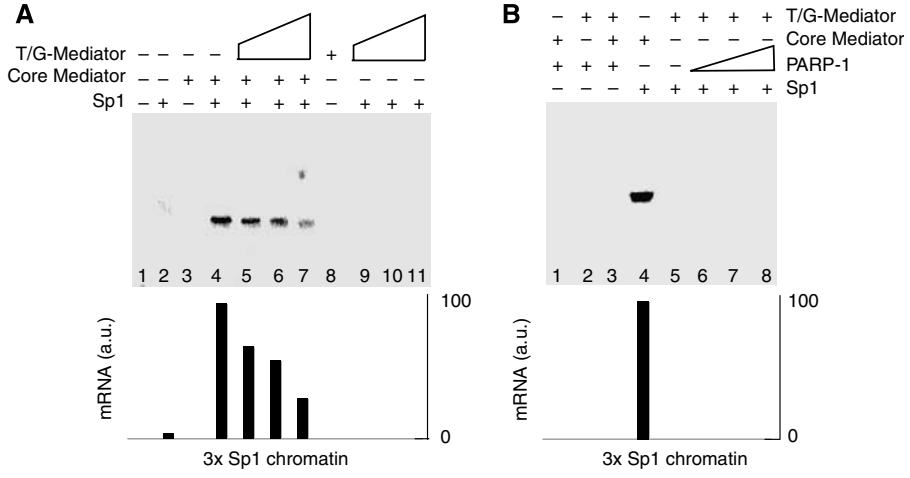


**Figure 6** *In vitro* transcription on chromatin templates. (**A**) Unlike core Mediator, T/G-Mediator does not directly activate transcription. Core Mediator is required to reconstitute activator-dependent transcription *in vitro* on chromatin templates (compare lanes 1–4); T/G-Mediator blocks core Mediator-dependent activation in a dose-dependent manner (compare lanes 4–7); T/G-Mediator is unable to activate transcription even in the presence of activator (lanes 8–11). (**B**) PARP-1 does not activate T/G-Mediator. Titration of PARP-1 (20, 50 or 120 ng) in the presence of T/G-Mediator and Sp1 (lanes 6–8). Quantitation of RNA transcripts is shown in the bar graph below each lane.

the selective interaction of TRRAP/GCN5L with cdk8-Mediator instead of core Mediator. Indeed, numerous studies describe a functional cooperativity between H3S10 phosphorylation and H3K14 acetylation, at least at a subset of genes (Clayton *et al*, 2000; Lo *et al*, 2000; Anest *et al*, 2003; Yamamoto *et al*, 2003). This functional coordination, coupled with the ability of phosphorylated H3S10 to stimulate H3K14 acetylation by GCN5, led to the hypothesis that an H3S10 kinase/H3K14 acetyltransferase might reside within the same multi-subunit complex (Cheung *et al*, 2000). To our knowledge, T/G-Mediator represents the first demonstration that an H3S10 kinase (cdk8) and an H3K14 acetyltransferase (GCN5L) can in fact function within a single stable, multi-subunit complex.

By coordinating post-translational modifications within histone H3, T/G-Mediator may work to direct early events in the activation of gene expression (that is, before full PIC assembly). Importantly, the observation that cdk8 phosphorylates H3S10 offers a biochemical rationale for past studies showing a correlation between cdk8 recruitment and transcription activation. Paradoxically, this positive role for cdk8 remains consistent with its well-established function as a negative regulator of transcription, provided the biochemical function of cdk8 is considered at different stages of initiation. As a histone kinase within T/G-Mediator, cdk8 may work together with other chromatin-modifying/remodelling factors to establish a chromatin environment favorable for transcription. However, by blocking stable pol II assembly within the PIC and inhibiting TFIIH (Akoulitchev *et al*, 2000; Näär *et al*, 2002; Elmlund *et al*, 2006), cdk8 would concomitantly inhibit transcription initiation at this stage. Actual transcription initiation likely occurs only upon dissociation of the cdk8 subcomplex (and perhaps TRRAP/GCN5L) from Mediator, which would enable pol II recruitment via its stable association with core Mediator and other PIC components (for example, TFIIB). This model is consistent with our *in vitro* transcription data, which clearly shows that T/G-Mediator, in contrast to core Mediator, is unable to activate transcription. Furthermore, previous studies in human cells demonstrate that dissociation of cdk8 subcomplex components—but not core Mediator itself—occurs immediately before transcriptional activation at RARβ- and CEBPβ-regulated genes (Mo *et al*, 2004; Pavri *et al*, 2005).

Notably, dissociation of the cdk8 submodule was shown to be dependent upon PARP-1 at RAR/RXR-regulated genes *in vitro* and *in vivo*; however, PARP-1 was unable to similarly 'activate' T/G-Mediator. This suggests that PARP-1 dissociation of the cdk8 subcomplex may be promoter specific, as proposed previously (Pavri *et al*, 2005). Indeed, PARP-1 was shown to interact directly with RARβ, and similar interactions may not occur with the activators tested here (Sp1, SREBP-1a, VDR/RXR). Alternately, the presence of TRRAP/GCN5L may block PARP-1-dependent dissociation of the cdk8 subcomplex from T/G-Mediator to prevent transcription activation. In either case, it is likely that an activity distinct from PARP-1 is required to dissociate the cdk8 submodule within T/G-Mediator. Future work will be directed towards identification of such factors.

The regulatory role of combined H3S10 phosphorylation and H3K14 acetylation appears to vary in different promoter contexts, and it is not established whether these tandem modifications are a general phenomenon of active gene expression. Furthermore, it is likely that tandem H3 S10/K14 phosphoacetylation does not occur cooperatively at all genes that require this modification (Thomson *et al*, 2001). Yet it is clear that each modification can facilitate transcription activation via recruitment of co-activators (for example, TAF1 or GCN5L itself) and/or loss of co-repressors (for example, HP1) (Kasten *et al*, 2004; Fischle *et al*, 2005; Hirota *et al*, 2005; Hassan *et al*, 2007). In mammalian cells, phosphorylation of H3S10 has been linked to activation of specific genes in response to growth factors or inflammation (Sassone-Corsi *et al*, 1999; Thomson *et al*, 1999; Anest *et al*, 2003; Yamamoto *et al*, 2003); moreover, H3S10 phosphorylation correlates with expression of at least a subset of RARβ and myc target genes (Lefebvre *et al*, 2002; Zippo *et al*, 2007). In several of these cases, distinct kinases were directly or at least indirectly responsible for H3 phosphorylation (for example, IKKα, MSK2/1, PIM1), suggesting that each may play gene-specific roles in transcriptional activation (Soloaga *et al*, 2003).

Our examination of modified H3 in human cells reveals that knockdown of cdk8 has a major impact on global levels of phosphoacetylated H3. Although these effects could be indirect, our *in vitro* results overwhelmingly support a direct role for cdk8 in the phosphoacetylation of H3. In fact, the striking correlation between the levels of cdk8 and phosphoacetylated H3 suggests that T/G-Mediator is a prominent regulator of this H3 modification. Further studies involving genome-wide approaches in human cells will be required to assess which genes may be specifically dependent upon the chromatin-modifying function of T/G-Mediator. Although cdk8 and Mediator itself appear to be globally recruited to promoter and enhancer regions in yeast (Andrau *et al*, 2006; Zhu *et al*, 2006), we predict that tandem phosphoacetylation by T/G-Mediator will be critical for expressing only a subset of human genes due to redundant histone kinase and acetyltransferase activities; furthermore, it is not evident that tandem H3 phosphoacetylation is generally required for expression of all protein-coding genes. Indeed, like cdk8, GCN5 is also globally recruited to gene promoters in yeast (Robert *et al*, 2004; Pokholok *et al*, 2005), yet in higher organisms, changes in histone acetylation are not uniformly observed for all genes upon activation (Nowak and Corces, 2000; Lefebvre *et al*, 2002). Notably, cdk8 can also negatively regulate transcription via phosphorylation of TFIIH (Akoulitchev *et al*, 2000); thus, precise control of cdk8 substrate specificity may be critical for regulating transcription initiation in humans.

Interestingly, TRRAP and GCN5L are also subunits of the STAGA/TFTC complex in humans, which is biochemically similar to yeast SAGA (Baker and Grant, 2007; Nagy and Tora, 2007). Because human TRRAP/GCN5L are shared between STAGA/TFTC and T/G-Mediator, these subunits (particularly TRRAP) may help orchestrate recruitment of STAGA/TFTC and Mediator at human genes. Indeed, ChIP studies in yeast and human cells have shown that recruitment of SAGA and Mediator can occur simultaneously or sequentially (Bhoite *et al*, 2001; Cosma *et al*, 2001; Bryant and Ptashne, 2003; Govind *et al*, 2005), and that SAGA may aid in Mediator recruitment, at least at some genes (Bhaumik *et al*, 2004; Qiu *et al*, 2005; Liu *et al*, 2008). Genetic studies in yeast also suggest functional interactions between SAGA and Mediator (Roberts and Winston, 1997), specifically with Mediator

 

containing the cdk8 submodule (Larschan and Winston, 2005). Although a potential mechanism for this interdependent recruitment is not established, the results shown here suggest that STAGA/TFTC and Mediator co-association or exchange may be dependent upon the 430 kDa TRRAP polypeptide. Notably, this is consistent with the perceived biochemical role of TRRAP as a molecular scaffold/architectural factor within the PIC (Murr *et al*, 2007).

## Materials and methods

### *Purification of T/G-Mediator*

A typical purification starts with nuclei isolated from approximately 100 l of HeLa cells, as outlined in Figure 2B. For the affinity purification step, after binding QFT, the resin was washed with 50 column volumes (CV) 0.5 M KCl HEGN (pH 7.9, 20 mM HEPES, 0.1 mM EDTA, 10% glycerol, 0.1% NP-40) and 10 CV 0.15 M KCl HEGN (0.02% NP-40). T/G-Mediator was then eluted from the GSH resin with 2 CV buffer containing 30 mM glutathione (pH 7.6, 20 mM Tris, 0.1 mM EDTA, 10% glycerol, 0.15 M KCl). This material was then loaded onto a 2 ml glycerol gradient (15–40% glycerol in 0.15 M KCl HEGN) and centrifuged at 55K r.p.m. for 6 h at 4°C. Fractions were collected from the top of the gradient in 100 μl aliquots.

### *LC MS/MS analysis*

Mass spectrometry experiments were completed on a PE Sciex API QSTAR Pulsar MS with capillary electrospray interface, essentially as described (Knuesel *et al*, 2005). In-gel trypsin digestion of proteins was completed following reduction (DTT) and alkylation (iodoacetamide) using standard techniques.

### *Antibodies*

Santa Cruz Biotechnology: Med1, GCN5L, cdk8, pol II; Bethyl Inc.: Med23; Upstate: phospho-H3T3, -H3S10, -H3S28, phosphoacetyl-H3S10/K14; Lab stocks: TRRAP, Med15, Med26, Med14.

### *Core histone purification*

Purification of core histones was completed with 140 g of 12–24 h *Drosophila* embryos, essentially as described (Laybourn and Kadonaga, 1991). Expression and purification of recombinant core histones was carried out as described (Luger *et al*, 1999).

### *QRT–PCR and ChIP assays*

HCT116 were maintained in McCoys 5A media (Gibco-Invitrogen) supplemented with 10% fetal bovine serum and antibiotic/antimycotic mix. For *CDKN1A* induction, cells were treated with 10 μM Nutlin-3 (Cayman Biochemicals). For *egr1* and *c-fos* induction, cells were serum-starved for 24 h before serum replenishment. QRT–PCRs and ChIPs assays were performed essentially as described (Gomes *et al*, 2006). Briefly, for RT–PCR, total RNA was isolated using the RNeasy Mini Kit (Qiagen), and cDNA was generated with the SuperScriptase II (Invitrogen). For ChIP assays, cells were fixed with 1% formaldehyde and harvested for whole cell lysate preparation. A quantity of 1 mg of protein lysate was used per ChIP with antibodies against pol II, TRRAP and cdk8. ChIP-enriched DNA was analysed by Q-PCR as described (Gomes *et al*, 2006). See Supplementary Table 1 and 2 for primer sequences.

### *shRNA knockdown of cdk8*

Control or anti-cdk8 shRNA sequences were cloned into a derivative of the pLL3.7 vector containing a G418 resistance cassette. After packaging of lentiviral particles in HEK293FT cells, HCT116 cells were transduced, selected in G418 and individual stable clones were grown and tested for cdk8 knockdown by Western blot.

### *Chromatin assembly*

For kinase or acetyltransferase assays, chromatin was assembled using purified *Drosophila* core histones and a DNA template (G5E4) derived from the sea urchin 5S rRNA gene, essentially as described (Ikeda *et al*, 1999). The quality of the assembled chromatin was determined by micrococcal nuclease digestion. For *in vitro* transcription, supercoiled template plasmids were assembled into chromatin using *Drosophila* embryo cytosolic extract (S-190), purified *Drosophila* core histones and an ATP-regenerating system as described (Näär *et al*, 1998). Chromatin assembly was carried out at 27°C for 5 h.

### *In vitro transcription*

Chromatin templates were incubated with activators (Sp1, SREBP-1a or gal4-VP16: 4 nM each; or VDR/RXR: 10 nM each plus ligand) for 30 min, followed by addition of purified and recombinant general transcription factors (40 nM TFIIA, 10 nM TFIIB, 0.8 nM TFIID, 10 nM TFIIE, 10 nM TFIIF, 0.5 nM TFIIH and 2 nM pol II) with/without non-limiting amounts of Mediator and/or T/G-Mediator (0–15 nM). Note that acetyl CoA (5 μM) was also added in some experiments and had no effect (not shown). After allowing 15 min for PIC assembly, NTPs (0.5 nM final concentration) were added; 30 min later, transcription was terminated by adding three volumes of stop solution (20 mM EDTA, 0.2 M NaCl, 1% SDS). RNA was then isolated and analysed by primer extension.

### *Supplementary data*

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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