Supplementary Information

Supplementary Results

T/G-Mediator acetylates histone H3K14
Having established that TRRAP/GCN5L can stably associate with cdk8-Mediator in human cells, we examined whether T/G-Mediator would display HAT activity. Using highly-purified core histones, we observed that T/G-Mediator displayed potent HAT activity (Supplementary Figure 5a). Importantly, core Mediator (which lacks the cdk8 submodule and TRRAP/GCN5L—see Supplementary Figure 4) was inactive in these assays (Supplementary Figure 5a, compare lanes 2 and 3). The HAT assay with T/G-Mediator suggested that H3 was its preferred substrate; subsequent acetylation experiments with recombinant H3 confirmed that H3 was targeted by T/G-Mediator (Supplementary Figure 5a, lane 5). We next examined the acetylation specificity of T/G-Mediator within histone H3. It is well-known that the N-terminal tail of histone H3 is a prominent site for post-translational modifications and that GCN5L itself typically targets lysine 14 within the H3 tail (Kuo et al., 1996). Therefore, we expressed and purified a GST-fusion of the H3 N-terminal tail (residues 1-37) as well as its K14R point mutant. As expected, T/G-Mediator efficiently acetylated the wild-type H3 tail but not the K14R point mutant (Supplementary Figure 5b). Similar acetyltransferase activity was observed with purified recombinant GCN5L alone (Supplementary Figure 5c), demonstrating that the substrate specificity of GCN5L is not altered within T/G-Mediator.

T/G-Mediator phosphorylates histone H3S10
The experiments in Supplementary Figure 5 indicate that T/G-Mediator possesses HAT activity and modifies lysine-14 within histone H3. Because T/G-Mediator also contains a kinase—cdk8—we tested whether T/G-Mediator might also phosphorylate histones. Significantly, we observed that within T/G-Mediator, cdk8 is a potent histone kinase, with clear specificity for histone H3 (Supplementary Figure 6a). To further demonstrate this, we performed an anti-cdk8 immunoprecipitation experiment from HeLa nuclear extract and tested bound material for histone H3 kinase and acetyltransferase activity. As shown in Supplementary Figure 6b, the cdk8-immunoprecipitated material was capable of acetylating and phosphorylating recombinant histone H3.

The results summarized in Supplementary Figure 5 and 6 suggested that T/G-Mediator could both acetylate and phosphorylate histone H3. Although histone kinase activity has never been observed with yeast or metazoan cdk8-Mediator complexes, it is well-understood that phosphorylation of H3 serine-10 stimulates H3K14 acetylation by GCN5 (Clements et al., 2003; Lo et al., 2000). In fact, the binding specificity of GCN5 for the phosphorylated H3S10 substrate is 10-fold higher than unmodified H3 (Cheung et al., 2000). Given that T/G-Mediator specifically acetylates H3K14, we hypothesized that the cdk8 target might be H3S10. We tested this hypothesis in a number of ways. First, we generated an S10A point mutant of the H3 tail and completed kinase assays that compared the H3S10A mutant with the wild-type H3 tail. As shown in Supplementary Figure 7a, T/G-Mediator efficiently phosphorylated wild-type H3, but not the H3S10A point mutant (compare lanes 1 and 2). By contrast, core Mediator was unable to phosphorylate the wild-type H3 tail, as expected (Supplementary Figure 7a, lane 3). Second, immunoblotting experiments demonstrated T/G-Mediator phosphorylation specificity for H3S10. Using recombinant core histone octamers, we performed kinase assays in the presence or absence of T/G-Mediator. Each reaction was then run on a polyacrylamide gel and probed with an antibody specific for phosphorylated H3T3, H3S10, or H3S28—three H3 sites known to be phosphorylated in vivo. As shown in Supplementary Figure 7b, only H3S10 phosphorylation was detected in the presence of T/G-Mediator. Together, these results demonstrate that T/G-Mediator phosphorylates serine-10 on histone H3.

T/G-Mediator modifies chromatin templates
Although T/G-Mediator displayed potent acetyltransferase and kinase activity against core histones, we also wanted to determine whether T/G-Mediator would display similar activity on chromatin templates. Using a DNA template with sequential nucleosome positioning sites (Ikeda et al., 1999), we assembled core histones
ChIP analysis against tandem phosphoacetyl-H3 at CDKN1A, egr1, and c-fos.

ChIP analysis using antibodies recognizing tandem H3S10 phosphorylation/K14 acetylation showed high levels of this modification pattern at the CDKN1A, egr1, and c-fos loci both before and after induction (data not shown). The fact that ChIP signals for tandem H3 phosphoacetylation do not change significantly upon activation of these genes may be due to: 1) the presence of reduced but significant levels of cdk8 before activation; 2) the action of other H3S10 kinases creating a high background of S10 phosphorylation; 3) epitope masking by additional H3 modifications (e.g. H3K9Ac) or by S10P and/or K14Ac binding proteins; or 4) changes in the H3 modification patterns that are too dynamic or are occurring at nucleosomes not covered by the small amplicons used in our qChIP assays.

Supplementary Discussion

A wealth of ChIP-chip data from yeast has provided a detailed view of how Mediator recruitment and histone modifications relate to transcriptional activity. A few points from this work are discussed here. In a pair of genome-wide studies, the Holstege and Gustafsson labs recently observed recruitment of yeast Mediator at inactive genes and also just prior to transcriptional activation (Andrau et al., 2006; Zhu et al., 2006). Moreover, they noted that cdk8 (also called srb10 in yeast) did not preferentially bind repressed genes; rather, cdk8 recruitment mirrored that of core Mediator yet its presence at promoters was more transient. These observations are consistent with cdk8-Mediator maintaining a promoter in an inactive state that is poised for rapid induction (Malik and Roeder, 2005). The isolation of T/G-Mediator and its histone modifying activity, coupled with previously published experiments describing activation of cdk8-Mediator at RARβ- and CEBPPβ-regulated genes (Mo et al., 2004; Pavri et al., 2005) provide further support for this model. Earlier microarray analysis in yeast indicated that GCN5 was required for activation of only about 4% of genes (Holstege et al., 1998; Lee et al., 2000). However, subsequent work revealed that, despite its absolute requirement at only a subset of genes, GCN5 was recruited to most yeast promoters and its recruitment correlated with transcription (Govind et al., 2007; Pokholyok et al., 2005; Robert et al., 2004). Because GCN5 is a component of the yeast co-activator SAGA, it is possible that redundant HAT activities (e.g. Nut1 or TAF1) could compensate at most genes and that GCN5 recruitment instead represents a general requirement for SAGA. Yet this is unlikely because disruption of the SAGA complex in yeast affects activation of only a subset of protein-coding genes (Lee et al., 2000). Notably, SAGA also contains Tra1, the yeast ortholog of TRRAP. Because Tra1 interacts with various transcriptional activators (Brown et al., 2001), Young and co-workers proposed that Tra1 may serve as an intermediary in the recruitment of GCN5 to yeast promoters (Robert et al., 2004). Our data with human Mediator certainly supports that view, albeit that TRRAP can also function to recruit GCN5L in the context of Mediator. Whether Tra1/GCN5 associate with yeast Mediator is not known, but such an association would provide a simple explanation for global GCN5 recruitment to yeast promoters. Alternately, the TRRAP/GCN5L-Mediator interaction may be specific to metazoans. Mediator possesses at least 8 metazoan-specific subunits and even the relatively well-conserved cdk8 subunit has demonstrated distinct activities in higher organisms (Akoulitchev et al., 2000; Sun et al., 1998). In fact, the yeast ortholog of cdk8 (srb10) does not phosphorylate histones (Hengartner et al., 1997).
Antibody production and peptide elution
The TRRAP peptides to the C-terminus (peptide 1), residues 1997-2017 (peptide 2), or residues 764-789 (peptide 3) were conjugated to rabbit albumin and injected into New Zealand white rabbits (200 µg peptide/injection) for antibody production. Serum collected from the rabbits was then tested by immunoblot and IP. Antibodies from one rabbit injected with TRRAP peptide 3 were very effective in IP experiments. For elution of TRRAP or TRRAP-containing Mediator complexes following IP, we attached 12-methoxy-4,7,10-trioxadodecanoate (Quanta Biodesign) to the C-terminus of peptide 3 to increase its solubility to 20 mg/mL in 0.15M KCl HEGN buffer.

Immunoprecipitation
Antibody affinity resins were prepared with a 1:1 mixture of protein A: protein G (GE Healthcare). The appropriate antibody (anti-TRRAP, -cdk8, -Med26) was incubated with the resin in batch for 90 minutes at 4°C. The resin was then washed with 4 x 20 volumes 0.5M KCl HEGN and 1 x 20 volumes 0.15M KCl HEGN. This washed antibody resin was then used for immunoprecipitation from partially purified extracts (e.g. P0.5M) that were mixed in batch with the resin for 2-3h at 4°C, washed with 5 x 20 volumes 0.5M KCl HEGN and 1 x 20 volumes 0.15M KCl HEGN, and eluted with 2% sarcosyl or with the appropriate peptide.

Electron microscopy
EM samples were purified according to Figure 2b except that an additional anti-TRRAP purification step was included after affinity purification. T/G-Mediator was then eluted from the anti-TRRAP resin with peptide, followed by glycerol gradient sedimentation. This additional step was included for EM to ensure all complexes contained the TRRAP polypeptide. Purified T/G-Mediator was applied to glow-discharged carbon-coated 400-mesh grids and negatively stained with a 4% uranyl acetate solution. Samples were buffer-exchanged on the grid with a 5% trehalose solution (0.15M KCl, 20 mM HEPES, 0.1 mM EDTA, pH 7.9) prior to negative staining. Untilted (0°) micrographs were used for alignment and classification of single-particle images. Micrographs were obtained on a Tecnai F20 FEG electron microscope at 29,000x magnification and scanned with an Agfa Duoscan f40 scanner at 13.3 µm, corresponding to a 4.6 Å pixel size at the specimen level. Image processing was completed with the SPIDER and WEB software package (Frank et al., 1996). Single-particle images (2391) were selected and windowed into 160 x 160 pixel images prior to multi-reference alignment and classification of the 2D images (Taatjes et al., 2004).

HAT assays
T/G-Mediator was pre-incubated in Acetyl CoA buffer (50mM HEPES-HCl, pH 7.9, 10 µg/mL BSA, 0.1mM EDTA, 1mM DTT, 70 µM [14C]-Acetyl CoA) for 30 minutes at 30°C. Acetylation was allowed to proceed at 30°C for 30 min. after addition of substrate. Reactions were then quenched with SDS sample buffer and analyzed on 15% acrylamide gels. Gels were stained and visualized by autoradiography.

Kinase assays
Kinase assays used 2.5 µCi/rxn 32γ-ATP in kinase buffer (25mM Tris-HCl, pH 8.0, 2mM DTT, 0.1mM cold ATP, 0.1M KCl, 10mM MgCl2), together with T/G-Mediator or core Mediator and substrate. Reactions were allowed to proceed at 30°C for 30 min. Reactions were then quenched with SDS loading buffer and analyzed on acrylamide gels by silver stain and autoradiography.

Supplementary Figures:
Figure 2

A

B

p53

SREBP
**Figure 3**

A. Western blot analysis using antibodies against various proteins.

B. Control IPs using α-cdk8 and α-Med26 antibodies.

- α-cdk8
- IgH
- Med1
- Med14
- Med23
- Med15, Med24
- TRRAP
- GCN5L, Med16
- Med26
- SAP130
- TAF9
- Med12, Med13
- 0.5M elute (lane 1)
- 0.5M elute (lane 2)
- 0.5M elute (lane 3)

**Control IPs**

- 0.5M fraction
- α-TRRAP antibody column
- α-cdk8 antibody column
- α-Med26 antibody column
- 0.5M elute (lane 1)
- 0.5M elute (lane 2)
- 0.5M elute (lane 3)
Figure 4
Figure 5

Silver stain

[14C] Acetyl CoA

WT H3 tail
K14R H3 tail

T/G-Mediator
rGCN5L

WT H3 tail
rGCN5L

[14C] Acetyl CoA

T/G-Med + rH3
T/G-Med + rH3

K14R H3 tail

WT H3 tail

rH3 coomassie

[14C] Acetyl CoA

T/G-Med + rH3
T/G-Med + rH3

K14R H3 tail

WT H3 tail

rH3 coomassie
Figure 6

Silver

[\textsuperscript{32P}]ATP

\[ \text{cdk8 ip} \]

\[ r \text{H3} \]

\[ 20 \text{kDa} \]

[\textsuperscript{14C}]AcCoA

\[ 15 \text{kDa} \]
Figure 7

Western blot

<table>
<thead>
<tr>
<th></th>
<th>α H3S28P</th>
<th>α H3S10P</th>
<th>α H3T3P</th>
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<tr>
<td>CTRL</td>
<td>+</td>
<td>+</td>
<td>--</td>
</tr>
<tr>
<td>T/G-Med</td>
<td>+</td>
<td>+</td>
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</tbody>
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Silver stain

32P[γ-ATP]

WT H3 tail
S10A H3 tail
WT H3 tail

α H3S10P
α H3T3P
α H3S28P
Figure 8
**Supplementary Figure legends**

**Supplementary Figure 1.** TRRAP/GCN5L associate with human Mediator. Co-IP experiments: Mediator subunits (Med1, Med15, cdk8), TAF4 (a component of TFIIID and STAGA), TBP, TRRAP, and GCN5L were probed in immunoblotting experiments following immunoprecipitation with anti-TRRAP or anti-cdk8 antibody resins.

**Supplementary Figure 2.** Purification of T/G-Mediator with alternate activation domains: SREBP-1a (residues 1-50) or p53 (residues 1-73). Silver-stained gels showing odd-numbered fractions pulled from a glycerol gradient. The purification protocol outlined in Figure 2b was used, with the activation domains of (A) SREBP-1a or (B) p53 for the affinity purification step. T/G-Mediator is enriched in lanes 13-15 in A and 11-13 in B. Subunit identities are indicated at the right of each gel.

**Supplementary Figure 3.** TRRAP/GCN5L associate with cdk8-Mediator but not core Mediator. (A) A P0.5M-derived, anti-TRRAP purified sample was immunoprecipitated with anti-cdk8 or anti-Med26. After washing each resin with 5 x 20 column volumes of high-salt buffer (0.5M HEGN), each resin was eluted with 2% sarcosyl and analyzed by western blot. Note that SAP130 and TAF9 are both components of the human STAGA complex. (B) Both Med26 and cdk8 antibody resins can IP Mediator. Silver-stained gels showing typical IP experiments with anti-Med26 or anti-cdk8. Because very little Med26 is present in the P0.5M fraction, the Med26 IP shown is from the P1M fraction.

**Supplementary Figure 4.** Purification of core Mediator. (A) Purification protocol. (B) Silver-stained gel (7% acrylamide) of core Mediator. In this case, the activation domain of SREBP-1a was used for the affinity purification step. (C) Western blot of core Mediator and T/G-Mediator highlighting their subunit differences.

**Supplementary Figure 5.** T/G-Mediator acetylates histone H3 lysine 14. (A) T/G-Mediator preferentially acetylates histone H3. Lane 1: loading control; lane 2 and 3: acetylation assays (detecting [14C] acetyl coA) with core Mediator or T/G-Mediator; lane 4: coomassie stain of recombinant histone H3; lane 5: acetylation assay ([14C] acetyl coA) with T/G-Mediator and recombinant H3. (B) Acetylation assay with recombinant, wild-type H3 tail (lane 1) or a K14R point mutant (lane 2). A loading control is shown below the autoradiograph. (C) Purification and activity of recombinant GCN5L. Protein expression was completed in insect cells following baculovirus infection; recombinant GCN5L is GST-tagged. The purified, recombinant GCN5L shows strong HAT activity in our in vitro acetylation assay.

**Supplementary Figure 6.** T/G-Mediator is a histone H3 kinase. (A) Kinase assay with purified core histones. Lane 1: T/G-Mediator only; lane 2 and 3: titration (5 or 10 ng) of T/G-Mediator with core histones. (B) cdk8 IP possesses H3 kinase/acetyltransferase activity. Anti-cdk8 beads were tested for H3 kinase/acetyltransferase activity following immunoprecipitation from HeLa NE. Lanes 1-2: kinase assay; lanes 3-4: acetyltransferase assay. Loading controls are shown below each lane.

**Supplementary Figure 7.** T/G-Mediator phosphorylates H3S10. (A) Kinase assay showing T/G-Mediator activity against wild-type (lane 1) or S10A mutant (lane 2) H3 tails. Lane 3: kinase assay with core Mediator. Loading controls are shown below each lane. (B) Kinase assays with T/G-Mediator and purified recombinant core histones followed by immunoblotting with phospho-specific H3 antibodies. Lane 4 (control) contains core histones purified from Drosophila embryos.

**Supplementary Figure 8.** T/G-Mediator acetylates and phosphorylates H3 within a chromatin template. Lane 1: chromatin with [32P]-ATP only; lanes 2-3: chromatin with [32P]-ATP + T/G-Mediator (10 or 20 ng). Lane 4: T/G-Mediator (10 ng) with [14C]-acetyl CoA only; lane 5: chromatin with [14C]-acetyl CoA + recombinant GCN5L; lanes 6-7: chromatin with [14C]-acetyl CoA + T/G-Mediator (10 or 20 ng). Histone loading references (silver stain) are shown for each lane.
Supplementary Figure 9. T/G-Mediator is transcriptionally inert and is not activated by PARP-1. The potential co-activator function of T/G-Mediator was tested at promoters responsive to VP16 (A), VDR/RXR (B), or SREBP/Sp1 (C). In each case, core Mediator displayed potent, activator-dependent co-activator activity whereas T/G-Mediator did not co-activate at any of the concentrations tested (up to 3-fold greater than core Mediator). PARP-1 also had no effect on T/G-Mediator activity when tested at the VDR/RXR or SREBP/Sp1 promoters.

References


Pavri, R., Lewis, B., Kim, T.K., Dilworth, F.J., Erdjument-Bromage, H., Tempst, P., de Murcia, G., Evans,


