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14. ABSTRACT
Dysregulated estrogen receptor (ER) function underlies many forms of breast cancer. This proposal is aimed at understanding how ERα activates its target genes from distal enhancers in a Mediator-dependent fashion. We have hypothesized that ER-Mediator interactions would be critical for signal transduction at ERα target genes through establishment of chromatin loops that facilitate long-range enhancer-promoter communication. The main aims of the proposal are thus to establish both cell based and cell-free (in vitro) transcription systems to recapitulate and mechanistically dissect Mediator-dependent ERα function from a distal enhancer and further to develop peptidomimetic inhibitors of the ERα-MED1 interaction to disrupt enhancer-promoter communication. In the current phase of the project we have completed reconstitution (via baculovirus expression of constituent subunits) of a recombinant Mediator complex that in addition to basal activity also shows p53 activator function. This sets the stage for incorporating ERα-interacting MED1 and its mutant variants into this core Mediator for further ERα-based functional studies. In view of the emerging importance of topologically associating domains (TADs) in enhancer-promoter communication, we also report the development of new functional assays in which linker histone H1-compacted higher order chromatin can be transcribed in vitro. These studies have revealed new factor dependencies that will inform our studies on ERα–dependent activation from distal enhancers. Towards pharmacological targeting of the ERα-MED1 interaction we have begun implementing a computationally driven approach for design and testing of peptidomimetics.

15. SUBJECT TERMS
estrogen receptor; breast cancer; cohesin; FoxA1; Mediator coactivator complex; enhancers; chromatin looping; higher order chromatin; peptidomimetics

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INTRODUCTION

Like many other transcriptional activators, liganded estrogen receptor (ERα) induces expression of its target genes in multiple steps entailing a series of coactivator exchanges that first make the chromatin more accessible and then lead to the establishment of functional Pol II complexes at core promoters. This proposal is aimed at understanding mechanisms whereby ERα activates its target genes from distal enhancers in a Mediator-dependent manner. Recent studies have shown chromatin looping at ERα-dependent genes and for a role of cohesin, a chromosome segregation factor, in ERα-dependent gene expression. Studies of the Mediator coactivator complex, which has emerged as an integrative hub for transcriptional regulation, indicate that it also plays critical roles in generating a distinct chromatin architecture at active loci through interactions with activators and cohesion. Given previous demonstrations of ERα interaction and function through the MED1 subunit of the Mediator, our working hypothesis is that ER-Mediator interactions would be critical for signal transduction at ERα target genes through establishment of chromatin loops that facilitate long-range enhancer-promoter communication. The proposal is thus aimed at (i) establishing cell-free (in vitro) transcription systems to recapitulate and mechanistically dissect Mediator-dependent ERα function from a distal enhancer; (ii) establishing complementary cell-based assays to analyze putative ERα-Mediator stabilized chromatin loop in the context of living cells; and (iii) developing peptidomimetic inhibitors of the ERα-MED1 interaction to modulate ERα-dependent gene expression by targeting enhancer-promoter communication. These studies are expected to provide mechanistic insights into normal transcription regulatory processes that go awry in breast cancer. Furthermore, the proposed peptidomimetics, which are expected to target novel druggable targets in the ERα activation pathway, may prove to be useful in breast cancer therapy since, unlike the widely used SERMs, they may have minimal off-target effects.

KEYWORDS

Estrogen receptor; breast cancer; cohesin; FoxA1; Mediator coactivator complex; enhancers; chromatin looping; higher order chromatin; peptidomimetics.

OVERALL PROJECT SUMMARY

As described below, this phase of the project (Year 2) continues our efforts from the previous year to have in hand essential reagents that will be needed for the proposed biochemical mechanistic studies and to establish key functional assays for reconstituting transcriptional activation from distal enhancers.

Reconstitution of an active core of the Mediator complex for functional studies (SOW Task 1)

As previously indicated, we have undertaken the reconstitution of the active core of the Mediator complex to facilitate our current studies on ERα, as well as other activators. Although thus far we have successfully relied on Mediator preparations obtained from cultured mammalian cell lines that express selected epitope-tagged Mediator subunits, isolation of the complex is cumbersome and suffers from limited yields. Furthermore, given that nearly all of the 30 Mediator subunits function only as part of the intact complex, mutational analysis of any given subunit is also greatly hampered. The large Mediator complex is composed of more or less discrete modules (“head”, “middle”, “tail” and “kinase”) (1). This modular organization in large part reflects the multiple layers of functions associated with the Mediator. Thus, whereas the head and middle modules are

responsible for the essential core functions of the Mediator, such as interaction with RNA polymerase II (Pol II) and support of basal (activator-independent) transcription, the tail is involved primarily in specialized functions dependent on specific activators. MED1, the subunit that interacts with ERα and other nuclear receptors and is essential for their activator function, is also dispensable for core Mediator functions. The kinase module is also required only conditionally.

Previously we had reported progress on the reconstitution of a Mediator core that was active in basal (activator-independent) transcription. Briefly, we first subcloned cDNAs encoding head and middle subunits into baculovirus vectors for overexpression in the MultiBac system developed by Dr. T. Richmond and colleagues (2). We isolated a stable bi-modular complex containing the subunits of the head and middle modules but found that it also needed the MED14 subunit for its function in basal transcription. More recent mechanistic studies showed that the recombinant complex containing stoichiometric amounts of the head and middle subunits plus MED14 was active in basal transcription because, unlike the head-middle bi-modular complex, it efficiently interacts with Pol II. In collaboration with Dr. Brian Chait at The Rockefeller University, parallel structural studies using newly developed mass spectrometric approaches in conjunction with protein-protein cross-linking (CX-MS) have revealed further details of the subunit architecture of the head-middle-MED14 Mediator subcomplex. Importantly, consistent with our functional data, these studies show that MED14 is critical both for the integrity of this complex and potentially for facilitating conformational changes necessary for Pol II binding and transcription. These conclusions are consistent with studies on the yeast Mediator complex that were published in the last year (3, 4).

Because the head-middle-MED14 complex lacks activator-targeted tail subunits it would not ordinarily be expected to support transcription by a range of activators. Fortuitously, a key component of the head module, MED17, is a target of the tumor suppressor p53 (5). We thus were able to demonstrate that this subcomplex efficiently supports p53-dependent transcription in vitro. These results established that Mediator harbors multiple functionalities that, minimally, are related to basal functions and transmission of activating signals. They also showed that by reconstituting larger derivatives of the core Mediator complex, we can expand the range of functions that can be elicited. However, and as expected, this complex did not support nuclear receptor function, which is mediated through MED1 interactions. Preliminary attempts to confer nuclear receptor function to the complex through addition of ectopic purified MED1 to transcription reactions were not successful. Interestingly, during the course of functional tests for activated transcription in a nuclear-extract based system, we found an additional requirement for the metazoan-specific MED26 subunit in overcoming the effect of negatively acting cellular factors. The results further highlight the possibility that still other subunits, in addition to MED1 and MED26, may be necessary to elicit ERα-dependent activation in vitro. We therefore are proceeding with reconstitution of Mediator complexes in which additional subunits, including tail subunits, are also present. Overall, these results set the stage for incorporating MED1 and its mutant derivatives into the core complex for our ongoing studies on ERα function through the Mediator.

The above results have now been published ((6), see also Publications, Abstracts, and Presentations, below). A copy of the paper is included as an appendix.

Establishment of an in vitro transcription system for transcription activation from distal sites (SOW Tasks 1, 2)

In our previous Progress Report we described our efforts to establish ERα-dependent transcription activation from distal sites. For this purpose we used a series of templates in which the EREs were placed at progressively distant sites relative to the core promoter/TSS. After assembly into chromatin using the recombinant histone/ACF1/NAP1 system (7), and ascertaining template quality by MNase1 digestion, the templates were subjected to p300-mediated histone acetylation

and subsequent transcription in unfractionated HeLa nuclear extract in the presence or absence of baculovirus-expressed and purified ERα. We had found that even in a template in which the ERα is over 1.0 kb away from the core promoter, ERα-dependent transcription was readily detectible. This indicated the existence of long-range ER-PIC interactions that characterize physiological enhancer functions. Nonetheless, the several-fold diminution of signal relative to the parent template, in which EREs are juxtaposed next to the core promoter, implied that certain critical factors might be limiting. An alternative possibility was that the system was partially compromised due to the absence of suitable higher-order template structures.

Indeed, very recent genome-wide studies that utilize variations of the chromatin conformation capture (3C) approaches have highlighted the presence of distinct higher-order chromatin structures, referred to as topologically associating domains (TADs), that could be especially important for effective enhancer-promoter looping (8). Given the likelihood that the chromatin in these structures exists in the form of compacted 30-nm fiber, in contrast to the 11-nm bead-on-a-string configuration of the chromatin that we typically use for our transcription assays (above), we have gone on to establish methods to generate linker histone H1.4-containing templates. Electron microscopy confirmed that inclusion of this linker histone in our modified chromatin assembly reactions leads to formation of characteristic 30-nm templates (Fig. 1B vs. Fig. 1A).

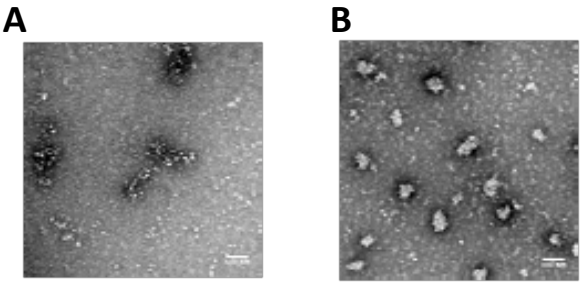

Fig. 1. Electron microscopy of GAL4-VP16 responsive templates chromatinized in without (A) or with linker histone H1.4 (B).

For the interim, we have focused on templates that carry binding sites for the potent hybrid transcriptional activator GAL4-VP16. Subjecting the H1.4-containing templates to modifications by histone acetyltransferase p300 followed by transcription in nuclear extract has allowed us to define factor requirements for opening and modification (acetylation) of the template in an activator-dependent fashion (Fig. 2). As expected, the templates exhibit a requirement for ATP-dependent chromatin remodelers (ACF or Swi/Snf) and the acetyltransferase p300. Interestingly, however, an unanticipated absolute requirement for the histone chaperone NAP1 that, under the conditions of the assay, is specific for the H1.4-containing template but not for the standard 11-nm chromatin template has also become evident (Fig. 2, lanes 3, 5, and 7 in the bottom panel vs. corresponding lanes in the top panel). Building on this assay, we plan to generate templates in which the activator binding sites are located distal from the core promoter. Contributions of Mediator and cohesin will also be assessed -- for example, through use of extract from which Mediator has been immunodepleted (9). Having optimized the assays, we will begin analyses of ERE-containing 30-nm templates and their responsiveness to ERα, which is a significantly weaker activator and therefore requires a better grasp of the experimental conditions.

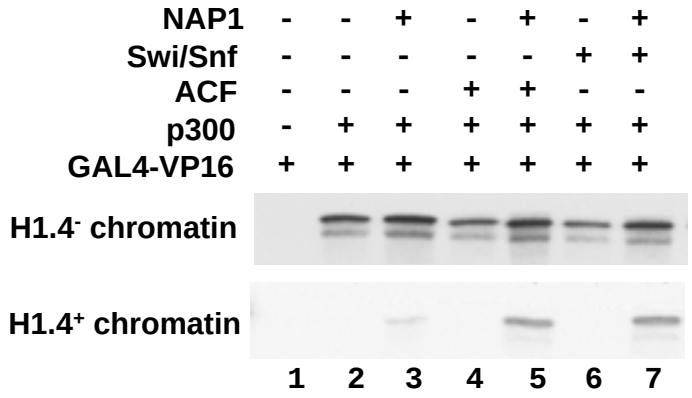

Fig. 2. In vitro transcription of GAL4-VP16 responsive templates chromatinized without (top panel) or with H1.4 (lower panel). Reactions were done with nuclear extract and additions were as indicated.

In parallel, we are proceeding with the reconstitution of long range activator-dependent transcription with purified components (9). In pilot studies we have used another strong activator, p53, in conjunction with a chromatinized template containing the *p21* gene regulatory region in its natural configuration in which the p53 binding sites are relatively distal from the core promoter. Initial data (not shown) suggest that the set of purified factors that might suffice for p53-dependent activation when multimerized p53 binding sites are juxtaposed next to the core promoter are not sufficient to support transcription of the natural p21 gene. However, spiking the systems with cruder fractions elicits activation of the gene, indicating a requirement for additional factors that might specifically be needed for optimal transcriptional activation from distal locations. This series of experiments will also guide us in selecting cofactors to include in assays that will be performed with ERα.

Pharmacological targeting of the ERα–MED1 interaction (SOW Task 3)

A goal of the proposal is to develop pharmacological inhibitors of the ERα-MED1 interaction with a view to destabilizing the chromatin loops bridging enhancers and promoters on ERα target genes. Our rationale is that even though Mediator might function at most, if not all, Pol II genes, its structural modularity, and potentially unique mechanisms for interactions with individual activators, offers opportunities for selectively targeting these interactions. With respect to nuclear receptors, which mainly interact with MED1 via its NR boxes, we reasoned that individual interactions would be different enough (owing to different flanking sequences, among other parameters) to allow for design of inhibitors that would discriminate between ERα-MED1 versus other NR-MED1 interactions. Although our original plan was to undertake a somewhat detailed structure-function study to delineate the potential basis for such selectivity prior to synthesis of peptidomimetics, recent developments in computational tools now allow for a more direct approach. In collaboration with Dr. Paramjit Arora (Department of Chemistry, New York University), who is both a leader in using computational approaches to identify protein interfaces that act as efficient side chain scaffolds for protein-protein interactions and an expert in chemical synthesis of corresponding

peptidomimetics, we are designing small molecules that target the ERα-MED1 interaction. We hope to be testing these candidate agents in our various functional assays soon.

KEY RESEARCH ACCOMPLISHMENTS

- Publication of reconstitution of the active core of the Mediator complex, the largest recombinant multiprotein complex reported to date to be so reconstituted.

CONCLUSION

The project remains focused on generating reagents and establishing functional assays. In addition to the reagents described in the previous report, we now have completed reconstitution of a recombinant Mediator complex that supports p53 activator function. Efforts are under way to impart ERα–responsiveness through inclusion of additional Mediator subunits. Towards analyzing ERα function from enhancers, we have also set up in vitro transcription assays for highly compacted chromatin templates and identified key factor requirements. Finally, we will be moving ahead with computationally designed small molecules to target ERα-MED1 interaction.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

Publication in peer-reviewed scientific journal:

Cevher, M.A., Shi, Y., Li, D., Chait, B.T., Malik, S., and Roeder, R.G. (2014) Reconstitution of active human core Mediator complex reveals a critical role of the MED14 subunit. *Nat. Struct. Mol. Biol. 21,*1028-34. doi: 10.1038/nsmb.2914*,* PMID*:* 25383669.

INVENTIONS, PATENTS, AND LICENSES

Nothing to report.

REPORTABLE OUTCOMES

Nothing to report.

OTHER ACHIEVEMENTS

Nothing to report since last reporting period.

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nature structural & molecular biology

Reconstitution of active human core Mediator complex reveals a critical role of the MED14 subunit

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The evolutionarily conserved Mediator complex is a critical coactivator for RNA polymerase II (Pol II)-mediated transcription. Here we report the reconstitution of a functional 15-subunit human core Mediator complex and its characterization by functional assays and chemical cross-linking coupled to MS (CX-MS). Whereas the reconstituted head and middle modules can stably associate, basal and coactivator functions are acquired only after incorporation of MED14 into the bimodular complex. This results from a dramatically enhanced ability of MED14-containing complexes to associate with Pol II. Altogether, our analyses identify MED14 as both an architectural and a functional backbone of the Mediator complex. We further establish a conditional requirement for metazoan-specific MED26 that becomes evident in the presence of heterologous nuclear factors. This general approach paves the way for systematic dissection of the multiple layers of functionality associated with the Mediator complex.

Activation of genes transcribed by eukaryotic RNA polymerase II (Pol II) entails a complex functional interplay between general transcription factors (GTFs), gene- and cell type–specific activators and an array of coactivators[1]. Whereas Pol II and GTFs can form a preinitiation complex (PIC) on core promoter elements that exhibits low-level (basal) activity *in vitro*, activators can greatly stimulate PIC function through coactivator recruitment. Among the diverse types of coactivators described, the multisubunit Mediator complex has emerged as perhaps the most critical coactivator that facilitates PIC establishment and function[2]. Although initially identified and characterized as a cofactor that bridges activators and the Pol II machinery[2], the metazoan Mediator has also been shown to stimulate basal (activator-independent)[3–5] and negative (co-repressor)[2,6] functions under certain conditions. More recently, given the multistep nature of the transcription process, Mediator has been further implicated in coordinating mechanistic transitions from the chromatin opening to the PIC-establishment phase[7–9] and, potentially, from the initiation to the elongation phases[10–12]. Additionally, evidence exists to suggest Mediator involvement in other transcriptionally relevant processes such as facilitation of enhancer-promoter communication by stabilization of chromatin loops through interactions with long noncoding RNA[13] or cohesin[14] and transcription-coupled DNA repair[15]. Mediator's critical role in the cell is also underscored by reports that tie mutations in its various subunits to human disease[16,17].

These diverse Mediator-associated functions are reflected in its complex subunit architecture. The 2-MDa metazoan Mediator consists of 30 subunits, many of which are evolutionarily conserved in eukaryotes from yeast to humans[18]. However, in agreement with the increased complexity of metazoan transcriptional programs relative to those in yeast, the extent of homology ranges from about 50% for a handful of the most conserved subunits (for example, MED7 and MED31) to much weaker relationships for the remainder[18]. Further, the metazoan complex contains additional metazoan-specific subunits (for example, MED26 and MED30). The overall structure of the complex, both in yeast and humans, is modular, with the subunits organized into head, middle, tail and kinase subcomplexes[2]. The subunits composing the head and middle modules are tightly associated with each other and constitute a stable core; they have been implicated in interactions with the Pol II machinery. By contrast, the individual subunits of the tail module are relatively loosely associated with each other; specific promoter- or enhancer-bound activators mainly, but not exclusively, target individual tail subunits[19]. The kinase module reversibly associates with the core complex and broadly tends to confer repressive properties to the Mediator.

Substantial progress has been made in the understanding of structure-function relationships for the Mediator, especially in yeast. Thus, previous studies of yeast Mediator have provided crystal structures for both the head and partial middle modules[20–24] and a model, based on cross-linking, for protein interactions within the middle module[25]. Yeast two-hybrid screens also have led to predictions for the protein interaction networks within the head and middle modules[26]. Most recently, EM analyses of the yeast Mediator have suggested a model for how individual subunits are organized within the complex[27,28]. However, without any demonstration of the minimal set of subunits required for the assembly of transcriptionally active Mediator or the identification and pinpointing of the critical roles of individual essential subunits, these studies have not led to an understanding of the identity and mechanism of action of the active core Mediator components. Furthermore, understanding of the metazoan complex has also been hampered, in part, by technical difficulties in manipulating this complex. These relate to its large size and heterogeneity, its many essential subunits and its limited yields upon purification from cell extracts.

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Thus far, the metazoan Mediator complex has been functionally characterized mainly in *in vitro* biochemical assays using preparations obtained from nuclear extracts of HeLa cell lines that stably express wild-type or mutant versions of selected subunits. However, in order to obtain a detailed structure-function understanding of the metazoan Mediator complex, it is necessary to dissect it at the level of individual subunits, modules and multimodule assemblies, and to make correlations with their roles in the transcriptional processes. The inherent modularity of the Mediator and the ability to isolate an active form (the PC2 complex) that lacks the kinase module and several tail subunits, but is enriched with respect to the metazoan-specific MED26 (refs. 4,29), makes it feasible to undertake a reconstitution-based approach to establish structure-function relationships for the Mediator.

Here, to generate a minimal active human core Mediator complex and to isolate homogeneous preparations in desirable yields, we used the efficient MultiBac baculovirus expression system[30] to jointly express Mediator subunits that are found in the active PC2 form of the Mediator. We first separately reconstituted the head and middle modules. We found that although these modules can stably associate with each other, the resulting bimodular complex is inactive in transcriptional assays unless MED14 is also incorporated. Mechanistically, we show that MED14 addition to the complex markedly enhances its interaction with Pol II. However, this complex is unable to support activity in extract-based assay systems unless complemented with MED26, thus suggesting that this subunit allows the Mediator to operate in the context of additional factors present in the extract. We also report an in-depth cross-linking–coupled MS (CX-MS) analysis of the reconstituted core complex that, while also revealing other interactions, further highlights the key structural role of MED14 in bridging all the main modules of the Mediator complex. Our results are discussed in the context of a recent study focused solely on the architecture of yeast and human Mediator[27].

RESULTS

Reconstitution of the head–middle bimodular complex

We initiated the reconstitution by first generating the middle module through coexpression of Flag-tagged MED7 (f-MED7), MED19, MED4, myc-MED21, MED31, MED9 and histidine-tagged MED10 (His-MED10) in insect cells. Because of the conditional requirement for MED1 (ref. 31), we did not include this subunit in our initial analysis. Sequential affinity chromatography (**Supplementary Fig. 1a**,**b**) yielded a MED4–MED7–MED10–MED21–MED31 complex containing all essential subunits of the middle module (**Fig. 1a**,**b**). The MED9 subunit (nonessential in yeast[32,33]) failed to express and was not required for Mediator function in our transcription assays (below) and thus was omitted in further reconstitutions. MED19, although expressed, showed no association with the middle module. We similarly reconstituted the head module of the human Mediator by coexpressing f-MED17, MED6, MED8, MED11, MED18, MED19, MED20, MED22 and the metazoan-specific MED30, which previously was not assigned to any module. After purification, we obtained a head-module complex (MED6–MED8–MED11–MED17–MED18–MED20–MED22–MED30) (**Fig. 1c**,**d**) that contained all of the input subunits except MED19, whose association with the complex is probably dependent on one or more metazoan-specific subunits not included in our reconstitutions.

To reconstitute a complex containing both the head and middle modules (H + M), we coexpressed the subunits of the two modules (**Fig. 1**). Sequential selection through f-MED17 (head module) and hemagglutinin-tagged MED7 (HA-MED7) (middle module) subunits and subsequent Superose 6 gel filtration revealed a stable interaction between the head and middle modules (**Fig. 1e** and **Supplementary Fig. 1c**,**d**). The resulting H + M preparation (**Supplementary Fig. 1d**) contained stoichiometric amounts of all the subunits except MED18 and MED20, which in yeast are nonessential[32,33] and form a labile heterodimer[23] and thus tend to dissociate upon gel filtration (**Supplementary Fig. 1e**; also described further below). Interestingly, we observed that separately purified head and middle modules do not associate to form a bimodular complex when mixed together (data not shown), results potentially indicative of a strict requirement for coexpression of subunits constituting the modules.

MED14 is critical for basal and activated transcription

Natural Mediator purified from human cells stimulates both basal and activator-dependent transcription in nuclear extract[3–5]. We therefore tested whether the H + M preparation stimulated basal transcription in our two standard *in vitro* transcription assays[34] containing either (i) purified general transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF

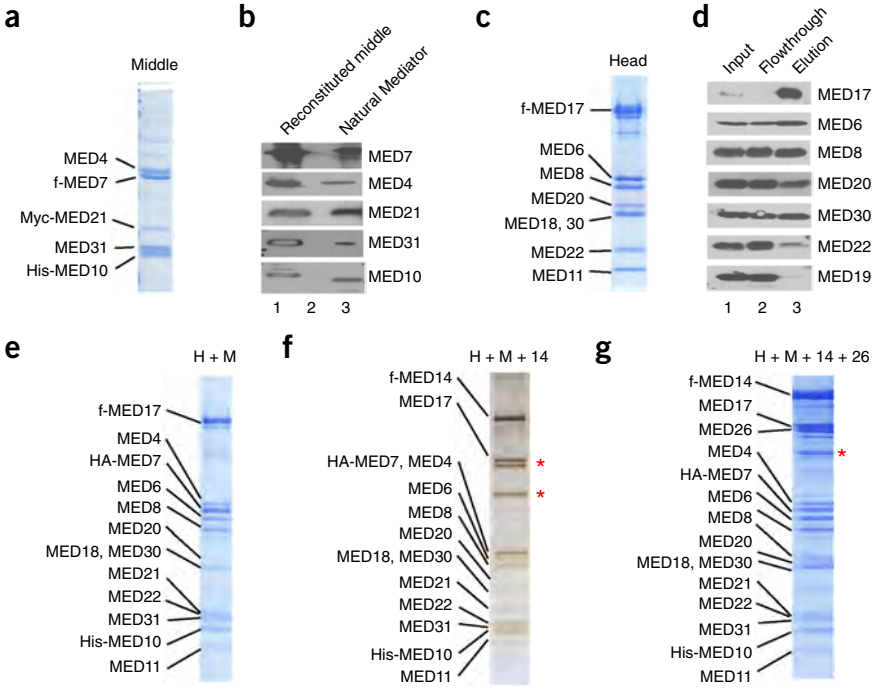

Figure 1 Reconstitution of human Mediator subcomplexes. (**a**) SDS-PAGE analysis (Coomassie blue staining) of baculovirus-expressed and reconstituted middle module. (**b**) Western blot analysis of middle-module subunits. (**c**) SDS-PAGE analysis (Coomassie blue staining) of the reconstituted head module. (**d**) Western blot analysis of head-module subunits. (**e**) SDS-PAGE analysis (Coomassie blue staining) of the bimodular head + middle (H + M) complex after purification on M2 agarose (via f-MED17) and HA agarose (via HA-MED7). (**f**) SDS-PAGE analysis (silver staining) of a MED14-containing head + middle (H + M + 14) complex purified as in **e**, except that the MED14 was Flag tagged. (**g**) SDS-PAGE analysis (Coomassie blue staining) of the H + M + 14 + 26 complex purified as in **f**. Asterisks in **f** and **g** point to contaminating polypeptides. Uncropped gel images are shown in **Supplementary Data Set 1**.

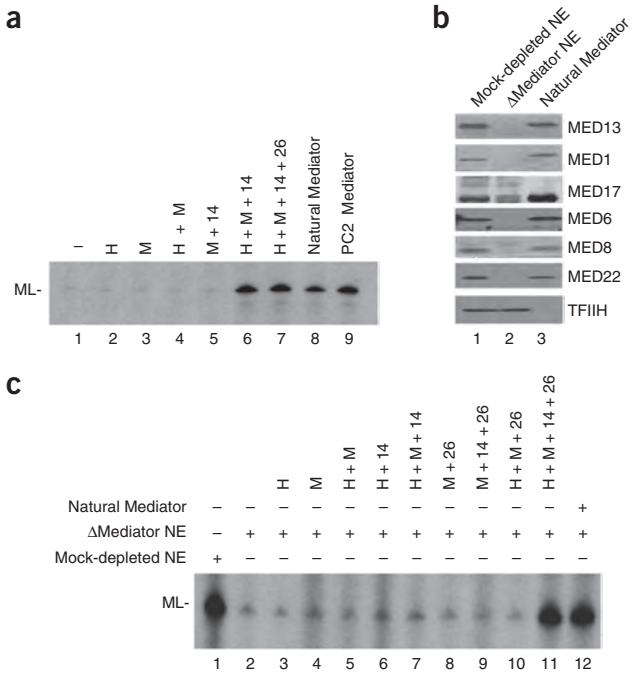

Figure 2 Critical roles of MED14 and MED26 in Mediator-stimulated basal transcription. (**a**) Autoradiogram of *in vitro* transcription reactions from a template (ML) containing the adenovirus major late core promoter. Reactions were performed with purified GTFs (IIA, IIB, IID, IIE, IIF and IIH), Pol II and PC4 and the indicated Mediator subcomplexes (H, head; M, middle; 14, MED14; 26, MED26). (**b**) Western blot analysis of HeLa nuclear extract (NE) immunodepleted of Mediator by anti-MED30 antibody (ΔMediator NE). (**c**) Autoradiogram of *in vitro* transcription reactions from the ML template with control (mock-depleted) or ΔMediator NE. Mediator subcomplexes were added to the transcription reactions as indicated. Uncropped gel images are shown in **Supplementary Data Set 1**.

and TFIIH), coactivator PC4 and Pol II (**Fig. 2a**) or (ii) unfractionated HeLa cell nuclear extract (NE) immunodepleted for the Mediator complex (**Fig. 2b**, lane 1 versus lane 2). Because the H + M preparation, as well as the independent head and middle modules, failed to show any activity in either assay (**Fig. 2a**, lanes 1–4; **Fig. 2c**, lane 5 versus lane 2), we sought to include additional subunits in our reconstitution. We started with MED14, which, despite its previous assignment to the tail module[35], is present in stoichiometric amounts in our PC2 preparations that otherwise tend to be deficient in tail components[4].

We reconstituted an H + M complex containing MED14 (H + M + 14) and performed affinity selection via MED14 to ensure that the resulting homogeneous preparation contained stoichiometric MED14 (**Fig. 1f**). When tested in the *in vitro* transcription assay with purified factors (GTFs, Pol II and PC4), this 14-subunit complex effected a strong stimulation of basal transcription (**Fig. 2a**, lane 6 versus lane 1). Importantly, the fold stimulation was equivalent to that elicited both by a natural Mediator preparation containing a complete set of subunits (lane 6 versus lane 8) and by its PC2 form (lane 6 versus lane 9). We therefore conclude that the subunits contained in the H + M + 14 preparation define the active human core Mediator complex.

MED26 requirement for Mediator function in nuclear extract

Although active in the defined assay system, the H + M + 14 preparation was unable to restore basal transcription when added back to Mediator-depleted nuclear extract (**Fig. 2c**, lane 7 versus lane 1). Because the extract contains a more natural complement of various nuclear factors, this result indicated a requirement for another Mediator subunit to overcome an apparent constraint by a negative cofactor(s). Although MED26-containing PC2 is a small fraction of the total cellular Mediator population in HeLa cells, a previous study has shown that extracts from which this subpopulation is depleted fail to support *in vitro* transcription[4]. Further, MED26-containing Mediator preparations have a higher Pol II content[4,36], and MED26 can recruit the super elongation complex to promoters[12,37]. We therefore generated variant complexes containing MED26. We found that MED26 associates with the middle module but not the head module (**Supplementary Fig. 2a**,**b**), in agreement with the recent report from Tsai *et al.*[27], and that it can be stably incorporated into an H + M + 26 complex (**Supplementary Fig. 2c**) and an H + M + 14 + 26 complex (**Fig. 1g** and **Supplementary Fig. 3**). Importantly, in the Mediator-depleted extract (**Fig. 2c**, lane 11 versus lane 12), as in the pure system (**Fig. 2a**, lane 7 versus lanes 8 and 9), the H + M + 14 + 26 complex restored basal transcription to the same level as did a natural Mediator preparation. Inclusion of MED26 into other partial complexes failed to restore transcription in the extract-based assay (**Fig. 2c**, lanes 8–10), thus suggesting that the additional requirement for MED26 in this context is superimposed upon a more fundamental structural dependency on MED14. Importantly, in the extract-based assay, the H + M + 14 + 26 (but not the H + M + 14) complex exhibited a clear coactivator function for the transcriptional activator p53, which is known to interact with the MED17 subunit[38] (**Fig. 3a**, lane 8 versus lanes 6 and 4). In control experiments, we saw no coactivator function for the thyroid-hormone receptor (TR) (**Fig. 3b**, lane 12 versus lane 6), which functions as a heterodimer with the retinoid X receptor (RXR) and targets the missing MED1 subunit[39].

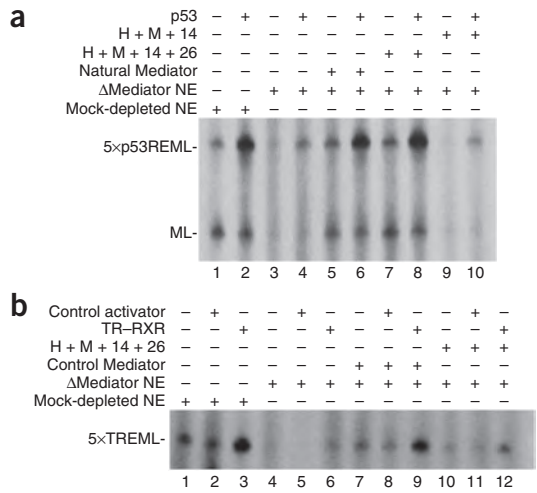

Figure 3 Critical roles of MED14 and MED26 in Mediator coactivator function. (**a**) Autoradiogram of *in vitro* transcription reactions performed as in **Figure 2c**. Extract-based reactions contained the p53-responsive template 5×p53REML and a control template (ML). The activator (p53) and Mediator subcomplexes (H + M + 14 and H + M + 14 + 26) were added as indicated. (**b**) Autoradiogram of *in vitro* transcription reactions performed as in **a**, except that the TR–RXR heterodimer was used as the activator together with the TR-responsive template 5×TREML. An irrelevant activator (AML1-ETO) was included as a control. Uncropped gel images are shown in **Supplementary Data Set 2**.

MED14 is crucial for Mediator–Pol II interaction

To understand the mechanism whereby MED14-containing Mediator complexes are rendered active, we used coimmunoprecipitation to investigate

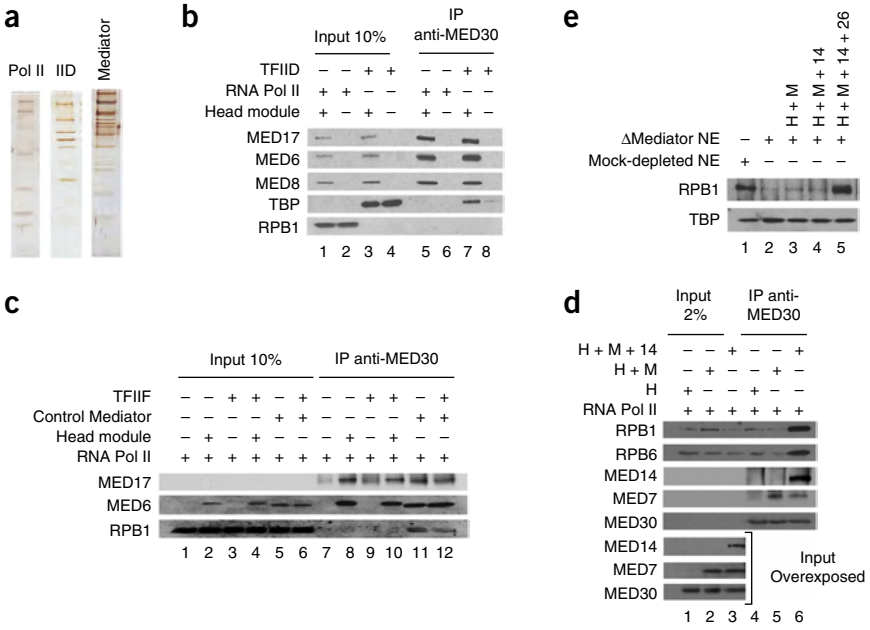

Figure 4 MED14-dependent Mediator–Pol II interaction and MED26-dependent Pol II recruitment in nuclear extract. (**a**) SDS-PAGE analysis (silver staining) of purified preparations of Pol II, TFIID (IID) and natural Mediator used in the binding assays. (**b**) Western blot analysis of Mediator interaction assays. Binding reactions included the Mediator head module and Pol II or TFIID, as indicated. Anti-MED30 immunoprecipitates (IP) were probed for TFIID (TBP), Pol II (RBP1) and selected Mediator subunits. (**c**) Western blot analysis of Mediator-head interaction assays in the presence of TFIIF. Binding reactions were as in **b**, except that they also included TFIIF as indicated. (**d**) Western blot analysis of Mediator–Pol II interaction assays. Binding reactions included Pol II and the indicated recombinant Mediator subcomplexes (H, H + M, or H + M + 14). Anti-MED30 immunoprecipitates were probed for Pol II (RPB1 and RBP6) and Mediator (MED7, MED14 and MED30) subunits. For lanes 1–3 (inputs), longer western blot exposures are also included. (**e**) Western blot analysis of an immobilized template recruitment assay to assess Pol II recruitment. Reactions were done with control or Mediator-depleted HeLa NE (ΔMediator NE) and were supplemented with various recombinant Mediator subcomplexes. Recruitment of Pol II (RPB1) and TFIID (TBP) was monitored. Uncropped gel images are shown in **Supplementary Data Set 2**.

the interaction of the head with TFIID and the interaction of the head, H + M and H + M + 14 complexes with Pol II (**Fig. 4**). In agreement with results from previous studies[40], the head module alone interacted with TFIID (**Fig. 4b**, lane 7). However, in contrast to the case in yeast[41], neither the head module (whether in the presence (**Fig. 4c**, lane 10) or absence (**Fig. 4b**, lane 5 and **Fig. 4c**, lane 8) of TFIIF) nor the H + M complex (**Fig. 4d**, lane 5) was able to bind to Pol II. By contrast, and importantly, the H + M + 14 complex bound up to 75% of input Pol II (**Fig. 4d**, lane 6). Hence, a critical function of MED14 is to render H + M capable of efficiently interacting with Pol II, thereby stimulating transcription.

MED26 overcomes a Pol II–recruitment restriction

To understand the basis for the conditional requirement of MED26 for Mediator function in nuclear extract, we performed an immobilized template assay in which we monitored Pol II recruitment to a promoter (**Fig. 4e**). For this purpose, we incubated DNA-bound beads with control or Mediator-depleted nuclear extract. In the latter case, we further supplemented the reactions with our various Mediator preparations. In agreement with our previous results[42], Pol II recruitment was abolished in Mediator-depleted extracts (**Fig. 4e**, lane 2 versus lane 1). Interestingly, neither the H + M complex (lane 3) nor the H + M + 14 complex (lane 4), the latter of which interacted strongly with purified Pol II (**Fig. 4d**), was able to induce Pol II recruitment. By contrast, and paralleling the results of the *in vitro* transcription experiment (**Figs. 2c** and **3**), the H + M + 14 + 26 complex was able to induce Pol II recruitment (**Fig. 4e**, lane 5 versus lane 1). Therefore, we conclude that the conditional requirement of MED26 in nuclear extract reflects a restriction, at the level of Pol II recruitment to the promoter, that MED26 allows the Mediator to overcome.

Molecular architecture of the core Mediator complex

To dissect the molecular architecture of the Mediator complex, we chemically conjugated the reconstituted H + M + 14 + 26 complex by amine-specific, isotopically labeled disuccinimidyl suberate (DSS)

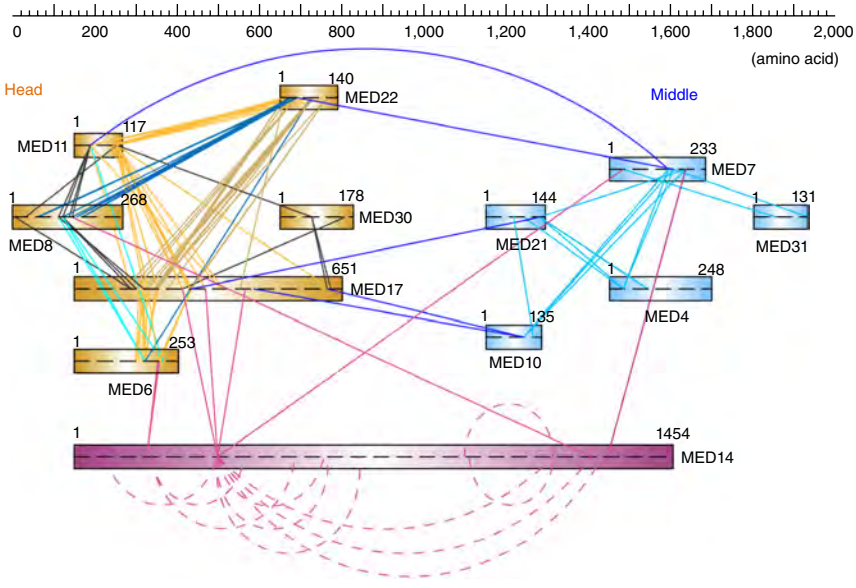

Figure 5 Molecular architecture of the reconstituted Mediator complex revealed by chemical cross-linking and MS (CX-MS). Residue-specific cross-linking map of the Mediator complex obtained by CX-MS. Except for MED14, for which intrasubunit cross-links (>200 residues apart) are shown, only intersubunit cross-links are depicted. Light blue, middle-module subunits; gold, head-module subunits; purple, MED14. **Supplementary Tables 1** and **2** show the complete cross-linking data set.

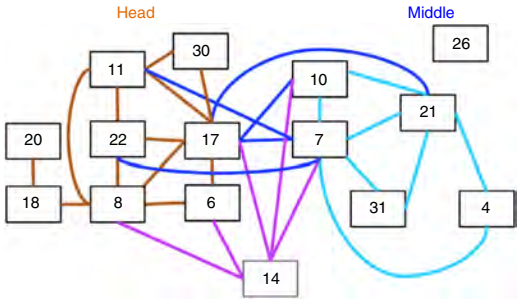

Figure 6 Schematic representation of subunit interactions in the human core Mediator complex, based on composite data from CX-MS and biochemical approaches. Light blue, intramodule interactions in the middle module; brown, intramodule interactions in the head; purple, MED14 interactions; dark blue, intermodule interactions between head and middle modules.

(**Supplementary Fig. 4a**) and applied high-resolution MS (CX-MS)[43] to identify cross-linked peptides. We identified 277 unique cross-links (**Supplementary Tables 1** and **2**), which we used to build a spatial connectivity map of the complex (**Fig. 5**). Remarkably, the cross-linked lysines represent 60% of the total lysines of the reconstituted H + M + 14 + 26 complex (**Supplementary Fig. 4b**,**c**). The data reveal an extensive network of contacts between subunits within each of the head and middle modules as well as between subunits of the two modules (**Fig. 5**). The intramodular cross-linking data are in good agreement with the published studies of yeast Mediator modules[22–25]. Especially for the head module, a region (amino acids ~150–300) toward the N-terminus of human MED17 is also a structural hub within the module, cross-linking with MED6, MED8, MED11, MED22 and MED30 (**Supplementary Table 2**). In agreement with their previously proposed hinge function within the middle module[20,25], MED7 and MED21 contact each of the other constituent subunits. MED18, MED20 and MED26, being substoichiometric, were not scored by CX-MS.

Importantly, the CX-MS data reveal intermodular contacts between MED17 in the head module and MED10 and MED21 in the middle module. Furthermore, relevant to the critical role of MED14 in Mediator function, this subunit cross-linked to both head (MED6, MED17) and middle (MED7) components (**Supplementary Table 2**), thus serving to further bridge the two modules. We also identified several intrasubunit cross-links between N- and C-terminal residues of MED14 in the active core Mediator complex, thus indicating that this large (170-kDa) subunit may potentially fold back upon itself and facilitate its interaction with the head and middle modules and perhaps also with Pol II (**Supplementary Table 2**). Alternatively, this cross-linking pattern might arise from a tendency of MED14 to form (transient) dimers.

In complementary experiments to validate the CX-MS data, we generated a series of partial derivatives of the head and middle modules by selective omission of subunits and performed immunoprecipitations with selected subunit combinations (**Supplementary Fig. 5**). We confirmed CX-MS–identified interactions of MED7 and MED21 with various middle subunits (**Supplementary Fig. 5a–c**) and detected an additional interaction between MED21 and MED31 (**Supplementary Fig. 5a**, lane 8). Similarly, for the head module (**Supplementary Fig. 5d**), we identified complex formation between MED11 and MED22 (**Supplementary Fig. 5d**, lanes 6, 7), between MED11, MED22 and MED17 (**Supplementary Fig. 5d**, lane 6), between MED6 and MED17 (**Supplementary Fig. 5d**, lane 4), between MED8 and MED17 (**Supplementary Fig. 5d**, lane 3 versus lanes 4 and 5) and between MED8 and MED18 (**Supplementary Fig. 5d**, lane 3 versus lane 5). Importantly, as they do in yeast, MED18 and MED20 formed a heterodimer that is anchored to the head via MED8 (**Supplementary Fig. 5d**, lane 8 and lane 3 versus lane 4).

In agreement with the cross-linking data for MED17, H + M formation was dependent on the presence of MED17 (**Supplementary Fig. 2b**). Moreover, MED17 also copurified with the middle module (**Supplementary Fig. 5f**), thus identifying it as a major link between the head and middle modules. Relatedly, this series of analyses also identified an additional interaction (between MED17 and MED7) that contributes to the head-middle interaction (**Supplementary Fig. 5g**).

Notably, through coexpression of MED14 with either head- or middle-module subunits, we established that MED14 could independently associate with the head and middle modules (**Supplementary Fig. 6a**,**b**), in agreement with the cross-linking data. Also of note, MED14 interacted with the MED24 and MED16 subunits of the tail module, which, however, were not included in our reconstitutions (**Supplementary Fig. 6c**). These results further implicate MED14 as the essential backbone of the Mediator that bridges its three main modules (composite subunit interaction network for the human core Mediator complex deduced from various approaches in **Fig. 6**). At a gross level, the deduced interactions among the subunits and the general architecture of the human core Mediator complex from these data are in good agreement with the data of Tsai *et al.*[27].

DISCUSSION

In this paper, we describe a reconstitution-based approach aimed at the generation of Mediator subcomplexes that display various functionalities previously ascribed to Mediator. We also generated a detailed spatial connectivity map of the active core Mediator complex through CX-MS and pairwise interaction analyses of selected subunits. These structural and functional studies converge to highlight a critical role for MED14 in Mediator architecture and activity. Although head-middle interactions yield a stable complex, MED14 association with these two modules is necessary to reconstitute a functionally active 14-subunit core Mediator complex. In this complex, the MED14 subunit is the one most critical for facilitating a very strong Pol II interaction that correlates with the acquisition of both basal and selective activator (p53)-dependent transcription activity. Our results also show that MED26, although not required for core Mediator function in an assay with purified factors, is essential (along with MED14) for core Mediator function in a nuclear extract. Thus, our approach has allowed us, uniquely, to identify the minimal components of the active core Mediator complex and to understand the underlying mechanisms and roles of Mediator subunits in a minimal purified system versus a nuclear extract containing a more natural complement of nuclear factors.

MED14 has been viewed as a tail component, albeit one that bridges the tail to the bulk complex[35]. Our protein-protein–interaction and CX-MS data establish that MED14 interacts with tail subunits (MED16 and MED24) as well as head-module (MED6, MED8 and MED17) and middle-module (MED7 and MED10) subunits. Thus, MED14 appears to furnish the architectural features necessary for integrating three separate modules of the Mediator into a single functional entity. This model of MED14 as an architectural backbone of the Mediator complex is in good agreement with the recent cryo-EM analysis of Tsai *et al.*[27], which revealed that density attributable to MED14 spans the length of the natural yeast Mediator complex and makes multiple contacts with subunits of the tail, middle and head modules. Neither our present study nor the study by Tsai *et al.*[27] addressed how MED14 relates to the dissociable kinase module.

In a major extension of the solely architectural focus of the study by Tsai *et al.*[27], we show further that MED14 is critically required for the function of the core Mediator. Previously, the isolated head module of the yeast Mediator was reported to interact with Pol II (via MED17)[44] and to stimulate basal activity[41]. Therefore, it initially was somewhat surprising that our reconstituted head–middle bimodular complex was unable to support even the most rudimentary Mediator activity of stimulating basal transcription. Indeed, the metazoan head–middle complex (as well as the head complex alone) failed to interact with Pol II in our hands. Only when MED14 was incorporated into this assembly through coexpression did the complex interact with Pol II and acquire basal transcription activity. MED14 is also required (along with MED26) for forming a Mediator complex (H + M + 14 + 26) that exhibits a selective coactivator function for p53, which interacts with the head subunit MED17. Thus, MED14 is not simply an architectural backbone of the Mediator complex but also is a critical subunit in facilitating transduction of the necessary signals within the Mediator–PIC assembly. Reciprocally, the results suggest that the tail module may serve principally as an activator target site for Mediator recruitment, with no additional role in core Mediator–enhanced transcription.

How might MED14 contribute to Pol II interaction and ultimately to stimulation of transcription? Most simply, this could result from a direct physical interaction between Pol II and the large surface furnished by the MED14 backbone. However, whereas their most recent cryo-EM study[27] did not shed additional light on which features of the yeast Mediator complex are responsible for holoenzyme formation, Tsai *et al.*[45] previously proposed a multistep model in which the Pol II CTD first interacts with the head module and then comes to rest within a cavity formed by the head, middle and tail modules of the remodeled Mediator complex. Although the EM analyses did not allow precise delineation of contacts, the density now identified as the MED14 backbone does not seem to be in direct contact with Pol II in the published images[45]. Furthermore, to our knowledge, neither prior yeast genetic studies nor other studies have implicated MED14 in Pol II interactions. Therefore, the alternative possibility remains that MED14 effects on Pol II binding are indirect and are related to the documented intermodule movements that occur upon Pol II binding[45,46]. It is likely that in the absence of MED14, the otherwise stable head–middle complex, which may yet be responsible for a majority of the Pol II contacts, is incapable of acquiring the necessary conformation on its own.

With its structural complexity, and in addition to its overlapping core functions of stimulating basal transcription and mediating activation signals, Mediator can coordinate the action of numerous cofactors that impinge upon the transcriptional machinery[2]. Thus, in contrast to its activity in transcription assays reconstituted with pure factors, the H + M + 14 complex failed to function in HeLa cell nuclear extract. Previous studies have shown that whereas Mediator acts mainly to stimulate transcription in the purified systems, its requirement in extracts is absolute[3,10]. This suggests that in the cellular milieu an important role of the Mediator is to overcome the effects of negatively acting cofactors that may include DSIF[10], Gdown1 (ref. 47) and potentially NC2 (ref. 48). Thus, our finding that a MED26-containing complex can function in nuclear extract to stimulate both basal and activator-dependent transcription suggests a role for this subunit in counteracting negative cofactors. This is consistent with our prior observation that even though the MED26-containing subpopulation of the Mediator (PC2) constitutes a very small fraction of the total Mediator, its depletion from HeLa cell nuclear extract leads to abrogation of transcription activity[4].

MED26 has been implicated in interactions with TFIID and the P-TEFb- and ELL-containing super elongation complex, thus leading to a model in which this subunit functions in a handoff from the initiation to the elongation machinery[12]. However, our mechanistic dissection reveals that, collectively, the cofactors in the extract impose an even earlier restriction at the level of Pol II recruitment to the promoter and that MED26-containing Mediator overcomes the restriction. This observation suggests a function for MED26 at the earliest stages of the transcription process, which precede involvement of the elongation machinery. It does not, however, preclude a subsequent additional role for MED26 at the initiation-to-elongation transition or the elongation stages. The precise mechanism whereby MED26-containing Mediator overcomes the effect of negative factors is unclear. However, its localization in the middle module relatively distant from the Pol II–binding cavity[27] argues against direct interactions with Pol II. Possibilities include MED26-dependent recruitment of activities that neutralize the negative cofactors or freezing of Mediator in conformations that favor Pol II interactions and disallow negative cofactor interference. Of note, even in this context, the MED14 requirement persists, in agreement with its mechanistically distinct and essential role.

A recent study in *Drosophila* has suggested that the MED26 requirement is stage specific[49]. Thus, it remains unclear whether our results reflect a general MED26 requirement or cell type-specific (HeLa) regulation. Nonetheless, our ability to generate compositionally defined Mediator complexes that carry out functions over and above Mediator's core functions nicely illustrates the feasibility of recapitulating increasingly complex metazoan-specific regulatory functions by building ever-larger Mediator derivatives. As we expand the scope of these studies and reconstitute larger derivatives of the core Mediator complex, we hope to obtain a better understanding of the full range of Mediator functions, including those that go awry in disease states.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

M.A.C., S.M., R.G.R., Y.S. and B.T.C. designed the experiments and wrote the manuscript. M.A.C. carried out biochemical experiments including cDNA preparations, reconstitutions, *in vitro* transcriptions and coimmunoprecipitation experiments. D.L. helped M.A.C. in the generation of partial head-module complexes (in **Supplementary Fig. 5d**). Y.S. carried out the CX-MS experiments.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

cDNA cloning of Mediator subunits. For subcloning into baculovirus expression vectors, we used existing cDNAs for MED4, MED6, MED7, MED10, MED14, MED16, MED18, MED20, MED21 and MED24 (refs. 4,29,38,50). For the remaining subunits, we isolated new cDNA clones from HeLa cells. Total RNA from HeLa cells was purified and cDNA prepared by reverse transcription with oligo-dT primers. The resulting cDNA was amplified with appropriate PCR primers to generate individual clones for MED8, MED9, MED11, MED19, MED22, MED26, MED30 and MED31. Interestingly, at least two variants were seen for MED8 and MED22. We selected the shortest variant cDNAs of each for expression.

Reconstitution of human Mediator complexes. In order to obtain near-stoichiometric complexes, Mediator subunit cDNAs were cloned into pFBDM and pUCDM transfer vectors[30]. Various tags (histidine, myc, HA or Flag) were inserted into different subunits of the Mediator to facilitate downstream purification. The transfer vectors were integrated into a single bacmid (through both transposition and Cre-Lox recombination) for generation of viruses. The resulting viruses were amplified in Sf9 cells. For protein production, Hi5 cells were infected with the amplified viruses. Infected cells were homogenized in BC500 (500 mM KCl, 10 mM Tris-Cl, pH 7.9, 20% glycerol, 0.1 mM EDTA, 3.5 mM β-mercaptoethanol and 0.1 mM PMSF supplemented with protease inhibitors pepstatin (0.5 μg/ml) and leupeptin (0.5 μg/ml). After ultracentrifugation (20,000 r.p.m. in a Beckman Type 45 Ti rotor for 30 min), the lysate was diluted to 300 mM KCl. The extract was then purified through various combinations of affinity (anti-Flag M2 agarose and anti-HA agarose for Flag-tagged and HA-tagged subunits, respectively), ion-exchange (typically SP-Sepharose) and gel-filtration (Superose 6) chromatography. For both M2 and anti-HA beads, elution was with 0.5 mg/ml of the corresponding peptide.

Optimization of the reconstitution protocol entailed extensive viral titrations, as well as identification, by trial and error, of which subunit to tag. The following summarizes our reconstitution protocol for one of the largest Mediator variants reported here. The individual cDNAs for subunits of the Mediator head module (MED6, MED8, MED11, MED18, MED19, MED20, MED22 and MED30) were inserted into the pFBDM and pUCDM transfer vectors, and the resulting transfer vectors were integrated into a single bacmid for virus generation. Individual cDNAs for subunits of the middle module were also inserted into the pFBDM and pUCDM transfer vectors (HA-MED7, MED4, MED21, His-MED10, MED31, MED9 and MED26) and integrated into another bacmid for production of the second virus. pFBDM-MED17 and pFBDM-Flag-MED14 were integrated into two different bacmids to form the third and fourth viruses, which were amplified in Sf9 cells. For protein production in Hi5 cells, scaled-up cultures were infected with the virus cocktail. A typical yield of pure core Mediator complex from 500 ml of infected cells was 100 μg.

Purification of transcription factors, activators and coactivators. Purification of the general transcription factors was essentially as previously described[34]. Recombinant TFIIB, TFIIE and TFIIF were expressed in bacteria and purified as described before[34]. Baculovirus-expressed TFIIA was purified from insect cells, as were the various transcriptional activators (p53, TRα and RXRα). Pol II, TFIIH and TFIID were purified from corresponding HeLa cell lines that stably express epitope-tagged subunits. For routine use, Mediator was also similarly affinity purified from a HeLa cell line that stably expresses the core subunit MED10 (ref. 34). The PC2 form of Mediator was affinity purified from the phosphocellulose P11 0.85 M fraction of nuclear extract from a cell line that expresses Flag-tagged MED26 (ref. 4).

***In vitro* transcription assays.** *In vitro* transcription assays with purified factors or nuclear extract were performed essentially as described previously[34]. Transcription reactions typically contained 50 ng of test templates. All templates contained G-less cassettes downstream of the adenovirus major late (ML) core promoter. The template 5×p53REML further contained five copies of a p53 response element, and the 5×TREML template contained five copies of a thyroid response element. Reactions were initiated by addition of protein factors to the reaction mixes, which contained [α-^{32}P]UTP or [α-^{32}P]CTP as the labeled NTP. Reactions took place for 50 min at 30 °C and then were processed and analyzed by electrophoresis on 5% polyacrylamide, 50% urea gels and autoradiography.

For reactions with Mediator-depleted nuclear extract, HeLa cell nuclear extract[51] was immunodepleted with antigen-purified anti-MED30 antibody (below), as previously described[34].

Immunoprecipitation assays. Antigen-purified MED30 antibody was coupled to Protein A–Sepharose beads. The beads were washed with BC200 and added to binding reactions containing various Mediator derivatives and either Pol II or TFIID. After incubation for 2 h, the beads were washed again in BC200 plus 0.1% NP-40 and eluted. The immunoprecipitates were analyzed by western blotting.

Immobilized template recruitment assays. A PCR-generated biotinylated adenovirus major late (Ad ML) promoter–containing DNA fragment was bound to Dynabeads M-280 Streptavidin (Invitrogen 11205-D), as recommended by the manufacturer. The beads were incubated with either mock-depleted or Mediator-depleted nuclear extracts in the presence or absence of variant Mediator preparations. The reaction mixes were set up as for *in vitro* transcription but were scaled up ten-fold, as previously described[52]. After incubation and washing, the bound material was eluted by boiling in SDS-PAGE sample buffer and characterized by immunoblotting.

Antibodies. Antibodies against most of the Mediator subunits were from our laboratory's previously published collection and have been validated for Western blotting[4]. Antibodies to MED18 (sc-161835), MED8 (sc-103619), MED22 (sc-107739) and MED14 (sc-9419) were purchased from Santa Cruz (validation on manufacturer's website). Except for anti-MED18, which was used at 1:100, all the primary-antibody dilutions were 1:1,000. Antibody to MED30, which has been validated for immunodepletion and coimmunoprecipitation[3] was affinity purified by chromatography against bacterially expressed antigen[34].

Chemical cross-linking and mass spectrometry. The purified complex was chemically cross-linked by 1 mM isotopically labeled disuccinimidyl suberate (d0:d12 with 1:1 ratio, Creative Molecules) for 45 min at 4 °C with constant agitation. The reaction was then quenched in 50 mM ammonium bicarbonate. After disulfide reduction and cysteine alkylation, the cross-linked complex was digested both in solution and in gel with trypsin to identify cross-linked peptides[53,54]. For in-solution digestion, ~50–100 μg of purified complex was digested with 2 μg of trypsin (Promega) in 1M urea with ~2% acetonitrile (ACN) and 0.1% Rapigest (Waters) at 37 °C. After 12–16 h of incubation, an additional 1–2 μg trypsin was added to the digest and was incubated for a further 4 h. The resulting proteolytic peptide mixture was purified with a C18 cartridge (Sep-Pak, Waters), lyophilized and fractionated by peptide size-exclusion chromatography[55]. For in-gel digestion, ~50 μg purified complex was resuspended and heated in 2× LDS loading buffer. The sample was cooled at room temperature for cysteine alkylation and separated by electrophoresis in a 4–12% SDS PAGE gel. The gel region above ~220 kDa was sliced, crushed into small pieces and digested in gel by trypsin. After extraction and purification, the resulting proteolytic peptide mixture was dissolved in 20 μl of a solution containing 30% ACN and 0.2% formic acid (FA) and fractionated by peptide SEC (Superdex Peptide PC 3.2/30, GE Healthcare) with offline HPLC separation with an auto sampler (Agilent Technologies). Three SEC fractions in the molecular-mass range of ~2.5 kDa to 8 kDa were collected and analyzed by LC/MS.

Purified peptides were dissolved in the sample loading buffer (5% MeOH, 0.2% FA) and loaded onto a self-packed PicoFrit column with an integrated electrospray ionization emitter tip (360 O.D, 75 I.D., with 15-μm tip, New Objective). The column was packed with 8 cm of reverse-phase C18 material (3-μm porous silica, 200-Å pore size, Dr. Maisch GmbH). Mobile phase A consisted of 0.5% acetic acid and mobile phase B of 70% ACN with 0.5% acetic acid. The peptides were eluted in a 150-min LC gradient (8% B to 46% B, 0–118 min, followed by 46–100% B, 118–139 min, equilibrated with 100% A until 150 min) with a HPLC system (Agilent) and analyzed with an LTQ Velos Orbitrap Pro mass spectrometer (Thermo Fisher). The flow rate was ~200 nl/min. The spray voltage was set at 1.9–2.2 kV. The capillary temperature was 275 °C, and ion transmission on Velos S lenses was set at 35%. The instrument was operated in the data-dependent mode, where the top eight most abundant ions were fragmented by higher-energy collisional dissociation/HCD (HCD energy 27–33, 0.1-ms activation time) and analyzed in the orbitrap mass analyzer. The target resolution was 60,000 for MS1 and 7,500 for MS2. Ions (370–1,700 m/z) with charge state of >3 were selected for

fragmentation. A dynamic exclusion of (15 s/2/55 s) was used. Other instrumental parameters include: 'lock mass' at 371.1012 Da, the minimal threshold of 5,000 to trigger an MS/MS event, and ion-trap accumulation limits of 10^5 and 10^6, respectively, for the linear ion trap and orbitrap. The maximum ion-injection time for the LTQ was set at 200 ms. The maximum ion-injection time for the orbitrap was 500 ms for full scan and 500–700 ms for MS2.

The raw data were transformed to mascot generic format (MGF) and searched by pLink software[56] with a database containing sequences of the protein subunits of human Mediator complex and BSA. Other search parameters included: mass accuracy of MS1 ≤10 p.p.m. and MS2 ≤20 p.p.m. for the initial database search, cysteine carboxymethylation as a fixed modification, methionine oxidation as a variable modification, and a maximum of two trypsin miscleavages. The results were filtered at 5% false discovery rate (FDR) and were subjected to manual verification of the resulting MS/MS spectra on the basis of the following criteria: for positive identifications, both peptide chains must contain at least five amino acids, and for both peptide chains the major MS/MS fragmentation peaks must be assigned and must follow a pattern that contains a continuous stretch of fragmentations. The appearance of dominant fragment ions N terminal to proline and C terminal to aspartate and glutamate for arginine-containing peptides was generally expected[57,58]. A total of 277 unique cross-linked peptides were identified as a result.

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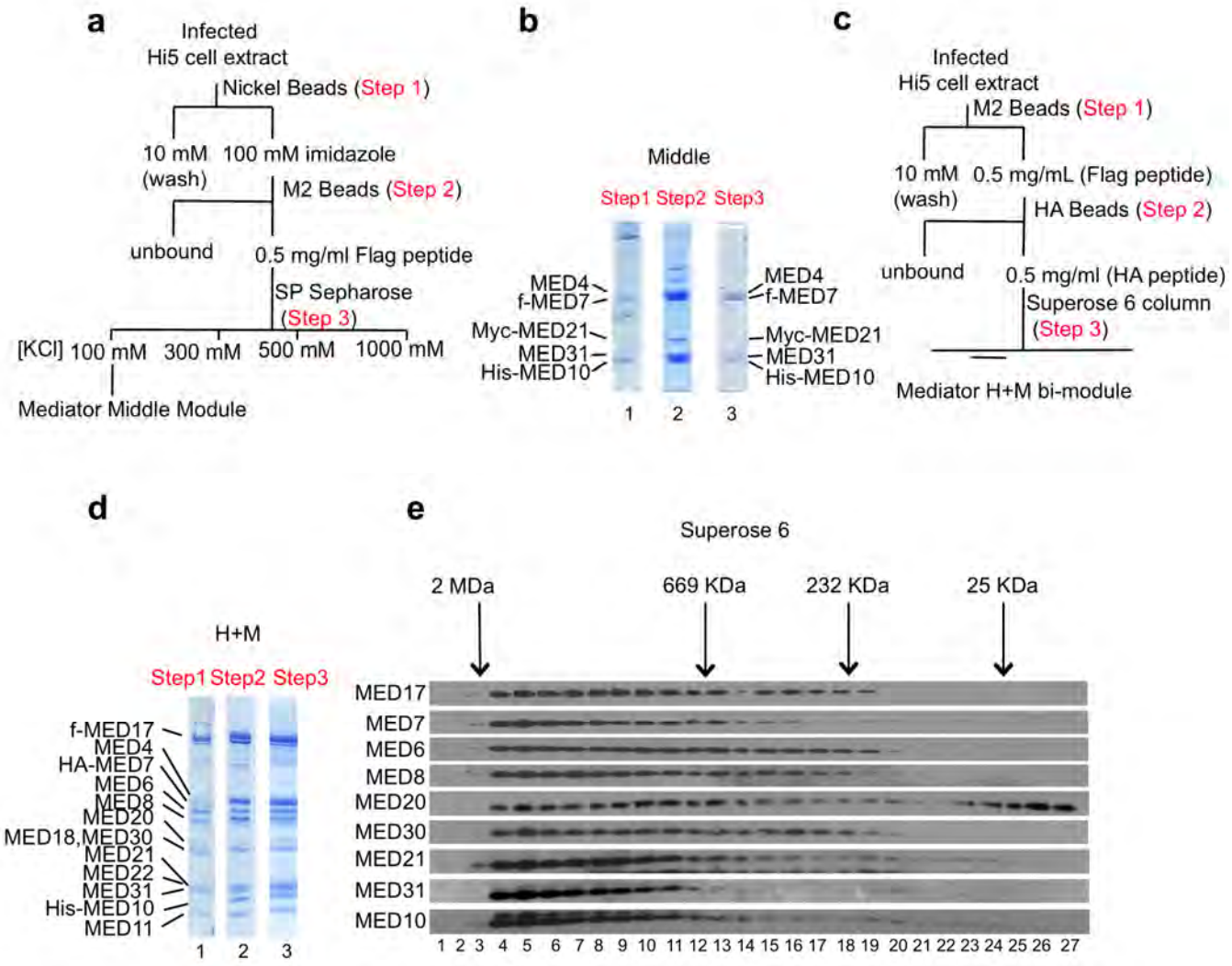
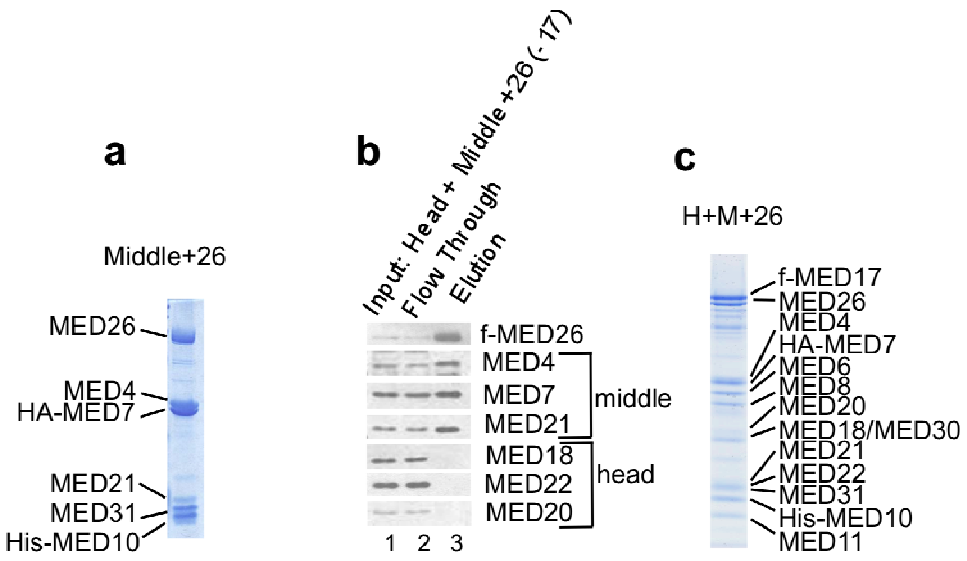


Supplementary Figure 1

Isolation of the recombinant middle and head + middle modules.

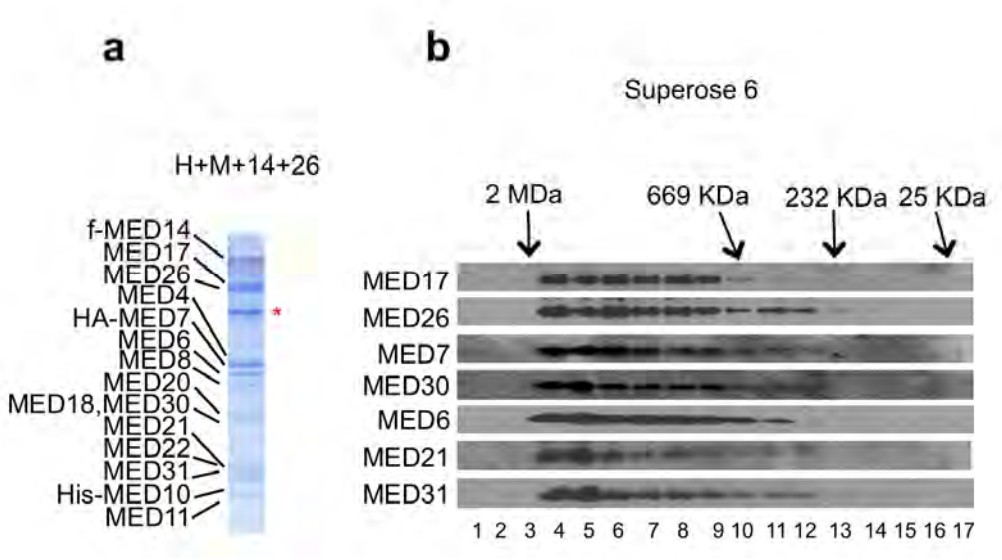
(a) Scheme illustrating the multi-step purification protocol for the reconstituted middle module. Extract from infected cells co-expressing middle module subunits was incubated with nickel-NTA agarose for purification via His-MED10, followed by M2 agarose for selection via f-MED7. Further purification was achieved through SP-Sepharose chromatography. **(b)** SDS-PAGE analysis (Coomassie staining) of middle module preparations after each of the three purification steps. **(c)** Scheme illustrating the multi-step purification protocol for the reconstituted H+M complex. Extract from infected cells co-expressing middle and head subunits was purified over M2 agarose for selection via f-MED17 followed by chromatography on an anti-HA resin for selection via HA-MED7. Further purification was achieved through AKTA Superose 6 gel filtration chromatography. **(d)** SDS-PAGE analysis (Coomassie staining) of H+M preparations after each of the three purification steps. **(e)** Superose 6 gel filtration profile of the reconstituted H+M complex. Column fractions were analyzed by immunoblotting with the indicated Mediator antibodies. Fractions at which the various molecular mass standards elute are identified.



Supplementary Figure 2

MED26 is part of the middle module.

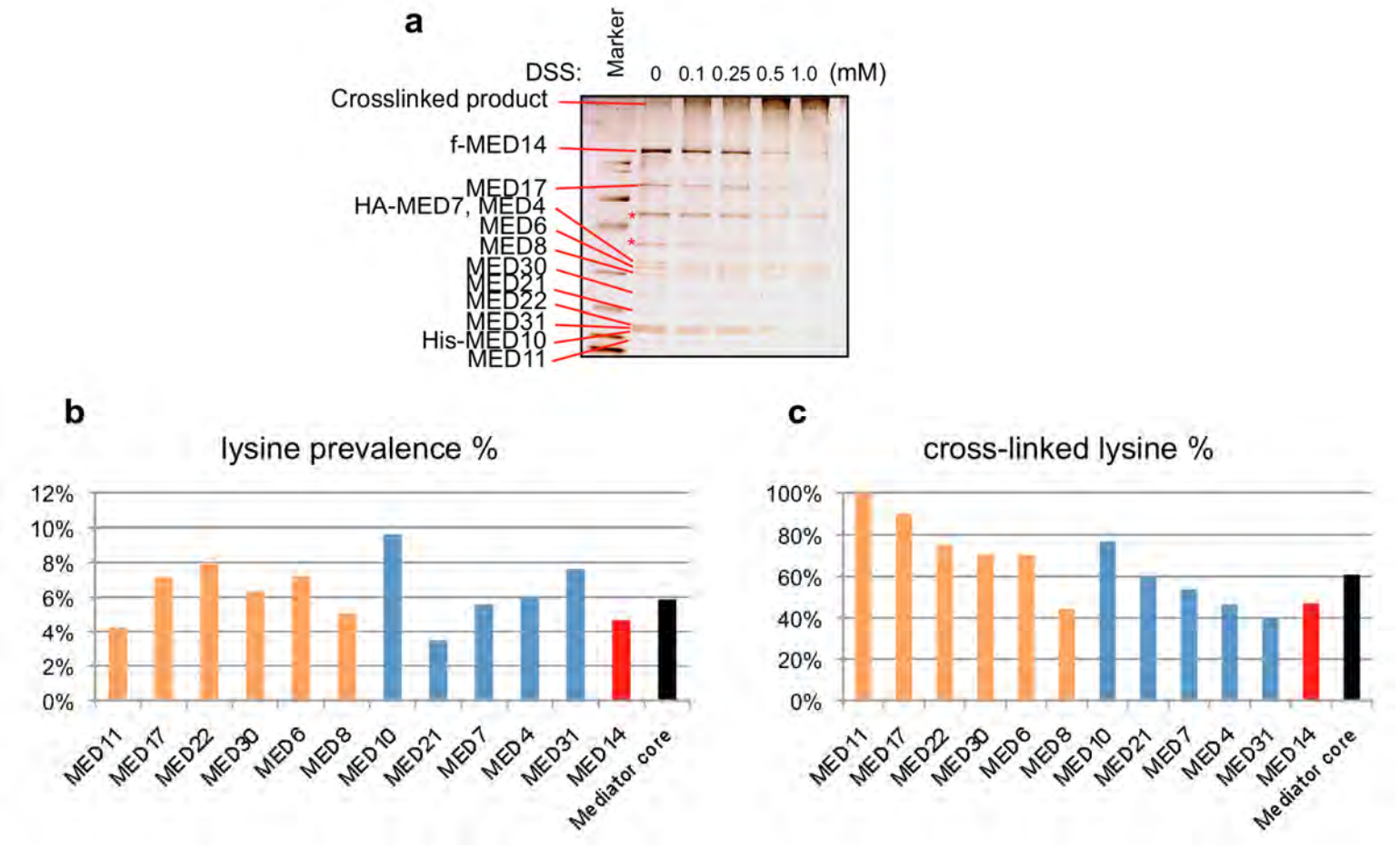
(a) Extract from infected cells co-expressing MED26 plus all middle module subunits was subjected to purification over an anti-HA resin (through HA-MED7). Coomassie staining shows strong enrichment of MED26. **(b)** Extract from infected cells co-expressing f-MED26 plus all middle module subunits together with all head module subunits except MED17 (which precludes H+M formation) was purified over M2 agarose and probed for representative head and middle subunits. Middle module subunits were selectively enriched in the eluates. **(c)** SDS-PAGE analysis of a reconstituted H+M+26 complex following M2 agarose chromatography.



Supplementary Figure 3

Purification of reconstituted head + middle + MED14 + MED26 complex.

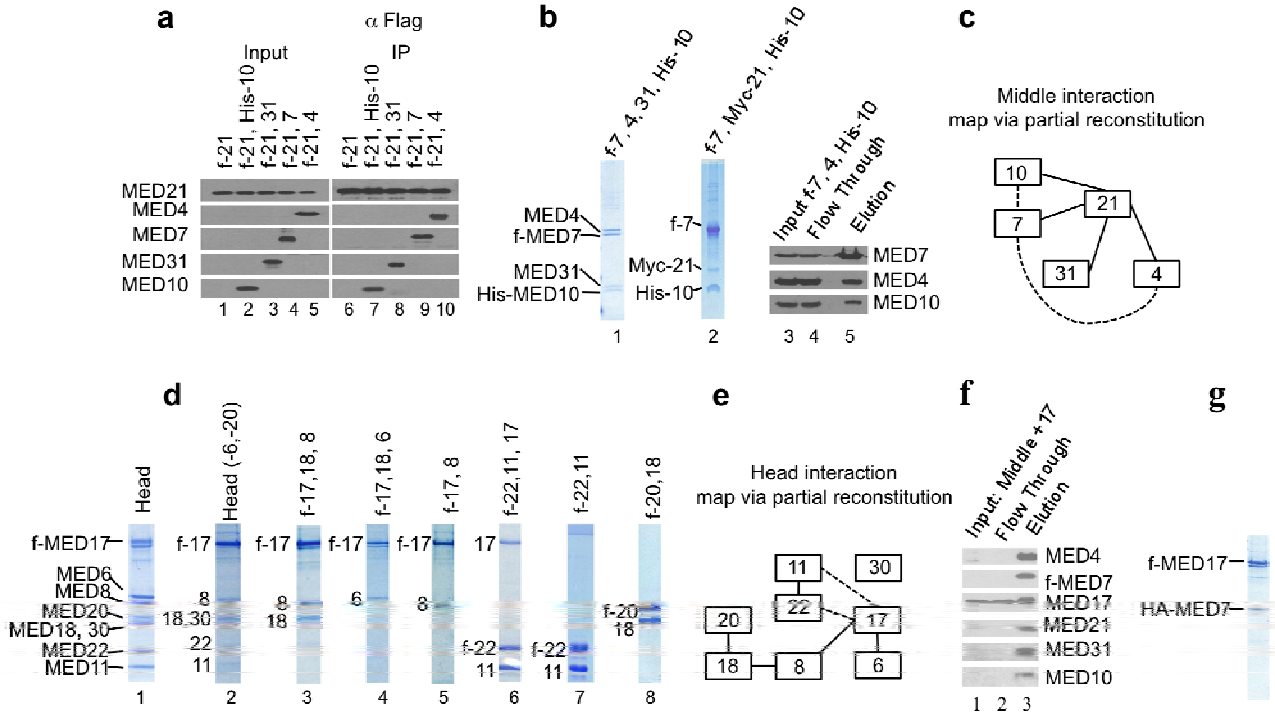
Recombinant H+M+14+26 complex was purified according to the three-step scheme in **Supplementary Fig**. **1c-e**. **(a)** SDS-PAGE analysis. **(b)** Superose 6 elution profile (immunoblotting of fractions) as in figure S2c.



Supplementary Figure 4

Cross-linking efficiency of lysine residues in CX-MS.

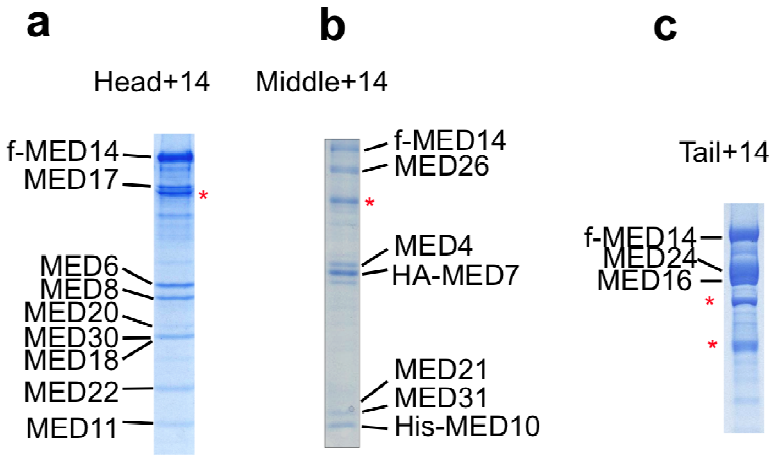
(a) Reconstituted complexes were treated with various concentrations of DSS crosslinker prior to SDS-PAGE and silver staining. A DSS concentration of 1 mM (20 min at 4^{O}C) was chosen for scaled-up CX-MS experiments. **(b)** Graph showing lysine prevalence within the cross-linked Mediator subunits. **(c)** Graph showing percentage of cross-linked lysines within the scored Mediator subunits.



Supplementary Figure 5

Subunit organization of the middle and head modules, based on immunoprecipitations and partial reconstitutions.

(a) MED21 interacts with all subunits of the middle module. Extracts from insect cells co-expressing f-MED21 and either MED10, MED31, MED7 or MED4 were incubated with M2 agarose and the eluates were analyzed by Western blot. **(b)** MED7 co-purifies with all tested middle module subunits. f-MED7 was co-expressed with: (i) MED4, MED31, and MED10 (lane 1); (ii) MED21 and MED10 (lane 2); and (iii) MED4 and MED10 (lane 5). Infected cell extracts were purified over M2 agarose and characterized by SDS-PAGE/Coomassie stain (lanes 1 and 2) or by Western blot (lane 5). **(c)** Schematic representation of the interaction pattern of the middle module subunits based on the results from panels **a** and **b**. Pairwise interactions established in these assays are shown by solid lines; interactions implied, but not established, are shown by broken lines. **(d)** Co-purification of MED17 with the majority of the subunits of the head module and heterodimer formation by MED11 and MED22 and by MED18 and MED20. Complexes were isolated from cell extracts co-expressing the following combinations: all head subunits (lane 1); all head subunits except MED6 and MED20 (-6, -20; lane 2); f-MED17, MED18, and MED8 (lane 3); f-MED17, MED18, and MED6 (lane 4); f-MED17 and MED8 (lane 5); f-MED22, MED11, and MED17 (lane 6); f-MED22 and MED11 (lane 7); and f-MED20 and MED18 (lane 8). In purifications (M2 agarose followed by SDS-PAGE of eluates) shown in lanes 1-5, complexes were selected through f-MED17. In lanes 6-8, selections were through f-MED22 (lanes 6 and 7) or f-MED20 (lane 8). MED18 fails to interact with MED17 in the absence of MED8 (compare lane 3 [MED18 in the presence of MED8] vs. lane 4 [MED18 in the absence of MED8]). However, MED18 and MED20 form a strong heterodimer. Further, leaving out MED20 does not affect MED18 incorporation into the complex (lane 2), which copurifies with MED17 and MED8 (lane 3). Thus, MED20 is anchored to MED17 via MED8. **(e)** Schematic representation of the interaction pattern of the head module subunits based on the results from panel **d**. Pairwise interactions established in these assays are shown by solid lines; interactions implied, but not established, are shown by broken lines. **(f)** MED17 co-purifies with middle module. Extracts with co-expressed f-MED17 and all middle subunits were purified over M2 agarose and eluates were characterized by Western blot. **(g)** MED17 interacts with MED7: Extract from Hi5 cells co-expressing f-MED17 and HA-MED7 subunits was incubated with M2 agarose and the eluate characterized by SDS-PAGE with Coomassie staining.



Supplementary Figure 6

Copurification of MED14 with all three major Mediator modules.
Extracts from infected cells co-expressing f-MED14 and either the complete middle module **(a)**, the complete head **(b)**, or the tail subunits MED16, MED23, and MED24 that are known to form a sub-module **(c)** were affinity purified over M2 agarose and analyzed by SDS-PAGE and Coomassie staining.