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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

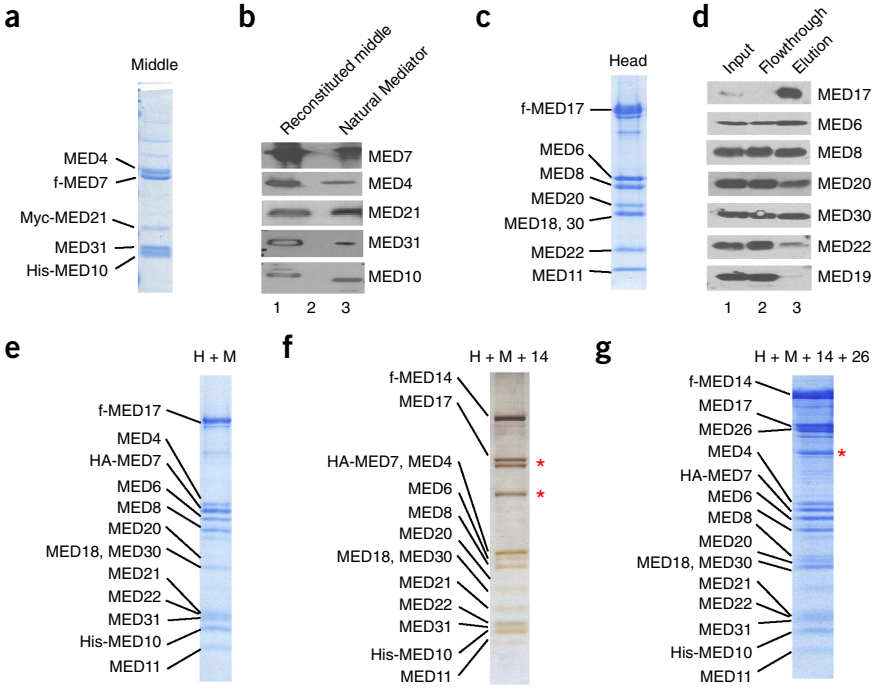

Figure 1 Reconstitution of human Mediator subcomplexes. (**a**) SDS-PAGE analysis (Coomassie blue staining) of baculovirus-expressed and reconstituted middle module. (**b**) Western blot analysis of middle-module subunits. (**c**) SDS-PAGE analysis (Coomassie blue staining) of the reconstituted head 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**.

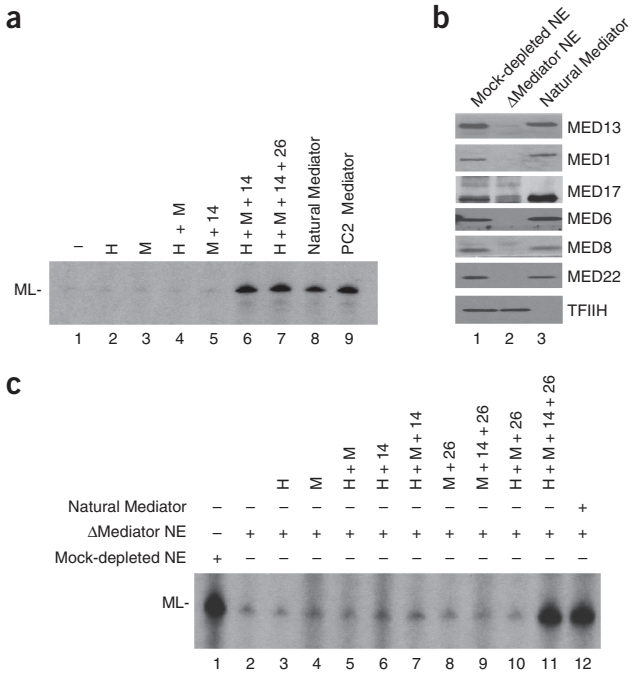

Figure 2 Critical roles of MED14 and MED26 in Mediator-stimulated basal transcription. (**a**) Autoradiogram of *in vitro* transcription reactions from a template (ML) containing the adenovirus major late core promoter. Reactions were performed with purified GTFs (IIA, IIB, IID, IIE, IIF and IIH), Pol 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].

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

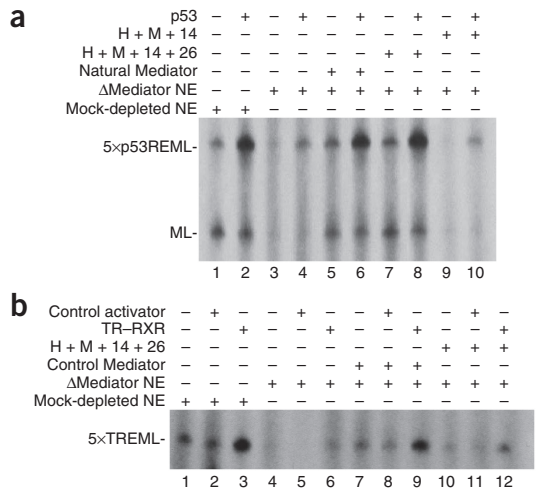

Figure 3 Critical roles of MED14 and MED26 in Mediator coactivator function. (**a**) Autoradiogram of *in vitro* transcription reactions performed as in **Figure 2c**. Extract-based reactions contained the p53-responsive template 5×p53REML and a control template (ML). The activator (p53) and Mediator 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**.

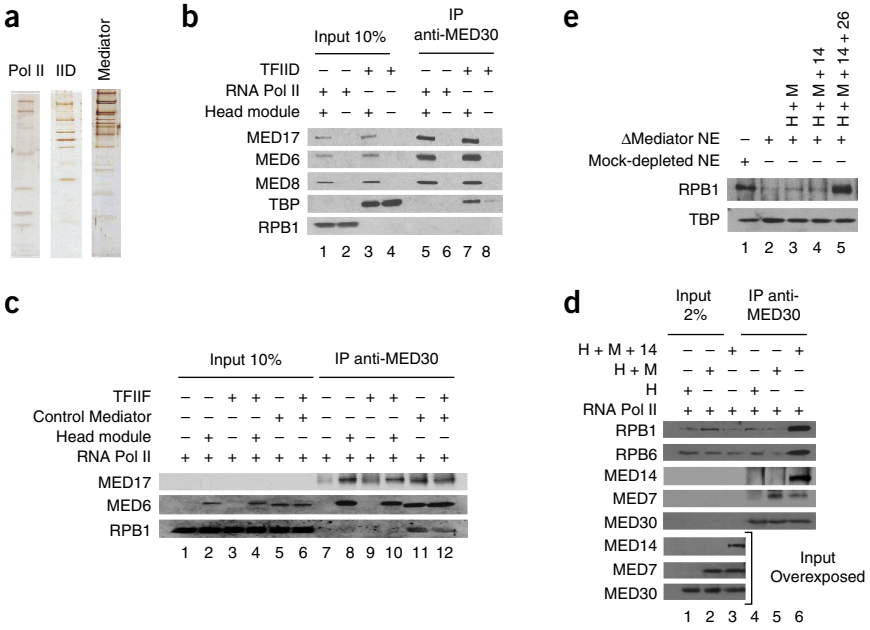

Figure 4 MED14-dependent Mediator–Pol II interaction and MED26-dependent Pol II recruitment in nuclear extract. (**a**) SDS-PAGE analysis (silver staining) of purified preparations of Pol II, TFIID (IID) and natural Mediator used in the binding assays. (**b**) Western blot analysis of Mediator interaction 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)

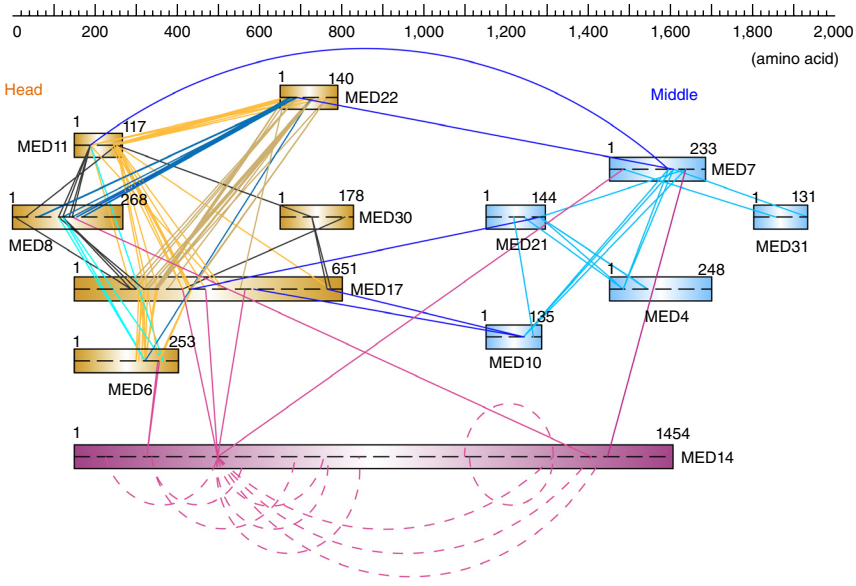

Figure 5 Molecular architecture of the reconstituted Mediator complex revealed by chemical cross-linking and MS (CX-MS). Residue-specific cross-linking map of the Mediator complex obtained by CX-MS. Except for MED14, for which intrasubunit cross-links (>200 residues apart) are shown, only intersubunit 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.

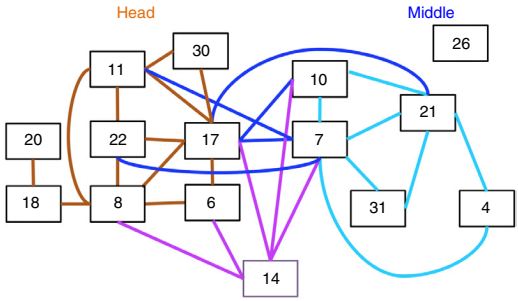

Figure 6 Schematic representation of subunit interactions in the human core Mediator complex, based on composite data from CX-MS and biochemical approaches. Light blue, intramodule interactions in the middle module; brown, intramodule interactions in the head; purple, MED14 interactions; dark blue, intermodule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

1. Roeder, R.G. Transcriptional regulation and the role of diverse coactivators in animal cells. *FEBS Lett.* **579**, 909–915 (2005).
2. Malik, S. & Roeder, R.G. The metazoan Mediator co-activator complex as an integrative hub for transcriptional regulation. *Nat. Rev. Genet.* **11**, 761–772 (2010).
3. Baek, H.J., Malik, S., Qin, J. & Roeder, R.G. Requirement of TRAP/mediator for both activator-independent and activator-dependent transcription in conjunction with TFIID-associated TAF(II)s. *Mol. Cell. Biol.* **22**, 2842–2852 (2002).
4. Malik, S., Baek, H.J., Wu, W. & Roeder, R.G. Structural and functional characterization of PC2 and RNA polymerase II-associated subpopulations of metazoan Mediator. *Mol. Cell. Biol.* **25**, 2117–2129 (2005).
5. Mittler, G., Kremmer, E., Timmers, H.T. & Meisterernst, M. Novel critical role of a human Mediator complex for basal RNA polymerase II transcription. *EMBO Rep.* **2**, 808–813 (2001).
6. Poss, Z.C., Ebmeier, C.C. & Taatjes, D.J. The Mediator complex and transcription regulation. *Crit. Rev. Biochem. Mol. Biol.* **48**, 575–608 (2013).
7. Black, J.C., Choi, J.E., Lombardo, S.R. & Carey, M. A mechanism for coordinating chromatin modification and preinitiation complex assembly. *Mol. Cell* **23**, 809–818 (2006).
8. Wallberg, A.E., Yamamura, S., Malik, S., Spiegelman, B.M. & Roeder, R.G. Coordination of p300-mediated chromatin remodeling and TRAP/mediator function through coactivator PGC-1α. *Mol. Cell* **12**, 1137–1149 (2003).
9. Lin, J.J. *et al.* Mediator coordinates PIC assembly with recruitment of CHD1. *Genes Dev.* **25**, 2198–2209 (2011).
10. Malik, S., Barrero, M.J. & Jones, T. Identification of a regulator of transcription elongation as an accessory factor for the human Mediator coactivator. *Proc. Natl. Acad. Sci. USA* **104**, 6182–6187 (2007).
11. Nock, A., Ascano, J.M., Barrero, M.J. & Malik, S. Mediator-regulated transcription through the +1 nucleosome. *Mol. Cell* **48**, 837–848 (2012).
12. Takahashi, H. *et al.* Human mediator subunit MED26 functions as a docking site for transcription elongation factors. *Cell* **146**, 92–104 (2011).
13. Lai, F. *et al.* Activating RNAs associate with Mediator to enhance chromatin architecture and transcription. *Nature* **494**, 497–501 (2013).
14. Kagey, M.H. *et al.* Mediator and cohesin connect gene expression and chromatin architecture. *Nature* **467**, 430–435 (2010).
15. Eyboulet, F. *et al.* Mediator links transcription and DNA repair by facilitating Rad2/XPG recruitment. *Genes Dev.* **27**, 2549–2562 (2013).
16. Schiano, C. *et al.* Involvement of Mediator complex in malignancy. *Biochim. Biophys. Acta* **1845**, 66–83 (2014).
17. Spaeth, J.M., Kim, N.H. & Boyer, T.G. Mediator and human disease. *Semin. Cell Dev. Biol.* **22**, 776–787 (2011).
18. Bourbon, H.M. Comparative genomics supports a deep evolutionary origin for the large, four-module transcriptional mediator complex. *Nucleic Acids Res.* **36**, 3993–4008 (2008).
19. Blazek, E., Mittler, G. & Meisterernst, M. The mediator of RNA polymerase II. *Chromosoma* **113**, 399–408 (2005).
20. Baumli, S., Hoeppner, S. & Cramer, P. A conserved mediator hinge revealed in the structure of the MED7.MED21 (Med7.Srb7) heterodimer. *J. Biol. Chem.* **280**, 18171–18178 (2005).
21. Koschubs, T. *et al.* Identification, structure, and functional requirement of the Mediator submodule Med7N/31. *EMBO J.* **28**, 69–80 (2009).
22. Larivière, L. *et al.* Structure of the Mediator head module. *Nature* **492**, 448–451 (2012).
23. Imasaki, T. *et al.* Architecture of the Mediator head module. *Nature* **475**, 240–243 (2011).
24. Robinson, P.J., Bushnell, D.A., Trnka, M.J., Burlingame, A.L. & Kornberg, R.D. Structure of the mediator head module bound to the carboxy-terminal domain of RNA polymerase II. *Proc. Natl. Acad. Sci. USA* **109**, 17931–17935 (2012).
25. Larivière, L. *et al.* Model of the Mediator middle module based on protein cross-linking. *Nucleic Acids Res.* **41**, 9266–9273 (2013).
26. Guglielmi, B. *et al.* A high resolution protein interaction map of the yeast Mediator complex. *Nucleic Acids Res.* **32**, 5379–5391 (2004).
27. Tsai, K.L. *et al.* Subunit architecture and functional modular rearrangements of the transcriptional mediator complex. *Cell* **157**, 1430–1444 (2014).
28. Wang, X. *et al.* Redefining the modular organization of the core Mediator complex. *Cell Res.* **24**, 796–808 (2014).
29. Malik, S., Gu, W., Wu, W., Qin, J. & Roeder, R.G. The USA-derived transcriptional coactivator PC2 is a submodule of TRAP/SMCC and acts synergistically with other PCs. *Mol. Cell* **5**, 753–760 (2000).
30. Berger, I., Fitzgerald, D.J. & Richmond, T.J. Baculovirus expression system for heterologous multiprotein complexes. *Nat. Biotechnol.* **22**, 1583–1587 (2004).
31. Ge, K. *et al.* Transcription coactivator TRAP220 is required for PPARã2-stimulated adipogenesis. *Nature* **417**, 563–567 (2002).
32. Nonet, M.L. & Young, R.A. Intragenic and extragenic suppressors of mutations in the heptapeptide repeat domain of *Saccharomyces cerevisiae* RNA polymerase II. *Genetics* **123**, 715–724 (1989).
33. Thompson, C.M., Koleske, A.J., Chao, D.M. & Young, R.A. A multisubunit complex associated with the RNA polymerase II CTD and TATA-binding protein in yeast. *Cell* **73**, 1361–1375 (1993).
34. Malik, S. & Roeder, R.G. Isolation and functional characterization of the TRAP/mediator complex. *Methods Enzymol.* **364**, 257–284 (2003).
35. Dotson, M.R. *et al.* Structural organization of yeast and mammalian mediator complexes. *Proc. Natl. Acad. Sci. USA* **97**, 14307–14310 (2000).
36. Sato, S. *et al.* A set of consensus mammalian mediator subunits identified by multidimensional protein identification technology. *Mol. Cell* **14**, 685–691 (2004).
37. Conaway, R.C. & Conaway, J.W. The Mediator complex and transcription elongation. *Biochim. Biophys. Acta* **1829**, 69–75 (2013).
38. Ito, M. *et al.* Identity between TRAP and SMCC complexes indicates novel pathways for the function of nuclear receptors and diverse mammalian activators. *Mol. Cell* **3**, 361–370 (1999).
39. Yuan, C.X., Ito, M., Fondell, J.D., Fu, Z.Y. & Roeder, R.G. The TRAP220 component of a thyroid hormone receptor-associated protein (TRAP) coactivator complex interacts directly with nuclear receptors in a ligand-dependent fashion. *Proc. Natl. Acad. Sci. USA* **95**, 7939–7944 (1998).
40. Larivière, L. *et al.* Structure and TBP binding of the Mediator head subcomplex Med8–Med18–Med20. *Nat. Struct. Mol. Biol.* **13**, 895–901 (2006).
41. Cai, G. *et al.* Interaction of the mediator head module with RNA polymerase II. *Structure* **20**, 899–910 (2012).
42. Baek, H.J., Kang, Y.K. & Roeder, R.G. Human Mediator enhances basal transcription by facilitating recruitment of transcription factor IIB during preinitiation complex assembly. *J. Biol. Chem.* **281**, 15172–15181 (2006).
43. Leitner, A. *et al.* Probing native protein structures by chemical cross-linking, mass spectrometry, and bioinformatics. *Mol. Cell. Proteomics* **9**, 1634–1649 (2010).
44. Soutourina, J., Wydau, S., Ambroise, Y., Boschiero, C. & Werner, M. Direct interaction of RNA polymerase II and mediator required for transcription *in vivo*. *Science* **331**, 1451–1454 (2011).
45. Tsai, K.L. *et al.* A conserved Mediator–CDK8 kinase module association regulates Mediator–RNA polymerase II interaction. *Nat. Struct. Mol. Biol.* **20**, 611–619 (2013).
46. Näär, A.M., Taatjes, D.J., Zhai, W., Nogales, E. & Tjian, R. Human CRSP interacts with RNA polymerase II CTD and adopts a specific CTD-bound conformation. *Genes Dev.* **16**, 1339–1344 (2002).
47. Jishage, M. *et al.* Transcriptional regulation by Pol II(G) involving mediator and competitive interactions of Gdown1 and TFIIF with Pol II. *Mol. Cell* **45**, 51–63 (2012).
48. Lemaire, M., Xie, J., Meisterernst, M. & Collart, M.A. The NC2 repressor is dispensable in yeast mutated for the Sin4p component of the holoenzyme and plays roles similar to Mot1p *in vivo*. *Mol. Microbiol.* **36**, 163–173 (2000).
49. Marr, S.K., Lis, J.T., Treisman, J.E. & Marr, M.T. II The metazoan-specific Mediator Subunit 26 (Med26) is essential for viability and is found at both active genes and pericentric heterochromatin in *Drosophila melanogaster*. *Mol. Cell. Biol.* **34**, 2710–2720 (2014).

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

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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.

50. Gu, W. *et al.* A novel human SRB/MED-containing cofactor complex, SMCC, involved in transcription regulation. *Mol. Cell* **3**, 97–108 (1999).
51. Dignam, J.D., Lebovitz, R.M. & Roeder, R.G. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11**, 1475–1489 (1983).
52. Malik, S., Wallberg, A.E., Kang, Y.K. & Roeder, R.G. TRAP/SMCC/mediator-dependent transcriptional activation from DNA and chromatin templates by orphan nuclear receptor hepatocyte nuclear factor 4. *Mol. Cell. Biol.* **22**, 5626–5637 (2002).
53. Shi, Y. *et al.* Structural characterization by cross-linking reveals the detailed architecture of a coatomer-related heptameric module from the nuclear pore complex. *Mol. Cell. Proteomics* doi:10.1074/mcp.M114.041673 (26 August 2014).
54. Algret, R. *et al.* Molecular architecture and function of the SEA complex, a modulator of the TORC1 pathway. *Mol. Cell. Proteomics* doi:10.1074/mcp.M114.039388 (29 July 2014).
55. Leitner, A. *et al.* Expanding the chemical cross-linking toolbox by the use of multiple proteases and enrichment by size exclusion chromatography. *Mol. Cell. Proteomics* **11** M111.014126 (2012).
56. Yang, B. *et al.* Identification of cross-linked peptides from complex samples. *Nat. Methods* **9**, 904–906 (2012).
57. Qin, J. & Chait, B.T. Matrix-assisted laser desorption ion trap mass spectrometry: efficient isolation and effective fragmentation of peptide ions. *Anal. Chem.* **68**, 2108–2112 (1996).
58. Michalski, A., Neuhauser, N., Cox, J. & Mann, M. A systematic investigation into the nature of tryptic HCD spectra. *J. Proteome Res.* **11**, 5479–5491 (2012).