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

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Abstract

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 mass spectrometry (CX-MS). Whereas the reconstituted head and middle modules can stably associate, only with incorporation of MED14 into the bi-modular complex does it acquire basal and coactivator functions. 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 systematically dissecting the multiple layers of functionalities associated with the Mediator complex.

INTRODUCTION

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 multi-subunit 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[1], the metazoan Mediator has also been

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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 co-IP experiments. D.L. helped M.A.C. in the generation of partial head module complexes in the Fig. 6d. Y.S. carried out the CX-MS experiments. The authors declare no competing financial interests.

shown to stimulate basal (activator-independent)[3-5] and negative (co-repressor)[2,6] functions under certain conditions. More recently, given the multi-step 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 elongation phase[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 lncRNA[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 from yeast to human[18]. However, consistent 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 (e.g., MED7 and MED31) to much weaker relationships for the remainder[18]. Further, the metazoan complex contains additional, metazoan-specific subunits (e.g., MED26 and MED30). The overall structure of the complex, both in yeast and human, is modular, with the subunits organized into head, middle, tail and kinase subcomplexes[2]. The subunits comprising 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; and 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 our understanding of structure-function relationships for the Mediator, especially in yeast. Thus, previous studies of yeast Mediator provided crystal structures for both the head and partial middle modules[20-24] and a model for protein interactions within the middle module based on cross-linking[25]. Yeast two-hybrid screens also 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 pin-pointing 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 due to technical difficulties in manipulating this complex. These include its large size, heterogeneity, the presence of many essential subunits and limited yields upon purification from cell extracts.

Thus far, the metazoan Mediator complex has been functionally characterized mainly in in vitro biochemical assays using preparations obtained from nuclear extract 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 multi-module

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[4,29], makes it feasible to undertake a reconstitution-based approach to establish structure-function relationships for the Mediator.

Here, toward the generation of a minimal active core Mediator complex and the isolation of 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 reconstituted separately the head and middle modules. We found that although these modules can stably associate with each other, the resulting bi-modular 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 -- 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 mass spectrometric analysis (CX-MS) 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 bi-modular complex

We initiated the reconstitution by first generating the middle module through co-expression of Flag-tagged-MED7 (f-MED7), MED19, MED4, Myc-MED21, MED31, MED9 and His-MED10 in insect cells. Because of the conditional requirement for MED1[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 (non-essential 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, while expressed, showed no association with the middle module. We similarly reconstituted the head module of the human Mediator by co-expressing f-MED17, MED6, MED8, MED11, MED18, MED19, MED20, MED22, and the metazoan-specific MED30, which previously was not assigned to any module. Following purification, we obtained a head module complex (MED6--MED8--MED11--MED17--MED18--MED20--MED22--MED30) (**Fig. 1c,d**) that contains all of the input subunits except MED19, whose association with the complex is likely dependent on a metazoan-specific subunit(s) not included in our reconstitutions.

To reconstitute a complex containing both the head and middle modules (“H+M”), we co-expressed the subunits of the two modules. Sequential selection through f-MED17 (head module) and HA-MED7 (middle module) subunits followed by Superose 6 gel filtration revealed a stable interaction between the head and middle modules (**Fig. 1e and**

Supplementary Fig. 1d). The resulting H+M preparation (**Supplementary Fig. 1d**) contained stoichiometric amounts of all the subunits except MED18 and MED20, which in yeast are non-essential[32,33] and form a labile heterodimer[23] and thus tend to dissociate upon gel filtration (**Supplementary Fig. 1e**; see further below). Interestingly, we observed that separately purified head and middle modules do not associate to form a bi-modular complex when mixed together (data not shown), possibly indicating the strict requirement for co-expression 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 if 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, and TFIIH), coactivator PC4 and RNA Pol II (**Fig. 2a**) or (ii) unfractionated HeLa cell nuclear extract (NE) immuno-depleted for the Mediator complex (**Fig. 2b,** lane 1 vs. lane 2). Since 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 vs. 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[2].

An H+M+14 complex was reconstituted and affinity-selected via MED14 to ensure that the resulting homogeneous preparation contains 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 considerable stimulation of basal transcription (**Fig. 2a,** lane 6 vs. lane 1). Importantly, the fold-stimulation was equivalent to that elicited both by a natural Mediator preparation that contains a complete set of subunits (lane 6 vs. lane 8) and by its PC2 form (lane 6 vs. 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

While 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 vs. lane 1). Because the extract contains a more natural complement of various nuclear factors, this result indicated a requirement for another Mediator subunit(s) to overcome an apparent constraint by a negative cofactor(s) present in the nuclear extract[36]. Although MED26-containing PC2 is a small fraction of the total cellular Mediator population in HeLa cells, extracts from which this sub-population is depleted fail to support in vitro transcription[4]. Further, MED26-containing Mediator preparations have a higher Pol II content[4,37] and MED26 can recruit the super elongation complex to promoters[12,38]. We therefore generated variant complexes containing MED26. We found that MED26 associates with the middle, but not the head, module (**Supplementary Fig. 2a and 2b**), consistent with the recent report from the Asturias group[27], and that it could be stably incorporated into H+M+26 (**Supplementary Fig. 2c**) and H+M+14+26 (**Fig. 1g** and **Supplementary Fig. 3a**) complexes. Importantly, in the Mediator-depleted extract (**Fig. 2c,** lane 11 vs. lane 12), as in

the pure system (**Fig. 2a,** lane 7 vs. lanes 8 and 9), the H+M+14+26 complex restored basal transcription to the same level as 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), 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[39] (**Fig. 3a,** lane 8 vs. lanes 6 and 4). In control experiments, no coactivator function was seen for the thyroid hormone receptor, which functions as a heterodimer with RXR and targets the missing MED1 subunit[40] (**Fig. 3b,** lane 12 vs. lane 6).

MED14 is crucial for Mediator-Pol II interaction

To understand the mechanism whereby MED14-containing Mediator complexes are rendered active, we used co-immunoprecipitation to investigate 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**). Consistent with previous studies[41], the head module alone interacted with TFIID (**Fig. 4b,** lane 7). However, in contrast to the case in yeast[42], 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 and 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, DNA-bound beads were incubated with control or Mediator-depleted nuclear extract. In the latter case, the reactions were further supplemented with our various Mediator preparations. Consistent with our previous results[43], Pol II recruitment was abolished in Mediator-depleted extracts (**Fig. 4e,** lane 2 vs. lane 1). Interestingly, neither the H+M (lane 3) nor the H+M+14 (lane 4) complex, 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 (**Fig. 2c and Fig. 3**), the H+M+14+26 complex was able to induce Pol II recruitment (**Fig. 4e,** lane 6 vs. 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) (**Supplementary Fig. 5a**) and applied high-resolution mass spectrometry (CX-MS)[44] to identify cross-linked peptides. We identified 277 unique cross-links (**Supplementary Table 1, 2**), which were 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. 5b, 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 intra-modular 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 (**Supplementary Table 2**), MED11, MED22, and MED30. Consistent 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 sub-stoichiometric, were not scored by CX-MS.

Importantly, the CX-MS data reveal inter-modular 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 [**Supplementary Table 2**]) and middle (MED7) components, thus serving to further bridge the two modules. We also identified several intra-subunit cross-links between N- and C-terminal residues of MED14 in the active core Mediator complex, 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 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. 6**). We confirmed CX-MS-identified interactions of MED7 and MED21 with various middle subunits (**Supplementary Fig. 6a-c**) and detected an additional interaction between MED21 and MED31 (**Supplementary Fig. 6a,** lane 8). Similarly, for the head module (**Supplementary Fig. 6d**), we identified complex formation between MED11 and MED22 (**Supplementary Fig. 6d,** lanes 6, 7), between MED11, MED22 and MED17 (**Supplementary Fig. 6d,** lane 6), between MED6 and MED17 (**Supplementary Fig. 6d,** lane 4), between MED8 and MED17 (**Supplementary Fig. 6d,** lane 3 vs. lanes 4 and 5) and between MED8 and MED18 (**Supplementary Fig. 6d,** lane 3 vs. lane 5). Importantly, as in yeast, MED18 and MED20 were found to form a heterodimer that is anchored to the head via MED8 (**Supplementary Fig. 6d,** lane 8 and lane 3 vs. lane 4).

Consistent with the cross-linking data for MED17, H+M formation was dependent on the presence of MED17 (**Supplementary Fig. 2b**). Moreover, MED17 also co-purified with the middle module (**Supplementary Fig. 6f**), identifying it as a major link between the head and middle modules. Related, this series of analyses also identified an additional interaction (between MED17 and MED7) contributing to the head-middle interaction (**Supplementary Fig. 6g**).

Notably, through co-expression of MED14 with either head or middle module subunits, and consistent with the crosslinking data, we established that MED14 could independently associate with the head and middle modules (**Supplementary Fig. 4a-b**). Also of note,

MED14 also interacted with the MED24 and MED16 subunits of the tail module, which, however, were not included in our reconstitutions (**Supplementary Fig. 4c**). These results further implicate MED14 as the essential backbone of the Mediator that bridges its three main modules. A composite subunit interaction network for the human core Mediator complex deduced from various approaches is shown in **Fig. 6**. At a gross level, the deduced interactions among the subunits and the general architecture of the human core Mediator complex 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 complexes that display various functionalities previously ascribed to the Mediator. We also generated a detailed spatial connectivity map of the active core Mediator complex through CX-MS and pair-wise 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, while 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 mechanism and dissect the 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, MED24), as well as head (MED6, MED8, MED17) and middle (MED7, MED10) module 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 conformity 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 Tsai et al study[27] addressed how MED14 relates to the dissociable kinase module.

In a major extension of the solely architectural focus of the Tsai et al study[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) and to stimulate basal activity[42,45]. It therefore was initially somewhat surprising that our reconstituted head-middle bi-modular 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. Beyond its stimulation of basal transcription, the resulting complex also 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 plays a critical role 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 be due to a direct physical interaction between Pol II and the large surface furnished by the MED14 backbone. However, and although the most recent study cryo-EM study from the Asturias group does not shed additional light on which features of the yeast Mediator complex are responsible for holoenzyme formation, they previously proposed[46] 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. While 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[46]. Furthermore, to our knowledge, neither prior yeast genetic 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 inter-module movements that occur upon Pol II binding[46,47]. 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.

Consistent with its structural complexity, and superimposed on 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 include DSIF[10], Gdown1[48], and potentially NC2[49]. 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[50].

MED26 has been implicated in interactions with TFIID and the P-TEFb- and ELL-containing Super Elongation Complex, leading to a model in which this subunit functions in a hand-off 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 that precede involvement of the elongation machinery. It does not, however, preclude a subsequent additional role for MED26 at the initiation-to-elongation transition or 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. Note that even in this context, the MED14 requirement persists, consistent with its mechanistically distinct and essential role.

A recent study in Drosophila has suggested that the MED26 requirement is stage-specific[51]. 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 how it is feasible to recapitulate 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 diseased states.

ONLINE METHODS

cDNA cloning of Mediator subunits

For sub-cloning into baculovirus expression vectors, we used existing cDNAs for MED4, MED6, MED7, MED10, MED14, MED16, MED18, MED20, MED21, and MED24[1-4]. For the remaining, we isolated new cDNA clones from HeLa cells. Total RNA from HeLa cells was purified and cDNA prepared by reverse transcription using 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[5]. 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 single virus generation. 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 pH7.9, 20% glycerol, 0.1 mM EDTA, 3.5 mM β-mercaptoethanol and 0.1 mM PMSF supplemented protease inhibitors pepstatin (0.5 μg/ml) and leupeptin (0.5 μg/ml). After ultracentrifugation (20,000 rpm in a

Type 45 Ti rotor for 30 minutes), 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 HA beads, elution was with 0.5 mg/ml of the corresponding peptide.

Optimization of the reconstitution protocol entailed countless viral titrations, as well as identifying, by trial-and-error, 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 the 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 described[6]. Recombinant TFIIB, TFIIE and TFIIF were expressed in bacteria and purified as described before[6]. 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 stable HeLa cell lines that expressed epitope-tagged subunits. For routine use, Mediator was also similarly affinity purified from a HeLa cell line that stably expressed the core subunit MED10[6]. 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[1].

In vitro transcription assays

In vitro transcription assays using purified factors or nuclear extract were performed essentially as described previously[6]. 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 5Xp53REML further contained 5 copies of a p53 response element and the 5XTREML template 5 copies of a thyroid response element. Reactions were initiated by adding protein factors to the reaction mixes, which contained α^{32}P-UTP or α^{32}P-CTP as the labeled nucleotide triphosphate. Reactions took place for 50 minutes at 30°C and then were processed and analyzed by electrophoresis on 5% polycrylamide-50% urea gels and autoradiography. For reactions with Mediator-depleted nuclear extract, HeLa cell nuclear extract[7] was immunodepleted using antigen-purified anti-MED30 antibody (below), as described[6].

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 hours, 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 10-fold, as described[8]. Following 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[1]. MED18 (sc-161835), MED8 (sc-103619), MED22 (sc-107739), and MED14 (sc-9419) were purchased from Santa Cruz. MED30 antibody used for immunodepletion and co-immunoprecipitation was affinity purified by chromatography against bacterially expressed antigen[6].

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 minutes at 4°C with constant agitation. The reaction was then quenched in 50 mM ammonia 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[10,11]. 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 hours of incubation an additional 1-2 μg trypsin was added to the digest and incubated for a further 4 hours. The resulting proteolytic peptide mixture was purified using a C18 cartridge (Sep-Pak, Waters), lyophilized and fractionated by peptide size exclusion chromatography[9]. For in-gel digestion, ~50 ug purified complex was resuspended and heated in 2X 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) using off-line 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 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 angstrom 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% B -100% B, 118-139 min and equilibrated with 100% A until 150 min) using a HPLC system (Agilent), and analyzed with a LTQ Velos Orbitrap Pro mass spectrometer (Thermo Fisher). The flow rate was ~200 nl/min. Spray voltage was set at 1.9-2.2 kV. 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 for MS1 was 60,000 and 7,500 for MS2. Ions (370- 1700 m/z) with charge state of >3 were selected for fragmentation. A dynamic exclusion of (15”/ 2 / 55”) was used. Other instrumental parameters include: “lock mass” at 371.1012 Da, the minimal threshold of 5,000 to trigger an MS/MS event, Ion trap accumulation limits were 10^5 and 10^6 respectively for the linear ion trap and orbitrap. The max ion injection time for the LTQ was set at 200 ms. The max ion injection time for orbitrap was 500 ms for full scan, and 500-700 ms for MS2.

The raw data were transformed to MGF (mascot generic format) and searched by pLink software[12] with a database containing sequences of the protein subunits of human Mediator complex and BSA. Other search parameters included: mass accuracy of MS1 ≤ 10 ppm (parts per million) and MS2 ≤ 20 ppm for the initial database search, cysteine carboxymethylation as a fixed modification, methionine oxidation as a variable modification, and a maximum of two trypsin miscleavages was allowed. The results were filtered at 5% false discovery rate (FDR) and were subjected to manual verification of the resulting MS/MS spectra based on 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 followed a pattern that contains a continuous stretch of fragmentations. The appearance of dominant fragment ions N-terminal to proline and C-terminal to aspartic acid and glutamic acid for arginine-containing peptides was generally expected[13,14]. A total of 277 unique cross-linked peptides were identified as a result.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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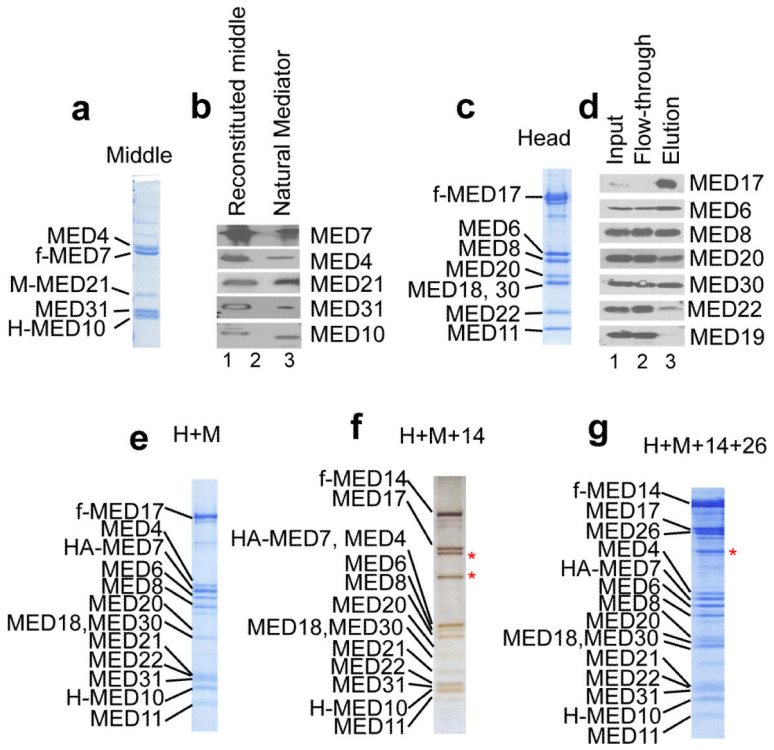

Fig. 1. Reconstitution of human Mediator sub-complexes

(a) SDS-PAGE analysis (Coomassie-blue staining) of baculovirus-expressed 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 bi-modular head+middle (H+M) complex following 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 panel 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 panel **f**. Asterisks in panels **f** and **g** point to contaminating polypeptides. See also **Supplementary Data Set 1.**

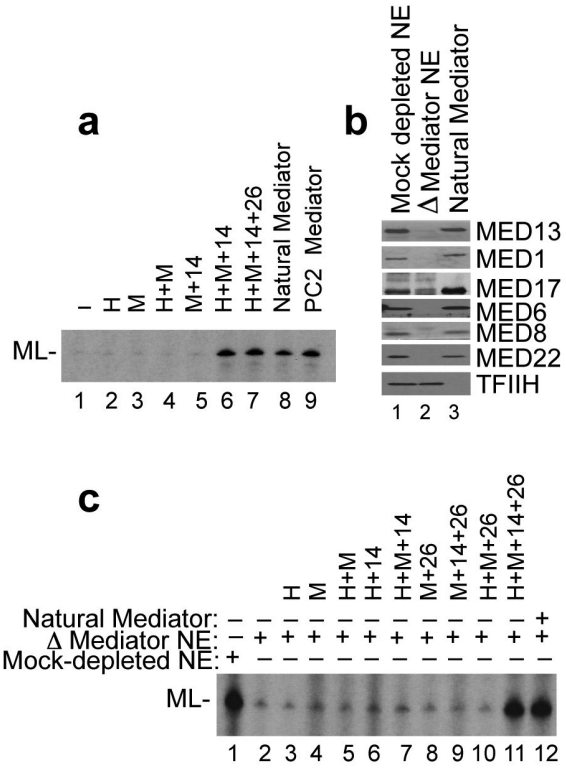

Fig. 2. Critical roles of MED14 and MED26 in Mediator-stimulated basal transcription

(a) Autoradiogram of in vitro transcription reactions from a template (ML) containing the adenovirus major late core promoter. Reactions were performed using purified GTFs (IIA, IIB, IID, IIE, IIF, and IIH), Pol II, and PC4 and the indicated Mediator sub-complexes (H, head; M, middle; 14, MED14; 26, MED26). **(b)** Western blot analysis of HeLa nuclear extract (NE) immuno-depleted of Mediator by anti-MED30 antibody (ΔMediator NE). **(c)** Autoradiogram of in vitro transcription reactions from the ML template using control (mock-depleted) or ΔMediator NE. Mediator sub-complexes were added to the transcription reactions as indicated. See also **Supplementary Data Set 1.**

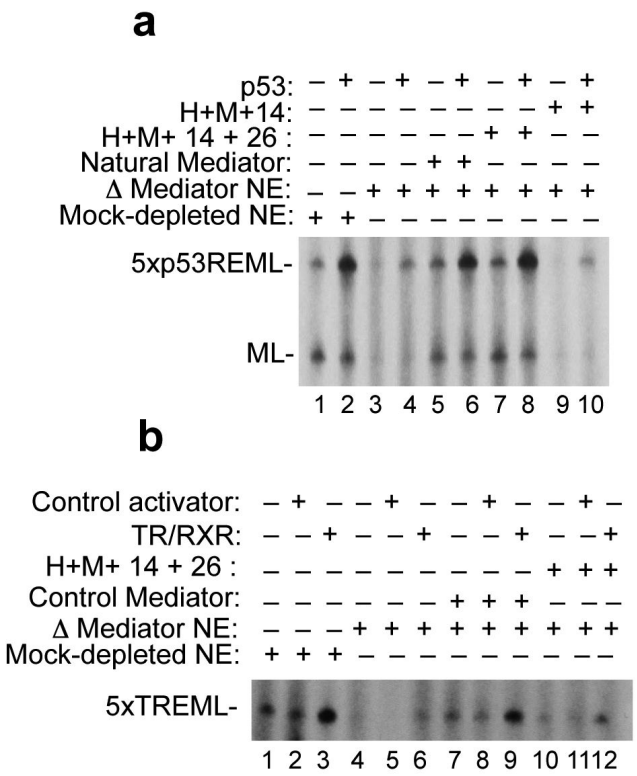

Fig. 3. Critical role of MED14 and MED26 in Mediator coactivator function

(a) Autoradiogram of in vitro transcription reactions performed as in **Fig. 2C**. Extract-based reactions contained the p53-responsive template 5×p53REML and a control template (ML). The activator (p53) and Mediator sub-complexes (H+M+14 and H+M+14+26) were added as indicated. **(b)** Autoradiogram of in vitro transcription reactions performed as in panel a**,** except that the TR--RXR heterodimer was used as the activator together with the TR-responsive template 5xTREML. An irrelevant activator (AML1-ETO) was included as control. See also **Supplementary Data Set 2.**

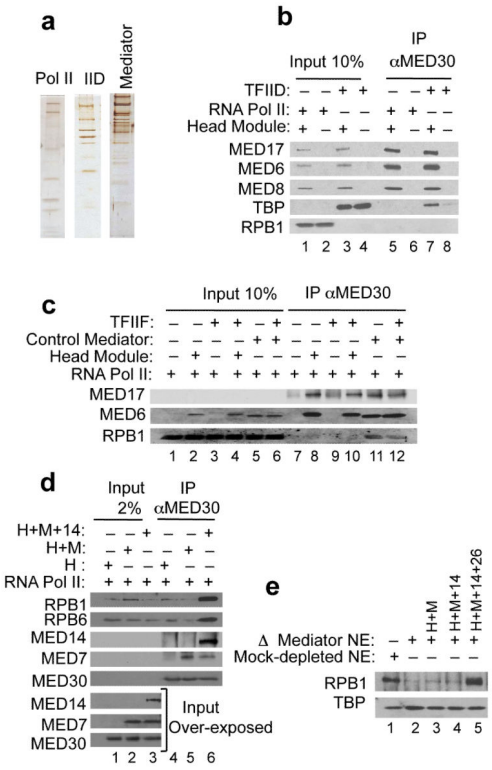

Fig. 4. MED14-dependent Mediator-Pol II interaction and MED26-dependent Pol II recruitment in nuclear extract

(a) SDS-PAGE analysis (silver staining) of purified preparations of Pol II, TFIID 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 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 panel 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 sub-complexes (H, H+M, or H+M+14). Anti-MED30 immunoprecipitates were probed for Pol II (RPB1 and RBP6) and Mediator (MED7, MED14, 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 sub-complexes. Recruitment of Pol II (RPB1) and TFIID (TBP) was monitored. See also **Supplementary Data Set 2.**

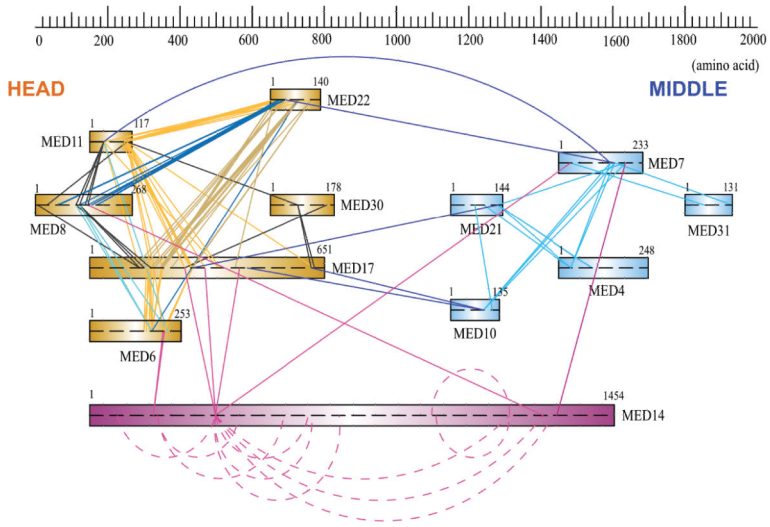

Fig. 5. Molecular architecture of the reconstituted Mediator complex revealed by chemical cross-linking and mass spectrometry (CX-MS)

Residue-specific cross-linking map of the Mediator complex obtained by cross-linking and mass spectrometry. Except for MED14, for which intra-subunit cross-links (> 200 residues apart) are shown, only inter-subunit cross-links are depicted. Middle module subunits are depicted in light blue; head module subunits are in gold; MED14 in purple. See **Supplementary Tables 1 and 2** for the complete cross-linking dataset.

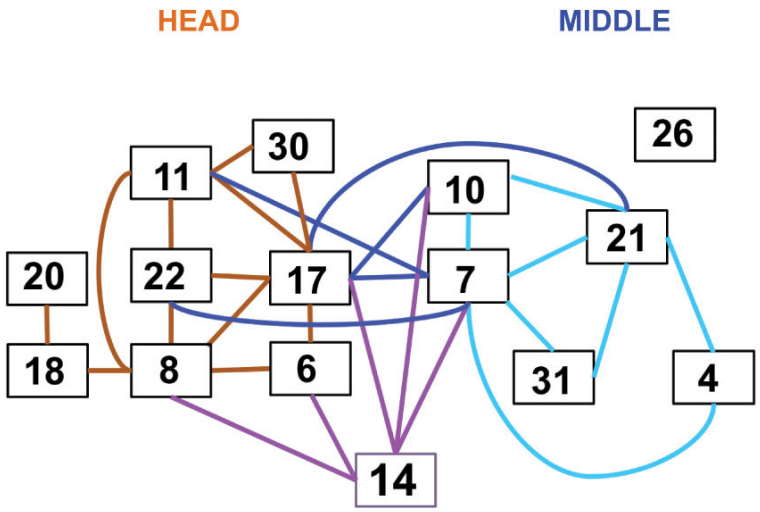

Fig. 6. Schematic representation of subunit interactions in the human core Mediator complex based on composite data from CX-MS and biochemical approaches.

Intra-module cross-links in the middle module are in light blue; intra-module cross-links in the head are in brown; MED14 cross-links are in purple; inter-module cross-links between head and middle modules are in dark blue.