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Mutual Exclusivity of MED12/MED12L, MED13/13L, and CDK8/19 Paralogs Revealed within the CDK-Mediator Kinase Module

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Abstract

The macromolecular complex Mediator plays important roles in regulation of RNA Polymerase II (RNAPII) activity by DNA-binding proteins, non-coding RNAs, and chromatin. Structural and biochemical studies have shown that human Mediator exists in two forms; a core complex of 26 proteins, termed Mediator, and a larger complex containing the CDK8 kinase module, termed CDK-Mediator. Interestingly, 3 subunits of the kinase module have undergone independent gene duplications in vertebrates to generate the paralog pairs MED12/MED12L, MED13/MED13L, and CDK8/CDK19. Each has been shown to interact with Mediator, yet clearly defining the composition of CDK kinase module has been challenging due to the large size (~600 kD), the similarities between paralogs, and potential combinatorial nature of complexes. In this study, we performed a systematic proteomic analysis using HaloTag technology to isolate each kinase module member (MED12, MED12L, MED13, MED13L, CDK8, CDK19, and Cyclin C) and their interacting partners from HEK293T cells. Using LC-MS/MS we were able to differentiate paralogs within samples by specific analysis of unique peptides. We found that the paralogs assemble into CDK-Mediator in a mutually exclusive fashion, thus allowing for up to eight different assemblies of the CDK module, with potential for functional specialization of Mediator complexes. Interestingly, we found that MED13L complexes carry the MED26 subunit, which has been shown to be either absent or in very low abundance in CDK-Mediator. This unique variant of the Mediator complex may reconcile recent observations demonstrating roles for MED26 and CDK8 in positive control of RNAPII elongation. Together these data expand the understanding of CDK-Mediator complexes and composition.

Keywords: Proteomics; Protein interactions; Mediator; RNA Polymerase II; Mass spectrometry; Cellular imaging; HaloTag technology

Introduction

RNA polymerase II (RNAPII) activity is tightly regulated by numerous factors in a highly complex and concerted fashion to achieve specific cellular responses and developmental programs [1-5]. In eukaryotes, a conserved multi-subunit complex, Mediator is an essential regulator of RNAPII transcription, important in initiation and elongation steps of the transcription cycle, functioning also as a repressor by prevention of RNAPII re-initiation [6-9]. Mediator interacts directly with RNAPII [10-16], DNA-binding transcription factors and other components of the transcriptional machinery [3,6,17-26]. Not surprisingly given its size and numerous subunits, further studies have implicated Mediator in roles beyond transcriptional activation and repression, and these include alteration of chromatin structure through interactions with cohesions [24], gene activation by long non-coding RNAs [27] and regulation of epigenetic marks on histones [28,29].

Biochemical, proteomic, and biophysical studies have shown Mediator exists primarily in two complex configurations, a smaller, ~1.2 MDa core complex, termed Mediator, and a larger, ~1.8 MDa complex consisting of the core bound to the CDK kinase module, termed CDK8-Mediator [7,16,30-34]. Notably, CDK-Mediator is mostly devoid of the MED26 subunit, which has been shown to bind the TFIID complex, part of the Pre-Initiation Complex (PIC), and the Super Elongation Complex [35]. Therefore the inclusion or exclusion of MED26 could have significant consequences on downstream transcriptional events. Initial studies focused upon understanding the roles of core Mediator as compared to CDK-Mediator concluded Mediator is a transcriptional co-activator, tightly bound to RNAPII [7-9,34], while CDK-Mediator is a repressive complex [16,25,36], as binding of the kinase module occludes interaction with RNAPII [25,32,36]. CDK8 could further act in a repressive fashion as it was found to phosphorylate RNAPII C-terminal domain repeats [20,35], inhibiting transcription initiation. Recent studies reveal however that CDK-Mediator has added layers of complexity. It has been shown to be required for effective transactivation within diverse transcriptional programs [23], including those controlled by p53 [21], the thyroid hormone receptor [37], Smads [38], serum response factors [20], and HIF1A [39]. The CDK kinase module also has activity as a separate complex, phosphorylating many targets including histones [28]. These data together suggest that the CDK kinase module can greatly change Mediator function, yet the precise mechanisms underlying these effects are poorly understood.

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In yeast, the kinase module is comprised of four components; MED12 (SRB8), MED13 (SRB9), CDK8 (SRB10), and Cyclin C. In vertebrates, the kinase module is more complex as paralogs have arisen from gene duplications for three of the four components, MED12, MED13, and CDK8 [40]. These are respectively named MED12-like (MED12L), MED13-like (MED13L) and CDK8-like (CDK19). Each paralog has been shown to interact with Mediator and are therefore thought to be components of the CDK kinase module [30,31], yet the combination of these is not clear. A recent study comparing CDK8 and CDK19 in human cells revealed that they associate with Mediator in a mutually exclusive fashion and contribute to the regulation of different sets of genes [39]. In order to more fully understand the organization of the human CDK kinase module and its interaction with core Mediator, we performed a systemic proteomic analysis in HEK293T cells by expressing and capturing via HaloTag each kinase module member (MED12, MED12L, MED13, MED13L, CDK8, CDK19, and Cyclin C) and their respective complexes. Each was then analyzed by LC-MS/MS to distinguish the presence or absence of paralogs within the complex mixtures. We show here that all of the paralogs are mutually exclusive of each other but not with other CDK-module subunits, which enables the assembly of up to 8 different combinations of the CDK-module, with great potential for functional specialization. Furthermore, we found a high abundance of MED26 only in the MED13L isolation, suggesting that when MED13L is present in the kinase module, MED26 is not excluded. Together our data indicate that CDK-Mediator could present itself in many forms, allowing for an interchangeable, rapid means to diversify its function.

Methods

Vectors and cell line

Full-length human MED12 (NM_005120.2), MED12L (Q86YW9), MED13 (NM_005121.2), MED13L (NM_015335.4), CDK8 (P49336), CDK19 (Q9BWU1), Cyclin C (P24863), and MED26 (NM_004831.3) were obtained from Kazusa DNA Research Institute as pFN21AHaloTag CMV Flexi Vector (catalog numbers FHC12080, FHC31710, FHC12078, FHC12079, FHC24966, FHC01935, FHC10447, and FHC05963). For the HaloTag experiments, the HaloTag Control Vector (Promega #G6591) was used as a negative control. HEK293T cells (ATCC #CRL-11268) were maintained in DMEM supplemented with 10% FBS at 37°C in an atmosphere of 5% CO_2. Cells were transfected using FuGENE HD transfection reagent (Promega) according to manufacturer's protocols.

HaloTag mammalian pull-down protocol

For these experiments, HEK293T cells (1.2×10^7) were plated in a 15 cm plate. After reaching 70-80% confluency, typically 18-24 hours later, cells were transfected with the various Mediator-HaloTag fusion constructs or HaloTag control vector. Twenty four hours post-transfection, cells were harvested and frozen at -80°C until processing. Cells were lysed in mammalian lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1%Na deoxycholate, and Protease Inhibitor cocktail (Promega) supplemented with 1.5 mM $MgCl_2$ and 10mM KCl, incubated on ice for 5 minutes, homogenized using a syringe, and then centrifuged at 14,000xg to clear the lysate. Resulting lysate supernatant were added directly to HaloLink Resin (Promega) equilibrated in 1X TBS and 0.1% IGEPAL-CA630, and allowed to bind for 15 minutes at 22°C with rotation. Resin was then washed 5 times with wash buffer (1X TBS, 0.1% IGEPAL-CA630, 1.5 mM $MgCl_2$ and 10 mM KCl), and protein interactors were eluted with SDS elution buffer (50 mM Tris-HCl, pH 7.5, and 1% SDS). Affinity purified complexes were then analyzed by nano LC/MS/MS and Western blotting.A detailed version of the HaloTag Mammalian Pull-Down protocol can be found at: http://www.promega.com/tbs/tm342/tm342.pdf.

Cellular imaging

HEK293T cells were transfected using FuGENE HD (Promega) according to manufacturer's recommendation. Twenty-four hours post-transfection cells were labelled with 5μM HaloTag TMR ligand (Promega) in complete media (DMEM and 10%FBS) for 15 minutes at 37°C and 5% CO_2. Media containing HaloTag-TMR ligand was then replaced twice with fresh complete media; cells were placed back at 37°C and 5% CO_2 for 30 minutes, and then imaged. Images were acquired on an Olympus Fluoview FV500 confocal microscope (Olympus, Center Valley, PA, USA) containing a 37°C+CO_2 environmental chamber (Solent Scientific Ltd., Segensworth, UK) using appropriate filter sets.

Mass spectrometry analysis

Two biological replicates were analysed by mass spectrometry for each Mediator-HaloTag fusion and the control expressing HaloTag protein only, and processed by MS Bioworks, LLC (Ann Arbor, Michigan, USA). The samples were separated on a SDS-PAGE gel which was subsequently Coomassie stained and cut into 10 fragments. Each gel piece was processed with the Progest Protein Digestion Station (Digilab). Briefly, gel slices were washed using 25 mM ammonium bicarbonate and acetonitrile, followed by reduction with 10 mM dithiothreitol, and alkylation with 50 mM iodoacetamide. Proteins were digested with trypsin (Promega) for 4 h and digestion was quenched with formic acid. Gel digests were analyzed directly by nano LC-MS/MS with a NanoAcquity HPLC (Waters) interfaced with an Orbitrap Velos Pro (Thermo Fisher) tandem mass spectrometer. Digested peptides were loaded on a trapping column and eluted over a 75 μm analytical column packed with Jupiter Proteo Resin (Phenomenex) at 350 nL/min. The mass spectrometer was operated in data-dependent mode, with MS performed in the Orbitrap at 60,000 Full Width at Half Maximum (FWHM) resolution, and MS/MS performed in the LTQ. The 15 most abundant ions were selected for MS/MS. The data were searched with Mascot (Matrix Science) against the concatenated forward/decoy UniProt Human Database, and Mascot DAT files were visualized and filtered by Scaffold (Proteome Software). Data were filtered using a minimum protein value of 90%, a minimum peptide value of 50% (Protein and Peptide Prophet scores), and required at least two unique peptides per protein. Spectral counting was performed and normalized spectral abundance factors determined. Data were reported at less than 1% false discovery rate (FDR) at the protein level based on counting the number of forward and decoy matches.

Western blot analysis and antibodies

Eluted proteins were separated by SDS-PAGE (8%) and transferred to PVDF membranes (0.45 μm, Thermo Fisher) at 300 mA for 90 min. Membranes were blocked in TBST (Tris-buffered saline with 0.05% Tween-20) containing 5% non-fat dry milk for 1 hr at room temperature before incubation overnight at 4°C with primary antibodies in TBST with 5% milk. Membranes were then washed five times with TBST and incubated for 1 hr at room temperature with horse radish peroxidase-coupled secondary antibodies (Santa-Cruz, 1:2000) in TBST with 5% milk, followed by a further five washes with TBST. Detection was by enhanced chemiluminescence and images were captured using an ImageQuant LAS4000 digital camera system (GE Healthcare). Antibodies used were as follows: CDK8: Santa Cruz sc -1521 (1:5000);

CDK19: Sigma HPA007053 (1:1000); MED12: Bethyl A300-774A (1:500); MED13: Bethyl A301-278A (1:500); MED13L: Bethyl A302-421A (1:500); MED26: Santa Cruz sc–48776 (1:500).

Results

Isolation of complexes and identification by mass spectrometry

HaloTag technology has previously been shown to efficiently isolate large multiprotein complexes, such as human RNA Polymerases I-III [41], the ribosome [42], and Mediator [39], as well as uncovering novel interactions [41,43]. For these studies, eight different Mediator-HaloTag fusion protein constructs were transiently expressed in HEK293T cells, then cells were lysed, and Mediator complexes were covalently captured via HaloTag on HaloLink resin (Figure 1). Gentle washes were employed to remove non-specific proteins, and interacting proteins were eluted with SDS. These complex mixtures were pulsed into a denaturing gel using electrophoresis, gel slices were thinly cut to separatefractions of proteins based upon size, then processed for analysis by liquid chromatography mass spectrometry (LC-MS/MS) (Figure 1). As a control for the experiment, the HaloTag protein alone was expressed and processed in parallel throughout the entire pull down protocol (Figure 1). As the paralogs within the human kinase module share overlapping peptides, searches were performed to specifically identify unique peptides to each paralog. Using this approach, we were able to determine with confidence the presence or absence of each paralog contained within the complex mixture.

Capture of mediator complexes and cellular localization

To systematically study the composition of the human CDK8 kinase module, HaloTag fusion proteins were constructed with each member; MED12, MED12L, MED13, MED13L, CDK8, CDK19, and Cyclin C. Each HaloTag fusion protein was initially tested for expression after transient transfection in HEK293T cells (data not shown) and proper cellular localization (Figure 2a). Treatment of cells with a covalent, fluorescent HaloTag ligand followed by confocal imaging revealed the Mediator-HaloTag fusions are nuclear (Figure 2a), indicating the presence of the HaloTag protein does not alter their expected physiological localization. Complex isolations were then performed following the protocol in Figure 1. Interacting proteins found in SDS elutions for each member were detected by silver-staining after gel electrophoresis (Figure 2b). Numerous bands, particularly at high molecular weight, are visible and specific enrichment of these is observed as compared to the HaloTag protein alone control (Figure 2b). As MED26 interactions have been previously characterized using FLAG-CoIP followed by MudPIT mass spectrometry, MED26-HaloTag complex isolations were additionally performed to serve as a positive control of the process [30,31] (Figure 2b).

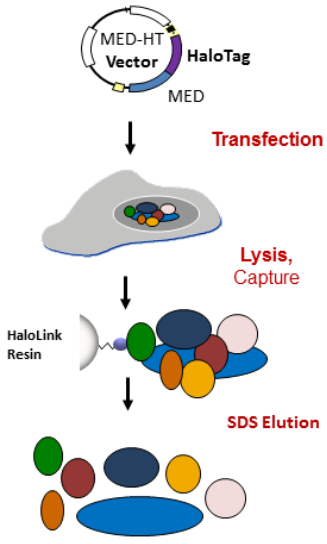

Figure 1: Diagram of the Mediator-HaloTag pulldown process. A genetic construct encoding the HaloTag (HT) protein fused N-terminally to a Mediator subunit (MED) was transiently expressed in HEK293T cells, where it incorporates into Mediator. After cellular lysis, MED-HaloTag complexes were covalently captured on HaloLink resin. Gentle washes were performed to remove non-specific interactions while maintaining complexes. Interacting protein partners were eluted with 1% SDS and analysed either by mass spectrometry or Western blot. As a control, HaloTag alone protein was expressed and processed through the entire pull-down protocol identically to the MED-HT sample.

Identification of mediator components

Each Mediator-HaloTag pull-down (MED12, MED12L, MED13, MED13L, CDK8, CDK19, Cyclin C, and MED26) and the HaloTag alone control was performed in biological duplicates. Identity of proteins isolated was determined and compared amongst the various Mediator-HaloTag fusion proteins. As is apparent from Figure 2b, numerous proteins are bound to each Mediator-HaloTag protein and unique banding patterns are present. Indeed the overall list of interactors identified by mass spectrometry is different for each (data not shown), but all show capture of Mediator subunits. For this paper, we focus on only the core Mediator and kinase module subunits identified using spectral counting and normalized spectral abundance factors (NSAFs), which account for size and relative abundance of each component [44,45] (Supplementary Table 1). NSAF values were averaged (Supplementary Table 1) and are presented for core Mediator and kinase module components in three categories corresponding to NSAF values $>1\times10^{-3}$ (Dark Blue), 1×10^{-3}-1×10^{-4} (Blue), and $<1\times10^{-4}$ (Light Blue) (Figure 3a). Blank squares indicate no peptides were found for that particular protein in the indicated complex isolation (Figure 3a). All isolations show excellent capture of core Mediator components and as expected, MED26 shows limited capture of CDK kinase module members [31] (Figure 3a). In contrast, all of kinase module isolations show high abundance of other kinase module proteins in addition to core Mediator components (Figure 3a). Further specificity is observed as the HaloTag alone controls did not show enrichment of Mediator subunits (Supplementary Table 1).

Within the overall NSAF analysis, several trends are apparent. The first is the finding that each of the paralogs is mutually exclusive with each other (Figure 3a lower panel), for example, MED12L is not present in MED12 isolations and vice versa, MED12 is not present in MED12L isolations. While paralogs are exclusive of each other, they are not exclusive of the other paralogs within the module, i.e. MED12 interacts with both MED13 and MED13L, as well as with CDK8 and CDK19 (Figure 3a lower panel). None of the kinase module members efficiently co-precipitate MED9 and MED21 core subunits (Figure 3a upper panel) which has been previously observed [32]. To further investigate this issue, isolations of HaloTag-MED9 and MED21 were performed followed by mass spectrometry, which revealed isolation of kinase module components (data not shown). These data support the hypothesis that MED9 and MED21 have weakened affinities with CDK-Mediator and are likely lost during the process of complex isolation [32].

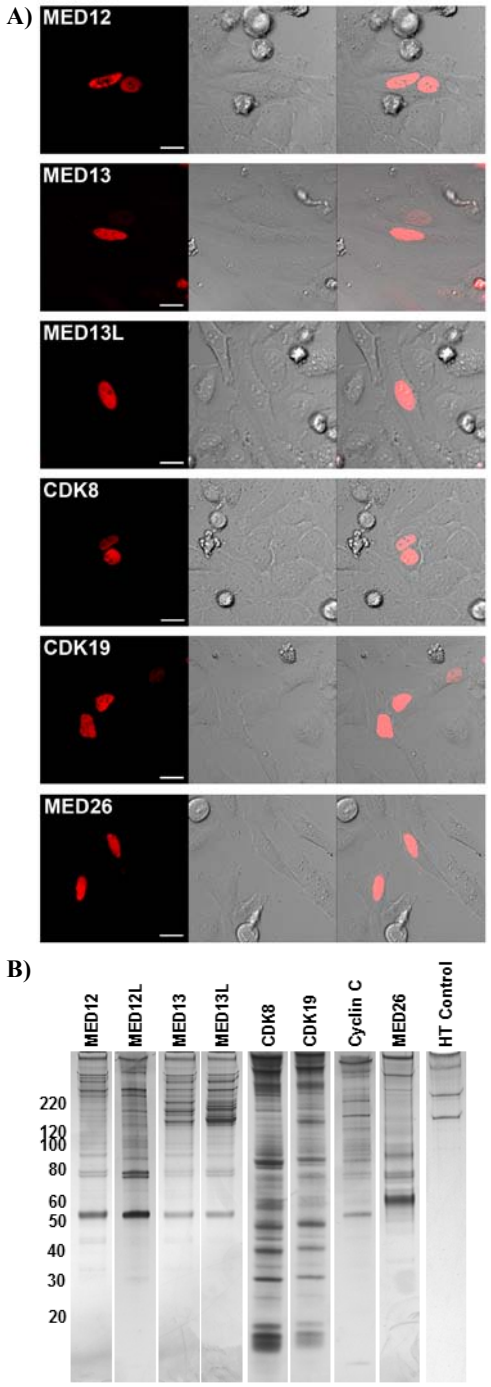

Figure 2: Cellular localization of Mediator-HaloTag fusions and complex isolations. A) Confocal cellular images of HEK293T cells expressing HaloTag-MED12, HaloTag-MED13, HaloTag-MED13L, HaloTag-CDK8, HaloTag-CDK19, and HaloTag-MED26. All are labelled covalently with red TMR fluorescent HaloTag ligand and shown unclear localization. The far right panel of each shows the TMR staining, the middle panel shows the bright field image of the cells, and the far left panel is both the respective bright field with the TMR staining. B) Silver stained SDS gels of proteins eluted from pulldowns of HaloTag-MED12 (Panel 1), HaloTag-MED12L (Panel 2), HaloTag-MED13 (Panel 3), HaloTag-MED13L (Panel 4), HaloTag-CDK8 (Panel 5), HaloTag-CDK19 (Panel 6), HaloTag-Cyclin C (Panel 7), HaloTag-MED26 (Panel 8), and HaloTag alone control (Panel 9) in HEK293T cells with respective molecular weight (kDa) marker numbers indicated.

Lastly, a significant difference between MED13 and MED13L isolations is observed with regards to MED26, which is thought to be present in only a minority of CDK-Mediator complexes. In these two isolations the NSAF values across kinase module members and core Mediator complex are nearly identical with the striking exception of a high abundance of MED26 in the MED13L isolation, whereas no peptides for MED26 are found in the MED13 isolation (Figure 3a upper panel and Supplementary Table 1). MED13L was not identified in the MED26 isolation (Figure 3a), but we believe this is due to low abundance of CDK-Mediator containing MED26 within the cell as compared to core Mediator [31]. Western blots were performed of the MED13 and MED13L samples to confirm the mass spectrometry results (Figure 3b). Indeed they show the mutual exclusivity of MED13 and MED13L, the presence of other kinase module members (MED12, CDK8, CDK19) in both samples, and the presence of MED26 only within the MED13L sample (Figure 3b). These data support previously published Western blots showing the mutual exclusivity of CDK8 and CDK19 [39]. Together, the proteomics data for each of the kinase module members reveals several interesting aspects, which could potentially have significant impact on the function and composition of CDK-Mediator.

Discussion

In this comprehensive proteomics study of the human CDK-Mediator kinase module members, we find all of the paralogs to be mutually exclusive of each other, though not exclusive of the other kinase module members (Figure 4). This indicates the paralogs do not form heterodimers, nor would they simultaneously be present in a CDK-Mediator complex. These data suggest the four component kinase module associated with Mediator exists in several configurations, anywhere between a minimum of 4 complexes to a maximum of 8. It is likely however that even if all eight possible combinations of the kinases module exist, they would not be equally represented within the cell. We predict some combinations would be favoured over others due to differences in affinities, and cell types may govern possible complexes by regulating the expression of the possible kinase module complexes. Of note, we have previously observed that CDK8 and CDK19 display a significant degree of tissue- and cell type-specific expression both at the RNA and protein level, yet they are also commonly co-expressed in many cell types [39].

Similarly, an interesting and significant difference observed between paralogs that may also have very important downstream transcription consequences is the finding that MED26 co-purifies with MED13L, but not MED13 (Figure 3a). This finding is supported by early studies of MED26 isolations wherein MED13 was barely detectable, yet MED13L was enriched [31]. As mentioned, MED26 is a critical subunit of Mediator as it facilitates direct interactions with two critical transcriptional activation machinery complexes, TFIID and the Super Elongation complex [35]. While the majority of CDK-Mediator is known to lack MED26 and likewise, Mediator isolations via MED26 show little or no kinase module subunits [30,31] (Figure 3a), this mutual exclusivity is not absolute [19,31,39]. Of note, we recently observed that both CDK8 and MED26 are recruited to HIF1A target genes during hypoxia, and that CDK8 is required for MED26 recruitment, suggesting a Mediator variant containing both subunits could be regulating these genes [39]. MED13/13L is the subunit which connects the kinase module to Mediator core complex [25,32], therefore it plays a critical structural role not only in the attachment of the CDK kinase module to Mediator but potentially also in the overall

A)

Mediator-HaloTag Fusions

Core Mediator Subunits	MED26	MED12	MED12L	MED13	MED13L	CDK8	CDK19	CyclinC
MED1	2.8E-03	3.2E-04	9.6E-04	2.6E-03	2.9E-03	5.2E-04	2.7E-04	7.0E-04
MED4	5.4E-03	8.3E-04	1.3E-03	7.8E-03	6.5E-03	1.9E-03	2.1E-04	1.0E-03
MED6	1.7E-03	3.9E-04	1.2E-03	2.7E-03	4.1E-03	7.9E-04	3.8E-04	1.1E-03
MED7	6.8E-04	1.0E-04	9.1E-04	5.3E-04	3.1E-04	3.9E-04		1.4E-03
MED8	7.2E-04	3.9E-04	9.7E-04	9.7E-04	2.4E-03	6.3E-04		9.7E-04
MED9	5.7E-04							
MED10	1.1E-03	5.2E-04	2.2E-04	8.9E-04	2.4E-03			1.0E-03
MED11	2.5E-03	8.3E-04	4.5E-04	5.5E-04	2.0E-03	3.3E-04		8.9E-04
MED14	2.9E-03	6.9E-04	1.4E-03	3.3E-03	3.4E-03	1.1E-03	7.2E-04	1.4E-03
MED15	1.9E-03	3.0E-04	4.7E-04	2.0E-03	2.7E-03	3.9E-04	3.5E-04	2.3E-04
MED16	2.0E-03	3.6E-04	7.5E-04	2.0E-03	1.8E-03	5.1E-04	2.5E-04	7.0E-04
MED17	2.8E-03	7.0E-04	9.1E-04	3.6E-03	4.0E-03	7.3E-04	5.1E-04	1.0E-03
MED18	1.2E-03	5.2E-04	1.5E-03	3.1E-03	2.7E-03	7.6E-04	1.8E-04	1.6E-03
MED19	9.4E-04		3.4E-04		3.2E-04	3.9E-04		1.2E-04
MED20	4.9E-03	1.1E-03	2.4E-03	7.0E-03	5.8E-03	2.2E-03	1.6E-03	2.7E-03
MED21	6.3E-04		2.2E-04					8.2E-04
MED22	2.8E-03	6.4E-04	1.5E-03	2.2E-03	4.3E-03	8.3E-04		1.9E-03
MED23	1.2E-03	6.9E-04	9.5E-04	3.2E-03	2.4E-03	7.6E-04	6.8E-04	8.5E-04
MED24	1.9E-03	4.7E-04	8.6E-04	3.0E-03	2.2E-03	7.9E-04	5.1E-04	1.1E-03
MED25	5.3E-04		1.9E-04	7.9E-04	6.9E-04		8.0E-05	2.3E-04
MED26	2.3E-02				1.5E-03			
MED27	1.2E-03	4.7E-04	1.5E-03	3.8E-03	3.0E-03	1.2E-03	1.8E-04	7.5E-04
MED28	1.0E-03	5.4E-04	1.2E-03	1.1E-03	1.8E-03			3.4E-04
MED29	2.1E-03	3.3E-04	5.3E-04			7.8E-04		2.5E-04
MED30	1.3E-03	8.5E-04	9.3E-04	3.3E-03	3.3E-03	2.0E-04	3.2E-04	1.4E-03
MED31	4.6E-03	2.0E-03	3.4E-03	3.4E-03	3.6E-03	1.2E-03	8.1E-04	2.0E-03

Kinase Module	MED26	MED12	MED12L	MED13	MED13L	CDK8	CDK19	CyclinC
MED12	1.4E-04	9.4E-03		3.6E-03	3.6E-03	1.7E-03	9.7E-04	1.2E-03
MED12L			1.2E-02	5.3E-04	5.9E-04	1.9E-04	1.2E-04	1.1E-04
MED13		2.3E-04	5.5E-04	1.6E-02		6.0E-04	3.4E-04	3.2E-04
MED13L		1.1E-04	2.2E-04		1.9E-02	1.7E-04	5.3E-05	5.3E-04
CDK8		2.3E-03	2.5E-03	2.6E-03	3.8E-03	3.4E-02		5.4E-03
CDK19		1.7E-03	1.2E-03	1.2E-03	2.3E-03		4.0E-02	4.4E-03
CyclinC	1.8E-04	2.7E-03	2.0E-03	3.9E-03	4.6E-03	3.5E-03	2.4E-03	3.1E-03

NSAF Values
> 1.0E-03
1.0E-03-1.0E-04
< 1.0E-05

B)

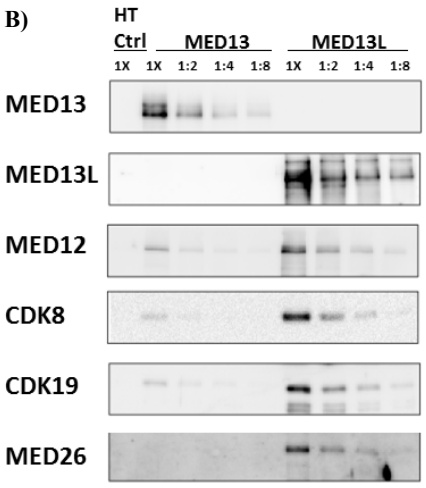

Figure 3: Proteomics NSAF analysis of Mediator core and kinase module subunits identified from HaloTag-Mediator complex isolations and Western blot analysis. A) Biological replicates of pulldowns from Mediator-HaloTag fusions indicated were analysed by LC-MS/MS and shown are averaged NSAF values for all Mediator core and kinase module components from the respective isolations. NSAF values are depicted in three categories; in Dark Blue are values greater than 1×10^{-3}, in Blue are values between 1×10^{-3} - 1×10^{-4}, and in Light Blue are values less than 1×10^{-4}. B) Western blots of HaloTag alone control (HT), HT-MED13 and HT-MED13L complex isolations. HT-MED13 and HT-MED13L samples were diluted 1X, 1:2, 1:4, and 1:8 as indicated. The HT control sample was used without dilution at 1X. Antibodies against MED13, MED13L, MED12, CDK9, CDK19, and MED26 were used to probe HT, HT-MED13, and HT-MED13L samples and results for each are shown in the indicated panels.

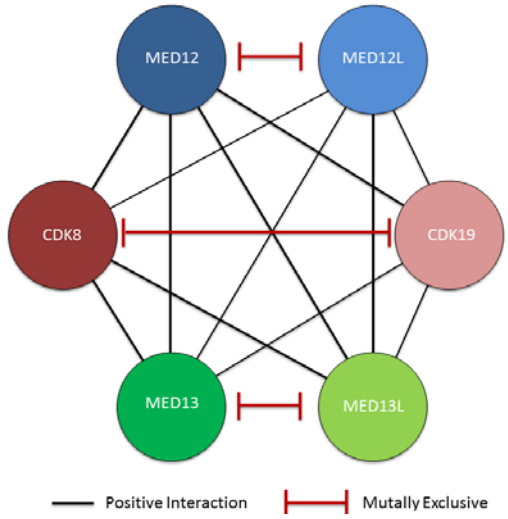

Figure 4: Model of interactions within the Mediator kinase module. All of the paralogs of the Mediator kinase module are shown (MED12/12L, MED13/13L, and CDK8/19). Positive interactions observed in Mediator-HaloTag complex isolations by mass spectrometry and Western blots (Figures 3a and b) are depicted with a solid black line, and those which are mutually exclusive are shown in red. Each of the paralogs are found to be mutually exclusive of one another, yet are not exclusive of any other kinase module member. This interaction map allows for a maximum of possible 8 complex configurations of the kinase module.

conformational change Mediator undergoes after binding the module [32]. Our finding that MED13L co-purifies MED26, yet MED13 does not, suggests it is either the choice of MED13 paralog that regulates the inclusion or exclusion of MED26 within CDK-Mediator complexes or MED13L is more structurally compatible with existing core Mediator complexes. Further experiments should be able to confirm CDK-Mediator populations containing MED26 are enriched forMED13L and more interestingly if there is a higher degree of genomic binding overlap between MED26 with MED13L in respect to MED13.

In summary, we find using HaloTag protein complex isolations in combination with high resolution mass spectrometry techniques we are able to resolve the mutual exclusivity of Mediator kinase module paralogs and CDK-Mediator complex composition in the context of a 1.8MDa transcription complex. Together these data indicate the human CDK kinase module itself is highly modular and perhaps paralogs were evolved thatconserved structural regions preserving module formation stoichiometry, yet changed signalling regions to exponentially broaden and diversify CDK-Mediator regulatory function.

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