Original Article

Proteomic Interactome of *C. elegans* Mediator Complex Subunit 28 (MDT-28) Reveals Predominant Association with a Restricted Set of Core Mediator Subunits and an Affinity to Additional Structural and Enzymatic Proteins

(*Caenorhabditis elegans* / GPF-TRAP / interactome / Head module / mass spectrometry / Mediator complex / MED28 / MDT-28 / proteomic)

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Abstract. Transcription factors exert their regulatory potential on RNA polymerase II machinery through a multiprotein complex called Mediator complex or Mediator. The Mediator complex integrates regulatory signals from cell regulatory cascades with the regulation by transcription factors. The Mediator complex consists of 25 subunits in *Saccharomyces cerevisiae* and 30 or more subunits in multicellular eukaryotes. Mediator subunit 28 (MED28), along with MED30, MED23, MED25 and MED26, belong to presumably evolutionarily new subunits that seem to be absent in unicellular eukaryotes and are likely to have evolved together with multicellularity and cell differentiation. Previously, we have shown that an originally uncharacterized predicted gene, *F28F8.5*, is the true *MED28* orthologue in *Caenorhabditis elegans* (*mdt-28*) and showed that it is involved in a spectrum of developmental processes. Here, we studied the proteomic interactome of MDT-28 edited as GFP::MDT-28 using Crispr/Cas9 technology or MDT-28::GFP expressed from extrachromosomal arrays in transgenic *C. elegans* exploiting the GFP-TRAP system and mass spectrometry. The results show that MDT-28 associates with the Head module subunits MDT-6, MDT-8, MDT-11, MDT-17, MDT-20, MDT-22, and MDT-30 and the Middle module subunit MDT-14. The analyses also identified additional proteins as preferential MDT-28 interactants, including chromatin-organizing proteins, structural proteins and enzymes. The results provide evidence for MDT-28 engagement in the Mediator Head module and support the possibility of physical (direct or indirect) interaction of MDT-28 with additional proteins, reflecting the transcription-regulating potential of primarily structural and enzymatic proteins at the level of the Mediator complex.

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Abbreviations: FDR – false discovery rate, HCD – higher-energy collisional dissociation, MMTS – methyl methanethiosulphonate, SDC – sodium deoxycholate, TEAB – triethylammonium bicarbonate, TFs – transcription factors.

Introduction

The Mediator complex, also called Mediator, is a large multiprotein complex that connects regulation by transcription factors (TFs) with the basal transcription machinery. This complex serves as a hub for integration of diverse regulatory signals such as kinase cascades

with the regulation of gene expression (Asturias et al., 1999; Allen and Taatjes, 2015; Soutourina 2018). Mediator can be divided into four structural modules, Middle module, Head module, Tail module, and Kinase module and this modular arrangement can be recognized from yeast to mammals (Bourbon, 2008; Jiang et al., 1998; Dotson et al., 2000; Tsai et al., 2014; Harper and Taatjes, 2017). The Mediator complex in *Saccharomyces cerevisiae* contains fewer subunits than its mammalian or plant equivalents (Soutourina, 2018). Four subunits seem to be specific for multicellular organisms and are absent in *S. cerevisiae*: MED23, MED25, MED26, MED28. Structural studies revealed the relative position of most core Mediator subunits (Asturias et al., 1999; Dotson et al., 2000; Mathur et al., 2011; Robinson et al., 2012, 2015, 2016; Verger et al., 2019), but also several distinct steric conformations of Mediator dependent on its interaction with specific transcription factors (SREBP, VP16, TR, VDR of p53AD) (Poss et al., 2013). The exact position of MED28 and MED30 has not yet been sufficiently elucidated (Soutourina, 2018).

The Mediator complex exists in two main forms, a larger complex probably containing most or all subunits of all four modules and a smaller complex lacking the Kinase module. Mediator that lacks the Kinase module is often linked to the formation of a more transcriptionally active Mediator. Some subunits have paralogues whose incorporation into Mediator have sub-specialization functions for transcription regulation (Allen and Taatjes, 2015). Besides that, the existence of complexes lacking one or more subunits was reported in the yeast that yield strains viable under laboratory conditions despite the lack of some subunits. While Med17 and Med21 were indispensable for wide transcription (Holstege et al., 1998; Thompson and Young, 1995), others including Med1, Med2, Med3, Med5, Med9, Med15, Med16, Med18, Med19, Med20, Med31, and CDK module subunits Med12 (srb8), Med13 (srb9), srb10 (CDK8), and srb11 were found non-essential as single mutants could have been maintained under laboratory conditions (Dettmann et al., 2010). Although under special conditions (in the yeast (Robinson et al., 2016) and in HeLa cells (Paoletti et al., 2006; Tsai et al., 2014)) most subunits were found in equimolar ratios, suggesting a fixed number of main Mediator subunits, some subunits immunoprecipitated at higher amounts (Paoletti et al., 2006). In tumours, numerous studies report up-regulation or down-regulation of individual subunits with links to the biological behaviour of cancer cells (Bragelmann et al., 2016; Syring et al., 2016).

MED28 has a special position in-between Mediator subunit proteins for its early discovered dual regulatory role, one as a Mediator subunit (Sato et al., 2004; Beyer et al., 2007) and the other, which is cytoplasmic, at the level of the cytoskeleton (Gonzalez-Agosti et al., 1996; Wiederhold et al., 2004; Lee et al., 2006; Lu et al., 2006; Huang et al., 2012).

In this study, we explored the protein interactome of *C. elegans* MDT-28 using its edited form GFP::MDT-28 or a transgenic form MDT-28::GFP and the GFP-Trap system. The data show predominant association of MDT-28 with the core Mediator subunits and support the engagement of MDT-28 in the Head module of the Mediator complex.

Material and Methods

Strains and transgenic lines

Two *Caenorhabditis elegans* lines expressing MDT-28 tagged with GFP were used:

1. The KV4 (8419) line with edited F28F8.5 in the form *gfp*::F28F8.5 in its normal genomic position $P_{F28F8.5}$ (V:15573749)::*gfp*::*F28F8.5* on both alleles (Kostrouchova et al., 2017) expressing edited GFP::MDT-28;
2. The *C. elegans* line 7949 with P*F28F8.5*(400 bp):: *F28F8.5*::*gfp* carrying extrachromosomal arrays with a construct containing the 400 bp internal promoter of *F28F8.5*, the genomic sequence of *F28F8.5* fused with the sequence coding for GFP with artificial introns (amplified from the pPD95.75 vector together with the 3′ UTR originally from *unc-54*) and the pRF4 selection marker (Kostrouchova et al., 2017).

For control, a *C. elegans* line carrying extrachromosomal arrays with the promoter of *nhr-180* regulating the expression of *gfp* alone (Kostrouchova et al., 2018) was used (Fig.1).

Laboratory cultures for large-scale nematode preparations

For immunoprecipitation experiments, the *C. elegans* lines were cultured on Petri dishes (diameter 60 mm) containing Nematode growth medium agar covered with OP50 *E. coli* as described (Brenner, 1974). *C. elegans* cultures were incubated in an air-conditioned laboratory with dim illumination (tempered to 22.5 °C) until the cultures reached visually similar population density and contained similar representation of individual larval stages, adults, and laid embryos; this was usually reached in four days. The mixed stage nematodes were controlled

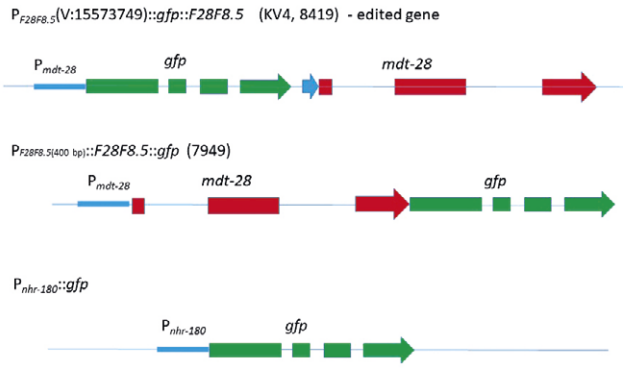

Fig. 1. Schematic representation of the edited alleles and constructs used in this study

for the expression of GFP-tagged MDT-28 in the line 7949 and showed the tendency to gradually lose GFP-tagged MDT-28, and were therefore checked for the proportion of nematodes positive for GFP fluorescence. Only cultures with more than 60 % of GFP-positive animals were used for further experiments. The nematodes were washed with deionized water, pelleted by centrifugation in 12 ml tubes (1,400 rpm, in Hettich Universal 32 R centrifuge, r_{max} = 120 mm, approximate maximal centrifugal force 176 × *g*, 4 °C, 2 min) (Hettich states 150 × *g* in these conditions in the swing-out rotor). Nematodes were resuspended in deionized water and pelleted to final samples that were stored at –80 °C until further processing.

GFP-Trap pull-down experiments

The GFP-Trap system available from Chromotek (https://www.chromotek.com/products/nano-traps/gfp-trap/) was used for pull-down of GFP-tagged MDT-28 and of GFP alone used as a control as described (Kostrouchova et al., 2018) with modifications regarding the scale of individual precipitations and the amount of lysate from mixed cultures of nematodes. Briefly, the *C. elegans* line 8419 with the *mdt-28* gene edited in the form of $P_{F28F8.5}$ (V:15573749)::*gfp*::*F28F8.5* on both alleles (Kostrouchova et al., 2017), the transgenic line carrying extrachromosomal arrays $P_{F28F8.5}$ (400 bp):: *F28F8.5*::*gfp* and the control line carrying extrachromosomal arrays containing $P_{nhr\text{-}180}$ (1361bp)::*gfp* were cultured on agar Petri dishes. The expression of GFP alone or GFP-tagged proteins was checked under an Olympus SZX12 microscope (Olympus, Tokyo, Japan). The mixed stage nematodes were washed with Dilution – Wash buffer (50 mM HEPES pH 7.4, 1 mM EGTA, 1 mM $MgCl_2$, 100 mM KCl). The nematodes were pelleted by centrifugation in ice-cold lysis buffer (50 mM HEPES pH 7.4, 1 mM EGTA, 1 mM $MgCl_2$, 100 mM KCl, 10% glycerol (v/v), 0.5% NP-40 (v/v)); all purchased from Sigma-Aldrich (St. Louis, MO). The maximum obtainable amount of packed nematodes (ranging from 1 to 2.6 ml) was used for each individual GFP-Trap preparation. Samples were resuspended in an equal volume of lysis buffer with protease and phosphatase inhibitors (Halt™ Protease and Phosphatase Inhibitor Cocktail (100x), Thermo Fisher Scientific, Waltham, MA) to obtain a final 1× solution of inhibitors.

Nematodes were sonicated 10 times for 15 s (with 45 s pauses) on ice (UP50H Ultrasonic Processor, Hielscher Ultrasound Technology, Teltow, Germany). Aliquots were controlled for complete lysis under the microscope. The samples were centrifuged for 25 min at 18,600 × *g* and 4 °C. The supernatant was transferred to a pre-cooled tube and 500 µl of Dilution – Wash buffer supplemented with 1× Protease inhibitors was added. GFP-Trap magnetic beads (GFP-Trap®_MA); purchased from ChromoTek, Planegg-Martinsried, Germany) were prepared according to the manufacturer's recommendations. Fifty microliters of bead slurry was resuspended in 500 µl of ice-cold dilution buffer, the beads were pulled-down using the magnetic separator and this step was repeated two more times. The diluted lysate was mixed with the equilibrated beads and the cleared supernatant was incubated at 4 °C for 12 h. Beads with bound proteins were pulled-down using the magnetic separator and washed three times using Dilution – Wash buffer.

Elution

Proteins bound to the beads were eluted by 50 µl 0.2 M glycine (pH 2.5) during 30 s under constant stirring followed by magnetic separation. The supernatant was transferred to a new tube and 5 µl 1 M Tris base (pH 10.4) was used for sample neutralization. The remaining beads were stored in wash buffer and also analysed by mass spectrometry.

Mass spectrometry

For mass spectrometry, the eluates and in some cases also the remaining beads were acetone precipitated and resuspended in 100 mM triethylammonium bicarbonate (TEAB) containing 1% sodium deoxycholate (SDC). Cysteines were reduced with 5 mM (final) Tris(2-carboxyethyl)phosphine (TCEP) (60 °C, 60 min) and blocked with 10 mM (final) methyl methanethiosulphonate (MMTS) (all Thermo Fisher Scientific) (10 min, 22 °C).

Proteins were cleaved with 1 µg of trypsin at 37 °C overnight, centrifuged, supernatants collected and acidified with TFA (1% final concentration). SDC was removed by extraction with ethyl acetate. Peptides were desalted on a Peptide Optitrap column (Optimize Technologies, Oregon City, OR). A nano reversed phase column (EASY-Spray column, 50 cm × 75 µm ID, PepMap C18, 2 µm particles, 100 Å pore size) (Thermo Fisher Scientific) was used for LC/MS/MS analysis. The mobile phase buffers were: A – water and 0.1% formic acid, B – acetonitrile and 0.1% formic acid. The samples were loaded onto the trap column (Acclaim PepMap300, C18, 5 µm, 300 Å Wide Pore, 300 µm × 5 mm, 5 Cartridges) for 4 min at 15 µl/min. The loading buffer was composed of water, 2% acetonitrile and 0.1% trifluoroacetic acid. Peptides were eluted with the mobile phase B gradient from 4% to 35% B in 60 min. Eluted peptide cations were converted to gas-phase ions by electrospray ionization and analysed in Thermo Orbitrap Fusion (Q-OT- qIT, Thermo Fisher Scientific).

Survey scans of peptide precursors from 400 to 1600 m/z were performed at 120K resolution (at 200 m/z) with a 5×10^5 ion count target. Tandem MS was performed by isolation at 1.5 Th with the quadrupole, higher-energy collisional dissociation (HCD) fragmentation with normalized collision energy of 30, and rapid scan MS analysis in the ion trap. The MS 2 ion count target was set to 10^4 and the max injection time was 35 ms. Only those precursors with charge states 2–6 were sampled for MS 2. The dynamic exclusion duration was set to 45 s with a 10 ppm tolerance around the selected precursor and its isotopes. Monoisotopic precursor selection was turned on. The instrument was run in top speed

mode with 2 s cycles. All data were analysed and quantified with the MaxQuant software (version 1.5.3.8) (Cox et al., 2014). The false discovery rate (FDR) was set to 1 % for both proteins and peptides and a minimum length of seven amino acids. The Andromeda search engine was used for the MS/MS spectra search against the *C. elegans* database (downloaded from Uniprot in April 2015, containing 25,527 entries) and further updates. Enzyme specificity was set as C-terminal to Arg and Lys, also allowing cleavage at proline bonds and a maximum of two missed cleavages. Dithiomethylation of cysteine was selected as fixed modification, and N-terminal protein acetylation and methionine oxidation as variable modifications. The "match between runs" feature of MaxQuant was used to transfer identifications to other LC-MS/MS runs based on their masses and retention time (maximum deviation 0.7 min) and used in quantification experiments. Quantifications were performed with the label-free algorithms and data analysis was performed using the Perseus 1.5.2.4 software (Masuda et al., 2008; Cox et al., 2014; Hebert et al., 2014; Tyanova et al., 2016).

The raw data were deposited to an open database https://www.ebi.ac.uk/pride/archive/ with accession number PXD015552 (Project DOI: 10.6019/PXD015552; Username: reviewer73133@ebi.ac.uk; Password: 2l2cgHCZ). The raw data concerning relevant proteins are also provided in four Excel files that list the raw data analysed by the Perseus program (http://coxdocs.org/doku.php?id=perseus:start version Perseus_1.6.1.3) and are accessible at the Folia Biologica Website as Supplementary data:

20180226_Yilma_C1_E1,E6.ods
20180504_Yilma_C2_E2.ods
20180829_Yilma_C3_E3,E4.ods
20190224_Yilma_C4_E5.ods

The experiments are marked as in Table 1 and Table 2: C1 to C4 are control samples and E1 to E6 are experimental samples.

Results

Identification of Mediator complex subunits associating with MDT-28 from mixed stage C. elegans cultures

The detection of proteins preferentially interacting with MDT-28 *in vivo* was performed using two *C. elegans* strains expressing MDT-28 tagged by GFP and a commercially available system GFP-Trap for isolating GFP-tagged proteins. Four control experiments were performed in order to exclude proteins binding non-specifically to GFP or the beads containing the anti-GFP single-chain antibody. Proteins identified in extracts prepared from animals expressing MDT-28 tagged with GFP on their N- or C-terminus were compared with the controls. As a positive result for protein detection, the intensity threshold of 18 and identification of at least two peptides were set as the criteria for reliable identification. To classify the detected protein as an MDT-28 interactor, only proteins falling under the threshold and classified as undetected by the Perseus program in all four control experiments but identified in lysates from lines with tagged MDT-28 were taken into account.

Prominently, a set of proteins classified as Mediator subunits were identified as MDT-28 interactants (Table 1). They mostly included subunits of the Head module: MDT-6, MDT-8, MDT-11, MDT-17, MDT-20, MDT-22, and MDT-30. The set of MDT-28 interactants further included MDT-14 of the Middle module. The other Middle module subunits, Tail module subunits, or Kinase module subunits were not identified in our screens as MDT-28

Table 1. Mediator complex subunits identified in MDT-28 pull-down experiments

	Ctrl 1	Ctrl 2	Ctrl 3	Ctrl 4	Exp 1	Exp 2	Exp 3	Exp 4	Exp 5	Exp 6
	GFP	GFP	GFP	GFP	C-term (7949)	C-term (7949)	C-term (7949)	N-term (8419)	N-term (8419)	N-term (8419)
MDT-6	–	–	–	–	+	–	–	–	+	+
MDT-8	–	–	–	–	+	–	–	+	+	+
MDT-11	–	–	–	–	+	–	–	+	+	+
MDT-14	–	–	–	–	–	–	–	+	+	+
MDT-17	–	–	–	–	+	+	+	+	+	+
MDT-20	–	–	–	–	–	–	–	–	+	+
MDT-22	–	–	–	–	–	–	–	+	+	–
MDT-28	–	–	–	–	+	+	+	+	+	–
MDT-30	–	–	–	–	+	–	–	+	+	+

Legend to Table 1. Detection of predicted Mediator subunits in immunoprecipitated samples from control experiments (marked as Ctrl 1 to 4) based on *C. elegans* expressing GFP alone, C-terminally tagged MDT-28::GFP expressed from extrachromosomal arrays (marked as Exp 1 to 3 in yellow table cells) and N-terminally tagged GFP::MDT-28 expressed from *C. elegans* line with edited *mdt-28* (marked as Exp 4 to 6 in green table cells). While control experiments did not show the presence of any known Mediator subunit, lines with GFP-tagged MDT-28 contained interacting Mediator complex subunits.

interactants under the experimental conditions and given selection criteria. MDT-18 was identified in two out of four experiments; however, this identification is statistically less significant since it is based only on one unique peptide.

MDT-28 associates with additional structural and enzymatic proteins

Comparison of the data from all experiments yielded a relatively small number of proteins that could have been classified as MDT-28 interactants under the chosen selection criteria (Table 1). The set of MDT-28 interacting proteins included proteins falling under the category of proteins likely to be involved in the regulation of transcription or other nuclear events and proteins that are not classified as primarily transcription-regulating proteins. Some identified MDT-28 interactants belong to the category of structural or enzymatic proteins or proteins with contemporarily unknown functions (Table 2). This set of potential MDT-28 interactants includes chromatin-organizing proteins, known nuclear transporters, nucleolar proteins and enzymes. Two proteins classified as RPL proteins, RPL-24.2 and RPL-29, were identified as strong binders in the immunoprecipitated samples.

Additionally, a set of mitochondrial ribosomal proteins of the small subunit were detected as potential interactants of MDT-28. This set included MRPS-2, MRPS-5, MRPS-6, MRPS-7, MRPS-15, and MRPS-26. The set of proteins immunoprecipitated with MDT-28 also included components of the Ubiquinol-cytochrome C oxidoreductase complex (UCR-1.1, UCR-2.1, UCR-2.2, and UCR-2.3) and UCR-11, which is also predicted to be a component of this complex.

Discussion

MDT-28 has tight interactions with selected Mediator Head subunits and Middle module subunit MDT-14 (and Middle module subunit MDT-9 in some experiments)

Our experiments show that MDT-28 associates with a set of core Mediator complex subunits mainly consisting of Head module subunits and Middle module subunit MDT-14. MDT-28 is a small protein comprising only 200 or 202 amino acids, and it is therefore likely that the identified proteins are extracted from the cellular material as a protein complex or multiple versions of smaller

Table 2. Additional MDT-28 interactants

	Ctrl 1	Ctrl 2	Ctrl 3	Ctrl 4	**Exp 1**	**Exp 2**	**Exp 3**	**Exp 4**	**Exp 5**	**Exp 6**	**GO/ notes**
	GFP	GFP	GFP	GFP	C-(7949)	C- (7949)	C- (7949)	N- (8419)	N- (8419)	N-(8419)	
ARX-1		–	–	–	–	+	+	+	+	–	Actin binding
DHC-1	–	–	–	–	–	–	–	+	+	–	Enzyme Cytoskeleton
EKL-4	–	–	–	–	–	–	+	+	–	–	Corepressor
GLY-7	–	–	–	–	–	+	+	+	+	–	N/C enzyme Golgi
HECD-1	–	–	–	–	–	+	–	–	+	–	E3 ligase Nuclear
HRP-2	–	–	–	–	+	–	+	–	+	+	Ribonucleo-protein
IMA-3	–	–	–	–	–	+	+	–	+	–	Nuclear import signal receptor activity
LET-716	–	–	–	–	–	+	+	+	+	–	Nucleic acid binding
NGP-1 GNL2 homol.	–	–	–	–	–	+	+	+	+	–	Enzyme GTPase, NMD
RPL-24.2	–	–	–	–	+	+	+	+	+	–	
RPL-29	–	–	–	–	+	+	+	+	+	+	
SWAN-1	–	–	–	–	–	+	+	+	+	–	N/C enzyme GTPase
VBH-1	–	–	–	–	+	+	+	–	+	+	Helicase

Legend to Table 2. Detection of MDT-28 interactants in immunoprecipitated samples from control experiments (marked as Ctrl 1 to 4) based on *C. elegans* expressing GFP alone, C-terminally tagged MDT-28::GFP expressed from extrachromosomal arrays (marked as Exp 1 to 3 in yellow table cells) and N-terminally tagged GFP::MDT-28 expressed from *C. elegans* line with edited *mdt-28* (marked as Exp 4 to 6 in green table cells). GO – gene ontology related to possible nuclear and cytoplasmic functions.

complexes. Our results further support the classification of MDT-28 as the orthologue of MED28, which is known to form contacts with the Head module subunits in proteomic studies (Tsai et al., 2014). Our results thus support the classification of MDT-28 and MED28 orthologues as a Head module subunit. The interactions of two subunits, MDT-30 and MDT-6, were reported earlier based on *in vitro* methods. The interaction with MDT-30 is likely to be direct since it was observed earlier on isolated proteins made in bacteria (Kostrouchova et al., 2017). The results support the prediction of MDT-30 as an MED30 orthologue. In contrast to other orthologues of Mediator subunits in *C. elegans* that have a similar size as the yeast and vertebrate counterparts, MDT-30 is substantially larger than its mammalian orthologues. MDT-30 contains 466 amino acids compared to human and mouse MED30, which contain only 143 and 178 amino acids, respectively. The Clustal analysis indicates a relatively high degree of conservation of the C-terminal half of MDT-30 with the almost entire sequence of human MED30 (isoform 1) (E Value 6 e-04, 26.83 % identity over the conserved region spanning 218 amino acids and three gaps for the total of eight amino acids). While the conserved region has a globular character in both nematode and human proteins, the N-terminal region present in the nematode MED30 orthologue has a character of intrinsically disordered protein.

The superposition of Mediator subunits identified as MDT-28 preferential interactants suggests the possibility of a complex that may be prevalent in lysates obtained from mixed stage *C. elegans*. The starting material that was used in our experiments is likely to contain a large proportion of tissues forming the majority of *C. elegans* bodies, such as gut, muscles and gonads.

It can be expected that the binding proteins may differ for N-terminally and C-terminally tagged MDT-28 for two reasons; first due to the steric blocking of interacting domains of MDT-28, and secondly because of the relatively higher cytoplasmic expression of C-terminally tagged MDT-28 expressed from extrachromosomal arrays.

The C-terminal globular domain of MDT-28 is likely to be important for interactions with other Mediator subunits. The N-terminal IDR is likely to mediate interactions with multiple proteins and assume its actual conformation during MED-28 assembly in the protein complex. The two variants of GFP-tagged MDT-28 are also expressed differently. The edited N-terminally tagged protein is expressed ubiquitously in a pattern that is likely to represent the natural expression of untagged MDT-28 including expression in gonads, while the C-teminally tagged MDT-28 is expressed strongly in the cytoplasm and in the nuclei of gut and epidermal cells but not in gonads, where the expression from extrachromosomal arrays is silenced. Nevertheless, the interaction with Mediator subunits was detected in both arrangements.

Since the Mediator subunits immunoprecipitated with MDT-28 are more likely to be precipitated in our experiments as a protein complex rather than proteins associated with MDT-28 individually, the immunoprecipitated complex is likely to represent a core MDT-28-containing Mediator complex (Fig. 2). Although some components of the MDT-28-containing Mediator complex may be removed from the complex during purification steps, the remaining complex is likely to reflect the most resistant and thus preferential protein complex that contains MDT-28.

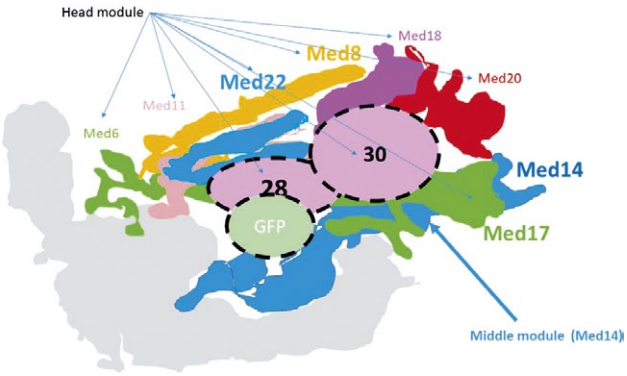

Fig. 2. Schematic representation of MDT-28 core Mediator A hypothetical MDT-28-containing core Mediator based on the known structure of the yeast core Mediator (Nozawa et al., 2017) and Mediator subunits immunoprecipitated with MDT-28. The domains occupied by individual proteins are indicated with colours corresponding to indicated Mediator subunits. MDT-28 tagged by GFP and MDT-30 are positioned arbitrarily to the central region of the yeast Mediator complex that is less densely filled by Mediator subunits in the core Mediator complex determined by Nozava et al. (2017).

Nuclear and cytoplasmic proteins of diverse nature interact with MDT-28

While the validity of the Mediator complex subunits that were identified as MDT-28 interactors is supported by numerous proteomic and functional studies, the possibility of functional relevance of additional MDT-28 interactors is supported only indirectly. Since MDT-28 as well as MED28 are both nuclear and cytoplasmic proteins (Wiederhold et al., 2004; Lee et al., 2006; Kostrouchova et al., 2017, 2018), they are likely to interact with proteins mediating their actual subcellular localization in both main cellular compartments.

Our screens were performed at relatively very stringent conditions that were necessary for disintegration of nematodes prior to the immunoprecipitation steps. This is in keeping with the fact that only a part of the core Mediator subunits were immunoprecipitated with MDT-28. This also gives presumption that possible cytoplasmic proteins identified in our screens may be directly involved in protein interactions at the cytoskeleton, which is well documented, but the proteins directly binding MED28 to the cytoskeleton are not known (Wiederhold et al., 2004).

A candidate protein identified in our screens that can mediate interaction with actin cytoskeleton is ARX-1, the orthologue of human ACTR3 (Actin-related protein 3), which is a constituent of the Arp2/3 complex. This complex is located at the cell surface and regulates cell shape and motility together with F-actin and Cortactin (Weed et al., 2000). ARP2/3 is also localized in the nuclei and has a known function in the regulation of actin polymerization linked to gene expression and DNA break clustering in homology-directed DNA repair (Schrank et al., 2018), an event that involves the presence of the Mediator complex (Soutourina and Werner, 2014).

Another primarily cytoplasmic protein that was identified in our screens as an MDT-28 interactor is dynein heavy chain DHC-1. It was identified in two out of three experiments exploiting N-terminally tagged MDT-28 with high intensities and 22 identified unique peptides. Dynein is involved in numerous protein interactions both in the cytoplasm and in the nucleus. It has been shown to interact with P53 protein and the glucocorticoid receptor (Harrell et al., 2004)

Additional MDT-28 interactors including EKL-4, GLY-7, HECD-1, HRP-2, IMA-3, LET-716, NGP-1, VBH-1, DHC-1, RPL-24.2, and RPL-29 may also be functionally relevant, since their nuclear and often transcription-regulating functions are listed in molecular biology databases. The two proteins classified as ribosomal proteins, RPL-24.2 and RPL-29, are not classical ribosomal proteins, but are related based on sequence similarity. Both RPL-29 and RPL-24.2 may have transcription regulation functions. RPL-29 is homologous to HIP/RPL29, which was shown to be present in the cytoplasm as well as in the nucleus and to play more than one role during adult mammary gland development (Kirn-Safran et al., 2002). RPL-24.2 is an orthologue of RSL24D1, which has a known primary nuclear localization, but at the present with unknown function (The Human Protein Atlas, https://www.proteinatlas.org/ENSG00000137876-RSL24D1/cell).

Our experiments identified a set of mitochondrial ribosomal proteins of the small ribosomal subunit as likely interactants with MDT-28 or MDT-28-containing Mediator complex. This subset was identified in experiments exploiting both forms of the MDT-28 GFP-tagged protein. It seems more likely that these proteins form a protein complex rather than being bound individually, since small subunit components are represented more than other mitochondrial ribosomal proteins. It is a question whether the identified proteins are kept together by another protein or some other factor, e.g., mitochondrial rRNA or other molecules of similar nature. It is unlikely that the complex be in fact a complete small mitochondrial ribosomal subunit, since other proteins of the complete small mitochondrial ribosomal subunit are missing in our screens. It is possible that mitochondrial ribosomal proteins interacted with the immunoprecipitated MDT-28 during our experimental procedures at the steps of cell disintegration. The observed situation may, however, reflect a physiologically relevant process, the mitochondrial stress response (Hill et al., 2018). The interaction of mitochondrial ribosomal proteins with proteins central to the regulation of transcription may have functional consequences, which is supported by the concept of the regulation of plant development by ribosomal proteins (Robles and Quesada, 2017). It may be compatible with the proposed concept of "the ribosome filter redux" or "the ribosome filter hypothesis" (Mauro and Edelman, 2002, 2007).

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References

Allen, B. L., Taatjes, D. J. (2015) The Mediator complex: a central integrator of transcription. *Nat. Rev. Mol. Cell Biol.* **16**, 155-166.

Asturias, F. J., Jiang, Y. W., Myers, L. C., Gustafsson, C. M., Kornberg, R. D. (1999) Conserved structures of mediator and RNA polymerase II holoenzyme. *Science* **283**, 985-987.

Beyer, K. S., Beauchamp, R. L., Lee, M. F., Gusella, J. F., Naar, A. M., Ramesh, V. (2007) Mediator subunit MED28 (Magicin) is a repressor of smooth muscle cell differentiation. *J. Biol. Chem.* **282**, 32152-32157.

Bourbon, H. M. (2008) Comparative genomics supports a deep evolutionary origin for the large, four-module transcriptional mediator complex. *Nucleic Acids Res.* **36**, 3993-4008.

Bragelmann, J., Klumper, N., Offermann, A., von Massenhausen, A., Bohm, D., Deng, M., Queisser, A., Sanders, C., Syring, I., Merseburger, A. S., Vogel, W., Sievers, E., Vlasic, I., Carlsson, J., Andren, O., Brossart, P., Duensing, S., Svensson, M. A., Shaikhibrahim, Z., Kirfel, J., Perner, S. (2016) Pan-cancer analysis of the Mediator complex transcriptome identifies CDK19 and CDK8 as therapeutic targets in advanced prostate cancer. *Clin. Cancer Res*. **23**, 1829-1840.

Brenner, S. (1974) The genetics of Caenorhabditis elegans. *Genetics* **77**, 71-94.

Cox, J., Hein, M. Y., Luber, C. A., Paron, I., Nagaraj, N., Mann, M. (2014) Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. *Mol. Cell. Proteomics* **13**, 2513-2526.

Dettmann, A., Jaschke, Y., Triebel, I., Bogs, J., Schroder, I., Schuller, H.-J. (2010) Mediator subunits and histone methyltransferase Set2 contribute to Ino2-dependent transcriptional activation of phospholipid biosynthesis in the yeast Saccharomyces cerevisiae. *Mol. Genet. Genomics* **283**, 211-221.

Dotson, M. R., Yuan, C. X., Roeder, R. G., Myers, L. C., Gustafsson, C. M., Jiang, Y. W., Li, Y., Kornberg, R. D., Asturias, F. J. (2000) Structural organization of yeast and mam-

malian mediator complexes. *Proc. Natl. Acad. Sci. USA* **97**, 14307-14310.

Gonzalez-Agosti, C., Xu, L., Pinney, D., Beauchamp, R., Hobbs, W., Gusella, J., Ramesh, V. (1996) The merlin tumor suppressor localizes preferentially in membrane ruffles. *Oncogene* **13**, 1239-1247.

Harper, T. M., Taatjes, D. J. (2017) The complex structure and function of Mediator. *J. Biol. Chem*. **293**, 13778-13785.

Harrell, J. M., Murphy, P. J., Morishima, Y., Chen, H., Mansfield, J. F., Galigniana, M. D., Pratt, W. B. (2004) Evidence for glucocorticoid receptor transport on microtubules by dynein. *J. Biol. Chem.* **279**, 54647-54654.

Hebert, A. S., Richards, A. L., Bailey, D. J., Ulbrich, A., Coughlin, E. E., Westphall, M. S., Coon, J. J. (2014) The one hour yeast proteome. *Mol. Cell. Proteomics* **13**, 339-347.

Hill, S., Sataranatarajan, K., Remmen, H. V. (2018) Role of signaling molecules in mitochondrial stress response. *Front. Genet.* **9**, 225.

Holstege, F. C., Jennings, E. G., Wyrick, J. J., Lee, T. I., Hengartner, C. J., Green, M. R., Golub, T. R., Lander, E. S., Young, R. A. (1998) Dissecting the regulatory circuitry of a eukaryotic genome. *Cell* **95**, 717-728.

Huang, C. Y., Chou, Y. H., Hsieh, N. T., Chen, H. H., Lee, M. F. (2012) MED28 regulates MEK1-dependent cellular migration in human breast cancer cells. *J. Cell. Physiol.* **227**, 3820-3827.

Jiang, Y. W., Veschambre, P., Erdjument-Bromage, H., Tempst, P., Conaway, J. W., Conaway, R. C., Kornberg, R. D. (1998) Mammalian mediator of transcriptional regulation and its possible role as an end-point of signal transduction pathways. *Proc. Natl. Acad. Sci. USA* **95**, 8538-8543.

Kirn-Safran, C. B., Julian, J., Fongemie, J. E., Hoke, D. E., Czymmek, K. J., Carson, D. D. (2002) Changes in the cytologic distribution of heparin/heparan sulfate interacting protein/ribosomal protein L29 (HIP/RPL29) during in vivo and in vitro mouse mammary epithelial cell expression and differentiation. *Dev. Dyn.* **223**, 70-84.

Kostrouchova, M., Kostrouch, D., Chughtai, A. A., Kassak, F., Novotny, J. P., Kostrouchova, V., Benda, A., Krause, M. W., Saudek, V., Kostrouchova, M., Kostrouch, Z. (2017) The nematode homologue of Mediator complex subunit 28, F28F8.5, is a critical regulator of C. elegans development. *PeerJ.* **5**, e3390.

Kostrouchova, M., Kostrouchova, V., Yilma, P., Benda, A., Mandys, V., Kostrouchova, M. (2018) Valproic acid decreases the nuclear localization of MDT-28, the nematode orthologue of MED28. *Folia Biol. (Praha)* **64**, 1-9.

Lee, M. F., Beauchamp, R. L., Beyer, K. S., Gusella, J. F., Ramesh, V. (2006) Magicin associates with the Src-family kinases and is phosphorylated upon CD3 stimulation. *Biochem. Biophys. Res. Commun.* **348**, 826-831.

Lu, M., Zhang, L., Sartippour, M. R., Norris, A. J., Brooks, M. N. (2006) EG-1 interacts with c-Src and activates its signaling pathway. *Int. J. Oncol.* **29**, 1013-1018.

Masuda, T., Tomita, M., Ishihama, Y. (2008) Phase transfer surfactant-aided trypsin digestion for membrane proteome analysis. *J. Proteome Res.* **7**, 731-740.

Mathur, S., Vyas, S., Kapoor, S., Tyagi, A. K. (2011) The Mediator complex in plants: structure, phylogeny, and expression profiling of representative genes in a dicot (Arabidopsis) and a monocot (rice) during reproduction and abiotic stress. *Plant Physiol.* **157**, 1609-1627.

Mauro, V. P., Edelman, G. M. (2002) The ribosome filter hypothesis. *Proc. Natl. Acad. Sci. USA* **99**, 12031-12036.

Mauro, V. P., Edelman, G. M. (2007) The ribosome filter redux. *Cell Cycle* **6**, 2246-2251.

Nozawa, K., Schneider, T. R., Cramer, P. (2017) Core Mediator structure at 3.4 A extends model of transcription initiation complex. *Nature* **545**, 248-251.

Paoletti, A. C., Parmely, T. J., Tomomori-Sato, C., Sato, S., Zhu, D., Conaway, R. C., Conaway, J. W., Florens, L., Washburn, M. P. (2006) Quantitative proteomic analysis of distinct mammalian Mediator complexes using normalized spectral abundance factors. *Proc. Natl. Acad. Sci. USA* **103**, 18928-18933.

Poss, Z. C., Ebmeier, C. C., Taatjes, D. J. (2013) The Mediator complex and transcription regulation. *Crit. Rev. Biochem. Mol. Biol.* **48**, 575-608.

Robinson, P. J., Bushnell, D. A., Trnka, M. J., Burlingame, A. L., Kornberg, R. D. (2012) Structure of the mediator head module bound to the carboxy-terminal domain of RNA polymerase II. *Proc. Natl. Acad. Sci. USA* **109**, 17931-17935.

Robinson, P. J., Trnka, M. J., Pellarin, R., Greenberg, C. H., Bushnell, D. A., Davis, R., Burlingame, A. L., Sali, A., Kornberg, R. D. (2015) Molecular architecture of the yeast Mediator complex. *Elife* **4**, pii: e08719.

Robinson, P. J., Trnka, M. J., Bushnell, D. A., Davis, R. E., Mattei, P. J., Burlingame, A. L., Kornberg, R. D. (2016) Structure of a complete Mediator-RNA polymerase II preinitiation complex. *Cell* **166**, 1411-1422.

Robles, P., Quesada, V. (2017) Emerging roles of mitochondrial ribosomal proteins in plant development. *Int. J. Mol. Sci.* **18**, pii: 18122595

Sato, S., Tomomori-Sato, C., Parmely, T. J., Florens, L., Zybailov, B., Swanson, S. K., Banks, C. A., Jin, J., Cai, Y., Washburn, M. P., Conaway, J. W., Conaway, R. C. (2004) A set of consensus mammalian mediator subunits identified by multidimensional protein identification technology. *Mol. Cell* **14**, 685-691.

Schrank, B. R., Aparicio, T., Li, Y., Chang, W., Chait, B. T., Gundersen, G. G., Gottesman, M. E., Gautier, J. (2018) Nuclear ARP2/3 drives DNA break clustering for homology-directed repair. *Nature* **559**, 61-66.

Soutourina, J., Werner, M. (2014) A novel link of Mediator with DNA repair. *Cell Cycle* **13**, 1362-1363.

Soutourina, J. (2018) Transcription regulation by the Mediator complex. *Nat. Rev. Mol. Cell Biol.* 19, 262-274.

Syring, I., Klumper, N., Offermann, A., Braun, M., Deng, M., Boehm, D., Queisser, A., von Massenhausen, A., Bragelmann, J., Vogel, W., Schmidt, D., Majores, M., Schindler, A., Kristiansen, G., Muller, S. C., Ellinger, J., Shaikhibrahim, Z., Perner, S. (2016) Comprehensive analysis of the transcriptional profile of the Mediator complex across human cancer types. *Oncotarget* **7**, 23043-23055.

Thompson, C. M., Young, R. A. (1995) General requirement for RNA polymerase II holoenzymes in vivo. *Proc. Natl. Acad. Sci. USA* **92**, 4587-4590.

Tsai, K. L., Tomomori-Sato, C., Sato, S., Conaway, R. C., Conaway, J. W., Asturias, F. J. (2014) Subunit architecture

and functional modular rearrangements of the transcriptional mediator complex. *Cell* **157**, 1430-1444.

Tyanova, S., Temu, T., Sinitcyn, P., Carlson, A., Hein, M. Y., Geiger, T., Mann, M., Cox, J. (2016) The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nat. Methods* **13**, 731-740.

Verger, A., Monte, D., Villeret, V. (2019) Twenty years of Mediator complex structural studies. *Biochem. Soc. Trans*. **47**, 399-410.

Weed, S. A., Karginov, A. V., Schafer, D. A., Weaver, A. M., Kinley, A. W., Cooper, J. A., Parsons, J. T. (2000) Cortactin localization to sites of actin assembly in lamellipodia requires interactions with F-actin and the Arp2/3 complex. *J. Cell Biol.* **151**, 29-40.

Wiederhold, T., Lee, M. F., James, M., Neujahr, R., Smith, N., Murthy, A., Hartwig, J., Gusella, J. F., Ramesh, V. (2004) Magicin, a novel cytoskeletal protein associates with the NF2 tumor suppressor merlin and Grb2. *Oncogene* **23**, 8815-8825.