Brief Report

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Human mediator subunit MED15 promotes transcriptional activation

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Summary **In eukaryotes, the Mediator complex is an essential transcriptional cofactor of RNA polymerase II (Pol II). In humans, it contains up to 30 subunits and consists of four modules: head, middle, tail, and CDK/Cyclin. One of the subunits, MED15, is located in the tail module, and was initially identified as Gal11 in budding yeast, where it plays an essential role in the transcriptional regulation of galactose metabolism with the potent transcriptional activator Gal4. For this reason, we investigated the function of the human MED15 subunit (hMED15) in transcriptional activation. First, we measured the effect of hMED15 knockdown on cell growth in HeLa cells. The growth rate was greatly reduced. By immunostaining, we observed the colocalization of hMED15 with the general transcription factors TFIIE and TFIIH in the nucleus. We measured the effects of siRNA-mediated knockdown of hMED15 on transcriptional activation using two different transcriptional activators, VP16 and SREBP1a. Treatment with siRNAs reduced transcriptional activation, and this reduction could be rescued by overexpression of HA/Flag-tagged, wild-type hMED15. To investigate hMED15 localization, we treated human MCF-7 cells with the MDM2 inhibitor Nutlin-3, thus inducing p21 transcription. We found that hMED15 localized to both the p53 binding site and the p21 promoter region, along with TFIIE and TFIIH. These results indicate that hMED15 promotes transcriptional activation.**

Keywords: Mediator complex, transcriptional activation, MED15, RNA polymerase II, TFIIE, TFIIH

1. Introduction

The Mediator complex (Mediator) is a transcriptional cofactor that is highly conserved among eukaryotes, has up to 30 subunits, and consists of four modules: head, middle, tail, and CDK/cyclin (*1-3*). Of these, the tail module has been the least extensively studied to date. The tail module, which recruits various transcriptional regulators to the transcription machinery, consists of eight subunits: MED2 (also known as MED29), MED3 (also known as MED27), MED5 (also known as MED24), MED14, MED15, MED16, MED23, and MED25 (*1,4*). One of the subunits, MED15, was originally identified as Gal11 in the yeast *Saccharomyces cerevisiae*. There, it functions in galactose metabolism in conjunction with the acidic transcriptional activator Gal4 and in amino acid and vitamine biosynthesis in conjunction with the other acidic transcriptional activator Gcn4 (*5,6*).

Human MED15 (hMED15, also called ARC105) consists of 788 amino acids and contains a KIX domain in the N-terminus along with a glutamine (Q)-repeat region in the middle (*7*). The KIX domain of hMED15 binds the sterol regulatory element binding protein (SREBP), allowing hMED15 to regulate cholesterol and fatty acid homeostasis (*7*). The KIX domain was initially found in the CREB-binding protein (CBP)/p300 (*8*). Whereas the CBP KIX domain binds SREBP, CREB, and c-Myb activators, the hMED15 KIX binds only SREBP, but not CREB or c-Myb (*7*). Thus,

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although their structures share a striking resemblance, their binding specificities differ (*7,9,10*).

In eukaryotes, the expression of protein-coding genes is strictly regulated at the level of transcription by RNA polymerase II (Pol II) (*11-13*). Pol II requires five general transcription factors, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH. TFIIE consists of two subunits, α and β, and functions in transcription initiation, as well as in the transition from initiation to elongation (*14*). Human TFIIH (hTFIIH) consists of ten subunits (XPB, XPD, p62, p52, p44, p34, Cdk7, Cyclin H, MAT1, and p8), which possess multiple catalytic activities that are required not only for transcription by Pol II but also for nucleotide excision repair (NER) (*15*). Human TFIIE (hTFIIE) recruits hTFIIH through the interaction between the acidic domain of hTFIIEα and the pleckstrin-homology domain of hp62. This may be an essential step for Pol II activation upon its C-terminal domain (CTD) phosphorylation (*16*).

Since yeast MED15 (Gal11) binds TFIIE (*17*), it is possible that hMED15 carries out some essential roles by interacting with hTFIIE. Thus, we studied the function of hMED15 in transcriptional activation. For the siRNA knockdown of hMED15, we designed three different siRNAs, all of which reduced hMED15 expression at the protein level. Knockdown of hMED15 caused slow growth and reduced transcriptional activation. With immunofluorescence microscopy, we observed hMED15 primarily in the HeLa cell nucleus, mostly co-localized with hTFIIE and hTFIIH. We investigated the localization of hMED15, TFIIE, and TFIIH on the p53 target gene *p21* by chromatin immunoprecipitation followed by quantitative PCR (ChIP-qPCR); these experiments revealed that all three proteins primarily localized to both the upstream p53 binding site (p53BS1) and the core promoter region. These results suggest that hMED15 promotes transcriptional activation in collaboration with the general transcription factors TFIIE and TFIIH.

2. Materials and Methods

2.1. *Plasmids*

Full-length human Med15 cDNA (transcript variant 2, NCBI Accession No. NM_015889) was cloned into the pOTB7 mammalian expression vector (Life Technologies Japan). The VP16 (amino acids 413–490) and SREBP1a (amino acids 1–50) activation domains were fused to the Gal4 N-terminal DNA binding domain (amino acids 1–147) and cloned into the pM mammalian expression vector (Clontech Laboratories, Mountain View, CA, USA).

2.2. *Antibodies*

Anti-hMED15 (11566-1-AP, Proteintech group, Chicago, IL, USA), anti-hTFIIEα (C-17, Santa Cruz Biotechnology, Dallas, Tx, USA), anti-p62 (Q-19, Santa Cruz Biotechnology), and anti-Pol II (N-20, Santa Cruz Biotechnology) rabbit polyclonal antibodies were used. Goat anti-rabbit IgG polyclonal antibody (AP132, Chemicon Interantional Inc., Billerica, MA, USA) was used as a secondary antibody.

2.3. *siRNAs*

Three siRNAs were used for siRNA transfection. The siRNA sequences that were synthesized by Invitrogen are shown below.

Non-target siRNA:
sense strand: 5'-AUUCUAUCACUAGCGUGACUU-3'
antisense strand: 5'-GUCACGCUAGUGAUAGAAUUU-3'

hMED15-191:
sense strand: 5'-AACAUGGCUCUCCAUAUCCUUGCUG-3'
antisense strand: 5'-CAGCAAGGAUAUGGAGAGCCAUGUU-3'

hMED15-304:
sense strand: 5'-UUCAUAGGAUCACUGACGGAAGCUU-3'
antisense strand: 5'-AAGCUUCCGUCAGUGAUCCUAUGAA-3'

hMED15-1558:
sense strand: 5'-UUCGACAGCUGCUUCAGCUUGUCCA-3'
antisense strand: 5'-UGGACAAGCUGAAGCAGCUGUCGAA-3'

2.4. *Luciferase reporter assay*

HeLa S3 and MCF-7 cells were cultured at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM), supplemented with 7 mg/mL of penicillin-streptomycin, 5% calf serum, and 29.2 mM glutamine.

2.5. *Cell culture*

HeLa S3 cells were seeded in 24-well plates at a density of 4 x 10^4 cells/well. After 1 day, siRNA oligos (final 12nM) were transfected into cells using Lipofectamine 2000 (Life Technologies Japan, Tokyo, Japan). Cells were cultured for 2.5 days, and, after washing with PBS, were cotransfected with 100ng of pE1b-TATA-luciferase reporter plasmid, 0.5ng of pRL-TK (with *Renilla* luciferase used as an internal control), and 0.5 ng of VP16-pM or 10 ng of SREBP1a-pM. After 1 day, the cells were lysed and their transcription activities were quantitated using a PicaGene Dual SeaPansy Luminescence kit (Wako Pure Chemical Industries, Osaka, Japan).

2.6. *Immunofluorescence*

To examine the subcellular colocalization of hMed15, hTFIIEβ, and hCDK7, HeLa S3 cells were seeded on coverslips and were transfected with HA/Flag-Med15-pIRESneoII using Lipofectamine 2000. The cells were then double-immunostained with anti-hTFIIEβ or hCDK7 rabbit polyclonal antibody, and anti-HA mouse

monoclonal antibody (12CA5). Axela Fluor 488 Goat anti-rabbit IgG and Axela Fluor 555 Goat anti-mouse IgG (Life Technologies Japan, Tokyo, Japan) were used as secondary antibodies. For the immunofluorescence analysis, confocal laser microscope, LSM 700 (Zeiss, Jena, Germany), was used to examine the intracellular localization of the red and green fluorescence.

2.7. *IChromatin immunoprecipitation (ChIP) and reverse-transcription quantitative polymerase chain reaction (RT-qPCR)*

MCF-7 cells grown in 10 cm dishes to 80–90% confluence were fixed in 1% formaldehyde for 10 min at room temperature. The cross-linking reaction was stopped by addition of 125 mM glycine, and cells were incubated for 5 min at room temperature before being washed with PBS. Fixed cells were collected in a 1.5 mL tube by scraping, and then 300 µL of lysis buffer (50 mM Tris•HCl [pH 8.1], 10 mM EDTA, and 1% SDS) was added to each tube. Cells were completely disrupted by chromatin shearing using a Bioruptor sonication device (UCD-250, Cosmo Bio, Tokyo, Japan) at 200W for 25–30 cycles (30 sec on, 30 sec off). Cell lysates were centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was diluted 10-fold with dilution buffer (16.7 mM Tris•HCl [pH 8.1], 167 mM NaCl, 1.2 mM EDTA, and 1.1% Triton X-100), then incubated overnight at 4°C with 2 µg of the indicated antibodies. Forty microliters of protein G Dynabeads were suspended in Dynabeads blocking buffer (10 mM Tris•HCl [pH 7.5], 1 mM EDTA, 1 mg/mL BSA, and 0.4 mg/mL salmon sperm DNA) and incubated for 3h at 4°C. The beads were then washed with 1 mL of low-salt buffer (20 mM Tris•HCl [pH 8.1], 150 mM NaCl, 2 mM EDTA, 1% TritonX-100, and 0.1% SDS), 1 mL of high-salt buffer (20 mM Tris•HCl [pH 8.1], 500 mM NaCl, 2 mM EDTA, 1% TritonX-100, and 0.1% SDS), 1 mL of LiCl buffer (10 mM Tris•HCl [pH8.1], 250 mM LiCl, 1 mM EDTA, 1% NP-40, and 1% sodium deoxycholate), and 1 mL of TEN buffer (16 mM Tris•HCl [pH 7.5], 1 mM EDTA, and 0.5% NP-40). After washing, immunocomplexes were eluted by incubation in 100 µL of elution buffer (1% SDS and 100 mM $NaHCO_3$) for 1 h at room temperature, and the eluate was collected. Cross-links were reversed by overnight incubation at 65°C. Next, the eluate was treated with RNaseA for 1 h at 37°C, followed by treatment with Proteinase K for 2 h at 37°C. For qPCR, DNA templates were purified using the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA, USA), and the purified DNA was quantified using SYBR® *Premix Ex* Taq™ II (TaKaRa, Otsu, Japan) on an Mx3000P QPCR system (Agilent Technologies, Santa Clara, CA, USA). Total RNA (350 ng) purified from siRNA-transfected cells using the Nucleo Spin RNA II kit (TaKaRa) was subjected to reverse transcription (RT) using PrimeScript™ RT Master Mix (TaKaRa). Synthesized cDNA was quantitated using SYBR® *Premix Ex* Taq™ II (TaKaRa) on an Mx3000P QPCR system (Agilent Technologies).

2.8. *Primer sets for qPCR*

The following primer sets were used in qPCR analyses of the $p21^{WAF1}$ gene:

Forward Primers

-2283	5'-AGCAGGCTGTGGCTCTGATT-3'
-20	5'-TATATCAGGGCCGCGCTG-3'
+4001	5'-AGTCACTCAGCCCTGGAGTCAA-3'

Reverse Primers

-2283	5'-CAAAATAGCCACCAGCCTCTTCT-3'
-20	5'-GGCTCCACAAGGTGACTTC-3'
+4001	5'-GGAGAGTGAGTTTGCCCATGA-3'

3. Results and Discussion

3.1. *Knockdown of hMED15 reduces cell growth rate*

Yeast MED15 (yMED15) was initially called Gal11 in *Saccharomyces cerevisiae* (*5*). yMED15 involves in galactose metabolism in tight collaboration with yeast transcriptional activator GAL4 and its deficiency causes slow growth (*12*). Since MED15 was determined to be one of the Mediator subunit conserved among eukaryotes, we could easily imagine that hMED15 plays essential roles in human. Therefore, to study the hMED15 functions using human living cells, three siRNAs (hMED15-191, hMED15-304, and hMED15-1558) were designed to knockdown its expression (Figure 1A). Treatment of HeLa cells with each siRNA clearly knocked down hMED15 expression at the protein level (Figure 1B). Among three, siRNA

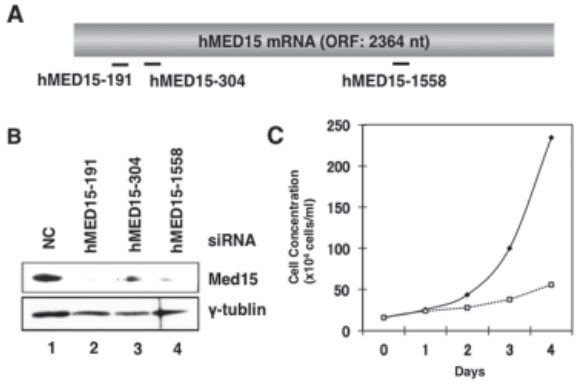

Figure 1. Effects of siRNA-mediated knockdown of hMED15 on HeLa S3 cell growth. (A) The positions of sequences targeted by three different hMED15 siRNAs (hMED15-191, hMED15-304, and hMED15-1558) are indicated on the hMED15 mRNA. **(B)** The effects of siRNA-mediated knockdown on hMED15 protein expression. Western blot analyses of hMED15 and γ-tubulin (used as a control) levels after siRNA treatment. NC: Non-targeting control siRNA. **(C)** The effects of siRNA-mediated knockdown on cell growth. HeLa S3 cells were treated with siRNA and cell growth was monitored for 4 days.

hMED15-191 showed the best knockdown efficiency and thus was used for further studies.

Since yMED15 deletion causes slow growth, the effects of hMED15 knockdown on HeLa cell growth were tested (Figure 1C). As expected, the growth rate was reduced to one fifth of the non-target siRNA treated cells. It will be easily imagined that deletion of hMED15 may cause reduction of the galactose metabolism because yMED15 (GAL4) has been demonstrated to be involved that. And this may cause the reduction of energy production in cells and, ultimately, may reduce the growth rate.

3.2. *Knockdown of hMED15 reduced Mediator-dependent transcription activation*

In yeast, yMED15 assists the transcriptional activation activity of GAL4. We investigated the effects of hMED15 on the activity of two different transcription activators (Gal4-VP16 and Gal4-SREBP1a) in HeLa cells (Figure 2). After the knockdown of hMED15 using hMED15-191 siRNA, transcription was drastically reduced (lane 2 versus lane 4 in Figures 2A and 2B). Expressing increasing amounts of hMED15 restored transcription (lane 4 versus lanes 6 and 8 in Figures 2A and 2B). It is worth noting that we used Gal4-VP16, a transcription factor with no direct interaction with hMED15, and Gal4-SREBP1a, a transcription factor that does interact. These results suggest that hMED15 promotes transcriptional activation.

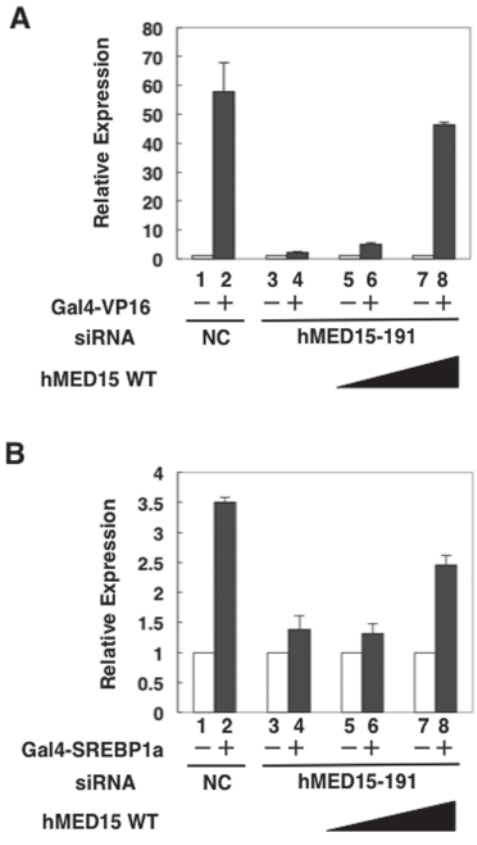

Figure 2. Effects of siRNA-mediated knockdown of hMED15 on transcriptional activation. (A) The effects of siRNA-mediated knockdown on transcriptional activation by Gal4-VP16. Lanes 3–8: siRNA hMED15-191 was introduced in HeLa S3 cells and to knockdown hMED15. Lanes 5–8: hMED15 wild-type was overexpressed in increasing amounts. **(B)** The effects of siRNA-mediated knockdown on transcriptional activation by Gal4-SREBP1a. Lanes 3–8: siRNA hMED15-191 was used for knockdown. Lanes 5–8: hMED15 wild-type was overexpressed in increasing amounts.

3.3. *hMED15 colocalizes with TFIIE and TFIIH in the nucleus*

Because our results suggest hMED15 functions in transcriptional activation, we next tested whether hMED15 colocalizes with the general transcription factors TFIIE and TFIIH (Figure 3). HeLa S3 cells were immunostained with antibodies against hMED15 and hTFIIEβ (Figure 3A). As Figure 3 shows, hMED15 was localized in the nucleoplasm (Figures 3A, left panels), while hTFIIEβ was both in the nucleoplasm and on the nuclear envelope (Figure 3A, second panel). The similar localization was observed for hCDK7 subunit of TFIIH (Figure 3B, second panel). These results suggest that hMED15 (and the Mediator complex that contains it) promotes transcriptional activation in the nucleoplasm together with the general transcription factors TFIIE and TFIIH.

3.4. *hMED15 colocalizes with TFIIE and TFIIH at the p53 binding site and the promoter region of the p21 gene*

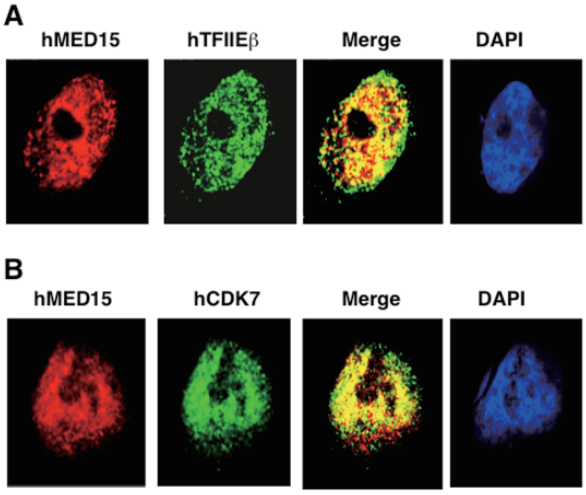

Figure 3. Localization of hMED15, hTFIIE, and hTFIIH in HeLa S3 cells. (A) Immunostaining of cellular hMED15 and the hTFIIEβ subunit of hTFIIE. hMED15 was stained in red and hTFIIEβ was stained in green, using Alexa-Fluor 594- or Alexa-Fluor 488-conjugated secondary antibodies, respectively. Those stained cell pictures were merged. Nuclei were stained with DAPI (shown in dark blue). **(B)** Immunostaining of cellular hMED15 and the hCDK7 subunit of hTFIIH. hMED15 was stained in red and hCDK7 was stained in green, using Alexa-Fluor 594- or Alexa-Fluor 488-conjugated secondary antibodies, respectively. Those stained cell pictures were merged. Nuclei were stained with DAPI (shown in dark blue).

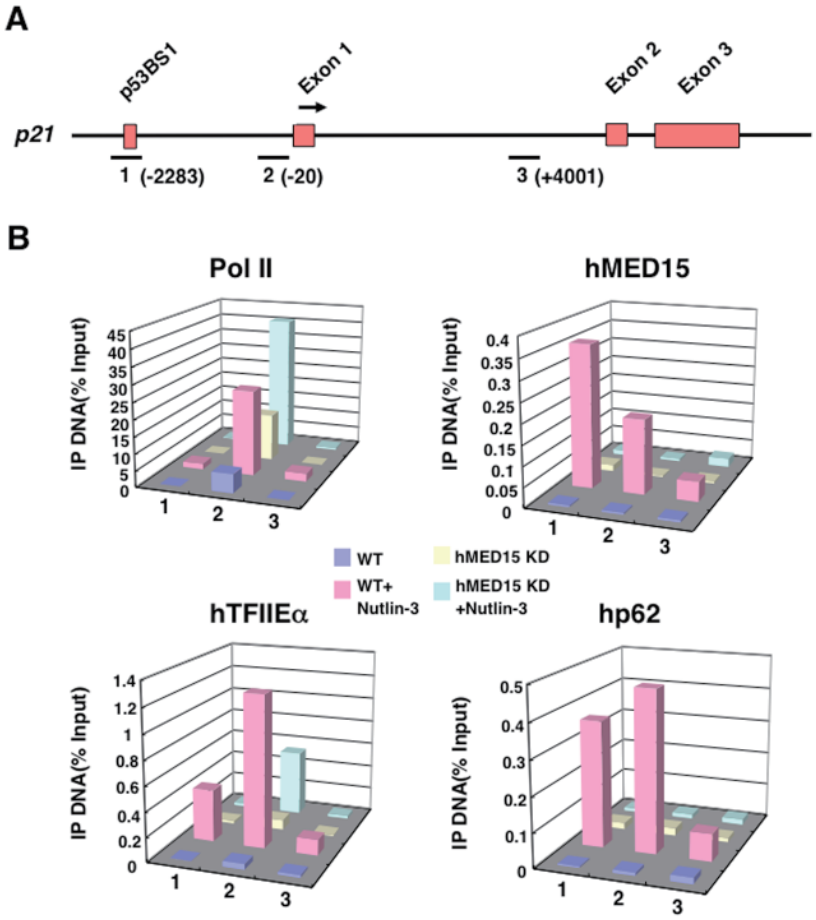

Figure 4. Localization of Pol II, hMED15, hTFIIE, and hTFIIH on the *p21* gene locus. (A) *p21* gene locus is illustrated with the positions of the p53 binding site (p53BS1), the exons (Exon 1–3), the transcription start site (arrow on Exon 1), and the positions of primer sets for qPCR. **(B)** ChIP analyses of Pol II, hMED15, hTFIIEα, and the hp62 subunit of hTFIIH on the *p21* gene. After a ChIP assay with the antibodies of four proteins, real-time quantitative PCR (RT-qPCR) was performed to measure the amount of each factor bound to the three primer sites (1; -2283, 2; -20, and 3; +4001). Assay was repeated three times. Each amount is shown as percent input (total immunoprecipitated DNA amount is defined as 100%).

To study the functional association of hMED15 with two general transcription factors, TFIIE and TFIIH, we used the human cyclin-dependent kinase inhibitor *p21*, which is dependent on p53 for transcriptional activation (Figure 4). When MCF-7 cells are treated with the MDM2 inhibitor Nutlin-3, p53 binds to the p53-binding site (p53BS1) and stimulates *p21* transcription. Figure 4A illustrates the *p21* gene locus structure and the positions of the primer sets used for qPCR. The Nutlin-3 treatment recruited hMED15, hTFIIEα, and the hp62 subunit of TFIIH to p53BS1 (position 1) and the promoter region (position 2). Pol II was recruited only to the promoter region (position 2). Knockdown of hMED15 abolished the binding of hMED15, hTFIIEα, and hp62 to those sites, whereas Pol II remained bound, although the amount was slightly reduced. When hMED15 was knocked down during Nutlin-3 treatment, the binding of Pol II to the promoter increased, and a smaller amount of hTFIIEα bound there. These results demonstrate that, during transcriptional activation of *p21*, hMED15 colocalizes with TFIIE and TFIIH at the upstream p53-binding site and the promoter region. Given MED15's established role in yeast transcription activation, as well as its effects on both the *p21* gene and the genome overall, we believe this study provides compelling evidence that hMED15 plays a key role in transcription activation.

Recently, we extensively studied the human Mediator head module subunit hMED17 (Kikuchi and Ohkuma, in preparation). This hMED17 also binds to the general transcription factors and functions positively in transcriptional activation. We assume that the Mediator tail subunit hMED15 and hMED17 function collaboratively in response to the transcriptional activator. These two subunits may evoke drastic conformational change of the whole Mediator complex upon transcriptional activator binding. We will study this mutual association in the near future.

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