MINT REPRESSES TRANSACTIVATION OF THE TYPE II COLLAGEN GENE ENHANCER THROUGH INTERACTION WITH αA-CRYSTALLIN-BINDING PROTEIN 1*
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Running title: MINT represses Col2a1 through interaction with CRYBP1
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Collagen type II is an extracellular matrix protein important for cartilage and bone formation, and its expression is controlled by multiple cis- and trans-acting elements including the zinc finger transcription factor αA-crystallin-binding protein 1 (CRYBP1). Here we show that MSX2-interacting nuclear target protein (MINT), a conserved transcriptional repressor, associates with CRYBP1 and negatively regulates the transactivation of the collagen type II gene (Col2a1) enhancer. We identified CRYBP1 as a binding partner of MINT by screening a mouse embryonic cDNA library using the yeast two hybrid system. We demonstrated that the C terminus of MINT interacts with the C terminus of CRYBP1 using the mammalian cell two hybrid assay, GST pull-down, and coimmunoprecipitation analyses. Furthermore, MINT and CRYBP1 form a complex on the Col2a1 enhancer, as shown by chromatin immunoprecipitation and gel shift assays. In the presence of CRYBP1, overexpression of MINT or its C terminal fragment in cells repressed a reporter construct driven by the Col2a1 enhancer elements. This transcription repression is dependent on HDAC, the main co-repressor recruited by MINT. The present study shows that MINT is involved in CRYBP1-mediated Col2a1 gene repression, and may play a role in regulation of cartilage development.

Cartilage is a translucent dense connective tissue functioning in both adult body and embryos where it acts as a model for endochondral ossification. There are essentially three types of cartilages, namely, the hyaline cartilage, the white fibrocartilage, and the yellow or elastic cartilage. Collagen type II is the predominant extracellular matrix (ECM) component in the hyaline cartilage. The collagen type II gene (Col2a1) is expressed primarily by proliferating chondrocytes but not by hypertrophic chondrocytes (1). Mutations in the human Col2a1 causes skeletal abnormalities characterized by changes in ECM structure and morphology of cartilage and the growth plate, demonstrating the importance of proper expression of Col2a1 for the
formation and integrity of these structures (2).

Transcriptional regulation of Col2a1 is mediated by cis-acting tissue-specific regulatory elements located within the promoter and the first intron enhancer, and trans-acting factors recruited to the promoter and the enhancer (3, 4). Using transgenic mice as well as cultured cells, it has been demonstrated that, within the first intron of the Col2a1 gene, a 48-bp segment containing three repeats of a consensus sequence recognized by high mobility group (HMG)-domain proteins is the minimal sequence sufficient for high-level and cell type-specific expression of Col2a1 in chondrocytes (5-9). Several transcription factors including SOX9 (Sry-type high-mobility-group box) have been shown to bind to these HMG-like motifs and transactivate Col2a1 expression (10, 11). SOX9 is a HMG domain transcription factor that is expressed in all cartilage primordia and cartilages during embryonic development, coincident with the expression of Col2a1 (12-14). In chondrocytes as well as non-chondrocytic cells such as fibroblasts, SOX9 binds to and transactivates the Col2a1 enhancer and a number of other chondrocyte-specific enhancers, supporting that SOX9 may work as a master gene for chondrogenesis (11, 15, 16). Mutations in the SOX9 gene cause campomelic dysplasia (CD), a severe dwarfism syndrome affecting all cartilage-derived structures (17-20). In addition to SOX9, two other HMG domain transcription factors, SOX5L and SOX6L, are also implicated in regulation of Col2a1 expression and chondrogenesis (23).

Alpha A-crystallin-binding protein 1 (CRYBP1), a ubiquitously expressed zinc finger DNA-binding protein, has been identified as a negative regulator of Col2a1 expression (24, 25). CRYBP1 has two sets of C2-H2 type zinc finger domains located in the amino and carboxyl termini, respectively, and was initially identified by its ability to interact with a functionally important sequence in the mouse alpha A-crystallin gene promoter (24, 26). Homologs of the mouse CRYBP1 have been identified in Drosophila (Schnurri) (27), C. elegans (SEM-4) (28), rat (AT-BP2) (29), and human (PRDII-BF1/MBP1/HIV-EP1) (30, 31), suggesting a conserved role of CRYBP1 through development. The full length CRYBP1 gene encodes a 300 kD protein. However, alternatively spliced mRNA also generates truncated molecules including a 200 kD N terminal fragment, and 68 kD, 50 kD, and 90 kD fragments containing the C terminal zinc finger (32, 33). The function of these molecules are elusive, but Tanaka et al (25) reported that expression of a C terminal fragment (2023-2688) of CRYBP1 in NIH3T3 or a rat chondrosarcoma cell line inhibits Col2a1 enhancer, which is transactivated by SOX9. Electrophoretic mobility shift assays (EMSA) showed that CRYBP1 binds to a specific sequence within the Col2a1 enhancer and inhibits the binding of SOX9 to the enhancer. Recently, Yamagiwa et al
provided further evidence to support that CRYBP1 binds to the Col2a1 enhancer and represses its transactivation (34). However, the molecular mechanism underlying CRYBP1-mediated repression of Col2a1 has not yet been elucidated.

MINT (MSX2-interacting nuclear target protein) is a nuclear matrix protein originally cloned as an interacting protein of MSX2, a homeodomain transcription repressor functioning in the craniofacial skeletal and neural development (35). MINT belongs to the Spen (split end) protein family which plays an essential role in multiple developmental events (36-38). Spen proteins vary in a wide range in size (90-600 kD), but are nevertheless characterized by a conserved domain structure, including three repeated RNA recognition motifs (RRMs) near the N terminus and a conserved SPOC (Spen paralog and ortholog C-terminal domain) domain at the C terminus, which mediates interaction with the SMRT/NcoR corepressors (39). The human homolog of MINT, SHARP, has been identified as a component in transcriptional repression complexes recruited by nuclear receptors (40). The MINT/SHARP-mediated repression was sensitive to the HDAC inhibitor TSA, and SHARP is a novel component of the HDAC corepressor complex, suggesting that MINT/SHARP represses transcription in an HDAC-dependent fashion (40). Recently, Kuroda et al and Oswald et al demonstrated that MINT also interacts with RBP-J, a key transcription factor downstream of Notch receptor, and represses the RBP-J-mediated transactivation by competing for the binding site and by recruiting co-repressors including SMRT/N-CoR and HDAC, and may thus regulate the Notch signaling pathway (41, 42). Kuroda et al also showed that targeted disruption of MINT lead to embryonic lethality, with a developmental retardation of multiple organs [42]. However, as a widely expressed protein, possible functions of MINT in other systems remain to be revealed.

In the present study, we identify CRYBP1 as a MINT-interacting protein by yeast two hybrid screening. We show that MINT physically interacts with CRYBP1 in vitro and vivo. Chromatin immunoprecipitation (CHIP) and EMSA showed that MINT and CRYBP1 forms a complex with a specific sequence within the Col2a1 enhancer. In cotransfection experiments, MINT or its C terminal fragment represses the transactivation of Col2a1 enhancer through association with the C terminus of CRYBP1 in an HDAC-dependent fashion. Our data suggest that MINT take part in CRYBP1-mediated transcription repression of Col2a1 by recruiting HDAC, and may therefore regulate cartilage development.

EXPERIMENTAL PROCEDURES

Plasmids - Bait plasmids for the yeast two hybrid assay were constructed by inserting different fragments of the full length MINT cDNA (generously provided by T. Honjo) into pGBK7T (Clontech, Palo Alto, CA) (pGBK-MINTF1-F6, amino acids 1-364, 365-1016, 1017-1663, 1664-2225, 2226-2959, 2960-3576, respectively). MINTF6 was further truncated into two segments and inserted into pGBK7T.
(pGBK-MINTF6-N, amino acids 2960-3214, and pGBK-MINTF6-C, amino acids 3215-3576). Prey plasmids containing the C terminus of CRYBP1 (CRYBP1-C, amino acid 2018-2688, see RESULTS) were generated by cloning CRYBP1-C into pGADT7 (Clontech, Palo Alto, CA) (pGADT7-CRYBP1-C). This fragment was further divided into two fragments and inserted into pGADT7 (pGADT7-CRYBP1-C1, amino acids 2018-2469, and pGADT7-CRYBP1-C2, amino acids 2470-2688).

Plasmids for the mammalian two hybrid assay were generated by subcloning MINTF6 and MINTF6-C fragments into pCMX-GAL4DBD (pCMX-GAL4DBD-MINTF6 and pCMX-GAL4DBD-MINTF6-C, respectively). CRYBP1-C was inserted into pCMX-VP16(NLS) to construct the expression vector pCMX-VP16-CRYBP1-C.

Expression vectors pCMV-Myc-MINTF6, pCMV-Flag-CRYBP1-C, and pCMV-HA-CRYBP1-C expressing the Myc-tagged MINTF6, Flag-tagged CRYBP1-C and HA-tagged CRYBP1, respectively, were generated by subcloning MINTF6 and CRYBP1-C into pCMV-Myc, pCMV-Flag2 and pCMV-HA vectors separately. pCMV-Myc-Luc construct was described previously (43). Prokaryotic vectors expressing His-Trx-tagged MINTF6-C (His-Trx-MINTF6-C) and GST-tagged CRYBP1-C (GST-CRYBP1-C) fusion proteins were constructed by inserting MINTF6-C and CRYBP1-C into pET32a (pET-MINTF6-C) and pGEX4T-2 (pGEX-CRYBP1-C), respectively.

The mouse SOX9 was cloned by PCR with a mouse embryonic cDNA library as a template, using primers 5′-tcagggctcagtagctgctgatagac-3′ and 5′-agatcctgtgacaccccttcatgaag-3′. The amplified fragment was subcloned by T-cloning using pMD-18T (Takara, Dalian, China) and sequenced, and inserted into pCMV-Flag2 to construct the expression vector pCMV-Flag-SOX9.

The reporter construct for the Col2a1 enhancer (pGL3-Col2α1) was generated according to Tanaka et al (25). Three repeats of the core sequence of the Col2a1 enhancer (5′-tgtatgcctgtgagacentcccttcga-3′) was synthesized and inserted into pGL3-promoter (Promega Co., Madison, WI). TK MH100 × 4 Luc used in the mammalian two hybrid assay was described previously (44). All the newly constructed plasmids were confirmed by sequencing.

Yeast Two Hybrid Assay - To screen a cDNA library using the yeast two hybrid system, the bait plasmid (pGBK-MINTF6) was used to co-transform the yeast strain AH109 with a mouse embryonic cDNA library constructed in pAD-GAL4-2.1 (Stratagene) using the LiAc method. The resulting yeast clones were plated on SD/-trp-leu plates, and then selected on SD/-trp-leu-his-ade plates. Single colonies were subjected to membrane β-gal assay. Plasmid DNA was recovered from positive clones and sequenced after amplification in E. coli.

To test protein-protein interaction in yeast, bait and prey plasmids were co-transformed into yeast AH109 and plated on SD/-trp-leu-his plates. Grown colonies were tested for nutritional phenotypes. Positive clones were
transferred onto nitrocellulose membrane and lysed by repeated freeze and thaw in liquid nitrogen. The membrane was incubated at 37°C with X-gal to test the activity of β-galactosidase.

**Cell Culture and Transfection** - The human embryonic kidney (HEK) 293, 293T, NIH3T3, and COS7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM), containing 10% fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin, in 5% CO2 at 37°C. Cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA), according to the manufacturer's instructions. Transfection was allowed to proceed for 6 h, and DMEM containing 20% FCS was added. Cells were collected for further experiments later.

**GST Pull-down Assay** - His-Trx-MINTF6-C and GST-CRYBP1-C fusion proteins were produced in E. coli using pET-MINTF6-C and pGEX-CRYBP1-C, respectively. Fusion proteins as well as control proteins (His-Trx and GST) were purified by the Ni2+-chelating resin (Invitrogen) or glutathione-Sepharose 4B beads (Pharmacia), respectively, following the supplier’s protocols. GST pull down assay was performed as described previously (43).

**Co-immunoprecipitation (IP)** - Cells (293T) cultured in 6 cm dishes were co-transfected with 5 µg of pCMV-Myc-MINTF6 and pCMV-Flag-CRYBP1-C, with pCMV-Luc-Myc expressing a Myc-tagged luciferase as a negative control. Cell lysates were prepared 60 h after the transfection, and immunoprecipitation was carried out as described in (43).

**Chromatin Immunoprecipitation (CHIP)** - NIH3T3 cells were transfected with plasmids for 48 h, and fixed by addition of formaldehyde to 1%. Cells were incubated at 37°C for 10 min, washed twice with ice cold PBS containing 1 mM PMSF, 1 µg/ml aprotinin, and 1 µg/ml pepstatin A, and then collected by centrifugation after scraping. Cells were resuspended in an SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH8.1), and disrupted by sonication. Cells were centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was collected and diluted in a CHIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 2 mM EDTA, 167 mM NaCl, 16.7 mM Tris-HCl, pH8.1) containing protease inhibitors as above. Chromatin was pre-cleared with the salmon sperm DNA-coated protein A-agarose, followed by immunoprecipitation using 5 µg of anti-Flag or anti-Myc antibodies overnight at 4°C with rotation. Antibody complexes were collected with the salmon sperm DNA-coated protein A-agarose, followed by extensive washing with the CHIP dilution buffer. Precipitated CHIP complexes were reverse cross-linked by incubation at 65°C for 4 h. The samples were then treated with proteinase K for 2 h at 42°C and the precipitated DNA was amplified by PCR using primers (5’-agagctctgtatgcgcttgaga-3’ and 5’-aagatctcatgaatggggcttt-3’). Preimmune serum was used as a negative control.

**EMSA** - NIH3T3 cells were transfected with pCMV-Flag-CRYBP1-C and/or pEF-BOS-MINT, and collected 48 h after transfection. Nuclear extracts were
preparing by resuspending cells \((1 \times 10^7)\) in buffer A \((10 \text{ mM HEPES, pH 7.8, 10 mM KCl, 0.1 mM EDTA, and 0.1% Nonidet P-40})\) containing protease inhibitors, incubating on ice for 10 min, and homogenizing. After centrifugation at 12,000 rpm for 5 min, cell pellets were resuspended in buffer B \((50 \text{ mM HEPES, pH 7.8, 420 mM KCl, 5 mM MgCl}_2, 0.1 \text{ mM EDTA, and 20% glycerol})\) and rotated at 4°C for 2 h. Supernatants with nuclear proteins were recovered by centrifugation at 12,000 rpm for 30 min. The \(Col2a1\) enhancer probe was generated by PCR using primers \(5'-\text{aagatctcatgaatggggcttt}-3'\) and \(5'-\text{cagatctcacagaatggaggaa}-3'\), and labeled with \([\gamma-^{32}\text{P}]-\text{ATP}\) using T4 polynucleotide kinase. Nuclear proteins \((3 \mu\text{g})\) were incubated with 35 fmol of the labeled probe, and DNA-protein complexes were separated on 6% nondenaturing polyacrylamide gel in 0.5 x TBE with 2.5% glycerol. Gels were dried and subjected to autoradiography. For competition and antibody supershift, unlabeled probes or 2 μg of the anti-Flag or anti-Myc antibody was added to the reaction mixture for 1 h at room temperature before addition of the labeled probe.

**RESULTS**

**Identification of CRYBP1 as a Binding Protein of MINT Using the Yeast Two Hybrid Assay** - MINT is a conserved and ubiquitously expressed transcription repressor that has been implicated in regulation of transcription mediated by nuclear receptors (40), MSX2 (35, 44), and RBP-J (41, 42). To look at other functions of MINT, we tried to isolate other possible interacting proteins of MINT using the yeast two hybrid system. With the C terminal fragment of MINT \((\text{MINTF6, amino acids 2960-3576})\) as a bait, we picked up several potential MINT-interacting molecules. Upon DNA sequencing, one of them turned out to be the C terminal fragment of CRYBP1 \((\text{CRYBP1-C, amino acids 2018-2688})\), another clone was the C terminus of the SMRT/NcoR (data not shown), consistent with previous findings (39).

The isolated CRYBP1-C fragment was tested for interaction with other regions of MINT in yeast. The result
showed that CRYBP1-C only interacted with MINTF6 (Fig. 1). To further specify regions mediating the interaction between MINT and CRYBP1, we truncated MINTF6 into MINTF6-N (2906-3214) and MINTF6-C (3215-3576), and tested their interaction with CRYBP1-C in yeast. The result showed that while MINTF6-C retained the binding ability with CRYBP1-C, MINTF6-N did not. Moreover, CRYBP1-C was truncated into CRYBP1-C1 (2018-2469) and CRYBP1-C2 (2470-2688), and tested for interaction with MINTF6-C and its derivatives. The result showed that CRYBP1-C1 interacted with MINTF6-C (Fig. 1). Based on these observations, we concluded that the MINTF6-C (3215-3576) fragment of MINT and the CRYBP1-C1 (2018-2469) fragment of CRYBP1 might be responsible for the interaction between the two molecules in yeast.

**Interaction between MINT and CRYBP1**

To examine the interaction between MINTF6 and CRYBP1-C in mammalian cells, we performed mammalian two hybrid assay by co-transfecting pCMX-GAL4DBD-MINTF6 and pCMX-VP16-CRYBP1-C into COS-7 or HEK293 cells, together with a luciferase reporter plasmid driven by a promoter containing multiple GAL4-binding sites (TK MH100x4 Luc). Luciferase activity was examined in cell lysates 48 h after transfection. As show in Fig. 2A and B, while pCMX-GAL4DBD-MINTF6 or pCMX-VP16-CRYBP1-C alone did not induce reporter gene expression in cells, co-transfection with the two plasmids stimulated luciferase activity in a dose-dependent manner in both HEK 293 (Fig. 2A) and COS-7 (Fig. 2B) cells. Similarly, consistent with the results in yeast, pCMX-GAL4DBD-MINTF6-C and pCMX-VP16-CRYBP1-C showed positive interaction in HEK 293 and COS-7 cells after co-transfection (Fig. 2, C and D, respectively).

The physical interaction between MINTF6 and CRYBP1-C was further tested by co-immunoprecipitation. Cells (293T) were co-transfected with pCMV-Myc-MINTF6 and pCMV-Flag-CRYBP1-C, which express Myc-tagged MINTF6 and Flag-tagged CRYBP1-C proteins, respectively. Whole-cell extracts were prepared 60 h after transfection, and immunoprecipitated with the anti-Myc antibody, followed by immunoblotting with the anti-Flag antibody. The result showed that Flag-CRYBP1-C was co-immunoprecipitated with Myc-MINTF6, but not with Myc-luciferase that was used as a negative control (Fig. 3A). On the other hand, when cell lysates from co-transfected cells were immunoprecipitated with the anti-Flag antibody and blotted with the anti-Myc antibody, similar results were achieved as shown in Fig. 3B. These results suggested that MINT interacted with CRYBP1 in cells.

The direct physical interaction between MINTF6 and CRYBP1-C was verified in vitro by GST pull-down analysis. The His-Trx-MINTF6-C fusion protein and the His-Trx tag were expressed in E. coli and purified. The purified proteins were separately incubated with glutathione-Sepharose beads coated with purified GST-CRYBP1-C1 fusion protein or GST. Associated proteins were pulled down,
and detected by Western blotting with the anti-His antibody. The result (Fig. 3C) showed the MINTF6-C was pulled down by association with CRYBP1-C1. No interaction could be detected between GST protein and MINTF6-C, or between His-Trx and GST-CRYBP1-C1. The result further suggested that the C terminus of MINT could associate directly with the C terminus of CRYBP1.

**MINT Is Recruited by CRYBP1 to the Col2al Enhancer** - The CRYBP1-C fragment (2018-2688) cloned in this study is almost the same as the C terminus of CRYBP1 isolated by Tanaka et al (2023-2688), which has been shown to bind to and repress the Col2al enhancer in NIH3T3 cells (25). We have shown that the C terminus of MINT interacts with CRYBP1-C, CRYBP1-C thus might recruit MINT to the Col2al enhancer. We employed CHIP and EMSA experiments to examine this speculation. As shown in Fig. 4A, in cells transfected with pCMV-Flag-CRYBP1-C (lane 1-3), the Col2al enhancer fragment was immunoprecipitated with the anti-Flag antibody, but not with the preimmune serum, consistent with that CRYBP1-C associates with the Col2al enhancer (25). In cells co-transfected with pCMV-Flag-CRYBP1-C and pEFBOS-Myc-MINT (lane 4-6), the Col2al enhancer fragment was precipitated with both the anti-Flag and anti-Myc antibodies. These results suggested that MINT may be recruited to the Col2al enhancer through interaction with CRYBP1-C.

Formation of a complex by CRYBP1-C and MINT on the Col2al enhancer was further demonstrated by EMSA. Cells (NIH3T3) were transfected with pCMV-Flag-CRYBP1-C and/or pEFBOS-Myc-MINT, and nuclear extracts were prepared 48 h after transfection. A radio-labeled Col2al enhancer probe was incubated with the nuclear extracts, and DNA-protein complexes were analyzed with PAGE. As shown in Fig. 4B, a shifted band (lane 2, band 1) was detected with nuclear extracts from cells transfected with pCMV-Flag-CRYBP1-C. This band disappeared when competitive cold probes were included in the reaction (lane 3), and supershifted by addition of the anti-Flag antibody (lane 4, band 1'), demonstrating that the band was a specific complex formed by Flag-CRYBP1-C and the Col2al enhancer probe. When cell lysates from cells transfected with both pCMV-Flag-CRYBP1-C and pEFBOS-Myc-MINT were used, while the band 1 was visible (lane 5), another band was also detected (band 2). Both the two bands disappeared in the presence of specific cold competitor (lane 6). The band 2 was supershifted with the anti-Myc antibody (lane 7, band 2'), indicating that it was formed by the Col2al enhancer probe, Flag-CRYBP1, and Myc-MINT, because no shift was detected with only Myc-MINT (lane 8). These results further demonstrated that CRYBP1-C is associated with the Col2al enhancer, and MINT is recruited to the Col2al enhancer by CRYBP1-C.

**MINT Represses Transactivation of the Col2al Enhancer by Association with CRYBP1 and in a HDAC-dependent manner** - CRYBP1 is a DNA-binding protein and binds to the Col2al enhancer and represses the
transactivation of Col2a1. The C-terminal part of CRYBP1 was shown to be sufficient to bind to and repress the Col2a1 enhancer (25). Our data presented above have shown that CRYBP1-C could recruit MINT to the Col2a1 enhancer. As MINT has been proved to repress transcription (40), we hypothesized that MINT represses the Col2a1 enhancer by association with CRYBP1-C.

As a master gene for chondrocyte differentiation in vivo, SOX9 transactivates the Col2a1 enhancer not only in chondrocytes, but also in non-chondrocytic cells such as fibroblasts (11, 15, 16), and this transactivation is repressed by a C-terminal fragment of CRYBP1 (25). We therefore employed mouse fibroblast cell line NIH3T3 to investigate the role of MINT in regulation of the Col2a1 enhancer and its functional relationship with CRYBP1. We constructed a reporter plasmid (pGL-Col2α1) bearing three repeats of the core sequences of the Col2a1 enhancer, according to previous reports (25). pGL-Col2α1 was transfected into NIH3T3 cells with expressing vectors for SOX9, CRYBP1-C, and/or MINT. Transactivation of the reporter construct in NIH3T3 cells was strictly dependent on SOX9 and repressed by high level of CRYBP1-C (data not shown), consistent with previous reports (25). While low level of CRYBP1-C moderately down-regulated the transactivation of the reporter construct, with increasing amount of MINT, transactivation was repressed in a dose-dependent manner (Fig. 5A). Because in the absence of CRYBP1, MINT alone did not exhibit significant level of transcription repression of pGL-Col2α1 (data not shown), these results suggested that MINT repressed the Col2a1 enhancer by association with CRYBP1-C.

MINT interacts with HDACs (40), and repression of the transcription factor RBP-J by MINT is dependent on HDACs (42). We therefore tested whether transcriptional repression of the Col2a1 enhancer by MINT might depend on HDAC activity using TSA, a specific inhibitor of HDACs. Reporter assays were performed as described above using pGL-Col2α1, in the presence or absence of TSA, and the luciferase activity was examined. The data showed that the transcription repression mediated by CRYBP1-C and MINT was abrogated by TSA in a dose-dependent way (Fig. 5B). These results suggested that MINT repressed the Col2a1 enhancer through recruitment of HDACs.

**MINTF6 is Sufficient for Repression of the Col2a1 Enhancer** - The MINTF6 fragment accommodates a SPOC domain, which interacts with the universal transcription repressor SMRT/NcoR and HDACs (39, 40). Our results have demonstrated that the MINTF6 fragment also associates with CRYBP1. We therefore tested potential effects of MINTF6 on transactivation of the Col2a1 enhancer by SOX9 using the reporter assay. The expression plasmids pCMV-Myc-MINTF6 and pCMV-HA-CRYBP1-C were co-transfected into NIH 3T3 cells with pCMV-Flag-SOX9 and pGL-Col2a1, and luciferase activity in cell lysates was examined 60 h after transfection. The data showed that MINTF6 could repress the transcription of the reporter gene in the presence of CRYBP1-C (Fig. 6) in a
dose-dependent way. Similarly, repression of the transactivation of the \textit{Col2a1} enhancer by MINTF6 was abrogated by the addition of TSA in a dose-dependent manner (Fig. 6). These results indicate that MINTF6 is sufficient to repress the \textit{Col2a1} enhancer, and the repression is also dependent on its recruitment of HDACs.

DISCUSSION

MINT is a widely expressed transcription repressor, and is involved in the regulation of multiple transcription factors including nuclear receptors, the homeodomain transcription factor MSX2, and RBP-J downstream of Notch, through protein-protein interaction and recruiting HDACs (35, 39-43). MINT belongs to the Spen protein family, which is characterized by N terminal RRM domains and the C terminal SPOC domain (39). In this study, we identified CRYBP1 as a new binding protein of the transcription repressor MINT. The interaction between MINT and CRYBP1 was demonstrated both in vitro and in vivo. We show that MINT and CRYBP1 form a complex through their C termini, and bind to the enhancer of \textit{Col2a1} that encodes collagen type II, as revealed by the CHIP assay and the gel shift assay. In the presence of CRYBP1, MINT represses the \textit{Col2a1} enhancer transactivated by SOX9 in NIH3T3 cells. These results provide evidence that MINT participates in the regulation of \textit{Col2a1} gene expression. Moreover, we show that the repression of the \textit{Col2a1} enhancer by MINT is dependent on the activity of HDACs. We also found the C terminal fragment of MINT (MINTF6) is able to repress the \textit{Col2a1} enhancer transactivation. Because the C terminus of MINT could associate with CRYBP1 (this study), and recruit multiple HDACs, we propose that MINT works as a molecular scaffold to recruit HDACs to the \textit{Col2a1} enhancer by association with CRYBP1, which recognizes a sequence in the \textit{Col2a1} enhancer.

The collagen type II is an early and abundant marker of the chondrocyte, and its expression is dynamically regulated by multiple factors. \textit{Col2a1} is expressed in proliferating chondrocytes but not in hypertrophic chondrocytes (1). The chondrocyte-specific \textit{Col2a1} enhancer is located within the first intron of the \textit{Col2a1} gene, and is necessary and sufficient for tissue specific expression of \textit{Col2a1} (2, 3). Positive regulators of the \textit{Col2a1} gene enhancer have been well elucidated (2, 3). It has been well demonstrated that SOX9 (17-22), as well as SOX5L and SOX6L (23), are essential transcription factors responsible for the \textit{Col2a1} expression in chondrocytes. On the other hand, as a negative regulator of the \textit{Col2a1} enhancer, CRYBP1 was initially identified by its ability to recognize the sequence GGGAAATCCC in the promoter of the mouse $\alpha$A-crystallin (24). It was further proved that CRYBP1 is involved in transcriptional regulation of the $\alpha$A-crystallin promoter, as well as other promoters including the $\alpha$1-antitrypsin promoter, the interferon $\beta$ promoter, the MHC H2-K$^b$ gene promoter, and the HIV-1 viral enhancer (26-31). CRYBP1 possesses two C$_2$H$_2$-type zinc finger DNA binding domains, located at the N terminal and the C terminal ends of the molecule, respectively. In addition to the 300 kD
full length protein, alternative splicing of the primary transcript generated 200 kD and 68 kD molecules, which contain the N terminal zinc finger and the C terminal zinc finger, respectively. Moreover, molecules with molecular weight of 50 kD and 90 kD, and containing the C terminal zinc finger have also been found (32, 33). One C terminal fragment with the C terminal zinc finger domain was shown to repress the Col2a1 enhancer by Tanaka et al (25, 34). In the Col2a1 enhancer, CRYBP1 recognizes a sequence of GAGAAAGGCC, located 3’ to the SOX9 recognition sites. These authors further demonstrated that association of CRYBP1 with its recognition site in the Col2a1 enhancer excludes SOX9 from the enhancer, and represses the transactivation of the enhancer by SOX9. Our results reported in this work suggested a second mechanism of transcriptional repression of the Col2a1 enhancer by CRYBP1, namely, the recruitment of co-repressor such as HDACs by the interaction with MINT. The MINT-CRYBP1 repression complex may collaborate with other repression mechanisms, such as the Krab-KAP1-HP1 system reported by Ayyanathan et al (45), to regulate chondrocyte-specific gene expression and chondrocyte differentiation.

Chondrogenesis during embryonic development is initiated with the recruitment of mesenchymal chondroprogenitor cells. These mesenchymal cells undergo condensation and then differentiate into chondrocytes by modifying their genomic expression pattern, resulting in a phenotypic change of the cells such as expression of ECM proteins. In the growth plate of skeletal elements that undergo endochondral ossification, layers of chondrocytes become flattened and continue to proliferate, and finally become hypertrophic. This hypertrophic differentiation of chondrocytes is accompanied with ECM changes, e.g., the down-regulation of the collagen type II. Thyroid hormones have been shown to act directly on the growth plate chondrocytes and promote their hypertrophic differentiation (46-48). In juvenile hypothyroidism and in some patients with resistance to thyroid hormones, growth arrest, delayed bone age, and epiphyseal dysgenesis occurs; whereas childhood thyrotoxicosis causes accelerated growth and skeletal maturation. However, how thyroid hormones performed this physiological function has not been fully understood. Given that MINT functions downstream of hormone receptors and participate in thyroid hormone signal transduction (40), we propose that thyroid hormones probably regulate hypertrophic chondrocyte differentiation by down-regulation of chondrocyte-specific genes such as Col2a1 expression through MINT. More studies are necessary to test this hypothesis. Involvement of MINT-CRYBP1 in the signaling of other regulators of hypertrophic differentiation of chondrocytes, such as the negative feedback loop composed of Indian hedgehog (IHH) and parathyroid hormone-related protein (PTHrP) (49), and/or bone morphogenesis proteins (BMPs) (50), is also an open question.
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FOOTNOTES

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1Abbreviations used are: ECM, extracellular matrix; Col2a1, type II collagen gene; CRYBP1, αA-crystallin-binding protein 1; MINT, MSX2-interacting nuclear target protein; SHARP, SMRT/HDAC1-associated repressor protein; SPOC domain, Spen paralog and ortholog C-terminal domain; HDAC, histone deacetylase; SOX, Sry-type high-mobility-group box; SMRT/NCoR, silencing mediator for retinoid and thyroid receptors/nuclear receptor corepressor; TSA, trichostatin A.

FIGURE LEGENDS

Fig. 1. Interaction between MINTF6 and CRYBP1-C in yeast. AH109 was transformed with different plasmids and plated on SD/-trp-leu-his-ade. +, grow within 48 h; -, no grow within 48 h. Positive colonies were tested for β-galactosidase activity using the membrane β-gal assay. +, turn blue within 2 h; -, not turn blue within 2 h. ND, not down.

Fig. 2. Interaction between MINTF6 and CRYBP1-C in mammalian cells. HEK293 (A and C) and COS7 (B and D) cells were transfected with expression vectors as indicated, together with the reporter construct MH TK100x4 luc (44). Cell lysates were prepared 48 h after transfection, and the luciferase activity was examined. pSV-β-gal was included in transfection as an internal control of the transfection efficiency. Results are expressed as means ± SD of more than three duplicated experiments. A and B, Interaction between MINTF6 and CRYBP1-C. C and D, Interaction between MINTF6-C and CRYBP1-C.

Fig. 3. Physical interaction between MINTF6 and CRYBP1-C. A and B, Co-immunoprecipitation assays. Cells (293T) were transfected with expression
vectors as indicated. IP was carried out using the anti-Myc (A) or anti-Flag (B), and immunoblotted with the anti-Flag (A) or anti-Myc (B). Expression of the Myc-tagged (A) or Flag-tagged (B) proteins were detected by immunoblotting with the anti-Myc (A) or anti-Flag (B) antibody (lower panels). Noticed that Myc-MINTF6 and Myc-Luc have very similar molecular weights (66.48 versus 60.75 kD) and could not be separated on the SDS-PAGE. C, GST-pull down. E. coli-expressed His-Trx-MINTF6 and His-Trx were purified and incubated with purified GST or GST-CRYBP1-C immobilized on glutathione-Sepharose 4B beads. Bound proteins were analysed by Western blotting using the anti-His antibody.

Fig. 4. MINT is recruited to the Col2a1 enhancer by CRYBP1-C. A, CHIP. NIH3T3 cells were transfected with expression vectors as indicated. CHIP was performed using the anti-Flag or anti-Myc, and co-precipitated DNA was amplified using primers targeting the Col2a1 enhancer. The amplified fragment is indicated by an arrow. B, EMSA. Cell lysates were prepared from NIH3T3 cells transfected with plasmids as indicated, and incubated with the 32P-labeled Col2a1 enhancer probe. Protein-probe complexes were analyzed using PAGE. For the competition assay, the cold probe was included in the reaction. For the supershift assay, the anti-Flag or anti-Myc antibody was added into the incubation. Free probes and different protein-probe complexes were indicated.

Fig. 5. MINT represses transactivation of the Col2a1 enhancer through CRYBP1-C in an HDAC-dependent manner. A, Repression of the Col2a1 enhancer transactivated by SOX9. NIH3T3 cells were transfected with pGL3-Col2α1 (0.1 µg), pCMV-Flag-SOX9 (0.2 µg), pCMV-HA-CRYBP1-C (0.2 µg), and increasing amount of pEFBOS-Myc-MINT (0.2, 0.4, 0.8 µg), in combinations as indicated. The luciferase activity in cell lysates was examined 48 h after transfection. The results were calibrated by co-transfected pSV-β-gal, and expressed as mean ± SD. B, Repression of the Col2a1 enhancer is dependent on HDAC. Cells were transfected with plasmids as in A, and cultured in the presence of 100, 200, and 400 nM of TSA. The luciferase activity was assayed and analyzed as above.

Fig. 6. The C terminus of MINT is sufficient for repression of the Col2a1 enhancer. NIH3T3 cells were transfected in a similar way as in Fig. 5, with pEFBOS-Myc-MINT replaced by pCMV-Myc-MINTF6, and the luciferase activity in cell lysates was examined 48 h after transfection. The TSA treatment was similar as in Fig. 5B.
### Fig. 1

| Baits | Preys | Growth | β-gal |
|-------|-------|--------|-------|
| MINT  |       | 2018   | +     |
|       | MINTF6| 2056   | +     |
| MINTF1|       | 2018   | +     |
| MINTF2|       | 2018   | +     |
| MINTF3|       | 2018   | +     |
| MINTF4|       | 2018   | +     |
| MINTF5|       | 2018   | +     |
| MINTF6-N|     | 2018   | +     |
| MINTF6-C|     | 2018   | +     |
| MINTF6-C|     | 2018   | +     |
| MINTF6-C|     | 2018   | +     |

*Note: Growth is indicated as + for growth, ND for no data.*
Fig. 2
Fig. 3

A

|      | Input | IP |
|------|-------|----|
| +    | +     | +  |
| -    | +     | -  |
| -    | -     | +  |

IP: anti-Myc
WB: anti-Flag

WB: anti-Myc

B

|      | Input | IP |
|------|-------|----|
| +    | +     | +  |
| -    | +     | -  |
| -    | -     | +  |

IP: anti-Flag
WB: anti-Myc

WB: anti-Flag

C

|      | Input | GST | GST-CRYBP1-C |
|------|-------|-----|--------------|
| -    | +     | -   | +            |
| +    | -     | +   | -            |

WB: anti-His
Fig. 4
MINT represses transactivation of the type two collagen gene enhancer through interaction with αA-crystallin-binding protein 1
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