Human THAP7 Is a Chromatin-associated, Histone Tail-binding Protein That Represses Transcription via Recruitment of HDAC3 and Nuclear Hormone Receptor Corepressor*

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The identities of signal transducer proteins that integrate histone hypoacetylation and transcriptional repression are largely unknown. Here we demonstrate that THAP7, an uncharacterized member of the recently identified THAP (Thanatos-associated protein) family of proteins, is ubiquitously expressed, associates with chromatin, and represses transcription. THAP7 binds preferentially to hypoacetylated (un-, mono-, and di-acetylated) histone H4 tails in vitro via its C-terminal 77 amino acids. Deletion of this domain, or treatment of cells with the histone deacetylase inhibitor TSA, which leads to histone hyperacetylation, partially disrupts THAP7/chromatin association in living cells. THAP7 coimmunoprecipitates with histone deacetylase 3 (HDAC3) and the nuclear hormone receptor corepressor (NCoR) and represses transcription as a Gal4 fusion protein. Chromatin immunoprecipitation assays demonstrate that these corepressors are recruited to promoters in a THAP7-dependent manner and promote histone H3 hypoacetylation. The conserved THAP domain is a key determinant for full HDAC3 association in vitro, and both the THAP domain and the histone interaction domain are important for the repressive properties of THAP7. Full repression mediated by THAP7 is also dependent on NCoR expression. We hypothesize that THAP7 is a dual function repressor protein that actively targets deacetylation of histone H3 necessary to establish transcriptional repression and functions as a signal transducer of the repressive mark of hypoacetylated histone H4. This is the first demonstration of the transcriptional regulatory properties of a human THAP domain protein, and a critical identification of a potential transducer of the repressive signal of hypoacetylated histone H4 in higher eukaryotes.

The dynamic nature of chromatin modification clearly remains at the core of the process of transcription. The acetylation of histone H3 and H4 tails is strongly associated with transcriptional activation, whereas hypoacetylated histones are a mark of transcriptionally silent heterochromatin (1, 2). Consistent with this, proteins that acetylate histones (histone acetyltransferases) are generally involved in transcriptional activation, whereas enzymes that remove acetyl groups from histones (histone deacetylases (HDACs)) function in transcriptional repression. In contrast, methylation of histone tails can have both positive and negative effects on transcription (3).

The number, variety, and interdependence of histone modifications has led to the histone code hypothesis that predicts that modifications acting in combinatorial or sequential fashion on histone tails specify unique downstream functions (4–6). More recently, a signaling network model of chromatin has been proposed that suggests that multiple chromatin modifications combine to confer bistability, robustness, and adaptability to transcriptional signaling networks (7). Both models predict the existence of cellular proteins that would either bind or be released from histones based on their modification status. Consistent with both models, histone H3 methylated at lysine 9 recruits heterochromatin protein 1 (8, 9), which plays an essential role in the establishment and maintenance of transcriptionally silent heterochromatin.

Acetylated histones can also serve as specific docking sites for proteins. Acetylated histone H3 and H4 are able to recruit bromodomain-containing proteins, including the chromatin re-modeling enzymes Swi/Snf and the transcriptional coactivator TAF(I) 250 and GCN5 to localized regions of chromatin (10–15). This is considered to be a critical mechanism whereby acetylated histones are translated into transcriptionally active domains. Histone acetylation also has proposed roles in addition to the signaling role, such as reducing higher order chromatin structure by neutralizing the positive charge on histone tails and preventing their association with the negatively charged phosphate backbone of DNA and altered interaction with neighboring nucleosomes (16, 17).

Sir3 and Tup1 are two yeast proteins that specifically bind hypoacetylated histones and have been linked to transcriptional repression. In yeast, at telomeres and the silent mating-type loci, Sir2 functions along with Sir3, Sir4, and Rap1 to mediate silencing (18–20). Sir3 interacts more efficiently with acetylated histones than are hypoacetylated by Sir2 (21). In addition, the yeast corepressor Tup1 interacts directly with histone H3 and H4 tails via a domain that is also required for tran-

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§ The abbreviations used are: HDAC, histone deacetylase; CBP, CREB-binding protein; DBD, DNA binding domain; HID, histone interacting domain; INHAT, inhibitor of acetyltransferases; GST, glutathione S-transferase; TK, thymidine kinase; THAP, Thanatos-associated protein; siRNA, small interfering RNA; luc, luciferase; TAF-Iα, template-activating factor-1α; TAF-Iβ, template-activating factor-Iβ; TSA, trichostatin A.
scriptional repression (22). This interaction is weakened by high levels of histone acetylation, suggesting a mechanism whereby repression can be relieved. The Snf6-Tup1 corepressor complex also interacts with class I histone deacetylases that are required for its function (23). In higher eukaryotes, however, the mechanisms whereby un- or hypoacetylated histones mediate transcriptional repression are largely unknown.

We have previously shown that INHAT subunits pp32, TAF-Iα, and TAF-Iβ bind to histones and consequently prevent them from serving as substrates for the histone acetyltransferase coactivators p300/CBP and p300/CBP-associated factor, a process we refer to as histone masking (24, 25). Recently we and others have shown that TAF-Iβ and pp32 associate with histone deacetylases and can bind to histone tails, but not if lysines are hyperacetylated, making them candidate human proteins that can translate the transcriptionally repressive marks of unacetylated histones (26, 27).

In this work we characterize THAP7, a novel human protein we isolated in an interaction screen with TAF-Iβ. This protein is a member of a large class of proteins containing the novel THAP domain, a C2-CH signature (Cys-Xaa2−4-Cys-Xaa3−5-gly Cys-Xaa4−His) zinc finger domain sharing significant similarity to the site-specific DNA binding domain of the Drosophila P element transposase (28). There are 12 distinct human proteins that contain the THAP domain, and all share a similar N-terminal location of the domain as well as its size of ~90 residues. To date, only two members of this family have been characterized; THAP0 (DAP4/p52ZIP1), a protein that was isolated in a genetic screen for genes involved in interferon-γ-induced apoptosis in HeLa cells (29) and in a screen for activators of the interferon-induced protein kinase R (30), and THAP1, a protein that potentiates both serum withdrawal and tumor necrosis factor α-induced apoptosis (31). Genetic evidence in Caenorhabditis elegans and Drosophila indicates that THAP domain proteins may have roles in chromatin-based processes, including transcription (28, 32, 33). However, no transcriptional regulatory properties of any member of the THAP domain family have been reported. Here we demonstrate that THAP7 binds to histones and nucleosomes in a tail-dependent manner and binds to histone H4 in an acetylation sensitive manner. Transfected THAP7 associates with chromatin in living cells and promoter-targeted THAP7 represses transcription of transiently transfected and chromosomally integrated reporters and associates with HDAC3 and NCOR. This is the first demonstration of chromatin targeting and transcriptional regulatory function of a human THAP family protein.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Screen**—TAF-Iβ was cloned into the yeast pBD-Gal4 (Stratagene) vector and transformed into YRG-2 His-yeast. These yeast were then transformed with a cDNA library from human vascular smooth muscle cells cloned into the yeast pAD-Gal4 (Stratagene) vector and plated onto SD-Trp-Leu-His plates (34). Approximately 100 yeast colonies grew and these yeast cells were spread onto one plate and then replica plated onto SD-Trp-His plates containing 5 mM 3-amino-triazole. Only 20 colonies still grew and plasmids were isolated from these yeast and sequenced. Half of the sequenced clones were novel human protein THAP7.

**Northern Analysis**—A 32P-THAP7 probe was generated by high prime DNA labeling, and incubated with a poly(A) mRNA multiple tissue Northern Array (normalized using six different housekeeping genes) and a multiple tissue blot (Clontech). After sensitive washing, the array and blot were analyzed by a phosphorimaging device.

**Plasmids and Proteins**—THAP7 and its truncation mutants were PCR amplified from the yeast pAD-Gal4–2.1 (Stratagene) THAP7 clone and inserted into the pCMX vector, the pCDNA3.1myc His vector (Invitrogen), the pCMX Gal4 (amino acids 1–147) vector, the pEGFP C2 vector (Clontech), and pGEX-4T2. GST fusion proteins were expressed in BL21 cells, and purified utilizing glutathione-Sepharose beads. Sequences of recombinant DNA were verified by automated sequencing.

**In Vitro Interaction Assay**—For histone binding assays, 10 μg of histones purified from HeLa cells were preincubated with a pan-monoclonal anti-histone antibody (Roche) in the presence of protein G-Sepharose beads and in vitro translated [35S]methionine-labeled proteins generated using the TnT in vitro translation system (Promega). For GST pull-down assays, in vitro translated [35S]methionine-labeled proteins or purified histones (Roche) were mixed with GST fusion proteins purified from BL21 cells in buffer containing 150 mM KCl, 20 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 1 mM dithiothreitol, 10 μg/ml bovine serum albumin, protease inhibitor mixture, and 0.5% Nonidet P-40 for 30 min, washed extensively, and bound proteins were collected on glutathione-Sepharose beads and resolved by SDS-PAGE. Gels were fixed, dried, and analyzed by a phosphorimaging device. For nucleosome and histone H3 binding assays, 270 mM KCl and 1% Nonidet P-40 were used.

**Cell Culture and Transfections**—293T cells or 293T cells containing a chromosomally integrated luciferase gene with Gal4 DNA binding sites under control of an SV40 or TK promoter were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum with penicillin/streptomycin and puromycin where necessary. Cells were seeded and transfected with Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen). For communoprecipitations, cells were harvested after transfection and luciferase assay 48 h after transfection according to the manufacturer’s instructions (Promega). TSA treatment for 16 h (200 nM or 1 μM) was performed 24 h after transfection.

**Communoprecipitations**—THAP7-transfected 293T cells were lysed in hypotonic buffer A on ice for 5 min (10 mM HEPES-KOH, pH 7.4, 1.5 mM MgCl2, 10 mM KCl, 1 mM phenylmethylsulfonyl fluoride, plus protease inhibitor mixture). Nuclei were isolated by centrifugation at 2000 rpm and resuspended in CoIP buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, protease inhibitors, plus 1% Nonidet P-40) and sonicated. Nuclear lysates or whole cell lysates generated by harvesting cells directly in CoIP buffer and sonicated as above were incubated at a 1:1000 dilution with monoclonal anti-myc antibody (Cell Signaling), anti-FLAG antibody (Santa Cruz), or anti-NCOR antibody (Santa Cruz) overnight, pulldown with GMM dynabeads (Dynal) and washed with CoIP buffer. Bound proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were then immunoblotted with anti-myc antibody, anti-FLAG antibody, or anti-NCOR antibody as indicated.

**Histone Peptide Binding Assays**—Biotinylated histone peptides from Upstate Biotech or Synpep were quantified by dot blot onto nitrocellulose membrane and probed with streptavidin-horse radish peroxidase to ensure equal loading. Three μg of the indicated peptide was incubated with in vitro translated THAP7 in buffer containing 150 mM KCl, 20 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 1 mM dithiothreitol, 10 μg/ml bovine serum albumin, protease inhibitor mixture, and 0.5% Nonidet P-40. Peptides were pulled down with streptavidin-agarose beads, washed, and bound proteins were separated by SDS-PAGE and analyzed by a phosphorimaging device.

**Nucleosome Preparation/Chromatin Association Assays**—Nucleosomes (chromatin fraction) were prepared essentially as described by Nielsen et al. (35). In short, ~107 293T cells were lysed in NIB buffer (15 mM Tris-HCl, pH 7.4, 60 mM KCl, 15 mM MgCl2, 15 mM NaCl, 1 mM CaCl2, protease inhibitor mixture, 1 mM phenylmethylsulfonyl fluoride) plus 0.3% Nonidet P-40, some of which was saved (whole cell extract). Following centrifugation of nuclei at 2000 rpm, supernatant was saved (cytoplasmic extract) and nuclei were washed with NIB plus 0.6% Nonidet P-40 buffer (wash 1). Nuclear lysates were then treated with micrococcal nuclease for 8 min and spun at 10,000 rpm. The supernatant was saved (wash 2) and the pellet was resuspended in ice-cold 2 mM EDTA, pH 8.0. After centrifugation at 10,000 rpm, the supernatant containing solubilized nucleosomes was collected (chromatin fraction). The presence of nucleosomes in the chromatin fraction was confirmed by DNA agarose gel electrophoresis and SDS-PAGE followed by Coomassie staining. All collected fractions were then subjected to immuno blot analysis with anti-myc (Cell Signaling), anti-Hpl1 (Upstate), and histone H1 (Upstate) antibodies as indicated or were subjected to treatment digestion for 10 min followed by addition of >20 soybean trypsin inhibitor where indicated.

Alternatively, transfected 293T cells were lysed in buffer A (described above) containing 0.5% Nonidet P-40 on ice for 10 min. Nuclei were spun down for 10 min at 2000 rpm. The nuclei were then extracted with buffer A with increasing amounts of NaCl to solubilize chromatin-associated proteins. Extracted proteins were then subjected to SDS-PAGE and immunoblot analysis as described above.
REPORTER GENE STUDIES—293T cells or 293T cells containing an intact integrated SV40 or TK luciferase reporter gene containing upstream Gal4 DNA binding sites (Gal4 SV40-luc and Gal4 TK-luc) were seeded into 48-well plates to be 50–70% confluent the next day. 293T cells were transfected with Gal4 TK-Luc reporter and pRL, Renilla luciferase (Promega). Gal4 DNA-binding protein (DBD) alone, Gal4 DBD-THAP7, or the indicated Gal4 DBD-THAP7 truncation mutant. Cells were lysed after 48 h and luciferase activity was measured according to the manufacturer’s instructions (Promega). Western blots utilizing anti-Gal4 antibody (Santa Cruz) were used to verify expression of mutant constructs. For the NCoR siRNA experiment, cells were first transfected with siRNA for 24 h, followed by transfection with reporter constructs. For dose response and repressor mapping, the data from three independent experiments were averaged.

CHROMATIN IMMUNOPRECIPITATION ASSAY—293T cells were fixed with 1% formaldehyde for 15 min followed by addition of 0.125 mM glycine for 5 min at room temperature. Cells were then washed in phosphate-buffered saline, spun down at 1,000 rpm, and resuspended in chromatin immunoprecipitation lysis buffer (1% SDS, 1 mM EDTA, 50 mM Tris-HCl, pH 8.0). Cells were then sonicated for a total of 30 s at level 3. After protein G-Sepharose beads and 20 μl of elution buffer (1% SDS, 0.1M sodium bicarbonate). Cross-links were reversed by incubating with protein G-Sepharose for 3 h and beads were washed once with wash buffer 1 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.0) and blocked with protein G-Sepharose beads and 20 μg of sonicated salmon sperm DNA. After washing down the beads at 3,000 rpm for 1 min, the supernatant was incubated overnight with anti-acetylated histone H3, anti-acetylated histone H4 (Upstate), anti-Gal4, anti-NCoR, anti-HDAC3, or anti-HDAC4 antibodies (Santa Cruz). The IP material was pulled down by incubating with protein G-Sepharose for 3 h and beads were washed once with wash buffer 1 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.0), again with wash buffer 1 plus 350 mM NaCl, once with wash buffer 3 (0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0), and twice with TE, pH 8.0. Immune complexes were eluted in 200 μl of elution buffer (1% SDS, 0.1 M sodium bicarbonate). Cross-links were reversed after addition of 200 mM NaCl by incubation overnight at 65 °C. DNA was then isolated using Qiagen spin columns and analyzed by PCR utilizing primers generated against the promoter of the MH100 TK-luc plasmid.

RESULTS

THAP7 IDENTIFICATION, HOMOLOGUES, AND EXPRESSION—To search for novel TAF-Iβ interactors and potential histone-binding proteins, we performed a yeast two-hybrid screen using TAF-Iβ as the bait and a human vascular smooth muscle cell library. Of the 20 strongest interacting clones, half were the THAP domain proteins. Human THAP7 is 309 amino acids long and contains a proline-rich region and a basic hypothetic human protein THAP7. THAP7 in a yeast two-hybrid screen with the INHAT subunit contains a tubulin domain not present in the human and mouse homologues. Of the 20 strongest interacting clones, half were the THAP domain proteins. Human THAP7 is 309 amino acids long and contains a proline-rich region and a basic hypothetic human protein THAP7. The rat homologue represents a chromosomal translocation that resulted in an extended N terminus that contains a RanGAP essential for THAP7-histone/nucleosome interactions. Importantly, tail-less nucleosomes did not bind to GST-THAP7 (Fig. 2E, right panel). These results together indicate that histone tails are essential for THAP7-histone/nucleosome interactions.

To further examine whether the histone binding properties of THAP7 are mediated by histone tails, we utilized histone H3 and H4 N-terminal tail peptides conjugated to biotin. These peptides were incubated with in vitro translated THAP7, washed, and pulled down with streptavidin agarose. Full-length THAP7-(1–309) bound to both histone H3 and H4 tails (Fig. 3B, middle panel). We next mapped the histone interaction domain (HID) of THAP7. For this purpose, we incubated a series of in vitro translated and radiolabeled THAP7 mutants (Fig. 3B, left panel, schematic shown in Fig. 3A) with biotinylated histone H3 or H4 tails in vitro. All of the THAP7 mutants that contained the C-terminal 77 amino acids, rich in positively charged residues, could bind to histone H3 and histone H4 tails, but a mutant (1–231) lacking this region could not (Fig. 3B, middle and right panels). These binding results (summarized in Fig. 3A) indicate that the THAP domain is not necessary and that the C-terminal 77 amino acids of THAP7 are necessary and sufficient for histone H3 and H4 tail binding in vitro. This region is thus referred to as the HID.

THAP7 ASSOCIATES WITH CHROMATIN IN INTACT CELLS—Because THAP7 associated with nucleosomes, total histones, and histone tails in vitro, it was important to demonstrate that THAP7
THAP7 in Transcriptional Repression

FIG. 1. THAP7 sequence and expression. A and B, alignment of human, mouse, and rat THAP7. A, human and mouse THAP7 are 309 amino acids long with three broad domains, a highly conserved THAP zinc finger domain at the N terminus, a proline-rich central region, and a basic stretch at the C terminus. The rat homologue has an extended N terminus with a RanGAP domain that contains a leucine-rich repeat, a tropomodulin domain, and a tubulin domain. B, THAP7 sequence alignment (human top, mouse middle, rat bottom). Mouse and human THAP7 share 93.3% identity at the amino acid sequence, whereas the mouse and rat share 92.3% identity. C and D, THAP7 is a ubiquitously expressed mRNA. A multiple tissue Northern Array (C) or a multiple tissue Northern blot (D) was probed with a high prime-labeled THAP7 probe.
does indeed associate with chromatin in living cells. Immunofluorescence studies indicate that myc-tagged THAP7 is found primarily in the nucleus in HeLa, NIH 3T3, and 293T cells (data not shown). To determine whether THAP7 interacts with chromatin, we fractionated 293T cells transfected with full-length THAP7 and subjected the nuclear extract to SDS-PAGE by Coomassie Blue and utilized in subsequent binding assays. C, THAP7 associates with histone H3 and histone H4. Purified histones were incubated with GST or GST-THAP7 immobilized on glutathione-Sepharose in buffer containing 150 mM KCl and 0.5% Nonidet P-40 (for histones H2A and H2B) or 270 mM KCl and 1% Nonidet P-40 (for histones H3 and H4). Bound proteins were subject to SDS-PAGE and stained with Coomassie. D, THAP7 interacts specifically with histone H3 tail. In vitro translated 35S-labeled THAP7 was incubated with equal amounts of GST, GST-H3, GST-H3 N terminus (amino acids 1–46), or GST-H3 C terminus lacking the N-terminal tail. Purified GST proteins were run on SDS-PAGE and stained with Coomassie Blue (left panel). Bound proteins were washed and separated by SDS-PAGE and analyzed by a phosphorimaging device (right panel). E, THAP7 interacts with nucleosomes and the interaction is dependent upon histone tails. Soluble nucleosomes were prepared from 293T cells and were treated with limited trypsin digestion for 10 min followed by addition of soybean trypsin inhibitor. Input nucleosomes were then subjected to SDS-PAGE and stained with Coomassie Blue (left panel). Tryptsinized tailless nucleosomes (right panel) or intact nucleosomes (middle panel) were then incubated with GST or GST-THAP7. Bound proteins were washed and separated by SDS-PAGE and analyzed by Coomassie Blue staining.

THAP7 in Transcriptional Repression

THAP7 binds to histone H3 and H4 tails, but not to hyperacetylated histone H4 tails in vitro—The histone code hypothesis and signal transducer model predict the existence of cellular proteins that recognize histones in a modification sensitive manner (4–7). The recruitment to or release of proteins from chromatin domains is proposed to play a critical role in gene regulation. It has recently been demonstrated that INHAT proteins pp32 and TAF-I bind to histone tails in a modification sensitive manner (26, 27). Because THAP7 also binds to histone tails in vitro and chromatin in vivo (Figs. 2–4), we set out to determine whether THAP7 bound to histone tails in a modification sensitive manner. To test this hypothesis, we incubated radiolabeled in vitro translated THAP7 with biotinylated histone H4 tail peptides that were mono-, di-, tri-, or tetraacetylated. THAP7 bound to unmodified, monoacetylated, and diacetylated histone H4, but did not bind triacetylated or
tetraacetylated histone H4 (Fig. 5A). This binding profile to histone H4 is completely opposite to what has been observed for the dual bromodomain containing protein Brd4, which binds preferentially to hyperacetylated histones (38), further supporting the notion of the existence of cellular proteins serving as transducers of chromatin signaling. This binding pattern is also different from what has been observed for INHAT proteins, which can only bind to completely unacetylated histone H4 peptides (26, 27).

We next tested whether THAP7 has a binding preference for modified histone H3 tails. Surprisingly, under our binding conditions, THAP7 bound to all histone H3 peptides tested, including unmodified histone H3, histone H3 that is mono-, di-, tri-, or tetraacetylated, phosphorylated at serine 10, and methylated at lysine 9 (Fig. 5B). This is completely different from what has been observed with INHAT proteins, which can bind to unacetylated histone H3 and methylated H3, but not to hyperacetylated histone H3 (26, 27). These results indicate that the pattern and extent of acetylation is an important factor in determining the robustness of THAP7 association with histone H4 but not with histone H3. This modification insensitive THAP7 binding to the histone H3 tail also suggests that its acetylation sensitive binding to histone H4 is specific and that THAP7 may play a dual role in establishing and translating the transcriptional regulatory marks of histones H3 and H4.

Because THAP7 associated with un- and hypoacetylated histone H4 tails but not hyperacetylated histone H4 tails, we hypothesized that THAP7 may not associate with hyperacetylated chromatin as tightly as with hypoacetylated chromatin. To test this hypothesis, we transfected cells with myc-tagged THAP7 and treated the cells with or without the HDAC inhibitor TSA, which leads to bulk histone hyperacetylation. Should THAP7 bind less tightly to hyperacetylated chromatin, lower salt concentrations will be sufficient to extract the protein from chromatin. Nuclei were isolated after hypotonic lysis of cells, and were subjected to extraction in buffer containing increasing amounts of NaCl as described to demonstrate the acetylation sensitive binding of Brd4 (38). In untreated cells, THAP7 was solubilized by 100 mM NaCl, and to a greater extent with increasing salt (Fig. 5C). But in cells treated with TSA, which contained hyperacetylated histones as observed by immunoblot (data not shown), THAP7 was solubilized with lower salt (10 mM), and was more greatly solubilized with 100 mM salt when compared with untreated cells (Fig. 5C, compare middle panel with top panel), indicating that it is less tightly associated with hyperacetylated chromatin than with hypoacetylated chromatin. In addition, when the HID was deleted from THAP7 (THAP7-(1–231)), THAP7 was also solubilized under low salt conditions, indicating that it is less tightly bound to chromatin than full-length THAP7, consistent with Fig. 4B. These data thus demonstrate that THAP7 association with histone H4 tails and chromatin is at least partially acetylation dependent, and that the HID domain is an important determinant in chromatin association.
THAP7 Associates with HDAC3—It is proposed that in yeast the chromatin-recruited Sir2 deacetylates histones and the resulting hypoacetylated histones are subsequently bound by the Sir2-associated Sir3 (21). Therefore, deacetylation of target sites (by Sir2) followed by recruitment of a histone-binding protein (Sir3) provides a mechanism of establishment and maintenance of transcriptional repression by the Sir complex in yeast. Second, the nuclear hormone receptor corepressor SMRT associates with histone deacetylase 3 and hypo- but not hyperacetylated histone H4 and functions in a feed-forward mechanism in HDAC-mediated histone deacetylation and transcriptional repression (39). We have previously demonstrated that THAP7 is a chromatin-associated protein that preferentially binds to hypoacetylated histone H4 in vitro. Based on the above discussion, we hypothesized that THAP7 may associate with HDAC3 in intact cells and that the region encompassing the THAP domain may have a novel HDAC3 association function.

Promoter-Targeted THAP7 Represses Transcription—in C. elegans, the THAP domain containing Lin15B protein has been implicated in a lin-35 Rb parallel repression pathway (32). Additionally, several Drosophila THAP domain proteins are putative transcription factors (28). However, there is no direct experimental demonstration of transcriptional regulatory properties of Lin15B or Drosophila THAP proteins. Based on the studies in C. elegans and Drosophila and our observation that THAP7 associates with chromatin and HDAC3, we reasoned that human THAP7 may have transcriptional regulatory properties. Because most of the THAP family members are largely uncharacterized, the identity of THAP7 target sites/genes on chromatin is currently unknown. To circumvent this problem and to rapidly study the transcriptional regulatory property of THAP7, we generated and analyzed the effect of a Gal4-DNA binding domain (DBD) fusion of THAP7 on a reporter plasmid containing four Gal4 DNA binding sites. Consistent with our finding of an association with HDAC3, Gal4-THAP7 repressed basal transcription in a dose dependent manner (between 4- and 10-fold, Fig. 7A), and at the highest dose tested, repressed 10-fold compared with basal levels (compare lane 1 with lane 5) and 60-fold with respect to an equal
amount of Gal4 DBD alone, as we consistently observed a modest activation by Gal4 DBD alone (data not shown).

To map the repression domains of THAP7, we generated and analyzed the effect of a Gal4-DBD fusion of THAP7 or THAP7 truncation mutants in 293T cells. Expression of each mutant was confirmed by immunoblot analysis with anti-Gal4 antibody (Fig. 7C). The schematic of mutants tested and percent repression for each THAP7 mutant is summarized in Fig. 7B, which is set at 100% for Gal4 DBD-THAP7 full-length. Deletion

of the THAP domain from amino acids 1–100 relieved the repression function of THAP7 by ~50%. Deletion of the HID from amino acids 232–309 also relieved repression by ~50%. Deletion of both the THAP and HID domains completely relieved repression mediated by THAP7-(101–231). These domains are designated as repressor domains 1 and 2 (RD1 and RD2). (It is important to note that although there were subtle differences in the expression of the THAP7 mutants, expression of transcriptional repression defective mutant THAP7-(101–231) is at comparable levels to mutants that still repress transcription.) This repressor domain mapping is consistent with our results of Figs. 3 and 6 in which we show that HID-(232–309) is the major histone interacting domain, whereas the THAP domain (1–100) is the major HDAC3 association site. The HDAC3 association domain and histone binding domain of THAP7 thus directly correlate with repression domains. These results also suggest that the novel THAP domain may function as an HDAC association and transcriptional repression domain.

Fig. 5. THAP7 binds to histones H3 and H4 tails but not to hyperacetylated histone H4 tail. A, THAP7 preferentially binds hypoacetylated histone H4 tails. The indicated biotinylated histone H4 tail peptide was incubated with in vitro translated 35S-labeled THAP7 and pulled down with streptavidin-agarose. Bound proteins were washed, separated by SDS-PAGE, and analyzed by a phosphorimaging device. B, THAP7 has no binding preference for either modified or unmodified histone H3 tails. The indicated biotinylated histone H3 tail peptide was incubated with in vitro translated 35S-labeled THAP7 and pulled down with streptavidin-agarose. Bound proteins were washed, separated by SDS-PAGE, and analyzed by a phosphorimaging device. C, THAP7 associates less tightly with hyperacetylated chromatin. Myc-tagged THAP7 or the THAP7 HID-deleted (1–231) mutant was transfected into 293T cells that were subsequently mock treated or treated with 1 μM TSA overnight. Cells were lysed in hypotonic buffer, and nuclei were then extracted with increasing NaCl to solubilize chromatin-associated proteins. Extracted proteins were subjected to SDS-PAGE and immunoblot analysis with anti-myc antibodies.

Fig. 6. THAP7 interacts with HDAC3. A, THAP7 coimmunoprecipitates with HDAC3. THAP7-FLAG or FLAG vector alone was co-transfected with HDAC3-myc or HDAC4-myc into 293T cells. Whole cell lysates were immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-myc or anti-FLAG antibody. B, HDAC3 associates with THAP7. HDAC3 (left panel) or HDAC4 (middle panel) were in vitro translated and incubated with the indicated GST fusion proteins. Bound proteins were washed, subjected to SDS-PAGE and phosphorimaging analysis. Input GST proteins are shown in the right panel. C, HDAC3 associates with the THAP domain. Association assays were performed as in B with the indicated GST fusion proteins. Input GST proteins are shown in the right panel.
FIG. 7. Promoter-targeted THAP7 represses transcription. A, THAP7 represses transcription of a transfected reporter. Increasing amounts of Gal4 DBD-THAP7 (lanes 2–5) were transfected into 293T cells along with the Gal4 TK-luc reporter and a control Renilla luciferase reporter. Luciferase assays were performed 48 h after transfection and luciferase values were reported relative to Renilla luciferase. B, saturating amounts of Gal4 DBD, Gal4 DBD-THAP7, or mutants 1–65, 62–309, 101–309, 232–309, 1–231, or 101–231 were transfected into 293T cells along with Gal4 TK-luc and the Renilla luc internal control plasmid as described in A. Luciferase assays were performed 48 h after transfection. Shown is a schematic of THAP7 mutants tested for repressive function and outcome of experiments. Repression by full-length THAP7 is set at 100% compared with empty vector-transfected cells and mutants were compared relative to full-length. C, expression of Gal4 DBD-THAP7 and truncation mutants. Gal4 DBD, Gal4 DBD-THAP7, or the indicated Gal4 DBD-THAP7 mutant were transfected into 293T cells. Cells were harvested 48 h later and expression of proteins was analyzed by immunoblot with anti-Gal4 antibody. D, THAP7 represses transcription of an integrated reporter. Increasing amounts of Gal4 THAP7 were transfected into the Gal4 SV40 luc integrated 293T cell line. Luciferase assays were performed 48 h after transfection.
The use of a transfected reporter gene assay provided a convenient and widely used way to rapidly establish the transcriptional repressive function of THAP7 and to map the repression domains of THAP7. To confirm the above results and to determine whether THAP7 can repress transcription in a more physiologically relevant context, we tested the effect of Gal4 DBD-THAP7 on cell lines containing either of two chromosomally integrated Gal4 responsive luciferase reporters (Gal4 TK-luc and Gal4 SV40-luc). These cell lines have recently been utilized to demonstrate the well established transcriptional repression and activation function by unliganded and liganded thyroid receptors and transcriptional repression by proliferating cell nuclear antigen (40, 41). When transfected into cells, Gal4 DBD-THAP7 repressed transcription of the Gal4 SV40 luciferase reporter 2–5-fold at the doses tested (Fig. 7D). Similar results were also obtained when the Gal4 TK-luc reporter was analyzed (data not shown). These results together demonstrate that promoter-targeted THAP7 represses transcription in a chromatin context. The use of three independent reporter systems (transfected and integrated) further confirms the transcriptional repressive function of THAP7.

THAP7 Associates with NCoR, and NCoR Is Required for THAP7-mediated Repression—NCoR/SMRT are classical transcriptional corepressors that associate with HDAC3 to mediate transcriptional repression including that of nuclear hormone receptors (42–45). Additionally, SMRT binds to hypoacetylated histones, associates with HDAC3, and promotes histone deacetylation in a feed-forward mechanism (39). Therefore we analyzed whether THAP7 associates with NCoR/SMRT. Consistent with our finding of an association between HDAC3 and THAP7, we found that myc-tagged THAP7 coimmunoprecipitated with endogenous NCoR in 293T cell nuclear lysates (Fig. 8A). To further analyze the requirement of NCoR in THAP7-mediated transcriptional repression, we transfected cells with siRNA against NCoR (40). We confirmed by immunoblot analysis that siRNA against NCoR significantly reduced NCoR protein expression when compared with scramble siRNA-transfected cells (Fig. 8B). HDAC3 expression was used as a control to demonstrate that NCoR knockdown is specific. In cells with reduced NCoR levels, we saw a reduction in repression by Gal4 DBD-THAP7 (Fig. 8C). These data indicate that NCoR is at least partially required for transcriptional repression by THAP7.

THAP7 Targets HDAC3 and NCoR to Promoters and Promotes Histone H3 Hypoacetylation—Because THAP7 associates with HDAC3 and can bind to hyperacetylated histone H3 we reasoned that THAP7 should target histone H3 hypoacetylation to promoters. To test this hypothesis, we transfected 293T cells with the reporter luciferase construct containing four Gal4 DNA binding sites along with Gal4 DBD alone or Gal4 DBD-THAP7 constructs. We then performed chromatin immunoprecipitation with anti-acetylated histone H3, anti-acetylated histone H4, Gal4, HDAC3, HDAC4, and NCoR, antibodies. We subsequently compared the level of histone acetylation and recruitment of the indicated proteins to the promoter in cells transfected with either Gal4 DBD or Gal4 DBD-THAP7 (Fig. 9A). Under conditions where the inputs are approximately equal (lane 1), comparable amounts of Gal4 DBD and Gal4 DBD-THAP7 were recruited to the promoter (lane 3). Control IgG antibodies were used to show the level of background of the PCR amplified promoter region (lane 2). As expected, Gal4 DBD-THAP7-transfected cells were significantly hypoacetylated on histone H3 (lane 5), but not histone H4 (lane 4). When we examined the presence of HDACs and NCoR at the promoter we found a significant increase in HDAC3 and NCoR recruitment to the promoter in Gal4 DBD-THAP7-transfected cells (lanes 6 and 8, respectively) compared with Gal4 DBD-transfected cells, but there was no significant change in HDAC4 recruitment (lane 7). This is consistent with our in vitro binding and coimmunoprecipitation studies, and suggests that THAP7 represses transcription by recruiting HDAC3 and NCoR to promoters to deacetylate histone H3.

DISCUSSION

In this article we characterize THAP7, a member of the newly identified THAP domain family of proteins (28). The
The novelty of this study lies in the demonstration that human THAP7 is a chromatin-associated protein and that the novel THAP domain is important for transcriptional repression. We demonstrate that human THAP7 binds to histones and nucleosomes in a tail-dependent manner, and associates with chromatin in intact cells. Strikingly, THAP7 binds to un-, mono-, and diacetylated histone H4 tails, but not tri- or tetraacetylated histone H4 tails, whereas it binds to histone H3 tails independently of their modification status. This is the first report demonstrating that a human THAP domain protein possesses transcriptional repression capacity.

**Fig. 9.** THAP7 recruits NCoR and HDAC3 to promoters and causes hypoacetylation of histone H3. **A.** The Gal4 TK luciferase reporter construct was transfected into 293T cells along with Gal4 DBD or Gal4 DBD-THAP7. Cells were cross-linked with 1% formaldehyde, and chromatin immunoprecipitation assays were performed with the indicated antibodies. PCR primers generated against the promoter of the reporter luciferase were used to determine specific recruitment of the indicated proteins. **B.** Proposed model of THAP7 function. THAP7 may be recruited to chromatin by one or a combination of three mechanisms that are not mutually exclusive: 1) by association with hyperacetylated histone H3 tail; 2) by association with hypoacetylated histone H4 tail; 3) by direct DNA binding by the THAP domain or additional mechanisms yet to be discovered. Once targeted to a promoter, THAP7 recruits corepressors HDAC3 and NCoR and possibly other proteins to repress transcription. For more details, see “Discussion.”
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THAP7 associates with histones in vitro and with chromatin in intact cells. The tails of histones are required for this association in vitro. It is interesting to note that whereas THAP7 uses a predominantly positively charged C-terminal domain (18 positively charged residues compared with 8 negatively charged residues) to associate with histone/nucleosomes, TAF-Iβ primarily uses a highly negatively charged domain for histone binding (24, 25). In living cells, deletion of the HID partially disrupts THAP7/chromatin association, which indicates that while the HID is involved in nucleosome association, additional domains of THAP7 are likely also to contribute to chromatin association. Future studies should clarify the importance, if any, of the THAP domain of THAP7 and other THAP proteins in direct DNA binding and chromatin targeting.

Another important feature of THAP7 is that it binds to un-, mono-, and di- but not tri- or tetraacetylated histone H4. Unexpectedly the binding to the histone H3 tails is insensitive to modification status. THAP7 has distinct histone binding properties from that of INHAT proteins pp32 and TAF-Iβ, which can only bind to completely unacetylated histone H4 and hypoacetylated histone H3 (26, 27). Consistent with these results, we found that THAP7 is solubilized from chromatin at lower salt concentrations in TSA-treated cells compared with untreated cells, indicating that THAP7 is less tightly bound to hyperacetylated chromatin, and is dependent upon electrostatic interactions.

In yeast, replacement of a single acetylatable lysine on the histone H4 tail does not abrogate induction of the GAL1 gene (46). However, replacement of three or four of these lysines with arginine can lead to a 5–50-fold reduction in activation. In addition, it has been suggested that the gene regulatory potential of the H3 and H4 N termini is substantially (but not entirely) contained in these modifiable lysine residues (47). Based on our in vitro binding results, we hypothesize that THAP7 release from histone H4 is likely to occur after acetylation of three or four lysine residues of histone H4. The acetylation sensitive THAP7/histone H4 interaction may help explain the need for seemingly redundant acetylatable lysines on histone H4. The identity of promoters that undergo tri- or tetraacetylation on histone H4 is not currently known and THAP7 may be a useful tool to characterize such genes, should they exist. Because THAP7 can directly repress transcription, its recruitment to hypoacetylated histone H4 may provide a mechanism for transcriptional repression observed for hypoacetylated histone H4. It is currently unclear what roles individual histone tails and their modifications play in THAP7 association in the context of chromatin and addressing this question would require the assembly of recombinant nucleosomes lacking individual histone tails or modifications, which is beyond the scope of the present study.

There is genetic evidence that suggests that THAP domain proteins may have a role in transcription. In C. elegans, the THAP domain containing lin15B acts in the same multifluvial pathway as lin-35 Rb indicating its potential role in transcriptional repression (32). In Drosophila, three of seven THAP domain containing proteins are putative transcription factors, and a fourth interacts with dorsal, a transcription factor of the NF-κB family (28, 48). A fifth Drosophila THAP7 domain protein contains a motif with homology to the DNA binding domain of the centromere protein B (CENPB) located close to the THAP domain (49). The THAP domain also has significant similarity to the site-specific DNA binding domain of Drosophila P element transposase. Mutations within the conserved C2-CH signature or deletion of the AVPTIF consensus box abrogates site-specific DNA binding of the P element transposase (50).

These results strongly suggest that THAP domain may represent a novel and putative DNA binding domain.

While we remain actively engaged in determining whether THAP7 directly binds DNA, several lines of evidence obtained in the present study establish a novel role of THAP7 in transcriptional repression. First, the use of both transfected and integrated reporters uncovers the transcriptional repressive function of THAP7. Importantly, there are two distinct transcriptional repression domains. Whereas the novel THAP domain is the primary association site for HDAC3, the C-terminal 77-amino acid domain binds to histones. Deletion of either of these domains results in a protein with reduced repressor activities, but deletion of both completely alleviates transcriptional repression by THAP7, indicating the importance of multiple repression domains in its function. Second, THAP7 recruits HDAC3 and NCoR to promoters in Gal4 fusion reporter gene assays and is dependent on NCoR protein expression. Thus we propose that THAP7 can repress transcription at least in part by recruiting known corepressor proteins to a chromatin domain, and actively deacetylating histone H3. It is also possible that THAP7 associates with other transcription factors or corepressors as part of its physiologic function. Nonetheless, our results provide the first direct experimental evidence supporting the transcriptional regulatory role of human THAP7. Our results also provide supporting evidence for the proposed transcriptional regulatory roles of C. elegans lin-15B and several Drosophila THAP proteins.

We hypothesize that THAP7 is a multifunctional repressor. THAP7 binds directly to un- and hypoacetylated histone H4 tails (see model, Fig. 9B). Acetylation of histone H4 by histone acetyltransferase proteins may then provide a mechanism to dissociate THAP7 from the histone H4 tail and allow for subsequent transcriptional activation. But THAP7 can also bind to histone H3 tails that are un- or hyperacetylated, and may also bind to DNA directly to target it to a promoter. When targeted to promoters, THAP7 promotes the deacetylation of histone H3, most likely via recruitment of HDAC3 and NCoR. Once recruited, THAP7 can directly repress transcription, and both the THAP domain and the HID are required. Future studies should clarify whether direct DNA binding by the THAP domain is required for chromatin targeting during transcriptional repression.

In summary, we define a transcriptional regulatory role of the human THAP7 protein and show that the novel THAP domain contributes to transcriptional repression. The ability of THAP7 to bind histones and associate with HDAC3 and NCoR provides a mechanism for the establishment of transcriptional repression by THAP7. Defining the chromosomal sites of interaction and potential target genes of THAP7 will become essential to more fully understand its in vivo role. It will also be necessary to demonstrate that THAP7 recruits the corepressors HDAC3 and NCoR to these sites to repress transcription. Based on our results, we predict that other THAP domain proteins will be involved in regulating transcription and other chromatin-based processes. Our results also highlight the emerging role of histone-binding proteins in chromatin signaling.

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