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Cell growth suppression by thanatos-associated protein 11(THAP11) is mediated by transcriptional downregulation of c-Myc

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Thanatos-associated proteins (THAPs) are zinc-dependent, sequence-specific DNA-binding factors involved in cell proliferation, apoptosis, cell cycle, chromatin modification and transcriptional regulation. THAP11 is the most recently described member of this human protein family. In this study, we show that THAP11 is ubiquitously expressed in normal tissues and frequently downregulated in several human tumor tissues. Overexpression of THAP11 markedly inhibits growth of a number of different cells, including cancer cells and non-transformed cells. Silencing of THAP11 by RNA interference in HepG2 cells results in loss of cell growth repression. These results suggest that human THAP11 may be an endogenous physiologic regulator of cell proliferation. We also provide evidence that the function of THAP11 is mediated by its ability to repress transcription of c-Myc. Promoter reporter assays indicate a DNA binding-dependent c-Myc transcriptional repression. Chromatin immunoprecipitations and EMSA assay suggest that THAP11 directly binds to the c-Myc promoter. The findings that expression of c-Myc rescues significantly cells from THAP11-mediated cell growth suppression and that THAP11 expression only slightly inhibits c-Myc null fibroblasts cells growth reveal that THAP11 inhibits cell growth through downregulation of c-Myc expression. Taken together, these suggest that THAP11 functions as a cell growth suppressor by negatively regulating the expression of c-Myc.
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The THAP proteins (Thanatos-associated protein), a novel family of cellular factors, are characterized by the presence of an evolutionarily conserved protein motif. The motif designated as the THAP domain presents striking similarities with the site-specific DNA-binding domain of *Drosophila P* element transposase.[1] Analysis of the *Drosophila* THAP family has revealed several interesting features. One of the *Drosophila* THAP proteins, dorsal-interacting protein 2, was identified earlier in a two-hybrid screen with dorsal, a transcription factor from the NF-κB family.[2] The third multi-THAP protein, designated as HIM-17, has been shown to play a critical role in chromosome segregation during meiosis by linking chromatin modification with competence to initiate meiotic recombination by double-strand breaks.[3] In addition, several proteins containing similar or consensus THAP domain were identified in *C. elegans*.[4,5]

There are 12 distinct human proteins that contain the THAP domain (THAP 0–11).[1] The first THAP protein characterized was the death-associated protein DAP4/P52^{rIPK} (THAP0).[6] Overexpression of THAP0 restored PKR activity and eIF-2α phosphorylation, thus suppressing the effects of P58^{IPK}.[7] THAP1 is a novel nuclear proapoptotic factor associated with promyelocytic leukemia nuclear bodies (PML NBs). THAP1 interacts with prostate-apoptosis-response-4 (Par-4) and potentates both serum withdrawal- and TNFα-induced apoptosis.[8] An *in vitro* DNA site selection strategy showed that the THAP domain of THAP1 could bind directly to a specific DNA sequence.[9] Recently, THAP1 was also identified as an endogenous physiologic regulator of endothelial cell proliferation and G1/S cell-cycle progression.[10] THAP7 was characterized as a protein with both histone-binding and putative DNA-binding motifs. THAP7 repressed transcription by recruiting NCoR and HDAC3 to promoters and promoting the deacetylation of histone H3.[11] The study also showed that THAP7 possessed additional mechanisms of repressing transcription by recruiting the INHAT subunit TAF-Iβ to promoters and masking histone acetylation.[12] These studies, together with the data obtained in *C. elegans*, suggested that THAP proteins are sequence-specific DNA-binding factors involved in cell proliferation, apoptosis, chromatin modification and transcriptional regulation.

THAP11 maps to a locus on chromosome 16q22.1 and was suggested as a putative candidate for polyglutamine disorders based on polymorphism and protein-folding simulation studies.[13] Most recently, the mouse homolog of THAP11, Ronin, was identified as an essential factor underlying embryogenesis and ES cell pluripotency, and was associated with sequence-specific DNA binding and epigenetic silencing of several gene expressions.[14] However, the function of human THAP11 still remains unknown. Here, we provide

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Abbreviations: ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; GFP, green fluorescence GFP; HCC, hepatocellular carcinoma; ODC, ornithin decarboxylase; RNAi, RNA interference; RT-PCR, reverse transcription-PCR; THAP, Thanatos-associated protein
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convincing evidence that THAP11 suppresses cell growth through downregulation of c-Myc.

Results

THAP11 is expressed in normal human tissues and downregulated in human tumors. Full-length cDNA coding human THAP11 was isolated from human bone marrow cDNA according to the sequence (GenBank accession no. BC012182). The putative THAP11 protein has a conserved THAP zinc-finger domain at the N terminus and a 29 residue repeat polyglutamine motif basic domain in the middle of the sequence. The region from 98 to 103 amino acids is also predicted to contain a weak nuclear localization signal. To confirm whether the nuclear localization signal in THAP11 protein was functional, THAP11 cDNA was fused into the N terminus of EGFP in pEGFP vector and transfected into COS-7 cells (Supplementary Figure S1). Fusion of THAP11 with GFP resulted in nuclear localization (Supplementary Figure S2). In contrast, the GFP signal distributed throughout the cytoplasm and nuclei of cells transfected with pEGFP plasmids. Moreover, the expression of the endogenous THAP11 in nucleus was shown by western blotting of subcellular fractions (Figure 1a). These results suggested that THAP11 encodes a nuclear protein.

To examine the expression profile of THAP11 in human adult tissues, reverse transcription-PCR (RT-PCR) analysis was performed using the Multiple Tissue cDNA. As Figure 1b shows, THAP11 was ubiquitously expressed in the tissues indicated. We also evaluated the expression pattern of THAP11 in several tumors and their corresponding normal tissues by hybridizing a THAP11-specific probe onto the Cancer Profiling Array II. As shown in Figure 1c, THAP11 was downregulated in six types of tumor tissue derived from the liver (3/3), thyroid gland (7/10), vulva (3/5), skin (5/10), pancreas (4/7) and kidney (4/10). In contrast, a significant upregulation of THAP11 was observed in tumor samples derived from the lung (7/10) and ovary (4/10) when compared with the corresponding normal cDNAs.

As THAP11 was downexpressed in liver cancer tissues, we further examined the expression status of THAP11 in primary HCCs. A real-time PCR was performed to detect the mRNA level of THAP11 in 12 pairs of HCC specimens and their adjacent non-cancerous liver tissues. The result showed that THAP11 was frequently downregulated in 75% (9/12) of HCC specimens as compared with adjacent non-cancerous liver tissues (Figure 1d).

THAP11 is involved in regulation of hepatoma cell growth. As THAP11 was downexpressed in HCC, we next

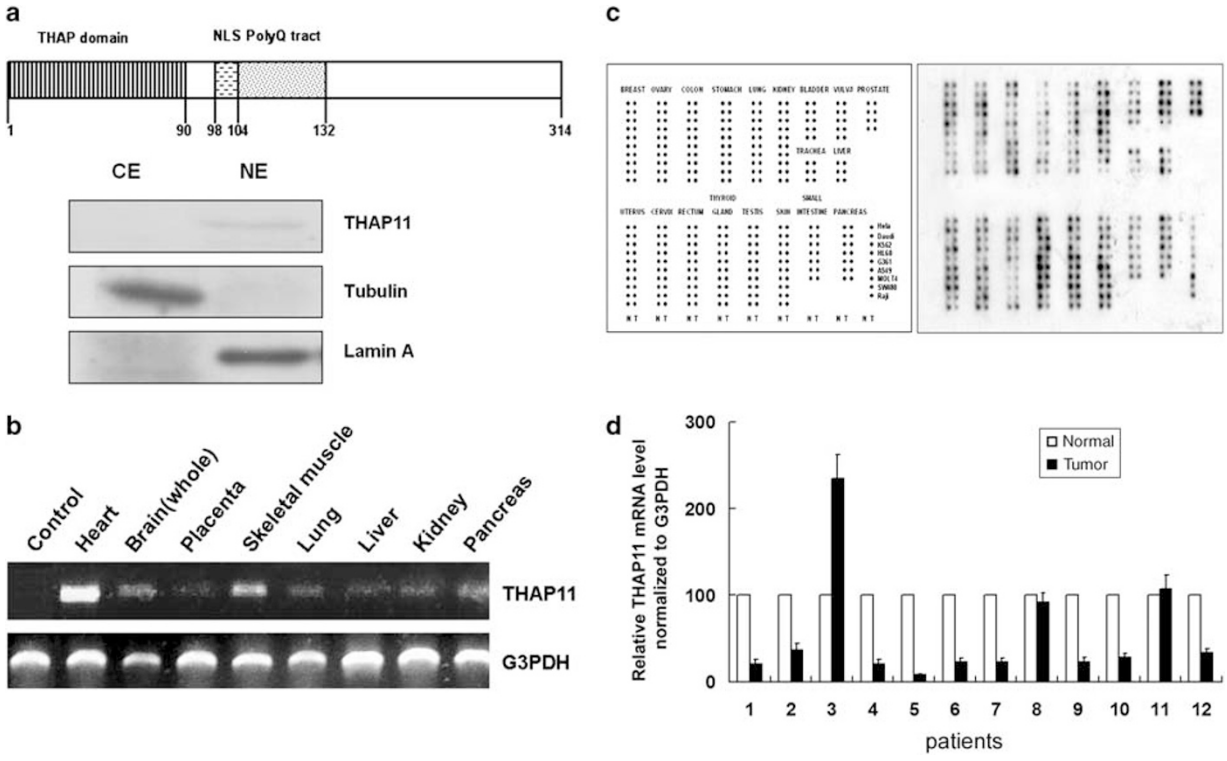

Figure 1 Cellular location and expression profile of THAP11. (**a**) Endogenous expression of THAP11 in nuclear extracts. Cytoplasm extract (CE) and nuclear extract (NE) were isolated from HepG2 cells and western blotting analysis was performed using THAP11 polyclonal antibody. Tubulin was used as a positive control of cytoplasm extract and Lamin A was used as a positive control of nuclear extract. The structure of human THAP11 protein was indicated as the upper panel. (**b**) THAP11 mRNA transcripts were detected by RT-PCR analysis using the Multiple Tissue cDNA assay (Clontech). G3PDH was used as the internal control. (**c**) Differential expression pattern of THAP11 in human tumor tissues and corresponding normal tissues. The radioactive THAP11-specific probe was hybridized onto an array containing 154 cDNA pairs of samples derived from multiple human tumors and corresponding normal tissue from individual patients. N: normal tissue sample. T: tumor tissue sample. (**d**) THAP11 is downregulated in HCC patients. Total RNA was extracted from 12 pairs of HCC specimens (tumor) and their adjacent non-cancerous liver tissues (normal) for real-time PCR analysis. The expression levels of THAP11 in non-cancerous tissues were set to 100%. Results were expressed as relative mRNA level with cancerous tissues and normalized to G3PDH mRNA

explored the role of THAP11 in HCC cell proliferation. Human hepatoma cell lines 7721 were transfected with pcDNA-THAP11 or the control vector. The efficiency of each transfection was monitored by GFP cotransfection in other wells after 48 h. All of the transfections displayed a similar efficiency (data not shown). At 12 h after transfection, 1×10^3 cells were seeded in each well of 96-well plates. MTS assay was performed from 1 day to 6 days after the transfection. As shown in Figure 2a, the proliferation rate of 7721 cells transfected with THAP11 gene was significantly lower than that of cells transfected with pcDNA3.1 and wild-type 7721 cells. Moreover, 7721 cells were transfected with pcDNA-THAP11 or pcDNA 3.1, and the growth of G418-resistant clones was measured. As Figure 2b shows, THAP11 expression led to reductions in colony formation of approximately 60%. No dead or apoptotic cell with THAP11 expression was observed for 3 days, which indicated that the ectopic expression of THAP11 was not immediately toxic (data not shown).

To further confirm the effect of THAP11 on cell proliferation *in vivo*, we performed RNA interference assays to investigate whether decreased THAP11 expression altered cell proliferation. To rule out off-target effects, two siRNA oligos were used (siTHAP11-1 and siTHAP11-2). Transfection of HepG2 cells with siTHAP11-1 or siTHAP11-2 resulted in an up to 84% or 65% decrease in the THAP11 mRNA level (Supplementary Figure S3) and a 70% or 53% decrease in the THAP11 protein level (Figure 2c) within 72 h after transfection. As shown in Figure 2d, when endogenous THAP11 expression was knocked down, HepG2 cells showed an increased proliferation rate compared with the control cells. Taken together, these results suggest that THAP11 might play a key role in the proliferation of HCC cells.

Overexpression of THAP11 represses the proliferation of several other cell lines. To study THAP11-induced cell growth inhibition in more detail, we developed a system for conditional expression of THAP11 in HEK293 cells. Three clones (clone 3, 6, and 15) that expressed THAP11 on treatment with Ponasterone A (Pon A), an ecdysone analog used as an inducing reagent, were identified using V5 epitope-specific antibody (Figure 3a). The inducible expression pattern of THAP11 was further analyzed in clone 3 cells. Although the protein was undetectable in the absence of PonA, the addition of PonA led to the detection of THAP11 24 h later and to its accumulation thereafter in a dose- and time-dependent manner (Figure 3b). The same inducible expression pattern of THAP11 was observed in clone 6 and 15 cells (data not shown). To analyze whether the expression of THAP11 affected cell proliferation, two cell clones (293-THAP11 clones 3 and 6) and control transfectants (parental 293EcR cells) were cultured in the presence or absence of PonA. No significant difference was observed in the growth kinetics of the cell lines in the absence of PonA (data not shown). However, the proliferation of 293-THAP11 cells was markedly inhibited in a dose-dependent manner when PonA was added to induce expression of THAP11 from days 3 to 6 (Figure 3c). Further, the inhibition of DNA synthesis following induction of THAP11 expression was confirmed using a [^{3}H] thymidine

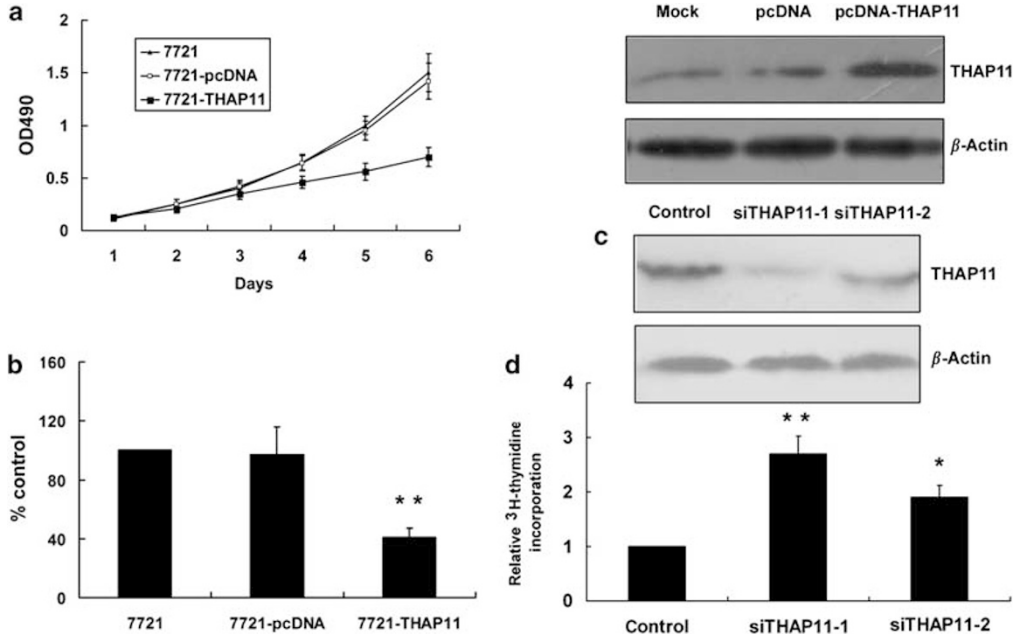

Figure 2 THAP11 is involved in regulation of hepatoma cell growth. (**a**) Human hepatoma cell line 7721 cells were transfected with pcDNA-THAP11 or control vector pcDNA3.1 together with pEGFP plasmid to normalize the transfection efficiency. At 12 h after transfection, 1×10^3 cells were seeded in each well of 96-well plates. MTS assay was performed from 1 day to 6 days after the transfection. The expression levels of THAP11 protein was determined by western blotting analysis (right panel). (**b**) THAP11 overexpression inhibits colony formation of 7721 cells. Cells (7721) were transfected with pcDNA-THAP11 or pcDNA 3.1 and the G418-resistant clones were isolated for the colony formation assay. (**c**) Knockdown of THAP11 by siRNA oligos (siTHAP11) or negative control siRNA (control) was analyzed by western blotting analysis. β-actin was used as internal control. (**d**) [^{3}H]-thymidine incorporation assay was performed after downregulation of THAP11 by siRNA oligos. Results represented mean ± S.D. of three independent experiments. The statistical difference between the samples was demonstrated as $^*P \leqslant 0.05$ or $^{**}P \leqslant 0.001$

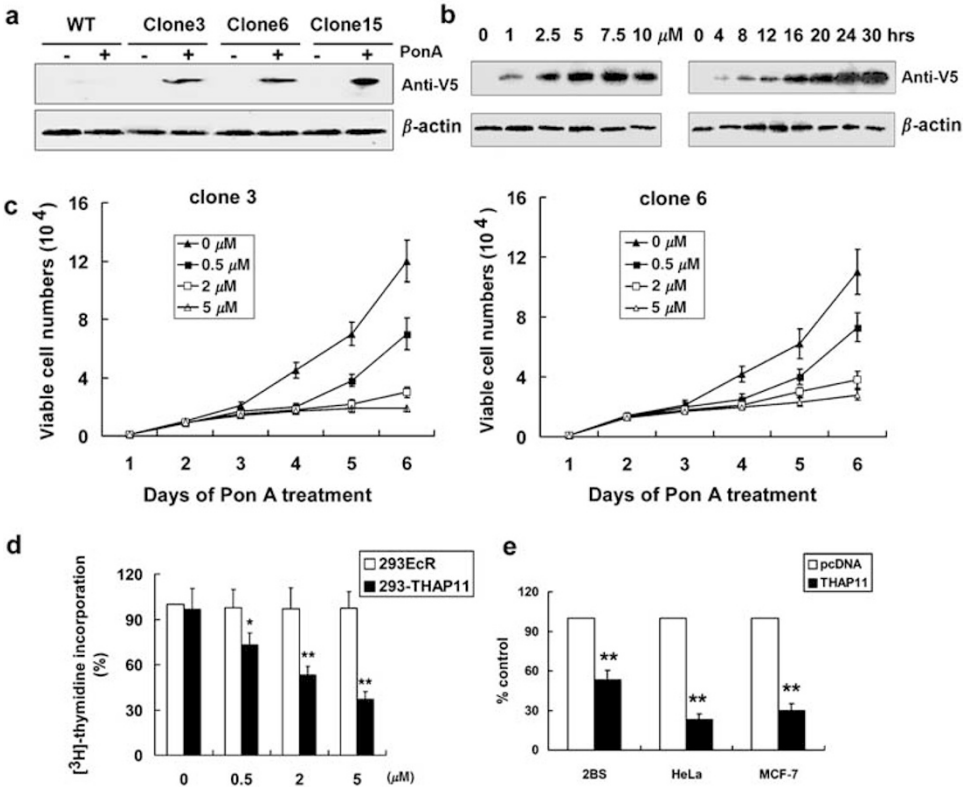

Figure 3 THAP11 expression results in cell growth inhibition. (**a**) Construction of inducible THAP11-expressing cell line. Differential cell clones were cultured with or without 5 μM PonA for 24 h. Cells were harvested and the levels of THAP11 protein were detected by western blotting analysis using anti-V5 antibody. β-actin was used as internal control. (**b**) 293-THAP11 Clone3 cells were exposed to 5 μM of PonA for the indicated time (Right) or to different concentrations of PonA for 24 h (Left). Cell lysates were prepared and THAP11 protein was detected by western blotting analysis. β-actin was used as internal control. (**c**) 293-THAP11 clone3 and 293-THAP11 clone 6 cells were seeded at 2×10^4/ml in complete medium with or without different concentrations of PonA. Cell numbers were counted every indicated day. (**d**) [^{3}H]-thymidine incorporation assay was performed in the absence or presence of PonA with 293EcR or 293-THAP11 clone3 cells after 2 days induction. These data represent relative [^{3}H]-thymidine incorporation in the presence and absence of PonA. (**e**) pcDNA-THAP11 or pcDNA3.1 vector were transfected into the cell lines indicated and colony formation assay were performed. Results represented mean $\pm$ S.D. of 3 independent experiments. The statistical difference between the samples was demonstrated as $^*P \leqslant 0.05$ or $^{**}P \leqslant 0.001$

incorporation assay. As shown in Figure 3d, induction of THAP11 in clone 3 cells reduced DNA synthesis by about 50% at 2 μM and 65% at 5 μM 3 days after the addition of PonA. Apoptosis analysis by Annexin-V/FACS assays revealed that the induction of THAP11 was not associated with the appearance of increased numbers of apoptotic cells (data not shown).

To further evaluate the potential role of THAP11 as a growth suppressor, we introduced THAP11 into the human diploid fibroblasts 2BS, human cervix adenocarcinoma cell line HeLa, and the human breast cancer cell line MCF-7 and measured the growth of G418-resistant clones harboring the pcDNA-THAP11 vector or pcDNA 3.1 vector. As shown in Figure 3e, THAP11 expression led to reductions in colony formation of approximately 65%, 80%, and 75%, respectively, in these cell lines. Taken together, these results indicated that THAP11 might function as a growth suppressor.

Overexpression of THAP11 suppresses c-Myc expression. Much evidence links c-Myc gene expression with cell proliferation, and reduction of its expression correlates with cell growth inhibition.[15,16] To examine whether the overexpression of THAP11 can reduce c-Myc expression, we investigated the expression of c-Myc during the induction of THAP11 expression. As shown in Figure 4a, 293-THAP11 cells expressed high levels of c-Myc protein. However, c-Myc protein expression decreased by 50% when the cells were cultured in the presence of PonA for 36 h to induce THAP11. The levels of c-Myc were comparable with those in control cells when the cultures were grown in the absence of PonA. We further examined the expression of c-jun and c-fos, which are also involved in cell growth and proliferation. In contrast to the changes seen in c-Myc expression, the expression of c-jun and c-fos was not suppressed at all in PonA-treated cells (Supplementary Figure S4). These data suggest that the c-Myc expression is inhibited selectively by THAP11. To confirm this result, hepatoma cell line HepG2 cells were transfected with siRNA oligos. As shown in Figure 4b, c-Myc expression was increased 2–2.5-fold when the endogenous THAP11 was knocked down.

To determine the molecular mechanism of c-Myc repression by THAP11, a c-Myc promoter reporter construct was transfected into the 293-THAP11 cells and cultured in the presence of different concentrations of PonA. As shown in Figure 4c, induction of THAP11 repressed c-Myc promoter

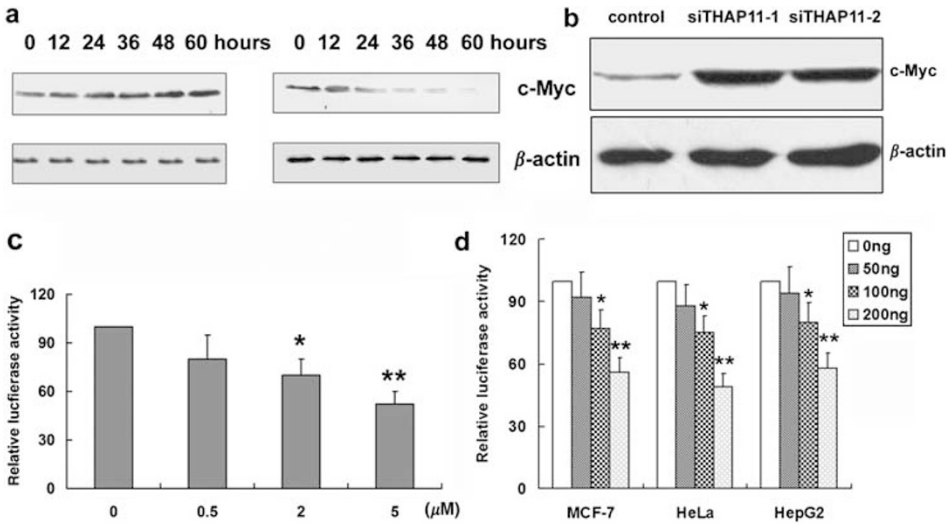

Figure 4 THAP11 downregulates c-Myc expression. (**a**) Proteins were extracted at the indicated time points from 293-THAP11 cells cultured in the absence (left) or presence (right) of 5 μM PonA. Western blotting was carried out with anti c-Myc antibody. β-actin was used as an internal control. (**b**) HepG2 cells were transfected with a negative control siRNA (control) or THAP11-specific siRNA oligos (siTHAP11-1 and siTHAP11-2) and then the cell lysates were prepared 24 h post-transfection for western blotting analysis using c-Myc antibody. (**c**) 293-THAP11 cells were transfected simultaneously with c-Myc promoter luciferase reporter plasmid, and pRL-TK as a control for transfection efficiency. Luciferase activity was measured after 24 h in the absence or presence of PonA as described in Materials and Methods. (**d**) The c-Myc promoter luciferase reporter construct was transfected with different doses of pcDNA-THAP11 plasmids into the indicated cell lines. Twenty-four hours later, the luciferase activity was measured. Results represented mean ± S.D. of three independent experiments. The statistical difference between the samples was demonstrated as $^*P \leqslant 0.05$ or $^{**}P \leqslant 0.001$

activity in a dose-dependent manner. Incubation with 5 μM PonA for 24 h reduced by approximately 52% the activity of c-Myc promoter. The same results were obtained in MCF-7, HeLa, and HepG2 cells by co-transfection of the c-Myc promoter reporter construct and pcDNA-THAP11 (Figure 4d). These data suggest that THAP11 represses the transcriptional activity of c-Myc.

THAP11 physically interacts with the c-Myc promoter. To further understand the mechanism by which THAP11 repressed c-Myc transcriptional activity in detail, a series of deletions of the c-Myc promoter were constructed to map the THAP11-responsive region (Figure 5a). We used 293-THAP11 cell line with reporter genes to test the activity of these deletion constructs, and found that fragments from -710 to -463 bp upstream of the c-Myc P2 promoter were required to mediate a 50–60% reduction in luciferase expression in response to THAP11 expression.

To determine whether THAP11 bound to the c-Myc promoter *in vivo*, chromatin was immunoprecipitated from 293-THAP11 clone 3 cells without Pon A treatment or at 24 h after PonA treatment, with either V5 monoclonal antibody or the unrelated FLAG M2 monoclonal antibody. The precipitated chromatin was queried by PCR with primers that amplified the region of -710 to -463 bp or other regions within the c-Myc promoter (-1218 to -918 bp) as a negative control. As shown in Figure 5b, a single amplification was produced by chromatin from 293-THAP11 cells treated with PonA and immunoprecipitated with V5 antibody. This binding was not observed in 293-THAP11 cells without PonA treatment or amplication of other distal region within the c-Myc promoter (-1218 to -918 bp). Furthermore, the levels of THAP11 occupancy at the c-Myc promoter were analyzed by quantitative ChIP assay. As Figure 5c shows, treatment of the cells with PonA resulted in high occupancy by THAP11 at the region of -710 to -463 bp showing an approximately 30-fold increase compared with the control. However, no significant difference was observed in the occupancy level of THAP11 at the region of -1218 to -918 bp. These results indicated that THAP11 binds to the c-Myc promoter *in vivo* and that this binding was restricted to the region of -710 to -463 bp.

As the THAP domain (DNA-binding domain) in THAP11 shows very high identity with Ronin,[14] we propose that the consensus binding sequence of THAP11 should be similar to that of Ronin. Analysis of the c-myc promoter revealed a consensus Ronin-binding sequence at -604 bp/-584 bp (Supplementary Figure S5). To confirm whether THAP11 bound to this sequence, electrophoretic mobility shift assay (EMSA) was performed by using HEK293 cells transfected with the pCMV-THAP11(Flag) vector and biotin-labeled probes indicated. As shown in Figure 5d, the shifted bands shown with an arrowhead were apparently observed in the presence of the probe Ronin-binding sequence (RBS) and oligo A that encompasses the 20 bp fragment, suggesting that nuclear proteins recognizing the probes were present in nuclear extracts of cells transfected with THAP11. To confirm whether the protein complexes that bound to the probe were indeed THAP11, gel supershift assays were undertaken with Flag monoclonal antibody. As expected, the bands were weakened by the addition of the anti-Flag antibody. Competition experiments showed that these bindings were effectively inhibited by a 100-fold molar excess of the probes. These results showed that THAP11 interacts with the c-Myc promoter.

Overexpression of c-Myc reverses THAP11-mediated cell growth inhibition. To address whether c-Myc overexpression could reverse THAP11-mediated cell

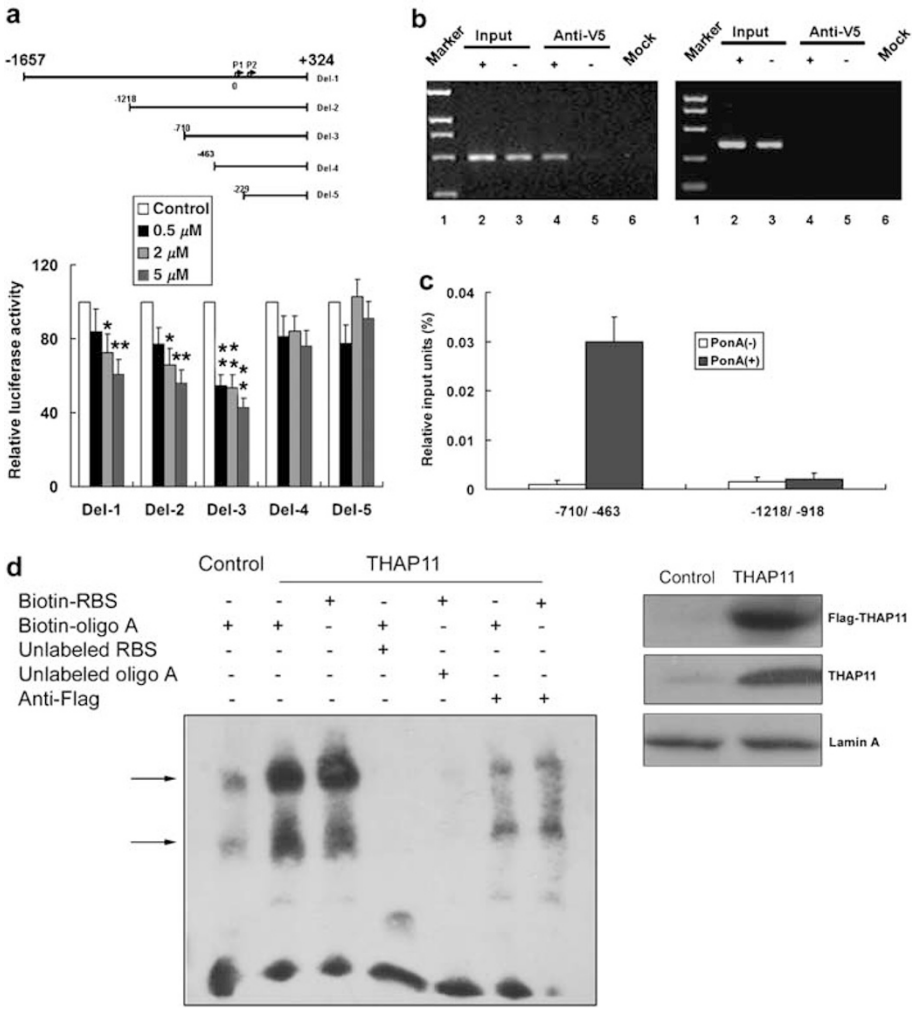

Figure 5 THAP11 binds to c-Myc promoter. (**a**) The THAP11-responsive region was located in the region from −710 to −463 bp of the c-Myc promoter . The horizontal lines represent the sequence in each reporter construct. P1 and P2 are start sites of transcription; P2 is the major start site. The location of each fragment was marked from the P2 transcription start site. The deletion mutants were respectively cotransfected into 293-THAP11 cells. Luciferase activity was measured after 24 h in the absence or presence of PonA at the indicated concentration. Results represented mean ± S.D. of three independent experiments. The statistical difference between the samples was demonstrated as $*P \leqslant 0.05$ or $**P \leqslant 0.001$. (**b**) Total chromatin was isolated from 293-THAP11 cells cultured in the presence (lanes 2, 4, 6) or absence (lanes 3, 5) of 5 μM PonA after 24 h induction and ChIP was performed as described in Materials and Methods. Presence of −710 to −463 region of c-Myc promoter (left) was determined by carrying out the ChIP assay with anti-V5 antibody (lanes 4 and 5). PCR of −1250 to −910 bp region of c-Myc promoter was performed as a negative control (right). The mock lane represents a ChIP assay performed on a sample inmmunoprecipitated with unrelated FLAG M2 monoclonal antibody. The amounts of specific DNA fragments that were present in the immunoprecipitates were quantitated by real-time PCR. (**c**) The bars indicate the percentage of the input DNA fragment in specific immunoprecipitates after subtracting the percentage in immunoprecipitates using control antibody. Error bars indicate the s.d. of duplicate PCRs. Similar results were obtained in three repeat experiments. (**d**) HEK293 cells were transfected with pCMV-THAP11(Flag) vector or control vector and nuclear extracts were prepared for EMSA. Then 10 μg nuclear protein was combined with biotin-labeled RBS and Oligo A. The arrowhead pointed to the specifically shifted bands. For supershift assay, anti-Flag monoclonal antibody was added. For competition experiments, 100-fold excess of unlabeled oligonucleotides indicated were added. The expression of THAP11 was determined by western blotting (right panel) using anti-Flag antibody and THAP11 antibody

growth inhibition, we cloned c-Myc and THAP11 into pIRES, a mammalian expression vector that can simultaneously express two genes of interest at high levels. As shown in Figure 6a, transfecting this plasmid resulted in significant overexpression of both c-Myc and THAP11, and the level of THAP11 expression was comparable with the cells transfected with pIRES-THAP11. For protection assays, HEK293 cells, MCF-7 cells, HepG2 cells, and HeLa cells were transfected with pIRES-Myc-THAP11, pIRES-THAP11, or the pIRES vector, and the growth of the cells was analyzed by colony formation assays. As Figure 6b shows, overexpression of c-Myc provided partial but significant protection against THAP11-mediated cell growth inhibition. From these findings, we concluded that growth arrest induced by THAP11 was at least partially associated with loss of c-Myc expression.

Overexpression of THAP11 only slightly inhibits c-Myc null fibroblasts cell growth. To further show that c-Myc is indeed required for the anti-proliferative effect of THAP11, we detected the effects of THAP11 on the growth of c-Myc null fibroblasts. Embryonic fibroblasts derived from wild type

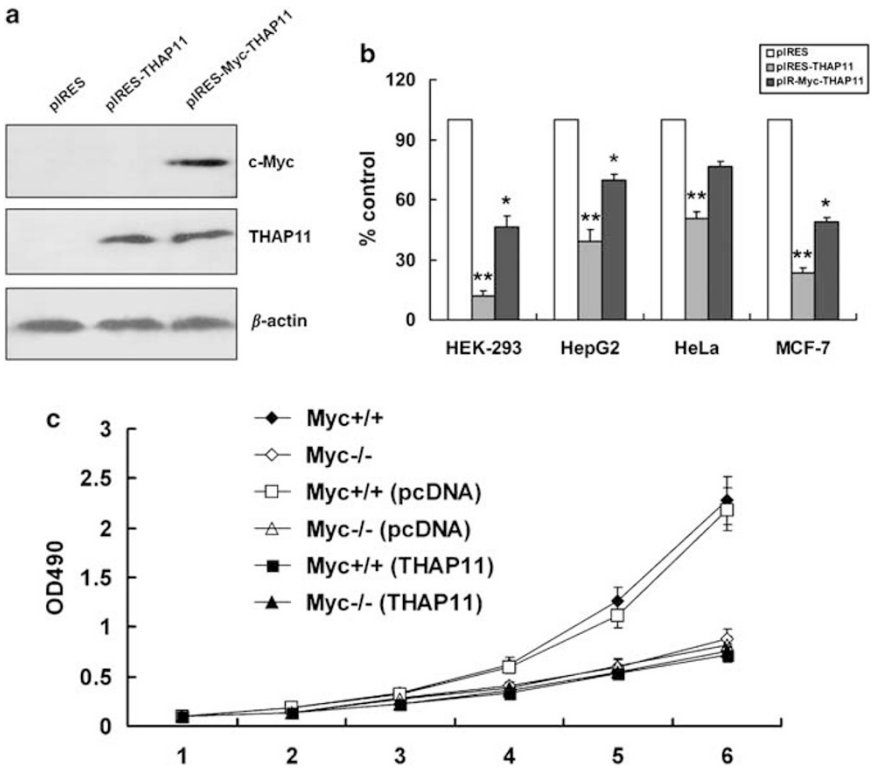

Figure 6 Cell growth inhibition by THAP11 is mediated by c-Myc. (**a**) HEK-293 cells were transfected with pIRES, pIRES-THAP11 or pIRES-Myc-THAP11 vector respectively. The cells were harvested after 36 h and the expression of c-Myc and THAP11 were analyzed by western blotting analysis using anti-Myc and anti-THAP11 antibody. (**b**) The cell lines were transfected with pIRES, pIRES-THAP11 or pIRES-Myc-THAP11. The average number of colonies after 14 days was determined in three independent experiments. $*P<0.05$; $**P<0.01$. (**c**) Overexpression of THAP11 only slightly inhibit c-Myc null fibroblasts cells growth. Embryonic fibroblasts derived from wild-type ($Myc^{+/+}$) or knockout ($Myc^{-/-}$) mice were transfected with pcDNA-THAP11 or the control vector pcDNA3.1. At 12 h after transfection, 1×10^3 cells were seeded in each well of 96-well plates. Viable cells were determined by MTS assay at the indicated time points after the transfection

($c\text{-}Myc^{+/+}$) or knockout ($c\text{-}Myc^{-/-}$) mice were transfected with pcDNA-THAP11 or the control vector pcDNA3.1. At 12 h after transfection, 1×10^3 cells were seeded and viable cells were determined by MTS assay at the indicated time points. The results showed that the growth of c-Myc wild-type fibroblast cells was markedly inhibited after transfection with pcDNA-THAP11 compared with control vector. In contrast, the proliferation of $c\text{-}Myc^{-/-}$ fibroblasts was unaffected by the overexpression of THAP11 despite their poor proliferation rate, as compared with $c\text{-}Myc^{+/+}$ fibroblasts (Figure 6c). These results clearly show that cell growth inhibition by THAP11 is mediated by c-Myc transcriptional repression.

THAP11 overexpression regulates c-Myc target genes. As a transcription factor, c-Myc binds to the promoters of a large number of genes controlling metabolic processes, macromolecular synthesis, the cell cycle and apoptosis and regulates their expression. These genes include cyclinD1,[17] p27Kip1,[18] p21,[19] Nucleolin,[20] ODC,[21] and hTERT.[22] We therefore examined the effect of THAP11 on the expression of these genes in 293-THAP11 cells by western blotting (Figure 7a) or RT-PCR (Figure 7b). The protein expression levels of p27*KIP1*, p53, did not change over time in the presence of PonA. However, the addition of PonA caused a marked decrease in Cyclin D1 protein and a significant increase in p21 protein, which is negatively regulated by c-Myc. The upregulation of p21 expression became apparent 24 h after THAP11 expression, and downmodulation of cyclin D1 expression became apparent 48–60 h after THAP11 expression. Moreover, RT-PCR analysis suggested that Nucleolin and ODC were significantly downregulated and that hTERT was slightly downregulated. No significant difference in the mRNA level of Cyclin A was observed.

Discussion

This study shows, for the first time, that THAP11 is downregulated in tumor tissues and has growth inhibitory properties. Overexpression of THAP11 markedly inhibits growth of a number of different cell lines. Silencing of THAP11 by RNA interference resulted in loss of cell growth repression. These results verified that human THAP11 may be an endogenous physiological regulator of cell proliferation. The finding that expression of c-Myc rescued significantly cells from THAP11-mediated cell growth suppression and THAP11 expression only slightly inhibits $c\text{-}Myc^{-/-}$ fibroblasts cell growth revealed that THAP11 inhibited growth through downregulation of c-Myc expression. ChIP and EMSA assays revealed that THAP11 binds to c-Myc promoter, indicating that c-Myc is one of the target genes of THAP11 and providing important

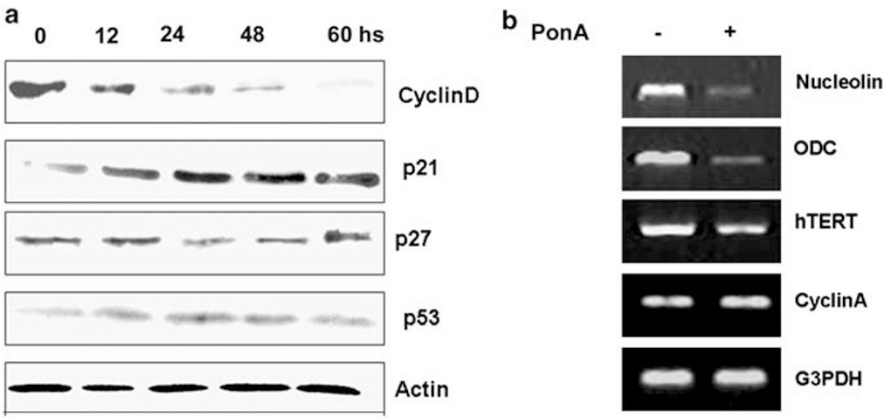

Figure 7 THAP11 overexpression regulates c-Myc target genes. Cells (293-THAP11) were cultured in the absence or presence of 5 μM PonA for 60 h. Total lysates were prepared for western blotting analysis (**a**) with β-actin as internal control and total RNA was extracted for RT-PCR analysis with G3PDH as internal control (**b**)

mechanistic insights into the role of THAP11 in the regulation of cell proliferation.

The proto-oncogene c-Myc is involved in a wide range of cellular processes including proliferation, differentiation, apoptosis and tumorigenesis. Several key target genes have also been identified in c-Myc-induced cell growth, transformation and tumorigenesis, including cyclins and cyclin-dependent kinases (Cdks),[23] c-Myc-dependent upregulation of metabolic enzymes and ribosomal proteins.[24] The data presented here show that THAP11-mediated reduction in c-Myc protein levels resulted in significantly decreased cyclin D1, Nucleolin and ODC expression and upregulation of p21. Various studies suggest that aberrant overexpression of cyclinD1 links to loss of cell cycle control and a wide variety of malignancies.[25,26] In contrast, downregulation of cyclin D1 expression induces the inhibition of proliferation and the reversion of the transformed phenotype. Nucleolin is a multifunctional protein shown to be involved in ribosome assembly, nucleo-cytoplasmic transport and cell proliferation.[27] ODC, a housekeeping enzyme involved in polyamine synthesis, is perhaps the best studied target of c-Myc and is necessary for cell proliferation.[28] Inhibition of either Nucleolin or ODC has been found to cause cell growth inhibition.[29] $p21^{WAF1/CIP1}$ is a well-characterized cyclin-dependent kinase (cdk) inhibitor that belongs to the Cip/Kip family of cdk inhibitors.[30] A study using microarray analysis has implicated p21 as one of the major targets of c-Myc repression[23] and the downregulation of p21 was associated with restoration of cell proliferation control.[31] Therefore, downregulation of cyclin D1, Nucleolin and ODC and upregulation of p21 resulting from the down-modulation of c-Myc may be involved in a key mechanism of THAP11-mediated growth arrest.

Many transcription factors have been shown to bind to the c-Myc promoter directly and alter its expression; these include CTCF, E2F4, MBP-1, MAZ, and BLIMP-1.[32–36] We demonstrated here that THAP11 represses c-Myc promoter activity by binding to the c-Myc promoter. The binding consensus sequence has the same conserved sites with the binding sites of Ronin.[14] It is reported that Ronin interacts with HCF-1 which is involved in epigenetic modification, raising the intriguing possibility that Ronin suppresses the activity of multiple genes by binding directly to DNA and then recruiting HCF-1 and thus chromatin-modifying proteins. Furthermore, among the THAP family, THAP7 is also reported to interact with chromatin-modifying elements including histone and HDACs.[12] In this paper, we provided evidence that THAP11 suppresses c-Myc transcription by binding directly to the c-Myc promoter; however, whether THAP11 represses c-Myc transcription by the same epigenetic modification mechanism as Ronin or THAP7 remains to be determined.

The gene encoding THAP11 maps to a region of human chromosome 16 (16q22.1) that is one of the most frequently altered region in human malignancies.[37,38] Consistent with these findings, many tumor suppressor candidate genes have been mapped to this region including E-cadherin and CTCF.[39,40] In this study, we find that THAP11 is a transcriptional repressor of c-Myc oncogene, and that knockdown of endogenous THAP11 increases both cell proliferation and c-Myc expression. Array hybridization revealed that THAP11 is frequently expressed at low levels in HCC patients, and that overexpression of THAP11 induces growth inhibition. All of these findings supported the hypothesis that THAP11 may be a candidate for a tumor suppressor.

Recently, Ronin, the mouse homolog of THAP11, was identified as a novel type of pluripotency factor. Conditional knockout of Ronin prevents the growth of murine ES cells whereas forced expression of Ronin promotes the self-renewal of murine ES cells.[14] Previous studies also have reported that sustained c-Myc activity maintains murine ES cell self-renewal in the absence of pro-maintenance factors such as LIF.[41] However, the self-renewal of ES cells in mouse and human are regulated by different pathways and mechanisms. The LIF/STAT3 and BMPs pathways, which are sufficient to support murine ES cell self-renewal, do not support human ES cells self-renewal.[41] In contrast to murine ES cells, c-Myc promotes both apoptosis and differentiation in human ES cells, but does not support self-renewal.[42] So, it is a very interesting question that whether THAP11 and the suppression of c-Myc by THAP11 play a role in self-renewal of human ES cells. Furthermore, although THAP11 shows high homology to Ronin, the expression patterns of THAP11 and Ronin are quite different. Ronin is expressed primarily

during the earliest stages of murine embryonic development whereas THAP11 is ubiquitously expressed in various human tissues, suggesting a complex transcriptional regulation mechanism of THAP11 in human beings. Further studies are required to clarify these notions.

Materials and Methods

Expression analysis using the tumor tissue array. The Cancer Profiling Arrays II (BD Biosciences Clontech, Palo Alto, CA, USA) consists of 154 paired cDNA samples generated from the total RNA of 19 tissue types. Each pair consists of a tumor sample and a corresponding normal tissue sample obtained from the same patient. This array, when hybridized with a probe for a housekeeping gene, yielded a fairly consistent hybridization signal for all samples. A THAP11-specific cDNA fragment was radio-labeled using a Prime-a-Gene labeling assay (Promega Corp., Madison, WI, USA.), hybridized overnight at 68 °C using ExpressHyb Hybridization Solution (Clontech), washed, and exposed to Biomax MS X-ray film with an intensifying screen (Eastman Kodak Co.).

Tissue specimens. All human HCC specimens were obtained from those patients who underwent surgical resection of their diseases and were informed consent before operation on their liver. The primary tumor specimens were immediately frozen at −80°C until RNA extraction. Both tumor and adjacent non-tumor tissues were sampled respectively, with approximate 1 cm^3 size of each specimen, and were proved by pathological examination.

Cell culture. The human breast cancer cell line MCF-7, human hepatoma cell line HepG2, human cervix adenocarcinoma cell line HeLa, and monkey kidney cell line COS-7 were obtained from the American Type Culture Collection (ATCC). 293EcR is a cell line derived from HEK293 cells that constitutively expresses a modified form of the ecdysone receptor (Invitrogen, Carlsbad, CA, USA). Mouse embryonic fibroblasts derived from wild-type ($Myc^{+/+}$) or knockout ($Myc^{-/-}$) mice is a gift from Dr. John Sedivy.[43] Human fetal lung diploid fibroblasts 2BS and human hepatoma cell line 7721 was obtained from the National Institute of Biological Products (Beijing, China). Cells were maintained in Dulbecco's modified Eagle's medium (Gibco Invitrogen, CA, USA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 100 μg/ml penicillin and 100 μg/ml streptomycin.

Plasmids. The full-length cDNA coding human THAP11 was obtained through RT-PCR from human bone marrow cDNA (Clontech) and cloned into ecdysone-inducible eukaryotic expression vector pIND-LacZ (His/V5) (Invitrogen) (pIND-THAP11), the eukaryotic expression vector pcDNA3.1 (Invitrogen) (pcDNA-THAP11), pCMV-Flag (Sigma) (pCMV-THAP11(Flag)), pEGFP-N1 (Clontech), (pTHAP11-GFP), and pM (Clontech) (pM-THAP11). To construct the pIRES-THAP11, the full-length of THAP11 was cloned into the *Xbal/Sal*I sites of MCS B of pIRES (BD Biosciences). pIRES-Myc-THAP11 was obtained by cloning the c-Myc cDNA into the *Eco*RI sites within MCS of pIRES-THAP11 in correct orientation; the internal ribosome entry site (IRES) from the encephalomyocarditis virus (ECMV) is located between these two genes. c-Myc promoter luciferase reporter vector was kindly provided by Dr. Kinzler of Johns Hopkins Oncology Center. The deletion series of human c-Myc promoter luciferase reporter constructs Del-2, Del-3, Del-4 and Del-5 were generated by subcloning the corresponding genomic fragments of c-Myc promoter into the *Mlu*/*Bgl*II site of the luciferase expression vector pGL3-basic (Promega). Sequences of recombinant DNA were verified by automated sequencing. The sequences of the primers are provided in Supplementary Table S1.

Production of THAP11 polyclonal antibody. Human THAP11 fragment (from 217 residue to 314 residue in N terminus of THAP11) coding cDNA was cloned into the expression vector pGEX-4T-2 (Amersham Pharmacia Piscataway, NY, USA), recombinant proteins were expressed and purified according to the manufacturer's protocol. A protein polyclonal antibody against THAP11 was raised by using the recombinant fusion protein GST- N98-THAP11 as antigen and fractionated from the rabbit anti-serum.

Generation of a HEK293 cell line expressing inducible THAP11. The pIND-THAP11 plasmids, as well as an empty vector control, were transfected into the 293EcR cell line by Lipofectamine 2000 (Invitrogen) and selected for stable transfection with 400 μg/ml G418 (Sigma) and 400 μg/ml Zeocin (Invitrogen) for 2 weeks. Clones of the transfectants were obtained by limiting dilution and identified by western blotting. Ponasterone A (PonA, Invitrogen) was dissolved in 100% ethanol before adding to cells for induction.

Reverse transcription-PCR and quantitative real-time RT-PCR. Total RNA was reverse-transcribed and amplified using reverse transcription (Promega) and PCR (Promega) kits, respectively, according to the manufacturer's instructions. Multiple-tissue cDNA (Clontech) was amplified according to the manufacturer's instructions. The sequences of the primers are provided in Supplementary Table S1. The PCR products were electrophoresed on 1% agarose gels and photographed under ultraviolet light. Quantitative real-time RT-PCR was done by ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA).

Analysis of cellular proliferation

Growth curves. Cells (1×10^3) were seeded in each well of 96-well plates and left overnight to adhere. Cell number was then counted with the use of a cytometer after 1, 2, 3, 4, 5 and 6 days of incubation at 37°C. Each point represents the average of four independent determinations.

MTS. Cells (1×10^3) were seeded in each well of 96-well plates and left overnight to adhere. Absorbance was determined by using CellTiter 96 Aqueous One Solution Reagent (Promega) according to the manufacturer's protocol at indicated time points. All experiments were conducted three times.

Colony formation assays. Cells were transfected with indicated plasmids with Lipofectamine 2000 (Invitrogen), and then plated on a 60-mm tissue culture dish (2×10^3 cells per well) and cultured in DMEM supplemented with 10% FBS. The cells were allowed to grow for 10 days in the presence of neomycin with medium changes every 2 days, after which they were washed, fixed with methanol, and stained with Giemsa (Sigma). Colonies 2-mm or greater in size were scored. All experiments were conducted three times.

[^{3}H]-thymidine incorporation assay. Cells were seeded into 96-well plates. On the next day, an inducing reagent (or ethanol as control) was added. After induction of 2 days, cellular proliferation was measured by adding 0.5 μCi of [^{3}H]-thymidine (ICN Biomedicals Inc.) for 3 h, after which cells were harvested onto glass fiber filters. Incorporated radioactivity was quantitated by scintillation counting.

Western blotting. For western blots, 10^6 cells were lysed with 30 μl TNT buffer (20 mM Tris-HCl [pH 7.5], 200 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitors leupeptin, aprotinin, pepstatin A, chymostatin, and antipain each at a final concentration of 10 μg/ml). Then, 20 μg of protein from each sample was loaded onto the gel. After separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred to nitrocellulose membranes and probed with various antibodies at the following concentrations: THAP11, 1 : 200; c-Myc (Santa Cruz, sc-42), 1 : 1000; p21 (Santa Cruz, sc-6246), 1 : 1000; p27 (Santa Cruz, sc-1641), 1 : 1000; p53 (Santa Cruz, sc-126), 1 : 1000; cyclinD1 (Santa Cruz, sc-8196), 1 : 1000; c-Jun (Santa Cruz, sc-1694), 1 : 500; c-Fos (Santa Cruz, sc-413), 1 : 500; β-actin (Santa Cruz, sc-1616), 1 : 1000; and anti-V5 (Invitrogen), 1 : 1000. Chemiluminescent detection was conducted using supersignal substrate (Pierce), according to the manufacturer's specifications.

Luciferase reporter assays. Luciferase assays were carried out at 24 h post-transfection with the Dual-luciferase Reporter Assay System (Promega). Transfection efficiencies were normalized using cotransfected plasmid pRL-TK measured by *Renilla* luciferase activity (Promega). For ecdysone-inducible expression cell line, cells were transfected with c-Myc promoter constructs and pRL-TK as internal control. After 6 h transfection, the PonA or ethanol was added to the medium to start to induce the expression of THAP11. Twenty-four hours later luciferase activity was measured.

RNAi. Two small interfering RNA (siRNA) oligos of THAP11 were synthesized in GenePharma Biotechnology, the sequences are as follows: siTHAP11-1, sense: GCUGCACUUCUACACGUUUTT; antisense: AAACGUGUAGAAGUGCAGCTT; siTHAP11-2, sense: CAUCGAUCUCACAGUGCAATT; antisense: UUGCACU GUGAGAUCGAUGGG. The negative control (non-silencing) siRNA were obtained from Qiagen (Germantown, MD, USA). siRNA were transfected into HepG2 cells using Lipofectamine 2000 (Invitrogen) at a concentration of 20 nM.

Quantitative chromatin immunoprecipitation assays. The chromatin immunoprecipitation procedure was adapted from the methods of Eberhardy *et al.*[44] Briefly, 293-THAP11 cells were treated with PonA or ethanol as control to induce THAP11 expression. After 24 h of induction, cells were crosslinked in 0.4% formaldehyde/PBS for 10 min at room temperature. Crosslinking was stopped with 0.125 M glycine. Chromatin was sonicated, precleared with irrelevant antibodies, and immunoprecipitated with anti-V5 antibody (2 μg, Invitrogen) or unrelated FLAG M2 antibody (Sigma) pre-bound to protein A/G beads. Beads were washed seven times, and the bound proteins were eluted into 100 mM $NaHCO_3$/1% SDS. Crosslinks were reversed at 65°C for 4 h, and protein was digested with proteinase K (0.3 mg/ml) for 2 h at 45°C. DNA was purified by phenol/chloroform extraction and ethanol precipitation. PCRs containing 3 μl of the above. DNA preparation were performed with the initial denaturation at 95°C for 4 min, followed by 30 PCR cycles (95°C for 45 s, 55°C for 30 s and 72°C for 30 s) and a final extension at 72°C for 10 min. The sequences of the primers used for the PCR are given in Supplementary Table S1. The amounts of the specific DNA fragment in immunoprecipitates were determined by quantitative PCR. The copy number of a specific DNA fragment in each immunoprecipitation was compared with the copy number of that fragment in the DNA before immunoprecipitation (input DNA) and a percentage of the input was calculated. Percentage of input was also determined for each DNA fragment in immunoprecipitation using appropriate control antibodies and these values were subtracted from the values obtained with the specific antibodies.

Electrophoretic mobility shift assay. Nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents, and EMSA was performed using the LightShift Chemiluminescent EMSA kit, according to the manufacturer's instructions (Pierce, Rockford, IL, USA). The biotin-labeled oligonucleotides used were as follows: Ronin-binding strand (RBS): 5′-ATCA ACTGTATTACAAGCTAGGACAGCACCCTAATGTC-3′; Oligo A: 5′-TCTTTCCT CCACTCTCCCTGGGACTCTTGATCAAAGCG-3′. For the competitor assay, a 100-fold excess of various unlabeled double-stranded DNA was added to the reaction mixture prior to the addition of the labeled probe. For the supershift assay, antibodies against Flag (Sigma) were used.

Statistical analysis. All experiments were performed at least three times. Data were reported as means ± S.D. and the statistical significance was assessed by a one-way analysis of variance followed by Students–Newman–Keuls tests. A value of $P \leqslant 0.05$ was considered to be significant.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)