ORIGINAL ARTICLE

Par-4/THAP1 complex and Notch3 competitively regulated pre-mRNA splicing of CCAR1 and affected inversely the survival of T-cell acute lymphoblastic leukemia cells

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Although the intensification of therapy for children with T-cell acute lymphoblastic leukemia (T-ALL) has substantially improved clinical outcomes, T-ALL remains an important challenge in pediatric oncology. Here, we report that the cooperative synergy between prostate apoptosis response factor-4 (Par-4) and THAP1 induces cell cycle and apoptosis regulator 1 (CCAR1) gene expression and cellular apoptosis in human T-ALL cell line Jurkat cells, CEM cells and primary cultured neoplastic T lymphocytes from children with T-ALL. Par-4 and THAP1 collaborated to activate the promoter of CCAR1 gene. Mechanistic investigations revealed that Par-4 and THAP1 formed a protein complex by the interaction of their carboxyl termini, and THAP1 bound to CCAR1 promoter though its zinc-dependent DNA-binding domain at amino terminus. Par-4/THAP1 complex and Notch3 competitively bound to CCAR1 promoter and competitively modulated alternative pre-mRNA splicing of CCAR1, which resulted in two different transcripts and played an opposite role in T-ALL cell survival. Despite Notch3 induced a shift splicing from the full-length isoform toward a shorter form of CCAR1 mRNA by splicing factor SRp40 and SRp55, Par-4/THAP1 complex strongly antagonized this inductive effect. Our finding revealed a mechanistic rationale for Par-4/THAP1-induced apoptosis in T-ALL cells that would be of benefit to develop a new therapy strategy for T-ALL.

INTRODUCTION

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive malignancy of T-cell precursors.[1] The prostate apoptosis response factor-4 (Par-4) is a pro-apoptotic factor and induces apoptosis in cancer cells,[2,3] which urges us to explore whether it can be utilized as an effective therapy for T-ALL.

Par-4 selectively gives rise to apoptosis in a wide variety of cancers, whereas leaving normal cells unaffected. This selective nature suggests Par-4 may be an attractive therapeutic option. The leucine zipper domain of par-4 spans amino acids 290–332, of which the primary function is to allow protein–protein and protein–DNA interactions.[3] It was reported that arsenic treatment induced the upregulation of par-4 and led to apoptosis in the leukemia cell lines HL-60 and K562.[4] However, its exact molecular mechanisms remain to be fully elucidated.

Cell cycle and apoptosis regulator 1 (CCAR1) is a transcriptional coactivator for nuclear receptors and exerts its functions as a key intracellular signal transducer of apoptosis signaling pathways.[5] Recent studies showed that CCAR1 could suppress the growth of human breast cancer and resulted in cell apoptosis by the activation of caspase-9.[6] However, it is unknown whether CCAR1 is involved in Par-4-induced apoptosis of neoplastic T lymphocytes.

Notch signaling is an evolutionarily conserved pathway implicated in various functions, including the regulations of cell apoptosis and proliferation. In mammals, there are four Notch receptors (Notch1–4). Once membrane-bound receptor interacts with its ligand, Notch receptor is cleaved and the intracellular domain (ICD) is translocated to the nucleus that activates the transcription of target genes.[7] Deregulated activation of Notch pathways markedly contributes to the generation of T-ALL.[8] Therefore, detailing potential functions of Notch molecule and blocking aberrant Notch signaling is essential for developing new effective therapeutic approaches against T-ALL.

THAP1, a sequence-specific DNA-binding factor, belongs to a large family of transcription factors sharing a common zinc-finger motif responsible for DNA binding.[9] THAP1 can modulate G1/S cell-cycle progression and cellular proliferation.[10] To date, no research has demonstrated that THAP1 participates in the regulation of survival and apoptosis in neoplastic T lymphocytes.

In this study, we define a novel molecular mechanism underlying Par-4/THAP1 protein complex-induced CCAR1 expression and cellular apoptosis in T-ALL cells.

RESULTS

Cooperative synergy between Par-4 and THAP1 induced CCAR1 gene expression and cellular apoptosis in T-ALL cells

As Par-4 is a pro-apoptotic transcription factor and CCAR1 is involved in cellular apoptosis,[3] we examined first whether over-expression of Par-4 might stimulate CCAR1 expression in T-ALL cells. Figure 1a and Supplementary Figure S1 showed that expression of CCAR1 protein was markedly increased in Jurkat

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Figure 1. Par-4 and THAP1 cooperatively induced CCAR1 gene expression and cellular apoptosis in T-ALL cells. (**a**) pcDNA3-Par-4 was transfected into Jurkat cells and CEM cells with increasing dosages and CCAR1 protein was detected with western blotting. * and #: $P<0.05$, compared with transfection with empty pcDNA3. (**b**) Interaction of Par-4 with THAP1 was determined by two-hybrid testing. The β-galactosidase activity of reporter gene was measured. *$P<0.01$, compared with Bar 1~6. (**c**) Jurkat cells and primary neoplastic T lymphocytes from three patients were transfected with either pcDNA3-Par-4 or pcDNA3-THAP1 or a combination of both. CCAR1 protein was detected with western blotting. Compared with transfection with empty pcDNA3, ■, Δ, *, #: $P<0.01$. This figure shows the results of three representatives of patients with T-ALL. (**d**) Jurkat cells and primary neoplastic T lymphocyes were transfected with either pcDNA3-Par-4 or pcDNA3-THAP1 or a combination of both. The percentage of apoptotic cells was measured by FACS (fluorescence-activated cell sorter) analysis. *, #, ★, Δ: $P<0.01$, compared with transfection with empty pcDNA3. This figure shows the results of three representatives of patients with T-ALL. (**e**) Both pcDNA3-Par-4 and THAP1 siRNA were co-transfected into Jurkat cells and the primary neoplastic T lymphocytes. Similarly, Jurkat cells and the primary neoplastic T lymphocyes were transfected with pcDNA3-THAP1 together with Par-4 siRNA. CCAR1 protein was detected with western blotting.

cells and CEM cells after transient transfection with Par-4 expression vectors.

Because Par-4 exerted its transcriptional control on target genes by interacting with some Par-4-binding partner proteins,[11] we next performed a yeast two-hybrid screen of a human T-ALL cell cDNA library using Par-4 as a bait to identify cellular targets of Par-4. Sequencing of positive clones demonstrated two identical library plasmids that corresponded to a partial cDNA coding sequences for some amino acids of the proapoptotic factor THAP1. The liquid β-galactosidase assay showed that co-transformed with Par-4 bait and THAP1 prey vectors led to a marked induction of lacZ reporter gene activity. This observation validated an interaction between Par-4 and THAP1 (Figure 1b).

It thus seemed feasible to test the hypothesis that Par-4 cooperated with THAP1 to induce CCAR1 expression and promote T-ALL cells apoptosis. As shown in Figures 1c and d and

Supplementary Figure S2, the co-transfection with both expression plasmids Par-4 and THAP1 increased significantly CCAR1 expression and enhanced the percentage of apoptotic cells to a median of 44% from a median of 9% or 11% (transfection with expression plasmids Par-4 or THAP1 alone, respectively) in Jurkat cells and the primary cultured neoplastic T lymphocytes from children with T-ALL. Moreover, the activity of caspase-9, which mediates cellular apoptosis, was upregulated by co-transfection with both the expression plasmids Par-4 and THAP1 (Supplementary Figure S3).

Further, western blotting analysis demonstrated that the suppression of THAP1 expression by small interfering RNA (siRNA) significantly attenuated Par-4-induced CCAR1 protein expression (Figure 1e, Supplementary Figures S4A, B and C). Similarly, Par-4 siRNA markedly attenuated THAP1-induced CCAR1 protein expression as well.

Together, these data indicated that cooperative synergy between Par-4 and THAP1 induced CCAR1 gene expression and apoptosis in T-ALL cells.

Par-4 and THAP1 cooperated to activate the promoter of CCAR1

Reverse transcriptase–PCR (RT–PCR) analysis revealed that CCAR1 mRNA level increased in response to the transfection with Par-4 plasmids, indicating that Par-4 regulated the level of CCAR1 protein at transcriptional level (Figure 2a). Thus, we hypothesized that Par-4 might directly or indirectly activate CCAR1 promoter.

To address this hypothesis, we used 5′ RACE (Rapid Amplification of cDNA Ends) technique to map the transcription start sites of CCAR1 basing the sequences of Gene ID 55749 and NM_018273.2. With the Promoter 2.0 Prediction Program and deletion analysis, the promoter P1 of CCAR1 was determined, which was located at −286 bp upstream from transcription start sites (Figures 2b and c).

Further, we investigated the effects of Par-4 and THAP1 on CCAR1 promoter. We built a reporter construct pGL3-CCAR1-P1, which contained CCAR1 promoter P1. Figure 2d showed that co-transfection with both pcDNA3-Par-4 and pcDNA3-THAP1 resulted in a significant increased activity of the CCAR1 promoter P1, compared with the transfection of either pcDNA3-Par-4 or pcDNA3-THAP1 alone.

Collectively, these results are consistent with the hypothesis that Par-4 and THAP1 cooperate to activate the promoter of CCAR1.

Par-4 and THAP1 formed a protein complex and bound to CCAR1 promoter P1 by a THAP1-binding motif

We further identified the physical interaction among these molecules. Immuno-cytochemical analysis showed that an increased co-localization of Par-4 and THAP1 was observed in the nucleus of Jurkat cells, primary T-ALL cells and 293T cells after co-transfection with both pcDNA3-Par-4 and pcDNA3-THAP1 (Figure 3). Moreover, Figure 4a demonstrated that Par-4 co-immunoprecipitated with THAP1 protein in nuclear extracts from the cells exposed to co-transfection. These data indicated the direct interaction between Par-4 and THAP1.

Having found that Par-4 generally exerts indirect transcriptional control on target genes though interacting with other partner protein, rather than direct binding to DNA,[11] we focused on investigating the regulatory effect of THAP1 on CCAR1 promoter P1. The previous research revealed that the consensus sequence of THAP1-binding site comprised a core 'GGCA' motif.[12] On the basis of this, two candidate DNA-binding sites within the region of CCAR1 promoter for THAP1, THAP1-S1 (−387 bp ahead of ATG) and THAP1-S2 (−270 bp ahead of ATG) were presumed. Luciferase activity assays revealed that the mutation of the THAP1-S1 did not significantly affect the activity of CCAR1 promoter P1. However, co-transfection of the reporter plasmids containing a THAP1-S2 mutant and pcDNA3-THAP1 resulted in a decrease of luciferase activity, indicating that THAP1-S2 was indispensable for CCAR1 promoter P1 responding to THAP1 stimulation (Figure 4b).

Electromobility shift assay (EMSA) experiments *in vitro* were further performed with the nuclear extracts from the Jurkat cells exposed to transfection with pcDNA3-THAP1. The results revealed that the wild-type THAP1-S2 appeared to form a DNA–protein complex following the transfection of pcDNA3-THAP1 (Figure 4c, Lane 3). Moreover, the DNA–protein complex formed between the THAP1 protein and THAP-S2 was supershifted by an anti-THAP1 antibody (Figure 4c, Lane 4), indicating that this binding was specific. Then, EMSA experiments were performed with the nuclear
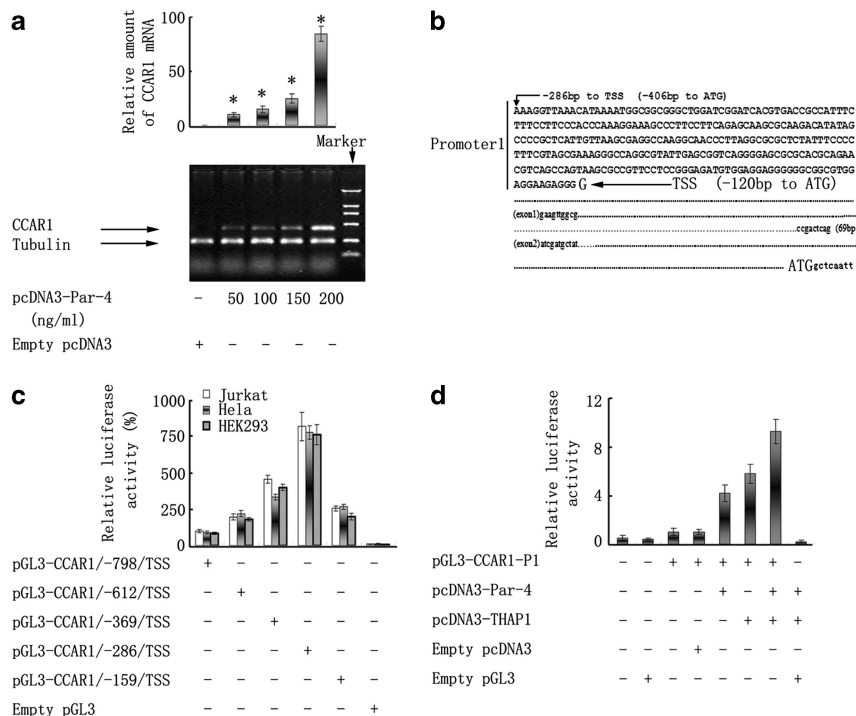
Figure 2. Par-4 and THAP1 cooperated to activate an alternative promoter of CCAR1. (**a**) pcDNA3-Par-4 was transfected into Jurkat cells with increasing dosages, and relative amount of CCAR1 mRNA was detected with RT–PCR. *$P < 0.05$, compared with transfection with empty pcDNA3. (**b**) 5′ RACE experiments were performed to map the transcription start sites (TSS) of CCAR1 in Jurkat cells. Nucleotide sequences of a promoter P1 of CCAR1 are shown. (**c**) Various deletion constructs of the putative CCAR1 promoter P1 were transfected into Jurkat cells, Hela cells and HEK293 cell lines. Luciferase activity assays were performed for characterization of CCAR1 promoter P1. The reporter activities were expressed as the percentage of transfection of the pGL3-CCAR1/-798/TSS, in which the DNA fragment spanning from −798 bp to TSS was contained. (**d**) Jurkat cells were co-transfected with pGL3-CCAR1-P1 reporter. Luciferase activity was determined and normalized for expression of Renilla luciferase and expressed as X-fold induction relative to the activity of pGL3-CCAR1-P1 reporter co-transfected with empty pcDNA3.
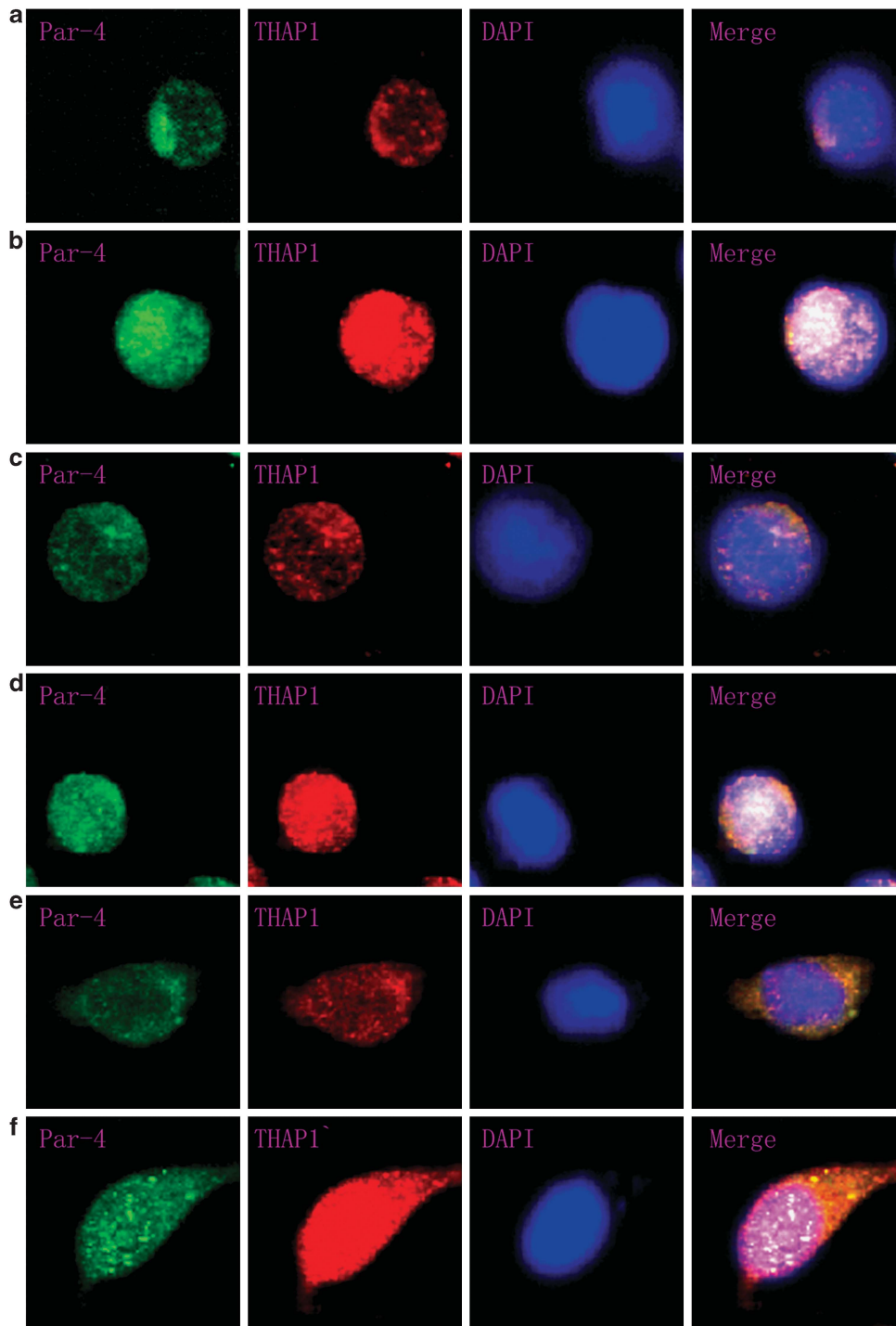
Figure 3. Co-localization of Par-4 and THAP1 in cell nuclei. Jurkat cells (**b**), primary T-ALL cells (**d**) and 293T cells (**f**) were co-transfected with both pcDNA3-Par-4 and pcDNA3-THAP1. Par-4 and THAP1 were stained with anti-Par-4 (Green) and anti-THAP1 (Red) antibodies, respectively. DAPI (4′,6-diamidino-2-phenylindole; Blue) was used to show cell nucleus. Co-localization is shown in Merge. Blue, green and red overlap results in white. Jurkat cells (**a**), primary T-ALL cells (**c**) and 293T cells (**e**) were co-transfected with empty plasmids as controls.

extracts from the Jurkat cells exposed to co-transfection with both pcDNA3-Par-4 and pcDNA3-THAP1. As shown in Figure 4d (Lane 4), two complexes were formed with the THAP1-S2 probe. Competitive EMSA experiments with unlabeled THAB-S2 oligonu-cleotides demonstrated that both of the two complexes were specific (Figure 4d, Lane 5). To identify the presence of Par-4 and THAP1 in these complexes, nuclear extracts were incubated with THAP1-S2 probe along with blocking antibodies directed against Par-4 or THAP1. This anti-Par-4 antibody has been demon-strated previously as a neutralizing antibody,[11] which caused only disappearance of the slower migrating complex, suggesting the presence of Par-4 (Figure 4d, Lane 6). The blocking antibody against THAP1 was screening by EMSA (Supplementary Figure S5), which caused disappearance of both the faster and the slower migrating complex, suggesting that THAP1 protein was present in both the complexes (Figure 4d, Lane 7). These observations
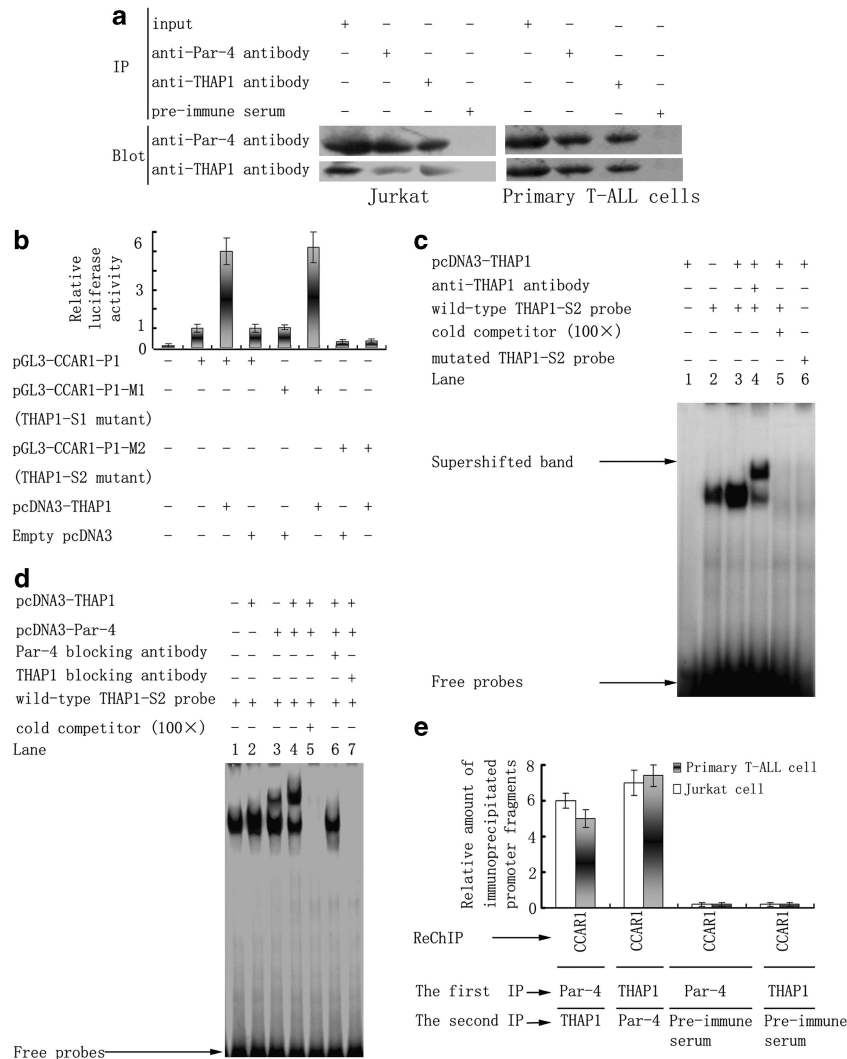
Figure 4. Par-4 and THAP1 formed a protein complex and bound to CCAR1 promoter P1 by a THAP1-binding motif. (**a**) Jurkat cells and the primary T-ALL cells were transfected with pcDNA3-Par-4 and pcDNA3-THAP1. Immunoprecipitations and western blotting analysis were performed with anti-Par-4 antibody and anti-THAP1 antibody. (**b**) Mutation analysis of two candidate DNA-binding sites for THAP1, THAP1-S1 (-387 bp head of ATG) and THAP1-S2 (-270 bp ahead of ATG) within the region of CCAR1 promoter P1. The reporter vectors having mutations (THAP1-S1 mutant or THAP1-S2 mutant) were transiently transfected into Jurkat cells together with pcDNA3-THAP1. The reporter activities were expressed as the fold increases over the co-transfection of the wild-type pGL3-CCAR1-P1 and empty pcDNA3 vectors. (**c**) DNA-binding activities of THAP1-S2 within CCAR1 promoter P1 assessed by EMSA. After the transfection of pcDNA3-THAP1, nuclear extracts were incubated with either wild-type or mutated THAP1-S2 probes in the presence or absence of anti-THAP1 antibody. The arrows indicate supershifts of the bands by antibodies against THAP1. (**d**) Jurkat cells were transfected with pcDNA3-Par-4 and pcDNA3-THAP1 alone or together. Nuclear extracts were incubated with THAP1-S2 probe along with blocking antibodies directed against Par-4 or THAP1. DNA-binding activity was detected by EMSA. (**e**) Jurkat cells and the primary T-ALL cells were transfected with pcDNA3-Par-4 and pcDNA3-THAP1. With anti-Par-4, anti-THAP1 or control pre-immune antibodies, ChIP-reChIP assay was carried out as described under MATERIALS AND METHODS. The immunoprecipitated DNA fragments were analyzed by real-time PCR with primers for CCAR1 promoter P1. The relative amounts of immunoprecipitated promoter fragments after normalizing to their respective levels in the input are shown. Data are presented as mean ± s.e. of three separate experiments.

indicated that it was THAP1, not Par-4, in Par-4–THAP1 proteins complex that directly bound to CCAR1 promoter P1.

Next, sequential chromatin immunoprecipitation (ChIP-reChIP) assays were used to investigate whether Par-4 and THAP1 associated *in vivo* with the chromatin of endogenous CCAR1 promoter. With specific antibodies against Par-4 and THAP1, we immunoprecipitated chromatin from the cells with the co-transfected of both pcDNA3-Par-4 and pcDNA3-THAP1. With PCR primers for CCAR1 promoter P1, genomic DNA fragments bound to Par-4 or THAP1 were detected. Analysis of genomic DNA immunoprecipitated with either anti-Par-4 antibody or anti-THAP1 antibody revealed the presence of CCAR1 promoter P1 sequences

(Figure 4e). Our results clearly indicated that Par-4 and THAP1 could occupy together *in vivo* CCAR1 promoter P1 as a part of a complex.

Taken together, we concluded that multimolecular complex of Par-4 and THAP1 binding to CCAR1 promoter by a THAP1-binding motif contributed to transcriptional regulation of CCAR1 gene.

Both the death domain at the C-terminus of Par-4 and the zinc-dependent DNA-binding domain of THAP1 are necessary for Par-4/THAP1 protein complex to activate CCAR1 promoter P1

Next, we examined whether the death domain at the C-terminus of Par-4 was required for the formation of Par-4/THAP1 protein

complex. Co-immunoprecipitation assays showed that Par-4 deletion mutant, which lacked the death domain at COOH terminus of the wild-type Par-4 protein, failed to associate with THAP1 (Figure 5a, Lane 5). Figure 5b showed further that deletion of Par-4 death domain failed to bring about noticeable activation of CCAR1 promoter P1, whereas co-transfection with increasing amounts of full-length Par-4 led to in an increased P1 activity in a dose-dependent way. These results indicated that the death domain of Par-4 was responsible for the functional effects of Par-4 on the activation of CCAR1 promoter.

Because the first 90 amino-terminal residues of THAP1 displayed a C2-CH signature (Cys-X2-4-Cys-X35-50-Cys-X2-His), which contained a zinc finger structure and possessed zinc-dependent DNA-binding activity,[9,12] we next defined the functional role of the zinc-dependent DNA-binding domain of THAP1 on CCAR1 promoter activation. The zinc-dependent DNA-binding domain of human THAP1 (amino acids 1–90), designated THAP1/1-90aa, was amplified by RT–PCR and the recombinant protein was expressed and purified. Similarly, the carboxy terminus of THAP1 (amino acids 91–213), designated THAP1/91-213aa, and full-length THAP1 protein were prepared. Competitive EMSA experiments demonstrated that purified recombinant THAP1/1-90aa bound to the THAP1-S2 target sequence specifically (Figure 5c, Lane 2). Moreover, the DNA-binding activity was gradually inhibited by incubation of the THAP1/1-90aa with increasing amounts of metal-chelating agent 1, 10-o-phenanthroline before EMSAs (Figure 5c, Lanes 4–6). Particularly, the DNA-binding activity of THAP1/1-90aa was restored by the addition of zinc for pre-incubation (Figure 5c, Lane 7), indicating that the binding reaction was dependent on zinc. However, THAP1/91-213aa failed to bind to THAP1-S2 probes (Figure 5c, Lane 8). Therefore, the zinc-dependent DNA-binding domain was essential for THAP1 to bind to specific DNA sequences in CCAR1 promoter P1.

To define the regions of THAP1 interacting with Par-4, we carried out glutathione S-transferase (GST)-pulldown experiments in which in vitro synthesized Par-4 was incubated with GST-THAP1 deletion constructs purified from Escherichia coli. As shown in Figure 5d (Lane 5), Par-4 interacted strongly with the carboxy terminus of THAP1 (amino acids 91–213), whereas the interactions with the amino terminus of THAP1 (amino acids 1–90) could not be detected (Figure 5d, Lane 4). These results suggested that both carboxy termini of THAP1 and Par-4 were requisite for their interaction.

Further, both pGL3-CCAR1-P1 reporter and pcDNA3-Par-4 were co-transfected into Jurkat cells with pcDNA3-THAP1, pcDNA3-GFP-THAP1 (amino acids 1–90) or pcDNA3-FLAG-THAP1 (amino acids 91–213). Luciferase activity assays demonstrated that co-transfection with pcDNA3-FLAG-THAP1 (amino acids 91–213) failed to give rise to the activation of CCAR1 promoter P1, and moderate activation were detected following the co-transfection with pcDNA3-GFP-THAP1 (amino acids 1–90) (Figure 5e). However, although Par-4 pulled down truncated THAP1 (amino acids 91–213; Figure 5d, Lane 5), ChIP and reChIP analyses showed that FLAG-THAP1 (amino acids 91–213) could not bind to CCAR1 promoter P1 (Figure 5f). These results suggested that THAP1 associated with Par-4 through its carboxy terminus and activated CCAR1 promoter P1 through its amino terminus.

Collectively, these data indicated that Par-4 and THAP1 formed a protein complex by the interaction of their carboxy termini, and THAP1 bound to CCAR1 promoter P1 though its zinc-dependent DNA-binding domain at amino terminus.

Par-4/THAP1 complex and Notch3 competitively regulated alternative pre-mRNA splicing of CCAR1 and affected inversely T-ALL cell survival

Based on the fact that deregulated activation of Notch signals markedly contributed to the generation of T-ALL,[13] our next step was to determine the effects of Notch on Par-4/THAP1-regulated expression of CCAR1. Western blotting showed that enforced expression of exogenous Notch3-ICD resulted in the most striking attenuation of Par-4/THAP1-induced enhancement of CCAR1 protein (Figure 6a). Moreover, transfection with increasing amounts of Notch3-ICD induced a gradually increasing amount of a short band at the ∼90 kDa position besides a full-length CCAR1 protein at the 130 kDa position (Figure 6b). To address whether the novel band was a protein isoform or non-special band, CCAR1 isoform profiles were investigated at 48 h after co-transfection of pcDNA3-Par-4 and pcDNA3-THAP1 together with pcDNA3-Notch3-ICD by northern blotting assays for CCAR1 mRNA. Two transcripts, approximately 3.5 kb and 2.5 kb in length, were found (Figure 6c). RACE experiments and sequencing analysis confirmed that one was corresponding to a full-length CCAR1 mRNA and the short one was a novel transcript, which produced a putative truncated 91-kDa protein. The 2.5-kb mRNA transcript contained a deletion in exon 15—exon 22 of full-length CCAR1 cDNA, where sequences mapped to 1196–3120 bp were missed. We cloned this 2.5-kb fragment into an expression plasmid and transfected it into Jurkat cells. Western blotting confirmed that it produced a protein band at the ∼90 kDa position (Figure 6d), indicating that the 90 kDa variant represented a new spliced CCAR1 isotype. It was designated as truncated-CCAR1. Full-length CCAR1 protein and truncated-CCAR1 protein shared a common N-terminus and a common C-terminal end, but the latter had a deletion of SAP domain. As a DNA/RNA-binding domain, the SAP motif is involved in chromosomal organization and apoptosis.[14,15] Western blotting showed that truncated CCAR1 protein was detectable in T-ALL primary cells and T-ALL cell lines (Supplementary Figures S6A and S7). The overexpression of Notch3-ICD induced an increase of the truncated CCAR1 isoform along with a decrease of apoptosis rate (Supplementary Figure S6A and B). Notch3 silencing led to the disappearance of the truncated protein (Supplementary Figure S6A). These finding indicated that Par-4/THAP1 and Notch3 might competitively regulate alternative pre-mRNA splicing of CCAR1, resulting in two protein variants.

Based on the present finding that pro-apoptotic Par-4/THAP1 and pro-survival Notch3 modulated inversely the expressions of full-length CCAR1 and truncated-CCAR1, we carried out experiments to determine whether these variants functioned differently in T-ALL cell survival. Figure 6e demonstrated that truncated-CCAR1 inhibited full-length CCAR1-induced cellular apoptosis. Conversely, full-length CCAR1 antagonized the pro-survival roles of truncated-CCAR1 in T-ALL cells (Figure 6f, Supplementary Figure S8 and Supplementary Table S1). Hence, we concluded that full-length CCAR1 and truncated-CCAR1 had opposite effects on T-ALL cell survival.

The recent research that ICD of the Notch3 bound to promoter of target gene and regulated transcription[16] suggested the possibility of Notch3-ICD binding to CCAR1 promoter P1. To confirm this reasoning, we used TRANSFAC database to search potential sequences. Two putative Notch3-ICD-binding sites (Notch3-BS) were obtained within CCAR1 promoter P1, Notch3-BS1 and Notch3-BS2 located, respectively, at −171 bp and −262 bp upstream from translation initiation site ATG. For further experimental validation, EMSA and ChIP experiments were performed. Figures 7a and b showed that Notch3-ICD was able to bind to Notch3-BS2 in vitro and in vivo, which contained a TTAGGC core motif.

Next, we investigated whether the binding of Notch3-ICD to CCAR1 promoter P1 affected the interaction between Par-4/THAP1 and CCAR1 promoter P1. ChIP experiments demonstrated that enforced expression of exogenous Notch3-ICD suppressed Par-4/THAP1 protein complex binding to CCAR1 promoter P1 (Figure 7c). In contrast, co-transfection with pcDNA3-Par-4
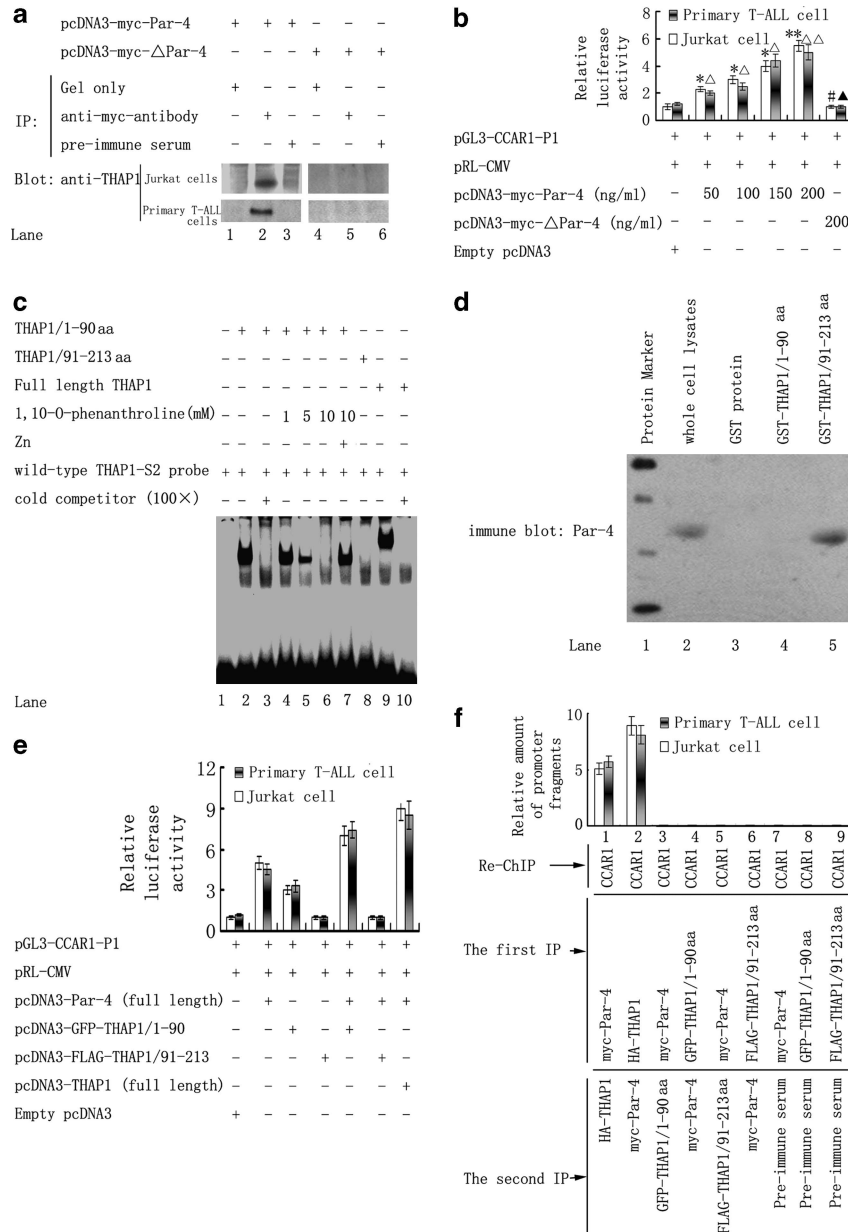
Figure 5. The death domain at the C-terminus of Par-4 and the zinc-dependent DNA-binding domain of THAP1 were both required for Par-4/THAP1 protein complex to activate CCAR1 promoter P1. (**a**) The pcDNA3-myc-ΔPar-4 was constructed, which encoded a myc fusion protein lacking the death domain at the COOH terminus of Par-4. Jurkat cells and the primary T-ALL cells were transfected with either pcDNA3-myc-ΔPar-4 or pcDNA3-myc-Par-4, of which the latter encoding the full-length Par-4 protein. Co-immunoprecipitation assays and western blot were performed with anti-myc antibody and anti-THAP1 antibody. (**b**) Both pGL3-CCAR1-P1 reporter and pcDNA3-THAP1 were co-transfected into Jurkat cells and the primary T-ALL cells with pcDNA3-myc-ΔPar-4 or pcDNA3-myc-Par-4. Luciferase activities were measured and pGL3-CCAR1-P1 reporter activities were normalized to pRL-CMV internal standard. All values are expressed as X-fold induction relative to the activity of pGL3-CCAR1-P1 under conditions where empty pcDNA3 was co-transfected. *$P < 0.05$, **$P < 0.01$, compared with the transfection with empty pcDNA3. #, ▲: $P > 0.05$, compared with the transfection of empty pcDNA3. (**c**) EMSA was performed to evaluate the DNA–protein interaction *in vitro* between ^{32}P-end-labeled THAP1-S2 probes and the recombinant THAP1/1-90aa and THAP1/91-213aa. The DNA-binding activity was detected after the incubation of the THAP1/1-90aa with increasing amounts of metal-chelating agent 1, 10-o-phenanthroline in the absence and presence of zinc pre-incubation. (**d**) GST pull-down experiment was performed with recombinant GST, GST-THAP1/1-90aa and GST-THAP1/91-213aa. Co-precipitation of Par-4 was detected by western blotting using an anti-Par-4 antibody. (**e**) The pGL3-CCAR1-P1 reporter and pcDNA3-Par-4 were co-transfected into Jurkat cells and the primary T-ALL cells with pcDNA3-THAP1, pcDNA3-GFP-THAP1/1-90 (amino acids 1–90) or pcDNA3-FLAG-THAP1/91-213 (amino acids 91–213). Luciferase activity assays were performed to detect the activity of CCAR1 promoter P1. All values were normalized for the expression of Renilla luciferase and expressed as X-fold induction relative to the activity of pGL3-CCAR1-P1. (**f**) Jurkat cells and the primary T-ALL cells were transfected with pcDNA3-myc-Par-4, pcDNA3-HA-THAP1, pcDNA3-GFP-THAP/1-90 and pcDNA3-FLAG-THAP1/91-213 alone or in combination. Pre-immune serum was used as a control. ChIP-reChIP assay were carried out as described under Materials and methods. The immunoprecipitated DNA fragments were analyzed by real-time PCR with primers for CCAR1 promoter P1. The relative amounts of immunoprecipitated promoter fragments after normalizing to their respective levels in the input are shown.
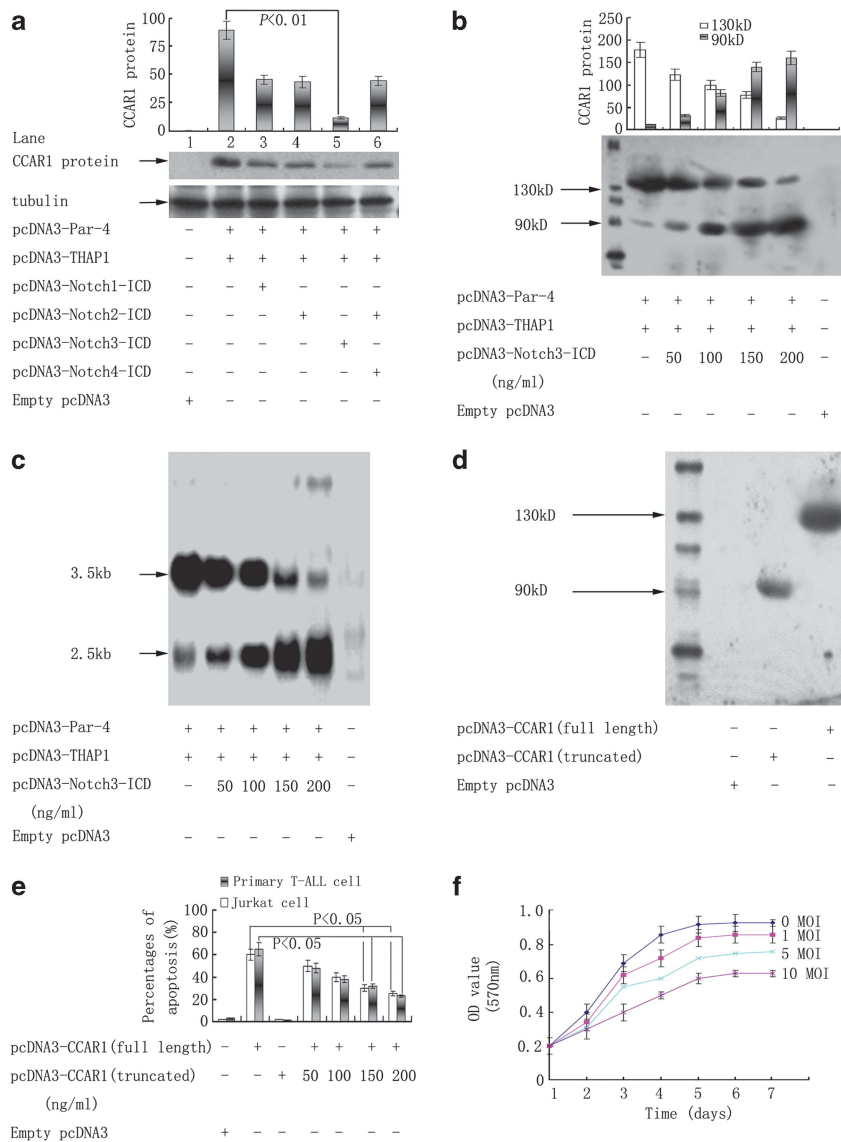
Figure 6. Par-4/THAP1 complex and Notch3 competitively regulated alternative pre-mRNA splicing of CCAR1 and affected inversely T-ALL cell survival. (**a**) Jurkat cells were co-transfected with both pcDNA3-Par-4 and pcDNA3-THAP1, together with plasmid expressing Notch1-ICD, Notch2-ICD, Notch3-ICD or Notch4-ICD. CCAR1 protein was detected by western blotting. (**b**) Jurkat cells were co-transfected with both pcDNA3-Par-4 and pcDNA3-THAP1, together with an increasing amount of pcDNA3-Notch3-ICD. CCAR1 protein was detected by western blotting. (**c**) Jurkat cells were co-transfected with both pcDNA3-Par-4 and pcDNA3-THAP1, together with an increasing amount of pcDNA3-Notch3-ICD. Northern blotting analysis was used to determine CCAR1 mRNA. (**d**) The full-length CCAR1 mRNA (3.5 kb) and the truncated transcript (2.5 kb) were cloned into expression plasmid pcDNA3 and were transfected into Jurkat cells. Western blotting was used to detect CCAR1 protein. (**e**) Jurkat cells and the primary T-ALL cells were transfected with both pcDNA3-CCAR1 (full length) and an increasing amount of pcDNA3-CCAR1 (truncated). The percentages of apoptosis were detected with FACS (fluorescence-activated cell sorter) analysis. (**f**) Jurkat cells were transducted with both recombinant lentivirus containing CCAR1 (truncated) cDNA and an increasing amount of recombinant lentivirus containing CCAR1 (full length) cDNA with a multiplicity of infection of 0, 1, 5 or 10. The cell proliferation was assessed with the MTT assay, and the growth curves are shown.

and pcDNA3-THAP1 suppressed Notch3-ICD binding to CCAR1 promoter P1 (Figure 7d). These results indicated that the competitive regulation between the Par-4/THAP1 complex and Notch3 on CCAR1 pre-mRNA alternative splicing was due to, at least partly, their competitive and exclusive bindings to CCAR1 promoter.

Collectively, our findings suggested that Par-4/THAP1 complex and Notch3 modulated competitively alternative pre-mRNA splicing of CCAR1 and played an opposite role in T-ALL cell survival, which was related with their competitive bindings to CCAR1 promoter.

Splicing factor SRp40 and SRp55 were involved in the competitive regulation of alternative pre-mRNA splicing of CCAR1 by Par-4/THAP1 and Notch3

We attempted further to elucidate the mechanisms specifically triggered by Par-4/THAP1 and Notch3 that would account for the shift in CCAR1 alternative mRNA splicing. Based on the previous research that the effects of Notch3 on alternative splicing of target gene was dependent on some splicing factors,[17] we analyzed the alternatively spliced CCAR1 exons 15–22 and intron 14–23 sequences using online ESE finder to identify putative binding motifs of known splicing factors. We found two high score motifs
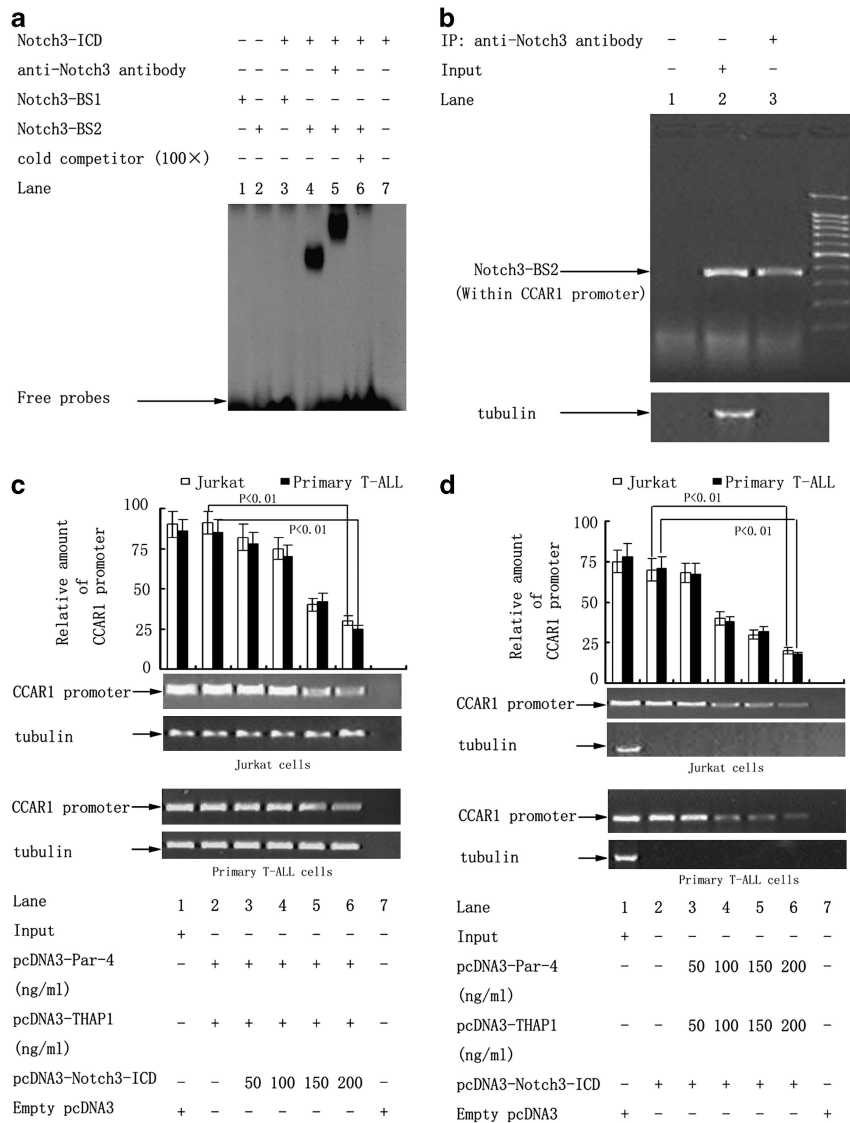
Figure 7. Notch3-ICD and Par-4/THAP1 complex competitively and exclusively bind to CCAR1 promoter. (**a**) DNA-binding activity of Notch3-BS within CCAR1 promoter P1 was assessed by EMSA. After the transfection of pcDNA3-Notch3-ICD, nuclear extracts were obtained from the Jurkat cells. Nuclear extracts were incubated with either Notch3-BS1 or Notch3-BS2 probes in the presence or absence of anti-Notch3 antibody. In competitive studies, a 100-fold molar excess of unlabeled probes was added to the binding reaction mixture before the addition of the labeled probes. Results shown are representative of three independent experiments. (**b**) Jurkat cells were transfected with pcDNA3-Notch3-ICD. ChIP experiments were performed with nuclear extracts. The precipitated DNA fragment containing Notch3-BS2 within the region of CCAR1 promoter was amplified by PCR. (**c**) Jurkat cells and primary T-ALL cells were co-transfected with both pcDNA3-Par-4 and pcDNA3-THAP1, together with an increasing amount of pcDNA3-Notch3-ICD. The precipitated THAP1-associated DNA fragment of CCAR1 promoter was amplified by PCR. (**d**) Jurkat cells and primary T-ALL cells were co-transfected with both pcDNA3-Notch3-ICD and the increasing amounts of pcDNA3-Par-4 and pcDNA3-THAP1. The precipitated Notch3-ICD-binded DNA fragment of CCAR1 promoter was amplified by PCR.

for SRp40 (TGTCAGG) and SFD2 (GTCAGGA) proteins in intron 14, which might be responsible of a donor splice site. Putative binding sites for SRp55 (TGAGTA) and SC35 (GGTCTGAG) proteins were found in intron 23, in which an acceptor splice site might be located. We designed ribooligonucleotides containing these binding motifs for use in RNA EMSAs to determine the functionality of these motifs in splicing binding. Figure 8a demonstrated that SRp40 bound specifically to wild-type binding motifs. A similar result was observed for SRp55-specific binding (Figure 8b). However, SFD2 and SC35 failed to associate with wild-type binding sites. Therefore, we concluded that SRp40 and SRp55 were involved in alternative splicing of CCAR1 pre-mRNA and might contribute to exons 15–22 skipping.

Next, real-time RT–PCR showed that the silencing of either SRp40 or SRp55 abrogated the effect of Notch3-ICD on CCAR1 isoform modulation and facilitated Par-4/THAP1 to induce more full-length transcript isoform of CCAR1. In contrast, the over-expression of either SRp40 or SRp55 resulted in an increasing shift toward short transcript isoform of CCAR1 in Notch3-ICD-trans-fected cells, importantly, which could be reversed by co-transfection with Par-4 and THAP1 expression plasmids and induce an increasing shift toward full-length transcript isoform (Figures 8c–f). These results demonstrated that splicing factor SRp40 and SRp55 were involved in the competitive regulation of alternative pre-mRNA splicing of CCAR1 by Par-4/THAP1 and Notch3.
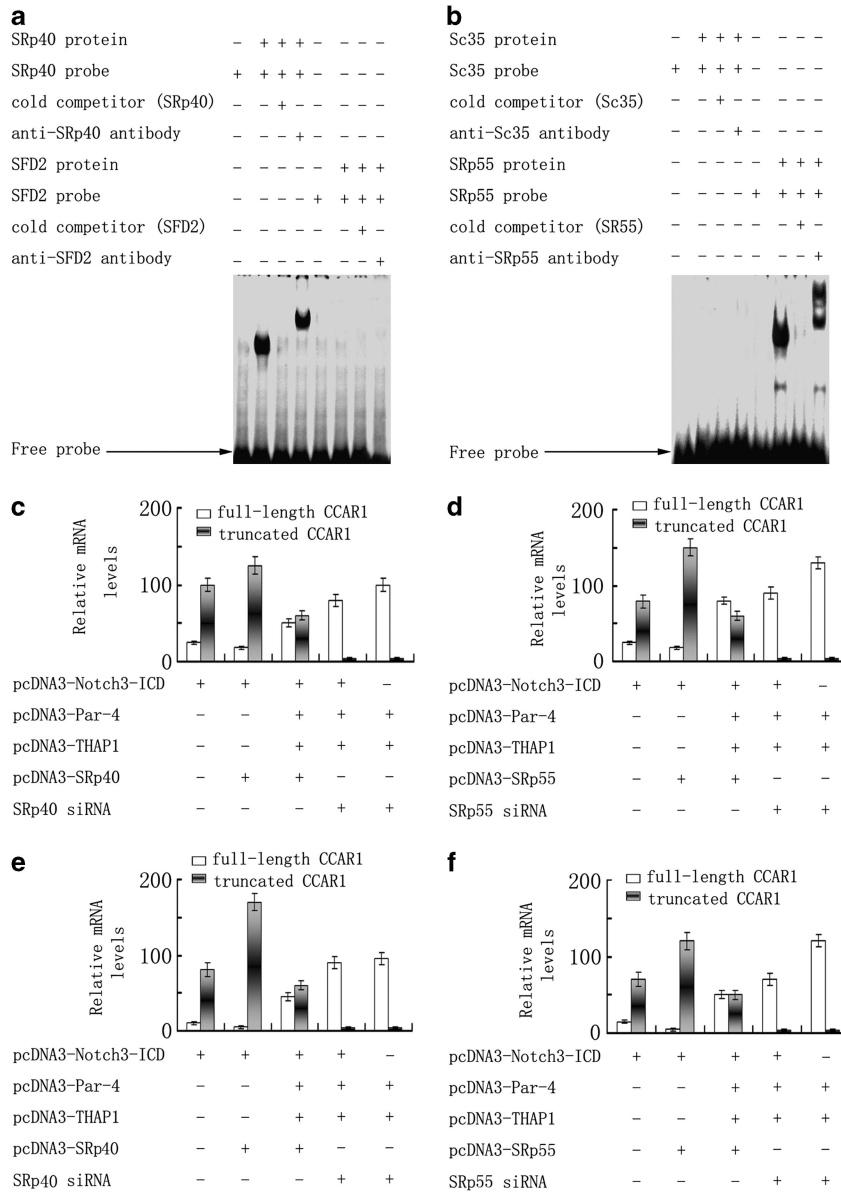
a

SRp40 protein	− + + + − − −
SRp40 probe	+ + + + − − −
cold competitor (SRp40)	− − + − − − −
anti-SRp40 antibody	− − − + − − −
SFD2 protein	− − − − + + +
SFD2 probe	− − − + + + +
cold competitor (SFD2)	− − − − − + −
anti-SFD2 antibody	− − − − − − +

Free probe →

b

Sc35 protein	− + + + − − −
Sc35 probe	+ + + + − − −
cold competitor (Sc35)	− − + − − − −
anti-Sc35 antibody	− − − + − − −
SRp55 protein	− − − − + + +
SRp55 probe	− − − + + + +
cold competitor (SR55)	− − − − − + −
anti-SRp55 antibody	− − − − − − +

Free probe →

c

Relative mRNA levels

☐ full-length CCAR1
▨ truncated CCAR1

pcDNA3-Notch3-ICD	+	+	+	+	−
pcDNA3-Par-4	−	−	+	+	+
pcDNA3-THAP1	−	−	+	+	+
pcDNA3-SRp40	−	+	+	−	−
SRp40 siRNA	−	−	−	+	+

d

Relative mRNA levels

☐ full-length CCAR1
▨ truncated CCAR1

pcDNA3-Notch3-ICD	+	+	+	+	−
pcDNA3-Par-4	−	−	+	+	+
pcDNA3-THAP1	−	−	+	+	+
pcDNA3-SRp55	−	+	+	−	−
SRp55 siRNA	−	−	−	+	+

e

Relative mRNA levels

☐ full-length CCAR1
▨ truncated CCAR1

pcDNA3-Notch3-ICD	+	+	+	+	−
pcDNA3-Par-4	−	−	+	+	+
pcDNA3-THAP1	−	−	+	+	+
pcDNA3-SRp40	−	+	+	−	−
SRp40 siRNA	−	−	−	+	+

f

Relative mRNA levels

☐ full-length CCAR1
▨ truncated CCAR1

pcDNA3-Notch3-ICD	+	+	+	+	−
pcDNA3-Par-4	−	−	+	+	+
pcDNA3-THAP1	−	−	+	+	+
pcDNA3-SRp55	−	+	+	−	−
SRp55 siRNA	−	−	−	+	+

Figure 8. Splicing factor SRp40 and SRp55 contributed to Notch3-induced shift in alternative splicing of CCAR1 pre-mRNA, which was antagonized by Par-4/THAP1. RNA EMSAs were used to determine the binding of splicing factor SRp40 (**a**) and SRp55 (**b**) to CCAR1 mRNA. Further verification of RNA–protein complexes was accomplished by supershift assays using anti-SRp40 and anti-SRp55 antibodies. SRp40 and SRp55 were either overexpressed or knocked down with either co-transfection of pcDNA3-SRp40 and pcDNA3-SRp55 or co-transfection of SRp40 siRNA and SRp55 siRNA. The real-time RT–PCR was used to detect the relative mRNA levels of full-length and truncated CCAR1 in the Jurkat cells (**c**, **d**) and the primary T-ALL cells (**e**, **f**) exposed to transfection with pcDNA3-Notch3-ICD alone or together with pcDNA3-Par-4 and pcDNA3-THAP1.

Together, our findings suggested that splicing factor SRp40 and SRp55 were responsible for the shift splicing from the full-length isoform toward a shorter form of CCAR1 mRNA by Notch3, which was antagonized by Par-4/THAP (Figure 9).

DISCUSSION

In the present study, we demonstrated for the first time that Par-4 collaborated with THAP1 to induce CCAR1 gene expression, which in turn caused cellular apoptosis in T-ALL cells. Our results demonstrated that Par-4 and THAP1 could form a protein complex and bound to CCAR1 promoter P1 by a THAP1-binding motif. This conclusion was supported by several experimental evidences. First, interaction of Par-4 with THAP1 was confirmed with a yeast two-hybrid screen of a human T-ALL cell cDNA library. Second,

competitive EMSA experiments verified protein–protein complex of Par-4 and THAP1 binding to CCAR1 promoter by a THAP1-binding site. Third, in vivo ChIP-reChIP experiments provided compelling evidence for this conclusion as well. Consistent with the previous data,[2,11] our finding indicated that the death domain at the COOH-terminus of Par-4 was responsible of the interaction between Par-4 and THAP1. Furthermore, the zinc-dependent DNA-binding domain was essential for THAP1 to bind to specific DNA sequences in CCAR1 promoter P1.

In this study, we found that Par-4/THAP1 complex and Notch3 modulated competitively alternative pre-mRNA splicing of CCAR1 and, the two variants played an opposite role in T-ALL cell survival. Similar observation for different variants of the same gene was shown by Ling et al.[18] that survivin-2B and survivin-ΔEx3 were two isoforms of survivin and played opposite roles in the modulation
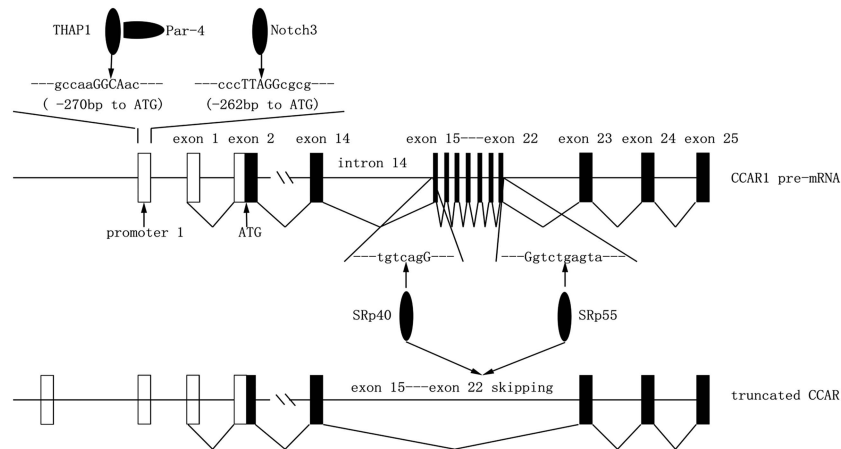
Figure 9. Schematic drawing showing the spliced structure of the CCAR1 pre-mRNA.

of cancer cell viability and patient survival in non-small-cell lung cancer.[18] We further verified that splicing factor SRp40 and SRp55 contributed to shift in alternative splicing of CCAR1 pre-mRNA by Par-4/THAP1 and Notch3. SRp40 and SRp55 are two splicing factors and have previously been researched for their functions in alternative splicing.[19] Our results showed that Notch3-ICD promoted SRp40 and SRp55 binding to 5' and 3' splice sites, respectively, at exon/intron boundaries, leading to the skipping of exons 15–22 and the production of a truncated variant. Conversely, the silencing of either SRp40 or SRp55 abolished the effect of Notch3-ICD on CCAR1 isoform regulation and facilitated Par-4/THAP1 to induce full-length variant of CCAR1, which promoted T-ALL cellular apoptosis. Aberrant and constitutively active Notch signaling has crucial role on oncogenic transformation of T lymphocytes and is a hallmark of T-ALL.[13] The counter regulatory effect of Par-4/THAP1 on Notch signaling revealed that Par-4 and THAP1 could be used for developing a novel therapeutic strategy for T-ALL. This conclusion was supported by previous data that Par-4 increased sensitivity to TRAIL-induced apoptosis in neoplastic lymphocytes and imatinib induced apoptosis in CLL lymphocytes with high expression of Par-4.[20,21]

In summary, we here provided the first evidence that cooperative synergy between Par-4 and THAP1 induced CCAR1 gene expression and cellular apoptosis in T-ALL cells. Our finding revealed a mechanistic rationale for Par-4/THAP1-induced apoptosis in T-ALL cells that would be of benefit to develop a new therapy strategy for T-ALL.

MATERIALS AND METHODS

Antibodies and patients' material
Antibodies to THAP1 were generated by immunization of rabbits with synthetic peptide, corresponding to the amino acids of the zinc finger structure sequence of THAP1, which were conjugated to keyhole limpet hemocyanin. Its immunoreaction specificity was verified after affinity purification by reaction with the immunizing antigen and characterized by western blotting. EMSA was used to screen the blocking antibody, which could block the binding of THAP1 to THAP1-S2 site (Supplementary Figure S5). Primary T-ALL cells were obtained from the children with T-ALL. All patients and guardians were informed of the fact, and written informed consent was obtained. The study was approved by the Ethic Committee of the First Affiliated Hospital of Nanjing Medical University.

Construction of expression plasmids and transfection
As described previously,[2] Par-4-expressed vector was constructed and was transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The production of recombinant lentivirus particles was performed as described previously as well.[22]

Yeast two-hybrid assay and quantitative β-galactosidase assay
Yeast two-hybrid assay and quantitative β-galactosidase assay were performed as described previously by McFie et al.[23] Yeast two-hybrid screening was conducted with the MATCHMAKER GAL4 Two-hybrid System, which was purchased from BD Biosciences Clontech (Palo Alto, CA, USA).

Flow cytometry analysis
Cells were collected by trypsinization and washed in phosphate-buffered saline. After incubation with Annexin V-fluorescein isothiocyanate and propidium iodide, cells were analyzed by flow cytometry.

Immunofluorescence and confocal microscopy
Immunofluorescence staining of nontransfected cells and cells transfected with both pcDNA3-Par-4 and pcDNA3-THAP1 was performed as previously described.[24]

MTT assay for cell survival
Cells were treated with 5 mg/ml colorimetric 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) in RPMI 1640 medium for 3 h at 37 °C, and cell proliferations were measured at 570 nm in a microplate reader. The cell growth curves were obtained with the optical density values.

Western blot analysis
Cell lysates were prepared and separated on 10% SDS–polyacrylamide gel. Proteins were transferred to nitrocellulose membranes. Membranes were blotted with the primary antibodies and developed after secondary antibody incubation using the ECL Kit (Amersham International, Amersham, UK) according to the manufacturer's protocols.

Luciferase assay
For construction of pGL3-CCAR1-P1 reporter, the CCAR1 promoter fragment (-286 bp to -1 bp upstream transcription start site), which contained THAP1-binding BS2 site, was cloned into pGL3-Basic plasmid. Jurkats cells were transiently co-transfected with pGL3-CCAR1-P1 reporter plasmids together with either pcDNA3-myc-Par-4 or pcDNA3-myc-THAP1. The luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) following the manufacturer's protocol.

ChIP and ChIP-reChIP assays
ChIP and ChIP-reChIP assays were performed as described previously.[25] Briefly, Jurkat cells were incubated in 1% formaldehyde. Sonication of cross-linked chromatin was performed, and chromatin fragments obtained ranged from 500 to 2000 bp in size. Soluble chromatin was subjected to overnight immunoprecipitation with either anti-Par-4 antibody or anti-THAP1 antibody. The immunoprecipitated DNA was obtained by heating to reverse formaldehyde cross-linking followed by PCR

purification. For ChIP-reChIP assays, cross-linked protein–DNA complexes were eluted from primary immunoprecipitates by incubation with 10 mM dithiothreitol for 45 min at 37 °C. The elutes were subjected to immunoprecipitation with the secondary antibodies. Then, PCR was performed to purify CCAR1 promoter sequences.

siRNA experiments

The sequences of siRNA for Par-4 were used as previously described.[2] Specific siRNAs for THAP1, SRp40 and SRp55 and the scrambled control sequences were designed with BLOCK-iT RNAi Designer online and shown in Supplementary Figure S8 and Supplementary Table S1.

RT–PCR and real-time quantitative RT–PCR

RT–PCR and real-time quantitative RT–PCR was performed with TaKaRa's PrimeScript RT–PCR Kit (Takara, Tokyo, Japan) and SYBR Green I Master PCR Kit. Gene expression levels were normalized to the level of housekeeping gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase) or tubulin.

GST pull-down experiment

The cDNA constructs of the GST fusion proteins of GST-THAP1/1-90aa and GST-THAP1/91-213aa were generated in the pGEX-4T-1 bacterial expression system (Amersham Biosciences, Piscataway, NJ, USA) and were transformed into *E. coli* BL21-(DE3)-RIL cells (Stratagene, La Jolla, CA, USA). The pull-down proteins were separated by SDS–PAGE and detected with anti-Par-4 antibodies by western blot analysis.

5′ RACE

5′ RACE was performed using a 5′/3′ RACE Kit (Roche Diagnostics, Indianapolis, IN, USA), according to the manufacturer's instructions. Three antisense CCAR1 gene-specific primers were needed for the first-strand cDNA synthesis and nested PCRs, including 5′-AGTCGGGTCCCGGCTAA CATCGAAGC-3′, 5′-CGAAGCCATTTCAAACCCGTCAGCT-3′, and 5′-TCAGCTTT AGGCGCATGCGCCA-3′.

Nuclear extract preparation and EMSAs

EMSA was performed with a Gel Shift Assay Kit (Promega). The double-stranded oligonucleotides used for EMSA of THAP1-binding sites (BS) in CCAR1 promoter are shown as follows: THAP1-binding site BS1 (underlined), 5′-GCGGCGCGGGCTGGATCG-3′, THAP1-binding site BS2 (underlined), 5′-GCGAGGCCAAGGCAACCC-3′; and mutated THAP1-binding site BS2 (underlined and in boldface), 5′-GCGAGGCCAAG**T**CAACCC-3′. The oligonucleotides were 5′-end-labeled with [γ-^{32}P]dATP. For the blocking reaction, 500 ng anti-Par-4 antibody (R334) and the blocking antibody against THAP1 were used. RNA EMSAs were used to determine the blocking of splicing factor SRp40 and SRp55 to CCAR1 mRNA. GpppG-capped and [$^{-32}$P]UTP-labeled RNA oligonucleotides containing specific motifs for splicing factors were synthesized and incubated with recombinant SRp40, SFD2, Sc35 or SRp55. RNA–protein complexes were resolved on non-denaturing acrylamide gels. Gels were autoradiographed.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1 Schrappe M, Hunger SP, Pui CH, Saha V, Gaynon PS, Baruchel A. Outcomes after induction failure in childhood acute lymphoblastic leukemia. *N Engl J Med* 2012; **366**: 1371–1381.

2 Lu C, Chen JQ, Zhou GP, Wu SH, Guan YF, Yuan CS. Multimolecular complex of Par-4 and E2F1 binding to Smac promoter contributes to glutamate-induced apoptosis in human bone mesenchymal stem cells. *Nucleic Acids Res* 2008; **36**: 5021–5032.

3 Hebbar N, Wang C, Rangnekar VM. Mechanisms of apoptosis by the tumor suppressor Par-4. *J Cell Physiol* 2012; **227**: 3715–3721.

4 Glienke W, Chow KU, Bauer N, Bergmann L. Down-regulation of wt1 expression in leukemia cell lines as part of apoptotic effect in arsenic treatment using two compounds. *Leuk Lymphoma* 2006; **47**: 1629–1638.

5 Ou CY, Kim JH, Yang CK, Stallcup MR. Requirement of cell cycle and apoptosis regulator 1 for target gene activation by Wnt and beta-catenin and for anchorage-independent growth of human colon carcinoma cells. *J Biol Chem* 2009; **284**: 20629–20637.

6 Zhang L, Levi E, Majumder P, Yu Y, Aboukameel A, Du J. Transactivator of transcription-tagged cell cycle and apoptosis regulatory protein-1 peptides suppress the growth of human breast cancer cells *in vitro* and *in vivo*. *Mol Cancer Ther* 2007; **6**: 1661–1672.

7 Kent S, Hutchinson J, Balboni A, Decastro A, Cherukuri P, Direnzo J. ΔNp63α promotes cellular quiescence via induction and activation of Notch3. *Cell Cycle* 2011; **10**: 3111–3118.

8 Van Vlierberghe P, Ferrando A. The molecular basis of T cell acute lymphoblastic leukemia. *J Clin Invest* 2012; **122**: 3398–3406.

9 Campagne S, Saurel O, Gervais V, Milon A. Structural determinants of specific DNA-recognition by the THAP zinc finger. *Nucleic Acids Res* 2010; **38**: 3466–3476.

10 Cayrol C, Lacroix C, Mathe C, Ecochard V, Ceribelli M, Loreau E. The THAP-zinc finger protein THAP1 regulates endothelial cell proliferation through modulation of pRB/E2F cell-cycle target genes. *Blood* 2007; **109**: 584–594.

11 Cheema SK, Mishra SK, Rangnekar VM, Tari AM, Kumar R, Lopez-Berestein G. Par-4 transcriptionally regulates Bcl-2 through a WT1-binding site on the bcl-2 promoter. *J Biol Chem* 2003; **278**: 19995–20005.

12 Clouaire T, Roussigne M, Ecochard V, Mathe C, Amalric F, Girard JP. The THAP domain of THAP1 is a large C2CH module with zinc-dependent sequence-specific DNA-binding activity. *Proc Natl Acad Sci USA* 2005; **102**: 6907–6912.

13 Tzoneva G, Ferrando AA. Recent advances on NOTCH signaling in T-ALL. *Curr Top Microbiol Immunol* 2012; **360**: 163–182.

14 Kim JH, Yang CK, Heo K, Roeder RG, An W, Stallcup MR. CCAR1, a key regulator of mediator complex recruitment to nuclear receptor transcription complexes. *Mol Cell* 2008; **31**: 510–519.

15 Aravind L, Koonin EV. SAP—a putative DNA-binding motif involved in chromosomal organization. *Trends Biochem Sci* 2000; **25**: 112–114.

16 Park JT, IeM Shih, Wang TL. Identification of Pbx1, a potential oncogene, as a Notch3 target gene in ovarian cancer. *Cancer Res* 2008; **68**: 8852–8860.

17 Bellavia D, Mecarozzi M, Campese AF, Grazioli P, Talora C, Frati L. Notch3 and the Notch3-upregulated RNA-binding protein HuD regulate Ikaros alternative splicing. *EMBO J* 2007; **26**: 1670–1680.

18 Ling X, Yang J, Tan D, Ramnath N, Younis T, Bundy BN. Differential expression of survivin-2B and survivin-DeltaEx3 is inversely associated with disease relapse and patient survival in non-small-cell lung cancer (NSCLC). *Lung Cancer* 2005; **49**: 353–361.

19 Yan XB, Tang CH, Huang Y, Fang H, Yu ZQ, Wu LM. Alternative splicing in exon 9 of glucocorticoid receptor pre-mRNA is regulated by SRp40. *Mol Biol Rep* 2010; **37**: 1427–1433.

20 Boehrer S, Nowak D, Puccetti E, Ruthardt M, Sattler N, Trepohl B et al. Prostate-apoptosis-response-gene-4 increases sensitivity to TRAIL-induced apoptosis. *Leuk Res* 2006; **30**: 597–605.

21 Chow KU, Nowak D, Hofmann W, Schneider B, Hofmann WK. Imatinib induces apoptosis in CLL lymphocytes with high expression of Par-4. *Leukemia* 2005; **19**: 1103–1105.

22 Wei B, Feng N, Zhou F, Lu C, Su J, Hua L. Construction and identification of recombinant lentiviral vector containing HIV-1 Tat gene and its expression in 293T cells. *J Biomed Res* 2010; **24**: 58–63.

23 McFie PJ, Wang GL, Timchenko NA, Wilson HL, Hu X, Roesler WJ. Identification of a co-repressor that inhibits the transcriptional and growth-arrest activities of CCAAT/enhancer-binding protein alpha. *J Biol Chem* 2006; **281**: 18069–18080.

24 Roussigne M, Cayrol C, Clouaire T, Amalric F, Girard JP. THAP1 is a nuclear proapoptotic factor that links prostate-apoptosis-response-4 (Par-4) to PML nuclear bodies. *Oncogene* 2003; **22**: 2432–2442.

25 Zhang D, Jiang P, Xu Q, Zhang X. Arginine and glutamate-rich 1 (ARGLU1) interacts with mediator subunit 1 (MED1) and is required for estrogen receptor-mediated gene transcription and breast cancer cell growth. *J Biol Chem* 2011; **286**: 17746–17754.

Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)