Oncogene (2012) 31, 2876–2887

www.nature.com/onc

ORIGINAL ARTICLE

Prolyl isomerase Pin1 stabilizes and activates orphan nuclear receptor TR3 to promote mitogenesis

H-Z Chen[1], L Li[1], W-J Wang[1], X-D Du[1], Q Wen[1], J-P He[1], B-X Zhao[1], G-D Li[1], W Zhou[2], Y Xia[2], Q-Y Yang[2], C-L Hew[2], Y-C Liou[2] and Q Wu[1]

[1]*State Key Laboratory of Cellular Stress Biology, School of Life Sciences, Xiamen University, Fujian, PR China and* [2]*Department of Biological Sciences, National University of Singapore, Singapore, Republic of Singapore*

Pin1 regulates a subset of phosphoproteins by isomerizing phospho-Ser/Thr-Pro motifs via a 'post-phosphorylation' mechanism. Here, we characterize TR3 as a novel Pin1 substrate, and the mitogenic function of TR3 depends on Pin1-induced isomerization. There are at least three phospho-Ser-Pro motifs on TR3 that bind to Pin1. The Ser95-Pro motif of TR3 is the key site through which Pin1 enhances TR3 stability by retarding its degradation. Pin1 can also catalyze TR3 through phospho-Ser431-Pro motif, which is phosphorylated by extracellular signal-regulated kinase 2 (ERK2), resulting in enhanced TR3 transactivation. Furthermore, Pin1 not only facilitates TR3 targeting to the promoter of cyclin D2, a novel downstream target of TR3, but also promotes TR3 to recruit p300, thereby inducing cell proliferation. Importantly, we found that Pin1 is indispensable for TR3 to promote tumor growth both *in vitro* and *in vivo*. Our study thus suggests that Pin1 has an important role in cell proliferation by isomerizing TR3.
Oncogene (2012) **31,** 2876–2887; doi:10.1038/onc.2011.463; published online 17 October 2011

Keywords: prolyl isomerase pin1; orphan receptor TR3; phosphorylation; cell proliferation

Introduction

Phosphorylation of proteins on serine or threonine residue preceding a proline (Ser/Thr-Pro) is a major mode of post-translational modification in signaling pathways. Pin1 is a unique peptidyl-prolyl *cis*/*trans* isomerase (PPIase) that specifically catalyzes phosphorylated Ser/Thr-Pro motifs (Lu *et al*., 1996; Ranganathan *et al*., 1997). The Pin1-medicated post-phosphorylation mechanism provides a novel and important aspect in regulating many cellular events (Ryo *et al*., 2003; Lu and Zhou, 2007; Yeh and Means, 2007). Structurally, Pin1 contains two separate domains, an N-terminal WW-domain and a C-terminal PPIase-domain (Ranganathan *et al*., 1997). The WW-domain functions as a binding module that targets specific phosphorylated proteins. The PPIase-domain is the catalytic region that induces conformational changes in its substrates, thereby exerting a wide variety of functions, such as protein stability, subcellular localization, protein–protein interactions, phosphorylation and transcriptional activation (Lu and Zhou, 2007; Yeh and Means, 2007).

Pin1 regulates many oncogenic proteins such as c-Jun, β-catenin, cyclin D1 and NF-κB and thus takes part in the tumor formation and development (Lu and Zhou, 2007; Fan *et al*., 2009). For example, Pin1 binds and isomerizes phosphorylated Ser246-Pro motif in β-catenin and blocks the interaction of β-catenin with its negative regulator APC, resulting in an accumulation of β-catenin in nucleus and its transactivation on cyclin D (Ryo *et al*., 2001). Furthermore, Pin1 binds to cyclin D1 at phosphorylated Thr286-Pro motif and catalyzes its isomerization, thus resulting in stabilization and accumulation of cyclin D1 in the nucleus (Liou *et al*., 2002). Overall, the protein stability and transactivation regulation mediated by Pin1 provide a unique regulatory mechanism in cell proliferation and tumorigenesis.

TR3 (also known as Nur77, NGFI-B and NR4A1) belongs to the NR4A subfamily of the nuclear receptor superfamily. Each member of this subfamily possesses a highly conserved structural organization with an amino-terminal region encoding a transactivation domain, followed by a DNA-binding domain, and a ligand-binding domain (Hazel *et al*., 1988; Chang *et al*., 1989). Since its biophysiological ligands have not yet been identified, TR3 is classified as an orphan receptor.

TR3 has been approved to have opposing biological functions in different cell types. A number of growth factors potently and rapidly induce the expression of TR3 in a variety of cancer cells (Hazel *et al*., 1988; Chang *et al*., 1989; Uemura and Chang, 1998; Kolluri *et al*., 2003), suggesting that this receptor may have a positive role in regulating cancer cell growth. The mitogenic effect of TR3 was reported to mainly occur in the nucleus and requires its DNA-binding and transcriptional activity (Kolluri *et al*., 2003). As a transcription factor, TR3 can bind to its specific

Correspondence: Dr Q Wu, State Key Laboratory of Cellular Stress Biology, School of Life Sciences, Xiamen University, Xiamen, Fujian 361005, PR China.
E-mail: qiaow@xmu.edu.cn or Dr Y-C Liou, Department of Biological Sciences, National University of Singapore, Singapore 117543, Republic of Singapore.
E-mail: dbslyc@nus.edu.sg
Received 3 April 2011; revised 17 August 2011; accepted 6 September 2011; published online 17 October 2011

response elements, NBRE (NGFI-B response element) or NurRE (Nur77 response element), and stimulates the expression of its downstream target genes (Martinez-Gonzalez and Badimon, 2005). However, the exact mechanism through which TR3 regulates cell proliferation is still largely unknown. On the other hand, the expression of TR3 is found to be required for apoptosis induction in cancer cells of prostate, lung and stomach (Li *et al*., 1998, 2000; Liu *et al*., 2002), indicating that it also has an important role in apoptosis. TR3 mainly exerts its pro-apoptotic functions through translocation from the nucleus to the mitochondria, where it converts the function of Bcl-2 from an anti-apoptotic to a pro-apoptotic role (Li *et al*., 2000; Lin *et al*., 2004; Chen *et al*., 2008). Hence, further elucidation of novel regulatory functions of TR3 would be of great interest to cancer biologists.

TR3 has been found to be hyperphosphorylated in various cell types, suggesting that the phosphorylation regulation is an important mechanism for its biological functions. The N-terminal of TR3 is the major region for the phospho-modification (Davis *et al*., 1993). For example, we previously showed that the Ser95 residue in TR3 is a novel phosphorylation site for c-Jun N-terminal kinases 1 (JNK1) upon anisomycin treatment and this JNK-induced phosphorylation is related with the stability and transcriptional activity of TR3 (Liu *et al*., 2007). Importantly, a total of 17 Ser/Thr-Pro motifs exist in TR3, 15 of which are N-terminally located and two of which are found at the C-terminus. Such structural features led us to hypothesize that Pin1 might bind to phosphorylated TR3 and regulate its functions through isomerization. In the current study, we demonstrate that Pin1 interacts with TR3 in a phosphorylation-dependent manner and regulates the mitogenic function of TR3 through isomerization.

Results

TR3 is a novel substrate for Pin1 isomerization

Given that TR3 is a hyperphosphorylated protein containing multiple Ser/Thr-Pro motifs, it is possible that TR3 may be a substrate of Pin1. To test this, Pin1 and TR3 were co-transfected into HEK293T cells, and co-immunoprecipitation (co-IP) experiments were performed. The results showed that Pin1 and TR3 pulled down each other reciprocally (Supplementary Figure S1A), suggesting an association between TR3 and Pin1 at exogenous levels. We further confirmed that endogenous Pin1 protein could be immunoprecipitated by anti-TR3 antibodies, but not the IgG control (Figure 1a). Furthermore, glutathione *S*-transferase (GST) pull-down assay demonstrated that GST-Pin1 but not GST alone could pull down HA-TR3 that was expressed in 293T cells (Figure 1b). Immunostaining also revealed that green fluorescent protein (GFP)-TR3 overlapped well with Flag-Pin1 in the nucleus (Supplementary Figure S1B). Together, these results demonstrate that TR3 is a novel Pin1-interacting protein.

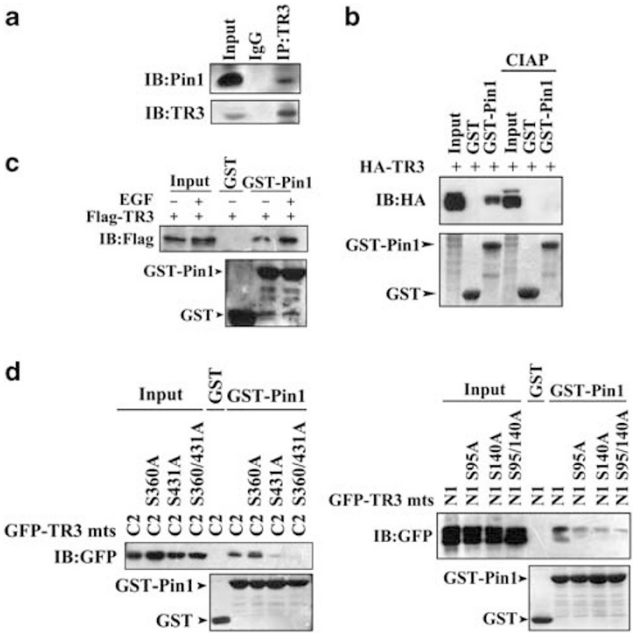

Figure 1 Pin1 interacts with TR3. (**a**) Pin1 interacts with TR3 in endogenous level. Cell lysates from 293T cells were immunoprecipitated with anti-TR3 antibody, followed by immunoblotting with anti-Pin1 antibody. IgG was used as a control. (**b**) Pin1 binds to phosphorylated TR3. HA-TR3 was transfected into 293T cells and cell lysates were prepared. After preincubation with or without CIAP (0.2 U/μl) for 1 h, the lysates were incubated with GST-Pin1, and then subjected to immunoblotting. (**c**) EGF treatment enhances the TR3–Pin1 interaction. Flag-TR3 was transfected into 293T cells, and then treated with EGF (200 ng/ml) for 1 h. The cell lysates were then subjected to a GST pull-down assay using GST-Pin1. (**d**) Identification of the Pin1-binding sites on TR3. Different TR3 mutants as indicated were transfected into 293T cells. GST pull-down analyses were then carried out.

As Pin1 preferentially interacts with phosphoproteins (Lu and Zhou, 2007), we next asked whether the phosphorylation of TR3 is required for Pin1 interaction. When cell lysates containing HA-TR3 were incubated with calf intestinal alkaline phosphatase (CIAP), which catalyzes protein dephosphorylation, GST-Pin1 failed to pull down TR3 (Figure 1b). Conversely, the treatment of epidermal growth factor (EGF), which has been reported to induce TR3 phosphorylation (Slagsvold *et al*., 2002; Jacobs *et al*., 2004), evidently enhanced the interaction between TR3 and Pin1 (Figure 1c). These results strongly suggest that Pin1 prefers to interact with phosphorylated TR3.

We next sought to map the mutual interaction domains of Pin1 and TR3. GST pull-down assays revealed that GST-WW-domain could pull down Myc-TR3 efficiently, whereas GST-PPIase-domain fusion proteins failed to do so (Supplementary Figure S1C), indicating that the WW-domain of Pin1 is responsible for TR3 binding. As for TR3, its truncation mutants N1 (residues 1–194), N2 (residues 1–264) and C2 (residues 265–598), but not C1 (residues 440–598), was effectively pulled down by GST-Pin1 (Supplementary Figure S1D). Similar results were also obtained in co-IP experiments (Supplementary Figure S1E). Therefore, it is likely that

Pin1 has multiple binding sites outside of the C-terminus (aa 440–598) of TR3.

We further attempted to identify the critical pSer/Thr-Pro motif(s) in TR3 for Pin1 binding and isomerization. Since the C2 mutant contains only two Ser-Pro motifs (residues 360–361 and 431–432), we mutated both of these sites with a serine-to-alanine substitution either individually (C2 S360A and C2 S431A) or in combination (C2 S360/431A). As shown in Figure 1d, TR3 C2 S360A was still pulled down by GST-Pin1, whereas C2 S431A or C2 S360/431A abolished this association (left panel), indicating that Ser431 is critical for the Pin1–TR3 interaction. In the N1 region of TR3, two Ser-Pro motifs (residues 95–96 and 140–141) were reported to be phosphorylated by JNK and extracellular signal-regulated kinase (ERK), respectively (Katagiri *et al*., 2000; Liu *et al*., 2007). We again constructed two single mutants (N1 S95A and N1 S140A) and one double mutant (N1 S95/140A), and tested their capability of Pin1 binding. The results showed that TR3 N1 S95A and N1 S140A had a significantly reduced affinity for Pin1, which was more pronounced in the case of the double mutant (Figure 1d, right). Moreover, TR3 3A (S95/140/431A) lost most of its ability to bind to Pin1 (Supplementary Figure S1F). Taken together, the Ser95-Pro, Ser140-Pro and Ser431-Pro residues of TR3 are important for Pin1 interaction.

Pin1 stabilizes TR3 protein by blocking its degradation

To determine the physiological consequences of the interaction between Pin1 and TR3, we detected the expression profile of both TR3 and Pin1 proteins in different cell lines and found them co-expressed in many cell lines (Supplementary Figure S2A). This finding led us to speculate that there may be a correlation between the expression of Pin1 and TR3. We further evaluated the endogenous protein levels of TR3 in 293T cells and found that the expression levels of TR3 were elevated with increased amount of Pin1 transfection (Figure 2a, left), while declined by transfection of Pin1-siRNA (Figure 2a, right). Importantly, this effect of Pin1 on TR3 relied on its isomerase activity, as a Pin1 isomerase-dead mutant K63A (Lu *et al*., 1999; Zhou *et al*., 2000) lost its ability to enhance TR3 expression (Figure 2a, left). Similar phenomena was also observed in HeLa cells (Supplementary Figure S2B). Thus, Pin1 positively regulates the protein level of TR3.

To further address whether Pin1 stabilizes TR3, we treated 293T cells with CHX (cycloheximide), a protein synthesis inhibitor, and monitored the stability of TR3. As shown in Figure 2b, the half-life of TR3 was significantly reduced from about 9 h to about 5 h upon the transfection of Pin1-siRNA (top). The reintroduction of Pin1 into the Pin1 knockdown (Pin1-KD) 293T cells significantly restored the half-life of TR3 to >9 h, whereas Pin1 K63A mutant did not show such effect (Figure 2b, bottom). Obviously, Pin1 enhances the stability of TR3 by blocking protein degradation.

The stability of different TR3 point mutants was then analyzed in the absence or presence of Pin1. When these mutants were individually transfected into 293T cells, the expression levels of S140A, S360A and S431A, but not that of S95A, were found to be significantly enhanced by the co-expression of Pin1 (Figure 2c). The S95A mutant was then chosen to assess the effects of Pin1 on its half-life. As expected, Pin1 no longer affected the half-life of this TR3 mutant (Figure 2d). Therefore, Ser95 is the key residue through which Pin1 stabilizes TR3.

Pin1 promotes transactivation of TR3 by enhancing its ability to recruit p300

We then investigated whether Pin1 modulates the transactivation function of TR3. Luciferase reporter assays showed that overexpression of Pin1 obviously increased the reporter activity of NurRE-Luc in 293T cells. In contrast, transfection of Pin1-siRNA evidently repressed it (Figure 3a). Similar results were also observed in HeLa cells (Supplementary Figure S3A), suggesting that Pin1 may induce the transactivation activity of TR3. Importantly, the effects of Pin1 upon this transcriptional activation are mediated by TR3. Once endogenous TR3 was knocked down, Pin1 lost its effect on the reporter activity (Figure 3a). In addition, both Pin1 W34A mutant that cannot bind phosphoprotein substrates and K63A mutant that lacks isomerase activity failed to activate the transactivation activity of TR3 (Figure 3b). These results demonstrate that both the substrate binding ability and isomerase activity of Pin1 are essential for activating TR3.

To identify the key phosphorylation site within TR3 that is responsible for Pin1 regulation, we again introduced different TR3 mutants into 293T cells. Interestingly, all TR3 mutants still showed Pin1-inducible transcriptional activities except S431A (Figure 3c), demonstrating that Ser431-Pro motif is critical for the activation of TR3 transcriptional activity by Pin1. Although Pin1 did not stabilize TR3 S95A mutant, Pin1 could still enhance its transactivation activity. However, when both the Ser95-Pro and Ser431-Pro motifs were mutated, the transactivation activity of TR3 could not be regulated by Pin1 (Supplementary Figure S3B). These results suggest that the increased protein level of TR3 by Pin1 only partly contributes to the Pin1-induced TR3 transactivation.

We thus hypothesized that Pin1-induced TR3 transactivation may be associated with the recruitment of co-activators, such as p300, SRC1, SRC2 and SRC3, all of which were reported to enhance TR3 transactivation activity (Wansa *et al*., 2002; Maira *et al*., 2003). The results showed that p300 and the SRCs each promoted TR3 transactivation activity to a moderate degree. Importantly, co-transfection of Pin1 significantly increased p300-mediated TR3 transactivation, whereas SRCs did not show such abilities (Supplementary Figure S3C). In accordance, the interaction of TR3 with p300 was enhanced by Pin1 (Supplementary Figure S3D). Moreover, the TR3 transactivation was found to be associated with the TR3 phosphorylation site Ser431 but not Ser95 (Figure 3d). Collectively, these results demonstrate that Pin1 may activate the TR3 transactivation by facilitating the recruitment of p300.

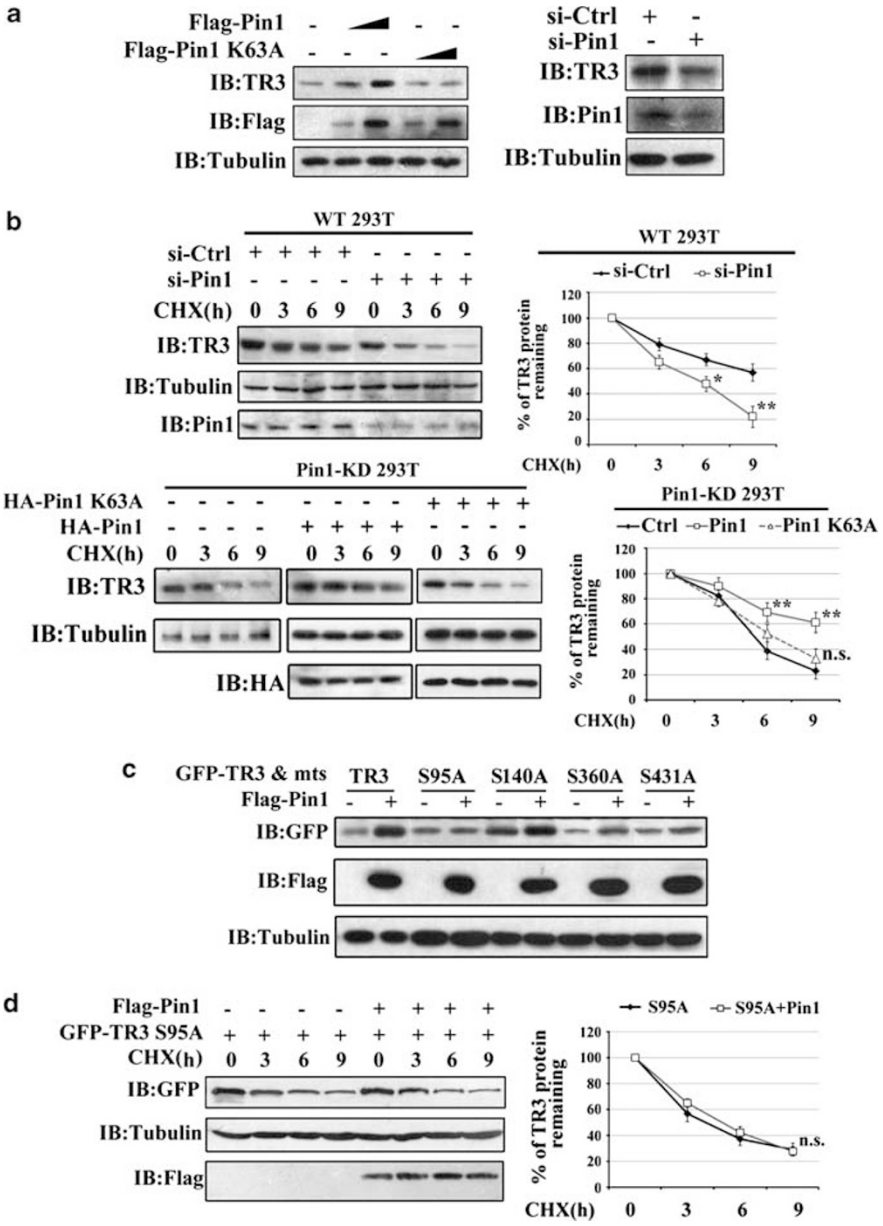

Figure 2 TR3 is stabilized by Pin1. (**a**) Pin1 induces TR3 expression. Pin1-siRNA, Ctrl-siRNA, Flag-Pin1 or a Pin1 activity-defective mutant (K63A) was transfected into 293T cells as required and the expression levels of TR3 were analyzed by western blotting. Tubulin was used as a loading control. (**b**) Pin1 enhances the stability of TR3 protein. Pin1-siRNA, Ctrl-siRNA, HA-Pin1 or HA-Pin1 K63A was transfected into 293T or Pin1 knockout (Pin1-KD) 293T cells, and then treated with CHX (100 g/ml) for the indicated times. The experiments were performed at least three times. The TR3 expression levels were assessed by western blotting and quantified by densitometry. $*P<0.05$; $**P<0.01$. (**c**) Pin1 affects the stability of various TR3 mutants. Different TR3 mutants as indicated were transfected with Pin1 into 293T cells. The expression levels of TR3 mutants were then analyzed by western blotting. (**d**) Effects of CHX on TR3 S95A expression. The TR3 S95A expression levels were assessed by western blotting and quantified by densitometry.

Pin1 promotes TR3-mediated expression of cyclin D2 and E2F1

If Pin1 regulates TR3 transcriptional activity, whether it will affect the expression of TR3 downstream targets as a consequence? Cyclin D2, a member of the cyclin protein family, has been reported to be upregulated upon TR3 overexpression in macrophages (Pei *et al*., 2006). However, whether it acts as a direct downstream target of TR3 has not been elucidated. After sequence analysis of the human cyclin D2 promoter, we found a putative TR3 response element NBRE and two NBRE-like sequences within this region (Supplementary Figure S4A), and these potential NBREs are conserved in human, mouse and rat (Supplementary Table S1).

To verify the direct association between TR3 and cyclin D2 promoter, an electrophoretic mobility shift assay (EMSA) was carried out. The binding of TR3 to the cyclin D2 promoter (using CCND2, which contains an NBRE, as a probe), but not to its mutant was clearly observed (Supplementary Figure S4B). This association

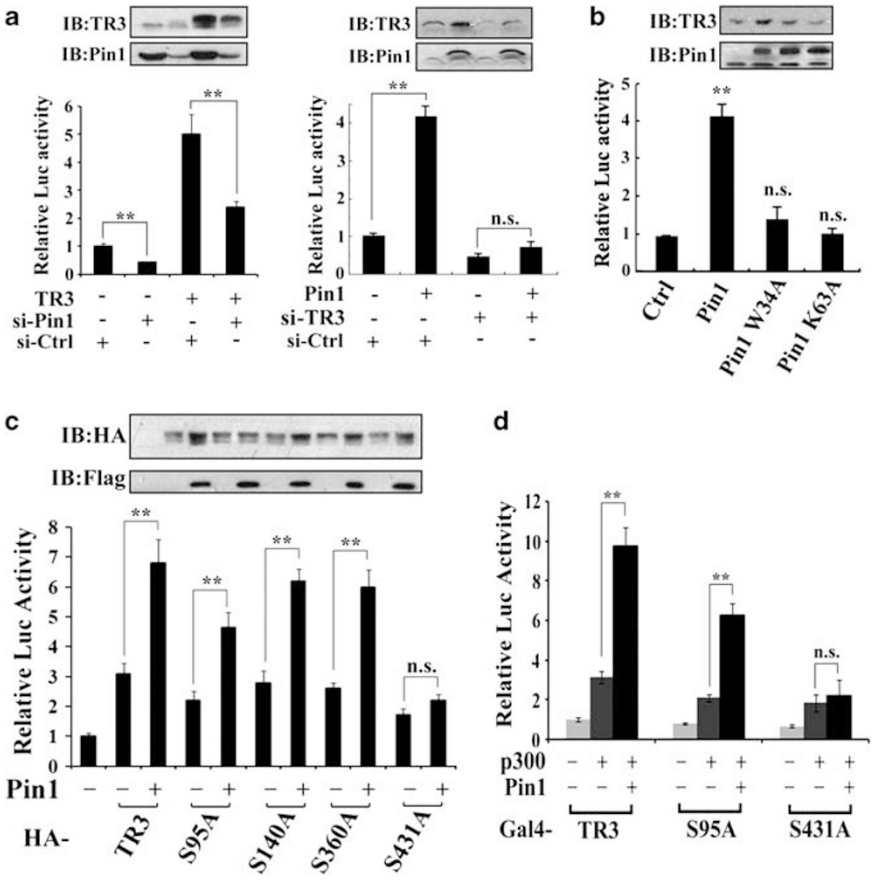

Figure 3 Pin1 stimulates the transactivation activity of TR3 by facilitating p300 recruitment. (**a**) Pin1 enhances TR3 transactivation activity. NurRE-luciferase reporter and β-gal gene expression vectors, together with Pin1-siRNA, TR3-siRNA, Ctrl-siRNA or Pin1 as indicated, were transfected into 293T cells. Reporter gene activity was then determined. The expression level of TR3 and Pin1 were shown at top panel. All the experiments were done at least three times. Error bars indicate the s.d. from three independent experiments. $^{**}P<0.01$. (**b**) Both WW-domain and PPIase-domain are important for Pin1 to activate TR3. Different Pin1 mutant expression vectors W34A and K63A were co-transfected with reporters into 293T cells, and the luciferase activities were then determined. The expression level of TR3 and different Pin1 mutants were shown at top panel. $^{**}P<0.01$. (**c**) Ser431-Pro of TR3 is required for Pin1-induced transactivation activity. Different TR3 mutant together with Pin1 were transfected into 293T cells. The transactivation activities of TR3 were assessed by luciferase assay. $^{**}P<0.01$. (**d**) p300-enhanced transactivation activity of TR3 relies on the Ser431-Pro motif. pGAL4 reporter and β-gal gene expression vectors, together with Pin1, p300, TR3 or TR3 mutants as indicated were transfected into 293T cells. Reporter gene activity was then determined. $^{**}P<0.01$.

between TR3 and cyclin D2 promoter *in vivo* was also obtained by chromatin immunoprecipitation (ChIP) assay (Supplementary Figure S4C). Consistently, the activity of cyclin D2 promoter was clearly induced by transfection of TR3 in a dose-dependent manner (Supplementary Figure S4D). Thus, cyclin D2 is a direct downstream target of TR3. Furthermore, co-transfection of Pin1 enhanced the TR3 binding to the cyclin D2 promoter, as detected by EMSA and ChIP assays (Figure 4a). In contrast, knocking down of Pin1 diminished the association of TR3 with cyclin D2 promoter (Figure 4c, right). Notably, the other two NBRE-like elements within the cyclin D2 promoter were also found to be involved in the regulation of cyclin D2 transcription by TR3, and only the deletion of all NBRE and NBRE-like sequences abolished Pin1-enhanced cyclin D2 promoter activity (Figure 4b).

As Pin1 and p300 synergically activate TR3, we further detected whether Pin1 facilitates the recruitment of p300 by TR3 to the cyclin D2 promoter using ChIP assay. Indeed, the transfection of Pin1 efficiently enhanced TR3-recruited p300 on the cyclin D2 promoter (Figure 4c, left). In contrast, knocking down of Pin1 almost abolished TR3 effect on p300 recruitment (Figure 4c, right). Furthermore, TR3 S431A no longer recruited p300 to the cyclin D2 promoter even in the presence of Pin1, while S95A still maintained this ability (Figure 4c). Hence, these results indicate that Pin1 enhances TR3 ability to recruit the co-activator p300 to the cyclin D2 promoter, and that the post-phosphorylation regulation by Pin1 through the Ser431-Pro motif of TR3 is vital for this event.

As a consequence, Pin1 may influence the expression of cyclin D2 mediated by TR3. Overexpression of TR3 clearly increased the protein level of cyclin D2, which was further enhanced upon Pin1 co-transfection (Figure 4d, left). In contrast, TR3 3A (S95/140/431A), in which all potential Pin1-binding motifs were mutated,

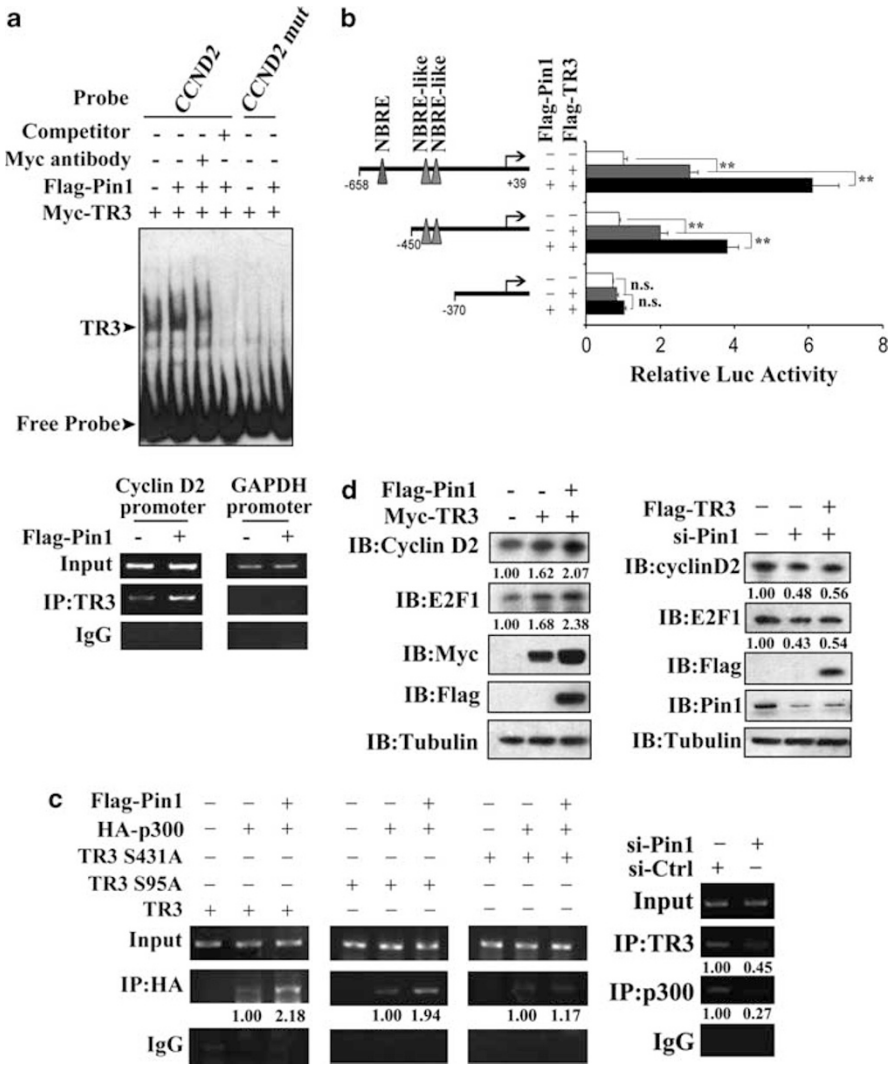

Figure 4 Pin1 enhances TR3-mediated cyclin D2 expression. (**a**) Pin1 augments TR3 binding to the cyclin D2 promoter. (Top panel) Flag-Pin1 and Myc-TR3 were transfected into 293T cells; the nuclear extracts were prepared, and then incubated with competitor or an anti-myc antibody, finally subjected to the EMSA assay using CCND2 as a probe. A CCND2 mutant probe was used as a control. (Bottom panel) 293T cells were transfected with or without Flag-Pin1. The cell lysates were incubated with anti-TR3 antibody and IgG was used as a negative control. Eluted DNA from the immunoprecipitates was amplified by polymerase chain reaction (PCR) using cyclin D2 promoter primers. The glyceraldehyde 3-phosphate dehydrogenase promoter was used as a negative control. (**b**) Activation of TR3-induced cyclin D2 promoter by Pin1 in 293T cells. Cells were transfected with Pin1 and TR3, together with constructs harboring different regions of the cyclin D2 promoter as indicated. The reporter gene activities were determined by luciferase assay. $^{**}P<0.01$. (**c**) Pin1 increases the TR3 ability to recruit p300 to the cyclin D2 promoter. (Left panel) TR3 or its mutants and p300 were transfected into 293T cells in the absence or presence of Pin1. The levels of p300 targeting to the cyclin D2 promoter were then determined by ChIP. (Right panel) Pin1-siRNA or Ctrl-siRNA was transfected into 293T cells. The levels of TR3 and p300 targeting to the cyclin D2 promoter were then determined by ChIP. The amount of p300 or TR3 associated with cyclin D2 promoter was quantified by densitometry and indicated at the bottom. The experiments were done at least three times. (**d**) Pin1 is important for TR3-induced cyclin D2 and E2F1 expression. TR3, Pin1 or si-Pin1 was transfected into 293T cells as indicated. The cell lysates were then subjected for western blotting. The expression levels of cyclin D2 and E2F1 were quantified by densitometry. The experiments were done at least three times.

lost most of its effect on the expression of cyclin D2 (Supplementary Figure S4F). Moreover, when endogenous Pin1 was knocked down via Pin1-siRNA, TR3 can no longer influence cyclin D2 expression (Figure 4d, right). In addition, Pin1 also influenced the association of TR3 with the promoter of E2F1 (Supplementary Figure S4E), another target gene of TR3 (Mu and Chang, 2003), accompanying with the upregulation of E2F1 expression (Figure 4d; Supplementary Figure S4F). Together, these results consistently indicate that the prolyl *cis*/*trans* isomerization catalyzed by Pin1 is a prerequisite for TR3 to stimulate the expression of its downstream target genes.

Pin1 is required for TR3 to stimulate cell proliferation

Our previous study has shown that the induction of TR3 transactivation is associated with its mitogenic properties (Liu *et al*., 2007). Since Pin1 regulates the transcriptional activity of TR3 and promotes TR3-induced cyclin

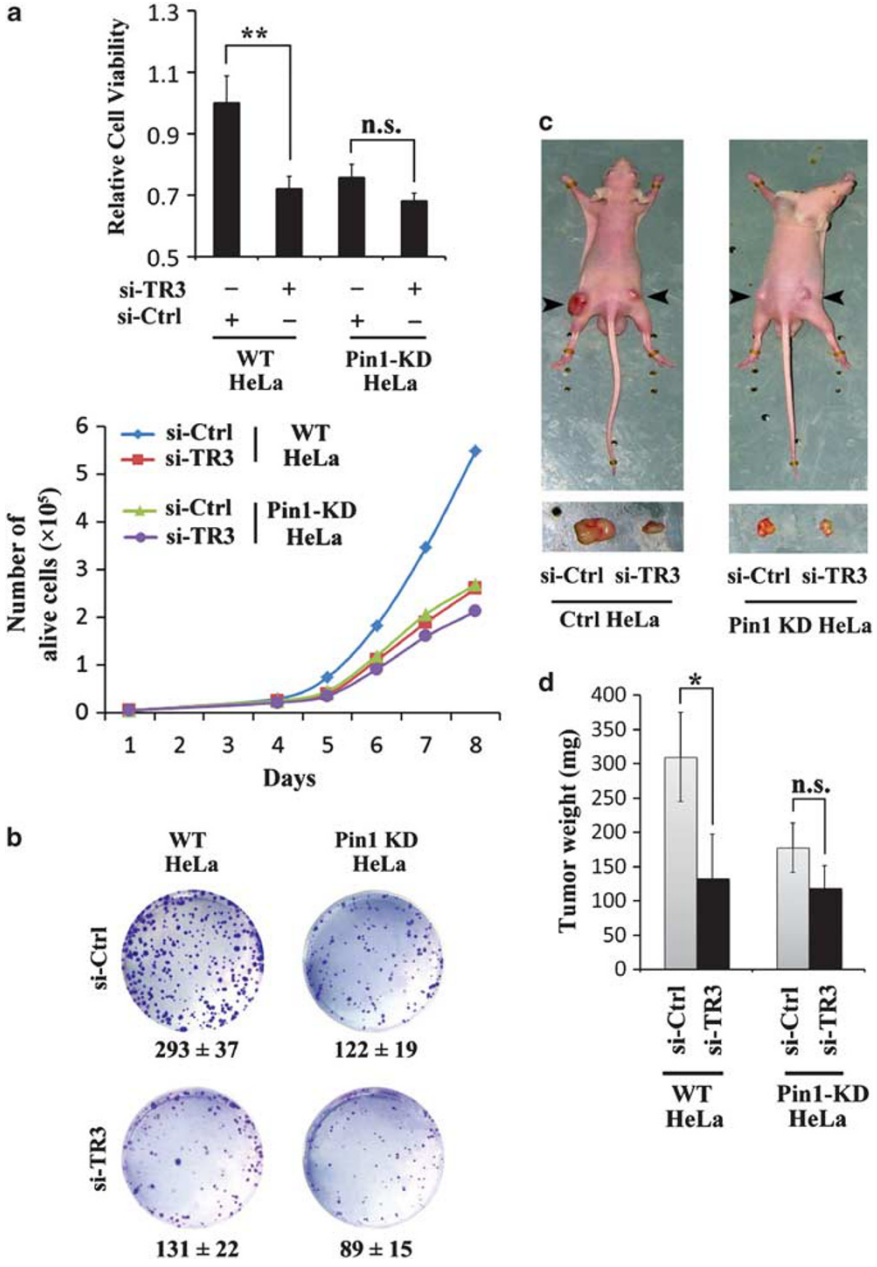

Figure 5 Pin1 is indispensable for the mitogenic function of TR3. (**a**) Pin1 is important for TR3-induced cell proliferation. TR3-siRNA or Ctrl-siRNA was transfected into WT or Pin1 knockdown (Pin1-KD) HeLa cells. The cell proliferation was determinate by MTT (top) or cell-counting assay (bottom). $^{**}P<0.01$. (**b**) Effect of Pin1 on TR3-induced colony formation. TR3-siRNA or Ctrl-siRNA was transfected into WT or Pin1-KD HeLa cells. The cells were then subjected to colony formation assay. Microscopic colonies composed of >50 cells were counted. The experiments were done at least three times. (**c**, **d**) TR3 promotes xenograft tumor growth in a Pin1-dependent manner. TR3-siRNA or Ctrl-siRNA transfected WT and Pin1-KD HeLa cells (5×10^6) were injected subcutaneously into the either posterior flank of the same nude mice, respectively. Photographs illustrate representative features of tumor growth 21 days after inoculation (**c**). The analysis of tumor weight was shown (**d**). $^{*}P<0.01$.

D2 and E2F1 expression, it is possible that Pin1 may regulate the mitogenic effect of TR3. To test this, we carried out 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays and found that knocking down of TR3 significantly reduced proliferation of HeLa cells. However, in Pin1 knocked down HeLa cells, TR3 did not influence cell proliferation (Figure 5a, top), suggesting that Pin1 is required for the pro-proliferative function of TR3. Similar results were also obtained by a cell-counting assay (Figure 5a, bottom). However, TR3 3A lost most of its ability to induce cell proliferation detected with above methods (Supplementary Figures S5A and B), demonstrating the important role of Pin1-induced TR3 isomerization in this process. Furthermore, knocking down of TR3 obviously repressed HeLa cells to form colonies, and

this effect of TR3 relied on Pin1 existence to some extent, as further silence of TR3 no longer influenced colony formation in Pin1 knocked down HeLa cells (Figure 5b). These results thus imply a linkage between Pin1 regulation and TR3 mitogenic effect.

To more extensively verify this linkage, we examined the effect of Pin1 on the mitogenic function of TR3 *in vivo* using nude mice. Wild-type (WT) and Pin1 knocked down HeLa cells that were pretransfected with scramble or TR3-siRNA were injected into mice. Three weeks after injection, the mice were killed and the tumor weights were determined. As shown in Figures 5c and d, knocking down of TR3 significantly restrained tumor growth in WT cell-injected mice; however, silence of TR3 only showed slight effect on the tumor growth when Pin1 was knocked down. Together, all the data from both *in vivo* and *in vitro* demonstrate that Pin1 positively regulates TR3 mitogenic function.

ERK2 and JNK1 are key kinases for TR3 phosphorylation that facilitates Pin1 recognition

Finally, we investigated the principal kinase(s) that phosphorylates and triggers TR3 isomerization by Pin1. Since EGF that affects TR3–Pin1 interaction (Figure 1c) can activate both ERK and JNK (Lim and Cao, 1999), we speculated that ERK and (or) JNK may be involved in the phosphorylation of TR3. Indeed, the phosphorylation of TR3 at serine sites induced by EGF could be obviously inhibited by an MEK-specific inhibitor PD98059 (which inhibits the activation of ERK) and a JNK inhibitor, but not that of p38-specific inhibitor SB202190 (Supplementary Figure S6A). Concordantly, the treatment of PD98059 or JNK inhibitor also impaired the interaction between TR3 and Pin1 (Figure 6a). Therefore, it is likely that the phosphorylation of TR3 by ERK and JNK facilitates Pin1 isomerization.

To further confirm this, ERK2 or JNK1 that has been reported to phosphorylate TR3, together with their upstream activators MEK2 CA (a constitutively active form of MEK2) or MKK4 was transfected into 293T cells, respectively. As a result, TR3 phosphorylation was significantly induced (revealed by band-shift), which was abolished by CIAP treatment (Supplementary Figure S6B, top). In contrast, transfection of an MEK inactive mutant (MEK2 mut) or a dominant-negative mutant of MKK4 (MKK4 DN) failed to induce TR3 band-shifted (Supplementary Figure S6B, bottom). Importantly, the ERK2 and JNK1-induced phosphorylations of TR3 were found to be associated with the enhancement of the Pin1–TR3 interaction (Supplementary Figure S6C). Therefore, the phosphorylation of TR3 by ERK2 and JNK1 enhances the binding of TR3 with Pin1.

Although both MEK and JNK inhibitors abolished the Pin1 effect on TR3 expression (Supplementary Figure S6D), only the MEK inhibitor could impair Pin1-enhanced TR3 transcriptional activity (Figure 6b), suggesting that the phosphorylation of TR3 by ERK, but not JNK, is a prerequisite for Pin1 to enhance TR3 transcriptional activity. Since the Ser431 residue of TR3 was shown to be important in Pin1-enhanced TR3 transactivation, we speculated that Ser431 may be a phosphorylation site of ERK. To verify this, ERK2 and MEK2 CA were co-expressed with TR3 C2 S360A or C2 S431A in 293T cells and the phosphorylation levels were detected. As shown in Figure 6c, a clear phosphorylation signal was detected in the TR3 C2 S360A transfected cells but not C2 S431A transfected cells (left panel). Moreover, EGF induced the phosphorylation of TR3 at Ser431 in an ERK-dependent manner, revealed by addition of PD98059 (right panel). When ERK2 was knocked down by siRNA, the EGF-induced phosphorylation of TR3 C2 was obviously impaired (Supplementary Figure S6E). Thus, the Ser431 residue is a novel site for ERK2 phosphorylation.

Finally, we studied the function of Pin1 on EGF-induced cell proliferation by 5-bromo-2′-deoxyuridine (BrdU) incorporation assay. EGF treatment significantly enhanced TR3-mediated cell proliferation in WT HeLa cells; however, this function of EGF was dramatically impaired by knocking down of Pin1 (Supplementary Figure S6F). Therefore, Pin1 does participate in EGF-induced cell proliferation.

Discussion

Pin1 has been shown to regulate the function of a variety of transcription factors by its unique phosphorylation-dependent isomerization. In our current study, we not only characterized TR3 as a novel target of Pin1, but also proposed a working model for the regulation of Pin1 in TR3 functions (Figure 6d). Upon binding to the Ser95-Pro motif of TR3, which was reported to be phosphorylated by JNK1 (Liu *et al*., 2007), Pin1 efficiently enhances the protein stability of TR3; while phosphorylation of TR3 on Ser431 by ERK2 facilitates Pin1 recognition, resulting in augment of TR3 transcriptional activity by promoting the recruitment of p300. This in turn increases the expression of cyclin D2 and E2F1, downstream targets of TR3, and stimulates cell proliferation. Our study thus demonstrates for the first time that Pin1-induced post-phosphorylation modification is an important event in TR3-mediated cell proliferation.

In TR3 molecule, at least three different pSer-Pro sites are responsible for Pin1 interaction and isomerization. The pSer95-Pro motif of TR3 is required for Pin1-induced stabilization; while the isomerization of pSer431-Pro by Pin1 promotes the transactivational function of TR3. Although the exact function of the pSer140-Pro isomerization remains to be elucidated, we may concluded that Pin1 exerts distinct regulatory functions upon TR3 by isomerizing different Ser-Pro motifs, and these isomerizations of TR3 at multiple sites finally promote the mitogenic function of TR3 in coordination.

The post-phosphorylation isomerization induced by Pin1 is initiated through the activities of various protein kinases. TR3 is a hyperphosphorylated protein that can be phosphorylated by a number of protein kinases

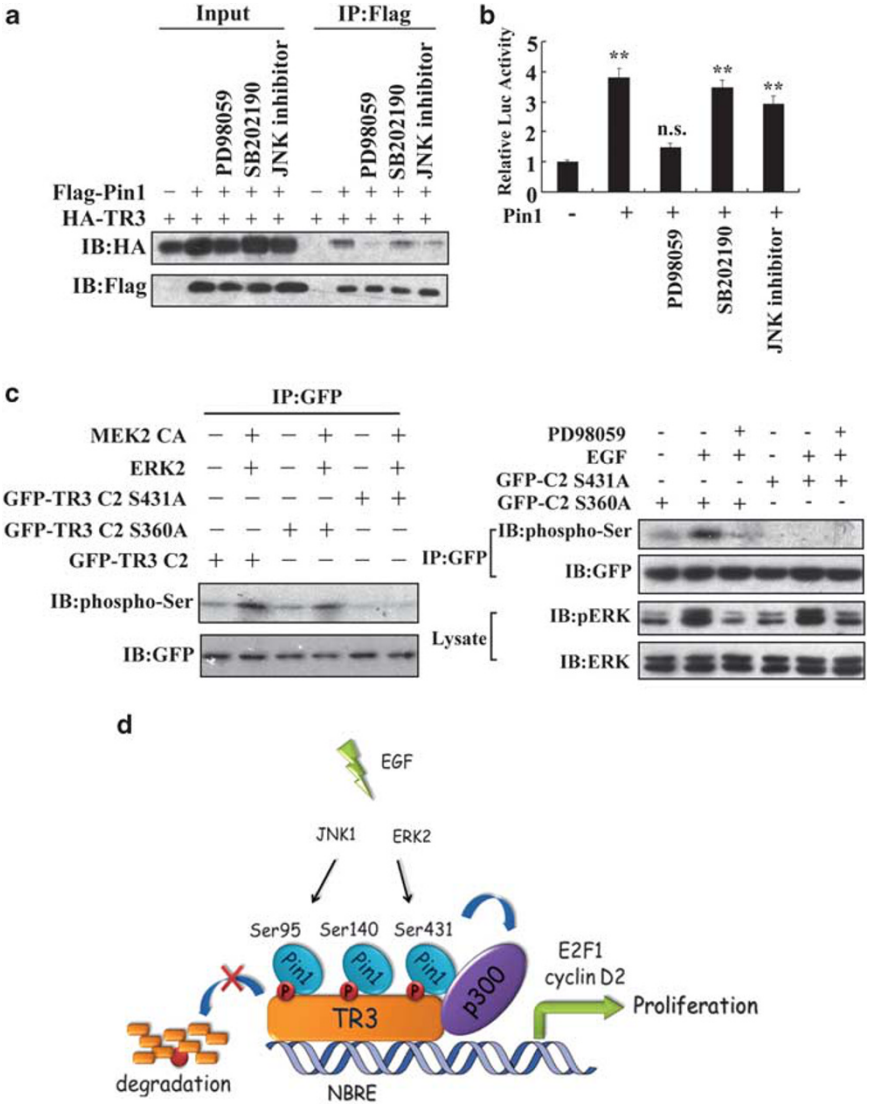

Figure 6 ERK and JNK are involved in regulating the Pin1–TR3 cross-talk. (**a**) Effects of different inhibitors on the TR3–Pin1 interaction. HEK293T cells were transfected with TR3 and Pin1, treated with PD98059 (20 μM), SB202190 (5 μM) or JNK inhibitor (10 μM), respectively, for 3 h as indicated and subjected to co-IP assays. (**b**) Effects of different inhibitors on Pin1-induced TR3 transactivation. Flag-Pin1 together with NurRE-luciferase reporter was transfected into 293T cells and then treated with different inhibitors for 3 h. The transactivation activity of TR3 was detected using a luciferase assay. $^{**}P<0.01$. (**c**) TR3 Ser431 is a novel phosphorylation site for ERK2. (Left panel) Different TR3 mutants together with ERK2 and MEK2 CA were transfected into 293T cells as indicated. The phosphorylation of TR3 was detected using an anti-phospho-serine antibody. (Right panel) After serum starvation overnight, cells were treated with EGF (200 ng/ml) for 1 h. Cell lysates were then collected and subjected to western blotting. The phosphorylation of TR3 was determined as described above. (**d**) A working model for the effect of Pin1 on TR3-mediated cell proliferation.

including ERK2 (Katagiri *et al*., 2000; Slagsvold *et al*., 2002; Jacobs *et al*., 2004) and JNK1 (Kolluri *et al*., 2003; Han *et al*., 2006; Liu *et al*., 2007). Several studies have highlighted the importance of the ERK signal pathway in controlling TR3 translocation (Katagiri *et al*., 2000; Jacobs *et al*., 2004). In the present study, we showed that ERK also regulates the transactivity of TR3 through Pin1 mediation. The Ser431 residue of TR3 was identified as a novel ERK2 phosphorylation site and the phosphorylation of this site is indispensable for Pin1 to regulate TR3 transactivation activity. This transactivation effect may owe to enhancement of the p300 recruitment. We have previously reported that the FLELFIL sequence located at aa 443–449 of TR3 is important for the TR3–p300 interaction (Li *et al*., 2007). Thus, the isomerization of pSer431-Pro by Pin1 may induce the conformational change of TR3, leading to the exposure of the nearby FLELFIL sequence, and further enhancing the interaction of TR3 with p300.

JNK1 is another kinase that phosphorylates TR3 and further facilitates the recognition of Pin1. Previously, we found that phosphorylation of TR3 by JNK1 at Ser95 site is involved in the ubiquitination regulation of TR3 upon anisomycin treatment (Liu *et al*., 2007). In our

current analyses, pSer95-Pro motif is required for Pin1 to stabilize TR3. Interestingly, both PD98059 and JNK inhibitor treatment abolished Pin1-induced TR3 expression, implying that ERK and JNK may cooperate in phosphorylating TR3 at Ser95 site. Since some ubiquitin E3 ligases or co-factors have a structural preference and specifically bind to phospho-Ser/Thr-Pro motifs in a *trans* conformation (Orlicky *et al*., 2003; Siepe and Jentsch, 2009; Zhou *et al*., 2009), we therefore speculate that Pin1 may convert the phospho-Ser95-Pro motif from a *trans* isomer to a *cis* conformation, which is unfavorable for the interaction of E3 ligase to TR3, resulting in stabilizing the TR3 protein. Therefore, identification of E3 ligase for TR3 will be important for further revealing the molecular mechanism of TR3 degradation.

The mitogenic function of TR3 has been reported by many studies (Hazel *et al*., 1988; Chang *et al*., 1989; Kolluri *et al*., 2003). Recently, it has been reported that TR3-induced survivin expression is important for pancreatic cancer cell growth (Lee *et al*., 2010). However, survivin is not the direct target of TR3. The exact molecular mechanisms for the pro-proliferative function of TR3 remained elusive. Cyclin D2 is a D-type cyclin that promotes the activity of CDK4 and CDK6, and thus accelerates G1 progression of cultured mammalian cells (Sherr and Roberts, 2004). In this study, we identify that cyclin D2 is one of novel downstream targets for TR3 to regulate cell proliferation. Induction of cyclin D2 by TR3 relies on the existence of Pin1 to some extent. When all the Pin1-binding motifs were mutated, TR3 no longer stimulates cyclin D2 expression. In addition, E2F1, which has an important role in cell proliferation (Polager and Ginsberg, 2009), was also found to be upregulated by TR3 in a Pin1-dependent manner. These regulatory functions of Pin1 toward TR3 were further confirmed *in vivo* with the use of nude mice. Silence of TR3 significantly suppresses the growth of xenograft tumor; however, knocking down of Pin1 nearly abolished the mitogenic effect of TR3. Therefore, Pin1 participates in TR3-activated mitogenic event.

In summary, our study indicates Pin1 acting as a mitogenic amplifier through isomerization of the orphan nuclear receptor TR3 at multiple phosphorylation sites and thus provides a significant increase in our understanding of the oncogenic functions of Pin1.

Materials and methods

Cell culture

HEK293T cells were purchased from ATCC (Manassas, VA, USA). Pin1 knockdown HEK293T and HeLa cells were established previously (Ryo *et al*., 2007). These cell lines were all maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U penicillin and 100 mg/ml streptomycin.

Reagents and siRNA

LightShift Chemiluminescent EMSA Kit, TurboFect transfection reagent, DharmaFect transfection reagents, goat anti-rabbit and anti-mouse secondary antibody conjugated to horseradish peroxidase are purchased from Thermo Fisher Scientific (Bremen, Germany); protein A/G agarose, polyclonal anti-TR3, anti-GFP, anti-HA, phycoerythrin-conjugated anti-BrdU antibodies are purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-flag, anti-tubulin antibodies and PD98059, SB202190, JNK inhibitor II, CHX are purchased from Sigma (St Louis, MO, USA); anti-phospho-serine antibody and EGF are purchased from Invitrogen (Carlsbad, CA, USA); monoclonal anti-TR3, anti-cyclin D2, anti-E2F1, anti-ERK, anti-phospho-ERK antibodies are purchased from Cell Signaling Technology (Beverly, MA, USA). ERK2 siRNA is purchased from Sigma, and transfected using DharmaFect transfection reagents. The sequence of TR3 targeted by pLL3.7 lentivirus system is 5′-GGGCATGGTGAAGGA AGTT-3′; the sequences of control siRNA in pLL3.7 lentivirus system is 5′-GCGCGCTTTGTAGGATTCG-3′.

GST pull-down assay

GST beads (Sigma) bound GST-tagged proteins were incubated with cell lysates from transfected HEK293T cells for 3 h. For CIAP treatment, the cell lysates were preincubated with 0.2 U/μl CIAP in 37 °C for 1 h. The beads were washed five times with lysis buffer, boiled in sodium dodecyl sulfate sample buffer, fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotted.

Luciferase activity assay

Cells were transfected with various plasmids, including a luciferase-linked reporter gene (NurRE) (Liu *et al*., 2007), a β-galactosidase (β-gal) expression vector and vectors expressing Pin1 or TR3 as required. At 36 h after transfection, cells were lysed and measured for luciferase and β-gal activities. The β-gal activity was used to normalize for transfection efficiency. The data are presented as the mean ± s.d. of at least three independent experiments.

Electrophoretic mobility shift assay

Nuclear proteins were prepared and analyzed by EMSA as described previously (Liu *et al*., 2010). Biotin-labeled *CCND2* (5′-ACTTCAGGGACA*TGACCTTT* ATCTCTGGGT-3′) and *CCND2 mut* (5′-ACTTCAGGGACA*TGTTTTTT* ATCTCTG GGT-3′) oligonucleotides were purchased from Invitrogen for use as probes.

ChIP assay

ChIP assays were carried out as described previously (Lei *et al*., 2009). The primer sequences are shown as below:

Cyclin D2 promoter: sense: 5′-CCTCCCTCTTCTTCCTCT GC-3′; anti-sense: 5′-TCCTAATCCTCCTGCCCTTG-3′

E2F1 promoter: sense 5′-ACGCTCTTCCAGGACGTG AG-3′; anti-sense 5′-GGTGACCGCTGCTGTGATTG-3′

glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoter: sense: 5′-GAGATGCCAGGAGCCAGGAGATG-3′; anti-sense: 5′-GAAACAGGAGGACTTTGGGAACG-3′.

MTT assay

Cells were transfected with different expression vectors and seeded in 96-well plate. Seventy-two hours later, the viable cell numbers were determined by determination of the activity of enzymes that reduce MTT as previously described (Zhan *et al*., 2008).

Colony formation assay

Twenty-four hours after transfection, 500 transfected cells were placed in a fresh six-well plate and maintained in Dulbecco's modified Eagle's medium containing 10% FBS for 2 weeks.

Colonies were fixed with methanol and stained with 0.1% crystal violet in 20% methanol for 15 min.

BrdU incorporation assay

WT and Pin1 knockdown HeLa cells that were transfected with TR3 were serum starved overnight. After 18 h of EGF (200ng/ml) treatment, cells were incubated with 5-BrdU (20 μmol/l; Sigma) for 2 h. After washing with phosphate-buffered saline, cells were fixed with 4% paraformaldehyde for 30 min at 4 °C, and then incubated with saponin (0.1%) for another 10 min. The cells were washed twice with phosphate-buffered saline containing 0.1% saponin and digested with 30 μg of DNase I. After incubation with phycoerythrin-conjugated anti-BrdU antibody (Santa Cruz) for 1 h, cells were washed with phosphate-buffered saline, and analyzed by flow cytometer (Beckman Coulter, Fullerton, CA, USA).

Xenograft experiments

Athymic nude mice (BALB/c, SPF grade, 18–20 g, 6–7 weeks old) were housed under sterilized conditions. WT and Pin1 knockdown HeLa cells (5×10^6 cells), that were pretransfected with scramble or TR3-siRNA, were suspended in Dulbecco's modified Eagle's medium and injected subcutaneously into the either posterior flanks of the animals. Mice were killed by cervical dislocation 3 weeks after inoculation, and the tumor weights were recorded. Animals were maintained at Xiamen University Laboratory Animal Centre (Xiamen University, China) in accordance with the institution guidelines.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by grants from Ministry of Education, China (Project 111, B06016) to WQ and YCL; grants from the Ministry of Science and Technology, China (2007CB914402) to WQ; the National Natural Science Foundation of China (30810103905 and 30871281 to WQ, 31000620 to CHZ); grants from the National Key New Drug Creation Program of China (2009ZX09103-083) to WQ; grants from the Fundamental Research Funds for the Central Universities, China (2010121096) to CHZ; Science Planning program grant of Fujian Province, China (2009J1010); and the grant (09/1/21/19/604) from the Biomedical Research Council, the Agency for Science, Research and Technology, Singapore to YCL.

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