HHS Public Access

Author manuscript

Oncogene. Author manuscript; available in PMC 2013 September 21.

Published in final edited form as:

Oncogene. 2013 March 21; 32(12): 1488–1496. doi:10.1038/onc.2012.178.

Prolyl isomerase Pin1 down-regulates tumor suppressor RUNX3 in breast cancer

Ying-Hung Nicole Tsang[1,2], **Xue-Wei Wu**[1], **Jing-Shan Lim**[1], **Chee Wee Ong**[3,4], **Manuel Salto-Tellez**[3,4], **Kosei Ito**[5], **Yoshiaki Ito**[2,4], and **Lin-Feng Chen**[1,6,†]

[1]Department of Biochemistry, Singapore 138673, Singapore

[2]Institute of Molecular and Cell Biology, Singapore 138673, Singapore

[3]Department of Pathology, National University of Singapore, Singapore

[4]Cancer Science Institute of Singapore, National University of Singapore, Singapore 138673, Singapore

[5]Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki 852-8588, Japan

[6]College of Medicine, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

Abstract

Emerging evidence demonstrates that RUNX3 is a tumor suppressor in breast cancer. Inactivation of RUNX3 in mice results in spontaneous mammary gland tumors, and decreased or silenced expression of RUNX3 is frequently found in breast cancer cell lines and human breast cancer samples. However, the underlying mechanism for initiating RUNX3 inactivation in breast cancer remains elusive. Here, we identify prolyl-isomerase Pin1, which is often over-expressed in breast cancer, as a key regulator of RUNX3 inactivation. In human breast cancer cell lines and breast cancer samples, expression of Pin1 inversely correlates with the expression of RUNX3. In addition, Pin1 recognizes four phosphorylated Ser/Thr-Pro motifs in RUNX3 via its WW domain. Binding of Pin1 to RUNX3 suppresses the transcriptional activity of RUNX3. Furthermore, Pin1 reduces the cellular levels of RUNX3 in an isomerase activity-dependent manner by inducing the ubiquitination and proteasomal degradation of RUNX3. Knocking down Pin1 enhances the cellular levels and transcriptional activity of RUNX3 by inhibiting the ubiquitination and degradation of RUNX3. Our results identify Pin1 as a new regulator of RUNX3 inactivation in breast cancer.

Keywords

breast cancer; degradation; Pin1; RUNX3; tumor suppressor; ubiquitination

[†]**Corresponding author:** Lin-Feng Chen, Department of Biochemistry, College of Medicine, MC-714, University of Illinois at Urbana-Champaign, Urbana, IL 61801 Tel: 217-333-7764; Fax:217-244-5858 lfchen@life.illinois.edu.

Conflict of interest The authors declare no conflict of interest

Introduction

The increased expression of estrogen receptor α (ERα) and the subsequently enhanced cellular responses to estrogen are the major risk factors in breast carcinogenesis (1). Enhanced estrogen signaling through ERα is associated with initiation and progression of breast cancer (2). Genetic alterations including the activation of dominant-acting oncogenes and the inactivation of specific tumor suppressor genes also contribute to breast cancer progression (3). We have recently identified RUNX3 as a tumor suppressor in breast cancer and demonstrated that RUNX3 elicits its tumor suppressor function by targeting ERα for proteasome-mediated degradation (4).

RUNX3 belongs to the family of Runt-related transcription factors (*RUNX*), which play essential roles in development and carcinogenesis (5). Functional inactivation of RUNX3 is frequently observed in a variety of cancers including breast cancer. Inactivation of RUNX3 in mammary epithelial cells leads to the development of ductal carcinoma in more than 20% of *Runx3*$^{+/-}$ female mice (4). The expression of RUNX3 generally decreases during breast cancer progression, and inactivation of RUNX3 constitutes an early event in breast cancer progression (6-8). Furthermore, loss of RUNX3 expression correlates with poor survival in breast cancer (7). Therefore, the expression level of RUNX3 is closely associated with the initiation and progression of breast cancer and has been suggested as a potential biomarker for cancer diagnosis (9, 10).

Inactivation of RUNX3 could occur at the genetic, epigenetic or cellular levels (11). Hemizygous deletion of the *Runx3* gene is found in many breast cancer cell lines (12). The promoter of *Runx3* is often hypermethylated, which correlates to the expression of RUNX3 in breast cancer cell lines and breast cancer tissues (8, 12). Additionally, cytoplasmic sequestration of RUNX3 is a frequent occurrence in breast cancer (8). While the detailed mechanisms for the genetic and epigenetic silencing of *Runx3* are not clear, posttranslational modifications of RUNX3 appear to be the key regulatory mechanism for inactivation of RUNX3 at the cellular level. Various posttranslational modifications, especially phosphorylation, have been demonstrated to control the cellular functions of RUNX3 (13). RUNX3 is a serine (Ser), threonine (Thr) and proline (Pro) rich protein, and many of these residues are subject to phosphorylation by different kinases (14). Phosphorylation of RUNX3 alters the functional properties of RUNX3, including its subcellular localization, protein stability, and its interaction with other proteins (14). However, how phosphorylation actually changes different properties of RUNX3 is unclear.

Peptidyl-prolyl *cis-trans* isomerase (PPIase) Pin1 only binds to peptide motifs containing phosphorylated Ser/Thr residues preceding a Pro (pSer/Thr-Pro). It contains an N-terminal WW domain involved in protein interaction, and a catalytic C-terminal PPIase domain (15). Upon binding to the pSer/Thr-Pro motif via the WW domain, it catalyzes the *cis-trans* isomerization of the bond N-terminal to the proline residue. This isomerization changes the conformation and the functional properties of the substrates. Pin1-mediated isomerization regulates the substrates' stability, phosphorylation status, protein-protein interaction, and subcellular localization in diverse cellular processes (15, 16).

Pin1 is a key signaling molecule involved in breast development and breast cancer (17, 18). Pin1 is overexpressed in breast cancer, and its levels positively correlate with the tumor grade in invasive breast cancer (18). In tandem with its overexpression in breast cancer, Pin1 is capable of mediating multiple oncogenic pathways and contributes to the tumorigenic potential of cells during mammary carcinogenesis. For example, overexpression of Pin1 leads to the upregulation of cyclin D1 and the transformation of breast epithelial cells (18, 19). Pin1 also upregulates estrogen responses by targeting ERα and its coactivator SRC-3 (20, 21). Furthermore, Pin1 enhances Notch1 transcription activation and tumorigenic potential in breast cancer (22). Additionally, Pin1 downregulates the tumor suppressor promyelocytic leukemia protein (PML) to promote the proliferation of breast cancer cells (23, 24).

We have recently identified RUNX3 as a novel tumor suppressor in breast cancer (4); however, its regulation in breast cancer is largely unknown. RUNX3 is a phosphorylated protein with multiple Ser/Thr-Pro motifs, raising the possibility that RUNX3 might be a target of Pin1. In an effort to understand the regulation of RUNX3 in breast cancer, we found that phosphorylated RUNX3 is specifically recognized by the WW domain of Pin1. Binding of Pin1 to four pSer/Thr-Pro motifs induces the ubiquitination, degradation, and inactivation of RUNX3. Our results reveal a mechanism by which RUNX3 is inactivated by Pin1 in breast cancer and identify a novel function of Pin1 as a regulator of tumor suppressor RUNX3.

Results

Pin1 levels inversely correlate with RUNX3 levels in human breast cancer cell lines and tissues

To investigate the possibility that Pin1 might be involved in the inactivation of RUNX3 in breast cancer, we first employed immunohistochemistry to examine the possible pathological correlation of the expression of Pin1 and RUNX3 in human normal breast and breast cancer samples. In human normal breast, the expression of Pin1 was barely detectable (Figure 1a) but RUNX3 was highly expressed in these tissues. In contrast, in human breast cancer the expression of Pin1 was significantly enhanced while the expression of RUNX3 was dramatically reduced (Figure 1a). When we further examined the expression of Pin1 and RUNX3 in a cohort of 80 human breast ductal carcinoma samples, we observed that more than 90% of samples (19 out of 21) with high expression of Pin1 displayed low or no expression of RUNX3 (Figure 1b). Conversely, approximately 78% of samples (46 out of 59) with low levels of Pin1 had higher expression of RUNX3. Statistic analysis reveals an inverse correlation between the expression of Pin1 and RUNX3 in these cancer samples with a Spearman coefficient for correlation (Pin1 and RUNX3) of −0.615 ($p<0.01$) (Figure 1b).

To further explore the possible relationship between expression levels of Pin1 and RUNX3, we examined the expression of Pin1 and RUNX3 in a panel of cell lines that include nontumorigenic breast epithelial cells and breast cancer cells (Figure 1c). In the non-tumorigenic MCF-12A cells, expression of Pin1 was barely detectable, and RUNX3 expression was moderately high (Figure 1c). However, in most breast cancer cell lines, Pin1 was highly expressed while the expression of RUNX3 was extremely low except for in

HCC70 and MDAMB-157 (Figure 1c). The overall expression of Pin1 seems to inversely correlate with the expression of RUNX3 in non-tumorigenic breast epithelial cells and in some breast cancer cell lines (Figure 1c), although the mRNA levels of Pin1 and RUNX3 in these cells do not precisely correspond to their protein levels (Supplementary Figure 1).

To better examine the correlation between Pin1 and RUNX3 expression, we next determined the effects of Pin1 inhibition on RUNX3 expression in breast cancer cells. First, we knocked down the expression of Pin1 using small interfering RNA (siRNA) against Pin1 in RUNX3-MCF-7 cells, in which exogenous RUNX3 is stably expressed (4). Ablation of Pin1 enhanced the cellular levels of exogenously expressed RUNX3 (Figure 1d). Next, we knocked down the expression of Pin1 in MDA-MB-361 cells that express both Pin1 and RUNX3. Similar to the enhanced expression of RUNX3 in RUNX3-MCF-7 cells, depletion of Pin1 increased the levels of endogenous RUNX3 (Figure 1e). All together, these results indicate that Pin1 regulates the expression of RUNX3.

Pin1 interacts with RUNX3 in a phosphorylation-dependent manner

Since Pin1 regulates protein functions through its prolyl-isomerase activity by directly binding to phosphorylated substrates (15), we then investigated whether Pin1 bound to RUNX3. In an immunoprecipitation assay with ectopically expressed Pin1 and RUNX3, we found that immunoprecipitation of Pin1 co-immunoprecipitated RUNX3 from transfected cells (Figure 2a). Furthermore, when the physical interaction between endogenous Pin1 and the endogenous RUNX3 was examined in MDA-MB-157 cells that express both Pin1 and RUNX3 (Figure 1c), Pin1 coimmunoprecipitated endogenous RUNX3 (Figure 2b). Conversely, an IgG control did not immunoprecipitate RUNX3 (Figure 2b). These data indicate that Pin1 and RUNX3 interacts in vivo. The interaction occurs likely in the nucleus since both RUNX3 and Pin1 expressed in the nucleus and a significant amount of each co-localized (Supplementary Figure 2). To further explore the interaction between Pin1 and RUNX3, we employed an in vitro binding assay using purified recombinant glutathione-S-transferase (GST)-fusion proteins of Pin1. GST-Pin1 fusion proteins but not GST proteins pulled down a significant amount of RUNX3 from cell lysates of HEK293T transfected with Flag-RUNX3 (Figure 2c), further confirming a specific interaction between Pin1 and RUNX3.

Pin1 selectively binds to substrates which contain a pSer/Thr-Pro motif (15). To determine whether the interaction between Pin1 and RUNX3 is also through such a mechanism, we pre-treated the cell lysates with the calf intestinal alkaline phosphatase (CIP) prior to the GST pull-down assay. Treatment of cell lysates with CIP completely abolished the interaction between Pin1 and RUNX3, suggesting a phosphorylation-dependent interaction between Pin1 and RUNX3 (Figure 2d). The phosphorylation appears to occur on the Ser/Thr-Pro motif of RUNX3 since Pin1 associated RUNX3 was recognized by the anti-pSer/Thr-Pro (MPM-2) antibodies (25, 26) and the CIP treatment significantly reduced the phosphorylated RUNX3 (Figure 2d). These data suggest that RUNX3 might contain a pSer/Thr-Pro motif which is recognized by Pin1. To furtehr explore the interaction between RUNX3 and Pin1, we used MPM-2 antibodies to immunoprecipitate RUNX3 from RUNX3-MCF-7 cell lysates. As shown in Figure 2e, RUNX3 was co-immunoprecipitated with the

MPM-2 antibodies but not by the IgG control, confirming that RUNX3 indeed contains a pSer/Thr-Pro motif. Collectively, these data demonstrate that Pin1 binds to RUNX3 in a phosphorylation-dependent manner, likely through the pSer/Thr-Pro motif.

Pin1 binds to four Ser/Thr-Pro motifs within RUNX3

Pin1 contains an N-terminal WW domain and a C-terminal catalytic PPIase domain (15). The WW domain forms a pocket that specifically binds to phosphorylated substrates using side chains of serine (S) 16, arginine (R) 17, tyrosine (Y) 23 and tryptophan (W) 34 (27). Since Pin1 binds to phosphorylated RUNX3 (Figure 2d), we then investigated whether the WW domain of Pin1 was involved in the binding to RUNX3. Mutation of R17 to alanine (A) significantly impaired Pin1's binding to RUNX3 and mutation of S16 or W34 to alanines completely abolished Pin1's binding to RUNX3 (Figure 3a). In contrast, mutation of cysteine (C) 115 to alanine, which destroys the *cis-trans* isomerization catalytic activity of Pin1 (27), had no effect on Pin1's binding to RUNX3. These data indicate that WW domain of Pin1 is essential for its interaction with RUNX3.

Since WW domain of Pin1 specifically binds to the pSer/Thr-Pro motif, we next assessed whether RUNX3 possessed such a motif that could be recognized by the WW domain of Pin1. To identify the specific pSer/Thr-Pro motif in RUNX3, we first determined which region of RUNX3 was involved in its association with Pin1. When GST-Pin1 was used to pull-down cell lysates containing full-length RUNX3 or its various deletion mutants (Figure 3c, left panel), we found that full-length RUNX3 as well as its four C-terminal deletion mutants (deleted up to amino acid 234) retained the ability to associate with Pin1 (Figure 3c, right panel). Further deletion to amino acid 187 completely eliminated RUNX3's interaction with Pin1 (Figure 3c, right panel), indicating that the region from amino acids 188 to 234 of RUNX3 is critical for the interaction with Pin1.

Analysis of RUNX3 sequence from amino acids 188 to 234 reveals that there are four Ser/Thr-Pro motifs within this region (Figure 3d), including threonines 209, 212, 231 and serine 214. To further determine which motif is in fact involved in Pin1 binding, we mutated each serine or threonine to alanine alone or in combination and examined the abilities of these mutants to bind to Pin1. RUNX3 retained its ability to bind to Pin1 when any of these motifs was mutated individually (Figure 3d). The binding of RUNX3 to Pin1 was completely abolished only when four motifs were mutated together (Figure 3d). An in vivo co-immunoprecipitation assay further confirmed the involvement of the four pSer/Thr-Pro motifs in the interaction since mutation of all four motifs disrupted RUNX3's interaction with Pin1 (Figure 3e). Collectively, these data demonstrate the possible redundancy of the pSer/Thr-Pro motifs of RUNX3 and that all four motifs are involved in the interaction with Pin1.

Pin1 negatively regulates the transcriptional activity of RUNX3

Having identified that Pin1 specifically binds to four pSer/Thr-Pro motifs of RUNX3, we sought to determine the functional consequence of this interaction. Since RUNX3 is a transcription factor and the function of RUNX3 is closely associated with its ability to regulate the expression of a variety of genes (5), we first examined the effect of Pin1 on the

transcriptional activity of RUNX3. RUNX3 activates the TβRE luciferase reporter, which contains three RUNX binding sites (28), in the presence of constitutively activated TGF-β type I receptor (TβRI) in HEK293T cells (Figure 4a). However, the transcriptional activity of RUNX3 was inhibited by the co-expression of wild-type Pin1 in a dose-dependent manner but not by Pin1-C115A or Pin1-W34A (Figure 4a). These data demonstrate that Pin1 negatively regulates the activity of RUNX3 and that the inhibitory effect of Pin1 relies on its binding to RUNX3 via the WW domain and its *cis-trans* isomerase activity. A similar inhibitory effect of RUNX3 by Pin1 was also observed in the more physiologically relevant MCF-7 breast cancer cells (Figure 4b). RUNX3 displayed lower transcription activity in MCF-7 cells than in HEK293T cells likely due to the lower transfection efficiency in MCF-7 cells (Figure 4b). Consistent with the finding that the inhibition relies on the binding of Pin1 to RUNX3 (Figure 4a), Pin1 failed to attenuate the activity of RUNX3-4A, although the transcriptional activity of RUNX3-4A was lower than that of wild-type RUNX3 (Figure 4c).

To further confirm the inhibitory effect of Pin1 on RUNX3, we knocked down the expression of Pin1 in MCF-7 cells and examined the transcriptional activity of RUNX3. Treatment of the MCF-7 cells with Pin1 siRNA but not the control siRNA enhanced the transcriptional activity of RUNX3 (Figure 4d). Together, these data suggest that Pin1 negatively regulates the transcriptional activity of RUNX3.

Pin1 reduces the stability of RUNX3

Pin1 regulates many protein functions by affecting the stability of its target proteins (16). The inverse correlation between RUNX3 and Pin1 expression (Figure 1) and the ability of Pin1 to down-regulate the activity of RUNX3 (Figure 4) prompted us to investigate whether Pin1 might regulate the stability of RUNX3. To test this hypothesis, we ectopically expressed wild-type Pin1 or the enzymatically inactive Pin1-C115A in RUNX3-MCF-7 cells and examined the cellular levels of RUNX3. Expression of Pin1 in RUNX3-MCF-7 cells significantly reduced the cellular levels of RUNX3. However, co-expression of Pin1-C115A had little effect on the cellular levels of RUNX3, indicating that catalytic activity of Pin1 is required for this down-regulation (Figure 5a). When we treated the cells with proteasome inhibitor MG-132, we found that MG-132 reversed the decreased levels of RUNX3 (Figure 5b), suggesting that reduced expression of RUNX3 is due to proteasome-mediated degradation of RUNX3. Consistently, the transcription of RUNX3 was not affected by Pin1 since RUNX3 mRNA remained at a similar level with or without Pin1 (Figure 5c). Furthermore, Pin1 binding defective RUNX3-4A mutant was resistant to Pin1-mediated degradation (Figure 5d), indicating that the specific interaction is essential for the degradation of RUNX3 by Pin1.

To further evaluate the Pin1-mediated degradation of RUNX3, we compared the half-life of wild-type RUNX3 and RUNX3-4A in the absence or presence of Pin1. Consistent with the finding that Pin1 stimulates RUNX3 degradation (Figure 5a), expression of Pin1 reduced the half-life of RUNX3 from >4 hours to ~2 hours (Figures 5e & 5f). However, the half-life of RUNX3 was only slightly reduced when RUNX3 was co-expressed with Pin1-C115A (Figures 5e), confirming that catalytic activity is important for Pin1-mediated degradation of

RUNX3. When we examined the effect of Pin1 on the half-life of RUNX3-4A, we found that RUNX3-4A was unstable, with a much shorter half-life even in the absence of Pin1 (Figures 5e). Nevertheless, RUNX3-4A was resistant to Pin1-induced degradation since co-expression of Pin1 barely changed the half-life of RUNX3-4A (Figures 5e). These data suggest that phosphorylation of the four Ser/Thr-Pro motifs of RUNX3 plays an essential role in regulating the stability of RUNX3, and that Pin1 decreases the stability of RUNX3 via direct physical interaction with RUNX3.

Pin1 induces the ubiquitination of RUNX3

Given that Pin1 stimulates the degradation of RUNX3, we next determined whether Pin1 promoted the ubiquitination of RUNX3, an event that is required for proteasome-mediated degradation. RUNX3 was moderately ubiquitinated in the absence of Pin1 when RUNX3 was co-transfected with ubiquitin (Figure 6a). However, co-expression of Pin1 significantly enhanced the ubiquitination of RUNX3 (Figure 6a), indicating that Pin1 promotes RUNX3 ubiquitination in vivo. Pin1-induced ubiquitination of RUNX3 relied on its isomerase activity since co-expression of the catalytically inactive Pin1-C115A mutant failed to enhance the ubiquitination of RUNX3 (Figure 6a).

Since the interaction between Pin1 and RUNX3 via the pSer/Thr-Pro motifs is essential for Pin1-induced degradation of RUNX3 (Figure 5), we next assessed whether the interaction was also important for Pin1-induced ubiquitination of RUNX3 by examining the ubiquitination of RUNX3-4A mutant. Compared to wild-type RUNX3, the ubiquitination of RUNX3-4A was significantly enhanced even in the absence of Pin1 (Figure 6b). This enhanced ubiquitination of RUNX3-4A in the absence of Pin1 likely accounts for the reduced stability of RUNX3-4A with a shortened half-life (Figure 5e). However, ubiquitination of RUNX3-4A remained unchanged in the presence of Pin1 (Figure 6b). These data suggest that the Ser/Thr-Pro motifs are required for Pin1-mediated degradation of RUNX3 (Figure 6b).

To further confirm the Pin1-induced RUNX3 ubiquitination, we examined the ubiquitination of RUNX3 in RUNX3-MCF-7 cells by knocking down the expression of Pin1. Exogenously expressed RUNX3 was ubiquitinated in RUNX3-MCF-7 cells, which have high expression levels of Pin1 (Figure 6c). Depletion of Pin1 by siRNA dramatically reduced the ubiquitination of RUNX3, indicating that Pin1 is indeed involved in the ubiquitination of RUNX3. Furthermore, when we examined the ubiquitination of RUNX3 in another breast cancer cell line, MDA-MB-361, which expresses both RUNX3 and Pin1, we found that the ubiquitination of endogenous RUNX3 was also diminished by the depletion of Pin1 (Figure 6d). Collectively, these data suggest that Pin1 induces the ubiquitination of RUNX3 via its binding to the four Ser/Thr-Pro motifs through its WW domain.

Discussion

Inactivation of RUNX3 is closely related to the initiation and progression of breast cancer (4, 6). RUNX3 is inactivated by a variety of mechanisms including hemizygous deletion of the *Runx3* gene, hypermethylation of the *Runx3* promoter and mis-localization of the RUNX3 protein to the cytoplasm (5, 9). In this study, we have identified peptidyl prolyl

isomerase Pin1-induced degradation as a new mechanism for the inactivation of RUNX3 in breast cancer. Pin1 directly associates with phosphorylated RUNX3 and induces RUNX3's ubiquitination and degradation, thus inhibiting the activity of RUNX3. The inverse correlation between elevated levels of Pin1 and reduced expression of RUNX3 in human breast cancer underscores the relevance of our findings for human carcinogenesis.

It is well recognized that Pin1 regulates protein functions by binding to the pSer/Thr-Pro motif within the substrates via its WW domain (15). We find that Pin1 binds to RUNX3 both in vitro and in vivo and that the WW domain of Pin1 is essential for this interaction. Mutation of the key residues within the WW domain demolishes Pin1's binding to RUNX3 and its ability to suppress RUNX3 activity (Figures 3&4). We also identify four pSer/Thr-Pro motifs in RUNX3 that are specifically recognized by Pin1 (Figure 3). Any one of the four motifs seems to be sufficient for Pin1 binding since mutation of each individual motif had no effect on Pin1 binding but mutation of all four motifs completely abolished RUNX3's binding to Pin1 (Figure 3), reflecting a redundancy of these motifs of RUNX3. The regulation of RUNX3 by Pin1 might be conserved among other RUNX proteins since RUNX proteins are Ser/Thr-Pro rich and RUNX1 and RUNX2 also contain multiple putative Pin1 recognition motifs (5). Supporting this, we find that Pin1 also binds to RUNX1 and RUNX2 (data not shown). However, the four Pin1 binding motifs are uniquely present in RUNX3 but not in other RUNX proteins, raising the possibility that Pin1 might differentially regulate distinct functions of RUNX proteins by binding to different phosphorylated sites.

Phosphorylation of RUNX3 by various kinases plays an important role in regulating the functions of RUNX3 including the subcellular localization and protein stability (13). In this study, we identify four unique pSer/Thr-Pro motifs within RUNX3 that are recognized by Pin1. However, the identity of the kinase mediating the phosphorylation of these Ser/Thr-Pro motifs remains unclear. It has been proposed that cyclin-dependent kinases (CDKs) and mitogen activated protein kinases (MAPKs) are the major kinases mediating the phosphorylation of Ser/Thr-Pro motifs of Pin1 substrates (29). It is possible that cyclin D1 or cyclin E-dependent kinases might be involved in Pin1-induced degradation of RUNX3. Cyclin D1 has been shown to be involved in PTHrP-induced RUNX3 phosphorylation and degradation in chondrocytes, and these kinases are frequently overexpressed in breast cancers (30, 31). It is also possible that a MAPK targets these Ser/Thr-Pro motifs and initiates Pin1-induced ubiquitination and degradation of RUNX3. Additionally, other kinases besides CDKs and MAPKs have been shown to be able to phosphorylate pSer/Thr-Pro motifs and might phosphorylate the Ser/Thr-Pro motifs of RUNX3 as well (15).

The phosphorylation of Ser/Thr-Pro motifs by proline-directed kinases plays a key role in controlling various cellular responses in which Pin1 serves as a post-phosphorylation regulatory factor by changing the conformation of its target proteins (15). Binding of Pin1 to the four Ser/Thr-Pro motifs of RUNX3 markedly impairs its transcriptional activity and induces its degradation. These results raise the question of how binding of Pin1 at these phosphorylated motifs promotes ubiquitination and degradation of RUNX3. These four motifs are located immediately C-terminal of the runt domain, a region has been shown to be important for RUNX3 stability (32). Binding of Pin1 to these phosphorylated motifs

might induce the *cis-trans* isomerization of RUNX3 since *cis-trans* ismomerase activity of Pin1 is essential for Pin1-mediated ubiquitination and inactivation of RUNX3 (Figures 4 & 6). Isomerization-mediated conformational change of RUNX3 might result in the engagement or disengagement of some RUNX3 binding proteins. For example, conformational change might lead to an increased accessibility of a RUNX3 E3 ligase. Previous studies have shown that binding of Pin1 to phosphorylated Smad2/3 enhances the interaction of Smad2/3 with the E3 ligase Smurf2, which then triggers the ubiquitination and degradation of Smad2/3 and the down-regulation of transforming growth factor-β signaling (33). In this regard, Smurfs are also E3 ligases for RUNX3, and the binding of Pin1 to RUNX3 might similarly enhance the binding of these E3 ligases (13, 32). Alternatively, binding of Pin1 to RUNX3 might prevent the binding of coactivators such as p300, which mediates the acetylation of RUNX3. Acetylation of RUNX3 is known to prevent its ubiquitination and enhance its stability and activity (13). Our results also indicate that Pin1 inhibits the coactivation function of p300 on RUNX3 (data not shown). Therefore, binding of Pin1 to phosphorylated RUNX3 might inhibit p300-mediated acetylation, allowing the same lysines to undergo ubiquitination. It is also possible that binding of Pin1 affects its dimerization with CBFβ, which has been shown to be important for maintaining the stability of RUNX proteins (34).

Our results clearly demonstrate that binding of Pin1 to phosphorylated RUNX3 induces its ubiquitination and degradation (Figures 5&6). However, Pin1 binding-defective RUNX3-4A mutant displays similarly enhanced ubiquitination, reduced stability and transcriptional activity even in the absence of Pin1 (Figures 4, 5&6). These data suggest that phosphorylation of the four Ser/Thr-Pro motifs is crucial for the stability and function of RUNX3, and that binding of Pin1 might also contribute to the dephosphorylation of RUNX3. Such a function of Pin1 is not unprecedented. Pin1 has been shown to be involved in the recycling of phosphorylation/dephosphorylation of Cdc25C and tau proteins (16, 35). By a similar mechanism, Pin1 might promote a conformation-specific phosphatase-mediated dephosphorylation of RUNX3. As such, Pin1 appears to serve as a molecular switch that dictates the fate of RUNX3 by controlling its phosphorylation status. It is likely that, when the levels of Pin1 are relatively low in normal breast cells, proper phosphorylation of RUNX3 at the four motifs stabilizes and activates RUNX3. However, when the Pin1 levels are increased through the progression of breast cancer, Pin1 binds to the phosphorylated RUNX3 and induces its degradation and inactivation. RUNX3 has been shown to be a tumor suppressor in a variety of cancers including lung and prostate cancers, and Pin1 is highly expressed in many of these cancers (9, 36). Whether Pin1 similarly regulates RUNX3 function in these different cancers remains an interesting question and needs to be further explored.

Overall, our studies have explored the possible functional consequence of the physical interaction between Pin1 and RUNX3 in breast cancer. The specific interaction between the WW domain of Pin1 and the four pSer/Thr-Pro motifs of RUNX3 suppresses the activity of RUNX3 by inducing its ubiquitination and proteasomal degradation. The over-expression of Pin1 in many cancers and its ability to regulate a variety of target proteins suggest that Pin1-specific inhibitors may have potential as anti-cancer drugs (15, 37). Our findings further

support this notion, since inhibiting the activity of Pin1 by siRNA or Pin1 inhibitor Juglone in breast cancer results in enhanced expression and activation of RUNX3 (Figures 1&4 and Supplementary Figure 3). Restoration of RUNX3 in breast cancer cells suppresses the proliferation and invasiveness of cancer cells (4, 8). Regulation of the interaction between Pin1 and RUNX3 might also be a potential target for the prevention of breast cancer. Therefore, identification of Pin1 as a novel regulator of RUNX3 not only provides new insights into the tumor suppressor function of RUNX3 and its regulation in breast cancer, but also provides potential alternative approaches for the prevention and treatment of breast cancer by inhibiting Pin1.

Materials and Methods

Cell lines, recombinant proteins, and plasmids

Human breast cancer cell lines MCF-7, BT-20, HS587T, HCC70, T-47D, MDA-MB-157, MDA-MB-361, MDA-MB-231, human mammary epithelia cell line MCF-12A and human embryonic kidney HEK293T cells were purchased from ATCC and were maintained according to ATCC culture conditions. GST-Pin1 expression vector was kindly provided by Dr. H.Y Kao (Case Western Reserve University). The GST-Pin1 fusion proteins were expressed in *Escherichia coli* BL-21 and purified according to the manufacturer's instructions (GE Healthcare, Piscataway, NJ, USA). Expression vectors for wild-type RUNX3 and its deletion mutants have been previously described (38). Expression vector for Flag-Pin1 was kindly provided by Dr. K.P. Lu (Harvard Medical School). Pin1-C115A, Pin1-W34A and RUNX3 Ser/Thr-Pro mutants were generated using Quikchange site-mutagenesis (Stratagene, San Diego, CA, USA) and mutations were confirmed by sequencing.

Antibodies

Antibodies against Flag, HA, Myc, ubiquitin and tubulin were from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). Antibody against Pin1 was from R&D systems (Minneapolis, MN, USA), and antibody against RUNX3 was from Abcam (Cambridge, MA, USA). Anti-p-Ser/Thr-Pro antibodies were from EMD Millipore (Billerica, MA, USA)

Human tissue samples and immunohistochemical staining

Paraffin blocks of mastectomy specimens from a total of 80 invasive ductal carcinomas were obtained from the pathology department of the National University Hospital, Singapore after approval of ethical issues by the Domain Specific Research Board of the National Health Care Group (NHG) of Singapore (approval code B06/006). The detection of the expression of RUNX3 and Pin1 of the human tissue microarray was performed as previously described (39). RUNX3 staining intensity was graded as previously described with a score 0 to 3 (6). Samples with a score 0 were graded as negative, samples with a score 1-3 were graded as positive. Pin1 staining intensity was graded the same as RUNX3.

siRNA treatment, immunoprecipitation and immunoblotting analysis

The pre-designed siRNA targeting Pin1 was purchased from Ambion (Austin, TX, USA). These siRNAs were used to transfect MCF-7 cells and MDA-MB-361 cells using

Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen, San Diego, CA, USA). Immunoprecipitation and immunoblotting analysis were performed as previously described (40).

Transient transfection and the luciferase reporter assay

MCF-7 cells were transfected with Fugene 6 HD according to the manufacturer's protocol (Roche, Indianapolis, IN, USA) with 0.5 μg of TβRE3-luc (28), 0.5 μg of TβR-I(CA), 0.25 μg of Flag-RUNX3, and respective dosage of Flag-Pin1 or its mutants. HEK293T cells were transfected using calcium phosphate transfection method. 48 hr post-transfection, firefly and renilla luciferase activities were measured with the dual luciferase assay system from Promega (Madison, WI, USA).

GST pull-down Assay

Agarose beads containing GST or GST-Pin1 were incubated with HEK293T cell lysates transfected with various RUNX3 expression vectors in 500 μl IP buffer (50 mM HEPES, pH 7.4; 250 mM NaCl; 1 mM EDTA; 1% Nonidet P-40; protease inhibitor cocktail (Roche); 1 mM PMSF), followed by rotation at 4°C for 2 hr. Binding of the Pin1 to RUNX3 was detected by immunoblotting with anti-Flag antibody.

Pulse-Chase Analysis

Pulse-chase experiments were performed as previously described (38). Basically, HEK293T cells were transfected with the indicated combination of expression vectors for RUNX3 and Pin1 for 24 hr before being subjected to treatment with cycloheximide (Sigma, St. Louis, MO, USA) to a final concentration of 100ng/ml for a period of 0, 1, 2, or 4 hr. Cells were lysed and analyzed with immunoblotting for the expression of RUNX3.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Drs Lu KP and Kao HY for the gift of reagents and members in the Chen lab for discussion. This work is supported in part by fund provided by UIUC (to L.F.C.) and NIH grants DK-085158 (to L.F.C.). Y.H.T. is an A*STAR-Illinois Partnership fellow.

References

1. Katzenellenbogen BS, Katzenellenbogen JA. Estrogen receptor transcription and transactivation: Estrogen receptor alpha and estrogen receptor beta: regulation by selective estrogen receptor modulators and importance in breast cancer. Breast Cancer Res. 2000; 2:335–44. [PubMed: 11250726]
2. Cheskis BJ, Greger JG, Nagpal S, Freedman LP. Signaling by estrogens. J Cell Physiol. 2007; 213:610–7. [PubMed: 17886255]
3. Lee EY, Muller WJ. Oncogenes and tumor suppressor genes. Cold Spring Harb Perspect Biol. 2010; 2:a003236. [PubMed: 20719876]
4. Huang B, Qu Z, Ong CW, Tsang YH, Xiao G, Shapiro D, et al. RUNX3 acts as a tumor suppressor in breast cancer by targeting estrogen receptor alpha. Oncogene. 2011; 31:527–34. [PubMed: 21706051]

5. Ito Y. Oncogenic potential of the RUNX gene family: 'overview'. Oncogene. 2004; 23:4198–208. [PubMed: 15156173]
6. Subramaniam MM, Chan JY, Soong R, Ito K, Ito Y, Yeoh KG, et al. RUNX3 inactivation by frequent promoter hypermethylation and protein mislocalization constitute an early event in breast cancer progression. Breast Cancer Res Treat. 2009; 113:113–21. [PubMed: 18256927]
7. Jiang Y, Tong D, Lou G, Zhang Y, Geng J. Expression of RUNX3 gene, methylation status and clinicopathological significance in breast cancer and breast cancer cell lines. Pathobiology. 2008; 75:244–51. [PubMed: 18580070]
8. Lau QC, Raja E, Salto-Tellez M, Liu Q, Ito K, Inoue M, et al. RUNX3 is frequently inactivated by dual mechanisms of protein mislocalization and promoter hypermethylation in breast cancer. Cancer Res. 2006; 66:6512–20. [PubMed: 16818622]
9. Subramaniam MM, Chan JY, Yeoh KG, Quek T, Ito K, Salto-Tellez M. Molecular pathology of RUNX3 in human carcinogenesis. Biochim Biophys Acta. 2009; 1796:315–31. [PubMed: 19682550]
10. Chen LF. Tumor suppressor function of RUNX3 in breast cancer. J Cell Biochem. 2012 Epub ahead of print 2012 Jan 13. doi: 10.1002/jcb.24074.
11. Chuang LS, Ito Y. RUNX3 is multifunctional in carcinogenesis of multiple solid tumors. Oncogene. 2010; 29:2605–15. [PubMed: 20348954]
12. Hwang KT, Han W, Bae JY, Hwang SE, Shin HJ, Lee JE, et al. Downregulation of the RUNX3 gene by promoter hypermethylation and hemizygous deletion in breast cancer. J Korean Med Sci. 2007; 22(Suppl):S24–31. [PubMed: 17923751]
13. Bae SC, Lee YH. Phosphorylation, acetylation and ubiquitination: the molecular basis of RUNX regulation. Gene. 2006; 366:58–66. [PubMed: 16325352]
14. Bae JS, Jang MK, Hong S, An WG, Choi YH, Kim HD, et al. Phosphorylation of NF-kappa B by calmodulin-dependent kinase IV activates anti-apoptotic gene expression. Biochem Biophys Res Commun. 2003; 305:1094–8. [PubMed: 12767944]
15. Lu KP, Zhou XZ. The prolyl isomerase PIN1: a pivotal new twist in phosphorylation signalling and disease. Nat Rev Mol Cell Biol. 2007; 8:904–16. [PubMed: 17878917]
16. Liou YC, Zhou XZ, Lu KP. Prolyl isomerase Pin1 as a molecular switch to determine the fate of phosphoproteins. Trends Biochem Sci. 2011; 36:501–14. [PubMed: 21852138]
17. Wulf G, Ryo A, Liou YC, Lu KP. The prolyl isomerase Pin1 in breast development and cancer. Breast Cancer Res. 2003; 5:76–82. [PubMed: 12631385]
18. Wulf GM, Ryo A, Wulf GG, Lee SW, Niu T, Petkova V, et al. Pin1 is overexpressed in breast cancer and cooperates with Ras signaling in increasing the transcriptional activity of c-Jun towards cyclin D1. EMBO J. 2001; 20:3459–72. [PubMed: 11432833]
19. Liou YC, Ryo A, Huang HK, Lu PJ, Bronson R, Fujimori F, et al. Loss of Pin1 function in the mouse causes phenotypes resembling cyclin D1-null phenotypes. Proc Natl Acad Sci U S A. 2002; 99:1335–40. [PubMed: 11805292]
20. Rajbhandari P, Finn G, Solodin NM, Singarapu KK, Sahu SC, Markley JL, et al. Regulation of ERalpha N-terminus conformation and function by peptidyl prolyl isomerase Pin1. Mol Cell Biol. 2011; 32:445–57. [PubMed: 22064478]
21. Yi P, Wu RC, Sandquist J, Wong J, Tsai SY, Tsai MJ, et al. Peptidyl-prolyl isomerase 1 (Pin1) serves as a coactivator of steroid receptor by regulating the activity of phosphorylated steroid receptor coactivator 3 (SRC-3/AIB1). Mol Cell Biol. 2005; 25:9687–99. [PubMed: 16227615]
22. Rustighi A, Tiberi L, Soldano A, Napoli M, Nuciforo P, Rosato A, et al. The prolylisomerase Pin1 is a Notch1 target that enhances Notch1 activation in cancer. Nat Cell Biol. 2009; 11:133–42. [PubMed: 19151708]
23. Reineke EL, Lam M, Liu Q, Liu Y, Stanya KJ, Chang KS, et al. Degradation of the tumor suppressor PML by Pin1 contributes to the cancer phenotype of breast cancer MDA-MB-231 cells. Mol Cell Biol. 2008; 28:997–1006. [PubMed: 18039859]
24. Lim JH, Liu Y, Reineke E, Kao HY. The MAPK ERK2 phosphorylates and promotes Pin1-dependent promyelocytic leukemia protein (PML) turnover. J Biol Chem. 2011; 286:44403–11. [PubMed: 22033920]

25. Davis FM, Tsao TY, Fowler SK, Rao PN. Monoclonal antibodies to mitotic cells. Proc Natl Acad Sci U S A. 1983; 80:2926–30. [PubMed: 6574461]
26. Yaffe MB, Schutkowski M, Shen M, Zhou XZ, Stukenberg PT, Rahfeld JU, et al. Sequence-specific and phosphorylation-dependent proline isomerization: a potential mitotic regulatory mechanism. Science. 1997; 278:1957–60. [PubMed: 9395400]
27. Lu PJ, Zhou XZ, Liou YC, Noel JP, Lu KP. Critical role of WW domain phosphorylation in regulating phosphoserine binding activity and Pin1 function. J Biol Chem. 2002; 277:2381–4. [PubMed: 11723108]
28. Hanai J, Chen LF, Kanno T, Ohtani-Fujita N, Kim WY, Guo WH, et al. Interaction and functional cooperation of PEBP2/CBF with Smads. Synergistic induction of the immunoglobulin germline Calpha promoter. J Biol Chem. 1999; 274:31577–82. [PubMed: 10531362]
29. Schutkowski M, Bernhardt A, Zhou XZ, Shen M, Reimer U, Rahfeld JU, et al. Role of phosphorylation in determining the backbone dynamics of the serine/threonine-proline motif and Pin1 substrate recognition. Biochemistry. 1998; 37:5566–75. [PubMed: 9548941]
30. Zhang M, Xie R, Hou W, Wang B, Shen R, Wang X, et al. PTHrP prevents chondrocyte premature hypertrophy by inducing cyclin-D1-dependent Runx2 and Runx3 phosphorylation, ubiquitylation and proteasomal degradation. J Cell Sci. 2009; 122:1382–9. [PubMed: 19351720]
31. Butt AJ, Caldon CE, McNeil CM, Swarbrick A, Musgrove EA, Sutherland RL. Cell cycle machinery: links with genesis and treatment of breast cancer. Adv Exp Med Biol. 2008; 630:189–205. [PubMed: 18637492]
32. Jin YH, Jeon EJ, Li QL, Lee YH, Choi JK, Kim WJ, et al. Transforming growth factor-beta stimulates p300-dependent RUNX3 acetylation, which inhibits ubiquitination-mediated degradation. J Biol Chem. 2004; 279:29409–17. [PubMed: 15138260]
33. Nakano A, Koinuma D, Miyazawa K, Uchida T, Saitoh M, Kawabata M, et al. Pin1 down-regulates transforming growth factor-beta (TGF-beta) signaling by inducing degradation of Smad proteins. J Biol Chem. 2009; 284:6109–15. [PubMed: 19122240]
34. Huang G, Shigesada K, Ito K, Wee HJ, Yokomizo T, Ito Y. Dimerization with PEBP2beta protects RUNX1/AML1 from ubiquitin-proteasome-mediated degradation. EMBO J. 2001; 20:723–33. [PubMed: 11179217]
35. Zhou XZ, Kops O, Werner A, Lu PJ, Shen M, Stoller G, et al. Pin1-dependent prolyl isomerization regulates dephosphorylation of Cdc25C and tau proteins. Mol Cell. 2000; 6:873–83. [PubMed: 11090625]
36. Bao L, Kimzey A, Sauter G, Sowadski JM, Lu KP, Wang DG. Prevalent overexpression of prolyl isomerase Pin1 in human cancers. Am J Pathol. 2004; 164:1727–37. [PubMed: 15111319]
37. Wulf G, Finn G, Suizu F, Lu KP. Phosphorylation-specific prolyl isomerization: is there an underlying theme? Nat Cell Biol. 2005; 7:435–41. [PubMed: 15867923]
38. Tsang YH, Lamb A, Romero-Gallo J, Huang B, Ito K, Peek RM Jr. et al. Helicobacter pylori CagA targets gastric tumor suppressor RUNX3 for proteasome-mediated degradation. Oncogene. 2010; 29:5643–50. [PubMed: 20676134]
39. Zhang DH, Salto-Tellez M, Chiu LL, Shen L, Koay ES. Tissue microarray study for classification of breast tumors. Life Sci. 2003; 73:3189–99. [PubMed: 14561524]
40. Chen LF, Mu Y, Greene WC. Acetylation of RelA at discrete sites regulates distinct nuclear functions of NF-κB. EMBO J. 2002; 21:6539–48. [PubMed: 12456660]

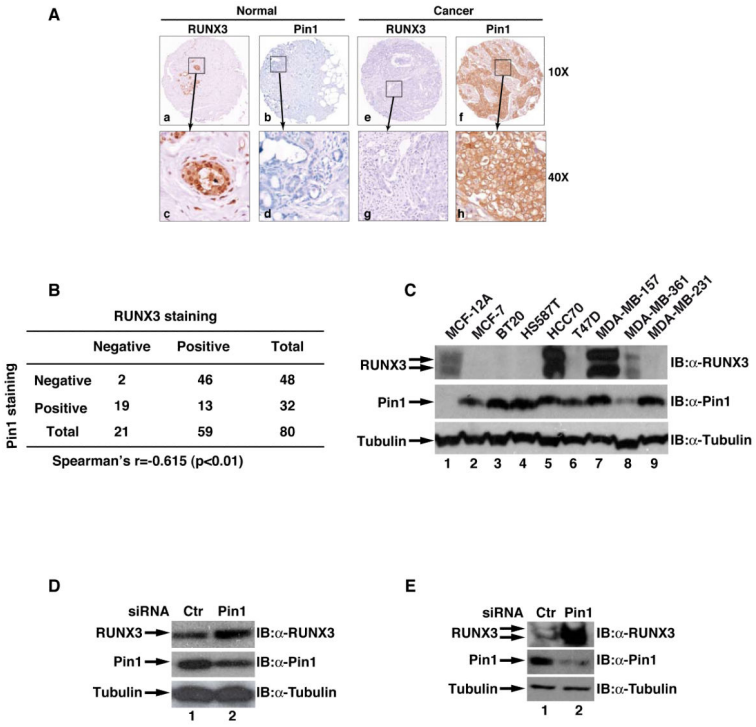

Figure 1. Expression of Pin1 inversely correlated with RUNX3 expression

(a) Representative immunohistochemical staining of Pin1 and RUNX3 in normal and tumor breast tissues. Boxed regions are enlarged below. The detection of the expression of RUNX3 and Pin1 of the human tissue microarray was performed as previously described (39). **(b)** Tissue sections of 80 breast cancer samples were immunostained with anti-Pin1 or anti-RUNX3 antibodies for the expression of Pin1 and RUNX3 and their correlation was analyzed by Spearman rank correlation test ($p<0.01$). **(c)** Expression levels of RUNX3 and Pin1 in various normal mammary and breast cancer cells. **(d)** RUNX3-MCF-7 cells stably expressing RUNX3 were transfected with control or Pin1 siRNA for 48 hr, whole cell lysates were immunoblotted as indicated for the expression levels of RUNX3 and Pin1. **(d)** MDA-MB-361 cells were transfected with control or Pin1 siRNA for 48 hr, the whole cell lysates were immunoblotted for the expression of endogenous RUNX3 and Pin1.

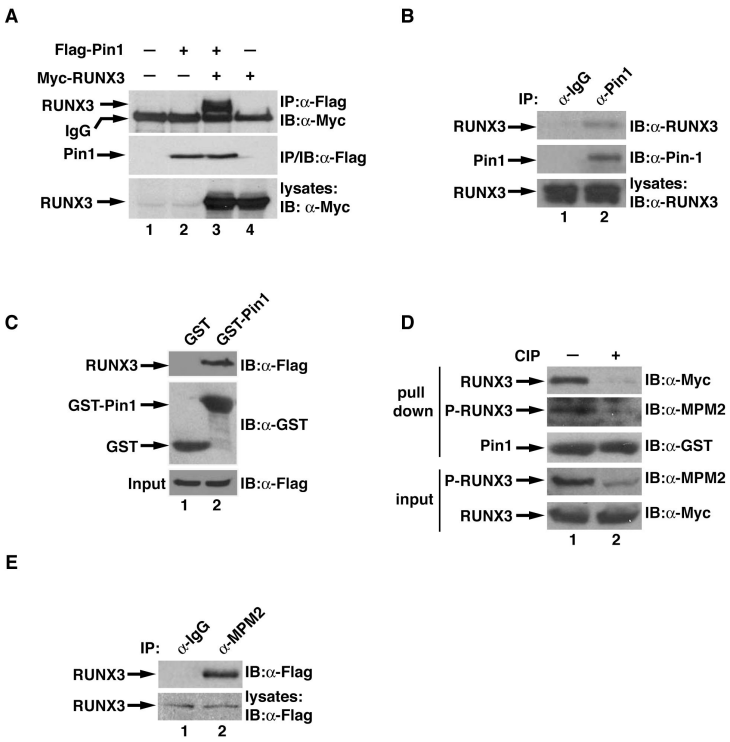

Figure 2. Pin1 interacts with phosphorylated RUNX3

(a) Pin1 interacts with RUNX3 in vivo. HEK293T cells were transfected with Flag-tagged Pin1 and Myc-tagged RUNX3 as indicated. 40 hr post-transfection, Flag-Pin1 immunoprecipitates were prepared from whole-cell lysates and immunoblotted for Myc-RUNX3. **(b)** Endogenous Pin1 interacts with endogenous RUNX3. Endogenous Pin1 immunoprecipitates were prepared from whole-cell lysates of MDA-MB-157 cells and immunoblotted for associated RUNX3. IgG was used as a control. **(c)** Pin1 interacts with RUNX3 in vitro. GST or GTS-Pin1 conjugated agaroses were incubated with HEK293T cell lysates transfected with Flag-RUNX3. The recovered materials were immunoblotted for Flag-RUNX3. **(d)** Pin1 interacts with phosphorylated RUNX3 in vitro. HEK293T cell lysates containing Flag-RUNX3 were pretreated with calf intestinal phosphatase (CIP) for 30 min followed by the incubation with GST-Pin1 conjugated agarose. GST-Pin1 associated RUNX3 was detected as in (c). Phosphorylated RUNX3 was immunoblotted with anti-pSer/Thr-Pro (MPM2) antibody. **(e)** RUNX3 contains pSer/Thr-Pro motif. Cell lysates from RUNX3-7 cells were immunoprecipitated with IgG or anti-MPM2 antibody and immunoblotted for the associated RUNX3 proteins.

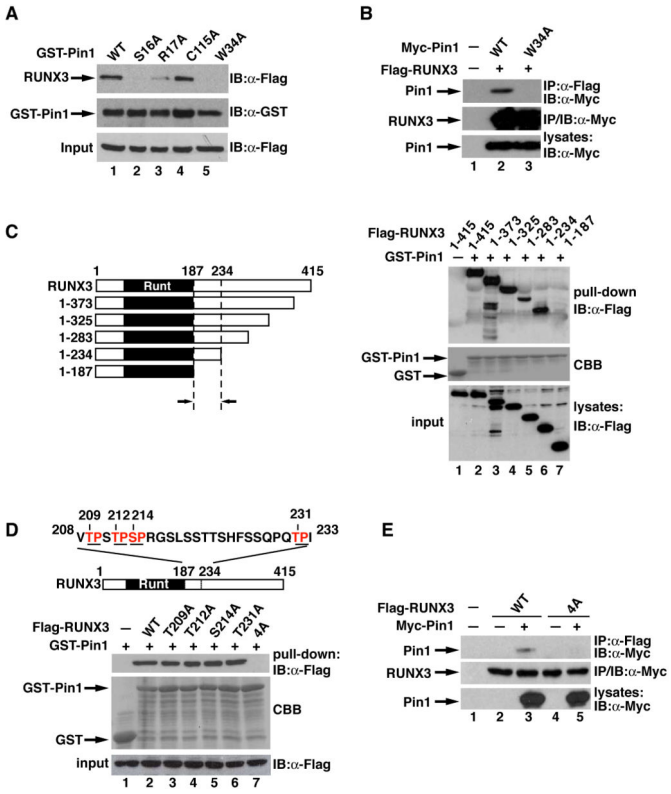

Figure 3. Pin1 interacts with four pSer/Thr-Pro motifs of RUNX3

(a) WW domain of Pin1 is involved in its association with RUNX3. HEK293T cell lysates containing transfected Flag-RUNX3 were subjected to GST pull-down assay with wild-type Pin1 and various Pin1 mutants. **(b)** HEK293T cells were co-transfected with expression vectors for Myc-Pin1 or Myc-Pin1-W34A and Flag-RUNX3 as indicated. Flag-RUNX3 immunoprecipitates were prepared from whole-cell lysates and immunoblotted for Myc-Pin1 and its mutant. **(c)** The region of RUNX3 from amino acids 188 to 234 is important for its interaction with Pin1. Left: Schematic showing the domain structure of RUNX3 and various deletion mutants of RUNX3. Right: GST-Pin1 conjugated agarose was incubated with cell lysates containing transfected Flag-RUNX3 or its various deletion mutants as indicated. The recovered materials were immunoblotted for RUNX3 and its deletion mutants with anti-Flag antibodies. Levels of GST or GST-Pin1 were shown by coomassie brilliant blue staining (CBB). **(d)** Four Ser/Thr-Pro motifs in RUNX3 are important for its interaction with Pin1. GST-Pin1 conjugated agarose was incubated with cell lysates containing transfected Flag-RUNX3 or its various point mutation mutants as indicated. The recovered materials were immunoblotted for RUNX3 and its mutants with anti-Flag antibodies. **(e)** HEK293T cells were transfected with expression vectors for Myc-Pin1 and Flag-RUNX3 or Flag-RUNX3-4A as indicated. 40 hr post-transfection, Flag-RUNX3 immunoprecipitates were prepared from whole-cell lysates and immunoblotted for Pin1 with anti-Myc antibodies.

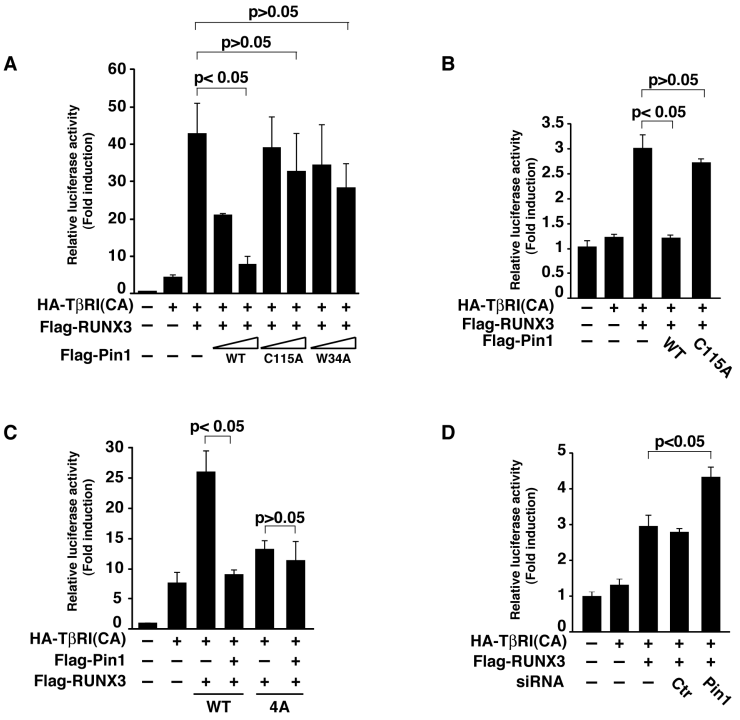

Figure 4. Pin1 abolishes the function of RUNX3

(a) Pin1 inhibits the transcriptional activity of RUNX3 in a prolyl isomerase activity-dependent manner. TβRE reporter plasmids were cotransfected with TβRI(CA) and RUNX3 expression vectors, alone or in combination with increasing amounts of Pin1 and its mutants into HEK293T cells. Luciferase activity was measured 30 hr after transfection. In each experiment, cells were also co-transfected with *Renilla* luciferase reporter plasmid which was used as an internal control. Results represent the average of three independent experiments +/− SD. Statistical analysis (p values) was performed using the Analysis of Variance (ANOVA) test. **(b)** Pin1 inhibits the transcriptional activity of RUNX3 in MCF-7 cells. MCF-7 cells were transfected, luciferase assay and statistical analysis were performed as in (a). **(c)** Pin1 inhibits the transcriptional activity of RUNX3 but not RUNX3-4A. TβRE reporter plasmids were co-transfected with TβRI(CA) and expression vectors for RUNX3 or RUNX3-4A, alone or in combination with Pin1 into HEK293T cells. Luciferase assay was performed as in (a). Statistical analysis was performed using a student's t-test for 2 variables. **(d)** Depletion of Pin1 enhances transcriptional activity of RUNX3. MCF-7 cells were transfected with control or Pin1 siRNA. 48 hr after transfection, TβRE reporter plasmids were co-transfected with TβRI(CA) and expression vectors for RUNX3. Luciferase assay and statistical analysis were performed as in (a).

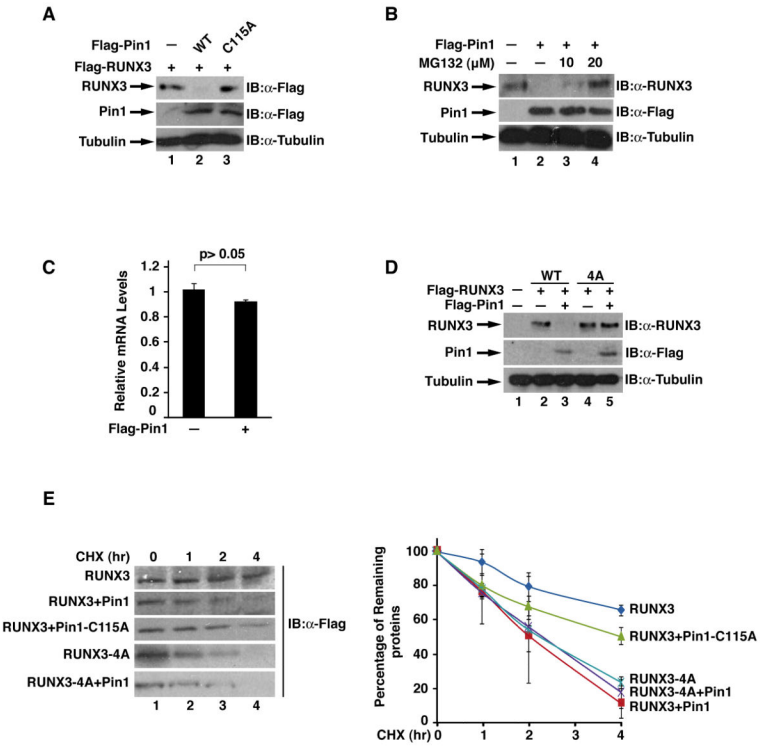

Figure 5. Pin1 targets RUNX3 for proteasome-mediated degradation

(a) Pin1 reduces the cellular levels of RUNX3. RUNX3-MCF-7 cells were transiently transfected with expression vectors for Flag-Pin1 or Flag-Pin1-C115A. Levels of RUNX3 were detected by immunoblotting the whole-cell extracts with anti-Flag antibodies. Levels of Pin1 and tubulin were shown as indicated. **(b)** Pin1 induces proteasome-mediated degradation of RUNX3. RUNX3-MCF-7 cells were transfected with Flag-Pin1 as in (a). 24 hr after transfection, cells were treated with increasing doses of MG132 for 8 hr. Cell lysates were then immunoblotted for expression of RUNX3, Pin1 and tubulin. **(c)** Pin1 does not alter the mRNA levels of RUNX3. RUNX3-MCF-7 cells were transfected with Flag-Pin1 for 48 hr before total RNA was extracted. Complementary DNA was synthesized and quantitative real-time PCR was performed. Levels of RUNX3 mRNA were normalized with the expression of hGAPDH. Statistical analysis was performed using a student's t-test. **(d)** Interaction between Pin1 and RUNX3 is critical for the degradation of RUNX3. MCF-7 cells were transiently transfected with expression vectors for Flag-Pin1, Flag-RUNX3 or Flag-RUNX3-4A. Cell lysates were immunoblotted for RUNX3 and Pin1 as in (a). **(e)** Pin1 reduces the half-life of RUNX3. HEK293T cells were transfected with wild-type RUNX3 or RUNX3-4A with or without Pin1 or Pin1-C115A as indicated. 24 hr after transfection, cells were treated with 100 ng/ml of cycloheximide (CHX) for the indicated time points, and immunoblotted for the expression of Flag-RUNX3. A representative result from three independent experiments is shown in the left panels. Quantification of the results is shown in the right panel. Data represent the average of three independent experiments +/− SD.

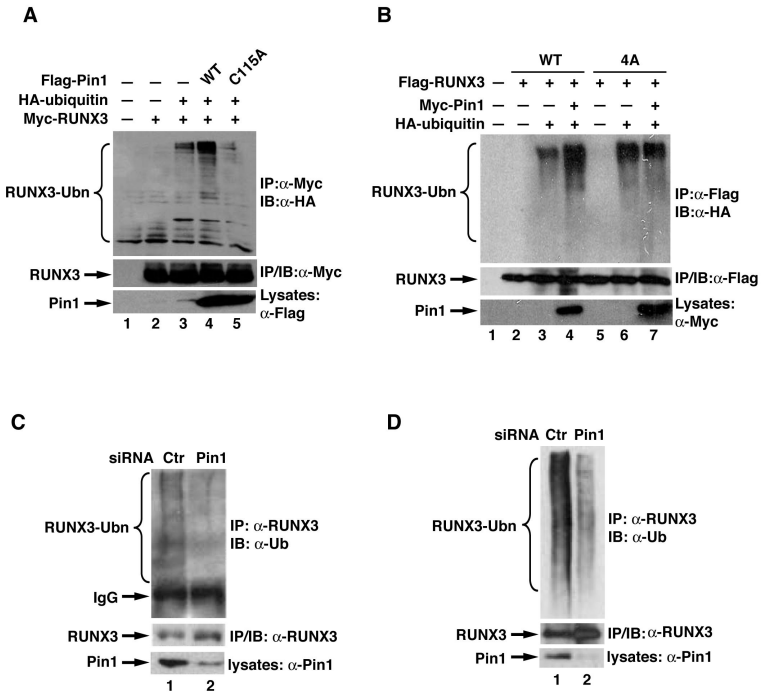

Figure 6. Pin1 stimulates the ubiquitination of RUNX3

(a) Pin1 enhances ubiquitination of RUNX3. HEK293T cells were transfected with indicated combinations of plasmids expressing Flag-RUNX3, HA-ubiquitin, Myc-Pin1 or Myc-Pin1-C115A. RUNX3 immunoprecipitates were immunoblotted for ubiquitination with anti-HA antibodies. Levels of RUNX3 and Pin1 are shown in the lower two panels. **(b)** Interaction between Pin1 and RUNX3 is important for the Pin1-induced ubiquitination of RUNX3. HEK293T cells were transfected with a combination of Flag-RUNX3 or Flag-RUNX3-4A, HA-ubiquitin and Myc-Pin1 as indicated. RUNX3 ubiquitination was detected as in (a). **(c)** Down-regulation of Pin1 decreases the ubiquitination of RUNX3. RUNX3-MCF-7 cells were transfected with control or Pin1 siRNA. RUNX3 immunoprecipitates were immunoblotted for ubiquitination with anti-ubiquitin antibodies. Levels of RUNX3 and Pin1 are shown in the bottom two panels. **(d)** MDA-MB-361 cells were transfected with control or Pin1 siRNA. Endogenous RUNX3 immunoprecipitates were immunoblotted for ubiquitination as in (c).