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ORIGINAL ARTICLE

Pin1 is required for the Ser727 phosphorylation-dependent Stat3 activity

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Signal transducer and activator of transcription 3 (Stat3) belongs to a family of latent cytoplasmic transcription factors important for cytokine signaling. Stat3 is constitutively activated in various tumors, and activated Stat3 itself also acts as an oncogene. Transcriptional activity of Stat3 is controlled by Tyr-phosphorylation, followed by dimerization and nuclear translocation. However, phosphorylation on Ser727 is indispensable for its maximal transcriptional activity with unclear mechanism. Here, we report that peptidyl-prolyl *cis/trans* isomerase 1 (Pin1), which specifically recognizes the pSer/Thr-Pro motifs on its target proteins, interacts with Stat3 upon cytokine/growth factor stimulation. Overexpression of Pin1 promotes Stat3 transcriptional activity and target gene expression, as well as recruitment of transcription coactivator, p300. These effects, however, were compromised in the Pin1-deficient cells, and were totally dependent on the Ser727 phosphorylation site. Finally, we showed that Pin1 enhances Stat3-mediated epithelial–mesenchymal transition in breast cancer cells induced by oncostatin M. Our data reveal a novel, Ser727 phosphorylation-dependent, post-translational regulation mechanism for Stat3.
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Keywords: Pin1; serine phosphorylation; Stat3; transcriptional activity

Introduction

Signal transducers and activators of transcription (STATs) are a family of latent cytoplasmic transcription factors, which are key components for cytokine signaling and effects (Darnell, 1997). Among seven mammalian STATs, Stat3 is the most pleiotropic member that is activated by various cytokines and certain growth factors, including the interleukin-6 (IL-6) cytokine family and epithelial growth factor (EGF), and is involved in multiple cellular events (Levy and Lee, 2002). Stat3 is constitutively activated in oncogenic tyrosine kinase v-Src- or v-abl-transformed cells (Danial *et al*., 1995; Yu *et al*., 1995; Cao *et al*., 1996), and various primary tumors and tumor cell lines. When it is activated, Stat3 itself also acts as an oncogene, causing cell transformation in NIH-3T3 cells and induction of tumor in nude mice (Bromberg *et al*., 1999). Stat3 has also been reported to play an essential role in the regulation of cell migration (Sano *et al*., 1999; Ng *et al*., 2006) and oncostatin M (OSM)-induced cell growth inhibition and morphological changes (Zhang *et al*., 2003).

Similar to other STAT proteins, Stat3 responds to cytokine stimulation by recruitment to the cytokine receptors and undergoes subsequent phosphorylation on its Tyr705 residue at the C terminus by the receptor-associated Janus kinases. Tyr-phosphorylation of Stat3 leads to dimerization via reciprocal interaction between the SH2 domain and the phosphorylated Tyr705, then nuclear transport, DNA binding and regulation of target gene expression (Darnell *et al*., 1994). In addition to the Tyr-phosphorylation site, a conserved serine phosphorylation site (Ser727) was also identified within the C terminus of Stat3 and several other STAT members. Ser-phosphorylation further enhances the transcriptional activities of Stat3 and Stat1 (Wen *et al*., 1995). Mutations of the conserved Ser727 residues of Stat3 and Stat1 lead to various degrees of compromise in their transactivation and downstream effects (Wen *et al*., 1995; Decker and Kovarik, 2000). In contrast to many studies exploring the role of Ser/Thr kinases in STAT phosphorylation, the underlying mechanisms governing Ser-phosphorylation itself on the transcriptional enhancement of STATs are less known.

Peptidyl-prolyl isomerase (PPIase) peptidyl-prolyl *cis/trans* isomerase 1 (Pin1) was initially cloned as a NIMA kinase interacting protein (Lu *et al*., 1996). Unlike other classes of PPIases, Pin1 specifically recognizes a proline bond preceded by a phosphorylated serine or threonine residue (phosphoSer/Thr-Pro motif). It comprises two known domains – a WW domain at the N terminus of the protein, which was assigned as the binding module for phosphorproteins, and a C terminal PPIase domain, which catalyses the *cis-trans* isomerization of the target peptidyl-prolyl bonds (Wulf *et al*., 2005).

Since its discovery, various proteins have been identified as substrates of Pin1, implicating it in the regulation of different biological processes. Pin1 plays

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an important role in the regulation of cell-cycle progression. For instance, Pin1 inhibits phosphorylated Cdc25 and controls the replication checkpoint in the cell cycle (Crenshaw *et al*., 1998; Winkler *et al*., 2000). It also regulates the protein stability and activity of tumor suppressors p53 (Zacchi *et al*., 2002; Zheng *et al*., 2002). Moreover, Pin1 is overexpressed in breast cancer and crucially linked to the mammary epithelial cell transformation (Wulf *et al*., 2003, 2004).

All the STAT proteins except Stat2 and Stat6 contain conserved Ser-phosphorylation sites followed by a proline residue, which are complementary to the phosphoSer-Pro element necessary for Pin1 recognition, making STAT proteins attractive candidates as substrates of Pin1. In this study, we demonstrate Pin1's role as a novel, positive regulator of Stat3 activity. Pin1 interacts with Ser727 phosphorylated Stat3 in a ligand-dependent manner. Furthermore, Pin1 is required for optimal DNA binding of Stat3 and recruitment of coactivator p300, all of which collectively lead to the enhancement of serine phosphorylation-dependent Stat3 transcriptional activity. Finally, Pin1 also enhances Stat3-mediated, OSM-induced epithelial–mesenchymal transition (EMT) in breast cancer cells, which raises the possibility that such positive regulation of Pin1 on Stat3 may be involved in breast cancer progression and invasion processes.

Results

Association of Stat3 and Pin1 in vivo *and* in vitro

To study the possible interaction of Stat3 and Pin1 in mammalian cells, immunoprecipitation between Flag-tagged Stat3 and Myc-tagged Pin1 was performed in COS-1 cells. As shown in Figure 1a, Stat3 was detected only in the immunoprecipitates from EGF-stimulated cells and only when Pin1, not the control plasmid, was co-transfected, suggesting that Pin1 interacts with activated Stat3. We next examined whether the interaction of Stat3 and Pin1 was detectable at the endogenous level and looked for the site of interaction. Both cytoplasmic and nuclear portions of HeLa cells were subjected to immunoprecipitation with anti-Pin1 antibody. Endogenous Stat3 co-immunoprecipitated with Pin1 in the nuclear portion of OSM-stimulated cells, but neither in the cytosolic portion of OSM-stimulated cells nor in the unstimulated cells (Figure 1b). These data suggest that Pin1 interacts physically with Stat3 *in vivo*.

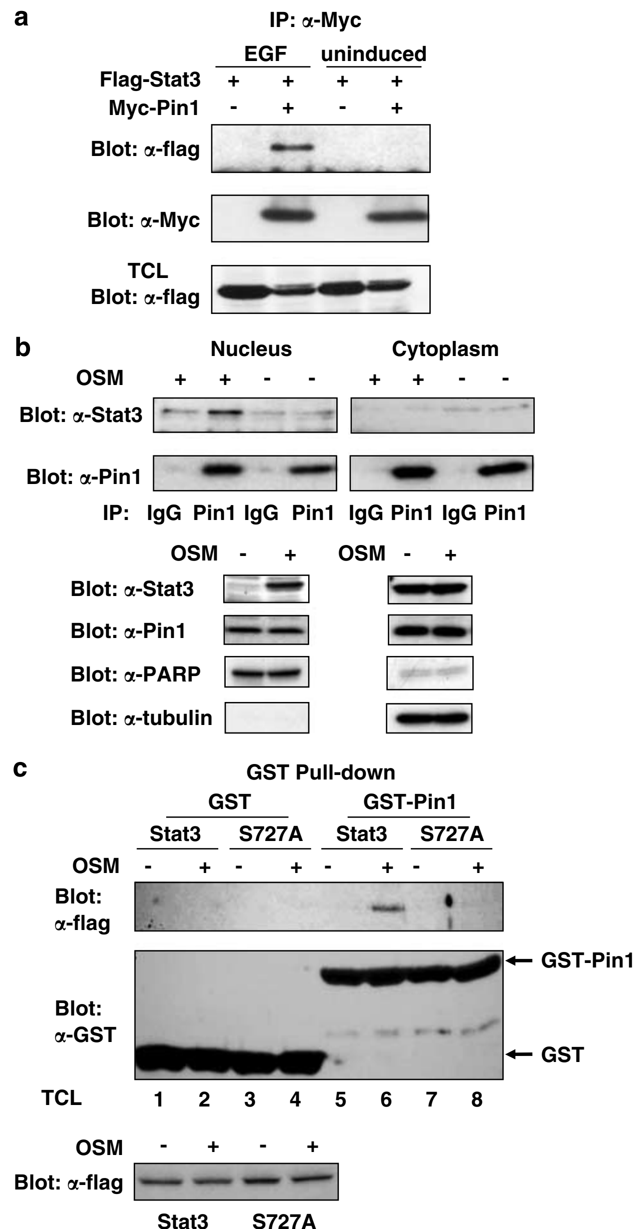

Figure 1 Interaction of Stat3 and Pin1. (**a**) Interaction of Stat3 and Pin1 *in vivo*. COS-1 cells were transfected with Flag-tagged Stat3 and/or Myc-tagged Pin1, and either left untreated or stimulated with epithelial growth factor (EGF) for 20 min. The cell lysates were immunoprecipitated with anti-Myc antibody, and the precipitates were fractionated by SDS–polyacrylamide gel electrophoresis and blotted with anti-Flag (top panel). The blot was stripped and reprobed with anti-Myc (middle panel). Total cell lysates (TCL) were subjected to western blot analysis with anti-Flag to monitor the expression of Stat3 (bottom panel). (**b**) Association of endogenous Stat3 and Pin1. HeLa cells were either left untreated or treated with oncostatin M (OSM) for 20 min. Cells were fractionated into nuclear and cytoplasmic portions. Both fractions were incubated with either normal IgG or anti-Pin1 antibody as labeled and blotted with anti-Stat3 (first panels), then reprobed with anti-Pin1 antibody (second panels). The lysates of nuclear and cytoplasmic portions were also directly probed with Stat3 and Pin1 antibodies to monitor the endogenous levels of these two proteins (third and fourth panels), with the endogenous poly (ADP-ribose) polymerase and tubulin levels as controls for the fractionation and loading efficiency (fifth and sixth panels). (**c**) *In vitro* glutathione-*S*-transferase (GST) pull-down assay. PC3 cells were transfected with Flag-tagged Stat3 or point mutant S727A and left untreated or induced with OSM for 20 min. The cell lysates were incubated with GST (lanes 1–4) or GST-Pin1 fusion protein (lanes 5–8), followed by addition of the GST beads. The precipitates were immunoblotted with anti-Flag to show the bound Stat3 (top panel) and reprobed with anti-GST to show the precipitated GST and GST-Pin1, as indicated (middle panel). Western blot analysis of Stat3/S727A in TCL is shown in the bottom panel.

Glutathione-*S*-transferase (GST) pull-down assays using bacterially produced GST-Pin1 fusion protein were performed to further analyse the Pin1–Stat3 interaction. Only the wild-type (WT) Stat3 from OSM-induced cells was co-precipitated with GST–Pin1, whereas the S727A mutant, in which the Ser727 residue was substituted with Ala to prevent phosphorylation, was not detectable (Figure 1c). This indicates that the ligand-induced Ser727 phosphorylation of Stat3 plays a crucial role in mediating the Pin1–Stat3 interaction.

Pin1 binding promotes endogenous Stat3 transcriptional activity

Because Pin1 interacts specifically with the ligand-activated form of Stat3 within the nucleus, we speculated that this binding might affect Stat3 transcriptional activity. In unstimulated HepG2 cells, basal Stat3 transcriptional activity for a high-affinity Stat3 binding site SIE (hSIE)-luciferase reporter was detectable at low levels in cells, but increased (~25-fold) upon IL-6 stimulation. With the overexpression of Pin1, the IL-6-induced Stat3 activity was further enhanced as much as three times as the empty vector control (Figure 2a, left panel). We next examined the effect of Pin1 on the Stat3 target gene α_2-macroglobulin (α_2-M). Similarly, Pin1 also increased the IL-6-inducible α_2-M promoter activity (right panel).

Substitution of the Tyr23 residue of Pin1 to Ala abolishes its phosphoSer/Thr-Pro motif binding ability (Lu *et al*., 1999). In HepG2 cells, expression of the Y23A point mutant failed to increase the IL-6-stimulated Stat3 transcriptional activity. In contrast, three reported PPIase-defective point mutants of Pin1, R68, 69A, K63A and C109A (Zhou *et al*., 2000), all failed to inhibit the Pin1-dependent positive regulation on Stat3 activity (Figure 2b). Together, these results demonstrate that the positive regulatory effects of Pin1 on Stat3 activity depend on the phosphorprotein-binding capacity of Pin1 but not its PPIase enzymatic activity.

Stat3 transcriptional activity is compromised in the absence of Pin1

We further investigated the endogenous Stat3 transcriptional activity in Pin1-deficient (KO) and WT murine embryonic fibroblasts (MEFs) (Fujimori *et al*., 1999). Two types of cells were transfected with either hSIE-luciferase (Figure 3a, left panel) or α2-M promoter-luciferase reporter gene construct (right panel). In both cases, OSM-induced Stat3 transcriptional activity was upregulated in the WT MEFs by 4.5- and twofold, respectively, in comparison with those in Pin1 KO cells. The loss of Pin1 expression which led to compromised Stat3 activity suggested the importance of Pin1 in the regulation of Stat3 transactivation. Such effects were further confirmed by the observation that the expression

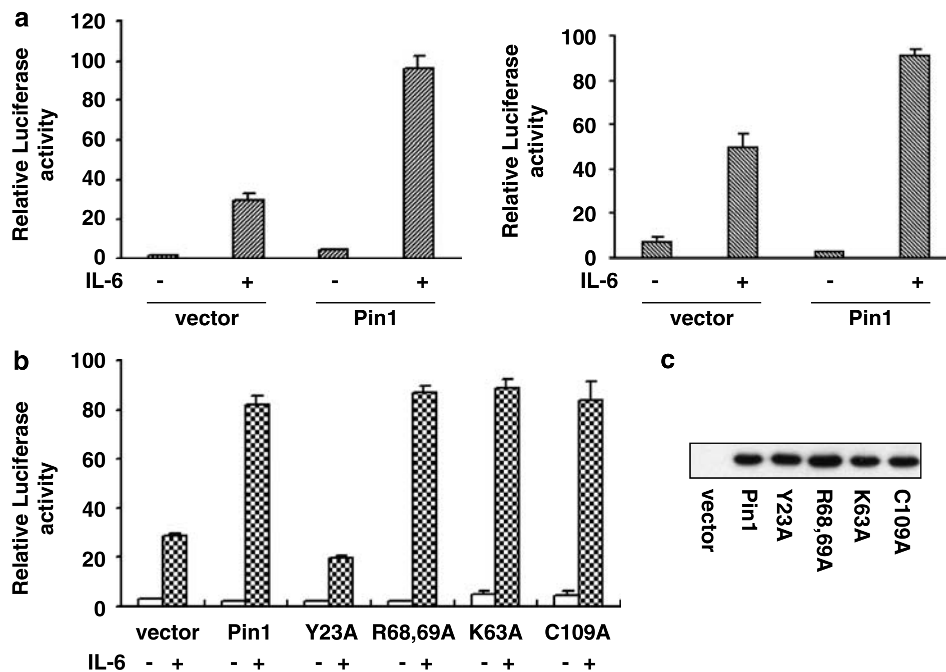

Figure 2 Pin1 promotes Stat3 transcriptional activity. (**a**) HepG2 cells were co-transfected with control vector, or Pin1 together with m67-luc (left panel) or pGL3-α2M-215luc (right panel) luciferase reporter plasmid. Thymidine kinase promoter-dependent *Renilla* luciferase construct, pRL-TK, was co-transfected as a control for transfection efficiency. Cells were either left uninduced or stimulated overnight with interleukin-6 (IL-6) before luciferase assays. Relative luciferase activity values are indicated, which represent a mean from three samples, with the standard deviation given as the error bars. (**b**) HepG2 cells were co-transfected with the control vector, Pin1, its Y23A, R68, 69A, K63A or C109A point mutants, together with pGL3-α2M-215luc and pRL-TK. Cells were either left uninduced or stimulated with IL-6, and luciferase assays were performed. (**c**) Expression of wild-type (WT) and mutant Pin1 in (**b**) was examined by western blot analysis.

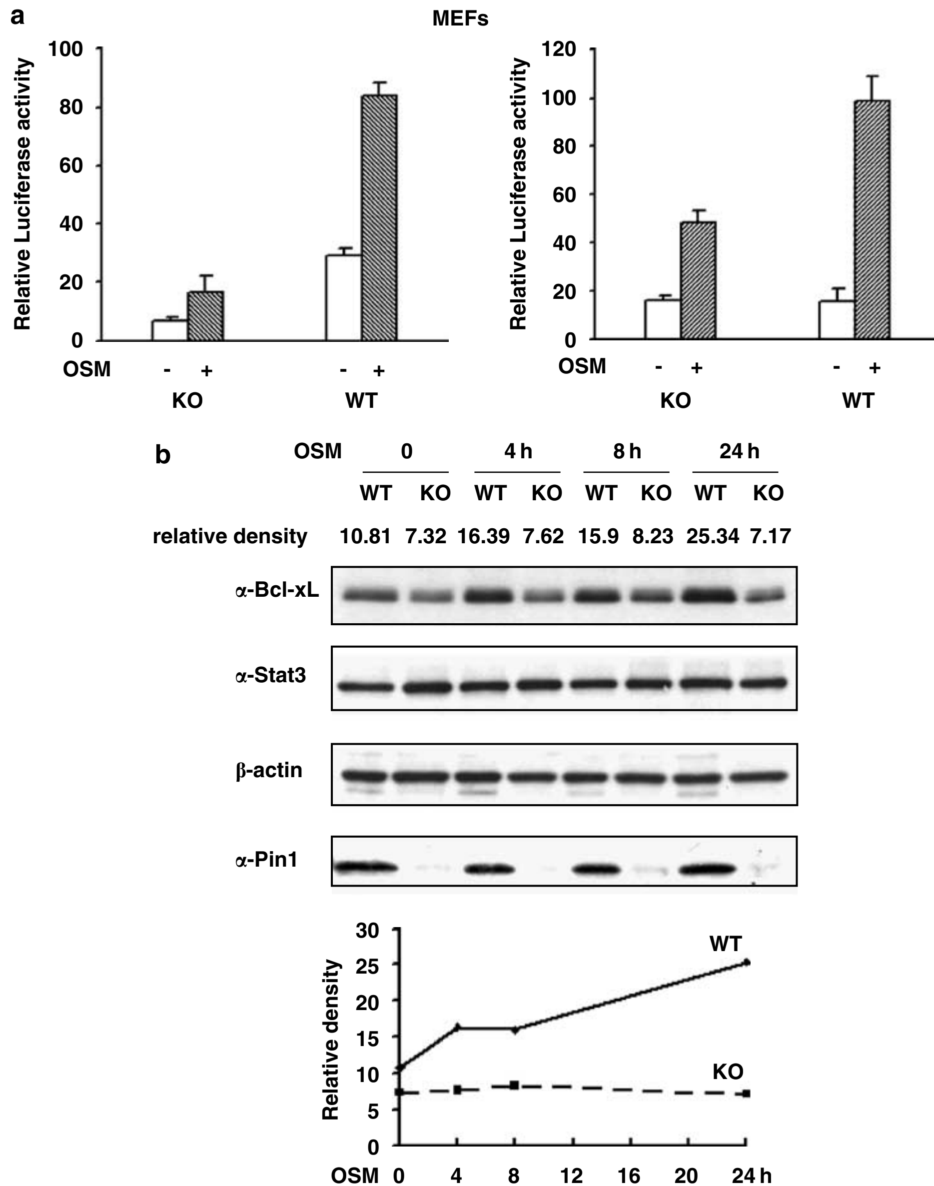

Figure 3 Stat3 transcriptional activity and target gene expression is impaired in Pin1-deficient murine embryonic fibroblasts (MEFs). (**a**) The wild-type (WT) and Pin1 KO MEFs were co-transfected with m67-luc (left panel) or pGL3-α2M-215luc (right panel) together with pRL-TK. Cells were either left untreated or stimulated overnight with oncostatin M (OSM), and luciferase assays were performed as described in Figure 2. (**b**) The WT and Pin1 KO MEFs were treated with OSM for various lengths of time as indicated. The total cell lysates (TCL) were subjected to western blot analysis with anti-Bcl-xL, anti-Stat3 and anti-Pin1 antibodies. The blot was also probed by actin antibody to serve as loading controls. The relative density of the Bcl-xL bands was indicated and also shown as the line chart normalized with actin (bottom panel).

of Bcl-xL, one of the Stat3 downstream genes, was enhanced in the WT, but not the KO, cells, by OSM induction for various lengths of time (Figure 3b).

Pin1 upregulates Stat3 transcriptional activity via Ser727 residue of Stat3

Serine phosphorylation of STATs has been reported to be required for the maximal STAT-dependent transcriptional activation. In EGF-stimulated COS-1 cells, Stat3 activity was increased by eightfold compared to the unstimulated cells. It was further enhanced by 1.8-fold in the presence of Pin1. However, upon EGF stimulation, the activity of the S727A mutant of Stat3 was increased by only 3.6-fold, representing ~45% of the WT Stat3 activity, which was not further increased by Pin1. As a control, the Y705A mutant shows only minimal level of Stat3 transcriptional activity (less than 10%), which is close to the basal level of Stat3 activity in the empty vector control (Figure 4).

Stat3 DNA binding ability is impaired in the Pin1 knockout MEFs

OSM-induced Stat3 Tyr/Ser-phosphorylation in HeLa cells was analysed, with the overexpression of Pin1 or its

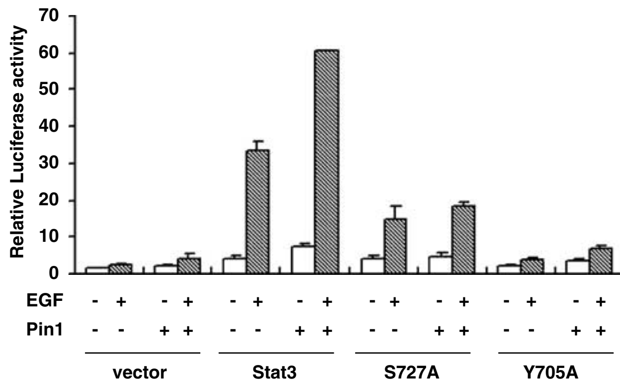

Figure 4 Pin1 does not promote the transcriptional activity of Stat3 S727A point mutant. COS-1 cells were transfected with the empty vector, wild-type (WT) or mutant (S727A, Y705A) Stat3 in the presence (+) or absence (−) of Pin1, together with pGL3-α2M-215luc and pRL-TK. Cells were either left untreated or stimulated with epithelial growth factor (EGF) (100 ng/ml) overnight, and luciferase assays were performed.

Y23A point mutant, and the results showed that there was no significant difference in Stat3 phosphorylation level between the Pin1-transfected cells and the control cells (Figure 5a). The WT and Pin1 KO MEFs did not differ obviously in their levels of Stat3 nuclear translocation either, upon OSM stimulation (Figure 5b).

Next, we monitored the activity of Stat3 dimers inside the nucleus by assessing the Stat3 DNA-binding ability in the presence or absence of Pin1. As shown in Figure 5c, the DNA-binding ability of Stat3 in the WT cells was strongest after 15 min of OSM stimulation and, it was significantly reduced thereafter. Strikingly, the DNA-binding ability of nuclear Stat3 was ∼50% reduced in the Pin1 KO cells compared with the WT control (compare lane 8 with lane 4). The specificity of Stat3–DNA complex was verified by its supershift with Stat3, but not Stat1, antibody. These data indicate that expression of Pin1 is required for optimal DNA binding of Stat3 to its DNA response element.

Pin1 increases Stat3 and p300 binding

To further illustrate the underlying mechanism of the upregulation effects of Pin1 on Stat3 transactivation, we next investigated whether Pin1 had a role in the regulation of the interaction between Stat3 and p300 coactivator. The interaction between endogenous Stat3 and p300 was increased drastically upon Pin1 overexpression (Figure 6a, top panel, lane 8), and such effect was observed only in cells with, but not without, OSM treatment (compare lane 4 with lane 8). We also examined the Stat3–p300 interaction in Pin1 WT and KO MEFs. The OSM-induced interaction between endogenous Stat3 and p300 was detected in the Pin1 WT, but was compromised in the Pin1 KO, MEFs (Figure 6b, top panel, compare lane 4 with lane 8).

Effects of Pin1 on OSM-stimulated cell growth inhibition and morphological changes

EMT describes the early morphological transition associated with an increased motility and invasiveness in epithelial cancer cells that are reminiscent of the differentiation of the first embryonic mesodermal epithelials during development (Thiery, 2003). OSM induces cell proliferation inhibition and morphological changes in breast cancer cells, which are similar to EMT in many aspects. Stat3 activation plays a key role in mediating this process (Zhang *et al*., 2003). To study the physiological consequences of Pin1 regulation in Stat3 activity, we investigated its role in the Stat3-mediated, OSM-induced cell proliferation inhibition and morphological changes in breast cancer cells. In MCF-7 cells, cell growth was inhibited by more than 40% with OSM treatment (Figure 7a, left panel). In the presence of overexpressed Pin1, the inhibited cell growth was further reduced to 50% (middle panel). In contrast, knockdown of the endogenous Pin1 expression by siRNA released such inhibitory effects (right panel). We also examined the effect of Pin1 on the OSM-induced morphological changes. Without OSM stimulation, MCF-7 cells observed under phase-contrast microscopy were typical of epithelial cells displaying morphology with tight cell-to-cell contacts in tightly adhered groups (Figure 7b, i–iv). However, prolonged OSM exposure induced the control cells undergoing morphological changes by showing scattered and elongated morphology and membrane protrusions (v and vii). Strikingly, Pin1 overexpression promoted more drastic morphological changes with further reduced cell–cell adhesion and enhanced cell scattering (panel vi). In contrast, Pin1 siRNA inhibited such morphological changes and reduced the occurrence of the scattered phenotype (panel viii). These results indicate a good correlation between the Pin1's regulatory roles in Stat3 activity and its function in EMT in the breast cancer cells.

Discussion

Pin1 has been identified in various cellular events and responses since its discovery. Involvement of Pin1 in multiple signaling pathways has been reported and activities of various transcription factors are differentially regulated by Pin1 via direct physical association (Wulf *et al*., 2005). However, whether Pin1 also regulates the STAT family was unknown. In this study, we reported a novel interaction between Pin1 and Stat3. We demonstrate that Pin1 binds to Stat3 only in ligand-stimulated cells, and the endogenous interaction was observed only in the nucleus, suggesting that Pin1 may selectively target the activated form of Stat3. Ligand-stimulated phosphorylation of the Ser727 residue in the C terminus of Stat3 mediates the Stat3–Pin1 interaction, and replacement of this crucial residue with phosphorylation-resistant alanine totally abolishes the protein associations (Figure 1c). Based on these results, it is possible that Pin1 interacts only with Ser-phosphorylated and dimerized Stat3 in the nucleus. In addition, taking into consideration the results in Figures 5 and 6 also raises the possibility that Pin1 binds only to DNA-bound and p300-bound Stat3 dimers, which are present

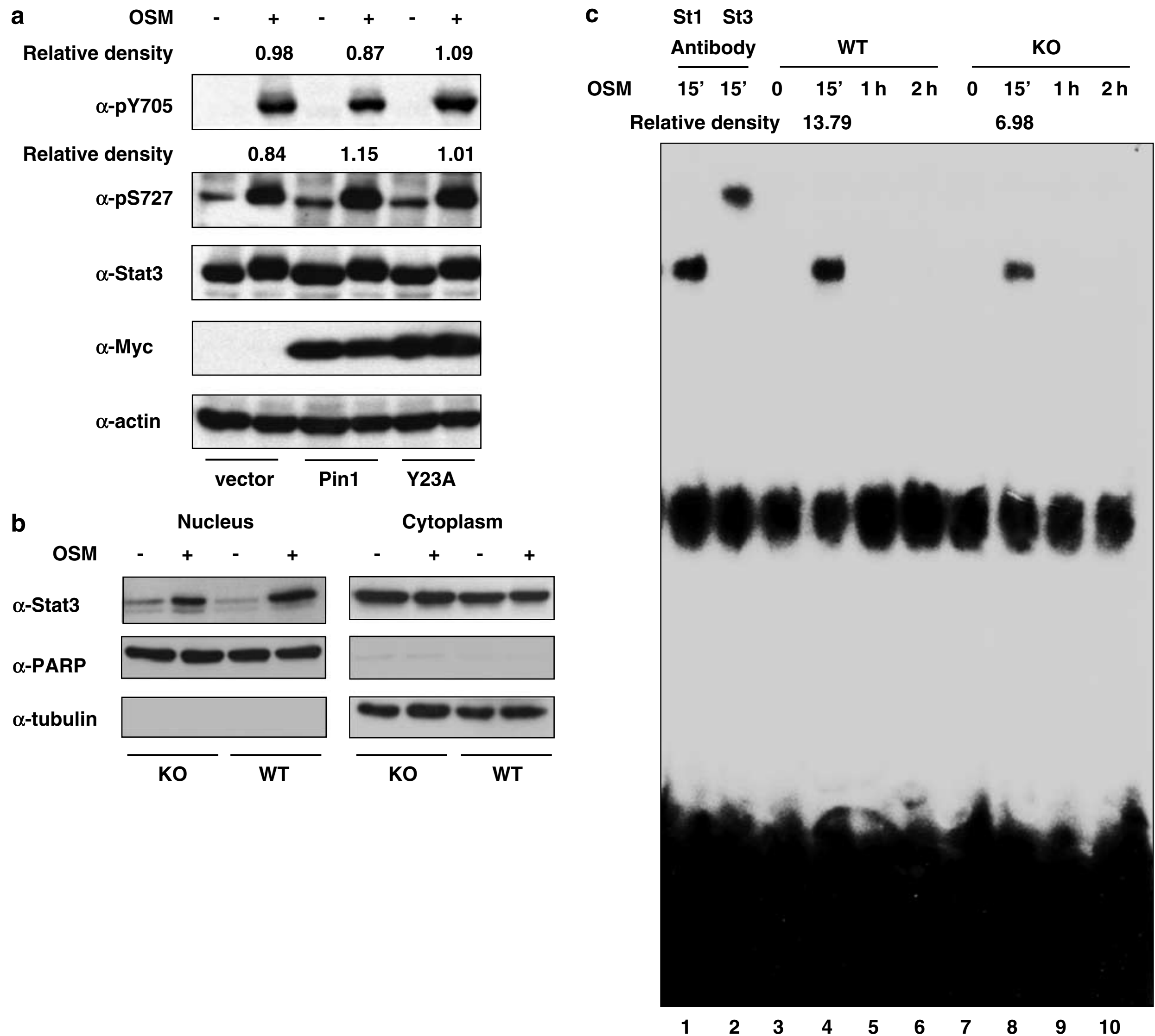

Figure 5 Stat3 DNA-binding ability is impaired in the Pin1 KO murine embryonic fibroblasts (MEFs). (**a**) HeLa cells were transfected with Myc-tagged Pin1, Y23A point mutant or control plasmids, and left uninduced or stimulated with oncostatin M (OSM) for 15 min. Phosphorylation of Stat3 Tyr705 and Ser727 was detected by the specific anti-phospho-Tyr705 and anti-phospho-Ser727 antibody, and the blot was stripped and reprobed with anti-Stat3 and anti-Myc antibodies. The blot was also probed by actin antibody to serve as loading controls. The intensity of the bands was measured and normalized with the total Stat3 protein level, and is indicated on top of the bands. (b) The wild-type (WT) and KO MEF cells were stimulated with OSM for 15 min and subjected to nuclear extraction. Endogenous Stat3 amounts in the nuclear/cytoplasmic fractions were analysed with anti-Stat3 antibody, with the endogenous poly (ADP-ribose) polymerase and tubulin as controls for the fractionation and loading efficiency. (**c**) Pin1 WT and KO MEF cells were induced with OSM for different lengths of time as indicated. The nuclear extracts were subjected to electrophoretic mobility shift assay. Super-shift assays with Stat1 or Stat3 antibody are shown in lanes 1 and 2 as indicated.

only in the nucleus, although currently we have no direct evidence showing that to be the case. The Stat3–Pin1 interaction leads to an increase of the endogenous Stat3 transcriptional activity in various cell lines, whereas the absence of Pin1 leads to significant impairment in the ligand-induced Stat3 activity and downstream gene expression (Figures 2 and 3). These results suggest that Pin1 is a positive regulator of Stat3 activity.

Generally, in addition to the phosphorylation of Tyr705, phosphorylation of the Ser727 residue also contributes to the activation of Stat3. While tyrosine phosphorylation plays a key role in all the basic events required for Stat3 activation, such as dimer formation, nuclear translocation and DNA binding, serine phosphorylation is required for its maximal transcriptional activity. Serine phosphorylation, therefore, probably represents a second level in the regulation mechanism through which, with the assistance of Pin1, maximal Stat3 transcriptional activity is achieved. Pin1 may, in fact, serve as a precise molecular switch, specifically devoted to serine phosphorylation, by converting Stat3 from its 'primarily activated' form to 'optimally

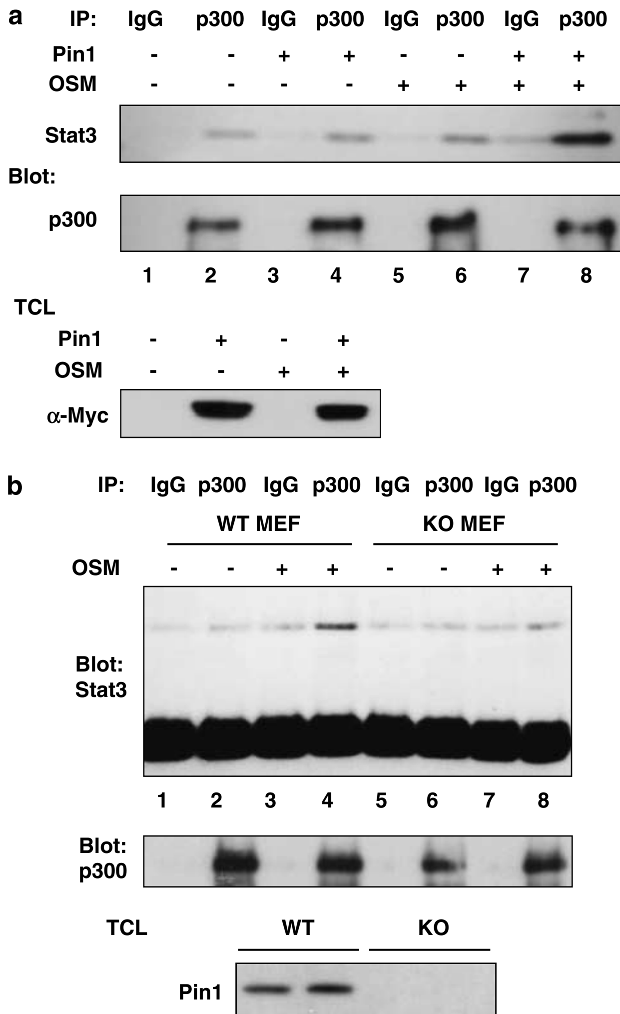

Figure 6 Pin1 increases Stat3 and p300 interaction. (**a**) MCF-7 cells were transfected with a control vector (−) or Myc-tagged Pin1 (+), and left untreated or stimulated with oncostatin M (OSM) for 20 min. The cell lysates were immunoprecipitated with rabbit IgG or anti-p300 antibody as labeled and blotted with anti-Stat3 (top panel). The blot was stripped and reprobed with anti-p300 (middle panel). Expression of Myc-Pin1 is shown in the bottom panel. (**b**) Pin1 wild-type (WT) and KO murine embryonic fibroblasts (MEFs) were either left untreated or stimulated with OSM for 20 min. The experiment was performed as described in (**a**).

activated' form. This second stage of regulation may involve various events such as enhanced Stat3 DNA binding and coactivator recruitment.

Identification of Stat3–Pin1 interaction via the Ser727 residue also provides a potential explanation for the long-undetermined mechanism of the serine phosphorylation-mediated enhancement to Stat3 transcription. In view of the fact that the interaction of Pin1 and Stat3 leads to upregulated Stat3 transactivation and that such interaction requires phosphorylated Ser727, we suspect that the transcription-enhancing effect of Stat3 serine phosphorylation may result from the specific binding of Pin1 via the phosphoSer727 residue. Considering the presence of the invariant serine residue in most STAT proteins, like Stat1 and Stat4, it is possible that Pin1 also accounts for the transcription-enhancing effects of serine phosphorylation in other STAT proteins. Our preliminary immunoprecipitation result has shown interaction between Stat1 and Pin1 in ligand-stimulated cells, which further supports this speculation.

Three PPIase-defective point mutants of Pin1 and treatment of HepG2 cells with PPIase inhibitor juglone at 1 μM failed to inhibit the Pin1-dependent positive regulation on Stat3 activity (Figure 2b and see Supplementary Figure S1). Based on these results, it seems that PPIase activity of Pin1 is not required for its positive regulatory roles in Stat3 transcriptional activity and that Pin1 may affect Stat3 through a mechanism other than its enzymatic activity. The actual mechanism remains to be further characterized.

Activation of STAT proteins, especially Stat3, is frequently associated with various tumors. For instance, constitutive activation of Stat3 has been observed in various breast carcinoma cell lines (Bowman *et al*., 2000), and significantly elevated Stat3 activation was detected in many human malignant breast cancer tissues (Clevenger, 2004). On the other hand, overexpression of Pin1 has also been reported in multiple human cancers (Bao *et al*., 2004). Roles of Pin1 in the mammary gland development, breast cancer and the transformation of mammary epithelial cells have been intensively studied (Wulf *et al*., 2003, 2004). In our study, Pin1 has been shown to be involved in various steps regulating the Stat3 activity. Hence, it will be interesting to know whether there is a correlation between the prevalent Pin1 overexpression and constitutive Stat3 activation in various tumors and cancer cells, especially human breast cancers. On the other hand, as Pin1 is involved in human cancers by regulating various proteins, it is difficult to specifically prove the role of Pin1 on Stat3 in the tumorigenesis. Recently, accumulating evidence suggests that OSM, an IL-6 family cytokine, induces EMT-like responses in various breast carcinoma cells, which may potentially contribute to tumor progression and metastasis *in vivo* (Holzer *et al*., 2004; Jorcyk *et al*., 2006). Stat3 has been shown to play a special role in this process by inhibiting cell growth and causing morphological changes (Zhang *et al*., 2003). In our study, Pin1 has been shown to promote OSM-induced cell growth inhibition and morphological changes in MCF-7 breast cancer cells, and suppression of Pin1 expression by siRNA attenuates such effects (Figure 7). These data suggest that Pin1 positively regulates Stat3-mediated, OSM-induced EMT in breast cancer cells and raises the possibility that such positive regulation of Pin1 on Stat3 may also be involved in breast cancer progression and invasion processes.

Materials and methods

Construction of expression plasmids
Murine Stat3 and its point mutant Ser727Ala were cloned into pXJ40-FLAG as described previously (Lim and Cao, 1999; Zhang *et al*., 2002). Full-length Pin1 was generated by PCR from human cDNA library (Clontech, Mountain View, CA,

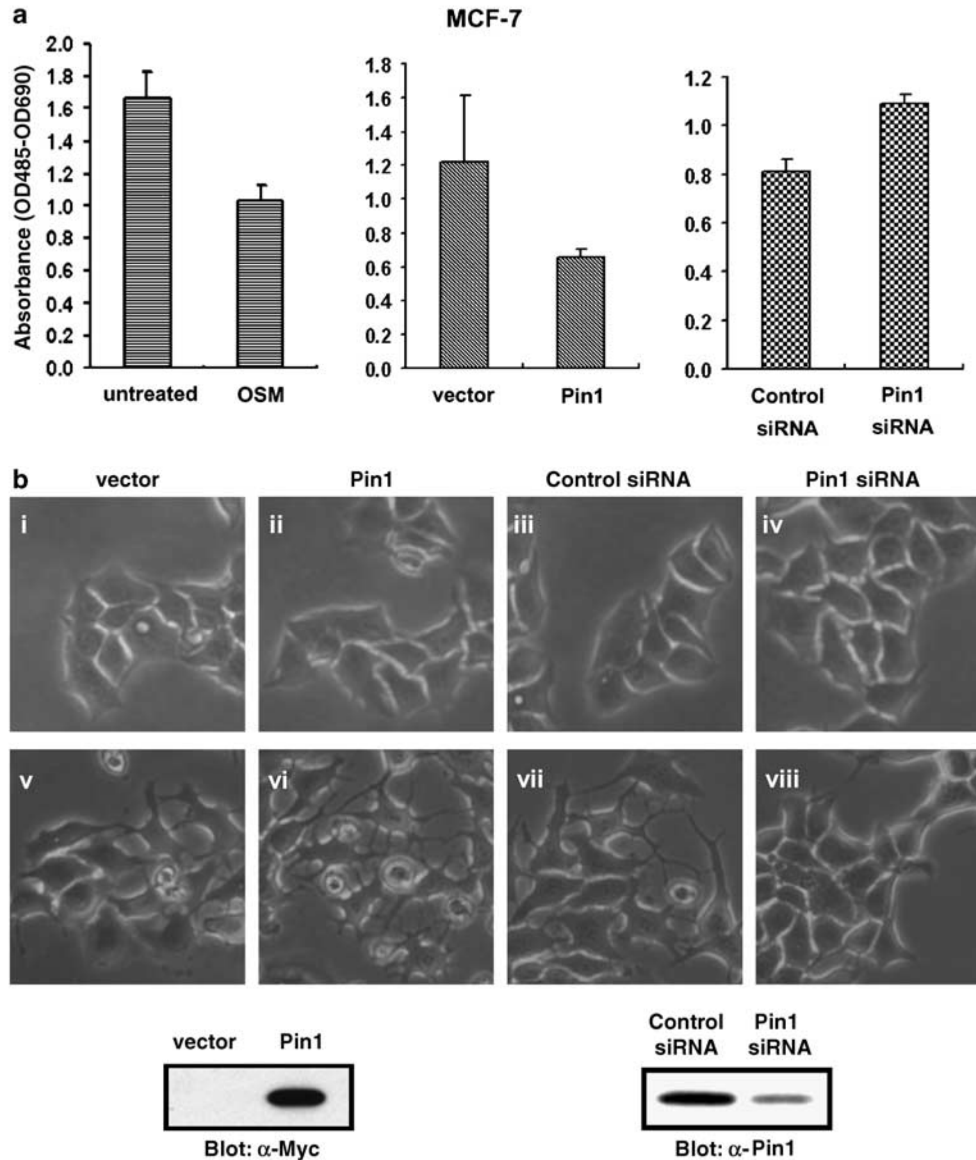

Figure 7 Effects of Pin1 on Stat3-mediated cell proliferation and morphological change. (**a**) In the left panel, MCF-7 were grown in a 24-well plate and left untreated or treated with oncostatin M (OSM) (10 ng/ml) for 72 h. In the middle and right panels, MCF-7 cells were transfected with control vector, Pin1, control siRNA or Pin1 siRNA, and all treated with OSM for 72 h. The cell proliferation assay was performed using the Cell Proliferation Kit II (XTT). Spectrophotometric absorbance was measured at 485 nm wavelength and reference wavelength 690 nm, which presents a mean of three samples, with the standard deviation given as the error bars. (**b**) MCF-7 cells were transfected as indicated, and left unstimulated (panels i–iv) or stimulated with OSM for 72 h (v–viii). The cell morphology was observed and recorded by phase-contrast microscopy. Expression of Pin1 is shown in the bottom panels.

USA), and the respective PCR product was sequenced and cloned into pXJ40-Myc and pXJ40-HA expression plasmids. The point mutant for Pin1 was generated by site-directed mutagenesis using the WT Pin1 as template.

Immunoprecipitation, GST pull-down and western blotting
Cell lysates containing 0.5–1 mg of total proteins were subjected to immunoprecipitation/blotting as described previously (Cao *et al*., 1996). For GST pull-down experiments, cell lysates containing 1 mg of proteins were incubated with 20 μg of bacterially produced GST fusion proteins, followed by 40 μl of glutathione–Sepharose 4B beads (Amersham Pharmacia Biotech, Uppsala, Sweden). The precipitates were washed and subjected to western blot analysis with the respective antibodies.

Luciferase assay
The firefly luciferase reporter gene constructs m67-luc and the pGL3-α2M-215luc were kindly given by Dr J Bromberg and Dr PC Heinrich, respectively. The luciferase assays were carried out as described previously (Lufei *et al*., 2003).

Cell culture and DNA transfections, production and purification of bacterial GST fusion protein, cell fractionation and electrophoretic mobility shift assay, and cell growth assay are described in Supplementary Materials and methods.

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