

The Peptidyl-Isomerase Pin1 Regulates p27^{kip1} Expression through Inhibition of Forkhead Box O Tumor Suppressors

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Abstract

The Forkhead box O (FOXO) protein family is an evolutionarily conserved subclass of transcription factors recently identified as bona fide tumor suppressors. Preventing the accumulation of cellular damage due to oxidative stress is thought to underlie its tumor-suppressive role. Oxidative stress, in turn, also feedback controls FOXO4 function. Regulation of this process, however, is poorly understood but may be relevant to the ability of FOXO to control tumor suppression. Here, we characterize novel FOXO4 phosphorylation sites after increased cellular oxidative stress and identify the isomerase Pin1, a protein frequently found to be overexpressed in cancer, as a critical regulator of p27^{kip1} through FOXO4 inhibition. We show that Pin1 requires these phosphorylation events to act negatively on FOXO4 transcriptional activity. Consistent with this, oxidative stress induces binding of Pin1 to FOXO, thereby attenuating its monoubiquitination, a yet uncharacterized mode of substrate modulation by Pin1. We have previously shown that monoubiquitination is involved in controlling nuclear translocation in response to cellular stress, and indeed, Pin1 prevents nuclear FOXO4 accumulation. Interestingly, Pin1 acts on FOXO through stimulation of the activity of the deubiquitinating enzyme HAUSP/USP7. Ultimately, this results in decreased transcriptional activity towards target genes, including the cell cycle arrest gene p27^{kip1}. Notably, in a primary human breast cancer panel, low p27^{kip1} levels inversely correlated with Pin1 expression. Thus, Pin1 is identified as a novel negative FOXO regulator, interconnecting FOXO phosphorylation and monoubiquitination in response to cellular stress to regulate p27^{kip1}. [Cancer Res 2008;68(18):7597–605]

Introduction

Forkhead box O (FOXO) transcription factors, consisting of mammalian FOXO1, FOXO3a, FOXO4, and FOXO6 are important downstream targets of the evolutionarily conserved phosphoinosi-

tide-3-kinase/Akt (PKB) signaling pathway (1, 2). Akt/PKB negatively regulates FOXO activity through direct phosphorylation by inducing their nuclear exclusion (3). FOXOs play a critical role in longevity, first shown in the nematode *Caenorhabditis elegans*, which had an extended life span upon deletion of the *daf-2*/insulin receptor (reviewed in ref. 4). This requires the FOXO orthologue *daf-16* and is characterized by an increase in stress resistance, consistent with the notion that resistance to cellular stress correlates with longevity (5).

Stress resistance is also closely related to the onset of age-related diseases such as cancer (5, 6). Indeed, FOXOs were recently shown to be tumor suppressors in a number of cancers (1, 7). Mice depleted for FOXO1, FOXO3, and FOXO4 are characterized by the appearance of thymic lymphomas and hemangiomas (8). Intriguingly, cellular stress in turn, also changes FOXO activity towards its target genes, thus allowing for an adaptive response to cellular stress (reviewed in ref. 9). Regulation of this process, however, is poorly understood but may be relevant to the ability of FOXO to control tumor suppression.

FOXOs regulate a number of transcriptional targets involved in stress resistance, survival, and cell proliferation (reviewed in ref. 7). A key transcriptional FOXO target is the cell cycle arrest gene p27^{kip1} (8, 10). The cyclin-dependent kinase inhibitor p27^{kip1} is a haploinsufficient tumor suppressor that regulates the entry of cells from quiescence to cell cycle through inhibition of CDK2 (11). Interestingly, activation of FOXO in cells induces cell cycle arrest and quiescence, involving p130 and p27^{kip1} expression (12). Re-entry into the cell cycle involves down-regulation of p27^{kip1}, a process that is poorly understood but is thought to involve the phosphorylation and degradation of p27^{kip1} (11). In human cancer, expression of p27^{kip1} is often found deregulated and numerous therapies are being developed to restore its function. Localization defects and degradation are thought to be the main cause of p27^{kip1} deregulation. However, it was recently shown that p27^{kip1} levels in human cancer are transcriptionally regulated as well, albeit through unknown mechanisms (13).

Regulation of protein activity often involves signaling through posttranslational modifications. These modifications either induce a structural change in the protein, thereby altering its activity, or induces the exposure of sites recognized by regulatory proteins. Pin1 is a peptidyl-prolyl isomerase that specifically recognizes phosphorylated serines and threonines flanked by a COOH-terminal proline residue (14). Pin1-mediated isomerization induces conformational changes in the peptide backbone. This leads to the altered function of its protein substrates and has been shown to be involved in numerous processes, including the regulation of cell proliferation and death (14). Pin1 is found to be overexpressed in many human cancers and is linked to tumorigenesis (15).

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi:10.1158/0008-5472.CAN-08-1059

Here, we identify a novel regulatory pathway for FOXO signaling. In response to cellular stress, FOXOs are phosphorylated and recognized by Pin1. Evidence is provided that Pin1 negatively regulates FOXO monoubiquitination at the level of deubiquitination through HAUSP/USP7. This inhibits nuclear FOXO translocation in response to hydrogen peroxide-induced stress and ultimately leads to decreased transcription and expression of FOXO4 transcriptional targets, including *p27^{kip1}*. Notably, in a panel of primary human breast cancers, we found an inverse correlation between low *p27^{kip1}* levels and Pin1 expression.

Materials and Methods

Cell culture and transfection. HEK293T and A14 cells (3T3 fibroblasts stably expressing the insulin receptor) were maintained in DMEM (Cambrex), 10% FCS, penicillin/streptomycin and 0.05% glutamine.

Constructs and RNAi. pMT2-HA-FOXO4, pMT2-Flag-FOXO4, pMT2-GFP-FOXO4, CMV-*p27^{kip1}*, His₆-Ubi, and pBabePuro have been described (16). pcDNA-His-Pin1, pcDNA-His-Pin1^{W34A}, pGEX-GST-Pin1, and pGEX-GST-Pin1^{W34A} were gifts from Drs. C. Fila and P. van der Sluijs (Department of Cell Biology, University Medical Centre Utrecht, Utrecht, The Netherlands). Pin1 was Flag-tagged NH₂-terminally by PCR, using oligonucleotide sequences to Flag-tag Pin1 (forward oligonucleotide, 5'-CCGGATCCATG-GACTACAAGGATGACGACGACAAGCGGACGAGGAGAAGCTG; reverse oligonucleotide, 5'-CGAATTCTCACTCAGTCGGAGATGATG-3'). PCR products were digested with *Bam*HI and *Eco*RI and cloned in pcDNA3.1.

Pin1^{K63A} was made by site-directed mutagenesis oligonucleotide sequences to make K63A mutant to Pin1 (forward, 5'-CGTGACTGGCTG-TGCGCCACCAGCAGGTGCGA-3' and its reverse complement strand). Nontargeting RNAi duplex (C), RNAi oligonucleotides specific for Pin1 (Pin1 #1: sense, 5'-GCCAUUUGAAGACGCGUCUGdTdT-3'; Pin1 #2: sense, 5'-CGUCCUGGCGGCAGGAAUdTdT-3') were purchased from Dharmacon. RNAi was transfected with OligofectAMINE (Invitrogen). Additional cotransfections were performed 8 h after RNAi transfection.

Antibodies. The antibodies against FOXO4 (834) and HA (12CA5) have been described (10). The following antibodies were purchased: MPM2 (Upstate), FOXO4-phospho-Thr28 (Upstate), Pin1 (R&D systems), *p27^{kip1}* (BD Biosciences), FOXO4-N19, 14-3-3, and glutathione *S*-transferase (GST; Santa Cruz), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon), tubulin, and Flag-M2 (Sigma).

GST pulldown assays, coimmunoprecipitations, and Western blot analysis. GST proteins were coupled to glutathione agarose beads, washed twice with radioimmunoprecipitation assay buffer [RIPA; 20 mmol/L Tris-HCl (pH 8.0), 1% TX-100, 0.5% NaDoc, 5 mmol/L EDTA, 150 mmol/L NaCl, protease and phosphatase inhibitors]. Cells were lysed in RIPA, cleared and incubated with the glutathione agarose beads for 2 h at 4°C. Subsequently, beads were washed, boiled in Laemmli sample buffer, and analyzed by Western blotting. Lambda phosphatase was purchased from New England Biolabs. For coimmunoprecipitation studies, 50 µL of Protein-A Sepharose beads were precoupled to 1 µg of the indicated antibody. Cells were lysed in RIPA or, in the case of endogenous coimmunoprecipitation, in 20 mmol/L of Tris-HCl (pH 8.0), 1% NP40, 10% glycerol, 1 mmol/L of MgCl₂, 1 mmol/L of EDTA, 150 mmol/L of NaCl, protease and phosphatase inhibitors, and incubated as described previously (17).

Mass spectrometry. Purified Flag-FOXO4 from hydrogen peroxide-treated HEK293T cells (200 µmol/L for 1 h) was digested with trypsin, subtilisin, and/or elastase (Roche). If required, samples were enriched for phosphorylated peptides using TiO₂ microcolumns, as described (18). Samples were subjected to nanoflow LC (Agilent 1100 series) coupled to a quadrupole time-of-flight tandem mass spectrometer (Micromass Waters). Data were processed and subjected to database searches using MASCOT software (Matrixscience). The identified peptides were confirmed by manual interpretation of the spectra.

Reverse transcriptase quantitative PCR. The expression of endogenous *p27^{kip1}* and *Gadd45a* genes in A14 cells was examined by reverse transcription of total RNA followed by real-time quantitative PCR (qPCR),

on an ABI cycler using Sybr Green (ABI), with oligonucleotides specific to *p27^{kip1}*, *Gadd45a* and *PBGD* oligonucleotides specific for mouse *p27^{kip1}* (forward, 5'-CTGGGTTAGCGGAGACTGT-3'; reverse, 5'-GGAAAA-CAAAACGCTTCTTCTAG-3'), mouse *Gadd45a* (forward, 5'-AGACC-GAAAGGATGGACACG-3'; reverse, 5'-TGACTCCGAGCCTTGCTGA-3'), mouse *PBGD* (forward, 5'-GGCAATGCGGCTGCAA-3'; reverse, 5'-GGGTA-CCACGCGAATCAC-3').

Protein stability assay. HEK293T cells were transfected with a CMV-driven *p27^{kip1}* construct and/or Pin1. Next, cells were treated with 10 µg/mL of cycloheximide for the indicated times. Protein levels were detected and corrected for GAPDH levels by Odyssey.

Immunohistochemistry. Paraffin blocks containing formaldehyde-fixed breast cancer tissues were sectioned, deparaffinized, and rehydrated and stained essentially as described (19) with the primary antibodies for Pin1, FOXO3a, FOXO4, and *p27^{kip1}*. These were detected using a poly-Hrp anti-Ms/Rb/Rt (ImmunoLogic) and developed with diaminobenzidine, followed by counterstaining with hematoxylin.

Ubiquitination assay. The monoubiquitination assay was performed as described (16). HEK293T cells were transfected with RNAi oligonucleotides and/or the indicated constructs. Forty-eight hours posttransfection, cells were treated as indicated and lysed in 8 mol/L of urea, 10 mmol/L of Tris-HCl (pH 8.0), 100 mmol/L of Na₂HPO₄/NaH₂PO₄, 0.2% TX-100, 5 mmol/L of NEM, and protease inhibitors. Ubiquitinated proteins were precipitated using Ni-NTA agarose beads and the experiment was analyzed by Western blotting.

Immunofluorescence. A14 cells were plated and transfected on coverslips. Immunostaining was performed 40 h after transfection (3). Paraformaldehyde-fixed cells were incubated with anti-FOXO4 (834) or anti-Pin1, followed by goat anti-mouse IgG conjugated to Alexa488 or goat anti-rabbit IgG conjugated to Alexa568. Nuclei were visualized with 4',6-diamidino-2-phenylindole. Fluorescence was captured using a Zeiss Axioskop microscope.

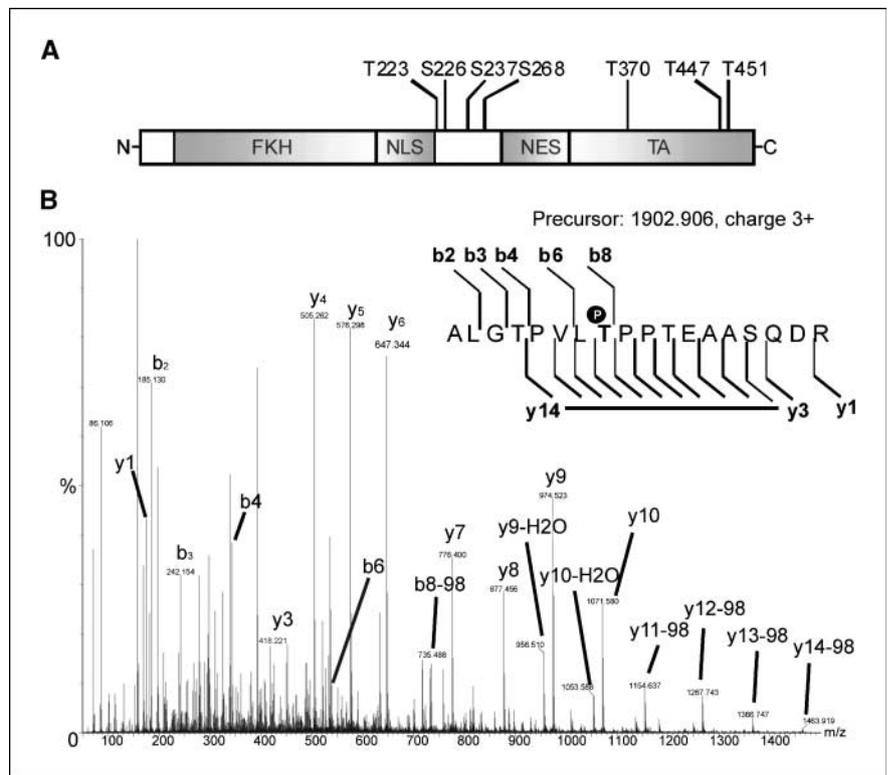
Results

Identification of seven phosphorylated Ser/Thr-Pro sites on FOXO4. To better understand the FOXO4 adaptive response under conditions of cellular stress, a search for posttranslational modifications was initiated on FOXO4 by employing tandem mass spectrometry (MS). Flag-FOXO4 was purified from hydrogen peroxide-treated human embryonic kidney 293T (HEK293T) cells and subjected to proteolytic digestion. Five novel phosphorylated sites for FOXO4 were characterized, in addition to the previously characterized oxidative stress-sensitive phosphorylation sites Thr⁴⁴⁷/Thr⁴⁵¹ (20), suggesting that at least some of these seven sites are regulated through oxidative stress signaling (Fig. 1; Supplementary Table S1). TiO₂ columns, which specifically enrich for phosphorylated peptides (18), were used in MS analysis and confirmed these sites. In addition, we identified two double-phosphorylated peptides, Thr⁴⁴⁷/Thr⁴⁵¹ and Thr²²³/Ser²²⁶ (Supplementary Table S1), indicating that FOXO4 can be multiphosphorylated.

Interestingly, all these sites consisted of phosphorylated Ser/Thr residues, followed by a Pro residue. These phosphorylated Ser/Thr-Pro motifs in proteins are potential recognition sequences for the peptidyl-prolyl isomerase Pin1, which has been proposed to regulate protein function through catalyzed *cis-trans* isomerization, thereby changing the local structure of its substrates (21). This prompted us to test the hypothesis that FOXO4 activity may be regulated by Pin1.

The FOXO4 transcriptional activity on *p27^{kip1}* is inhibited by Pin1. Previously, we have shown that FOXO4 regulates transcriptional activity of the tumor suppressor gene *p27^{kip1}* (10). Real-time reverse transcriptase qPCR was performed to test if Pin1 could affect FOXO4 transcriptional activity. Expression of FOXO4 increased the abundance of *p27^{kip1}* mRNA by approximately 3-fold

Figure 1. FOXO4 is phosphorylated on multiple Ser/Thr-Pro sites in conditions of oxidative stress. **A**, schematic representation of FOXO4, with the MS-identified phosphorylated residues. *FKH*, Forkhead domain; *NLS*, nuclear localization signal; *NES*, nuclear export signal; *TA*, transactivation domain. **B**, representative tandem MS sequence of a FOXO4 tryptic peptide spanning amino acids 444 to 461. b and y ions are shown as well as the -98 Da H₃PO₄ loss and the phosphorylated Thr⁴⁵¹ residue.



(Fig. 2A), similar to previous findings (10). Interestingly, coexpression of Pin1 inhibited the FOXO4-induced increase in p27^{kip1} mRNA levels (Fig. 2A). Pin1 harbors two functional domains, an NH₂-terminal WW domain, involved in substrate interaction (22), and a COOH-terminal PPIase domain critical for its isomerase activity (23). The Pin1 mutant Pin1^{W34A}, which fails to bind phosphorylated substrates (23), no longer inhibited p27^{kip1} transcription, indicating that the WW domain is required for the Pin1-mediated p27^{kip1} down-regulation. Similar results were obtained by using primers specific for the *Gadd45a* gene (Fig. 2A, right), another FOXO transcription target (24).

To determine if p27^{kip1} protein levels change accordingly, FOXO4 was transiently expressed in the absence or presence of Pin1 and the mutant Pin1^{W34A}. Consistent with the reverse transcriptase qPCR results and previous data (10), FOXO4 increased p27^{kip1} protein expression. However, Pin1 coexpression, but not mutant Pin1^{W34A}, abolished this increase in a dose-dependent manner (Fig. 2B). Similar results were found for p21 expression (Supplementary Fig. S1), another FOXO4 transcriptional target (25). Aside from FOXO-regulated transcription of p27^{kip1}, Pin1 could also affect p27^{kip1} expression at other posttranscriptional levels. To test whether Pin1 could also have a direct effect on p27^{kip1} protein stability, we determined the p27^{kip1} half-life time by performing cycloheximide experiments. Coexpression of Pin1 did not change the p27^{kip1} half-life (Fig. 2C), indicating that Pin1 does not affect p27^{kip1} protein stability.

Finally, we wanted to address if the regulation of p27^{kip1} is mediated by the isomerase activity of Pin1. For this, we coexpressed FOXO4 and a previously described isomerase-defective Pin1 mutant, Pin1^{K63A} (21). Whereas Pin1 inhibited FOXO4-induced p27^{kip1} expression, this mutant did not (Fig. 2D), indicating that the isomerase activity of Pin1 is required for Pin1-mediated inhibition of FOXO4 transcriptional activity. Taken together, these

results show that Pin1 expression inhibits FOXO4 transcriptional activity, which is dependent on both the Pin1 substrate interaction domain and the isomerization domain. This prompted us to test if Pin1 and FOXO4 physically interact.

Cellular stress induces Pin1 binding to phosphorylated FOXO4. Glutathione-S-transferase (GST) pulldown experiments were performed to examine if Pin1 could physically interact with FOXO4. As shown in Fig. 3A, GST-Pin1, but not GST alone, specifically precipitated FOXO4 from HEK293T cell lysates. Interestingly, treatment of cells with increasing amounts of hydrogen peroxide prior to lysis strongly enhanced this interaction in a dose-dependent manner. In addition to cellular stress generated by hydrogen peroxide, we tested other stressors as well. GST pulldown experiments were performed on FOXO4-expressing cells that were treated with anisomycin, which is known to activate stress-activated protein kinases (26), doxorubicin and UV, both used to induce DNA damage. Binding of FOXO4 increased only when cells were treated with hydrogen peroxide or anisomycin, indicating specificity for these stressors (Supplementary Fig. S2). Next, GST pulldown experiments were performed to test if Pin1 could also interact with FOXO3a, a closely related FOXO family member. Like FOXO4, FOXO3a was found to bind to Pin1 (Supplementary Fig. S3), suggesting that the interaction is conserved among the FOXO family members. Moreover, consistent with the inability to reduce FOXO4 transcriptional activity, the Pin1^{W34A} mutant, incapable of binding Pin1 substrates, could no longer interact with FOXO4 and FOXO3a (Fig. 3B; Supplementary Fig. S3). The above experiments indicate that Pin1 interacts with FOXO4 via its WW domain, an interaction that is enhanced in response to hydrogen peroxide. As mentioned, Pin1 binding is specific for phosphorylated Ser/Thr-Pro sites, of which MS on FOXO4 identified seven. Therefore, we analyzed whether the Pin1-FOXO4 interaction is actually dependent on the phosphorylation of FOXO4

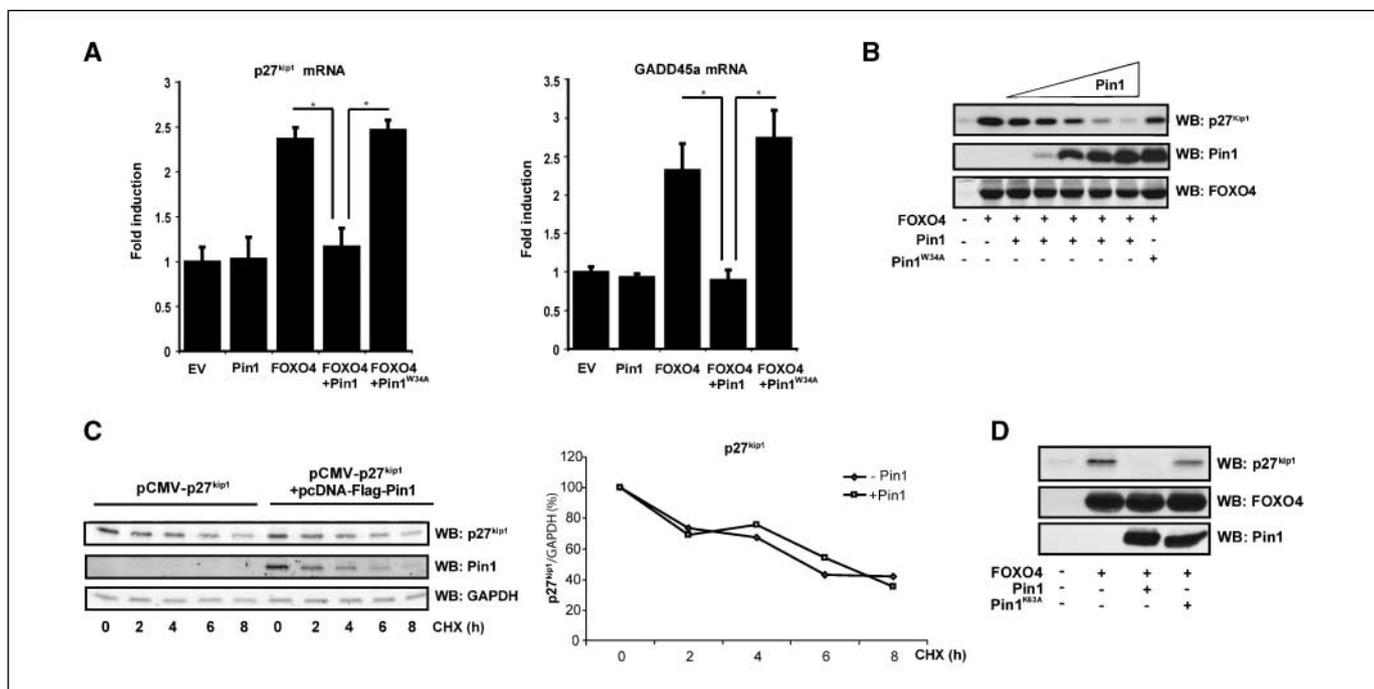


Figure 2. Pin1 inhibits FOXO4-induced p27^{kip1} expression. *A*, Pin1 inhibits FOXO4 transcriptional activity. A14 cells were transfected as indicated together with pBabePuro. Total RNA extracted from puromycin-resistant cells was subjected to real-time reverse transcriptase-qPCR, using specific oligonucleotides for p27^{kip1} or Gadd45a mRNA. Samples were normalized against PBDG mRNA. Columns, mean of triplicates; bars, SD (*, $P < 0.05$, t test). *B*, transient expression of Pin1 reduces p27^{kip1} protein levels. HEK293T cells were transfected as indicated together with pBabePuro. Puromycin-selected cells were analyzed by Western blotting. *C*, Pin1 has no direct effect on p27^{kip1} protein levels. HEK293T cells were transfected with a CMV-driven p27^{kip1} construct and/or Flag-Pin1. Prior to lysis, cells were treated for the indicated times with cycloheximide. Protein levels relative to GAPDH were detected and quantified (representative experiment). *D*, the Pin1 effects are dependent on its isomerase activity. HEK293T cells were transfected as indicated together with pBabePuro. Puromycin-selected cells were analyzed by Western blotting.

on these sites. Pretreatment of hydrogen peroxide-treated FOXO4 with lambda phosphatase, leading to FOXO4 dephosphorylation as indicated by the loss of reduced motility in SDS-PAGE, abolished the ability of FOXO4 to interact with Pin1 (Fig. 3*B*), indicating that FOXO4 needs to be phosphorylated for its interaction with Pin1.

Next, we set out to determine which phosphorylated FOXO4 Ser/Thr-Pro sites are involved in the Pin1-FOXO4 interaction. Mutational analysis of single phosphorylation sites to Ala residues did not result in significantly decreased Pin1 binding upon peroxide stress, indicating that multiple phosphorylation sites are likely to be involved (data not shown). Further analysis using combinations of multiple Ala mutations consistently showed that the Pin1-FOXO4 interaction decreased progressively (data not shown) and is impaired for the FOXO4 mutant, in which all putative Pin1 binding sites as identified by MS, are mutated to Ala and is therefore referred to as FOXO4-7S/TA (Fig. 3*C*).

Finally, coimmunoprecipitation assays on endogenous FOXO4 and Pin1 in HEK293T cells showed that Pin1 specifically interacts with FOXO4 *in vivo*, an interaction that is increased after hydrogen peroxide-induced stress (Fig. 3*D*). Taken together, these results show that Pin1 and FOXO4 interact *in vitro* and *in vivo*. This interaction is sensitive to cellular stress and is dependent on a functional WW domain of Pin1 as well as phosphorylation of FOXO4.

Pin1 regulates peroxide-induced FOXO4 monoubiquitination and nuclear localization through inhibition of HAUSP/USP7. Regulation of FOXO activity is often mediated through a change in cellular distribution. For instance, signaling through PKB/Akt inactivates FOXO4 through phosphorylation, resulting in nuclear exclusion (3). Alternatively, increased oxidative stress as

generated by hydrogen peroxide results in increased nuclear localization (17, 27). The observed Pin1-dependent decrease in FOXO4 transcriptional activity could therefore be the result of a changed nuclear-cytoplasmic FOXO4 localization. Immunofluorescence of FOXO4 in A14 cells shows that FOXO4 is distributed in both the cytoplasm and nucleus under normal growth conditions but is redistributed predominantly to the nucleus when stimulated with hydrogen peroxide, consistent with previous observations (Fig. 4*A*; refs. 17, 27). However, ectopic expression of Pin1 prevented the redistribution of FOXO4 to the nucleus in response to oxidative stress. This phenotype depends on the interaction of FOXO4 with Pin1, as cells coexpressing Pin1^{W34A} do not inhibit FOXO4 relocalization. Furthermore, the FOXO4-7S/TA mutant that is impaired in binding to Pin1 had a nuclear localization in normal serum-containing medium (Fig. 4*A*). Treatment of cells with hydrogen peroxide and Pin1 coexpression did not change FOXO4-7S/TA localization (data not shown), indicating that phosphorylation of these sites is required to retain FOXO4 in the cytoplasm. Taken together, these results indicate that Pin1 inhibits FOXO4-mediated transcriptional effects by inhibiting its nuclear localization.

The nuclear-cytoplasmic shuttling of FOXO factors by growth factor signaling is regulated through binding of 14-3-3 proteins, which leads to the export of FOXO in a Ran/Crm1-dependent manner to the cytoplasm (3). To uncover the mechanism by which Pin1 regulates FOXO4 localization, the interaction between 14-3-3 and FOXO4 was explored. FOXO4, but not a mutant in which all three PKB/Akt sites are mutated (FOXO4-A3), could bind 14-3-3 (Fig. 4*B*; ref. 3). In the presence of Pin1, however, the interaction of FOXO4 with 14-3-3 is unchanged. Altered 14-3-3 binding therefore,

could not explain the Pin1-mediated effects observed on FOXO4 localization. Importantly, phosphorylation of FOXO4 on Thr28 which is mediated by PKB/Akt did not change upon Pin1 expression, indicating that Pin1 does not affect PKB-mediated FOXO4 signaling.

Recently, we have shown that FOXO4 can be monoubiquitinated in response to oxidative stress, a process that is reversed by the deubiquitinating enzyme HAUSP/USP7 (16). Monoubiquitination of proteins is a distinct modification leading to a change in cellular localization/signaling, clearly different from polyubiquitination that leads to proteasome-mediated degradation of protein substrates (28, 29). Indeed, monoubiquitinated FOXO4 leads to nuclear localization of FOXO4 independent of protein turnover (16). As shown in (Fig. 4C), low amounts of hydrogen peroxide induce FOXO4 monoubiquitination. However, expression of Pin1 completely inhibits hydrogen peroxide-induced FOXO4 monoubiquitination. Because HAUSP/USP7 is the enzyme responsible for deubiquitinating FOXO4, we tested if the Pin1 binding impaired mutant FOXO4-7S/TA mutant, could still be deubiquitinated by USP7. Surprisingly, whereas USP7 can efficiently and completely deubiquitinate FOXO4, deubiquitination is impaired for FOXO4-7S/TA (Fig. 4D). This observation suggests that USP7-mediated deubiquitination on FOXO4 is in part dependent of Pin1. USP7-mediated deubiquitination of FOXO4 involves binding of USP7 to FOXO4. Therefore, we tested whether FOXO4-7S/TA still binds to USP7. In coimmunoprecipitation assays, USP7 was found to interact equally well with both FOXO4 and FOXO4-7S/TA (Supplementary Fig. S4). Because FOXO4-7S/TA shows strongly impaired binding to Pin1, this result suggests that Pin1 enhances

substrate, i.e., monoubiquitinated FOXO4 recognition of USP7. This also explains the small amount of FOXO4-7S/TA deubiquitination because USP7 still interacts with FOXO4. Together, these results show that Pin1 prevents hydrogen peroxide-induced FOXO4 nuclear localization and this likely results from the ability of Pin1 to inhibit hydrogen peroxide-induced FOXO4 monoubiquitination through stimulation of HAUSP/USP7-mediated FOXO4 deubiquitination.

Pin1 regulates p27^{kip1} through FOXO4. FOXOs are known regulators of p27^{kip1} expression, which as shown here, can be inhibited by Pin1. However, to our knowledge, Pin1 has not been described to affect p27^{kip1} expression. Therefore, we wished to address the role of endogenous Pin1 in regulating p27^{kip1} expression. To this end, we used small interfering RNA (siRNA) oligonucleotides against Pin1. Knockdown of Pin1 by two independent siRNAs increased the expression of p27^{kip1} protein, in agreement with the role of Pin1 in p27^{kip1} regulation and also in agreement with Pin1 acting as a negative regulator of FOXO (Fig. 5A). To exclude the possibility that the observed increase in p27^{kip1} expression is the result of off-target knockdown, we generated a siRNAi-insensitive Pin1 construct by introducing a silent mutation in the RNAi recognition sequence of oligonucleotide no. 1 (Pin1-SM). Expression of this mutant in a Pin1 RNAi #1 background significantly rescued the RNAi-induced p27^{kip1} protein levels, indicating that the observed effects are specific for Pin1 (Fig. 5A, right). These results indicate that depletion of Pin1 increases p27^{kip1} expression *in vivo*. FOXO4-Thr28 phosphorylation did not change upon Pin1 depletion, consistent with the notion that Pin1 does not affect PKB-mediated FOXO4 signaling.

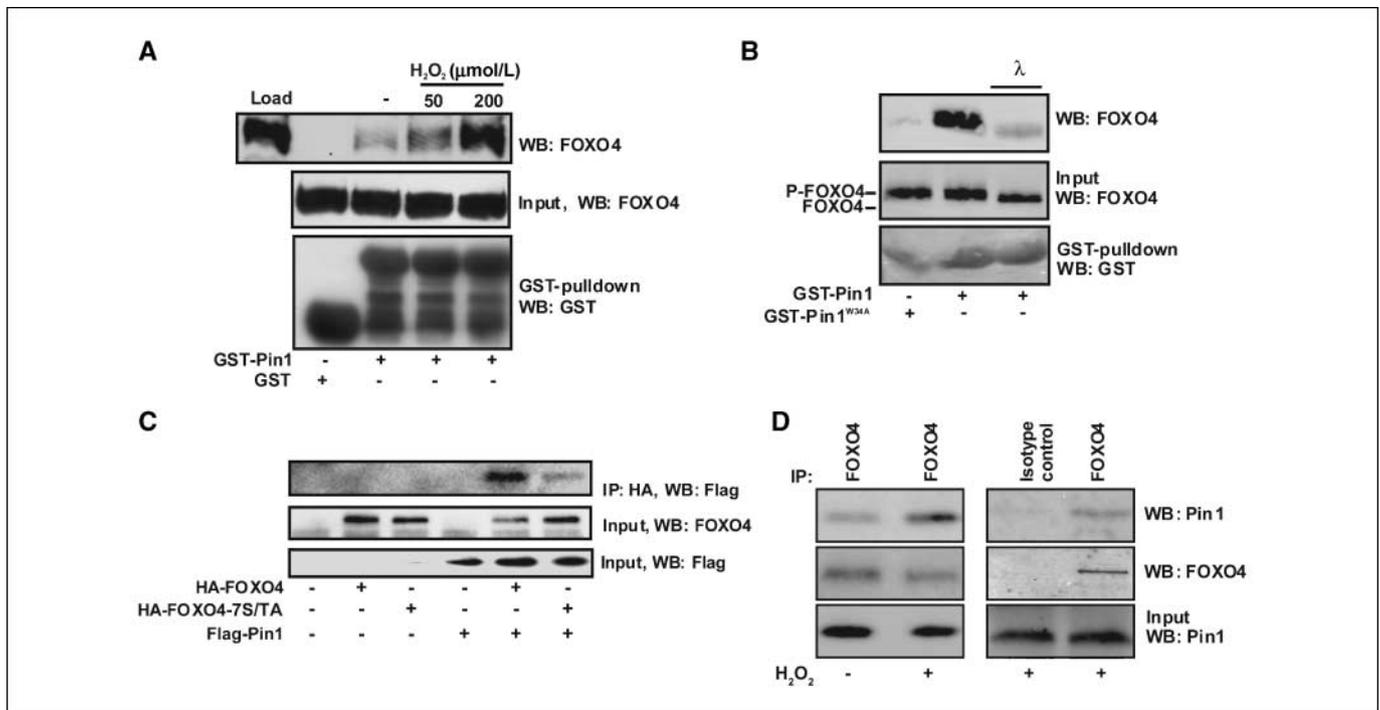


Figure 3. Pin1 interacts with FOXO4 *in vitro* and *in vivo*. **A**, FOXO4 binds Pin1 in a GST pull-down assay. HEK293T cells were transfected with empty vector or Flag-FOXO4. Cells were treated for 1 h as indicated and lysates were subjected to a GST pull-down assay. **B**, FOXO4 dephosphorylation prevents Pin1 binding. HEK293T cells were transfected with Flag-FOXO4. One hour prior to lysis, cells were treated with 200 μmol/L of H₂O₂. FOXO4 was purified with an anti-Flag antibody, eluted off with Flag peptide, and treated with lambda phosphatase (λ) for 30 min, or left untreated and subjected to GST pull-down. **C**, multiple phosphorylation sites are involved in FOXO4-Pin1 binding. HEK293T cells were transfected as indicated, stressed with 200 μmol/L of H₂O₂ for 1 h, and subjected to coimmunoprecipitation. **D**, endogenous FOXO4 and Pin1 interact. HEK293T cells were treated as indicated, lysed, and either immunoprecipitated with a specific antibody for FOXO4 (N19) or an isotype control.

of Pin1 (30). Moreover, Pin1 contributes to oncogenic transformation as, for instance, Pin1 is essential for the transformation of mammary epithelial cells induced by Neu/Ras (31). Loss of p27^{Kip1} expression is also often found in many human cancers and correlates with poor survival (32, 33). This prompted us to determine if p27^{Kip1} expression correlates with Pin1 levels in human cancer. We had access to a panel of 100 human invasive ductal breast cancer tumors and stained them for p27^{Kip1} and Pin1. In agreement with previously published work, Pin1 was found to be overexpressed to various degrees in the majority of tumors ($n = 77/100$), as compared with normal ductal breast tissue, in which only weak staining of Pin1 was found (ref. 34 and Supplementary Fig. S5). We also stained for FOXO3 and FOXO4 and found no changes in expression staining, indicating that FOXO expression is not affected in our set of tumors (data not shown).

Expression of p27^{Kip1} was highly variable, varying from a complete loss to a staining comparable with normal breast tissue (Supplementary Fig. S5). Because loss of p27^{Kip1} is highly correlated with tumorigenesis and poor prognostic outcome, we divided the tumors into two distinct groups; one with normal p27^{Kip1} levels (defined as tumors with stronger staining than the statistical median, $n = 61/100$) and one with low p27^{Kip1} levels ($n = 39/100$). In the population with low p27^{Kip1} expression, a significant inverse correlation with Pin1 expression was found ($P < 0.01$, $r = -0.42$ Spearman correlation test, two-tailed). Importantly, this observation unlikely represents an artifact of Pin1 overexpression in the majority of tumors because we observed no correlation with the high p27^{Kip1}-expressing group (Table 1). These results support the notion that Pin1 is important in p27^{Kip1} regulation and extends our observations to human breast cancer.

Discussion

Here, we provide evidence that the peptidyl-prolyl isomerase Pin1 acts as a negative regulator of FOXO transcriptional activity. Pin1, through its WW domain, binds FOXO directly in a phosphorylation-dependent manner. Furthermore, Pin1 regulation of FOXO requires, next to binding, the isomerase activity of Pin1. This indicates that Pin1-induced conformational changes within FOXO underlie the regulatory action of Pin1. Previously, it had been suggested that Pin1 catalyzes the dephosphorylation

Table 1. p27^{Kip1} and Pin1 expression inversely correlate in low p27^{Kip1}-expressing breast cancers

	Low p27 ^{Kip1}	High p27 ^{Kip1}
<i>N</i> (100)	39	61
<i>r</i>	-0.41	-0.013
<i>P</i>	0.007*	0.81

*Indicates significance, $P < 0.01$ Spearman correlation test, two-tailed.

of the phosphorylated threonine/serine residues involved in Pin1 binding (14). Thus, Pin1-induced isomerization is thought to facilitate access of phosphatases like PP2A to the phosphorylated threonine/serine residues. Using phosphospecific antibodies against the PKB phosphorylation sites and phosphospecific antibodies against the sites identified in this study, we were unable to obtain any conclusive evidence that Pin1 binding to FOXO4 resulted in the dephosphorylation of these sites. In contrast, we observed that Pin1 binding to FOXO4 regulates its monoubiquitination. Previous studies have indicated that Pin1 can modulate polyubiquitination and subsequent protein degradation of substrates with a short-lifetime, such as Myc, p53, p73, and β -catenin (reviewed in ref. 14). However, FOXOs are relatively stable proteins and monoubiquitination, in contrast, does not lead to proteasome-mediated degradation but instead often results in changes in signaling and subcellular localization (28, 29). Indeed, monoubiquitination of FOXO4 leads to nuclear localization and increased transcriptional activity but does not affect FOXO stability (16). Deubiquitination of FOXO4 is mediated by HAUSP/USP7 and similar to phosphatases, deubiquitinating enzymes such as USP7 require a cysteine within their catalytic activity to remove the ubiquitin moiety. We provide evidence that Pin1 increases USP7-mediated FOXO4 deubiquitination, which at least in part leads to decreased FOXO4 monoubiquitination. Thus, analogous to its role in regulating phosphatase activity towards its substrates, Pin1 may also regulate the activity of USP7 towards FOXO4. Nevertheless, Pin1-mediated attenuation of monoubiquitination represents, to the best of our knowledge, a

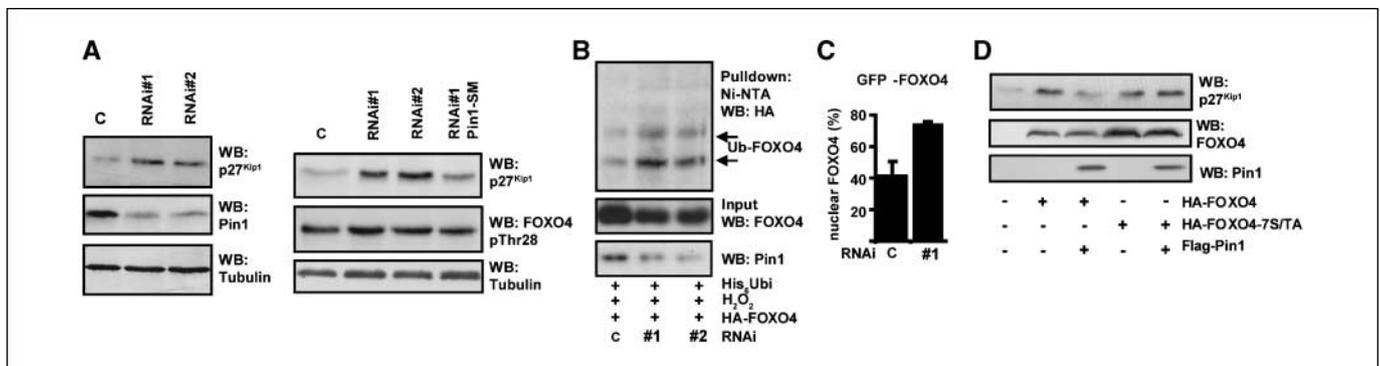


Figure 5. Pin1 regulates p27^{Kip1} through FOXO4. **A**, Pin1 depletion increases p27^{Kip1} protein expression. HEK293T cells were transfected with either nontargeting RNAi oligonucleotides (C), or Pin1 RNAi oligonucleotides (#1 or #2) or in combination with pBabePuro, EV, or the Pin1 RNAi-silent mutant (Flag-Pin1-SM; right). Cell lysates were detected for p27^{Kip1} and Pin1 expression by Western blotting. **B**, knockdown of Pin1 increases FOXO4 monoubiquitination. HEK293T cells were treated with the indicated RNAi oligonucleotides and were subsequently cotransfected with His₆-Ubi and HA-FOXO4. A ubiquitination assay was performed. **C**, knockdown of Pin1 increases FOXO4 nuclear localization. A14 cells were transfected with GFP-FOXO4 and control or Pin1 RNAi #1. Transfected cells were scored for FOXO4 localization. **D**, Pin1 inhibits p27^{Kip1} expression through FOXO4. HEK293T cells were cotransfected with pBabePuro, EV, HA-FOXO4, HA-FOXO4-7S/TA, and/or Flag-Pin1. Cells were selected with puromycin for 48 h.

previously uncharacterized mechanism of Pin1 substrate activity regulation.

Previously, we have shown that $p27^{kip1}$ is a key transcriptional FOXO target gene in the regulation of cell cycle arrest and quiescence (10, 12), a notion that was recently supported by *in vivo* experiments (8). To maintain cell cycle arrest, FOXOs increase the cellular antioxidant capacity by up-regulating genes like MnSOD and catalase (9). In turn, increased cellular oxidative stress activates FOXOs, thereby creating a feedback loop that can prevent excessive cellular oxidative stress. Recent studies on conditional FoxO1/3/4 knockout mice underscore the critical importance of oxidative stress management by FOXOs in the hematopoietic stem cell compartment in keeping stem cells quiescent (35). Deletion of all three FOXO genes in hematopoietic stem cells results in stem cell depletion due to increased proliferation and concomitant increased intracellular levels of oxidative stress. Thus, FOXOs are important players in cellular oxidative stress management, and as such, it is of importance to understand how cellular oxidative stress impinges on FOXO. Under conditions of cellular stress, FOXO is phosphorylated, and here, we identified several novel sites of phosphorylation, which we show are involved in Pin1 binding after hydrogen peroxide treatment. Therefore, regulation of FOXO activity through Pin1 could present a novel mechanism of how cells re-enter the cell cycle from a quiescent state especially in response to cellular stress. In this respect, it is interesting to note that embryonic fibroblasts derived from Pin1 knockout mice show defective G_0 - G_1 entry (36). In line with this, we observe that knockdown of Pin1 increases $p27^{kip1}$ expression.

In contrast, overexpression of Pin1 inhibits FOXO4-induced $p27^{kip1}$ expression. $p27^{kip1}$ is a haploinsufficient tumor suppressor gene and decreased $p27^{kip1}$ expression can result in loss of cell cycle control, a hallmark of carcinogenesis (37). Loss of $p27^{kip1}$ is

prevalent in human cancer and correlates with poor survival (33). Unlike loss of, for instance, the tumor suppressor p53, $p27^{kip1}$ function is lost because of a transcriptional or posttranslational down-regulation rather than a genetic defect (11, 13). Moreover, it has been shown that Pin1 is overexpressed in numerous cancer tissues, notably in breast cancer and prostate cancer, and contributes to the malignant transformation of cancer cells (15). The significance of our data, in which Pin1 inhibits $p27^{kip1}$ expression is underscored by our findings in human breast cancers, where we find that loss of $p27^{kip1}$ expression strongly correlates with high Pin1 expression. Thus, in some percentage of tumors, loss of $p27^{kip1}$ may result from increased Pin1 expression inhibiting FOXO function. Reconstitution of nuclear FOXO activity has been shown to arrest both normal and transformed cells in G_1 , to inhibit soft agar growth, and to inhibit xenograft growth in nude mice (38, 39). Disruption of the Pin1-FOXO interaction aimed at increasing the nuclear FOXO pool and restoring $p27^{kip1}$ transcription could therefore represent a potential therapeutic point for intervention.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 3/21/2008; revised 6/3/2008; accepted 6/9/2008.

Grant support: European Commission Transfog Consortium (LSHC-CT-2004-503438; A.B. Brenkman) and the VICI grant (P.L.J. de Keizer and B.M.T. Burgering; NWO).

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We thank Drs. T. Dansen, K.W. Mulder, and G.J. Kops for critical reading of the manuscript.

References

- Accili D, Arden KC. FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. *Cell* 2004;117:421-6.
- Burgering BM, Medema RH. Decisions on life and death: FOXO Forkhead transcription factors are in command when PKB/Akt is off duty. *J Leukoc Biol* 2003;73:689-701.
- Brownawell AM, Kops GJ, Macara IG, Burgering BM. Inhibition of nuclear import by protein kinase B (Akt) regulates the subcellular distribution and activity of the forkhead transcription factor AFX. *Mol Cell Biol* 2001;21:3534-46.
- Wolff S, Dillin A. The trifecta of aging in *Caenorhabditis elegans*. *Exp Gerontol* 2006;41:894-903.
- Kirkwood TB, Austad SN. Why do we age? *Nature* 2000;408:233-8.
- Schumacker PT. Reactive oxygen species in cancer cells: live by the sword, die by the sword. *Cancer Cell* 2006;10:175-6.
- Greer EL, Brunet A. FOXO transcription factors at the interface between longevity and tumor suppression. *Oncogene* 2005;24:7410-25.
- Paik JH, Kollipara R, Chu G, et al. FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. *Cell* 2007;128:309-23.
- van der Horst A, Burgering BM. Stressing the role of FoxO proteins in lifespan and disease. *Nat Rev Mol Cell Biol* 2007;8:440-50.
- Medema RH, Kops GJ, Bos JL, Burgering BM. AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through $p27^{kip1}$. *Nature* 2000;404:782-7.
- Kaldis P. Another piece of the $p27^{kip1}$ puzzle. *Cell* 2007;128:241-4.
- Kops GJ, Medema RH, Glassford J, et al. Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors. *Mol Cell Biol* 2002;22:2025-36.
- Timmerbeul I, Garrett-Engle CM, Kossatz U, et al. Testing the importance of p27 degradation by the SCFskp2 pathway in murine models of lung and colon cancer. *Proc Natl Acad Sci U S A* 2006;103:14009-14.
- Wulf G, Finn G, Suizu F, Lu KP. Phosphorylation-specific prolyl isomerization: is there an underlying theme? *Nat Cell Biol* 2005;7:435-41.
- Ryo A, Liou YC, Lu KP, Wulf G. Prolyl isomerase Pin1: a catalyst for oncogenesis and a potential therapeutic target in cancer. *J Cell Sci* 2003;116:773-83.
- van der Horst A, Vries-Smits AM, Brenkman AB, et al. FOXO4 transcriptional activity is regulated by mono-ubiquitination and USP7/HAUSP. *Nat Cell Biol* 2006;8:1064-73.
- van der Horst A, Tertoolen LG, Vries-Smits LM, Frye RA, Medema RH, Burgering BM. FOXO4 is acetylated upon peroxide stress and deacetylated by the longevity protein hSirt1(SIRT1). *J Biol Chem* 2004;279:28873-9.
- Larsen MR, Thingholm TE, Jensen ON, Roepstorff P, Jorgensen TJ. Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. *Mol Cell Proteomics* 2005;4:873-86.
- Michalides R, van Tinteren H, Balkenende A, et al. Cyclin A is a prognostic indicator in early stage breast cancer with and without tamoxifen treatment. *Br J Cancer* 2002;86:402-8.
- Essers MA, Weijzen S, Vries-Smits AM, et al. FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. *EMBO J* 2004;24:4802-12.
- Pastorino L, Sun A, Lu PJ, et al. The prolyl isomerase Pin1 regulates amyloid precursor protein processing and amyloid- β production. *Nature* 2006;440:528-34.
- Lu PJ, Zhou XZ, Shen M, Lu KP. Function of WW domains as phosphoserine- or phosphothreonine-binding modules. *Science* 1999;283:1325-8.
- Yaffe MB, Schutkowski M, Shen M, et al. Sequence-specific and phosphorylation-dependent proline isomerization: a potential mitotic regulatory mechanism. *Science* 1997;278:1957-60.
- Tran H, Brunet A, Grenier JM, et al. DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein. *Science* 2002;296:530-4.
- Seoane J, Le HV, Shen L, Anderson SA, Massague J. Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. *Cell* 2004;117:211-23.
- Hazzalin CA, Cano E, Cuenda A, Barratt MJ, Cohen P, Mahadevan LC. p38/RK is essential for stress-induced nuclear responses: JNK/SAPKs and c-Jun/ATF-2 phosphorylation are insufficient. *Curr Biol* 1996;6:1028-31.
- Brunet A, Sweeney LB, Sturgill JF, et al. Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. *Science* 2004;303:2011-5.
- Di Fiore PP, Polo S, Hofmann K. When ubiquitin meets ubiquitin receptors: a signalling connection. *Nat Rev Mol Cell Biol* 2003;4:491-7.
- Salmena L, Pandolfi PP. Changing venues for

- tumour suppression: balancing destruction and localization by monoubiquitylation. *Nat Rev Cancer* 2007;7:409–13.
30. Wulf G, Garg P, Liou YC, Iglehart D, Lu KP. Modeling breast cancer *in vivo* and *ex vivo* reveals an essential role of Pin1 in tumorigenesis. *EMBO J* 2004;23:3397–407.
31. Ryo A, Liou YC, Wulf G, Nakamura M, Lee SW, Lu KP. PIN1 is an E2F target gene essential for Neu/Ras-induced transformation of mammary epithelial cells. *Mol Cell Biol* 2002;22:5281–95.
32. Slingerland J, Pagano M. Regulation of the cdk inhibitor p27 and its deregulation in cancer. *J Cell Physiol* 2000;183:10–7.
33. Philipp-Staheli J, Payne SR, Kemp CJ. p27(Kip1): regulation and function of a haploinsufficient tumor suppressor and its misregulation in cancer. *Exp Cell Res* 2001;264:148–68.
34. Wulf GM, Ryo A, Wulf GG, 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.
35. Tothova Z, Kollipara R, Huntly BJ, et al. FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. *Cell* 2007;128:325–39.
36. Fujimori F, Takahashi K, Uchida C, Uchida T. Mice lacking Pin1 develop normally, but are defective in entering cell cycle from G(0) arrest. *Biochem Biophys Res Commun* 1999;265:658–63.
37. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57–70.
38. Ramaswamy S, Nakamura N, Vazquez F, et al. Regulation of G1 progression by the PTEN tumor suppressor protein is linked to inhibition of the phosphatidylinositol 3-kinase/Akt pathway. *Proc Natl Acad Sci U S A* 1999;96:2110–5.
39. Nakamura N, Ramaswamy S, Vazquez F, Signoretti S, Loda M, Sellers WR. Forkhead transcription factors are critical effectors of cell death and cell cycle arrest downstream of PTEN. *Mol Cell Biol* 2000;20:8969–82.

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Cancer Res 2008;68:7597-7605.

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