Published in final edited form as:
Mol Cell. 2011 April 22; 42(2): 147–159. doi:10.1016/j.molcel.2011.03.005.

Death Associated Protein Kinase 1 Phosphorylates Pin1 and Inhibits its Prolyl Isomerase Activity and Cellular Function

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SUMMARY

Pin1 is a phospho-specific prolyl isomerase that regulates numerous key signaling molecules and whose deregulation contributes to disease notably cancer. However, since prolyl isomerases are often believed to be constitutively active, little is known whether and how Pin1 catalytic activity is regulated. Here we identify death associated protein kinase 1 (DAPK1), a known tumor suppressor, as a kinase responsible for phosphorylation of Pin1 on Ser71 in the catalytic active site. Such phosphorylation fully inactivates Pin1 catalytic activity and inhibits its nuclear location. Moreover, DAPK1 inhibits the ability of Pin1 to induce centrosome amplification and cell transformation. Finally, Pin1 pSer71 levels are positively correlated with DAPK1 levels and negatively with centrosome amplification in human breast cancer. Thus, phosphorylation of Pin1 Ser71 by DAPK1 inhibits its catalytic activity and cellular function, providing strong evidence for an essential role of the Pin1 enzymatic activity for its cellular function.

INTRODUCTION

Protein phosphorylation on certain serine or threonine residues preceding proline (pSer/Thr-Pro) is a central signaling mechanism in diverse cellular processes, especially cell growth and proliferation. Interestingly, pSer/Thr-Pro motifs in polypeptides exist in two completely distinct cis and trans conformations, whose conversion is markedly slowed down upon phosphorylation, but specifically catalyzed by the peptidyl-prolyl isomerase (PPIase) Pin1 (Lu and Zhou, 2007). Pin1 is a unique PPIase that binds to and isomerizes specific phosphorylated Ser/Thr-Pro motifs in a subset of proteins, having a profound impact on diverse phosphorylation signaling (Lu and Zhou, 2007).

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The striking substrate specificity of Pin1 towards certain pSer/Thr-Pro bonds results from its unique N-terminal WW domain and C-terminal PPIase domain, which form a “double-check” mechanism (Lu and Zhou, 2007). The WW domain of Pin1 binds only to specific pSer/Thr-Pro-motifs, targeting the Pin1 catalytic domain close to its substrates, where the PPIase domain isomerizes specific pSer/Thr-Pro motifs and induces conformational changes in proteins (Lu and Zhou, 2007). It is these Pin1-induced conformational changes after phosphorylation that have been proposed to control many protein functions, including their catalytic activity levels, phosphorylation status, protein interaction, subcellular location and protein stability because the catalytic inactive Pin1 mutations abolish Pin1 function (Lu and Zhou, 2007). However, since these Pin1 mutations do not naturally occur and it is impossible to directly detect Pin1-catalyzed prolyl isomerization in cells, a major unanswered question remains whether Pin1 catalytic activity is required for its cellular function under physiological conditions (Lu and Zhou, 2007).

Functionally, Pin1 is important in diverse biological processes involving Pro-directed phosphorylation and its deregulation contributes to some pathological conditions, notably aging, cancer and Alzheimer’s disease (Lu and Zhou, 2007; Yeh and Means, 2007). In human cancers, Pin1 is prevalently overexpressed and its overexpression correlates with poor clinical outcome (Ayala et al., 2003; Bao et al., 2004; Ryo et al., 2001; Wulf et al., 2001). Pin1 activates and inactivate close to 30 oncogenes and tumor suppressors, respectively (Liou et al., 2002; Lu and Zhou, 2007; Reineke et al., 2008; Rustighi et al., 2009; Ryo et al., 2001; Stanya et al., 2008; Wulf et al., 2001). Pin1 overexpression disrupts coordination between DNA synthesis and centrosome duplication, causing centrosome amplification, chromosome instability and tumorigenesis in vitro and in vivo (Ryo et al., 2002; Suizu et al., 2006). In contrast, Pin1 inhibition or ablation suppresses cell transformation in vitro (Ryo et al., 2002) and prevents breast cancer in Neu or Ras transgenic mice (Wulf et al., 2004). Thus, Pin1 promotes tumorigenesis by acting on multiple targets in many oncogenic pathways and offers an attractive anticancer target (Lu, 2003; Lu and Zhou, 2007). However, little is known about whether and how Pin1 enzymatic activity is regulated during growth regulation.

Death associated protein kinase 1 (DAPK1) is a Ser/Thr kinase that was originally identified by a functional cloning based on its involvement in interferon-γ induced apoptosis (Bialik and Kimchi, 2006; Deiss et al., 1995). Subsequent studies have shown that DAPK1 functions as a positive mediator of apoptosis induced by various other stimuli (Bialik and Kimchi, 2006; Bovellan et al., 2010; Tu et al., 2010).

DAPK1 is a well-known tumor suppressor. First, DAPK1 loss or inactivation is frequently observed in cancer tissues (Bialik and Kimchi, 2006; Michie et al., 2010; Raval et al., 2007). Second, DAPK1 loss correlates with recurrence and metastasis incidence (Raveh and Kimchi, 2001). Third, DAPK1 has the direct anti-tumorigenic function in the Lewis lung carcinoma system (Inbal et al., 1997). Finally, DAPK1 is capable of suppressing c-Myc- and E2F-induced oncogenic transformation (Raveh et al., 2001). However, only a handful of direct DAPK1 substrates have been identified, and the molecular mechanisms of its tumor suppression are poorly understood.

In this paper, we demonstrate that DAPK1 directly phosphorylates Pin1 on Ser71 in the catalytic active site and inhibits its catalytic activity and cellular function, and provide a direct evidence for an essential role of the catalytic activity for Pin1 function in cells.

RESULTS

Phosphorylation of Pin1 on Ser71 fully inhibits its phospho-specific PPIase activity

Pin1 is mainly phosphorylated in normal tissues and cells, but hypophosphorylated in cancer tissues and cells (Wiegand et al., 2009; Wulf et al., 2001), suggesting a regulatory role for Pin1 phosphorylation in oncogenesis. To test this hypothesis, we first performed mass spectrometric analysis of Pin1 immunoprecipitated from normal breast cells and found phosphorylation of Pin1 on Ser71 in the PPIase domain (Figure 1A).

To examine the effects of this phosphorylation, we phosphorylated Pin1 by PKA in vitro because Ser71 is surrounded by the PKA consensus sequence (RRP**S**^{71}), similar to Ser16 in the WW domain (KRM**S**^{16}) that is phosphorylated by PKA (Lu et al., 2002). Phosphorylation of Pin1 by PKA fully inhibited the PPIase activity toward the pSer-Pro bond (Figure 1B and S1A). PKA also abolished the activity of isolated Pin1 PPIase domain and Pin1^{S16A}, but not Pin1^{S71A} mutant (Figure 1B and S1A). Thus, Ser71 phosphorylation is likely to be responsible for inhibiting PPIase activity.

To confirm this possibility, we treated Pin1 phosphorylated by PKA with commonly used phosphatases, and mutated Ser71 to Ala or Asp. With the exception of PP2C, PP1, PP2A, and PP2B fully restored the PPIase activity of phosphorylated Pin1 in vitro (Figure 1C). Moreover, while Pin1^{S71A} was fully active, Pin1^{S71D} had little PPIase activity toward the pSer-Pro bond, (Figure 1D). Importantly, neither S71A nor S71D mutation had any effects on the basal PPIase activity towards the Ala-Pro bond (Figure 1D). Phosphorylation of Pin1 on Ser71 thus fully inhibits the phospho-specific catalytic activity.

To determine the structural basis of Pin1 inhibitory phosphorylation, we performed structural modeling of pSer71-containing Pin1 based on the crystal structure of Pin1 in complex with a pThr-containing peptide inhibitor (Zhang et al., 2007). In this Pin1 complex, hydrogen bonds between the phosphate group from the pThr-containing peptide forms with the side chain of Arg69 in the PPIase domain are the key determinant of the Pin1 unique specificity toward a phosphorylated substrate (Figure 1E and S1B). Importantly, Ser71 is located at the center of the pSer/Thr-binding pocket of the PPIase domain and the phosphate group from pSer71 likely formed two H-bonds with the side chain of Arg69 (Figure 1E and S1B), the most important residue for conferring phospho-specific PPIase activity (Yaffe et al., 1997; Zhang et al., 2007). Thus, Ser71 phosphorylation likely prevents a phosphorylated substrate from entering the catalytic active site, explaining why such phosphorylation abolishes the phospho-specific, but not basal PPIase activity.

Phosphorylation of Pin1 on Ser71 inhibits its cellular function

To examine whether Pin1 Ser71 phosphorylation may inhibit its function in the cell, we first used Ser71 point mutants to examine the effects of Pin1 Ser71 phosphorylation first on its transcriptional activation towards the well established Pin1 targets cyclin D1, NF-κB or β-catenin (Liou et al., 2002; Ryo et al., 2001; Ryo et al., 2003; Wulf et al., 2001). Co-transfection with Pin1 resulted in a 9.3-fold activation of the cyclin D1 promoter (Figure 1F). Importantly, while Pin1 and Pin1^{S71A} were fully active, neither Pin1^{S71D} nor Pin1^{S71E} was able to activate the promoter of cyclin D1 (Figure 1F), β-catenin (Figure S1C) or NF-κB (Figure S1D).

To examine whether Pin1 Ser71 phosphorylation affects cyclin D1 turnover in vivo, we monitored stability of endogenous and exogenous cyclin D1 proteins in Pin1−/− MEFs, as described (Liou et al., 2002). In contrast to Pin1- or Pin1^{S71A}-reexpressing Pin1−/− cells, both cyclin D1 proteins were highly unstable in Pin1^{S71D}-or Pin1^{S71E}-reexpressing cells,

identical to vector controls (Figure 1G, 1H, S1E and S1F). Thus, phosphorylation of Pin1 on Ser71 abolishes its ability to regulate transcription and protein stability.

DAPK1 interacts with and phosphorylates Pin1 on Ser71 in vitro and in vivo

Given that Pin1 Ser71 phosphorylation inhibits its PPIase activity and cellular function, the next question is what kinases are responsible for such phosphorylation. Although PKA could phosphorylate Pin1 on Ser71 in vitro, its biological relevance in regulating Pin1 function remains unclear because there is no clear consensus linking PKA to cancer or Alzheimer's disease, where Pin1 function is well defined (Lu and Zhou, 2007). We thus performed database mining for Pin1 inhibitory kinases using three major criteria: 1) kinases can phosphorylate Pin1 Ser71 (RRP**S**^{71}S), 2) kinases have a known role in cell growth and transformation and are frequently lost in breast cancer because Pin1 become hypophosphorylated in breast cancer tissues and cell lines, and 3) kinases have some neuronal role because Pin1 inhibition contributes to Alzheimer's disease. We found death associated protein kinase 1 (DAPK1) to be most intriguing. DAPK1 is a Ser/Thr kinase that phosphorylates on RRxS (Bialik et al., 2008; Pike et al., 2008) and a tumor suppressor that is reduced or absent in many tumors (Bialik and Kimchi, 2006; Michie et al., 2010). DAPK1 is also genetically linked to Alzheimer's disease and memory loss (Li et al., 2006; Yukawa et al., 2006).

To examine whether DAPK1 is a possible Pin1 kinase, we first tested whether DAPK1 interacts with Pin1 in cells. FLAG-DAPK1 or its kinase-impaired DAPK1^{K42A} was reciprocally co-immunoprecipitated by anti-HA-Pin1 (Figure 2A and 2B). Moreover, endogenous DAPK1 and Pin1 were formed a complex in normal breast cells (Figure 3J and 3K). Thus, Pin1 forms stable complexes with DAPK1 independently of its kinase activity in cells.

We further examined the Pin1 and DAPK1 interaction in vitro using GST pulldown experiments (Yaffe et al., 1997). GST-Pin1, but not control GST, bound to FLAG-DAPK1, its constitutively active DAPK1$^{\Delta CaM}$ and inactive DAPK1^{K42A} (Figure 2C). This interaction was not affected by arresting cells at mitosis using nocodazole (Figure 2D). To map the domains in Pin1 and DAPK1 important for their interaction, a series of Pin1 (Figure S2A) and DAPK1 (Figure 2I) mutants were generated and used in vitro binding assay. In contrast to all known Pin1-binding proteins, which bind only to the WW domain (Lu and Zhou, 2007; Lu et al., 1999b), DAPK1 bound only to the PPIase domain (Figure 2E and S2A). As for the DAPK1-binding domain in Pin1, the DAPK1 fragment from 637 to 1423 efficiently bound to Pin1 (Figure 2F and 2I), suggesting that cytoskeleton-binding domain (637–847, Cyto) is likely to bind Pin1. Indeed, all DAPK1 mutants lacking this region could not bind to Pin1 (Figure 2G and 2I). Moreover, Pin1 interacted with DAPK-Cyto, as shown by co-immunoprecipitation (Figure 2H) and GST-Pin1 pulldown assay (Figure S2B). Thus, DAPK1 specifically binds to the PPIase domain of Pin1 via its Cyto domain.

Given the DAPK1 and Pin1 specific interaction, a central question is whether DAPK1 phosphorylates Pin1 in vitro and in vivo. Pin1 contains two conserved serine-containing peptides (KRM**S**^{16} and RRP**S**^{71}) that are similar to the DAPK1 consensus phosphorylation site. To determine whether Pin1 is a DAPK1 substrate, we incubated GST-Pin1 with purified recombinant DAPK1 or immunoprecipiated FLAG-DAPK1, using GST-MLC as a positive control. Both Pin1 and MLC were efficiently phosphorylated by DAPK1, but not DAPK1^{K42A} in a time-dependent manner (Figure 3A–C). Importantly, DAPK1 phosphorylated only to the C-terminal PPIase domain, but not the WW domain of Pin1 (Figure 3D). Furthermore, DAPK1 phosphorylated Pin1 and Pin1^{S16A}, but not Pin1^{S71A} or Pin1$^{S16/71A}$ (Figure 3E). DAPK3, a DAPK1-related kinase, has been shown to use a +1 pS/pT as a priming phosphate (Pike et al., 2008). To test whether DAPK1 can use Ser72 as a

priming phosphate, we introduced Ala or Asp into Ser72 in Pin1, but neither of the mutations had any obvious effects on Ser71 phosphorylation (Figure 3F), suggesting that Ser72 phosphorylation unlikely serves as a priming site. The failure of DAPK1 to phosphorylate Pin1 on Ser16 was further confirmed using antibodies specifically against phosphorylated Ser16-Pin1 and unphosphorylated Ser16-Pin1 (Figure 3G). These results indicate that unlike PKA (Figure 1), DAPK1 phosphorylates Pin1 only on Ser71 motif, but not on Ser16 in vitro, which is consistent with finding that DAPK1 binds only to the PPIase domain (Figure 2E and S2A).

To detect Pin1 Ser71 phosphorylation in vivo, we raised antibodies against the pSer71-containing peptide of Pin1. The antibodies specifically recognized pSer71-containing Pin1 that was phosphorylated by DAPK1 in vitro (Figure 3G). To detect Pin1 phosphorylation by DAPK1 in cells, HeLa cells were co-transfected with HA-Pin1 and FLAG-DAPK1 or $DAPK1^{K42A}$ constructs, and immunoprecipitated with anti-HA antibody, followed by immunoblotting analysis with anti-pSer71 antibodies. DAPK1, but not $DAPK1^{K42A}$, phosphorylated Pin1 in vivo (Figure 3H). Moreover, pretreatment of the lysates with a phosphatase completely abolished the recognition of Pin1 by anti-pSer71 antibodies (Figure S3A). To confirm these results, we used lentiviral expression system to establish stable NIH3T3 cells that inducibly overexpressed DAPK1 or its mutants by addition of doxycycline (Figure S3B), as described (Gossen et al., 1995). When $DAPK1^{\Delta CaM}$ was induced, pSer71-specific antibodies robustly recognized Pin1, but not Ser71Ala point mutant (Figure 3I). These results demonstrate the specificity of pSer71-specific antibodies and a critical role of DAPK1 in phosphorylating Pin1 at Ser71 in vivo.

To detect Ser71 phosphorylation of endogenous Pin1 in cells, we analyzed DAPK1 expression and Pin1 Ser71 phosphorylation in a few human normal and breast cancer cell lines because Pin1 is mainly phosphorylated in normal cells (Wiegand et al., 2009; Wulf et al., 2001) and DAPK1 expression is often lost in many cancer cells (Bialik and Kimchi, 2006; Michie et al., 2010; Raval et al., 2007). Indeed, DAPK1 expression was readily detected in normal cell lines MCF10A and HMLE, but not in cancer cell lines, MCF7 and HCC1937 (Figure 3J). Importantly, Ser71 phosphorylation of endogenous Pin1 was detected in normal, but not cancer cell lines (Figure 3J). Moreover, knocking down DAPK1 using two different shRNA constructs fully abolished endogenous Pin1 Ser71 phosphorylation in normal cells (Figure 3K). In contrast, inhibition of PKA using H89 did not reduce Pin1 Ser71 phosphorylation in these cells (Figure S3D), although PKA and DAPK1 phosphorylated Pin1 to similar extents in vitro (Figure S3C). Moreover, activation of PKA using foskolin did not induce Pin1 Ser71 phosphorylation in DAPK1 knockdown cells, although it caused robust phosphorylation of PKA substrates, as detected by PKA phosphoryation-specific antibodies (Figure 3L). Thus, DAPK1 binds to the Pin1 PPIase domain, and also phosphorylates on Ser71 in this domain in vitro and in vivo.

DAPK1 inhibits Pin1 nuclear localization and cellular function

Given the specific phosphorylation of Pin1 on Ser71 by DAPK1, the next question is whether this phosphorylation has any biological significance or pathological consequence. To address this question, we first examined the effects of DAPK1 on the ability of Pin1 to stabilize cyclin D1 protein and to activate transcription. In cells expressing vector control or $DAPK1^{K42A}$, cyclin D1 was rather stable. However, cyclin D1 was much less stable in cells expressing $DAPK1^{\Delta CaM}$ (Figure 4A and 4B), indicating that DAPK1 inhibits the ability of Pin1 to stabilize cyclin D1. Furthermore, DAPK1 or $DAPK1^{\Delta CaM}$ also reduced the ability of Pin1 to activate cyclin D1 promoter (Figure 4C). Interestingly, $DAPK1^{K42A}$ was less potent, but not completely inactive in inhibiting Pin1 activity (Figure 4C), which might be due to $DAPK1^{K42A}$ residual kinase activity (Figure 3B). To further examine this possibility, we determined the ability of DAPK1 Cyto deletion mutants that could not bind Pin1 to

phosphorylate Pin1 and to inhibit cyclin D1 promoter. Cyto deletion completely abolished the ability of DAPK1 or its mutants to phosphorylate Pin1 on Ser71 (Figure S4A) and to inhibit cyclin D1 promoter (Figure 4D). Given that the isolated Cyto domain of DAPK1 had no obvious effect on Pin1 PPIase activity (Figure S4B), these results suggest that the Cyto domain targets DAPK1 to Pin1 to allow DAPK1 to phosphorylate Pin1 on Ser71 to inhibit cellular function.

During the above experiments, we often noted that Pin1 subcellular localization was changed in DAPK1- or DAPK1$^{\Delta CaM}$-overexpressing cells. Furthermore, a recent report shows that Arg68/69 of Pin1 is a major part of nuclear localization signal (NLS) of Pin1 (Lufei and Cao, 2009). Ser71 phosphorylation might disrupt Pin1 nuclear localization due to possible H-bonds between pSer phosphate and the side chain of Arg69 (Figure 1E), suggesting that DAPK1 might affect Pin1 localization. To examine this possibility, we first overexpressed DAPK1 or its mutants in cells, followed by immunoflourescence microscopy. As shown (Lu et al., 1996; Lu et al., 2002), endogenous Pin1 is mainly localized in the nucleus, especially in nuclear speckles in cultured cells (Figure 4E). When DAPK1 or DAPK1$^{\Delta CaM}$ was introduced, however, Pin1 was not concentrated in the nucleus, but distributed both in the nucleus and cytoplasm, although kinase-impaired mutants were much less effective in driving Pin1 to the cytoplasm (Figure 4E). Furthermore, nuclear fractionation experiments showed that nuclear Pin1 was significantly reduced when DAPK1 or DAPK1$^{\Delta CaM}$ was induced, as compared with vector controls (Figure S4C). Moreover, the Cyto domain deletion completely abolished the ability of DAPK1 not only to phosphorylate Pin1 on Ser71 (Figure S4A), but also to inhibit Pin1 nuclear localization (Figure 4E and S4C). These results suggest that Ser71 phosphorylation regulates Pin1 localization. To confirm these results, we generated stable cell lines inducibly expressing Pin1^{S71A} or Pin1^{S71D} (Figure S4D) or inducibly expressing DAPK1 (Figure S3B) with Pin1^{S71A} overexpression. Although Pin1 or Pin1^{S71A} was nuclear, Pin1^{S71D} was largely excluded from the nucleus (Figure 4F). Moreover, Pin1^{S71A} remained nuclear even after DAPK1 was overexpressed (Figure 4F). Thus, Ser71 phosphorylation by DAPK1 also inhibits Pin1 nuclear localization.

DAPK1 inhibits Pin1-induced centrosome amplification and cell transformation

DAPK1 is often downregulated in most human cancers and correlates with recurrence and metastasis (Bialik and Kimchi, 2006; Michie et al., 2010). Furthermore, tight regulation of Pin1 function during the cell cycle in normal cells is critical for the coordination of DNA synthesis and centrosome duplication (Ryo et al., 2002; Suizu et al., 2006). As a result, constitutive Pin1 overexpression disrupts this coordination, leading to centrosome amplification, abnormal spindle formation, chromosome instability and cell transformation in vitro and in mice leading to breast cancer (Suizu et al., 2006). This Pin1 function might be suppressed by Pin1 Ser71 phosphorylation by DAPK1.

To examine this possibility, we first examined the effects of Pin1 phosphorylation on centrosome duplication by transfecting NIH3T3 cells with Pin1, Pin1^{S71A}, Pin1^{S71D} or vector, as described (Suizu et al., 2006). Indeed, more than 60% of Pin1-transfected S-arrested cells contained >2 centrosomes (Figure 5A and 5B). Importantly, although Pin1^{S71A} was also fully active like wild-type Pin1, such centrosome amplification was rarely observed in control or Pin1^{S71D} cells (Figure 5A and 5B). These results suggest that Ser71 phosphorylation of Pin1 inhibits its ability to induce centrosome amplification.

To determine whether DAPK1 inhibits Pin1-drived centrosome amplification, we used retroviral and lentiviral expression systems to generate double stable NIH3T3 cells expressing DAPK1 or its mutants in a tetracycline-inducible manner and expressing Pin1 constitutively (Figure S5). Again, constitutive Pin1 overexpression resulted in centrosome

amplification in ~60% of S arrested cells (Figure 5C and 5D). However, this activity was significantly reduced by co-expression of DAPK1 or DAPK1$^{\Delta CaM}$ (Figure 5C and 5D). Interestingly, while DAPK1^{K42A} has much reduced activity, Cyto domain deletion completely abolishes DAPK inhibitory activity (Figure 5C and 5D). Importantly, the inhibitory effects of DAPK1 on Pin1 were highly specific because they did not occur at all when DAPK1 or its mutants was not induced (Figure 5C and 5D). Thus, Pin1 Ser71 phosphorylation by DAPK1 suppresses Pin1-induced centrosome amplification.

To examine whether Ser71 phosphorylation affects the ability of Pin1 to induce abnormal mitotic spindle during mitosis, NIH3T3 cells stably expressing either control vector, Pin1, Pin1^{S71A} or Pin1^{S71D} were first arrested in S phase by aphidicolin and then released for 24 hours (about one cell cycle), followed by immunostaining with tubulin antibodies to detect the mitotic spindle. As reported (Suizu et al., 2006), ~20% of Pin1-overexpressing cells contained supernumerary centrosomes and displayed multipolar spindle formation and nucleation (Figure 6A and 6B). Importantly, Pin1^{S71A} also induced abnormal spindle formation like wild-type Pin1, but Pin1^{S71D} was completely inactive (Figure 6A and 6B). Thus, Ser71 phosphorylation of Pin1 inhibits Pin1-induced abnormal chromosome separation.

To examine whether Ser71 phosphorylation also have any effects on Pin1-inducd transforming phenotypes, NIH3T3 cells stably expressing Pin1, its Ser71 mutants or control vector were seeded on plastic plates or in soft agar. Cells expressing Pin1 or Pin1^{S71A}, but not Pin1^{S71D}, had a significantly higher number of foci on plastic plates (Figure 6C and 6D) and colonies in soft agar (Figure 6E and 6F). These results together demonstrate that Pin1 Ser71 phosphorylation suppresses its ability to induce centrosome amplification, chromosome instability and cell transformation.

Given the ability of DAPK1 to phosphorylate and inhibit Pin1-induced cell transformation, we wondered whether Pin1 inhibition contributes to tumor suppress activity of DAPK1. Since a known mechanism of DAPK1 tumor suppression is to inhibit cancer cell migration (Kuo et al., 2006) and Pin1 knockdown suppresses cancer cell migration (Ryo et al., 2005), we examined whether Pin1 affects the antimigratory function of DAPK1 in breast cancer cells. As compared with vector control, knockdown of DAPK1 or Pin1 in breast cancer cells significantly increased or inhibited cell migration, respectively, as assayed by wound healing (Figure 6G, 6H and S6), consistent with the previous results. Importantly, Pin1 knockdown greatly attenuated increased cell migration in DAPK1 knockdown cells (Figure 6G, 6H and S6). Thus, Pin1 inhibition likely contributes to tumor suppress function of DAPK1.

Pin1 Ser71 phosphorylation correlates positively with DAPK1 levels, but negatively with centrosome amplification in human breast cancer

DAPK1 is a tumor suppressor commonly downregulated in human malignancies including human breast cancer cells (Bialik and Kimchi, 2006; Michie et al., 2010) and Pin1 plays a major role in the development of breast cancer, with Pin1 overexpression correlating with centrosome amplification in human breast cancer tissues (Suizu et al., 2006; Wulf et al., 2004; Wulf et al., 2001). Given that DAPK1 inhibits Pin1 function through Ser71 phosphorylation and that Pin1 overexpression induces centrosome amplification, chromosome instability and cell transformation, we asked whether Pin1 Ser71 phosphorylation correlates with DAPK1 levels or centrosome amplification in human normal and breast cancer tissues.

To investigate the relationship between DAPK1 and Pin1 Ser71 phosphorylation in human tissues, we performed immunohistochemistry using antibodies specifically against pSer71-Pin1 and DAPK1 on serial sections of tissue microarrays from human normal and breast

cancer tissues. Thirteen out of fourteen normal breast samples that contained low DAPK1 also had a low expression of Pin1 Ser71 phosphorylation (Figure 7A and 7B). In contrast, pSer71 Pin1 levels in 21 out of 23 normal samples that contained high DAPK1 levels were high, indicative of inactive Pin1 (Figure 7A and 7B). Interestingly, most tumor containing low levels of DAPK1 also had marked reduced levels of pSer71 of Pin1 (Figure 7C and 7D). In contrast, most tumors containing high DAPK1 had high pSer71 Pin1 (Figure 7C and 7D). There was a significant correlation between DAPK1 expression and pSer71 Pin1 levels in tumors, as determined by the Spearman rank correlation test ($P<0.001$). These results indicate a positive correlation between DAPK1 expression and Pin1 pSer71 levels in human tissues.

To examine the relationship between Pin1 Ser71 phosphorylation and centrosome amplification in human breast cancer tissues, we performed immunohistochemistry using antibodies specifically against pSer71-Pin1 and γ-tubulin on serial sections of tissue microarrays from human breast cancer tissues. More than 30% cells contained more than 2 centrosomes per cell in most tumors containing low pSer71-Pin1 (29 out of 31) (Figure 7C and 7E). However, in most of the tumors that contained very high level of pSer71 Pin1 (21 out of 26), less than 10% cells contained more than 2 centrosomes per cell (Figure 7C and 7E). These results in human breast cancer tissues are consistent with the above findings that phosphorylation of Pin1 on Ser71 inhibits its ability to induce centrosome amplifications and further support the notion that the tumor suppressor DAPK1 phosphorylates Pin1 on Ser71 and inhibits its catalytic activity and oncogenic function.

DISCUSSION

Pin1 regulates close to 30 oncogenes and tumor suppressors, but little is known about the regulation of its catalytic activity in oncogenesis. Here, we found that phosphorylation of Pin1 on Ser71 inactivates its phospho-specific PPIase activity, nuclear localization and cellular function and also identified DAPK1 as an enzyme that is responsible for such phosphorylation in vitro and in vivo. Importantly, Ser71 phosphorylation by DAPK1 inhibits the ability of Pin1 to activate oncogenic transcription factors, increase cyclin D1 protein stability and induce centrosome amplification, chromosome instability and cell transformation. Moreover, Pin1 Ser71 phosphorylation correlates positively with DAPK1 expression, but inversely with centrosome amplification in breast cancer tissues. These results demonstrate that Pin1 phosphorylation by DAPK1 inhibits its catalytic activity and cellular function and provide the strongest evidence yet for an essential role of the catalytic activity for Pin1 function in vivo.

Pin1 contains a WW domain and a PPIase domain, which binds to and isomerize specific pSer/Thr-Pro motifs, respectively (Lu and Zhou, 2007). The essential role of the WW domain for Pin1 function in vivo has been demonstrated by multiple approaches, including inactivating phosphorylation of the WW domain on Ser16 (Lu and Zhou, 2007; Lu et al., 2002; Lu et al., 1999b). However, it has been much more challenging to document the significance of the Pin1 phospho-specific PPIase activity because such activity can only be demonstrated in small peptides in vitro (Pastorino et al., 2006; Yaffe et al., 1997). The only approach that has widely been used to address the role of the Pin1 catalytic activity is to use Pin1 mutations in the PPIase domain that inactivate its catalytic activity (Lu and Zhou, 2007; Winkler et al., 2000; Zhou et al., 2000). However, these mutations are not naturally occurring. Moreover, so far there is not good tool available to directly detect Pin1-catalyzed prolyl isomerization in a protein in vitro or in vivo. Our findings provide strong evidence for a crucial role of the catalytic activity for Pin1 function.

Emerging evidence suggests that Pin1 function may be regulated at multiple levels. With a few exceptions such as neurons (Liou et al., 2003; Lu et al., 1999a), Pin1 expression is generally correlated with cell proliferation in normal human tissues, but further upregulated in many human cancer tissues (Bao et al., 2004; Liou et al., 2002; Ryo et al., 2001; Wulf et al., 2001). Indeed, Pin1 expression is subject to E2F-mediated transcriptional regulation in response to growth factors (Ryo et al., 2002). In addition, Pin1 is one of the genes that are most drastically suppressed by up-regulation of BRCA1 (MacLachlan et al., 2000). Pin1 is also subject to post-translational modifications, including Ser16 phosphorylation, which abolishes the ability of Pin1 to interact with its substrates (Lu et al., 2002) and Ser65 by PLK, which appears to increase Pin1 protein stability (Eckerdt et al., 2005). However, little is known about the regulation of Pin1 phospho-specific PPIase activity.

We have now demonstrated that Pin1 catalytic activity is regulated in vitro and in vivo. First, Pin1 is phosphorylated on Ser71 in the catalytic domain in vitro and in vivo. Second, this phosphorylation fully inactivates Pin1 phospho-specific PPIase activity without affecting the basal catalytic activity, likely due to that phosphorylation of Ser71 would prevent a phosphorylated substrate from entering the catalytic active site. Third, Ser71 phospho-mimicking mutations (S71D, or S71E) inactivate Pin1 phospho-specific PPIase activity in vitro, and also inhibit Pin1 nuclear localization and cellular function in cells. Fourth, unlike all known Pin1-binding proteins, DAPK1 interacts uniquely with the PPIase domain and importantly, DAPK1 phosphorylates Pin1 on Ser71 in vitro and in vivo. Fifth, inducible DAPK1 expression fully inhibits the ability of Pin1 to activate transcription factors, stabilize proteins and induce centrosome amplification, chromosome instability and cell transformation. In contrast, DAPK1 mutants that fail to phosphorylate Pin1 are completely inactive in inhibiting Pin1 nuclear localization and cellular function. Finally, Pin1 Ser71 levels positively correlated with DAPK1 levels, but negatively with centrosome amplification in breast cancer tissues. Thus, Ser71 phosphorylation by DAPK1 fully inhibits Pin1 catalytic activity, nuclear localization and cellular function.

These findings offer an exciting mechanism to control Pin1 catalytic activity in human disease. The most notable example is cancer, where Pin1 and DAPK1 have been shown to have many opposite functions in tumorigenesis, although they have not been studied together before. For example, Pin1 is upregulated (Bao et al., 2004; Ryo et al., 2001; Wulf et al., 2001), but DAPK1 is downregulated in many human cancers (Bialik and Kimchi, 2006; Michie et al., 2010; Raval et al., 2007; Raveh et al., 2001). Furthermore, Pin1 upregulation, or DAPK1 downregulation strongly correlates with tumor recurrence and metastasis incidence (Ayala et al., 2003; Raveh and Kimchi, 2001). Moreover, reducing Pin1 overexpression or restoring DAPK1 downregulation in cancer cells effectively suppresses tumorigenic phenotypes (Inbal et al., 1997; Wulf et al., 2004). We have now demonstrated that DAPK1 directly inhibits Pin1 catalytic activity and nuclear translocation via phosphorylation. Furthermore, DAPK1 and Pin1 Ser71 phosphorylation are readily detected in human normal, but not cancer breast cells in vitro. Moreover, DAPK1 effectively neutralizes the ability of Pin1 to transactivate multiple oncogenes and to induce chromosome instability and cell transformation. Importantly, Pin1 effectively suppresses the antimigratory effects of DAPK1, a mechanism through which DAPK1 suppresses tumors (Kuo et al., 2006). Finally, Pin1 Ser71 levels are positively correlated with DAPK1 levels but negatively with centrosome amplification in human breast cancer tissues. Thus, DAPK1 suppresses Pin1 oncogenic function. Given that Pin1 activates and inactivate numerous oncogenes and tumor suppressors, respectively, this connection between DAPK1 and Pin1 provides insight into tumorigenesis and might eventually lead to more effective therapeutic strategies for cancer.

Given the importance of Pin1 in diverse cell functions, DAPK1 likely regulates Pin1 function in other cellular processes and/or human diseases. Another notable example is Alzheimer's disease, where Pin1 is pivotal for protecting against age-dependent neurodegeneration by regulating many phosphoproteins such as well known proteins tau and APP (Liou et al., 2003; Lu and Zhou, 2007; Lu et al., 1999a; Pastorino et al., 2006; Zhou et al., 2000). Furthermore, Pin1 knockout or inhibition has been shown to contribute to the development of tangle and plaque pathologies, and neurodegeneration both in mouse models as well as human Alzheimer's brains (Liou et al., 2003; Lu and Zhou, 2007; Pastorino et al., 2006). This is especially exciting because DAPK1 is highly expressed in the brain (Bialik and Kimchi, 2006) and its kinase activity-deficient mice are more efficient learners and have better spatial memory to wild-type mice (Yukawa et al., 2006). Moreover, DAPK1 polymorphisms are associated with susceptibility to Alzheimer's disease (Li et al., 2006). These results suggest that aberrant DAPK1 activation might contribute to age-dependent neurodegeneration in Alzheimer's disease by inhibiting Pin1 function.

EXPERIMENTAL PROCEDURES

Plasmids

DAPK1 constructs with C-terminal Flag tag were described (Chen et al., 2005), as were Pin1 constructs (Lu et al., 2002). pRK5-Flag DAPK1 deletion and DAPK1$^{\Delta cyto}$ mutants were generated by PCR. Inducible lentiviral DAPK1 constructs were subcloned to pTight vector. Pin1 S71 mutants were subcloned to pLenti6/V5-GW/lacZ vector.

PPIase assay

Pin1 and its variants were phosphorylated to completion by PKA catalytic subunit (New England Biolabs), as described (Lu et al., 2002). Subsequently, the PPIase activity of each sample was determined using the protease free or chymotrypsin coupled PPIase activity assay with the substrate Suc-Ala-pSer-Pro-Phe-pNA, Suc-Ala-Glu-Pro-Phe-pNA or Suc-Ala-Ala-Pro-Phe-pNA (50 μM) in 35 mM HEPES pH 7.8 at 10°C, as described previously (Yaffe et al., 1997). The activity of Pin1 treated with phosphatases is compared to the activity of the unphosphorylated Pin1, which was used as a control (100%) in all the experiments.

Protein stability assay

For protein stability assay, cells were transfected stably or transiently with expression plasmids as indicated. Cycloheximide (100 μg/ml) was added to the media to block new protein synthesis. Cells were harvested at each time points, and total lysates were analyzed by immunoblot with anti-cyclin D1, -Pin1, -tubulin, or -β-actin antibodies. The blots were scanned and semi-quantitated by using the software NIH image 1.6.2, as described previously (Lee et al., 2009). The results from at least three independent experiments are plotted such that the protein levels at 0 h time point is set at 1.

Kinase assay

Kinase assays were performed, as described previously (Chen et al., 2005). Briefly, HeLa cells were transfected with FLAG-DAPK1 or its mutants, followed by immunoprecipitation with anti-FLAG antibodies. Immunoprecipitates were incubated with His-Pin1 in the kinase reaction buffer at 25°C for 30 min, detected by autoradiography. Purified recombinant DAPK1 or PKA was incubated with GST-Pin1 or MLC in the kinase reaction buffer at 25°C for 30 min, detected by autoradiography.

GST pulldown, immunoprecipitation and immunoblotting

GST pulldown, immunoprecipitation, and immunoblotting analyses were performed as described previously (Lee et al., 2009; Lu et al., 1999a). Briefly, relevant proteins were expressed in 293T or HeLa cells by transient transfection, followed by lysis or dilution in a buffer. The cellular supernatants were incubated with 1 μM GST or GST fusion proteins or specific antibodies for 1 hr at 4°C and 15 μl of glutathione agarose beads or protein A agarose were then added, followed by further incubation for 2 h at 4°C. The precipitated proteins were washed 4–6 times in the same buffer and subjected to immunoblotting analysis.

Immunohistochemistry and Immunostaining analyses

Formalin-fixed and paraffin-embedded tissue microarrays of human breast cancer tissue were purchased from Imgenex. Immunohistochemical staining for Pin1 was performed as described previously (Ryo et al., 2003). For immunofluorescent analysis of centrosome staining, the slides were dewaxed in xylene and rehydrated through a series of ethanol to water gradients and 0.3% H_2O_2 in water was then used to block endogenous peroxidase. The sections were further blocked with 5% normal goat sera for 1 hr at room temperature, followed by incubation with anti-γ-tubulin (Sigma) at 1: 800 dilution overnight at 4 °C. After extensive wash with PBS, slides were incubated with Alexa546-labeled anti-mouse IgG (Molecular Probe, Eugene, OR) diluted 1:500 for 30 min at room temperature. After washing with PBS, the nuclei were stained with DAPI for 10 min at room temperature. The ratio of centrosomes to nuclei was scored in each samples from >200 nuclei under a fluorescent microscope. To detect centrosomes in cultured cells, cells were fixed with 3.7% buffered formaldehyde for 5 min and stained with anti-γ-tubulin (Sigma), as described (Suizu et al., 2006).

Analysis of centrosome duplication during S phase

Centrosome duplication assays in NIH3T3 cells were performed, as described previously (Suizu et al., 2006). Briefly, cells were arrested in G1/S phase by adding aphidicolin at a final concentration of 10 μg/ml for 24 hr. Cells were then fixed with cold ethanol for 5 min and stained for centrosomes with anti-γ-tubulin antibodies and analyzed by fluorescent microscopy

Soft agar colony formation assay

Soft agar assays were done by seeding cells at a density of 10^3 in 60-mm culture dishes containing 0.3% top low-melt agarose −0.5% bottom low-melt agarose, as described (Ryo et al., 2002). Cells were fed every 4 days and colonies were counted and measured after 3 weeks.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

T.H.L and C-H.C. are the recipients of NIH Pathway to Independence (PI) Award (K99AG033104) and DOD Breast Cancer Research Program Postdoctoral Award (W81XWH09-1-0481), respectively. The work was supported by NIH grants R01GM56230 and R01AG017870 to K.P.L.

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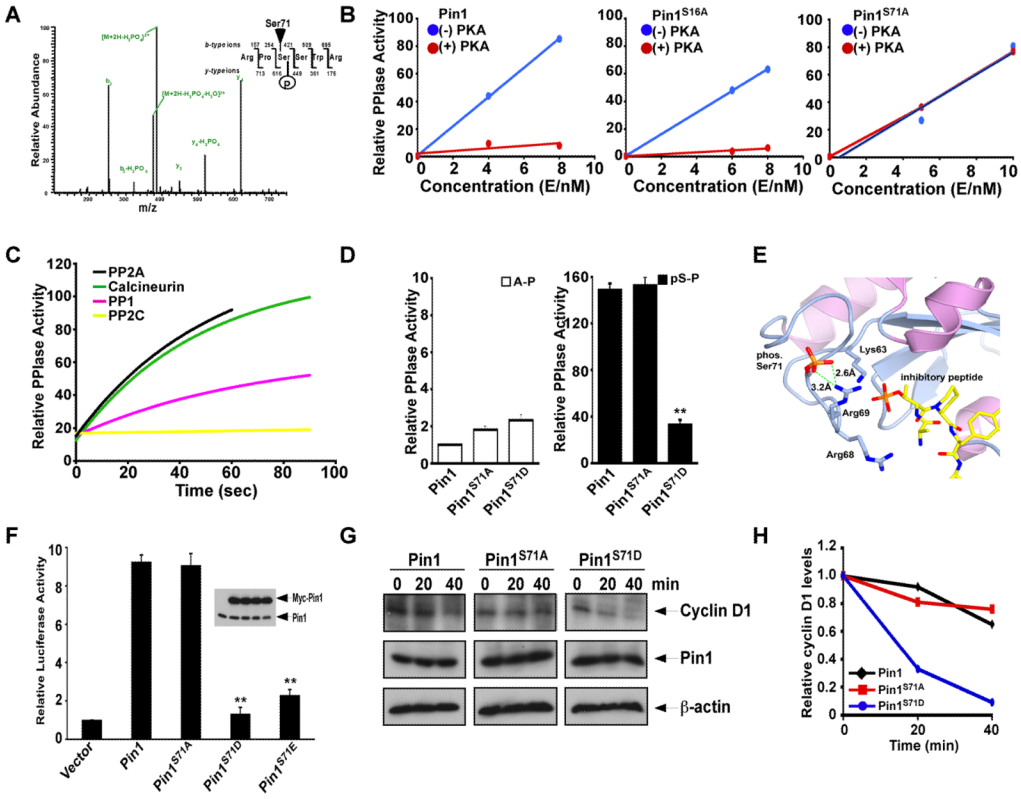


Figure 1. Phosphorylation of Pin1 on Ser71 inhibits its PPIase activity and cellular function

(A) Identification of Ser71 phosphorylation in Pin1 immunoprecipitated from normal breast cells.

(B) Pin1 Ser71 phosphorylation inhibits its PPIase activity. Pin1 and its mutants were phosphorylated by PKA catalytic subunit and then determined their PPIase activities.

(C) Pin1 pSer71 dephosphorylation fully restores its PPIase activity. Dephosphorylation of phosphorylated Pin1 was performed with the Ser/Thr phosphatases PP1, PP2A, PP2B or PP2C.

(D) Pin1-S71D, but not Pin1-S71A mutation abolishes the pSer-Pro specific PPIase activity, without affecting basal Ala-Pro PPIase activity. Results shown are mean ± SEM, n = 3. **, p <0.002.

(E) Structure model of Ser71 inhibitory phosphorylation, with a full view in Figure S1B.

(F) Pin1-S71D and Pin1-S71E mutations abolish the ability to activate cyclin D1 promoter in cells. HeLa cells were co-transfected with Pin1, its mutants, reporter constructs or vector control, followed by assaying the luciferase activity. Results shown are mean ± SEM, n = 3. **, p <0.001.

(G and H) Pin1-S71D mutation abolishes the ability to stabilize endogenous cyclin D1 protein in cells. Pin1−/− MEFs expressing Pin1 or its S71 mutants were treated with cycloheximide, followed by immunoblot with anti-cyclin D1, Pin1 or β-actin antibodies (G). Cyclin D1 levels were semi- quantitated using β-actin as a loading control and the relative levels at time 0 defined as 1 (H).

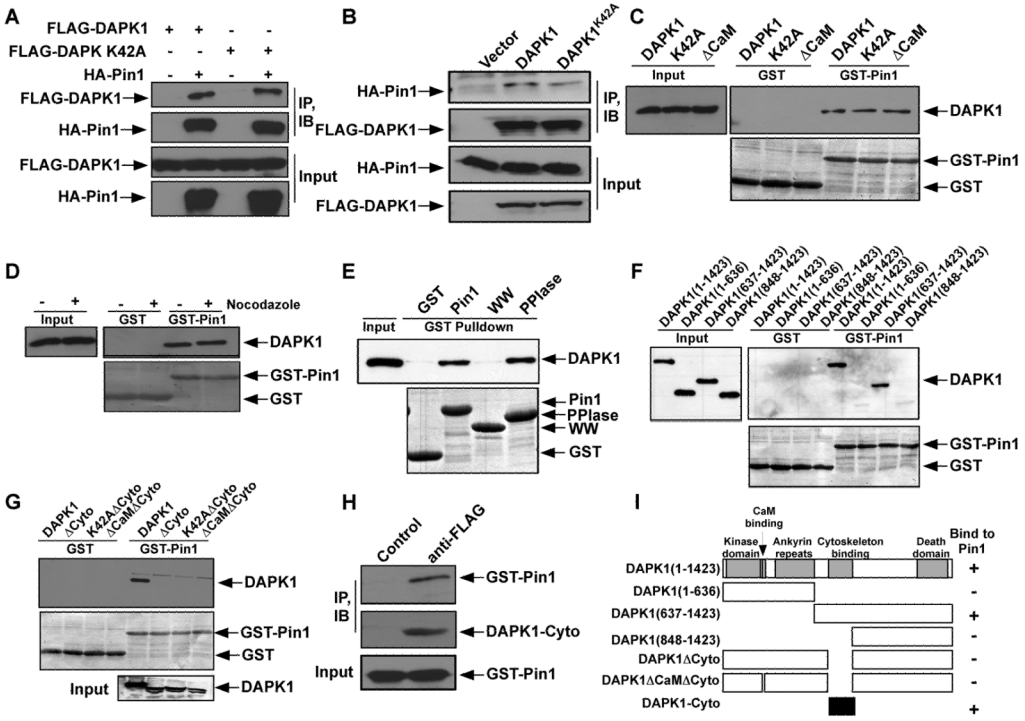


Figure 2. DAPK1 interacts with Pin1 in vitro and in vivo

(A and B) Co-immunoprecipitation of expressed DAPK1 and Pin1 in vivo. 293T cells expressing FLAG-DAPK1 or K42A mutant and HA-Pin1 were subjected to immunoprecipitation with anti-HA (A) or FLAG (B) antibodies, followed by immunoblot with anti-FLAG or HA antibodies.

(C) DAPK1 and Pin1 interaction in vitro. 293T cells expressing DAPK1 or its mutants were subjected to GST pulldown assay.

(D) Cell cycle-independent DAPK1 and Pin1 interaction. 293T cells expressing DAPK1 were incubated with or without nocodazole before GST pulldown assay.

(E) DAPK1 binding to the PPIase domain, but not WW domain of Pin1. 293T cells expressing DAPK1 were subjected to GST pulldown assay.

(F) Pin1 binding to the Cyto domain of DAPK1. 293T cells expressing DAPK1 or its truncated mutants were subjected to GST pulldown assay.

(G) A deletion of the Cyto domain of DAPK1 fails to bind to Pin1. 293T cells expressing DAPK1 or its Cyto deletion mutants were subjected to GST pulldown assay.

(H) The Cyto domain of DAPK1 is sufficient to interact with Pin1. 293T cells expressing DAPK1-Cyto were subjected to GST pulldown assay.

(I) Schematic representation of full length DAPK1 and its truncated mutants.

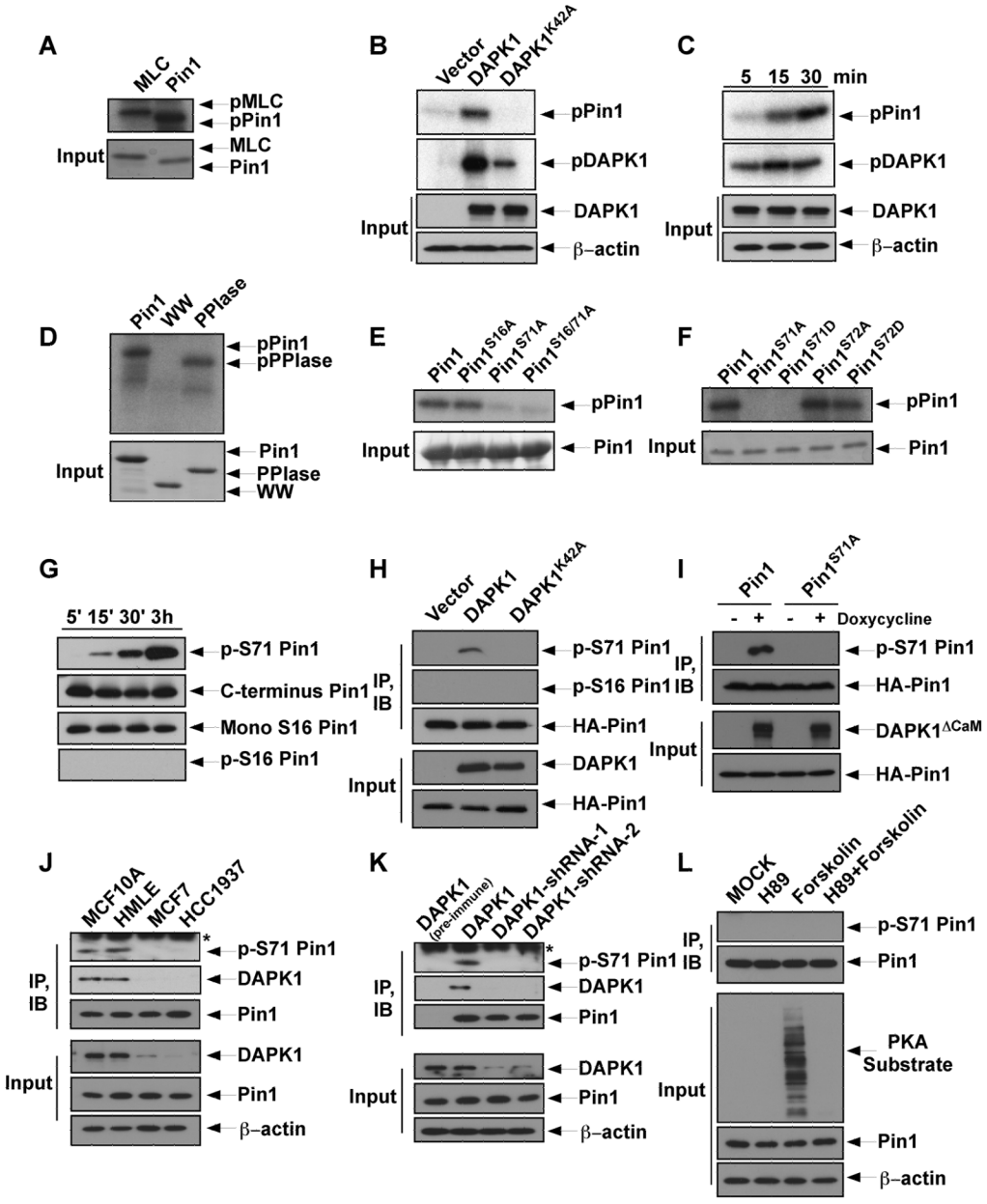


Figure 3. DAPK1 phosphorylates Pin1 on Ser71 in vitro and in vivo

(A) DAPK1 phosphorylates Pin1 in vitro. Purified recombinant DAPK1 was incubated with GST Pin1 or MLC and ^{32}P-ATP, detected by autoradiography.

(B) Failure of kinase-impaired DAPK1 mutant to phosphorylate Pin1. HeLa cells expressing DAPK1, DAPK1^{K42A} or vector control were immunoprecipitated with anti-FLAG antibodies and then incubated with His-Pin1 and ^{32}P-ATP, detected by autoradiography.

(C) Time-dependent phosphorylation of Pin1 by DAPK1.

(D) DAPK1 phosphorylates Pin1 PPIase domain, but not WW domain. Purified recombinant DAPK1 was incubated with GST-Pin1, WW or PPIase domain and ^{32}P-ATP, detected by autoradiography.

(E) DAPK1 phosphorylates Pin1 on Ser71, but not Ser16 in vitro.

(F) DAPK1 does not phosphorylate Pin1 on Ser72 in vitro.

(G) DAPK1 phosphorylates Pin1 on Ser71 in vitro. Purified recombinant DAPK1 was incubated with GST-Pin1, followed by immunoblotting with anti-pSer71, C-terminus, Ser16 or pSer16 Pin1 antibodies.
(H) DAPK1 phosphorylates Pin1 on Ser71 in vivo. HeLa cell expressing FLAG-DAPK1 or K42A mutant and HA-Pin1 were subjected to immunoprecipitation with anti-HA, followed by immunoblotting with anti-pSer71 Pin1 antibodies.
(I) Failure of DAPK1 phosphorylation to phosphorylate Pin1 S71A mutant. HeLa cells expressing FLAG-DAPK1$^{\Delta CaM}$ and HA-Pin1 or its mutant S71A were induced FLAG-DAPK1$^{\Delta CaM}$ expression using doxycycline and then subjected to immunoprecipitation with anti-HA antibodies, followed by immunoblot with anti-pSer71 Pin1 antibodies.
(J) Endogenous phosphorylation of Pin1 on Ser71 by DAPK1. Various breast cell lines were subjected to immunoprecipitation with anti-Pin1, followed by immunoblot with anti-pSer71 Pin1 or DAPK1 antibodies. * Immunoglobulin light chain.
(K) DAPK1 knockdown abolishes endogenous Pin1 on Ser71 phosphorylation. MCF10A cells stably expressing DAPK1 shRNAs were subjected to immunoprecipitation with pre-immune serum or anti-Pin1, followed by immunoblot with anti-pSer71 Pin1 or DAPK1 antibodies.
(L) PKA does not phosphorylate Pin1 on Ser71 in vivo. DAPK1 shRNA MCF10A cells were treated with H89, forskolin or both, followed by immunoprecipitation with anti-Pin1 and immunoblot with anti-pSer71 Pin1 antibodies.

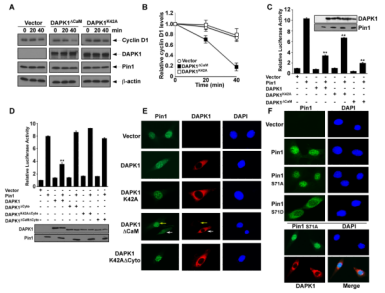


Figure 4. DAPK1 inhibits Pin1 nuclear localization and cellular function

(A and B) DAPK1 inhibits the ability of Pin1 to stabilize cyclin D1 protein. Pin1−/− MEFs expressing Pin1 and DAPK1$^{\Delta CaM}$, DAPK1^{K42A} or vector control were treated with cycloheximide, followed by immunoblot analysis (A) and semi-quantitated using β-actin (B). Results shown are mean ± SEM, n = 3.

(C) DAPK1 inhibits the ability of Pin1 to activate cyclin D1 promoter in cells. HeLa cells were transiently transfected with Pin1, reporter constructs and DAPK1, its mutants or vector control, followed by assaying the luciferase activity. Results shown are mean ± SEM, n = 3. **, p <0.01.

(D) DAPK1 and Pin1 binding is required for DAPK1 to inhibit Pin1-mediated cyclin D1 activation. Results shown are mean ± SEM, n = 3. **, p <0.01.

(E) DAPK1 prevents Pin1 nuclear localization. Cells expressing DAPK1, its mutants or control were immunostained with anti-Pin1 (green), anti-DAPK1 (red) antibodies and DAPI (blue). White and yellow arrows point to DAPK1$^{\Delta CaM}$-transfected and non-transfected cells, respectively.

(F) Ser71 phosphorylation regulates Pin1 localization. Cells inducibly expressing Pin1 or its Ser71 mutants or inducibly expressing DAPK1 with Pin1^{S71A} overexpression were stained with anti-HA (green) or FLAG (red) antibodies and DAPI (blue).

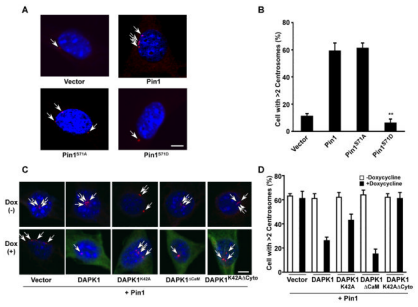


Figure 5. DAPK1 suppresses Pin1-induced centrosome amplification

(A and B) S71D, but not S71A abolishes the ability of Pin1 to induce centrosome amplification. NIH3T3 cells stably expressing Pin1, its Ser71 mutants or control were arrested at the G1/S boundary by aphidicolin. Cells were stained with anti-γ-tubulin antibody (red) and DAPI (blue). Bar, 10 μm (A). Cells containing >2 centrosomes were scored in 300 transfected cells (B). Results shown are mean ± SEM, n = 3. **, p <0.001. **(**C and D**)** DAPK1 inhibits Pin1-induced centrosome amplification. Stable cell lines were arrested at the G1/S boundary by aphidicolin in the presence or absence of doxycycline. Cells were stained with anti-γ-tubulin antibody (red) and DAPI (blue). Bar, 10 μm (C). Cells containing >2 centrosomes were scored in 300 transfected cells (D). Results shown are mean ± SEM, n = 3.

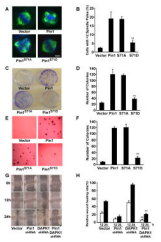
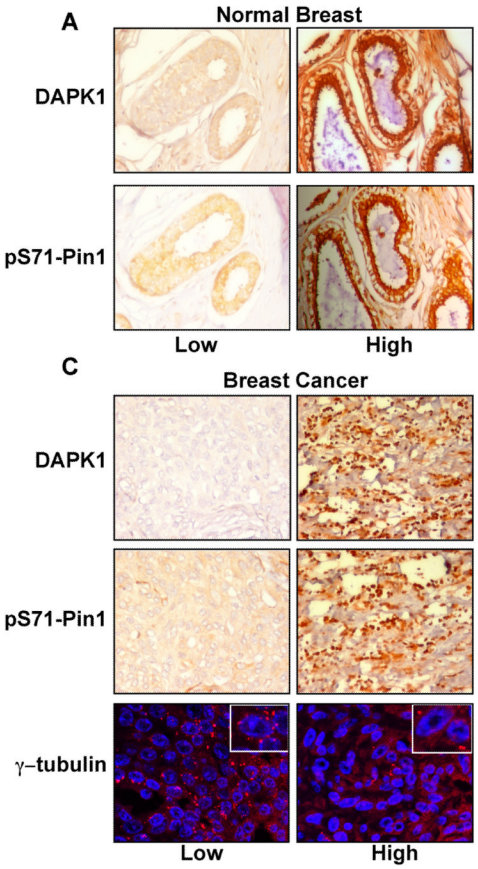


Figure 6. Phosphorylation of Pin1 on Ser71 inhibits Pin1-induced chromosome instability and cell transformation

(A and B) Ser71 phosphorylation suppresses the ability of Pin1 to induce the formation of multipolar spindles. NIH3T3 cells stably expressing Pin1 or its Ser71 mutants were released from the aphidicolin block for 24 hr, then fixed and co-stained with anti-α-tubulin (green) and anti-γ-tubulin (red) antibodies followed by DAPI staining (blue). Representative mitotic cells with bipolar or multipolar spindle poles were shown. Bar, 10 μm (A). Cells containing >2 mitotic spindle poles were scored in 200 mitotic cells (B). Results shown are mean ± SEM, n = 3. **, p <0.01.

(C–F) Ser71 phosphorylation abrogates the ability of Pin1 to induce anchorage-dependent cell growth on plastic plates (C and D) and soft agar (E and F). Stable NIH3T3 cells were released from the aphidicolin block and seeded on plastic plates (C) or in soft agar (E) for 3 weeks, followed by crystal violet staining (C) or P-iodonitrotetrazolium violet staining (E). The number of colonies formed per 1000 cells was scored (D, F). Colony numbers are mean ± SEM, n = 3.**, p <0.002.

(G and H) Effect of DAPK1 and Pin1 on wound-healing migration. MDA-MB-231 cells expressing Pin1 shRNA, DAPK1 shRNA or both were assayed for wound-healing migration, and cell migration into wounds was monitored by time-lapse microscopy. Still images were captured at the indicated times after wounding. Bar, 200 μm. Results shown are mean ± SEM, n = 3. **, p <0.01.



B

DAPK1 staining (Normal)

pS71-Pin1	Low level (n=14)	High level (n=23)	Total
Low	13 (35%)	2 (5%)	15 (40%)
High	1 (3%)	21 (57%)	22 (60%)
Total	14 (38%)	23 (62%)	37 (100%)

P<0.001

D

DAPK1 staining (Cancer)

pS71-Pin1	Low level (n=40)	High level (n=38)	Total
Low	31 (40%)	10 (13%)	41 (53%)
High	9 (11%)	28 (36%)	37 (47%)
Total	40 (51%)	38 (49%)	78 (100%)

P<0.001

E

Centrosome staining (Cancer)

pS71-Pin1	Cells with >2 Centrosomes (%) <10	10-30	>30	Total
Low	5 (6%)	7 (9%)	29 (37%)	41 (53%)
High	21 (27%)	14 (18%)	2 (3%)	37 (47%)
Total	26 (33%)	21 (27%)	31 (40%)	78 (100%)

P<0.001

Figure 7. Pin1 S71 phosphorylation positively correlate with DAPK1 levels, but negatively with centrosome amplification in human breast cancer

(A and B) Serial sections of tissue arrays of 37 normal breast specimen were subjected to immunohistochemistry using anti-DAPK1 (upper panel) or anti-pSer71 Pin1 antibodies (low panel), and visualized by the DAB staining (A). In each sample, DAPK1 expression and pSer71 levels were semi-quantified in a double-blind manner as high or low according to the standards presented in (**A**) and summarized in (**B**). Their correlation was analyzed by Spearman rank correlation test (P<0.001).

(C–E) Serial sections of tissue arrays of 78 breast cancer specimen were subjected to immunohistochemistry using anti-DAPK1 (upper panel), anti-pSer71 Pin1 antibodies (middle panel) and visualized by the DAB staining (brown) and different sections of the same tissues immunostained with anti-γ-tubulin antibodies (low panel) and visualized by Alexa488-conjugated secondary antibody (red) along with DAPI (blue) (C). In each sample, DAPK1 expression and pSer71 levels were semi-quantified in a double-blind manner as high or low according to the standards presented in (C) and summarized in (D). The ratio of centrosomes to nuclei was scored under a fluorescent microscope (C) and summarized in (E). Their correlation was analyzed by Spearman rank correlation test (P<0.001).