Phosphoinositides and lipid kinases in oxidative stress signalling and cancer
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CHAPTER 3

Regulation of Phosphatidylinositol-5-Phosphate Signaling by Pin1 Determines Sensitivity to Oxidative Stress

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Abstract

Oxidative signaling and oxidative stress contribute to aging, cancer, and diseases resulting from neurodegeneration. Pin1 is a proline isomerase that recognizes phosphorylated substrates and regulates the localization and conformation of its targets. Pin1−/− mice show phenotypes associated with premature aging, yet mouse embryonic fibroblasts (MEFs) from these mice are resistant to hydrogen peroxide (H₂O₂)-induced cell death. We found that the abundance of phosphatidylinositol-5-phosphate (PtdIns5P) was increased in response to H₂O₂, an effect that was enhanced in Pin1−/− MEFs. Reduction of H₂O₂-induced PtdIns5P compromised cell viability in response to oxidative stress, suggesting that PtdIns5P contributed to the enhanced cell viability of Pin1−/− MEFs exposed to oxidative stress. The increased PtdIns5P in the Pin1−/− MEFs stimulated the expression of genes involved in defense against oxidative stress and reduced the accumulation of reactive oxygen species. Pin1 and PtdIns5P 4-kinases (PIP4Ks), enzymes that phosphorylate and thereby reduce the amount of PtdIns5P, interacted in a manner dependent on the phosphorylation of PIP4K. Although reintroduction of Pin1 into the Pin1−/− MEFs reduced the amount of PtdIns5P produced in response to H₂O₂, in vitro assays indicated that the isomerase activity of Pin1 inhibited PIP4K activity. Whether this isomerase-mediated inhibition of PIP4K occurs in cells remains an open question, but the data suggest that the regulation of PIP4K by Pin1 may be complex.
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Introduction

Oxidative signaling by hydrogen peroxide ($H_2O_2$) or other reactive oxygen species (ROS) has an important role in aging, cancer, and diseases associated with neuronal dysfunction (1). $H_2O_2$ is produced within the cell as a consequence of electron leakage during oxidative phosphorylation (2) and also through the specific activation of oxidases, such as the NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) oxidases (3). The intracellular generation of $H_2O_2$ also occurs downstream of oncogenic signaling induced by activated Ras (4). $H_2O_2$ generated by tumor cells has been implicated in the remodeling of the tumor and stromal microenvironment to increase nutrient availability and facilitate tumor cell growth (5, 6). By modifying critical amino acid residues, intracellular $H_2O_2$ can regulate enzyme activity and thus regulate signaling pathways (7). For example, the tumor suppressor protein and lipid phosphatase PTEN (phosphatase and tensin homolog) is inactivated by $H_2O_2$, leading to an increase in cellular phosphatidylinositol (3,4,5)-triphosphate [PtdIns(3,4,5)P₃] and the subsequent activation of the kinase PKB (also known as Akt) (8).

However, oxidative stress, induced by excessive exposure to $H_2O_2$, can lead to DNA damage, trigger senescence, or induce apoptosis. The importance of ROS in disease is exemplified by the development of pathologies in mice genetically manipulated to mismanage ROS (9–11) and by the demonstration that overexpression of enzymes that induce ROS is sufficient to induce cell transformation (12). Therefore, defining the signaling pathways and identifying protein targets that are regulated by ROS will enable better understanding of disease processes associated with aging and of tumorigenesis.

Polyphosphoinositides are a minor phospholipid constituent of cell membranes, and these lipids function in cell signaling and are often deregulated in diseases (13). The synthesis and degradation of the seven different polyphosphoinositides are controlled by an array of kinases, phosphatases, and lipases. In response to changes in the extracellular environment, intracellular environment, or both, these enzymes can establish subcellular profiles of phosphoinositides that interact with and regulate proteins containing specific lipid-binding domains to control cell proliferation, survival, and migration, as well as trafficking of proteins through the subcellular membrane compartments and gene transcription (14).

The abundance of the phosphoinositide phosphatidylinositol-5-phosphate (PtdIns5P) is increased in response to ultraviolet (UV) irradiation (15) and osmotic shock (16) in mammalian cells and by dehydration and osmotic stress in plant cells (17, 18). Furthermore, the amount of PtdIns5P is constitutively high in some leukemia cells (19). By interacting with proteins containing the plant homeobox domain finger motif, PtdIns5P increases the transcriptional activity of the stress-dependent tumor suppressor p53 in mammalian cells (20) and causes the relocalization of the histone H3 lysine 4 methylase ATX1 from the nucleus to the cytoplasm in response to dehydration.
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stress in plants (17). The abundance of PtdIns5P can be increased by the stress-activated lipid kinase PIKfyve (21, 22) and by two families of phosphatases (17, 23–26). By phosphorylating PtdIns5P to produce PtdIns(4,5)P₂, the PtdIns5P 4-kinases (PIP4Ks) can reduce the abundance of cellular PtdIns5P (27). In response to activation of the proline-directed kinase p38, PIP4Kβ becomes phosphorylated at Ser₁326 (human), which decreases its activity, leading to an increase in the nuclear abundance of PtdIns5P (15). Thus, PtdIns5P appears to function downstream of cellular stressors.

Protein phosphorylation by proline-directed kinases, such as p38, can be transduced into conformational changes by a phosphorylation-dependent interaction with the evolutionarily conserved rotamase Pin1 (28, 29). Pin1 contains a WW motif, which specifically interacts with target proteins phosphorylated on serine or threonine residues situated next to proline residues, and a proline isomerase activity (PPI) that induces conformational changes (30, 31). The PPI activity of Pin1 can regulate protein catalytic activity, localization, stability, dephosphorylation, and interaction with other proteins (31). Although Pin1 has been implicated in control of cell cycle, transcription, splicing, DNA replication checkpoint and DNA damage response, survival of neurons, and development and migration of germ cells (31), Pin1-deficient mice are viable and fertile, developing to young adulthood apparently normally (32). However, they display various premature phenotypes of aging (31), such as reduced body size, atrophy of the testis and retina, motor coordination and behavioral defects, neuron degeneration and loss, osteoporosis, lordokyphosis, skin atrophy, and accelerated telomere loss (31), suggesting a role for age-dependent accumulation of cellular damage. However, Pin1⁻/⁻ mice are not cancer-prone and do not develop tumors in model systems (32, 33). The abundance of Pin1 is often increased in human tumors, suggesting a possible oncogenic role for Pin1 (34–36).

Unexpectedly, compared to wild-type mouse embryonic fibroblasts (MEFs), MEFs derived from Pin1⁻/⁻ mice are resistant to cell death caused by oxidative stress induced by exogenous H₂O₂ (37). Because of this stress-resistant phenotype and because phosphoinositides have roles in oxidative signaling (38), we investigated whether Pin1 regulated the phosphoinositide abundance to control oxidative stress responses and the sensitivity of cells to oxidative stress. Using Pin1 to affinity-purify lipid kinases, we identified the PIP4K family of lipid kinases as interacting with Pin1 in a p38-dependent manner. The interaction between Pin1 and PIP4K depended on the phosphorylation of PIP4K at Ser₁326 or Thr³²² and was stimulated by exposure of cells to UV radiation or H₂O₂. Compared to MEFs from wild-type mice, MEFs isolated from Pin1⁻/⁻ mice displayed increased PtdIns5P in response to exposure to H₂O₂. Reducing the amount of H₂O₂-induced PtdIns5P in Pin1⁻/⁻ MEFs reduced cell viability in response to oxidative stress. The expression of genes encoding proteins required for eliminating ROS was increased in Pin1⁻/⁻ MEFs, leading to an increase in the cellular ability to cope with oxidative stress. Reducing the amount of PtdIns5P in Pin1⁻/⁻ MEFs decreased the expression of these genes and increased the accumulation of cellular
ROS. Thus, PtdIns5P is a redox- and Pin1-regulated second messenger, which alters gene expression to calibrate the sensitivity of cells to ROS and determine cell fate in response to oxidative stress.

**Results**

**Pin1 interacts with PIP4 kinases**

The WW domain of Pin1 interacts with proteins that are phosphorylated on serine or threonine residues situated next to a proline residue. We performed affinity purification using glutathione S-transferase (GST) fused to wild-type Pin1 (GST-Pin1) or GST fused to Pin1 mutated in the WW domain (GST–Pin1-WW) to reduce its interaction with phosphopeptides to identify lipid kinases that interacted with Pin1 in a phosphorylation-dependent manner. Using a general lipid kinase substrate (Folch lipid fraction from brain with the addition of synthetic PtdIns5P because it is normally present at low concentrations) in the presence of \( \gamma^{32}P\)ATP, we identified an enzyme that synthesized PtdInsP2 that interacted with GST-Pin1, but not with the GST–Pin1-WW (Fig. 1A).

PtdIns(4,5)P2 can be generated by two distinct families of lipid kinases (39): the phosphatidylinositol-4-phosphate 5-kinases (PIP5Ks) phosphorylate PtdIns4P on the 5' position (39), and the PIP4Ks phosphorylate PtdIns5P on the 4' position (27, 40, 41) (Fig. 1A). To discriminate between the two families, we incubated the GST pull-downs either with the Folch lipid fraction as a source of the PIP5K substrate phosphatidylinositol-4-phosphate or with the synthetic PtdIns5P as a substrate for PIP4K and found that GST-Pin1 affinity-purified a PIP4K in a manner dependent on the WW domain of Pin1 and thus likely dependent on phosphorylation of PIP4K (Fig. 1B).

To determine which of the three different isoforms of PIP4K—PIP4K\(\alpha\) (42), PIP4K\(\beta\) (43), or PIP4K\(\gamma\) (44)—interacted with Pin1, we knocked down each individual isoform with short hairpin RNA (shRNA) in U2OS cells and subjected the cell lysates to affinity purification with GST-Pin1. Suppression of either PIP4K\(\alpha\) or PIP4K\(\beta\) expression reduced the amount of PIP4K activity affinity-purified by GST-Pin1, whereas suppression of PIP4K\(\gamma\) did not (Fig. 1C). Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) demonstrated that the shRNA constructs had the largest effect on the abundance of the transcripts of the targeted PIP4Ks, although the shRNA targeting PIP4K\(\beta\) and PIP4K\(\gamma\) also reduced PIP4K\(\alpha\) expression to a lesser extent. PIP4Ks can homo- and heterodimerize, and the activity of the \(\alpha\) isoform is at least two to three orders of magnitude greater than the activity of the \(\beta\) or \(\gamma\) isoform (40, 45). The increase in PIP4K activity associated with GST-Pin1 after suppression of PIP4K\(\gamma\) might result from PIP4K\(\alpha\) that is no longer part of a heteromeric complex. The reduced PIP4K activity observed upon knockdown of PIP4K\(\beta\) might be a consequence of the decrease in PIP4K\(\alpha\) mRNA or might suggest that PIP4K\(\alpha\) can interact with Pin1 through its interaction with PIP4K\(\beta\).
Figure 1. PIP4K and PIP5K interact with Pin1 in a phosphorylation-dependent manner. (A) The indicated GST fusion protein (1 μg) coupled to glutathione beads was used to affinity-purify lipid kinases from U2OS cell lysates (1 mg), and then a lipid kinase assay was performed. Labeled lipids were extracted and chromatographed by thin layer chromatography (TLC) together with a standard radiolabeled PtdIns(4,5)P2 marker (PIP2). Bottom: a Coomassie-stained gel of the fusion proteins coupled to the beads. Data are representative of three experiments. The schematic shows the two pathways for lipid kinase–mediated synthesis of PtdIns(4,5)P2. (B) Affinity-purified lipid kinases were extracted and chromatographed by thin layer chromatography (TLC) (1 mg), and then a lipid kinase assay was performed. Labeled lipids were extracted and chromatographed by thin layer chromatography (TLC) together with a standard radiolabeled PtdIns(4,5)P2 marker (PIP2). (C) PIP2, TLC origin, and PtdIns(4,5)P2. (D) To establish whether phosphorylation at a single site was sufficient and independent experiments. (E) The right graph shows the abundance of the indicated mRNA, using qRT-PCR, after knockdown by the indicated shRNA (average ± SD of triplicate samples representative of two experiments).
To confirm that PIP4Kα and PIP4Kβ interacted with Pin1, we overexpressed the Myc-tagged forms of the α or β isoforms in human embryonic kidney (HEK) 293 cells and assessed their interaction with GST-Pin1, GST–Pin1-WW, or a fusion of GST with Pin1 lacking the PPI activity (GST–Pin1–PPI) by Western blotting (Fig. 1D). Both PIP4Kα and PIP4Kβ associated with GST-Pin1 in a phosphorylation-dependent and PPI-independent manner (Fig. 1D). We also observed coimmunoprecipitation of hemagglutinin (HA)–tagged Pin1 and Myc-tagged PIP4Kα or PIP4Kβ (Fig. 1E) expressed in HEK293 cells. Thus, we have identified an interaction between Pin1 and PIP4Kα and PIP4Kβ and showed that the interaction required the intact phosphopeptide binding site of Pin1.

Pin1 interacts with PIP4Kβ phosphorylated at Thr^{322} and Ser^{326}

We previously demonstrated that PIP4Kβ is phosphorylated at Thr^{322} and Ser^{326} in response to UV irradiation (arrows in Fig. 2A) (15). Phosphorylation at Ser^{326} is dependent on the proline-directed stress kinase p38 (15); however, the kinase responsible for phosphorylation at Thr^{322} is unknown. When phosphorylated, either of these sites can form potential interaction sites for Pin1, although the presence of glycine at residue 321 and proline at residue 324 (numbering based on PIP4Kβ) might suggest a stronger affinity to Ser^{326} (30) (Fig. 2A). Therefore, we investigated whether the Pin1/PIP4K interaction depended on p38 signaling. Coexpression of PIP4Kα with a constitutively active form of mitogen-activated protein kinase kinase 6 (MKK6), a specific upstream activator of p38, increased the interaction of both PIP4Kα and PIP4Kβ with GST-Pin1 (Fig. 2B). In contrast, PIP4Kγ did not interact with Pin1 under basal conditions, and this interaction was not detected in lysates from cells coexpressing MKK6 (Fig. 2B), which may be explained by the lack of the equivalent TP and SP motifs in PIP4Kγ present in PIP4Kβ and conserved in PIP4Kα.

To determine the importance of phosphorylation of Thr^{322} and Ser^{326} in the interaction with Pin1, we expressed mutated forms of PIP4Kβ with or without coexpression of constitutively active MKK6 and assessed the interaction with Pin1.
Figure 2. The interaction between PIP4Kβ and Pin1 requires the phosphorylation of Thr\textsuperscript{322} and Ser\textsuperscript{326}. (A) Sequence alignment of the region around the Thr\textsuperscript{322} and Ser\textsuperscript{326} phosphorylation sites of PIP4Kβ in all three PIP4K isoforms. Arrows denote Thr\textsuperscript{322} and Ser\textsuperscript{326} from PIP4Kβ. (B) Myc-tagged PIP4Kα, PIP4Kβ, and PIP4Kγ were overexpressed in HEK293 cells alone or together with constitutively active MKK6. The lysates were then used for affinity purification with GST-Pin1, and bound proteins were analyzed by Western blotting with an antibody recognizing Myc. The graph below shows the average binding and SD of Pin1 with constitutively active MKK6. Myc-tagged PIP4K proteins that interacted with GST-Pin1 were detected with antibodies specifically recognizing Myc or HA. The inset shows the average binding and SD of wild-type (WT) and PIP4K\textsuperscript{–T322A, PIP4K\textsuperscript{β–S326A, and PIP4K\textsuperscript{β–T322A/S326A}}. (C) Myc-tagged PIP4Kβ, PIP4Kβ-T322A, PIP4Kβ-S326A, and PIP4Kβ-T322A/S326A
were overexpressed in HEK293 cells alone or together with constitutively active MKK6. Myc-tagged PIP4K proteins that interacted with GST-Pin1 were detected as described in (B). Phosphorylation of Thr322 and Ser326 was detected with antibodies specifically recognizing each residue (P-T322–PIP4Kβ and P-S326–PIP4Kβ respectively). (D) Myc-tagged PIP4Kβ or the indicated mutant forms were coexpressed with HA-Pin1 or HA-Pin1-WW in HEK293 cells. Lysates were immunoprecipitated with antibody recognizing HA, separated by SDS-PAGE, and probed with antibodies recognizing Myc or HA. The inset shows the average binding and SD of wild-type (WT) and PIP4Kαβ mutants to GST-Pin1 from three independent experiments. Samples of the total lysates were probed with the indicated antibodies. (E) Surface plasmon resonance of PIP4Kβ peptides with GST-Pin1. The following peptides [100 response units (RU)] were coupled through their cysteine residues to a Bio-Rad GLM sensor chip: T322, CLCSYGTPDSP; P-T322, CLCSYG(T)pFDPDSP; P-T322/P-S326, CLCSYG[pFDPDSP]pSPGNL; P-S326, CSYGTPDSP[pSPGNL]; S326, CSYGTPDSP[pSPGNL]; and Empty, no peptide. The peptides were then probed for interaction with varying concentrations of GST-Pin1 as indicated. Data are representative of three to five different injections and have been carried out using two different SPR chips.
Regulation of Phosphatidylinositol-5-Phosphate Signaling by Pin1 Determines Sensitivity... (Fig. 2C). In the absence of coexpressed MKK6, the interaction between PIP4Kβ and Pin1 was reduced by mutation of Thr^{322} but not by mutation of Ser^{326}. Mutation of both residues to alanine also reduced the interaction with GST-Pin1. When PIP4Kβ and its mutants were coexpressed with MKK6, the interaction with GST-Pin1 was increased compared to the interaction in the absence of MKK6, and mutation of Ser^{326} to alanine or Thr^{322} to alanine reduced the interaction (Fig. 2C, right). Thus, both residues appear to contribute to the interaction with Pin1 and that phosphorylation enhanced the interaction.

To confirm the importance of Thr^{322} and Ser^{326} in the interaction of Pin1 with full-length PIP4Kβ in cells, we mutated Thr^{322}, Ser^{326}, or both residues to alanine residues and coexpressed them with HA-tagged Pin1 or HA-tagged Pin1-WW mutant (HA–Pin1-WW). When overexpressed in HEK293 cells, we detected constitutive phosphorylation of both Thr^{322} and Ser^{326} with antibodies that recognized each phosphorylated site specifically [Fig. 2, C (left) and D]. Mutation of neither Thr^{322} nor Ser^{326} to alanine strongly affected phosphorylation of the other residue (Fig. 2, C and D). PIP4Kβ coimmunoprecipitated with HA-Pin1, whereas PIP4Kβ failed to coimmunoprecipitate with HA–Pin1-WW (Fig. 2D, compare lanes 2 and 3), consistent with a phosphorylation-dependent interaction. Mutation of Ser^{326} or Thr^{322} to alanine reduced the interaction, and the interaction was not further reduced by mutation of both residues (see inset graph in Fig. 2D). Consistent with the affinity purification data, these coimmunoprecipitation data suggested that both Thr^{322} and Ser^{326} contribute to the interaction with Pin1, and that phosphorylation enhanced the interaction.

To establish whether phosphorylation at a single site was sufficient for the interaction of PIP4Kβ with Pin1 and to confirm a direct interaction with Pin1, we synthesized nonphosphorylated peptides or peptides phosphorylated at Thr^{322} or Ser^{326} and peptides phosphorylated at both residues and assessed their interaction with GST-Pin1 or Pin1 mutants by surface plasmon resonance (SPR). GST-Pin1 did not interact with the nonphosphorylated peptides, whereas it exhibited a concentration-dependent interaction with the doubly phosphorylated Thr^{322}/Ser^{326} peptide (Fig. 2E). Unexpectedly, Pin1 exhibited a concentration-dependent interaction with the Thr^{322}-phosphorylated peptide but not with the Ser^{326}-phosphorylated peptide (Fig. 2E). We confirmed the specificity of binding by SPR using GST–Pin1-WW, which did not interact with any of the peptides (Supplementary Fig. 1). A mutant of Pin1 lacking catalytic activity, GST–Pin1-PPI, interacted to a similar extent and with similar specificity as GST-Pin1 (Supplementary Fig. 1).

We obtained similar results with phosphorylated and nonphosphorylated peptides coupled to Sepharose beads and used to affinity-purify Pin1 from HeLa cell lysates (Supplementary Fig. 2A), suggesting that the dependence on phosphorylation of Thr^{322} was not an artifact of peptide coupling to the SPR chip surface. Thus, phosphorylation of Thr^{322} is sufficient for binding of PIP4K peptides to Pin1 in vitro. Because mutation of either Ser^{326} or Thr^{322} reduced the interaction when Pin1 and PIP4Kβ were overexpressed
Figure 3. Pin1 and PIP4Kβ interact in vivo. (A) pRetroSuper-shRNAi (short hairpin–mediated RNA interference) constructs targeting PIP4Kβ (RNAi PIP4Kβ) or Pin1 (RNAi Pin1) or a negative control (pRetro) were used to generate stable HEK293 cell lines, and cell lysates were immunoblotted with antibodies recognizing the indicated proteins. The graph shows the relative abundance of PIP4Kβ and Pin1 in the cell lines stably expressing the indicated RNAi. (B) Cell lines expressing the indicated RNAi were untreated or treated with UV irradiation (100 J/m²) as indicated. Nuclear lysates were immunoprecipitated (IP) with either preimmune antisera or antibodies recognizing PIP4Kβ, and precipitates were immunoblotted with antibodies recognizing the proteins as indicated to the right. (C) Cell lines expressing the indicated RNAi were untreated or treated with UV irradiation (100 J/m²), and then nuclear lysates were immunoprecipitated with antibodies recognizing PIP4Kβ, and precipitates were immunoblotted with antibodies recognizing the proteins as indicated to the right. (D) Cell line in which PIP4Kα or Pin1 was knocked down was untreated or treated with UV irradiation (100 J/m²). Nuclear lysates were immunoprecipitated with a PIP4Kβ-specific antibody, and the indicated proteins were detected in the immunoprecipitates. The gel below shows the extent to PIP4Kβ knockdown (lower panel) compared to PIP4Kβ knockdown (upper panel). (E) The indicated cell lines were subjected to UV irradiation in the presence or absence of the p38 inhibitor SB203580 as indicated. Nuclear lysates were immunoprecipitated with a PIP4Kβ-specific antibody, and the indicated proteins were detected in the immunoprecipitates. The immunoblots are representative of two independent experiments.
in cells, it is likely that the interaction between these two proteins involves more than just a single phosphorylated site and depends on a specific conformation of PIP4Kβ.

To confirm that Pin1 and PIP4Kβ interact directly and that Ser^{326} contributed to the interaction with Pin1, we used a protein overlay assay with wild-type HA-tagged PIP4Kβ or HA-tagged PIP4Kβ-S326A purified from HEK293 cells before and after UV irradiation, which would stimulate p38 activity. The purified proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose, and overlaid with GST-Pin1 (Supplementary Fig. 2B). GST-Pin1 interacted with PIP4Kβ, and this interaction appeared to increase after UV irradiation. Consistent with a role for the phosphorylation of Ser^{326}, the UV irradiation–dependent interaction with Pin1 was reduced when Ser^{326} was mutated to alanine (Supplementary Fig. 2B). We also confirmed the WW domain–dependent interaction between Pin1 and PIP4Kβ when

**Figure 4.** Pin1 modulates the in vitro activity of PIP4Kα and PIP4Kβ. (A) Top: Abundance of the indicated Myc-tagged protein in lysates expressing PIP4Kα, PIP4Kβ, or empty vector (EV) used for affinity purification. Bottom: Amount of Myc-tagged PIP4Kα or PIP4Kβ present after affinity purification with the indicated GST-Pin1 fusion protein. (B) Lipid kinase activity affinity-purified with the indicated GST-Pin1 fusion protein. Data are representative of multiple experiments. (C) Average data of multiple lipid kinase activity assays for PIP4Kα (left) and PIP4Kβ (right) affinity-purified with the indicated GST-Pin1 fusion proteins. Data are the averages ± SD of multiple experiments (PIP4Kα, n = 2, and PIP4Kβ, n = 3) each performed in triplicate.
the proteins were coexpressed in HeLa cells (Supplementary Fig. 3C) and showed that mutation of either Thr\textsuperscript{322} or Ser\textsuperscript{326} to alanine did not compromise the interaction with Pin1, but mutation of both sites abolished the interaction (Supplementary Fig. 3C). Thus, the cellular data suggest that phosphorylation of either Ser\textsuperscript{326} or Thr\textsuperscript{322} can mediate the direct interaction between PIP4K and Pin1 and that conditions that promote Ser\textsuperscript{326} phosphorylation, such as UV stress, may enhance the interaction.

*Pin1 interacts with endogenous PIP4K in a stress- and p38-dependent manner*

UV irradiation induces the phosphorylation of PIP4K\textsubscript{β} at Ser\textsuperscript{326} by p38 (15). To examine whether this stress stimulated the interaction between endogenous Pin1 and endogenous PIP4K\textsubscript{β}, we generated HEK293 cell lines in which endogenous PIP4K\textsubscript{β} or Pin1 was stably knocked down by RNA interference (RNAi) and compared the interaction in control cells with that in cells subjected to UV irradiation. Targeted RNAi reduced PIP4K\textsubscript{β} to 33% and Pin1 to 20% of their respective abundance in cells with control RNAi (Fig. 3A). In HEK293 cells, UV irradiation increased Ser\textsuperscript{326} phosphorylation 1.77 ± 0.16-fold over nonstimulated cells (average ± SD, representative data are shown in Fig. 3B, lanes 5 and 6). Endogenous PIP4K\textsubscript{β} only interacted with endogenous Pin1 after UV irradiation, and the interaction was lost upon RNAi-mediated suppression of the expression of either Pin1 (Fig. 3B, compare lane 4 with lane 6) or PIP4K\textsubscript{β} (Fig. 3C).

Because our data suggested that both PIP4K\textsubscript{α} and PIP4K\textsubscript{β} interacted with Pin1 and because PIP4K\textsubscript{α} can interact with β (46), we assessed whether the interaction of PIP4K\textsubscript{β} with Pin1 was dependent on PIP4K\textsubscript{α} by suppressing its expression using RNAi. Although coimmunoprecipitation experiments indicated that in HEK293 cells, the association between endogenous Pin1 and endogenous PIP4K\textsubscript{β} depended on UV irradiation, the interaction was not affected by knockdown of PIP4K\textsubscript{α} (Fig. 3D).

Pretreatment of HEK293 cells with SB203580, a specific inhibitor of the kinase activity of p38, appeared to reduce the phosphorylation of PIP4K\textsubscript{β} at Ser\textsuperscript{326}, as previously shown (15), and to reduce the UV irradiation–induced interaction between Pin1 and PIP4K\textsubscript{β} (Fig. 3E). The increase in Pin1 association with PIP4K\textsubscript{β} appeared much stronger than the actual increase in PIP4K\textsubscript{β} phosphorylation at Ser\textsuperscript{326}. This increased binding may reflect phosphorylation of Thr\textsuperscript{322}, the extent of which we have been unable to monitor for the endogenous protein. Furthermore, using SPR, we found that the Ser\textsuperscript{326} phosphorylation–specific antibody exhibited a reduced ability to recognize a peptide phosphorylated at both Ser\textsuperscript{326} and Thr\textsuperscript{322} (Supplementary Fig. 3), suggesting that Western blotting may underestimate the extent of phosphorylation of PIP4K\textsubscript{β} Ser\textsuperscript{326} phosphorylation when both sites are phosphorylated. These experiments with endogenous proteins indicated that UV irradiation can induce the association between PIP4K\textsubscript{β} and Pin1.

*Pin1 interaction regulates PIP4K activity*

The interaction between Pin1 and its targets can modulate their rate of dephosphorylation, stability, or localization. We did not detect an appreciable difference in the abundance of PIP4K\textsubscript{β} in Pin1 knockdown cells compared to cells expressing the
control RNAi before or after treatment with UV irradiation (Supplementary Fig. 4A), nor was there any difference in abundance of PIP4Kα or PIP4Kβ in Pin1−/− MEFs compared to their abundance in wild-type MEFs (Supplementary Fig. 4B). No differences were observed in the rate of degradation (Supplementary Fig. 4C) or in the gross subcellular localization (nucleus, cytosol, and membrane fractions) of PIP4Kβ (Supplementary Fig. 4D) between wild-type and Pin1−/− MEFs.

To assess the effect of Pin1 on the activity of PIP4K, we overexpressed PIP4Kα and PIP4Kβ; affinity-purified the proteins with either GST-Pin1, GST–Pin1-WW, or GST–Pin1-PPI; and determined the amount of PIP4K purified by Western blotting and the PIP4K activity by a lipid kinase activity assay (Fig. 4). As expected, lipid kinase activity was not present in samples affinity-purified with the Pin1-WW mutant because the PIP4K did not interact with this mutant (Fig. 1D). Although similar amounts of PIP4K were affinity-purified by either the wild-type Pin1 or the PPI mutant, as assessed by Western blotting (Fig. 4A), we found ~2.5 times more lipid kinase activity associated with the PPI-deficient Pin1 compared to that associated with wild-type Pin1 (Fig. 4, B and C). These data suggested that the isomerase activity of Pin1 inhibits the catalytic activity of PIP4Kα and PIP4Kβ in vitro.

**Pin1 reduces the cellular amount of PtdIns5P in response to H2O2.**

Although Pin1-deficient mice show premature aging–associated phenotypes, MEFs isolated from the mice counterintuitively show decreased sensitivity to oxidative stress compared to wild-type MEFs (37). We monitored the amount of PtdIns5P, the substrate for PIP4K, in MEFs derived from control or Pin1−/− mice in response to oxidative stress. Because PtdIns5P cannot easily be measured by high-performance liquid chromatography (HPLC), we calculated the relative amount of PtdIns5P with a mass assay based on the phosphorylation of PtdIns5P to PtdIns(4,5)P2. In Pin1−/− MEFs, H2O2 treatment increased PtdIns5P in a dose-dependent manner, with 125 μM stimulating a measurable increase in PtdIns5P (Supplementary Fig. 5A). The increase in PtdIns5P in response to H2O2 was greater in Pin1−/− MEFs compared to the increase in wild-type MEFs (Fig. 5A). Reintroduction of Pin1 into Pin1−/− MEFs significantly reduced the increase in PtdIns5P in response to H2O2 compared with cells transfected with an empty vector control (Fig. 5B). Pin1 deletion did not change the abundance of PIP4Kα or PIP4Kβ (Supplementary Fig. 4B and Fig. 5C), and H2O2 triggered a similar fold increase in PIP4Kβ phosphorylation at Ser326 in both Pin1−/− MEFs (1.67 ± 0.16 average fold increase after stimulation with H2O2 for 10 min, n = 3) and wild-type MEFs (1.60 ± 0.14 average fold increase after stimulation with H2O2 for 10 min, n = 3) (Fig. 5C). Stimulation of wild-type MEFs with H2O2 increased the amount of Pin1 that coimmunoprecipitated with PIP4Kα (2.2 ± 0.15) or PIP4Kβ (2.1 ± 0.13) (quantified as the mean ± range of two experiments and presented as the fold increase above cells not treated with H2O2) (Fig. 5D). The observed H2O2-induced interaction between Pin1 and PIP4K might suggest a role for PIP4K in regulating the abundance of PtdIns5P, and
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**Figure 5.** Redox-regulated changes in the amount of PtdIns5P are modulated by the presence of Pin1. (A) WT or Pin1−/− MEFs were stimulated with 1 mM H2O2 as indicated for 45 min, after which lipids were extracted and used to measure the amount of PtdIns5P. Data represent the fold increase (average and SD) and are representative of three different experiments. The fold increase by H2O2 shown is relative to the control (absence of H2O2) for each genotype. (B) Pin1−/− MEFs were transduced with either empty vector (EV) or HA-Pin1 and kept as controls or stimulated with 1 mM H2O2 for 45 min. Lipids were extracted and used to measure PtdIns5P. The immunoblot shows the abundance of HA-Pin1 and actin after transduction. Data represent the averages ± SD of three independent experiments. (C) WT or Pin1−/− MEFs were stimulated with 1 mM H2O2 for the indicated times. Lysates were immunoprecipitated with a PIP4Kβ-specific antibody, and the indicated proteins were detected in the immunoprecipitates. The indicated proteins in the lysates were also immunoblotted. Data are representative of two independent experiments. (D) WT MEFs were stimulated with 1 mM H2O2 as indicated, after which the lysates were immunoprecipitated with PIP4Kα- or PIP4Kβ-specific antibodies and the indicated proteins were detected in the immunoprecipitates. The indicated proteins in the total lysates were immunoblotted. Data are representative of two independent experiments.

RNAi-mediated suppression of PIP4Kα and PIP4Kβ in Pin1−/− MEFs increased the basal and H2O2-stimulated increase in PtdIns5P (Supplementary Fig. 5B).

To determine the intracellular location where Pin1 might modulate PtdIns5P, we overexpressed PIP4Kβ tagged with green fluorescent protein (GFP) in HeLa cells. GFP-PIP4Kβ was detected in both the nucleus and the cytoplasm; however, immunofluorescence microscopy showed that PIP4Kβ and Pin1 colocalized predominantly in a punctuate pattern in the nucleus (Supplementary Fig. 6), suggesting that Pin1 likely regulates a pool of nuclear PtdIns5P. Although we were unable to colocalize endogenous PIP4Kβ and Pin1 using the available antibodies, the presence of both endogenous PIP4Kβ and Pin1 in nuclear fractions (Supplementary Fig. 4, D and E) and their coimmunoprecipitation from nuclear fractions (Fig. 3) suggested that this colocalization was unlikely a consequence of overexpression.
Increased PtdIns5P in Pin1−/− MEFs enhances their resistance to H₂O₂

We next investigated whether PtdIns5P contributed to responses of MEFs to H₂O₂. In response to stimulation with concentrations of H₂O₂ between 50 and 200 μM, cells can undergo cell cycle arrest and induce a senescent phenotype (47), which can be easily analyzed using clonogenic survival and proliferation assays (47). Exposure of wild-type MEFs to H₂O₂ reduced the number of colonies from 1000 to 200, demonstrating their sensitivity to H₂O₂. In the absence of H₂O₂, Pin1−/− MEFs grew slower and generated fewer colonies than wild-type MEFs (Fig. 6A). However, there was no reduction in the number of colonies obtained after exposure of the Pin1−/− MEFs to H₂O₂ (Fig. 6A). We next sought to determine whether the generation of PtdIns5P was important in regulating the cellular sensitivity to H₂O₂.

Because PIP4Kα exhibits higher catalytic activity than PIP4Kβ (40), we overexpressed PIP4Kα in wild-type and Pin1−/− MEFs, which significantly reduced H₂O₂-induced PtdIns5P (Fig. 6B). In accordance with a role for PtdIns5P in the resistance to H₂O₂ toxicity, overexpression of PIP4Kα in Pin1−/− MEFs or in wild-type MEFs significantly reduced the number of viable colonies after treatment with H₂O₂ (Fig. 6A), whereas there was no reduction in viable colonies for Pin1−/− MEFs expressing the empty vector. Because H₂O₂ stimulation leads to changes in various phosphoinositides (48, 49), we measured their abundance in control and PIP4Kα-overexpressing cells untreated or treated with H₂O₂ (Fig. 6C). H₂O₂ induced a decrease in PtdIns(4,5)P₂ in both wild-type and Pin1−/− MEFs, and this reduction was not affected by overexpression of PIP4Kα. The only difference, besides PtdIns5P generation, between wild-type and Pin1−/− MEFs was an increase in H₂O₂-induced PtdIns(3,4)P₂, which was only observed in Pin1−/− MEFs. However, the increase in PtdIns(3,4)P₂ induced by H₂O₂ was not significantly different in cells overexpressing PIP4Kα (Fig. 6C). Reintroduction of Pin1 into Pin1−/− MEFs increased the number of colonies in untreated cells and resulted in sensitivity to treatment with H₂O₂ (Supplementary Fig. 7). Therefore, our data indicated that an increase in PtdIns5P has a role in the increased cell viability in response to H₂O₂ observed in Pin1−/− MEFs, although we cannot exclude the possibility that other lipids that change in response to alterations in PtdIns5P may also play a role.

Pin1-regulated changes in PtdIns5P modulate ROS accumulation

Cell viability in response to oxidative stress can be regulated by many different mechanisms, including the evolutionarily conserved FOXO and NRF2 transcription pathways, which stimulate the expression of genes that are involved in the detoxification of ROS (50–53). Previous studies have demonstrated a role for Pin1 in the regulation of both pathways (38, 54). In Pin1−/− MEFs, transcripts of the NRF2 target genes NQO1 [encoding NAD(P)H:quinone oxidoreductase 1] and GSTA1 (encoding GST α1) were increased in response to H₂O₂ (Fig. 7A). The expression of GSTA1 was also increased after H₂O₂ treatment in wild-type MEFs, and its abundance was greater in Pin1−/− MEFs than in wild-type MEFs in the absence of stimulation with...
Figure 6. PtdIns5P plays a key role in modulating the sensitivity of Pin1−/− MEFs to oxidative stress. (A) WT or Pin1−/− MEFs were transduced with the indicated constructs, and then 1000 cells were plated in 10-cm dishes. The following day, H2O2 (final concentration, 100 μM) was added, and the cells were allowed to grow for 10 days. The colonies were stained with crystal violet and quantified. The immunoblot shows the abundance of PIP4Kα and actin in the two cell lines. Data are the averages ± SD and are representative of three different experiments. (B) WT or Pin1−/− MEFs transduced with the indicated constructs were stimulated with 1 mM H2O2 as indicated, after which lipids were extracted and used to measure the amount of PtdIns5P. Data are the averages ± SD of three independent experiments. Student’s t test was performed for statistical analysis. (C) WT or Pin1−/− MEFs transduced with the indicated constructs (EV, empty vector) were labeled with [32P]-orthophosphate, after which lipids were extracted, dried, deacylated, and chromatographed using HPLC. Data represent the averages ± SD of three independent experiments. Compared to control cells, no significant changes in any phosphoinositide were observed in cells overexpressing PIP4Kα. Student’s t test was performed for statistical analysis.

H2O2 (Fig. 7A). In accordance with the role for PtdIns5P in the regulation of these redox-controlled genes, overexpression of PIP4Kα reduced H2O2-induced expression of NQO1 and GSTA1 in Pin1−/− MEFs (Fig. 7A). Expression of PIP4Kα also reduced the increased expression of GSTA1 in Pin1−/− MEFs in the absence of H2O2 stimulation (Fig. 7A). Tertiary butylhydroquinone (TBHq) is an activator of the NRF2 pathway (55),
Figure 7. Pin1-regulated changes in PtdIns5P modulate ROS accumulation. (A) WT or Pin1^−/− MEFs transduced with EV or PIP4Kα were exposed to 100 μM H_2O_2 for 8 hours as indicated. qRT-PCR was performed with primers for NQO1 and GSTA1. The relative amounts of each transcript were normalized to the abundance of actin mRNA. Data are the averages ± SD of triplicate samples and are representative of two experiments. (B) The indicated MEFs transduced with EV or PIP4Kα were stimulated with 20 μM TBHQ, 100 μM TBHQ, or 100 μM H_2O_2 for 2.5 hours, and the abundances of NQO1 and GSTA1 transcripts were measured by qRT-PCR and presented normalized as in (A). Data are the averages ± SD of triplicate samples and are representative of two experiments. (C) The indicated MEFs transduced with EV or PIP4Kα were stimulated with 100 μM H_2O_2 or 100 μM TBHQ for 8 hours as indicated. qRT-PCR was performed with primers for SOD2 and GADD45. The relative amounts of each transcript were normalized to the abundance of actin mRNA. Data are the averages ± SD of three independent experiments. (D) The indicated MEFs were labeled with CM-H_2DCFDA, and both basal (top) and H_2O_2-induced (middle) ROS were
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and the increase in expression of NQO1 and GSTA1 by TBHq was also attenuated by PIP4Kα overexpression in both wild-type and Pin1−/− MEFs (Fig. 7B).

FOXO transcription factors are inhibited by Pin1 (38), and expression of the FOXO target genes SOD2 (encoding superoxide dismutase 2) and GADD45 (encoding growth arrest and DNA damage 45) was increased in Pin1−/− MEFs, compared to wild-type MEFs, under basal conditions and after stimulation with either TBHq or H2O2 (Fig. 7C). However, their expression was not decreased by PIP4Kα overexpression in either wild-type or Pin1−/− MEFs in the absence or presence of TBHq or H2O2 (Fig. 7C). Thus, although Pin1 inhibited the expression of SOD2 and GADD45, this regulation did not appear to involve PtdIns5P. Although increased PtdIns5P can promote the activation of the kinase Akt, which stimulates NRF2 (56, 57) and inhibits FOXO (58) transcription, the activation of Akt in response to either insulin or H2O2 stimulation was comparable between wild-type and Pin1−/− MEFs (Supplementary Fig. 8).

NRF2-induced expression of genes encoding proteins, such as NQO1 and GSTA1, has been associated with an increase in the capability of cells to prevent ROS accumulation (52, 59). We monitored ROS accumulation in wild-type and Pin1−/− MEFs with and without overexpression of PIP4Kα. Cells were labeled with CM-H2DCFDA [5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester], which, upon oxidation, generates a fluorescent signal that accumulates within the cell and serves as an indication of the oxidative environment of the cell. The rate of basal fluorescence accumulation was reduced in Pin1−/− MEFs compared to the rate in wild-type MEFs, and this reduction was reversed by the overexpression of PIP4Kα (Fig. 7D). Exposure of the cells to 100 μM H2O2 increased the rate of fluorescence accumulation in wild-type MEFs, which was less in the Pin1−/− MEFs. In response to H2O2, overexpression of PIP4Kα in the Pin1−/− MEFs resulted in a rate of fluorescence accumulation that was indistinguishable from that of wild-type MEFs (Fig. 7D). The decreased basal ROS accumulation could be a consequence of the slower rate of proliferation observed in Pin1−/− MEFs; however, PIP4Kα overexpression in Pin1−/− MEFs, despite increasing the rate of ROS production, did not alter their proliferation rate (Supplementary Fig. 9A). Furthermore, Pin1−/− MEFs have decreased amounts of cyclin D1 (32) and increased amounts of p27 (38), both of which reduce cell cycle progression. Neither of the genes encoding these proteins was regulated by PIP4Kα overexpression (Supplementary Fig. 10). These data suggested that increased PtdIns5P generated in Pin1−/− MEFs increased cell viability in response to oxidative stress by stimulating the expression of genes that limit cellular ROS accumulation.

measured by 2,7-dichlorofluorescein diacetate (DCF) fluorescence. The bar graph shows the area under the curve for fluorescence accumulation in the indicated MEFs. Data are the averages ± SD and are representative of three independent experiments. Statistical significance in all graphs was determined using Student’s t test, and calculated P values are shown. NS, not significant.
Discussion

Here, we showed that PtdIns5P is a redox-regulated second messenger that increases upon exposure of cells to H₂O₂. Furthermore, in Pin1−/− MEFs, which are more resistant to oxidative stress than their wild-type counterparts, the increase in PtdIns5P is enhanced. Overexpression of PIP4Kα to reduce the increase in PtdIns5P induced by Pin1 deletion sensitized Pin1−/− MEFs to the toxic effects of H₂O₂. PtdIns5P may contribute to resistance to oxidative stress by promoting the expression of genes that limit the cellular accumulation of ROS.

Our strategy to purify lipid kinases (phosphoinositide and diacylglycerol kinases) that interact with Pin1 in a phosphorylation-dependent manner identified the PIP4K family of enzymes that reduce the abundance of PtdIns5P. In cells, the interaction between PIP4Kβ and Pin1 required phosphorylation at either Thr³²² or Ser³²⁶. Because phosphorylation of Thr³²² and Ser³²⁶ is likely controlled by different kinases, we speculate that the interaction between PIP4K and Pin1, and therefore the amount of PtdIns5P, will be regulated by different inputs. We found that in cells, Pin1 interacted with either the high-activity isoform PIP4Kα or the low-activity PIP4Kβ isoform, which can interact with and target PIP4Kα to the nucleus (46). Furthermore, we showed that the interaction between PIP4K and Pin1 was enhanced by exposure of cells to H₂O₂-induced oxidative stress. We suggest that the redox-regulated phosphorylation of PIP4K stimulates the interaction between PIP4K and Pin1 to reduce stress-induced PtdIns5P generation, which is consistent with our observation that PtdIns5P is increased in cells lacking Pin1. However, H₂O₂ still increased PtdIns5P in MEFs in which both PIP4Kα and PIP4Kβ were knocked down, suggesting that the Pin1-PIP4K interaction was not the major driver for the increase in PtdIns5P in response to H₂O₂. How H₂O₂ increases PtdIns5P synthesis is not clear, but it may require the activity of myotubularins or PIKfyve, both of which could increase the abundance of PtdIns5P. Surprisingly, we found that in vitro, the isomerase activity of Pin1 inhibited PIP4K activity. This suggests that the Pin1 interaction with PIP4K may facilitate the removal of PtdIns5P, and Pin1 isomerase activity may keep PIP4K activity in check.

The regulation of PtdIns5P likely occurs in the nucleus because Pin1 and PIP4Kβ colocalized in punctate nuclear structures and were coimmunoprecipitated from nuclear fractions. Consistent with a nuclear function for PtdIns5P, we found that reducing PtdIns5P by overexpression of PIP4Kα reduced the induction of NRF2 target genes by H₂O₂. The NRF2 and FOXO transcription pathways are implicated in the response to oxidative stress by stimulating genes encoding proteins involved in limiting ROS and are inhibited by Pin1 (38, 54). In response to H₂O₂ or TBHq treatment, Pin1 deficiency increased the expression of genes that are downstream targets for either NRF2 (NQO1 and GSTA1) or FOXO (SOD2, GADD45, and p27). However, the reduction in PtdIns5P by the overexpression of PIP4Kα only attenuated the increase in NQO1 and GSTA1 expression, suggesting that PtdIns5P might play a
role in regulating NRF2-mediated transcription. NRF2 activation enhances the cell's capability to manage ROS accumulation (52, 60–62), and we found that cellular ROS accumulation was decreased in Pin1−/− MEFs, consistent with their increased resistance to oxidative stress. Overexpression of PIP4Kα, to reduce the amount of PtdIns5, increased the rate of ROS accumulation in Pin1−/− MEFs and reduced their resistance to oxidative stress.

The product of GSTA1 can also act as a scaffolding protein and reduce the activation of the c-Jun N-terminal kinase pathway (63, 64), which may be part of the mechanism by which increased PtdIns5P enhances cell viability in response to oxidative stress. We suggest that ROS-mediated increases in nuclear PtdIns5P may recalibrate the cellular response to subsequent changes in oxidative stress, possibly by stimulating NRF2-mediated gene transcription. Multiple mechanisms regulate NRF2 activity, including signals that affect its stability (65–67) or its nuclear import and export (68, 69). In addition, NRF2 activity is regulated by multiple pathways, including the extracellular signal–regulated kinase (70), p21 kinase (71), or phosphoinositide 3-kinase (PI3K) pathways (72). We did not find evidence for PtdIns5P acting through the kinase Akt, which is activated by the PI3K pathway and which could stimulate NRF2 (56, 57) and inhibit FOXO (58) signaling.

Pin1-deficient mice display widespread phenotypes of premature aging (31), and mutations in enzymes that regulate cellular PtdIns5P have been associated with the development of diseases with similar phenotypes. Mutations in PIP4Kα have been associated with the development of schizophrenia (73–77), and mutations in myotubularins, which can generate PtdIns5P, are associated with the development of various severe diseases, including X-linked myotubular myopathy and Charcot-Marie-Tooth syndrome (78, 79). Furthermore, the phenotypes observed in Pin1-knockout mice also suggest that Pin1 might regulate the activity of other phosphoinositide-metabolizing enzymes.

We analyzed the amounts of different phosphoinositides in the wild-type and Pin1−/− MEFs in the absence and presence of H2O2. In addition to the increase in PtdIns5P, we found that the amounts of PtdIns(3,4)P2 induced by H2O2 were significantly increased in Pin1−/− MEFs, suggesting that Pin1 might regulate either the dephosphorylation of PtdIns(3,4,5)P3 or PtdIns(3,4)P2 or the synthesis of PtdIns(3,4)P2. PtdIns(3,4)P2 is important in Akt signaling (80), and its regulation by Pin1 warrants further investigation. The use of the affinity purification strategy described here followed by the specific measurement of lipid kinase or phosphatase activity should be useful in identifying how PtdIns(3,4)P2 might be regulated by proline-directed phosphorylation and Pin1.

Signaling by intracellular H2O2 leads to pleiotropic phenotypes. Low concentrations of H2O2 can stimulate proliferation and enhance cell function, whereas higher concentrations can induce cell senescence and apoptosis. The modification of specific amino acid residues in the context of the correct protein microenvironment enables tight regulation of specific signaling pathways by H2O2. Our studies suggest
that Pin1 and PtdIns5P signaling modulates enzymes that regulate the cellular accumulation of ROS, which in turn dictates the cellular sensitivity to oxidative stress and thus may be important for aging, inflammatory responses, tumor development, and neurodegeneration.

References


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68. W. Li, M. R. Jain, C. Chen, X. Yue, V. Hebb, R. Zhou, A. N. Kong, Nrf2 Possesses a


Supplementary materials

Fig. S1. Binding of PIPK4β peptides to Pin1 requires the Pin1-WW domain.

Fig. S2. Phosphorylation of Thr_{322} and Ser_{326} of PIP4Kβ contributes to its interaction with Pin1.

Fig. S3. Antibodies recognizing phosphorylated Ser_{326} peptide show reduced recognition of peptides phosphorylated at both Thr_{322} and Ser_{326}.

Fig. S4. The localization, abundance, and rate of degradation of PIP4Ks are not altered when Pin1 abundance is reduced.

Fig. S5. H_{2}O_{2} treatment increases the abundance of PtdIns5P, which is further increased by knockdown of PIP4Kα and PIP4Kβ.

Fig. S6. PIP4Kβ and Pin1 colocalize in the nucleus.

Fig. S7. Reexpression of Pin1 in Pin1−/− MEFs reconstitutes sensitivity to H_{2}O_{2}.

Fig. S8. Knockout of Pin1 does not alter the kinetics of Akt activation in response to insulin or H_{2}O_{2}.

Fig. S9. Overexpression of PIP4Kα in wild-type or Pin1−/− MEFs does not alter their rate of proliferation.

Fig. S10. Overexpression of PIP4Kα in wild-type or Pin1−/− MEFs does not alter the expression of p27 or cyclin D1.

Table S1. shRNA targeting sequences and qRT-PCR primer sequences.
Supplementary Figure 1. Binding of PIPK4β peptides to Pin1 requires the Pin1-WW domain. The following peptides were coupled to the SPR chip: 1. control peptide CLCSYGTPPDSP, 2. P-Thr^{322}CLCSYGT(p)PPDSP, 3. P-Thr^{322}/P-Ser^{326}CLCSYG(p)TPPD(p)SPGNL, 4. P-Ser^{326}CSYGTPPDSP(p)PGNLL, 5. CSYGTPPDSPGNLL, 6. empty. Interaction with different concentrations of either GST-Pin1-WW or GST-Pin1-PPI was measured. The binding curves were referenced by subtracting the response units obtained in the reference channel (empty channel). GST-Pin1-PPI interacted similarly to wild-type Pin1; however, no interaction was observed with GST-Pin1-WW.
**Supplementary Figure 2.** Phosphorylation of Thr\textsuperscript{322} and Ser\textsuperscript{326} of PIP4K\textbeta{} contributes to its interaction with Pin1. (A) Agarose beads alone or beads coupled to nonphosphorylated or phosphorylated peptides as described below were used to affinity purify Pin1 from lysates of HeLa cells expressing HA-Pin1 (1 mg). Bound proteins were separated by SDS-PAGE and immunoblotted with an HA antibody coupled to HRP (top). Total lysates expressing HA Pin1 were immunoprobed for HA Pin1 were immunoprobed for HA (bottom). The last lane is lysate from nontransfected HeLa cells. Beads, bead without a peptide; S326, CSYGTPPDSPGNLL; P-326, P-Ser\textsuperscript{326}CSYGTPPDS(p)PGNLL; T322, CLCSYGTPPDSP; P-322, P-Thr\textsuperscript{322}CLCSYGTPPDSP; P-322/P-326, P-Thr\textsuperscript{322}/P-Ser\textsuperscript{326}CLCSYG(p)TPPD(p)SPGNL. (B) HA-tagged wild-type PIP4K\textbeta{} or PIP4K\textbeta{} mutated at Ser\textsuperscript{326} to an alanine were expressed and stimulated with UV irradiation as indicated and immunopurified using an HA antibody. The eluted proteins were separated by SDS-PAGE and blotted to nitrocellulose. The blot was then overlaid with GST-Pin1 and the direct interaction was visualized using a GST-antibody (bottom). The blot was stripped and re-probed with an HA antibody to visualize total PIP4K\textbeta{} (top). These data show that Pin1 interacts directly with PIP4K\textbeta{} in a manner enhanced by the presence of phosphoSer\textsuperscript{326} and UV irradiation. (C) HeLa cells were transfected with the indicated cDNAs and irradiated with 100J/m\textsuperscript{2} UV irradiation or left as control. Lysates were immunoprecipitated with an HA antibody and immunoblotted with the indicated antibodies. These data show that in HeLa cells mutation of both Thr\textsuperscript{322} and Ser\textsuperscript{326} to alanine are required to reduce Pin1 binding to PIP4K\textbeta{}.
Supplementary Figure 3. Antibodies recognizing phosphorylated-Ser\(^{326}\) peptide show reduced recognition of peptides phosphorylated at both Thr\(^{322}\) and Ser\(^{326}\). Peptides [P-Ser\(^{326}\) CSYGTPPDS(p) PGNLL (top panel) or P-Thr\(^{322}\)/P-Ser\(^{326}\) CLCSYG(p)TPPD(p)SPGNL (bottom panel)] were coupled to the SPR chip and tested for their interaction with the indicated dilutions of the phospho-S326 specific antibody. The antibody interacted well with the singly phosphorylated peptide but the interaction with the doubly phosphorylated peptide was attenuated.

Supplementary Figure 4. The localization, abundance, and rate of degradation of PIP4KS are not altered when Pin1 abundance is reduced. (A) U2OS cells were transfected with nontargeting or Pin1-targeting siRNAi as indicated and the cells were treated with UV irradiation for the times shown. Lysates were then immunoblotted with the indicated antibodies (right). (B) Lysates (1 mg) from wild-type or Pin1\(^{-/-}\) MEFs were immunoprecipitated with the indicated antibodies (top) and precipitates were immunoblotted with either a PIP4K\(\beta\)-specific antibody (left blot) or a PIP4K\(\alpha\)-specific antibody (right blot). These data show that the levels of PIP4K\(\alpha\) and \(\beta\) are not changed in Pin1\(^{-/-}\)-MEFs. (C) U2OS cells were transfected with nontargeting or Pin1-targeting siRNA as indicated and were treated with cycloheximide for the times shown to determine the rate of degradation of PIP4K\(\beta\). Lysates were then immunoblotted with the indicated antibodies. (D) Wild-type or Pin1\(^{-/-}\)-MEFs were used to derive nuclear (N), cytosolic (C) or membrane (M) fractions before (-) or after (+) treatment with UV irradiation. Each fraction was separated by SDS-PAGE and immunoblotted as indicated.
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Supplementary Figure 5. H$_2$O$_2$ treatment increases the abundance of PtdIns5P, which is further increased by knockdown of PIP4K$\alpha$ and PIP4K$\beta$. (A). H$_2$O$_2$ dose-dependently increases the level of PtdIns5P in Pin1$^{-/-}$ MEFs. Cells were treated with increasing concentrations of H$_2$O$_2$ as indicated and then the cellular amount of PtdIns5P was determined. Assays were carried out in biological triplicates and the data represent mean ± SD. (B). Knockdown of PIP4K$\alpha$ and PIP4K$\beta$ increases both basal and H$_2$O$_2$–induced PtdIns5P in Pin1$^{-/-}$ MEFs (right graph) qRT-PCR was performed to confirm knockdown of both PIP4K$\alpha$ and PIP4K$\beta$ (left graph). The data represent the average ± SD of biological triplicates. Statistical significance was determined using a Student t-test.
Supplementary Figure 6. PIP4Kβ and Pin1 colocalize in the nucleus. HeLa cells were transfected with GFP-PIP4Kβ and HA-Pin1 and cells were fixed and stained with an HA antibody and DAPI to visualize the nuclei. The channels are shown individually and the merge represents the GFP and HA channels. These data demonstrate that Pin1 and PIP4Kβ predominantly colocalize in nuclear speckles.
Supplementary Figure 7. Re-expression of Pin1 in Pin1$^{-/-}$ MEFs reconstitutes sensitivity to H$_2$O$_2$. Pin1$^{-/-}$ MEFs transduced with the constructs shown above [empty vector (EV) or Pin1 (HA-Pin1)] were assessed for their sensitivity to H$_2$O$_2$ using a clonogenic assay. Typical images of the colonies are shown and quantitated and presented in graphical form below. The immunoblot demonstrates the transduction and expression of Pin1 and actin as control. These data show that re-expression of Pin1 induces oxidative stress sensitivity in Pin1$^{-/-}$ MEFs. These data are representative of two experiments and show the average ± SD of triplicate measurements.
Supplementary Figure 8. Knockout of Pin1 does not alter the kinetics of Akt activation in response to insulin or H$_2$O$_2$. Wild-type or Pin1$^{-/-}$ MEFs were stimulated with insulin (A) or H$_2$O$_2$ (B) for the indicated times. Lysates were prepared and immunoblotted with the antibodies as shown (right). These data demonstrate that insulin and H$_2$O$_2$ stimulation led to phosphorylation of Akt with comparable kinetics in both wild-type and Pin1$^{-/-}$ MEFs.

Supplementary Figure 9. Overexpression of PIP4Kα in wild-type or Pin1$^{-/-}$ MEFs does not alter their rate of proliferation. Growth curve of MEFs measured using Alamar Blue. Pin1$^{-/-}$ MEFs grow slower than wild-type MEFs. Overexpression of PIP4Kα does not influence cell growth.
Supplementary Figure 10. Overexpression of PIP4Kα in wild-type or Pin1−/− MEFs does not alter the expression of p27 or cyclin D1. Wild-type or Pin1−/− MEFs transduced with empty vector or PIP4Kα were stimulated as indicated with 100 µM H₂O₂ for 8 hours or kept as control. qRT-PCR was performed using primers for p27 (A) or cyclin D1 (B). These data demonstrate that PIP4Kα overexpression does not influence the changes in p27 or cyclin D1 expression induced by the deletion of Pin1.
**Supplementary Table 1.** shRNA targeting sequences and qRT-PCR primer sequences. Human genes are designated by h and mouse by m.

### pLKO.1 shRNA targeting sequences:

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### RT-PCR primer sequences:

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<td>mP27</td>
<td>5'-gaggaggtgtcagggagag-3’</td>
<td>3'-tctgtcgttgtgccttc-5’</td>
</tr>
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