Promyelocytic Leukemia Protein Controls Cell Migration in Response to Hydrogen Peroxide and Insulin-like Growth Factor-1

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The promyelocytic leukemia protein (PML) was originally identified as part of a chromosomal translocation in acute promyelocytic leukemia (APL). Since its discovery, PML has been found to play diverse roles in different cellular processes. Notably, PML has anti-proliferative and pro-apoptotic activity that supports its role as a tumor suppressor. We have previously shown that the peptidyl-prolyl isomerase Pin1 is able to affect cell proliferation and hydrogen peroxide (H$_2$O$_2$)-mediated cell death through modulation of the steady-state levels of PML. We have extended these studies to show that the interaction between PML and Pin1 is targeted by multiple extracellular signals in the cell. We show that H$_2$O$_2$ up-regulates and IGF-1 down-regulates PML expression in a Pin1-dependent manner. Interestingly, we found that H$_2$O$_2$- and IGF-1-mediated alteration in PML accumulation regulate MDA-MB-231 cell migration. Furthermore, we show that the control of cell migration by PML, and thus H$_2$O$_2$ and IGF-1, results from PML-dependent decreased expression of integrin β1 (ITGB1). Knockdown of Pin1 leads to decreased cell migration, lower levels of ITGB1 expression and resistance to IGF-1- and H$_2$O$_2$-induced changes in cell migration and ITGB1 expression. Taken together, our work identifies PML as a common target for H$_2$O$_2$ and IGF-1 and supports a novel tumor suppressive role for PML in controlling cell migration through the expression of ITGB1.

The promyelocytic leukemia protein (PML)$^3$ was originally discovered as part of a chromosomal translocation in acute promyelocytic leukemia (APL) that results in expression of the PML-RARα fusion oncoprotein (1). Since then, PML has been found to be involved in numerous cellular mechanisms including transcriptional regulation, viral infection, replicative senescence and apoptosis (2–4). Importantly, PML has been implicated as a tumor suppressor in APL and in other forms of cancer. PML protein levels are down-regulated in human cancers of many histological origins without a corresponding decrease in its transcript levels (5). Loss of PML sensitizes mice to physically and chemically induced tumorigenesis. This sensitization correlates with defects of PML-$^{-}$/- mouse embryo fibroblasts (MEFs) in apoptotic responses to stimuli such as type I and II interferons, gamma radiation, and ceramide (6, 7). Additionally, several mouse models of cancer have shown that loss of PML sensitizes mice to increased tumorigenesis, whereas increased expression of PML has a preventative effect on tumor growth (8–13).

PML has no known enzymatic activity and is a member of the RING-B-box coiled-coil (RBCC) family of proteins (14). The RBCC motif of PML is important for protein-protein interactions and mediates post-translational modifications that regulate PML activity and stability. One of the best characterized of these post-translational modifications is sumoylation of PML. Sumo modification of PML is an important mediator of PML nuclear body (NB) dynamics (15). PML NBs are unique subnuclear domains that act as depots for more than 50 known proteins including p53, CBP, NFκB, HDAC7, Pin1, and Daxx (16–20). The localization of most proteins to PML NBs is signal-dependent, though a few proteins, such as Daxx, appear to be constitutive members of these structures. Importantly, these structures are lost in PML-$^{-}$/- mice and can be reconstituted with exogenous PML, suggesting a critical role for PML in their formation (16, 21–23). PML NBs are disrupted in several diseases including APL, some disorders involving DNA damage and a few neurodegenerative diseases (24). The roles of PML in most cellular mechanisms rely on the presence of PML NBs. Because PML is the only known protein required for PML NB formation, it is important to understand how PML is regulated to gain better insight into NB dynamics.

We have previously characterized an interaction between PML and the peptidyl-prolyl cis-trans isomerase Pin1 (19). The result of this interaction is a decrease in the stability and steady-state levels of PML protein. Interestingly, the interaction of PML with Pin1 occurs in a phosphorylation-dependent manner, whereas sumoylation of PML by Sumo1 appears to prevent the interaction. Functionally, we have shown that modulating this interaction in MDA-MB-231 breast cancer cells affects cell proliferation and cell death in response to hydrogen peroxide (H$_2$O$_2$). Hydrogen peroxide is a reactive oxygen species that exists in the cell due to product release from normal metabolic processes as well as from the uptake of environmental causes.
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FIGURE 1. H$_2$O$_2$ treatment results in increased PML protein accumulation. A, MDA-MB-231 cells were treated with H$_2$O$_2$ for 60 min followed by immunostaining with anti-PML antibodies. B, MDA-MB-231 cells were treated with H$_2$O$_2$ for 2 h followed by immunostaining with anti-PML antibodies. Numbers of PML NBs in each cell were scored. The data are derived from two independent experiments; each includes 12 fields from a total of more than 200 cells. C, MDA-MB-231 cells were treated with H$_2$O$_2$ for the times and concentrations indicated. Whole cell lysates were prepared and subjected to SDS-PAGE followed by immunoblotting with anti-PML and anti-α-tubulin antibodies. Tubulin served as a loading control. D, MDA-MB-231 cells were treated with or without 100 μM H$_2$O$_2$ for 120 min. RNA was isolated and analyzed by standard RT-PCR and real time RT-PCR to analyze changes in the expression of PML. There was no significant difference between the treated and untreated samples. GAPDH was used as an internal control. Error bars indicate S.D.

including cigarette smoke, coffee, radiation, and visible and ultraviolet light. Oxidative stress occurs from the accumulation of reactive oxygen species in the cell. If these species are not cleared, the cell will undergo cell death (25). Specifically, PML antagonizes cell proliferation and promotes hydrogen peroxide-induced cell death. Presumably, many of these cellular responses are a result of changes in PML NB number, and composition.

Cancer progression often results in metastasis, which relies on the ability of the tumor cells to migrate into the blood stream. Cell migration can be stimulated extracellularly by many factors such as growth factors and ultimately leads to cell movement due to changes in cell surface adhesion proteins, such as the integrins. For example, insulin-like growth factor-1 (IGF-1) stimulates not only proliferation, but also migration, invasion and adhesion in several cell types (26). IGF-1 has been implicated in cancer risk in human prostate, colorectal, and breast cancer, though its role in breast cancers is the best studied (27–30). Furthermore, IGF-1 has been shown to increase Pin1 expression, which suggests it may control PML expression (31). In MDA-MB-231 cells, which are ERα-negative, IGF-1 is a potent stimulator of cell migration and invasion, but has no mitogenic or anti-apoptotic activities (32). Thus, IGF-1 treatment of MDA-MB-231 cells provides an excellent system in which to study the possible effects of PML on cell migration.

Our current work focuses on determining upstream stimuli that modulate the interaction between PML and Pin1, and what role PML plays in the effects of these stimuli on cell activity. Importantly, we have found that hydrogen peroxide and insulin-like growth factor (IGF-1) can both regulate the interaction between PML and Pin1 and the steady-state protein levels of PML. In doing so, we have identified a previously uncharacterized role for PML in regulating cell migration.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—MDA-MB-231 cells were grown at 37 °C in 7% CO$_2$ in 1× Dulbecco’s modified Eagle’s medium (DMEM) with 4.5g/liter glucose, l-glutamine, and sodium pyruvate (Cellgro) supplemented with 10% charcoal-stripped fetal bovine serum (FBS), 50 units/ml of penicillin G, and 50 μg/ml of streptomycin sulfate. MDA-MB-231 control short hairpin RNA (shRNA) and pSuper-shPin1 cell lines were a generous gift from Dr. Anthony Means and were created as described in Ref. 19. These were grown in the same medium as regular MDA-MB-231 cells with the addition of 0.5 μg/ml puromycin to maintain selection for cells with stably integrated DNA. MCF-7 cells were obtained from the ATCC and were maintained in Dulbecco’s modified Eagle’s medium containing 10% FBS, 50 units/ml of penicillin G, and 50 μg/ml of streptomycin sulfate at 37 °C and 7% CO$_2$. IGF-1 was used at either 20 or 100 ng/μl as described and was purchased from Sigma. Prior to IGF-1 treatment, cells were grown for 24 h in serum-free medium (0.5% FBS). Hydrogen peroxide was purchased from Sigma, filter-sterilized, and used at the indicated concentrations in regular medium.

Plasmid Construction—CMX-HA-PML 4, CMX-HA-PML 4 (4×), pGEX4T-1-Pin1, and CMX-GFP have been described previously (19).

Immunofluorescence Microscopy—Immunostaining was carried out as described previously with the following changes (19). Primary antibody incubation with anti-PML (Santa Cruz Biotechnology, PG-M3) was carried out at 4 °C overnight. Secondary antibodies used are Alexa Fluor 488 (Invitrogen). All images were taken with a Leica Wetzlar Gmbh microscope. Data acquisition was done with SPOT camera and software (Diagnostic Instruments, Inc.).
Western Blotting—MDA-MB-231 cells were treated with the indicated drug or transfected as indicated with \( \geq 5 \) µg of plasmid DNA using Lipofectamine 2000 following the manufacturer’s protocol (Invitrogen). Whole-cell lysates were prepared at the indicated time after \( \text{H}_2\text{O}_2 \) or IGF-1 treatment or 48 h after transfection using radioimmunoprecipitation assay (RIPA) buffer (1X phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS plus protease inhibitors. After 90 min of incubation with the RIPA buffer, insoluble components were removed, and the resulting lysates were separated by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes, and products were visualized by immunoblotting. Blocking was performed in 12.5% milk in 1X phosphate-buffered saline with 0.1% Tween (PBST) and primary and secondary antibodies were incubated in 5% milk/PBST. Primary antibody incubation was carried out overnight at 4°C. Detection was performed using an ECL detection kit (Pierce). Antibodies used are: PML (Santa Cruz Biotechnology, H-238), \( \alpha \)-tubulin (Sigma, B-5-1-2), GFP (Santa Cruz Biotechnology, B-2), HA-HRP (Roche), and ITGB1 (Santa Cruz Biotechnology, N-20).

**GST Pulldown**—The GST pull-downs were performed as previously described using GST alone or GST-Pin1 proteins (19).

**RNA Isolation and RT-PCR**—Total RNA was isolated from MDA-MB-231 cells using a PrepEase RNA Spin kit (USB). cDNA was synthesized using Superscript III reverse transcriptase according to the manufacturer’s protocol (Invitrogen). PCR was performed using Taq polymerase and standard techniques according to the manufacturer’s protocol (Invitrogen). Sequences for PML, ITGB1, and GAPDH primers are available upon request. For semi-quantitative real-time PCR, reactions were performed using iQ SYBR Green Supermix (Bio-Rad) and run and analyzed as previously described (33).

**siRNA**—MDA-MB-231 cells were grown to 60–70% confluence in 6-well cell culture plates in the medium noted above without the antibiotics. Cells were treated with either a control random small interfering RNA (siRNA) oligonucleotide or siRNA directed against PML or Pin1 (Dharmacon), according to the manufacturer’s protocol using the Dharmafect 1 reagent. Each well received 10 ng total of siRNA. 48 h after transfection, the medium was changed. 72 h after transfection, the cells were treated and harvested as indicated.

**Wound-healing Assays**—Cells were grown to 100% confluence in 12-well cell culture plates. At time 0, a 200-µl pipette tip was used to create a scratch in the monolayer. Images of the scratch were taken with a Leica Wetzlar microscope. The cells placed at 37°C and 7% CO₂ for 6 h, and then pictures of the scratch were taken again. Images at 0 and 6 h were analyzed.
lyzed using Adobe Photoshop for the width of the scratch at each time point. Only images of the exact same field were directly compared. The % of migration was calculated as the difference between the width of the scratches divided by the initial width of the scratch times 100%. At least three fields were analyzed for each scratch and each sample was performed in duplicate in each experiment. When anti-ITGB1 (BD Pharmingen, Ha 2/5) was used, it was placed in the medium at time 0 at a concentration previously shown to block ITGB1 function, 10 μg/ml (34, 35). For experiments using siRNA-transfected cells, the cells were split at 72 h after siRNA transfected for the wound assay. Because knockdown of PML or Pin1 increases or decreases cell proliferation, respectively, siControl, siPML-, or siPin1-treated cells were analyzed at equal confluencies. For MDA-MB-231-shLuc and MDA-MB-231-shPin1 cells, cells were treated with H2O2 prior to wounding. H2O2 was not present during the migratory phase. IGF-1 was placed into the medium at time 0 and remained present throughout the assay. Images were recorded at 0 and 8 h.

RESULTS

Hydrogen Peroxide Up-regulates PML Protein Levels—We have previously shown that hydrogen peroxide (H2O2) is capable of regulating cell death of MDA-MB-231 breast cancer cells in a PML- and Pin1-dependent manner (19). We hypothesized that hydrogen peroxide may regulate PML protein levels. To examine this possibility we observed changes in PML expression due to H2O2 treatment by immunofluorescence and immunoblotting. We found that H2O2 treatment resulted in increased PML NB size and number, while there was no apparent change in PML localization (Fig. 1, A and B). A similar trend was observed in MCF-7 cells. Furthermore, the effects of H2O2 on PML were dose- and time-dependent at the concentrations tested. These increases in PML NB number correlated with increased PML protein levels (Fig. 1C). However, we did not observe any changes in PML mRNA (Fig. 1D). Similar data were obtained using MCF-7 breast cancer cells (supplemental Fig. S1).

H2O2 Negatively Regulates the Interaction between PML and Pin1—Because we previously observed regulation of cell death in a PML- and Pin1-dependent manner in response to H2O2, we hypothesized that Pin1 may regulate PML expression in response to H2O2. To investigate this hypothesis, cell lysates of MDA-MB-231 cells expressing HA-PML 4 were treated with hydrogen peroxide and used in GST pulldown assays with bac-
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We have previously shown that IGF-1 down-regulates PML protein levels (31). Therefore, we hypothesized that IGF-1 treatment might result in decreased expression of PML by up-regulating Pin1. Indeed, we found that IGF-1 treatment at 100 ng/μl decreased PML protein levels (Fig. 3A), without altering PML mRNA expression (Fig. 3B). This decreased PML expression was maintained for at least 6 h of treatment. A lower dose of IGF-1, 20 ng/μl, was also capable of down-regulating PML expression (data not shown). Furthermore, immunofluorescence microscopy confirmed that the changes in PML expression due to IGF-1 correlated with changes in PML NB number (Fig. 3C). We also observed the effects of IGF-1 on PML in MCF-7 cells (supplemental Fig. S1). Taken together, IGF-1 treatment of MDA-MB-231 cells results in decreased PML protein and PML NB number.

IGF-1 Positively Regulates the Association of PML with Pin1—We hypothesized that IGF-1 may affect PML protein levels by regulating the association between PML and Pin1. To test this hypothesis, GST pulldown experiments were performed to analyze exogenous HA-PML 4 association with GST-Pin1 in the presence and absence of IGF-1. As expected, we found that there was a modest increase in the amount of PML associated with Pin1 when cells were treated with H2O2 (Fig. 2A). We further investigated if the effects of hydrogen peroxide and IGF-1 on PML would be consistent with a negative regulatory relationship. We observed that when cells were treated with H2O2 (4×) mutant that has a decreased association with Pin1 (Fig. 4B). Likewise, IGF-1 treatment resulted in decreased PML protein levels in the control cells, but did not affect cells expressing shRNA against Pin1 (Fig. 4C). Overall, these data strongly support the idea that IGF-1 down-regulates PML protein levels in a Pin1-dependent manner.

H2O2 and IGF-1 Regulate Cell Migration in a PML-dependent Manner—H2O2 is generally toxic to cells, while IGF-1 has anti-apoptotic and pro-proliferative activity. Because these factors have opposing effects on PML, we hypothesized that they may oppositely regulate some of the same cellular pathways that PML mediates. In MDA-MB-231 cells, IGF-1 does not have strong proliferative activity, but potently stimulates cell migration (32). Whereas H2O2 is not known to control migration, it has been shown to have many effects on tumor cells. We therefore tested whether H2O2 could affect the migration of MDA-MB-231 cells. Using wound-healing assays to assess cell migration, we found that H2O2 was able to inhibit cell migration over a 6-h time period (Fig. 5A, p < .005). As a control, we found no significant difference in cell death between hydrogen peroxide-treated and untreated cells (data not shown).

We further investigated if the effects of hydrogen peroxide and IGF-1 on cell migration were PML-dependent, as we
PML Negatively Regulates Integrin β1 Expression — The effects of PML on migration have not previously been investigated. One main mediator of cell migration in MDA-MB-231 cells is integrin β1 (ITGB1) (36–38). We hypothesized that PML may control cell migration by regulating the expression of ITGB1. To test this, we examined the effects of PML knockdown on ITGB1 expression by siRNA. When PML expression was decreased, we observed increases in both ITGB1 mRNA and protein accumulation by real-time RT-PCR and immunoblotting, respectively (Fig. 6, A and B, p < .005). Similar results were observed in MCF-7 cells (supplemental Fig. S1). These data indicate that changes in PML expression negatively regulate ITGB1 expression. As we have previously shown that Pin1 promotes PML degradation (19), we hypothesize that knockdown of Pin1 will decrease ITGB1 expression and cell migration. Consistent with our hypothesis, we found that MDA-MB-231 cells transfected with siPin1 expressed lower ITGB1 mRNA and protein levels and exhibited decreased migration (Fig. 6, C–E).

PML-dependent Down-regulation of Integrin β1 Expression Mediates PML-induced Changes in Cell Migration — The above data suggest that the effects of PML on cell migration are mediated, in part, by regulating ITGB1 levels. To test this, we inactivated ITGB1 activity by using a well-characterized ITGB1 antibody (34, 35). Our results show that when ITGB1 activity is blocked, knockdown of PML is no longer capable of promoting cell migration (Fig. 7A, compare a 2-fold change for siControl versus a 5.5-fold change for siPML). We observed similar results in MCF-7 cells (supplemental Fig. S1). These results suggest that H2O2 controls cell migration in a PML- and ITGB1-dependent manner. Indeed, H2O2 (Fig. 7B, p < .05) inhibited the expression of ITGB1.

FIGURE 7. The effects of H2O2 on cell migration are mediated through PML control of ITGB1 expression. A, MDA-MB-231 cells transfected with control oligonucleotide or a combination of two siRNAs against PML were subjected to wound-healing assays in the presence of control IgG or anti-ITGB1 antibodies. The results shown indicate changes in six independent fields and are representative of two independent experiments. Results for each independent siPML construct are found in supplemental Fig. S2. B, MDA-MB-231 cells were treated with H2O2, as described in Fig. 1C. Cells were harvested and whole cell lysates or RNA prepared. Whole cell lysates were analyzed by immunoblotting with anti-ITGB1 and anti-α-tubulin antibodies (left panel). Tubulin served as a loading control. Total RNA was analyzed by real-time RT-PCR for ITGB1 expression. GAPDH served as an internal control (right panel). C, MDA-MB-231 cells transfected with control oligonucleotide or siRNA against PML were treated with or without 100 μM H2O2 for 120 min as indicated. Whole cell lysates were analyzed by immunoblotting with anti-ITGB1 and anti-α-tubulin antibodies. Tubulin served as a loading control. D, MDA-MB-231-shLuc and MDA-MB-231-shPin1 cells were treated with H2O2 followed by whole cell lysate preparation and immunoblotting with anti-ITGB1 and anti-α-tubulin antibodies. E, confluent MDA-MB-231-shLuc and MDA-MB-231-shPin1 cells were treated with H2O2 prior to scratch wound-healing assays. Error bars indicate S.D.
Taken together, these data indicate that H$_2$O$_2$ and IGF-1 control ITGB1 expression in a PML-dependent manner, which is likely mediated through Pin1. They further imply that PML-dependent changes in ITGB1 expression are responsible for the effect of hydrogen peroxide and IGF-1 on cell migration.

DISCUSSION

Our results define a novel mechanism whereby PML inhibits MDA-MB-231 cell migration through negative regulation of integrin β1 expression. We show that hydrogen peroxide (H$_2$O$_2$) treatment of MDA-MB-231 cells decreases the association between PML and Pin1, leading to an increase in PML protein levels. This increase correlates with H$_2$O$_2$-mediated decreases in ITGB1 expression and inhibition of cell migration. Conversely, IGF-1 treatment results in decreased PML protein levels in a Pin1-dependent manner, which is important for IGF-1-mediated increased ITGB1 expression and cell migration.

We have previously shown that Pin1 is a negative regulator of PML (19). Our current work supports the idea that modulation of PML expression by Pin1 is a pathway that can be important under multiple circumstances. Consistent with these observations, we demonstrate that Pin1 is required for IGF1-induced ITGB1 expression and cell migration. Similarly, knockdown of Pin1 abrogates H$_2$O$_2$-mediated inhibition of ITGB1 expression and cell migration. H$_2$O$_2$ and IGF-1 do not have effects on PML protein accumulation when Pin1 expression is knocked down, suggesting that modulation of the interaction between PML and Pin1 is a major mode of action by these agents. This is the first report of both a death-inducing agent (hydrogen peroxide) and a pro-proliferation agent (IGF-1) acting through a Pin1-dependent mechanism to control PML protein levels. Our data raise the possibility that other signals may regulate PML expression by modulating its interaction with Pin1 (19). Our current work supports the idea that modulation of PML expression by Pin1 is a pathway that can be important under multiple circumstances.

The data suggest that PML can regulate accumulation of ITGB1 and that H$_2$O$_2$ and IGF-1 also regulate ITGB1 expression. Therefore, we hypothesized that H$_2$O$_2$ and IGF-1 alter ITGB1 abundance by regulating PML. To test this, we examined the effect of H$_2$O$_2$ or IGF-1 on levels of ITGB1 in cells transfected with either control or PML siRNA. As expected, H$_2$O$_2$ decreased ITGB1 expression in the presence of control siRNA, but not in the presence of siRNA against PML (Fig. 7C). Notably, the ability of H$_2$O$_2$ to decrease ITGB1 expression and cell migration was blocked in Pin1 knockdown cells (Fig. 7, D and E).

To test whether ITGB1 mediates IGF-1-induced cell migration, we employed anti-ITGB1 antibodies. We found that the ability of IGF-1 to increase cell migration was blocked by anti-ITGB1 antibodies (Fig. 8A). This observation correlates the ability of IGF-1 to induce ITGB1 expression (Fig. 8B, p < 0.005). Because IGF-1 treatment results in decreased PML protein expression, we hypothesized that exogenous expression of PML would block the effects of IGF-1 on ITGB1 expression. As expected, the addition of exogenous HA-PML 4 to MDA-MB-231 cells abrogated the IGF-1-dependent increase of ITGB1 expression (Fig. 8C). Consistent with the notion that Pin1 negatively regulates PML protein levels, IGF-1 effects on ITGB1 expression (Fig. 8D), and cell migration were abolished when Pin1 was knocked down (Fig. 8E).
sion of PML by siRNA knockdown or IGF-1 leads to lower expression of ITGB1 mRNA and protein accumulation. Furthermore, inactivating ITGB1 function with anti-ITGB1 antibodies blocks PML knockdown-induced cell migration. Conversely, overexpression of PML or induction of PML by H2O2 down-regulates ITGB1 and inhibits cell migration. Collectively, these data support a model in which PML inhibits cell migration, in part, by regulating ITGB1 expression. While there is no evidence to suggest a direct association between PML and the ITGB1 promoter, negative regulation of expression of some genes by PML may reflect a function of PML NBs. For example, the NFκB family member RelA/p65 can be sequestered by PML in PML NBs (20). This prevents it from binding to the DNA, thus inhibiting expression of its target genes. It will be interesting to investigate the mechanisms by which PML inhibits ITGB1 mRNA accumulation.

The negative regulation of migration by PML is one more tumor suppressor function of PML that can be added to its control of proliferation, cell cycle arrest, apoptosis, and senescence. Interestingly, many of the effects of PML on tumor growth are thought to rely on its pro-apoptotic activity (6). Our work suggests that PML may slow cancer progression and metastasis through its modulation of cell migration. A recent study indicated that transplants of mouse embryonic fibroblasts prone to develop fibrosarcomas into nude mice resulted in increased tumor vascularization when PML was also knocked out (8). Because blood vessel formation requires cell migration, it is possible the lack of PML stimulated cell migration contributing to the increased tumor vascularization. Whereas all of our work was performed in MDA-MB-231 breast cancer cells, we anticipate that the findings are applicable to other cell types as well. We have reproduced many of our results in MCF-7 breast cancer cells (supplemental Fig. S1), and we have also observed the interaction between PML and Pin1 in HeLa cervical cancer cells. Because each of these cell lines will respond to extracellular signals differently, the consequences of the interaction between PML and Pin1 and the effect of signaling on PML levels will likely be varied. However, we anticipate that the overall mechanisms will be conserved.

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