PHOSPHORYLATION-DEPENDENT PEPTIDYL-PROLYL CIS/TRANS ISOMERASE PIN1

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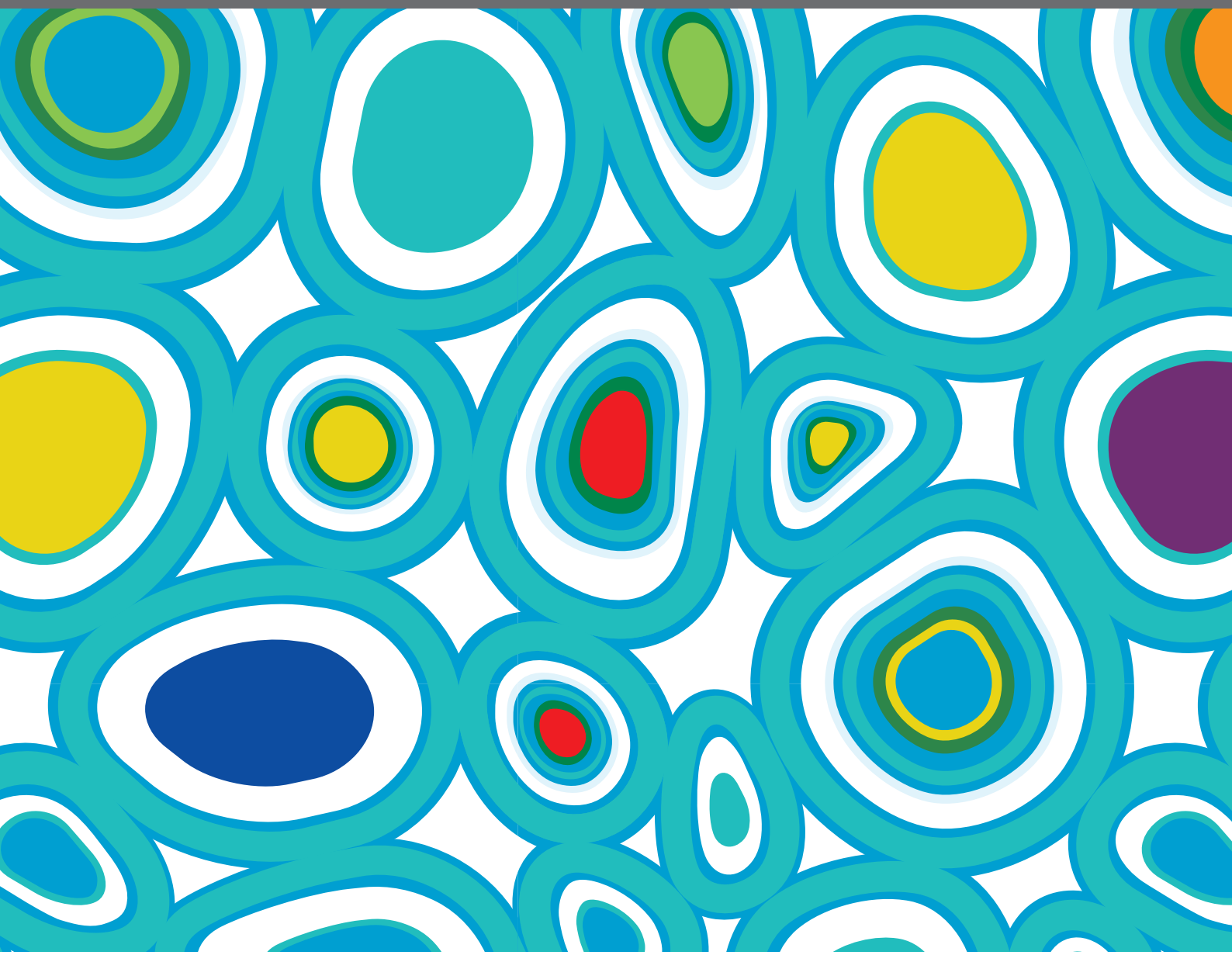

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PHOSPHORYLATION-DEPENDENT PEPTIDYL-PROLYL CIS/TRANS ISOMERASE PIN1

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EDITORIAL
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Editorial: Phosphorylation-Dependent Peptidyl-Prolyl Cis/Trans Isomerase PIN1

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Keywords: PIN1, peptidyl prolyl isomerization, cancer, Alzheimer's disease, phosphorylation

Editorial on the Research Topic

Phosphorylation-Dependent Peptidyl-Prolyl Cis/Trans Isomerase PIN1

PIN1 has been known to isomerize the phosphorylated serine/threonine-proline (pS/pT-P) motif and exert its physiological function by regulating multiple phosphorylated signaling proteins via different mechanisms for one ultimate goal of the cell, for instance osteogenic differentiation, an example not covered by the articles in this Research Topic. In particular, PIN1 regulates osteogenesis via stabilizing RUNX2 or OSX from ubiquitination, or by increasing nuclear retention of β-catenin in Wnt3a-induced osteoblast (Yoon et al., 2013; Lee et al., 2015; Shin et al., 2016). Targeting the role of PIN1 in osteogenesis through pharmacological inhibition has been applied in the study of craniosynostosis due to FGFR2 mutation (S252W) in mice modeling Apert syndrome, suggesting that PIN1 is important for FGFR2 mutant (S252W)-induced RUNX2 activation (Shin et al., 2018). This is one of the most rewarding outcomes of the intensive research into PIN1 since the original discovery of its function in mitosis (Lu et al., 1996). With much accumulated and ever-expanding knowledge, we need up-to-date summaries organized into tables and charts to describe the ever-increasing roles of PIN1 and its binding partners, so that more studies are inspired.

The idea that PIN1 could be used as a molecular switch and the concept of PIN1 catalyzing by lowering the energy barrier for cis- and trans- isomerization for pS/pT-P, accelerating the conversion up to a 1,000-fold, has been previously proposed (Liou et al., 2011). In this Research Topic, Chen et al. summarizes the understanding of how PIN1 is regulated, with a comprehensive table on the post-translational modifications (PTMs) of PIN1, including phosphorylation, oxidation, SUMOylation, and ubiquitination. How PTMs regulate PIN1 enzymatic activity, binding ability, localization, and function, and how the deregulation of PIN1 PTMs contribute to the development of cancer and Alzheimer's disease (AD), are reiterated and conceptualized. Therapeutic options and the challenge for targeting PIN1 PTMs with possible drug candidates are also discussed.

PIN1 promotes cell growth and proliferation, and its aberrant expression and activity are associated with cancer development. The mini review written by Pu et al. highlights PIN1's biological mechanisms, listing the deregulatory factors of PIN1 at transcriptional, translational, and post-translational levels. PIN1's roles manifest in its substrates and their biological activity, protein stability, and cellular localization in different types of cancer. The authors discuss the multifaceted roles of PIN1 in proliferative signaling, growth, invasion, metastasis, and angiogenesis

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during oncogenesis, with an impressive list of the substrates' specific phosphorylation sites. Cheng and Tse's review emphasizes the importance of PIN1 in regulating hepatocellular carcinoma (HCC). PIN1 meddles in HCC development by impairing microRNA biogenesis, enhancing cell proliferation, inhibiting apoptosis, and promoting migration, molecularly involving and interacting with cyclin D1, Hepatitis B virus X-protein, exportin-5, and GLI1. The development of PIN1 inhibitors, such as sorafenib, all-trans retinoic acid (ATRA), arsenic trioxide, and API-1, for HCC treatments are organized in a table. Yu et al. focuses on recent cancer drug development targeting PIN1 in a variety of human malignancies, especially small-molecule compounds. They meticulously list more than 20 PIN1 inhibitors developed by PPIase relevant binding assays, structure-based rational design, and mechanism-based high-throughput screens, revealing the properties of recently discovered PIN1 inhibitors, working mechanisms, kinetics, and limitations in different cancer types.

In addition to the reviews, Huang et al., show in their original research that the treatment of the PIN1 inhibitor, ATRA, reduces PIN1 levels, thereby promoting ERα degradation and decreasing ERK1/2 and AKT activity in tamoxifen-resistant human breast cancer cells. These results suggest that ATRA inhibits multiple PIN1-drived cancer promoting pathways and provide a potential therapeutic strategy for treating drug-resistant cancers.

However, PIN1 sometimes acts in a double-edged sword manner. Makinwa, Musich et al. further elaborate on the complexity of PIN1 in cancer development and provide an anti-tumor role of PIN1 under certain conditions. Nevertheless, they focus on ataxia telangiectasia- and Rad3-related (ATR), recently discovered as a novel PIN1 substrate that plays an important role in DNA damage response. They discuss the PIN1-mediated *cis* to *trans* induced conformational change of ATR that promotes its anti-apoptotic function in certain types of cancer cells. Makinwa, Cartwright et al. have also found that protein phosphatase 2A (PP2A) dephosphorylates the PIN1 binding site of ATR, thereby accumulating the anti-apoptotic *cis* ATR in the cytoplasm. These data describe that PP2A may regulate PIN1 by depleting phosphorylated ATR, eventually causing cell death upon DNA damage.

PIN1 regulates a plethora of transcription factors and transcription cofactors including c-Myc, p53, and b-catenin. These substrates are organized into a table by Hu and Chen. The gene transcription governed by PIN1 substrates contributes to the diverse pathophysiological functions of PIN1, including cancer, neurodegenerative disorders, inflammation, and immune response. Understanding the PIN1-related transcriptional regulation of cell cycles provides new insights into the pathophysiological function of PIN1, and new strategies of therapeutic treatment for several PIN1 disfunction-related diseases. Cohn et al. provides a new perspective and they found that PIN1 interferes with the spatiotemporal dynamics of Myc via stabilizing its pS62. Of special interest is that this association facilitates the localization of phosphorylated Myc in the inner basket of the nuclear pore, thereby affecting the euchromatin, implying an epigenetic regulation.

PIN1 expression and activity are significantly suppressed in the brains of people with Alzheimer's disease (AD). Wang et al., addresses a major impact of PIN1 deregulation in AD development and the many ways in which PIN1 acts on based on currently recognized molecular mechanisms. They also discuss the developing diagnostic and therapeutic strategies targeting PIN1 and its upstream regulators.

In the dynamic interplay between pathogens, such as viruses or parasites, and the host in infectious diseases, host PIN1 can bind in an inter-species manner to the virus core protein. Nishi et al., discover that hepatitis B virus core protein (HBc) is a unique substrate of host PIN1. HBc is stabilized by PIN1 in a phosphorylation-dependent manner. They also show that the pyruvate dehydrogenase phosphatase catalytic subunit 2 (PDP2) can dephosphorylate HBc at the PIN1-binding sites, thereby suppressing PIN1-mediated HBc stabilization, implicating the possibility of designing new antiviral therapeutics based on targeting PIN1.

On the other hand, the parasite-derived PIN1 protein can isomerize host proteins, such as transcription factors. Medjkane and Weitzman discuss the function of parasitic protists *Theileria annulata* secreted PIN1 (TaPIN1), which regulates the activity of host transcription factors c-Jun and HIP1a by manipulating the ubiquitination of host interactor proteins Fbw7 and PKM2. *Theileria* hijacks the host transcriptional pathways to boost host cell proliferation and metabolic activity and maintains their critical nutrients for parasitic proliferation and survival within host cells. Therefore, TaPIN1 can be a critical target for treating infectious disease.

While the Research Topic collections summarize important recent findings of PIN1-mediated biological mechanisms and attempt to organize the vast amount of information relevant to the PIN1 interactions with its substrates, we have to admit that this series does not cover all the PIN1 research fields; as an example, PIN1 regulations of lipid and glucose metabolism (Nakatsu et al., 2020) is not discussed. We hope this Research Topic attracts attention to and questions on the further investigation of PIN1 biology.

AUTHOR CONTRIBUTIONS

JL coordinates, integrates, re-organizes, and finalizes the content of editorials. THL summarizes the manuscripts relevant to cancer and Alzheimer's diseases. FS summarizes the manuscripts relevant to pathogen-host interspecies PIN1 and substrate interactions and transcriptional factors and regulations. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Targeting Pin1 by All-Trans Retinoic Acid (ATRA) Overcomes Tamoxifen Resistance in Breast Cancer via Multifactorial Mechanisms

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Breast cancer is the most prevalent tumor in women worldwide and about 70% patients are estrogen receptor positive. In these cancer patients, resistance to the anticancer estrogen receptor antagonist tamoxifen emerges to be a major clinical obstacle. Peptidyl-prolyl isomerase Pin1 is prominently overexpressed in breast cancer and involves in tamoxifen-resistance. Here, we explore the mechanism and effect of targeting Pin1 using its chemical inhibitor all-trans retinoic acid (ATRA) in the treatment of tamoxifen-resistant breast cancer. We found that Pin1 was up-regulated in tamoxifen-resistant human breast cancer cell lines and tumor tissues from relapsed patients. Pin1 overexpression increased the phosphorylation of ERα on S118 and stabilized ERα protein. ATRA treatment, resembling the effect of Pin1 knockdown, promoted ERα degradation in tamoxifen-resistant cells. Moreover, ATRA or Pin1 knockdown decreased the activation of ERK1/2 and AKT pathways. ATRA also reduced the nuclear expression and transcriptional activity of ERα. Importantly, ATRA inhibited cell viability and proliferation of tamoxifen-resistant human breast cancer cells *in vitro*. Slow-releasing ATRA tablets reduced the growth of tamoxifen-resistant human breast cancer xenografts *in vivo*. In conclusion, ATRA-induced Pin1 ablation inhibits tamoxifen-resistant breast cancer growth by suppressing multifactorial mechanisms of tamoxifen resistance simultaneously, which demonstrates an attractive strategy for treating aggressive and endocrine-resistant tumors.

Keywords: ATRA, Pin1, breast cancer, tamoxifen, ERα

INTRODUCTION

Breast cancer is a leading cause of cancer-related death in female (Chen et al., 2016). In all breast cancer patients, approximately 70% patients are estrogen receptor positive (Deroo and Korach, 2006; Yager and Davidson, 2006; Nilsson et al., 2011). Although selective estrogen receptor modulator such as tamoxifen are effective for ER positive patient, approximately 30% of patients

are not sensitive to tamoxifen treatment at the beginning, and over 50% of initial effective patients finally suffer from tamoxifen-resistance (TAMR) (Osborne and Schiff, 2011). The mechanism of TAMR is still not completely known. The possible molecular mechanisms include, but not limited to, the alteration of estrogen receptor transcriptional co-regulatory proteins (Shao et al., 2004; Girault et al., 2006), cross-talk between receptor tyrosine kinase signaling and estrogen receptor (Stenoien et al., 2001), non-canonical transcriptional activation of estrogen receptor (Anbalagan and Rowan, 2015), the expression of specific microRNAs (Miller et al., 2008), etc. Given that many studies have demonstrated that estrogen receptors play a central role in TAMR (Wijayaratne and McDonnell, 2001; Marsh et al., 2017; Zhang et al., 2017), blocking estrogen receptor related pathways is an attractive strategy to treat TAMR breast cancer.

Pin1 is a peptidyl-prolyl cis/trans isomerase (PPIase), which specifically recognizes pSer/Thr-Pro motifs of proteins and catalyzes their *trans-cis* conformational change (Lu and Zhou, 2007). Pin1 plays a vital role in cancer development by regulating more than 40 oncoproteins and over 20 tumor suppressors, therefore promoting cancer growth and cancer stem cell tumorigenesis (Zhou and Lu, 2016). Pin1 has been found to be up-regulated in tamoxifen-resistant breast cancer (Stanya et al., 2008; Namgoong et al., 2010; Khanal et al., 2012). Overexpression of Pin1 reduces the protein stability of estrogen receptor transcriptional co-regulatory protein SMRT (Stanya et al., 2008), as well as regulates the transcription function of ERα (Rajbhandari et al., 2012, 2015). Knockdown of Pin1 by siRNA inhibits the viability of TAMR breast cancer cells (Namgoong et al., 2010), indicating that Pin1 might be a promising therapeutic target for tamoxifen-resistant breast cancer. However, due to the lack of appropriate Pin1 inhibitors, it is challenging to evaluate the effect of targeting Pin1 on overcoming TAMR. Recently, Wei et al. has discovered all-trans retinoic acid (ATRA) as a specific Pin1 chemical inhibitor (Wei et al., 2015). ATRA has been used to induce differentiation and treat acute promyelocytic leukemia (APL). In APL, ATRA facilitates PML–RAR-α degradation, thereby suppresses APL stem cells (Huang et al., 1988; de The and Chen, 2010; Sanz and Lo-Coco, 2011). Wei et al. (2015) has found that besides RAR, Pin1 is a key target of ATRA in APL and breast cancer. ATRA directly and selectively binds to and degrades active Pin1, thereby inhibiting multiple Pin1-regulated cancer driving pathways.

In the current study, we explored the effects of ATRA in inhibiting Pin1 and treating tamoxifen-resistant breast cancer *in vitro* and *in vivo*. Our experiments showed that Pin1 was up-regulated in tamoxifen-resistant cells and increased ERα protein stability. ATRA treatment accelerated ERα protein turnover, reduced ERα transcriptional activity, and decreased the phosphorylation of AKT and ERK1/2 simultaneously, which further inhibited ERα activation. Thus, ATRA induced the degradation of Pin1 and suppressed cell viability and proliferation of tamoxifen-resistant breast cancer cells. More importantly, slow-releasing ATRA tablets showed remarkable anti-tumor effects in the tamoxifen-resistant xenograft model. Therefore, targeting Pin1 by ATRA promised a new potential approach to treat tamoxifen-resistant breast cancer.

MATERIALS AND METHODS

Cell Culture

The human breast cancer cell lines MCF7 and T47D were purchased from American Type Culture Collection (Manassas, VA, United States) and cultured in DMEM (Thermo Fisher Scientific, Waltham, MA, United States) supplemented with 10% fetal bovine serum (FBS, GBICO). Tamoxifen resistant cell lines (MCF-7R and T47DR) were kindly provided by Dr. Qiang Liu as gift, and were cultured in no-phenol red 1640 medium (Life Technologies, United States) supplemented with 10% charcoal-stripped FBS (cFBS) (HyClone, United States) and 1 μM 4-hydroxytamoxifen (Sigma-Aldrich, St. Louis, MO, United States). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Antibodies, Reagents, and Sequences

Antibodies of ERα was from cell signaling technology (8644) and Abcam (16660). Antibodies of Pin1 was from R&D (MAB2294) and Abnova (MAB12340). Phospho-ERα S118 antibody was from Abcam (ab32396). Phospho-ERα S167 antibody was from cell signaling technology (64508). ATRA was from Sigma (R2625). Tamoxifen was from Sigma (H6278). Pin1 shRNA targeting sequence: CCACCGTCACACAGTATTTAT; Pin1 siRNA-1 targeting sequence: TCAGGCCGAGTGTACTACT; Pin1 siRNA-2 targeting sequence: GCTCAGGCCGAGTGTACTA; RARα siRNA-1 targeting sequence: CCAGCTCACAGAACTGCTT; RARα siRNA-2 targeting sequence: TTCCGCACGTAGACCTT TAGC; ERα siRNA-1 targeting sequence: CAGGCCAAATTCA GATAAT; ERα siRNA-2 targeting sequence: GGTCCAC CTTCTAGAATGT.

Colony-Forming Assays

Six-well plates were seeded 2000 cells per well. Cells were treated with vehicle (DMSO), 10 μM ATRA, 10 μM tamoxifen (TAM) or ATRA plus TAM, and medium were changed every 3 days. 14 days later cells were fixed with methanol, stained with 0.5% crystal violet.

Cell Viability Assays

Cell viability was measured using the Cell Titer Glo reagent (Promega). The cells were plated in 96-well plates at 1×10^3 cells per well and maintained at 37°C. At the indicated time points, cell viability was measured according to the manufacturer's instructions.

Quantitative RT-PCR

Total RNA was extracted from cells using Trizol (life, United States). MMLV kit (life, United States) was used to generate cDNA. Real time PCR were performed using Toyobo SYBR GREEN. The primers used were as follows: Pin1 (forward, 5′- AGCTCAGGCCGAGTGTACTA-3′; reverse, 5′-CCTTGGTCCGGGTGATCTTC -3′); growth regulation by estrogen in breast cancer 1 (GREB1) (forward, 5′-GTGGT AGCCGAGTGGACAAT-3′; reverse, 5′-ATTTG TTTCCAGCC CTCCTT-3′); progesterone receptor (PGR) (forward, 5′-GG

CATGGTCCTTGGAGGT -3′; reverse, 5′-CCACTGGCTGTGG GAGAG-3′); c-Myc (forward, 5′- TACAACACCC GAGC AAGGAC-3′; reverse, 5′-GAGGCTGCTGGTTTTCCACT-3′); β-actin (forward, 5′-GGAAGGGGGACGGGGACAGC-3′; reverse, 5′- GGAGGAGCAAG GAGCGGGAG-3′).

Immunoblot Analysis

Cells were lysed with RIPA buffer containing 0.1% protease inhibitors or phosphatase inhibitors (Life, United States). The supernatant of lysate was separated by electrophoresis and blotted onto a PVDF membrane, then blocked with 5% skim milk at room temperature for 1 h. The blots were incubated with the following antibody at 4°C overnight: ERα (1:1000, CST, #8644); Pin1 (1:1000, R&D, #MAB2294); phospho-ERα S118 (1:1000, CST, #ab32396); AKT (1:1000, CST); Flag-tag (1:5000, Sigma, United States); phospho-AKT (1:1000, CST); phospho-c-Raf (1:1000, CST); phospho-MEK1/2 (1:1000, CST); ERK1/2 (1:1000, CST); phospho-ERK1/2 (1:1000, CST); phospho-ERαS167 (1:1000, CST, #64508); β-Actin (1:2000, CST); GAPDH (1:2000, proteintech). After incubation with HRP-conjugated secondary antibodies at room temperature for 1 h, all blots were detected by an enhanced chemiluminescence (ECL) and were scanned using ChemiDoc^{TM} XRS + imaging system (Bio-Rad, Hercules, CA, United States).

Immunofluorescence

MCF-7R and T47DR cells were fixed in 4% polyoxymethylene at 4°C for 20 min, washed with PBS and permeabilized in 0.1% Triton X-100 at room temperature for 10 min. Cells were then blocked in 10% goat serum at room temperature for 30 min, and incubated with ERα antibody (1:100, Abcam, #16660) in 10% goat serum at 4°C overnight. Cells were washed, incubated with secondary antibodies at room temperature for 1 h, washed, incubated with DAPI at room temperature for 15 min. Slides were then covered with fluorescently quencher 30 μl, sealed and photographed with an Olympus confocal microscope.

Animal Experiments

Nude mice were purchased from Laboratory Animal Service Center, Sun Yat-sen University. The experiment protocol was approved by the Animal Care and Use Committee of Sun Yat-sen University. 2×10^6 MCF-7R cells were mixed with an equal volume of matrigel (Corning) and injected into the mammary fat pads of 4 week-old female BALB/c nude mice. One week later, when tumor size reached ∼100 mm^3, the tumor-bearing mice were randomized into treatment groups. 21-days ATRA tablets were implanted under neck skin. Tamoxifen was injected at 4 mg/kg per day. Tumor volume was measured every 3 days.

Patients and Immunohistochemistry

Tumor samples were obtained from patients with ER positive breast cancer who underwent tamoxifen therapy in Sun Yat-sen Memorial Hospital. All samples were collected from patients with informed consent, and all related procedures were performed with the approval of the internal review and ethics boards of Sun Yat-sen Memorial Hospital. Immunohistochemistry staining for Pin1 and ERα was performed as described previously (Luo et al., 2015; Zhang et al., 2016). Briefly, sodium citrate was used to repair tissue antigen. Incubation of primary antibodies (Pin1, 1:50, Abnova, #MAB12340; ERα, 1:50, CST, #16660) was carried out at 4°C overnight. The slides were incubated with HRP-conjugated secondary antibodies at room temperature for 1 h, washed, visualized with DAB solution, followed by staining with hematoxylin. Immunostaining results was analyzed by ImageJ software.

Statistical Analyses

All data are presented as the means ± SD. Student's *t*-test was used to analysis the significance between two experimental groups, and ANOVA test was used to analysis among three or more groups. $P < 0.05$ was considered significant. All the statistical analyses were performed using SPSS20.

RESULTS

Pin1 Is Up-Regulated in Tamoxifen-Resistant Breast Cancer and Correlates With ERα Expression in Human Breast Cancer Cell Lines and Cancer Tissues

We established tamoxifen-resistant human breast cancer cell lines MCF-7 and T47D by long-term exposure to tamoxifen (Herman and Katzenellenbogen, 1996; Knowlden et al., 2003; Chu et al., 2015). We confirmed the resistance of these cells by showing that the viability of resistance cells was significantly higher than parental cells and apoptosis were remarkable lower in the presence of 1 μM tamoxifen (Chu et al., 2015). We found that both Pin1 protein and mRNA were up-regulated in tamoxifen-resistant MCF-7 (MCF-7R) and T47D (T47DR) cells, comparing to parental cells (**Figures 1A–E** and **Supplementary Figure S5**), which was consistent with previous reports that Pin1 was overexpressed in TAMR human breast cancer tissues (Namgoong et al., 2010; Khanal et al., 2012).

Although ERα was not so indispensable for TAMR cells as for parental cells, depleting ERα still further limited the growth of TAMR cells (Xiong et al., 2017). Indeed, through a variety of mechanisms, TMAR breast cancer cells made full use of remaining ERα to escape from the impact of tamoxifen (Osborne and Schiff, 2005; Johnston, 2010; Marsh et al., 2017). Here we examined the ERα level in TAMR cells, and found that ERα protein was down-regulated in TAMR cells (**Figures 1F–H** and **Supplementary Figure S5**), as shown previously (Stone et al., 2013; Lu et al., 2016). Given that ERα was a known Pin1 substrate which was positively regulated by Pin1 (Rajbhandari et al., 2012, 2015). We asked why Pin1 level was high while ERα level was low in TAMR cells. We found that Pin1 knockdown further decreased ERα level in TAMR cell lines (**Figures 1I–L** and **Supplementary Figure S5**). These results suggest that Pin1 is up-regulated and helps maintain ERα levels in TAMR cells even although ERα levels in these cells are low.

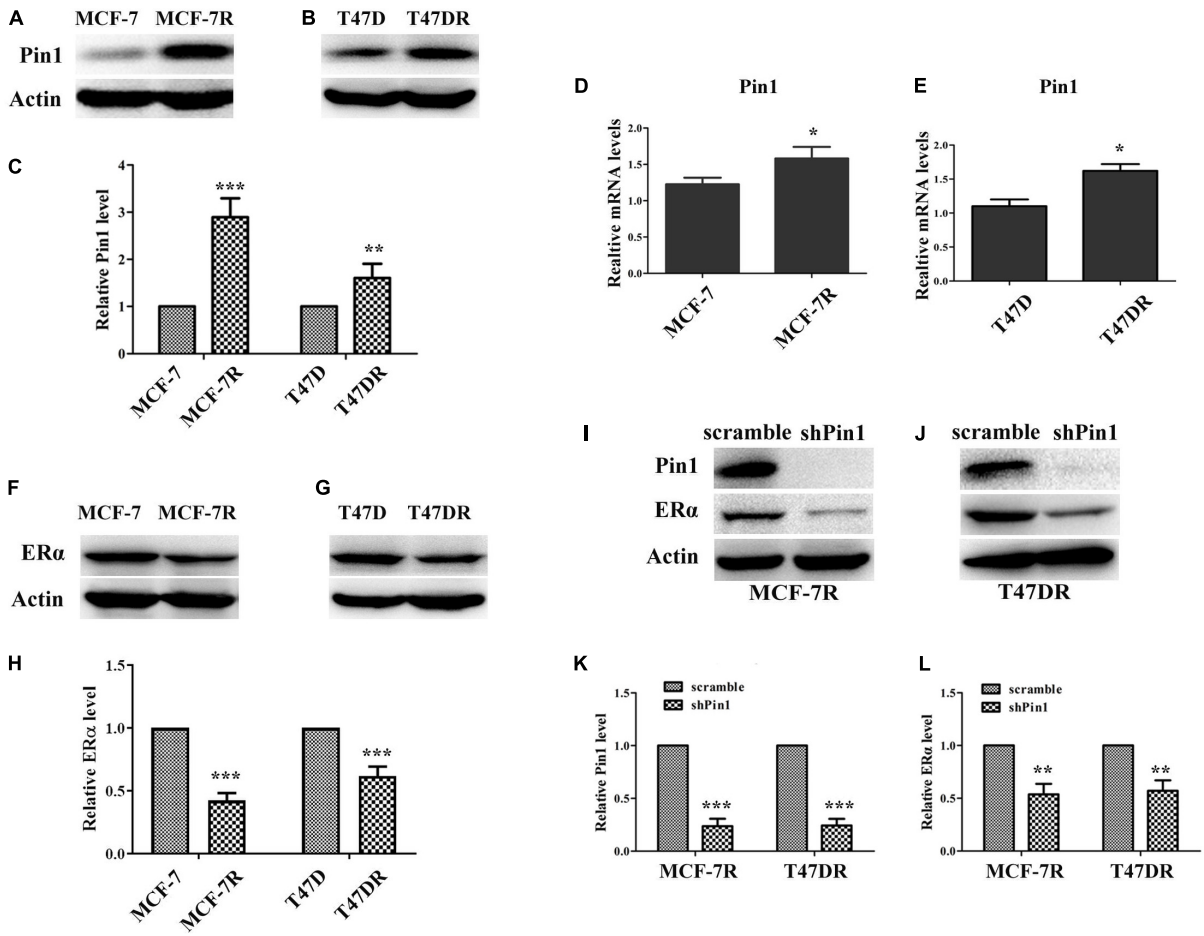


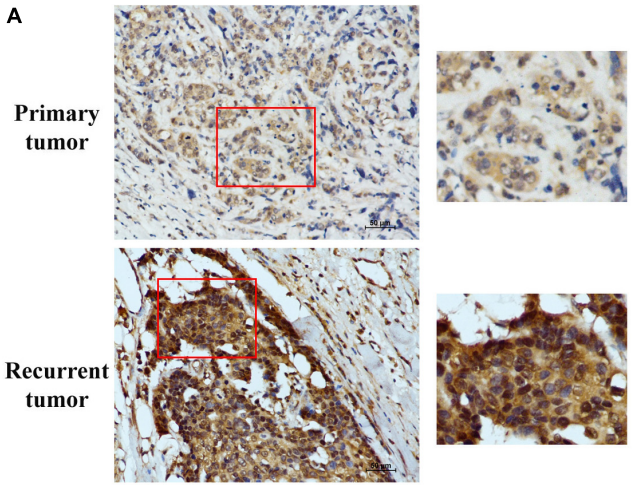
FIGURE 1 | Pin1 is overexpressed in tamoxifen resistant breast cancer cells. **(A,B)** Pin1 is up-regulated in tamoxifen resistant breast cancer cells. Pin1 protein was detected by western blot in parental (MCF-7 and T47D) and tamoxifen resistant (MCF-7R and T47DR) cells. **(C)** Quantification of Pin1 levels in parental and tamoxifen resistant cells. Western blot bands in panels **(A,B)** were quantified by densitometric scan and represented as a relative ratio to control samples. Data are represented as means ± SD for three independent experiments. **(D,E)** Pin1 mRNA is up-regulated in tamoxifen resistant breast cancer cells, as detected by qRT-PCR. **(F–H)** The ERα protein level in parental and resistant breast cancer cells. Western blot bands were quantified in panel **(H)**. **(I–L)** Pin1 knockdown decreases the level of ERα in MCF-7R and T47DR cells. Western blot bands were quantified in panels **(K,I)**. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Next, we detected Pin1 and ERα protein levels in tumor tissues of recurrent ER positive breast cancer patients, who have received tamoxifen treatment. Pin1 protein level was significantly higher in recurrent tumors comparing with primary tumors ($P = 0.004$) (**Figures 2A,B**). More importantly, the expression level of Pin1 was associated with ERα in these tissues (**Figures 2C,D**). Together, Pin1 was up-regulated in both TAMR human breast cancer cell lines and relapsed tumor tissues, which positively correlated with ERα expression.

ATRA-Induced Pin1 Ablation Promotes ERα Protein Degradation

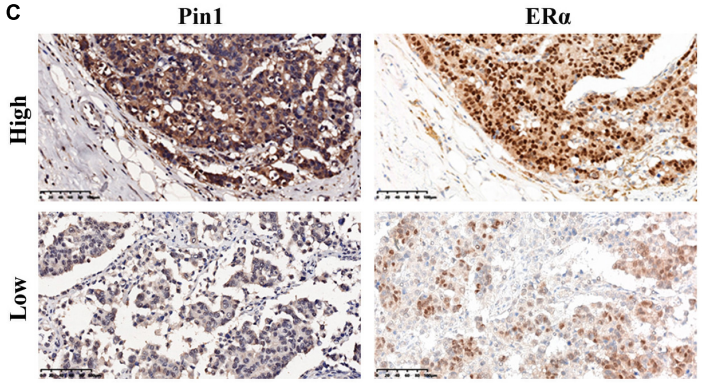
To explore the effect of Pin1 on regulating ERα, and more importantly to test whether ATRA was effective in inhibiting Pin1's function on ERα, we first examined whether overexpressing Pin1 affected ERα protein level. As expected, estradiol (E2) could induce down-regulation of ERα protein (**Figures 3A,B** and **Supplementary Figure S6**), which was due to ligand-dependent degradation (Wijayaratne and McDonnell, 2001; Nonclercq et al., 2004). We found that not only enforced Pin1 expression (Flag-Pin1) rescued the ERα expression, but the Pin1 inhibitor ATRA reversed this effect in both MCF-7 and MCF-7R cells (**Figures 3A,B** and **Supplementary Figures S1A,B**), suggesting that ATRA specifically inhibiting Pin1 from protecting the degradation of ERα.

Next, to confirm the effect of ATRA on ERα protein degradation, MCF-7 and MCF-7R cells stably knocking down Pin1 with shRNA were treated with or without proteasome inhibitor MG132. Contrary to overexpression experiments, Pin1 knockdown promoted E2-induced ERα degradation (**Figures 3C,D** and **Supplementary Figures S1C,D**). Notably, ATRA had the same effects as shPin1 both in MCF-7 and MCF-7R cells (**Figures 3C,D** and **Supplementary Figures S1C,D**). One of the vital regulatory element governing ERα protein turnover is Ser118 phosphorylation of the N-terminus, which is



B

Type of breast cancer	Pin1 staining		Statistics
	High	Low	
Primary	35	43	X^2=8.708
Recurrent	58	20	*P*=0.004



D

		Pin1 staining		
		High	Low	Total
ERα staining	High	42	9	51
	Low	16	11	27
	Total	58	20	78

Pearson correlation coeffient = 0.244 (*P*=0.024)

FIGURE 2 | Pin1 is up-regulated in recurrent tumors and positively correlates with ERα in breast cancer tissues. **(A)** Representative immunohistochemistry image shows that Pin1 expression is higher in recurrent tamoxifen treated tumors than primary breast cancer tissues. Tissues in the red frames in left panels were magnified in the right panels. Scale bars, 50 μm. **(B)** Statistic analysis shows that Pin1 expression is significantly different in primary and recurrent tumors. *P* = 0.004. **(C,D)** Pin1 expression correlates with ERα expression in recurrent breast cancer tissues. Representative image **(A)** showed high and low levels of Pin1 and ERα. Pearson correlation of Pin1 and ERα was analyzed in panel **(B)**. Scale bars, 100 μm.

phosphorylated by ERK1/2 as well as other kinases, and regulated by Pin1 (Rajbhandari et al., 2012, 2014, 2015). Therefore we speculated that ATRA promoted ERα protein turnover through ERα-pS118. Indeed, overexpressing Pin1 increased the phosphorylation of S118 as well as total ERα level, suggesting that Pin1 prevented the turnover of pERα, whereas ATRA reversed Pin1's effect (**Figure 3E** and **Supplementary Figures S1E,F**).

To directly examine whether ATRA could promote the degradation of Pin1 and ERα in tamoxifen-resistant cells, we treated MCF-7R and T47DR cells with ATRA, followed by cycloheximide (CHX) and detected the protein levels at different time points. Our data showed that ATRA promoted the degradation of both Pin1 and ERα in TAMR breast cancer cells in a dose dependent manner (**Figures 3F,G** and **Supplementary Figures S1G–J**). In addition, we treated MCF-7R and T47DR cells with increasing doses of ATRA for different length of time, and found that both Pin1 and ERα protein levels indeed reduced (**Supplementary Figures S2A,B, S9**). Together, these data demonstrate that overexpressing Pin1 in breast cancer cells protects the ERα protein from degradation. ATRA blocks the up-regulated Pin1 in tamoxifen-resistant cells, thereby promoting the degradation of remaining ERα in tamoxifen-resistant cells, which suggests that ATRA may be able to overcome TAMR by eradicate ERα.

ATRA Blocks ERK1/2 and AKT Pathways in TAMR Breast Cancer Cells

Several kinase pathways have been reported to involve in the growth of TAMR breast cancer cells, including AKT and ERK1/2 (Svensson et al., 2005; Garcia-Becerra et al., 2012). AKT phosphorylates ERα on S167 (Sun et al., 2001), and ERK1/2 phosphorylates ERα on S104/S106, S167, and S118 (Ali et al., 1993; Arnold et al., 1995; Endoh et al., 1999; Sun et al., 2001; Sheeler et al., 2003). Notably, phosphorylation of S118 and S167 induces estrogen-independent activation of ERα (Garcia-Becerra et al., 2012). Moreover, AKT and ERK1/2 activity are also regulated by Pin1 (Liao et al., 2009; Luo et al., 2015). Thus,

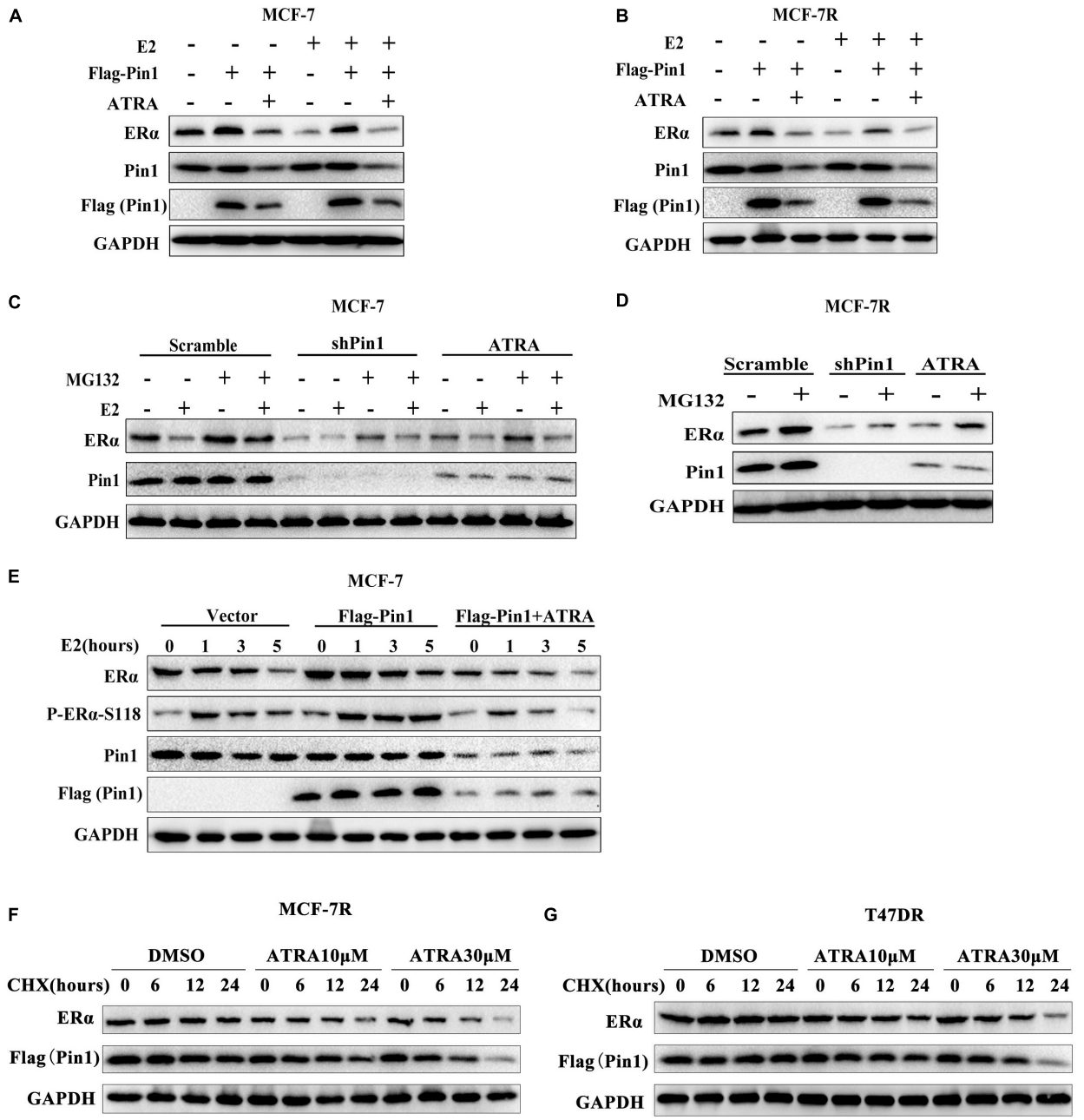


FIGURE 3 | ATRA promotes proteasome-mediated degradation of ERα by blocking Pin1. **(A,B)** Ectopic expression of Pin1 up-regulates ERα, but ATRA abrogates the effect. MCF-7 **(A)** and MCF-7R **(B)** cells transfected with Flag-Pin1 or empty vector were pretreated with 10 μM ATRA for 72 h, followed by 10nM E2 or EtOH treatment for 5 h. **(C,D)** Pin1 knockdown or ATRA treatment promotes ERα degradation. MCF-7 **(C)** or MCF-7R **(D)** were treated with 10 μM MG132 for 4 h before harvesting. **(E)** Overexpression of Pin1 stabilizes pS118-ERα, but ATRA abrogates the effect. MCF-7 cells transfected with Flag-Pin1 or empty vector were pretreated with 10 μM ATRA for 72 h, followed by 10nM E2 treatment for 0, 1, 3, and 5 h. **(F,G)** ATRA promotes Pin1 and ERα degradation in MCF-7R and T47DR cells. Cells were pretreated with ATRA and treated with CHX for indicated time course.

we explored the effects of ATRA in inhibiting these pathways in MCF-7R and T47DR cells. ATRA treatment didn't alter the total expression of AKT or ERK1/2, but reduced the level of phosphorylated AKT, MEK1/2, ERK1/2 and Raf (**Figures 4A,B**). In consistence with decreased activity of these pathways, phosphorylation of ERα on S167 and S118 were also inhibited, resembling the effect of Pin1 knockdown (**Figures 4A,B** and **Supplementary Figure S7**).

To assess the effect of ATRA on ER signaling in normal cells, we treated immortalized mammary epithelial cells MCF-10A and HMLE with different doses of ATRA. These two cell lines expressed very low level of Pin1, comparing to breast cancer cell

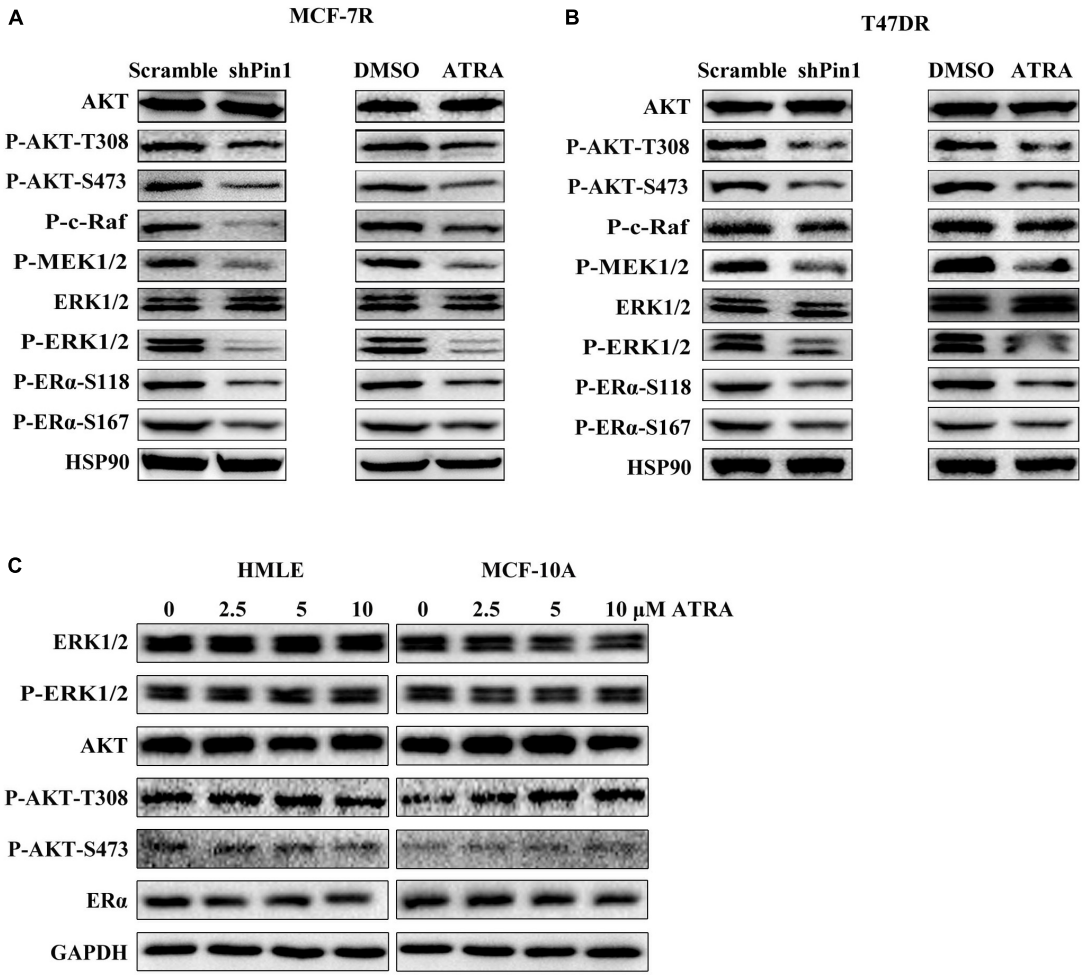


FIGURE 4 | ATRA and Pin1 knockdown inhibits the activation of AKT and ERK1/2 signal pathways. **(A)** Pin1 knockdown or ATRA treatment inhibits the activation of AKT and ERK1/2 signal pathways simultaneously in MCF-7R cells. Cells were infected with lentivirus expressing scramble or Pin1 shRNA, or treated with 10 μM ATRA for 72 h. **(B)** Pin1 knockdown or ATRA treatment decreases the activation of AKT and ERK1/2 signal pathways simultaneously in T47DR cells. **(C)** ATRA treatment doesn't affect ERα, AKT, and ERK1/2 in HMLE and MCF-10A cells.

lines (Wei et al., 2015). We found that ATRA almost had no effect on the protein level of ERα, or P-ERK1/2 and P-AKT (**Figure 4C**). Hence, ATRA had the unique potential to simultaneously block multiple signal pathways in TAMR breast cancer cells.

In addition, RARα, another ATRA target, has been indicated to play a role in tamoxifen resistance of breast cancer (Johansson et al., 2013). Using siRNAs, we knocked down either Pin1 or RARα in MCF-7R cells with or without ATRA treatment (**Supplementary Figures S3A,B**). The total and phosphorylated levels of ERα only decreased in Pin1-silencing, but not RARα-silencing cells (**Supplementary Figures S3C, S10**). As ATRA can still target other proteins to regulate ERα, our data indicate that ATRA may mainly act on Pin1 to regulate ERα.

ATRA Inhibits ERα Transcriptional Activity

To determine whether ATRA affected the transcriptional function of ERα in tamoxifen-resistant cells, we first examined ERα subcellular expression by immunofluorescence. Parental or resistant MCF-7 and T47D cells were treated with 10 μM ATRA for 72 h. The nuclear staining of ERα was dramatically reduced by ATRA treatment in all cell lines (**Figures 5A–D**), indicating a decreased transcriptional activity of ERα. Next, we detected the transcription of three known ERα regulatory genes, including PGR, GREB1, and c-Myc (Lee and Gorski, 1996; Bosch et al., 2015; Wu et al., 2018). The mRNA levels of these three genes were decreased after ATRA treatment (**Figures 5E–H**). These data suggest that ATRA suppresses ERα transcriptional function *in vitro*.

ATRA Inhibits the Viability and Proliferation of Parental and Tamoxifen-Resistant Breast Cancer Cells

Although our data demonstrated that ATRA targeted Pin1 to promote ERα protein degradation, decrease ERα transcriptional

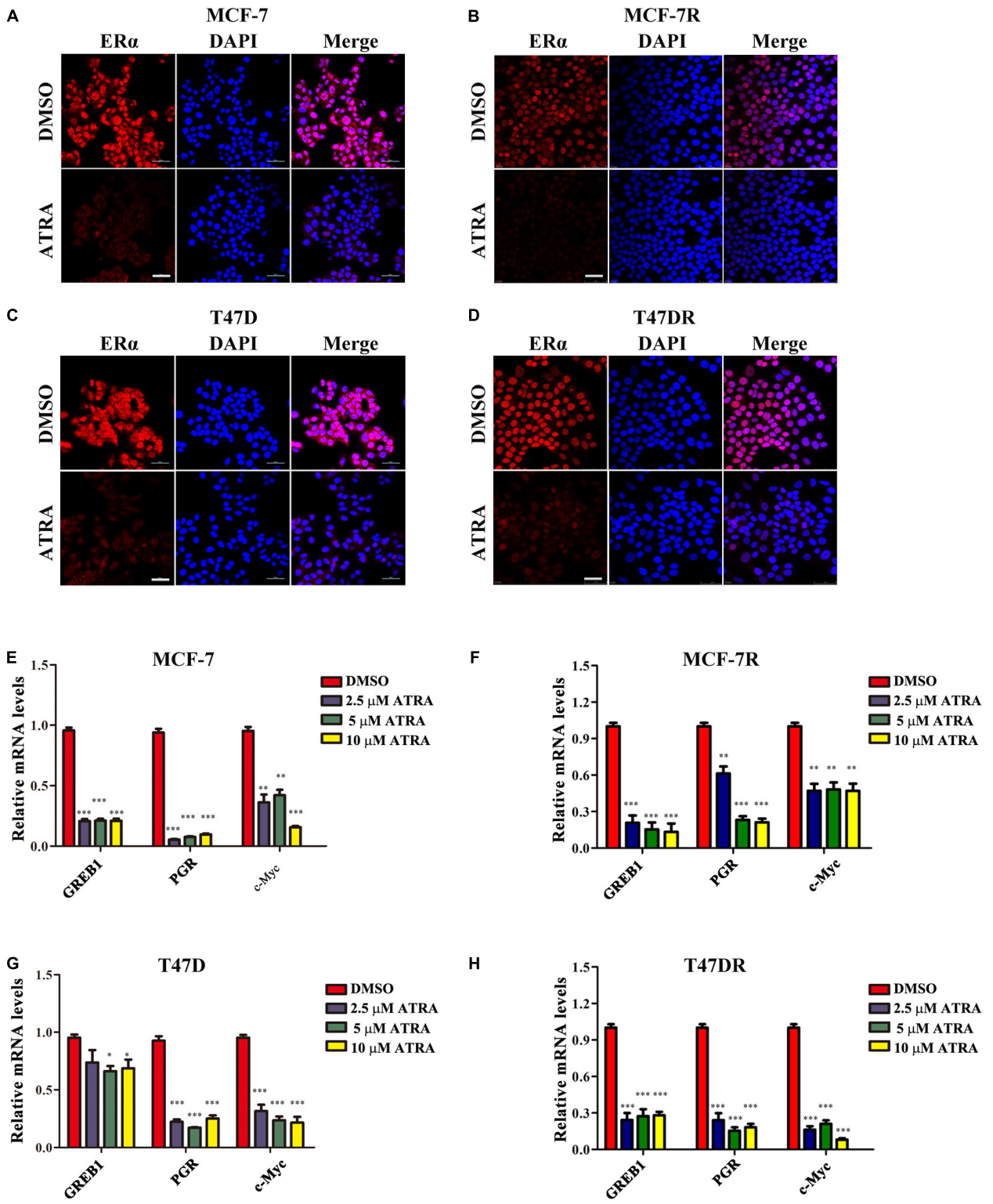


FIGURE 5 | ATRA suppresses nuclear expression and transactivation of ERα. **(A,B)** ATRA decreases the level of nuclear ERα in MCF-7 and MCF-7R. Cells were treated with 10 μM ATRA for 72 h before immunofluorescence staining. Scale bars, 50 μm. **(C,D)** ATRA decreases the level of nuclear ERα in T47D and T47DR. Cells were treated with10 μM ATRA for 72 h before immunofluorescence staining. Scale bars, 50 μm. **(E–H)** ATRA suppresses the transcription of ERα target genes GREB1, PGR, and c-Myc. Cells were treated with ATRA (2.5, 5.0, and10 μM) for 48 h. Expression of ERα downstream genes were detected by qRT-PCR, and normalized to β-ACTIN expression in DMSO treated cells. Error bars denote the SD of three biological replicates, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

activity, and inhibit AKT and ERK1/2 pathway, the therapeutic potential of ATRA in treating tamoxifen-resistant breast cancer was still not clear. We thus evaluated the effects of ATRA on cell viability and foci formation of parental and TAMR cells. As expected, tamoxifen treatment reduced the growth of parental cells, but not the TAMR cells, whereas ATRA suppressed the proliferation of both parental and TAMR cells (**Figures 6A–D**). Moreover, ATRA potentiated tamoxifen therapeutic effect in both parental and TAMR cells (**Figures 6A–D**). In the colony formation experiments, ATRA showed similar effects as in the proliferation assay (**Figures 6E–G**).

We have shown that ATRA-induced Pin1 degradation reduces the protein expression of ERα in tamoxifen-resistant breast cancer cells. To confirm that ERα contributes to tamoxifen resistance in our TAMR cell model, we used siRNAs to knock down ERα in MCF-7R and T47DR. ERα siRNAs dramatically

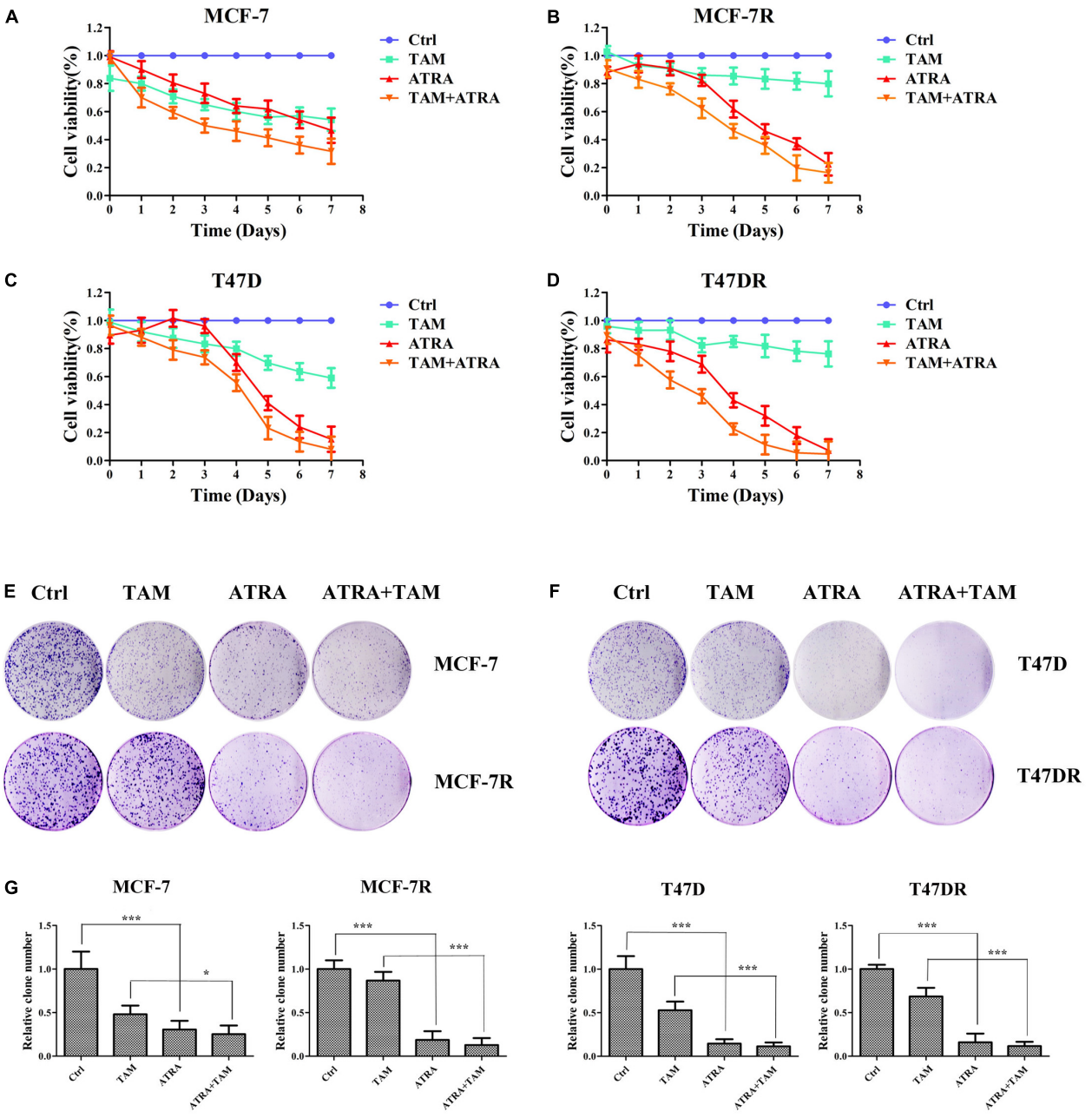


FIGURE 6 | ATRA inhibits the cell growth of tamoxifen resistant breast cancer cells. **(A–D)** The inhibitory effect of ATRA and tamoxifen on cell viability of parental and tamoxifen resistant breast cancer cells. Viability of drug treated cells was normalized to control untreated cells (Ctrl). **(E,F)** The inhibitory effect of ATRA and tamoxifen on foci formation of parental and tamoxifen resistant breast cancer cells. **(G)** Quantification of foci formation by ImageJ software. $^{*}P < 0.05$ and $^{***}P < 0.001$, as determined by Student's *t*-test. Bar graphs are means ± SD by three independent experiments.

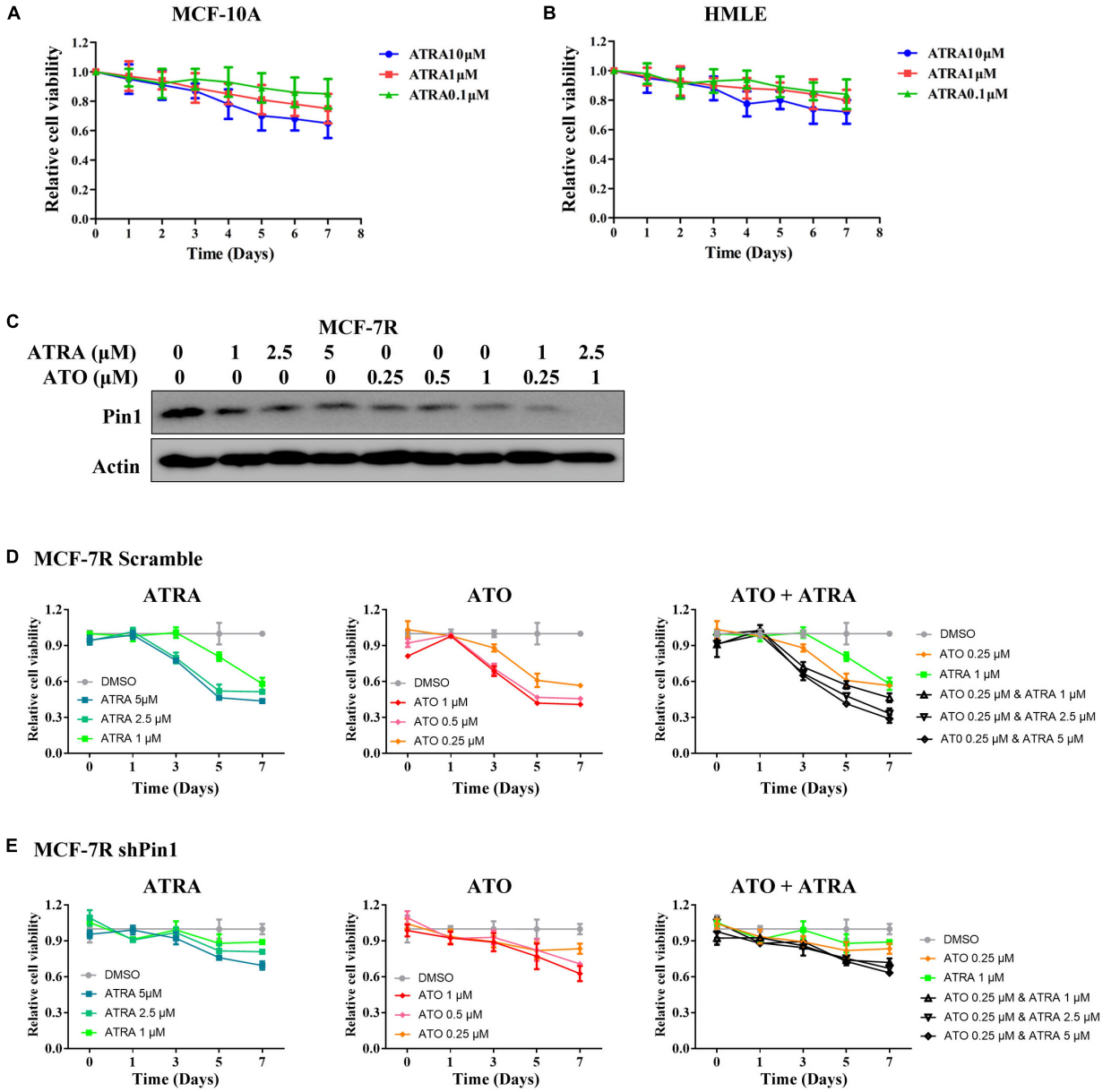


FIGURE 7 | ATRA and other Pin1 inhibitor has limited inhibitory effect on Pin1-low expressing cells. **(A,B)** ATRA shows little inhibitory effect on the viability of MCF-10A and HMLE cells. Cells were treated with increasing doses of ATRA for 7 days. **(C)** The effect of ATRA and ATO on reducing the level of Pin1 protein in MCF-7R cells. The concentration of ATRA and ATO were added to the culture medium as indicated for 48 h. **(D,E)** ATRA and ATO show much less inhibitory effect on the proliferation of shPin1 cells than that of control MCF-7R cells. MCF-7R cells stably expressing scramble or Pin1 shRNA were treated with ATRA and ATO for 7 days.

suppressed the proliferation of these cells upon tamoxifen treatment (**Supplementary Figures S4A,B, S10**), suggesting that ERα indeed contributed to TAMR in these cells.

To investigate the effects of ATRA on Pin1-low cells, we treated MCF-10A and HMLE with different doses of ATRA. ATRA exhibited very limited inhibitory effects on cell viability of these epithelial cells (**Figures 7A,B**), likely because ATRA selectively targets active Pin1 in cancer cells, but not in normal cells with low Pin1 levels (Wei et al., 2015). Thus these results demonstrated that ATRA inhibits cell growth of TAMR breast cancer, with little effects on normal cells.

We also treated MCF-7R and T47DR cells that had knocked down Pin1 with ATRA and ATO, a newly identified Pin1 inhibitor (Kozono et al., 2018). Both ATRA and ATO showed much less inhibitory effect on the proliferation of shPin1 cells than that of control MCF-7R cells (**Figures 7C–E** and **Supplementary Figure S8**). Notably, although either ATRA or ATO could inhibit the proliferation of TAMR cells, the

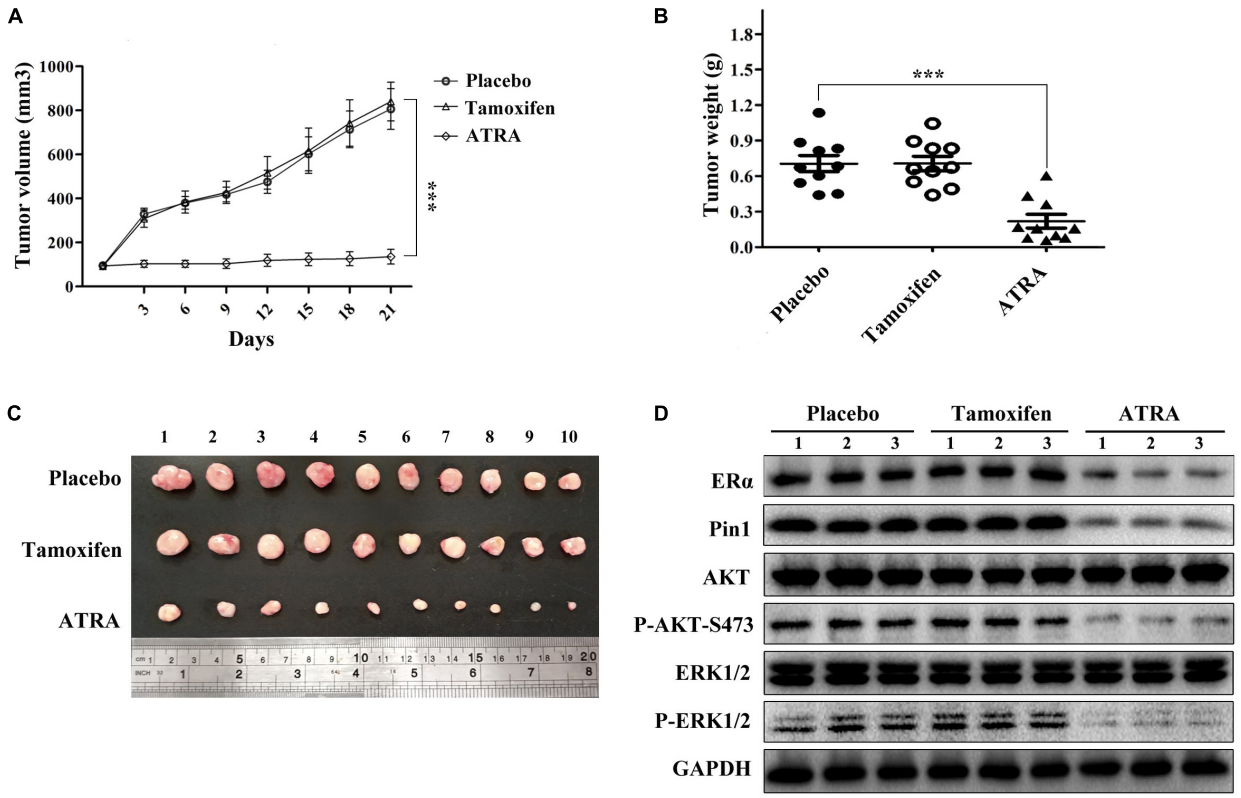


FIGURE 8 | ATRA suppresses the growth of TAMR breast cancer *in vivo*. **(A)** Growth curve of MCF-7R xenografts in nude mice. Tumor volumes were measured every 3 days. Error bars represent SD of ten mice, $^{***}P < 0.001$. **(B)** Slow-releasing ATRA pellet inhibits the tumor growth of MCF-7R xenografts in nude mice. The tumor weight represented the mean ± SD of each group, $^{***}P < 0.001$. **(C)** MCF-7R xenografts were collected 3 weeks after treatment. **(D)** Representative immunoblots show Pin1, ERα, P-AKT, and P-ERK1/2 levels in MCF-7R xenografts.

combination of ATRA + ATO effectively suppressed the cell viability in low dose (**Figures 7D,E**).

ATRA Suppresses the Growth of TAMR Breast Cancer *in vivo*

Given the remarkable effects of ATRA on inhibiting ERα, AKT, and ERK1/2, as well as cell proliferation in tamoxifen-resistant breast cancer *in vitro*, we next asked whether ATRA had therapeutic effect against TAMR breast tumors *in vivo*. We established MCF-7R xenografts and implanted 21-day slow-releasing ATRA tablets in nude mice. Tamoxifen showed no therapeutic effect on TAMR xenografts, whereas ATRA remarkably inhibited the growth of TAMR breast cancer cells *in vivo* (**Figures 8A–C**). In addition, ATRA significantly suppressed Pin1, ERα, as well as the phosphorylation of AKT and ERK1/2 in the xenografts (**Figure 8D** and **Supplementary Figure S8**). Therefore, ATRA is effective in overcoming tamoxifen resistance *in vivo*.

DISCUSSION

Tamoxifen resistance is one of the major hurdles in treating breast cancer. A large body of evidence suggests that modulation of ERα pathway and activation of pro-survival pathways are important factors of tamoxifen resistance (Cui et al., 2015; Ferraiuolo et al., 2017; Marsh et al., 2017; Zhang et al., 2017). Our study demonstrated that Pin1 was up-regulated in tamoxifen-resistant breast cancer cells and relapsed breast cancer tissues. ATRA-induced Pin1 degradation decreased the protein stability and transcription activity of ERα, as well as reduced the phosphorylation of pro-survival kinases AKT and ERK1/2 in tamoxifen-resistant breast cancer cells. Moreover, targeting Pin1 by ATRA inhibited cell growth *in vitro*, and exhibited anti-tumor effects *in vivo* against tamoxifen-resistant breast cancer. Our data suggest that ATRA is a potent drug in treating tamoxifen-resistant breast cancer via suppressing multifactorial mechanisms of tamoxifen resistance.

Compelling evidence has demonstrated that decreased ERα expression and function contributes to intrinsic and acquired tamoxifen resistance (Cui et al., 2015; Ferraiuolo et al., 2017; Marsh et al., 2017; Zhang et al., 2017). Various clinical and experimental models suggest that tumor cells with acquired resistance to tamoxifen express low level of ERα (Stone et al., 2013; Lu et al., 2016). In the presence of tamoxifen, the resistant cells can still activate ERα, but through a ligand-independent way (He et al., 2018), or rely on non-ERα growth-promoting pathways for survival (Hur et al., 2004; Cannings et al., 2007; Mohseni et al.,

2014). Thus the low level of ERα is one of the key resources of growth signal that are available for the resistant cells to utilize. Previous study showed that Pin1 inhibited phosphorylation-dependent ubiquitination and degradation of ERα in breast cancer cells (Rajbhandari et al., 2014). Here we found that Pin1 was up-regulated in tamoxifen-resistant breast cancer cells. This up-regulated Pin1 prevented ERα from degradation, which substantially enhanced the ERα level in tamoxifen-resistant cells. Although ERα level was low in these resistant cells, it would be even lower if Pin1 was not up-regulated. In our clinical samples, ERα expression was high in more than 60% of recurrent breast cancer tissues. This may be because Pin1 is frequently highly expressed in recurrent tumors, therefore preventing ERα from degradation, which substantially enhances the ERα level in relapsed tumors. Notably, this increased ERα, just as the low level of ERα in the resistant cells, is very likely activated via ligand independent way. This is supported by the evidence that phosphorylation of key serine residues of ERα, in particular serine 118 and 167, promotes re-activation of ERα in a ligand-independent manner (Garcia-Becerra et al., 2012). Pin1 has been reported to bind specifically to pS118 ERα to isomerize the serine118-proline119 bond (Rajbhandari et al., 2012). Therefore, Pin1 overexpression promotes the growth of tamoxifen-resistant breast cancer cells by up-regulating the ligand-independent ERα activity.

In addition to the effects on ERα stabilization, isomerization of phosphorylated ERα by Pin1 directly increases endogenous ERα DNA binding activity (Rajbhandari et al., 2015). Our study showed that inhibiting Pin1 by ATRA suppressed nuclear ERα expression and the transcription of ERα target genes. Moreover, previous data suggest that besides affecting ERα, Pin1 may promote tamoxifen resistance of breast cancer by activating growth-promoting pathways (Khanal et al., 2012), down-regulating SMRT (Stanya et al., 2008) and cyclin dependent kinase (Khanal et al., 2012), facilitating tumor angiogenesis (Kim et al., 2009b, 2012) and epithelial–mesenchymal transition (Kim et al., 2009a). However, few studies assess the potential of Pin1 inhibitor in treating TAMR breast cancer *in vitro* and *in vivo*. Our study showed that ATRA decreased ERα level both *in vitro* and *in vivo*. Moreover, ATRA down-regulated phosphorylation of ERα at S118 and two important pro-survival kinases ERK1/2 and AKT. Thus, ATRA inhibits Pin1 to overcome tamoxifen resistance in breast cancer cells at least at three levels: (1) promotes the degradation of ligand independent ERα, (2) suppresses the transactivation of ERα, (3) inhibits alternative growth pathways. Indeed, our results showed that ATRA exhibited potent anti-tumor activity against tamoxifen-resistant breast cancer *in vitro* and *in vivo*.

All-Trans Retinoic Acid has been used to treat APL for a long period of time. Recently Wei et al. (2015) has discovered that ATRA is a Pin1 inhibitor which binds to Pin1's active site and accelerated its degradation. These findings make it possible to expand the application of ATRA to treat more types of cancer, especially solid tumors, because Pin1 is overexpressed in a wide range of human cancers and regulates multiple cancer-driving pathways (Lu and Hunter, 2014; Zhou and Lu, 2016). Besides Wei et al. demonstrated that ATRA-induced Pin1 ablation inhibits triple-negative breast cancer cell growth (Wei et al., 2015), Liao et al. also reported the anti-tumor effect of ATRA in hepatocellular carcinoma (HCC) *in vitro* and *in vivo* (Liao et al., 2017; Yang et al., 2018). New Pin1 inhibitors have also been discovered to suppress the growth of cancer cells (Campaner et al., 2017; Kozono et al., 2018), even the tumor-initiating cells, as Pin1 promotes the self-renewal of these stem-like cancer cells (Luo et al., 2014; Rustighi et al., 2014, 2017). Our data showed that ATRA suppressed the cell proliferation of tamoxifen-resistant breast cancer cells, and effectively reduced the tumor growth of tamoxifen-resistant xenografts by promoting ERα degradation, decreasing ERα transactivation, and inhibits the activation of ERK1/2 and AKT. Given that multiple survival pathways and factors contribute to tamoxifen resistance, blocking a single pathway may be ineffective to overcome the resistance. Therefore, ATRA may have the advantage of suppressing multifactorial mechanisms of tamoxifen resistance simultaneously.

Currently there are still obstacles of using ATRA to treat solid tumors in human. Regular ATRA has a half-life of only 45 min in humans. Although slow-releasing pellets can be implanted subcutaneously in mice, the formulation of ATRA pellet is different from that used for oral administration or intravenous injection in human therapies, and can't be applied to human yet. Novel controlled releasing formulation of ATRA for effective cancer therapy are being developed actively (Westervelt et al., 2002; Tsimberidou et al., 2006; Yang et al., 2018). In addition, ATRA concentration is high in treating solid tumors. Similar to the previous report that the combination of ATRA + ATO inhibited tumor-initiating cells (Kozono et al., 2018), we found that this combination could reduce ATRA concentration and effectively inhibited the growth of TAMR cells. Thus, studies are ongoing to increase the efficacy of ATRA by improving its formulation, or using ATRA as part of combination therapies.

In summary, our data have shown for the first time that targeting Pin1 by ATRA effectively inhibits the growth of tamoxifen resistant breast cancer. This new approach represents a potential therapeutic strategy for intrinsic tamoxifen-resistant patients and relapsed ERα-positive breast cancer patients. Our findings shed new light on the molecular mechanism of ATRA in overcoming tamoxifen resistance and warrant future preclinical and clinical studies of ATRA in treating the tamoxifen resistant breast cancers.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Internal review and ethics boards of Sun Yat-sen Memorial Hospital. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Animal Care and Use Committee of Sun Yat-sen University.

AUTHOR CONTRIBUTIONS

M-LL and SH: conception and design. YL and YZ: development of methodology. YC, Z-ML, N-NL, and DL: acquisition of data. YC and SH: analysis and interpretation of data. M-LL and YY: writing, review, and/or revision of the manuscript. XZ and KL: technical and material support. M-LL and YY: study supervision.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2019.00322/full#supplementary-material

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REVIEW
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Targeting PIN1 as a Therapeutic Approach for Hepatocellular Carcinoma

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PIN1 is a peptidyl-prolyl *cis/trans* isomerase that specifically binds and catalyzes the *cis/trans* isomerization of the phosphorylated serine or threonine residue preceding a proline (pSer/Thr-Pro) motif of its interacting proteins. Through this phosphorylation-dependent prolyl isomerization, PIN1 is involved in the regulation of various important cellular processes including cell cycle progression, cell proliferation, apoptosis and microRNAs biogenesis; hence its dysregulation contributes to malignant transformation. PIN1 is highly expressed in hepatocellular carcinoma (HCC). By fine-tuning the functions of its interacting proteins such as cyclin D1, x-protein of hepatitis B virus and exportin 5, PIN1 plays an important role in hepatocarcinogenesis. Growing evidence supports that targeting PIN1 is a potential therapeutic approach for HCC by inhibiting cell proliferation, inducing cellular apoptosis, and restoring microRNAs biogenesis. Novel formulation of PIN1 inhibitors that increases *in vivo* bioavailability of PIN1 inhibitors represents a promising future direction for the therapeutic strategy of HCC treatment. In this review, the mechanisms underlying PIN1 over-expression in HCC are explored. Furthermore, we also discuss the roles of PIN1 in HCC tumorigenesis and metastasis through its interaction with various phosphoproteins. Finally, recent progress in the therapeutic options targeting PIN1 for HCC treatment is examined and summarized.

Keywords: PIN1, phosphorylation, hepatocellular carcinoma, inhibitor, hepatocarcinogenesis

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in men and the ninth in women worldwide. It is the third leading cause of cancer death, with a reported mortality of more than 780,000 per year (Bray et al., 2018). Patients with HCC have poor outcome and have an inferior 5-year overall survival of 18% as compared with that of other common cancers including breast (90%), colon (65%), prostate (98%), and stomach (31%) cancers (American_Cancer_Society, 2019). Chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), heavy alcohol consumption and the presence of liver cirrhosis are important risk factors for the development of HCC (Rawla et al., 2018). At the cellular level, metabolic dysregulation and genetic aberrations contribute to HCC development through the activation of multiple cancer-driving signaling pathways. The interplay of these signaling pathways results in a complex cancer cell circuitry that

leads to the aggressive clinical course and poor treatment outcomes of HCC. Early stage HCCs are amenable to several curative and effective therapies including orthotopic liver transplantation, surgical liver resection, transcatheter arterial chemoembolization (TACE) and radiofrequency ablation (RFA) (Lurje et al., 2019). However, only a small proportion of HCC cases are detected at an early stage, owing to the lack of sensitivity of the conventional HCC surveillance techniques such as ultrasonography (60%) and computed tomography (68%) (Colli et al., 2006). As a result, HCCs are frequently diagnosed at advanced stage and curative treatment options are not available for these patients. Conventional chemotherapy and molecular targeting therapy for advanced HCC have modest efficacy only. Clinical studies have demonstrated that treatment with doxorubicin or tyrosine kinase inhibitor sorafenib extend the survival of patients with advanced HCC for merely 3 and 12 weeks, respectively (Lai et al., 1988; Llovet et al., 2008). These treatments are also associated with development of drug resistance and ultimate disease progression (Chow et al., 2013; Nishida et al., 2015; Zheng, 2017). Recently, several molecular targeting drugs, including lenvatinib, regorafenib, and cabozantinib, have been approved by the US Food and Drug Administration (FDA) for the treatment of advanced HCC. Similar to sorafenib, lenvatinib is recommended as a first-line therapeutic agent for patients with advanced HCC. The other two molecular targeting drugs are approved as a second-line treatment in the presence of sorafenib resistance. Lenvatinib, regorafenib and cabozantinib are tyrosine kinase inhibitors and through inhibiting different sets of tyrosine kinases, treatment with these inhibitors results in better overall survival benefit as compared with sorafenib (**Table 1**; Bruix et al., 2017; El-Khoueiry et al., 2017; Abou-Alfa et al., 2018; Kudo et al., 2018). Thus, it is imperative to understand the various signaling pathways involved in hepatocarcinogenesis to facilitate the development of effective molecular targeting drugs.

Cancer-driving signaling pathways are often regulated by protein phosphorylation and dephosphorylation. Phosphorylation of serine or threonine residues preceding proline (pSer/Thr-Pro) motif of many regulatory proteins is mediated by cyclin-dependent kinases (CDKs) and mitogen-activated protein kinases (MAPKs). The phosphorylated Ser/Thr-Pro motif provides a potential binding site for the peptidyl-prolyl *cis/trans* isomerase PIN1 that catalyzes a *cis/trans* isomerization of the prolyl peptide bond (Lu et al., 1996; Lu, 2000). PIN1 is mainly localized in the nucleus and consists of two structurally and functionally distinct domains (Lee et al., 2011). Its N-terminal WW domain is responsible for specific binding to the pSer/Thr-Pro motifs of its protein substrates while its C-terminal prolyl isomerase (PPIase) domain is responsible for catalyzing *cis/trans* isomerization of the pSer/Thr-Pro peptide bonds (Lu et al., 1999; Lu P. J. et al., 2002; Behrsin et al., 2007). PIN1-mediated isomerization induces conformational changes of its bound proteins, thereby fine-tuning their cellular functions, interactions with other proteins, stability and subcellular localization (Lu K. P. et al., 2002). Through this mechanism, PIN1 is involved in various cellular processes, including apoptosis, cell cycle progression, cell proliferation, differentiation and transformation. As a result, PIN1 plays an important role in many human diseases including Alzheimer's disease (AD) and cancers (Zhou and Lu, 2016).

TABLE 1 | Molecular targeting drugs for hepatocellular carcinoma.

Drug	Targets	Study phase	Overall survival	References
First-line treatment				
Sorafenib	VEGFR1/2/3; PDGFR; RAF/MEK/ERK	III	10.7 months	Llovet et al., 2008; Cheng et al., 2009
		III	6.5 months	
Lenvatinib	VEGFR1/2/3; FGFR1/2/3/4; FGF; PDGFR; RET	III	13.6 months	Kudo et al., 2018
Second-line treatment				
Regorafenib	VEGFR1; RET; RAF1; TIE2; BRAF; PDGFR; FGFR	II	13.8 months	Bruix et al., 2017
Cabozantinib	VEGFR1/2/3; c-MET	III	10.2 months	Abou-Alfa et al., 2018
Nivolumab	PD-1	I/II	15 months	El-Khoueiry et al., 2017

VEGFR, Vascular endothelial growth factor receptor; PDGFR, Platelet-derived growth factor receptor; FGFR, Fibroblast growth factor receptor; RET, Glial cell-derived neurotrophic factor receptor; TIE2, Angiopoietin receptor; c-MET, Hepatocyte growth factor receptor; PD-1, Programmed cell death protein 1.

In cancer, PIN1 has been shown to promote carcinogenesis through its interaction with cell-cycle regulatory proteins and apoptosis-related proteins including β-catenin, cyclin D1, nuclear factor-kappa B (NF-κB)-p65, p53, and myeloid cell leukemia-1 (Mcl-1) (Ryo et al., 2001; Liou et al., 2002; Zacchi et al., 2002; Ryo et al., 2003; Ding et al., 2008). These PIN1-interacting proteins are frequently deregulated in cancers, and their oncogenic potential is enhanced through PIN1-dependent isomerization. Consequently, PIN1 over-expression has been linked to dysregulated cell proliferation, malignant transformation and tumor development. Indeed, PIN1 over-expression has been found in many cancers, including hepatocellular carcinoma (HCC). Several studies have shown that PIN1 is over-expressed in more than 50% of HCC tissues (Pang et al., 2004; Cheng et al., 2013; Shinoda et al., 2015; Leong et al., 2017). In addition, PIN1 over-expression not only promotes malignant transformation of hepatocytes (Pang et al., 2006), but also enhances hepatocarcinogenesis through interaction with the x-protein of hepatitis B virus (HBx), the inhibitor of apoptosis protein survivin, and the cycle-dependent kinase inhibitor p27 (Pang et al., 2007; Cheng et al., 2013, 2017). Notably, compelling evidence shows that inhibition of PIN1 suppresses the proliferation of HCC cells *in vitro* and *in vivo* (Liao et al., 2017; Zheng et al., 2017; Pu et al., 2018; Yang et al., 2018; Sun et al., 2019). Currently, there is no effective conventional chemotherapy and molecular targeting therapy for advanced HCC. Thus, PIN1 inhibition may be a promising therapeutic strategy for HCC treatment. In this article, we review the role of PIN1 in HCC and discuss the therapeutic potential of targeting PIN1.

REGULATION OF PIN1 EXPRESSION IN HEPATOCELLULAR CARCINOMA

Many studies have demonstrated a high prevalence of PIN1 over-expression in HCC. The expression of PIN1 is regulated by a number of transcriptional factors and microRNAs (miRNAs). miRNAs are a family of small non-coding RNAs that negatively regulate gene expression by binding to the 3′UTR of target mRNA, resulting in the target mRNA degradation or translational repression. Currently, six miRNAs (miR-140-5p, miR-200b/c, miR-296-5p, miR-370, and miR-874-3p) (**Table 2**) have been found to bind PIN1 mRNA directly and inhibit its expression in cancers (Zhang et al., 2013; Lee et al., 2014; Luo et al., 2014; Leong et al., 2017; Yan et al., 2017; Chen et al., 2018). Experiments have confirmed that over-expression of these miRNAs reduces PIN1 protein expression in cancer cells and reverses PIN1-mediated cellular effects, including cell proliferation, apoptosis, migration and invasion. Among these PIN1-targeting miRNAs, the expression of miR-140-5p and miR-874-3p are significantly down-regulated and inversely correlated with PIN1 overexpression in primary human HCC samples, suggesting that the down-regulation of miR-140-5p and miR-874-3p contributes to PIN1 over-expression during hepatocarcinogenesis.

PIN1 expression is also transcriptionally regulated by retinoblastoma protein (Rb)-E2F pathway. E2F protein is a transcription factor that activates PIN1 expression by binding to the E2F-binding sites of the *PIN1* gene promoter (Ryo et al., 2002). Hypophosphorylated Rb binds to and sequesters E2F transcription factor, leading to transcriptional inactivation of PIN1 expression. After phosphorylation by CDK kinases, hyperphosphorylated Rb dissociates E2F transcription factors from Rb-E2F complex, resulting in increased E2F transcriptional activity and PIN1 expression. Therefore, the E2F-induced PIN1 expression mainly depends on the release of E2F transcription factor from the hyperphosphorylated Rb. As a higher nuclear expression of E2F protein is found in HCC tissues (Palaiologou et al., 2012), it is speculated that a higher E2F expression may contribute to PIN1 over-expression in HCC pathogenesis.

The relationships between PIN1 expression and clinical factors in HCC have also been studied (Shinoda et al., 2015). Higher PIN1 expression is significantly associated with larger tumor size, increased intrahepatic metastasis and portal vein invasion. Compared with patients with low PIN1 expression, patients with high PIN1 expression show significantly inferior prognosis, shorter overall survival and higher early recurrence rate. These findings support the notion that deregulated PIN1 expression may play an important role in determining the clinical course of HCC.

ROLES OF PIN1 IN HEPATOCARCINOGENESIS

The first evidence showing the role of PIN1 in promoting HCC pathogenesis is the finding of malignant transformation of non-tumorigenic human liver cells by PIN1 over-expression (Pang et al., 2006). Both *in vitro and in vivo* experiments have demonstrated that PIN1 over-expression in non-tumorigenic liver cells induces the colony formation in soft agar and tumor formation in nude mice. Conversely, PIN1 depletion by shRNA reduces HCC tumorigenicity (Pang et al., 2006; Cheng et al., 2013). Through PIN1-mediated isomerization, PIN1 contributes to hepatocarcinogenesis by fine-tuning the oncogenic functions of its interacting proteins (**Figure 1**).

PIN1 AND CYCLIN D1

One of the most well studied oncogenic proteins regulated by PIN1 is cyclin D1, an important cell cycle regulator. Deregulation of cyclin D1 is associated with various types of cancers. Cyclin D1 functions to trigger the cell entering cell cycle and its expression is critical for promoting cell cycle progression and cell proliferation (Hunter and Pines, 1994). Notably, PIN1 has been found to interact with cyclin D1 in cancer cells. Through PIN1-dependent isomerization, PIN1 increases the protein stability of cyclin D1, resulting in the increase of nuclear accumulation of cyclin D1 (Liou et al., 2002). In addition to its post-translational regulation, PIN1 also increases cyclin D1 expression at the transcriptional level. Firstly, PIN1 interacts with β-catenin to inhibit nuclear export and protein degradation of β-catenin (Ryo et al., 2001). Increased nuclear accumulation of β-catenin leads to an increase of β-catenin transcriptional activity on the downstream target genes, including *cyclin D1*. Moreover, PIN1 has been found to bind c-Jun and the p65/RelA subunit of NF-κB, leading to the activation of c-Jun and NF-κB transcriptional activities toward the *cyclin D1* gene (Wulf et al., 2001; Ryo et al., 2003). Consequently, PIN1 over-expression increases cyclin D1 protein expression level through PIN1-mediated protein stabilization of cyclin D1 and PIN1-induced transcriptional activation of β-catenin, c-Jun and NF-κB. Increased cyclin D1 expression in turn promotes cell proliferation. In fact, several studies have demonstrated that PIN1 expression is positively correlated with the cyclin D1 expression in human HCC tumors (Pang et al., 2004; Shinoda et al., 2015), further confirming the role of PIN1 in promoting hepatocarcinogenesis by up-regulation of cyclin D1 expression.

PIN1 AND HBX

Chronic infection with HBV contributes to hepatocarcinogenesis through several mechanisms. These mechanisms involve the integration of HBV-DNA into the host genome to induce chromosome instability, insertional mutagenesis of diverse cancer-related genes to alter their expression, and the expression of viral regulatory protein HBx to modulate apoptosis and cell proliferation of the infected cells (Brechot et al., 1980; Feitelson and Duan, 1997; Paterlini-Brechot et al., 2003). HBx is known to interact with p53 and inhibits its translocation into the nucleus, resulting in the suppression of p53-dependent apoptosis (Ueda et al., 1995). HBx is also a viral transactivator that promotes cell proliferation by up-regulating the expression

TABLE 2 | Identification of PIN1-targeting microRNAs.

Name of miRNAs	Target position on PIN1 3′UTR	Cancer types
miR-140-5p	Position 477-483 of PIN1 3′UTR 5′... CCCAAUUAAACCCAGAACCACUG...3′ ||||||| miR-140-5p 3′ GAUGGUAUCCCAUUUUGGUGAC 5′	Hepatocellular carcinoma (Yan et al., 2017)
miR-200b	Position 111-117 of PIN1 3′UTR 5′... CCUGCCACCGUCACACAGUAUUU...3′ ||||||| miR-200b 3′ AGUAGUAAUGGUCCGUCAUAAU 5′	Metastatic lymph node from breast cancer (Zhang et al., 2013)
miR-200c	Position 111-117 of PIN1 3′UTR 5′... GCCACCGUCAC - - - ACAGUAUUU...3′ ||| | | || ||||||| miR-200c 3′ AGGUAGUAAUGGGCCGUCAUAAU 5′	Breast cancer stem cells (Shimono et al., 2009; Luo et al., 2014)
miR-296-5p	Position 140-147 of PIN1 3′UTR 5′... GGAGGGGGCCCUUCCA...3′ |||||||| miR-296-5p 3′ UGUCCUAACUCCCCCCCGGGA 5′ Position 157-164 of PIN1 3′UTR 5′... GAUUGGGGGCCCUGGG...3′ |||||||| miR-296-5p 3′ UGUCCUAACUCCCCCCCGGGA 5′	Prostate cancer (Lee et al., 2014)
miR-370	Not Available	Esophageal squamous-cell carcinoma (Chen et al., 2018)
miR-874-3p	Position 30-36 of PIN1 3′UTR 5′... GGCCUGGCCUCGGGGCAGGGCAG...3′ ||||||| miR-874-3P 3′ AGCCAGGGAGCCCGGUCCCGUC 5′ Position 263-269 of PIN1 3′UTR 5′... GGAGGCUCCCAGACCCAGGGCAG...3′ ||||||| miR-874-3P 3′ AGCCAGGGAGCCCGGUCCCGUC 5′	Hepatocellular carcinoma (Leong et al., 2017)

miRNA, microRNA; UTR, Untranslated region.

of several oncogenes including c-Jun and NF-κB. Previously, our group demonstrates an interaction between PIN1 and HBx (Pang et al., 2007). PIN1-dependent isomerization of HBx results in stabilization of the HBx protein and augmentation of the HBx transactivating activity. Enhanced transactivation activity of HBx resulting from PIN1 and HBx interaction up-regulates the expression of its downstream target gene NF-κB. As a result, co-expression of PIN1 and HBx synergistically promotes cell proliferation and xenograft tumor growth in HCC as compared with the expression of PIN1 or HBx alone (Pang et al., 2007). In addition, PIN1 over-expression is strongly associated with HBV-related HCC tumors, suggesting that PIN1 is critical for HBV-induced hepatocarcinogenesis through its upregulation of the transactivating activity of HBx protein.

PIN1 AND SURVIVIN

Deregulation of apoptosis is also involved in HCC pathogenesis. PIN1 has been found to interact with the anti-apoptotic protein survivin in HCC cells (Cheng et al., 2013). During mitotic progression of a proliferating cell, phosphorylation of survivin on Thr^{34}-Pro motif occurs to facilitate its binding to hepatitis B X interacting protein (HBXIP) and pro-caspase-9, thereby preventing caspase-9 activation and inhibiting apoptosis (Marusawa et al., 2003). Interestingly, this survivin phosphorylation site (Thr^{34}-Pro) is also a binding site for PIN1. Through PIN1-dependent isomerization, PIN1 increases the binding between survivin and pro-caspase-9 via HBXIP, resulting in the suppression of caspase-dependent apoptosis in HCC cells (Cheng et al., 2013). Inhibition of apoptosis by PIN1 results in an increase of tumor growth in HCC xenograft mouse model. In addition, both PIN1 and survivin protein expression levels are higher in human HCC tumors as compared with adjacent non-timorous liver tissues, and there is a positive correlation between PIN1 and survivin expression in HCC (Cheng et al., 2013). These findings suggest that PIN1 over-expression promotes hepatocarcinogenesis by enhancing the anti-apoptotic function of survivin.

PIN1 AND XPO5

As discussed earlier, several miRNAs have been found to inhibit PIN1 expression in cancers. Conversely, PIN1 also regulates miRNAs expression through its interaction with precursor-miRNA (pre-miRNA) transporter exportin-5 (XPO5) (Li et al., 2018). The biogenesis of miRNA starts with the transcription of a primary miRNA (pri-miRNA) by RNA polymerase II, followed by processing of the pri-miRNA to generate pre-miRNA by Drosha in the nucleus. Nuclear pre-miRNA with short hairpin structure only becomes a functional mature miRNA after cytoplasmic processing by Dicer (Krol et al., 2010). The function of XPO5 is to mediate the export of pre-miRNA from nucleus to cytoplasm for miRNA maturation. As pre-miRNA export is a rate-limiting step in miRNA biogenesis, XPO5 plays a critical role in the regulation of miRNA expression (Yi et al., 2005).

In HCC cells, phosphorylation of XPO5 by ERK has been shown to promote its interaction with PIN1 (Sun et al., 2016). Through PIN1-mediated isomerization, PIN1 impairs the pre-miRNA binding capacity of XPO5, resulting in the

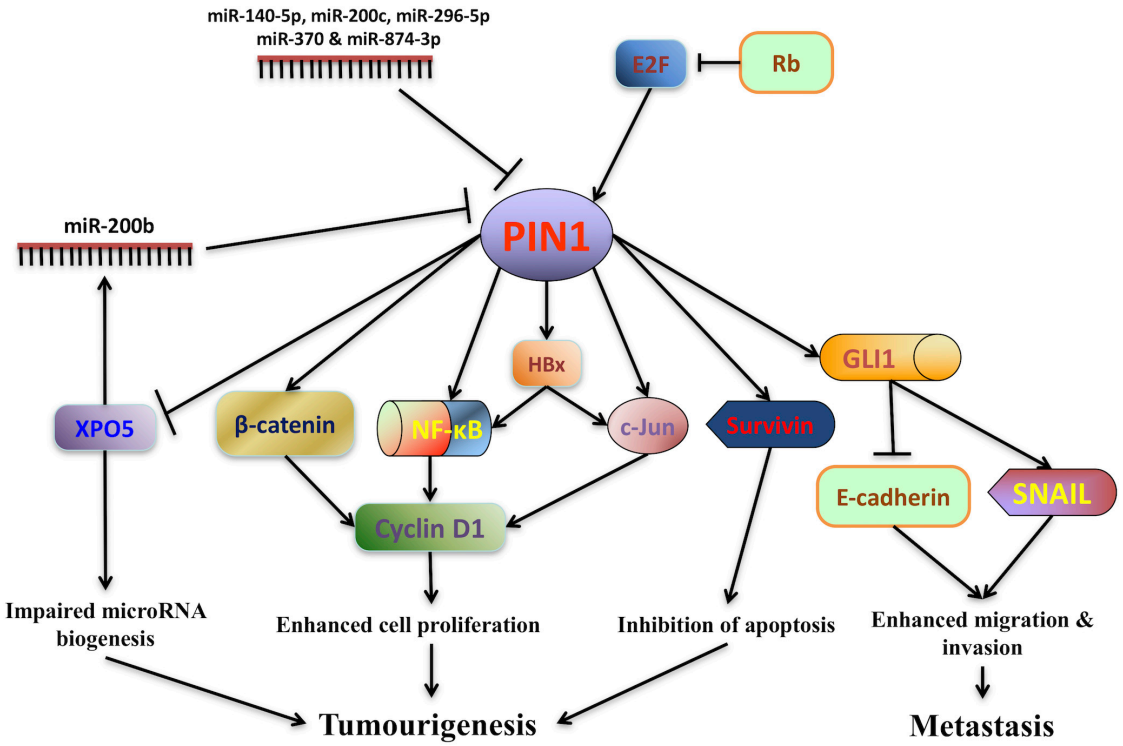

FIGURE 1 | PIN1 involvement in hepatocarcinogenesis. The expression of PIN1 is up-regulated by E2F transcription factors through the retinoblastoma protein (Rb)-E2F pathway while its expression is down-regulated by a number of microRNAs (miR-140-5p, miR-200b/c, miR-296-5p, miR-370, and miR-874-3p). Through phosphorylation-dependent prolyl isomerization, PIN1 fine-tunes the oncogenic functions of various phosphoproteins involved in HCC tumorigenesis. PIN1 increases cyclin D1 protein level through both transcriptional and translational regulation. At the transcriptional level, PIN1 increases the transcriptional activities of β-catenin, c-Jun and nuclear factor-kappa B (NF-κB), resulting in an increase in cyclin D1 transcription. At the translational level, PIN1 binds and stabilizes cyclin D1 protein. In addition, PIN1 stabilizes Hepatitis B virus X-protein (HBx) and augments HBx transactivating activity on downstream targets c-Jun and NF-κB that up-regulate cyclin D1 expression. Increased cyclin D1 expression in turn contributes to enhanced cell proliferation and tumorigenesis. PIN1 also enhances the anti-apoptotic function of survivin to inhibit caspase-dependent apoptosis and promote tumorigenesis. Furthermore, PIN1 impairs pre-miRNA binding capacity of exportin-5 (XPO5), decreases nuclear to cytoplasmic export of pre-miRNA and finally reduces miRNA biogenesis. Global down-regulation of miRNA expression leads to HCC tumorigenesis. Through interaction with GLI1, PIN1 enhances migration and invasion of HCC cells by up-regulating mesenchymal cell marker SNAIL and down-regulating epithelial cell marker E-cadherin.

suppression of nuclear-cytoplasmic export of pre-miRNA (Li et al., 2018). Reduced cytoplasmic pre-miRNA finally reduces miRNA biogenesis and miRNA expression. By impairing pre-miRNA binding capacity of XPO5, PIN1 has been found to reduce the expression of tumor suppressive miRNAs, such as miR-122, miR-200b, and miR-146a. Through decreasing the expression of these miRNAs in HCC cells, PIN1 promotes cell proliferation *in vitro* and tumor growth *in vivo*. In fact, a global down-regulation of miRNA expression is tightly associated with HCC progression (Wong et al., 2012). Given the critical role of nuclear-cytoplasmic export of pre-miRNA in miRNA biogenesis, the regulation of pre-miRNA binding ability of XPO5 by PIN1 has profound effects on miRNA expression, which in turn contributes to hepatocarcinogenesis (Li et al., 2018). Interestingly, PIN1 has been shown to suppress the expression of miR-200b that is one of the PIN1-targeting miRNAs (Zhang et al., 2013; Li et al., 2018). Further study is required to investigate this potential positive feedback loop for PIN1 over-expression through the regulation of miR-200b biogenesis in HCC.

PIN1 AND GLI1

There is increasing evidence to indicate that PIN1 enhances migration and invasion of cancer cells by promoting epithelial-mesenchymal transition (EMT). PIN1 over-expression in breast cancer cells has been found to increase expression of mesenchymal cell markers such as N-cadherin, SNAIL and vimentin, and decrease expression of epithelial cell marker E-cadherin (Kim et al., 2009; Luo et al., 2014). The first evidence showing the role of PIN1 in enhancing migration and invasion ability of HCC cells is the identification of its interaction with GLI1, an effector of the Hedgehog pathway (Wang et al., 2019). GLI1 is a transcriptional factor that activates Hedgehog signals, thereby promoting EMT and HCC metastasis (Li et al., 2016). PIN1 interacts with and stabilizes GLI1 in HCC cells, leading to an increase in GLI1 protein expression. Although the mechanism underlying the regulation of EMT by GLI1 remains undefined, PIN1-induced GLI1 stabilization results in an altered expression of EMT regulating proteins with up-regulation of SNAIL and down-regulation of E-cadherin

(Wang et al., 2019). Experiments have confirmed that PIN1 over-expression promotes HCC cell migration and invasion *in vitro* while its depletion by shRNA inhibits lung metastasis of HCC cells *in vivo* (Wang et al., 2019). Interestingly, PIN1 expression is not only positively correlated with GLI1, but also with SNAIL in human HCC tissues. Moreover, PIN1 expression is also negatively correlated with E-cadherin in HCC (Wang et al., 2019). These findings have demonstrated that PIN1 contributes to migration and invasion of HCC cells through stabilization of GLI1 and modulation of EMT regulating proteins expression.

DEVELOPMENT OF PIN1 INHIBITORS FOR HCC TREATMENTS

Given the importance of PIN1 in HCC pathogenesis, PIN1 is an attractive drug target for HCC treatment. Our previous studies have shown that suppression of PIN1 by RNA interference in HCC cells reduces cell proliferation, inhibits colony formation in soft agar and enhances caspase-dependent apoptosis (Pang et al., 2006; Cheng et al., 2013). Moreover, PIN1 depletion results in a suppression of tumor growth and induction of tumor apoptosis in xenograft mouse model of HCC. Conceivably, targeting PIN1 is a potential therapeutic approach for HCC. Through screening of various chemical compound libraries, numerous PIN1 inhibitors have been identified to exert varying degrees of anti-proliferative effect on cancer cells by inhibiting PIN1 PPIase activity. PIN1 inhibitors can be subdivided into two groups (covalent and non-covalent) based on their binding to the PIN1 PPIase domain. After binding to the PIN1 PPIase domain, covalent PIN1 inhibitor (Juglone and KPT-6566) induces a covalent modification of thiol group of the cysteine residues in the PPIase domain (Hennig et al., 1998; Campaner et al., 2017). Through this structural modification of the catalytic domain, covalent PIN1 inhibitor irreversibly blocks the PIN1 PPIase domain and inhibits its activity. Most of the PIN1 inhibitors are non-covalent PIN1 inhibitors (e.g., PiB, ATRA, ATO, and API-1). Similar to covalent PIN1 inhibitor, non-covalent PIN1 inhibitor also directly binds to the PIN1 PPIase domain, but inhibits PIN1 activity in a competitive manner (Uchida et al., 2003; Wei et al., 2015; Kozono et al., 2018; Pu et al., 2018). However, there are limitations of these PIN1 inhibitors that restrict their clinical application (**Table 3**).

The first identified PIN1 inhibitor is juglone, which is a covalent PIN1 inhibitor exerts *in vitro* and *in vivo* anti-proliferative effect against various types of cancer cells by irreversibly inhibiting PIN1 PPIase activity (Hennig et al., 1998). Juglone not only inhibits cell proliferation in HCC and prostate cancer cells *in vitro*, but also suppresses tumor growth of prostate cancer in xenografting experiments (Lee et al., 2009; Kanaoka et al., 2015). Notwithstanding its potent anti-tumor effect, juglone is not suitable for clinical use due to the lack its specificity on PIN1 inhibition. Other than PIN1, Juglone has been found to inhibit the cellular functions of RNA polymerase II and Rab4 (Chao et al., 2001; Fila et al., 2008).

Several non-covalent PIN1 inhibitors such as PiB, dipentamethylene thiuram monosulfide (DTM) and TME-001 have been identified to inhibit PIN1 PPIase activity and suppress cancer cell proliferation. PiB and DTM are effective against cell proliferation in colon cancer while TME-001 is found to exert anti-proliferative effect on cervical cancer (Uchida et al., 2003; Tatara et al., 2009; Mori et al., 2011). In addition to directly inhibiting PPIase activity, 5′-nitro-indirubinoxime (5′-NIO) has been shown to reduce PIN1 protein expression, resulting in suppression of lung cancer cell proliferation (Yoon et al., 2012). A novel covalent PIN1 inhibitor, KPT-6566, has been found to induce PIN1 protein degradation, leading to inhibition of proliferation in cancer cells including breast, prostate, lung and pancreatic cancer (Campaner et al., 2017). Moreover, KPT-6566 exerts a higher anti-proliferative effect on PIN1-expressing cells than PIN1-silenced cells, suggesting that KPT-6566 has a more specific PIN1 inhibitory activity. Despite the promising anti-proliferative effects on cancer cells, the efficacy and safety of these inhibitors for cancer treatment in human remain to be verified in animal models and patients.

In addition to the development of more potent and specific PIN1 inhibitors, there were studies to demonstrate that several drugs, which are approved for other clinical indications, also possess inhibitory activity against PIN1. The potential values of these agents for HCC treatment are further discussed in the following sections (**Table 4**).

PIN1 AND SORAFENIB

Sorafenib is a multi-tyrosine kinase inhibitor that is FDA-approved for the first-line treatment of advanced HCC. Through inhibition of the RAF/MEK/ERK and VEGF receptor tyrosine kinase signaling pathways, sorafenib has been shown to induce cell apoptosis, suppress cell proliferation, and inhibit tumor growth and angiogenesis in HCC cells (Liu et al., 2006). Given its inhibitory effect on ERK phosphorylation, sorafenib reduces the PIN1-induced stabilization of anti-apoptotic protein Mcl-1 by inhibiting the ERK-mediated phosphorylation of Mcl-1. This results in reduction of Mcl-1 protein expression, promotion of apoptosis, and inhibition of cell proliferation. Although a positive correlation between PIN1 and Mcl-1 has only been reported in human breast cancer, deregulated Mcl-1 expression is also commonly found in HCC (Fleischer et al., 2006; Sieghart et al., 2006). Theoretically, sorafenib may enhance apoptosis of HCC cells by impairing the interaction between PIN1 and Mcl-1. Thus, sorafenib may indirectly inhibit PIN1 function through targeting the phosphorylation of PIN1-interacting proteins.

In addition, sorafenib also reduces PIN1 mRNA and protein expression in HCC cells by inhibiting Rb phosphorylation (Zheng et al., 2017). As phosphorylated Rb releases E2F transcription factor and activates PIN1 expression, sorafenib may down-regulate PIN1 expression through targeting the Rb-E2F pathway. Furthermore, HCC cells with PIN1 depletion are more sensitive to sorafenib induced cell death, suggesting that some of the PIN1-interacting proteins associated with HCC pathogenesis may not be the targets of sorafenib.

TABLE 3 | Limitations of potential PIN1 inhibitors.

Drug	Covalent or non-covalent	Details	Tested cancer types	Limitations
Juglone	Covalent	Irreversible inhibits PIN1 PPIase activity Inhibits cell proliferation and xenograft tumor growth	HCC Prostate cancer	Non-specific inhibition of RNA polymerase II and Rab4
PiB	Non-covalent	Inhibits PIN1 PPIase activity and cell proliferation	Colon cancer	No testing in animal model
Dipentamethylene thiuram monosulfide (DTM)	Non-covalent	Inhibits PIN1 PPIase activity and cell proliferation	Colon cancer	No testing in animal model
TME-001	Non-covalent	Inhibits PIN1 PPIase activity and cell proliferation	Cervical cancer	No testing in animal model
5′-nitro-indirubinoxime (5′-NIO)	N.A.	Reduce PIN1 protein expression Inhibits cell proliferation	Lung cancer	No testing in animal model
KPT-6566	Covalent	Induce PIN1 protein degradation Inhibits cell proliferation More specific PIN1-inhibitory activity	Breast cancer Prostate cancer Lung cancer Pancreatic cancer	No testing in clinical trial

HCC, Hepatocellular carcinoma; N.A., Not applicable.

TABLE 4 | Potential PIN1 inhibitors for HCC treatment.

Drug	Covalent or non-covalent	Mechanisms of action	Clinical limitations
Sorafenib	N.A.	FDA-approved for advanced HCC treatment Inhibits RAF/MEK/ERK and VEGF receptor tyrosine kinases Suppresses PIN1-mediated Mcl-1 protein stabilization Reduces PIN1 expression by Inhibiting Rb phosphorylation Enhances apoptosis and inhibits cell proliferation	Unsatisfactory response rate with only 12 weeks survival advantage (Llovet et al., 2008) Developing sorafenib resistance or enhancing metastatic traits (Chow et al., 2013)
All-trans retinoic acid (ATRA)	Non-covalent	Induces PIN1 protein degradation Inhibits cell proliferation, migration, invasion and metastasis of HCC cells Demonstrates an enhanced anti-cancer effect by encapsulated in a slow-releasing pellet and PLLA microparticle	Poor overall survival and unsatisfactory response rate (Meyskens et al., 1998)
Arsenic trioxide (ATO)	Non-covalent	Induces PIN1 protein degradation Inhibits HCC cancer cell proliferation and xenograft tumor growth Combined with ATRA to exert a synergistic effect in inhibiting HCC cell proliferation	Ineffective in a phase II clinical study (Lin et al., 2007)
API-1	Non-covalent	Restores PIN1-impaired microRNA biosynthesis by enhancing XPO5 pre-miRNA binding ability Inhibits HCC cancer cell proliferation and xenograft tumor growth Shows an enhanced anti-cancer activity by liposomal formulation (API-LP)	No testing in clinical trial

N.A., Not applicable; FDA, US Food and Drug Administration; HCC, Hepatocellular carcinoma; Rb, Retinoblastoma; PLLA, Poly L-lactic acid; AQP9, Aquaporin 9; XPO5, Exportin-5.

Therefore, it is speculated that PIN1 inhibitor together with sorafenib may have synergistic therapeutic effects against HCC. A recent study by Zheng et al. (2017) has demonstrated that combined treatment of sorafenib with a PIN1 inhibitor, all-*trans* retinoic acid (ATRA) [details will be discussed below], exerts a synergistic effect in inhibiting cell proliferation and xenograft tumor growth in HCC as compared with sorafenib or ATRA alone. Moreover, combination of sorafenib and ATRA results in a synergistic inhibition of PIN1 protein expression and various PIN1-mediated oncogenic pathways. In a clinical study, although HCC patients receiving sorafenib monotherapy have longer overall survival, the response rate remains unsatisfactory with only 12 weeks survival advantage (Llovet et al., 2008). Sorafenib monotherapy also involves in development of sorafenib resistance or in enhancing the metastatic traits of HCC cells (Chow et al., 2013; Liang et al., 2013). Thus, the study of combination therapy with sorafenib and various PIN1 inhibitors may improve the overall survival in HCC patients and minimize the risk of drug resistance and metastasis.

PIN1 AND ATRA

ATRA is first identified as a therapeutic agent for acute promyelocytic leukemia (APL). It inhibits cell proliferation of APL cells by inducing terminal differentiation of APL cells. A recent study by Wei et al. (2015) has demonstrated a PIN1-inhibitory function of ATRA against cancer cells. ATRA directly

binds to the PIN1 PPIase domain, causing PIN1 isomerase inhibition and PIN1 protein degradation. ATRA-induced PIN1 degradation results in inhibition of multiple cancer-driving pathways and suppression of proliferation in APL cells *in vitro* and *in vivo*. For HCC cells, ATRA has been demonstrated to exert a profound inhibitory effect on cell proliferation *in vitro* (Cui et al., 2016). In addition, HCC cells with PIN1 depletion are more resistant to the cytotoxic effect of ATRA than control cells, suggesting that ATRA-induced PIN1 degradation plays a critical role in the suppression of HCC cell proliferation (Yang et al., 2018). Moreover, ATRA does not show any growth-inhibitory effect on normal liver cells, further demonstrating its specificity toward HCC cancer cells. Importantly, ATRA also exhibits an inhibitory effect on migration, invasion, and lung metastasis of HCC cells by inducing protein degradation of PIN1 (Wang et al., 2019).

However, previous clinical study has demonstrated that ATRA was ineffective in patients with HCC, as evidenced by demonstration of a poor overall survival and unsatisfactory response rate (Meyskens et al., 1998). The efficacy of ATRA in HCC treatment is limited because of its short half-life of 45 min in humans and its rapid metabolism by liver. Therefore, it is necessary to develop a more stable ATRA formulation for clinical application. A slow-releasing ATRA formulation has been developed by encapsulating ATRA in the acid form of vitamin A pellets. In contrast to free ATRA, the slow-releasing ATRA pellet is more stable in animals and can maintain ATRA plasma concentration in a steady level (Liao et al., 2017). Therefore, a minimum effective dose of the slow-releasing ATRA formulation can be applied to minimize its toxic effects to animals. More importantly, the slow-releasing ATRA formulation has been found not only to induce PIN1 degradation, but also reduce tumorigenicity in xenograft mouse model of HCC. A more recent study has identified a novel controlled release formulation of ATRA that exerts a more potent anti-proliferative effect against HCC cells (Yang et al., 2018). This novel formulation is processed through encapsulation of ATRA into the poly L-lactic acid (PLLA) microparticles. In comparison with slow-releasing ATRA formulation, the treatment of ATRA-PLLA microparticles shows a more significant inhibition of xenograft tumor growth and reduction of PIN1 protein expression. Moreover, injection of ATRA-PLLA microparticles into mice achieves a higher ATRA plasma concentration in a steady level as compared with implantation of slow releasing ATRA pellet. Notably, ATRA-PLLA microparticle showing more potent anti-cancer efficacy is encapsulated with a lower concentration of ATRA (2 mg) while slow releasing ATRA pellet has a higher ATRA concentration (5 mg). Thus, these findings demonstrate that ATRA is an attractive PIN1-targeting therapeutic drugs against HCC and the development of stable encapsulated ATRA is a promising strategy to improve the efficacy and safety of its use.

PIN1 AND ATO

Arsenic trioxide (ATO), is a FDA approved drug used for the treatment of APL that is refractory to or relapsed after ATRA therapy. The anti-cancer effect of ATO mainly depends on its ability to induce ubiquitin-dependent proteasomal degradation of various oncogenic proteins, including promyelocytic leukemia-retinoic acid receptor-alpha (PML-RARA) in APL, cyclin D1 in mantle cell lymphoma, and nucleophosmin-anaplastic lymphoma kinase (NPM-ALK) in anaplastic large cell lymphoma (Shao et al., 1998; Lo and Kwong, 2014; Piao et al., 2017). Through the proteasome pathway, ATO has also been found to induce PIN1 degradation by directly binding to the PIN1 PPIase domain (Kozono et al., 2018). ATO-induced PIN1 degradation results in the suppression of multiple PIN1-mediated oncogenic pathways and inhibition of breast cancer cell proliferation *in vitro* and *in vivo*. For HCC cells, ATO inhibits cell proliferation and xenograft tumor growth through triggering caspase-dependent apoptosis (Kito et al., 2002, 2003; Sadaf et al., 2018). However, a phase II clinical study has showed that ATO monotherapy is ineffective as a treatment for HCC (Lin et al., 2007).

The cytotoxic effect of ATO partly depends on its cellular uptake that is mediated by a transmembrane arsenic transporter, aquaporin 9 (AQP9) (Leung et al., 2007). The ATO-induced cell death is highly associated with expression level of AQP9, and AQP9 expression level varies between different types of cancer cells. Up-regulation of AQP9 expression may enhance the cytotoxic effect of ATO against cancer cells. In addition to its PIN1-inhibitory activity, ATRA has been shown to increase AQP9 expression to promote ATO uptake into the cells. As a result of enhancing cellular uptake of ATO, combined treatment of ATO with ATRA synergistically reduces PIN1 expression, suppresses multiple PIN1-regulated oncogenic pathways, and inhibits breast cancer cell proliferation *in vitro* and *in vivo* as compared with ATO or ATRA alone (Kozono et al., 2018). Although a clinical study for ATO-ATRA combination therapy against HCC has yet to be conducted, experiments have demonstrated that this combined treatment exerts a synergistic effect in inhibition of cell proliferation and promotion of apoptosis in HCC cells *in vitro* (Lin et al., 2005; Wei et al., 2014).

PIN1 AND API-1

Most of the identified PIN1 inhibitors exert their anti-proliferative effect against cancer cells in a PIN1-dependent manner with a higher inhibition of cell proliferation in PIN1-expressing cells than PIN1-depleted cells. A recent study has discovered a novel PIN1 inhibitor, API-1, with a potent anti-proliferative effect in HCC cells, and its anti-proliferative activity is dependent on both PIN1 expression and XPO5 phosphorylation (Pu et al., 2018). HCC cells with higher PIN1 expression and enhanced XPO5 phosphorylation are more sensitive to API-1 treatment than those with low PIN1 expression and/or reduced XPO5 phosphorylation. As previously described, XPO5 is responsible for the nuclear-cytoplasmic export of pre-miRNA to facilitate miRNA maturation. PIN1 interacts with phosphorylated XPO5 to impair its pre-miRNA binding ability, resulting in reduction of pre-miRNA export and miRNA expression. Due to its PIN1-inhibitory activity, API-1 has been

found to increase XPO5-mediated pre-miRNA export from the nucleus to the cytoplasm and restore biosynthesis of tumor suppressive miRNAs in HCC cells. Consequently, treatment of API-1 in HCC cells results in reduction of cell proliferation and suppression of xenograft tumor growth through restoration of PIN1-impaired miRNA biosynthesis.

In addition, liposomal formulation of API-1 further enhances its anti- HCC effects *in vitro* and *in vivo* (Sun et al., 2019). Encapsulation of API-1 by liposome (API-LP) results in an increase of API-1 solubility and a slower rate of API-1 drug release, leading to an improved bioavailability of API-LP in animals. Similar to unencapsulated API-1, API-LP restores XPO5-mediated pre-miRNA nuclear-cytoplasmic export and miRNA biogenesis through the inhibition of PIN1 activity. Due to its improved bioavailability, API-LP shows a more significant suppression of tumor growth of HCC xenografts as compared with unencapsulated API-1. More importantly, API-LP shows no apparent toxicity to the mice as it does not cause any necrotic damage to the tissues in mouse major organs such as heart, liver, spleen, lung and kidney. Although further study is required to evaluate the clinical efficacy and safety of API-LP for HCC patients, the liposomal formulation provides new insights into the development of a potent PIN1 inhibitor with enhanced bioavailability against HCC in animals and humans.

CONCLUSION AND FUTURE PERSPECTIVES

Given the oncogenic role of PIN1 in promoting hepatocarcinogenesis, targeting PIN1 is a potential therapeutic approach against HCC. Through high-throughput screening technology, it is possible to identify novel and potent PIN1 inhibitors from different chemical compound libraries. However, the clinical use of PIN1 inhibitor is not only determined by its anti-cancer activity, but also depends on its *in vivo* bioavailability. Poor aqueous solubility and chemical instability of various identified PIN1 inhibitors limit their clinical applications. The development of microparticle (ATRA-PLLA) or liposome (API-LP) encapsulated PIN1 inhibitors show great superiority in the inhibition of HCC xenograft tumor growth by improving the bioavailability of PIN1 inhibitors in animals. Further study and work are required to develop various encapsulation methods for enhancing the anti-cancer effect of PIN1 inhibitors.

In recent years, a number of new molecular targeting drugs have been approved to treat patients with advanced-stage HCC (**Table 1**). Regorafenib is one of these new drugs that has been recommended as a second-line treatment option for sorafenib-resistant unresectable HCC. In a randomized clinical trial, regorafenib has been shown to improve overall survival in HCC patients that progressed following first-line sorafenib treatment (Bruix et al., 2017). However, similar to sorafenib treatment, regorafenib resistance will develop with time in HCC patients. A recent study has demonstrated that the development of regorafenib-resistant HCC cells by continuous low-dose treatment of regorafenib results in up-regulation of PIN1 (Wang et al., 2019). Although the relationship between PIN1 expression and acquired regorafenib-resistance in HCC remains to be further investigated and confirmed, the role of PIN1 in drug resistance in HCC would be an interesting topic that is worth exploring. A better understanding of the molecular mechanisms of PIN1 that lead to acquired drug resistance is critical to support the potential use of various PIN1 inhibitors as a second-line treatment option for drug-resistant HCC.

AUTHOR CONTRIBUTIONS

C-WC and ET wrote and approved the review.

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Prolyl Isomerase Pin1 Regulates the Stability of Hepatitis B Virus Core Protein

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The dynamic interplay between virus and host proteins is critical for establishing efficient viral replication and virus-induced pathogenesis. Phosphorylation-dependent prolyl isomerization by Pin1 provides a unique mechanism of molecular switching to control both protein function and stability. We demonstrate here that Pin1 binds and stabilizes hepatitis B virus core protein (HBc) in a phosphorylation-dependent manner, and promotes the efficient viral propagation. Phos-tag gel electrophoresis with various site-directed mutants of HBc revealed that Thr160 and Ser162 residues within the C terminal arginine-rich domain are phosphorylated concomitantly. GST pull-down assay and co-immunoprecipitation analysis demonstrated that Pin1 associated with phosphorylated HBc at the Thr160-Pro and Ser162-Pro motifs. Chemical or genetic inhibition of Pin1 significantly accelerated the rapid degradation of HBc via a lysosome-dependent pathway. Furthermore, we found that the pyruvate dehydrogenase phosphatase catalytic subunit 2 (PDP2) could dephosphorylate HBc at the Pin1-binding sites, thereby suppressing Pin1-mediated HBc stabilization. Our findings reveal an important regulatory mechanism of HBc stability catalyzed by Pin1 and may facilitate the development of new antiviral therapeutics targeting Pin1 function.

Keywords: virus-host interaction, phosphorylation, prolyl isomerization, lysosome, hepatitis B virus

INTRODUCTION

Virus–host interactions play important roles in virus replication and pathogenesis (Brito and Pinney, 2017). Viruses have evolved a number of ways of hijacking host machinery and cellular regulatory mechanisms to produce progeny viruses, as well as counteracting host immune systems (Mitra et al., 2018). Understanding these elaborate interactions may provide insight into the basic host elements indispensable for the viral life cycle, as well as antiviral host factors counteracting viral propagation. Moreover, the accumulation of information relevant to the molecular basis of virus–host interactions could be of great use in the development of new antiviral strategies.

Hepatitis B virus (HBV), a globally leading infectious agent, is the main cause of hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) (McMahon, 2005; Baumert et al., 2007).

Despite the availability of an HBV vaccine, approximately 350 to 400 million people are constantly infected with the virus in the world (Cryer and Imperial, 2019). Epidemiological studies suggest that persistent HBV infection is the major factor for the development of HCC (Lee and Ahn, 2016; Wang et al., 2017). HCC is a chief cause of cancer-associated deaths, highlighting the requirement for understanding the molecular mechanisms that regulate HBV replication in chronically infected HBV patients.

The genome structure of HBV is composed of circular partially double-stranded DNA, which is approximately 3.2 kb long and encodes four genes designated C (core), X, P (polymerase), and S (surface) (Beck and Nassal, 2007). Among these viral proteins, HBV core protein (HBc) plays pivotal roles in the viral replication processes, acting as the basic unit for capsid assembly, and is involved in HBV genome replication and progeny virion biosynthesis (Zheng et al., 2019). An essential structural element of HBV is the spherical capsid, which consists of multiple copies of a single HBc that contains viral pre-genomic RNA (pgRNA) and polymerase. HBc is a 21.5-kDa protein and composed of two specific domains, the N-terminal self-assembly domain (amino acids 1–140) and the C-terminal arginine-rich domain (CTD, amino acids 150–185) for the nucleic acid–binding (Nassal, 1992; Newman et al., 2003; Steven et al., 2005). The CTD plays an essential role in the specific encapsidation of pgRNA and polymerase during replication. Moreover, the phosphorylation of serine (Ser) or threonine (Thr) residues within the CTD can modulate multiple stages of HBV replication, such as viral core formation and subcellular localization (Diab et al., 2018). Although the accumulated evidence has emphasized the functional significance of the phosphorylation of CTD, it is still unknown whether phosphorylated HBc (pHBc) is subjected to further post-phosphorylation regulation.

The phosphorylation of proteins on serine or threonine residues that immediately precede proline (Ser/Thr-Pro) provides a unique signaling mechanism regulating a plethora of cellular processes, including cell proliferation, differentiation, and cell death (Lu et al., 2002). Peptidyl-prolyl *cis-trans* isomerase NIMA-interacting 1 (Pin1) is a regulator that specifically interact with phosphorylated Ser/Thr-Pro motifs and catalyzes the *cis* and *trans* amide isomer interconversion, leading to the conformational changes of its substrates (Lu and Zhou, 2007). This Pin1-mediated prolyl isomerization can provide further post-phosphorylation modifications that control various protein functions, such as protein stability, catalytic activity, protein–protein interactions, dephosphorylation and/or subcellular localization (Wulf et al., 2005; Lu et al., 2007; Liou et al., 2011; Nakamura et al., 2012). Recent studies have demonstrated that a number of viral proteins are also regulated by Pin1-mediated prolyl isomerization (Kojima and Ryo, 2010).

Here, we demonstrate that Pin1 binds pHBc and regulates its stability to sustain efficient viral replication. Specifically, we show that the targeted inhibition of Pin1 facilitates the prompt degradation of HBc via the lysosomal pathway. Furthermore, using NanoBRET technology, we showed that PDP2 serves as a negative regulator for HBc by selectively dephosphorylating HBc, thereby inhibiting the Pin1–HBc interaction. Our findings reveal an important molecular mechanism of HBc stabilization by Pin1-dependent prolyl isomerization and might provide insight into new antiviral therapeutics targeting Pin1 function.

RESULTS

Identification of Phosphorylation Sites in HBc CTD

Because HBc CTD contains multiple phospho-acceptor sites at Ser/Thr residues, we generated site-directed mutants in which Ser/Thr residues were replaced by alanine (**Figure 1A**). The wild-type (WT) HBc and the mutant proteins were expressed in cells, and cell lysates were subjected to Phos-tag polyacrylamide gel electrophoresis followed by immunoblot analysis. In a Phos-tag gel, the migration speed of phosphorylated proteins is reduced, separating them from non-phosphorylated proteins (specifically, the bands shift upward) (Kinoshita et al., 2006). WT HBc exhibited the most prominently shifted broad bands, reflecting its phosphorylation at multiple sites. On the other hand, HBc harboring a T160A or S162A mutation yielded relatively lower molecular weight bands than WT HBc and other site-directed mutants (S155A and S170A). Notably, the T160A/S162A double mutant yielded a much lower molecular weight band, implying that both sites are phosphorylated within HBc (**Figure 1B**). To further confirm phosphorylation at Thr160 and Ser162, we produced a phospho-specific HBc antibody (anti-pHBc) that exclusively detects phosphorylated Thr160/Ser162. Cells expressing either HA-tagged WT HBc or the T160A/S162A mutant were processed for the immunoblot analysis with anti-pHBc or anti-HA antibody. We observed phosphorylation of HBc only in WT HBc, but not in the T160A/S162A mutant (**Figure 1C**). Importantly, the phosphorylation signal was also detected in stably HBV-producing HepG2.2.15.7 cells, but this signal was diminished when the cell lysate was pre-treated with calf intestine alkaline phosphatase (CIAP) (**Figure 1D**). These results indicate that Thr160 and Ser162 are distinct phosphorylation sites within HBc.

Pin1 Interacts With Phosphorylated HBc

The results described above indicate that HBc is phosphorylated at Thr160 and Ser162, both of which are potential Pin1-binding sites (pSer/Thr-Pro). We next asked whether Pin1 directly binds to these sites within HBc. To this end, we generated recombinant GST-Pin1 and the WW domain mutant (W34A), which lacks pSer/Thr-Pro binding activity (Zhou et al., 2000). GST pull-down assay with whole-cell lysate from HepG2 cells expressing HA-HBc revealed that HBc co-precipitated with GST-Pin1 but not with GST-Pin1W34A or control GST (**Figure 2A**). The association between Pin1 and HBc was abolished by pretreatment of the cell lysates with CIAP prior to the GST pull-down analysis (**Figure 2B**), indicating that Pin1 can only interact with pHBc. The intracellular interaction between HBc and Pin1 was also confirmed by immunoprecipitation analysis where Pin1 was co-precipitated with HA-HBc (**Figure 2C**). We also observed that endogenous

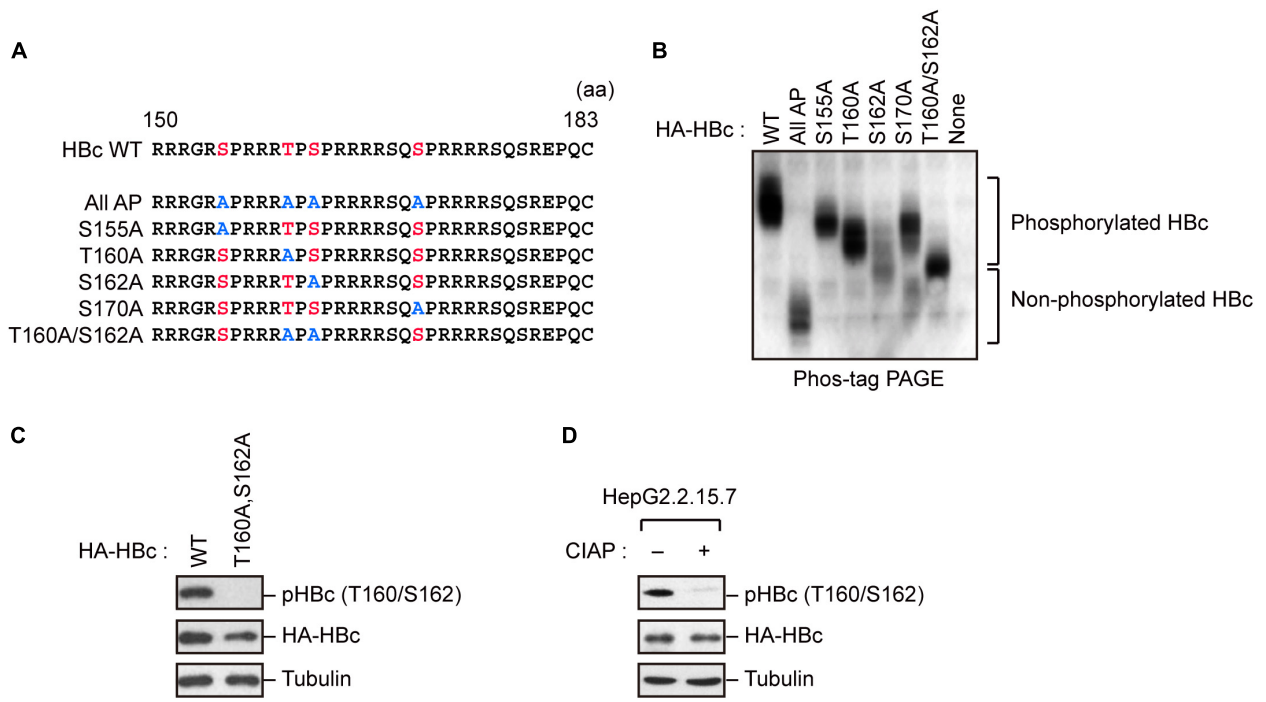

FIGURE 1 | Concomitant phosphorylation of HBc at Thr160 and Ser162. **(A)** Schematic representation of the HBc deletion mutants generated in this study. The sequence of the HBc CTD, with the four major phosphorylation sites (S155, T160, S162, and S170) and alanine substitutions, is shown. **(B)** Mobility shifts of HBc in Phos-tag Gel. HepG2 cells were transfected with plasmids encoding HA-HBc or its site-directed mutants. The transfected cells were harvested at 24 h post-transfection, and cell lysates were subsequently subjected to Phos-tag gel electrophoresis and analyzed by immunoblot analysis with anti-HA antibody. **(C)** Detection of phosphorylation of HBc by phospho-specific antibody. HepG2 cells were transfected with WT HBc or its site-directed (T160A/S162A) mutant for 48 h in the presence of protease inhibitors. Cell lysates were then subjected to immunoblot analysis with anti-phospho HBc (T160/S162), anti-HBc, or anti-α-tubulin antibodies. **(D)** Cell lysates from stably HBV-producing HepG2.2.15.7 cells were treated or not treated with calf intestine alkaline phosphatase (CIAP), and then subjected to immunoblotting with anti-phospho HBc (T160/S162), anti-HBc, and anti-α-tubulin antibodies.

Pin1 could bind HBc in HBV-producing cells (**Figure 2D**). We next attempted to determine the Pin1-binding sites for HBc. HepG2 cells were transfected with plasmid encompassing HA-HBc or its mutants (T160A, S162A, or T160A/S162A), and then subjected to GST pull-down assay. We found that a single site-directed mutation (T160A and S162A) resulted in the prominent reduction of co-precipitated HBc with GST-Pin1 (**Figure 2E**). Notably, HBc harboring a double mutation (T160A/S162A) completely lost the ability to bind GST-Pin1 (**Figure 2E**). These results illustrate that Pin1 directly interacts with pHBc at Thr160 and Ser162. Although above Phos-tag analysis indicated that S162 is the major site of the Pin1-binding phosphorylation site (**Figure 1B**), we found that a single site-directed mutant (S162A) still interacted with Pin1 with relatively lower binding activity. Since mutation in both sites (T160A/S162A) completely abolished its Pin1-binding, these results indicate that Thr160 is an another Pin1 binding-phosphorylated residue.

Given that HBc CTD can be phosphorylated by cyclin-dependent kinases (CDKs) (Ludgate et al., 2012), we next asked whether the inhibition of CDKs could affect the Pin1 interaction with HBc. We found that treatment with the broad-spectrum CDK inhibitor roscovitine significantly reduced Pin1–HBc binding along with decreased levels of pHBc (**Figures 2F,G**), indicating that CDKs contribute to the Pin1–HBc interaction, presumably by mediating the phosphorylation of Thr160/Ser162 residues.

Pin1 Regulates HBc Stability

Because Pin1 is a general regulator of protein stability, it is plausible that Pin1 could stabilize HBc. To test this proposition, we knocked down Pin1 in HepG2.2.15.7 cells by stable transduction of Pin1-specific shRNA. Immunoblot analysis demonstrated that HBc expression was significantly decreased upon Pin1 depletion (**Figure 3A**). Notably, a parallel experiment showed that HBV mRNA levels were not significantly altered following Pin1 depletion (**Figure 3B**), indicating post-translational regulation of HBc. The reduced level of HBc was also observed in Pin1-knockdown HepG2.2.15.7 cells, and this reduction was rescued by transient expression of Pin1, but not Pin1W34A (**Figure 3C**). We also found that the Pin1 inhibitor juglone (Chao et al., 2001) prominently reduced the protein expression of HBc (**Figure 3D**). Cycloheximide analysis further revealed that the protein stability of HBc was prominently decreased in Pin1-knockdown cells as compared with control cells (**Figure 3E**). Together, these results suggest that Pin1 inhibition decreases HBc stability, thereby decreasing the HBc protein level in cells.

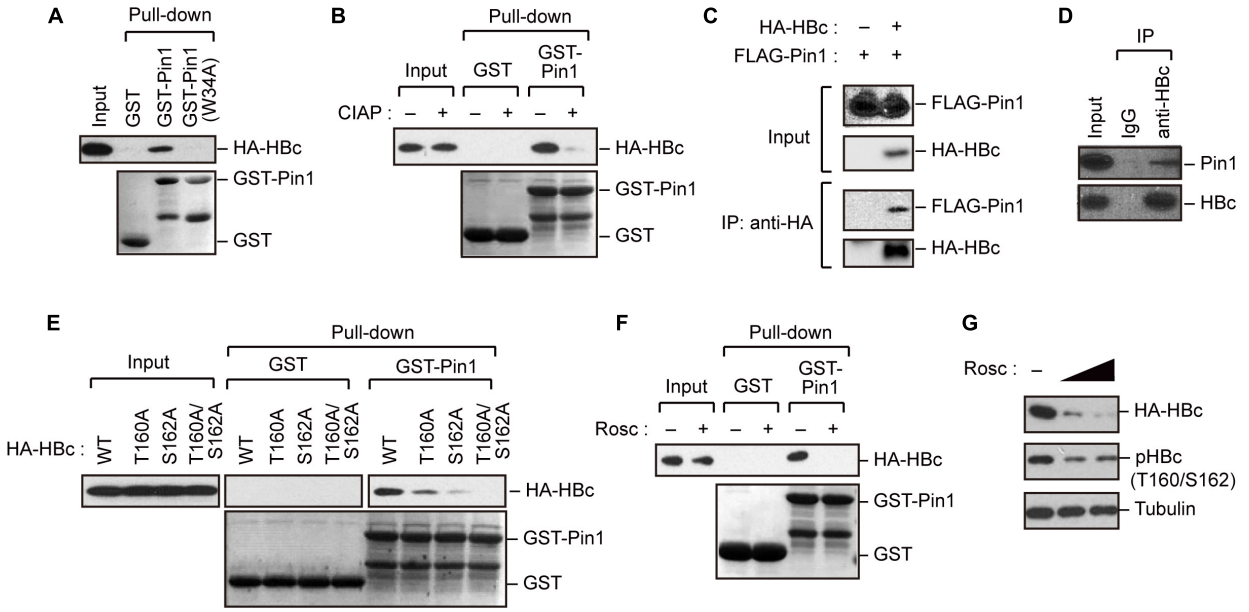

FIGURE 2 | Pin1 interacts with phosphorylated HBc. **(A)** HepG2 cells were transfected with plasmid encoding HBc. After 48 h, cell lysates were subjected to GST pull-down analysis with GST, GST-Pin1, or GST-Pin1W34A mutant followed by immunoblotting with anti-HA antibody. **(B)** Cell lysates derived from HepG2 cells transfected with HBc were treated or not treated with CIAP, followed by GST pull-down analysis as described in **(A)**. **(C)** HepG2 cells were transfected with HA-HBc and FLAG-Pin1 expression vectors. After 48 h, cell lysates were subjected to immunoprecipitation (IP) analysis with anti-HA or non-immunized IgG, followed by immunoblotting analysis with anti-FLAG or anti-HA antibodies. **(D)** HepG2.2.15.7 cell lysates were subjected to IP analysis with anti-HBc or non-immunized IgG, followed by immunoblotting analysis with anti-Pin1 or anti-HBc antibodies. **(E)** Pin1 interacts with HBc via its Thr160-Pro and Ser162-Pro motifs. HepG2 cells were transfected with WT HBc or the indicated mutants for 48 h in the presence of lysosome inhibitors. Cell lysates were then subjected to GST pull-down followed by immunoblot analysis. **(F)** HepG2 cells were transfected with HA-HBc expression plasmid. At 24 h following transfection, cells were treated with roscovitine (Rosc, 50 μM). After 15 h, cell lysates were harvested and subjected to GST pull-down analysis as shown in **(A)**. **(G)** HepG2 cells expressing HA-HBc were treated with roscovitine (Rosc, 25 or 50 μM). After 15 h, cell lysates were subjected to immunoblotting analysis with indicated antibodies.

To further delineate the functional implication of the Pin1–HBc interaction, we investigated the protein stability of the T160A/S162A mutant, which is unable to bind Pin1. Cycloheximide analysis demonstrated that the HBc-T160A/S162A mutant was conspicuously destabilized relative to WT HBc (**Figure 3F**), confirming that Pin1 indeed regulates the HBc stability by interacting with the phosphorylated Thr160-Pro and Ser162-Pro motifs.

Pin1 Inhibits Lysosomal Degradation of HBc

Given that Pin1 stabilizes HBc, we next attempted to clarify the molecular pathway by which HBc degraded. To this end, we utilized chemical inhibitors, bafilomycin and NH_4Cl (lysosome inhibitors) or MG132 (proteasome inhibitor). Pin1-depleted HepG2.2.15.7 cells were treated with each inhibitor for 24 h, and HBc protein levels were examined by immunoblotting. Our result demonstrated that bafilomycin and NH_4Cl, but not MG132, prominently reverted the HBc instability upon Pin1 knockdown (**Figure 4A**), indicating that Pin1 might inhibit the endo-lysosomal degradation of HBc. Immunofluorescence analysis demonstrated that HBc was colocalized with the lysosome, forming cytoplasmic foci in Pin1-knockdown cells whereas control cells exhibited a relatively diffuse pattern of HBc in the cytoplasm without lysosomal co-localization (**Figure 4B**). Together, these results indicate that Pin1 counteracts the lysosomal degradation of HBc.

Screening of Phosphatases for Pin1 Bindings Sites Within HBc

To better understand the regulation of HBc stability, we screened host phosphatases that remove phosphate(s) from pHBc. For this object, we performed the NanoBRET protein–protein interaction assay (Machleidt et al., 2015). This method employs a NanoLuc fusion protein as the bioluminescent donor and a fluorescently labeled HaloTag fusion protein as the acceptor. We cotransfected the NanoLuc-tagged HBc and 150 different HaloTag-conjugated phosphatases into HEK293 cells (**Figure 5A**, left). At 48 h post-transfection, the BRET signal was visualized, and a BRET ratio >0.2 was used as the threshold. We identified two phosphatases (SNAP23 and PDP2) whose BRET signals were much higher than those of other phosphatases (**Figure 5A**, right). Accordingly, we focused on SNAP23 and PDP2 for further functional analysis. To investigate the direct interaction of the phosphatases with HBc, we performed immunoprecipitation analysis using HepG2 cells co-transfected with plasmids encoding HA-HBc and either HT-PDP2 or SNAP23. Our result revealed that HBc was co-precipitated with PDP2, but not with SNAP23 (**Figure 5B**), indicating that PDP2 can physically associate with HBc.

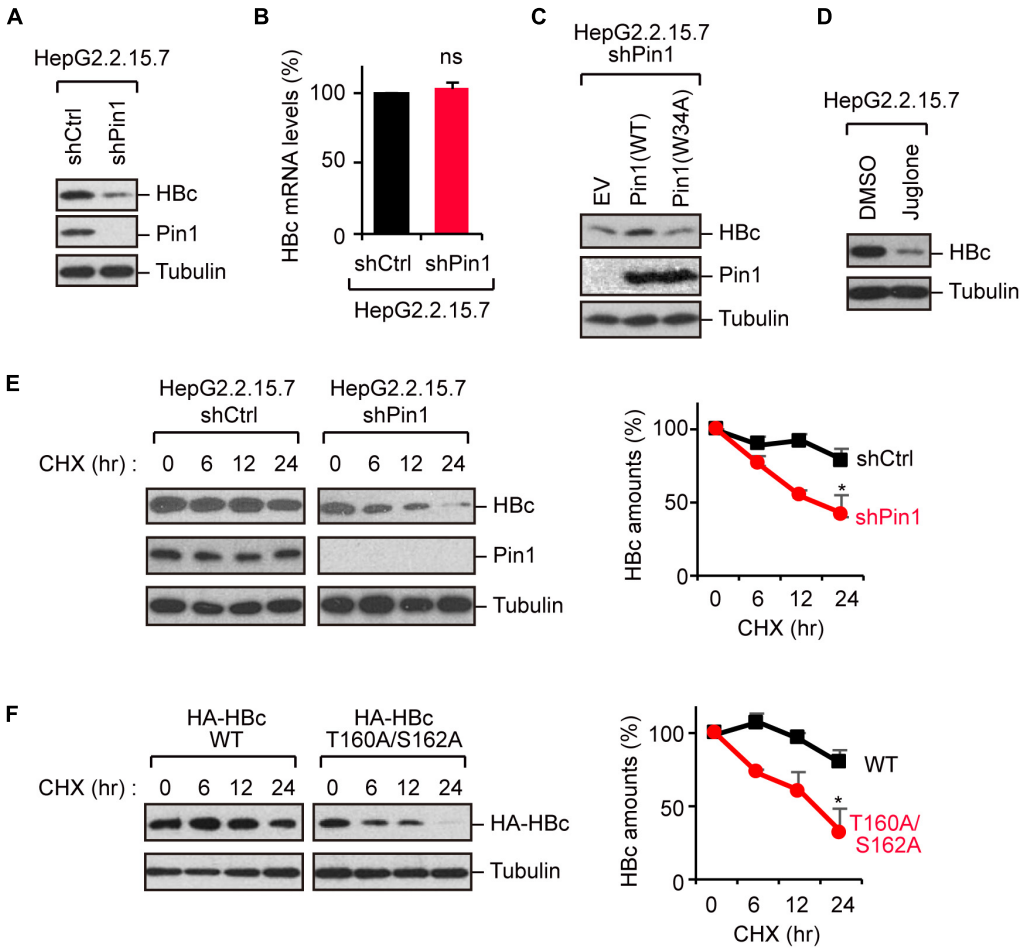

FIGURE 3 | Pin1 regulates HBc stability. **(A)** Lysates from HepG2.2.15.7 cells that were infected with retroviral vectors carrying control shRNA (shCtrl) or Pin1-specific shRNA (shPin1) were immunoblotted with anti-HBc, anti-Pin1, or anti-α-tubulin antibodies. **(B)** Total mRNA from indicated HepG2.2.15.7 cells were subjected to quantitative PCR for HBc mRNA. Data were normalized with the amounts of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). ns, not significant. **(C)** Pin1-depleted HepG2.2.15.7 cells were transfected with empty vector (EV), Pin1WT, or Pin1W34A mutant. After 48 h, cell lysates were subjected to immunoblotting with anti-HBc, anti-Pin1, or anti-α-tubulin antibodies. **(D)** HepG2.2.15.7 cells were treated with either DMSO or 5 μM juglone for 24 h. Cell lysates were then subjected to immunoblot analysis with anti-HBc or anti-α-tubulin antibodies. **(E)** HepG2.2.15.7 cells were treated with 100 μM cycloheximide (CHX) and harvested at the indicated time points, followed by immunoblotting analysis with anti-HBc, anti-Pin1, or anti-α-tubulin antibodies. Quantitative data are shown in the right panel. *$P < 0.05$, two-tailed unpaired *t*-test. **(F)** HepG2 cells were transfected with WT HBc or the T160A/S162A mutant followed by CHX assay as shown in **(D)**. Quantitative data are shown in the right panel. *$P < 0.05$, two-tailed unpaired *t*-test.

We next asked whether PDP2 could dephosphorylate HBc. HepG2 cells were co-transfected with HA-HBc and HT-PDP2. After 48 h, cells were harvested and cell lysates were subjected to immunoblot analysis Our data demonstrated that PDP2 expression decreased the level of HBc while dephosphorylating it in a dose-dependent manner (**Figure 5C**). Consistent with this, GST pull-down assay revealed that PDP2 overexpression inhibited the interaction between Pin1 and HBc (**Figure 5D**). These results were also confirmed in HepG2.2.15.7 cells; PDP2 was able to decrease pHBc and interfere with Pin1-HBc interaction (**Figures 5E,F**). Of note, we found that PDP2-mediated dephosphorylation of HBc could negatively regulate HBV particle production (**Figure 5G**). These results together indicate that PDP2-mediated HBc dephosphorylation results in the dissociation of Pin1 from HBc, thereby reducing HBc stability as well as HBV biosynthesis.

Effect of Pin1–HBc Interaction on HBV Propagation

To investigate the functional role of Pin1 in HBV replication, we attempted to knock down Pin1 in HepG2.2.15.7 cells that can stably secrete viral particles in culture supernatant. We then analyzed HBV DNA and virus core antigen (HBcAg) in the cell supernatant by quantitative PCR and ELISA, respectively. The results illustrated that Pin1 knockdown had no effect on cell proliferation (**Figure 6A**), but prominently decreased the levels of both viral DNA and HBcAg relative to control cells (**Figures 6B,C**), indicating a reduction in viral particle

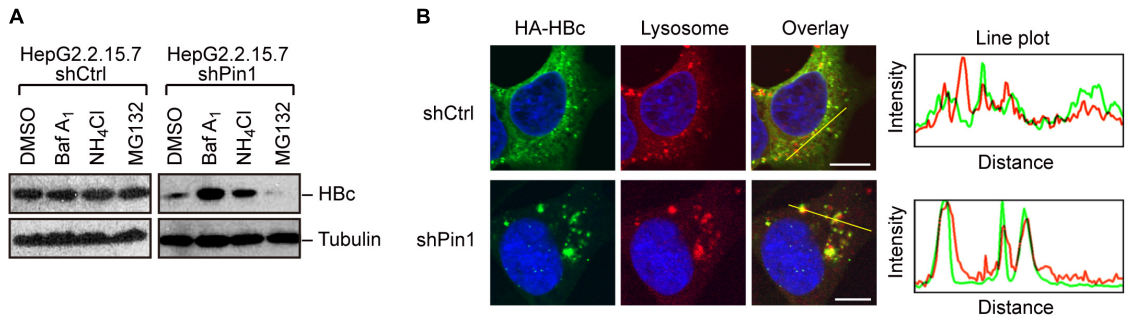

FIGURE 4 | Pin1 inhibits lysosomal degradation of HBc. **(A)** HepG2.2.15.7 cells transduced with shCtrl or shPin1 were treated with the indicated inhibitors for 24 h. Cell lysates were then subjected to immunoblot analysis with anti-HBc or anti-α-tubulin antibodies. The final concentration of inhibitors as follows; BafilomycinA1, 100 nM; NH_4Cl, 4 mM; MG132, 10 μM. **(B)** HepG2 cells transduced with shCtrl or shPin1 cells were transfected with HA-HBc expression vector. After 24 h, cells were fixed with 3% formaldehyde and immunostained with anti-HA (green), LysoTracker (red), and DAPI (blue). Cells were then subjected to confocal microscopy. Scale bar, 10 μm. Line plots indicate the fluorescence intensity of the left images.

production. To further delineate the biological importance of the Pin1–HBc interaction, we tested the efficiency of virus production of HBV encoding WT HBc or its T160A/S162A mutant. HepG2 cells were transfected with an HBV molecular clone (either WT or T160A/S162A), and supernatants were collected to analyze HBV DNA and HBcAg. Amounts of HBV DNA and HBcAg, but not HBeAg devoid of Pin1-binding site, were significantly reduced in the case of the T160A/S162A virus relative to the WT virus (**Figures 6D–F**). Together, these results indicate that the Pin1 interaction with HBc stabilizes HBc, eventually leading to efficient virus particle production in HBV infected cells.

DISCUSSION

Viral proteins are required to interact with host proteins to maintain the viral life cycle. Some host proteins act as antiviral factors to restrict viral propagation, whereas others interact with viral proteins in a manner that sustains viral replication. Understanding the molecular operations of the virus-host interaction will aid in identification of new therapeutic targets and to develop antiviral strategies. In this study, we revealed that the peptidyl-prolyl isomerase Pin1 is a potent host factor that binds HBc and facilitates viral biogenesis. Moreover, by screening a phosphatase library, we identified PDP2 as the phosphatase responsible for the dephosphorylation of Thr160/Ser162 residues within HBc. PDP2 counteracts Pin1-mediated HBc stabilization, thereby decreasing virus propagation (**Figure 6G**). Our current findings shed new light on a virus–host interaction mediated by viral protein phosphorylation and subsequent prolyl isomerization by Pin1.

Protein phosphorylation is a major fashion of post-translational modification and, by modulating intracellular signaling pathways, serves as an essential regulatory event for many cellular processes (Hunter, 1995). Phosphorylated proteins are likely to undergo a novel type of post-phosphorylation regulation by Pin1. Pin1 recognizes phosphorylated serine or threonine residue immediately preceding a proline residue (pSer/Thr-Pro) (Ryo et al., 2003). Following the binding to substrates, Pin1 catalyzes the conformation *via cis-trans* isomerization of the peptide bonds, which alters the catalytic activity, localization, and stability of target proteins (Ryo et al., 2003; Lu and Zhou, 2007). Our current observations show that Pin1 binds to phosphorylated HBc, thereby stabilizing the viral protein. Accordingly, Pin1 inhibition promotes HBc degradation via the lysosomal pathway to reduce progeny viral production. Our results reveal a previously undescribed role of Pin1 in the post-phosphorylation regulation of HBc and suggest that the Pin1 inhibition represents a promising new therapeutic option for treating HBV-related diseases.

The post-phosphorylation switch mediated by Pin1 is involved in the stability and function of several viral proteins. For example, Pin1 modulates DNA polymerase conformation of Epstein–Barr virus and is responsible for productive viral replication (Narita et al., 2013). Pin1 also binds to the non-structural NS5A/NS5B proteins of Hepatitis C virus, stabilizing them (Lim et al., 2011). The viral replication processes of HIV-1 in genome integration (Manganaro et al., 2010) and capsid uncoating (Misumi et al., 2010) are also regulated by Pin1. Moreover, Pin1 has been shown to enhance the stability of human T-cell leukemia virus type 1 Tax oncoprotein and facilitate the malignant transformation (Jeong et al., 2009). In the case of HBV infection, Pin1 binds HBx protein and increase its transcriptional competency to cell proliferation and oncogenesis (Pang et al., 2007). These studies demonstrate that Pin1 plays a pivotal function in viral replication for a broad range of viruses. However, the role of Pin1 in HBV replication, especially in viral core formation, has yet to be resolved. In our current report, we demonstrated that Pin1 also associate with phosphorylated HBc and stabilizes HBc, thereby promoting efficient virus propagation. Although we found that Pin1 suppressed HBc degradation through the inhibition of endo-lysosomal-mediated degradation pathway, its precise mechanism is still uncertain. A previous report showed that intracellular HBc proteins could be transported to early endosomes and lysosomes, depending on the adaptor protein Eps15 and the small GTPase Rab5 (Cooper and Shaul, 2006). Pin1 may prevent the association of these factors to HBc. Further

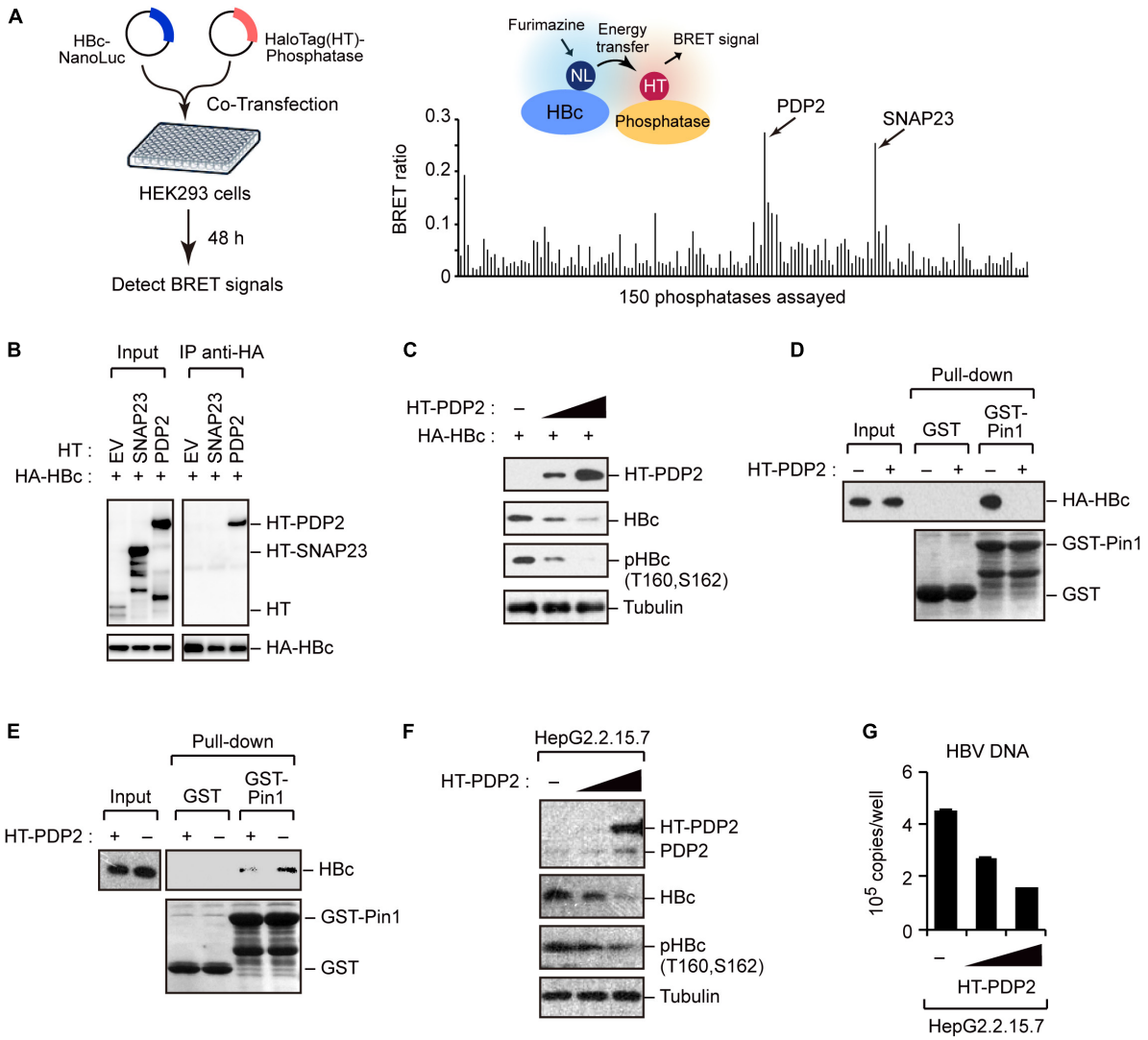

FIGURE 5 | Screening of phosphatases for Pin1 binding sites within HBc. **(A)** NanoBRET-based screen to identify HBc-interacting proteins in living cells. Schematic representation of the NanoBRET-based screening method (left panel). HEK293 cells were co-transfected with NanoLuc-tagged HBc and HaloTag-conjugated phosphatase expression vectors, followed by Halotag-620 ligand and furimazine substrate addition to the cells. If two proteins were within 200 nm of each other, BRET signals were detected. Two candidates with high BRET ratios (>0.2; right panel) were also shown. **(B)** HEK293 cells were co-transfected with HA-HBc together with HT empty vector (EV), HT-SNAP23, or HT-PDP2 and cultured for 24 h in the presence of protease inhibitors. Cell lysates were then subjected to immunoprecipitation with anti-HA antibody, followed by immunoblot analysis with the indicated antibodies. **(C)** PDP2 decreases HBc-T160/S162 phosphorylation. HepG2 cells were co-transfected with the expression vector encoding HA-HBc and Halotag (HT)-PDP2. At 48 h post-transfection, cells were harvested and subjected to immunoblot analysis with the indicated antibodies. **(D,E)** PDP2 interferes with HBc-Pin1 interaction. HepG2 cells expressing HA-HBc and HT-PDP2 **(D)** or HepG2.2.15.7 cells expressing HT-PDP2 **(E)** were lysed and subjected to GST pull-down analysis with GST or GST-Pin1, followed by immunoblot analysis with indicated antibodies. **(F,G)** HepG2.2.15.7 cells were transfected with expression vector encoding HT-PDP2. At 48 h post-transfection, cell lysates were subjected to immunoblot analysis with indicated antibodies. The levels of HBV DNA in the culture supernatants were measured by real-time PCR.

careful analysis will be required to more precisely determine the molecular function of Pin1 with regard to HBc turnover during HBV particle production.

Hepatitis B virus core protein contains several phosphorylation recognition motifs at Ser or Thr residues preceding Pro (Ser/Thr-Pro) in its CTD phospho-acceptor sites, which are remarkably well conserved among related viruses (Jung et al., 2014). A previous report have identified at least seven conserved serine and threonine residues subjected to phosphorylated *in vivo* (Chen et al., 2011). Especially, the Ser-Pro motifs at positions 155, 162, and 170, are highly retained, and phosphorylated by multiple host serine/threonine protein kinases (Daub et al., 2002; Ludgate et al., 2012). The HBc protein contains another three major phosphorylated serine residues (Ser155, 162, and 170), along with four additional phosphorylated serine residues (Ser168, 176, and 178) and one phosphorylated threonine residue (Thr160) (Lan et al., 1999; Steven et al., 2005; Jung et al., 2014; Ludgate et al., 2016). By

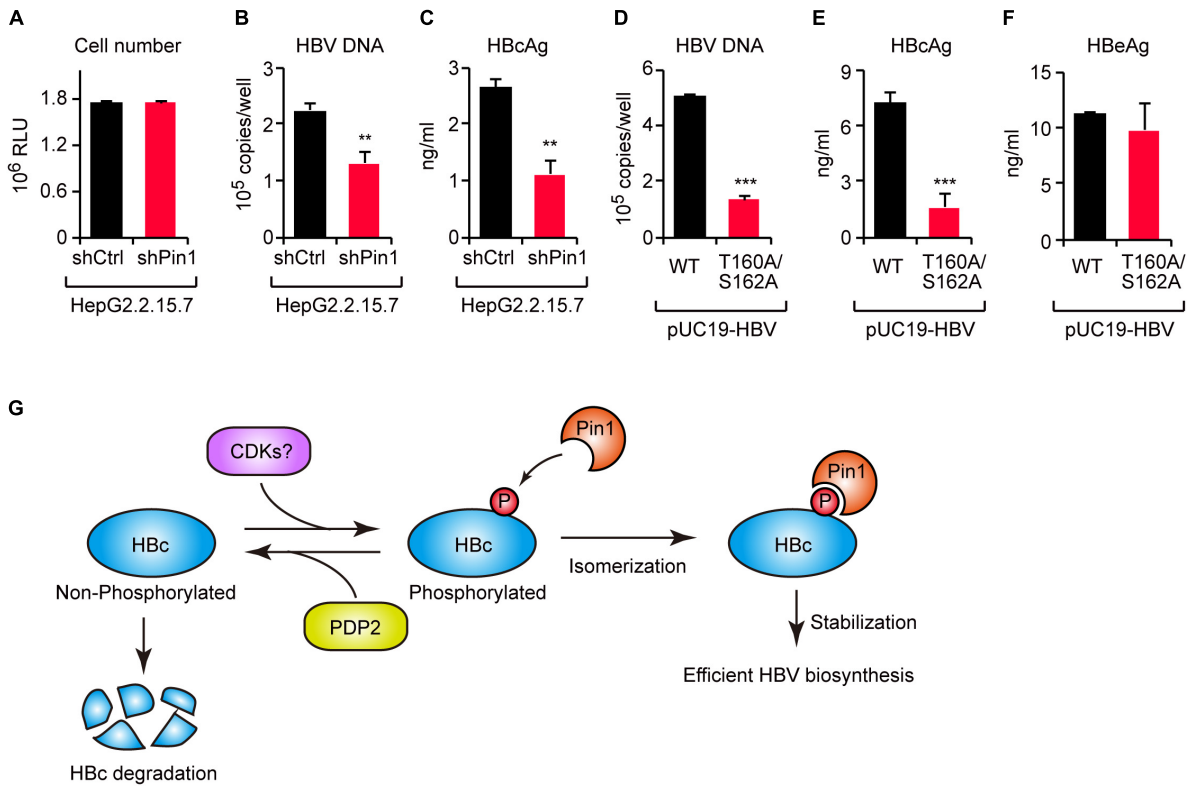

FIGURE 6 | The HBc–Pin1 interaction regulates HBV biosynthesis. **(A)** Cell viability analysis of HepG2.2.15.7 cells stably expressing shCtrl or shPin1. **(B,C)** The levels of HBV DNA **(B)** and HBcAg **(C)** in the culture supernatants of HepG2.2.15.7-shCtrl or HepG2.2.15.7-shPin1 cells were measured by real-time PCR and ELISA, respectively. $^{**}P < 0.01$, two-tailed unpaired *t*-test. **(D–F)** HepG2 cells were transfected with an HBV molecular clone (pUC19-C_JPNAT) and its site-directed mutant (T160A/S162A). After 24 h, the levels of HBV DNA in the culture the culture supernatants were measured by real-time PCR **(D)**, and the levels of HBcAg **(E)** or HBeAg **(F)** in the culture supernatants were measured by ELISA. $^{***}P < 0.001$, two-tailed unpaired *t*-test. **(G)** Schematic representation of the model proposed in this study. CDKs phosphorylate HBc to create Pin1-binding sites. Subsequently, Pin1 stabilizes HBc by preventing its lysosomal degradation, thereby promoting effective HBV biosynthesis. On the other hand, PDP2 dephosphorylates HBc to enhance its degradation.

screening the Ser/Thr phosphorylation of HBc CTD using Phos-tag gel, we also identified two concomitant phosphorylations at Thr160 and Ser162, consistent with previous results (Jung et al., 2014; Heger-Stevic et al., 2018). CTD phosphorylation of HBc is mediated by host cell kinases, including cyclin-dependent kinase 2 (CDK2) (Ludgate et al., 2012), protein kinase C (PKC) (Kann and Gerlich, 1994), cyclin-dependent protein kinase $p34^{cdc2}$ (also known as CDK1) (Yeh et al., 1993), the 46-kDa serine protein kinase (Kau and Ting, 1998), and serine/arginine-rich protein kinases 1 and 2 (SRPK1/2) (Daub et al., 2002; Heger-Stevic et al., 2018). PLK1 is also involved in CTD phosphorylation (Diab et al., 2017). However, it remains unclear whether phosphorylated HBc is conversely dephosphorylated by host phosphatases. Therefore, we screened a phosphatase library to uncover the molecular mechanism involved in the phosphorylation/dephosphorylation regulation of HBc. By screening 150 genes in the phosphatase library, we found that PDP2 interacts with phosphorylated HBc and dephosphorylates it, leading to HBc degradation and reduction of viral production. PDP2 dephosphorylates and reactivates the alpha subunit of the E1 component of the pyruvate dehydrogenase complex, and is thus involved in the enzymatic resetting of the pyruvate dehydrogenase complex (Huang et al., 1998). Therefore, it would be interesting to examine the relationship between glucose metabolism, HBc phosphorylation, and virus replication.

MATERIALS AND METHODS

Cell Culture

HEK293 cells (ATCC, CRL-1573) and HepG2 cells (ATCC, HB-8065) were cultured in DMEM (Fujifilm Wako) containing 10% FBS. HepG2.2.15.7 cells (Iwamoto et al., 2017) were cultured with DMEM/F-12, GlutaMAX (Thermo Fisher Scientific) supplemented with 10% FBS, 10 mM HEPES (Thermo Fisher Scientific), and 5 μg/ml insulin (Sigma-Aldrich). HepG2 and HepG2.2.15.7 cells were grown on collagen-coated dishes.

shRNA-Mediated Gene Silencing

To generate Pin1-depleted cells, cells were infected with retrovirus vector carrying Pin1-specific shRNA (Ryo et al., 2005). For the production of retroviruses, Plat-E cells (Morita et al., 2000) were transduced with pSUPER.retro vector and pVSV-G with Effectene reagent (Qiagen). After 48 h, cell

supernatants were filtrated with a 0.45-μm filter and added with 10 μg/ml Polybrene. Target cells were then selected with 1 μg/ml puromycin (InvivoGen).

GST Pull-Down, Immunoprecipitation, and Immunoblotting Analyses

GST pull-down assay was previously described (Nishi et al., 2011). Briefly, cells were treated with 100 nM bafilomycin A1 and 4 mM NH_4Cl for 15 h before harvesting, treated with GST pull-down buffer (50 mM HEPES pH 7.4, 200 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 100 mM NaF, 1 mM Na_3VO_4, 0.5 μg/ml leupeptin, 1 μg/ml pepstatin, and 0.2 mM PMSF), and incubated at 4°C for 3 h with glutathione-agarose beads containing either GST or GST-Pin1. The collected beads were then washed three times with GST pull-down buffer and processed for SDS-PAGE. To immunoprecipitate proteins, cells were harvested and lysed with NP-40 lysis buffer (10 mM Tris–HCl pH 7.4, 100 mM NaCl, 1% NP-40, 2 mM EDTA, 1% sodium deoxycholate, 50 mM NaF, 1 mM Na_3VO_4, 0.5 μg/ml leupeptin, 1 μg/ml pepstatin, and 0.2 mM PMSF). Cell lysates were then incubated for 1 h with protein A/G–Sepharose beads (GE Healthcare). Supernatant fractions were recovered and immunoprecipitated with 4 μg of mouse IgG or anti-HA (MBL) together with 20 μl of protein A/G–Sepharose at 4°C for 3 h. After washing three times with lysis buffer, the bound proteins were analyzed by immunoblotting, as previously described (Miyakawa et al., 2018, 2019). For Phos-tag PAGE, we used 12.5% acrylamide gel containing 50 μM Phos-tag (Fujifilm Wako). Source data are provided as a **Supplementary Material** (**Data Sheets S1, S2**).

Plasmids and Antibodies

The hepatitis B virus molecular clone pUC19-C_JPNAT (genotype C) has been described previously (Sugiyama et al., 2006). HBc cDNAs were amplified from pUC19-C_JPNAT with the appropriate primer pairs, followed by subcloning into the pcDNA-based N-HA vector (Thermo Fisher Scientific). The HBc derivatives were constructed using PCR-based mutagenesis. The primary antibodies used in this study were as follows: anti-HA (MBL), anti-FLAG and anti-α-tubulin (Sigma-Aldrich), anti-Pin1 (R&D System), anti-HaloTag (Promega), and anti-HBc monoclonal antibody (Kanto Chemical). A phospho-specific polyclonal antibody against HBc phosphorylated at Thr160 and Ser162 was generated by Scrum Inc. (Tokyo, Japan).

Protein Degradation Assay

Protein degradation assays were performed as described previously (Nishi et al., 2011). Briefly, 100 μM cycloheximide was added to the medium, and the cells were harvested at the indicated time points. Total cell lysates in SDS sample buffer were boiled and analyzed by immunoblotting.

Microscopic Analysis

Microscopic procedure was previously described (Miyakawa et al., 2017). Briefly, HepG2 cells were seeded onto glass cover slips 1 day before transfection. At 48 h post-transfection, the cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. The cells were then stained with anti-HA (MBL) and Alexa Fluor 488-conjugated secondary antibody (Thermo Fisher Scientific). For lysosome staining, cells were treated with Lysosomes-RFP reagents (Thermo Fisher Scientific) at 16 h prior to fixation. Microscopic imaging was performed with an FV1000-D confocal microscope (Olympus). Line plots of the fluorescence intensity were generated using the ImageJ software (NIH).

NanoBRET-Based Protein–Protein Interaction Assays

Expression vectors encoding N-terminally HaloTag-conjugated host proteins (human phosphatases) were prepared by Kazusa Genome Technologies (Chiba, Japan) or purchased from Promega. NanoBRET analysis were performed as described previously (Miyakawa et al., 2019). Briefly, HEK293 cells were transfected with vectors encoding HaloTag-fused protein and NanoLuc-fused HBc at a 100:1 ratio. At 48 h post-transfection, NanoBRET activity was measured using the NanoBRET Nano-Glo Detection System (Promega).

HBV Quantification Assays

Hepatitis B virus quantification procedure were previously described (Miyakawa et al., 2015). Culture supernatants of HepG2.2.15.7 cells or HepG2 cells expressing HBV molecular clone were cleared of cell debris by centrifugation at 3,000 rpm for 3 min. The HBcAg and HBeAg amounts in the culture supernatants were measured using HBcAg and HBeAg ELISA kit (Cell Biolabs), respectively. To remove the plasmid-derived DNA, culture supernatants were digested at 37°C for 2 h with 200 μg/ml DNase I, 100 μg/ml RNase A, and 6 mM MgOAc, and then centrifuged at 13,000 rpm for 1 min. The supernatants were then mixed with a buffer containing 10 mM EDTA, 1% SDS, 100 mM NaCl, and 200 μg/ml proteinase K (Roche), and incubated at 55°C for 1 h. These samples were extracted with phenol/chloroform, precipitated with ethanol, and dissolved in TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA). Amount of viral DNA was measured by real-time PCR using SYBR Premix Ex Taq II (Takara) as previously described (Miyakawa et al., 2015). For quantification of intracellular viral RNA, total RNA extraction was performed using the Trizol reagent (Thermo Fisher Scientific) and cDNA synthesis was conducted with ReverTra Ace (Toyobo), respectively.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

MN and KM designed and performed the research, analyzed the data, and wrote the manuscript. SM and YY performed the

research and analyzed the data. HKh analyzed the data and wrote the manuscript. KW and MS contributed reagents and analyzed the data. HKi and TW analyzed the data. AR directed the research, analyzed the data, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00026/full#supplementary-material

DATA SHEET S1 | Full images of the immunoblots presented in **Figures 1**–**4**.

DATA SHEET S2 | Full images of the immunoblots presented in **Figure 5**.

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REVIEW
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Post-translational Modifications of the Peptidyl-Prolyl Isomerase Pin1

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The peptidyl-prolyl *cis/trans* isomerase (PPIase) Pin1 is a unique enzyme that only binds to Ser/Thr-Pro peptide motifs after phosphorylation and regulates the conformational changes of the bond. The Pin1-catalyzed isomerization upon phosphorylation can have profound effects on substrate biological functions, including their activity, stability, assembly, and subcellular localization, affecting its role in intracellular signaling, transcription, and cell cycle progression. The functions of Pin1 are regulated by post-translational modifications (PTMs) in many biological processes, which include phosphorylation, ubiquitination, SUMOylation and oxidation. Phosphorylation of different Pin1 sites regulates Pin1 enzymatic activity, binding ability, localization, and ubiquitination by different kinases under various cellular contexts. Moreover, SUMOylation and oxidation have been shown to downregulate Pin1 activity. Although Pin1 is tightly regulated under physiological conditions, deregulation of Pin1 PTMs contributes to the development of human diseases including cancer and Alzheimer's disease (AD). Therefore, manipulating the PTMs of Pin1 may be a promising therapeutic option for treating various human diseases. In this review, we focus on the molecular mechanisms of Pin1 regulation by PTMs and the major impact of Pin1 PTMs on the progression of cancer and AD.

Keywords: Pin1, phosphorylation, oxidation, post-translational modification, SUMOylation, ubiquitination, Alzheimer's disease, cancer

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INTRODUCTION

Post-translational modifications (PTMs) of proteins play important roles in regulating protein conformation, localization, stability, and activity and ultimately induce a number of fundamental biological functions, including signal transduction, protein–protein interaction, protein trafficking, cell differentiation, and proliferation (Marcelli et al., 2018; Wu et al., 2019). To date, more than 450 PTMs have been identified, including phosphorylation, oxidation, ubiquitination, and SUMOylation (Venne et al., 2014). PTMs are reversible and tightly regulated during physiological conditions. However, gene mutations, increased cellular stresses, and deregulated cellular signals can modify PTMs or introduce non-specific PTMs and contribute to the development of human disease, notably cancer and neurodegeneration (Martin et al., 2011; Mowen and David, 2014; Marcelli et al., 2018; Wu et al., 2019).

The peptidyl-prolyl *cis/trans* isomerase (PPIase) Pin1 was first identified by a combined genetic and biochemical screening strategy based on its physical interaction with the *Aspergillus* mitotic kinase NIMA, and the function of which Pin1 suppresses to induce mitotic catastrophe

(Lu et al., 1996; Lu and Zhou, 2007; Zhou and Lu, 2016). Pin1 is a unique prolyl isomerase that specifically binds and isomerizes certain phosphorylated serine or threonine residues preceding proline (pSer/Thr-Pro) (Yaffe et al., 1997; Lu et al., 1999b; Lu and Zhou, 2007; Zhou and Lu, 2016). Pin1 induces conformational changes in phosphorylated target proteins because pSer/Thr-Pro motifs exist in two distinct *cis* and *trans* conformations (Yaffe et al., 1997; Lu et al., 2007). Pin1-induced conformational changes have been shown to play a crucial role in the cellular functions, including the cell cycle, cell signaling, transcription and splicing, DNA damage responses, germ cell development, and neuronal survival (Crenshaw et al., 1998; Shen et al., 1998; Fujimori et al., 1999; Winkler et al., 2000; Zhou et al., 2000; Wulf et al., 2001, 2002; Liou et al., 2002; Zacchi et al., 2002; Zheng et al., 2002; Atchison and Means, 2003; Atchison et al., 2003; Xu et al., 2003; Lu and Zhou, 2007; Moretto-Zita et al., 2010; Lee et al., 2011b). The expression and function of Pin1 are tightly controlled at multiple levels by transcriptional, post-transcriptional, and post-translational regulation under physiological conditions. In particular, Pin1 deregulation including PTMs is directly involved in an increasing number of pathological conditions, notably premature aging, cancer and Alzheimer's disease (AD) (Lu et al., 1999a; Ryo et al., 2001, 2002; Liou et al., 2003; Bao et al., 2004; Lu, 2004; Akiyama et al., 2005; Pastorino et al., 2006, 2012, 2013; Suizu et al., 2006; Yeh et al., 2006; Balastik et al., 2007; Lu and Zhou, 2007; Takahashi et al., 2007, 2008; Yeh and Means, 2007; Lee et al., 2009, 2011b; Teng et al., 2011; Nakatsu et al., 2016; Zhou and Lu, 2016; Han et al., 2017). This review focuses on the molecular mechanisms of Pin1 regulation by PTMs and discusses the major impact of Pin1 deregulation on the progression of cancer and AD (**Figure 1**).

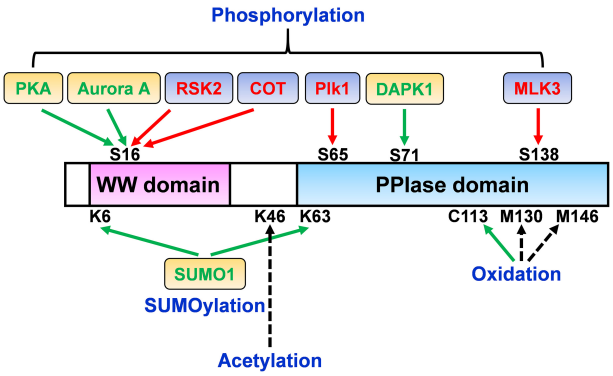

FIGURE 1 | Pin1 regulation perspective by post-translational modifications (PTMs). The molecular mechanisms and activity/function of Pin1 can be regulated by multiple signals through a variety of PTMs. While the phosphorylation of Ser16 in the WW domain of Pin1 by RSK2 and COT increases Pin1 functions, Ser16 phosphorylation by PKA and Aurora A negatively regulates Pin1 activity. Ser65 phosphorylation in the PPIase domain by Plk1 prevents Pin1 ubiquitination and increases its stability. Moreover, DAPK1 directly binds and phosphorylates Pin1 on Ser71, which suppresses Pin1 activity and functions. Furthermore, Pin1 phosphorylation at Ser138 by MLK3 increases Pin1 nuclear localization and functions. Pin1 SUMOylation in both the WW and PPIase domains (K6 and K63) decreases Pin1-induced proliferation and cell transformation. Pin1 oxidation on C113 is highly increased in the human AD brain and abolishes Pin1 enzymatic activity. Pin1 acetylation on Lys46 has been identified in human acute myeloid leukemia and neuroblastoma cell lines. The regulatory role of Pin1 acetylation on Lys46 and oxidation on Met130 and Met146 remains to be determined. Green arrow, positive regulation of Pin1 activity; red arrow, negative regulation of Pin1 activity; black dotted arrow, unknown.

PIN1 STRUCTURE

The human Pin1 is composed of 163 amino acids with a mass of 18 kDa (Lu et al., 1996). Pin1 contains two distinct major domains: an N-terminal WW domain (residues 1–39) and a C-terminal PPIase domain (residues 50–163), which are connected by a flexible linker region (residues 35–53) (Ranganathan et al., 1997; Lu et al., 1999b; Lu and Zhou, 2007; Lee and Liou, 2018). In addition, an interdomain interface between the two domains, consists of WW domain Loop II (residues 27–30) and part of the PPIase domain (residues 138–142). This domain interface has been reported to play an important role in the allosteric regulation of Pin1 functions (Namanja et al., 2007, 2011; Wilson et al., 2013).

The WW domain consists of a triple-stranded anti-parallel β-sheet and two conserved tryptophan residues that are essential for binding to the phosphorylated proteins (Ranganathan et al., 1997; Lee and Liou, 2018). Thus, the WW domain acts on the pSer/Thr-Pro motif binding module, which targets the Pin1 catalytic domain close to the substrate binding sites, where the PPIase domain isomerizes specific pSer/Thr-Pro motifs to induce conformational changes, in a type of "double-check" mechanism (Lu et al., 1996, 1999b, 2007; Ranganathan et al., 1997; Zhou et al., 2000; Lu and Zhou, 2007; Lee et al., 2011b). Although it is believed that the combined primary structure with sequence-specific dynamics is important for WW domain substrate specificity, it remains unclear why Pin1 binds only to specific pSer/Thr-Pro motifs in certain proteins.

The PPIase domain contains a PPIase binding domain that can bind to pSer/Thr-Pro motifs and a catalytic loop at the catalytic site (Ranganathan et al., 1997; Lee and Liou, 2018). A hydrophobic pocket within the PPIase domain is composed of the substrate proline that binds through the cyclic side chain of residues Leu122, Met130, and Phe134, and the peptidyl-prolyl bond that catalyzes the *cis*/*trans* isomerization surrounded by side chain residues His59, Cys113, Ser154, and His157 (Ranganathan et al., 1997). A phosphate-binding loop that undergoes substrate recognition consists of the residues Lys63, Arg68, and Arg69, and mediates the catalytic selectivity for the N-terminal side chain binding to the proline (Ranganathan et al., 1997; Behrsin et al., 2007).

Moreover, the WW domain can regulate PPIase activity depending on whether a peptide substrate is phosphorylated on a single site or on multiple sites. Subsequent studies have shown that most Pin1 substrates contain a single phosphorylation target for the WW domain and that the PPIase domain would have to act on the same pSer/Thr-Pro motif to accelerate its isomerization (Yaffe et al., 1997; Lu et al., 1999b; Lu and Zhou, 2007; Peng et al., 2007). For example, a Thr668-Pro motif in an amyloid precursor protein (APP) exists in

the *trans* conformations before phosphorylation, as indicated through nuclear magnetic resonance (NMR) analysis (Pastorino et al., 2006). However, the *cis* conformation appears only after phosphorylation due to the limitations imposed by the local structure (Ramelot and Nicholson, 2001). Pin1 binds to the pThr668-Pro motif in APP, which accelerates APP isomerization to the *trans* configuration, which results in the suppression of amyloidogenic APP processing and amyloid-β production (Pastorino et al., 2006, 2012; Ma et al., 2012) although there are conflicting results (Akiyama et al., 2005). Alternatively, Pin1 binds to multiple pSer/Thr-Pro motifs in a single substrate. Pin1 binds to phosphorylated p53 on Ser33 and Ser46 in response to DNA damage, and regulates the stability of p53 (Wulf et al., 2002; Zacchi et al., 2002; Zheng et al., 2002). Consistently, it has been found that both Ser33 and Ser46 residues are close to the MDM2-binding site and affect transcriptional activity of p53 (Kussie et al., 1996; Haupt et al., 1997; Kubbutat et al., 1997), suggesting that Pin1 binds to and isomerizes p53 on both the phosphorylated Ser33 and Ser46 sites. The isomerization may suppress the interaction of p53 with its ubiquitin ligase MDM2, affect the phosphorylation of p53 at other sites, and/or affect the p53 transcriptional mediation of p21. The evidence that Pin1 substrates may have multiple phosphorylation sites or form a multi-protein complex suggests that the WW domain and PPIase domain might act on different pSer/Thr-Pro motifs in the same protein or in different proteins. Further studies are needed to solve these important questions.

DUAL ROLES OF PIN1 IN THE DEVELOPMENT OF CANCER AND AD

Although cancer represents proliferating characteristics and AD shows degenerating features, two distinct diseases share common signaling mechanisms, including Pro-directed phosphorylation regulation (Driver and Lu, 2010). Pin1 has been shown to promote cell proliferation and has a protective role against neurodegeneration including AD, however, Pin1 exerts opposite effects on the development of cancer and AD (Yeh and Means, 2007; Takahashi et al., 2008; Zhou and Lu, 2016; Han et al., 2017).

In cancer, Pin1 expression and activity are aberrantly elevated in many malignancies, which are regulated in genetic, transcriptional, post-transcriptional, and PTMs levels (Bao et al., 2004; Takahashi et al., 2008; Lee et al., 2011b, 2014; Li et al., 2013; Lu and Hunter, 2014; Luo et al., 2014; Zhou and Lu, 2016). Upregulated Pin1 controls many Pro-directed phosphorylation signaling events and its modulation is involved in cell cycle coordination, chromosome instability, proliferation, migration, metastasis, and apoptosis in cancer cells (Zhou and Lu, 2016). Indeed, Pin1 is known to activate more than 50 oncogenes or growth-promoting regulators and suppress a number of suppressors or growth inhibitory regulators by regulating activity, protein interaction, stability, and cellular localization (Min et al., 2016). Moreover, Pin1 has been shown to increase self-renewal activity and promote breast cancer stem cell-mediated tumorigenesis (Luo et al., 2014, 2015; Rustighi et al., 2014). In animal models, Pin1 deficiency effectively prevents tumorigenesis by overexpressing Neu, but not c-Myc, and Pin1 overexpression in mammary gland induces chromosome instability and leads to malignant breast cancer (Wulf et al., 2004; Suizu et al., 2006). Furthermore, double Pin1 and p53 knockout (KO) mice are completely resistant to tumorigenesis although these mice show increased levels of thymic hyperplasia (Takahashi et al., 2007).

Although cumulative results suggest that Pin1 is strongly associated with cell proliferation and cancer development, Pin1 has been shown to have a tumor suppressor function (Yeh and Means, 2007; Takahashi et al., 2008; Han et al., 2017). It has been reported that the expression levels of Pin1 are downregulated in renal cell carcinoma due to gene deletion and Pin1 restoration reduces tumor growth of human renal cell carcinoma cells (Teng et al., 2011). Moreover, Pin1 ablation in mouse embryonic fibroblasts of C57BL6 background has been shown to increase cyclin E stability and accelerate genomic instability (Yeh et al., 2006). Furthermore, Pin1 stabilizes tumor suppressor p53 (Wulf et al., 2002; Zacchi et al., 2002; Zheng et al., 2002) and inactivate oncoprotein c-Myc (Yeh et al., 2004) although the same group has reported that Pin1 acts as a transcriptional coactivator for c-Myc and increases its tumorigenic activity (Farrell et al., 2013). These results suggest that Pin1 might be a conditional tumor suppressor in a certain context depending on genetic background, tissue and upstream regulation of Pin1, such as PTMs. More studies are needed to clarify the molecular mechanisms by which Pin1 has opposite effects on the development of cancer.

One of major features of AD is aggregated neurofibrillary tangles that consist of hyperphosphorylated tau (Binder et al., 2005; Ballatore et al., 2007). The phosphorylated forms of tau are dissociated from microtubules and disrupt microtubule structure integrity (Geschwind, 2003). Pin1 specifically binds to phosphorylated tau on Thr231-Pro motif and promotes its *cis* to *trans* conformation (Lu et al., 1999a; Liou et al., 2003). *Cis* phosphorylated tau, but not *trans*, appears very early in human brains with mild cognitive impairment (MCI), tends to be aggregated, and is associated with neurofibrillary degeneration (Nakamura et al., 2012a). Therefore, Pin1-catalyzed the isomerization of phosphorylated tau restores its ability to promote microtubule assembly and may prevent Alzheimer's tau pathology. Among sporadic AD patients, accumulation of senile plaques composed of amyloid beta (Aβ) peptides which derives from APP is regarded as another pathological hallmark (Tanzi and Bertram, 2005; Thinakaran and Koo, 2008). APP is known to be processed in two sequential cleavages by β-secretase in the extracellular domain of the full-length APP and γ-secretase in the transmembrane region, releasing the intact Aβ during the development of AD (Thinakaran and Koo, 2008). Cdk5- and GSK3β-mediated Thr668 phosphorylation may facilitate APP cleavage by β-secretase, thereby increasing Aβ secretion (Lee et al., 2003; Phiel et al., 2003; Cruz et al., 2006). Importantly, Pin1 binds to the phosphorylated Thr668-Pro motif of full length APP and accelerates the isomerization from *cis* to *trans* by over 1000-fold (Pastorino et al., 2006). Overexpression of Pin1 decreases Aβ secretion *in vitro* whereas Pin1 ablation in mice promotes amyloidogenic APP processing and increases insoluble toxic Aβ42 in an age-dependent manner (Pastorino et al., 2006, 2012; Ma et al., 2012). However, Akiyama et al. (2005)

found that Pin1 binds to phosphorylated Thr668 of β-secretase-cleaved C-terminus APP product, C99 rather than full length APP and promotes Aβ production in the mouse embryonic fibroblasts or COS7 cells. Moreover, soluble and insoluble Aβ levels are decreased in Pin1 deficient mice compared with WT mice (Akiyama et al., 2005). Since Pin1 is prominently localized with full length APP at the plasma membrane and presumably binds to C99 in the cytosol, these opposite results might be due to Pin1 cellular compartment, different APP metabolism, and/or feedback mechanisms (Takahashi et al., 2008; Pastorino et al., 2013). Further studies are needed to elucidate molecular mechanisms by which Pin1 regulates Aβ production and its binding capacity of different APP cleavage products in the development of AD.

POST-TRANSLATION MODIFICATIONS OF PIN

Phosphorylation of Pin1

Protein phosphorylation on Ser/Thr-Pro is a critical signaling mechanism in regulating many cellular processes by causing changes in protein conformation and its deregulation contributes to many human diseases including cancer and AD (Blume-Jensen and Hunter, 2001; Pawson and Scott, 2005; Olsen et al., 2006; Lee et al., 2011b). Pin1, including the WW and PPIase domains, is phosphorylated at multiple sites, and this phosphorylation regulates its binding ability, enzymatic activity, and function in both physiological and pathological conditions (Lu and Zhou, 2007).

It has been reported that cAMP-protein kinase A (PKA) phosphorylates Pin1 at Ser16 in the WW domain *in vitro* and *in vivo* (Lu et al., 2002). The phosphorylation of Ser16 abolishes the ability of Pin1 to interact with its substrate, MPM-2 antigen, and disrupts Pin1 nuclear speckle localization. Since Ser16 is one of the important amino acid residues which are responsible for the binding of phosphorylated substrate to the WW domain, this modification may affect the binding ability of Pin1 and its functions (Lu et al., 2002). Aurora A can directly interact with and phosphorylate Pin1 at Ser16 during G2/M progression, which markedly suppresses the function of Pin1 in G2/M, thus affecting the cell cycle (Lee et al., 2013). Pin1 overexpression also delays mitotic entry by inducing the premature degradation of Aurora A cofactor Bora in the G2 phase through the β-TrCP-mediated ubiquitin-proteasome pathway and alters the cytoplasmic translocation of endogenous Bora. However, Pin1 phosphorylation on Ser16 by Aurora A disrupts its binding ability to Bora, which increases Bora protein stability and ultimately suppresses mitotic progression (Lee et al., 2013). Since Pin1 plays a critical role as a cell cycle modulator to promote cell cycle progression and since cell cycle disorder is a common phenomenon in cancer (Lin et al., 2015), phosphorylation of Pin1 at Ser16 might be tightly regulated during the cell cycle such that its deregulation might cause cell cycle disruption in pathological conditions. While PKA- or Arora A-mediated Ser16 phosphorylation abolishes the ability of Pin1 to bind to its substrates, other kinases increase Pin1 function and/or binding ability through Ser16 phosphorylation. Ribosomal protein S6 kinase 2 (RSK2) forms a strong complex with Pin1 and phosphorylates Ser16 (Cho et al., 2012). Moreover, 12-O-tetradecanoylphorbol-13-acetate (TPA) promotes the interaction of RSK2 and Pin1, which promotes RSK2 phosphorylation, thereby increasing TPA-induced cell transformation (Cho et al., 2012). In addition, the MAP3K-related serine/threonine kinase COT has been reported to directly phosphorylate Pin1 at Ser16 in the presence of TPA (Kim et al., 2015). COT overexpression leads to Pin1 phosphorylation, which subsequently increases cyclin D1 abundance and enhances mammary gland tumorigenesis in MCF7 cells. Consistently, tumor growth has been abrogated in a nude mouse xenograft model treated with Pin1 inhibitor and/or COT kinase inhibitor. Furthermore, Pin1 pSer16 levels have been shown to be positively correlated with COT levels in human breast cancer (Kim et al., 2015). These opposite results of Pin1 Ser16 phosphorylation might be due to responsible kinases, different cellular locations, the selected cell lines or differences in the experimental sensitivity and specificity of the methods used (**Figure 2**). In addition, tau and GSK3β dephosphorylation promotes Pin1 phosphorylation at Ser16, suggesting that Ser16 phosphorylation of Pin1 in the brain might have a protective role against AD (Min et al., 2005). However, Ando et al. (2013) showed that the level of Ser16 phosphorylation of Pin1 is highly increased in human AD brain tissues compared to those of normal subjects. Therefore, the *in vivo* effects and mechanisms of Pin1 Ser16 phosphorylation on its substrate binding ability and function remain to be determined.

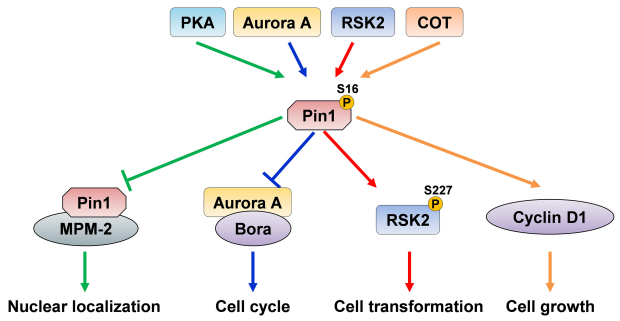

FIGURE 2 | The dual effects of Pin1 Ser16 phosphorylation. Pin1 phosphorylation on Ser16 by PKA inhibits the binding ability of Pin1 to MPM-2, thereby suppressing Pin1 nuclear localization. Aurora A directly binds to and phosphorylates Pin1 and suppresses its binding to Bora, and ultimately disrupts cell cycle, indicating that PKA and Aurora A inhibit Pin1 ability to interact with its substrate and function. However, Pin1 phosphorylation by RSK2 induces RSK2 phosphorylation and promotes cell transformation. COT directly phosphorylates Ser16 of Pin1 and increases cyclin D1 levels, and subsequently promotes cell growth, suggesting that RSK2- and COT-mediated Pin1 phosphorylation increase Pin1-induced cell proliferation.

Phosphorylation in the PPIase domain also regulates Pin1 functions. Eckerdt et al. (2005) showed that Polo-like kinase 1 (Plk1), a critical regulator of mitosis, phosphorylates Pin1 at Ser65 in the PPIase domain. However, neither the

phosphorylation of Pin1 by Plk1 nor mimicked phosphorylation at Ser65 has an effect on the catalytic activity of Pin1. Interestingly, phosphorylation of Pin1 by Plk1 enhances Pin1 stability by inhibiting its ubiquitination. In addition, inhibition of Plk1 activity by transfection of siRNA targets to Plk1 or by transfection of dominant negative Plk1 K82M or hyperactive Plk1 T210D enhances the ubiquitination of Pin1. Ubiquitination is the conjugation of proteins and ubiquitin, a highly conserved 76-amino-acid eukaryotic protein, and is essential for the degradation of proteins (Schlesinger and Goldstein, 1975; Ciechanover et al., 1980a,b; Hershko et al., 1980; Weissman, 2001; Pickart and Eddins, 2004). Aberrant ubiquitination impacts a wide range of eukaryotic biology, and its defective regulation results in extensive developmental diseases, including cancer and neurodegenerative diseases (Rape, 2018). Plk1 expression is dramatically upregulated in tumor cell lines and in human cancer tissues (Yuan et al., 1997; Liu et al., 2017). Since overexpression of Pin1 contributes to tumorigenesis, these observations indicate that the stabilization of Pin1 might be one of the mechanisms that contribute to Plk1-mediated tumorigenesis. However, it remains unclear whether Plk1 directly phosphorylates Pin1 *in vivo*.

Death-associated protein kinase 1 (DAPK1) directly binds to and phosphorylates Pin1 on Ser71, which is located in the substrate binding loop consisting of residues 63–80 in the PPIase domain (Bialik and Kimchi, 2011; Lee et al., 2011a,b). DAPK1 is a calcium/calmodulin-dependent Ser/Thr kinase that is involved in cell death, and its deregulation is implicated in cancer and AD (Cohen et al., 1997; Inbal et al., 1997; Kissil et al., 1997; Kim et al., 2014, 2016, 2019; Zhao et al., 2015; You et al., 2017; Chen et al., 2019). Pin1 phosphorylation on Ser71 by DAPK1 inhibits the catalytic activity of Pin1, specifically suppressing the ability of Pin1 to activate transcription factors and stabilize proteins, blocks Pin1 nuclear localization, and attenuates the centrosome amplification, chromosome instability and cell transformation induced by Pin1 (Lee et al., 2011a). Since Ser71 is located at the center of the PPIase domain binding pocket, this phosphorylation might prevent phosphorylated substrates to enter the catalytic active site (Lee et al., 2011a; Mahoney et al., 2018). Furthermore, DAPK1 increases tau protein stability and phosphorylation through Pin1 Ser71 phosphorylation (Kim et al., 2014). Pin1 ablation or inhibition induces the development of AD pathologies (Liou et al., 2003; Pastorino et al., 2006; Lu and Zhou, 2007; Lim et al., 2008), suggesting that aberrant DAPK1 activation might contribute to the age-dependent neurodegeneration in AD by inhibiting Pin1 function. Therefore, DAPK1 might be involved in the development of cancer and AD by regulating Pin1 function through its phosphorylation of Ser71 (**Figure 3**).

In contrast, Pin1 phosphorylation of Ser138 in the PPIase domain by mixed-lineage kinase 3 (MLK3) enhances the functions of Pin1 by increasing its catalytic activity and nuclear translocation (Rangasamy et al., 2012). The Ser138 phosphorylation of Pin1 promotes cell-cycle progression, cyclin D1 protein stability, and centrosome amplification. In breast cancer tissues, the levels of Pin1 phosphorylated at Ser138 are significantly upregulated, indicating that targeting MLK3 or Pin1 Ser138 might benefit cancer treatment (Rangasamy et al., 2012). Thus, Pin1 phosphorylation in both the WW and PPIase domains regulates its substrate binding ability, subcellular localization, and function, and this modification might contribute to tumorigenesis and neurodegeneration.

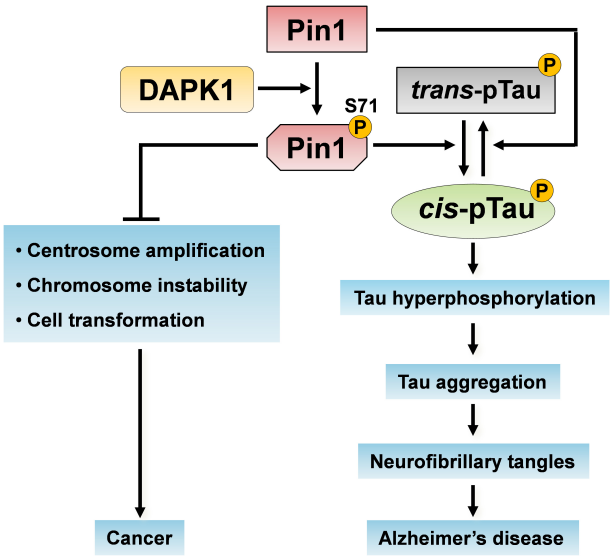

FIGURE 3 | Pin1 phosphorylation on Ser71 by DAPK1 in cancer and AD. The activation of DAPK1 directly phosphorylates Pin1 on Ser71 and inhibits Pin1 functions. Pin1 phosphorylation on Ser71 inhibits its catalytic activity and the ability of Pin1 to induce centrosome amplification, chromosome instability and cell transformation, thereby suppressing oncogenic pathways. Moreover, the inactivation of Pin1 promotes the *trans* p-tau to *cis* p-tau conversion, induces tau hyperphosphorylation and aggregation, which might eventually cause neurofibrillary tangles in the development of AD.

Oxidation of Pin1

Oxidative stress has been widely regarded as a contributing factor to AD and cancer, as indicated by protein oxidation, lipid peroxidation, nucleic acid oxidation, advanced glycation products, and reactive oxygen species (ROS) formation (Butterfield and Lauderback, 2002; Zhu et al., 2004; Gorrini et al., 2013; Tonnies and Trushina, 2017). It has been reported that Pin1 oxidation levels are significantly increased in patients with MCI and that Pin1 catalytic activity is decreased in the hippocampus region of their brains (Butterfield et al., 2006; Sultana et al., 2006). These results suggest that Pin1 is likely involved in the initial development of MCI to AD because MCI is an intermediate stage between normal cognitive aging and early dementia or clinically probable AD, and which eventually develops AD. Indeed, Pin1 has been found to be oxidized in the brains of AD patients, and Pin1 oxidation appeared to decrease Pin1 activity by reducing its isomerase activity (Butterfield et al., 2006; Sultana et al., 2006). Moreover, oxidized Pin1 could be recognized by the ubiquitination system for its degradation (Tramutola et al., 2018). However, the site(s) where Pin1 is oxidized was unknown, and how this oxidative modification affects Pin1 catalytic activity was unclear.

TABLE 1 | Pin1 is regulated by various post-translational modifications.

Modification type	Modification site	Enzyme	Effect on Pin1	References
Phosphorylation	S16	PKA	Inhibit binding ability and nuclear localization	Lu et al., 2002
Phosphorylation	S16	Aurora A	Inhibit binding ability	Lee et al., 2013
Phosphorylation	S16	RSK2	Increase binding ability	Cho et al., 2012
Phosphorylation	S16	COT	Increase binding ability	Kim et al., 2015
Phosphorylation	S71	DAPK1	Inhibit catalytic activity and nuclear localization	Lee et al., 2011a; Kim et al., 2014; Mahoney et al., 2018
Phosphorylation	S138	MLK3	Increase catalytic activity and nuclear translocation	Rangasamy et al., 2012
Phosphorylation	S65	Plk1	Inhibit ubiquitination and increase protein stability	Eckerdt et al., 2005
SUMOylation	K6, K63	SUMO1	Inhibit binding ability and catalytic activity	Chen et al., 2013
Oxidation	C113		Inhibit catalytic activity and nuclear localization	Chen et al., 2015
Oxidation	M130, M146		Unknown	Ando et al., 2013
Acetylation	K46		Unknown	Choudhary et al., 2009; Ando et al., 2013

PKA, cAMP-protein kinase A; RSK2, ribosomal protein S6 kinase 2; DAPK1, death-associated protein kinase 1; MLK3, mixed-lineage kinase 3; Plk1, polo-like kinase 1; SUMO1, small ubiquitin-like modifier 1.

Recently, two independent studies have identified the oxidation site in Pin1 by mass spectrometry and X-ray crystallization (Aluise et al., 2013; Chen et al., 2015). Pin1 is modified by an oxidative modification of Cys113 in the PPIase domain upon hydrogen peroxide (Chen et al., 2015). Although Pin1 that is oxidized on Cys113 can still effectively bind to substrates, Pin1 enzymatic activity is abolished, indicating that it can trap substrates. Moreover, Pin1 oxidation has been found to inhibit Pin1 nuclear localization, and increase tau/APP protein stability and Aβ secretion, and it is increased in human AD brains, as well as in AD mouse models (Chen et al., 2015). Thus, Pin1 oxidation of Cys113 causes Pin1 inactivation and mislocalization, thereby contributing to the development of AD. Other oxidative modification sites in Pin1 have been identified at Met130 and Met146 in human neuroblastoma SH-SY5Y cells stably expressing Pin1, indicating that Pin1 might have multiple oxidation sites through which its function is regulated (Ando et al., 2013).

SUMOylation of Pin1

A small ubiquitin-like modifier (SUMO) peptide on a lysine residue plays important roles in regulating a spectrum of protein functions, including protein activity, stability, and localization (Geiss-Friedlander and Melchior, 2007; Gareau and Lima, 2010; Hannoun et al., 2010). The SUMOylation and deSUMOylation of proteins is a highly dynamic process, and only a small fraction of a substrate is modified at a given time. Increasing evidence indicates that deregulation of either SUMO conjugation or deconjugation can contribute to tumorigenesis (Eifler and Vertegaal, 2015). Chen and coworkers found that Pin1 is SUMOylated on Lys6 in the WW domain and on Lys63 in the PPIase domain, as determined by mass spectrometric analysis and site-directed mutagenesis assay (Chen et al., 2013). Both Lys6 and Lys63 in Pin1 resemble the consensus SUMOylation site. Among mammalian SUMO isoforms such as SUMO1, SUMO2, and SUMO3, only SUMO1 specifically promotes SUMOylation of Pin1 at both sites. SUMOylation inhibits the substrate binding ability, phospho-specific PPIase activity, and cellular function of Pin1. Since SUMO1 that is conjugated at Lys6 is very close to the Trp34 residue of the WW domain, which is essential for Pin1 to bind to its phosphorylated substrates, and Lys63 is critical for anchoring the pSer/Thr-binding pocket in the PPIase domain, these modifications might affect Pin1 substrate binding and catalytic activity. Moreover, SUMO protease (SENP) 1 directly binds to the PPIase domain of Pin1 and promotes deSUMOylation of Pin1 after exposure to oxidative stress, suggesting that SENP1 may reverse Pin1 inhibition that had been induced by SUMOylation. Indeed, SENP1 promotes Pin1-induced centrosome amplification, chromosome instability, proliferation, and cell transformation (Chen et al., 2013). These results further demonstrate that SENP1 levels are positively correlated with Pin1 levels in human breast cancer tissues. Thus, in cancers, modifications of Pin1 by SENP1 may contribute to cell proliferation and tumorigenesis.

THERAPEUTIC TARGETING OF PIN1 PTMS

Since deregulation of Pin1 expression and activity has critical effects on the development of cancer and AD, and Pin1 has a specified substrate binding and active site, targeting of Pin1 has been an attractive druggable target. The widely used Pin1 inhibitors such as juglone, PiB, pTide, and TME-001, have been shown to inhibit Pin1 PPIase activity and suppress Pin1-mediated cell growth (Hennig et al., 1998; Uchida et al., 2003; Wildemann et al., 2006; Zhang et al., 2007; Mori et al., 2011). However, these inhibitors also suppress other PPIase activities as well as Pin1 and they have not been further studied whether they specifically affect Pin1 PTMs in the PPIase domain. Recently, two Pin1 inhibitors have been shown to directly bind to the catalytic domain, increase Pin1 degradation, and inhibit Pin1-mediated cell growth in cancer cells (Wei et al., 2015; Campaner et al., 2017; Liao et al., 2017; Zheng et al., 2017; Kozono et al., 2018). Food and Drug Administration (FDA)-approved all-trans retinoic acid (ATRA) has been identified to form salt bridges with Lys63 and Arg69 of Pin1 PPIase domain and may mimic Ser71 phosphorylation by DAPK1 because both amino acid residues

are critically involved in the phosphate binding to Pin1 Ser71 (Lee et al., 2011a; Wei et al., 2015). Wei et al. (2015) showed that Pin1 levels are inversely correlated with the expression of DAPK1 in human triple negative breast cancer tissues and ATRA sensitivity is also negatively correlated with Pin1 Ser71 levels. Since DAPK1 expression is dramatically suppressed in most of solid tumors, ATRA binding to the Pin1 active site inhibits its substrate isomerase activity, thereby ultimately leading to Pin1 degradation. Another small molecule, KPT-6566 covalently binds and transfers the sulfanyl-acetate to the Cys113 residue of the Pin1 catalytic domain, and inhibits Pin1 PPIase activity (Campaner et al., 2017). Moreover, KPT-6566-B, the byproduct of KPT-6566 after Pin1 interaction, produces ROS and increases cancer cell death. Since Pin1 Cys113 oxidation abolishes Pin1 catalytic activity, KPT-6566 binding to Pin1 may have similar effects as a result of Pin1 modification.

None of drug candidates targeting Pin1 PTMs for AD has been reported yet. Instead, conformational phospho-specific antibodies targeting Thr231 of tau which is the Pin1 binding site have been developed (Nakamura et al., 2012a; Kondo et al., 2015; Albayram et al., 2017). Recently, *cis* phosphorylated Thr231 tau (p-tau), but not *trans* p-tau, has been shown to disrupt microtubule structure and axonal mitochondrial transport, spread through the brain in a prion-like fashion, and is associated with neurofibrillary tangles (Albayram et al., 2016, 2018; Lu et al., 2016). Kondo et al. (2015) generated mouse monoclonal antibodies (mAbs) specifically distinguishing *cis* from *trans* p-tau and found that *cis* mAb is able to enter neurons and effectively blocks time-dependent induction of pathological *cis* p-tau without affecting physiological *trans* p-tau, suggesting that *cis* p-tau antibody therapy may offer new approaches to treat tau-related pathologies including AD.

However, Pin1 modulator as a drug target may be challenging since both activation and inhibition of Pin1 activity may contribute to pathological conditions. Mouse develops normally in the absence of Pin1 although Pin1 KO has defects on the differentiation of neuronal stem cell, testis, and breast (Fujimori et al., 1999; Liou et al., 2002; Atchison and Means, 2003; Atchison et al., 2003; Nakamura et al., 2012b; Luo et al., 2014). Moreover, Pin1 KO mice show age-dependent neurodegeneration and Pin1 transgenic mice exhibit malignancy in breast (Liou et al., 2003; Suizu et al., 2006; Lee et al., 2009). These results indicate that targeting Pin1 or its PTMs should be carefully considered due to possible side effects depending on specific diseases and modified for designing more selective pharmaceuticals with tissue specificity and fewer off-target liabilities in the future.

CONCLUSION AND PERSPECTIVE

Pin1 is the key protein isomerase that regulates cell growth, proliferation, cell cycle progression, apoptosis, and degeneration through the signal transduction pathways. The most important mechanism that regulates Pin1 enzymatic activity are mediated by PTMs. Pin1 consists of multiple domains with special PTM sites that are important for catalytic activation related to pathology. Pin1 PTMs affect the binding ability, stabilization and/or localization of Pin1 as well as its catalytic activity, ultimately resulting in regulating Pin1 cellular functions (**Table 1**). It is crucial to identify and characterize the role of PTMs in Pin1 signaling *in vivo* in future studies to determine its role as a switch between physiological and pathological conditions. Although single-site modification might precisely adjust Pin1 function only in a cell- or tissue-type-specific manner, the numerous possible combinations of different modifications could regulate Pin1 activity, enabling Pin1 to confer its various effects. Thus, the extensive cross-talk between different Pin1 modifiers, including those that mediate phosphorylation, ubiquitination, oxidation, acetylation and SUMOylation, need to be studied. Understanding the detailed degree and ratio to which modified and unmodified PTMs vary in human diseases such as cancer and AD could provide key therapeutic interventions that target Pin1. Furthermore, the discovery of novel types of PTMs in Pin1 and their potential roles are important for obtaining new insight into Pin1 related proliferation and degeneration and associated disorders. Indeed, the possibility of a Lys acetylation site in Pin1 has been identified, although the regulatory role of this acetylation of Pin1 remains to be determined (Choudhary et al., 2009; Ando et al., 2013). By elucidating the mechanism and significance of Pin1 PTMs in normal and pathological conditions, this study will provide not only a better idea of how these PTMs are induced but also a basis on which to determine whether they are viable targets for therapeutic intervention.

AUTHOR CONTRIBUTIONS

DC and TL conceptualized the manuscript. DC prepared the figures. LW professionally edited the manuscript. DC, LW, and TL wrote the manuscript.

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OPINION
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Intracellular *Theileria* Parasites PIN Down Host Metabolism

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INTRODUCTION

The word "*parasite*" has been used since the 16th century, stemming from the Greek *parasitos* meaning "*eating at another's table,*" from para- meaning "*alongside*" + sitos "*food.*" The relationship between parasites and their hosts is an inherently metabolic one. Intracellular parasites feed off their host and are therefore likely to perturb their metabolic pathways in the process. Indeed, there has been much recent interest in the role of metabolic exchange in host-parasite interactions (Blume and Seeber, 2018; Zuzarte-Luís and Mota, 2018; Krishnan et al., 2019). In recent years we have become fascinated by a remarkable metabolic host-parasite interaction; *Theileria* parasites can reprogram their host cells to drive a cancer-like metabolic state. And our most exciting discovery has been the critical role that the Peptidyl-Prolyl *Cis/Trans* Isomerase Pin1 plays in this relationship.

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TALENTED *THEILERIA* PARASITES

Theileria spp. are obligate, intracellular parasites belonging to the phylum of apicomplexa. Two *Theileria* species, *T. parva,* and *T. annulata* are bovine-specific pathogens that cause disease with considerable economic impact due to the high cost of treatment, the cost of anti-tick control, animal mortality, and decreased bovine production. Tropical Theileriosis kills over 1.1 million cattle per year and costs in the hundreds of millions of dollars. Infection by *Theileria* causes a lymphoproliferative disease in cows that has some clinical features of human leukemias (Tretina et al., 2015). *T. annulata* infects bovine B cells and macrophages, whereas the related species *T. parva* infects B and T lymphocytes. *Theileria*-infected cells are transformed and immortalized (Cheeseman and Weitzman, 2015; Tretina et al., 2015); they display cancer phenotypes such as uncontrolled proliferation, growth factor independence, and increased invasiveness and the ability to form metastases in immunodeficient mice (Tretina et al., 2015). Of particular interest, *Theileria*-dependent transformation is reversible; animals can be cured by treatment with the theilericidal drug Buparvaquone. Incubating *Theileria*-infected cells *in vitro* with Buparvaquone, diminishes the number of intracellular parasites in host leukocytes, which loose the transformed phenotypes, stop proliferating, and regain apoptosis sensitivity. To drive host cell transformation, the parasite manipulates the host cell signaling pathways that control cell proliferation and survival. Several signaling pathways were implicated, including c-Jun N-terminal Kinase (JNK) and host nuclear factors c-Myc, NF-κB, and AP-1 (Chaussepied et al., 1998; Heussler et al., 2002; Dessauge et al., 2005; Tretina et al., 2015). We showed that the Jun/AP-1 transcription factor maintains a critical oncogenic microRNA feedback loop (Marsolier et al., 2013). Another fascinating feature of *Theileria*-induced transformation is the induction of a metabolic signature characteristic of the "Warburg effect" observed in cancer cells (hereafter referred to as a Warburg-like effect) (Cairns et al., 2011; Medjkane and Weitzman, 2013; Medjkane et al., 2014; Metheni et al., 2015). The parasite-induced Warburg-like effect shows the classic signs of a shift from oxidative phosphorylation to aerobic glycolysis. We and others previously reported the central role of the Hypoxia-inducible factor 1 alpha (HIF1α) in driving the expression of glycolytic enzymes and

metabolic genes in infected cells (Medjkane et al., 2014; Metheni et al., 2015). Despite this progress in identifying host pathways underlying the transformed phenotype, it remained unclear how the intracellular parasite initiates the signaling events leadings to rewiring of the host transcriptome.

UNDERPINNING HOST-PARASITE INTERACTION

In order to identify potential secreted oncoproteins in the *T. annulata*, we mined the parasite genome looking for genes encoding proteins with signal peptides that might be secreted into the host cytoplasm and acts as "epigenators" (Cheeseman and Weitzman, 2015) of oncogenic signals to hijack host regulatory pathways. A bioinformatics pipeline led to a relatively restricted list of candidate genes and the most promising on the list was the parasite homolog of Phosphorylation-Dependent Peptidyl-Prolyl *Cis/Trans* Isomerase PIN1 (Marsolier et al., 2015). The role of human Pin1 in carcinogenesis and metabolic reprogramming offered a link between infection and transformation by *Theileria* parasites (Nakatsu et al., 2019). The parasite encoded isomerase, that we named TaPin1, is particularly interesting; it has a catalytic isomerase domain and the WW domain present in mammalian Pin1 is replaced by a putative signal peptide sequence. While several Pin1 homologs also lack the WW domain, the PPIase domain of TaPin1 is well conserved. Indeed, the TaPin1 PPIase domain shares 47% identity with hPin1, 45% with *Arabidopsis thaliana* AtPin1, and 43% with *Trypanosoma brucei* TbPin1 (Marsolier et al., 2015). Interestingly, the signal peptide is not conserved in non-transforming species of *Theileria* or in the related apicomplexan homologs in *Toxoplasma* or *Plasmodium* (Marsolier et al., 2015). We showed that the TaPin1 protein is a *bona fide* prolyl isomerase and that it is secreted into host cells (Marsolier et al., 2015). The importance of TaPin1 in the parasite-induced transformation process was highlighted by the discovery that TaPin1 isomerase activity can be inhibited by the anti-parasite drug Buparvaquone. An additional twist was the finding that Buparvaquone-resistant parasites have a mutation in the gene encoding TaPin1. The same A53P mutation has now been reported in drug-resistant isolates from both Tunisia and Sudan (Marsolier et al., 2015; Salim et al., 2019). This mutation affects the ability of Buparvaquone to enter into the active site and inhibit isomerase activity. Interestingly, the presence of a signal peptide was observed only in the transforming species (*T. annulata* and *T. parva*), but not in non-transforming species or closely related apicomplexan such as *Plasmodium* or *Toxoplasma* (Marsolier et al., 2015). Although there are likely to be other parasite-encoded proteins that contribute to the transformation of the host cells, TaPin1 represents a remarkable of example of how a prolyl isomerase has evolved to play a key role in host-parasite relationships.

TaPin1, A MOLECULAR LYNCHPIN

Once TaPin1 was identified as a critical parasite-secreted epigenator, the question remained how it could hijack host cell signaling pathways. Pin1 is a conserved enzyme that specifically isomerizes phosphorylated Ser/Thr-Pro bonds in a defined subset of proteins, thereby inducing conformational changes impacting their stability, localization and activity. Human Pin1 protein has multiple substrates involved in a wide range of cellular processes that contribute to transformation (Marsolier and Weitzman, 2014; Zhou and Lu, 2016). A search for TaPin1 interactors and host partner proteins identified at least two host pathways that are induced by the parasite isomerase (**Figure 1**). We showed that the TaPin1 protein interacts with host ubiquitin ligase Fbw7, leading to its auto-degradation (Marsolier et al., 2015). This interaction releases the host oncoprotein c-Jun from Fbw7-dependent ubiquitination and degradation. The c-Jun protein is part of the AP-1 transcription factor that induces the oncomiR-155 which drives host cell proliferation (Marsolier et al., 2013). AP-1 also induces the gene encoding the matrix metalloprotease MMP-9 which drives host cell invasive phenotypes (Cock-Rada et al., 2012). We also identified the host protein Pyruvate Kinase isoform M2 (PKM2), which is critical for the Warburg-like effect and the transcription of glycolytic enzymes in cancer cells, as a TaPin1 interactor (Marsolier et al., 2019). This time the consequence is the stabilization of PKM2 which leads to HIF-1α-dependent regulation of host metabolism. The TaPin1-PKM2-HIF-1α axis causes induction of host metabolic enzymes (such as GLUT1 and Hexokinase 2),

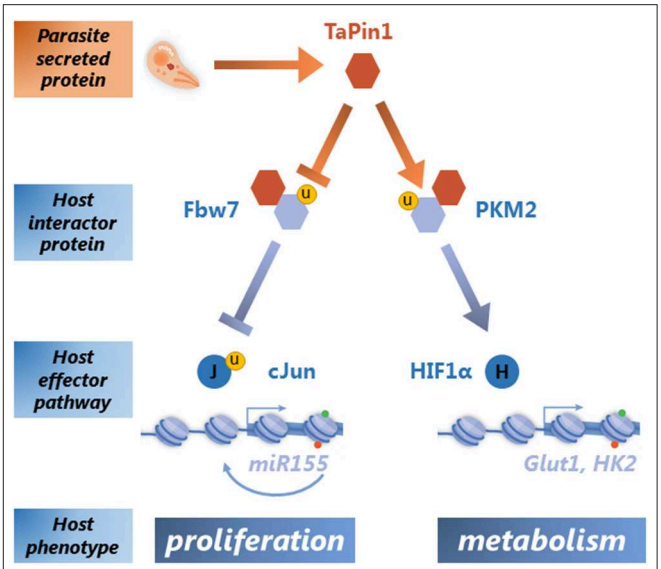

FIGURE 1 | Secreted TaPin1 isomerase regulates host signaling pathways leading to proliferative and metabolic phenotypes. A schematic representation of the role of the secreted TaPin1 molecule in host-parasite interactions. *Theileria* parasites (represented in orange) secrete TaPin1 proteins into the host cell (represented in blue). The TaPin1 interacts with two host signaling pathways: by destabilizing the Fbw7 ubiquitin ligase, TaPin1 activates the c-Jun transcription factor leading to regulation of proliferative genes such as the onco-miR-155 (Marsolier et al., 2013, 2015); in contrast, TaPin1 stabilizes the host PKM2 protein which drives host cell metabolic genes through the transcription factor HIF1α (Medjkane et al., 2014; Marsolier et al., 2019). The yellow circle indicates the importance of ubiquitination in the TaPin1-regulated pathways.

increased glucose uptake and the transformed phenotypes of parasite-infected cells (Medjkane et al., 2014; Marsolier et al., 2019). These are the combined features of the parasite-induced Warburg-like effect. The precise molecular mechanisms by which TaPin1 stabilizes host PKM2 protein, while promoting Fbw7 degradation, is unclear. We hypothesize that the prolyl isomerisation of PKM2 or Fbw7 could differentially affect the interaction with ubiquitin ligases or other factors that modulate protein stability.

DISCUSSION

Many studies on the role of the Pin1 phosphorylation-dependent Peptidyl-Prolyl *Cis/Trans* Isomerase have firmly placed the protein as a key regulator of oncogenic and metabolic pathways (Marsolier and Weitzman, 2014; Zhou and Lu, 2016; Nakatsu et al., 2019). The discovery and characterization of the parasite TaPin1 add parasite-host interactions to the list of effects of this multi-tasking enzyme. As described above, TaPin1 links parasitism to the regulation of host metabolism and host cell proliferation. Our findings on TaPin1 binding and isomerization of host substrates converge on the regulation of strategic host transcriptional reprogramming leading to two major biological processes that offer clear advantages for the parasite (**Figure 1**). First, TaPin1 contributes to host cell proliferation and tumor growth via stabilization of c-Jun which promotes transformation, thereby enabling parasite dissemination. Secondly, TaPin1 induces major metabolic reprogramming through activation of the PKM2-HIF1α axis. This shift in cellular glucose resources could potentially provide critical nutrients required for *Theileria* proliferation and maintenance within the host cells. Interestingly, the acquisition during evolution of a signal peptide for TaPin1 that is restricted to transforming *Theileria* species (*T. annulata* and *T. parva*) provides a compelling way to be secreted into the cytoplasmic host compartment in order to hijack transduction pathways and rewire host transcriptional programs. In this way TaPin1 is critical for parasite survival and is a promising drug target. Indeed, the observation in the field of Buparvaquone-resistant parasites and mutations in the *TaPin1* gene highlights the need for alternative Pin1 inhibitors that can still target mutant proteins. The levels of host bovine *BtPin1* transcripts and protein were unaffected by Buparvaquone treatment, suggesting that this drug specifically targets the parasite protein and this might explain the absence of toxicity in uninfected cells. Of note, Juglone, a well-characterized inhibitor of mammalian Pin1 can substitute for the treatment by Buparvaquone leading to a decrease in parasite burden and viability of host cells infected with *T. annulata* or *T. parva in vitro* (Marsolier et al., 2015). Clearly, Pin1 proteins from different species will continue to amaze us with their versatility and multi-tasking in the years ahead. This is likely to remain an exciting field, with clinical relevance for both cancer and infectious diseases.

AUTHOR CONTRIBUTIONS

SM and JW wrote the article.

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Function of PIN1 in Cancer Development and Its Inhibitors as Cancer Therapeutics

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Peptidyl-prolyl isomerase (PIN1) specifically binds and isomerizes the phosphorylated serine/threonine–proline (pSer/Thr–Pro) motif, which results in the alteration of protein structure, function, and stability. The altered structure and function of these phosphorylated proteins regulated by PIN1 are closely related to cancer development. PIN1 is highly expressed in human cancers and promotes cancer as well as cancer stem cells by breaking the balance of oncogenes and tumor suppressors. In this review, we discuss the roles of PIN1 in cancer and PIN1-targeted small-molecule compounds.

Keywords: cancer therapeutics, PIN1, PIN1 inhibitor, proline-directed phosphorylation, prolyl isomerase, tumorigenesis

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INTRODUCTION

Proline (Pro)-directed serine/threonine (Ser/Thr) phosphorylation is a modification of various signaling pathways. Proline is the unique amino acid with the ability to have either a cis or trans structure, and these isomerizations are catalyzed by peptidylprolyl isomerases (PPIases). The cis–trans isomerization of proline in the phosphorylated Ser/Thr–Pro motif is mediated by PIN1 (Liou et al., 2011). The PPIase superfamily contains FK506-binding proteins (FKBPs), cyclophilins and parvulins. FKBPs and cyclophilins are inhibited by the immunosuppressants FK506/rapamycin and cyclosporine A (CyA). PIN1 is a kind of parvulins and inhibited by juglone. PIN1 is well known PPIase that controls the isomerization of the phosphorylated Ser/Thr–Pro (pSer/Thr–Pro) motif.

PIN1 contains two domains including an WW domain in N-terminal and a PPIase domain in C-terminal (Lu et al., 1996, 1999). The N-terminal WW domain interacts with specific pSer–Pro or pThr–Pro motifs, which are the regulatory phosphorylation sites of substrate proteins (Lu et al., 1996, 1999). After interacting with its substrate, the PPIase domain isomerizes the pSer/Thr–Pro motifs, which affect the function of protein by the conformational changes of target protein (Lu et al., 1999; Lu P. -J. et al., 2002).

The post-translational modifications of PIN1, containing oxidation, sumoylation, phosphorylation, and ubiquitination, control the PPIase activity and stability of PIN1, and contribute to the high expression and/or activation of Pin1 in cancer development. PIN1 is involved in the cell cycle, synthesized protein folding, and DNA damage responses (Lu et al., 1996). PIN1 is overexpressed in several human cancers (Lee T. H. et al., 2011), including prostate cancer (Ayala et al., 2003; La Montagna et al., 2012), breast cancer (Wulf et al., 2001; Ryo et al., 2002), and oral squamous carcinomas (Miyashita et al., 2003). In cancer patients, a high expression of PIN1 correlates with a poor clinical outcome, lymph node metastasis in non-small cell lung cancer patients, and disease progression in patients with oral squamous carcinoma (Ryo et al., 2001; Ayala et al., 2003; Bao et al., 2004; Suizu et al., 2006). PIN1 overexpression induces chromosome instability and tumorigenesis. PIN1 inactivates and activates more than 26 tumor suppressors and 56 oncogenes, respectively.

In cancer stem cells, multiple PIN1 substrates play an important role. PIN1 regulates the tumorigenesis and expansion of CSCs in leukemia and breast cancer. However, it is not fully understood how PIN1 controls cancer and cancer stem cell development. Several studies have reported that some single nucleotide polymorphisms (SNPs) of the *Pin1* gene increases the cancer risk, whereas other variants function as protective factors (Segat et al., 2007; Lu et al., 2009; Li et al., 2013). In this review, we summarize the function of PIN1 in regulating cancer development and small-molecule compounds that exhibit anticancer activities by targeting PIN1.

TRANSCRIPTIONAL AND POST-TRANSLATIONAL REGULATION OF PIN1 IN CANCER

Oncogenes activating E2F transcriptional factor including H-Ras, Her2, p38, and PI3K increase the mRNA expression of *Pin1*, which appears to activate *Pin1* transcription by E2F, considering the existence of the E2F consensus sequence in the *Pin1* promoter region (Ryo et al., 2002, 2009; Kamimura et al., 2011). The transcriptional activation of PIN1 is induced by the E2F or by the binding of Notch1 with the *Pin1* promoter region (Ryo et al., 2002; Rustighi et al., 2009). In acute myeloid leukemia (AML), oncogenic CCAAT/enhancer binding protein-α ((C/EBPα)-p30) is a dominant negative isoform of the tumor suppressor C/EBPα that is generated by *CEBPA* mutations. C/EBPα-p30 recruits the E2F transcription factor to bind to the *PIN1* pro-moter.

On the contrary, p53 and AP4 act as transcriptional repressors and reduce the *Pin1* transcription (Mitchell and Smith, 1988; Jeong et al., 2014). Xbp1 induces the transcription of p53 via HEPN1 and represses E2F1 via NF-κB activation, resulting in reduced *Pin1* transcription (Chae et al., 2016). The transcription of PIN1 is repressed by *BRCA1*, a tumor suppressor gene (MacLachlan et al., 2000). BRCA1 interacts with some proteins to control DNA repair. During cancer development, BRCA1 is often mutated, resulting in the accumulation of DNA damage in cells (Mersch et al., 2015). The mRNA stability of *PIN1* is reduced by microRNAs, such as miR-200c (Luo et al., 2014), miR-200b (Zhang et al., 2013) and miR296-5p (Lee et al., 2014) in breast cancer, breast CSCs, and prostate cancer.

Under physiological conditions, the protein activity is generally regulated by post-translational modifications. Post-translational modifications at specific sites, including sumoylation, phosphorylation, ubiquitination, and oxidization, can regulate the PIN1 protein activity and function. The S65, S71, S138, and S16 residues in PIN1 protein sequence are reported as phosphorylation sites (Eckerdt et al., 2005; Rangasamy et al., 2012; Bhaskaran et al., 2013). The PIN1 phosphorylation at Ser16 in the N-terminal WW domain, inhibits the ability of PIN1 to bind with its substrates (Lu P. -J. et al., 2002), and it can be induced by ribosomal S6 kinase 2 (Cho et al., 2012), protein kinase A (Lu K. P. et al., 2002), and aurora kinase A (Lee et al., 2013). The PIN1 phosphorylation at Ser65 in the C-terminal PPIase domain by polo-like kinase (Plk1) (Eckerdt et al., 2005) induces the ubiquitination and stabilization of PIN1. The PIN1 phosphorylation at Ser138 by mixed-lineage kinase 3 induces its nuclear translocation and catalytic activity (Rangasamy et al., 2012). The PIN1 phosphorylation at Ser71 by death-associated protein kinase 1 (DAPK1) can reduce MYC and E2F-mediated oncogenic transformation.

PIN1 sumoylation at Lys6 in the N-terminal WW domain and Lys63 in the C-terminal PPIase domain suppresses its oncogenic function and enzymatic activity (Chen et al., 2013). PIN1 desumoylation at Lys6 and Lys63 by SUMO1/sentrin specific peptidase 1 (SENP1) recovers its substrate-binding and catalytic activity. Under oxidative stress, PIN1 is generally oxidized at Cys113 in the PPIase catalytic site, which can suppress the enzymatic activity of PIN1 (Chen et al., 2015).

PIN1 reduces the degradation of oncogenes and/or growth-promoting regulators, such as β-catenin, AKT, c-fos, cyclin D1, c-Jun, ER, HER2, Hbx, HIF-1, Mcl-1, NF-κB, Nanog, NUR77, PML-RARa, Oct4, Stat3, and Tax (Lu and Zhou, 2007; Gianni et al., 2009; Liao et al., 2009; Moretto-Zita et al., 2010; Lu and Hunter, 2014; Wei et al., 2015). On the contrary, PIN1 induces the degradation of tumor suppressors such as Daxx, FoxO4, Fbw7, GRK2, PML, KLF10, RARa, RUNX3, RBBP8, Smad, SUV39H1, SMRT, and TRF1 (Lu and Zhou, 2007; Lee T. H. et al., 2009; Ryo et al., 2009; de Thé et al., 2012; Lu and Hunter, 2014; Ueberham et al., 2014; Wei et al., 2015). ERα increases the tumor proliferation through regulating the expression of estrogen response element (ERE)-containing genes in breast cancer (Anderson, 2002). PIN1 induces the ERE-binding affinity and transcription activity, and reduces the ERα degradation mediated by E3 ligase E6AP in breast cancer (Rajbhandari et al., 2012, 2014, 2015). Through inhibiting ubiquitination and destabilizing the transcriptional corepressor SMRT, PIN1 increases HER2 activity (Lam et al., 2008; Stanya et al., 2008). PIN1 also increases the activity of NF-κB pathway via inducing the nuclear accumulation of c-Rel, RelA/p65, and v-Rel (Ryo et al., 2003; Fan et al., 2009). Furthermore, it inhibits the p65 ubiquitination mediated by SOCS-1 (Ryo et al., 2003). PIN1 directed NF-κB activation regulates the proliferation of AML, endometrial carcinoma, glioblastoma, and hepatocellular carcinoma (HCC) (Atkinson et al., 2009; Saegusa et al., 2010; Shinoda et al., 2015; Chen et al., 2016). An isoform of p63, ΔNp63 lacking an intact N-terminal transactivational domain is important for cancer development (Murray-Zmijewski et al., 2006). PIN1 reduces the ΔNp63 ubiquitination induced by WWP1 to enhance the proliferation of oral squamous cell carcinoma (Li et al., 2013). PIN1 stabilizes BRD4 protein to increase the migration and proliferation of gastric cancer (Hu et al., 2017). It also upregulates c-Jun, c-Myc, FoxM1, β-catenin, NUR77, and XBP1 (Chen et al., 2012; Helander et al., 2015; Chae et al., 2016; Kruiswijk et al., 2016; Zhu et al., 2016; Csizmok et al., 2018).

PIN1 AND SIGNAL TRANSDUCTION IN CANCER

PIN1 is associated with the development of various cancers, including melanoma, breast cancer, gastric cancer, cervical cancer, gallbladder cancer, pancreatic ductal carcinoma,

colorectal cancer, prostate cancer, ovarian cancer, non-small cell lung cancer, osteosarcoma, esophageal cancer, hepatitis B virus (HBV)-induced hepatocellular carcinoma, Burkitt lymphoma, and T cell acute lymphoblastic leukemia. PIN1 is reported to activate 56 oncogenes and/or growth-promoting regulators. Also, it is reported to inactivate 26 tumor suppressors and/or growth-inhibitory regulators (**Figure 1**).

In human breast cancer, PIN1 promotes oncogenesis via the cyclin D1 regulation (Ryo et al., 2001; Wulf et al., 2001). Studies have shown that PIN1 increases cyclin D1 transcription in association with the HER2–HRAS–JNK–AP1, WNT–β-catenin, and NF-κB pathways. PIN1 regulates HER2, NOTCH1, NOTCH3, androgen receptor (AR) and estrogen receptor α (ERα), which are cancer-driving receptors (La Montagna et al., 2012; Rajbhandari et al., 2012). Furthermore, PIN1 regulates AMPK, AKT93, MYC, PKM2, RAF1, SMAD2, SMAD3, STAT3, the RAS family member RAB2A28, FAK, protein tyrosine phosphatase, PTP-PEST, S6K, and SGK1, which act as intracellular signaling modulators (Lee N. Y. et al., 2009; Jo et al., 2015; Chen Y. et al., 2018). PIN1 induces the interaction of non-receptor type 12 (PTP-PEST) with FAK to increase the FAK Tyr397 dephosphorylation, which induces cancer metastasis (Zheng et al., 2009, 2011). PIN1 also promotes epithelial–mesenchymal transition (EMT) of MCF-7 cells by inducing the transcriptional activity of STAT3 and recruiting its transcription coactivator p300 (Lufei et al., 2007). PIN1 induces the cancer metastasis and invasion by activating β-catenin, BRD4, NF-κB, and p53M (Muller et al., 2009; Wang et al., 2012; Zhu et al., 2016; Hu et al., 2017). Overexpression of PIN1 increases the PTOV1 expression as a novel interactome of PIN1, and knockdown of both genes inhibits the expression of β-catenin, cyclin D1, and c-Myc in breast cancer MDA-MB-231 cells (Karna et al., 2019).

Pin1 transgenic mice in mammary glands induces mammary hyperplasia and malignant mammary tumors (Suizu et al., 2006). Pin1-deficient mice inhibit the massive proliferation of breast epithelium in pregnancy through reducing cyclin D1 levels (Liou et al., 2002) and decreases β-catenin expression in breast cancer (Ryo et al., 2001). Pin1 knockout mice show defects in breast development and induces retinal degeneration and neurodegenerative disorder in brain (Fujimori et al., 1999; Liou et al., 2002).

In human liver cancer, PIN1 is associated with the transcription levels of RhoC and RhoA, and co-overexpression of both genes correlates with metastasis and recurrence of HCC (Ng et al., 2019). All-trans retinoic acid (ATRA) is potent PIN1

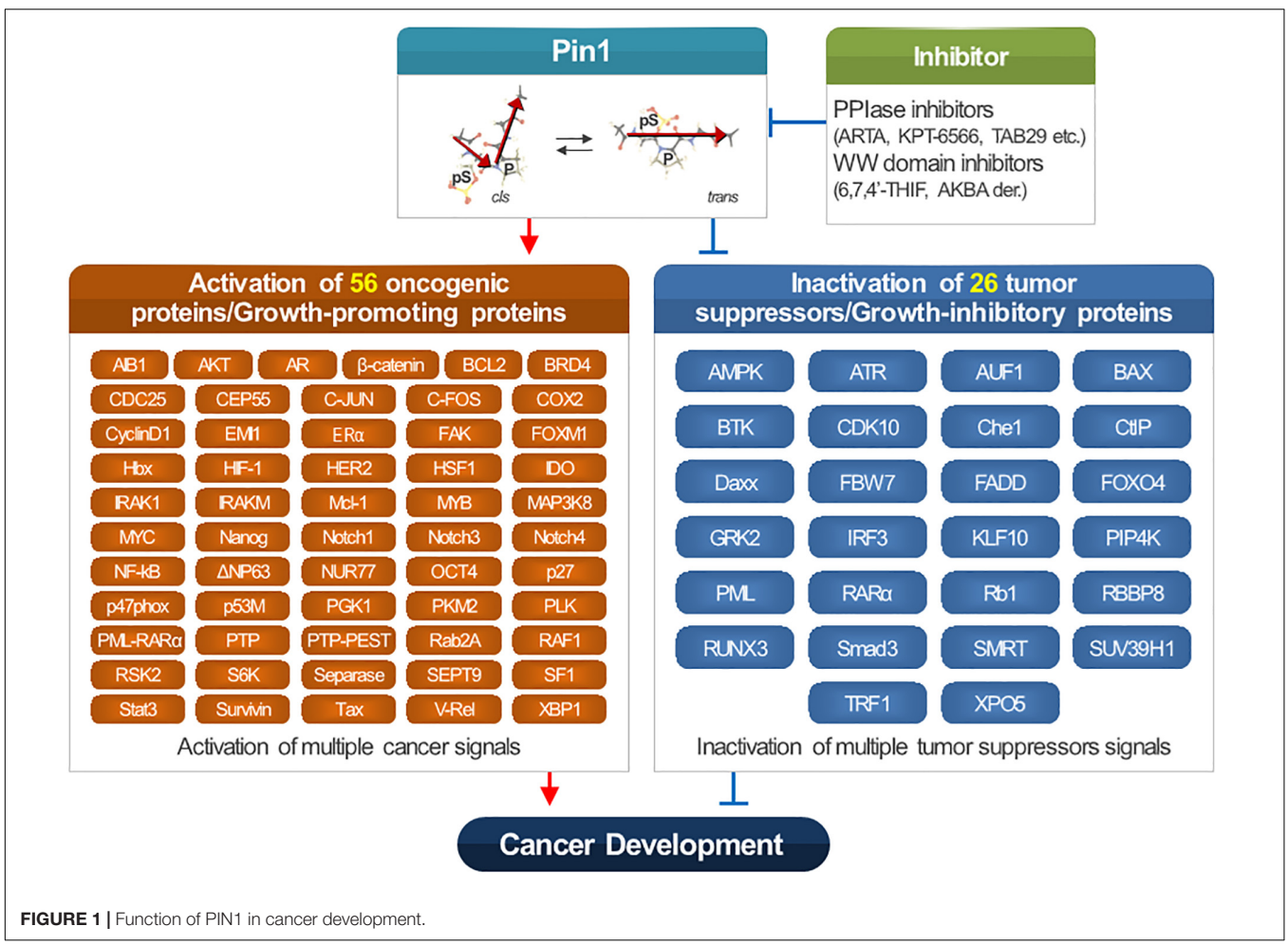


FIGURE 1 | Function of PIN1 in cancer development.

inhibitor in hepatocellular carcinoma (Liao X. -H. et al., 2017) and co-targeting p53-RS (p53-R249S) with CDK4, c-Myc, or PIN1 is more effective against the treatment of HCC (Liao X. -H. et al., 2017). PIN1 inhibitor (AF-39) significantly suppresses cell proliferation through the XPO5 subcellular distribution and miRNAs biogenesis in HCC cells (Zheng et al., 2019). Inhibition of Pin1 reverses regorafenib resistance in hepatocellular carcinoma (HCC) with reducing EMT, migration and metastasis (Wang et al., 2019).

In pancreatic cancer, PIN1 was highly expressed in pancreatic ductal adenocarcinoma (PDAC) tissues and significantly correlated with the worst outcomes in patients. PIN1 inhibition with specific siRNA or ATRA suppressed tumor growth in PDAC (Chen et al., 2019). Pin1 is overexpressed and correlated with poor prognosis in gastric cancer (Shi et al., 2015). Pin1 inhibition using small molecule inhibitor such as ATRA or short hairpin RNA, reduces cancer development by inhibiting Wnt/β-catenin and PI3K/AKT signaling pathways in gastric cancer (Zhang et al., 2019).

In nasopharyngeal carcinoma (NPC), Pin1 inhibition reduced NPC cell proliferation, colony formation and anchorage-independent growth through the decrease of cyclin D1 expression and the activation of caspase-3 (Xu et al., 2016). Pin1 enhances transcription activity of ATF1 and induces tumorigenesis in NPC (Huang et al., 2016). Using specific siRNA, Pin1-targeted inhibition suppresses transformed properties and prevents cell proliferation in prostate cancer cells (Ryo et al., 2005). In human melanoma metastasis, although the expression of cytoplasmic Pin1 is not associated with primary melanoma clinical outcome, Pin1 expression in cytosol is correlated with poor survival of melanoma patients (Chen X. et al., 2018). In human colorectal cancer, the expression of cytoplasmic Pin1 is importantly correlated with aggressive tumor behaviors and a worse prognosis in colorectal cancer (Pyo et al., 2018).

In osteosarcoma, PIN1 overexpression using adenovirus significantly stimulates MG-63 and U2-OS cell proliferation. Also, PIN1 inhibitor, juglone reduces cell proliferation in osteosarcoma cells (Zhou et al., 2013). In esophageal squamous cell carcinoma (ESCC), increased Pin1 expression is associated with worse outcome of ESCC patients. Also, Pin1 promotes the aggressiveness of ESCC via β-catenin and cyclin D (Lin et al., 2014). In human lung cancer, cancer patients without Pin1 overexpression has longer cancer-related survival than cancer patients with Pin1 overexpression. Pin1 knockdown in H1299 cell reduces cell invasion and migration (Tan et al., 2010).

In metastatic cancer, PIN1 level is considerably higher than that in primary cancer. The TGF-β signaling promotes the metastasis of cancer. PIN1 increases SMAD degradation mediated by E3 ligase Smurf-2 to repress TGF-β signaling (Nakano et al., 2009). In prostate cancer, PIN1 promotes TGF-β-induced metastasis (Matsuura et al., 2010). Inhibiting the phosphorylation of SMAD3 represses the aggressiveness of breast cancer by reducing the interaction with PIN1 (Thomas et al., 2017).

PIN1 is also involved in angiogenesis. It enhances the transcriptional activity and of stability HIF-1α in several cancer cells (Jalouli et al., 2014; Han et al., 2016). PIN1 promotes the VEGF expression mediated by NF-κB in HCC and regulates the transcriptional factors by VEGF including β-catenin and FoxM1 (Wang et al., 2007; Jiang et al., 2015; Shinoda et al., 2015). Overexpression of HIF-1α, VEGF, and *Pin1* is correlated to TAM-resistant MCF-7 cell lines (TAMR-MCF-7) (Oh et al., 2010; Lee T. H. et al., 2011). RNA interference of Pin1 inhibits the angiogenesis as well as the growth of prostate cancer. In TAMR-MCF-7 cells, PI3K/p38 signal pathways increase the *Pin1* expression through increasing E2F1 (Lee K. Y. et al., 2011).

PIN1 and Signal Transduction in Cancer Stem Cells (CSCs)

Studies have shown a role of PIN1 in stem cells of breast cancer and leukemia (Luo et al., 2014, 2015; Rustighi et al., 2014; Wei et al., 2015). PIN1 induces NOTCH1 cleavage by γ-secretase, leading to enhanced NOTCH1 transcriptional and tumorigenic activities. PIN1 increases NOTCH1 stability to promote self-renewal and metastasis of breast CSCs by reducing the ubiquitin ligase F-box/WD repeat-containing protein 7 (FBXW7)-mediated degradation of NOTCH1 and NOTCH4 (Rustighi et al., 2014). The deletion of *Pin1* decreases the NOTCH-induced invasion of T cell acute lymphoblastic leukemia (T-ALL) cells (Franciosa et al., 2016). PIN1 interacts with the AP1 transcription factors JUN and FOS to activate AP1-dependent RAB2A transcription to promote the expansion and tumorigenesis of breast CSCs (Luo et al., 2015). Overexpression of PIN1 converts normal human breast epithelial cells to cells with stem-like and EMT phenotypes, whereas PIN1 silencing reduces the tumorigenesis and self-renewal activity of breast CSCs in primary breast cancer tissue (Luo et al., 2014, 2015; Rustighi et al., 2014). PIN1 is a pivotal target of miR-200c, a key negative regulator of CSC function and EMT (Shimono et al., 2009; Luo et al., 2014). Inhibition of PIN1 induces the degradation of the fusion oncogene promyelocytic leukemia (PML)–retinoic acid receptor-α (PML–RARα) that drives leukemia stem cells (LSCs), and thereby, treats APL without inducing myeloid differentiation (Ito et al., 2008; de Thé and Chen, 2010). PIN1 controls the maintenance of stability of Nanog, octamer-binding protein 4 (OCT4), and MYC (Nishi et al., 2011; Farrell et al., 2013) and is important for the self-renewal of CSCs.

Pin1 Regulates the Cell Death Resistance and Inflammation of Cancer

Pin1 inhibits apoptosis through BAX as proapoptotic factor in human eosinophils (Shen et al., 2009) and regulates death-associated proteins DAXX to promote its degradation in human gliomas (Ryo et al., 2007). Pin1 induces cell death resistance function of BCL-2 and myeloid cell leukemia-1 (MCL-1) as anti-apoptosis factors (Basu and Haldar, 2002; Ding et al., 2008). Pin1 increases the survival of cisplatin-treated cervical cancer cells through Wnt/β-catenin and FoxM1 signaling (Wang et al., 2016). Pin1 increases the tamoxifen resistance upregulating LC-3 in breast cancer (Namgoong et al., 2010). Pin1 inhibit proapoptotic signals and activate antiapoptotic signals which consequently regulates the cell death resistance in cancer cells.

In allergen-injected rat, inhibition of Pin1 decreases the production of GM-CSF (Esnault et al., 2007). Pin1 induces the IL-22-induced proliferation and survival of breast cancer cells by activating c-Jun, and STAT3 (Kim et al., 2014). Pin1 is involved in inflammatory diseases such as non-alcoholic steatohepatitis (NASH) (Nakatsu et al., 2012), atherosclerosis (Paneni et al., 2015), rheumatoid arthritis (Jeong et al., 2009), and biliary cholangitis (Asuri et al., 2018).

THERAPEUTIC TARGETING OF PIN1

PIN1 is reported to be highly expressed in variety of human cancers, such as hepatic, prostate, lung, colorectal and esophageal cancers. It participates in diverse cancer-associated signaling pathways. Thus, the development of PIN1 inhibitors has been the focus of several research groups (**Table 1**).

The first PIN1 inhibitor discovered by low-throughput screening is juglone. Juglone functions to inhibit the PIN1 PPIase activity in C-terminal catalytic domain, and a high dose of juglone reduces PIN1 protein expression. In addition, juglone has also shown to reduce the prostate cancer cell growth by inhibiting PIN1 activity (Hennig et al., 1998; Chao et al., 2001; Jeong et al., 2009; Costantino et al., 2016; Wang et al., 2017; Shin et al., 2018). Nevertheless, juglone possesses a primarily simple structure that may affect diverse specificity.

A chemically synthesized library containing compounds having a double-ring structure was screened and PiB inhibiting PIN1 (IC50 = 1.5 μM) was identified. Unlike juglone, PiB has been shown to a competitive inhibitor that inhibits the growth of *Pin1*-containing cells, but not that of *Pin1*-deficient cells. Furthermore, the inhibition of PIN1 by PiB treatment destabilizes Nanog, transcription factor required for the essential survival of cancer stem cells (Uchida et al., 2003). Uchida et al. identified TME-001 (IC50 = 6.1 μM) for a PIN1 inhibitor by library screening using *in vitro* enzymatic assay. The results revealed that this compound prevents the growth of HeLa cells (Mori et al., 2011).

Like juglone and PiB, other PIN1 inhibitors have been screened by low-throughput or high-throughput screening. pTide peptide shows PIN1 inhibition at 1.2 nM *in vitro*, but it is inactive in cells (Wildemann et al., 2006). The specificity of pTide against PIN1 has been shown by the X-ray crystal structure (Zhang et al., 2007). Attachment of an PIN1 octaarginine sequence to the pTide fragment enhances the membrane permeable ability and inhibits the cell growth in cancer (Liu et al., 2010).

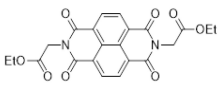
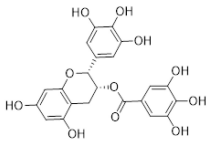
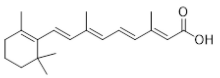
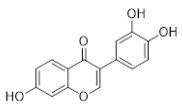
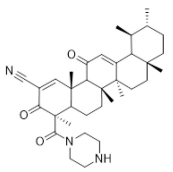
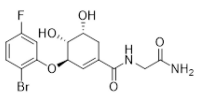
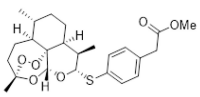
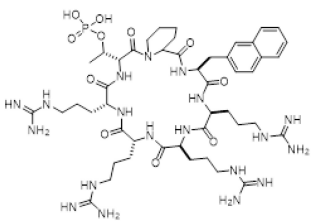
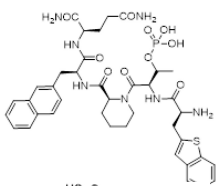
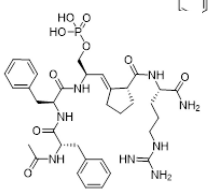
A cyclic peptide derivative with increased cell permeable ability repressed the activity of PIN1 (IC50 = 32 nM) and inhibited the BT-474 breast cancer cell proliferation (Liu et al., 2010). Treatment of 100 nM of this peptide in cancer cell lines (HeLa and BT-474) increases the levels of PML and SMRT, and inhibits intracellular PIN1 activity (Liu et al., 2010; Bedewy et al., 2017). A major flavonoid of green tea, epigallocatechin 3 gallate (EGCG) is widely known as chemo-preventive compound for cancer and one of PIN1 inhibitor. Urusova et al. found utilizing X-ray crystal co-structure that EGCG binds to the N-terminal WW as well as C-terminal PPIase domains of PIN1. EGCG inhibits the activity of PIN1 *in vitro* enzyme assay (IC50 = 20 μM) and reduces JNK signaling pathway, and Bcl-xL and cyclin D1 expression in MEF cells transformed by ErbB2 (Urusova et al., 2011).

Structure-based design induces the identification of cis-locked alkene peptidomimetics as PIN1 inhibitors. They exhibited anti-proliferation activities in A2780 ovarian carcinoma cell line (Wang et al., 2004; Xu and Etzkorn, 2009). Using structure-based drug design, Vernalis and Pfizer develop small molecules. These inhibitors often contain a phosphate or carboxylate as isostere or a phenyl imidazole core, which is required to target the phosphate-binding pocket of the PIN1 protein (Guo et al., 2009; Dong et al., 2010; Potter A. et al., 2010; Potter A. J. et al., 2010; Guo et al., 2014). Pfizer has identified an inhibitor that repressed the PPIase activity of PIN1 at nano-molar concentrations (IC50 = 6 nM) by investigating and exploring the protein crystal structure of PIN1 (Guo et al., 2009; Dong et al., 2010; Guo et al., 2014). Using 900-number fragment library, Vernalis has developed a NMR-based fragment screen to isolate PIN1 inhibitors through the protease-coupled *in vitro* enzyme assay. A PIN1 inhibitor was synthesized (Potter A. J. et al., 2010) and showed good nanomolar inhibition against PIN1 *in vitro* (IC50 = 830 nM). However, they are poorly active or inactive in cell lines since the phosphate or carboxylate renders the inhibitors poor cell permeable ability (Guo et al., 2009; Dong et al., 2010; Potter A. et al., 2010; Potter A. J. et al., 2010).

Leung et al. have identified a PIN1 inhibitor from natural-product library using structure-based virtual screening and they show that compound 1 targets PIN1 and interferes the interaction of PIN1 with the NF-κB p65 subunit in cells. Moreover, a natural-product compound induced apoptosis in PC-3 cell lines (Wu et al., 2018). Using the virtual screening analysis, PIN1 protein has been identified as a target of 6,7,4′-trihydroxyisoflavone (6,7,4′-THIF). 6,7,4′-THIF bound to PIN1 protein, but did not bind to the family proteins such as FKBP or cyclophilin A, suggesting a selective and specific binding with PIN1. 6,7,4′-THIF compound was analyzed for specific inhibitory activity for PIN1 using *Neu/Pin1* knockout (KO) and *Neu/Pin1* wild-type (WT) MEFs. This PIN1 inhibitor affected *Neu/Pin1* WT MEF cells, but not *Neu/Pin1* KO MEF cells. In addition, the result of a xenograft tumor growth assay in mice utilizing *Neu/Pin1* KO and WT MEF cells have been shown similar to the result from the *in vitro* enzyme assay (Lim et al., 2017).

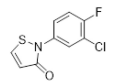
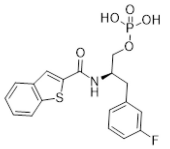
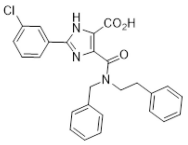
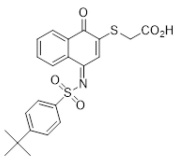
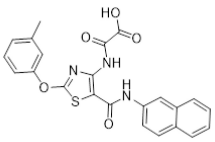
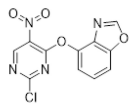
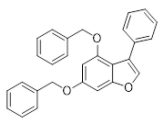
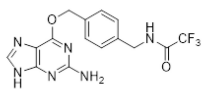
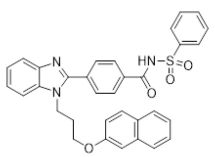
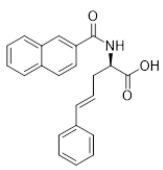
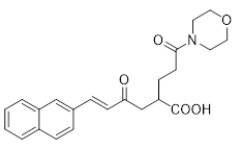
One of the reported inhibitors of PIN1, aetyl-11-keto-β-boswellic acid (AKBA) derivative has been shown to inhibit the growth of prostate cancer PC-3 (IC50 = 40 nM) and LNCaP (IC50 = 270 nM) cell lines. The compound inhibited the activity of PIN1, to stabilize cyclin D1, which improved anti-proliferative effects of prostate cancer treatment through new mechanisms (Li et al., 2017). In addition to the previous inhibitors of PIN1, thiazole derivatives (IC50 = 5.3 μM), pyrimidine derivatives (IC50 = 1.7 μM), benzimidazle derivatives (IC50 = 1.0 μM), 6-O-benzylguaninie derivative API-1 (IC 50 = 72 nM), and phenylbenzofuran derivative TAB29 (IC50 = 874 nM) have been reported as non-small molecule inhibitors (Zhao et al., 2016; Cui et al., 2018; Pu et al., 2018; Fan et al., 2019; Ma et al., 2019).

TABLE 1 | PIN1 inhibitors for cancer treatment.

Pin1 inhibitor	Structure	Group	Cancer type	Remark	References
Juglone			Prostate cancer	Irreversible inhibitor of Pin1 PPIase catal ytic domain	Hennig et al., 1998; Chao et al., 2001; Jeong et al., 2009; Cho et al., 2015; Costantino et al., 2016; Shin et al., 2018
PiB		Uchida group	Colon cancer	IC50 1.5 μM (enzyme assay) Competitive inhibitor of Pin1	Uchida et al., 2003
EGCG		Zigang group	Colorectal cancer (HCT116 cell)	IC50 20 μM (PPIase assay) Bind both WW and PPIase domains Actived cancer stem cell	Urusova et al., 2011
ATRA		Ping Lu group	APL and Breast cancer	IC50 820 nM (PPIase assay) Specific inhibitor of Pin1 PPIase catalytic domain Effected Pin1 WT MEFs but not Pin1 KO MEFs	Wei et al., 2015; Liao X. -H. et al., 2017; Zheng et al., 2017
6,7,4′-THIF		Chen group	Esophageal cancer	Directly interacts with Pin1 at the WW an d PPIase domains Effected Neu/Pin1 WT MEFs but not Neu/Pin1 KO MEFs	Lim et al., 2017
AKBA derivative		Zhao group	Prostate cancer	IC50 3.6 μM (PPIase assay) Bind WW domain	Li et al., 2017
Netural-product like		Leung group	Prostate cancer (PC3 cell, LNCaP cell)	IC50 1.1 μM (enzyme assay)	Wu et al., 2018
Artemisinin derivative		Pokharel group	Prostate cancer (PC3 cell)		Gour et al., 2019
Cyclic peptide		Pei group	Hela cell, Breast cancer (BT-474cell)	IC50 32 nM (PPIase assay) In creased the protein levels of PML and SMRT	Liu et al., 2010; Bedewy et al., 2017
pTide		Fischer group	Hela cell (G2/M phase)	IC50 1.2 nM (PPIase assay)	Wildemann et al., 2006; Zhang et al., 2007
Cis-locked alkene peptidomimetics		Etzkorn group	Ovarian cancer (A2780 cell)	IC50 1.3 μM (PPIase assay)	Wang et al., 2004; Xu and Etzkorn, 2009

(Continued)

TABLE 1 | Continued

Pin1 inhibitor	Structure	Group	Cancer type	Remark	References
TME-001		Uchida group	Hela cell	IC50 6.1 μM (PPIase assay) Dual inhibition of Pin1 and CypA Competitive inhibitor of Pin1 PPIase catal ytic domain	Mori et al., 2011
Benzothiophene		Guo group		IC50 6 nM (enzyme assay) Poor cell activity	Guo et al., 2009
Phenyl imidazoes		Moore group	PC3 cell	IC50 830 nM (enzyme assay) NMR-based fragment screening	Potter A. et al., 2010, p. 20, 586; Potter A. J. et al., 2010, p. 20, 6483
KPT-6566		Campanet group	Breast Cancer (MDA-MB-231 ce ll), Prostate cancer (PC3 cell)	IC50 625 nM (PPIase assay) Covalent inhibitor of Pin1 Curbs breast cancer stem cells Growth inhibition of lung metastasis in vivo	Campaner et al., 2017
Thiazole derivative		Xu group		IC50 5.4 μM (PPIase assay)	Zhao et al., 2016
Pyrimidine derivative		Xu group		IC50 1.7 μM (enzyme assay) Covalent inhibitor of Pin1	Cui et al., 2018
TAB29		Zhao group	Hepatocellular car cinoma (HCC) (SK-Hep-1, SNU-423 cell)	IC50 874 nM (PPIase assay) Bind PPIase domain	Fan et al., 2019
API-1		Pu group	Hepatocellular car cinoma (HCC)	IC50 72 nM (PPIase assay) Bind PPIase domain	Pu et al., 2018
Benzimidazole derivative		Zhao group	PC3 cell	IC50 1.0 μM (enzyme assay) Bind PPIase domain	Ma et al., 2019
Naphthylamido acid derivative		Guo group	PC3 cell	IC50 1.8 μM (enzyme assay) phosphate-containing inhibitor of Pin1	Dong et al., 2010
(S)-2		Nakagawa group	PC3 cell	IC50 3.2 μM (proteinase-coupled assay) Covalent inhibitor of Pin1	Ieda et al., 2019

In particular, *in silico* virtual screening was performed using the PIN1crystal structures and identified API-1 and TAB29 as small molecules that bind to the PPIase domain. Furthermore, PIN1 inhibition by API-1 and TAB29 upregulates miRNA biogenesis by maintaining the active XPO5 conformation and represses the development of hepatocellular carcinoma (HCC), suggesting that PIN1 mediates miRNA biogenesis mechanism, API-1 can be a drug candidate for therapy for *Pin1*-overexpressing or extracellular signal-regulated kinase-activated HCC (Pu et al., 2018; Fan et al., 2019).

The Food and Drug Administration (FDA) approved all-trans retinoic acid (ATRA) for acute promyelocytic leukemia (APL) therapy (Wei et al., 2015). ATRA was identified using a mechanism-based high-throughput screening system. ATRA inhibited the activity of PIN1 by binding with the C-terminal catalytic PPIase domain of PIN1. ATRA induces the degradation of PIN1 protein, but also suppresses the oncogenic function by decreasing the expression of cyclin D1. Furthermore, PIN1 inhibition mediated by ATRA induces the degradation of PML-RARA oncoprotein, resulting in anti-proliferative effect in APL cells and mouse models, as well as in humans. Moreover, a slow-release ATRA formulation induces the degradation of PIN1 and decreases tumorigenicity in mice xenograft model of HCC (Wei et al., 2015; Liao P. et al., 2017). Additionally, a combination of ATRA and sorafenib for the HCC treatment decreases the expression of PIN1 protein, increases cancer cell death, and represses the HCC growth compared with sorafenib or ATRA alone. These results provide an important rationale for further PIN1 inhibitor development to increase the therapeutic efficacy of general drug for HCC (Zheng et al., 2017).

A more recent study identifies KPT-6566, a novel PIN1 small molecule inhibitor, possessing high potency (IC50 = 625 nM) and specificity from a drug-like collection of 0.2 million commercial compounds (Campaner et al., 2017). Compounds capable of covalently binding to the C113 residue of the PIN1 catalytic domain were selected by virtual structure-based screening and cytotoxicity testing to select the final compounds. Structurally, the electrophile sulfonyl-acetate moiety of KPT-6566 directly faces the nucleophile sulfur atom of C113. Like ATRA, KPT-6566 also promotes the degradation of PIN1, resulting in the reduction of hyper-phosphorylated pRB and cyclin D1 levels. KPT-6566 increases the apoptosis and decreases the cancer cell proliferation such as pancreatic, lung, prostate, and breast cancers. It showed a better anti-proliferative effect on cancer cell lines than on normal cell lines. Furthermore, treatment with KPT-6566 inhibited the overexpression of *Pin1*, confirming the reduction of breast cancer stem cells. In addition, in *in vivo* studies, KPT-6566 has been shown to decrease the lung metastasis in breast cancer mouse models. Currently, KPT-6566 is the only PIN1 inhibitor in the preclinical stage of research.

A study reported by the Pokharel et al. shows that the artemisinin derivatives commonly used as antimalarial drugs are very effective in variety of cancer cell lines to inhibit cancer cell growth. Especially compound 9a, one of the artemisinin derivatives increases anti-proliferative, pro-apoptotic and anti-metastatic effect in PC-3 prostate cancer cells by decreasing the expression of Pin1, cyclin D1, c-Myc, elF4E, and PCNA (Gour et al., 2019).

Irreversible PIN1 inhibitor (*S*)-2 (IC50 = 3.2 μM), and its derivatives recently designed by Ieda et al. show the inhibition of Pin1 in protease-coupled *in vitro* assay and the reduction of cyclin D1 expression in PC-3 prostate cancer cell (Ieda et al., 2019).

CONCLUSION

PIN1 is a well-known PPIase that regulates the cis–trans isomerization of pSer/Thr–Pro, which highlights its importance in the control of Pro-directed phosphorylation. PIN1 regulates protein function via conformational changes of target protein and is associated with the oncogenic pathway activation by controlling tumor suppressors and oncogenes. PIN1 is overexpressed in cancer tissues and CSCs, and correlated with poor clinical outcome in various cancer patients. Inhibition of PIN1 plays an important role in the tumorigenesis and angiogenesis of cancer, thereby providing a new great therapeutic target. Recently, PIN1 inhibitors have been developed elsewhere using structure-based drug designs and natural compounds that inhibit the activity of cancer. PIN1 obviously can be an super attractive target for curing cancer and cancer stem cells.

AUTHOR CONTRIBUTIONS

All authors designed and wrote this manuscript.

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REVIEW
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Pinning Down the Transcription: A Role for Peptidyl-Prolyl *cis-trans* Isomerase Pin1 in Gene Expression

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Pin1 is a peptidyl-prolyl *cis-trans* isomerase that specifically binds to a phosphorylated serine or threonine residue preceding a proline (pSer/Thr-Pro) motif and catalyzes the *cis-trans* isomerization of proline imidic peptide bond, resulting in conformational change of its substrates. Pin1 regulates many biological processes and is also involved in the development of human diseases, like cancer and neurological diseases. Many Pin1 substrates are transcription factors and transcription regulators, including RNA polymerase II (RNAPII) and factors associated with transcription initiation, elongation, termination and post-transcription mRNA decay. By changing the stability, subcellular localization, protein-protein or protein-DNA/RNA interactions of these transcription related proteins, Pin1 modulates the transcription of many genes related to cell proliferation, differentiation, apoptosis and immune response. Here, we will discuss how Pin regulates the properties of these transcription relevant factors for effective gene expression and how Pin1-mediated transcription contributes to the diverse pathophysiological functions of Pin1.

Keywords: conformational change, isomerization, phosphorylation, Pin1, transcription, RNA polymerase II, transcription factor

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INTRODUCTION

Prolyl isomerases (PPIases) catalyze the *cis-trans* isomerization of the peptidy prolyl (X-Pro) bonds. There are three distinct families of PPIases: cyclophilins (CyPs), FK506-binding proteins (FKBPs), and parvulins (Zhou and Lu, 2016). Pin1 belongs to the parvulin family and is comprised of an N-terminal WW domain serving as a phosphoprotein-binding module and a C-terminal catalytic domain that is distinct from other conventional PPIases (Zhou and Lu, 2016). Because of its unique WW and PPIase domains, Pin1 specifically isomerizes the pSer/Thr-Pro motif and regulates the functions of a defined group of phosphoproteins by altering their conformations (Liou et al., 2011). Pin1-mediated post-phosphorylation regulation has profound effects on multiple cellular and biological processes, including cell cycle, cell differentiation and death, and metabolic and immune response (Liou et al., 2011; Zhou and Lu, 2016). Aberrant expression of Pin1 has been identified to be associated with many diseases, especially in cancer and neurodegenerative disorders, such as Parkinson's disease (PD) and Alzheimer's disease (AD) (Liou et al., 2011; Zhou and Lu, 2016). While Pin1 is highly expressed in the majority of cancers and promotes cancer progression, its

expression is down-regulated in neurodegenerative diseases (Liou et al., 2011; Zhou and Lu, 2016), highlighting the diverse regulatory functions of Pin1 in physiology and diseases.

The temporal and spatial eukaryotic gene expression is a highly orchestrated molecular event that is regulated at multiple levels and is responsible for the distinct cellular responses and functions. The multi-level regulation includes the signal-dependent activation of tissue-specific transcription factors, the remodeling of chromatin on promoters and enhancers, the pausing and release of RNAPII, the post-transcriptional processing of mRNA, and the translational regulation (Splinter and De Laat, 2011; Dong et al., 2012; Hensel and Xiao, 2013). Post-translational modifications, especially phosphorylation, play important roles in the multi-level regulation of gene expression (Pawson and Scott, 2005). Many transcription factors and transcription related proteins undergo phosphorylation and activate gene expression in response to intra- and extracellular stimuli (Hunter and Karin, 1992). The reversible phosphorylation on serine or threonine residues preceding a proline (pSer/Thr-Pro) has emerged as a pivotal switch for controlling the activities of participating transcription components in gene expression (Shaw, 2007; Hanes, 2015). Pin1's ability to regulate many cellular processes might rely on its ability to regulate the expression of various genes by binding to the phosphorylated transcription regulators. More than 40 different kinds of transcription related proteins, including transcription activators and general transcription machinery components, have been identified to be Pin1 substrates (**Table 1**). Pin1 binds to the pSer/Thr-Pro motifs of these proteins and regulates the gene transcription by altering the stability, subcellular localization, protein-protein interactions, and protein-DNA/RNA interaction of these factors (Xu and Manley, 2007a). In this review, we summarize how Pin1 controls the activity of these transcription regulators for the spatiotemporal expression of genes involved in cell cycle, cell proliferation and growth, metabolism and inflammation, thus contributing to the diverse functions of Pin1 in physiology and disease.

Pin1 AND DNA BINDING TRANSCRIPTION FACTORS

Eukaryotic gene expression is regulated by genomic enhancers and promoters that are recognized by various tissues specific DNA binding transcription factors (Patikoglou and Burley, 1997). Pin1 regulates the activities of a spectrum of transcription factors, many of which are involved in cancer cell proliferation and inflammatory response (Lu and Zhou, 2007).

Nucleocytoplasmic Shuttling of Transcription Factors

A key regulatory step in transcription is the nucleocytoplasmic shuttling of transcription factors, which are synthesized in the cytoplasm and need to be transported into the nucleus, where they bind to the promoters or enhancers to activate gene expression in response to different intra- or extracellular stimuli (Cartwright and Helin, 2000). A number of studies have shown that Pin1 regulates the nucleocytoplasmic shuttling of transcription factors for the activation or inactivation of transcriptional response. For example, Pin1 promotes nuclear localization of RelA subunit of NF-κB (Ryo et al., 2003) and β-catenin (Ryo et al., 2001). Upon cytokine stimulation, Pin1 binds to the phosphorylated Thr254-Pro motif in RelA and increases the nuclear accumulation of RelA by inhibiting its binding to IκBα (Ryo et al., 2003). IκBα, the inhibitor of NF-κB, is known to sequester NF-κB in the cytoplasm by masking the nuclear localization signal (NLS) of NF-κB (Chen and Greene, 2004). Thr254 of RelA is in the proximity of Ser238, Asp243, and Arg253, three key amino acids involved in the IκBα binding (Jacobs and Harrison, 1998). Phosphorylation of Thr254 and the subsequent of binding of Pin1 likely change the conformation of RelA, therefore preventing its interaction with IκBα□ (Ryo et al., 2003). NF-κB is a master regulator of inflammatory response and is also a key player in the cancer cell development (Chen and Greene, 2004). By regulating the activation of NF-κB, Pin1 promotes tumor progression and inflammatory cytokine production (Ryo et al., 2003; Atkinson et al., 2009; Fan et al., 2009; Shinoda et al., 2015).

The nucleocytoplasmic shuttling of β-catenin is regulated by its association with APC (the adenomatous polyposis coli), which contains two active nuclear export sequences (NES) for the nuclear export of β-catenin (Henderson, 2000). Pin1 recognizes phosphorylated Ser246-Pro motif of β-catenin. Interestingly, the Ser246-Pro motif is next to the APC binding site (Ryo et al., 2001). Therefore, Pin1-mediated isomerization of the pSer246-Pro peptide bond in β-catenin would affect its binding to APC, leading to the accumulation of β-catenin in the nucleus and the up-regulation of its target genes, such as cyclin D1 and c-Myc (Ryo et al., 2001). Since aberrant accumulation of β-catenin contributes to abnormal development and tumorigenesis, Pin1 regulates many processes in development and tumor formation, including osteoblast and neuronal differentiation, cancer cell proliferation, and drug resistance, via affecting the transcriptional activity of β-catenin (Ryo et al., 2001; Nakamura et al., 2012; Shin et al., 2016; Wang et al., 2016).

In addition to stimulating the nuclear accumulation of NF-κB and β-catenin, Pin1 can also sequester transcription factors in the cytoplasm to inactivate the target gene expression. The nuclear localization and the transcriptional activity of FOXO4, a tumor suppressor preventing the accumulation of cellular damage due to oxidative stress, are regulated by its monoubiquitination (Van Der Horst et al., 2006). In response to oxidative stress, Pin1 binds to phosphorylated FOXO4 and increases USP7-mediated FOXO4 deubiquitination, resulting in the decreased monoubiquitination and the increased cytoplasmic accumulation. Ultimately, binding of Pin1 to FOXO4 decreases its transcriptional activity toward its target genes, including the cell cycle arrest gene *p27kip1* (Brenkman et al., 2008).

Another example for the Pin1-mediated nucleocytoplasmic shuttling of transcription factor is nuclear factor activated T cell (NFAT), which is essential for T cell activation (Liu et al., 2001). Upon T cell activation, intracellular calcium is increased and NFAT is subject to dephosphorylation by the calcium- and calmodulin (CaM)-dependent protein phosphatase calcineurin,

TABLE 1 | List of Pin1 substrates in transcription regulation.

Substrates	Motif	Regulation by Pin1	Cellular consequence of Pin1 interaction	Evidence of isomerization	References
Transcription factors					
(1) Nucleocytoplasmic shuttling					
RelA	T254	Increased nuclear accumulation and stability	Cell survival, proliferation and inflammation	Yes	Ryo et al., 2003; Atkinson et al., 2009; Fan et al., 2009; Shinoda et al., 2015
β-catenin	S246	Increased nuclear accumulation and stability	Cancer cell proliferation, osteogenesis	Yes	Ryo et al., 2001; Nakamura et al., 2012
Fox04	N/A	Deubiquitylation and decreased nuclear accumulation	Cell cycle and cancer cell proliferation	Yes	Brenkman et al., 2008
NFAT	N/A	Decreased nuclear accumulation	T cell activation	N/A	Liu et al., 2001
(2) Protein stability					
p53	S33, S46, T81, S315	Increased stability and transactivation	DNA damage response, cancer cell cycle arrest and apoptosis	Yes	Wulf et al., 2002; Zacchi et al., 2002; Zheng et al., 2002
p63	T538	Increased or decreased stability	Cancer and limb development	N/A	Li et al., 2013; Restelli et al., 2014
p73	S412, T442, T482	Increased stability and transactivation	Apoptosis	Yes	Mantovani et al., 2004
c-Jun	S63, S73	Increased stability	Ras and JNK signaling	Yes	Wulf et al., 2001; Pulikkan et al., 2010
Naong	S52, S65, S71, T287	Increased stability	Stem cell pluripotency	Yes	Moretto-Zita et al., 2010
Oct4	S12	Increased stability	Stem cell pluripotency	Yes	Nishi et al., 2011
FoxMl	S331, S704	Increase stability	Drug resistance	N/A	Kruiswijk et al., 2016; Wang et al., 2016
Osterix	S76, S80	Increase stability and transactivation	Osteogenic differentiation	Yes	Jang et al., 2005
ATF1	T184	Increase stability and transactivation	NPC tumorigenesis	N/A	Huang et al., 2016
TR3	S95, S140, S431	Increase stability and transactivation	Mitogenesis	Yes	Chen et al., 2012
Runx2	T408, T449, S472, S510	Increase sub-nuclear area accumulation and stability	Skeletal development, Osteoblast differentiation.	Yes	Lee et al., 2013; Yoon et al., 2013
Runx3	T209, T212, T231, S214	Degradation, suppresses transactivation	Breast cancer cell proliferation	Yes	Nicole Tsang et al., 2013
Smad3	T179, S204, S208, S213	Decreased stability	Cell migration and invasion	Yes	Nakano et al., 2009
IRF3	S339	Decreased stability	Antiviral responses	Yes	Saitoh et al., 2006
RAR	S77	Decreased stability	Cancer cell proliferation	Yes	Gianni et al., 2009
MEF2C	S98, S110	Decreased stability	Muscle terminal differentiation	Yes	Magli et al., 2010
Fox03	N/A	Decreased stability	Drug resistance	No	Shimizu et al., 2016
(3) DNA binding activity and transcriptional activity					
c-Myc	T58, S62	Decreased stability, increased DNA binding	Cancer cell proliferation	Yes	Yeh et al., 2004; Farrell et al., 2013
ERα	S118, S294	Increased dimerization, stability and transactivation activity	Cancer cell proliferation	Yes	Rajbhandari et al., 2014

(Continued)

TABLE 1 | Continued

Substrates	Motif	Regulation by Pin1	Cellular consequence of Pin1 interaction	Evidence of isomerization	References
HIFIα	S641, S643	Increase stability and transactivation	Angiogenesis	Yes	Jalouli et al., 2014; Han H. J. et al., 2016
SP1	T739	Increased stability, decreased DNA binding	Cell cycle progression	Yes	Yang et al., 2014
c-Fos	T232, T325, T331	Increased interaction with other transcription factors	Mitogen response	Yes	Monje et al., 2005
GR	S203, S211	Increased transactivation	Inflammatory response	Yes	Poolman et al., 2013
PPARγ	S273	Increased stability and transactivation	Adipogenesis	N/A	Fujimoto et al., 2010; Han Y. et al., 2016
Nur77	S152	Increased DNA binding and transactivation	Vascular disease and metabolism	No	van Tiel et al., 2012
Stat3	S272	Increased DNA binding and transactivation	EMT and type 2 diabetes	No	Lufei et al., 2007; Lv et al., 2013; Nakada et al., 2019
Transcription cofactors					
SRC-3	multiple sites	Increased interaction with p300 and degradation	Brease cancer cell proliferation	Yes	Yi et al., 2005
Notch1	S2122, T2133, S2137	Enhanced Notch1 cleavage and transcriptional activity	Notch signaling	Yes	Rustighi et al., 2009
SMRT	S1241, S1445, S1469	Decreased stability	Cancer cell proliferation and response to tamoxifen	Yes	Stanya et al., 2008
CRTC2	S136	Decreased nuclear accumulation	Glucose metabolism	N/A	Nakatsu et al., 2010
PRDM16	N/A	Decreased stability	Thermogenesis	N/A	Chi and Cohen, 2016
RNA polymerase					
Rpbl	S2, S5 of CTD	Altered phosphorylation of CTD	Transcription	Yes	Xu et al., 2003; Zhang et al., 2012
Histone					
Histone HI	multiple sites	Increased dephosphorylation and binding to chromatin	Transcription	Yes	Raghuram et al., 2013
Transcription elongation regulators					
Spt5	multiple sites	Increased binding to transcription regulators	Transcription	N/A	Lavoie et al., 2001
Brd4	T204	Increased stability and transcriptional activity	Transcription and cancer	Yes	Hu et al., 2017
mRNA decay factors					
SLBP	T171	Increased dephosphorylation	Cell cycle	Yes	Krishnan et al., 2012
AUF1	S83	Decreased AUF1-mRNA interactions	Eosinophil survival, T cell activation, allergic inflammation	Yes	Shen et al., 2005; Esnault et al., 2006
KSRP	S181	Increased dephosphorylation and mRNA interaction	Hyperparathyroidism	N/A	Nechama et al., 2009
HuR	N/A	mRNA binding affinity	Transcription	N/A	Krishnan et al., 2014

triggering the translocation of NFAT into the nucleus where it binds to the promoter region of a number of cytokines and activates their transcription (Zhu and Mckeon, 2000). Pin1 has been reported to form a stable complex with the phosphorylated form of NFAT, which contains 3 Pin1 binding motifs (Liu et al., 2001). Therefore, by controlling the nucleocytoplasmic shuttling, Pin1 functions as a negative regulator of NFAT and T cell activation.

Stability of Transcription Factors

Another major mechanism of Pin1-mediated transcription factor regulation is through ubiquitin-mediated protein degradation (Liou et al., 2011; Dilworth et al., 2012; Hanes, 2015). Pin1 can either increase or decrease the stability of transcription factors, depending on the functionality of these transcription factors.

The tumor suppressor p53 is a key transcription factor regulating cellular pathways such as DNA repair, cell cycle, apoptosis and senescence and is a pivotal gatekeeper against cancer onset and progression (Zilfou and Lowe, 2009). A key regulatory mechanism for the transactivation of p53 is the E3 ligase MDM2-mediated ubiquitination and degradation (Zilfou and Lowe, 2009; Nag et al., 2013). In response to DNA damage, p53 is stabilized by its release from MDM2 and activates its downstream target genes to induce cell cycle arrest or cell death (Zilfou and Lowe, 2009). DNA damage induces the phosphorylation of p53 at several Ser/Thr-Pro residues, including Ser33, Ser46, Thr81 and Ser315 (Wulf et al., 2002; Zacchi et al., 2002; Zheng et al., 2002). Binding to Pin1 to phosphorylated p53 and the subsequent Pin1-mediated isomerization of p53 prevent the interaction of p53 with MDM2 since binding of Pin1 to pThr81-Pro motif of p53 disassociates p53 from MDM2, leading to stabilized p53 and the activation of p53 target genes (Zacchi et al., 2002).

The stability of p63 and p73, two other p53 gene family members, is also regulated by Pin1 (Mantovani et al., 2004; Li et al., 2013; Restelli et al., 2014). The conformation of p73 is altered by Pin1-mediated isomerization, promoting its interaction with p300 and the subsequent acetylation in a c-Abl dependent manner, likely preventing the ubiquitination of p73 on the acetylated lysine (Mantovani et al., 2004). As a result, Pin1 augments p73's ability to induce the expression of proapoptotic genes, including *Bax*, *Pig3*, and *p53AIP1* (Mantovani et al., 2004). On the other hand, Pin1 specifically interacts with Thr538-Pro of p63a and disrupts the interaction between p63a and WWP1, an E3 ligase for p63a, resulting in the enhanced transcriptional activity for the expression of proapoptotic gene *Bax* (Li et al., 2013). It appears that Pin1 represents a common mediator linking proapoptotic cooperative activity of the p53 family members. As a regulator of p53, Pin1 regulates many cellular responses related to cell cycle and cell death, including genotoxic response, apoptosis, and mitochondrial apoptotic function (Wulf et al., 2002; Zacchi et al., 2002; Zheng et al., 2002; Follis et al., 2015; Mantovani et al., 2015).

Pin1 has been demonstrated to increase the stability of c-Jun via inhibition of its ubiquitination (Pulikkan et al., 2010). Pin1 binds to c-Jun that is phosphorylated on Ser63/73-Pro motifs by JNK or Ras (Wulf et al., 2001). Similar to p53, Pin1-mediated isomerization and the conformation change of c-Jun weakens its binding to the E3 ubiquitin ligase Fbw7, therefore attenuating the degradation of c-Jun (Csizmok et al., 2018). A similar mechanism is also identified for Pin1-mediated stabilization of estrogen receptor a(ERa), a key player in the development of breast cancer. ERa is phosphorylated at Ser118-Pro119 and Pin1 binds to this specific phosphorylated serine and induces the *cis-trans* isomerization of Pro119. Binding of Pin1 to ERa disrupts the ubiquitination of ERa by interfering with its interactions with the E3 ligase, E6AP, which is shown to bind to phosphorylated Ser118 and degrade ERa (Rajbhandari et al., 2014).

While the above examples confirm a role for Pin1 in the stabilization of transcription factors, Pin1 also promotes the degradation of transcription factors. Phosphorylation of Thr58 of c-Myc is critical for its oncogenic potential, since a mutation at Thr58 is often identified in the amplified *c-myc* genes in Burkitt's lymphoma and Thr58 mutant of c-Myc demonstrates enhanced oncogenic potential with increased protein stability (Farrell and Sears, 2014). Phosphorylation of Thr58 is important for the recognition of c-Myc by Pin1 via the WW domain, which might lead to the conformational change of c-Myc, facilitating c-Myc dephosphorylation at Ser62 by PP2A and promoting c-Myc turnover by the ubiquitin-proteasome pathway (Yeh et al., 2004; Farrell et al., 2013). Therefore, Pin1 triggers the degradation of c-Myc by facilitating the dephosphorylation of c-Myc by PP2A. The increased protein stability and oncogenic potential of Thr58 mutant in Burkitt's lymphoma might result from the defect in Pin1-medaited dephosphorylation of c-Myc.

Pin1 also reduces the stability of tumor suppressive transcription factors. RUNX3, a tumor suppressor in breast cancer (Chen, 2012), has been identified as a Pin1 substrate. Pin1 recognizes four phosphorylated Ser/Thr-Pro motifs in RUNX3 via its WW domain and reduces the cellular levels of RUNX3 in an isomerase activity-dependent manner by inducing the ubiquitination and proteasomal degradation of RUNX3 (Nicole Tsang et al., 2013). These four motifs are located immediately C-terminal of the runt domain, a region has been shown to be important for RUNX3 stability. Binding of Pin1 to these phosphorylated motifs and the associated conformational change of RUNX3 might result in the recruitment of RUNX3 E3 ligases (Nicole Tsang et al., 2013). Therefore, Pin1-mediated protein degradation might partially account for the decreased RUNX3 expression, an early event in breast cancer progression (Chuang and Ito, 2010). Interestingly, Pin1 also regulates the activity of RUNX2, which is another key member of the Runt family proteins and the master transcription factors for bone formation (Lian and Stein, 2003). Different from RUNX3, binding of Pin1 to phosphorylated RUNX2 stabilizes RUNX2 protein by preventing RUNX2 ubiquitination and degradation (Lee et al., 2013; Yoon et al., 2013). Through modulating the stability and transcriptional activity of RUNX2, Pin1 regulates the osteoblast differentiation and skeletal development (Lee et al., 2013; Yoon et al., 2013).

Other transcription factors regulated by Pin1 at the level of protein stability include RelA (Ryo et al., 2003), β-catenin (Ryo et al., 2001), IRF3 (Saitoh et al., 2006), Naong (Moretto-Zita et al., 2010), Oct4 (Nishi et al., 2011), MEF2C (Magli et al., 2010),

SP1 (Yang et al., 2014), Osterix (Lee et al., 2015), ATF1 (Huang et al., 2016), TR3 (Chen et al., 2012), FoxM1 (Kruiswijk et al., 2016; Wang et al., 2016), Smad3 (Nakano et al., 2009), RAR (Gianni et al., 2009), FoxO3 (Shimizu et al., 2016), PPARγ (Fujimoto et al., 2010; Han Y. et al., 2016), and HIF-1a (Han H. J. et al., 2016) (**Table 1**). The detailed mechanisms for how Pin1 regulates their stability might be different for each factor, it appears that changing the accessibility of E3 ligases to the Pin1 substrates due to Pin1-medaited protein conformational change via isomerization might represent a general mechanism for the regulation of protein stability by Pin1. In this regard, Pin1 prevents the binding of E3 ligase RNF4 to SP1 and SPOP for Naong, respectively (Yang et al., 2014; Zhang et al., 2019). By changing the stability of these transcription factors and their transcriptional activities, Pin1 regulates diverse biological processes, including inflammatory response, cell proliferation, stem cell reprogramming, myogenesis, and bone formation (**Table 1**) (Liou et al., 2011).

DNA Binding and Transactivation

DNA binding domain and transactivation domain (TAD) are two essential protein domains that help define a transcription factor. Pin1 is able to modulate both the DNA binding and transcriptional activity of transcription factors. Pin1 binds to the N-terminal Ser118-Pro motif in the intrinsic activation function 1 (AF1) domain of ERα (Rajbhandari et al., 2012). Binding of Pin1 and the subsequent Pin1-mediated conformational change via isomerization increases ERα DNA binding activity with a concomitant increase in ERα transcriptional activity in estrogen activated breast cancer cells (Rajbhandari et al., 2015). Pin1 also promotes the binding of c-Myc to the DNA, independent of the protein stability regulated by Pin1 (Farrell et al., 2013). This regulation requires Pin1 PPIase activity and the phosphorylation of c-Myc on Ser62-Pro63. While Pin1 stimulates the DNA binding activity of ERa and c-Myc, but the Pin1 binding motifs on ERa or c-Myc are not within the DNA binding domain (Farrell et al., 2013; Rajbhandari et al., 2015). How Pin1-mediated isomerization in one region could affect the activity of the DNA binding domain on a different region? One possibility is that the conformation change-mediated recruitment of co-activators (e.g., p300 and GCN5) might alter the accessibility of the chromatin, leading to the enhanced DNA binding of the transcription factors and the enhanced transcription of target genes. In this regard, the AF1 domain of ERa is responsible for the recruitment of SRC1 and CBP (Dutertre and Smith, 2003). c-Myc's interaction with p300 and the recruitment of p300, GCN5, hSNF5, and pTEFb to promoters is also facilitated by the binding of Pin1 (Farrell et al., 2013; Sanchez-Arevalo Lobo et al., 2013). It has to be noted that Pin1 can also decrease the DNA binding activity of transcription factors. Binding of Pin1 to phosphorylated Thr739 of Sp1 has been reported to cause Sp1 to move out of the chromosome completely by decreasing its DNA binding activity during mitosis (Yang et al., 2014).

While changing the DNA binding affinity would affect the transcriptional activity of a transcription factor, Pin1 can also regulate the transcriptional activity by directly binding to the TADs of the transcription factors (Monje et al., 2005; Lufei et al., 2007; van Tiel et al., 2012; Lv et al., 2013; Poolman et al., 2013; Nakada et al., 2019) (**Table 1**). Phosphorylation of the carboxyl-terminal transactivation domain of c-Fos by extracellular signal-regulated kinases (ERK) in response to growth factors is essential for the transcriptional activation of AP-1, heterodimer of c-Jun and c-Fos (Monje et al., 2003). Pin1 binds to c-Fos through specific pSer/Thr-Pro sites within the c-Fos TAD, and this interaction results in an enhanced transcriptional response of c-Fos to polypeptide growth factors that stimulate ERK (Monje et al., 2005). The detailed mechanism for this enhanced transactivation is undetermined, but likely results from the change of interactions from transcription related proteins of the TAD (Monje et al., 2005).

Pin1 has also been shown to regulate the transcriptional activity of glucocorticoid receptor (GR) by binding to the TAD. Binding of Pin1 to the phosphorylated Ser203 and Ser221 within the TAD of GR enhances the transactivation of GR (Poolman et al., 2013). Interestingly, this enhanced transactivation appears to result from enhanced recruitment of GR to the promoters of its GR target genes but not directly from the transactivation (Poolman et al., 2013). How the binding of Pin1 to the TAD enhances the DNA binding activity of GR remains to be determined. It is possible that Pin1-mediated conformational change of TAD would affect the conformation of DNA binding domain, which is adjacent to the TAD (Poolman et al., 2013).

Pin1 also regulates the transcriptional activity of HIF-1a. Pin1 interacts with p42/p44 MAPK-mediated phosphorylation of HIF-1a at Ser641 and Ser643 of the transactivation region and promotes its conformational changes for the efficient expression of HIF-1a genes, including VEGF, GLUT1 and PGK1 (Jalouli et al., 2014). It has been speculated that the enhanced transactivation of HIF-1a might stem from the increased HIF-1a binding to DNA or transcriptional cofactors (Jalouli et al., 2014).

Pin1 and the Transcription Co-regulators

Transcription factors often recruit transcription co-activators for their full transcriptional potential and biological functions (Spiegelman and Heinrich, 2004). Pin1 regulates the activity of some transcription co-regulators to control the effective gene expression.

Steroid receptor-mediated transcription requires the ligand-dependent association of receptors with steroid receptor coactivator 3 (SRC-3) (Lydon and O'malley, 2011). Pin1 interacts with phosphorylated SRC-3 and regulates its co-activation function by enhancing its interaction with CBP/p300 and stimulating its cellular turnover, facilitating the cyclic recruitment of nascent phosphorylated SRC-3 to the promoter (Yi et al., 2005).

Pin1 also regulates CREB co-activator CRTC2 (CREB-regulated transcriptional co-activator 2) by binding to phosphorylated CRTC2 at Ser136, which locates within its nuclear localization signal (Nakatsu et al., 2010). Different from SRC-3, binding of Pin1 to phosphorylated CRTC2 suppresses the co-activation function of CRTC2 by attenuating its nuclear localization and cAMP-responsive element (CRE) transcriptional activity (Nakatsu et al., 2010).

A recent study also demonstrates that transcriptional co-activator PRDM16 is negatively regulated by Pin1 (Nakatsu et al., 2019). PRDM16 plays crucial roles in the determination and function of brown and beige fat as well as in hematopoiesis and cardiac development (Chi and Cohen, 2016). Pin1 interacts with phosphorylated PRDM16 at Ser44A, Ser52A, Thr61A and Ser66A, promotes its degradation and the suppression of the thermogenic response (Chi and Cohen, 2016). The detailed mechanism for Pin1-mediated PRDM16 degradation remains undetermined. Nevertheless, by regulating the activity of co-activators such as CRTC2 and PRDM16, Pin1 is involved in the regulatory mechanism governing the glucose metabolism and adipose thermogenesis (Nakatsu et al., 2016).

In the Notch1 signaling pathway, activation of CSL [CBF-1, Su(H), Lag-1] -target genes requires the co-activation function of the intracellular domain of the notch protein (NICD), which is released from the membrane-bound Notch1 protein processed by the γ-secretase (Bray, 2016). NICD has also been shown to be a co-activator for Notch-mediated activation of LEF-1 target gene independent of its co-activation function for CSL (Ross and Kadesch, 2001). Binding of Pin1 to Notch1 stimulates the processing of the Notch1 from its inactive transmembrane form to γ-secretase-processed, activated nuclear localized form (Rustighi et al., 2009). The catalytic activity of Pin1 is required for the cleavage of the Notch protein by γ-secretase for the release of NICD (Rustighi et al., 2009). By mediating the generation of NICD, Pin1 regulates gene expression in Notch signaling pathway.

In addition to the regulation of transcription co-activators, Pin1 can control gene expression by targeting transcription co-repressors. Silencing mediator for retinoic acid and thyroid hormone receptor (SMRT) is a transcriptional corepressor that participates in diverse signaling pathways and human diseases (Chen and Evans, 1995). Pin1 interacts with SMRT and regulates SMRT protein stability, thereby affecting SMRT-dependent transcriptional repression (Stanya et al., 2008). SMRT is phosphorylated by Cdk2 at Ser1241, Ser1445 and Ser1469. Cdk2-mediated phosphorylation of SMRT at these serines is required for Pin1 binding and the decreased SMRT stability. More importantly, ErbB2 destabilizes SMRT protein level via Cdk2-Pin1 axis, suggesting that ErbB2 signaling upstream of Cdk2 and Pin1 is a potential regulatory cascade involved in regulating the stability of SMRT (Stanya et al., 2008). Interestingly, two of the Cdk2 phosphorylation sites of the Pin1 binding motifs in SMRT are conserved in N-CoR, a closely related transcription repressor (Stanya and Kao, 2009), suggesting that the activity of N-CoR might be regulated by Pin1 via a similar mechanism.

Pin1 AND RNA POLYMERASE II

Transcription factors and transcription coactivators are essential for the recruitment of RNAPII to the promoters or enhancers to activate transcription. The C-terminal domain (CTD) of Rpb1, the largest subunit of RNAPII, which consists of 26-52 tandem heptapeptide repeats with the general consensus sequence Tyr_1-Ser_2-Pro_3-Thr_4-Ser_5-Pro_6-Ser_7 from yeast to human. The proline-rich CTD functions as a docking platform for numerous transcription regulatory proteins involved in transcription initiation, elongation, termination and post-transcription processing (Hahn, 2004). The CTD is marked by a number of post-translational modifications, including phosphorylation, glycosylation, methylation, and acetylation (Brookes and Pombo, 2009). During the early events of transcription initiation, unphosphorylated RNAPII, general transcription factors and a mediator complex are recruited onto the promoters to form the pre-initiation complex (PIC) (Thomas and Chiang, 2006). Phosphorylation of Ser5 promotes the dissociation of RNAPII from PIC and the promoter clearance, processes that are required for transition from initiation to early elongation (Phatnani and Greenleaf, 2006; Sogaard and Svejstrup, 2007). Different from phosphorylated Ser5, phosphorylation of Ser2 results in the recruitment of elongation, termination and 3′ end processing factors, allowing the coupling of transcription elongation with mRNA processing (Bentley, 2002; Ahn et al., 2004).

Pin1 binds to both pSer2-Pro3 and pSer5-Pro6 of the CTD (Xu et al., 2003; Zhang et al., 2012). Pin1's binding to CTD depends on the phosphorylation of Ser2 by kinases CDK2 and CDK9 (Komarnitsky et al., 2000; Bartkowiak et al., 2010), and the phosphorylation of Ser5 by CDK7 (Phatnani and Greenleaf, 2006). Pin1 induces the conformational changes of the CTD, leading to the recruitment of CTD-modifying enzymes and transcription regulatory proteins essential for RNAPII function (Hanes, 2015). The presence of two Ser-Pro motifs with the CTD repeats creates four possible *cis-trans* configurations, and thus expands the complexity of the CTD code signature by providing a scaffold for the recruitment of a variety of chromatin and RNA processing factors (Srivastava and Ahn, 2015). The *cis* or *trans* configuration of the Pin1 binding motifs on CTD determines the transcription outcome via the recruited factors. For example, Mce (capping enzyme), Pcf11 (3′ end processing factor), Scp1 (CTD phosphatases) and SCAF8 (splicing factor) bind to phosphorylated CTD with the prolines in *trans* configuration (Fabrega et al., 2003; Noble et al., 2005; Zhang et al., 2006; Becker et al., 2008; Ghosh et al., 2011). In contrast, Ssu72 and the termination factor Nrd1 bind CTD with phosphorylated Ser5-pro6 in the *cis* configuration (Xiang et al., 2010; Werner-Allen et al., 2011; Kubicek et al., 2012).

RNAPII is subject to regulatory control at all steps of transcription cycle, including initiation, elongation and termination. The high selectivity of transcription regulatory proteins for *cis* or *trans* isomers supports the idea that Pin1 serves as a key transcription regulator for gene expression. However, how Pin1 creates and maintains the *cis* or *trans* configuration of CTD in during transcription cycle remains obscure and merit further investigation.

Pin1 AND TRANSCRIPTION INITIATION

Transcription initiation encompasses multiple steps, including the exposure of promoters in chromatin, the association of

promoters with RNAPII and transcription regulatory proteins to form PIC, and the clearance of promoter for the release of RNAPII (Li et al., 2007). The wrapping of promoter DNA around a histone octamer in the nucleosome suppresses transcription initiation. The ordered disassembly of nucleosomes facilitates transcription by allowing RNAPII to interact with the promoters. Histone H1 plays a crucial role in maintaining higher order chromatin structure and reversible phosphorylation of H1 is closely correlated with transcription initiation with increased phosphorylation of H1 associating with a relaxed chromatin structure, allowing the access of RNAPII and DNA-binding proteins to the promoter region (Hohmann, 1983; Vicent et al., 2011).

Pin1 has been demonstrated to bind to H1 via phosphorylated S/T-Pro residues on the C-terminal of H1 (Raghuram et al., 2013). Pin1 promotes dephosphorylation of H1 and stabilizes H1's interaction with chromatin to facilitate condensation, implying that Pin1 may act as a suppressor of transcription initiation. The idea that Pin1 inhibits transcription initiation is supported by *in vitro* transcription assays demonstrating that Pin1 inhibits transcription initiation in nuclear extracts whereas an inactive Pin1 mutant stimulates transcription initiation (Xu and Manley, 2007a). Pin1 might also inhibit transcription initiation via dephosphorylation of Ser5 of the CTD of RNAPII (Werner-Allen et al., 2011). However, some studies indicate that Pin1 might have a positive effect on transcription initiation since Pin1 inhibitor Juglone disrupts the formation of functional PIC (Chao et al., 2001). The discrepancy in these studies might result from the different *in vitro* and *in vivo* assays and the approaches to inhibit Pin1. For example, Pin1 inhibitor Juglone is known to be toxic to the cells and might have off-target effects, which accounts for the initiation inhibition (Nakatsu et al., 2018). Pin1 has also been shown to regulate the chromosome condensation during mitosis targeting the topoisomerase (Topo) IIa (Xu and Manley, 2007a).

While Pin1 indirectly regulates the transcription initiation by affecting the chromosome structure, it is also possible that Pin1 might directly affect the activity of transcription initiation factors. The activity of transcription initiation factor TFIID is tightly regulated by phosphorylation. During mitosis, TFIID is phosphorylated at multiple sites and phosphorylated TFIID is unable to direct activator-dependent transcription (Segil et al., 1996). Considering that Pin1 is a major regulator of mitosis (Liou et al., 2011), Pin1 might target TFIID to regulate transcription during cell cycle. Interestingly, mice deficient in TAF4b, a gonad-specific subunit of TAFIID exhibit germ cell deficiency, a phenotype similar to $Pin1^{-/-}$ mice (Falender et al., 2005). These studies provide genetic evidence linking Pin1 to TFIID, but the detailed mechanism how Pin1 regulates TFIID for the transcription initiation needs to be further investigated.

Pin1 AND TRANSCRIPTION ELONGATION

After ∼20–60 bp RNA is synthesized, RNAPII is repressed by negative elongation factors, such as DSIF (DRB sensitivity-inducing factor) and NELF (negative elongation factor) at promoter-proximal pausing sites (Wada et al., 1998; Yamaguchi et al., 1999). CDK9, a catalytic subunit of P-TEFb (positive transcriptional elongation factor b), is recruited and activated by Brd4 (Bromodomain-containing protein 4), and phosphorylates NELF, DSIF and Ser2 in the CTD of RNAPII (Li Y. et al., 2018). Phosphorylation of NELF and DSIF by CDK9 removes these negative factors from the pausing sites, releasing the paused RNAPII into the productive elongation phase (Fujinaga et al., 2004). Pin1 seems to play a role in the transcription elongation by removing the negative elongation factor DSIF and activating the positive elongation factor P-TEFb.

Pin1 binds to the hSpt5 subunit of DSIF via its phosphorylated carboxyl terminal part 2 (CTR2) domain by Cdk9 (Lavoie et al., 2001). The CTR2 domain contains a p(T/S)PSP(Q/A)(S/G)Y motif, which resembles the CTD repeats of RNAPII (Hanes, 2015). hSpt5 is phosphorylated by CDK9 in interphase but not in mitosis and this interphase form of phosphorylated hSpt5 is bound to the nuclear matrix, indicating its involvement in transcription (Lavoie et al., 2001). Binding of Pin1 to phosphorylated hSpt5 induces the conformational change of hSpt5 via isomerization, leading to the subsequent change of its phosphorylation status and the conversion of DSIF from a repressor to an activator (Lavoie et al., 2001).

In mammalian cells, Brd4 regulates transcription elongation by recruiting P-TEFb to stimulate the phosphorylation of the CTD of RNAPII (Jang et al., 2005; Ai et al., 2011). Brd4 has emerged as an important factor in tumorigenesis by promoting the transcription of genes involved in cancer development (Muller et al., 2011; Wu et al., 2013; Basheer and Huntly, 2015; Jung et al., 2015). Our recent studies demonstrate that the stability and functions of Brd4 are positively regulated by Pin1 in cancer cells (Hu et al., 2017). Pin1 directly binds to phosphorylated Thr204 of Brd4 by an unidentified kinase and enhances Brd4's stability by inhibiting its ubiquitination. Pin1 also catalyzes the isomerization of Pro205 of Brd4 and induces its conformational change through a *cis-trans* isomerization, which leads to enhanced CDK9 binding to Brd4 and enhanced recruitment of CDK9 to a subset of promoters of Brd4-mediated tumor-promoting genes, including c-*MET* and *MMP9* (Hu et al., 2017). In addition to the enhanced CDK9 binding, Pin1-mediated conformational change might also decrease the accessibility of a Brd4 E3 ligase or increase the accessibility of a Brd4 deubiquitinating enzyme for the increased protein stability with reduced ubiquitination (Hu et al., 2017). Therefore, the overall tumor-promoting activity of Brd4 in cancer cells might result from the Pin1-mediated conformational change of Brd4, leading to more stabilized Brd4 and conformational change-associated transcriptional potential increase (Hu et al., 2017).

Pin1 AND TRANSCRIPTION TERMINATION

Termination of transcription involves the release of RNA transcripts, the dissociation of RNAPII and its binding proteins from the DNA, coupled with the cleavage of 3′ end of the nascent

transcript and the polyadenylation (Richard and Manley, 2009). Phosphorylation of RNAPII at Ser2 or Ser5 is closely related to transcription termination. Levels of Ser5 phosphorylation is high near the transcription start site, while Ser2 phosphorylation increases over the gene body, peaking near the transcription termination site (Hsin and Manley, 2012). Pin1 increases the dephosphorylation of Ser5, but not Ser2, by CTD phosphatase Ssu72 (Kops et al., 2002; Werner-Allen et al., 2011). Pin1 also inhibits the CTD dephosphorylation by affecting the activity of another CTD phosphatase, Fcp1, or increasing the CTD phosphorylation by Cdc2/Cyclin B (Xu and Manley, 2007a,b). As such, Pin1 can regulate the transcription termination by changing the phosphorylation status of CTD of RNAPII.

Various phosphorylation status of CTD of RNAPII creates a CTD code that dictates the assembly and disassembly of factors to the RNAPII and determines the transcription outcome. Pin1 has been implicated in the construction and deciphering the CTD code (Buratowski, 2003). In yeast, mRNA 3′-end processing factor Pcf11 binds to CTD repeats of RNAPII containing Pro3 in the *trans*-configuration (Noble et al., 2005), whereas the termination factor Nrd1 binds to the *cis* form of phosphorylated Ser5 (Kubicek et al., 2012). By changing the *cis*- or *trans*-configuration of prolines in CTD and coordinating the recruitment of the termination and/or 3′ -end mRNA processing factors, such as Pcf11, Rtt103 and Nrd1 (Noble et al., 2005; Lunde et al., 2010; Kubicek et al., 2012), Ess1 (yeast Pin1) might facilitate the transcription termination. A similar regulator mechanism might also occur in mammalian cells since these CTD binding factors are highly conserved in eukaryotic cells.

Pin1 AND THE POST-TRANSCRIPTIONAL REGULATION OF GENE EXPRESSION

mRNA levels are determined by a complex interplay between the rates of gene transcription and mRNA decay (Schoenberg and Maquat, 2012). mRNA decay is closely associated with the 3′ untranslated region (3′-UTR) of the mRNAs. Many early response genes contain AU-rich element (ARE) in 3′-UTRs (Barreau et al., 2005). AREs occur in up to 5–8% of all mRNA transcripts in human cells and these AREs are recognized by AU-binding proteins (AUBPs), which promote either decay or stabilization of mRNA on a gene- and cell type-specific manner (Barreau et al., 2005; Halees et al., 2008). Many AUBPs are phosphoproteins and their activity is tightly regulated through reversible phosphorylation (Shen and Malter, 2015). Via binding to the specific phosphorylated AUBPs, Pin1 controls mRNA decay of selective genes.

Histone mRNAs are rapidly degraded at the end of S phase, and a 26-nucleotide stem-loop in the 3′-UTR is a key determinant of histone mRNA stability (Heintz et al., 1983). This sequence is the binding site for stem-loop binding protein (SLBP), which helps to recruit components of the RNA degradation machinery to the histone mRNA (Wang et al., 1996). Pin1 binds to phosphorylated Thr171-Pro172 of SLBP and promotes its dephosphorylation by PP2A, causing its dissociation from histone mRNA hairpin, triggering the rapid degradation of histone mRNA (Krishnan et al., 2012). Another example for Pin1-mediated mRNA stability is the mRNA of the parathyroid hormone (PTH), which regulates the serum calcium via its effect on bone, kidney, and intestine (Nechama et al., 2009). The stability of *PTH* mRNA is decreased by the binding of K-homology splicing regulator protein (KSRP) to a *cis*-acting element in the 3′-UTR region of *PTH* mRNA (Nechama et al., 2008). Pin1 interacts with the phosphorylated Ser181 of KSRP and induces the *cis-trans* isomerization of the proline bond in KSRP. The conformational change of KSRP exposes the phosphorylated Ser181, triggering the dephosphorylation, an event that is required for the activation of KSRP. Activated KSRP then interacts with *PTH* mRNA and induces its decay (Nechama et al., 2009).

Pin1 can also regulate the mRNA stability of cytokine via binding to AUBPs. AUF1 typically functions as a destabilizing protein for AU-rich mRNAs, including *GM-CSF* and *c-Fos* (Loflin et al., 1999). Pin1 associates with phosphorylated AUF1 and disassociates AUF1 from the mRNA of *GM-CSF* in activated eosinophils and T cells (Shen et al., 2005; Esnault et al., 2006). Binding of Pin1 to AUF1 changes the conformation of AUF1 and attenuates its RNA binding activity, leading to the stabilization of *GM-CSF* mRNA by HuR or hnRNP C (Shen et al., 2005; Esnault et al., 2006). Via a similar mechanism, Pin1 regulates the stability of *TGF-β1* mRNA and *c-Fos* mRNA (Shen et al., 2008; Krishnan et al., 2014). Regulation of mRNA stability by targeting specific RNA binding proteins, including AUF1, KSRP, SLBP, and HuR, might represent another layer of gene regulation by Pin in cancer and inflammatory response.

MicroRNAs (miRNAs) are small, endogenous non-coding RNAs of 18–24 nucleotides in length and play significant roles in the regulation of gene expression and participate in numerous cellular processes, including cell cycle arrest, cell proliferation and death (Benhamed et al., 2012). miRNAs bind to the 3′-UTR of target mRNAs via nucleotide pairing and regulates the target gene expression by decreasing the mRNA stability or translation (Fabian et al., 2010). The biogenesis of miRNAs is tightly controlled at multiple steps, including RNAPII-dependent transcription of miRNA genes, Drosha- and Dicer-mediated processing of primary miRNAs (pri-miRNAs) or precursor miRNAs (pre-miRNA), and the nuclear export of (pre-miRNAs) to the cytoplasm by exportin-5 (XPO5) (Ha and Kim, 2014). Recent studies demonstrate that the biogenesis of miRNAs, especially the XPO5-mediated export of pre-miRNA, is regulated by Pin1 (Li J. et al., 2018; Pu et al., 2018). Pin1 binds to the ERK-mediated phosphorylated XPO5 in hepatocellular carcinoma (HCC) and changes XPO5's conformation through *cis-trans* isomerization, leading to the retention of XPO5 in the nucleus and the impaired nuclear export of pre-miRNAs (Li J. et al., 2018). As a result, several tumor suppressor miRNAs, including miR-200b, miR-146a and miR-122, are down-regulated in HCC (Li J. et al., 2018). Down-regulation of these miRNAs likely changes the expression of their target genes, promoting the development of HCC (Li J. et al., 2018; Pu et al., 2018). Therefore, Pin1 is also able to regulate gene expression at the post-transcriptional level via

controlling the biogenesis of miRNAs, adding another regulatory layer for mRNA stability.

SUMMARY AND PERSPECTIVES

Pin1 is involved in almost every step of gene expression, from activation of transcription factors to transcription initiation and termination by targeting a host of transcription factors and transcription regulatory proteins (**Table 1**). In many cases, Pin1 binds to the phosphorylated transcription factors and induces the protein conformational change via isomerization, although direct evidence for the isomerization-mediated conformational change is missing in some of the studies (**Table 1**). Conformation change accounts for the changes of various protein properties, including protein stability, subcellular localization, phosphorylation status, protein-protein interactions, protein-DNA interactions, leading to the increased or decreased transcriptional potential of these transcription factors (**Figure 1**). In addition, Pin1 also targets RNA polymerase II through interacting with the CTD of Rbp1. Pin1-mediated isomerization of prolines or phosphorylation status of CTD generates a CTD code for recruitment or disengagement of transcription regulatory proteins required for transcription initiation, elongation and termination (**Figure 2**). Finally, Pin1 controls mRNA decay by interacting with AUBPs (**Figure 2**). It has to be noted that Pin1 often affects the activity of a single substrate via multiple mechanisms. For example, Pin1 regulates the nucleocytoplasmic shuttling and the stability of RelA and β-catenin (Ryo et al., 2001, 2003). Pin1 also regulates both the stability and DNA binding activity ERa (Rajbhandari et al., 2014, 2015). Via these multi-level regulations, Pin1 might impose the spatiotemporal control of the expression of a subset of genes.

Epigenetics plays crucial roles in the regulation of gene expression by post-translational modifications of histone proteins and methylation of DNA (Dawson et al., 2012). Epigenetic regulation is mediated by various enzymes that add or remove various modifications (writers and erasers) and the proteins that recognize these modifications (readers) (Dawson et al., 2012). While some PPIases regulating histone modifying enzymes have been reported (Hanes, 2015), studies on epigenetic regulation of gene expression by Pin1 are largely missing. We have recently shown that epigenetic reader Brd4, which specifically binds to the acetylated lysine on histone and non-histone proteins, is a Pin1 substrate and the stability and transcriptional activity of Brd4 is regulated by Pin1-catalyzed isomerization (Hu et al., 2017). Whether and how Pin1 regulates gene expression via targeting these epigenetic regulators remain exciting questions and need to be further investigated. Many of these epigenetic factors are dysregulated in cancer and the highly expressed Pin1 in cancer might contribute to the dysregulation.

Pin1-catalyzed isomerization and the subsequent protein conformational change might accounts for all the functional changes of Pin1 substrates. Protein conformational change often leads to the engagement or disassociation of the interacting proteins. The alteration of protein stability after conformational change is largely affected by the changes in the accessibility to E3 ligases. Conformational change can also alter the accessibility of the NLS or NES to the nuclear import or export machinery, affecting the nucleocytoplasmic shuttling of the transcription factors. However, how Pin1 directly regulates the DNA binding activity via protein conformational change is not quite clear. Although it is generally believed that binding of Pin1 leads to the conformational changes of its substrates, many studies failed to include the isomerase inactive mutant of Pin1 (**Table 1**), an issues needs to be addressed in the future studies.

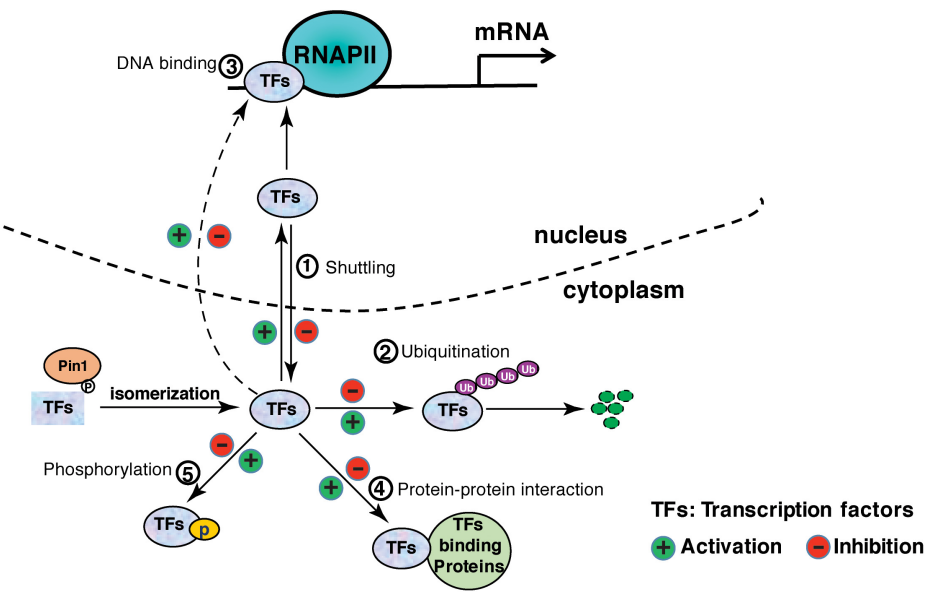

FIGURE 1 | Pin1 regulates the activation of transcription factors via distinct mechanisms: ① affects nucleocytoplasmic shuttling; ② affects protein stability by ubiquitination; ③ affects DNA-binding affinity; ④ affects protein interaction; ⑤ regulates phosphorylation or dephosphorylation.

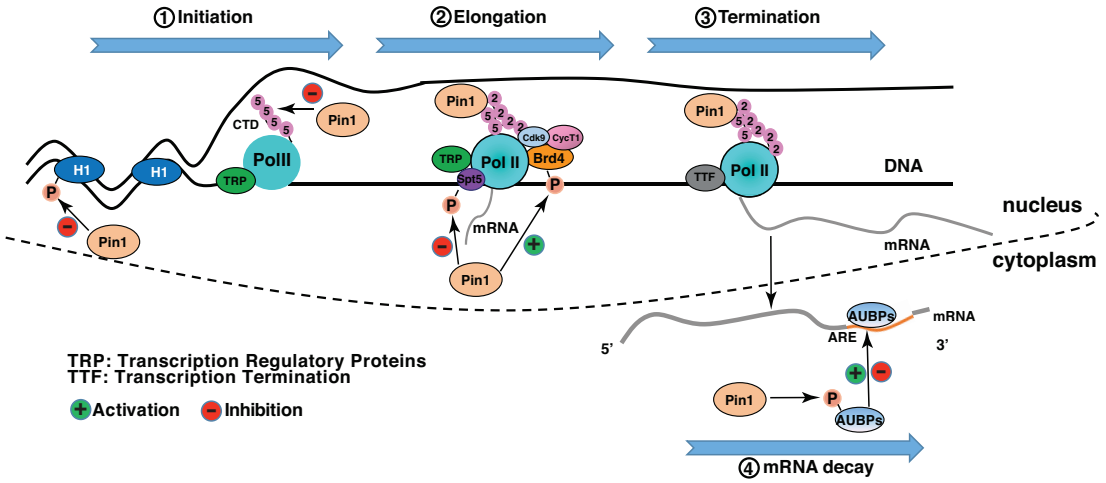

FIGURE 2 | Pin1 regulates gene expression network. During transcription initiation, Pin1 promotes dephosphorylation of Histone H1 and Ser5 in the CTD of RNA polymerase II, to inhibit the recruitment of TRP (transcription regulatory proteins) and pre-initiation complex, and promoter clearance, processes that are required to transcription initiation. During transcription elongation, on one hand, Pin1 binds to phosphorylated Spt5 and might facilitate conversion of DSIF from a negative elongation factor into a positive elongation factor. On the other hand, Pin1 enhances Brd4's stability and its interacting with CDK9 to phosphorylate Ser2 in the CTD of RNA polymerase II, and thus increases transcription elongation. In transcription termination, Pin1 binds to phosphorylated Ser2 in the CTD of RNAPII and facilitates coordinate recruitment of TTF (transcription termination factors). After the synthesis of mRNA, Pin1 binds to AUBPs (AU-binding proteins) to accelerate or slow mRNA decay.

Pin1 could have completely opposite effects on its substrates. Pin1 increases or decreases the stability of transcription factors in a similar phosphorylation and isomerization-dependent manner (**Table 1**). Pin1 regulates the activities of a spectrum of transcription factors, many of which are oncogenes and tumor suppressors (Lu and Zhou, 2007). Pin1 is aberrantly activated in most cancers and Pin1 generally activates the oncogenic transcription factors but inhibits the tumor suppressive transcription factors, reflecting Pin1's ability to promote cancer cells by activating caner promoting factors and inactivating cancer suppressive factors (Zhou and Lu, 2016). However, it is not clear how Pin1 imposes the opposite regulatory effects on oncogenic and tumor suppressive transcription factors. One possibility is that the expression of the target genes of these transcription factors and the resulting cellular functions might provide some feedback signals for Pin1 to determine the fates of these transcription factors.

While Pin1 is able to regulate gene expression at various levels, it is possible that Pin1 is not absolutely required for the transcription of whole genome. The success rate of $Pin1^{-/-}$ homozygous cross breeding was much lower that that of heterozygous mice, indicating a critical role of Pin1 in gene expression and cell division (Liou et al., 2002). Consistently, $Pin1^{-/-}$ fibroblasts grow normally but with defect in re-entering the cell cycle from G_0 arrest (Fujimori et al., 1999; Liou et al., 2002). Pin1-mediated transcription and gene expression is clearly a cell type-specific and signal-dependent event since many transcription factors and their target genes are inducible in response to specific stimuli. Studies on the transcription regulation by Pin1 were largely performed *in vitro* or in cultured cells with recombinant or overexpressed Pin1. The significance of these biochemical studies in gene regulation would be strengthened if similar regulatory mechanism would be confirmed in $Pin1^{-/-}$ or Pin1 conditional knockout mice in combination with mouse disease models. A great example is demonstrated in a recent study of the identification of Pin1 as a regulator of thermogenesis by targeting PRDM16 for degradation (Nakatsu et al., 2019).

While a great deal is known about how Pin1 regulates the activation transcription factors for gene expression in response to stimuli, much less is known about how Pin1 regulates the transcription machinery for the spatiotemporal control of gene expression except that Pin1 helps to construct the CTD code. It also remains to be determined whether these regulations on RNAPII and the associated transcription is a general mechanism that can apply to all genes or whether it is only a gene-specific and cell-specific phenomenon. Furthermore, the subcellular localization, the expression levels and the activity of Pin1 are subject to change in response to stimulation and in diseases conditions (Boussetta et al., 2010; Lee et al., 2011; Rangasamy et al., 2012; Zannini et al., 2019), adding another layer of complexity to Pin1-mediated gene expression. Overall, better understating the regulation of gene expression by Pin1 would provide new insights into the pathophysiological functions of Pin1 and new therapeutic approaches for the treatment of cancer and other human diseases by targeting Pin1 alone or in combination with targeting different transcription regulators.

AUTHOR CONTRIBUTIONS

XH drafted the manuscript. L-FC revised the manuscript. XH and L-FC reviewed and modified the manuscript. All authors agreed on the final version.

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MINI REVIEW
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Prolyl Isomerase Pin1 in Human Cancer: Function, Mechanism, and Significance

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Peptidyl-prolyl *cis-trans* isomerase NIMA-interacting 1 (Pin1) is an evolutionally conserved and unique enzyme that specifically catalyzes the *cis-trans* isomerization of phosphorylated serine/threonine-proline (pSer/Thr-Pro) motif and, subsequently, induces the conformational change of its substrates. Mounting evidence has demonstrated that Pin1 is widely overexpressed and/or overactivated in cancer, exerting a critical influence on tumor initiation and progression via regulation of the biological activity, protein degradation, or nucleus-cytoplasmic distribution of its substrates. Moreover, Pin1 participates in the cancer hallmarks through activating some oncogenes and growth enhancers, or inactivating some tumor suppressors and growth inhibitors, suggesting that Pin1 could be an attractive target for cancer therapy. In this review, we summarize the findings on the dysregulation, mechanisms, and biological functions of Pin1 in cancer cells, and also discuss the significance and potential applications of Pin1 dysregulation in human cancer.

Keywords: Pin1, PPIase isomerase, phosphorylation, *cis-trans* isomerization, cancer hallmarks

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INTRODUCTION

Cellular processes are spatially and temporally regulated by a number of molecular machineries consisting of proteins and nucleic acids (Csizmok et al., 2016; Koelwyn et al., 2017; Hentze et al., 2018). Diverse regulatory mechanisms have been well established to interpret cellular processes, such as epigenetic changes, allosteric regulations, and post-translational modifications (Aebersold and Mann, 2016; Changeux and Christopoulos, 2016; Luo et al., 2018). Among them, post-translational modifications are currently emerging as an important regulator of cell fate and thus have a strong potential to be implicated in cellular disorders (Barber et al., 2018; Steklov et al., 2018). As a dominative component of post-translational modifications, protein phosphorylation in response to extracellular or intracellular stimuli mainly controls the signal transduction within cells (Boss and Im, 2012), which often includes conformational changes in kinase-phosphorylated substrates (He et al., 2015; Martin et al., 2016). Therein, the conformational switch of peptide bonds precisely regulated by prolyl *cis-trans* isomerization plays a central role in many aspects of cellular processes (Lu et al., 2007; Marsolier et al., 2015).

Proline residues in proteins have *cis* and *trans* peptide bond conformations, which are tightly orchestrated by prolyl *cis-trans* isomerization (Lummis et al., 2005; Zosel et al., 2018). Proline conversion occurs very slowly in aqueous solution (Fischer and Aumuller, 2003). But in the presence of peptidyl prolyl *cis-trans* isomerases (PPIases), the *cis-trans* rotation of peptide bond is

stimulated, thereby adjusting the spatial arrangement of protein backbone segments (Theuerkorn et al., 2011). There are four evolutionally conserved PPIase subfamilies: cyclophilins, FK506-binding proteins (FKBPs), parvulins, and protein phosphatase 2A phosphatase activator (PTPA) (Thapar, 2015; Zhou and Lu, 2016). Peptidyl-prolyl *cis-trans* isomerase NIMA-interacting 1 (Pin1), a member of parvulins subfamily, was originally identified in 1996 (Lu et al., 1996), and is a unique enzyme that specifically catalyzes the isomerization of phosphorylated serine-proline or phosphorylated threonine-proline (pSer/Thr-Pro) motifs, representing a novel mechanism that protein conformation after Ser/Thr-Pro phosphorylation can be regulated by Pin1 to display alterable biological functions (Lu and Hunter, 2014; Zhou and Lu, 2016). Furthermore, the data from global mass spectrometry analysis have suggested a high percentage of serine/threonine phosphorylation in all phosphorylated proteins (Shi, 2009). Thus, Pin1 is of great interest to scientists committing to the research of molecular cell biology.

Emerging evidence has demonstrated that Pin1-mediated prolyl isomerization exerts a pivotal effect on multiple physiological processes including cell growth, cell cycle regulation, immune response, neuronal differentiation, and tumorigenesis (Sacktor, 2010; Tun-Kyi et al., 2011; Daza-Martin et al., 2019). In cancer—one of the leading causes of human death worldwide (Bray et al., 2018)—Pin1 is widely overexpressed and/or overactivated compared with normal cells or tissues (Pang et al., 2004; Pulikkan et al., 2010; Lu and Hunter, 2014). A high level of Pin1 overexpression/overactivation closely correlates to poor clinical prognosis of diverse cancers (Wang et al., 2015; Zhou and Lu, 2016). Through multiple regulatory mechanisms, Pin1 promotes tumor initiation, development, and drug resistance by acting as an activator of some oncogenes and growth enhancers, or as an inactivator of some tumor suppressors and growth inhibitors (Yeh and Means, 2007; Lu and Hunter, 2014; Zhou and Lu, 2016). Therefore, these achievements provide strong evidence that Pin1 is an attractive target for cancer therapy, leading to the discovery of Pin1 inhibitors for treating cancer and preventing drug resistance.

Given the critical role of Pin1 in cancer, here we review the recent findings about dysregulation, mechanisms, and biological functions of Pin1 in cancer cells, and also discuss the significance and potential applications of Pin1 dysregulation in human cancer.

PIN1 DYSREGULATION IN CANCER

The *PIN1* gene is located on chromosome 19p13.2 and encodes Pin1 isomerase, composed of 163 amino acids (Lu et al., 1996; Ranganathan et al., 1997; Modena et al., 2006). In normal tissues and cells, the level of Pin1 expression is usually closely correlated to the cell proliferation potential (Saegusa et al., 2010), and Pin1 level in tissues decreases with aging (Lee et al., 2011b). However, Pin1 is aberrantly upregulated or overactivated in many tumors or cells with a tendency to differentiate into tumors (Chen et al., 2018). Varied transcriptional, translational, and post-translational factors contribute to Pin1 dysregulation in cancer cells (**Table 1**).

Pin1 expression is regulated by a series of transcriptional factors. The E2F family are highly active in nearly all cancer types, regulating gene expression driven by cyclin-dependent kinase (CDK)-Rb-E2F axis (Dick et al., 2018; Kent and Leone, 2019). *PIN1* transcription is stimulated by the E2F family, which is located on the E2F binding sites of the *PIN1* promoter (Ryo et al., 2002). Additionally, E2Fs-mediated Pin1 transcription is also activated by other transcriptional factors. C/EBPα-p30, a mutant of transcription factor C/EBPα, which was found in around 9% of acute myeloid leukemia (AML) patients, induces Pin1 expression by recruiting E2F1 in the *PIN1* promoter and enhances leukemia (Pulikkan et al., 2010). *PIN1* promoter activity is also induced by Neu and Ras signaling via E2F activation in breast cancer (Ryo et al., 2002; Wulf et al., 2004). Unlike other transcriptional factors, Notch1 specifically binds the distal BS1 element of *PIN1* promoter and directly triggers *PIN1* transcription, where Pin1 potentiates Notch1 cleavage by γ-secretase to increase Notch1 transcriptional activity, thereby generating a positive loop to upregulate Pin1 expression in human breast cancer (Takahashi et al., 2007; Rustighi et al., 2009, 2013). Because transcriptional factors of Pin1 are generally overactivated by upstream oncogenic signaling (Pabst et al., 2001; Giuli et al., 2019; Kent and Leone, 2019), the above-mentioned evidence gives an explanation, at least in part, for the upregulation of Pin1 in cancer cells.

Along with transcriptional regulation, Pin1 expression is also controlled at post-transcriptional levels, including mRNA stability and protein translation. miRNAs are a class of small non-coding RNAs that regulate gene expression by repressing protein translation or destabilizing target mRNAs by forming a functional RNA-induced silencing complex (RISC) (Garzon et al., 2010; Inui et al., 2010). Diverse miRNAs are found to regulate Pin1 expression. For example, miR-200c is reported to directly target the 3′-UTR of Pin1 mRNA, thus decreasing Pin1 level in breast cancer (Luo et al., 2014). MiR-140-5p is also identified as a potential negative regulator of Pin1 expression by directly binding to the 3′-UTR of Pin1 mRNA, inhibiting Pin1 translation in hepatocellular carcinoma (Yan et al., 2017). Moreover, miR-200b, miR-296-5p, and miR-874-3p were found to be Pin1-targeted miRNAs (Zhang et al., 2013; Lee et al., 2014; Leong et al., 2017). Given the fact that global miRNA expression is downregulated in tumors (Lu et al., 2005; Hermeking, 2012; Zhang et al., 2015), this reduced miRNA expression could lead to Pin1 overexpression in cancer.

Post-translational regulation is another strategy affecting Pin1 dysregulation. PLK1, a trigger for G2/M transition, mediates phosphorylation of Ser65 in Pin1, stabilizing Pin1 by inhibiting its ubiquitination in human cancer cells (Eckerdt et al., 2005). MLK3, a MAP3K family member, phosphorylates Pin1 on a Ser138 site to activate its catalytic function and nuclear translocation, driving the cell cycle and promoting cyclin D1 stability and centrosome amplification of cancer cells (Rangasamy et al., 2012). By contrast, DAPK1, a known tumor suppressor, associates with and phosphorylates Pin1 on Ser71, which suppresses Pin1 nuclear localization and sustains cell cycle

TABLE 1 | Selected factors contribute to Pin1 dysregulation in cancer.

Regulators	Acting sites	Regulatory activity to Pin1 and cancer	Cancer types	References
Transcriptional regulators				
E2F	PIN1 promoter E2F site	Activation	Breast cancer	Ryo et al., 2002
C/EBPα-p30	PIN1 promoter E2F site	Activation	Leukemia	Pulikkan et al., 2010
Ras	PIN1 promoter E2F site	Activation	Breast cancer	Ryo et al., 2002; Wulf et al., 2004
c-Neu	PIN1 promoter E2F site	Activation	Breast cancer	Ryo et al., 2002; Wulf et al., 2004
Notch1	PIN1 promoter BS1 site	Activation	Breast cancer	Takahashi et al., 2007; Rustighi et al., 2009, 2013
Translational regulators				
miR-200c	3′-UTR of Pin1 mRNA	Inhibition	Breast cancer	Luo et al., 2014
miR-140-5p	3′-UTR of Pin1 mRNA	Inhibition	Hepatocellular carcinoma	Yan et al., 2017
miR-200b	3′-UTR of Pin1 mRNA	Inhibition	Breast cancer	Zhang et al., 2013
miR-296-5p	3′-UTR of Pin1 mRNA	Inhibition	Prostatic cancer	Lee et al., 2014
miR-874-3p	3′-UTR of Pin1 mRNA	Inhibition	Hepatocellular carcinoma	Leong et al., 2017
Post-translational regulators				
PLK1	Ser65 of Pin1 protein	Activation	Cervical cancer	Eckerdt et al., 2005
MLK3	Ser138 of Pin1 protein	Activation	Breast cancer Cervical cancer	Rangasamy et al., 2012
SENP1	Lys6, Lys63 of Pin1 protein	Activation	Breast cancer	Chen et al., 2013
DAPK1	Ser71 of Pin1 protein	Inhibition	Cervical cancer	Lee et al., 2011a

by activating cyclin D1 promoter in cells (Lee et al., 2011a). In addition, SENP1 binds to and deSUMOylates Pin1, leading to increased Pin1 stability and enhanced centrosome amplification and cell transformation during tumorigenesis (Chen et al., 2013). Collectively, Pin1 is aberrantly overexpressed/overactivated in multiple tumors through transcriptional, post-transcriptional, and post-translational regulations.

PIN1 PARTICIPATES IN TUMORIGENESIS VIA MULTIPLE MECHANISMS

Pin1 is mainly localized in the nucleus of both normal and cancer cells, colocalizing with a series of nucleoproteins, such as NEK6 (Chen et al., 2006), but its nuclear-cytoplasmic distribution could be changed upon phosphorylation by kinases including the above-mentioned DAPK1 and MLK3 (Lee et al., 2011a; Rangasamy et al., 2012). Recently, Chen et al. (2018) reviewed 81 Pin1 targets in human cancer. We have checked these targets based on published articles and found that Pin1 regulates 29 targets in the nucleus and 35 targets in the cytoplasm (the rest are unknown for their cellular localization), indicating that Pin1 has no apparent preference between its nuclear or cytoplasmic clients. Additionally, Pin1 participates in cancer development via transcriptional, post-transcriptional, and post-translational mechanisms, and these mechanisms operate in both the nucleus and cytoplasm (Lu and Hunter, 2014; Zhou and Lu, 2016). Thus, Pin1 has both nuclear and cytoplasmic functions, and is extensively involved in the initiation and progression of cancer.

Structurally, Pin1 contains an N-terminal WW domain and a C-terminal PPIase domain, and these two domains are connected by a flexible sequence (Yaffe et al., 1997). It is well-established that WW domain is responsible for specifically recognizing and binding the pSer/Thr-Pro segment of its substrates (Lu et al., 1999; Verdecia et al., 2000), while PPIase domain is the *bona fide* component catalyzing the conformation change of pSer/Thr-Pro's peptide bond (Yaffe et al., 1997; Lu et al., 2007). Recently, a new opinion has emerged that the WW domain is also an allosteric effector. Substrate binding to Pin1 WW domain changes the intra/inter domain mobility under a stereoselective manner, thereby altering the binding and catalysis in the distal PPIase domain (Namanja et al., 2011; Peng, 2015). The data from computational calculations also support this opinion and further predicts that Ile28 at the flexible sequence between the PPIase and WW domains is a potential key residue responsible for bridging the communication between the two domains to realize Pin1 allostery (Barman and Hamelberg, 2016; Momin et al., 2018). Considering the phosphorylated state of its substrates, Pin1 renders a functional diversity and/or pathological consequences of given substrates (Zhou and Lu, 2016; Chen et al., 2018), which is achieved mainly through three mechanisms: regulating biological activity, protein degradation, and nucleus-cytoplasm distribution of its substrates (**Table 2**).

Regulating Biological Activity of Pin1 Substrates

The biological activities of most human proteins are conformationally specific (Papaleo et al., 2016). Pin1-mediated conformational change significantly impacts their functions. The C-terminal domain (CTD) of the RNA polymerase (RNAP) II plays a critical role in pre-mRNA transcription (Bentley, 2014; Jeronimo et al., 2016). Pin1 affects CTD phosphorylation and RNAP II activity during initiation of

TABLE 2 | Regulatory mechanism of Pin1 in cancer.

Substrates	Motif	Phenotype	Cancer types	References
Regulating biological activity of Pin1 substrates				
RNAP II	Ser2-Pro	Regulates cell cycle	Cervical cancer	Kops et al., 2002; Xu and Manley, 2007
BRCA1-BARD1	Ser114-Pro (BRCA1)	Promotes replication fork protection	OsteosarcomaCervical cancer	Daza-Martin et al., 2019
B-Myb	Not available	Regulates cell cycle	Cervical cancer	Werwein et al., 2019
FAK	Ser910-Pro	Promotes cell migration, invasion, and metastasis	Breast cancerGlioblastoma	Zheng et al., 2009
PTP-PEST	Ser571-Pro	Promotes migration, invasion, and metastasis	Glioblastoma	Zheng et al., 2011
ATR	Ser428-Pro	Prevents apoptosis	Lung cancerColon cancer	Hilton et al., 2015
Rb	Ser608-Pro Ser612-Pro	Regulates cell cycle	OsteosarcomaLung cancer	Rizzolio et al., 2012 Tong et al., 2015
ERα	Ser118-Pro	Promotes proliferation	Breast cancer	Rajbhandari et al., 2012 Rajbhandari et al., 2015
Smad2/3	Thr179-Pro	Promotes migration and invasion	Prostate cancer	Matsuura et al., 2010
STAT3	Ser727-Pro	Induce EMT	Breast cancer	Lufei et al., 2007
Affecting protein degradation of Pin1 substrates				
NF-κB	Thr254-Pro	Promotes migration	GlioblastomaLeukemiaLymphomas	Ryo et al., 2003 Atkinson et al., 2009
Nanog	Ser52-Pro Ser65-Pro	Promotes cancer stem cell traits	Prostate cancer	Moretto-Zita et al., 2010 Zhang et al., 2019
BRD4	Thr205-Pro	Promotes proliferation, migration, and invasion	Gastric cancer	Hu et al., 2017
Fbw7	Thr205-Pro	Promotes proliferation and transformation	Colon cancer	Min et al., 2012; Bhaskaran et al., 2013
CDK10	Thr133-Pro	Induce tamoxifen resistance	Breast cancer	Khanal et al., 2012
ΔNp63	Thr538-Pro	Promotes proliferation	Oral squamous cell carcinoma	Li et al., 2013
c-Myc	Ser62-Pro	Promotes proliferation	Breast cancer	Farrell et al., 2013
PML	Ser403-Pro Ser505-Pro	Promotes proliferation	Breast cancer	Lim et al., 2011
RUNX3	Thr209-Pro Thr212-Pro Thr214-Pro Thr231-Pro	Promotes proliferation	Breast cancer	Nicole Tsang et al., 2013
HIF-1α	Ser641-Pro Ser643-Pro	Promotes angiogenesis	Colon cancer	Han et al., 2016
Altering nucleus-cytoplasmic distribution of Pin1 substrates				
PKM2	Ser37-Pro	Promotes Warburg effect and tumor growth	Glioblastoma	Yang et al., 2012; Yang and Lu, 2013
TRIM59	Ser308-Pro	Promotes tumor growth	Glioblastoma	Sang et al., 2019
p53-RS	Ser249-Pro	Regulates cell cycle	Hepatocellular carcinoma	Liao et al., 2017
XPO5	Ser497-Pro	Promotes proliferation, migration, and invasion	Hepatocellular carcinoma	Sun et al., 2016; Li et al., 2018
Cyclin D1	Thr286-Pro	Promotes cell cycle and proliferation	Nasopharyngeal carcinoma	Liou et al., 2002; Xu et al., 2016

the transcription cycle, and not during elongation, suggesting the functional role of Pin1 in RNA transcription (Kops et al., 2002; Xu et al., 2003; Xu and Manley, 2007). Pin1 also enhances BRCA1-BARD1 interaction with RAD51, thereby increasing the presence of RAD51 at stalled replication structures and governing replication fork protection during cancer development (Daza-Martin et al., 2019). Moreover, B-Myb phosphorylated by CDK is isomerized by Pin1, enabling PLK1 docking and subsequent PLK1-mediated B-Myb phosphorylation to stimulate transcription of late cell cycle genes (Werwein et al., 2019). In Ras-activated tumor cells, the function of FAK and PTP-PEST are also regulated by Pin1. Pin1 isomerizes both Ser910-phosphorylated FAK and Ser571-phosphorylated PTP-PEST to enhance the interaction between PTP-PEST and FAK, leading to the dephosphorylation of FAK Tyr397 by PTP-PEST and the promotion of migration, invasion, and metastasis of Ras-related tumor cells (Zheng et al., 2009, 2011).

In addition to activating substrate activity, Pin1 is also able to deactivate substrates. ATR, a PI3K-like protein kinase, has an antiapoptotic activity at mitochondria in response to UV-induced DNA damage. In cancer cells, this mitochondrial activity

is reduced by Pin1 that catalyzes ATR from *cis*-isomer to *trans*-isomer at the phosphorylated Ser428-Pro motif (Hilton et al., 2015). Moreover, the function of the tumor suppressor Rb is largely regulated by a dynamic balance of phosphorylation and dephosphorylation. Pin1 directly interacts with the spacer domain of Rb protein, and allows the interaction between CDK/cyclin complexes and Rb in mid/late G1, leading to the inactivation of Rb (Rizzolio et al., 2012; Tong et al., 2015). Subsequently, the Pin1-induced Rb inactivation leads to the dissociation of E2F from Rb and increased E2F transcriptional activity, triggering the expression of cell cycle regulatory proteins and promoting cell cycle progression through the G1 checkpoint in cancer cells (Cheng and Tse, 2018).

Affecting Protein Degradation of Pin1 Substrates

Pin1 has the ability to prevent protein degradation of oncogenes and growth-promoting regulators. For example, Pin1 associates with the pThr254-Pro motif of transcription factor NF-κB p65 subunit, leading to the increased protein stability of p65 and enhanced transcriptional activity of NF-κB in various cancers, including leukemia, lymphomas, and glioblastoma (Ryo et al., 2003; Atkinson et al., 2009). In prostate cancer, tumor suppressor SPOP interacts with Nanog and promotes Nanog poly-ubiquitination and subsequent degradation, but Pin1 functions as an upstream Nanog regulator and impairs its recognition by SPOP, stabilizing Nanog to promote the cancer stem cell traits and tumor progression (Zhang et al., 2019). Moreover, Pin1 directly binds to and isomerizes phosphorylated Thr204-Pro205 motif of BRD4 to enhance its stability by inhibiting its polyubiquitination, promoting BRD4's interaction with CDK9 and its transcriptional activity. Substitution of BRD4 with Pin1-binding-defective BRD4-T204A mutant reduces BRD4 stability, which attenuates BRD4-mediated gene expression and suppresses cell proliferation, migration, invasion, and tumor formation, suggesting the positive correlation of Pin1 function and BRD4 stability in gastric cancer cells (Hu et al., 2017).

Pin1 could also promote the protein degradation of tumor suppressors and growth-inhibitory regulators. Fbw7 is the substrate recognition component of the E3 ligase complex and is critical for ubiquitylation and degradation of given proteins (Ji et al., 2015). Pin1 interacts with Fbw7 and induces Fbw7 self-ubiquitination and protein degradation by disrupting Fbw7 dimerization, contributing to oncogenesis. By contrast, depletion of Pin1 in cancer cells leads to elevated Fbw7 expression, which subsequently reduces Mcl-1 abundance, sensitizing cancer cells to taxol treatment (Min et al., 2012; Bhaskaran et al., 2013). An inverse correlation between the expression of CDK10 and the degree of tamoxifen resistance suggests CDK10 could be an important determinant of tamoxifen resistance in breast cancer. Pin1 facilitates CDK10 degradation as a result of its interaction with, and subsequent ubiquitination of, CDK10, thereby suggesting that the Pin1-mediated CDK10 ubiquitination is a major regulator of tamoxifen-resistant breast cancer cell growth and survival (Khanal et al., 2012).

Altering Nucleus-Cytoplasmic Distribution of Pin1 Substrates

Changing the nucleus-cytoplasm distribution is another mechanism of Pin1 function. A typical example is PKM2. Upon the activation of EGFR signaling, Ser37-phosphorylated PKM2 recruits Pin1 for *cis-trans* isomerization and promotes PKM2 binding to importin α5 and translocating to the nucleus, where nuclear PKM2 acts as a coactivator of β-catenin to promotes the Warburg effect and tumorigenesis (Yang et al., 2012; Yang and Lu, 2013). This process is similar to the recently published mechanism of TRIM59 (Sang et al., 2019). In addition, the mechanism underlying the gain-of-function of p53-R249S (p53-RS), a p53 mutant frequently detected in hepatocellular carcinoma, is also mediated by Pin1. In detail, Pin1 isomerizes p53-RS phosphorylated by CDK4 in the G1/S phase and enhances nuclear localization of p53-RS, resulting in a p53-RS-c-Myc interaction and an elevated c-Myc-dependent rDNA transcription key for ribosomal biogenesis, which promotes cell cycle progression and cell growth of hepatocellular carcinoma (Liao et al., 2017).

Recently, we have demonstrated that Pin1 plays an important role in miRNA biogenesis. XPO5-mediated nucleus-to-cytoplasm export of precursor miRNAs (pre-miRNAs) is a post-transcriptional step in the process of miRNA biogenesis (Lin and Gregory, 2015; Peng and Croce, 2016; Wu et al., 2018). Pin1 blocks nucleus-to-cytoplasm export of XPO5 phosphorylated by ERK kinase, decreasing mature miRNA biogenesis in hepatocellular carcinoma (Sun et al., 2016; Li et al., 2018; Pu et al., 2018). Moreover, this impaired miRNA biogenesis in hepatocellular carcinoma could be restored by novel Pin1 inhibitors and their formulations (Pu et al., 2018; Fan et al., 2019; Sun et al., 2019; Zheng et al., 2019), giving new insight into the therapy of liver cancer.

SIGNIFICANCE OF PIN1 DYSREGULATION IN TUMOR

Following the epoch-making conclusion by Hanahan and Weinberg (2011), the major cancer hallmarks are summarized, such as sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, and inducing angiogenesis. Emerging evidence demonstrates that Pin1 promotes cancer by acting as an activator of numerous oncogenes and growth enhancers or as an inactivator of numerous tumor suppressors and growth inhibitors to affect cancer hallmarks (Zhou and Lu, 2016). In this section, we review the roles of Pin1 in these cancer hallmarks (**Figure 1**).

Pin1 Sustains the Proliferative Signaling

Cancer cells possess an excessive cell proliferation ability that sustains proliferative signaling (Samatar and Poulikakos, 2014; Bykov et al., 2018). Pin1 is initially identified as a regulator of mitosis and gives rise to sustaining proliferative signaling in multiple cancers.

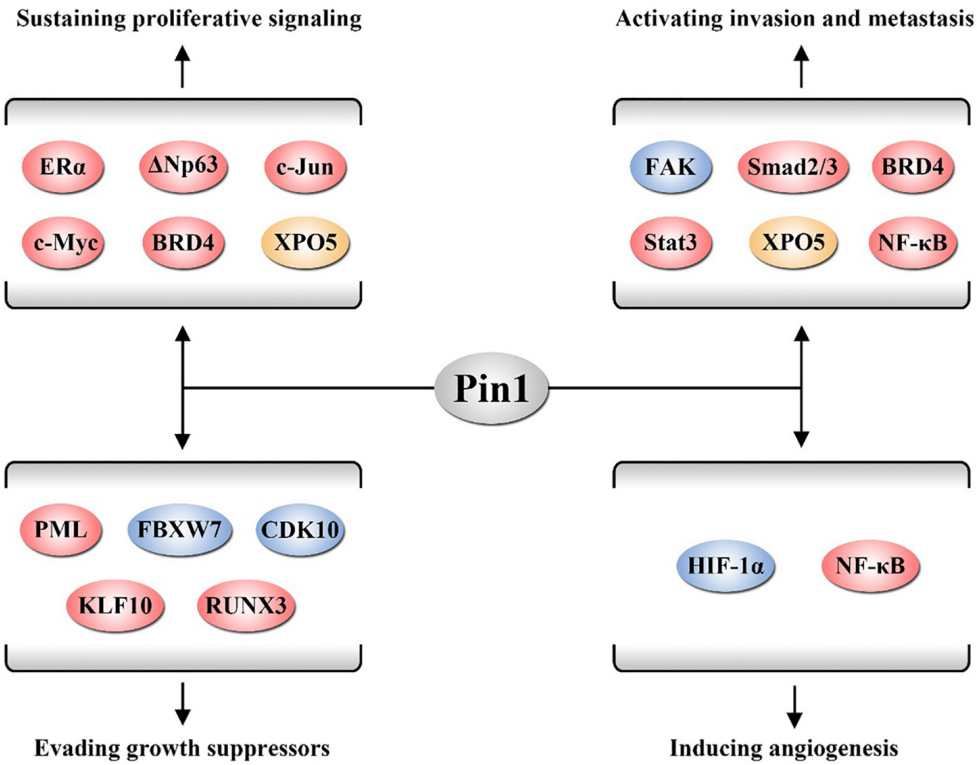

FIGURE 1 | Pin1 is involved in several cancer hallmarks. Pin1 activates a number of oncogenic proteins to sustain proliferative signaling, evade growth suppressors, activate invasion and metastasis, and induce angiogenesis. The proteins displayed in red, yellow, and blue participate in these hallmarks through regulating biological activity, affecting protein degradation, and altering nucleus-cytoplasm distribution of its substrates, respectively.

Cyclin D1, a pivotal cell cycle regulator, promotes cell cycle progression in human cancer (Sherr, 1996). Pin1 interacts with and isomerizes cyclin D1 in a phosphorylation-dependent manner, enhancing the nuclear accumulation of cyclin D1 and triggering cells into cell cycle, and promotes cell proliferation (Liou et al., 2002). Dysregulation of ERα expression also contributes to the proliferation of cancer, especially breast cancer (Brisken, 2013). Pin1 promotes ERα function through several mechanisms. Pin1 isomerizes the Ser118-Pro bond of ERα AF1 region to increase AF1 transcriptional activity, promoting the growth of tamoxifen-resistant breast cancer cells (Rajbhandari et al., 2012). Furthermore, Pin1 can directly regulate the adjacent DNA binding domain of ERα in an allosteric manner, enhancing the DNA binding function of ERα to drive breast cancer proliferation (Rajbhandari et al., 2015).

ΔNp63s, the N-terminal truncated isoforms of p63 lacking the transactivation domain, are associated with human tumorigenesis (Chen et al., 2017). Pin1 interacts with Thr538-Pro of ΔNp63α and disrupts p63α-WWP1 interaction to inhibit the proteasomal degradation mediated by E3 ligase WWP1, promoting ΔNp63α-induced cell proliferation of human oral squamous cell carcinoma (Li et al., 2013). Moreover, Pin1 enhances the stability of BRD4 by inhibiting its ubiquitination and increasing transcriptional activity of BRD4 to promote the proliferation of gastric cancer (Hu et al., 2017). In addition, Pin1 also activates many pro-proliferative proteins to enhance tumor cell proliferation and tumor growth, including c-Myc and XPO5 (Farrell et al., 2013; Li et al., 2018).

Pin1 Evades Growth Suppressors

There are a number of tumor suppressors that negatively regulate cancer progression within cells, but cancer cells are able to bypass these barriers via various mechanisms. Several works suggest that Pin1 is an expert in injuring tumor suppressors.

The promyelocytic leukemia (PML) is a tumor suppressor involved in apoptosis and DNA damage repair. Pin1 binds and targets PML for degradation in an ERK-dependent manner by targeting Ser403 and Ser505 of PML, inducing the development of breast cancer cells (Lim et al., 2011). Moreover, KLHL20 coordinates with Pin1 and CDK1/2 to mediate hypoxia-induced PML proteasomal degradation, thereby potentiating multiple tumor hypoxia responses in human prostate cancer (Yuan et al., 2011). Furthermore, Pin1 also stabilizes the oncogenic fusion protein PML-RARα, resulting in a decreased anti-proliferative activity of ATRA in AML (Gianni et al., 2009).

Runt-related transcription factor 3 (RUNX3) is an ERα inhibitor in breast cancer (Huang et al., 2012). Pin1 recognizes four phosphorylated Ser/Thr-Pro motifs in RUNX3 via its WW domain to suppress the transcriptional activity of RUNX3 and induce the ubiquitination and proteasomal degradation of RUNX3 in breast cancer (Nicole Tsang et al., 2013). KLF10 is a member of the Krüppel-like transcription factor family and acts

as a tumor suppressor, mimicking the anti-proliferative effect of TGF-β in various cancer cells. Pin1 interacts with KLF10 and promotes its protein degradation, blocking the anti-proliferative function of KLF10 in cancer cells (Hwang et al., 2013). Pin1 also interacts with Fbw7 and CDK10 in a phosphorylation-dependent manner and promotes their ubiquitination and degradation, which suppresses their function to trigger cell proliferation and transformation of cancer cells (Khanal et al., 2012; Min et al., 2012).

Pin1 Activates Invasion and Metastasis

Invasion and metastasis are the leading causes of death in cancer patients and remain the greatest challenges in the clinical management of cancer (Lambert et al., 2017). Mounting works have demonstrated the invasion- and metastasis-promoting function of Pin1 in human cancer.

The transforming growth factor β (TGF-β) signaling pathway is a key player in tumor development, modulating processes including cell motility, where Smad proteins are major downstream effectors of TGF-β signaling (Lamouille et al., 2014). Phosphorylated Thr179-Pro motif of Smad2/3 interacts with Pin1 in a TGF-β-dependent manner, inducing migration and invasion via N-cadherin in prostate cancer cells (Matsuura et al., 2010). In turn, Pin1–Smad3 interaction is reduced by the inhibition of CDK-mediated Smad3 phosphorylation, leading to the suppression of triple negative breast cancer cells (Thomas et al., 2017).

Ras and STAT3 signaling has a significant impact on tumor metastasis. Pin1 binding and prolyl isomerizing of FAK cause PTP-PEST to interact with and dephosphorylate FAK Tyr397, promoting Ras-induced cell migration, invasion, and metastasis of numerous cancers (Zheng et al., 2009, 2011). Pin1 associates with STAT3 upon cytokine/growth factor stimulation to promote STAT3 transcriptional activity and target gene expression as well as recruit transcription coactivator p300, inducing epithelial–mesenchymal transition of MCF-7 cells (Lufei et al., 2007). Additionally, Pin1 enhances the invasion and metastasis of multiple cancers by activating NF-κB, BRD4, and XPO5 (Hu et al., 2017; Li et al., 2018; Nakada et al., 2019).

Pin1 Induces Angiogenesis

Solid tumors rely on angiogenesis to supply sufficient nutrients and oxygen as well as to eliminate metabolic waste and carbon dioxide for rapidly expanded cancer cells (Chung et al., 2010). The angiogenesis is strictly controlled *in vivo*. Increasing evidence has illustrated that Pin1 is involved in cancer-associated angiogenesis.

Hypoxia-inducible factor 1α (HIF-1α) is responsible for promoting the expression of many genes involved in angiogenesis (Rosmorduc and Housset, 2010). Pin1 directly interacts with HIF-1α at both exogenous and endogenous levels to stabilize the HIF-1α protein in human colon cancer cells and upregulating expression of VEGF, a major contributor to angiogenesis (Han et al., 2016). Moreover, Pin1 cooperates with KLHL20 to induce the ubiquitin-dependent degradation of PML, an inhibitor of HIF-1α-induced angiogenesis, resulting in the activation of angiogenesis in many cancers (Yuan et al., 2011). Additionally, NF-κB is also triggered by Pin1 to promote angiogenesis in hepatocellular carcinoma (Shinoda et al., 2015). By contrast, inhibition of Pin1, through RNAi or small molecular inhibitors, significantly reduces the cancer-induced angiogenesis (Ryo et al., 2005; Kim et al., 2012), further supporting the crucial role of Pin1 in angiogenesis.

CONCLUSION

Pin1 is identified as a unique enzyme mediating the *cis-trans* isomerization of pSer/Thr-Pro motif of proteins specifically, extensively participating in the initiation and progression of many human cancers. In this article, we reviewed the existing works on the dysregulation, biological function, molecular mechanism, and significance of Pin1 in cancer cells. These works commonly report that Pin1 is an excellent target for the diagnosis and therapy of diverse cancers. Over the past two decades, diverse small-molecule Pin1 inhibitors were developed and some of them, such as ATRA, KPT-6566, arsenic trioxide, and API-1, exhibited attractive *in vitro* and *in vivo* activity toward human cancer, including acute PML, breast cancer, and hepatocellular carcinoma (Wei et al., 2015; Campaner et al., 2017; Kozono et al., 2018; Pu et al., 2018). However, to date, no Pin1 inhibitors are submitted to clinical trial for cancer treatment. Moreover, Pin1 is also not applied in clinical cancer diagnosis, even though Pin1 seems to be a potential cancer-specific biomarker. Therefore, more effort should be made to fill these gaps.

Despite these efforts, a number of highly relevant questions remain unanswered. First, Pin1 enrichment is precisely orchestrated by multiple regulatory mechanisms. However, the theory about the epigenetic regulation and protein decay of Pin1 is rarely studied. So, the origin of Pin1 dysregulation is not fully understood. Second, mounting data have indicated that non-coding RNAs, especially regulatory non-coding RNAs including miRNA, long non-coding RNA (lncRNA), and circular RNA (circRNA), construct a complex molecular network along with numerous functional proteins to regulate cellular processes as well as canceration (Anastasiadou et al., 2018). But the relationship of Pin1 and non-coding RNAs is still unclear. Third, post-translational modifications, such as phosphorylation, acylation, sumoylation, and glycosylation, could positively or negatively change protein activity without altering the sequences of proteins (Han et al., 2018). However, little is known on how the post-translational modifications modulate Pin1 function. We expect that the answers to these questions will be found in the coming years, pushing Pin1 toward a truly clinical application.

AUTHOR CONTRIBUTIONS

WP, YZ, and YP wrote the manuscript, performed revisions, and read and approved the submitted version.

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PERSPECTIVE
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PIN1 Provides Dynamic Control of MYC in Response to Extrinsic Signals

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PIN1 is a phosphorylation-directed member of the peptidyl-prolyl *cis/trans* isomerase (PPIase) family that facilitates conformational changes in phosphorylated targets such as c-MYC (MYC). Following signaling events that mediate phosphorylation of MYC at Serine 62, PIN1 establishes structurally distinct pools of MYC through its *trans-cis* and *cis-trans* isomerization activity at Proline 63. Through these isomerization steps, PIN1 functionally regulates MYC's stability, the molecular timing of its DNA binding and transcriptional activity, and its subnuclear localization. Recently, our group showed that Serine 62 phosphorylated MYC can associate with the inner basket of the nuclear pore (NP) in a PIN1-dependent manner. The poised euchromatin at the NP basket enables rapid cellular response to environmental signals and cell stress, and PIN1-mediated trafficking of MYC calibrates this response. In this perspective, we describe the molecular aspects of PIN1 target recognition and PIN1's function in the context of its temporal and spatial regulation of MYC.

Keywords: Pin1, c-Myc, nuclear pore complex, phosphorylation, isomerization

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INTRODUCTION

Proline isomerization of cellular proteins provides post-translational control of target protein structure, and therefore function, within the cell. Proline residues within peptides can exist in two distinct energetically stable states, *cis* or *trans*. While proline residues exhibit an intrinsic ability to isomerize, this process occurs on a very slow biomolecular timescale as a result of the high-energy barrier associated with this conformational change. This high-energy barrier isolates the *cis* and *trans* protein states, and rapidly switching between these two conformational states requires a catalyst. The evolutionarily conserved peptidyl-prolyl *cis*/*trans* isomerases (PPIases) catalyze this conformational change and are required to drive isomerization in a timeframe relevant to dynamic signaling cascades within the cell (Lu et al., 2007; Chen Y. et al., 2018). By functioning as molecular switches to toggle targets between their *cis* and *trans* conformations, these enzymes can affect target protein stability, localization, activity, and protein–protein interactions (Göthel and Marahiel, 1999; Lu et al., 2007; Takahashi et al., 2008).

The PPIase, NIMA-interacting 1 (PIN1) is the only known PPIase that specifically recognizes phosphorylated serine or threonine residues that immediately precede a proline (pSer/pThr-Pro). This pSer/pThr-Pro motif accounts for over 25% of all phosphorylation sites identified in a global phosphorylation study (Ubersax and Ferrell, 2007). The proline-directed kinases that target these

sites are central to extracellular stimuli responses (Pearson et al., 2001) and cell cycle progression (Morgan, 1997; Cheng and Tse, 2018). The selectivity of PIN1 for phosphorylated proteins provides it with the potential to modify and functionally regulate a variety of targets involved in these phospho-signaling cascades. Indeed, PIN1 has been shown to target important cell cycle phospho-proteins such as Cyclin D1 (Liou et al., 2002) as well as proteins in the NF-κB, WNT, and AKT pathways, where extrinsic signals result in phosphorylation-regulated cascades that ultimately alter gene transcription to affect cell phenotype (Ryo et al., 2001, 2003; Liao et al., 2009). Despite PIN1's involvement in critical signaling pathways, PIN1 null mice are viable. The major phenotype of mice lacking PIN1 is a defect in cellular proliferation that contributes to stunted body size and infertility (Fujimori et al., 1999; Liou et al., 2002). Consistent with this, mouse embryonic fibroblasts (MEFs) from PIN1 knockout mice, that exhibit similar proliferation relative to wildtype (WT) MEFs during asynchronous growth in culture, display significantly delayed proliferation relative to WT MEFs when stimulated with mitogens after being starved to G_0 arrest (Fujimori et al., 1999; Su et al., 2018). This result supports an important role for PIN1 in dynamic signaling pathways to elicit an efficient response to extracellular stimuli.

Loss of PIN1 also renders cells resistant to transformation and, strikingly, PIN1 knockout mice have delayed tumor formation when crossed with tumor-driving mutants of HER2 or RAS (Ryo et al., 2002; Wulf et al., 2004). Phospho-signaling is increased in cancer, often in a cell-intrinsic manner by oncogenic mutations in signaling pathways (e.g., RAS or HER2), but also through cell-extrinsic signals from the tumor microenvironment (e.g., TGFβ or FGF). These conditions lead to an abundance of proline-directed kinases driving oncogenic signaling cascades that control tumorigenic phenotypes (Gross et al., 2015). PIN1 regulates a large number of these cancer-related targets from extracellular receptors such as NOTCH1 (Rustighi et al., 2009) or HER2 (Lam et al., 2008), to intracellular effector proteins like RAF1 (Dougherty et al., 2005) or FAK (Zheng et al., 2009), and ultimately to transcription factors such as c-MYC (Farrell et al., 2013), β-catenin (Ryo et al., 2001), or NF-κB (Ryo et al., 2003). The overexpression of PIN1 is common in many types of cancer and is correlated with poor outcomes (Zhou and Lu, 2016; Cheng and Tse, 2018). For example, in pancreas cancer, elevated levels of PIN1 were shown to cooperate with MYC and NRF2 to maintain redox balance, allowing for tumor cell proliferation and survival (Liang et al., 2019). In a mouse model of B-cell lymphoma, loss of PIN1 suppresses MYC-driven proliferation and lymphomagenesis (D'artista et al., 2016). In breast cancer, the overexpression of PIN1 can regulate Notch signaling and increase cancer stem cell-like phenotypes, including tumorigenicity and drug resistance (Luo et al., 2014; Rustighi et al., 2014). PIN1 also enhances the tumorigenic characteristics of mutant p53 in breast cancer by co-activating aggressive oncogenic transcriptional programs. When PIN1 expression is decreased, the malignant activity of mutant p53 is remarkably reduced (Girardini et al., 2011). A more comprehensive list of oncogenes and tumor suppressors that PIN1 can target is reviewed elsewhere (Zhou and Lu, 2016).

Here, we discuss the role of PIN1 as a critical controller of dynamic phosphorylation signaling cascades in response to extrinsic signals that governs gene transcription to alter phenotypic responses in normal and diseased states. PIN1 affects a variety of target transcription factors in such cascades, but we focus on work describing PIN1's temporal and spatial control of the bHLH-LZ transcription factor c-MYC (hereafter MYC), which PIN1 functionally regulates in both physiologic and pathologic responses. We will describe how PIN1-dependent isomerization temporally and spatially influences the phosphorylation cascade that affects MYC stability and activity in the nucleus. Together, these roles frame PIN1 as a promising therapeutic target for controlling oncogenic MYC.

PIN1 REGULATES MYC STABILITY AND ACTIVITY

The proto-oncogene *MYC* encodes a critical transcription factor that influences transcription across the genome to control a multitude of cellular processes including proliferation, survival, metabolism, and morphology (Fernandez et al., 2003; Chen H. et al., 2018). In physiologic conditions, MYC protein levels are mitogen responsive and are influenced by two sequential and interdependent, proline-directed phosphorylation events on Ser62 (pS62) and Thr58 (pT58) in the conserved MYC Box 1 (MB1) region of MYC's transactivation domain. Phosphorylation at each site influences PIN1's interaction with the MB1 region of MYC and isomerization at Pro63 (Farrell et al., 2013; Helander et al., 2015). Briefly, MYC is stabilized and activated downstream of growth stimuli through RAS-induced kinases and/or cyclin-dependent kinases (CDKs), which phosphorylate MYC at Ser62 when Pro63 is in *trans* (Sears et al., 2004; Vervoorts et al., 2006). Phosphorylation of Ser62 primes MYC for subsequent phosphorylation at Thr58 by the processive GSK3 kinase (Gregory et al., 2003). Phosphorylation at Thr58 then facilitates the proline-directed, *trans*-specific phosphatase, PP2A-B56α, to remove the activating S62 phosphate (Arnold and Sears, 2006; Arnold et al., 2009). pT58-MYC is then targeted for ubiquitination by the E3 ubiquitin ligase Fbw7, resulting in MYC's degradation (Gregory and Hann, 2000; Welcker et al., 2004).

As depicted in **Figure 1**, PIN1 plays a critical role regulating MYC stability and activity, as the kinases and phosphatase that target Ser62 and Thr58 are *trans*-specific enzymes. Thus, PIN1 can interrupt the progression of pS62-MYC through its degradation cascade by stabilizing Pro63 in the *cis*-conformation. This sterically protects the Ser62 phosphate from PP2A-mediated dephosphorylation, allowing for prolonged pS62-MYC interaction with DNA and increasing target gene transcription (Farrell et al., 2013). However, PIN1 can also direct MYC toward degradation following GSK3 phosphorylation of Thr58, associated with subsequent Ser62 dephosphorylation by the *trans*-specific phosphatase, PP2A-B56α (Yeh et al., 2004). Like Ser62, Thr58 is followed by a proline; however, Proline 59 falls within a poly-proline domain, likely structured as a rigid *trans* isomer helix (Andresen et al., 2012). Thus, while

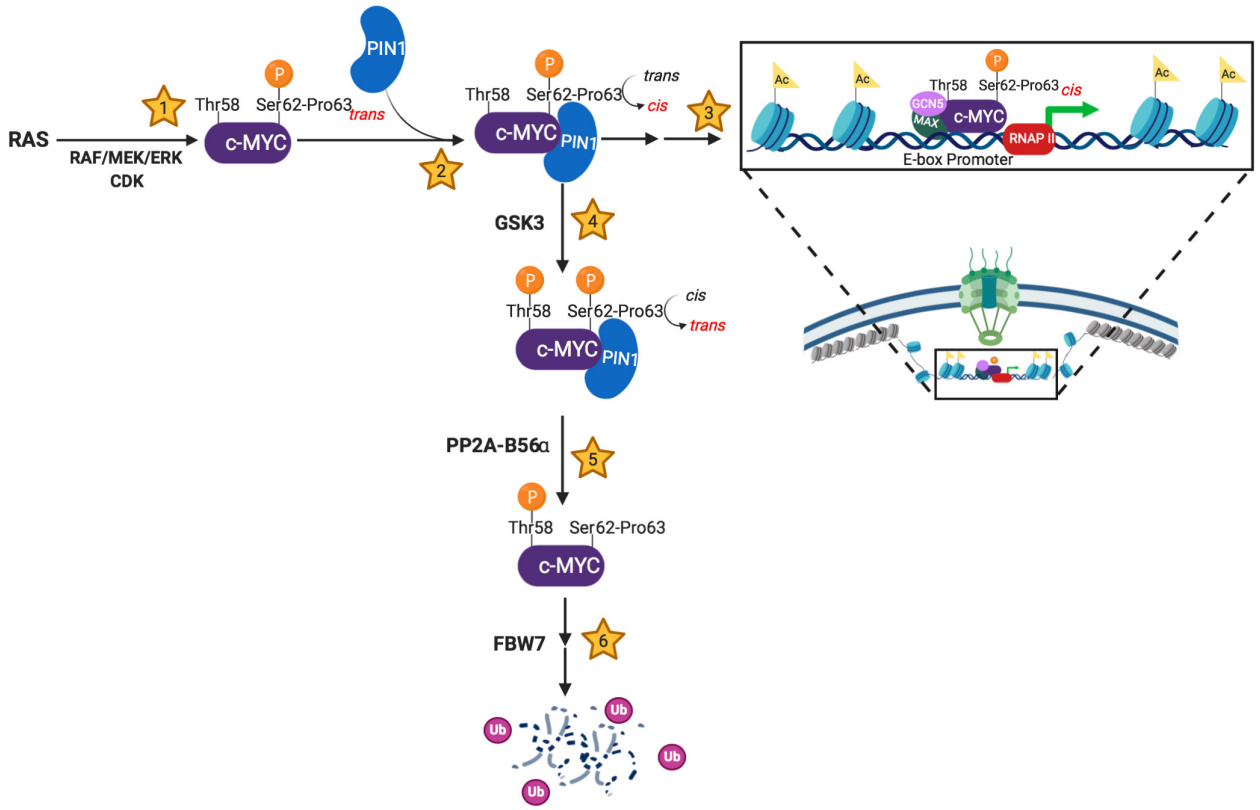

FIGURE 1 | Schema showing PIN1's involvement in the molecular events regulating MYC's stability and activity. **(1)** c-MYC becomes transcriptionally active following Ser62 phosphorylation by *trans*-specific RAS-induced kinases and/or cyclin-dependent kinases (CDKs). **(2)** PIN1 stabilizes pSer62-Pro63-MYC in the *cis* conformation, sterically preventing phosphatase activity. **(3)** The transcriptionally active, pSer62-*cis*-Pro63-MYC has increased DNA binding to E-box promoters and increased co-activator association (MAX, GCN5, etc.), which results in increased chromatin accessibility. Additionally, PIN1-directed isomerization of pSer62-MYC has been shown to locate MYC to the basket of the nuclear pore. **(4)** c-MYC is directed towards degradation via Thr58 phosphorylation by the GSK3 kinase. This phosphorylation event promotes phosphatase activity at pSer62, which requires a *cis* to *trans* isomerization of pSer62-Pro63-MYC. **(5)** The *trans*-specific phosphatase, PP2A-B56α, removes the activating phosphate on Ser62-MYC. **(6)** pThr58-MYC signals the E3-ubiquitin ligase, FBW7, to poly-ubiquitinate c-MYC, leading to proteasome degradation. Created with Biorender.com.

Thr58 phosphorylation introduces an additional binding site for PIN1, PIN1-mediated isomerization of MB1 is likely to center on the sterically more flexible Proline 63. From this, we speculate that the re-engagement of PIN1 with pT58 drives a *cis-trans* isomerization of Pro63, allowing for the function of PP2A at pSer62. However, additional research is required to understand precisely how Thr58 phosphorylation promotes the dephosphorylation of pSer62, and how this additional phosphorylation affects PIN1's activity on MYC.

Structural studies into PIN1's substrate interactions indicate that a flexible interdomain, which connects PIN1's WW phospho-substrate binding domain to its PPIase catalytic domain, can exist in different rigidity states that influence PIN1 target binding and isomerase activity (Namanja et al., 2011). Furthermore, a study involving molecular dynamic simulations of PIN1 binding suggests that the two subdomains are allosterically regulated in a two-step mechanism. Upon initial substrate binding, PIN1 is primed in an enzymatically quiescent state until the substrate becomes phosphorylated and engages PIN1's WW domain, triggering PIN1-dependent isomerization (Guo et al., 2015). In support of both primed and activated states for PIN1, a study specifically investigating PIN1's physical interactions with MYC demonstrated that PIN1 binds to unphosphorylated MYC at a conserved motif, designated MYC Box 0 (MB0), N-terminal to MB1 (Helander et al., 2015). This pre-anchoring of PIN1 to the MB0 region resembles the first quiescent state of PIN1's substrate engagement, which precedes Ser62 phosphorylation. Phosphorylation of Ser62 triggers PIN1's WW domain binding and subsequent isomerization of Pro63. However, phosphorylation of Ser62 also increases the dissociation rate of PIN1 from MB1, suggesting release following enzymatic conversion of Pro63 to *cis*. This dynamic interaction may provide a rational role for the additional phosphorylation at Thr58 to re-engage PIN1 with MB1 to mediate a second isomerization event from *cis* to *trans* at the more flexible Pro63. The dual function of PIN1 in promoting both MYC's activity and degradation through two isomerization events is supported by experiments assessing the effects of point mutations in the MB0 domain that disrupt PIN1 pre-anchoring or of PIN1 knockdown. Both conditions result a reduction in

MYC DNA binding and a corresponding decrease in target gene activation, cellular proliferation, and cellular transformation, even though there is an increase in pS62-MYC and MYC stability (Farrell et al., 2013; Helander et al., 2015).

In addition to directly controlling the conformation of MYC to affect its activity vs. ubiquitination, other proteins regulate and are regulated by PIN1 that contribute to the MYC degradation pathway. For example, PIN1 can downregulate the E3 ubiquitin ligase FBW7 (Min et al., 2012), which could disrupt MYC degradation. SENP1 is an enzyme that deSUMOylates MYC, which reduces MYC's FBW7-directed ubiquitination and degradation; SENP1 also deSUMOylates PIN1 (Chen et al., 2013), which increases PIN1's activity (Sun et al., 2018). PIN1 is also subject to phosphorylation that can decrease its catalytic activity (Lee et al., 2011). These additional players and levels of post-translational control likely contribute to the differential regulation of PIN1 on MYC in physiologic and pathologic conditions; however, the molecular details require additional research.

PIN1 REGULATES TEMPORAL AND SPATIAL DYNAMICS OF MYC

Understanding the dynamics of MYC regulation is critical in order to elucidate the pleiotropic effects of MYC in the genome and its control of diverse cellular phenotypes. PIN1 plays a key role in this regulation by imparting both temporal and spatial regulation of MYC activity in the nucleus. Temporal studies of MYC DNA binding revealed that MYC oscillates on and off DNA at E-box containing promoters in response to cell growth signaling (Farrell et al., 2013). This dynamic binding of MYC to DNA is dependent on Ser62 and Thr58 phosphorylation and PIN1-mediated Pro63 isomerization. Timed MYC DNA binding assays indicate that phosphorylation of Ser62 accelerates MYC E-box promoter binding in a PIN1-dependent manner while Thr58 phosphorylation accelerates the release of MYC from DNA. This mechanism creates an oscillatory binding of MYC to target gene promoters with a periodicity of approximately 20 min, and loss of PIN1 suppresses this cyclic DNA binding. The temporal control of MYC by PIN1 also regulates its association with its co-activators, which similarly oscillate on and off DNA, in a PIN1-dependent manner, with the same kinetics as MYC (e.g. p300, GCN5, CDK9, and SNF5). MYC's dynamic binding to coactivators and DNA affects subsequent gene expression by triggering RNA polymerase release and elongation (Jaenicke et al., 2016). Inhibition or reduction in PIN1 levels results in decreased MYC oscillation on DNA and decreased MYC-dependent gene expression, even with an observed increased in MYC protein levels (Farrell et al., 2013).

In addition to temporally regulating MYC activity, PIN1 regulates the subnuclear localization of MYC under normal mitogen stimulation conditions, during wound healing, and in cancer cell lines (Su et al., 2018). Initial observations of MYC at the nuclear periphery were recently extended to show that transcriptionally active pS62-MYC associated with Lamin A/C (Eisenman et al., 1985; Vriz et al., 1992; Myant et al., 2015). This observation is surprising since the majority of chromatin in lamin-associated domains (LADs) at the nuclear periphery is transcriptionally silent heterochromatin. At the nuclear pore (NP), however, there are regions of open chromatin that are poised for transcription (Blobel, 1985; Krull et al., 2010; Beck and Hurt, 2017). Using proximity ligation assay (PLA) with confocal microscopy and super-resolution stochastic optical reconstruction microscopy (STORM), we showed that pS62-MYC associated with the interior basket proteins of the NP complex (NPC) (Su et al., 2018). Although the mechanism of pS62-MYC trafficking to the NP remains unclear, PIN1-mediated isomerization is necessary for stabilizing pS62-MYC at the NPC. In addition, the recruitment of MYC-associated coactivators and epigenetic modifiers, such as GCN5, to the NPC is also PIN1-dependent. This PIN1-dependent spatial reorganization of MYC appears to impact epigenetic regulation in response to extrinsic signals. Upon serum stimulation in starved MEFs, the PIN1-dependent trafficking of pS62-MYC and its associated epigenetic modifiers to the NP results in increased histone acetylation and transcription of NPC-resident genes. Whether this also involves oscillatory DNA binding by MYC at these NPC-resident genes will require future research. Global chromatin accessibility assays indicate that early response chromatin site opening is PIN1-dependent and overlaps with MYC gene program activation, suggesting that these early events involve NPC-associated euchromatin. In the absence of PIN1, the cellular response to mitogen stimulation is delayed, which results in reduced cellular proliferation as well as decreased MYC-associated chromatin remodeling, supporting a critical role for PIN1-MYC regulation of NPC associated euchromatin for efficient response to cellular stimulation.

The PIN1-driven spatial reorganization of MYC to specific chromatin domains at the NP suggests that post-translational control of transcription factors in response to environmental signals may dictate their involvement in regulating specific topologically associated domains or TADs. Interestingly, the number and composition of NPs is increased and altered in cancer cells (Simon and Rout, 2014; Rodriguez-Bravo et al., 2018). In addition, the NP region is speculated to be a site of epigenetic memory for genes associated with rapid response to environmental signals (D'urso and Brickner, 2014). PIN1 drives a relocation of MYC to chromatin regions at the NP, and if these regions comprise a subset of rapid response genes, this could provide a mechanism for MYC's differential activity on subsets of cell-context specific genes (Sabò et al., 2014; Su et al., 2018).

These findings suggest that in response to extrinsic signals, PIN1 facilitates the generation of a distinct pool of post-translationally modified MYC that associates with chromatin near the inner basket of the NP. This pool may be distinct from the population of MYC within the nuclear interior that binds promoter regions in open chromatin. There is much discussion in the field for whether oncogenic MYC acts as a global transcriptional amplifier or if there is a more specific MYC-driven gene program that drives malignancies (Loven et al., 2012; Nie et al., 2012; Sabò et al., 2014; Caforio et al., 2018; Muhar et al., 2018). Our data suggest that the PIN1-dependent subnuclear reorganization of MYC into distinct pools might allow

a population of MYC to drive a specific subset of genes, while the PIN1-independent population may accomplish its global transcriptional amplification function. Future investigation into the dynamic distribution of MYC's transcriptional activity is necessary for bolstering this hypothesis.

CONCLUSION

Here we present a perspective of the role of PIN1 in regulating dynamic response phenotypes, focusing on its isomerization of MYC in multiple cellular contexts. PIN1's interaction with and isomerization of MYC supports the physiologic and oncogenic activity of MYC (Yeh et al., 2004; Farrell et al., 2013; Sanchez-Arévalo Lobo et al., 2013; Helander et al., 2015; Su et al., 2018). Mechanistically, this involves regulation of MYC stability, its DNA binding and transcriptional activity, and its subnuclear localization to the NP. In normal cells, PIN1's regulation of MYC contributes to increased proliferation, migration, and wound healing (Su et al., 2018). In cancer, PIN1's regulation of MYC has been shown to affect oncogenic transformation, proliferation, redox maintenance, and cell survival (Farrell et al., 2013; Helander et al., 2015; D'artista et al., 2016; Su et al., 2018; Liang et al., 2019). PIN1 fine-tunes the rapid spatial and temporal control of MYC by integrating isomerization of Pro63 with the sequential phosphorylation events at Ser62 and Thr58 (**Figure 1**). Whether the dynamic nature of PIN1-dependent regulation of MYC extends to PIN1-dependent regulation of other transcription factors will be of great interest.

Multiple efforts to therapeutically reduce or control MYC's oncogenic activity have been unsuccessful for several reasons, including an inability to specifically control MYC expression and the lack of an enzymatic region to target with small molecules (Chen H. et al., 2018). The direct targeting of PIN1 to modulate MYC activity provides a promising therapeutic opportunity with numerous drugs under investigation (Chen Y. et al., 2018). For example, the inhibition of PIN1 with PiB reduced the rate of MYC binding to target DNA promoters in MCF10A cells, leading to decreased expression of oncogenic gene signatures and decreased tumor growth (Farrell et al., 2013). In addition, Juglone (Kim et al., 2009) and ATRA (Wei et al., 2015) have been shown to potently reduce PIN1's oncogenic activity in breast cancer models; however, the efficacy of these drugs on reducing MYC's oncogenic activity remains to be studied. Furthermore, a recent covalent PIN1 inhibitor, KPT-6566, has shown potency for reducing PIN1-dependent cancer phenotypes (Campaner et al., 2017). Since PIN1 null mice are viable, taking advantage of the upstream functional control of phosphorylated MYC via PIN1 enzymatic blockade could reduce systemic toxicity associated with total loss of MYC, while specifically targeting signaling-activated oncogenic MYC. This specificity provides a compelling rationale for PIN1-dependent therapeutic strategies to treat MYC-dependent cancers.

AUTHOR CONTRIBUTIONS

GC contributed to writing all sections of the manuscript. DL and EL wrote sections of the manuscript. EL organized and oversaw the conceptual approaches. RS oversaw conceptual approaches, edited all sections of the manuscript, and approved the manuscript. All authors contributed to manuscript revision, read, and approved the submitted versions.

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REVIEW
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Phosphorylation-Dependent Pin1 Isomerization of ATR: Its Role in Regulating ATR's Anti-apoptotic Function at Mitochondria, and the Implications in Cancer

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Peptidyl-prolyl isomerization is an important post-translational modification of protein because proline is the only amino acid that can stably exist as *cis* and *trans*, while other amino acids are in the *trans* conformation in protein backbones. This makes prolyl isomerization a unique mechanism for cells to control many cellular processes. Isomerization is a rate-limiting process that requires a peptidyl-prolyl *cis/trans* isomerase (PPIase) to overcome the energy barrier between *cis* and *trans* isomeric forms. Pin1, a key PPIase in the cell, recognizes a phosphorylated Ser/Thr-Pro motif to catalyze peptidyl-prolyl isomerization in proteins. The significance of the phosphorylation-dependent Pin1 activity was recently highlighted for isomerization of ATR (*ataxia telangiectasia*- and Rad3-related). ATR, a PIKK protein kinase, plays a crucial role in DNA damage responses (DDR) by phosphorylating hundreds of proteins. ATR can form *cis* or *trans* isomers in the cytoplasm depending on Pin1 which isomerizes *cis*-ATR to *trans*-ATR. *Trans*-ATR functions primarily in the nucleus. The *cis*-ATR, containing an exposed BH3 domain, is anti-apoptotic at mitochondria by binding to tBid, preventing activation of pro-apoptotic Bax. Given the roles of apoptosis in many human diseases, particularly cancer, we propose that cytoplasmic *cis*-ATR enables cells to evade apoptosis, thus addicting cancer cells to *cis*-ATR formation for survival. But in normal DDR, a predominance of *trans*-ATR in the nucleus coordinates with a minimal level of cytoplasmic *cis*-ATR to promote DNA repair while preventing cell death; however, cells can die when DNA repair fails. Therefore, a delicate balance/equilibrium of the levels of *cis*- and *trans*-ATR is required to ensure the cellular homeostasis. In this review, we make a case that this anti-apoptotic role of *cis*-ATR supports oncogenesis, while Pin1 that drives the formation of *trans*-ATR suppresses tumor growth. We offer a potential, novel target that can be specifically targeted in cancer cells, without killing normal cells, to significantly reduce the adverse

effects usually seen in cancer treatment. We also raise important issues regarding the roles of phosphorylation-dependent Pin1 isomerization of ATR in diseases and propose areas of future studies that would shed more understanding on this important cellular mechanism.

Keywords: cytoplasmic ATR, Pin1, antiapoptotic ATR, apoptosis, prolyl isomerization, cancer, cis and trans

PEPTIDYL-PROLYL ISOMERIZATION OF PROTEINS AND Pin1

Individual proteins may perform multiple functions and have evolved to evade unnecessary degradation. These differing functions and survival skills involve posttranslational modifications of proteins. Apart from protein function, post-translational modifications (PTMs) of proteins also can affect their sub-cellular location, stability and inter-molecular interactions with other proteins (Gothel and Marahiel, 1999; Lu and Zhou, 2007; Lu et al., 2007). Of the various types of PTMs such as phosphorylation, ubiquitination, acetylation, and so on, peptidyl isomerization of a protein is a unique type of PTM (Tanford, 1968). Peptidyl isomerization is the reversible transformation of a molecule between *cis* and *trans* isomeric forms, such that the peptide or protein can exist in two distinct geometric conformations, *cis* and *trans* (**Figure 1**). This modification causes no change in the molecular weight of the peptide or protein; hence, the inability to detect this change by mass spectrometry; however, isomerization, especially of a proline residue, alters the affected protein's structure. The biological significance of prolyl isomerization, as compared to the other 19 non-proline amino acids, is that all non-proline amino acids are naturally stable in *trans* isomeric form whereas proline can be in either the *cis* or the *trans* isoform at the amide bond of proline with the preceding amino acid (Fischer and Schmid, 1990; Hinderaker and Raines, 2003; Song et al., 2006; Craveur et al., 2013; **Figure 1**). Thus, peptidyl isomerization of protein refers mostly to peptidylprolyl isomerization.

Most amino acid residues within a folded protein are thermodynamically more stable in the *trans* form (Stewart et al., 1990; Schmidpeter and Schmid, 2015). However, proline has the unique ability to exist as a *cis* or a *trans* residue in a protein's structural backbone as the side chain of proline forms part of the backbone of protein (Fischer and Schmid, 1990; Hinderaker and Raines, 2003; Song et al., 2006; Craveur et al., 2013). This potential to switch between isomeric forms (**Figure 1**) *via* isomerization allows proline to act as a molecular switch that affects the protein's structure and, hence, its physiological functions. The isomerization naturally occurs slowly and is rate limiting in the protein folding process. Hence, enzymes, such as peptidyl-prolyl *cis*/*trans* isomerases (PPIases) are required to overcome existing high-energy barriers between these protein isomers and to stabilize the transition between *cis*/*trans* isoforms. Protein isomerization is involved in many cellular processes such as apoptosis (Follis et al., 2015; Hilton et al., 2015), mitosis (Lu et al., 1996; Yaffe et al., 1997; Rippmann et al., 2000; Zhou et al., 2000; Yang et al., 2014), cell signaling (Brazin et al., 2002; Sarkar et al., 2007; Toko et al., 2013), ion channel gating (Antonelli et al., 2016), amyloidogenesis (Eakin et al., 2006), DNA damage repair (Steger et al., 2013), and neurodegeneration (Pastorino et al., 2006; Grison et al., 2011; Nakamura et al., 2012; Sorrentino et al., 2014).

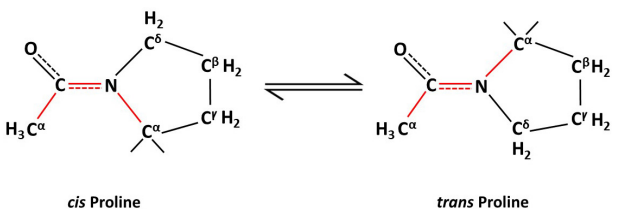

FIGURE 1 | Non-enzymatic proline isomerization within proteins is a slow, rate-limiting process in the folding pathway.

Pin1 is a member in the parvulin family of peptidyl prolyl isomerases (PPIases); it can catalyze proline isomerization only at a phosphorylated Ser/Thr-Pro (pSer/pThr-Pro) motif (Lu et al., 1996, 2007; Lu and Zhou, 2007). Structurally, Pin1 consists of an N-terminal WW protein interaction domain which binds its substrate at the pSer/pThr-Pro motif, a central flexible linker and a C-terminal PPIase domain to catalyze proline isomerization (Lu et al., 1996). Pin1's activity, stability, subcellular location and substrate binding can be regulated by its own PTMs, including Serine 71 phosphorylation by DAPK1 (inactivates Pin1; Lee et al., 2011; Hilton et al., 2015), ubiquitination (Eckerdt et al., 2005) oxidation (Chen et al., 2015), and sumoylation (Chen et al., 2013). Pin1 is involved in regulating multiple cellular processes including cell cycle transit and division (Rippmann et al., 2000), differentiation and senescence (Hsu et al., 2001; Toko et al., 2014) and apoptosis (Pinton et al., 2007; Follis et al., 2015; Hilton et al., 2015). To perform these cellular functions, Pin1 binds to many substrates within the cell (**Figure 2**). These substrates include proteins involved in cell cycle regulation (p53, cyclin E), transcriptional regulation (E2F, Notch1), DNA damage responses (DDR), and so forth (Lin et al., 2015; Chen et al., 2018). Pin1 expression and activity have been implicated in many diseases from neurodegenerative disorders such as Alzheimer disease and amyotrophic lateral sclerosis (Pastorino et al., 2006; Kesavapany et al., 2007; Nakamura et al., 2012, 2013), autoimmune diseases like systemic lupus erythematosus (Wei et al., 2016), to cancer (Ayala et al., 2003; Ryo et al., 2003; He et al., 2007; Yeh and Means, 2007; Finn and Lu, 2008; Nakamura et al., 2013; Lu and Hunter, 2014; Lin et al., 2015; Zhou and Lu, 2016; Chen et al., 2018; El Boustani et al., 2018; Nakatsu et al., 2019), etc. ATR (*ataxia*

telangiectasia- and Rad3-related) protein, a master regulator and phosphatidylinositol 3-kinase (PI3K-like) protein kinase in DDR (Zou and Elledge, 2003; Cimprich and Cortez, 2008; Flynn and Zou, 2011), was recently reported to be a substrate of Pin1 for prolyl isomerization (Hilton et al., 2015). Given that ATR phosphorylates hundreds of proteins in response to DNA damage (Matsuoka et al., 2007), isomerization of ATR by Pin1 represents a new paradigm in understanding Pin1's biological activities, which is the focus of this article (**Figures 2**, **3**).

POSTTRANSLATIONAL MODIFICATIONS OF ATR FOR ITS RESPECTIVE NUCLEAR AND CYTOPLASMIC FUNCTIONS

ATR is a key DDR protein kinase that the cell employs to sense replicative stress and DNA damage. Following replication arrest and formation of single-stranded DNA (ssDNA), RPA coats the ssDNA and recruits ATR-ATRIP complex via ATRIP (ATR interacting protein). ATRIP is the nuclear partner of ATR and carries bound ATR along to the DNA damage site, where ATR is autophosphorylated at its T1989 residue (Cortez et al., 2001). This phosphorylated residue serves as a docking site for TopBP1 to significantly enhance the activation of ATR's kinase activity (Burrows and Elledge, 2008; Mordes et al., 2008; Liu et al., 2011). ATR in turn activates several key downstream proteins, including p53 and other checkpoint kinases such as Chk1, leading to an S-phase cell cycle arrest for proper repair of the DNA damage or apoptosis in case of excessive damage (Cortez et al., 2001; Zou and Elledge, 2003; Sancar et al., 2004; Mordes and Cortez, 2008; Ciccia and Elledge, 2010; Nam and Cortez, 2011; Saldivar et al., 2017; Ma et al., 2019).

Recently, ATR was found to function in the cytoplasm and was described to play an important anti-apoptotic role directly at the mitochondria, independent of nuclear ATR and its kinase activity (Hilton et al., 2015). In contrast to nuclear ATR which always remains in *trans* form in complexing with ATRIP, cytoplasmic ATR in the absence of ATRIP exists in two forms, *cis* and *trans*, the existence of which depends on changing just one peptide bond orientation in ATR by prolyl isomerization. The balance between *cis* and *trans* cytoplasmic forms is regulated by Pin1, which catalyzes the conversion of *cis*-ATR to *trans*-ATR by recognizing the phosphorylated Serine 428-Proline 429 residues (pS428-P429) in the N-terminal region of ATR (**Figure 3**; Hilton et al., 2015). The activity of Pin1 favors the formation of *trans*-ATR, but inactivation of Pin1 by DAPK1 kinase upon DNA damage promotes *cis*-ATR accumulation at the mitochondria as *cis*-ATR appears to be naturally stable in cells. It is proposed that unlike its *trans* isoform, *cis*-ATR has an exposed BH3-like domain that allows it to bind to the pro-apoptotic tBid protein at the mitochondria. This binding prevents tBid from activating Bax-Bak polymerization which is necessary for the intrinsic apoptotic pathway. Hence, *cis*-ATR performs an anti-apoptotic role that allows the cells to survive long enough to repair its damaged DNA (**Figure 3**). However, this can be a double-edged sword that can play a role in carcinogenesis as discussed below. The newly discovered BH3 domain, a hallmark of apoptotic proteins, in ATR defines *cis*-ATR's role in the apoptosis pathway (**Figure 3**).

PHOSPHORYLATION-DEPENDENT ISOMERIZATION OF ATR BY Pin1

Pin1 has a high degree of phosphate specificity (Zhou et al., 1999; Lu, 2000; Liou et al., 2011). Due to the numerous amounts of phosphorylated substrates that Pin recognizes in the cell, Pin1 can be a potential target in treatment of many diseases (Ryo et al., 2003; Kesavapany et al., 2007; Finn and Lu, 2008; Liou et al., 2011; Lu and Hunter, 2014; Lin et al., 2015; Wei et al., 2015, 2016; Campaner et al., 2017; Chen et al., 2018). Since Pin1's activity on ATR requires the phosphorylation at Ser428 of ATR, this could serve as an important regulatory tool to influence the levels of the ATR isomer. Thus, phosphorylation at Ser428 may play a critical role in regulating ATR prolyl isomerization and, thus, ATR's anti-apoptotic activity at mitochondria.

Hilton et al. (2015) showed that when the serine 428 residue in human ATR is mutated to alanine (S428A), Pin1 is unable to recognize its motif to isomerize *cis*-ATR to *trans*-ATR; hence, cytoplasmic S428A ATR exists primarily as the anti-apoptotic *cis* isomer. In addition, when the proline 429 residue was mutated to alanine, the P429A ATR in the cytoplasm was in the *trans* form. This indicates that the type of ATR present in the cytoplasm can be regulated by targeting this phosphorylation-dependent Pin1-mediated isomerization of ATR (**Figure 4**). An accumulation of *cis*-ATR at mitochondria confers a survival signal that allows the cell to escape apoptosis even following DNA damage. The evasion of cell death may allow mutations that have occurred in these cells to be passed to daughter cells. Survival of an increasing number of cells with accumulating mutations over time can increase genomic instability and cause carcinogenesis. The alternative scenario where *trans*-ATR is dominant in the cytoplasm leads to an increase in free t-Bid since *trans* ATR is unable to bind and sequester t-Bid, allowing the programmed cell death that occurs when the cell is unable to repair DNA damage. In support of this mechanism proposed by Hilton et al. (2015), Lee et al. (2015) observed that a low expression of cytoplasmic pATR (S428; which implies higher levels of cytoplasmic *cis*-ATR) is associated with an advanced stage epithelial ovarian carcinoma (EOC) with poor disease prognosis and treatment outcomes. In contrast, no such correlations were found with nuclear pATR (S428) levels, implicating that cytoplasmic *cis*-ATR levels are uniquely important in the disease progression of EOC.

The level at Ser428 phosphorylation in ATR can be determined by two important classes of proteins: protein kinases and phosphatases. The former phosphorylates Ser428 while the latter dephosphorylates this residue. The balance between the two opposing activities is critical to controlling the *cis*/*trans* balance of ATR isomers and, thus, the health of the cells. Identification of the phosphatases which have activities at Ser428 is particularly important to cancer treatment as dephosphorylation of this residue leads to an increase of anti-apoptotic *cis*-ATR formation (Hilton et al., 2015) and poor prognosis for cancer treatment (Lee et al., 2015). Thus, the responsible phosphatase(s) would be

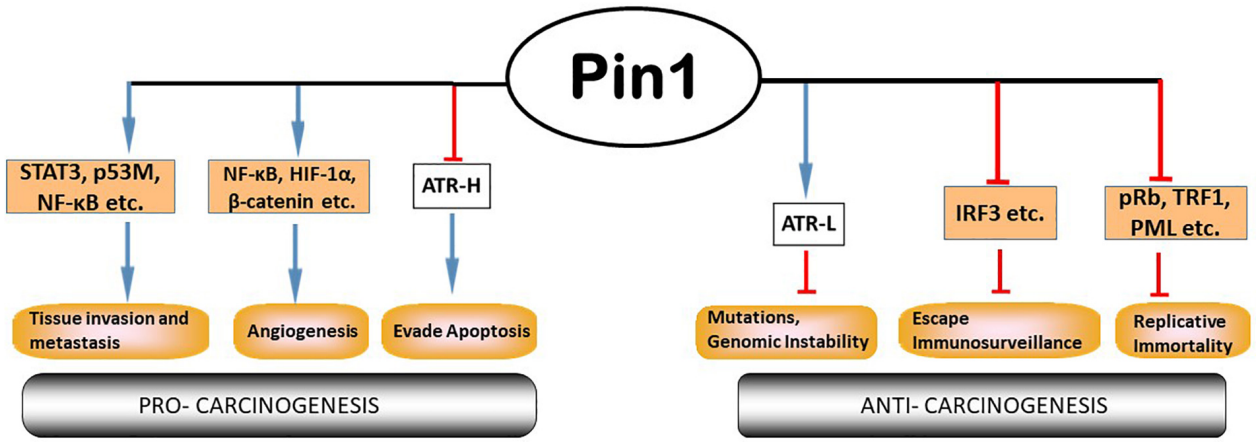

FIGURE 2 | Pin1 participates extensively in multiple cellular processes involved in cancer. Pin1 has many cellular substrates that participate in the multi-step tumor development processes. Pin1's roles can be contradictory: pro- or anti-tumor. Pin1 inhibits formation of *cis*-ATR and deprives the cell of *cis*-ATR's anti-apoptotic role at the mitochondria, while promoting the formation of *trans*-ATR in the nucleus where it is important for repair of genotoxic stress to prevent mutations and maintain genome stability. Modified from Chen et al. (2018).

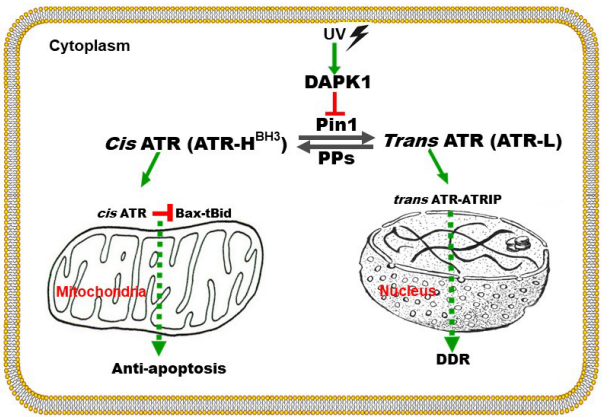

FIGURE 3 | Graphical representation of the proposed mechanism by which ATR plays a direct anti-apoptotic function at the mitochondria. UV damage inactivates Pin1's isomerization of ATR in the cytoplasm. *Cis*-ATR (ATR-H) then accumulates and binds to and sequesters t-Bid at the outer mitochondria membrane. Without tBid, Bax and Bak fail to polymerize, thus *cis*-ATR inhibits cytochrome c release and apoptosis. *Trans*-ATR (ATR-L) is the dominant isomer in the nucleus where it interacts with ATRIP, RPA and chromatin in the DNA damage repair (DDR) response. PPs (protein phosphatases) can dephosphorylate the Pin1 recognition motif and promote formation of *cis*-ATR (to be published elsewhere). Modified from Hilton et al. (2015).

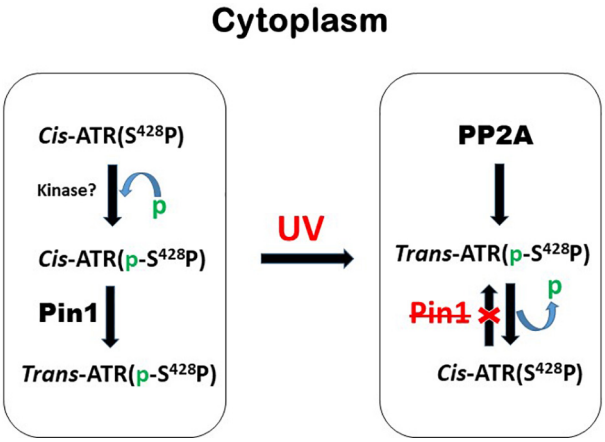

FIGURE 4 | A brief summary of the mechanism by which the levels of cytoplasmic *cis*- and *trans*-ATR isoforms are mediated by phosphorylation and dephosphorylation before and after UV irradiation. The red X stands for inhibition or inactivation of Pin1.

a reasonable target for inhibition to improve cancer treatment. Indeed, we recently identified PP2A (Protein Phosphatase 2A) as the protein phosphatase that dephosphorylates Ser428 in the Pin1 recognition motif of cytoplasmic ATR. When PP2A dephosphorylates this Ser428 residue, Pin1 can no longer recognize its motif to isomerize cytoplasmic ATR from the *cis* to the *trans* isoform (**Figure 4**). This key regulation was found to increase the level of *cis*-ATR in the cytoplasm and its accumulation at the mitochondria to bind tBid for its anti-apoptotic role (**Figure 3**). In addition, cells in which PP2A was inhibited were found to be significantly more sensitive to DNA damage agents. In contrast, a kinase that phosphorylates cytoplasmic ATR at Ser428 in the Pin1 recognition motif will cause an opposite effect; in the cytoplasm, there would be a relative abundance of phosphorylated substrate for Pin1 to perform its phosphorylation-dependent isomerization of *cis*-ATR to the *trans* form. Since the *trans* form has no direct anti-apoptotic benefit following DNA damage, the cells with a predominance of cytoplasmic *trans*-ATR will succumb more quickly to apoptosis. It is worth noting that UV irradiation reduces the Ser428 phosphorylation level of ATR in the cytoplasm (Hilton et al., 2015) while at the same time increasing the phosphorylation level at the same S428 residue of ATR in the

nucleus of cells. The former consistently leads to accumulation of *cis*-ATR at mitochondria. The latter's effect remains unknown as the nuclear phosphorylation of ATR-Ser428 has no effect on ATR checkpoint activation of Chk1 after UV damage (Liu et al., 2011). In addition, while the mechanism of ATR isomerization is defined with the cells treated with UV, Hilton et al. (2015) also show that other types of DNA damage agents such as hydroxyurea and camptothecin can induce formation of *cis*-ATR in the cytoplasm though less efficiently. This suggests that the mechanism defined by Hilton et al. (2015) may represent a universal pathway of ATR isomerization in response to DNA damage. By simply regulating a PTM event in the cytoplasmic ATR protein, i.e., addition or removal of a phosphate group in the Pin1 motif of ATR, one would be able to control how cells respond to a DNA damaging event: survival or death as summarized in **Figures 3**, **4**.

Cis-ATR'S ANTI-APOPTOTIC FUNCTION MAY SUPPORT AN ONCOGENIC PROCESS IN DIVIDING CELLS

Cancer is characterized with deregulated cell growth, where there is an imbalance in the inherent cell cycle regulation to check the rate and integrity of cell division and growth. *In addition, given that cis-ATR is antiapoptotic, we hypothesize that cis-ATR may perform an oncogenic role, while Pin1 might be tumor suppressive in terms of ATR's anti-apoptotic activity at the mitochondria.* If *cis*-ATR is the dominant cytoplasmic form, it may block mitochondrial apoptosis and allow damaged cells to survive and mutate, even when DNA damage repair is insufficient and the abnormal cells are supposed to die via apoptosis. This evasion of apoptosis is an important hallmark of cancer cells that, over time, allows them to accumulate the mutations that define genome instability and, eventually, leads to carcinogenesis. However, if Pin1's action is increased and *trans*-ATR is the dominant form of ATR in the cytoplasm, before mutations can be propagated, programmed death will occur in those cells that are too severely damaged for proper DNA repair. Thus, reduction of cytosolic *cis*-ATR discourages accumulation of cells with DNA damage that could be passed on to daughter cells and would promote carcinogenesis.

This hypothesis is interesting in and of itself, but is inconsistent with the existing literature which suggests other roles of Pin1 in cancer development (**Figure 2**). The current understanding stems primarily from observations that Pin1 is overexpressed/has increased activity in most cancers and cancer stem cells, with corresponding negative prognostic outcomes (Ayala et al., 2003; He et al., 2007; Tan et al., 2010; Girardini et al., 2011; Luo et al., 2014; Rustighi et al., 2014; Xu et al., 2016; Nakatsu et al., 2019). Also, Pin1 upregulates many oncogenes, while downregulating several tumor suppressor genes (Chen et al., 2018). Pin1 overexpression or its over activation can be inhibited by genetic approaches or chemically with juglone (Hennig et al., 1998), all-trans retinoic acid (ATRA; Toledo et al., 2011) or KPT-6566 (Campaner et al., 2017) and, when tested, Pin1 inhibitors were able to suppress cancers (Estey et al., 1997; Budd et al., 1998; Shen et al., 2004; Lu and Hunter, 2014; Wei et al., 2015; Zhou and Lu, 2016; Lian et al., 2018). However, there are many challenges to chemically inhibiting Pin1, especially with retinoids (e.g., ATRA), the most commonly used clinical inhibitor. These include low drug bioavailability, clinical relapse and retinoid resistance, etc. (Muindi et al., 1992; Decensi et al., 2009; Arrieta et al., 2010; Moore and Potter, 2013; Jain et al., 2014). In contrast, bioinformatic analyses of human tumors (Kaplan–Meier Plots) reported in the Human Protein Atlas (7,932 cases) found that low Pin1 RNA expression is largely associated with a lower survival profile for most types (12 types) of cancer patients while high expression correlates with a higher survival profile for three types of cancer (**Table 1**). For two other types of cancer the relationship of survival profile with Pin1 expression is non-determined. Interestingly, two types of male-only cancer, prostate and testis, are among the three types of minorities; these patients had a higher survival profile with low versus high Pin1 RNA expression. These results also are consistent with the 5-year survival probabilities (**Table 1**). However, of all the 17 cancer types analyzed, only in two types, renal and pancreatic, are Pin1 expression prognostic: high Pin1 expression is favorable for better prognosis as determined by Human Protein Atlas (**Table 1**). This appears to contradict a recent report on the prognostic value of Pin1 in cancer which analyzed the data from 20 published papers (2,474 patients) which concluded that Pin1 overexpression was significantly associated with advanced clinical stage of cancer, lymph node metastasis and poor prognosis, although no correlation with poor differentiation was found (Khoei et al., 2019). Interestingly, it is known that over 50% of cancers have mutations in p53, and Pin1 expression was found to promote mutant p53-induced oncogenesis (Girardini et al., 2011). Also, importantly, Pin1 isomerizes wild-type p53 in DDR and the wild type p53 functions are regulated by Pin1 (Wulf et al., 2002; Zacchi et al., 2002; Zheng et al., 2002). Thus, p53 status may affect the relationship between Pin1 expression and cancer as Pin1 appears to have different effects on cancer cells with mutant and wild-type p53 (Mantovani et al., 2015). It remains unknown if or how the p53 status would affect cancer prognosis in correlation with Pin1 expression levels, which is of great interest to determine. We propose that a wider role for Pin1 and its regulator partners in carcinogenesis needs to be considered and investigated further to provide better context (Han et al., 2017).

While it is logical to target Pin1 or the many processes that Pin1 regulates directly or indirectly via its substrates involved in carcinogenesis (see **Figure 2**), we propose that it would be significantly more effective to target the control of apoptosis, a common pathway always deregulated in carcinogenesis with uncontrolled proliferation. This is because apoptosis is the ultimate terminator and always has the final say in determining the fate, death or survival, of cells. This would tie in with the emerging idea of oncogene addiction, where the so-called "Achilles heel" of a cancer is used to deal a deathblow to that cancer (Weinstein, 2002; Weinstein and Joe, 2006, 2008). Oncogene addiction is one of the themes that has evolved in the study of tumor progression. There are innumerable

TABLE 1 | Pin1 RNA expression in caner patients analyzed by Kaplan-Meier Plot (Human Protein Atlas).

Cancer type	Male/female (n/n)	Max post-diagnosis years	Pin1 expression						
			Survival probability		5-year survival (%)		Expression Level cut-off	*P* score	Prognosis status (Prognosability)
			Lower	Higher	Low expression	High expression			
Renal	591/286	16	Low	High	64%	82%	9.65	0.000078	Yes
Pancreatic	96/80	7	Low	High	7%	48%	8.72	0.00032	Yes
Glioma	99/54	7	Low	High	5% (*)	12% (*)	15.74	0.022	No
Thyroid	135/366	15	Low	High	91%	100%	9.19	0.031	No
Lung	596/398	20	Low	High	40%	47%	6.16	0.029	No
Stomach	229/125	10	Low	High	26%	50%	8.03	0.022	No
Breast	12/1063	23	Low	High	81%	82%	7.16	0.25	No
Cervical	0/291	17	Low	High	59%	74%	10.81	0.0061	No
Endometrial	0/541	19	Low	High	70%	80%	8.61	0.044	No
Ovarian	0/373	15	Low	High	27%	38%	13.22	0.0072	No
Urothelial	299/107	14	Low	High	33%	43%	7.49	0.012	No
Head and Neck	366/133	17	Low	High	39%	57%	8.75	0.0065	No
Melanoma	60/42	5	High	Low	37% (*)	0 (*)	15.17	0.27	No
Prostate	494/0	14	High	Low	100%	97%	11.77	0.094	No
Testis	134/0	20	High	Low	100%	97%	8.63	0.26	No
Liver	246/119	10	Non-determined		53%	46%	5.4	0.190	No
Colorectal	322/275	12	Non-determined		63%	60%	8.76	0.065	No
Total Cases	3679/4253		Low:High=3:12		(*): 3-year Survival				

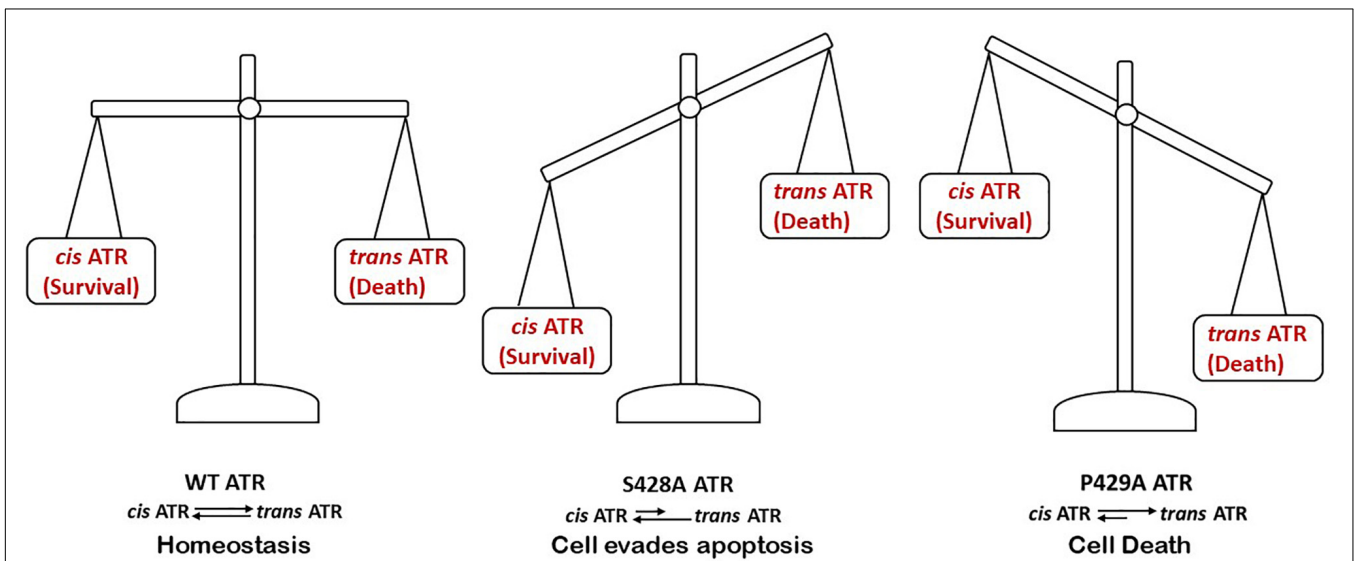

FIGURE 5 | An appropriate balance between cytoplasmic levels of *cis*- and *trans*- ATR is critical for the wellbeing of cells.

causes of cancer, hence the difficulties in identifying suitable treatment targets for developing effective therapies. Research has shown that oncogenes and tumor suppressor genes are constantly undergoing mutations in the background of genetic instability that can drive tumor progression. Oncogene addiction attempts to simplify the essence of carcinogenesis to a single, most important oncogenic protein that a tumor depends on for its survival, while the counterpart normal protein has little or no negative effects on normal cell survival. If this oncogenic pathway is targeted and switched off, cancer cells that are addicted to this pathway will be disproportionately affected, sparing normal cells (Weinstein, 2002; Weinstein and Joe, 2006, 2008). This is the ideal cancer treatment, with a surgical precision in its action, leaving negligible side effects that biomedical researchers have been working toward for decades.

POTENTIAL TARGETING OF ATR ISOMERIZATION IN CANCER THERAPIES

Prior to the elucidation of this anti-apoptotic role of *cis*-ATR in the cytoplasm, a wealth of knowledge already existed about the nuclear kinase roles of ATR which is a *trans* isomer and several cancer therapies have taken advantage of this by targeting the kinase function of ATR to promote cancer cell killing. ATR inhibitors, in combination with chemo- and radio-therapy, have been utilized in a synthetic lethality approach to sensitize cancer cells for cell death with varied results (Wagner and Kaufmann, 2010; Toledo et al., 2011; Fokas et al., 2014; Karnitz and Zou, 2015; Lecona and Fernandez-Capetillo, 2018). Challenges to this approach include: development of specific ATR inhibitors, delivery of the ATR inhibitors to achieve useful physiological concentrations in test subjects, and specificity in killing only cancer cells and not normal cells. VX-970, AZD6738, and other ATR inhibitors are in ongoing clinical trials, being used in conjunction with chemo- or radio-therapy for breast (Kim et al., 2017), ovarian (Huntoon et al., 2013), pancreatic (Prevo et al., 2012), and small cell lung cancers (Vendetti et al., 2015). Pin1 inhibitors also are being evaluated for their usefulness in cancer therapies (Zannini et al., 2019); however, it is possible that side effects could be a concern for this targeting due to the number and diversity of important Pin1 substrates in the cell.

It should be pointed out that the current ATR inhibitors used in cancer clinical trials are specific inhibitors of ATR kinase activity which is pivotal to the hallmark ATR's DNA damage checkpoint functions in the nucleus. Since the new anti-apoptotic activity of *cis*-ATR at mitochondria is independent of ATR kinase activity (Hilton et al., 2015), these inhibitors have no effect on *cis*-ATR's anti-apoptotic activity. *Cis*-ATR (ATR-H), potentially, can be such a target protein that is novel and could be effective in cancer treatment. *Cis*-ATR is not directly mutagenic, but it allows cancer cells to evade apoptosis, a very important hallmark of carcinogenesis. It is possible that cancerous cells, especially with chemo- or radio-therapeutic challenge, have a proportionally higher level of cytoplasmic *cis*-ATR and are resistant to killing due to a low level of Pin1 or a lower level of the phosphorylation of Ser428 in ATR than normal cells (Ibarra et al., 2017). In support, a reduced level of pSer428 ATR in the cytoplasm of advanced stage epithelial ovarian cancer cells correlates with a poor prognosis (Lee et al., 2015). Therefore, targeting *cis*-ATR as an adjuvant in treating cancers by irradiation or chemotherapy should preferentially kill *cis*-ATR-addicted cancer cells, with minimal effects on the normal functions of nuclear *trans*-ATR in cells. ATR is an essential protein (Brown and Baltimore, 2000) and its *cis* and *trans* isomers function normally and exist in a delicate balance to ensure cellular survival and normality (**Figure 5**). By utilizing the natural balance that exists in normal human cells between *cis*- and *trans*-ATR isoforms, we propose *cis*-ATR as a novel, potential target in cancer treatment. Also, *cis*-ATR might serve as a diagnostic marker of prognosis and treatment efficacy in cancer management.

Given the critical role of Pin1 in maintaining the balance between *cis*- and *trans*-ATR in the cytoplasm, manipulation of Pin1 subcellular level or activity could be another means to control *cis*-ATR formation for cancer therapeutics. Ibarra et al. recently reported different subcellular distribution of Pin1 in different cell types in zebrafish *in vivo*, suggesting specific mechanisms for regulating Pin1 subcellular activity are cell-type dependent (Ibarra et al., 2017). These authors also found dramatic reduction of Pin1 in the nucleus and high cytoplasmic Pin1 levels in some cell types *in vivo* (Ibarra et al., 2017). These findings could have important implications in terms of cytoplasmic *cis*-ATR formation.

PROSPECTIVE

There are still important questions remaining to be answered to validate the hypotheses put forward in this review, including a better understanding of (1) how the Ser428 residue is phosphorylated or dephosphorylated under different physiological and biological conditions. Phosphorylation status plays a critical role in the regulation of ATR isomerization and, thus, its antiapoptotic activities; (2) the structural differences between the *cis* and *trans* isomers; and (3) their specific folding for substrate recognition and binding. Are there specific binding partners of *cis*- and *trans*-ATR in the cytoplasm and nucleus, respectively, which help to energetically stabilize ATR in their isoforms? If so, what are these proteins and how are they regulated. Understanding the mechanisms of each isomer's formation and stabilization can help to define whether *cis*-ATR fulfils the criteria to be termed an oncoprotein. It also should be possible to develop drugs that can selectively increase or reduce the specific ATR isoform that is needed in the management of a disease, as elucidated earlier for cancer, for example.

The quest for an ideal cancer therapy began when cancer itself was described as a disease and many promising targets have been investigated in the past with varying results. Since a cancer cell starts as a normal cell that has become deregulated, the ability to selectively target only cancer cells by identification of proteins/processes unique to cancer cells remains elusive for many cancer types and stages. Such targeting should minimize adverse effects while obtaining an effective treatment. As a further complication, the pathways that lead to cancer are numerous and varied, with confounders like immunoediting, persistence of cancer stem cells, etc. Here we propose a target common to all cells: isomerization-mediated apoptosis, but in such a specifically targeted way that normal cells are spared. The isomerization of ATR by Pin1 is an important biological process that should be studied further since the existing evidence points to exciting possibilities for drug/genetic regulation of this singular process. There would be significant potential translational implications in disease diagnosis and treatment.

Finally, the ability to induce or prevent apoptosis in select groups of cells can be of importance in other diseases such as ischemia and inflammation where cell death is the major issue. Moreover, it is worth investigating if *cis*-ATR plays a role in elongating the life of

a cell in the context of aging since more cells would be able to successfully evade apoptosis by increasing the mitochondrial health of the cell.

AUTHOR CONTRIBUTIONS

YM wrote the draft of the manuscript based on the outlines made by YZ. YZ oversaw the process. All authors read and participated in revising the manuscript.

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REVIEW
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Peptidyl-Prolyl *Cis/Trans* Isomerase Pin1 and Alzheimer's Disease

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Alzheimer's disease (AD) is the most common cause of dementia with cognitive decline. The neuropathology of AD is characterized by intracellular aggregation of neurofibrillary tangles consisting of hyperphosphorylated tau and extracellular deposition of senile plaques composed of beta-amyloid peptides derived from amyloid precursor protein (APP). The peptidyl-prolyl *cis/trans* isomerase Pin1 binds to phosphorylated serine or threonine residues preceding proline and regulates the biological functions of its substrates. Although Pin1 is tightly regulated under physiological conditions, Pin1 deregulation in the brain contributes to the development of neurodegenerative diseases, including AD. In this review, we discuss the expression and regulatory mechanisms of Pin1 in AD. We also focus on the molecular mechanisms by which Pin1 controls two major proteins, tau and APP, after phosphorylation and their signaling cascades. Moreover, the major impact of Pin1 deregulation on the progression of AD in animal models is discussed. This information will lead to a better understanding of Pin1 signaling pathways in the brain and may provide therapeutic options for the treatment of AD.

Keywords: Alzheimer's disease, amyloid precursor protein (APP), Pin1, phosphorylation, tau

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INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia, accounting for 50–75% of all cases, and presents as a series of cognitive or behavioral symptoms including decline in memory (Mckhann et al., 2011; Lane et al., 2018; Alzheimer's Association, 2019). The progression of AD may drive or be exacerbated by various systemic abnormalities, such as abnormalities in systemic immunity, metabolic disorders, cardiovascular disease, and sleep disorders (Wang et al., 2017). Approximately 50 million people worldwide currently suffer from dementia, and this number is expected to triple in the next three decades due to the increasing number of aging people (Lane et al., 2018). The neuropathological hallmarks of AD are the intracellular aggregation of neurofibrillary tangles (NFTs) containing paired helical filaments (PHFs) consisting of hyperphosphorylated tau protein and the extracellular deposition of senile plaques (SPs) composed of beta-amyloid (Aβ) peptides derived from amyloid precursor protein (APP) (Hardy and Selkoe, 2002;

Binder et al., 2005; Goedert and Spillantini, 2006; Roberson and Mucke, 2006; Ballatore et al., 2007; Ittner and Gotz, 2011). However, the molecular link and mechanisms underlying the pathogenesis of AD are not fully understood. Therefore, understanding the early disease mechanisms responsible for neurodegeneration in AD is critical for identifying proper diagnostic approaches and new effective therapeutic targets.

Protein phosphorylation is one of the major post-translational modifications and is involved in diverse cellular processes regulating numerous physiological and pathological processes (Cohen, 1982; Nestler and Greengard, 1983; Oliveira et al., 2017; Butterfield, 2019). In particular, serine or threonine residues preceding proline (S/T-P) are the most frequently phosphorylated motifs in AD (Lu et al., 2002, 2003; Lu, 2004; Lu and Zhou, 2007; Iqbal et al., 2016). Interestingly, due to its unique five-carbonyl ring structure, proline is able to present as two strikingly distinct conformations, *cis* and *trans* (Lu et al., 1996; Ranganathan et al., 1997; Yaffe et al., 1997; Wulf et al., 2005; Lu et al., 2007; Lu and Zhou, 2007). The peptide bond dihedral angle ω of proline residue adopts either about 0° (*cis* conformation) or about 180° (*trans* conformation), which plays critical roles in the rate-determining steps of protein folding, thus controlling the biological activity of proteins and their cellular progression (Wedemeyer et al., 2002; Andreotti, 2003; Fischer and Aumuller, 2003; Cortes-Hernandez and Dominguez-Ramirez, 2017). The spontaneous interconversion of *cis*/*trans* isomerization occurs slowly but can be catalyzed by a number of peptidyl-prolyl *cis*/*trans* isomerases (PPIases), such as cyclophilins, FK506-binding proteins (FKBPs), and parvulin-type PPIases (Fischer and Aumuller, 2003; Lu and Zhou, 2007). Cyclophilins and FKBPs not only belong to immunophilins which are cellular targets for the immunosuppressive drugs, but also have relationships with tau-related and Aβ pathology (Blair et al., 2015). Cyclophilin D is one of the most unique and well-studied cyclophilins, and cyclophilin D deficiency can protect neurons from Aβ- and oxidative stress-induced toxicity (Du et al., 2008, 2014; Guo et al., 2013). FKBP with a molecular mass of ∼52 kDa (FKBP52) is one of the most well-studied FKBPs, and FKBP52 has been shown to be highly expressed in neurons and abnormally low in AD brains (Giustiniani et al., 2012, 2014, 2015). Nevertheless, the phosphorylation of an S/T-P motif further slows the spontaneous isomerization rate and renders the peptide bond against the catalytic action of known PPIases (Wulf et al., 2005; Lu and Zhou, 2007). Thus, the important discovery of Pin1 has shed light on the significance of this intrinsic conformational switch in human physiology and pathology.

Pin1 (protein interacting with NIMA (never in mitosis A)-1) was originally identified in a yeast genetic and biochemical screen for proteins involved in mitotic regulation (Lu et al., 1996, 2002). The yeast Pin1 homolog Ess1 has been found to be the only enzyme being essential for survival among 13 PPIases since its discovery (Hanes et al., 1989; Lu, 2004). The human Pin1 has 163 amino acids with a molecular mass of 18 kDa, containing an N-terminal WW domain (residues 1–39) characterized by two invariant tryptophans and a C-terminal PPIase domain (residues 50–163) which shares little similarity with cyclophilins and FKBPs (Lu et al., 1996; Ranganathan et al., 1997). Pin1 is a unique and conserved PPIase that binds to specific phosphorylated proline-directed serine or threonine (pS/T-P) motifs and catalyzes the *cis/trans* isomerization of peptidyl-prolyl peptide bonds (Lu et al., 1996, 1999b; Ranganathan et al., 1997; Yaffe et al., 1997; Schutkowski et al., 1998; Shen et al., 1998). The unique substrate specificity of Pin1 results from the organization of active site residues (Ranganathan et al., 1997; Lu et al., 2002). Specifically, the residues L122, M130, and F134 form a hydrophobic binding pocket for the substrate proline, and the cluster sequestering K63, R68, and R69 forms a positive charged phosphate binding loop which either interacts with a bound sulfate ion or facilitates binding to the pS/T-P motif (Ranganathan et al., 1997; Behrsin et al., 2007; Lee and Liou, 2018). Further studies revealed that mutation of R68 and R69 could abolish the striking phosphorylation-specificity completely but barely affect the basic enzymatic activity (Yaffe et al., 1997; Zhou et al., 2000; Lu et al., 2002). In addition, the WW domain has been shown to target Pin1 to the substrates since it has a higher affinity to phosphorylated peptides as compared to the PPIase domain (Lu et al., 1999b; Smet et al., 2005). This Pin1-mediated conformational change of its substrates regulates numerous cellular processes, such as cell-cycle progression, cellular stress responses, development, neuronal function, immune responses, and cell death (Zhou et al., 1999; Lu and Zhou, 2007). Notably, Pin1 deregulation is implicated in age-dependent human diseases, including cancer and AD (Lu and Zhou, 2007; Lee et al., 2011b; Zhou and Lu, 2016). Pin1 activity and expression are significantly inhibited in human AD brains and highly increased in diverse types of cancers, indicating that Pin1 might have important roles in both proliferation and degeneration (Lu et al., 1999a; Liou et al., 2003; Butterfield et al., 2006; Lu and Zhou, 2007; Lee et al., 2011b; Driver et al., 2012; Zhou and Lu, 2016; Chen et al., 2020).

This review focuses on the deregulation of Pin1 in AD brains, the currently understood mechanisms of tau hyperphosphorylation and APP processing associated with Pin1, and the major impact of Pin1 deregulation on AD development. This advanced understanding of the involvement of the Pin1 signaling pathway in phosphorylation will support Pin1 as a novel potential diagnostic and therapeutic target.

REGULATION OF PIN1 IN AD

Pin1 Expression in AD

The significantly different levels of soluble and functional Pin1 between the brain samples of patients with AD and the control brain samples from age-matched normal subjects suggest a possible protective role of Pin1 against AD. A large amount of soluble Pin1 is dramatically depleted and sequestrated in NFTs in the human AD brain but not in age-matched normal brains (Lu et al., 1999a). Pin1 expression has been further examined in the human hippocampus, a brain region that is particularly vulnerable to AD damage at early stages (Liou et al., 2003; Mu and Gage, 2011). In the hippocampus of normally aged brain samples, the expression of Pin1 in the CA1 region and subiculum

is relatively lower than that in the CA4, CA3, and CA2 regions and presubiculum (Liou et al., 2003; Lu et al., 2003). Notably, in AD brains, NFTs predominantly occur in the CA1 region and subiculum, consistent with the finding that these subregions are prone to pyramidal neuron loss in AD (Davies et al., 1992; Liou et al., 2003). Indeed, among a randomly selected pool of 1,000 pyramidal neurons in AD, 96% of pyramidal neurons with higher expression of Pin1 seem to avoid tau-related pathology, while 71% of neurons with lower expression of Pin1 are vulnerable to NFT formation (Liou et al., 2003). On the contrary, some groups also reported that Pin1 was localized to granular vesicles but not to tau aggregates in AD (Holzer et al., 2002; Ramakrishnan et al., 2003; Dakson et al., 2011; Ando et al., 2013). Recently, according to the hippocampal gene expression profiles of patients from three distinct age groups, the expression of Pin1 is decreased slightly in the aging group but is dramatically decreased in the AD group compared with the young group (Lanke et al., 2018). These results suggest that reduced expression of Pin1 may contribute to the development of AD, including neurofibrillary degeneration.

Pin1 Genetics in AD

The apolipoprotein E (APOE) ε4 allele was the first definitive gene to be implicated in late-onset AD (LOAD) and is located on chromosome 19q13.2 (Corder et al., 1993; Huq et al., 2019; Yamazaki et al., 2019). Although the human Pin1 gene is located on the same chromosome, this locus has been identified as a novel LOAD locus and is independent of APOE (Wijsman et al., 2004). Currently, three single nucleotide polymorphisms (SNPs) in the promoter region of the Pin1 gene have been identified to investigate their correlations with AD, including rs2287839 (-5185 G/C), rs2233678 (-842 G/C), and rs2233679 (-667 T/C). All of Pin1 polymorphism studies were conducted using genomic DNA from blood cells between AD patients and age-matched normal subjects. The polymorphism rs2233678 results in decreased Pin1 levels and is associated with a significantly raised risk of developing AD (Segat et al., 2007). The polymorphism rs2287839 leads to increased Pin1 expression and is correlated with 3-year delayed onset of LOAD (Ma et al., 2012b). However, other groups showed that polymorphisms in the promoter of Pin1, rs2233678 and rs2233679, were not associated with increased LOAD risk (Lambert et al., 2006; Nowotny et al., 2007; Cao et al., 2013). Interestingly, rs2233678 and rs2233679 have also been shown to decrease Pin1 expression and are implicated in the decreased risk of breast cancer, lung cancer, and nasopharyngeal carcinoma (Han et al., 2010; Lu et al., 2011, 2013). Therefore, since the controversial results remain to be elucidated, further validation of large prospective studies is needed to verify the roles of Pin1 polymorphisms in AD. Recently, a highly pathogenic and novel somatic single nucleotide variation (SNV) in Pin1 has been found in the hippocampal formation (HIF) of an AD patient (Park et al., 2019). Since the T152M mutation is located in the C-terminal PPIase domain of Pin1, the mutation might attenuate the enzymatic activity of Pin1 and increase tau hyperphosphorylation (Park et al., 2019). However, the molecular mechanism by which the somatic mutation regulates Pin1 activity and whether T152M knockin mice show tau-related and Aβ pathology remain to be elucidated.

Pin1 Post-translational Modification in AD

Pin1 activity is regulated by post-translational modifications, including oxidation and phosphorylation, in AD. Neurons in the human brain are vulnerable to oxidative stress, and increased oxidative damage has been shown to be an early event in AD (Markesbery, 1997; Nunomura et al., 2001; Halliwell, 2006). Notably, Pin1 is modified by oxidation, leading to the loss of its activity in the hippocampus in AD (Butterfield et al., 2006; Sultana et al., 2006). Besides, oxidized Pin1 may be recognized by the ubiquitinylation system, giving rise to the polyubiquitination (Tramutola et al., 2018). By employing antibodies specifically recognizing oxidized C113 of Pin1, Pin1 oxidation on C113 has been identified to inactivate the catalytic activity of Pin1, and C113-oxidized Pin1 is elevated in human AD brains compared with age-matched controls (Chen et al., 2015). It is possible that an increased percentage of C113-oxidized Pin1 in response to oxidative stress may result in the inhibition of enzymatic activity and reduction of Pin1 levels. The loss of Pin1 activity induced by oxidative stress may also result in the loss of synaptic plasticity, which is the structural basis for memory impairment in AD (Xu et al., 2017). These results suggest that the protective roles of Pin1 may be attenuated by a variety of reactive oxygen species, which are common in human AD brains.

Recently, death-associated protein kinase 1 (DAPK1) has been found to play essential roles in neuronal cell death and various neurodegenerative diseases, including AD (Chen et al., 2019; Kim et al., 2019). Importantly, DAPK1 is capable of phosphorylating Pin1 at S71 in the PPIase domain, thus inhibiting its nuclear localization, prolyl isomerase activity, and cellular function (Lee et al., 2011a,b). DAPK1 dramatically increases tau protein stability and hyperphosphorylation at multiple AD-related sites, which is mediated by the inhibition of Pin1 activity by phosphorylation (Kim et al., 2014). DAPK1 phosphorylates and activates N-myc downstream-regulated gene 2 (NDRG2), resulting in increased tau phosphorylation via a reduction in Pin1 expression (Rong et al., 2017; You et al., 2017). In summary, the existence of Pin1 in the normal brain may have certain protective functions against AD, as decreased expression or declined activity of Pin1 make neurons vulnerable to pathologies related to AD.

PIN1 AND TAU-RELATED PATHOLOGY

The intracellular aggregation of NFTs containing PHFs made of hyperphosphorylated tau is one of the neuropathological hallmarks of AD (Geschwind, 2003; Binder et al., 2005; Goedert and Spillantini, 2006; Roberson and Mucke, 2006; Ballatore et al., 2007). Compared with Aβ pathology, which may play a critical role in AD pathogenesis, the prevalence of NFTs has a strong correlation with the severity of cognitive impairment, indicating that tau-related pathology may indicate the status of cognitive deficits and dementia (Nelson et al., 2012). Encoded by a single gene, MAPT, located on human chromosome 17, tau is a type of microtubule-associated protein and is expressed predominantly in the brain (Lee et al., 1989;

Albayram et al., 2016; Iqbal et al., 2016). It is well-established that the physiological function of tau is to maintain microtubule-related functions, such as microtubule assembly and axonal transportation, and the abnormal hyperphosphorylation of tau inhibits normal microtubule functions and alters tau protein stability (Drubin and Kirschner, 1986; Bramblett et al., 1993; Alonso et al., 1994; Petrucelli et al., 2004; Shimura et al., 2004; Stoothoff and Johnson, 2005; Poppek et al., 2006). Specifically, abnormally phosphorylated tau is detached from microtubules and disrupts microtubule integrity (Iqbal et al., 2009, 2016). Hyperphosphorylated tau, but not normal tau, is a component of PHF-forming insoluble aggregates and further becomes NFTs (Lee et al., 1991; Goedert et al., 1992; Matsuo et al., 1994). These results indicate that the phosphorylation of tau is essential for the development of tau-related pathology.

The phosphorylation of T231 (pT231), among a number of tau phosphorylation sites, appears to be the first detectable event during AD pretangle formation (Luna-Munoz et al., 2007). pT231 may play a critical role in regulating the conformation and misfolding process of tau (Lee et al., 2011b; Iqbal et al., 2016). Notably, Pin1 colocalizes with phosphorylated tau, directly binds to pT231-tau, and can restore its biological activity by promoting tau dephosphorylation to bind microtubules and increase microtubule assembly (Lu et al., 1999a, 2003; Ramakrishnan et al., 2003; Lu, 2004). Pin1 facilitates tau dephosphorylation through the proline-directed phosphatase PP2A, which has conformational specificity and dephosphorylates only the *trans* pS/T-P motif (Zhou et al., 2000). Pin1 has been found to bind PHFs and be trapped in tangles in the AD brain, resulting in the depletion of soluble Pin1 (Lu et al., 1999a). A recent *in vitro* study showed that reduced Pin1 expression led to the increase of pT231-tau levels (Park et al., 2019). Pin1 has been shown to accelerate the *cis* to *trans* isomerization of pT231-tau, restore its function, and maintain tau levels via proteasome-dependent proteolytic pathway (Poppek et al., 2006; Lim et al., 2008). However, Pin1 has no effect on T231A mutant tau (Lim et al., 2008; Nakamura et al., 2012). Interestingly, when hippocampal cultured neurons are exposed to Aβ42 oligomers, Pin1 can be activated to dephosphorylate pT231-tau mediated by PP2A (Bulbarelli et al., 2009). Notably, studies have showed that microtubule assembly can be significantly increased by unphosphorylated wild-type tau, but not phosphorylated tau which can be restored by PP2A, while the phosphorylated T231A tau is still able to promote microtubule assembly and this ability is not affected by Pin1, suggesting that T231 phosphorylation is critical for Pin1 to maintain microtubule function of tau (Nakamura et al., 2012). Therefore, tau hyperphosphorylation might induce tau aggregation which further sequestrates Pin1, thereby preventing pT231-tau dephosphorylation mediated by PP2A (**Figure 1**). However, other studies have questioned the specificity of Pin1 targeting site, as they revealed that Pin1 recognized other pS/T-P sites such as pT212 and pS235 motifs in full-length tau, which were the preferred substrates over pT231 motif (Smet et al., 2004, 2005; Landrieu et al., 2006; Kimura et al., 2013; Eichner et al., 2016). Besides, other studies also indicated that Pin1 did not regulate the microtubule function of phosphorylated tau (Lippens et al., 2007; Landrieu et al., 2010, 2011; Kutter et al., 2016; Lu et al., 2016; Rogals et al., 2016). Therefore, the specificity of Pin1 targeting sites of tau and the regulatory function of Pin1 toward phosphorylated tau raise other possibilities which need further investigation.

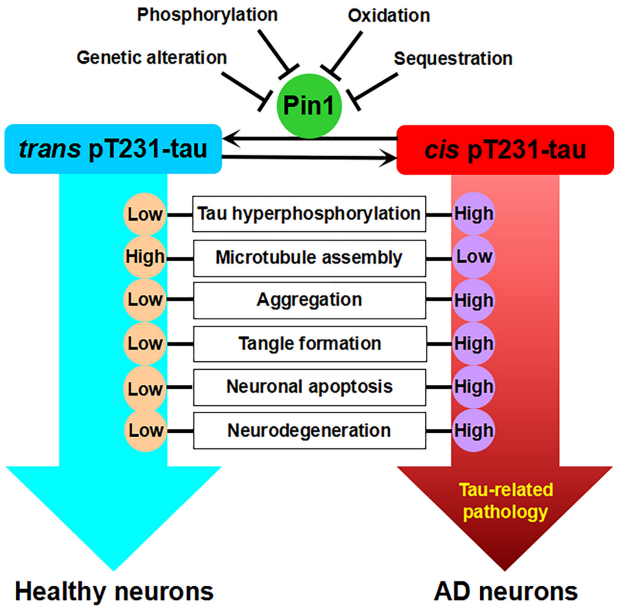

FIGURE 1 | Pin1-regulated isomerization of pT231-tau against tau-related pathology. There are two strikingly distinct *cis* and *trans* conformations of the pT231 motif of tau after phosphorylation. *Cis*, but not *trans*, pT231-tau promotes tau hyperphosphorylation, the disruption of microtubule assembly, tau aggregation, tangle formation, neuronal apoptosis, and neurodegeneration. Pin1 binds to the pT231-P motif and isomerizes the *cis* form of pT231 to the *trans* form. Low levels of Pin1 due to genetic alteration or the inhibition of Pin1 expression or activity by phosphorylation, oxidation, and sequestration increase the levels of *cis* pT231-tau and may contribute to AD.

Recently, the Lu laboratory developed conformation-specific *cis* and *trans* polyclonal and monoclonal pT231-tau antibodies (Nakamura et al., 2012; Kondo et al., 2015). Specifically, *cis* pT231-tau appears to be more responsible for resistance to tau dephosphorylation and degradation, the disruption of microtubule structure, and vulnerability toward aggregation, and Pin1 catalyzes the isomerization of pT231-tau from *cis* to *trans*, restoring its ability to bind microtubules (Lu et al., 1999a, 2016; Nakamura et al., 2012; Albayram et al., 2016, 2018). Indeed, *cis* pT231-tau, but not *trans* pT231-tau, is significantly increased and localized to dystrophic neurites in human mild cognitive impairment (MCI) and AD brains (Nakamura et al., 2012). Furthermore, *cis* pT231-tau, but not *trans* pT231-tau, strongly correlates with neurofibrillary degeneration, which is associated with decreased Pin1 levels in the AD hippocampus, in accord with the binding of Pin1 to PHFs leading to the depletion of soluble Pin1 (Lu et al., 1999a; Nakamura et al., 2012). In addition, *cis* pT231-tau is dramatically induced, facilitates the disruption of axonal microtubules and organelle transport, and finally leads to neuronal apoptosis under neuronal stress (Kondo et al., 2015). Therefore, the neurotoxic *cis* pT231-tau may function as

a critical driver of neurodegeneration, as it can spread among neurons in a prion-like fashion (Kondo et al., 2015; Albayram et al., 2018). In tau-overexpressing mice, while *trans* pT231-tau is barely detected in sarkosyl-insoluble fractions, *cis* pT231-tau levels are robustly increased in insoluble fractions in the brain (Nakamura et al., 2012). Interestingly, *cis* pT231-tau has been shown to be a major early driver of traumatic brain injury (Kondo et al., 2015; Albayram et al., 2017). These results suggest that the Pin1-regulated isomerization of the *cis* to *trans* conformations of phosphorylated tau is a key mechanism to protect against tau-related pathology. Nevertheless, the *cis* pT231-tau antibody raised against a peptide containing a chemically modified proline instead of a native *cis*-proline has also been questioned, and it is suggested that the specific pT231-P232 bond in phosphorylated tau be majorly in the *trans* conformation (Shih et al., 2012; Ahuja et al., 2016; Lippens et al., 2016).

Thus, Pin1 may maintain normal tau functions through the conformational change of pT231-tau, but its deregulation leads to tau-related pathology during AD development. However, Pin1 acts on different phosphorylation sites of tau and has opposite results of tau function. This discrepancy may be due to the different characteristics of the diverse physiological and pathological conditions. Therefore, more evidence is needed to clarify the role of Pin1 in phosphorylated tau and its function.

PIN1 AND APP PROCESSING

The extracellular deposition of SPs composed of Aβ peptide derived from APP is another neuropathological hallmark of AD (Hardy and Selkoe, 2002). The human APP gene is located on chromosome 21 and encodes a type I transmembrane protein that plays important roles in neuronal growth, survival, and repair (Thinakaran and Koo, 2008). Upon synthesis in the endoplasmic reticulum, APP undergoes trafficking through the Golgi/trans-Golgi network (TGN) toward the plasma membrane, where it accumulates and internalizes to the endosomes (Selkoe et al., 1996; Thinakaran and Koo, 2008; Pastorino et al., 2012). APP is processed by two different proteolytic processes, the non-amyloidogenic pathway and amyloidogenic pathway (Koo and Squazzo, 1994; Selkoe et al., 1996; Hardy and Selkoe, 2002; Nunan and Small, 2002; Vetrivel and Thinakaran, 2006). In the non-amyloidogenic processing pathway, APP is cleaved by α-secretase at a site within the sequence of Aβ at the plasma membrane, generating soluble extracellular sAPPα with neurotrophic properties and a C-terminal fragment, C83; C83 is further cleaved by γ-secretase to generate the APP intracellular domain (ACID) and a small p3 fragment, thus avoiding Aβ pathology (Esch et al., 1990; Sisodia et al., 1990; Selkoe et al., 1996; Hardy and Selkoe, 2002; Thinakaran and Koo, 2008). In the amyloidogenic processing pathway, APP is internalized to early endosomes through Fe65 and is cleaved by β-secretase to generate soluble sAPPβ and a C-terminal fragment, C99; C99 is further cleaved by γ-secretase in late endosomes to generate the ACID and intact Aβ, inducing Aβ pathology, which is elevated in AD (Selkoe et al., 1996; Wolfe et al., 1999; Yan et al., 1999; Cai et al., 2001; Hardy and Selkoe, 2002; Thinakaran and Koo, 2008).

APP processing and Aβ generation are regulated by the phosphorylation of the intracellular C-terminal fragment (Pastorino and Lu, 2005; Suzuki and Nakaya, 2008). Notably, the phosphorylation of APP at the T668-P motif is increased in the brains of AD patients compared with those of age-matched controls, facilitating the amyloidogenic processing pathway and Aβ generation (Lee et al., 2003). Importantly, Pin1 binds to APP specifically on the phosphorylated T668-P motif *in vitro* and *in vivo* (Pastorino et al., 2006). The binding of Pin1 to phosphorylated T668-P accelerates its isomerization from *cis* to *trans* by over 1,000-fold, as visualized by NMR spectroscopy (Ramelot et al., 2000; Ramelot and Nicholson, 2001; Pastorino et al., 2006). The overexpression of Pin1 significantly decreases Aβ secretion *in vitro*, while Pin1 ablation dramatically increases insoluble Aβ42 secretion in cell models and mouse models in an age-dependent manner (Pastorino et al., 2006). Pin1 is colocalized with APP at the plasma membrane and in clathrin-coated vesicles rather than endosomes, and Pin1 inhibition leads to reduced APP levels at the plasma membrane (Pastorino et al., 2006, 2012). Pin1 influences the levels of Fe65, which can interact with APP and facilitate amyloidogenic APP processing (Pastorino et al., 2012, 2013). Thus, Pin1 isomerizes APP to the *trans* conformation, controls the intracellular localization and internalization of APP, modulates AICD in a Fe65-dependent manner, and thus exerts a protective function against Aβ pathology, indicating that the Pin1-regulated prolyl isomerization of APP plays a key role in regulating Aβ pathology.

A number of protein kinases responsible for phosphorylating APP at the T668-P motif are abnormally elevated in the AD brain, such as GSK3β, SAPK1b/JNK3, Cdc2, and Cdk5 (Zhou et al., 2000; Lee et al., 2003, 2011b; Lu and Zhou, 2007; Ma et al., 2012a). Among them, GSK3β is a widely expressed proline-directed serine/threonine kinase that is implicated in a number of physiological processes in the nervous system (Jiang et al., 2005; Yoshimura et al., 2005; Castano et al., 2010). The aberrant regulation of GSK3β contributes to major neurological disorders, including both familial and sporadic AD (Hooper et al., 2008; Peineau et al., 2008). The hyperactivity of GSK3β increases Aβ production, while the inhibition of GSK3β reduces plaques *in vitro* and *in vivo* (Lovestone et al., 1994; Flaherty et al., 2000; Engel et al., 2006; Hurtado et al., 2012). Ma and colleagues showed that Pin1 directly binds to the phosphorylated T330-P motif in GSK3β and inhibits its kinase activity *in vitro* and *in vivo* (Ma et al., 2012a). The suppression of Pin1 causes GSK3β activation, leading to increased levels of T668-phosphorylated APP and amyloidogenic APP processing. In addition, Pin1 promotes APP protein degradation by binding to the phosphorylated T330-P motif of GSK3β (Ma et al., 2012a; Xiong et al., 2013). Thus, Pin1 promotes APP protein turnover by inhibiting GSK3β activity, suggesting a novel neuroprotective role of Pin1 against Aβ pathology. However, contrasting results showed that in the Pin1-deficient mice Aβ was lower and Pin1 promoted Aβ production *in vitro* (Akiyama et al., 2005). The discrepancy might due to the usage of β-cleaved carboxy-terminal frangment C99 instead of full-length APP and detection of the mouse brain at an unspecified age. Thus, in healthy neurons, sufficient levels of

Pin1 promote the non-amyloidogenic processing pathway of APP and its turnover, reducing Aβ secretion. However, when Pin1 expression is reduced or its activity is inhibited, amyloidogenic APP processing is increased, resulting in Aβ production and Aβ plaques in the AD brain (**Figure 2**).

PIN1 MOUSE MODELS OF AD

Animal models recapitulating the characteristics of AD are of vital importance not only for performing *in vivo* studies that explore molecular mechanisms but also for providing preclinical subjects for potential drug candidates. Due to research on familial forms of AD, transgenic mouse models have been developed and widely used (Games et al., 1995; Duff et al., 1996; Hsiao et al., 1996; Lewis et al., 2001; Gotz and Ittner, 2008). However, mutated tau and APP overexpression mice do not recapitulate all features of AD (Drummond and Wisniewski, 2017). Pin1 knockout (KO) mice were initially created to explore the function of Pin1 in mammalian cells, and they were viable and developed normally to adulthood (Fujimori et al., 1999). Importantly, Pin1 KO mice are the first mouse models to show both tau-related and Aβ pathology when a specific gene is deleted (Liou et al., 2003; Pastorino et al., 2006; Lu and Zhou, 2007; Lee et al., 2011b). Pin1 KO mice show tau hyperphosphorylation leading to age-dependent tau filament formation and NFT-like pathologies compared with their wild-type littermates (Liou et al., 2003). Pin1-KO mice also show neuronal loss and progressive age-dependent motor and behavioral deficits, such as abnormal limb-clasping reflexes, hunched posture, reduced mobility, and eye irritation (Liou et al., 2003). When neuron-specific Pin1 transgenic (Tg) mice are bred with wild-type tau Tg mice, Pin1 overexpression reduces tau hyperphosphorylation, NFT specific conformations, and aggregation (Lim et al., 2008). Surprisingly, in Tg mice overexpressing human P301L mutant tau, which causes frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), Pin1 KO drastically decreases the total tau levels and the hyperphosphorylation of tau, while Pin1-Tg promotes tau hyperphosphorylation, tau aggregation, and NFT conformation (Lim et al., 2008). This unexpected discrepancy was explained that the mutation might somehow render the pT231 motif in tau to be favored in the *trans* conformations (Lim et al., 2008). Therefore, Pin1 overexpression might accelerate the isomerization of the protective *trans* conformation to the pathogenic *cis* conformation, whereas Pin1 inhibition contributes to maintaining the *trans* conformation, facilitating P301L tau degradation (Lim et al., 2008). Since no tau mutations have been found in AD, tau-related pathology induced

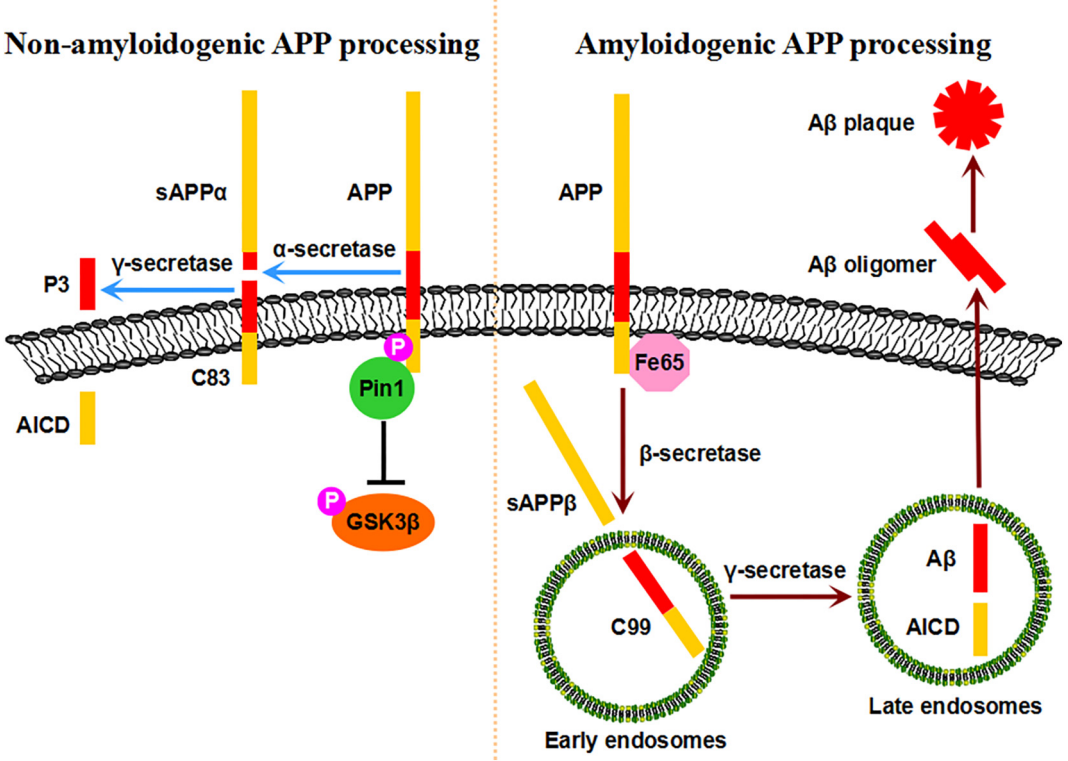

FIGURE 2 | Pin1-mediated APP processing. Pin1 binds to the pT668-P motif of APP, accelerates its isomerization from *cis* to *trans*, stabilizes APP at the plasma membrane, and ultimately promotes the non-amyloidogenic processing pathway. Moreover, conformational changes in APP may affect the binding of Fe65, thus preventing the amyloidogenic processing pathway. Furthermore, Pin1 promotes APP protein turnover by decreasing GSK3β activity. Under physiological conditions, sufficient levels of Pin1 protect healthy neurons against Aβ pathology. When Pin1 expression/activity is inhibited, the amyloidogenic processing pathway is dominant, resulting in Aβ production and plaques.

by exogenous overexpression of mutant tau may be different from human AD in terms of molecular regulatory mechanisms. In addition, when Pin1 KO mice have been bred with tau-Tg mice, these mice exhibit increased *cis* pT231-tau, but decreased *trans* pT231-tau levels, supporting the Pin1-mediated suppression of tau-related neurodegeneration in mice (Nakamura et al., 2012).

Pin1 ablation in mice also affects APP processing in APP-overexpressing mouse brains (Pastorino et al., 2006). Compared with the wild-type littermates, Pin1 KO mice exhibit increased levels of insoluble Aβ42, the major toxic species, at 15 months of age, but not at 6 months of age, suggesting that Pin1 KO promotes amyloidogenic APP processing in an age-dependent manner (Pastorino et al., 2006). Pin1 ablation in APP-Tg2576 mice significantly induces insoluble Aβ42 species and increases soluble APPβ levels at 6 months of age. These Aβ42 species are mainly localized to multivesicular bodies of neurons that experience Aβ plaque pathology (Pastorino et al., 2006). Thus, Pin1 is a unique protein, the deletion of which causes age-dependent tau-related and Aβ pathologies, suggesting evidence of a molecular link between tangles and plagues and a protective role of Pin1 against AD.

PIN1 IN DIAGNOSTIC AND THERAPEUTIC STRATEGIES FOR AD

Following a series of abnormal tau hyperphosphorylation that induces the formation of NFTs, pT231 appears to be the first detectable phosphorylation site of tau (Luna-Munoz et al., 2007). Due to the elevated sequestration of pT231-tau into the tangles and the decreased levels of pT231-tau that enter the cerebrospinal fluid (CSF), pT231-tau provides an early and specific biomarker of AD progression (Hampel et al., 2001; Spiegel et al., 2016). The assessment of pT231-tau in the CSF has been regarded as a good predictor of conversion from MCI to AD (Ewers et al., 2007). However, the presence of individual variations using pT231-tau might impede its application as a standardized test, whereas whether the existence of distinct forms (*cis* or *trans*) of pT231-tau helps to explain the variations remains to be investigated (Nakamura et al., 2012). Notably, *cis* and *trans* pT231-tau forms, which are regulated by Pin1, can be distinguished by recently developed conformation-specific antibodies (Nakamura et al., 2012; Kondo et al., 2015). Importantly, *cis* pT231-tau appears early in MCI, is pathologically more relevant, and contributes to AD (Nakamura et al., 2012). Current diagnostic approaches using CSF or positron emission tomography (PET) are either invasive or expensive, making it difficult to achieve early diagnosis using these approaches (Wang et al., 2017; Long and Holtzman, 2019). Commonly known markers used as diagnostic methods are often detectable months or years after the initiation of AD pathogenesis. Therefore, early detectable concentrations of *cis* pT231-tau, changes in *cis* pT231-tau levels, and the ratio of *cis* pT231-tau to *trans* pT231-tau in body fluids and blood from normal and AD patients might be better and more standardized biomarkers for early diagnosis.

To date, AD remains incurable, and a pool of issues remains to be solved. First and foremost, whether Aβ pathology occurs first and induces tau-related pathology, or vice versa, is still controversial. The answer to this question may influence the efficacy of targeted therapies specific for Aβ or tau. Moreover, available therapeutic strategies primarily focus on slowing down the progression of cognitive decline and neurodegeneration rather than targeting essential pathways (Pastorino et al., 2013). Furthermore, the administration of drugs at late stages due to the lack of early diagnosis may dramatically attenuate the efficacy since AD usually takes more than a decade to develop. Notably, the discovery of the Pin1-catalyzed *cis/trans* isomerization of phosphorylated S/T-P motifs in tau and APP and *cis* pT231-tau, but not *trans* pT231-tau, as an early and potent driver in MCI and AD, offers an attractive and promising therapeutic strategy for AD. A generation of mouse monoclonal antibodies specific for *cis* pT231-tau has been developed and shown to eliminate pathologic *cis* pT231-tau and prevent tau-related pathology development and spread (Kondo et al., 2015; Albayram et al., 2017). Importantly, immunotherapy employing this strategy specifically aims at the earliest possible pathogenic form of tau rather than the physiological *trans* form of pT231-tau with normal functions in AD (Kondo et al., 2015). Thus, further humanization of the *cis* pT231-tau antibody is conducive to developing novel therapeutic strategies for AD.

Because Pin1 plays an important role in preventing tau-related and Aβ pathologies in AD, the upregulation and/or activation of Pin1 could be a viable strategy for AD treatment. However, Pin1 overexpression contributes to a number of cancers, eliminating the possibility of direct administration (Zhou and Lu, 2016). Aberrant Pin1 elevation has been shown to be involved in many signaling events such as cell cycle coordination, chromosome instability, proliferation, migration, metastasis, and apoptosis in cancer (Zhou and Lu, 2016). Indeed, Pin1 is known to activate 56 oncogenes and inactivate 26 tumor suppressors by regulating their activity, protein interaction, stability, and cellular localization (Cheng and Tse, 2019; Yu et al., 2020). Pin1 overexpression in mammary gland induces chromosome instability and leads to breast cancer development and Pin1 ablation effectively prevents tumorigenesis by overexpressing Neu in animal models (Wulf et al., 2004; Suizu et al., 2006). Therefore, direct Pin1 activation in brain might cause malignant brain tumor. If we could specifically deliver Pin1 activator to neurons, it might be useful because neurons do not divide or proliferate. In addition, therapeutic strategies targeting Pin1 also focus on the upstream regulators of Pin1 such as DAPK1 or targets such as *cis* or *trans* pT231-tau. Indeed, the inhibition of DAPK1 has been shown to attenuate tau hyperphosphorylation and Aβ production (Kim et al., 2014, 2016, 2019; You et al., 2017; Chen et al., 2019). Thus, Pin1-related therapeutic strategies might be valuable in the treatment of AD.

CONCLUSION

The peptidyl-prolyl *cis/trans* isomerase Pin1 is a crucial regulator that is implicated in a wide variety of physiological and pathological activities. The deregulation of Pin1 expression and/or activity is associated with the development of cancer

and neurodegeneration, including AD. Interestingly, Pin1 regulates the conformational change of both tau and APP and has protective effects against tau-related and Aβ pathology, suggesting that Pin1 might be a novel and promising candidate for exploring the molecular mechanisms, diagnosis, and treatment of AD.

The availability of drug candidates largely depends on animal models. Currently, many types of single or biogenic Tg mice are broadly used to study tau-related and Aβ pathologies; however, these mice fail to recapitulate all aspects of human AD. Pin1 KO mice develop both tau-related and Aβ pathologies in an age-dependent manner by employing endogenous tau and APP proteins, providing an attractive *in vivo* model for AD research and drug testing. Novel therapeutic strategies such as *cis* pT231-tau antibodies that target conformation-specific phosphorylated tau or small molecules such as DAPK1 inhibitors might provide effective treatment for human AD. However, many questions, including how to regulate Pin1 levels due to its dual roles in cancer and AD, how to overcome the blood-brain barrier for antibody treatment, and how to validate a suitable time for drug administration in the early stage of AD, remain to be answered before clinical validation. Moreover, Pin1 has been shown to lead different direction of tau phosphorylation and APP processing depending on cellular context. More research is urgently needed to illuminate the underlying roles of Pin in the molecular regulation, early diagnosis, potential treatment, and possible prevention of AD.

AUTHOR CONTRIBUTIONS

This review manuscript was conceptualized by LW and TL. YZ prepared figures. DC professionally edited the manuscript. LW, YZ, and TL wrote the manuscript.

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PP2A Regulates Phosphorylation-Dependent Isomerization of Cytoplasmic and Mitochondrial-Associated ATR by Pin1 in DNA Damage Responses

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Ataxia telangiectasia and Rad3-related protein (ATR) is a serine/threonine-protein kinase of the PI3K family and is well known for its key role in regulating DNA damage responses in the nucleus. In addition to its nuclear functions, ATR also was found to be a substrate of the prolyl isomerase Pin1 in the cytoplasm where Pin1 isomerizes *cis* ATR at the Ser428-Pro429 motif, leading to formation of *trans* ATR. *Cis* ATR is an antiapoptotic protein at mitochondria upon UV damage. Here we report that Pin1's activity on *cis* ATR requires the phosphorylation of the S428 residue of ATR and describe the molecular mechanism by which Pin1-mediated ATR isomerization in the cytoplasm is regulated. We identified protein phosphatase 2A (PP2A) as the phosphatase that dephosphorylates Ser428 following DNA damage. The dephosphorylation led to an increased level of the antiapoptotic *cis* ATR (ATR-H) in the cytoplasm and, thus, its accumulation at mitochondria *via* binding with tBid. Inhibition or depletion of PP2A promoted the isomerization by Pin1, resulting in a reduction of *cis* ATR with an increased level of *trans* ATR. We conclude that PP2A plays an important role in regulating ATR's anti-apoptotic activity at mitochondria in response to DNA damage. Our results also imply a potential strategy in enhancing cancer therapies *via* selective moderation of *cis* ATR levels.

Keywords: ATR, Pin1, PP2A, UV irradiation, DNA damage response, ATR antiapoptotic activity at mitochondria, BID

INTRODUCTION

ATR is a phosphatidylinositol 3 kinase (PI3K)-like protein that plays a crucial role in sensing DNA damage for maintenance of genomic integrity (Zhou and Elledge, 2000; Zou and Elledge, 2003). It is an essential factor for cellular regulation of DNA damage responses in order to maintain cell homeostasis. Following DNA damage ATR, together with its nuclear partner ATR-interacting protein (ATRIP), senses and recognizes the presence of elongated regions of

replication protein A (RPA)-coated ssDNA resulting from DNA damage-induced replication stress. This recognition allows activation of the DNA damage checkpoint responses, including cell cycle arrest, gene expression alterations, DNA repair and/or apoptosis (Zou and Elledge, 2003; Sancar et al., 2004; Mordes and Cortez, 2008; Flynn and Zou, 2011; Nam and Cortez, 2011; Awasthi et al., 2016; Saldivar et al., 2017; Lecona and Fernandez-Capetillo, 2018). The cell cycle arrest that follows sensing of DNA damage ultimately leads to DNA repair or, if too severe, to apoptotic cell death. ATR also is involved in sensing mechanical stress to the cell (Baumann, 2014; Kumar et al., 2014), replication origin firing (Shechter et al., 2004; Moiseeva et al., 2019) and autophagy (Liu et al., 2018; Ma et al., 2018).

Hilton et al. (2016) reported that ATR has two peptidylprolyl isomeric forms, *cis* and *trans*, in cells. The cell nucleus contains only *trans* ATR, while the relative levels of *cis* and *trans* forms in the cytoplasm are DNA damage dependent. Upon DNA damage, the *trans* isomeric form of ATR in the nucleus is responsible for its DNA damage checkpoint functions. In contrast, the *cis* isomeric form generated in the cytoplasm after DNA damage directly acts at the mitochondria as an antiapoptotic protein. Thus, ATR can directly regulate apoptosis at the mitochondrion, a response distinct from the ATR-mediated DNA damage checkpoint pathway. However, it is believed that the *trans* and *cis* ATR activities in the nucleus and mitochondria, respectively, are coordinated and the balance of the activities plays a critical role in ATR-dependent DNA damage responses. It was reported that the interconversions between *cis*- and *trans*-isomeric forms of ATR are regulated by the prolyl isomerase Pin 1 (peptidylprolyl *cis*/*trans* isomerase NIMA-interacting 1) (Hilton et al., 2016). In cells, Pin1 isomerizes ATR at the motif of Ser428-Pro429, converting the protein from *cis* to *trans*. Upon DNA damage, Pin1 is phosphorylated at S71 which inactivates Pin1, leading to cytoplasmic accumulation of the antiapoptotic *cis* ATR (Hilton et al., 2016). It is believed that upon DNA damage, *trans*-ATR is activated in the nucleus to initiate DNA damage signaling and checkpoint pathways, which lead to cell cycle arrest and promote DNA repair. At the same time, coordinately, *cis*-ATR forms in the cytoplasm and is translocated to mitochondria to prevent activation of Bax, suppressing apoptosis (Hilton et al., 2016). The coordination between *trans*- and *cis*-ATR activities prevents premature cell death while DNA damage is removed, followed by resuming cell cycling.

Pin1 catalyzes the isomerization of specific phosphorylated Ser/Thr-Pro amide bonds in proteins. This phosphorylation-dependent isomerization can lead to major conformational changes in protein structure and function (Lu et al., 1996; Lu, 2000; Liou et al., 2011; Schmidpeter and Schmid, 2015). In the case of ATR, Pin1 recognizes the phosphorylated Ser428-Pro429 (pSer428-Pro429) motif in ATR and isomerizes ATR from the *cis*-isomeric form (ATR-H) to the *trans* form (ATR-L) (Hilton et al., 2016). The fact that Pin1 isomerization of ATR depends on the status of Ser428 phosphorylation suggests a mechanism by which the balance between kinase(s) and phosphatase(s) activities can regulate Pin1 activity toward ATR. While the regulation is expected to have an important effect on the ATR's antiapoptotic activity at mitochondria and DNA damage responses, the identities of the kinase(s) or phosphatase(s) remain unknown.

Protein phosphatase 2A (PP2A) plays an important role in regulating DNA damage responses (DDR) by dephosphorylating DDR proteins to change the phosphorylation status of proteins that are critical to genome stability (Freeman and Monteiro, 2010; Harris and Bunz, 2010; Palii et al., 2013; Ferrari et al., 2017; Merigliano et al., 2017; Ramos et al., 2019). Substrates of PP2A in DDR include ATR, ATM, DNA-PK, Chk1, Chk2, p53, and so on (Ramos et al., 2019). All these regulations are carried out in the nucleus where DNA damage checkpoint signaling occurs. For example, PP2A activation attenuates ATR/ATR-dependent DDR (Ferrari et al., 2017). Inhibition or knockdown of PP2A leads to an increase of γ-H2AX, an important DNA damage signal of DNA strand breaks (Nazarov et al., 2003; Chowdhury et al., 2005; Keogh et al., 2006). PP2A and Wip1 dephosphorylate ATM at S1981 and, thus, suppress ATM activity (Goodarzi et al., 2004; Shreeram et al., 2006). PP2A also is involved in regulating cell death (Andrabi et al., 2007; Guenebeaud et al., 2010; Lee et al., 2011; Sun and Wang, 2012; Zhou et al., 2017).

In this study, we examined how Pin1-mediated ATR isomerization is regulated. Specifically, we identified PP2A, which belongs to the Ser/Thr phosphoprotein phosphatase family, as the phosphatase responsible for the dephosphorylation of ATR at Ser428. We show that when PP2A is inhibited, phosphorylation of ATR at Ser428 significantly increases, promoting the formation of *trans* ATR with less *cis* ATR in the cytoplasm. This reduces the protection of cells from apoptotic death following UV-induced DNA damage. Our study reveals a potential pharmacological target in the regulation of mitochondria-associated ATR by phosphatase PP2A that may imply new therapeutic strategies against diseases involving apoptotic cell death and cancer.

MATERIALS AND METHODS

Cell Culture, UV Irradiation, siRNA, and Inhibitor Treatments

A549 and HCT 116 cell lines were used for all experiments. UV treatments were delivered at 40 J/m^2, followed by a 2 h recovery, except for the graduated UV doses and timed recoveries as indicated in the text and specific figure legends. The siRNAs targeting PP2A, PP4, and PP5 were directed against the catalytic subunits of PP2Ac, PP4c, and PP5c, respectively. The PP2A inhibitor (PP2Ai) used was LB-100 at a dose of 10 uM concentration for 1 h before and during all UV treatments. The CRISPR-generated knock-in cell lines (ATR-S428A and ATR-P429A) were generated with the human A375 melanoma cell line. Based on off-target profile analysis and distance to target site, the gRNA, GTGATGGAATATCACCCAAANGG was used to create single base substitutions in the ATR gene in A375 cell line to create the mutant A375 cell lines.

Cell Lysis and Immunoblotting

Cells were harvested by scraping or trypsinization and lysed with buffer [50 mM Tris–HCl, pH 7.8, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1X protease/phosphatase inhibitor cocktail

(Thermo 1861280)]. 2X SDS loading buffer was added to the lysates before heating at 95°C for 5 min.

Three to eight percentage Tris-Acetate SDS PAGE gradient gels (Invitrogen EA0378) were used to resolve ATR-H from ATR-L; otherwise, standard SDS PAGE gels were used. PVDF membranes (Amersham 10061-492) were used to capture proteins transferred from gels. Chemiluminescent signal was captured using the GE Amersham Imager 680. Antibodies and their dilutions for western blots include pATR S428 (Cell Signaling 2853) 1:1000, PP2Ac (Cell Signaling 2038) 1:1000, GAPDH (Cell Signaling 5174) 1:1000, PARP 1 (Cell Signaling 9532) 1:1000, Bid (Cell Signaling 2002) 1:1000, or (Santa Cruz Biotechnology sc6538) 1: 500, Beta Actin (Invitrogen MA1-140) 1:5000, mtHSP70 (Invitrogen MA3-028) 1:1000, ATR (Bethyl Laboratories, Inc., A300-137A, A300-138A) 1:8000, ATRIP (ABclonal A7139) 1:1000, PP2A-Cα/β (Santa Cruz Biotechnology sc-80665) 1:500, Cleaved Caspase-3 (Cell Signaling 9664) 1:1000, PP4c (Bethyl Laboratories, Inc., A300-835A) 1:8000, PP5c (Bethyl Laboratories, Inc., A300-909A) 1:8000.

RNAi and Plasmid Transfections

Transfections were performed using the Polyplus siRNA (409-10) and DNA transfection reagents (101-10) according to manufacturer's instructions. DNA transfection was into the HCT 116 $ATR^{flox/-}$ cell line.

Cellular Fractionation

Lysis buffers and differential centrifugation fractionated the cells into cytoplasmic and nuclear isolates at 4°C. 10 volumes of the hypo-osmotic cytoplasmic lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 3 mM $CaCl_2$, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 0.1% Triton X-100) with 1X protease and phosphatase inhibitor cocktail was added to resuspend 1 volume of packed cells for 10 min. The suspension was then centrifuged for 7 min at 600 × *g* and the supernatant collected as the cytoplasmic fraction. The pellet (nuclei) was washed twice in ice-cold cytoplasmic lysis buffer, then lysed with 1/10 volume of the nuclear lysis buffer (50 mM Tris–HCl, pH 7.9, 140 mM NaCl, 3 mM $CaCl_2$). After rotation for 15 min at 4°C the nuclear lysate was collected as the supernatant after centrifugation at 10,000 rpm for 10 min at 4°C.

To establish equal protein loading and the quality of all fractionation procedures the separation of cytoplasmic GAPDH from nuclear PARP was assessed by western blotting (WB).

Mitochondrial Isolation

Mitochondrial isolation was performed either by using the Qiagen mitochondrial extraction kit (Qiagen 37612) or according to the established protocol by Frezza et al. (2007). Mitochondrial HSP70 (mtHSP70) protein was used to assess the quality of protein loading from mitochondrial isolates in WB.

Co-immunoprecipitation Assays

The cytoplasm of A549 cells was isolated after PP2Ai pretreatment (where indicated) and UV irradiation. ATR antibody (Bethyl labs) was added at 1 μg/mL for overnight incubation at 4°C. ATR protein was immunoprecipitated (IPed) with magnetic beads (Pierce) for 3 h at 4°C, and then washed three times in co-IP wash buffer (50 mM Tris–HCl, pH 7.6, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.2% Tween-20). The final pellet was resuspended in 1X SDS-loading buffer. The mixture was boiled at 95°C for 5 min. The amounts of Bid and PP2A bound to the pulled-down ATR was determined by WB with Bid and PP2Ac antibodies, respectively (Cell signaling).

Duolink *in situ* Proximity Ligation Assays

The Duolink protein-protein interaction assay was performed according to the manufacturer's instructions (Sigma DUO 92101). Images were captured with a Life Technologies EVOS microscope.

Cellular Viability Assays

MTT assays were performed following the manufacturer's instructions (Cayman's MTT Proliferation Assay kit #10009365).

Statistical Analysis

The statistical analysis of samples was carried out with the student's *t*-test (two-tailed) and one-way ANOVA. A *p*-value of less than 0.05 was taken as significant.

RESULTS

PP2A Dephosphorylates Cytoplasmic ATR-L at Serine 428

To identify the phosphatase that dephosphorylates cytoplasmic ATR at Ser428 human A549 cells were transfected with siRNAs of several phosphatases. The siRNA-knocked down cells then were UV-treated. After a brief recovery, cellular fractionation was performed and cell lysates were analyzed by WB. As shown in **Figure 1A**, the level pATR(Ser428) was greatly reduced in mock-treated (control) cells after UV irradiation (lanes 1 vs. 5). In the siRNA-treated cells only those with PP2Ac knockdown abolished the UV-induced dramatic reduction of pATR(Ser428) level in the cytoplasm as compared to the UV-treated control cells (lanes 5 vs. 6). Interestingly, even in the absence of UV, siRNA knockdown of PP4c and PP5 reduced pATR(Ser428) level dramatically. However, there remained a significant decrease of pATR(Ser428) after UV irradiation with siPP4c and siPP5 knockdown (lanes 5 vs. 7 and 8). Thus, siRNAs targeting of these phosphatases, had little effect on the levels of pATR(S428) in the cytoplasm. Also, the cellular fractionation demonstrates that the effect of PP2A siRNA on pATR(S428) is limited to the cytoplasm of the cell (**Figure 1B**).

To demonstrate the specificity of PP2A in dephosphorylating ATR at Ser 428, an ATR expression construct in which the Ser 428 residue had been mutated to alanine (S428A) was introduced into an $ATR^{flox/-}$ cell line. As shown in **Figure 1C**, the mutation abolished the effect of UV-induced PP2A dephosphorylation on ATR S428 in the cytoplasm. In addition, the Duolink *in situ* proximity ligation assay (PLA) showed that PP2A interacted directly with ATR (**Figure 1D**) to perform its dephosphorylating activity. The increased interaction was highest at 1 h but was still

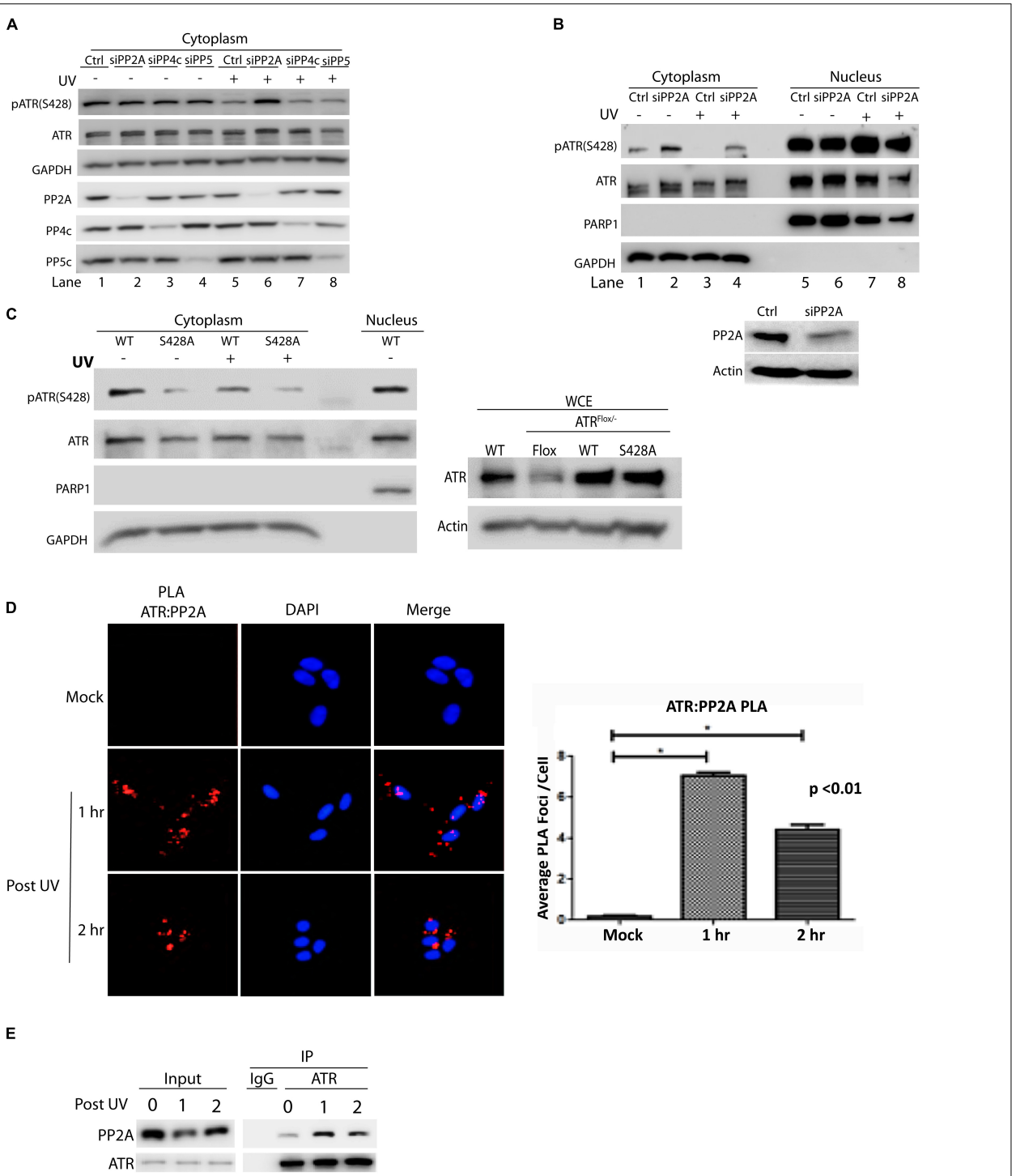

FIGURE 1 | Protein phosphatase 2A (PP2A) specifically dephosphorylates cytoplasmic ATR at S428 and its action involves direct binding to ATR. **(A)** A549 cells were treated with siRNAs against three protein phosphatases (PP2Ac, PP4c, and PP5c) followed by UV treatment at 40 J/m^2 with a 2 h recovery. Analysis of cytoplasmic extracts reveals that PP2A is required to dephosphorylate cytoplasmic pATR (S428). **(B)** siRNA knockdown of PP2Ac in A549 cells was followed by UV treatment at 40 J/m^2 with a 2 h recovery. pATR (S428) levels are increased in cytoplasm with PP2Ac knockdown while the phosphorylation status of ATR in the nucleus remains unchanged. WB of whole cell extracts shows PP2A siRNA knockdown efficiency in A549 cells **(C)** HCT 116 ATR$^{flox/-}$ cells were transfected with N-terminal Flag-tagged wild-type (WT) or S428A mutant ATR expression constructs and UV treated at 40 J/m^2 with a 2 h recovery. Analysis of the cytoplasmic extracts reveals that PP2A specifically targets the pATR (S428) residue. The pATR (S428) observed in the S428A cells reflects some phosphorylation of the residual endogenous ATR remaining in the ATR$^{flox/-}$ cells (right panel). **(D)** PLA revealing that in A549 cells there is a direct interaction between ATR and PP2A, especially after UV irradiation at 40 J/m^2. A DAPI-staining overlay is used to show location of the nuclei. The bar graph represents a statistical analysis of the PLA images. **(E)** A549 cells treated with UV at 40 J/m^2 were lysed, followed by co-immunoprecipitation of PP2A using anti-ATR antibody. *Stands for p value < 0.01.

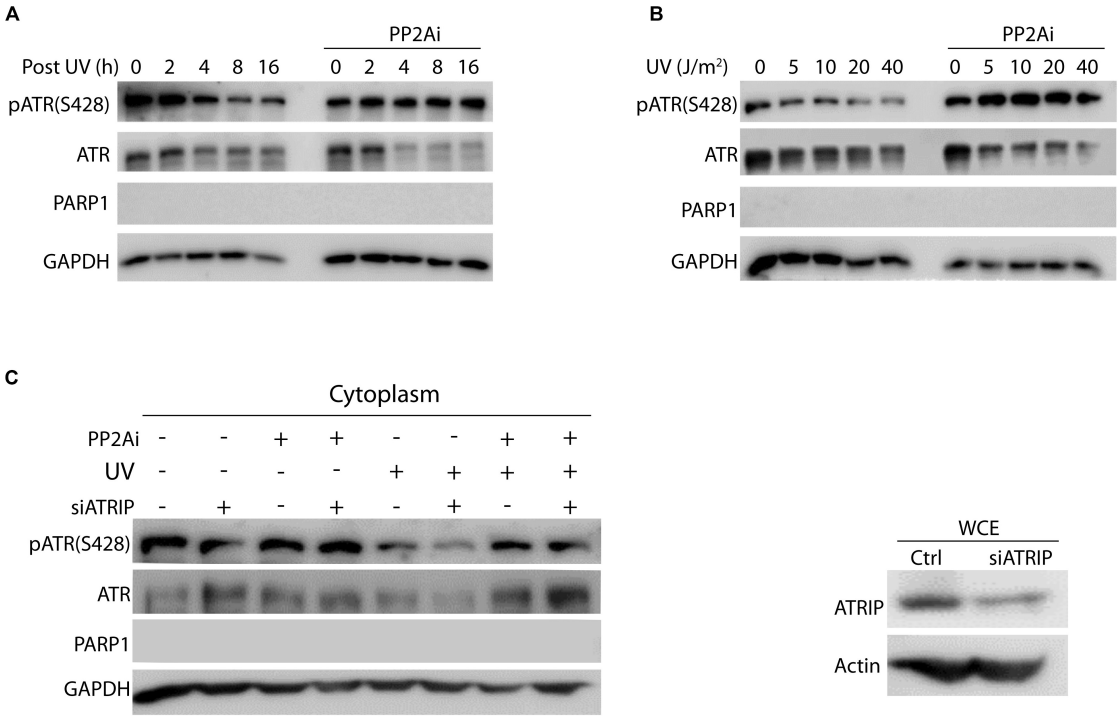

FIGURE 2 | Protein phosphatase 2A (PP2A) dephosphorylates cytoplasmic pATR (S428) in a UV dose- and recovery time-dependent manner and is independent of ATRIP. **(A)** Cytoplasmic pATR (S428) dephosphorylation by PP2A is dependent on post-irradiation recovery time in A549 cells. The WB shows that the level of ATR phosphorylation at S428 depends on the recovery time following UV irradiation at 40 J/m^2. PP2A inhibitor treatment (PP2Ai) abolishes this dephosphorylation at all recovery times. **(B)** Cytoplasmic pATR (S428) dephosphorylation by PP2A is UV dose dependent and is attenuated with PP2A inhibitor treatment of A549 cells. Control A549 cells show a reduction in the level of pATR (S428) that is dependent on the dose of UV irradiation used, but retain their level of pATR (S428). **(C)** A549 cells, depleted of ATRIP by siRNA knockdown, were divided into two groups with one treated with PP2A inhibitor and the other left as a control. WB assay shows that this ATRIP knockdown does not affect PP2A's dephosphorylation of pATR (S428) in the cytoplasm of A549 cells. The right panel shows the efficiency of the ATRIP knockdown.

significant at 2 h following UV irradiation. These results were confirmed with co-immunoprecipitation of PP2A with anti-ATR antibody (**Figure 1E**), indicating that PP2A did interact with the ATR to dephosphorylate phospho-S428.

PP2A Dephosphorylates Cytoplasmic ATR at S428 Independent of ATRIP and PP2A's Activity Is UV Dose-Dependent

It is well known that ATR's nuclear function in the DNA damage signaling is dependent on ATRIP. To determine if PP2A dephosphorylation of cytoplasmic pATR(S428) and, therefore, the formation of *cis* ATR requires ATRIP, ATRIP-knockdown cells were UV-treated, then fractionated and the cytoplasmic fraction analyzed by WB (**Figure 2**). The level of pATR(S428) was increased with PP2A inhibitor treatment in both the presence and absence of ATRIP (**Figure 2C**). Also, we observed no significant difference in dephosphorylation of pATR(S428) by PP2A in the cytoplasm between cells with or without ATRIP depletion (**Figure 2C**). This suggests that in the absence of ATRIP, the nuclear binding partner of ATR, PP2A in the cytoplasm was still able to dephosphorylate ATR at S428. This is consistent with ATRIP depletion having no effect on cytoplasmic ATR-H formation and its anti-apoptotic function at the mitochondria (Hilton et al., 2016). In addition, to investigate whether the activity of PP2A was dependent on post-irradiation recovery time or UV dose, cells were UV treated and allowed to recover for 0–16 h or irradiated at different doses of UV followed by a 2 h recovery before cellular fractionation and WB analysis. The UV-induced depletion of cytoplasmic pATR (S428) (shown in **Figure 1B**) occurred in both a recovery time- (**Figure 2A**) and a dose- (**Figure 2B**) dependent manner. This is consistent with the earlier reported data that ATR-H (*cis* ATR) formation in the cytoplasm was UV dose- and post-UV recovery time-dependent given that Ser428 dephosphorylation promotes ATR-H formation (Hilton et al., 2016). However, PP2A inhibitor reversed the pattern by significantly increasing the pATR level in both time- and dose-dependent manners, likely leading to the relatively more ATR-L formation than ATR-H (Hilton et al., 2016).

PP2A Dephosphorylation of ATR-L (*trans*-ATR) Promotes the Formation of ATR-H (*cis* ATR) in the Cytoplasm

To investigate whether phosphorylation of cytoplasmic ATR at S428 affects the formation of ATR-H (*cis* ATR) and ATR-L (*trans* ATR) in the cytoplasm, PP2A was knocked down by PP2A siRNA, followed by UV irradiation of the cells, cellular

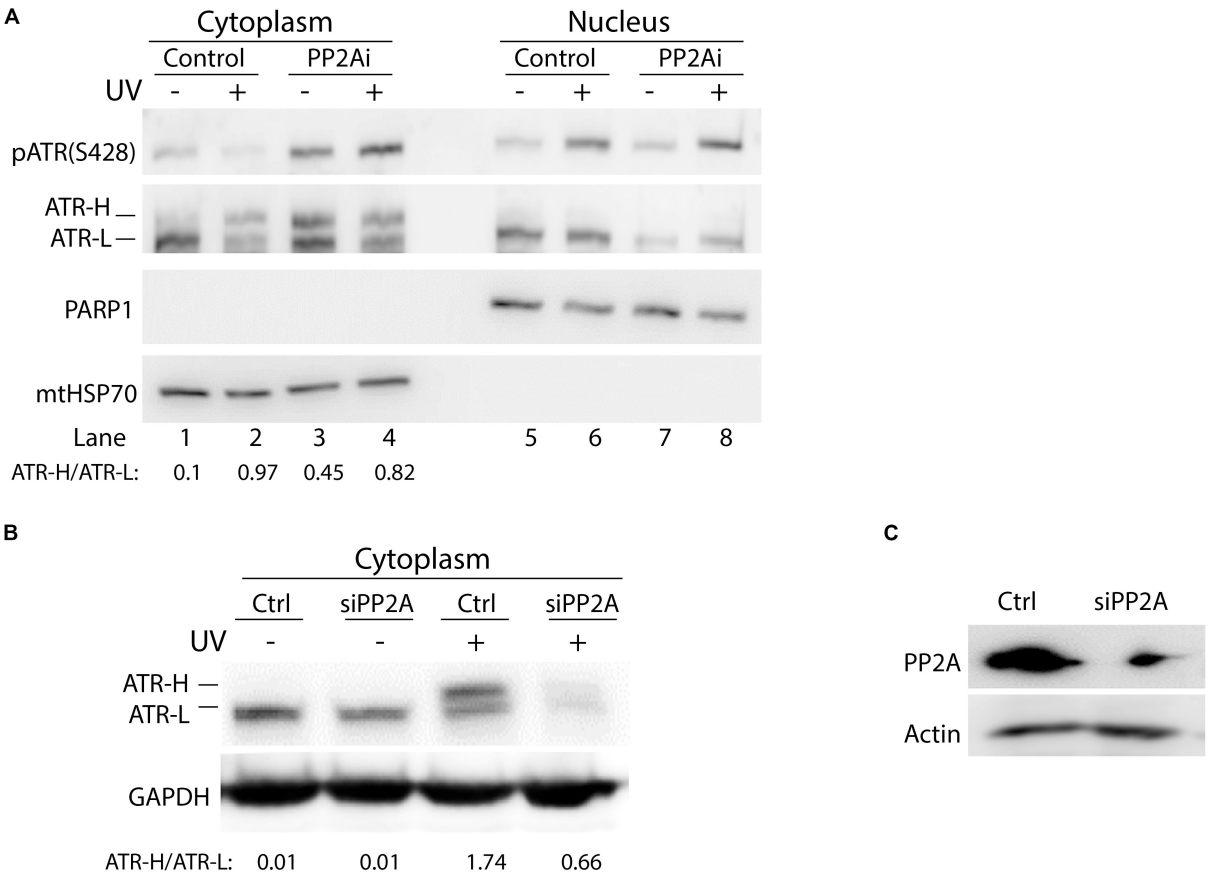

FIGURE 3 | Protein phosphatase 2A (PP2A) dephosphorylation of pATR changes ATR to the higher form (ATR-H). **(A)** UV irradiation of A549 cells at 40 J/m^2 increases the formation of ATR-H in the cytoplasm. Also, the ATR-H is not phosphorylated while the ATR-L band is phosphorylated in both the nucleus and the cytoplasm (cytoplasm to nucleus loading ratio = 3:1). The samples were analyzed on a 3–8% gradient gel. **(B)** A549 cells were pretreated with DMSO (Ctrl) or transfected with PP2A siRNAs, then UV-irradiated at 40 J/m^2 (–/+UV) followed by a 2 h recovery. The cytoplasmic fraction was analyzed by electrophoresis through a 3–8% gradient gel and WB to show the form of ATR in the cytoplasm. PP2A depletion reduces the levels of cytoplasmic ATR and the amount of ATR-H formed following UV treatment. **(C)** WB showing PP2A siRNA knockdown efficiency in A549 cells.

fractionation, 3–8% gradient gel electrophoresis and WB. In the control cells, as expected, pATR (S428) levels were reduced with UV damage as compared to that without UV (**Figure 3A**). More ATR was found in ATR-L form relative to the ATR-H form in the cells in the absence of UV treatment, while the level of ATR-H relative to ATR-L increased and accumulated in UV-treated cells (**Figures 3A,B**). The latter is due to the UV-induced inhibition of Pin1 activity (Hilton et al., 2016). However, ATR-H accumulation is reversed by PP2A depletion, which kept ATR in the *trans* form in non-irradiated cells, and also promoted *trans* ATR formation in UV-irradiated cells (**Figures 3B,C**). It should be noted that in **Figure 3A**, PP2A inhibition resulted in an unexpected amount of ATR-H in the cytoplasm of the cells without UV treatment. This is probably due to the known non-specific activity of LB-100 relative to the siRNA treatment, which is much more specific and showed no such effects (**Figure 3B**). Despite this, the patterns for the data of **Figures 3A,B** remain the same as either PP2A inhibition or siRNA depletion decreases the ATR-H/ATR-L ratios in UV-treated cells, while UV increases the ratio in cells without PP2A inhibition or depletion.

It is worth noting that in cells without PP2Ai treatment, UV irradiation consistently reduced cytoplasmic pATR (Ser428) (**Figure 1B**), but increased pATR (Ser428) in the nucleus. These observations suggest that phosphorylation of ATR on Ser428 is regulated differently in the cytoplasm and the nucleus of cells in response to DNA damage. This further confirms that ATR functions in the nucleus differ significantly from its roles in the cytoplasm.

Inhibition of PP2A Reduces the Association of ATR-H With tBid at Mitochondria

We reported previously that upon UV irradiation ATR-H directly interacts with proapoptotic protein tBid at mitochondria, blocking Bax-tBid interaction and thus preventing activation of apoptosis (Hilton et al., 2016). Given that PP2A dephosphorylates pATR-L (*trans*-ATR) at Ser428 and, thus, regulates ATR-H formation, we determined the effects of PP2A inhibition on this ATR-tBid interaction. We examined the ATR-tBid binding in two

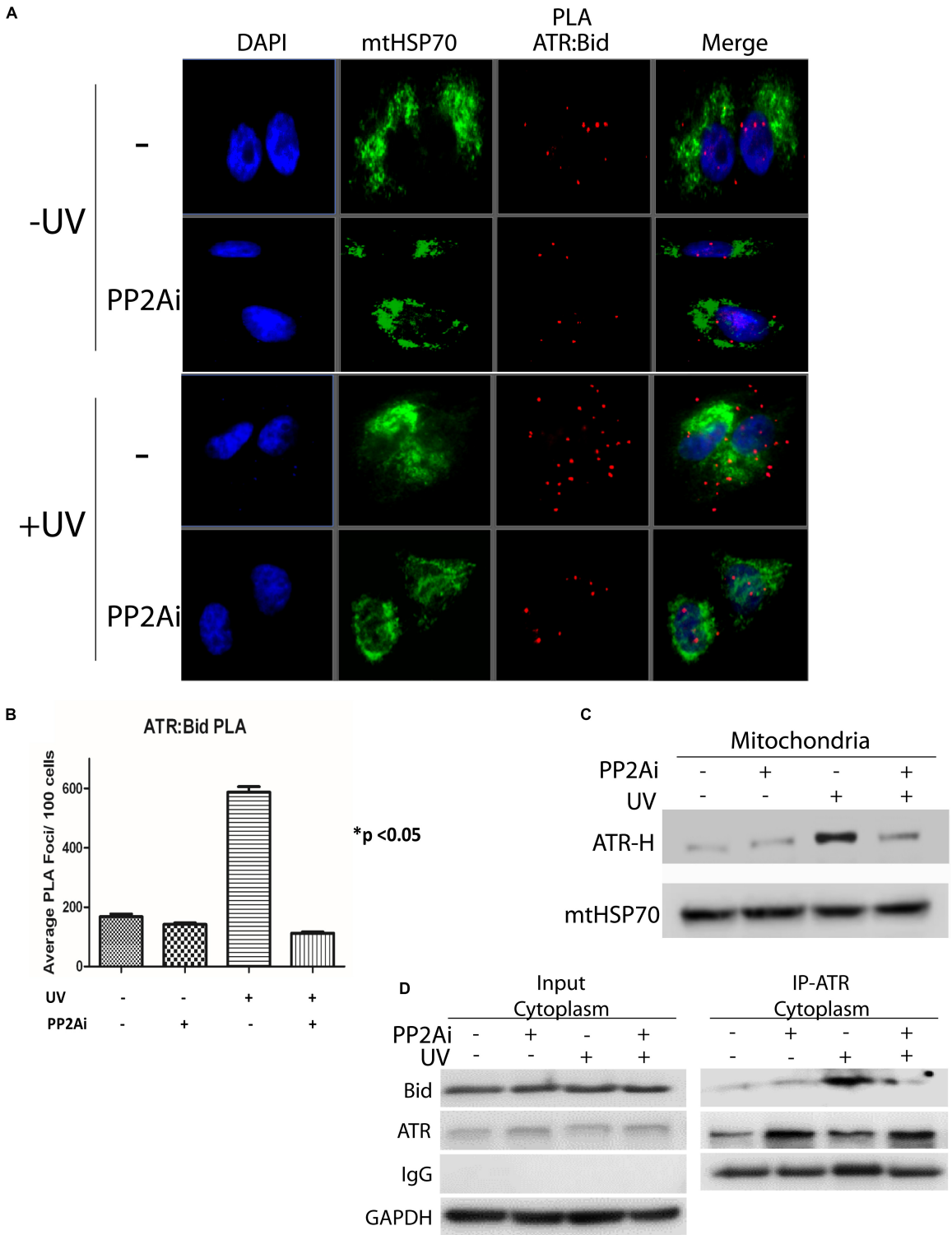

FIGURE 4 | Inhibition of PP2A's dephosphorylation of pATR and ATR-L accumulation in the cytoplasm leads to reduced association of ATR with tBid on mitochondria. **(A)** PLA shows that cytoplasmic ATR directly associates with Bid and that this association increases with UV treatment, but the UV-induced association is attenuated by PP2A inhibitor treatment. The nuclei are stained with DAPI and mitochondria are indicated by mtHSP70 immunofluorescence. A549 cells were UV irradiated at 40 J/m^2 with a 2 h recovery. **(B)** A graphic display of the PLA data shown in **(A)**. The $p < 0.05$ refers to the sample (+UV, −PP2Ai) versus any of other samples. **(C)** ATR-H accumulation at the mitochondria increases with UV treatment at 40 J/m^2 in A549 cells, but is reduced in cells treated with PP2A inhibitor. **(D)** Biochemical confirmation of the reduction in the cytoplasmic ATR:tBid association observed by PLA **(A,B)**. A549 cells were pretreated with PP2Ai followed by UV irradiation with a 2 h recovery. After cellular fractionation ATR was immunoprecipitated from the cytoplasmic fraction using C-terminal-specific ATR antibody. The association of tBid with ATR was confirmed by WB.

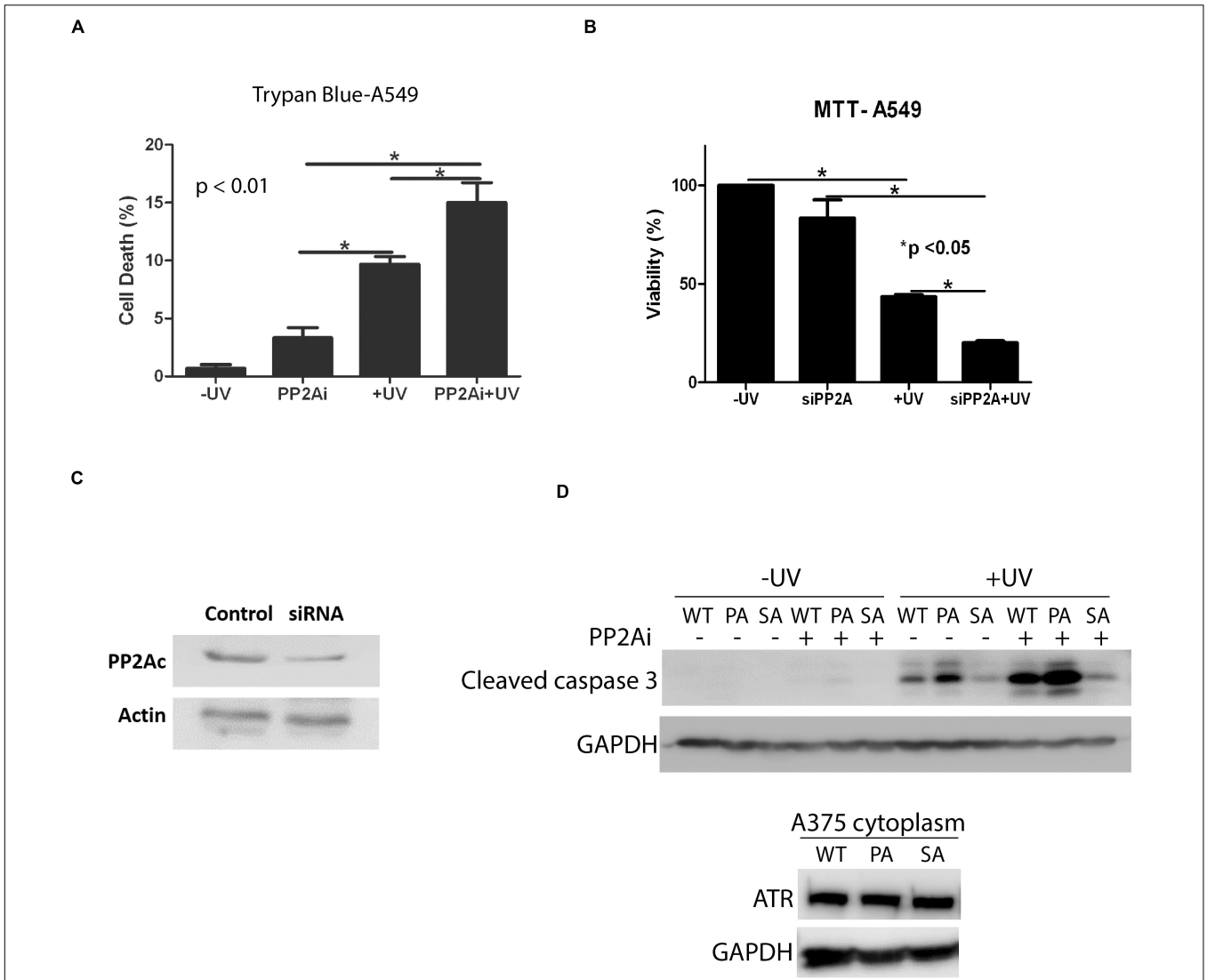

FIGURE 5 | Inhibition of PP2A decreases cellular viability which is enhanced UV-induced DNA Damage. **(A)** A trypan blue exclusion assay shows that PP2A depletion with siRNA knockdown increases apoptosis in A549 cells and that the level of apoptosis is increased further by UV irradiation at 40 J/m^2 with a 24 h recovery time. **(B)** MTT assay showing increased levels of apoptosis in A549 cells depleted of PP2A by siRNA knockdown which is enhanced by UV irradiation at 40 J/m^2 and a 24 h recovery time. **(C)** WB showing PP2A siRNA knockdown efficiency in A549 cells. **(D)** WB shows the caspase 3 cleavage assay which indicates the apoptosis activation, in transgenic A375 human melanoma cells expressing wild type ATR (WT), ATR-P429A (PA) or ATR-S428A (SA) 24 h following UV irradiation in the presence and absence of PP2Ai treatments. *Stands for *p* value < 0.01 or 0.05.

ways. First, using the Duolink *in situ* proximity ligation assay (PLA) we demonstrated that the UV-induced direct interaction between ATR and tBid is significantly reduced when PP2A is inhibited (**Figures 4A,B**). This finding was corroborated by isolating mitochondria from UV-irradiated cells with or without PP2A inhibition (**Figure 4C**). The results show that there is significantly less ATR-H at mitochondria when the PP2Ai is present. The inhibition significantly reduced the amount of mitochondria-associated ATR-H (**Figure 4C**). We further confirmed these findings with a co-immunoprecipitation (co-IP) assay of tBID-ATR complexes in the cytoplasmic fraction of treated cells (**Figure 4D**). The immunoprecipitated ATR from PP2A-inhibited UV-irradiated cells had less associated tBid protein than that from non-inhibited cells. These data suggest that PP2A inhibition would result in increased cell death since ATR-H needs to be associated with tBID at mitochondria for it to exert its anti-apoptotic function (Hilton et al., 2016).

Inhibition of PP2A Decreases Cellular Viability in Response to UV-Induced DNA Damage

Next, we investigated the effect of PP2A inhibition on the apoptotic cell death induced by UV. **Figure 5A** shows that depletion of active PP2A led to a significant increase in A549 cell death at 24 h following UV damage in comparison with

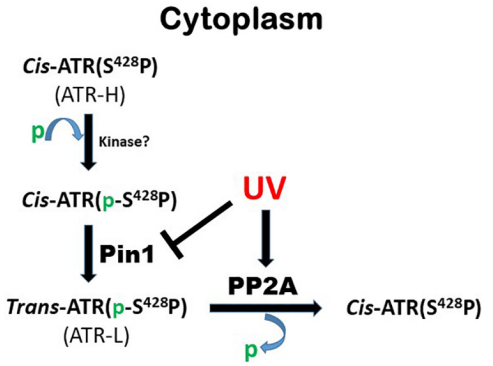

FIGURE 6 | Summary of role of PP2A in Pin1-mediated ATR isomerization in the cytoplasm.

the cells without PP2A inhibition. The results of trypan blue exclusion assay also were confirmed using the colorimetric MTT assay (**Figure 5B**). Note that PP2A depletion, either by inhibition or by knockdown, had a smaller but significant effect on cell viability without UV irradiation. Furthermore, we assess the cytoplasmic cleaved caspase-3 levels in transgenic knock-in ATR-S428A and ATR-P429A human melanoma cells (A375 cells) (**Figure 5D**). Opposite effects on apoptosis are observed between ATR-S428A and ATR-P429A cells because the ATR-P429A cells contained only *trans* ATR, and thus, there was little or no *cis* ATR (*cis* ATR null) in the cells. Since *cis* ATR is antiapoptotic, this means ATR-P429A is pro-apoptotic and ATR-P429A cells are more sensitive to apoptotic signals than wild-type A375 cells, thus resulting in greater cell death. In contrast, ATR-S428A cells contain an overwhelming amount of antiapoptotic *cis* ATR in the cytoplasm which significantly reduced the apoptotic activity. Together, these results strongly suggest that the effects of PP2A dephosphorylation of ATR at Ser428 may have a direct impact on the DNA damage-induced cell death.

DISCUSSION

As summarized in **Figure 6**, this work identifies PP2A as the phosphatase that dephosphorylates ATR at Ser428 following UV-induced DNA damage. PP2A mediated the reduction of pATR (S428) in the cytoplasm following UV treatment (**Figure 1A**). Since Pin1's activity requires the Ser428-Pro429 recognition site in ATR to be phosphorylated, PP2A antagonizes Pin1's action by depleting the amounts of cytoplasmic pATR at S428. This PP2A control over the phosphorylation status of ATR at the Pin1 recognition motif adds another level of regulatory complexity to the DDR process. Following DNA damage, PP2A interacts with ATR (**Figures 1D,E**) to dephosphorylate its Pin 1 recognition motif to prevent the further isomerization of *cis* to *trans* ATR in the cytoplasm, resulting in *cis* ATR-H accumulation. In addition, PP2A's dephosphorylation of ATR at S428 is ATRIP independent. This further highlights how unique and distinct cytoplasmic ATR is from its nuclear counterpart which requires its partner ATRIP to perform its kinase activities following DNA damage. We found that PP2A activity led to the accumulation of *cis* ATR-H in the cytoplasm and, then, at the mitochondria following DNA damage (**Figure 4C**). Mitochondrial *cis* ATR-H is an antiapoptotic protein that binds to tBid (**Figures 4A,B,D**) to prevent activation of apoptosis and to suppress DNA damage-induced apoptotic cell death (**Figures 5A–C**). It should be noted that PP2A inhibition appears to reduce the ATR protein level in cells treated with UV (**Figure 2A,B**). It is possible that the decrease in ATR level could be due to the degradation of ATR in the cells undergoing apoptosis caused by depleted PP2A activity. Importantly, however, the pATR level that signals the Pin1-mediated ATR isomerization pathway was significantly increased, leading to relatively more ATR-L formation than ATR-H (Hilton et al., 2016).

Protein phosphatase 2A depletion led to reduced levels of UV-induced antiapoptotic ATR-H in the cytoplasm. While this is true, the study also showed that a marginal level of ATR-H remained in the cytoplasm or at mitochondria, even after PP2A depletion (**Figure 3B**). This is consistent with our previous observation that UV can inactivate Pin1 via DAPK1 phosphorylation of Pin1 at Ser71 (Hilton et al., 2016). Therefore, a combination of effects from Pin1-Ser71 phosphorylation by DAPK1 (Lee et al., 2011) and PP2A dephosphorylation of ATR at Ser428 may result in the ultimate formation of antiapoptotic *cis* ATR-H at mitochondria. Unfortunately, the kinase(s) for phosphorylation of ATR-Ser428 remains unidentified. Overall, these processes should be considered as possible candidates in developing new targets for adjuvant chemotherapy.

Apoptosis is an important cellular process that is tightly regulated by the cell, and in the cytoplasm a balance between ATR-H and ATR-L levels needs to be maintained for proper homeostasis. Pin1 is central to the regulation of this balance since it catalyzes the phosphorylation-dependent isomerization that converts *cis* ATR-H to *trans* ATR-L in the cytoplasm. The proper balance ensures a functional and effective DNA damage response as the protection of cells from apoptosis is essential for the activities of cell cycle checkpoint arrest and DNA repair. Our findings reported in this study suggest that PP2A plays an important role in regulating this balance.

Our results reveal a new mechanism by which the cell can regulate mitochondrial apoptosis. Because deregulated apoptosis is a key hallmark in carcinogenesis, the involvement of PP2A in the regulation suggests the importance of the phosphorylation status of cytoplasmic Ser428 of ATR in carcinogenesis and cancer treatment. Indeed, this is supported by the significant correlation of the dephosphorylation status of cytoplasmic ATR (Ser428) with the severity of ovarian cancer in patients such as advanced stage, serous histology, large residual mass and so on (Lee et al., 2015).

Our findings indicate that ATR-mediated regulation of the mitochondrial cell death pathway can be altered by changing the phosphorylation status of pATR at S428. Because the PP2A regulation of pATR (S428) status is localized to the cytoplasm, modulating this ATR phosphorylation should have minimal

effects on the DNA damage-signaling pathway in the nucleus. This defines PP2A as a potential target in regulating ATR's anti-apoptotic activity. It is well documented that PP2A is a heterotrimeric protein of various subunit isoforms, particularly the regulatory subunit B, each with distinct functions (Janssens and Goris, 2001; Cho and Xu, 2007; Seshacharyulu et al., 2013; Sangodkar et al., 2016; Wlodarchak and Xing, 2016; Reynhout and Janssens, 2019). To achieve a specific inhibition of PP2A for its activity at ATR (Ser428) to reduce potential adverse side effects, identification of the regulatory subunit isoform specifically responsible for ATR (Ser428) dephosphorylation is necessary though it is out of scope of this study.

In summary, our results have allowed a better understanding of the mechanism of how ATR's anti- apoptotic activity at the mitochondria is regulated. This also opens up the possibilities of targeting PP2A for potential translational applications.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

YM performed most of the experiments and wrote the draft of the manuscript. BC started the project and generated the data during the early period of the study. HB performed some of the experiments during manuscript revision. PM participated in experimental design, data analysis, and manuscript preparation. ZL provided help to the experiments. YZ is the senior author who oversaw and directed this study from the beginning through the manuscript preparation. All authors contributed to the article and approved the submitted version.

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