

ORIGINAL ARTICLE

Protein kinase C α activation switches YAP1 from TEAD-mediated signaling to p73-mediated signaling

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Abstract

Yes-associated protein 1 (YAP1) interacts with TEAD transcription factor in the nucleus and upregulates TEAD-target genes. YAP1 is phosphorylated by large tumor suppressor (LATS) kinases, the core kinases of the Hippo pathway, at 5 serine residues and is sequestered and degraded in the cytoplasm. In human cancers with the dysfunction of the Hippo pathway, YAP1 becomes hyperactive and confers malignant properties to cancer cells. We have observed that cold shock induces protein kinase C (PKC)-mediated phosphorylation of YAP1. PKC phosphorylates YAP1 at 3 serine residues among LATS-mediate phosphorylation sites. Importantly, PKC activation recruits YAP1 to the cytoplasm even in LATS-depleted cancer cells and reduces the cooperation with TEAD. PKC activation induces promyelocytic leukemia protein-mediated SUMOylation of YAP1. SUMOylated YAP1 remains in the nucleus, binds to p73, and promotes p73-target gene transcription. Bryostatin, a natural anti-neoplastic reagent that activates PKC, induces YAP1/p73-mediated apoptosis in cancer cells. Bryostatin reverses malignant transformation caused by the depletion of LATS kinases. Therefore, bryostatin and other reagents that activate PKC are expected to control cancers with the dysfunction of the Hippo pathway.

KEYWORDS

Hippo pathway, promyelocytic leukemia protein, protein kinase C, SUMOylation, TEAD, TP73, YAP1

1 | INTRODUCTION

Yes-associated protein 1 (YAP1) was originally discovered as a WW domain-containing SRC kinase-binding protein.¹ YAP1 is distributed in the cytoplasm and in the nucleus.² YAP1 interacts with various transcription factors such as the TEAD family proteins, SMAD proteins, RUNX, and p73 to regulate gene transcription in the nucleus,

while YAP1 undergoes proteasome degradation in the cytoplasm.²⁻⁷ Therefore, cytoplasmic YAP1 is thought to be inactive, while nuclear YAP1 is considered to be active. The best characterized regulatory mechanism of the subcellular distribution of YAP1 is its phosphorylation mediated by large tumor suppressor kinases (LATS1/2), the core kinases of the tumor suppressive Hippo pathway. LATS1/2 phosphorylate YAP1 at 5 serine residues.² The phosphorylation at

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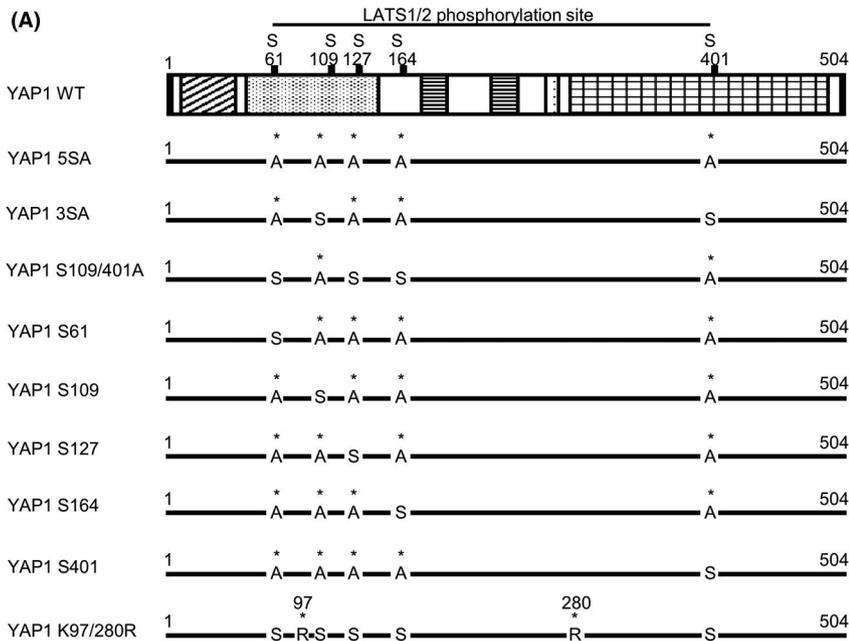


FIGURE 1 YAP1 is phosphorylated by protein kinase C α at serines 61, 127, and 164. **A**, Schematic drawings of YAP1 constructs. A, K, R, and S stand for alanine, lysine, arginine, and serine. In this study, YAP1 isoform 1 with 504 amino acids was used. **B**, HEK293FT cells were transfected with the pCIneoFHF-YAP1 constructs and pCIneoMCherry-PKC α as indicated. At 48 h later, cell lysates were run on Phos-tag (upper) and conventional SDS-PAGE (2 lower) gels immunoblotted with anti-FLAG and anti-mCherry antibodies. **C**, HEK293FT cells were transfected with pCIneoFHF-YAP1 or pCIneoFHF-YAP1 3SA. At 48 h later, the cells were treated with either control DMSO or TPA/A23187 (the mixture of 0.1 nmol/L TPA and 10 μ mol/L A23187) for 60 min. Cell lysates were run on Phos-tag (top) and conventional SDS-PAGE (bottom) gels and immunoblotted with anti-FLAG antibody. **D**, U2OS cells were pre-treated with 2 μ mol/L Go 6976 for 3 h and then treated with TPA/A23187. Endogenous YAP1 was detected with anti-YAP1 antibody

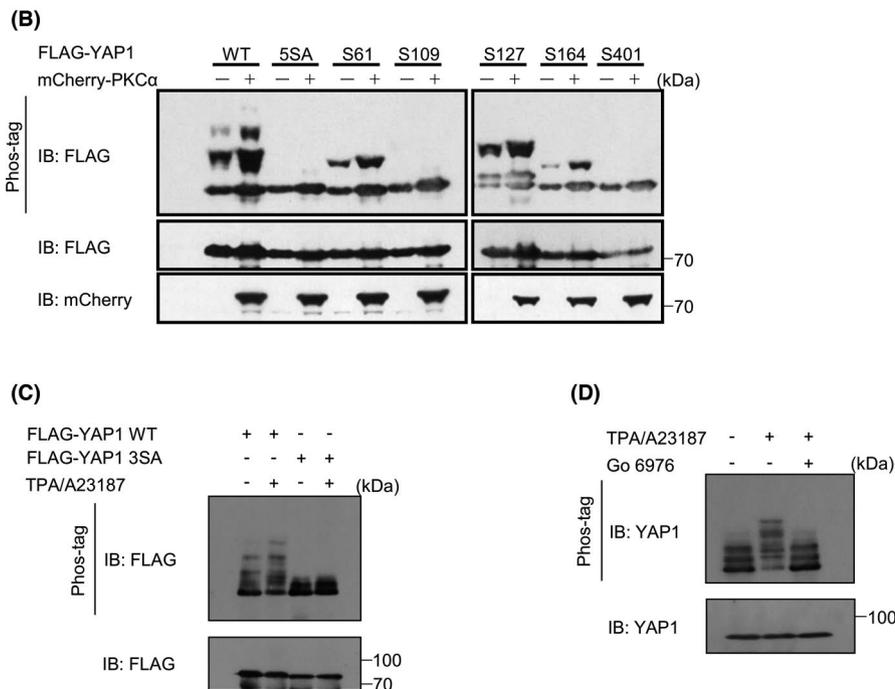
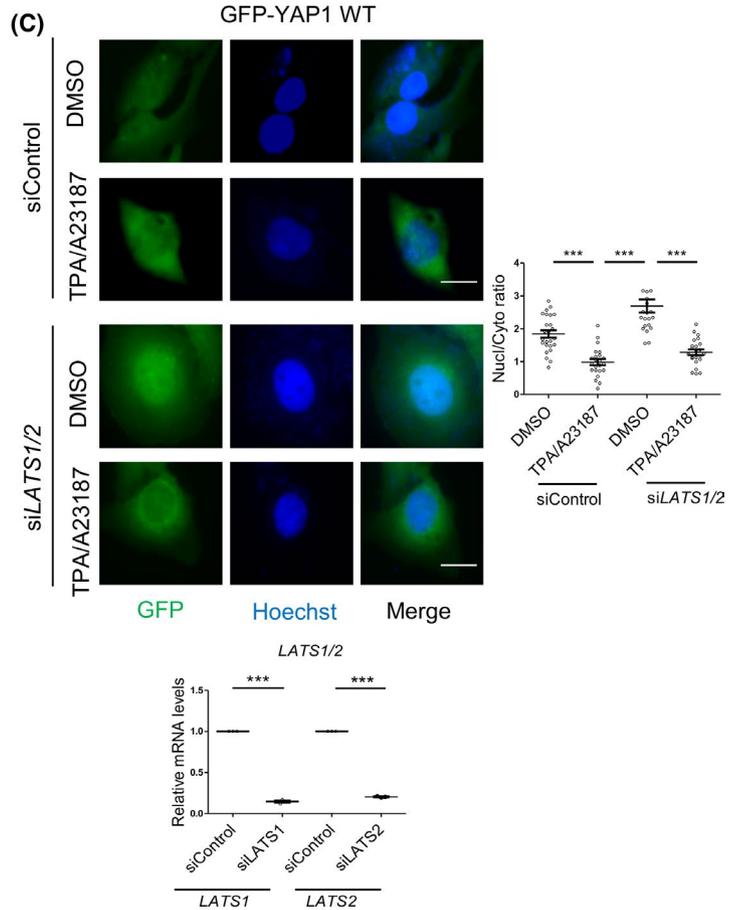
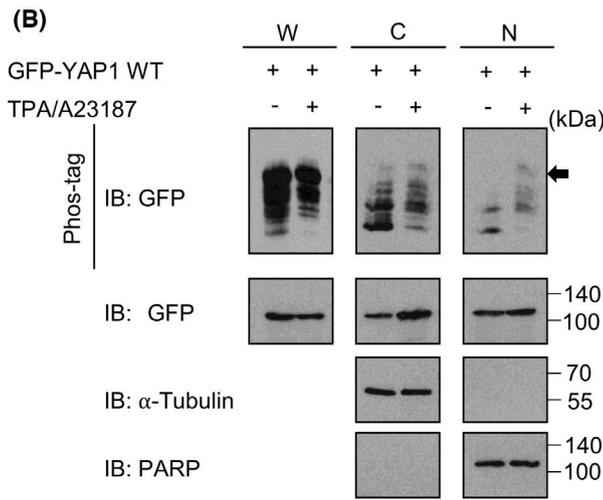
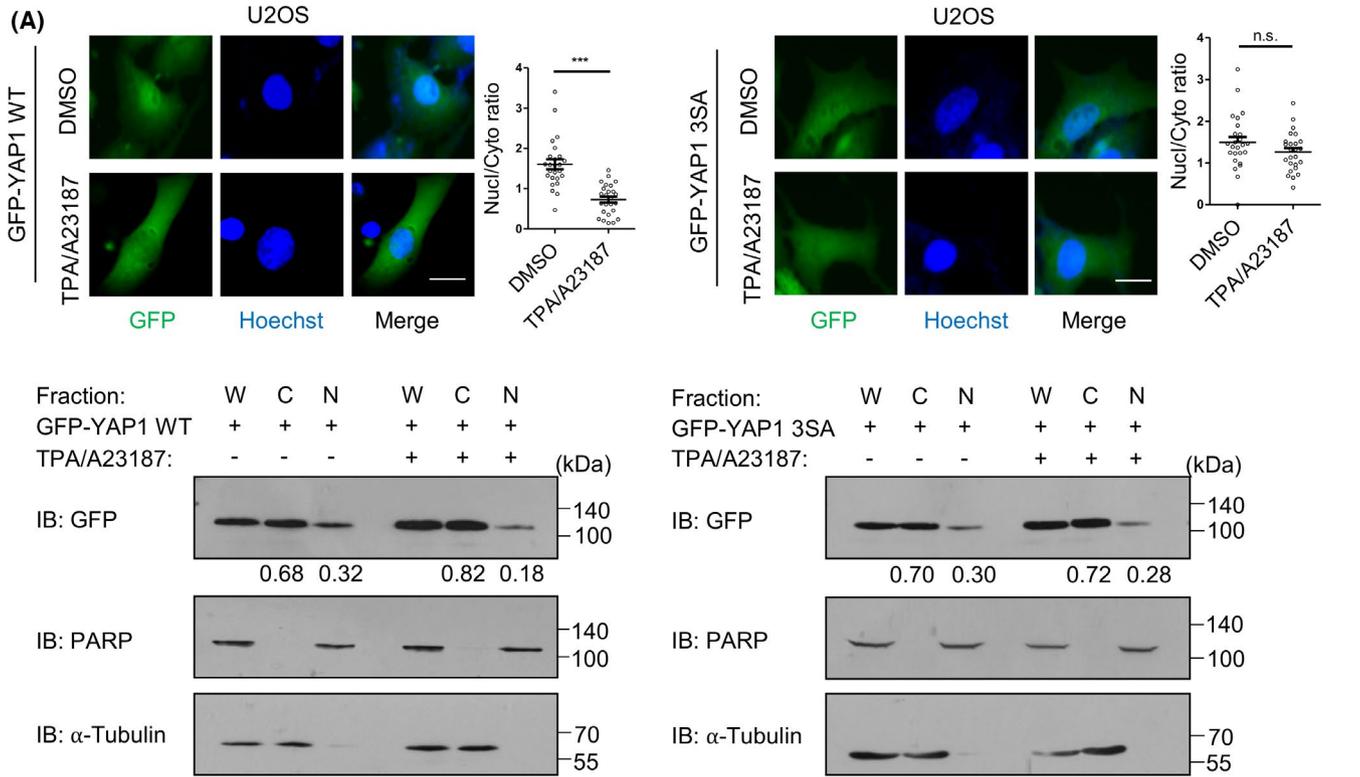


FIGURE 2 TPA/A23187 treatment shifts YAP1 to the cytoplasm. **A**, U2OS cells expressing GFP-YAP1 (GFP-YAP1 WT) or GFP-YAP1 3SA were plated at 3×10^5 cells/well in 6-well plates and were treated with TPA/A23187 for 1 h. Nuclei were visualized with Hoechst 33342 stain (Hoechst). Scale bar, 25 μ m. GFP-YAP1 in the cytoplasm and in the nucleus was evaluated by ArrayScan in 500 cells. n.s., not significant; and ***, $P < .001$. Subcellular fractionation was performed. Comparable amounts of the whole cell lysate (W), the cytoplasmic fraction (C), and the nuclear fraction (N) were immunoblotted with indicated antibodies. α -Tubulin and PARP were used as the cytoplasmic and nuclear markers. The signals were measured using ImageJ software. The values for the sum of the cytoplasmic and nuclear fractions were set at 1.00. The experiments were repeated 3 times and the representative results were demonstrated. **B**, Subcellular fractionation was performed as in (A) and the whole cell lysate, the cytoplasmic fraction, and the nuclear fraction were run on Phos-tag gels and immunoblotted with the indicated antibodies. Phosphorylated YAP1 was detected in the nuclear fraction of TPA/A23187-treated cells (arrow). **C**, 1×10^6 U2OS-GFP-YAP1 cells in a 10-cm dish were transfected with siRNAs against *LATS1* and *LATS2*. At 48 h later, the cells were replated at 3×10^5 cells/well in 6-well plates. At 24 h later, the cells were treated with DMSO or TPA/A23187 for 1 h. The distribution of GFP-YAP1 was evaluated as in (A). The suppression of *LATS1* and *LATS2* was confirmed by qRT-PCR. *** $P < .001$



serine 127 generates a 14-3-3-binding motif, so that YAP1 is trapped by 14-3-3 in the cytoplasm. The phosphorylation of serine 401 triggers YAP1 degradation. Therefore, LATS1/2 negatively regulate YAP1. YAP1 is also phosphorylated by other kinases. SRC- and YES-mediated tyrosine phosphorylation increases the nuclear YAP1 to enhance TEAD-dependent gene transcription, while ABL- or JNK-mediated phosphorylation promotes the cooperation with p73 to induce apoptosis.^{5,8,9} CDK1-mediated phosphorylation may be involved in the regulation of cytokinesis.¹⁰

When cells reach confluency or when cells are exposed to DNA damage, the Hippo pathway is switched on and YAP1 is recruited to the cytoplasm.^{2,11,12} Conversely, mechanical stretch and metabolic stimuli induce the nuclear accumulation of YAP1.¹³⁻¹⁵ Recently, we and others have reported that YAP1 is transiently accumulated in the nucleus after heat shock and spontaneously returns to the cytoplasm.^{16,17} These findings indicate that YAP1 activity is continuously modulated to adapt to changes in the cellular environment. After observing the effect of heat shock on YAP1, we raised a naïve question what would happen to YAP1 when cells are exposed to cold shock. It turned out that cold shock at 4°C triggers YAP1 phosphorylation and that protein kinase C (PKC) is involved in this phosphorylation. Atypical PKC is reported to repress YAP1.¹⁸⁻²⁰ In terms of classical PKC, it is discussed using myristoylated constitutive active PKC α that its activation suppresses LATS kinase activity and eventually activates YAP1.¹⁸ However, whether and how classical PKC-mediated phosphorylation of YAP1 affects YAP1 activity has not yet been directly examined. In this study, we report that PKC α phosphorylates YAP1 at 3 serine residues and that PKC-mediated phosphorylation promotes p73-dependent gene transcription. Moreover, we also demonstrate that bryostatin, a natural compound, which activates PKC, reverses the malignant properties of cancer cells caused by the dysfunction of the Hippo pathway.

2 | MATERIALS AND METHODS

2.1 | DNA constructions

pCIneoHA, pCIneoFHF, pCIneoGFP, pCIneoCherry, and pCIneoLuc were prepared from pCIneo (Promega) and described previously.²¹⁻²³ YAP1, p73, and TEAD4 constructs were prepared using these vectors. The YAP1 5SA mutant was generated in a previous study.²⁴ YAP1 3SA, YAP1 S61, YAP1 S109, YAP1 S127, YAP1 S164, YAP1 S401, YAP1 S109/401A, and YAP1 K97/280R were prepared based on either YAP1 5SA or YAP1 using PCR. The primers are listed in Table S1. For instance, PCR was performed with the indicated primers (YAP1 start/YAP1 S61 rev and YAP1 S61 for/YAP1 end) on YAP1 5SA to recover serine 61 and generate YAP1 S61. pLenti-EF-EmGFP-LATS1/2 KD has been described previously.²⁵ pLL3.7-ires-puro was prepared by replacing the blasticidin resistance gene of the previously described pLL3.7-ires-blast vector with the puromycin resistance gene.²⁶

pLL3.7-puro-FHF-YAP1 3SA was prepared by ligating FHF-YAP1 3SA into the pLL3.7-ires-puro vector. cDNA for PKC α was obtained by PCR on human lung and kidney cDNA libraries (Clontech). pLNCX-HA-hPML IV, 8xGT-IIC- δ 51LucII luciferase reporter (TEAD reporter), and pCMV alkaline phosphatase were gifts from Issay Kitabayashi (National Cancer Research Center Institute), Hiroshi Sasaki (Osaka University), and Sumiko Watanabe (The University of Tokyo), respectively.^{27,28} pCIneoHA-SUMO was generated from pBudCGFP-SUMO.²⁹

Other Materials and methods are described in Supplementary information (Doc S1) including Tables S2 and S3.

3 | RESULTS

3.1 | Cold shock induces phosphorylation of YAP1

We first confirmed using Phos-tag gels that YAP1, when cells were exposed to cold shock, was phosphorylated (Figure S1A). LATS1/2 silencing abolished serum deprivation-mediated, cell density-dependent, H₂O₂-induced or sorbitol-induced YAP1 phosphorylation, supporting the idea that LATS1/2 kinase activity was effectively suppressed (Figure S1B). Nevertheless, YAP1 was still phosphorylated in response to cold shock, indicating that not only LATS1/2 but also other kinase(s) contributed to the cold shock-induced phosphorylation of YAP1 (Figure S1C). BAPTA-AM treatment markedly attenuated the cold shock-induced phosphorylation (Figure S1D). Depletion of calcium from the medium also abolished the cold shock-induced phosphorylation (Figure S1E). Furthermore, the intracellular calcium concentration was enhanced after cold shock (Figure S1F). Therefore, we speculated that phosphorylation was mediated by calcium-dependent kinase(s). Indeed, Go 6976, an inhibitor of protein kinase C α/β , blocked phosphorylation, while KN-62, an inhibitor of calcium/calmodulin-dependent kinase, had no effect (Figure S1G). Therefore, we concluded that cold shock induced YAP1 phosphorylation through protein kinase C α/β in U2OS cells. Indeed, the recombinant YAP1 was phosphorylated by the immunoprecipitated PKC α in vitro (Figure S1H).

3.2 | Protein kinase C α phosphorylates YAP1 at serines 61, 127, and 164

We next attempted to determine which residues were phosphorylated by PKC. We prepared various YAP1 mutants (Figure 1A). We focused on protein kinase C α (from this point forwards described as PKC) and co-expressed it with YAP1 mutants in HEK293FT cells. YAP1 was phosphorylated, while YAP1 5SA mutant, in which serines 61, 109, 127, 164, and 401 were mutated to alanine, was not (Figure 1B, lanes 1-4). Moreover, YAP1 serines 61, 127, and 164 were phosphorylated, whereas YAP1 serine 109 or 401 was not (Figure 1B, lanes 5-14). To further confirm

the PKC-mediated phosphorylation sites, we expressed YAP1 and YAP1 3SA, in which serines 61, 127, and 164 were mutated to alanine, in HEK293FT cells, and treated the cells with 12-O-tetradecanoylphorbol-13-acetate (TPA)/A23187. YAP1 was phosphorylated in response to TPA/A23187 treatment, while YAP1 3SA was not (Figure 1C). Endogenous YAP1 in U2OS cells was also phosphorylated in response to TPA/A23187, which was blocked by Go 6976 (Figure 1D).

3.3 | TPA/A23187 treatment shifts YAP1 to the cytoplasm and inhibits TEAD-target gene transcription

We next examined whether and how TPA/A23187 treatment would affect the subcellular localization of YAP1. Immunofluorescence and subcellular fractionation demonstrated that YAP1 was shifted to the cytoplasm in response to TPA/A23187 treatment, while

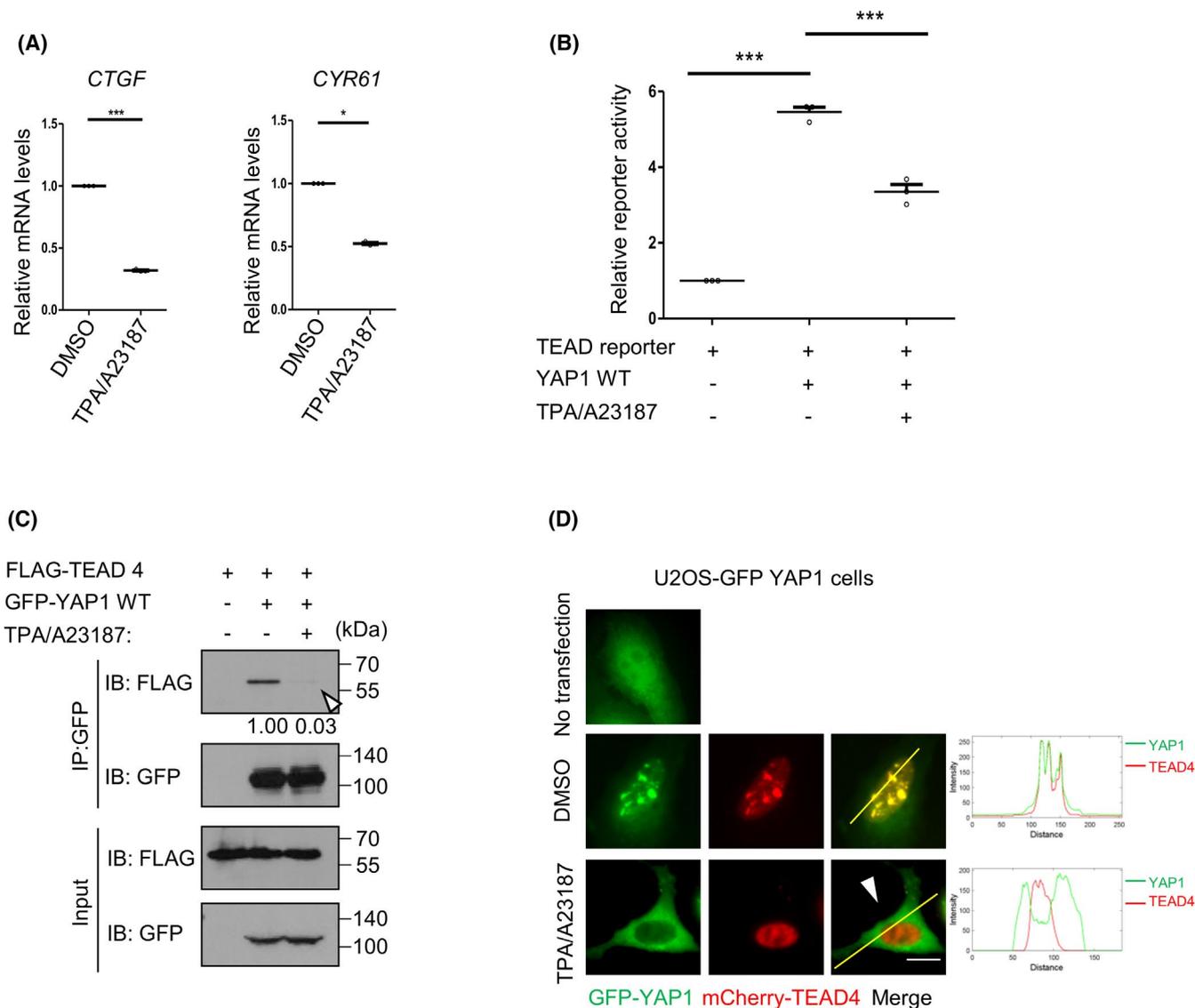


FIGURE 3 TPA/A23187 treatment reduces the interaction between YAP1 and TEAD4. A, HEK293FT cells were plated at 1×10^6 cells/well in a 6-well plate. At 48 h later, the cells were treated with DMSO or TPA/A23187 for 3 h. Total RNA was extracted and qRT-PCR was performed. The amounts of *CTGF* and *CYR61* were calibrated by the amount of *GAPDH*. B, HEK293 FT cells were transfected with 8xGT-IIC- δ 51LucII luciferase reporter (TEAD reporter) and pCneoFHF-YAP1. At 24 h later, cells were treated with TPA/A23187 for 3 h and the reporter assay was performed. The value of the samples that expressed only the reporter, was set at 1.00. * $P < .05$; *** $P < .001$. C, HEK293FT cells were transfected with pCneoFHF-TEAD4 and pCneoGFP-YAP1. At 48 h later, the cells were treated with TPA/A23187. Immunoprecipitation was performed with anti-GFP antibody and protein G Sepharose™ 4 Fast Flow beads. The inputs (Input) and the immunoprecipitates (IP) were immunoblotted with indicated antibodies. The signals detected with anti-GFP antibody in the immunoprecipitates were measured using ImageJ software. The value for the co-immunoprecipitated FLAG-TEAD4 without TPA/A23187 treatment was set at 1.00. The experiments were repeated 3 times and the representative result was demonstrated. D, U2OS-GFP-YAP1 cells were transfected with pCneomCherry-TEAD4. At 48 h later, cells were treated with DMSO or TPA/A23187 for 3 h, and then fixed. The colocalization of green and red signals was evaluated as described in the Materials and Methods section

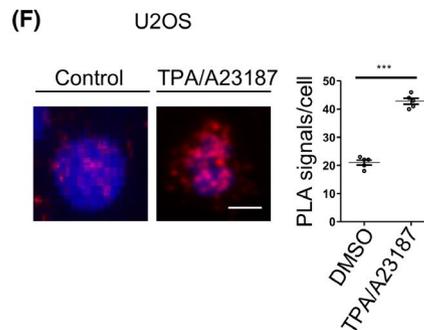
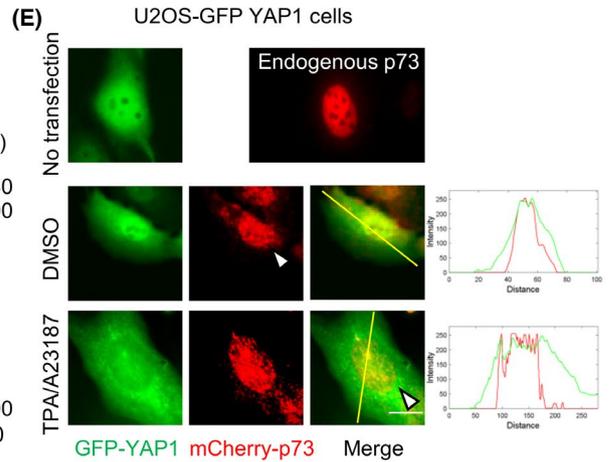
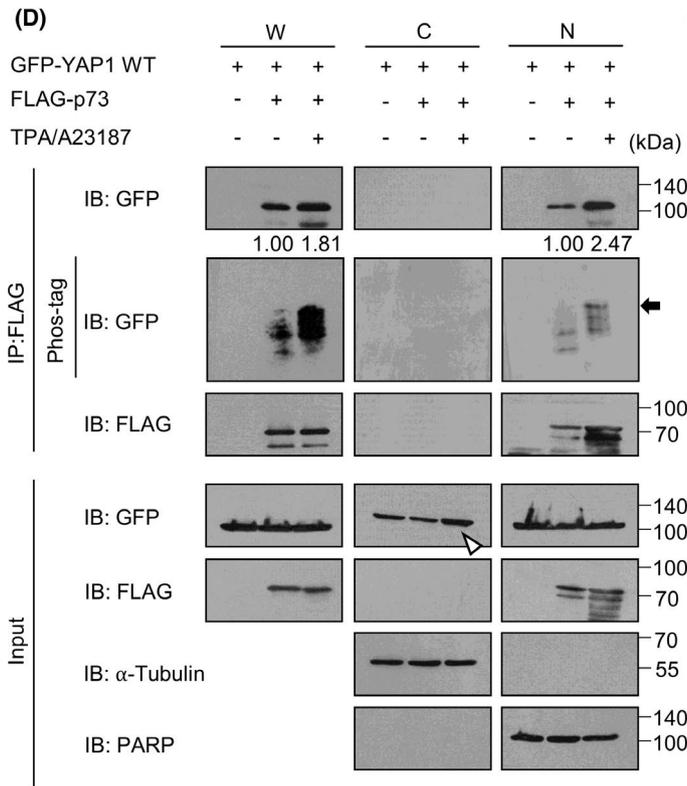
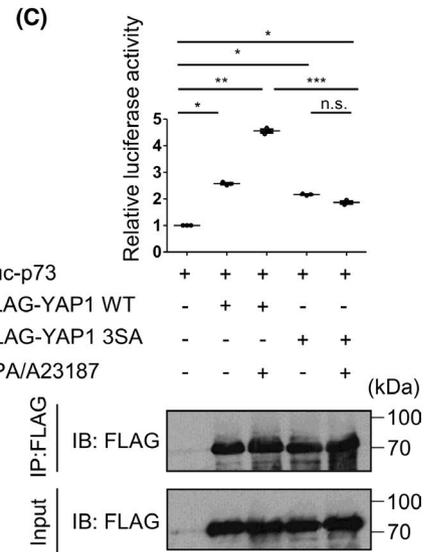
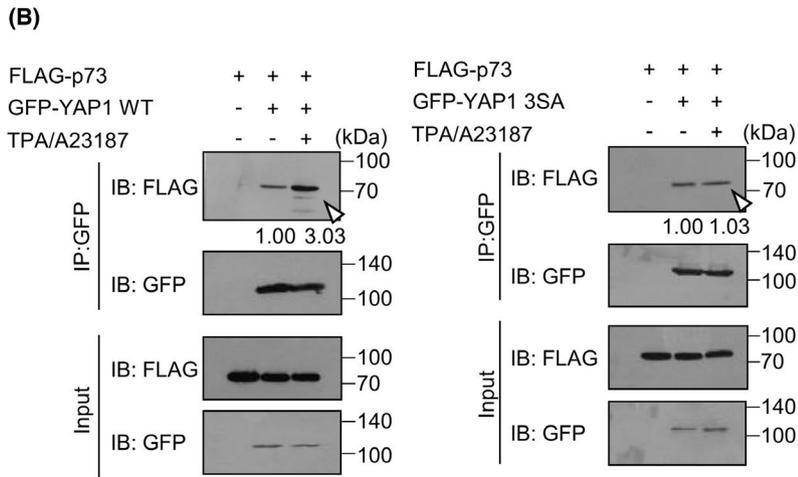
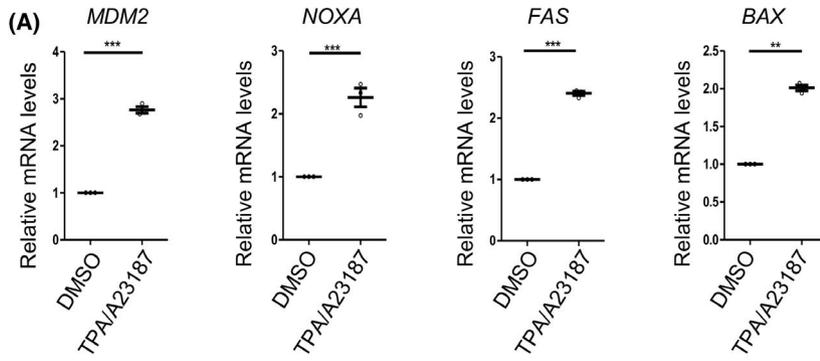


FIGURE 4 TPA/A23187 treatment enhances the expression levels of p73-target genes and the interaction between YAP1 and p73. A, The expression of p73-target genes was evaluated by qRT-PCR as described for TEAD-target genes in Figure 3A. $**P < .01$; $***P < .001$. B, D, The interaction between YAP1 and p73 or was evaluated using pCIneoFHF-p73 and pCIneoCherry-p73 as described for YAP1 and TEAD4 in Figure 3C. The interaction between YAP1 3SA and p73 was examined in (B), right. In (D), FLAG-p73 was immunoprecipitated from the whole cell lysate (W), the cytoplasmic fraction (C) and the nuclear fraction (N). The samples were run on conventional SDS-PAGE and Phos-tag gels. The arrowhead indicates the increase in cytoplasmic YAP1 in TPA/A23187-treated cells. The arrow indicates phosphorylated YAP1 co-immunoprecipitated with FLAG-p73. The value for the co-immunoprecipitated FLAG-p73 without TPA/A23187 treatment was set at 1.00. C, HEK293FT cells were transfected with pCIneoLuc-p73, pCIneoFHF, pCIneoFHF-YAP1, and pCIneoFHF-YAP1 3SA as indicated and the cells were treated with DMSO or TPA/A23187. Immunoprecipitation was performed with anti-DYKDDDDK-tag beads and a Lumier assay was performed. $*P < .05$; $**P < .01$; $***P < .001$. E, Colocalization between GFP-YAP1 and mCherry-p73 was evaluated as described for GFP-YAP1 and mCherry-p73 in Figure 3D. mCherry-p73 unlike endogenous p73 formed small clusters (arrowhead). F, The interaction between endogenous YAP1 and p73 was detected by using PLA in control and TPA/A23187-treated U2OS cells as described in the Materials and Methods section

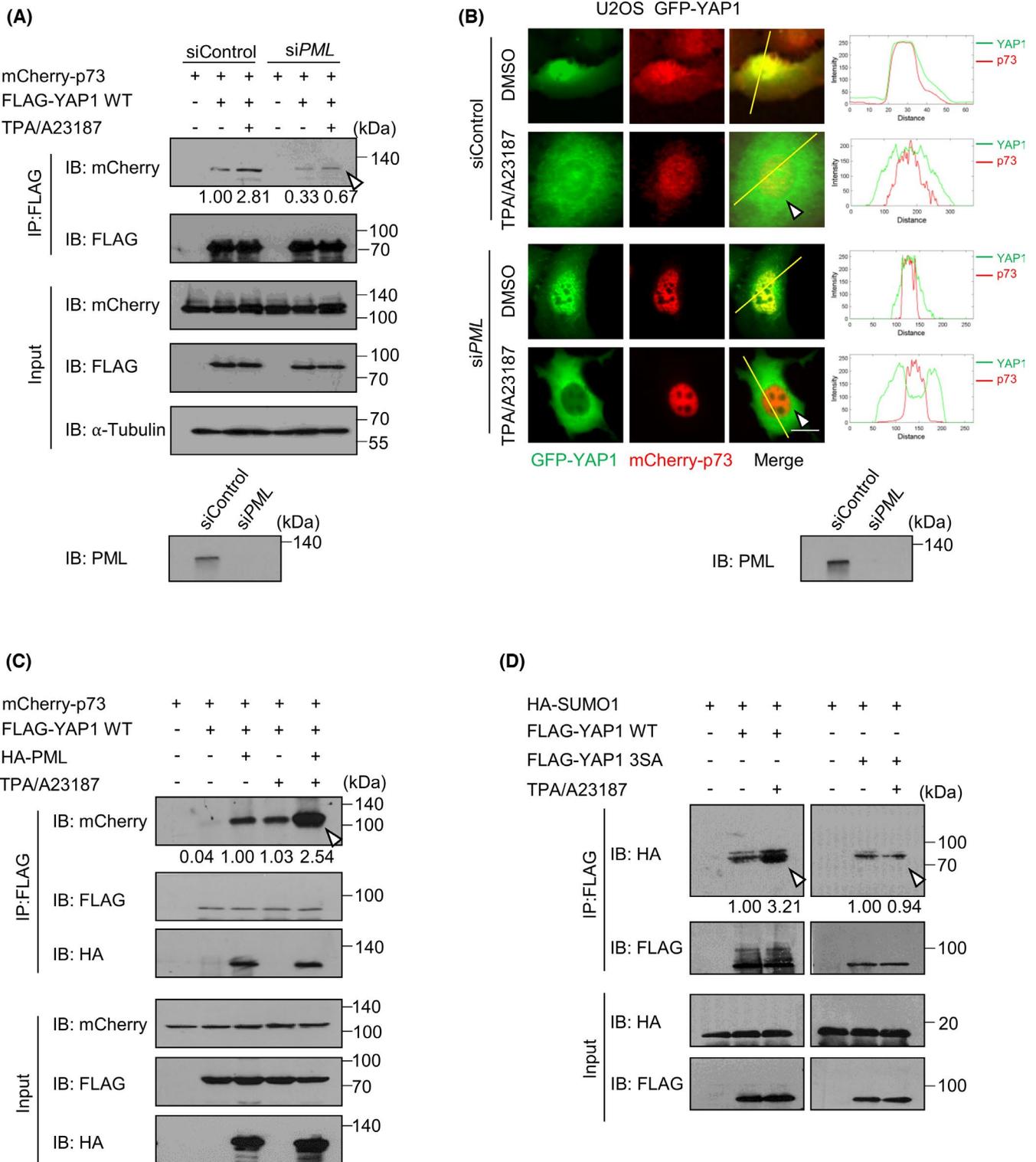
YAP1 3SA was not (Figure 2A). Subcellular fractionation demonstrated that phosphorylated YAP1 was detected in the nucleus of TPA/A23187-treated cells (Figure 2B, arrow). Importantly, TPA/A23187 treatment increased YAP1 in the cytoplasm even in LATS1/2-depleted cells (Figure 2C). Consistent with the change in YAP1 distribution, TPA/A23187 reduced the expression of CTGF and CYR61, the targets of TEAD in U2OS cells (Figure 3A), decreased TEAD-responsive reporter activity (Figure 3B), and attenuated the interaction between YAP1 and TEAD4 in HEK293FT cells (Figure 3C). When mCherry-TEAD4 was expressed in U2OS-GFP-YAP1 cells, GFP-YAP1 formed clusters in the nucleus, which supported the interaction between YAP1 and TEAD4 (Figure 3D, top and the middle panels). However, after TPA/A23187 treatment, the clusters composed of GFP-YAP1 and mCherry-TEAD4 disappeared, which supported the dissociation of YAP1 from TEAD4 (Figure 3D, the bottom panel, arrowheads).

3.4 | TPA/A23187 treatment enhances p73-mediated gene transcription and the interaction between YAP1 and p73

We next asked whether TPA/A23187 modified gene transcription mediated by other transcription factors. We were particularly

interested in p73, because p73 induces apoptosis in contrast with TEAD.⁶ TPA/A23187 treatment enhanced the expression of MDM2, NOXA, FAS, and BAX (Figure 4A). Consistently, TPA/A23187 treatment enhanced the co-immunoprecipitation of p73 with YAP1 but not with YAP1 3SA (Figure 4B, arrowheads). We also performed a Lumier assay to confirm that TPA/A23187 treatment augmented the amount of co-immunoprecipitated p73 with YAP1 but not with YAP1 3SA (Figure 4C). We performed co-immunoprecipitation from the nuclear fraction and confirmed that the nuclear phosphorylated YAP1 interacted with p73 in TPA/A23187-treated cells (Figure 4D, arrow). Although endogenous p73 was diffusely distributed in the nucleus, mCherry-p73 formed numerous small clusters in the nucleus (Figure 4E, middle panel, mCherry-p73, arrowhead). However, unlike mCherry-TEAD4, mCherry-p73 did not induce clustering of GFP-YAP1, which implied that YAP1 does not bind to p73 so tightly as to TEAD4 (Figure 4E, bottom panel, GFP-YAP1). Nevertheless, TPA/A23187 only partially reduced the colocalization of GFP-YAP1 and mCherry-p73 in U2OS cells (Figure 4E). Moreover, the interaction between endogenous YAP1 and p73 and its enhancement by TPA/A23187 were corroborated by PLA (Figure 4F). These findings support that although YAP1 is shifted to the cytoplasm after TPA/A23187 treatment, some population of YAP1 remains in the nucleus and interacts with p73.

FIGURE 5 PML is implicated in TPA/A23187-induced enhancement of the interaction between YAP1 and p73. A, HEK293FT cells were transfected with either control siRNA or siRNA against PML. At 48 h later, cells were transfected with pCIneoCherry-p73 and pCIneoFHF-YAP1 and immunoprecipitation was performed with anti-DYKDDDDK-tag beads. The arrowhead indicates that TPA/A23187 treatment fails to enhance the co-immunoprecipitation of mCherry p73 with FLAG-YAP1 after PML silencing. B, U2OS-GFP-YAP1 cells were transfected with either control siRNA or siRNA against PML. At 48 h later, cells were transfected with pCIneoCherry-p73. At 48 h later, cells were treated with TPA/A23187. The colocalization of GFP-YAP1 and mCherry-p73 was evaluated as described for Figure 3D. The suppression of PML protein was confirmed by immunoblotting. C, HEK293FT cells were transfected with pCIneoCherry-p73, pCIneoFHF-YAP1, and pLNCX-HA-hPML IV. At 48 h later, cells were treated with TPA/A23187 and immunoprecipitation was performed with anti-DYKDDDDK-tag beads. The signals for mCherry-p73 in the immunoprecipitates were measured using ImageJ software. The value of the co-immunoprecipitated mCherry-p73 with FLAG-YAP1 under the basal condition was set at 1.00. TPA/A23187 treatment more robustly enhanced the interaction in the presence of the exogenously expressed PML (arrowhead). D, HEK293FT cells were transfected with pCIneoHA-SUMO, pCIneoFHF-YAP1, and pCIneoFHF-YAP1 3SA. At 48 h later, cells were treated with TPA/A23187. Immunoprecipitation was performed with anti-DYKDDDDK-tag beads. The precipitated YAP1 was immunoblotted with anti-HA and anti-FLAG antibodies. TPA/A23187 treatment augmented the SUMOylation of YAP1 but not of YAP1 3SA (arrowheads)



3.5 | Promyelocytic Leukemia Protein (PML) is implicated in TPA/A23187-mediated enhancement of the interaction between YAP1 and p73

A previous study revealed that the *PML* gene is a target of YAP1 and p73 and that PML protein interacts with and stabilizes YAP1 to promote YAP1/p73-mediated gene transcription.³⁰ We hypothesized

that PML is involved in TPA/A23187-induced enhancement of the interaction between YAP1 and p73. As expected, *PML* silencing attenuated the effect of TPA/A23187 on the interaction between YAP1 and p73 (Figure 5A, arrowhead). *PML* silencing also attenuated the colocalization between YAP1 and p73 in TPA/A23187-treated cells (Figure 5B). Conversely, *PML* co-expression strengthened the interaction between YAP1 and p73 (Figure 5C, second and third lanes). TPA/A23187 further augmented the interaction (Figure 5C,

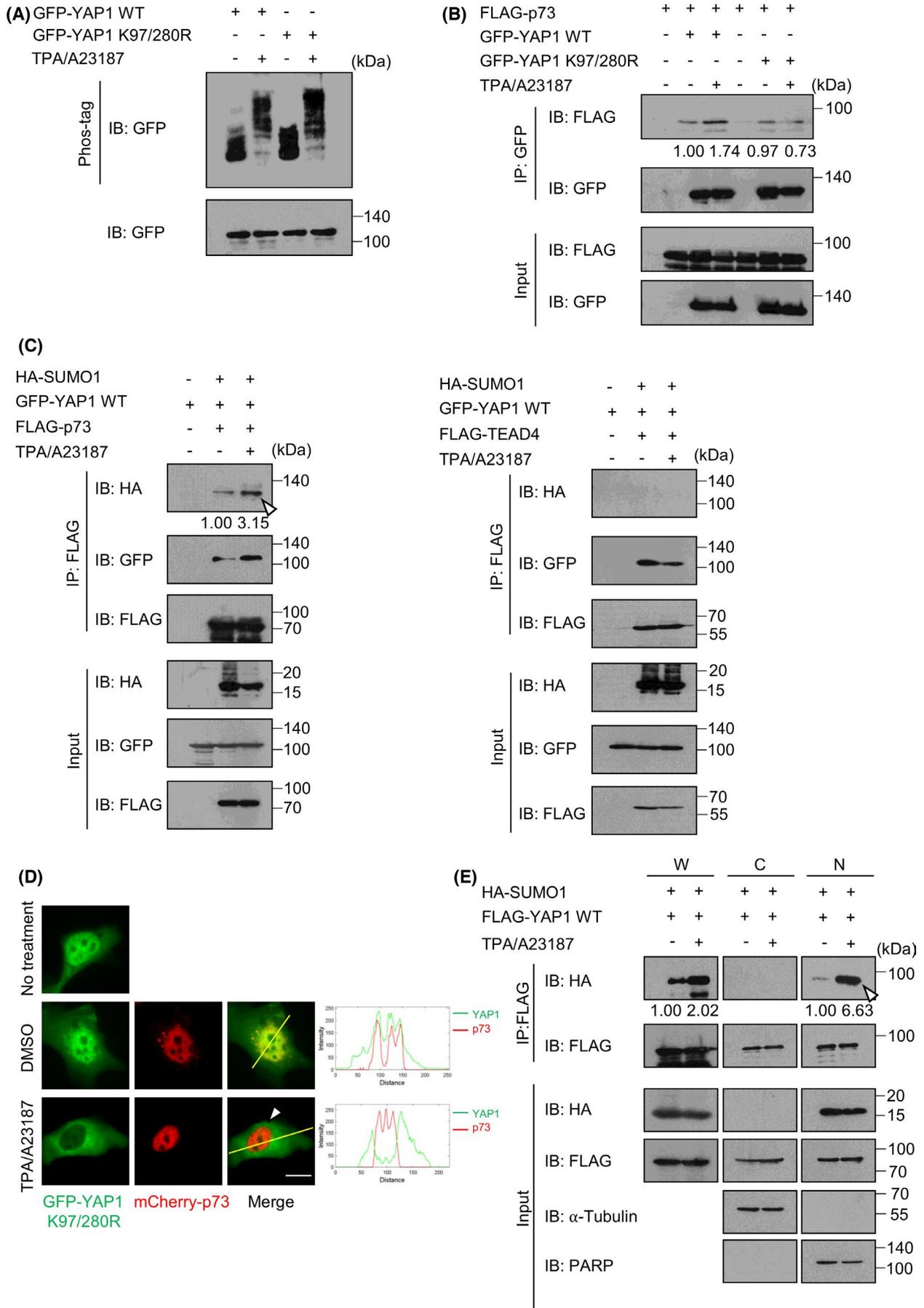


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FIGURE 6 SUMOylation is necessary for TPA/A23187-mediated enhancement of the interaction between YAP1 and p73. A, HEK293FT cells were transfected with pCIneoGFP-YAP1 or pCIneoGFP-YAP1 K97/280R. At 48 h later, cells were treated with DMSO or TPA/A23187. The cell lysates were run on conventional SDS-PAGE and Phos-tag gels. Immunoblotting was performed with anti-GFP antibody. B, HEK293FT cells were transfected with pCIneoGFP-YAP1, pCIneoGFP-YAP1 K97/280R, and pCIneoFHF-p73 as indicated. At 48 h later, cells were treated with DMSO or TPA/A23187. Immunoprecipitation was performed with anti-GFP antibody. C, GFP-YAP1 and HA-SUMO were co-expressed with either FLAG-p73 or FLAG-TEAD4 in HEK293FT cells. The cells were treated with TPA/A23187. Immunoprecipitation was performed with anti-DYKDDDDK-tag beads. YAP1 co-immunoprecipitated with FLAG-p73 was detected using anti-HA antibody (arrowhead). D, The colocalization of mCherry-p73 and GFP-YAP1 K97/280A was examined as described for Figure 3D. E, FLAG-YAP1 was co-expressed with HA-SUMO, immunoprecipitated from the whole cell lysate (W), the cytoplasmic fraction (C), and the nuclear fraction (N), and immunoblotted with the indicated antibodies. TPA/A23187 treatment increased the signal of nuclear YAP1 detected with HA antibody (arrowhead)

third and fifth lanes, arrowhead). As PML stabilizes YAP1 through SUMOylation, we speculated that PKC-mediated phosphorylation is involved in the regulation of SUMOylation. Indeed, TPA/A23187 treatment increased the SUMOylation of YAP1 but not of YAP1 3SA (Figure 5D, arrowheads).

3.6 | SUMOylation is necessary for TPA/A23187-mediated enhancement of the interaction between YAP1 and p73

A previous study revealed that YAP1 is SUMOylated at lysine residues 97 and 280.³¹ We examined whether SUMOylation was required for TPA/A23187-mediated phosphorylation of YAP1. YAP1 K97/280R, in which lysines 97 and 280 were mutated to arginine, was phosphorylated, indicating that PKC-mediated phosphorylation promotes the SUMOylation but that the SUMOylation is not necessary for PKC-mediated phosphorylation (Figure 6A). However, TPA/A23187 treatment failed to enhance the interaction between YAP1 K97/280R and p73 (Figure 6B). We wanted to know whether SUMOylated YAP1 would indeed bind to p73. To this end, we expressed HA-SUMO with GFP-YAP1 and FLAG-p73 and confirmed that the co-immunoprecipitated YAP1 was detected by anti-HA antibodies (Figure 6C, left). In contrast, YAP1 co-immunoprecipitated with TEAD4 was not SUMOylated (Figure 6C, right). Furthermore, after TPA/A23187 treatment, the nuclear YAP1 K97/280R was reduced and the colocalization with p73 became undetectable (Figure 6D). Subcellular fractionation demonstrated that nuclear YAP1, but not cytoplasmic YAP1, was SUMOylated (Figure 6E). Therefore, we conclude that PKC-mediated phosphorylation promotes SUMOylation of YAP1 and that the SUMOylated YAP1 interacts with p73 but not with TEAD4.

3.7 | TPA/A23187 treatment does not promote YAP1 degradation as rapidly as hydrogen peroxide treatment in the presence of PML

We also examined the effect of PML on the stabilization of YAP1. The degradation of YAP1 was facilitated by hydrogen peroxide treatment, which is widely used to activate the Hippo pathway (Figure

S2A). TPA/A23187 treatment also promoted YAP1 degradation but to a less extent (Figure S2A). Hydrogen peroxide treatment did not enhance the degradation of YAP1 S109/401A (Figure S2B). As PKC does not phosphorylate the phosphodegron and YAP1 S109/401A lacks the phosphodegron, these results are understandable. However, when PML was silenced, TPA/A23187 treatment facilitated YAP1 degradation as did hydrogen peroxide treatment (Figure S2C).

3.8 | Bryostatin induces PKC-mediated phosphorylation of YAP1 in U2OS cells

Our findings prompted us to hypothesize that PKC activation may switch YAP1 from TEAD-mediated signaling to p73-mediated signaling and confers a tumor suppressor property to YAP1. As nuclear YAP1 is reduced even on a LATS1/2-negative background, PKC activation is expected to be useful for suppressing the growth of cancers with the dysfunction of the Hippo pathway. With this in mind, we searched for natural compounds that could stimulate PKC. We selected 3 compounds, bryostatin, ingenol-3-angelate, and decursin (Figure S3A).³²⁻³⁵ Among these 3 compounds, bryostatin induced phosphorylation of YAP1, which was blocked by Go 6976 (Figure S3A, B). Bryostatin is a known naturally occurring anti-neoplastic agent. Therefore, we further characterized the effect of bryostatin. 0.1 nM bryostatin induced phosphorylation in YAP1 S61, S127, and S164, but not in YAP1 S109, S401, and 3SA (Figure S3C, D). All these findings support the idea that bryostatin triggers PKC-mediated phosphorylation of YAP1.

3.9 | Bryostatin treatment reduces nuclear YAP1 and shifts YAP1 from TEAD signaling to p73 signaling

We next confirmed that YAP1, but not YAP1 3SA, was shifted to the cytoplasm in bryostatin-treated U2OS cells (Figure 7A). Bryostatin treatment enhanced the binding of p73 to YAP1 and the expression of p73-target genes but reduced the expression of CTGF and CYR61 (Figure 7B, C). A chromatin immunoprecipitation experiment demonstrated that, in bryostatin-treated cells, YAP1 attached to the p73-target gene promoters (MDM2 and BAX) and was dissociated from TEAD-target gene promoters (CTGF) (Figure 7D).

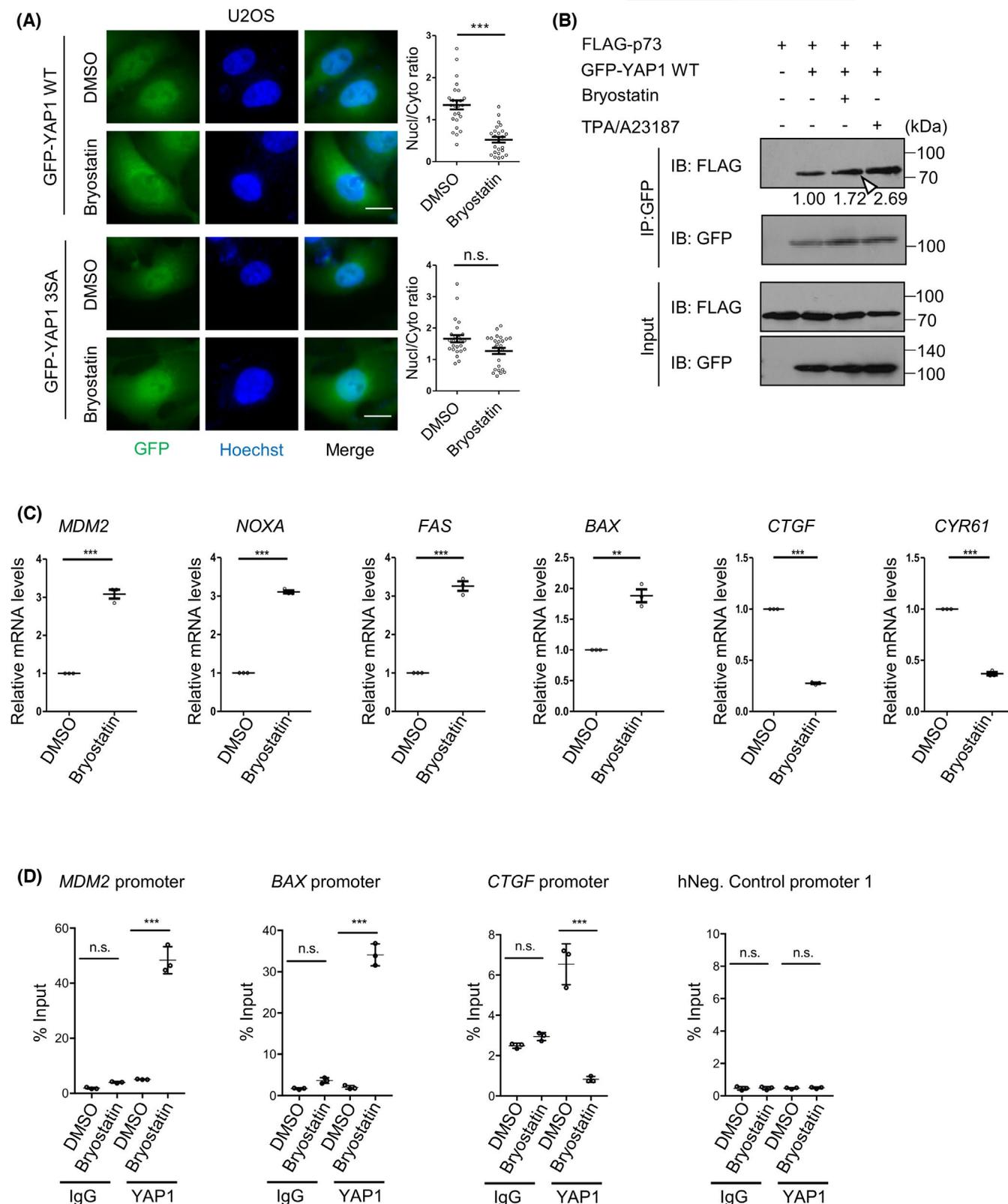


FIGURE 7 Bryostatin treatment induces the shift of YAP1 to the cytoplasm and enhances the interaction between YAP1 and p73. A-C, The effects of bryostatin treatment on the distribution of GFP-YAP1 and GFP-YAP1 3SA, the interaction between GFP-YAP1 and FLAG-p73, and the expression of p73-target and TEAD-target genes were evaluated as described for Figures 2A, 4B and A. Cells were treated with 0.1 nM bryostatin for 3 h. Bryostatin augmented the interaction between YAP1 and p73 (arrowhead). Scale bars, 25 μ m. D, Parent U2OS cells were treated with DMSO or 0.1 nmol/L bryostatin. Chromatin immunoprecipitation was performed with anti-YAP1 antibody. The association of GFP-YAP1 with the promoters of indicated genes was evaluated. n.s., not significant; ** $P < .01$; *** $P < .001$

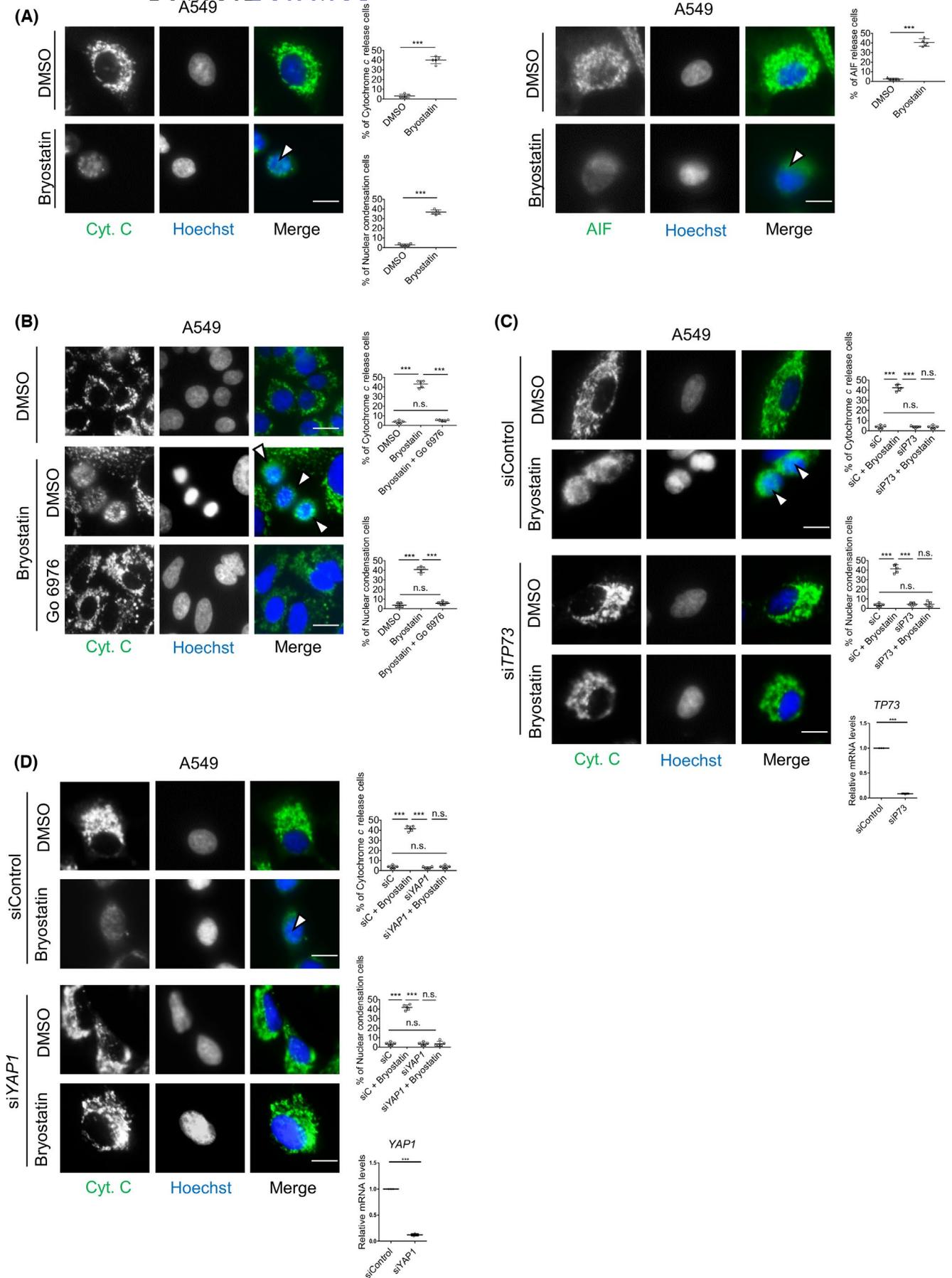


FIGURE 8 Legend on next page

FIGURE 8 Bryostatin induces apoptosis in human lung cancer A549 cells. A, A549 cells were treated with 1 nmol/L bryostatin for 24 h. Here, 100 cells were observed in each experiment and apoptosis was evaluated by the release of cytochrome c and apoptosis-inducing factor (AIF) (arrowheads). Data from 5 independent experiments are shown as mean \pm SD. B–D, Bryostatin-induced apoptosis was evaluated as described for (A). In (B), cells were also treated with Go 6976. In (C) and (D), cells were transfected with control siRNA, siRNA against YAP1, or siRNA against TP73 in advance. n.s., not significant; *** $P < .001$. Scale bars, 25 μ m

3.10 | Bryostatin induces apoptosis via PKC, p73, and YAP1 in human lung cancer cells

Consistently, bryostatin treatment induced apoptosis in A549 cells (Figure 8A) and Go 6976 blocked it (Figure 8B). TP73 silencing and YAP1 silencing attenuated bryostatin-mediated apoptosis (Figure 8C,D). Bryostatin also induced apoptosis in U2OS and H1299 cells and Go 6976 blocked it (Figure S4).

3.11 | Bryostatin treatment suppresses cell growth, migration, and invasiveness of human lung cancer cells

We subsequently examined whether bryostatin treatment antagonized malignant transformation in cancer cells with the dysfunction of the Hippo pathway. We prepared LATS1/2-depleted A549 and H1299 cells. LATS1/2 depletion facilitated cell proliferation, but bryostatin treatment abolished the effect of LATS1/2 depletion, although bryostatin treatment failed to suppress the cell proliferation of cells expressing YAP1 3SA (Figures 9A and S5A). Similarly, bryostatin treatment reduced migration and invasion in Transwell experiments in LATS1/2-depleted A549 cells but not in YAP1 3SA-expressing A549 cells (Figure 9B,C). We obtained similar results using H1299 cells (Figure S5B,C).

4 | DISCUSSION

We and others have reported that heat shock induces dephosphorylation of YAP1.^{16,17} We also demonstrated that heat shock switches YAP1 to NF- κ B-signaling.¹⁷ Therefore, we started this study with a simple question 'What would happen to YAP1 when cells are exposed to cold shock?' In contrast with heat shock, cold shock at 4°C induced YAP1 phosphorylation. We experience cold shock at 4°C in our daily lives. We identified PKC as a key kinase for the cold shock-induced phosphorylation of YAP1. Our findings were consistent with the previous report that PKC is activated in airway epithelial cells in response to cold stimuli.³⁶ The authors discussed in this previous paper that PKC inhibits cold shock-induced transient receptor potential Melastatin 8-mediated induction of inflammatory cytokines. We recently reported that YAP1 activates NF- κ B signaling in response to heat shock. Therefore, it might be possible that PKC activation recruits YAP1 to the cytoplasm and subsequently suppresses the inflammatory responses against cold stimuli. It is reasoned that when we drink cold water, YAP1 is phosphorylated in the oral cavity, pharynx, and upper esophagus. It may be interesting to study the physiological meaning of

YAP1 phosphorylation in response to cold shock. Even so, with PKC having many substrates, cold shock-induced phosphorylation is not supposedly specific for YAP1. Therefore, we shifted our focus from cold shock to PKC activation and asked the meaning of PKC-mediated phosphorylation of YAP1 in this study.

Although we did not directly determine phosphorylated residues, the experiments using YAP1 mutants suggested that PKC phosphorylates YAP1 at serines 61, 127, and 164. That is, PKC phosphorylates 3 serine residues among 5 serine residues that are phosphorylated by LATS kinases, but serine 401, the phosphodegron, is saved. Accordingly, PKC activation induces the accumulation of YAP1 in the cytoplasm but does not facilitate YAP1 degradation as does LATS kinase activation. Reflecting the cytoplasmic accumulation of YAP1, the interaction between YAP1 and TEAD is weakened and PKC activation reduces TEAD-dependent gene transcription. This finding is important because it implies that PKC activation negatively regulates YAP1/TEAD, even in cancer cells with the dysfunction of the Hippo pathway. Our findings are apparently inconsistent with a previous report that classical PKC induces dephosphorylation of YAP1.¹⁸ This discrepancy could be due to the difference in the cell types used in the experiments. However, more importantly, in the previous study, the researchers performed the experiments under the conditions that LATS kinase activity is high due to serum deprivation and used artificial constitutively active PKC α or a high concentration (10–100 nmol/L) of TPA without calcium ionophore to activate PKC. We speculated that the difference in the experimental conditions could explain this discrepancy.

Several studies have supported that YAP1 induces apoptosis through p73-dependent gene transcription and plays a tumor suppressive role.^{9,30,37} Interestingly, PKC activation enhances the interaction between YAP1 and p73 and upregulates p73-target genes. This finding is apparently inconsistent with the cytoplasmic accumulation of YAP1. Therefore, we speculated that, although most YAP1 is shifted to the cytoplasm, some populations of YAP1 remain in the nucleus and co-operates with p73. The study using the microscope supported this assumption. We could detect the colocalization of YAP1 and p73 in the nucleus even after PKC activation. The experiments using nuclear fraction corroborated the finding that phosphorylated YAP1 is detected in the nucleus and interacts with p73 after PKC activation. Previous studies have revealed that PML is a target of YAP1/p73 and that PML binds to YAP1 and stabilizes it through SUMOylation.^{30,36} PKC activation enhances the interaction between YAP1 and p73 in the presence of PML. We speculate that YAP1 phosphorylated by PKC is SUMOylated and binds to p73 in the presence of PML and that this may be the reason why phosphorylated YAP1 remains in the nucleus (Figure 10). Although we could not conclude yet whether SUMOylated YAP1 would bind

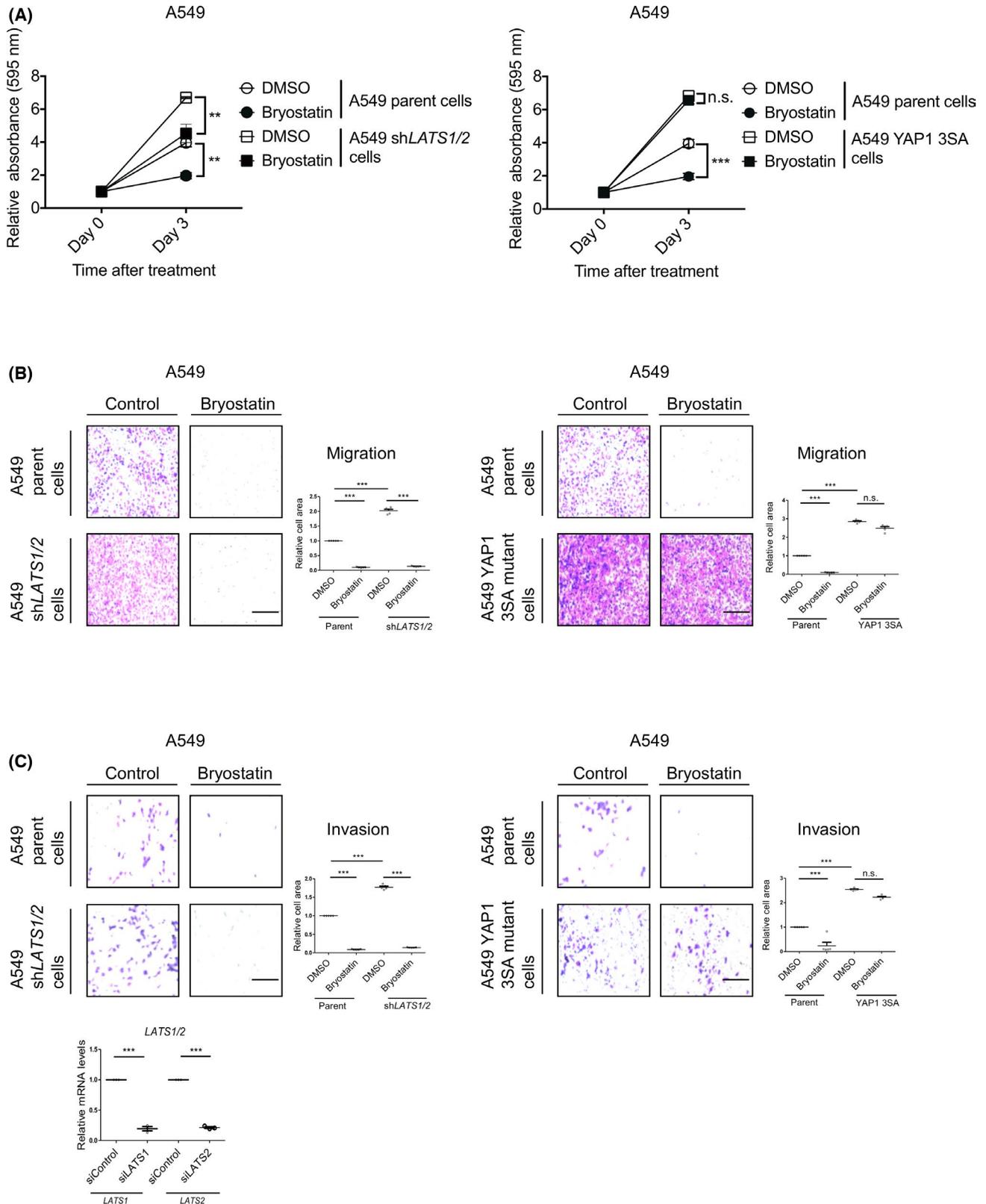
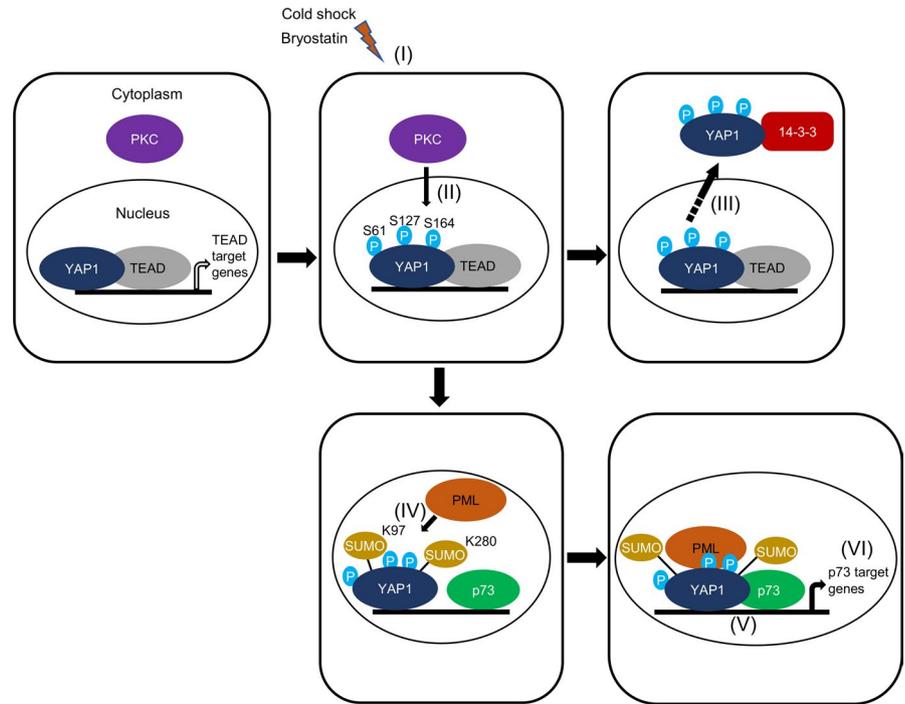


FIGURE 9 Bryostatin reverses the LATS1/2 depletion-induced malignant properties of human lung cancer A549 cells. **A**, Parent, LATS1/2-depleted and GFP-YAP1 3SA-expressing A549 cells were plated at 3000 cells/well in a 96-well plate. At 24 h later, the cells were treated with DMSO or 1 nmol/L bryostatin, and cultured for 72 h. A colorimetric assay was performed. **B**, **C**, Parent, LATS1/2-depleted, and YAP1 3SA-expressing A549 cells were serum-starved for 24 h. The cells were replated at 4×10^4 cells/insert in 8.0 μ m Transparent PET Membrane Insert. Migration (**B**) and invasion (**C**) assays were performed as described in the Materials and Methods section. At 40 h later, cells were stained with crystal violet. The migrating or invading cells were observed under a microscope in 3 independent fields for each condition and quantified using ImageJ software. The values for A549 parent cells treated with DMSO were set at 1

FIGURE 10 Protein kinase C activation switches YAP1 from TEAD-mediated signaling to p73-mediated signaling. (I) PKC is activated upon various stimuli such as cold shock and bryostatin treatment. (II) PKC phosphorylates YAP1 at serines 61, 127, and 164. (III) Phosphorylated YAP1 is trapped in the cytoplasm but is not degraded rapidly. (IV) Some part of phosphorylated YAP1 is SUMOylated in the presence of PML. (V) SUMOylated YAP1 remains in the nucleus and interacts with p73. (VI) p73-target genes are upregulated



more tightly to p73 compared with unSUMOylated YAP1, we could observe that SUMOylated YAP1 did not interact with TEAD4. As SUMOylated lysines reside in the TEAD-binding region, we speculated that SUMOylation might disturb the interaction between YAP1 and TEAD.

In the last part of this study, we evaluated the effect of bryostatin on cancer cells with the dysfunction of the Hippo pathway. Bryostatin is an activator of PKC and is a known anti-neoplastic natural product.^{32,33,38} Bryostatin reversed the malignant properties of cancer cells induced by silencing of *LATS* genes *in vitro*. It should also be noted that bryostatin is effective in H1299 cells lacking p53. We have not applied bryostatin to animal models in this study. However, bryostatin has been already tested in a Phase II clinical trial for Alzheimer disease, metastatic colorectal cancer, and advanced renal cancer.^{39–41} Although no complete or partial responses were obtained in the latter 2 studies, it may be meaningful to re-evaluate the effect of bryostatin on cancers, in which the Hippo pathway is compromised. Moreover, bryostatin analogs have been developed. It would be interesting to examine the effects of these compounds on human cancers with the dysregulation of the Hippo pathway.⁴²

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

CKS performed the experiments. JM, SN, KA-M, JAK, and HI helped CKS in the experiments. JM and YH designed the research and wrote the paper with CKS. HN critically read the manuscript.

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SUPPORTING INFORMATION

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