Retinoic acid-stimulated sequential phosphorylation, PML recruitment, and SUMOylation of nuclear receptor TR2 to suppress Oct4 expression

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We previously reported an intricate mechanism underlying the homeostasis of Oct4 expression in normally proliferating stem cell culture of P19, mediated by SUMOylation of orphan nuclear receptor TR2. In the present study, we identify a signaling pathway initiated from the nongenomic activity of *all-trans* retinoic acid (atRA) to stimulate complex formation of extracellular signal-regulated kinase 2 (ERK2) with its upstream kinase, mitogen-activated protein kinase kinase (MEK). The activated ERK2 phosphorylates threonine-210 (Thr-210) of TR2, stimulating its subsequent SUMOylation. Dephosphorylated TR2 recruits coactivator PCAF and functions as an activator for its target gene *Oct4*. Upon phosphorylation at Thr-210, TR2 increasingly associates with promyelocytic leukemia (PML) nuclear bodies, becomes SUMOylated, and recruits corepressor RIP140 to act as a repressor for its target, *Oct4*. To normally proliferating P19 stem cell culture, exposure to a physiological concentration of atRA triggers a rapid nongenomic signaling cascade to suppress *Oct4* gene and regulate cell proliferation.

The POU-domain transcription factor Oct-4 is essential for the maintenance of embryonic stem (ES) cell cultures and is an important ES cell marker (1–3). A fixed amount of Oct4 protein is also required for the normal proliferation status of pleuropotent embryonal carcinoma (EC) cell cultures such as P19 (4). The expression of Oct4 is regulated, primarily, by transcriptional events (5) involving transcription factor binding to a Sp1 site (6) and hormone response elements (HREs), including retinoic acid receptors (RARs) (7), steroidogenic factor 1 (SF-1) (8), germ cell nuclear factor (GCNF) (9), liver receptor homologue-1 (LRH-1) (10), chick ovalbumin upstream promoter transcription factor (COUP-TFs) (11), and testis receptor 2 (TR2) (4). RAR, GCNF, and COUP-TFs all repress the *Oct4* gene (also known as *Pou5f1*), whereas LRH-1 and SF-1 both activate the *Oct4* gene. TR2 can bind to this HRE to activate or repress the *Oct4* gene in P19 EC stem cell culture to regulate its proliferation, depending on its SUMOylation on Lys-238 (4).

The mouse TR2 orphan nuclear receptor (encoded by the mouse *Nr2c1* gene) is expressed, primarily, in peri-implanting embryos (12), germ cells (13), embryonal carcinoma cells (14, 15), and preadipocytes (16). Despite the absence of putative ligands, TR2 has been reported to affect various cellular processes, including differentiation, proliferation, and apoptosis (4, 17–19). At the molecular level, TR2 can be an activator or a repressor for the same target genes that carry HREs, such as DR5 of *Rarβ* (20) and DR1 of *Oct4* (4). However, the molecular basis of its activities has remained a mystery for a long time due to the lack of specific ligands. Only recently have we reported that TR2 can fine-tune *Oct4* gene expression in P19 stem cell culture through an intricate balancing between the activating function of the unSUMOylated TR2 that recruits coactivator PCAF and the repressive function of the SUMOylated TR2 that recruits corepressor RIP140. The critical step for TR2 SUMOylation is its association with PML nuclear bodies (4). As such, an important issue with respect to the homeostatic control of the *Oct4* gene is what signal triggers TR2 recruitment to PML nuclear bodies for SUMOylation.

In most cell proliferation/differentiation models, *all-trans* retinoic acid (atRA) is known primarily as a differentiation agent acting through the RAR family (21, 22) Activation of these receptors by atRA normally leads to stem cell differentiation (23–25). However, atRA has also been known to exert some nongenomic actions to affect several cellular processes, and involves, mostly, activation of extracellular signal-regulated kinase (ERK) (26–29).

In the present study we found that atRA could rapidly stimulate the SUMOylation of TR2 without acting through RARs, thereby rendering TR2 a repressor for the *Oct4* gene. We thus set up experiments to determine (*i*) if atRA exerts a nongenomic effect on TR2 SUMOylation to repress Oct4, (*ii*) which nongenomic signaling pathway mediates atRA's effect on TR2 SUMOylation, and (*iii*) how this pathway controls TR2 recruitment to PML. This study provides the evidence for a nongenomic signaling pathway of atRA and demonstrates how atRA triggers the activation of ERK2 through stimulating complex formation of ERK2 with its upstream kinase mitogen-activated protein kinase kinase (MEK). This is followed by ERK2-mediated phosphorylation of TR2 on a specific threonine residue, Thr-210, which stimulates its recruitment to PML. The hyperphosphorylated TR2 is then SUMOylated and becomes a repressor of *Oct4* gene. This result provides a unique insight into atRA-stimulated nongenomic signaling pathway that modulates molecular interaction and specific posttranslational modifications of proteins, which contributes to the homeostasis of important regulatory molecules such as Oct4.

Results

atRA Stimulates SUMOylation of TR2 and Modulates Its Activity in Regulating Oct4 Gene in P19 EC Culture. Previously (4), we showed that normally proliferating P19 stem cell culture expresses a basal level of unSUMOylated TR2 that functions as an activator for the *Oct4* gene by recruiting PCAF. We also detected a SUMOylated form of TR2 that could repress *Oct4* gene by recruiting RIP140, but the upstream trigger for TR2 SUMOylation was unclear. Interestingly, atRA could rapidly repress *Oct4* gene expression without going through RARs (see Fig. 1*C*). We thus determined whether the rapid repression of Oct4 by atRA involved TR2. atRA could stimulate TR2 SUMOylation (Fig. 1*A*, lane 3) and the Oct4 level was reduced to ≈10%. We conducted siRNA knockdown of endogenous TR2 in P19, with and without atRA treatment. As

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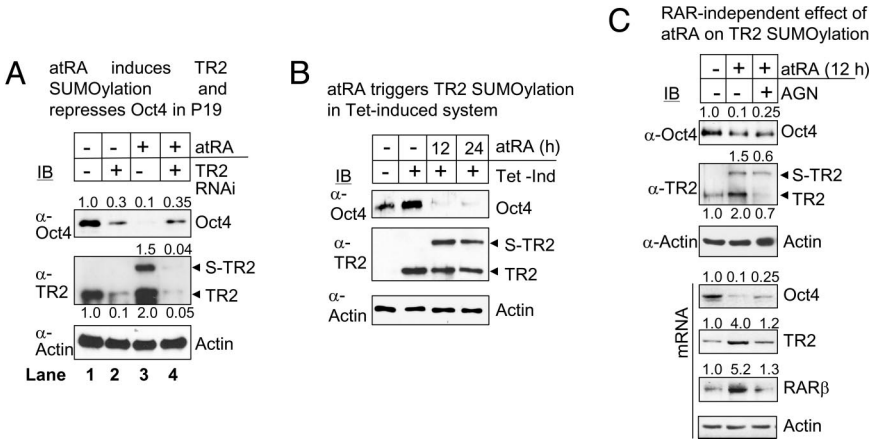

Fig. 1. atRA triggers TR2 SUMOylation, independent of nuclear RARs. (*A*) atRA (0.1 μM, 12 h) was tested for its ability to modulate Oct4 expression with or without TR2 knockdown (by 40 nM specific siRNA). Average values were obtained from three independent experiments. Differences between lanes 1 and 2; 2 and 3; 3 and 4, but not 2 and 4, are statistically significant ($P < 0.05$) for Oct4 and TR2 expression. (*B*) atRA stimulation of TR2 SUMOylation in a Tet-inducible TR2 P19 clone (induced by removing tetracycline for 36 h). The levels of TR2, SUMOylated TR2, and Oct4 were monitored (panel 1). (*C*) atRA-stimulated TR2 SUMOylation was not inhibited by the panantagonist to RARs (AGN193109, 0.1 μM, 2 h before atRA treatment for 12 h). S-TR2: SUMOylated TR2. Quantified data were shown above or under each panel.

predicted, siRNA knockdown of endogenous TR2 lowered the basal level of Oct4 mRNA because the activator TR2 was reduced (Fig. 1*A*, lane 2 to 1). Also, atRA repression of Oct4 seemed to partially recover (from a relative intensity of 0.1 to 0.35, lane 3 to 4) when the endogenous TR2 was knocked down by siRNA (lane 4). These findings suggest that the repressive effect of atRA on *Oct4* is probably mediated through its ability to regulate TR2 SUMOylation (i.e., turning activator TR2 into a SUMOylated repressor).

It is well known that atRA exerts many genomic and nongenomic effects, including its slight (≈2- to 4-fold after 24–48 h) induction of TR2 mRNA through the activity of RAR (15, 18, 30). The rapid stimulation of TR2 SUMOylation by atRA suggested that it might be triggered by the nongenomic/canonical activities of atRA. We examined this possibility by using a stable, tetracycline-inducible P19 system where TR2 level could be elevated by removing tetracycline (Tet), and Oct4 expression could be correspondingly induced (4). As shown in Fig. 1*B*, TR2 was elevated in the Tet-inducible clone without apparent SUMOylation. A shorter period of atRA treatment (12 h, or even 8 h) could stimulate TR2 SUMOylation as efficiently as that elicited by 24 h of treatment, suggesting a potential nongenomic/canonical action of atRA. This was confirmed in an experiment where a specific RAR panantagonist was used to block its genomic effect (RAR mediated) (31) (Fig. 1*C*). Despite the fact that the TR2 mRNA level, as well as a more sensitive RA target RARβ, was repressed by the specific RAR panantagonist (AGN193109) (second and third panels from the bottom), TR2 protein could still be SUMOylated (second panel from the top). Consistently, Oct4 expression was repressed (the top panel for protein and the fourth panel for mRNA).

All of the findings clearly demonstrate that atRA can rapidly stimulate TR2 SUMOylation, contributing to the repression of Oct4 without going through the canonical pathway mediated by RARs.

Phosphorylation of TR2 at Thr-210 Leads to Its SUMOylation. The nongenomic action of atRA on TR2 SUMOylation suggested that protein modification on TR2 might contribute to its SUMOylation. Mass spectrometry analyses showed extensively phosphorylation of TR2 (32, 33). A preliminary test using kinase inhibitors revealed effective ablation of atRA-stimulated TR2 SUMOylation by the inhibitor of mitogen-activated protein kinase (MAPK)/ERK, but not protein kinase C (PKC) [see supporting information (SI) Fig. S1]. We carefully examined the mass spectrum of TR2 protein and identified three MAPK/ERK-modified residues: Ser-203, Thr-208, and Thr-210 (Table S1 and Fig. S2). In an established TR2 reporter system (Fig. S3), activation of this reporter was also inhibited by atRA and an MAPK/ERK activator, but significantly enhanced by an ERK2 inhibitor, supporting that TR2 activity could be regulated by the ERK2 pathway. We then generated individual Ser-203, Thr-208, and Thr-210 phosphomimetic (mutating to Glu) and phosphonegative (mutating to Ala) TR2 mutants, and tested these mutants, as well as a previously generated TR2 mutant defected in PKC-mediated phosphorylation, for their SUMOylation in the COS1 system, which lacks endogenous TR2 and contains only limited endogenous SUMO1 for reconstitution of the relevant molecules such as SUMO and various forms of TR2. As shown in Fig. 2*A*, the WT TR2 was effectively SUMOylated by supplementing the culture with SUMO, and the TR2 was mutated at multiple PKC sites (PKC-CN, mutated at S170A, S185A, S461A, and S568A). Though the negative phosphomutants of Ser-203 (203CN) and Thr-208 (208CN) could still be efficiently SUMOylated, the negative phosphomutant at Thr-210 (210CN) totally failed to be SUMOylated. This finding revealed Thr-210 as the critical residue

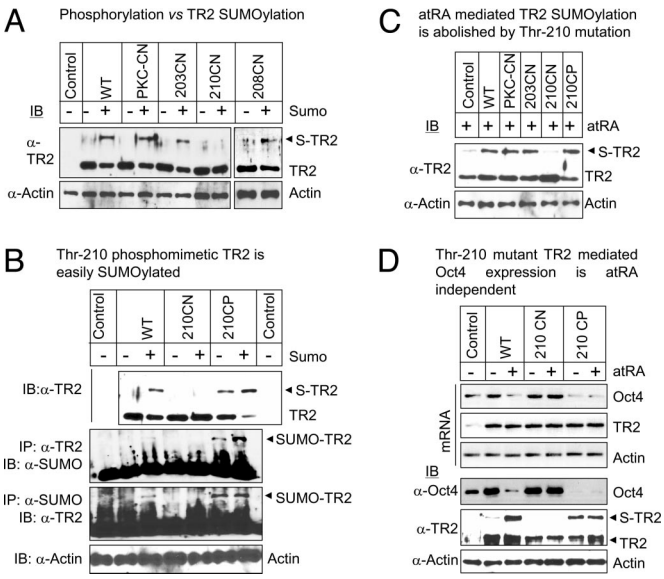

Fig. 2. Phosphorylation of Thr-210 is critical for TR2 to be SUMOylated. (*A*) Western blots of TR2 in cotransfection experiments of various TR2 phosphomutants and WT (0.5 μg/ml) with or without SUMO (0.2 μg/ml) in COS1. (*B*) SUMOylation of phosphomimetic TR2 (210CP mutant) but not the negative phosphorylation mutant (210 CN) with or without additional SUMO in COS1 (top panel). Association of SUMO moiety with 210CP validated in reciprocal coimmunoprecipitation experiments (panels 2 and 3). (*C*) SUMOylation of phosphorylation mutants transfected (0.5 μg/ml) in P19 stimulated by atRA (0.1 μM, 12 h). (*D*) Effects of WT TR2 and mutants 210CN and 210CP with or without atRA, with regards to *Oct4* expression in P19 detected by RT-PCR (mRNA levels) and Western blot (α-Oct4). TR2, S-TR2, and actin were monitored in all of these expressions.

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whose phosphorylation regulated the subsequent SUMOylation. To validate this finding, Thr-210 was mutated into a Glu to mimic phosphorylation (210CP), which was effectively SUMOylated in COS1 (Fig. 2*B*) even without additional SUMO. This could be due to stimulated SUMO conjugation by the phosphorylation signal, which allowed SUMOylation to efficiently take place even with limited SUMO in the cells. The significantly enhanced SUMOylation by additional SUMO substrate remained true for 210CP. Further, in reciprocal coimmunoprecipitation experiments (Fig. 2*B*, lower three panels), this phosphomimetic mutant was confirmed to be effectively associated with SUMO.

These mutated TR2s were then tested in P19 to determine whether atRA could stimulate their SUMOylation (Fig. 2*C*). As predicted, the WT, PKC-CN, 203CN, and 210CP were effectively SUMOylated after atRA treatment, whereas the 210CN mutant failed to be SUMOylated. The phosphomimetic 210CP mutant was effectively SUMOylated in P19 even without atRA stimulation (S-TR2; Fig. 2*D*). It could also efficiently repress Oct4 expression without atRA. The negative phosphorylation mutant 210CN failed to effectively repress Oct4 with or without atRA. The 210CN, which could not be SUMOylated, failed to perform its repressor function. These findings suggest that the repressive effect of atRA is mediated through its ability to regulate TR2 phoshporylation and SUMOylation.

Functional Role for ERK2 in atRA-Stimulated TR2 SUMOylation and the Kinetics of the Activation of This Pathway in atRA-Treated P19. To examine the ERK pathways triggering TR2 SUMOylation, we first used pharmacological agents to activate/block ERK. The preliminary result showed that SUMOylation could be effectively abrogated by the ERK2 inhibitor (Fig. S1). We then performed siRNA knockdown to validate the functional role for ERK2 in TR2 SUMOylation. As shown in Fig. 3*A*, siRNA knockdown of ERK2 very effectively blocked atRA-stimulated SUMOylation of endogenous TR2 (panel 2). In the control experiment, atRA indeed dramatically stimulated TR2 phosphorylation as detected with anti-phosphorylated Thr (panel 1).

To follow Thr-210 phosphorylation of TR2, triggered by nongenomic effects of atRA, the kinetics of activation/expression of the endogenous components in atRA-stimulated P19 were examined (Fig. 3*B*). Interestingly whereas the upstream kinase of ERK2, MEK1/2 remained relatively constant in its phosphorylation/activation or level of expression (panels 1 and 2), phospho-ERK2 (phospho-p42) was rapidly (15 min, panel 10) elevated (indicative of activated ERK2) by atRA stimulation, which was sustained till 16 h and subsided later (panel 3). The expression level of ERK2 was only slightly elevated, but the level of ERK1 remained relatively constant (panels 2 and 3). However, atRA very efficiently enhanced, or stabilized, the enzyme substrate, MEK/ERK, complex formation, which was in good agreement with the kinetics of ERK2 phosphorylation (panels 3 and 10). Consistently, Thr-phosphorylation of TR2 was also rapidly (30 min) stimulated (panel 12) and diminished at 16 h (panel 7). SUMOylation of TR2 became apparent at 8 h, and the level stayed high till 24 h, but sharply subsided at 48 h (panel 8). It is interesting that the *p*-Thr antibody could also detect, transiently, the SUMOylated TR2 (panel 7), suggesting that the Thr-phosphate group of TR2 was quickly removed from TR2 after its SUMOylation. The nongenomic nature of these events was further verified by using transcription and translation inhibitors, both failed to block these events (Fig. S4*A* and *B*). The delay in TR2 SUMOylation subsequent to phosphorylation was intriguing. It appeared that drugs inhibiting microtubule-dependent motors (kinesin and dynein) could block atRA-stimulated TR2 phosphorylation-SUMOylation cascade (Fig. S4*C*), suggesting that certain molecular transport was likely to be involved in converting phosphorylated TR2 into SUMOylated TR2.

We further used the Tet-inducible system to elevate TR2 level in P19, and treated the culture with atRA for 12 h in the presence or

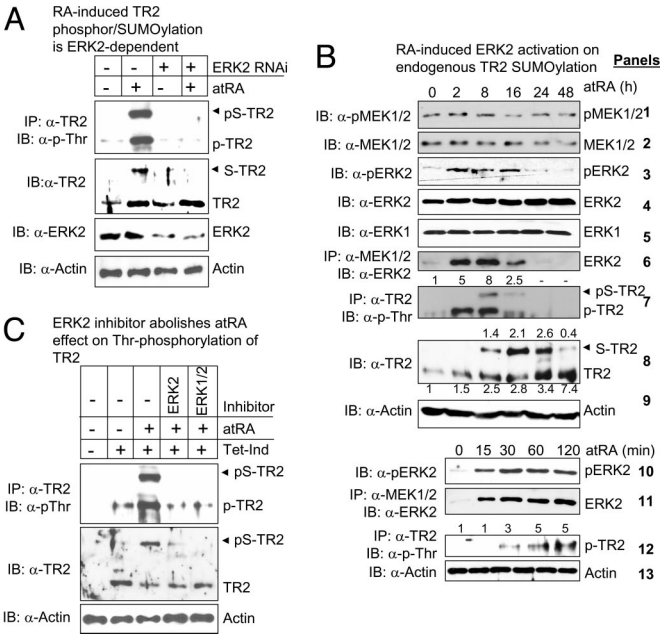

Fig. 3. atRA-stimulated TR2 SUMOylation, mediated by ERK2 that phosphorylates Thr-210 of TR2. (*A*) ERK2 silencing (20 nM siRNA) abolished atRA (0.1 μM, 12 h)-stimulated phosphorylation (panel 1) and SUMOylation (panel 2) of endogenous TR2 in P19. ERK2 silencing was monitored in panel 3. (*B*) A kinetic study of endogenous components in P19 cells after atRA treatment at 0, 2, 8, 16, 24, 48 h (panels 1–9) and early time points (0, 15, 30, 60, 120 min) (panels 10–13). IB indicates Western blot data. IP followed by IB shows immunoprecipitation of the first component followed by Western blot detection of the second component. P-TR2: phosphorylated TR2; pS-TR2: phosphorylated and SUMOylated TR2. Numbers above the images show quantified levels of TR2 or S-TR2. (*C*) Effects of atRA on TR2 phosphorylation (detected by α-pThr) and SUMOylation, with or without ERK2 or ERK1/2 inhibitors in Tet-inducible P19 clone.

absence of ERK inhibitors. As shown in Fig. 3*C* panel 2, elevated TR2 was unSUMOylated without atRA treatment, but was strongly stimulated by atRA for SUMOylation, which was effectively blocked by ERK2 or ERK1/2 inhibitors. As predicted, atRA also dramatically stimulated Thr-phosphorylation of TR2, which was completely ablated by ERK inhibitors (panel 1). The effect of atRA on Thr-210 phosphorylation was further supported by using the point mutants of TR2 (Fig. S4*D*). The WT TR2 was apparently phosphorylated at Thr in the atRA-stimulated culture, whereas both the 210CP and the 210CN mutants were only marginally phosphorylated at Thr residues (panel 1), confirming that Thr-210 was the principal target of atRA-stimulated Thr phosphorylation.

Together, these findings validate the nongenomic effect of atRA that stimulates enzyme-substrate (MEK/ERK) complex formation. The activated ERK2 is responsible for TR2 phosphorylation at Thr-210 and mediates the stimulating effect of atRA for TR2 SUMOylation in P19.

Thr-210 Phosphorylation-Dependent Association of TR2 with PML. We previously concluded that PML was required for TR2 SUMOylation because TR2 was recruited to PML for SUMOylation (4). We then investigated the relationship between Thr-210 phosphorylation, or Lys-238 SUMOylation, and the recruitment of TR2 to PML. We aimed to answer a principal question—that is, whether Thr-210 phosphorylation or Lys-238 deSUMOylation is more critical for TR2 to be associated with PML. We generated two combinations of double mutants that carry SUMOylation mutation (K238R) on the backbone of 210CN or 210CP mutant. As shown in Fig. 4*A* (a coimmunoprecipitation experiment), the WT Flag-TR2 and the Flag-210CP + K238R double mutant both could be

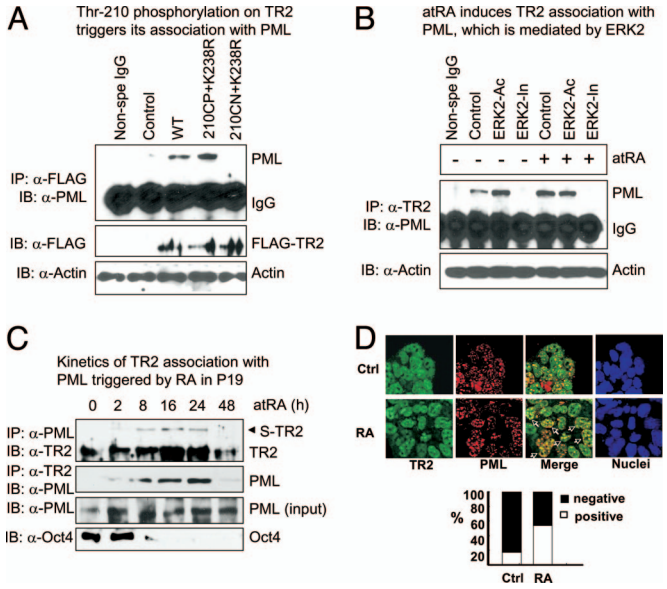

Fig. 4. Phosphorylation of Thr-210 of TR2 triggers its association with PML in P19. (*A*) P19 cells were transiently transfected (0.5 μg/ml, 16 h) with flag-tagged WT TR2, flag-tagged negative SUMOylation mutation in combination with phosphomimetic (210CP + K238R) or negative phosphorylation mutation (210CN + K238R). Association with PML was examined by coimmunoprecipitation experiment (panel 1). Transfection input was monitored in panel 2, and actin is shown in panel 3. (*B*) The association of endogenous TR2 with PML in P19 was examined in the presence of atRA (0.1 μM, 12 h) and/or reagents triggering ERK2 activation (ERK2-Ac) or inhibition ERK2-In. (*C*) A kinetic experiment monitoring endogenous TR2 association with PML (IP followed by IB) in P19 treated with atRA. Corresponding changes in *Oct4* gene expression were shown in the bottom panel. Total PML expression was also monitored. (*D*) Immunostaining of endogenous TR2 and PML in control cells, or cells treated with atRA (0.1 μM) for 6 h. Nuclei were stained with DAPI. Large colocalized TR2/PML puncta in stimulated cells are marked with arrows. Shown at the bottom is the statistical result by scoring the percentage of cells with colocalized TR2 and PML puncta (positive) among the total cells (positive + negative).

associated with PML. However, PML association was completely abolished for the 210CN + 238R double mutant. Thus, phosphorylation on Thr-210, but not deSUMOylation on Lys-238, triggered effective association of TR2 with PML. This was further supported by the behavior of TR2 in the presence of kinase activators or inhibitors as shown in Fig. 4*B*. In the presence of an MAPK/ERK activator, TR2 was increasingly associated with PML, which was completely abrogated by ERK2 inhibitor. In the presence of atRA, ERK2 activator failed to further stimulate TR2 association with PML. Moreover, atRA could not rescue the failure of PML recruitment caused by the ERK inhibitor, supporting that the effect of atRA was mediated by ERK2.

The behavior of endogenous TR2 in P19, in terms atRA-stimulated phosphorylation and association with PML, was validated during the course of early atRA stimulation by using reciprocal coimmunoprecipitation (Fig. 4*C*). TR2 and PML became associated at 2–8 h; the formation of this complex peaked at 16–24 h and sharply declined at 48 h. The kinetics follows very closely that of atRA-stimulated TR2 phosphorylation and SUMOylation as shown in Fig. 3*B*.

To validate whether atRA could facilitate TR2 association with PML, immunohistochemistry was conducted to monitor the distribution of TR2 and PML in the Tet-inducible P19, with and without a short-term (6 h) atRA treatment as shown in Fig. 4*D*. Without atRA (control cells), TR2 was only minimally ($< 20\%$ of the cells) colocalized with endogenous PML (upper panels). As predicted, in the atRA-treated culture (lower panels), TR2 colocalization with PML was enhanced ($\approx 60\%$ of the cells showing colocalization). This result further supports the effect of atRA to stimulate the association of TR2 with PML in this experimental system.

Effects of Thr-210 Phosphorylation on Coregulator Recruitment and the Biological Activity of TR2. To extend the finding in a mechanistic manner, we then determined whether the atRA-elicited signaling pathway could regulate the behavior of TR2 in terms of its ability to recruit appropriate coregulators for regulating target genes by using a previously established mammalian two-hybrid interaction assay as shown in Fig. S5. The interaction with PCAF was dramatically enhanced for 210CN, K238R, and the double mutants 210CP + K238R and 210CN + K238R. Only when Thr-210 was mutated into Glu (210CP) did the receptor then fail to interact with PCAF. Remarkably, the behavior of these mutants with respect to interaction with corepressor RIP140 was clearly opposite. This result confirmed that abolishing either phosphorylation on Thr-210 or SUMOylation on Lys-238 could effectively enhance TR2's interaction with PCAF (i.e., these mutants would behave as activators). In contrast, the 210CP phosphomimetic mutant would be SUMOylated, interact with RIP140, and behave as a repressor. Further, Thr-210 phosphorylation preceded Lys-238 SUMOylation, and therefore, atRA presented an efficient signal trigger to activate SUMOylation of TR2. The predicted biological activities of these mutants were evaluated on a previously established reporter in P19 (32, 33) (Fig. 5*A*) and on endogenous target gene *Oct4*. As shown in Fig. 5*B*, only the 210CP mutant (predicted to be heavily SUMOylated, confirmed in panel 5) significantly repressed Oct4 (panels 1 and 4). All other TR2 mutants could not be SUMOylated and, as predicted, lost their repressor activities for the endogenous target *Oct4*.

Discussion

We previously reported an intricate mechanism that controlled the homeostasis of *Oct4* gene expression mediated by switching the SUMOylation status of TR2 via recruitment to PML (4). An important issue remained to be addressed was whether and how TR2 recruitment to PML for SUMOylation could be regulated. We observed that atRA could stimulate TR2 SUMOylation and repress Oct4 expression through the activation of ERK2 that phosphorylates Thr-210 on TR2. The nongenomic effect of atRA on ERK2 activation was mediated by stimulating the association of ERK2 with its upstream kinase MEK (Fig. 2*B*).

The signal transduction pathway of atRA to regulate TR2 phosphorylation and SUMOylation was mediated by the activation of ERK2 that phosphorylated Thr-210 of TR2, which then became increasingly associated with PML where SUMOylation of TR2 took place (Fig. 4). However, we failed to detect a direct interaction of the WT TR2, or the 210CP, with PML (data not shown), suggesting that a third player could be involved in recruiting—specifically, the Thr-210 phosphorylated TR2 to PML. This is in line with a view that a phosphorylation-SUMOylation cascade might be involved in the recruitment of SUMOylation machinery (34–38). However, in the case of TR2, a novel intermediate step is the recruitment of phosphorylated TR2 to PML where SUMOylation then occurs. Preliminary observation suggests microtubule-assisted recruitment of TR2 to PML (Fig. S4*D*). This may explain the delay in TR2 SUMOylation subsequent to phosphorylation.

In several TR2-targeted gene models, the action of TR2 has been shown to be integrated with vitamin A signaling (14, 18, 39). All of these earlier studies, however, addressed the physiological connection of TR2 with the genomic action of atRA (i.e., by integration with the actions of RARs). To most of the reported TR2 reporters and putative target genes, the action of TR2 was attributed to its competing with RARs for binding to HREs of these gene promoters, thereby suppressing atRA activation of these target genes (40). With our recent proteomic endeavors, we

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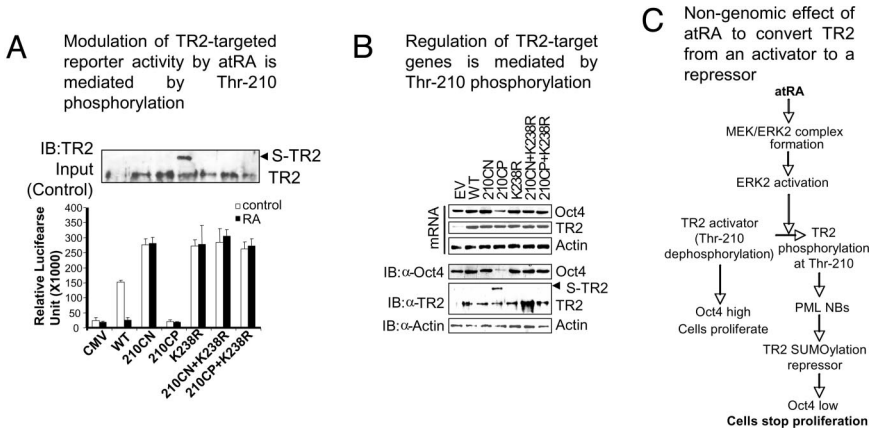

Fig. 5. The nongenomic action of atRA to modulate TR2 biological activity. (*A*) The biological activity of various TR2 mutants evaluated by using an established DR5 reporter in COS1. Cotransfection with WT or mutant (Mt) TR2 (0.1 μg/ml), together with DR5 reporter (0.25 μg/ml) and LacZ (0.05 μg/ml) internal control, followed by 16 h atRA (0.1 μM) treatment, was conducted. (*B*) The activity of various TR2 mutants, assessed by monitoring endogenous Oct4 expression (mRNA and protein) in P19 cells transfected with TR2 vectors (0.5 μg/ml, 16 h). The expression of TR2 input and its SUMOylation, as well as actin, were monitored by IB. (*C*) A schematic diagram for the signal transduction pathway of the nongenomic effect of atRA, which initiates with MEK/ERK2 complex formation that activate ERK2, which in turns phosphorylates TR2 at Thr-210. This facilitates its recruitment to PML and subsequent SUMOylation. The SUMOylated TR2 becomes a repressor for Oct4, reduces Oct4 level, suppresses stem cell proliferation, and prepares cells for the genomic effects of atRA on cellular differentiation.

have detected both activating and repressing functions of TR2, depending on its protein modifications, including PKC- and ERK-mediated phosphorylation (32, 33) and SUMOylation (4). PKC-mediated TR2 phosphorylation regulates, primarily, its protein stability via the ubiquitin pathway. Lys-238 SUMOylation is the primary signal triggering TR2 to recruit corepressor, thereby turning TR2 into a repressor. This current study establishes that the specific ERK2-mediated phosphorylation on Thr-210 activates TR2 SUMOylation pathway. Although TR2 SUMOylation can be triggered by the nongenomic action of atRA (Fig. S4*B*), its total level may be augmented by the genomic effect of atRA as previously reported that atRA could induce TR2 transcription (15). An important novelty in this nongenomic atRA signal transduction pathway is the stabilization of ERK2 with its upstream kinase MEK. Though atRA predominantly, and ultimately, acts through RARs to regulate target genes, it has also been known to elicit rapid nongenomic responses, primarily the ERK pathways. However, the often different/opposing downstream targets of the ERK enzyme system in various cell/tissue types casts serious doubts, and provokes heavy debates, on the specificity and the molecular targets of the intensively debated nongenomic action of atRA. This present study reveals a signal transduction pathway mediated by the nongenomic action of atRA to regulate kinase activation and specific protein phosphorylation and SUMOylation, which seems to involve molecular movement through microtubule-dependent motors.

This study focusses on atRA effects elicited during a short-term (no longer than 12 h) treatment and at a physiological concentration of atRA (0.1 μM). It is anticipated that in a physiological condition, the concentration of atRA is determined by multiple factors, such as its biosynthetic and degrading enzymes (41, 42), which are likely to fluctuate as cells change their property upon differentiation. Whether this pathway can be effectively executed in a prolonged, atRA-stimulated culture, or in cultures that have undergone differentiation, is unclear. A recent study reported surface RARα that was able to mediate a nongenomic action of atRA in hippocampal neurons, which also involved ERK activation (43). It was proposed that a nonconventional action of RARα played a role in neuronal translation. In the P19 stem cell system used in our present study, we could not detect apparent surface RARs. Therefore, it is likely that different pathways could be involved in mediating these observed effects of atRA in different experimental systems.

The P19 system is a widely adopted system to study stem cell proliferation and differentiation. Because the regulation of *Oct4* gene must be safeguarded in healthily proliferating stem cell cultures, it is not surprising that stem cells exploit various transcription factors (4, 7–11) to regulate Oct4 expression under a normal condition. As shown in the signal transduction pathway (Fig. 5*C*), upon exposure to atRA, TR2 would then serve to deliver the early nongenomic activity of atRA to rapidly repress Oct4 expression and slow down its proliferation, facilitating a cellular environment suitable for the subsequent differentiation programs that are expected to take place later in the atRA-exposed cultures. This can be important in maintaining stem cell cultures, or tumor cells, where the control of cell differentiation is often coupled to the slowdown in cell proliferation. Finally, the unexpected nongenomic functional role for atRA in facilitating molecular complex formation in the second messenger system suggests a potentially wider spectrum of biological activities of atRA, in addition to its well established nuclear function to regulate gene expression.

Materials and Methods

Plasmid constructs, site-directed mutagenesis, chemicals, RNA interference, and reverse-transcription PCR are provided in *SI Materials and Methods*.

TR2-Inducible P19 Clones (4) and TR2/PML Immunohistochemistry. TR2 was induced by removing tetracycline (2 μg/ml) for 36–48 h before atRA treatment for 6 h. Cells were fixed in 4% formaldehyde and a permeation buffer, blocked for 30 mins, and incubated with primary antibodies at 4°C overnight, followed by secondary fluorescence-conjugated antibodies, at room temperature for 3 h. Images were acquired with a fluorview confocal system (Olympus). Quantification was conducted by scoring the positive cells (showing >20% colocalized PML with TR2) versus total cell numbers. Transfection and reporter assay were as described (4, 32, 33, 46). Assays were conducted at 16–36 h.

Immunoprecipitation and Western Blot Analysis. As described previously (4, 16), antibodies were FLAG-M2 (F3165) from Sigma, SUMO-1 (OPA1–07200) from Affinity BioReagents, phosphothreonine (ab-9337) from Abcam, and PML (05–718) from Upstate Biotechnology. MEK1/2 (sc-436), phosphor-MEK1/2 (sc-7995), Oct 4 (sc-9081), TR2 (sc-9087), and PML (sc-5621) were from Santa Cruz Biotechnology. ERK1 (4372), ERK2 (9108), and phosphor-p42/p44 ERK (9101) were from Cell Signaling.

Statistics. All statistical data were from averages of three or more independent experiments. Two-tailed Student *t* test was performed to obtain *P* values. *P* value <0.05 was considered to be statistically significant.

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