Cell Cycle 12:8, 1251–1266; April 15, 2013; © 2013 Landes Bioscience

CDK1 interacts with RARγ and plays an important role in treatment response of acute myeloid leukemia

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Keywords: cell cycle, differentiation, leukemia, retinoic acid receptor, AML, CDK1, therapy

Alterations in cell cycle pathways and retinoic acid signaling are implicated in leukemogenesis. However, little is known about the roles of cyclin-dependent kinases (CDKs) in treatment response of leukemia. In this study, we observed that CDK1 expression was significantly higher in bone marrow from 42 patients with acute myeloid leukemia (AML) at recurrence than that at first diagnosis ($p = 0.04$). AML patients had higher level of nuclear CDK1 in their leukemic blasts tended to have poorer clinical outcome compared with those with lower levels. We showed that CDK1 function is required for all-trans retinoic acid (ATRA) to achieve the optimal effect in U-937 human leukemic cells. CDK1 modulates the levels of $P27^{kip}$ and AKT phosphorylation in response to ATRA treatment. Further, we show, for the first time, that RARγ in concert with ATRA regulates protein levels of CDK1 and its subcellular localization. The regulation of the subcellular content of CDK1 and RARγ by ATRA is an important process for achieving an effective response in treatment of leukemia. RARγ and CDK1 form a reciprocal regulatory circuit in the nucleus and influence the function and protein stability of each other and the level of $P27^{kip}$ protein. In addition, expression of wee1 kinase and Cdc25A/C phosphatases also coincide with CDK1 expression and its subcellular localization in response to ATRA treatment. Our study reveals a novel mechanism by which CDK1 and RARγ coordinate with ATRA to influence cell cycle progression and cellular differentiation.

Introduction

Acute myeloid leukemia (AML) is a common cancer type among children and the elderly population. The recurrence of the disease and poor response of certain leukemic patients to treatments remains a major clinical challenge.[1] It is therefore important to gain deeper understanding of the molecular mechanisms underlying cancer recurrence and treatment resistance. Further, identification of novel therapeutic targets and elucidation of their precise function will provide insight into the improvement of cancer therapies.

Cyclin-dependent kinases (CDKs) and their associated regulatory cyclins are required for cell cycle progression and DNA replication.[2] CDK1, but not CDK2, CDK4 or CDK6, is functionally required for embryonic development, as mice lacking CDK1-gene alleles fail to develop to the morula and blastocyst stages.[3] Alterations in the expression or activity of CDKs, in particular, CDK1, are frequently observed in various types of solid tumors including lung cancer, colon cancer and breast cancer.[4-6] Specific activity of CDK1, but not CDK2, significantly predicts distant metastases of colon cancer.[5] Further, the activity of CDK1 and CDK2 in combination is shown to be a significant prognostic indictor for breast cancer recurrence.[6] CDK1 itself is activated by A-type cyclins and is in complex with cyclin B1 to facilitate the onset of mitosis.[7]

It has been proposed that tumor cells with defective CDK signaling can escape the anti-mitogenic signals induced by chemotherapeutic drugs.[2] Protein phosphorylation strongly influences subcellular localization, activity and function.[8,9] CDK1 can be phosphorylated on Thr-14 and Tyr-15 residues.[10,11] CDK1 activity, substrate specificity and function also depend on its phosphorylation status and subcellular localization.[12,13] Recent study has revealed that subcellular localization of CDK1 has clinical importance in patients with lung cancer. Although the total level of CDK1 is highly expressed in agressive non-small cell lung cancer tissues, loss of cytoplasmic CDK1 is associated with a poor prognosis for NSCLC patients.[4] Thus, CDK1 has emerged as a potential novel cancer therapeutic target, and several clinical

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Submitted: 02/19/13; Accepted: 03/14/13
http://dx.doi.org/10.4161/cc.24313

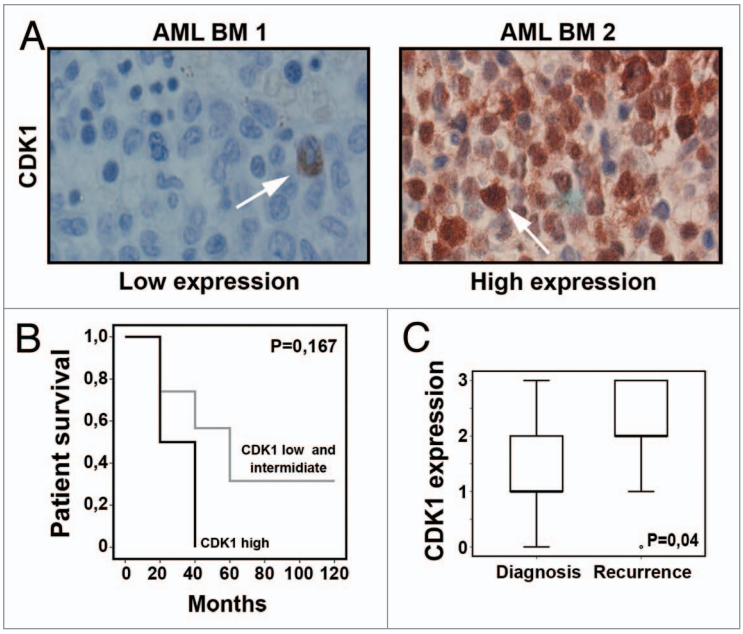

Figure 1. CDK1 expression in AML patients and its implication in disease recurrence and overall patient survival. (**A**) Immunohistochemical analysis shows the expression and subcellular localization of CDK1 in BM from two individual AML patients. CDK1 is either absent or little in AML BM, as shown in the left panel, or is highly nuclear in AML BM, as shown in the right panel. (**B**) Kaplan-Meier curve shows overall survival of AML patients (n = 42). Patients were divided into two groups based on the level of CDK1 expression. Patients who belonged to CDK1-high group (score 3–4) had predominantly high level of nuclear CDK1 expression (n = 5), whereas the remaining patients belonged to CDK1 low and moderate-group (score 0–2) (n = 37). (**C**) CDK1 expression in BM from first diagnosis is compared with that in BM from disease relapse in the same patients (n = 13).

trials that target CDK1 by using its inhibitors are currently under way.[14] However, little is known about the role of CDK1 during leukemogenesis and in treatment response.

ATRA has been successfully used for the treatment of subtypes of leukemia such as acute promyelocytic leukemia.[15] Because ATRA induces both terminal differentiation and growth arrest,[16,17] it has therefore been proposed as a candidate for therapy of different types of cancers.[18] ATRA inhibits cell cycle progression by both direct and indirect regulation of key components of the cell cycle apparatus, including cyclins, CDKs and CDK inhibitors such as $P21^{cip1}$ and $P27^{kip}$.[19-21] However, treatment resistance in leukemia using cytotoxic drugs and ATRA occurs in some cases, and the incidence of treatment resistance increases in patients who have had disease recurrence.[18]

ATRA exerts its biological effects through the activation of nuclear retinoid receptors, which regulate transcriptional activation of retinoid target genes.[22] Defects in retinoid signaling and metabolism are common in many human solid and hematological malignancies.[21] There are three retinoic acid receptors (RARα, RARβ and RARγ) that typically form heterodimeric complexes with one of three retinoid X receptor isoforms (RXRα, RXRβ and RXRγ).[22] In addition to its well-established nuclear function in regulating gene expression, ATRA can activate mitogen-activated protein kinases (MAPKs) through RAR receptors.[23] Loss, accumulation, mutations or aberrant modifications of RARs lead to uncontrolled proliferation and blockage of differentiation and subsequently carcinogenesis.[24] RARγ is an important regulator of cell reproduction, proliferation and differentiation and is associated with retinoid effects.[24] Treatment resistance might be the consequence of the alteration of retinoid signaling pathways, resulting in the failure of leukemic cells to respond to ATRA and cytotoxic treatment. However, the mechanism(s) by which leukemic cells with defective CDK pathways and RAR receptor signaling may escape ATRA-induced terminal differentiation and growth arrest are poorly understood.

In the present study, we elucidate the clinical importance of CDK1 in ATRA treatment response of AML patients. We further unravel a novel role of CDK1 and CDK2 along with the retinoic receptors, RARα RARβ and RARγ, and underlying mechanisms in ATRA treatment response in cancer cell lines. Our study shows that high levels of CDK1 expression are associated with poor treatment response of AML patients, and that CDK1 expression and subcellular localization is regulated by RARγ. Multiple cellular pathways cooperatively play important roles in mediating the effect of ATRA in cancer cells. Identification of molecular pathways and targets that mediate ATRA-induced proliferation and differentiation may provide novel tools for more effective and selected therapies for leukemia and various types of cancers.

Results

CDK1 expression, disease relapse and treatment resistance in AML patients. To delineate the clinical importance of CDK1 expression in disease progression and treatment response of AML, we analyzed CDK1 expression in bone marrow specimens from 42 patients with AML and in normal bone marrow from donors (n = 10) using immunohistochemical analysis. We observed a weak or moderate expression of CDK1 in both nuclear and cytoplasmic compartments of myeloid progenitor cells from normal bone marrows. In leukemic bone marrows from patients, CDK1 expression was either undetectable or was present at high level in the nucleus of leukemic blasts (**Fig. 1A**). We then investigated whether CDK1 expression correlated with therapeutic response and clinical outcome. Among the 42 patients, 12 patients achieved remission, 17 had disease-recurrence and the remaining 13 either died or did not respond to the initial therapy (**Table 1**). 66.6% (n = 6 out of 9) of the patients who did not respond to therapy had undetectable or very little CDK1 expression in their leukemic blasts. Kaplan-Meier overall-survival analysis with a median follow-up of 66 mo, ranging from 1 to 132 mo, revealed that patients with high level of nuclear CDK1 had poorer overall survivals (**Fig. 1B**). The complete remission rate (CR rate) tended to be lower in CDK1-high group (40.0%) compared with CDK1-moderate or low group (64.9%), although statistical significance was not achieved (p = 0.167) (**Fig. 1B**).

We next compared CDK1 expression in leukemic bone marrows at diagnosis paired-wise with those obtained at AML-recurrence

Table 1. Patient cohort

	Number of patient at diagnosis	Number of patients with relapse	Number of patients with remission	Number of normal BMs
Total number of patients	42	17	12	10
Subtype M1–2	34	15	11	-
Subtype M3	6	1	1	-
Subtype M5	2	1	-	-
Number of BM available	42	13	11	10
CDK1 negative or low (0–1)	26	6	7	10
CDK1 moderate or high (2–4)	16	11	5	-

from the same patients (n = 13). CDK1 level was significantly higher in bone marrow specimens at AML-recurrence than that at diagnosis (p = 0.04) (**Fig. 1C**). There were no significant differences in CDK1 expression between bone marrow specimens at diagnosis and that at remission (n = 11, p = 0.85). Collectively, these data suggests that CDK1 expression is associated with disease progression and treatment response in AML.

ATRA induces differentiation and growth arrest in U-937 leukemic cells, coinciding with a rapid decrease of nuclear CDK1 protein. To gain a deeper understanding of the molecular mechanisms and functional aspects of CDKs in treatment response of cancer cells, we first employed leukemic U-937 cell line and ATRA treatment as the model systems. We wanted to explore whether modulation of CDK1 expression may sensitize the responsiveness of non-acute promyelocytic leukemic cells to ATRA treatment. First we treated U-937 cells with ATRA at 1 μM for 24, 48 or 72 h to induce terminal differentiation. This led to morphological changes (**Fig. 2A**) and a ~3-fold increase in cells that expressed CD11c, a cell surface marker for differentiation, after 72 h of treatment as compared with the controls ($p < 0.01$) (**Fig. 2B**). In addition, ATRA induced cell cycle arrest, with a ~20% increase in the number of G_0/G_1 phase-cells compared with controls (**Fig. 2C and D**). These data confirm that ATRA induced terminal differentiation and cell cycle arrest at the G_0/G_1 phase in U-937 cells.

We then examined the expression and subcellular localization of CDK1 in U-937 cells. The equal amount of protein lysates from total cellular proteins, cytoplasmic vs. nuclear proteins were subjected to immunoblot analysis. We detected two forms of CDK1 attributed to differences in CDK1 phosphorylation, as described by Borgne et al.,[10] in nucleus and cytoplasm of U-937 cells (**Fig. 3A**). Next, we determined the changes in cellular and subcellular expression of CDK1 in U-937 cells following ATRA treatment. ATRA induced a significant time-dependent decrease in total CDK1 (p = 0.03, p = 0.009 and p = 0.03 after 24, 48 and 72 h, respectively) (**Fig. 3A and B**). The ATRA-induced decrease in nuclear CDK1 level was significantly pronounced (p = 0.037 and $p < 0.001$ after 48 and 72 h), while cytoplasmic CDK1 was slightly increased after 24 h, but was declined to a less extend after 48 to 72 h of ATRA treatment (**Fig. 3A and B**). This is the first report showing the relationship between subcellular CDK1 expression and the effect of ATRA treatment.

Because protein phosphorylation influences subcellular localization, activity and function,[8,9] and CDK1 can be phosphorylated on Thr-14 and Tyr-15 residues,[10,11] we next determined the status of CDK1 phosphorylation and subcellular distribution in relation to the cellular response to ATRA. We subjected nuclear and cytoplasmic protein lysates to anti-CDK1 immunoprecipitation analysis. Anti-phospho-tyrosine antibody detected CDK1 complexes exclusively in the nuclear fraction, confirming that nuclear CDK1, but not cytoplasmic CDK1, was tyrosine phosphorylated (**Fig. 3C**). ATRA treatment greatly reduced tyrosine phosphorylation of CDK1 readily after 48 h and throughout 72 h. Taken together, the phosphorylation status of CDK1 in the nucleus may be functionally linked to the ATRA effect on cell cycle arrest and differentiation.

CDK1 function is required for cell cycle control at G_0/G_1 phase and for mediating ATRA effect on differentiation and growth arrest in U-937 cells. We next elucidated the role of CDK1 alone or together with CDK2 in mediating response of cells to ATRA-induced cell cycle arrest and differentiation. To test this, we functionally depleted CDK1 and CDK2 individually or in combination in U-937 cells using siRNA-mediated knockdown. Cells with single or double knockdown of these proteins were referred to as siCDK1, siCDK2 and siCDK1+2, respectively. The successful reduction in CDK1, phosphorylation level of CDK1 and CDK2 protein expression by siRNAs throughout 24 to 72 h was confirmed by immunoblotting (**Fig. 4A**). Individual knockdown of CDK1 or double knockdown of CDK1+CDK2 in U-937 cells resulted in an approximately 10% decrease in the proportion of cells at G_0/G_1 phases (for siCDK1, p = 0.002; for siCDK1+2, p = 0.049) and a concomitant increase in the ratio of G_2/M phase (for siCDK1, p = 0.015; for siCDK1+2, p = 0.044) as determined by flow cytometry (**Fig. 4B**). Knockdown of CDK2 alone showed no measureable effect on cell cycle distribution (**Fig. 4B**). No major changes in the expression of proteins critical for DNA synthesis including cyclin E and cyclin A2 were detected in siCDK1, siCDK2 or siCDK1+2 cells as compared with controls (**Fig. S1**). This data suggests that CDK1 function is required for maintaining the balance of cell cycle. To test whether CDK1 function is required for effective cellular response to ATRA treatment, we treated siCDK1, siCDK2 or siCDK1+2 cells with ATRA for 48 h. ATRA treatment induced a lesser degree of G_0/G_1 arrest in siCDK1 cells compared with that in siControl cells (p = 0.001) (**Fig. 4C**). Further, a proportion of G_2/M phase cells was higher in ATRA-treated siCDK1 cells compared with ATRA-treated siControl cells (for siCDK1, p = 0.015; for siCDK1+2, p = 0.044) (**Fig. 4C**). This suggests

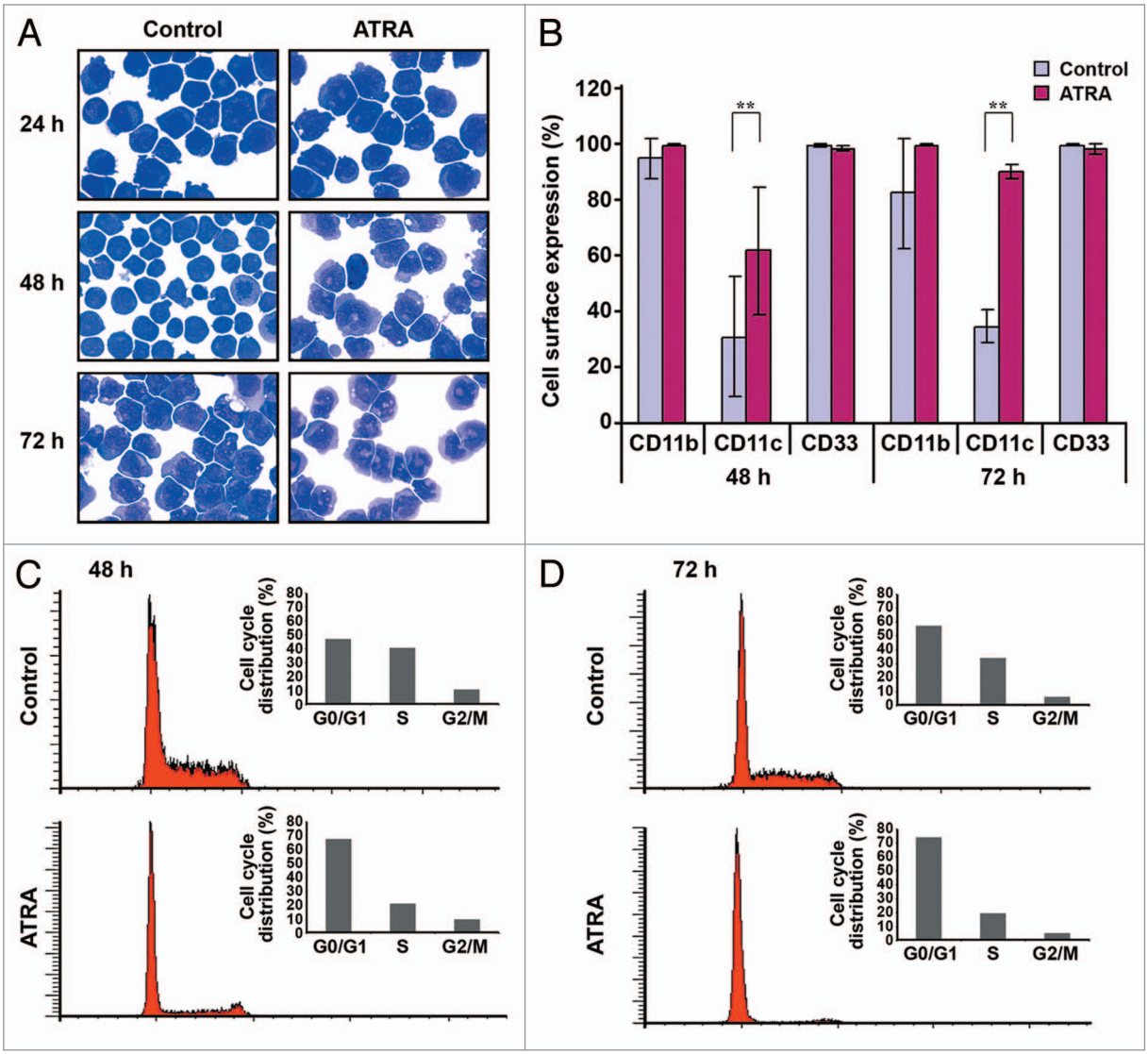

Figure 2. The effect of ATRA treatment on terminal differentiation and cell cycle distribution in U-937 cells. (**A**) U-937 cells were treated with ATRA (1 μM) or solvent DMSO/ethanol (control) for 24, 48 and 72 h, as indicated. The cells were fixed by cytospin and stained with Giemsa. (**B**) The expression of CD11b, CD11c and CD33 in U-937 cells treated with ATRA or solvents for 48 h or 72 h was measured by flow cytometry. Percentage of U-937 cells expressing the cell surface markers is indicated in the Y-axis. Mean ± SD represents three independent experiments. **p < 0.01 (paired t-test). (**C and D**) Cell cycle distributions of U-937 cells treated with ATRA (1 μM) or solvent control for 48 h or 72 h. The histogram of cell cycle profiles from flow cytometry analysis and the percentages of cells at onset of each cell cycle phase are shown.

that loss of CDK1 may allow cancer cells undergo active mitosis, thus reduce their sensitivity to ATRA-induced cell cycle arrest. Because $P27^{kip}$ is a cell cycle inhibitor that is required for the maintenance of quiescence at G_0/G_1 phase, and increased level of $P27^{kip}$ is an indicator of ATRA effect on cell cycle,[29] we therefore examined the effect of silencing CDK1 and CDK2 on $P27^{kip}$. There was a pronounced reduction (~35%) in $P27^{kip}$ expression in siCDK1 U-937 cells as compared with siControl U-937 cells (**Fig. 4D**), suggesting that CDK1 function is required for maintaining proper level of $P27^{kip}$ expression. ATRA increased the level of $P27^{kip}$ in siCDK2 or siCDK1+siCDK2 cells, which were comparable to control U-937 cells (**Fig. 4E**). However, ATRA-induced increase in $P27^{kip}$ was less pronounced in siCDK1 cells compared with the controls (**Fig. 4E**). The above data show that the reduced sensitivity of siCDK1 cells to ATRA treatment is likely due to the loss of the balance of cell cycle control mechanisms, including the decreased p27 expression concomitant with the increased mitotic activities that resulted from the loss of CDK1 function.

Since ATRA induces cellular differentiation in leukemic cells, we next investigated whether CDK1 and CDK2 may play a role in ATRA-induced differentiation. Inhibition of individual CDKs or CDK1 and CDK2 in combination had no effect on the basal expression of CD11c, the cell surface marker for differentiation (**Fig. S2**). However, siCDK1 U-937 cells displayed a reduced CD11c expression (~10%) following ATRA treatment as

compared with siControl cells (**Fig. 4F**), suggesting that cells lacking CDK1 expression were less responsive to ATRA induced differentiation. Collectively, these results show that CDK1 expression is associated with the sensitivity of U-937 cells to ATRA induced cell cycle arrest and differentiation. This novel finding supports our observations in AML patients that absence of CDK1 expression correlated with treatment resistance.

Functional link between CDK1, CDK2 and RARs in cellular response to ATRA. Next, we wanted to further investigate the underlying molecular mechanisms related to CDK1 in treatment response of cancer cells. Since ATRA effect is functionally mediated by the RARs, we therefore wanted to assess whether RARs may be functionally associated with CDK1 in mediating ATRA sensitivity of U-937 cells. We examined mRNA and protein expression of the three major RARs, RARα, RARβ and RARγ in U-937 cells treated with solvent control or ATRA. ATRA induced an increase in *RAR*α and *RAR*β mRNA levels (**Fig. 5A and B**), but a decrease in RARα and RARβ protein expression in U-937 cells as compared with controls (**Fig. 5E**). This suggests that ATRA modulated RARα and RARβ protein expression via post-transcriptional mechanisms. In contrast to what was observed for RARα and RARβ, RARγ mRNA and protein expression were both reduced upon ATRA treatment (**Fig. 5C and D**). Next, we examined the effect of CDK1 knockdown on the protein expression of the RARs in the absence or presence of ATRA treatment. RARγ was increased in siCDK1 cells compared with siControl cells (**Fig. 5D**). Knockdown of CDK1 also impaired ATRA-induced downregulation of RARγ protein (**Fig. 5D**). Consistent with this, there is evidence that ATRA induced degradation of RARγ is required for RARγ transcriptional activity of target genes.[30] Knockdown of CDK1 did not show pronounced effect on RARα and RARβ (**Fig. 5E**). Because the activity of phosphatidylinositol 3-kinase (PI3K)/Akt pathway is associated with cancer cell survival and treatment resistance, we therefore examined the effect of CDK1 knockdown on the phosphorylation of Akt in the absence or presence of ATRA. Expression of phospho-AKT was increased in siCDK1 cells compared with the control cells

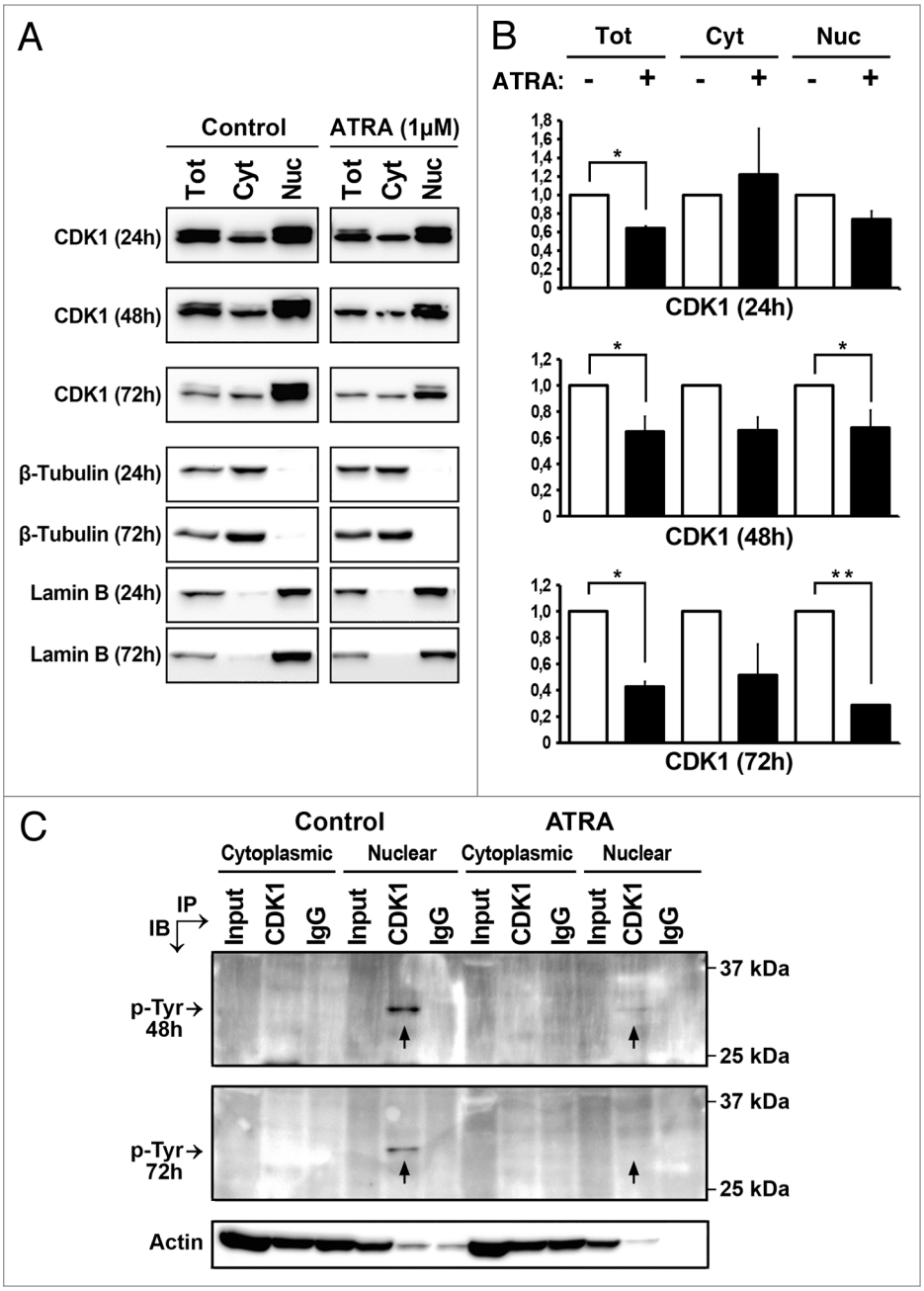

Figure 3. ATRA effects on CDK1 localization and phosphorylation. (**A**) Immunoblot (IB) analysis of CDK1 in the total cell lysates and in subcellular fractions of U-937 cells following ATRA treatment for 24, 48 and 72 h, as indicated. Blotting of β-tubulin served as loading control for total lysates and cytoplasmic fractions, and antibody against lamin B was used as a control for the nuclear fraction. (**B**) Densitometric quantification of the levels of total CDK1, cytoplasmic and nuclear fractions separated from U-937 cells treated with solvent or ATRA throughout 24 to 72 h. Mean ± SD represents two to three independent experiments. *$p < 0.05$ and **$p < 0.01$ (paired t-test) are indicated. (**C**) Cytoplasmic and nuclear fractions separated from U-937 cells treated with solvent or ATRA (1 μM) for 48 h or 72 h were subjected to immunoprecipitation (IP) assay. Antibody to CDK1 was used to pull down the immunocomplexes; antibody against phospho-Tyr was used for immunoblot analysis to detect tyrosine phosphorylation of CDK1 in cytoplasmic or nuclear compartment, and total cell lysates were used as controls (input). Antibody to IgG was used as negative control.

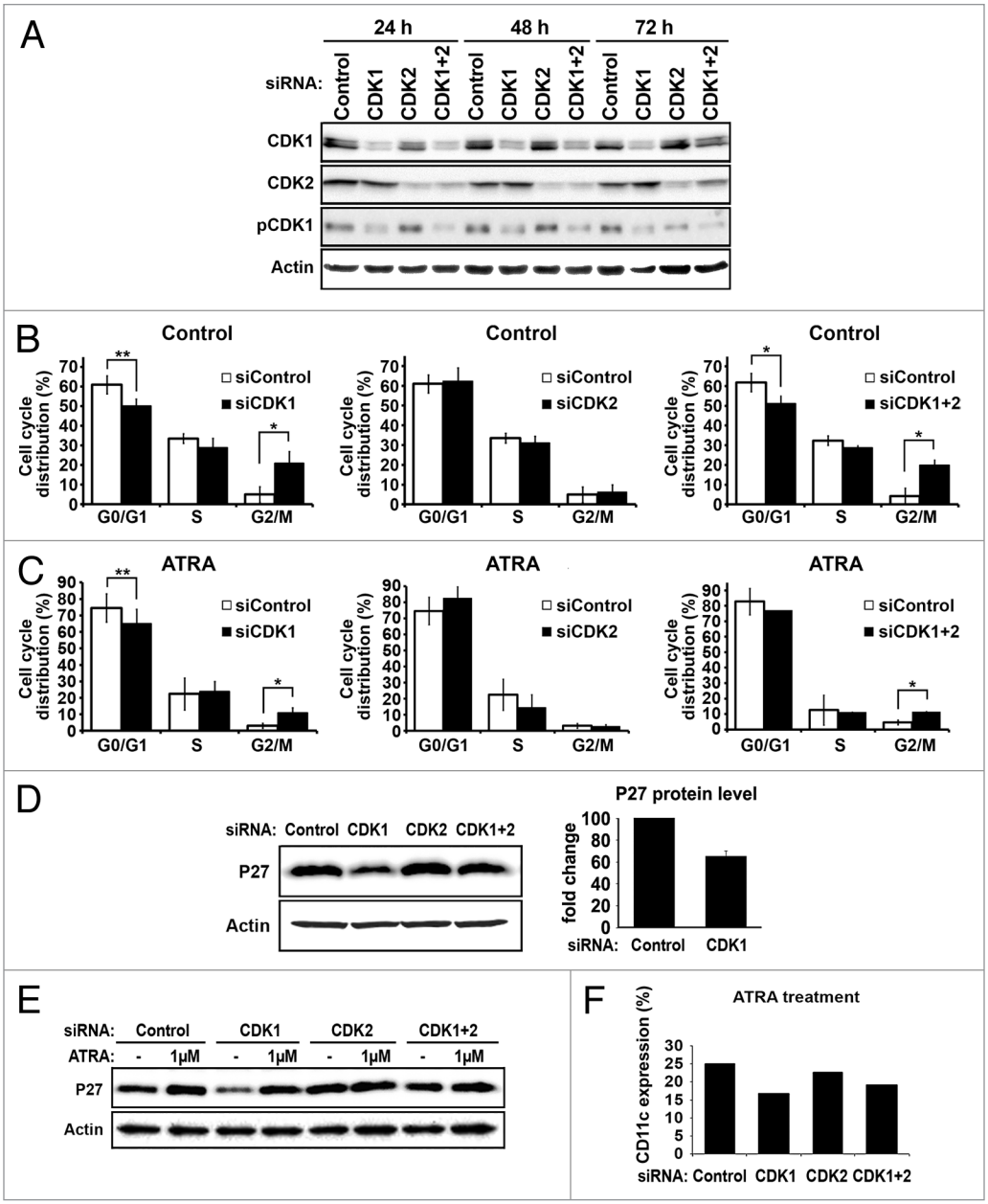

Figure 4. Functional analysis of the effects of siRNA mediated targeted depletion of CDK1, CDK2, or CDK1 + CDK2, on cell cycle and differentiation in human leukemic U-937 cells. (**A**) IB analysis of protein lysates from U-937 cells transfected with control siRNA or siRNAs targeting CDK1, CDK2 and CDK1 + CDK2 respectively, harvested at 24, 48 and 72 h. (**B**) Flow cytometry analysis of cell cycle distribution of U-937 cells that were transfected with control siRNA (siControl) or siRNA to CDK1 (siCDK1), CDK2 (siCDK2) and CDK1 + CDK2 (siCDK1+2), respectively. The percentage of cells at onset of each cell cycle phase is shown on the Y-axis. Mean ± SD represents three independent experiments. *$p < 0.05$ and **$p < 0.01$ (paired t-test). (**C**) Effect of ATRA on cell cycle distributions in siControl U-937 cells, siCDK1, siCDK2 and siCDK1+2 U-937 cells. Mean ± SD represents three independent experiments. *$p < 0.05$ and **$p < 0.01$ (paired t-test). (**D**) IB analysis shows the expression of $P27^{kip}$ in siControl U-937 cells, siCDK1, siCDK2 and siCDK1+2 U-937 cells (left panel). IB analysis shows the expression of $P27^{kip}$ in siControl U-937 cells, siCDK1, siCDK2 and siCDK1+2 U-937 cells in the absence or presence of 1 μM ATRA treatment for 48 h (right panel). (**E**) IB analysis shows the expression of $P27^{kip}$ in siControl U-937 cells, siCDK1, siCDK2 and siCDK1+2 U-937 cells, and the effect of ATRA treatment (1 μM) for 48 h. (**F**) The representative experiment showing the percentage of cells expressing CD11c in siControl U-937 cells, siCDK1, siCDK2 and siCDK1+2 U-937 cells that were treated with 1 μM ATRA for 72 h.

(**Fig. 5F**), suggesting that depletion of CDK1 is asscoaited with the increased activity of AKT survival pathway. Further, treatment of siCDK1 cells with ATRA greatly enhanced the level of AKT phosphorylation compared with the controls (**Fig. 5F**). This novel finding suggests that knockdown of CDK1 in U-937 cells reduced the sensitivity to ATRA treatment and may be linked to the increased activity of Akt survival pathways.

The complex formation between CDK1 and RARγ, and their similar subcellular distribution in response to ATRA treatment. It has been proposed that the level of ATRA receptor

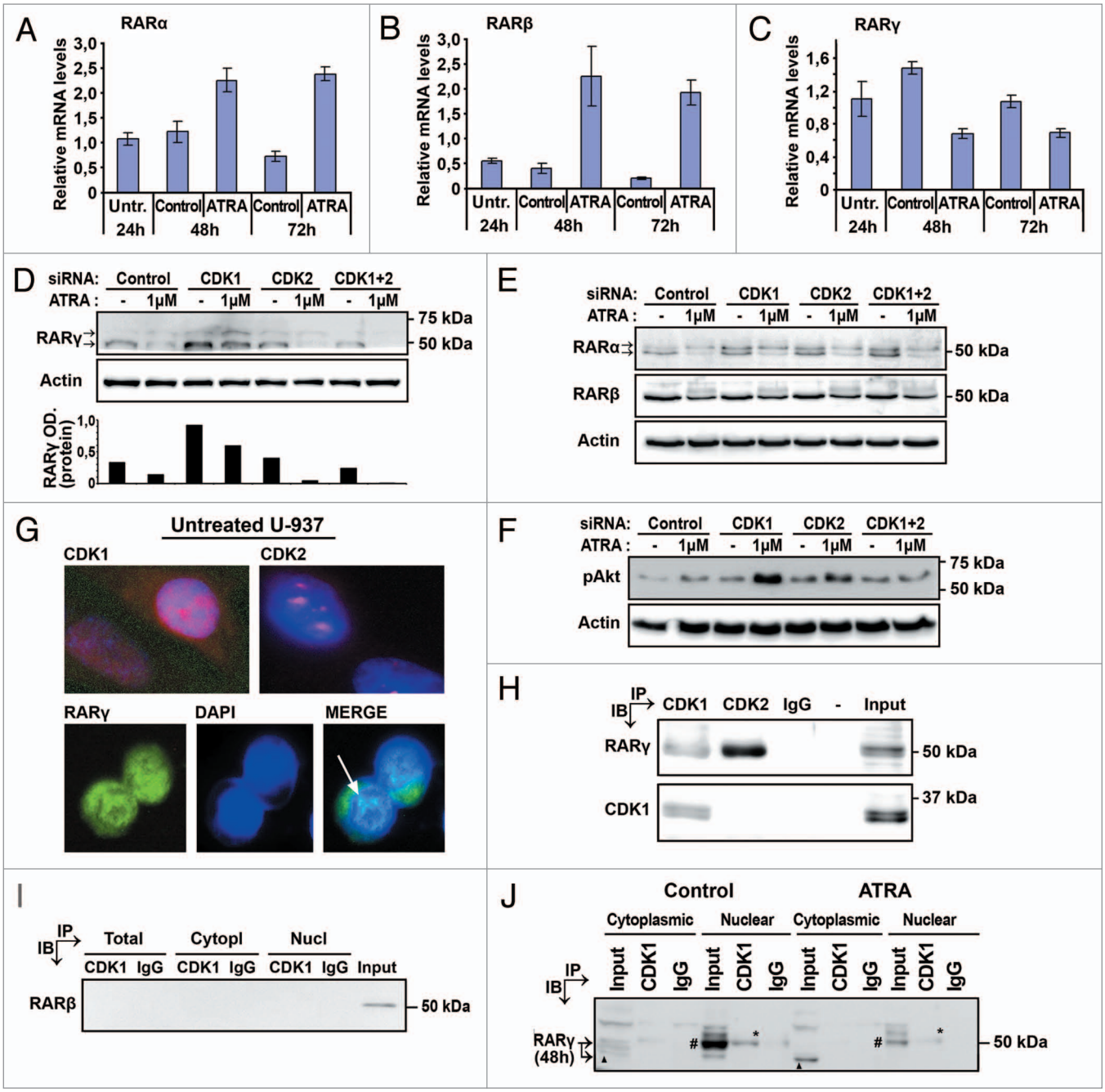

Figure 5. The effect of ATRA treatment and CDK downregulation on RAR expression. (**A–C**) mRNA expression of the ATRA receptors *RAR*α, *RAR*β and *RAR*γ in U-937 cells untreated cells: "Untr.," treated with solvent: "Ctrl," or with 1 μM ATRA: "ATRA" for 24, 48 and 72 h. (**D and E**) IB analysis to determine the expression of RARγ, RARα and RARβ protein levels in siControl, siCDK1, siCDK2 or siCDK1+2 treated with ATRA (1 μM) or solvents (-) for 48 h. (**F**) IB analysis to determine the expression of pAkt levels in siControl, siCDK1, siCDK2 or siCDK1+2 treated with ATRA (1 μM) or solvents (-) for 48 h. (**G**) Upper panels: Immunofluorescence (IF) staining of U-937 cells using Rhodamine-conjugated antibody against CDK1 (red) merged with DAPI (blue) and Rhodamine-conjugated antibody against CDK2 (red) merged with DAPI (blue). Lower panels: IF staining of RARγ subcellular localization using FITC-conjugated antibody against RARγ (green) merged with DAPI (blue). RARγ was predominantly detected in the nuclear compartments, and some signals were found in the subset of the nuclear bodies in U-937 cells. (**H**) CDK1- or CDK2-complexes were immunoprecipitated from total U-937 cell lysates, IgG was used as negative control. Antibodies against RARγ or CDK1 were used for detection of complexes between CDK1, CDK2 and RARγ. (**I**) Antibody against CDK1 was used to pull down the complexes in total lysate, nuclear fraction and cytoplasmic fraction of U-937 cells, antibody to RARβ2 was used to detect complexes by IB analysis. (**J**) CDK1 immunocomplexes were pulled down from nuclear and cytoplasmic fractions of U-937 cells treated with solvent or ATRA, and were detected using antibody against RARγ. The lysates from nuclear vs. cytoplasmic fractions were used as controls and were probed with antibody against RARγ on IB. The cytoplasmic RARγ is indicated with the symbol "▲" the nuclear RARγ is markered with "#." The nuclear CDK1-RARγ protein complexes are marked with "*."

expression itself does not determine the response to retinoids; the cellular response is instead determined by other co-regulating factors.[31,32] However, the co-regulators of the ATRA receptors remained to be identified. We next wanted to investigate whether CDK1 may act as co-regulator of RARs. We first performed immunofluorescence analysis to visualize the subcellular localizations of CDK1, CDK2 and RARγ. CDK1, CDK2 and RARγ were localized in the nucleus of U-937 cells (**Fig. 5G**). Immunoprecipitation assays were performed to determine whether there might be a physical interaction between RARγ and CDK1 in U-937 cells. RARγ was indeed present in CDK1-immunocomplexes, and in CDK2-immunocomplexes as well (**Fig. 5H**), whereas RARβ was not detected in CDK1- or CDK2-immunocomplexes (**Fig. 5I**). RARγ was abundant in the nucleus as marked with "#" (**Fig. 5J**), which is consistent with its role as the nuclear receptor.[23] RARγ protein was also observed in the cytoplasmic compartment (**Fig. 5J**). Immunoprecipitation of nuclear and cytoplasmic fractions using antibody against CDK1 showed that complexes between CDK1 and RARγ were located exclusively in the nucleus (marked "*" in **Fig. 5J**). ATRA treatment resulted in a remarkably reduced level of nuclear RARγ isoforms but led to an appearance of the truncated cytoplasmic RARγ (marked with "▲" in **Fig. 5J**). CDK1 and RARγ complexes persist, but at a reduced level following ATRA treatment of U-937 cells (**Fig. 5J**). This novel finding showed that CDK1 and RARγ formed protein-protein complexes in the nucleus of U-937 cells. ATRA treatment reduced the level of the complexes of CDK1 and RARγ.

RARβ and RARγ but not RARα are required for ATRA to influence the protein expression of CDK1. As mentioned above, high level of nuclear CDK1 expression was associated with low rate of complete remission in AML patients, and a downregulation of nuclear CDK1 is associated with ATRA-mediated treatment response. We next investigated whether RARs were able to modulate CDK1 expression in response to ATRA treatment. To this end, we used wild type mouse F9 teratocarcinoma cells (F9 WT), which are sensitive to ATRA-induced differentiation, and F9 cells harboring targeted deletions of both alleles of the genes encoding the retinoic acid receptors (F9 RARα$^{-/-}$, F9 RARβ2$^{-/-}$ and F9 RARγ$^{-/-}$). Targeted deletion of the RARγ gene completely abolished expression of RARγ protein as confirmed by immunoblot analysis (**Fig. S3**). *RARβ2* and *RARα* mRNA levels were increased, while *RARγ* mRNA was slightly decreased after ATRA treatment for 24 h, which was consistent with what was observed in U-937 cells (**Fig. 6A**). We next assessed the isoform-specific effects of RARs on *CDK1* mRNA level in F9 cells (F9 WT) and in F9 RARα$^{-/-}$, F9 RARβ2$^{-/-}$ and F9 RARγ$^{-/-}$ cells. Loss of RARγ led to a significant decrease in *CDK1* and *CDK2* mRNA levels. Similarly, loss of RARβ2 also resulted in a significant decrease in *CDK1* mRNA expression (**Fig. 6B**), suggesting that *CDK1* mRNA expression is regulated by RARγ and RARβ2. Interestingly, ATRA treatment had no effect on *CDK1* mRNA expression in F9 cells or in F9 cells that lack RARγ, RARβ or RARα receptors. A similar expression pattern was observed for *CDK2* mRNA and *cyclin B1* mRNA, respectively, in these cells before and after ATRA treatment (**Fig. 6B**; **Fig. S4**).

In contrast, ATRA induced a time-dependent decrease in CDK1 protein level in F9 WT cells, which was consistent with what was observed in U-937 cells (**Fig. 6C**). This suggests that ATRA-induced downregulation of CDK1 occurs at the post-transcriptional level. However, targeted deletion of the RARγ resulted in a reduced level of CDK1 protein. ATRA treatment significantly increased CDK1 protein level in RARγ mutant cells (**Fig. 6C**). This intriguing finding suggests that RARγ is required for maintaining the proper level of CDK1 in the absence and presence of ATRA. Loss of RARγ also impaired the response of CDK2 and the cyclin partners cyclin A2 and cyclin B1 to ATRA-mediated effects (**Fig. 6D**). Expression patterns of CDK1 as well as CDK2 protein in F9 RARα$^{-/-}$ cells resemble that in F9 WT cells, suggesting that RARα is not involved in the ATRA-related effects on CDK1 or CDK2 (**Fig. 6C and D**). Interestingly, loss of RARβ2 also impaired expression of CDK1, CDK2, cyclin A2 and cyclin B1 and their response to ATRA (**Fig. 6C and D**). Taken together, these intriguing findings suggest that RARγ mutant, but not that of RARα mutant reduces expression of CDK1 and CDK2 and thus may impair the cellular response to ATRA.

Functional consequences of the protein-protein interactions between CDK1 and RARγ and CDK1 phosphorylation and ubiquitination in response to ATRA treatment. As was the case for U-937 cells, we also observed a physical interaction between RARγ and CDK1 and CDK2 in F9 WT cells in the presence or absence of ATRA (**Fig. 7A**). This result suggests that the physical interaction between RARγ and CDK1 is required for their function. As mentioned above, tyrosine phosphorylated CDK1 was exclusively detected in the nucleus of U-937 cells (**Fig. 3C**). ATRA treatment decreased CDK1 phosphorylation and induced a rapid decrease in nuclear CDK1. Similar to what was observed in U-937 cells, anti-phospho-tyrosine antibody detected CDK1-complexes in F9 WT cells (**Fig. 7B**). We therefore tested whether RARγ influences CDK1 phosphorylation. The absence of RARγ indeed resulted in the enhanced level of CDK1 phosphorylation compared with the control (**Fig. 7B**). In the presence of ATRA treatment, the level of tyr phosphorylation of CDK1 was higher in F9 RARγ$^{-/-}$ cells than F9 control cells, suggesting that in the absence of RARγ, ATRA was unable to downregulate CDK1 expression and phosphorylation (**Fig. 7B**).

We next wanted to investigate whether RARγ function is involved in ubiquitin-mediated regulation of CDK1 protein. Immunoprecipitation assay was performed to assess whether CDK1 might be conjugated with ubiqutin ligase in U-937 cells. We observed a low level of ubiquitinated CDK1 in F9 cells, and the level of ubiquitinated CDK1 was slightly increased in F9 RARγ$^{-/-}$ cells (**Fig. 7C**). There was also a difference in the pattern of ubiquitinated CDK1 between F9 cells and F9 RARγ$^{-/-}$ cells, which were treated with ATRA for 48 h (**Fig. 7C**). In the absence of RARγ, ATRA is unable to induce ubiquitin-mediated degradation of CDK1. This finding indicates that loss of RARγ impaired CDK1 ubiquitination upon ATRA treatment.

Because the proper level of P27^{kip} is required for cells to achieve optimal response to ATRA treatment, as mentioned in

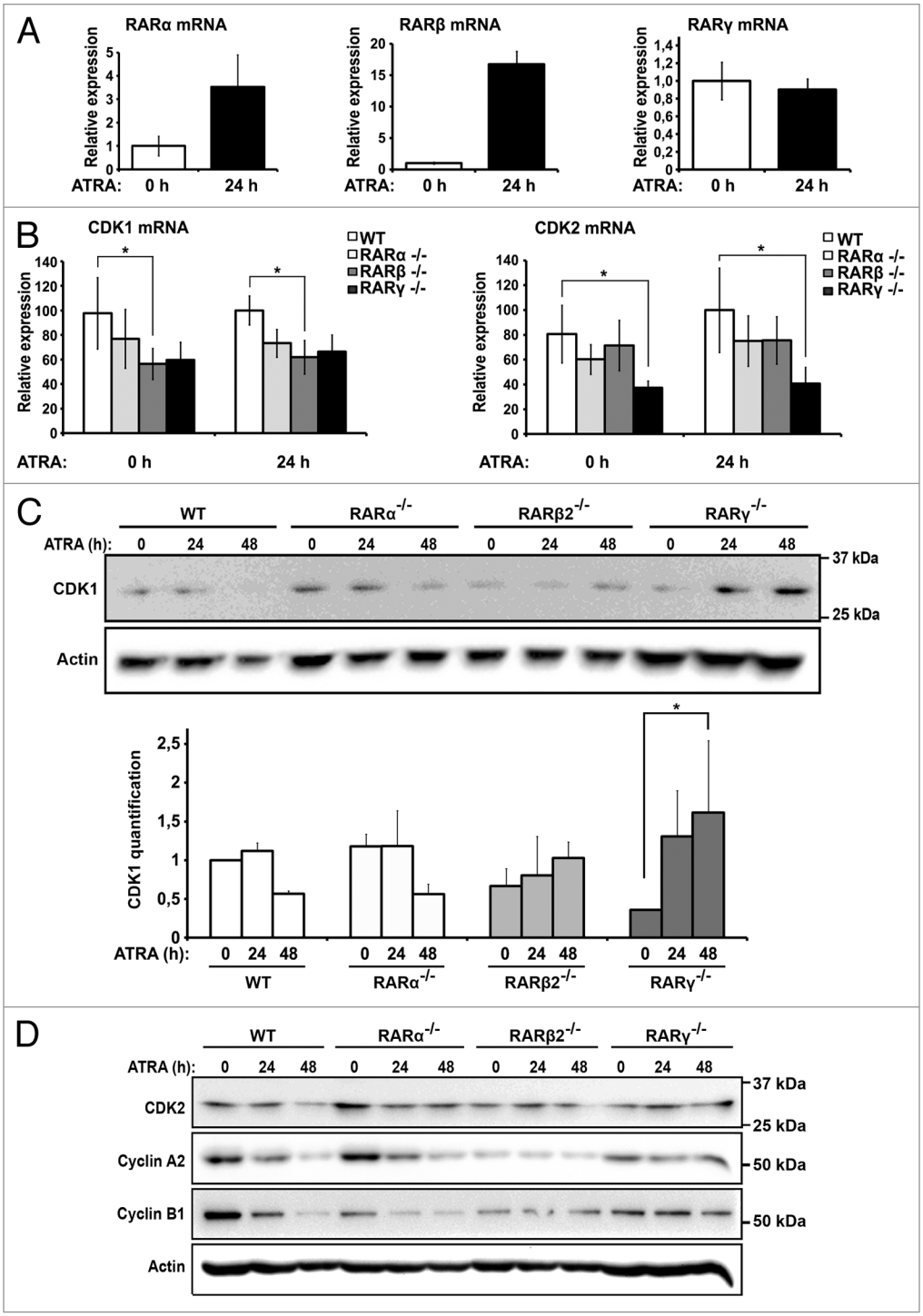

Figure 6. The effect of ATRA on RAR and CDK mRNA levels, and cell cycle regulatory proteins in F9 cells. (**A**) mRNA expression of the ATRA receptors *RARα*, *RARβ* and *RARγ* was determined by real-time RT-PCR using mRNA isolated from WT F9 cells, untreated (0 h), or treated with ATRA for 24 h. (**B**) *CDK1 and CDK2* mRNA expression in F9 WT cells, and in F9 RARα$^{-/-}$, F9 RARβ2$^{-/-}$ and F9 RARγ$^{-/-}$ cells, untreated (0 h), or treated with ATRA for 24 h. Mean ± SD represents three independent experiments. *p < 0.05 (paired t-test) is indicated. (**C and D**) IB analysis showing the protein expression of CDK1, CDK2, cyclin A2 and cyclin B1 in F9 control, F9RARα$^{-/-}$, F9 RARβ2$^{-/-}$ and F9 RARγ$^{-/-}$ cells, untreated (0 h) or treated with ATRA for 24 and 48 h, respectively. Densitometric quantification of CDK1 expression in cells mentioned above. Mean ± SD represents three independent experiments. *p < 0.05 (paired t-test) is indicated in (**C**).

Figure 4D and E, loss of CDK1 reduced P27^{kip} protein level, we next wanted to examine whether RARγ is also involved in the regulation of P27^{kip} protein expression. To this end, we determined P27^{kip} protein level in F9 WT cells and in F9 RARα$^{-/-}$ F9 RARβ2$^{-/-}$ and F9 RARγ$^{-/-}$ cells following ATRA treatment. P27^{kip} expression was decreased in F9 RARγ$^{-/-}$ cells compared

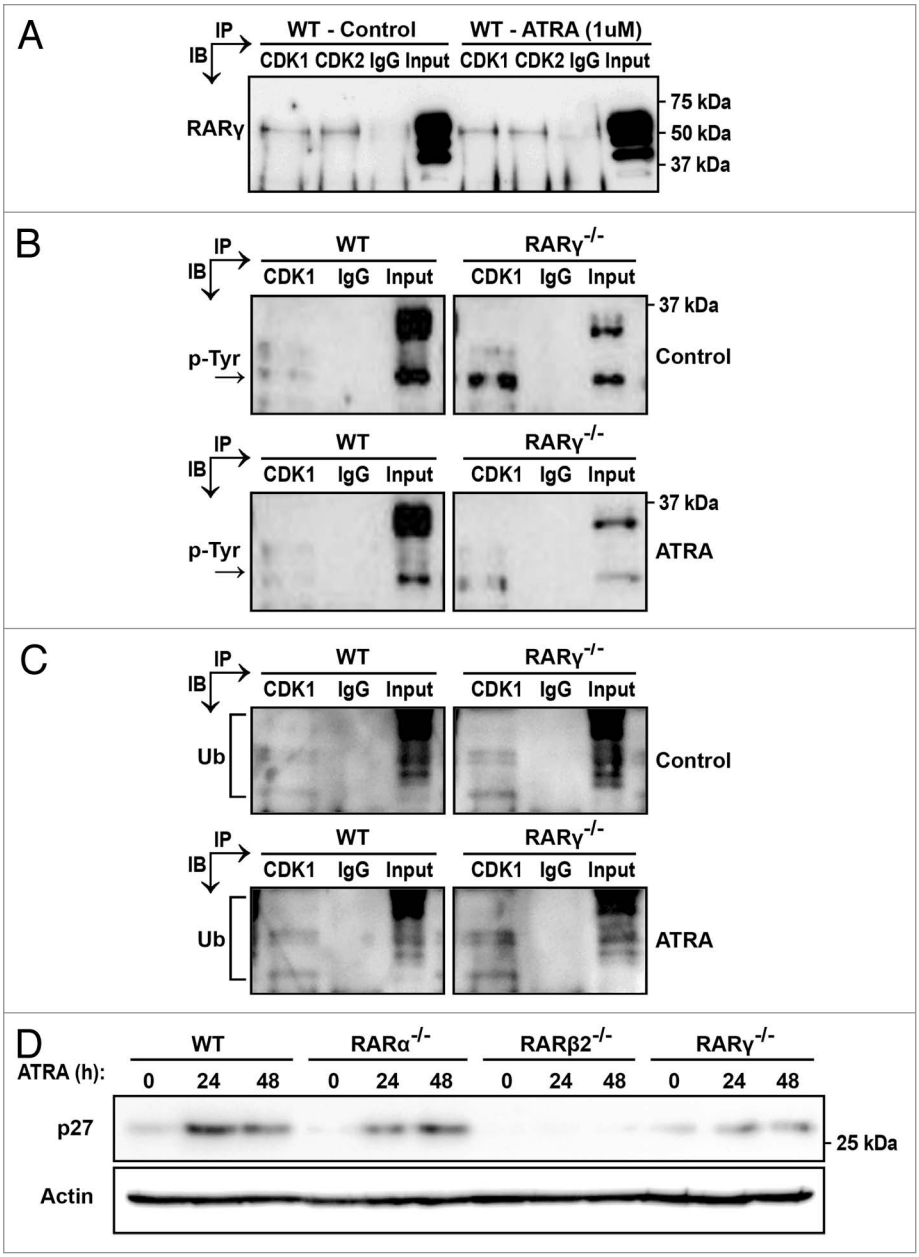

Figure 7. The effects of specific RAR isoform deletion in F9 cells, on CDK1 phosphorylation, P27 and CDK1 ubiquitination. (**A**) RARγ was detected in F9 WT total cell lysates and CDK1- or CDK2-containing immunocomplexes precipitated from F9 WT cells, treated with solvent or ATRA for 48 h, IgG was used as negative control. (**B**) Phospho-tyrosine was detected in F9 total cell lysates and CDK1-containing immunocomplexes that were precipitated from F9 WT cells and F9-RARγ$^{-/-}$, treated with ATRA (1 μM) for 48 h. (**C**) Ubiquitin was detected in F9 total cell lysates and CDK1-containing immunocomplexes that were precipitated from F9 WT cells and F9 RARγ$^{-/-}$, treated with ATRA (1 μM) for 48 h. Immunoprecipitation is indicated as "IP" and immunoblot analysis is indicated as "IB." (**D**) IB analysis was performed to examine the expression of P27^{kip} in F9 WT cells and in F9 RARα$^{-/-}$, F9 RARβ2$^{-/-}$ and F9 RARγ$^{-/-}$ treated with ATRA (1 μM) for 48 h.

with F9 controls (**Fig. 7D**). As anticipated, ATRA treatment increased P27^{kip} levels in F9 WT. However, ATRA-mediated increase in P27^{kip} was much less pronounced in F9 RARγ$^{-/-}$ cells compared with controls (**Fig. 7D**). Consistent with previously published study,[33] P27^{kip} was not detected in control treated or in ATRA treated F9 RARβ2$^{-/-}$ cells (**Fig. 7D**). Collectively, our data indicates that RARγ is also involved in the regulation of P27^{kip} protein expression in response to ATRA treatment.

Downregulation of nuclear CDK1 and nuclear RARγ is a critical event for sensitizing the cells to respond ATRA treatment. As mentioned above, CDK1 and RARγ form a reciprocal regulatory circuit and influence the protein stability of each other and affect the level of P27^{kip} protein. ATRA treatment led to a simultaneous reduction of both CDK1 and RARγ. We next wanted to investigate whether downregulation of CDK1 and RARγ protein levels in response to ATRA treatment has any functional significance. To this end, we used MG132, a proteosome inhibitor to prevent proteosome-mediated degradation of CDK1 and RARγ. MG132 treatment for 24 h enhanced total levels of CDK1 and RARγ in U-937 cells (**Fig. 8A**). MG132 enhanced levels of cytoplasmic vs. nuclear CDK1 and RARγ as determined by immunoblot analysis (**Fig. 8A**). Immunofluorescence analysis further showed that CDK1 co-localized with RARγ in nuclear and cytoplasmic compartments of U-937 cells (**Fig. 8C**). MG132 treatment enhanced the levels of CDK1 and RARγ (**Fig. 8C**). In the presence of MG132, ATRA was unable to effectively reduce the levels of CDK1 and RARγ (**Fig. 8A–C**). This suggests that MG132 partially blocked ATRA-mediated reduction of CDK1 and RARγ in U-937 cells.

We determined whether persistent expression of CDK1 and RARγ induced by MG132 may affect the cellular response to ATRA treatment. Treatment of U-937 cells with ATRA alone induced a higher rate of apoptosis compared with control cells (**Fig. 8D**). MG132 treatment alone did not induce apoptosis. However, combination of ATRA with MG132 induced less level of apoptosis compared with ATRA treatment alone. This suggests that cells are less sensitive to ATRA in the presence of MG132. This suggests that preventing the degradation of CDK1 and RARγ leads to a reduced sensitivity of cells to ATRA treatment.

Phosphorylation of CDK1 is regulated by the Wee1 kinase, while its dephosphorylation is mediated by Cdc25A and Cdc25C phosphatases.[34] We examined the cellular expression and subcellular distribution of Wee1 kinase and Cdc25A and Cdc25C

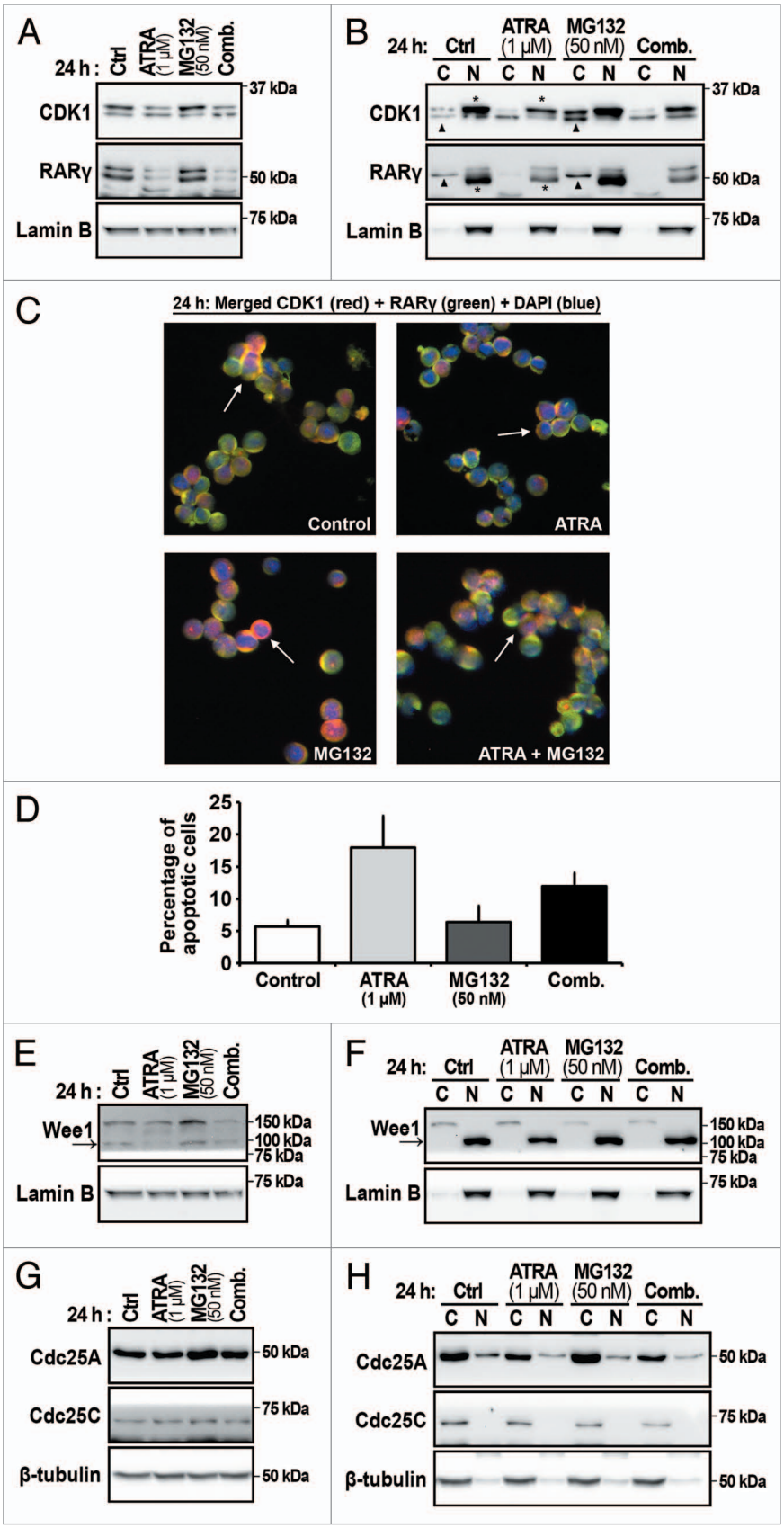

Figure 8. The effect of ATRA and proteasome inhibitor MG132 on the subcellular localization of CDK1 regulatory proteins in U-937 cells. (**A and B**) IB analysis of U-937 cells treated with DMSO, ATRA, MG132 (50 nM) or MG132 (50 nM) + ATRA (indicated as "Comb.") for 24 h, showing the total levels (**A**) and subcellular localization (**B**) of CDK1 and RARγ. Antibody to Lamin B was used as control for the nuclear fraction. The upper phosphorylated and nuclear CDK1 is marked "*," while cytoplasmic CDK is marked with "▲".The nuclear RARγ is marked with "*," while cytoplasmic RARγ is marked "▲". (**C**) Expression levels and subcellular localization of CDK1 and RARγ as evaluated by IF staining, in U-937 cells treated with DMSO, ATRA, MG132 (50 nM) or MG132 (50 nM) + ATRA for 24 h. (**D**) Apoptotic cell count in IF stained U-937 cells treated with DMSO, ATRA, MG132 (50 nM) or MG132 (50 nM) + ATRA for 24 h. Mean ± SD represents three independent experiments. (**E and F**) IB analysis of U-937 cells showing total levels (**E**) and subcellular localization (**F**) of Wee1 with the agents as mentioned in (**A**). Lamin B was used as control for the nuclear fraction. (**G and H**) IB analysis of U-937 cells showing total levels (**G**) and subcellular localization (**H**) of Cdc25A and Cdc25C was performed in U-937 cells treated with the agents as mentioned in (**A**). Antibody to β-tubulin was used as control for the cytoplasmic fraction.

phosphatases in control U-937 cells and in U-937 cells treated with ATRA for 24 h (**Fig. 8E–H**). The abundant nuclear expression of Wee1 was evident and correlated with the exclusive nuclear localization of the phosphorylated CDK1 (**Fig. 8F**). ATRA treatment resulted in a slight decrease in Wee1 expression (**Fig. 8F**), which was coincided with the decreased nuclear phosphorylation of CDK1. This suggests that ATRA inhibited the phosphorylated CDK1 in part by reducing the level of CDK1 kinase, Wee1 (**Fig. 8F**). The expression levels of phosphatases Cdc25A and Cdc25C were abundant in the cytoplasm of U-937 cells (**Fig. 8H**). ATRA treatment for 24 h decreased nuclear and cytoplasmic Cdc25A (**Fig. 8H**). However, ATRA had no pronounced effect on the level of Cdc25C, which was exclusively detected in the cytoplasm (**Fig. 8H**). Taken together, these data suggest that ATRA treatment also affected the expression levels of cell cycle proteins that are responsible for CDK1 activity (**Fig. 8F and H**). Because CDK1 is a downstream effect protein of Wee1, and Cdc25A and Cdc25C, the proper regulation of its expression and activity is essential for sensitizing the cells to respond to treatment.

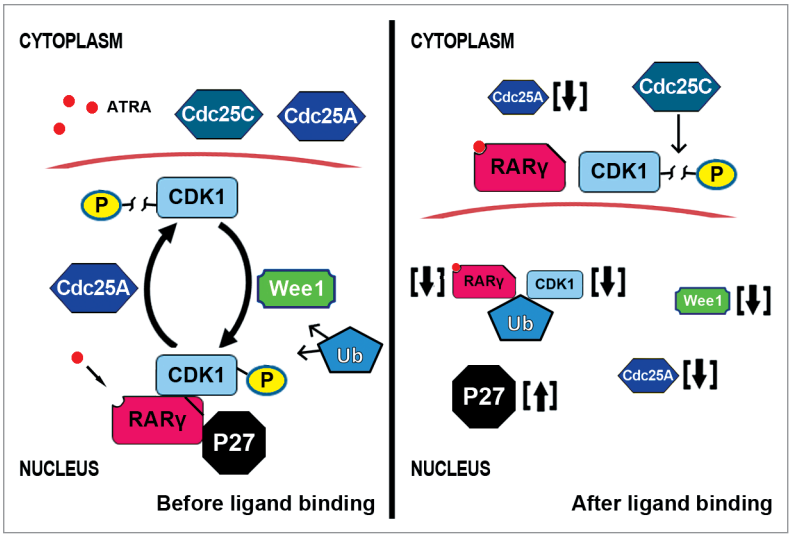

Figure 9. Proposed model suggests that a functional link between CDK1 and RARγ is important for ATRA to exert its effect on cancer cells. Nuclear CDK1 is phosphorylated by Wee1, and forms a complex with RARγ. ATRA treatment leads to dephosphorylation of CDK1 in both nuclear and cytoplasmic compartment, by Cdc25A and Cdc25C. The increase of both CDK1 and RARγ in cytoplasm after treatment suggests a shuttling mechanism between the two compartments, alongside Ub-mediated degradation. The specific retinoic acid receptor isoform gamma seems essential for ATRA induced cell cycle arrest and inhibition of CDK1, among other cell cycle regulatory proteins. CDK1 has a novel function to maintain G_0/G_1 cell cycle phase through $P27^{kip}$. RARγ function is required for the regulation of CDK1 phosphorylation status and subcellular localization.

Discussion

In the present study, we showed that CDK1 expression is associated with therapeutic resistance. First, loss of CDK1 expression is observed in certain numbers of AML patients who did not respond to therapy. Second, enhanced level of nuclear CDK1 correlates with poor outcome and lower CR rate. In addition, AML patients who experience the disease relapse have increased level of CDK1 expression. These data suggest that either loss of CDK1 expression or enhanced level of CDK1 is associated with treatment response and disease relapse in the AML with diverse subtypes. Our novel clinical finding in this study is in line with a recent reported study that provided strong evidence on the clinical importance of subcellular localization of CDK1 in lung cancer. It was shown that loss of cytoplasmic CDK1 correlated with treatment resistance of patients with non-small cell lung cancer (NSCLC) to standard chemotherapies.[4] Another recent study showed that enzymatic activity and protein expression of CDK1, but not CDK2, significantly predicted distant metastasis in colon cancer.[5] In addition, targeting CDK1 promoted FLT3-activated acute myeloid leukemia differentiation in cell lines as well as in patient blood samples.[35] The above mentioned studies together with our finding suggest that CDK1 activity and expression is linked to various types of cancers. Given that CDK1 is able to orchestrate with multiple cellular pathways and control the cell cycle and cellular checkpoints, alterations in CDK1 expression are likely to interfere with the effects of therapeutic drugs that target cell growth and differentiation.

One of our key findings is that functional depletion of CDK1 resulted in a decreased proportion of cells in the G_0/G_1 phase of the cell cycle, which is accompanied by a remarkable decrease in $P27^{kip}$ protein expression. In addition, depletion of CDK1 reduced level of ATRA-induced cell cycle arrest and terminal differentiation. Moreover, CDK1 expression is needed for ATRA to achieve the optimal effectiveness in inducing a growth arrest and terminal differentiation in U-937 leukemic cells. Expression of phospho-AKT was increased in siCDK1 cells treated with ATRA, suggesting that CDK1 is required for modulating the activity of PI3K/AKT pathway and affected ATRA-induced downregulation of RARγ. This novel finding suggests that loss of CDK1 expression in U-937 cells increases resistance to ATRA treatment by blocking ATRA-mediated downregulation of RARγ and by increasing the activity of PI3K/AKT survival pathway. In agreement with previous reported studies in which CDK1 is required for controlling proliferation of embryonic cells,[36,37] our present study emphasizes an important role for CDK1 in treatment response and suggests that normal level and activity of CDK1 is also required for cellular response to ATRA treatment.

Because we have observed that high level of nuclear CDK1 expression is associated with poor treatment response in AML patients, we investigated whether expression and subcellular localization of CDK1 may be functionally linked to treatment response in U-937 cells. Indeed, we demonstrated that ATRA specifically targets and eliminates the nuclear CDK1 and restores the cytoplasmic CDK1 expression. We further unraveled underlying cellular mechanisms. We found that RARγ, but not RARα or RARβ2 nuclear receptors, is a co-factor of CDK1. RARγ and CDK1, partly via their physical interactions, coordinate the cellular response to ATRA treatment.

In the present study, CDK1 functionally interacted with the RARγ nuclear receptor primarily in the nucleus. We have shown that RARγ but not RARα is critical for the ATRA induced regulation of CDK1 protein level, and thus RARγ exhibits a specific function in cell cycle regulation. Deletion of RARγ reduced protein stability and increased phosphorylation of CDK1 and blocked ATRA-mediated effect on CDK1 protein degradation. This suggests that RARγ and CDKs form a reciprocal regulatory circuit to influence each other at a post-transcriptional level. Strikingly, deletion of RARγ also led to a significant decrease in $P27^{kip}$ expression. Also, deletion of CDK1 function greatly reduced the level of $P27^{kip}$ expression. Thus, the nuclear CDK1 in complex with RARγ facilitates the regulation of $P27^{kip}$ expression and contributes to the sensitivity of the leukemic cells in response to ATRA treatment. This confirms that the physical and functional interaction specifically between RARγ and CDK1 are required for mediating cellular response to ATRA treatment.

Proteasome-mediated ubiqitination, phosphorylation and protein translocation between cytoplasm and nucleus is one of the

Table 2. Oligonucleotide primer sequences

Gene	Forward (5'-3')	Reverse (5'-3')
hSDHA	tgggaacaagagggcatctg	ccaccactgcatcaaattcatg
hUBC	atttgggtcgcggttctt	tgccttgacattctcgatggt
hYWHAZ	acttttggtacattgtggcttcaa	ccgccaggacaaaccagtat
hRARα	gaactctccaccaagtgcatca	cgatggtgagggtggtgaag
hRARβ	ccccagaacaagacaccatga	ccaaatccagcattgtgcat
hRARγ	ggcccctcacagaccttgt	agccctgtctcggtgtcatc
m36B4	agaacaacccagctctggagaaa	acaccctccagaaagcgagagt
mCDK1	ccgtcgtaacctgttgagtaactat	gtctacccttatacaccacaccgtaa
mCDK2	taccgagcacctgaaattcttc	ctccttgtgatgcagccactt
mCyclinB1	acttcctccgtagagcatc	gcagagttggtgtccattc
mRARα	ccatggagacccagtccagcagttcc	cctggtgcgctttgcgaacc
mRARβ	gatcctggatttctacaccg	cactgacgccatagtggta
mRARγ	ccatggagacacagtccaccagctcg	gatacagtttttgtcacggtgacat

features of the post-transcriptional regulation of protein function.[38,39] In the present study, ATRA greatly reduced the levels of phosphorylated and dephosphorylated CDK1 isoforms in the nucleus. MG132 enhanced multiple proteins including CDK1 and RARγ, the persistent expression of these proteins reduced the sensitivity of U-937 cell response to ATRA.

As illustrated in **Figure 9**, we have uncovered cellular mechanisms by which CDK1, with RARγ mediates ATRA effects on proliferation and terminal differentiation in cancer cells. We further show that RARγ and CDKs form a reciprocal regulatory circuit in the nucleus, which is functionally essential for balancing their own protein stability and activity. CDK1 and RARγ are both required for the regulation of $P27^{kip}$ expression that is crucial for proliferation and differentiation. ATRA treatment achieves G_0/G_1 cell cycle arrest and terminal differentiation by reducing the levels of nuclear CDK1 and RARγ complexes and so allowing an increased $P27^{kip}$ expression. ATRA specifically targets and eliminates the nuclear CDK1, most likely through RARγ, as RARγ forms a complex with CDK1 and exhibits similar patterns of changes in the subcellular localization. In addition, ATRA effectively abolishes the nuclear CDK1 by inhibiting the expression of its upstream Wee1 kinase and Cdc25A phosphatases in the nucleus. Conversely, ATRA maintains a constant level of CDK1 in the cytoplasm. This is coincided with a sustained level of cytoplasmic Cdc25C. The regulation of the subcellular content of CDK1 and RARγ by ATRA is likely essential for cellular response to proliferation and differentiation. Our study reveals a novel mechanism by which CDK1 and RARγ coordinate with ATRA to influence cell cycle progression and cellular differentiation.

Phosphorylation of CDK1 is regulated by the Wee1 kinase, while its dephosphorylation is mediated by Cdc25A and Cdc25C phosphatases. We showed that ATRA treatment resulted in a time-dependent decrease in Wee1 expression, which was also coincided with the decreased level of the phosphorylated nuclear CDK1. This suggests that the ATRA-mediated decrease in the level of the phosphorylated nuclear CDK1 is in part due to the decreased level of Wee1, the major kinase that phosphorylates CDK1 in the nucleus.

Our findings indicate the clinical importance of CDK1 expression in treatment response of AML patients. Our studies using cell line systems have further advanced our understanding of the role of CDK1 and the regulation of CDK1 level and subcellular distributions in relation to ATRA treatment. Such knowledge is important to enable the development of novel therapeutic approaches and to overcome ATRA resistance related to the altered cell cycle and differentiation pathways in cancer patients.

Materials and Methods

Patients and bone marrow samples. Bone marrow (BM) samples from 42 adult patients with de novo AML at time of diagnosis were obtained as archival specimens from the Department of Pathology, Lund University, University Hospital in Malmö. For each case, all available hematoxylin and eosin-stained sections were reviewed by National Board certified pathologist Lola Anagnostaki. Samples containing 70–90% blast cells were included and a representative block was selected for constructing tissue microarrays (TMAs) and for immunohistochemical analysis as previously described in reference 25. In addition to BM samples that were collected from 42 patients at diagnosis, BM samples were also collected from 13 out of 17 patients at disease relapse and 11 out of 12 patients at remission (**Table 1**). Follow-up information was missing from five patients, and the remaining patients died or had no remission. AML subtypes in this cohort: M1, n = 24; M2, n = 10; M3, n = 6; and M5, n = 2, according to the French American and British classification system. Patients with subtype M1 and M2 were treated with cytotoxic drugs (a combination of cytarabine and idarubicin), while all the M3 patients were treated with all-trans retinoic acid. Clinical characteristics of the patients have previously been described.[25] A median follow-up is 66 mo, ranging from 1 to 132 mo. Samples of normal bone marrow were obtained from

10 donors with no pathological diseases. The study was approved by the Ethics Committee at Lund University, and the Helsinki Declaration of Human Rights was strictly observed.

Immunohistochemistry. Deparaffinization of paraffin embedded tissue samples (7 μm) was performed, and was followed by antigen retrieval in which slides were boiled in 0.01 M citrate buffer, pH 6.0, for 10 min. The staining procedure was performed using a semiautomatic staining machine (Ventana ES, Ventana Inc.) as described.[25] The specimens were evaluated by four different specialists; one of them is a specialist on hematopathology. The staining intensity in AML bone marrows was normalized by comparing the staining in normal bone marrows and was scored as: 0, negative; 1, weakly positive; 2, moderately positive; and 3 and 4, strong or very strongly positive. The specimens were viewed and the microphotographs were taken under 40× magnification with a Nikon 800 microscope.

Cell culture and treatment. Human leukemic cell line U-937 (monoblasts) was maintained in RPMI 1640 medium (PAA Laboratories) supplemented with 10% fetal calf serum (FCS) and 1× PEST (streptomycin 18 μg/ml, pencillin 18 IU/ml) (Sigma-Aldrich). Mouse cell line F9 teratocarcinoma (or F9) cells that harbor the deletions of receptors RARα, RARβ2 or RARγ were propagated as described in reference 26. The genotypes RARα$^{-/-}$, RARβ2$^{-/-}$ and RARγ$^{-/-}$ cell lines were confirmed by PCR. For ATRA treatment, cells were cultured in medium containing 1 μM ATRA (Sigma-Aldrich) dissolved in DMSO/ethanol (5:1) for U-937 cells, or 1 μM ATRA dissolved in 0.1% ethanol for F9 cells. Cells were seeded at a density of 0.3 × 10^6 cells/ml and treated with ATRA for the indicated time points. For treatment of U-937 cells with the proteasome inhibitor MG132 (Sigma-Aldrich), two doses at 50 nM or 500 nM were used as indicated in the figure legend. For treatment of U-937 cells with ATRA, in combination with MG132, cells were pre-treated with MG132 for 30 min. before the addition of 1 μM ATRA.

Sources of antibodies for western blotting. The following antibodies were used: polyclonal antibodies against P27, Lamin B, RARα, RARβ, RARγ, Cyclin A2, Cdc25A, Wee1 and Cyclin E (Santa Cruz Biotechnology Inc.); monoclonal antibody against ubquitin, Cdc25C and p-Tyr (Santa Cruz biotechnology); monoclonal antibody to β-Tubulin (Sigma-Aldrich); Cdk1 and Cdk2 (BD, pharmingen), pCdc2, Cyclin B1 (Cell signaling, technology), RARγ (Abcam Inc.) and β-Actin (MP Biomedicals). Secondary antibodies: HRP-conjugated anti-mouse IgG and anti-rabbit IgG (GE Healthcare).

siRNA silencing of CDK1 and CDK2 in U-937 cells. For siRNA experiments, 2 × 10^6 U-937 cells were transfected with 100 nM siRNA non-targeting (Ctrl) smart-pool or single oligo sequences specific to CDK1 and/or CDK2 (D-001810-10-20, L-003224-13-0005, and L-003236-13-0005, respectively, Dharmacon) and cultured for 24, 48 or 72 h. Where levels of *CDK1* and *CDK2* mRNAs were targeted simultaneously, cells were transfected with 100 nM of both siRNAs. Transfections were performed with Microporator MP-100 (Digital-Bio Technology) according to the manufacturer's instructions.

Immunofluorescence. U-937 cells (1 × 10^6) were fixed in methanol on slides at –20°C for 5 min. The slides were washed in 1× PBS before permeabilization in 0.5% Triton X-100, then blocked in 2.5% horse serum. Primary antibody was incubated in a humid chamber overnight at at +4°C. Primary antibodies: CDK1 (BD, PharMingen), CDK2 (BD, 610145), pCDK1 (pCdc2) (Cell signaling), RARγ (Abcam Inc.). Secondary antibodies: Rhodamine (Chemicon/Millipore) and Alexa fluor 488 (Invitrogen). The nuclei were stained with DAPI. The slides were analyzed at 20× and 100× magnification.

Real-time RT-PCR. For real-time RT-PCR analysis in U-937 cells, RNA was extracted with a QIAshredder spin column kit and purified using a RNeasy Mini kit (Qiagen), according to the manufacturer's instructions. RNA (2 μg) was DNase treated with RQ1 RNase-free DNase (Promega) and desalted in Microcon-100 columns (Amicon). Reverse transcription reactions were performed in a PTC-100 PCR machine (MJ Research Inc.) using random hexamers and Superscript II RT enzyme. The synthesized cDNA (20 ng) was run on a 7300 Real Time PCR system machine (Applied Biosystems), in a 25 μl reaction of 1× SYBR Green PCR Master mix (Applied Biosystems) with 250 nM forward and reverse primers. The cycling conditions were 10 min. at 95°C, 40 cycles at 95°C for 15 sec and 60°C for 60 sec. Data analysis was performed using the 7300 Real-Time SDS Software, and the relative quantification of expression levels was performed using the comparative Ct method, with normalization to the succinate dehydrogenase complex, subunit A (SDHA), ubiquitin (UBC) and 5-monooxygenase activation protein zeta polypeptide (YWHAZ). Primer sequences are provided in **Table 2**. For the generation of cDNA and real-time PCR reactions with F9 cells, total cellular RNA was extracted using Trizol reagent (Invitrogen) and quantitated by optical density at 260 nm. Total RNA (1.5 μg) isolated from F9 cells was reverse transcribed (Quanta Biosciences) and then diluted 1:10 with H_2O. Real-time PCR reactions were performed using SYBR Green Supermix (Quanta Biosciences) in a 15 μl reaction containing reaction-mix (1×), 0.25 μM of each primer and 3 μl of cDNA template. The reactions were run on a Bio-Rad MyiQ™ Single-Color Real-time PCR Detection System (Bio-Rad). Amplification in the linear range was demonstrated by a serial dilution of cDNA from RA treated Wt cells included in each reaction (1:1, 1:5, 1:10, 1:50, 1:100, 1:500). Reactions with H_2O and template without RT enzyme, respectively, served as negative controls for primer-dimer and for amplification of residual genomic DNA (gDNA). Each expression analysis was performed using biological triplicates or quadruplicates (e.g., independently propagated cells, repeated 3–4×).[27]

Immunoblotting and immunoprecipitation. For immunoblotting, cells were lysed in ice-cold RIPA buffer 120 mM NaCl, 50 mM Tris-HCL pH 7.6, 50 mM NaF, 0.1 mM Na_3VO_4, 1% NP40, 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich) and 15% protease inhibitor cocktail Complete Mini (Roche) and centrifuged twice at 20,000 × g for 20 min at +4°C. Ten–50 μg of protein was used for blotting. For immunoprecipitation assays, an equal amount of 300–500 μg protein lysate/sample and Protein G Sepharose beads (GE Healthcare) equilibrated in RIPA buffer, were used. Protein was loaded and samples were separated on 12% SDS-PAGE gels and transferred onto

nitrocellulose membranes (Bio-Rad). Signals were visualized with Enhanced ChemiLuminescence detection system (ECL), using an AlphaImager CCD system. Selected blots were quantified using ImageJ, public domain software.

Subcellular fractionation. Subcellular fractionation was done as previously described.[28] Cell pellets were resuspended in ice-cold nuclei isolation buffer (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1% Triton X-100, 15% PI Complete Mini, 1 mM PMSF) and incubated on ice for 10 min, then centrifuged. The supernatant containing the cytoplasmic fraction was collected, while the pellet was resuspended in ice-cold RIPA buffer, followed by incubation on ice for 20 min. After centrifugation the supernatant containing the final nuclear fraction was collected. The nuclear and cytoplasmic fractions were subjected to immunoblot analysis as described above. The subcellular fractionation was controlled by detection of β-tubulin and Lamin B in the cytoplasmic and nuclear fractions, respectively.

Cell cycle analysis, flow cytometry-based analysis. For analysis of cell surface markers, U-937 cells were incubated with antibodies against phycoerythrin (PE)-conjugated CD33, fluorescin isothiocyanate (FITC)-conjugated CD11b, PE-conjugated CD11c and PE- or FITC-conjugated mouse IgG controls (Beckman Coulter) for 15 min. in the dark. The cells were then washed once with PBS before subjected to flow cytometry analysis (FACSCalibur). The WinList 5.0 software (Verity Software House Inc.) was used. For cell cycle analysis, U-937 cells were washed with PBS, and fixed in 70% ice-cold ethanol at –20°C overnight. Fixed cells were centrifuged at 400 × g and washed with PBS. The cells were stained with propidium iodide (Sigma-Aldrich) for 40 min. on ice in the dark, and the PI-elicited fluorescence of individual cells was measured using flow cytometry (FACSCalibur).

Statistical analysis. Statistical analyses were performed using SPSS version 20.0 (SPSS Inc.). The p values used for comparison of variables between groups of patients were based on the Student's t-test. Distributions of overall survival (OS) and disease-free survival (DFS) were estimated by the method of Kaplan-Meier, with 95% confidence intervals. Differences between survival curves were calculated using the log-rank test.

All procedures are in accordance with the Helsinki declaration of 1975.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work was supported by grants from the Swedish Cancer Society, The Swedish National Research Council, The Swedish Childhood Cancer Foundation, Malmö Hospital Cancer Foundation, Malmö Hospital Foundation, Gunnar Nilsson Cancer Foundation and Crafoord Foundation to J.L.P. This research was supported by NIH R01CA043796 to L.J.G., and by the University of Nottingham to N.P.M. We thank Åsa Ehlén for helping to perform experiments and for technical advice. We thank Dr Chambon for the constructs used to generate the F9 cell lines for the manuscript.

Supplemental Materials

Supplemental materials may be found here:
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