E2F-Dependent Repression of Topoisomerase II Regulates Heterochromatin Formation and Apoptosis in Cells with Melanoma-Prone Mutation

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Abstract

RB pathway mutations, especially at the CDK4 and INK4A loci, are hallmarks of melanomagenesis. It is presently unclear what advantages these alterations confer during melanoma progression and how they influence melanoma therapy. Topoisomerase II inhibitors are widely used to treat human malignancies, including melanoma, although their variable success is attributable to a poor understanding of their mechanism of action. Using mouse and human cells harboring the melanoma-prone p16Ink4a-insensitive CDK4R24C mutation, we show here that topoisomerase II proteins are direct targets of E2F-mediated repression. Drug-treated cells fail to load repressor E2Fs on topoisomerase II promoters leading to elevated topoisomerase II levels and an enhanced sensitivity of cells to apoptosis. This is associated with the increased formation of heterochromatin domains enriched in structural heterochromatin proteins, methylated histones H3/H4, and topoisomerase II. We refer to these preapoptotic heterochromatin domains as apoptosis-associated heterochromatic foci. We suggest that cellular apoptosis is preceded by an intermediary chromatin remodeling state that involves alterations of DNA topology by topoisomerase II enzymes and gene silencing via formation of heterochromatin. These observations provide novel insight into the mechanism of drug action that influence treatment outcome: drug sensitivity or drug resistance. (Cancer Res 2005; 65(10): 4067-77)

Introduction

Chemotherapy remains the primary treatment modality for cancer patients. However, the success of chemotherapy is variable due to either the inherent insensitivity of tumors to chemotherapeutic agents or due to acquired drug resistance in tumors upon relapse (1, 2). To overcome the problems associated with multidrug resistance, much needs to be known regarding the mechanisms of action of conventional chemotherapeutic agents. Cellular processes such as growth arrest, senescence, genomic instability, and apoptosis are intermediary stages that precede cell death in response to chemotherapy (3). Inherited or acquired mutations that disable one or more of these intermediary stages have the capacity to reverse or negate the efficacy of chemotherapeutic drugs, leading to drug resistance (1).

Mutations in cell cycle components are an important hallmark of a cancer cell thereby making the cell cycle machinery an important target for anticancer therapeutic strategies (4, 5). These alterations that include overexpression of cyclins, activation of cyclin-dependent kinases (CDK), inactivation of CDK inhibitors, or loss of retinoblastoma tumor suppressor protein (RB) expression provide cancer cells distinct advantage and allow them to bypass quiescence or cellular senescence pathways (4). At least one (and frequently more) of these proteins is deregulated in all major human cancer categories, which lead to changes in the levels of phosphorylation of RB proteins and inactivation of RB tumor suppressor function (4, 6). RB is principally involved in release of E2F-mediated repression of the relative importance of the E2F proteins in tumorigenesis. These observations suggest that E2F2-mediated repression may not be the primary mechanisms of RB-mediated tumor suppression and the role of activator E2Fs (especially E2F1) in RB-mediated tumor suppression remains undefined.

p16Ink4a is a CDK inhibitor that promotes cell cycle arrest via the retinoblastoma tumor suppressor pathway (11). The INK4a locus, which encodes the tumor suppressor p16Ink4a, is frequently mutated in human cancer, including melanoma, and has been linked to treatment sensitivity (11–13). Along with loss of function mutations in p16Ink4a, gain-of-function mutations via alterations in Cdk4 activity are also a feature of melanomagenesis (14). Inheritance of a CDK4R24C mutation was shown to predispose to familial melanoma primarily due to the inability of the CDK4R24C kinase to be inhibited by p16Ink4a (15, 16). To examine the consequences of inheritance of the Cdk4R24C mutation on development and carcinogenesis, we generated a mouse model that harbored the R24C mutation in the Cdk4R24C mutation (18, 19). Cdk4R24C mouse embryoblasts (MEF) have increased proliferation potential, escape cellular senescence, and have increased transformation properties (18, 19). Therefore, the Cdk4R24C mouse model serves as an excellent model to understand the mechanisms of the carcinogenic potential of this mutation.

In this article, we focus on understanding the mechanisms that underlie cancer cell sensitivity to established chemotherapy agents. Cdk4R24C mouse embryoblasts (17, 18) and melanocytes in addition to human melanoma cell lines (15) that harbor Cdk4R24C mutations constitute a convenient model to understand the
mechanism of action of oncogenic mutations, to evaluate the efficacy of anticancer agents and to study mechanisms that confer resistance to cancer therapy. Using this model system, we focused on target proteins that are deregulated in cancer and hence are the basis of established chemotherapy agents. Topoisomerase II proteins fit these criteria because topoisomerase II expression is deregulated in cancer and anti–topoisomerase II inhibitors are extensively used in cancer chemotherapy (20–22). Topoisomerase II proteins are critical regulators of DNA topology and chromatin structure that influences processes such as DNA replication, gene transcription, chromatin structure, chromosome segregation, and mitosis (20–22). We show that mouse and human cancer cells harboring the Cdk4R24C mutation inherit a distinct carcinogenic advantage and are exquisitely sensitive to topoisomerase II inhibitors. In trying to understand the mechanistic reasons that determine this sensitivity, we identify an important role for E2Fs, surprisingly for E2F1, in topoisomerase II transcriptional repression. We further elucidate that treatment with topoisomerase II inhibitors leads to profound chromatin reorganization driven by topoisomerase II proteins that involves heterochromatin formation. These results suggest that E2F1- and topoisomerase II–mediated chromatin reorganization precedes drug-induced apoptosis and thus provide novel insight into the mechanisms of drug action that influence treatment outcome.

Materials and Methods

Cell culture and flow cytometry assays. MEFs were isolated by standard techniques (17). Primary melanocyte cultures were prepared from neonatal litters of mice as described previously (23). Briefly, dorsal skin was surgically and aseptically removed, placed in trypsin (250 μg/mL) and EDTA (200 μg/mL) solution overnight at 4 °C. The epidermis was split from the dermis and discarded. The dermis containing the hair follicles was minced and placed for 1 week in “Tip medium” which contained 100 μg/mL geneticin (G418 sulfate, Life Technologies, Grand Island, NY) to eliminate contaminating keratinocytes and fibroblasts (23). Subsequently, for the entire study the culture medium was changed to “Maintenance medium” (23). Human melanoma cells SK-Mel-28, SK-Mel-29, and SK-Mel-39 cells were cultured in DMEM. Flow cytometry analysis of DNA content was done as described previously (17).

Semiquantitative reverse transcription-PCR. Reverse transcription-PCR (RT-PCR) was done as described previously with some modifications (17). Total RNA was extracted using the TRI Reagent Kit (Molecular Research Center, Inc., Cincinnati, OH). RT-PCR was done using One Step RNA PCR Kit (AMV; TaKaRa Biomedicals/Fisher, Medford, MA) according to the manufacturer’s protocol. Primer sequences were based on published cDNA sequences corresponding to each of the genes analyzed, and these primer pairs were selected with the help of PrimerExpress 1.0 software (Perkin-Elmer Co., Boston, MA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard. The densitometry data were normalized to mRNA levels of GAPDH. All samples were done at least in duplicate. Primer sequences are available upon request.

Immunoblotting and immunofluorescence assays. Western blots and immunofluorescence assays were done using standard methods (17). Antibodies were used anti–topoisomerase I (4E12, 1:250, anti–topoisomerase II) (3B6, 1:500, anti–α-tubulin (clone B-5-1-2, Sigma Chemical Co., St. Louis, MO), anti–pRb (1:4001A, BD PharMingen Biosciences, San Diego, CA), HP1α (Chemicon, Temecula, CA), HP1β (H-50, anti–E2F-1 (SC-193) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies to modified histones, H3-K9M (1:50) and H3-K20M (1:50) were from Thomas Jenuwein. Alexa-fluor antibodies were from Molecular Probes (Eugene, OR).

Chromatin immunoprecipitation assays. Cells were cross-linked with formaldehyde, harvested, and processed according to the chromatin immunoprecipitation Assay Kit instructions (Upstate, Lake Placid, NY) using E2F1, E2F4, and pRB antibodies. PCR amplification was done using topoisomerase IIβ promoter-specific oligonucleotide primers. Sequences of primers are available upon request.

Drug treatment and survival assays. The following chemotherapeutic agents were used: paclitaxel, cisplatin [cis-platinum(II)-diammine dichloride], etoposide (Ve-Pesid), and doxorubicin (Adriamycin); purchased from Sigma Chemical. Drugs were dissolved at 100 mmol/L in DMSO and stored at −20 °C. All drugs were diluted in culture medium before addition to the cell cultures. The cytotoxicity of drugs was determined by measuring the conversion of the tetrazolium salt WST-1 to formazan according to the manufacturer’s instructions (Roche, Indianapolis, IN). Briefly, cells were plated into 96-well plates in triplicate wells (5 × 104 cells per well) and exposed to doxorubicin, etoposide, cisplatin, and paclitaxel at various concentrations. After 2 days of incubation at 37 °C, WST-1 was added to each well and incubated at 37 °C for 1 hour. The number of viable cells was assessed by measuring the conversion of the tetrazolium salt WST-1 to formazan through measurement of absorbance at 415 nm. Results are expressed as the percentage of the absorbance of control (untreated) cells.

In vivo tumorigenesis. The SK-Mel-28, SK-Mel-29 human melanoma cells, and mouse Cdk4R24C MEFs were used for animal experiments. Cells (5 × 106) were injected s.c. into the bilateral flanks of athymic BALB/c nu/nu male mice (6-8 weeks of age, three mice in each group, Taconic Laboratories, Germantown, NY) to form tumors. One week after tumor development, to evaluate the ability of systemic etoposide or doxorubicin to suppress tumor growth, mice were treated with etoposide (33 mg/kg), Adriamycin (1 mg/kg), or 0.9% normal saline via the i.p. route once every 3 days for a total of four treatments. Tumors were measured every 3 days, and tumor volume was determined by measuring three orthogonal diameters of each tumor and calculated (D1 × D2 × D3/2). Mice were injected i.p. with bromodeoxyuridine (BrdUrd) for 1 hour, and tumors were subjected to anti-BrdUrd and terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) immunohistochemistry. Animal experiments were done in accordance with institutional guidelines and approved by the NIH Institutional Animal Care and Use Committee.

Transient transfection and reporter assays. Cells were seeded into 6-well plates 1 day before transfection. The pTop2b-1067-LUC, pTop2b-454-LUC, and pXP2 luciferase reporter plasmid (1 μg) was transfected together with 0.2 μg of E2F1 wild-type (WT) and mutant expression vectors into cells. Empty vectors were used to supplement equal amounts of DNA in each transfection. Transfection was carried out using FuGENE 6 (Roche). Twenty-four hours after transfection, cells were rinsed with PBS and lysed with reporter lysis buffer (Promega, Madison, WI). Lysates were assayed for luciferase activity and normalized to total protein concentration. All experiments were done in duplicate and repeated two to three times. The results shown are the mean ± SE.

Results

Cells harboring the Cdk4R24C mutation are sensitive to topoisomerase II inhibitors. We evaluated the sensitivity of Cdk4R24C mutation harboring mouse fibroblasts (Cdk4R24C MEFs) and human melanoma tumor cells (SK-Mel-28 and SK-Mel-29) to anti–topoisomerase II chemotherapy agents. Results were compared with cells harboring WT Cdk4 (Cdk4+/- MEFs and SK-Mel-29 human melanoma cells). Cells were exposed to different doses of two topoisomerase II inhibitors, etoposide (a nonintercalating agent) and doxorubicin (an intercalating agent), and the sensitivity of cells was determined. Cells with the Cdk4R24C mutation, Cdk4R24C MEFs (Fig. 1A and B, ●) and SK-Mel-28 and SK-Mel-29 cells (Fig. 1C and D, ○; data not shown) were sensitive to low doses of both topoisomerase II inhibitors. In contrast, cells with WT Cdk4, Cdk4+/- MEFS (Fig. 1A and B, ○) or SK-Mel-29 cells (Fig. 1C and D, ○) were resistant to the topoisomerase II inhibitors at low concentrations. Cdk4+/- cells were either sensitive to very high doses of doxorubicin (Fig. 1A and C, ○) or were resistant to...
maximal doses of etoposide (Fig. 1B and D). In contrast, exposure to non–topoisomerase II inhibitor agents, such as paclitaxel (that interferes with microtubule function at mitosis) or cisplatin (an alkylating agent which induces DNA damage), was ineffective in inducing cell death in Cdk4R24C–harboring cells (Fig. 1E and F). Analysis of cell sensitization using propidium iodide–based flow cytometry was indicative of an increased sub-G0 population (Fig. 1G, closed columns) in topoisomerase II inhibitor–treated Cdk4R24C cells (SK-Mel-28 and SK-Mel-29) with a proportionate reduction in cells with G1-DNA content (Fig. 1G, open columns). In contrast, cells with wild type Cdk4 (SK-Mel-39) were relatively unaffected by the topoisomerase II inhibitor agents with only a slight increase in sub-G0 population (Fig. 1G). These results indicate that exposure to anti–topoisomerase II agents sensitizes cells harboring the Cdk4R24C mutation to increased apoptosis.

To examine if the Cdk4R24C mutation sensitizes cells to topoisomerase II inhibitors in vivo, we conducted xenograft tumorigenesis assay. As expected, no tumor formation was observed in Cdk4+/+ MEFs and interestingly SK-Mel-39 melanoma cells that harbor WT Cdk4 also failed to induce tumors. In contrast, presence of the Cdk4R24C mutation in Cdk4R24C MEFs (Fig. 2A) was found to increase tumor sensitivity to topoisomerase II inhibitor treatment.

Figure 1. Cdk4R24C harboring cells are sensitive to topoisomerase II inhibitor treatment. A–F, viability of Cdk4+/+ (WT, ○) and Cdk4R24C (R24C, ●) MEFs (A and B) and human melanoma cells (C–F) in response to indicated concentrations of doxorubicin (A and C), etoposide (B and D), paclitaxel (E), or cisplatin (F) for 72 hours. G, treatment of Cdk4+/+ (SK-Mel-39) and Cdk4R24C (SK-Mel-28 and SK-Mel-29) melanoma cells with control (C), doxorubicin (D), or etoposide (E) for 0 to 72 hours. Number of cells (%) in sub-G0 phase (black columns), G1 phase (white columns), and S-G2M phase (shaded columns).

Figure 2. Cdk4R24C harboring tumors are sensitive to topoisomerase II (TopoII) inhibitor treatment. A–F, tumor chemosensitization of untreated (○) and topoisomerase II inhibitor–treated (●) MEFs (A and D) and human melanoma cell lines SK-Mel-28 (B and E) and SK-Mel-29 (C and F) to etoposide (A–C) or doxorubicin (D–F). Relative tumor volumes at indicated days post-chemotherapy treatment. Asterisk (A, B, D, and E) indicates that animals were euthanized at that time due to increased tumor burden. G, proliferation (BrDU) and cell death (TUNEL) levels in control and topoisomerase II inhibitor–treated Cdk4R24C harboring tumors derived from MEFs and melanoma cells (SK-Mel-28). Brown labeled nuclei are indicative of BrDU incorporation and TUNEL staining.
contrast to untreated tumors (Fig. 2A-F, ○) indicative of tumor cell death in response to the anti-topoisomerase II agents. We observed reduced anti-BrdUrd immunoreactivity and increased TUNEL-positive staining in topoisomerase II inhibitor–treated tumors in contrast to untreated tumors that retained a high anti-BrdUrd immunoreactivity and very low TUNEL staining (Fig. 2G). These assays indicated that Cdk4R24C–bearing tumors that were on the topoisomerase II inhibitor treatment had reduced proliferative and higher cell death rates.

**E2F mediated repression of the topoisomerase II promoter.** Our next studies focused on understanding the mechanisms associated with the increased sensitivity of Cdk4R24C mutation harboring cells to topoisomerase II inhibitors. Topoisomerase II RNA (Fig. 3A, RR) and protein levels (Fig. 3B, RR) are elevated in Cdk4R24C harboring MEFs, primary melanocytes, and human melanoma cells in comparison with Cdk4+/+ cells (Fig. 3A and B, WT). Sequence analysis revealed the presence of a single consensus E2F-binding site in the topoisomerase IIα promoter and not on the topoisomerase IIβ promoter, that suggested a role for E2Fs in topoisomerase IIβ regulation. To address the role of E2F-dependent regulation of topoisomerase IIβ, we studied mutants of the topoisomerase IIβ promoter (combined to a luciferase reporter; Fig. 3C). Results were compared with observations obtained with the promoter of Cyclin E, which is also a prominent E2F target gene. Consistent with the increased RNA levels, increased basal topoisomerase IIβ promoter activity was observed in Cdk4R24C harboring MEFs (Fig. 3D, closed columns) and human melanoma cells SK-Mel-28 and SK-Mel-29 cells (Fig. 3E, closed and shaded columns) compared with Cdk4+/+ harboring MEFs and SK-Mel-39 cells (Fig. 3D and E, open columns) suggesting that deregulated E2Fs in Cdk4R24C cells were responsible for increased topoisomerase IIβ levels (Fig. 3A and B).

To delineate the precise role of E2Fs in regulation of the topoisomerase IIβ promoter, we focused the subsequent studies on genetically defined primary mouse embryo fibroblast cells that are convenient to dissect the relative contributions of specific regulators. In contrast to activation of the Cyclin E promoter, the activating E2F2s (E2F1, E2F2, and E2F3) repressed the topoisomerase IIβ promoter to levels similar to repression by E2F4 (Fig. 4A and B) with or without coactivators DP1 or DP2 (Fig. 4C). Moreover, no significant repression was observed in the topoisomerase IIβ promoter fragment (Top2b-454) that lacked the E2F-binding site (Fig. 4D). Mutation of the E2F-binding site on the topoisomerase IIβ promoter led to a significant relief of repression that illustrated the importance of E2F in mediating the transcriptional repression (3- to 4-fold derepression; Fig. 4E), although it is possible that maximal repression of the topoisomerase IIβ promoter also involves contribution of other transcription factors. Because our observations suggested an important and an unexpected role for E2F1 in gene repression, we decided to further explore the mechanisms of E2F1-mediated repression of topoisomerase IIβ. To define the regions of E2F1 that mediate repression, we used mutants of E2F1 defective in DNA binding (E132), transactivation (Δ363), and binding to retinoblastoma (Δ18). Results indicate that the E2F1-DNA binding domain is required for E2F1-repressor activity as a double-point mutation in the E2F1-DNA binding domain (E132) nullifies E2F1 repressor activity (Fig. 4F). However, the E2F1 transactivation domain is dispensable for E2F1 repressor activity as a mutant that lacks the E2F1 transactivation domain (E2F1-1-363) retains repressor activity that is comparable to WT E2F1 (Fig. 4F).

**Figure 3.** Deregulated topoisomerase II (TopoII) levels in Cdk4R24C harboring cells. A, RNA levels of topoisomerase II isoforms α and β in Cdk4+/+ (WT) and Cdk4R24C (RR) primary fibroblasts (MEFs), primary melanocytes, and human melanoma cell lines. GAPDH control levels are shown. B, protein levels of topoisomerase II isoforms α and β in Cdk4+/+ (WT) and Cdk4R24C (RR) primary MEFs and melanocytes. Tubulin antibody control is provided. C, topoisomerase IIα promoter deletion constructs in front of a luciferase (Luc) reporter. The E2F-site is indicated in the –1067 and –864 fragments. The –454 fragment lacks E2F sites. D, topoisomerase IIβ promoter activity in Cdk4+/+ (open column) or Cdk4R24C (RR, closed column) MEFs. E, topoisomerase IIβ promoter activity in Cdk4+/+ (SK-Mel-39, open column) or Cdk4R24C (SK-Mel-28, closed column and SK-Mel-29, shaded column) human melanoma cells.

and D), SK-Mel-28 cells (Fig. 2B and E), and SK-Mel-29 cells (Fig. 2C and F) resulted in robust tumorigenesis. More importantly, treatment with topoisomerase II inhibitors, etoposide (Fig. 2A-C, ○) or doxorubicin (Fig. 2D-F, ●), lead to tumor regression in
motifs (24–27). Amino acids 1 to 374 of E2F1 are involved in the recently defined E2F1-specific retinoblastoma binding (25), whereas amino acids 374 to 437 of E2F1 incorporate the 18 amino acids required for interaction of all E2Fs including E2F1 with retinoblastoma (24). E2F1 (1-363) that retains the E2F1-specific retinoblastoma binding is as efficient in repression as WT E2F1 indicating that the E2F1-specific retinoblastoma-binding motif is sufficient for E2F1 repressor activity (Fig. 4F). The E2F1 mutant that lacks the 18 amino acids required for general retinoblastoma binding confers repression, albeit to a slightly reduced level and not to the same extent as either WT E2F1 or E2F1 (1-363; Fig. 4F), which is suggestive of a limited but not essential role for retinoblastoma in repression of the topoisomerase IIβ promoter. These results, taken together, thus confirm that E2F1 is a bona fide gene repressor for topoisomerase IIβ.

In agreement with this, exogenous E2F1 was able to repress topoisomerase IIβ expression levels (Fig. 5A). To further delineate the contribution of E2F1 in topoisomerase IIβ promoter regulation, we used MEFs nullizygous for E2F1 (E2F1−/−). Topoisomerase IIβ expression levels were elevated in E2F1−/− cells compared with both WT and Cdk4R24C (RR) cells (Fig. 5B), which is consistent with our hypothesis that E2F1 represses topoisomerase IIβ. Importantly, restoration of E2F1 expression was sufficient to repress the topoisomerase IIβ promoter activity in E2F1−/− cells (Fig. 5C). E2F1−/− cells have a high proliferative potential compared with WT and Cdk4R24C (RR) cells (Fig. 5D). To examine the effects of E2F1 deficiency on sensitivity to anti–topoisomerase II therapy, we monitored viability of E2F1−/− cells exposed to topoisomerase II inhibitors, etoposide and doxorubicin. E2F1−/− cells are exquisitely sensitive to the topoisomerase II inhibitors (Fig. 5E and F) with a
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A higher degree of susceptibility to cell death than topoisomerase II inhibitor–treated Cdk4R24C cells. These results are therefore consistent with an important gene repressor and tumor suppressor function for E2F1 by virtue of its regulatory role on topoisomerase II levels. Importantly, these observations also illustrate the influence of E2F activity on the success of anti–topoisomerase II therapy.

Loss of E2F promoter occupancy leads to topoisomerase II promoter derepression. Levels of E2F1 and E2F4 RNA and protein are higher in Cdk4R24C cells compared with Cdk4−/− cells (Fig. 6A). Because our studies identified the importance of E2F1-DNA binding in topoisomerase II promoter repression (see Fig. 4F), we decided to examine the importance of promoter occupancy of E2Fs in topoisomerase II promoter derepression. To this end, we did chromatin immunoprecipitation analysis using antibodies against E2F1 and E2F4 and primers that flank the E2F-binding sites on the topoisomerase II promoter (Fig. 6B). After adjusting for the alterations in E2F1 and E2F4 protein levels in Cdk4−/− and Cdk4R24C cells (based on levels seen in Fig. 6A), we observe a 55% reduction in E2F1 binding on the topoisomerase II promoter (Fig. 6B) indicating that a significantly large proportion of E2F1 in Cdk4R24C cells was not promoter bound in comparison with Cdk4−/− cells. Approximately 95% loss of binding of E2F4 on the topoisomerase II promoter in Cdk4R24C cells was observed in comparison with Cdk4−/− cells (Fig. 6B). These observations reveal an important role for appropriate promoter occupancy of E2F1 and E2F4 in repression of the topoisomerase II promoter. Based on these results, we infer that the elevation of topoisomerase IIβ levels in Cdk4R24C cells (Fig. 3) is due to partial and complete loss of E2F1 and E2F4 promoter occupancy, respectively.

Interestingly, treatment of Cdk4R24C cells with doxorubicin (D) or etoposide (E) resulted in further augmentation of topoisomerase IIβ levels (Fig. 6C) in contrast to control (C) treated Cdk4R24C fibroblasts. The increase was evident at 24 hours after treatment and persisted up to 48 hours. Treatment with the topoisomerase II inhibitors did not significantly alter E2F1 expression levels (Fig. 6C). Our previous results indicated that whereas E2F4 does not occupy the topoisomerase IIβ promoter, at least 45% E2F1 was promoter bound in Cdk4R24C cells (Fig. 6B). To examine if response to etoposide and doxorubicin alters the occupancy levels of the remaining repressor E2F1 and/or retinoblastoma on the topoisomerase IIβ promoter, we did chromatin immunoprecipitation experiments in control or drug-treated Cdk4R24C cells using genomic primers to topoisomerase IIβ promoters and antibodies against E2F1 and retinoblastoma. These assays revealed that etoposide and doxorubicin treatment of Cdk4R24C cells leads to a complete loss of E2F1 promoter occupancy (Fig. 6D, lanes 3 and 6), whereas, control Cdk4R24C cells maintained their basal levels of E2F1 promoter interaction (Fig. 6D, lane 9 and also see Fig. 6B described previously). In contrast to E2F1 protein, retinoblastoma did not occupy the topoisomerase IIβ promoter in either control or drug-treated Cdk4R24C cells (Fig. 6D, lanes 2, 5, and 8). Taken together, these results indicate that etoposide and doxorubicin treatment of Cdk4R24C cells leads to a complete loss of E2F1
occupancy on the topoisomerase IIβ promoter (Fig. 6D) resulting in increased derepression of the topoisomerase IIβ promoter and elevated topoisomerase II expression levels.

Drug-induced apoptosis is preceded by chromatin reorganization with features of silent heterochromatin. The amount of topoisomerase II is an important determinant of the activity of topoisomerase II inhibitors, because cytotoxic actions of topoisomerase II inhibitors greatly depend on a functional target enzyme in the same cellular compartment as the genomic DNA (20, 28). This is consistent with our observations of elevated topoisomerase II levels in drug-treated cells and the associated increased sensitivity of these cells to apoptosis. We next asked what are the cellular consequences of the elevated topoisomerase II levels in drug-treated Cdk4R24C cells. Because topoisomerase II isoforms are essential regulators of DNA topology that influences nuclear organization, we asked if the elevated topoisomerase II levels in drug-treated cells elicits chromatin reorganization during apoptosis. Recently, it was shown that a distinct heterochromatic structure accumulates in senescent human fibroblasts (29). This structure was shown dependent on the retinoblastoma tumor suppressor pathway and recruitment of heterochromatin proteins to E2F-repressive promoters resulting in stable repression of E2F target genes. We hypothesized that similar to the cellular senescence program, drug-induced apoptosis may also feature a gene silencing program. To explore this possibility and to determine if chromatin reorganization precedes the apoptosis response, we examined control and topoisomerase II inhibitor–treated Cdk4R24C cells using immunofluorescence assays (Fig. 7). Untreated Cdk4R24C cells display normal nucleoli and a typical pattern of 4’,6-diamidino-2-phenylindole (DAPI) staining with several irregularly shaped foci representing heterochromatin regions (Fig. 7A-C, control; DAPI). In contrast, etoposide- and doxorubicin-treated Cdk4R24C cells that are sensitive to these drugs displayed dramatically altered nuclear morphology. Drug-treated Cdk4R24C cells typically displayed what seemed fewer and enlarged nucleoli and more well defined, round DAPI-stained foci (Fig. 7A-C, doxorubicin and etoposide; DAPI). In addition in topoisomerase II inhibitor–treated Cdk4R24C cells, the heterochromatin marker proteins, HPIβ and HPIγ, critical adaptor molecules that are required for heterochromatin assembly and are involved in epigenetic gene regulation (30–32) accumulated in the enlarged heterochromatin foci. Whereas HPIβ was largely diffusely distributed in control cells and did not accumulate significantly in heterochromatin foci, the protein prominently

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**Figure 6.** Loss of E2F-binding to topoisomerase IIβ (TopoIIβ) promoter. A, RNA and protein levels of E2F1 and E2F4 in Cdk4**+/** (WT) and Cdk4**R24C** (RR) primary fibroblasts. GAPDH (RNA) and tubulin (protein) control levels. B, ChIP assay (lanes 1-12) using Cdk4**+/** (WT) and Cdk4**R24C** (RR) MEFs and primary melanocytes with no antibody (C) and antibodies to E2F1 (E1) or E2F4 (E4). Genomic DNA PCR (lanes 13-15) control is also shown. Amplified topoisomerase II products (closed arrow) and primers (open arrows). Quantified relative levels of protein expression based on (A) and percentage specific promoter binding based on the ChIP assay (below the respective lanes). C, protein levels of topoisomerase IIβ and E2F1 in Cdk4**R24C** MEFs after 24 and 48 hours treatment with control (C), doxorubicin (D), or etoposide (E). Tubulin antibody control is provided. D, loss of E2F1 binding to topoisomerase IIβ promoter in doxorubicin and etoposide-treated Cdk4**R24C** cells. Chromatin immunoprecipitation assay (lanes 1-9) using control, doxorubicin-, or etoposide-treated Cdk4**R24C** MEFs with no antibody (C) and antibodies to E2F1 (E1) or Rb. Genomic DNA PCR (lane 10) control. Amplified topoisomerase IIβ products.
localized and accumulated in the DNA foci of topoisomerase II inhibitor–treated Cdk4<sup>R24C</sup> cells (Fig. 7C, HP1<sup>β</sup>; data not shown). These enlarged foci indeed represented heterochromatin as indicated by their histone modification patterns. Control Cdk4<sup>R24C</sup> cells exhibited a low level of histones methylated on lysine 9 or lysine 20, which are characteristic marks for heterochromatin (Fig. 7B, H4K20; data not shown). In contrast, Cdk4<sup>R24C</sup> cells treated with doxorubicin and etoposide displayed increased levels and a distinctive accumulation of the modified histones in the enlarged heterochromatin foci. Therefore, both H3-K9M and H4-K20M (yellow) is evident in the merged image. C, AAHFs in doxorubicin and etoposide-treated cells accumulate heterochromatin protein, HP1<sup>β</sup> (green) whereas HP1<sup>α</sup> is generally dispersed throughout the nucleoplasm in untreated cells. Colocalization of topoisomerase II<sup>α</sup> and HP1<sup>β</sup> (yellow) is evident in the merged image.

**Discussion**

**Dual role of E2F1 as an activator and a repressor of gene transcription.** Cell culture models have greatly advanced our understanding of the importance of cell cycle proteins in normal development and cancer biology. However, the paradigms established by cell culture models have not always been substantiated in mouse models in which the expression of cell cycle genes is disrupted by gene targeting. For example, whereas such in vivo models have validated the tumor suppressor functions of retinoblastoma and p53 and the oncogenic potential of Cyclin D1, the minimal phenotypes associated with the loss of Cdk4, Cdk6, Cdk2, the D-Cyclins and Cyclin E have raised questions regarding the specific role of Cyclin D-Cdk4/6 and Cyclin E-Cdk2 complexes as essential regulators of the cell cycle machinery (17, 33–37). Importantly, these observations are consistent with earlier reports that cancer cells proliferate despite CDK2 inhibition, thereby questioning the suitability of CDK2 as a target for cancer therapy (38).

Similarly, E2F1 has been regarded as an activator of gene transcription that is critical for entry of cells into the S phase of DNA synthesis. However, loss of E2F1 led to increased tumorigenesis (8, 9) that instead suggested a tumor suppressor function for E2F1 most likely due to its role in mediating apoptosis. Likewise, although tissue culture systems have indicated a repressor role for E2F4, mice deficient in E2F4 are not tumor prone and instead have developmental defects and early lethality (10). Taken together, these results suggest that tumor suppression may not be entirely mediated by the repressive function of E2F4 (or the related E2F5). Instead, there seems an important role for the “activator” E2Fs (E2F1, E2F2, and E2F3) in tumor suppression. Continuing on the
E2F Regulates Topoisomerase IIβ

Figure 8. Loss of E2F repression results in topoisomerase II (TopoII) overexpression that triggers heterochromatin-associated chromatin reorganization during apoptosis. A, E2F1 and E2F4 normally repress the topoisomerase IIβ promoter. B, in Cdk4R24C cells moderate derepression of the topoisomerase IIβ promoter due to loss of E2F4 binding and 50% reduction in E2F1 binding leads to modest elevation of topoisomerase IIβ levels. C, treatment of Cdk4R24C cells with doxorubicin and etoposide initiates a cell death program, which includes complete derepression of the topoisomerase IIβ promoter due to loss of both E2F1 and E2F4 binding. The elevated topoisomerase IIβ levels trigger chromatin modifications that include formation of AAHFs. Free E2F1 can contribute to further chromatin alterations and independent apoptosis events (see text). This model proposes that chromatin modifications that include heterochromatin formation precede cell death in response to topoisomerase II inhibitors.
in this article, are indicative of a critical role for E2F1 in chromatin remodeling that influences the cellular apoptosis program (Fig. 8). Our findings suggest that AAHF formation precedes drug-induced apoptosis and identifies heterochromatin formation and gene silencing as a post-damage response to chemotherapy. We propose that heterochromatin formation may play a role during the apoptosis responses elicited by other anti-cancer modalities. These findings add to the impressive body of literature that is unraveling the importance of chromatin reorganization in complex cellular processes such as senescence and apoptosis. It has been shown that the retinoblastoma pathway plays an important role in heterochromatin formation and the silencing of E2F target genes during cellular senescence (29). This study elegantly illustrated the occurrence of senescence-associated heterochromatic foci (SAHF) that was dependent on an intact retinoblastoma pathway. It is plausible that the DNA foci that we refer as AAHFs could be similar to the SAHFs reported by Narita et al. Whereas this issue will require further characterization, an important distinction worth mentioning is that the Cdk4(R24C) cells are refractory to inhibition by p16(Ink4a) and escape cellular senescence due to inactivation of the p16(Ink4a)/retinoblastoma pathway (17, 18). This observation is not entirely consistent with SAHF formation that requires an intact RB pathway (29).

Topoisomerase II isoforms interact directly with chromatin modifier proteins such as histone deacetylase proteins (47) and the histone methyltransferase proteins and methylated histones (Fig. 7). These results are suggestive of a direct role for topoisomerase II in chromatin remodeling in response to chemotherapy treatment and provide new insight into the involvement of topoisomerase II in heterochromatin formation. We infer that topoisomerase II inhibitors engineer a program to elevate the levels of topoisomerase II by lowering the promoter occupancy levels of repressor E2F1. This in turn allows topology alterations that facilitate heterochromatin formation and gene silencing which shuts down the gene expression program in preparation for apoptosis. The precise mechanism responsible for the alterations in E2F occupancy on the topoisomerase II promoter is not known at this time. It is plausible that chromatin alterations due to elevated topoisomerase II proteins and/or the increased heterochromatin formation may contribute to the altered promoter occupancy of E2Fs. The increased topoisomerase II in the vicinity of the heterochromatin may lead to further accumulation of “cleavable complexes” that facilitate the activity of the topoisomerase II inhibitors to do their cytoidal action. The chromatin alterations that precede drug-induced apoptosis offer new insight into the mechanism of drug action and suggest development of novel cancer therapeutic strategies aimed at enhancing the action of chromatin modifiers capable of inducing a silent heterochromatic state in cancer cells.

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References

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E2F-Dependent Repression of Topoisomerase II Regulates Heterochromatin Formation and Apoptosis in Cells with Melanoma-Prone Mutation

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