Aberrant Regulation of Survivin by the RB/E2F Family of Proteins*

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Survivin is a putative oncogene that is aberrantly expressed in cancer cells. It has been hypothesized to play a central role in cancer progression and resistance to therapy in diverse tumor types. Although some of the transcriptional processes regulating its expression have been established, the diversity of genes that may be controlling the levels of its expression in both normal cells as well as in cancer cells has not been fully explored. The most common genetically mutated pathways in human malignancies are the p53 tumor suppressor pathway and the RB/E2F pathway. Both of these pathways, when intact, provide essential checkpoints in the maintenance of normal cell growth and protect the cell from DNA damage. Using non-transformed embryonic fibroblasts, we provide evidence of a molecular link between the regulation of survivin transcription and the RB/E2F family of proteins. We demonstrate that both pRb and p130 can interact with the survivin promoter and repress survivin transcription. We also show that the E2F activators (E2F1, E2F2, and E2F3) can bind to the survivin promoter and induce survivin transcription. Genetically modified cells that harbor deletions in various members of the RB/E2F family confirm our data from the wild-type cells. Our findings implicate several members of the RB/E2F pathway in an intricate mechanism of survivin gene regulation that, when genetically altered during the process of tumorigenesis, may function within cancer cells to aberrantly alter survivin levels and enhance tumor progression.

Survivin is expressed at the highest levels reported in G2/M and lower levels in G1 (1, 5). Survivin expression is cell cycle-regulated with the highest levels in G2/M and lower levels in G1 (1, 5). This cell cycle specificity has been attributed to two cell cycle-dependent elements (CDEs)1 that are upstream of the transcription start site (6, 7). Mutation within the CDEs abolishes the cell cycle specificity of survivin expression.

Survivin is highly expressed in a variety of transformed cell lines, including human cancers of the lung, colon, pancreas, breast, brain, and bone marrow (for review, see Ref. 8). High levels of expression within cancer cells correlate with an adverse clinical outcome (8). Data from an in vivo tumor model support a mechanism whereby its targeted disruption specifically eliminates tumor cells by a proapoptotic mechanism (9). Survivin also plays a critical role in normal development, as genetic disruption is lethal early in the embryo (65). Postnatally, survivin is expressed in a few select tissues, including bone marrow stem cells, vascular endothelial cells, colonic epithelium, and the ependyma and choroid plexus of the brain (10–13). The pattern of survivin expression suggests that it plays a critical role in the maintenance of actively dividing cells and tissues.

The RB/E2F pathway plays an essential role in cell cycle regulation and maintenance of cellular homeostasis (14, 15). Mutations in genes within this pathway are found at high rates in almost all types of cancer, attesting to their importance in cell growth control (15, 16). RB and its structurally and functionally related family members, p107 and p130, have similar properties of binding to the E2F family members and of inhibiting cell cycle progression when ectopically expressed (17). The E2F family of proteins includes both the transcriptional activators (E2F1, E2F2, and E2F3) and the repressors (E2F4 and E2F5) (18). These proteins bind to specific promoter regions within diverse gene targets. The interaction of RB with the E2F activators normally results in an inhibition of E2F-mediated transactivation and a cell growth arrest at the G1 phase of the cell cycle (19–21). RB family mutants that lack the ability to cause growth arrest also lack the ability to bind E2F and inhibit E2F-dependent transcription. Such mutants have been isolated from many different human tumors (22, 23). The viral oncoprotein E1A also binds and sequesters the RB family members, resulting in the release of E2F transcriptional activity, induction of DNA synthesis, and transformation of mammalian cells (24–26). Overexpression of E2F1, E2F2, and E2F3 can induce entry into the cell cycle and can lead to transformation of primary cells (27). Derepressed E2F transcriptional activity can also trigger apoptosis through p53-dependent and -independent pathways, although the individual mechanisms and E2F species that play the major role in these processes have not been identified (18).

Our data, in wild-type cells and in cells that have been genetically modified to delete pRb, the E2Fs, and p53, suggests...
that survivin and the RB/E2F proteins interact via a mechanism that involves multiple RB/E2F members acting as repressors and activators of survivin transcription. We hypothesize that disruption of upstream elements within the RB pathway such as the deletion of RB, the amplification of CDK4, or the deletion of p16INK4a, perhaps in the early stages of tumorigenesis, may result in the deregulation of survivin, leading to chromosomal instability and an accumulation of mutations that confer resistance to therapy.

EXPERIMENTAL PROCEDURES

**Cells and Culture Conditions**—Primary mouse embryonic fibroblasts (MEFs) harboring genetic deletions of the E2F activators (E2F1, E2F2, and E2F3), as well as pRB and p53, were derived from day 13.5 embryos after breeding mice harboring these genotypes (28). These MEFs were designated as follows: 1, pRB; 2, pRB/p53; 3, p53; E2F1 −/− 2 −/−; 4, pRB/E2F1 −/− 2 −/− 3 −/−; and 5, E2F3 −/−, with f indicating an allele derived by insertion of loxP sites around exons of the gene of interest. To delete the genes containing loxP sites in vitro, MEF cells were transduced with a retroviral construct capable of expressing Cre recombine, an enzyme that recognizes loxP sites within the genome and renders the cell incapable of transcribing these genes from either allele.

**In vitro Transfection Experiments**—Poly(A) RNA was isolated from 150 μg of total RNA, subjected to denaturing agarose electrophoresis, and transferred to a nylon membrane (GeneScreen Plus, PerkinElmer Life Sciences). Blots were hybridized with a 32P-labeled riboprobe of murine survivin or GAPDH. Signals were quantitated by phosphorimaging (31).

**Northern Blot Analyses**—Poly(A) RNA was isolated from 150 μg of protein was separated by electrophoresis on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad) using a Bio-Rad semidy transfer cell. The membrane was blocked in 20 μg Tris-buffered saline (pH 7.6) with 0.1% Tween 20 (TBST) containing 2% (w/v) blocking powder at 4 °C overnight. The membrane was incubated with primary antibody at a 1:5000 dilution (survivin, FL-142, or D-8, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature, washed, and incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody at a 1:5000 dilution (Santa Cruz Biotechnology). After TBST washes, the blot was incubated in detection reagent (ECLTM Advance Western blotting detection kit) and exposed to a Hyperfilm™ ECLTM (Amersham Biosciences). Membranes were exposed to x-ray film, and the autoradiogram was scanned to determine the signal intensity.

**Northern Blot Analyses**—50 μg of protein was separated by electrophoresis on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad) using a Bio-Rad semidy transfer cell. The membrane was blocked in 20 μg Tris-buffered saline (pH 7.6) with 0.1% Tween 20 (TBST) containing 2% (w/v) blocking powder at 4 °C overnight. The membrane was incubated with primary antibody at a 1:5000 dilution (survivin, FL-142, or D-8, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature, washed, and incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody at a 1:5000 dilution (Santa Cruz Biotechnology). After TBST washes, the blot was incubated in detection reagent (ECLTM Advance Western blotting detection kit) and exposed to a Hyperfilm™ ECLTM (Amersham Biosciences). Membranes were exposed to x-ray film, and the autoradiogram was scanned to determine the signal intensity.

**Chromatin Immunoprecipitation (ChIP)**—This procedure was modified from a protocol obtained from the laboratory of Dr. Peggy Farnham (33). WI-38 cells were serum-starved in EMEM with 2% FBS for 72 h after induction into the cell cycle with 15% FBS. Formaldehyde (final concentration, 1%) was added either at 0 (72 h after addition of low serum) or 20 h after readdition of 15% serum. Plates were incubated at room temperature for 10 min, and reactions were terminated by addition of glycine (final concentration, 125 mM). Cells were washed with cold phosphate-buffered saline, harvested by scraping, and placed in hypotonic buffer (10 mM HEPES, 1 mM MgCl2, 10 mM KCl, and 0.5 mM dithiothreitol) at 4 °C for 10 min. Cells were then harvested by centrifugation, resuspended in hypotonic buffer, and lysed with a Dounce homogenizer to release nuclei. Nuclei were collected by centrifugation, then resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet-P 40, 0.5% deoxycholate, 0.1% SDS) in the presence of protease inhibitors, and sonicated on ice for 30 min. Lysates were passed through a 21-gauge needle and then sonicated with a high intensity ultrasonic processor (Cole Parmer). After centrifugation, the soluble chromatin was diluted 1:10 in ChIP buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 μM Tris-HCI, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF), preincubated with protein A-agarose, and collected by centrifugation. Each tube was incubated with 5 μg of antibody (E2F1-C20, E2F2-N-20, E2F4-A20, E2F5-E19, p130-C20, or p107 (Santa Cruz Biotechnology), or pRB (Pharmingen)) at 4 °C overnight with agitation. Controls included no antibody and rabbit Ig. After incubation, preblocked protein A-agarose was added to each tube, and the mixture was incubated for 1 h at 4 °C. The immune complex-bound agarose beads were collected by centrifugation and washed sequentially. DNA was eluted with 1% SDS, 0.1 mM NaHCO3, and treated with RNase A. To reverse the cross-links, 5 μl NaCl was added, and the samples were incubated at 65 °C. The original, non-antibody-treated chromatin was used as total input for a positive control for the PCR reaction and was similarly purified, precipitated, and assessed by real time quantitative PCR. The position and sequence of human primers used to amplify ChIP-enriched DNA spanning the E2F-like binding sites were from -347 to -117, 5′-AGC CCC TTC TGC TGG TCA TAA CCT-3′, and +48 to +71, 5′-CCG GCC TAA CTC TCT ACT CTC-3′. The E2F-like binding site and the survivin transcription start site were identified in a previously published report (34). Reactions were performed in triplicate for each sample, and results were compared with total input DNA as the reference using the 2−ΔΔCT calculation method (35). Results were obtained at h 0 and h 20 after the addition of serum. For E2F1 and E2F3, zero-h results were used as the calibrator. For E2F4, E2F5, and pRB, results at 20 h were used as the calibrator.

**Real Time PCR**—Primer sets were designed according to the Applied Biosystems Primer Express software using the standardized reaction parameters used by the software to quantity relative cDNA expression levels (mouse survivin: forward, ATC CAC TGG ACC GAG AA, and reverse, CTT GGC TCT GTC TGT CTC.
A SYBR® Green kit (ABI) was used to quantitate mouse survivin RNA levels. GAPDH was used as the internal control (reference). TaqMan analysis was carried out according to the manufacturer’s instructions by using an Applied Biosystems 7700 sequence detection system. Experiments were performed in triplicate, and standard deviations were based on the average of three experiments. Values were calculated using the $2^{-\Delta\Delta CT}$ method (35) with the calibrator assigned as that value obtained after the addition of Cre ($p53^{FL}/E2F1^{00}/pRbf^{00}/E2F3^{00}$) or in the absence of Cre ($p53^{FL}$ and $pRbf^{00}$).

RESULTS

Cell Cycle-dependent Expression of Survivin Is E2F-dependent—To validate the use of non-transformed human embryonic fibroblasts as a model for our studies, we first confirmed the cell cycle dependence of survivin expression in these cells. Early passage human fibroblasts (WI-38) were grown to 50% confluence in EMEM, 10% FBS and then serum-deprived by replacing the medium with EMEM, 0.2% serum for 72 h. Cells were then restimulated with 15% FBS and harvested for protein at various time points (Fig. 2A). Although little or no survivin expression could be detected in G1-arrested cells (Fig. 2A, time 0), expression increased dramatically as cells entered the cell cycle (late G1/S phase), with maximal levels detected at 30 h after serum restimulation. At this time, the majority of cells were in G2/M phase, as indicated by flow cytometry analysis of cells plated in parallel (Fig. 2B). These experiments confirmed the cell cycle-dependent expression pattern of survivin in WI-38 cells, reported previously in other cell types.

To determine whether the cell cycle-dependent expression of survivin described above was mediated via transcriptional mechanisms, non-transformed fibroblasts were transfected with luciferase reporter constructs driven by a 400-bp fragment of the survivin promoter capable of responding to mitogenic signals through the CDE/CHR element (34, 40, 41).
Expression of survivin is cell-cycle dependent in non-transformed fibroblasts. A, Western blot analysis of survivin protein in human embryonic fibroblasts shows maximal induction in G2/M phase. Proliferating WI-38 cells were synchronized in G1 phase by serum starvation for 72 h. 15% serum was readded to the medium, and cells were harvested for protein at the times indicated. Protein blots were probed with a polyclonal antisurvivin antibody and a β-actin control antibody. B, cell cycle analysis of WI-38 cells at various time points before and after readdition of serum. WI-38 cells were synchronized in G1 phase, as described in A, and then harvested for cell cycle analysis at several time points after readdition of 15% serum. C, the CDE/CHR element containing the E2F-like site of mouse survivin is responsive to changes in the cell cycle. A luciferase reporter vector containing a 400-bp region of the mouse survivin promoter harboring either a wild-type (wt) CDE/CHR element and E2F-like site or a 2-bp mutant (mt) (diagrammed in Fig. 1) within this site was transfected into proliferating REF cells. Cells were brought to quiescence by serum starvation and then restimulated by addition of 15% FBS. Cells were harvested and assayed for luciferase activity at time points indicated below the graphs.
mutation within the CDE/CHR element (diagrammed in Fig. 1) resulted in a significant increase in reporter activity in quiescent cells. There was no further increase in luciferase activity observed after the readdition of serum (Fig. 2C). These results, consistent with those reported in other cell systems (6), suggest that in non-transformed quiescent cells transcriptional regulation of survivin occurs through active repression that requires an intact CDE/CHR regulatory region. Derepression of these promoter elements is sufficient to maximally induce its expression during entry into the cell cycle.

Considering that E2F is a major RB-regulated protein important for the expression of many cell cycle-regulated genes, we utilized an adenoviral vector to overexpress E2F1 to assess its role in the control of survivin expression. Overexpression of E2F1 in quiescent REF cells resulted in an induction of survivin expression (~30-fold) (Fig. 3A). To examine the possibility of a direct transcriptional effect of the E2F proteins on the survivin promoter, the ability of various E2F family members to activate a wild-type or mutant survivin reporter construct was evaluated in these cells. Transfection of increasing amounts of E2F1, E2F2, and E2F3a expression vectors led to a dose-dependent increase in wild-type survivin reporter activity that was 3–20-fold above basal levels (Fig. 3B). This activation was dependent on an intact CDE/CHR DNA element because survivin reporter constructs disrupted at this site were unresponsive to E2F overexpression (Fig. 3C). Moreover, DNA-binding mutants of E2F1 (E2F1E132) and E2F3a (E2F3aE132) were incapable of inducing survivin reporter activity (Fig. 3B), suggesting that E2F-mediated regulation of survivin expression requires direct contact of the E2F transcriptional domain with the CDE/CHR element on the survivin promoter. Interestingly, E2F1-overexpressing REF cells eventually undergo apoptosis despite deregulated expression of survivin (36). Presumably, proapoptotic signals induced by E2F1 override the potential antiapoptotic effects reported previously for survivin.

**RB Inactivation by E1A Leads to the Induction of Survivin Expression**—To investigate the possibility that survivin is repressed by the RB family of proteins, E1A, a potent transforming oncogene capable of binding to and inactivating RB and its family members, was introduced into both REF and MEF cells, and survivin expression was assayed by Northern blot assays (Fig. 4A). Ectopic expression of E1A in quiescent cells after adenoviral transduction led to a significant increase in survivin levels relative to an empty adenoviral control vector (~30-fold). To assess the specific components of E1A that led to this activation we utilized a mutant construct of E1A that rendered E1A incapable of binding to the RB family of proteins. We evaluated the transcriptional effects of wild-type E1A compared with those of the mutant E1A protein (125,135A) in survivin reporter assays (Fig. 4B). REF cells were transfected with these constructs, made quiescent by serum deprivation, and then harvested after 36 h. As shown in Fig. 4C, E1A expression led to a significant increase in survivin-specific reporter activity, whereas the RB family-binding mutant of E1A (124,135A) was incapable of inducing survivin reporter activity. The ability of E1A to further induce survivin activity was eliminated when the regulatory CDE/CHR promoter element was disrupted (data not shown). Together, these results suggest that wild-type E1A stimulates survivin expression through the inactivation of RB and/or its family members and that this regulation requires an intact CDE/CHR element. These data infer that the RB family of proteins can repress survivin transcription.

**Genetic Loss of the E2F Activators Represses Survivin Transcription**—To more carefully examine the molecular relationship between the RB/E2F pathway and survivin transcription, we used genetically modified MEFs that harbor conditional deletions of components of the RB/E2F pathway as well as the tumor suppressor p53. Primary MEF cells derived from mouse embryos harboring either conditionally or conventionally targeted genetic deletions in the p53, pRb, and E2F loci included the following: p53f, pRbf, E2F2f, E2F3f, p53f/E2F1−/− pRbf/E2F1−/−, and pRbf/E2F1−/− pRbf/E2F1−/− E2F3f, with f indicating an allele derived by insertion of loxP sites around exons of the gene of interest. In addition, two immortalized cell lines derived from p53f/E2F1−/− pRbf/E2F1−/− E2F3f primary MEFs were used to confirm our findings in the primary cells. Retroviral transduction of a Cre recombinase expression vector into proliferating primary MEF cells resulted in the deletion of the floxed alleles in vitro as confirmed by PCR (not shown). Transduction of empty retroviral vectors into the primary MEF cells served as controls for each cell type. Cells were harvested for protein and RNA 120 h after retroviral infection. Western blot analysis of survivin protein expression for each cell type is shown in Fig. 5A. Real time PCR using survivin-specific primers and a SYBR® Green PCR Master Mix reagent (ABI) was also used to quantitate survivin transcription levels for each condition (Fig. 5B). Consistent with previous publications that used expression constructs to overexpress p53 or cancer cell lines null for p53 (26, 41), homologous genetic deletion of p53 in non-transformed fibroblasts resulted in a significant induction of survivin RNA and protein (4-fold by real time PCR). Homologous deletion of RB also led to an induction of survivin in these cells. Interestingly, homologous deletion of p53 or pRb in combination with a deletion of the E2F activators, E2F1, E2F2, and E2F3, led to a repression (2-fold by real time PCR) of survivin transcription. This implies that one or more of the E2F activators play a dominant role in survivin activation.

We evaluated the role of E2F3 in survivin transcription using primary cells harboring a conditionally targeted homologous deletion for this gene. Loss of E2F3 has been implicated previously in centrosome amplification and aneuploidy, a phenotype also observed in cells with aberrant survivin expression (43). The absence of the E2F3 gene resulted in a 3-fold repression of survivin transcription, arguing for a role of E2F3 in the activation of survivin transcription.

The **E2F Proteins Bind to the Survivin Promoter in Vivo**—To provide evidence for a physical interaction between the survivin promoter and members of the RB/E2F family, we utilized in vivo DNA binding assays. Coprecipitation of the E2F activators, E2F1 and E2F3, the E2F repressors, E2F4 and E2F5, as well as of the RB family members RB and p130 with the survivin promoter was confirmed by ChiP assays in human embryonic fibroblasts (Fig. 6). WI-38 cells were starved by serum deprivation and then harvested at two time points, h 0 and 20 h after serum readdition. Based on our prior observations (see Fig. 2), WI-38 cells are arrested in G1 at h 0 and are in late G1–early S phase of the cell cycle 20 h after serum readdition. Human survivin primers designed around the E2F-binding like binding site (see “Experimental Procedures”) were used in real time PCR reactions to quantitate the binding affinities. Changes in binding levels as cells were induced from a quiescent to a proliferative state were then calculated. Results of the ChiP experiments showed that E2F1 and E2F3 bind to the survivin promoter with a 1000-fold change in binding occurring upon entry into the cell cycle (Fig. 6). In addition, p130 coprecipitates with the survivin promoter when the cells are in a quiescent state. pRB-mediated coprecipitation with the survivin promoter was also observed with an increase in binding (180-fold), when cells are quiescent. This binding activity is consistent with an RB family-mediated repression of survivin that likely occurs through binding of the RB proteins to the
FIG. 3. Expression of survivin is E2F-dependent. A, E2F induces survivin transcription. REF cells were arrested in G1 by serum starvation. Cells were transduced with adenoviral vectors containing either E2F1 or an empty vector control. RNA blots were hybridized with mouse survivin and mouse GAPDH riboprobes. B, the E2F activators induce survivin transcription through the E2F transactivation domain. REF cells were transfected with a wild-type survivin promoter construct, E2F1, E2F2, or E2F3a expression vectors or empty vector control (Con) and a β-galactosidase expression vector. Cells were brought to quiescence and then harvested 36 h after serum deprivation. To determine the requirement for the E2F transactivation domain, E2F1 and E2F3a mutant expression constructs containing mutations within the transactivation domains (E2F1E132 and E2F3aE132) were transfected in parallel with wild-type constructs. C, the CDE/CHR mutant derepresses survivin transcription. REF cells were transfected as in B except that a mutant (mt) survivin promoter construct (diagrammed in Fig. 3) was used instead of a wild-type (wt) construct. Luciferase reporter activity was normalized to β-galactosidase activity for all reporter assays.
E2F repressors. To evaluate binding of the E2F repressor proteins, E2F4 and E2F5 were also used in co-immunoprecipitation assays. Both E2F4 and E2F5 bind to the survivin promoter, when cells are quiescent (Fig. 6). Together, these findings suggest that survivin transcription is positively regulated by the E2F activator proteins and negatively regulated by the E2F repressors, the latter occurring in association with the RB proteins.

**DISCUSSION**

The functional activities of survivin within both normal cells and transformed tissue remain to be clarified. Conventional deletion targeting of the survivin locus results in early embryonic lethality (day 4.5) with a disruption of chromosomes and abnormal mitoses described in the surviving cells (3). This phenotype parallels that reported for chromosomal passenger proteins and the Aurora family of serine-threonine kinases, whose functions include the control of spindle-pole body duplication, bipolar spindle formation, chromosome segregation, and cytokinesis (37). It is also similar to that of BIR-1, a survivin homolog in *Caenorhabditis elegans*, whose disruption results in defects of chromosome alignment and separation and in cytokinesis (38). Cellular localization studies in nonmalig-
nant cells support a role for survivin in regulating mitotic processes (4). Studies in cancer cells suggest an additional function for survivin as a classic cell death inhibitor, exemplified by transgenic mouse models of survivin inhibiting ultraviolet B radiation-induced apoptosis through interference with caspase activation (39).

The RB/E2F pathway is disrupted in virtually all human malignancies (14, 15). Although survivin is aberrantly ex-

**Fig. 5. Loss of the E2F activators represses survivin.** A, Western blot analysis of primary MEF cells harboring targeted homozygous deletions in p53, pRb, E2F3a, and E2F3b, as well as quadruple deletions of pRb, E2F1, E2F2, E2F3, and p53, E2F1, E2F2, E2F3, E2F1, E2F2, and E2F3 were designated as follows: 1, p53\^f\^f; 2, pRb\^f\^f; 3, p53\^f\^f/E2F1\^f\^f/2\^f\^f/3\^f\^f; 4, pRb\^f\^f/E2F1\^f\^f/2\^f\^f/3\^f\^f; and 5, E2F3\^f\^f, with \^f\^f indicating an allele derived by insertion of loxP sites around exons of the gene of interest. Cells were grown under proliferating conditions and transduced with a retroviral vector expressing Cre recombinase (\^f\^f) or with an empty vector (\^f\^f) control. Two different cell lines created from p53\^f\^f/E2F1\^f\^f/2\^f\^f/3\^f\^f primary cells were chosen for comparison (designated 6 and 7). Blots were probed with a polyclonal antisurvivin antibody and \^f\^f actin control. B, quantitative PCR analysis of genetically modified MEF cells treated with Cre (\^f\^f) or empty vector (\^f\^f) control as designated. Total RNA was isolated from each cell line by affinity column chromatography and transcribed into cDNA using reverse transcriptase. Levels of survivin were normalized to GAPDH for each condition. The relative change in survivin expression was then calculated based on the 2^\(-\Delta\DeltaCT\) method (35). For graphical representation, the results for the p53\^f\^f and pRb\^f\^f cells were arbitrarily assigned a value of one in the absence of Cre and shown in comparison with the results after the addition of Cre; for the p53\^f\^f/E2F1\^f\^f/2\^f\^f/3\^f\^f, pRb\^f\^f/E2F1\^f\^f/2\^f\^f/3\^f\^f, and E2F3\^f\^f cells, the results after the addition of Cre were arbitrarily assigned a value of one and shown compared with the results in the absence of Cre.
The survivin gene has cell cycle-regulatory sequences (CDE/CHR elements) within its promoter that control maximal activation in the G2/M phase of the cell cycle. Mutation within these sequences results in the loss of cell cycle responsiveness and a significant transcription induction (6). This result, demonstrated with other genes that also contain CDE/CHR regulatory elements (40), suggests that survivin transcription is actively repressed throughout the G1 phase of the cell cycle. Relief of this repression mechanistically requires a restructuring of protein-DNA interactions within the CDE/CHR regulatory region. It has been shown previously that the p53 and p73 tumor suppressor genes can repress survivin transcription (34, 41, 42). This mechanism is dependent on an intact transactivation domain within these proteins and on a functional downstream target, p21. p21 has been speculated to function in survivin regulation by inactivating cyclin-dependent kinases (42). This would then result in the hypophosphorylation of RB, preventing the release of E2F activators but allowing for an interaction with the E2F repressors, as E2F4 and E2F5, to bind to E2F target sequences. If p53 were deleted or mutated, a frequent occurrence in human cancer, p21 would not be activated, and RB would be constitutively phosphorylated, leading to the release of the E2F activators and the induction of survivin transcription. Thus, these prior studies indirectly support the findings in this paper, linking RB to the regulation of survivin transcription.

Our experiments in non-transformed embryonic fibroblasts suggest that several RB/E2F proteins regulate survivin expression. We show that RB proteins can repress survivin transcription as demonstrated by a decrease in survivin reporter activity following transfection of an E1A-RB-binding mutant. We also show that both pRB and p130 coprecipitate an upstream survivin DNA promoter region that contains an E2F-like binding element. In addition, we show that primary cells derived from mice genetically lacking pRb have higher levels of survivin than control cells with an intact pRb gene.

In addition to the RB-mediated repression of survivin transcription, we demonstrate that several E2F activator proteins can induce survivin activity. E2F1 and E2F3 directly induce survivin through binding to its promoter as shown by both the reporter assays and by the ChIP studies. This E2F-mediated transcriptional induction is abrogated if the E2F DNA-binding domain is mutated, suggesting the importance of this domain in transcriptional activation.

The E2F3 locus controls the expression of two genes (31), E2F3a and E2F3b. Either one or both could be involved in survivin regulation during different phases of the cell cycle. Using an antibody to E2F3 (N-20) that recognizes the E2F3a but not the E2F3b protein, we show that E2F3a binds to survivin in vivo. Homozygous deletion of E2F3 in cells derived from mice with genetically targeted deletions of both the E2F3a and the E2F3b genes results in a repression of survivin transcription. As E2F3 has been shown recently to control centrosome duplication, its disruption may operate through survivin targeting to interfere with a centrosome duplication checkpoint, resulting in aneuploidy and further genetic instability in cancer cells (43).

In summary, our data support the hypothesis that dysregulation of survivin expression can occur as a consequence of genetic alterations within the RB/E2F pathway arising during the process of tumorigenesis. As these are common events in all human malignancies, it is reasonable to hypothesize that multiple alterations within these pathways, such as deletions or mutations involving pRB, p16, or p21 or amplifications of CDE4, for example, may be responsible for some of the downstream events regulating survivin expression. These findings
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will have implications in furthering the understanding of malignant transformation, especially in deciphering interventions in highly resistant disease.

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