E2F-3B Is a Physiological Target of Cyclin A*

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The E2F family of transcription factors controls the expression of numerous genes that are required for the G1/S transition. Among the mechanisms that modulate the activity of the E2F proteins, cyclin A has been found to be important for the down-regulation of E2F-1, -2, and -3A activity after cells have progressed through G1/S. Specifically, phosphorylation of these E2F proteins by cyclin A/Cdk2 ultimately results in their necessary degradation as cells progress through S phase. E2F-3B was recently identified as an alternatively spliced form of E2F-3A that was predicted to lack a functional cyclin A binding domain. In this paper, we present considerable evidence that contradicts this prediction. First, we demonstrate binding of cyclin A to E2F-3B as bacterially expressed proteins in vitro. Second, we demonstrate binding of cyclin A to E2F-3B in mammalian cells in vivo. Third, we show that co-expression of cyclin A with E2F-3B significantly reduces E2F-3B-mediated transcriptional activity. Finally, in synchronized cells, we observe down-regulation of E2F-3B protein expression coincident with the up-regulation of cyclin A. We conclude that E2F-3B is a physiological target of cyclin A.

The E2Fs represent a family of transcription factors whose activity plays a critical role in cell growth control (1–3). Specifically, the E2F family controls the expression of genes required for DNA synthesis at the G1/S phase boundary (4–7). Presently, the E2F family can be divided into two functional groups. The first group includes E2F-1, -2, and -3A. These factors represent the growth stimulatory segment of the family, since they are potent transcriptional activators and are required for the entrance of cells into S phase (8–11). Members of this group are expressed at low levels in G0 and early G1, and their expression is highly induced in late G1 (10, 12–16). Structural characteristics of this group include an extended N-terminal region of unknown function, a nuclear localization sequence (NLS), and overlapping the NLS, a cyclin A binding domain (17–23). The second group includes E2F-4, -5, and -6, which lack these three functional domains and induce S phase inefficiently. This group of E2Fs appears necessary for growth arrest and differentiation rather than S phase entry (24–26).

In normal cells, transcriptional activation by the E2F family appears to be largely restricted to late G1 and the G1/S boundary. In G0 and early G1, E2F activity is negatively regulated by one or more members of the pRb protein family (27, 28). Once cells reach late G1, the Rb family members become phosphorylated and release the E2Fs. There is then a surge of E2F activity (primarily E2F-1, E2F-2, and E2F-3A) that drives the expression of genes that are required for DNA synthesis. Once in S phase, E2F activity is no longer needed, and the cyclin A protein directs the phosphorylation of the three growth-promoting members of the E2F family by the bound Cdk2, leading to their degradation (21–23, 29). This down-regulation of E2F activity by cyclin A is required for orderly S-phase progression (30–32), and in its absence, apoptosis occurs (33, 34).

Although the E2F family can be divided into two functional groups, the newest member of the E2F family, E2F-3B, is not easily to classify. E2F-3B lacks the N-terminal domain present in E2F-3, which is also referred to as E2F-3A (35, 36). Thus, it resembles the non-growth-promoting group of E2Fs in structure. The expression pattern of E2F-3B is also consistent with its having a role in growth restraint, since E2F-3B is expressed at its highest levels in G0, where it associates with Rb, and its levels drop as cells enter S phase. This pattern of expression is the opposite of that of E2F-1, -2, and -3A (35, 36). However, E2F-3B differs from the growth-restraining E2Fs because it clearly encodes a nuclear localization sequence, as do the growth-promoting E2Fs (35).

Before the work described herein, it has not been clear whether E2F-3B contains a functional cyclin A binding domain. Fig. 1A highlights the nuclear localization sequence and putative cyclin A binding domain of E2F-3A and E2F-3B. E2F-3B transcription uses an alternative promoter and an alternative initiation exon (exon 1b) compared with E2F-3A (exon 1a) and shares the same exons from exon 2 onward (36). Nevins and co-workers (36) predict that E2F-3B will not interact with cyclin A, based upon experiments that mapped the E2F-1 cyclin A binding domain to a region that includes the 21 amino acids highlighted in Fig. 1A (21, 37). If true, this model would suggest that the cyclin A binding domain is encoded in part by exon 1a and in part by exon 2, which seems unlikely. Furthermore, Kaelin and co-workers (38) show that a significantly shorter sequence in E2F-1 (PAKKRLFL) is sufficient to bind to cyclin A. Because the shorter region is present in both E2F-3A and -3B, we have hypothesized that E2F-3B can still bind cyclin A.

In the present work, we test this hypothesis and present several lines of in vivo and in vitro evidence that clearly show that E2F-3B is a physiological target of the cyclin A protein. Because a number of cyclin-dependent kinase inhibitors are in various stages of clinical trials for the treatment of cancer (39, 40), we anticipate that E2F-3B may contribute to the activity of these drugs. Thus, the findings of this report may have important clinical ramifications.

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GST1 vector into the pcDNA3 vector using Moffitt Cancer Center). The cyclin A cDNA was subcloned from the cyclin A/pGEX fusion construct was a gift from Jack Pledger (H. Lee Moffitt Cancer Center) and was in Dulbecco’s modified Eagle’s medium supplemented with 5% calf serum. Cells were density-determined by the Bradford assay (Bio-Rad). For each IP, 40 µg of protein extract for 2 h at 4 °C. Beads were washed 3 times with the same protein lysate buffer, incubated with 0.5% Nonidet P-40, and the lysates containing the E2F proteins were subjected to SDS-PAGE followed by Western blotting. Antibodies used for Western blotting were E2F-3A (sc-866, Santa Cruz Biotechnology), cyclin A polyclonal antibody (BF643) (45), and cyclin A monoclonal antibody (BP643) (45).

Electromobility Shift Assays—For DNA binding assays, the E2F-1 expressing bacteria were lysed as previously described (41). Electrophoretic mobility shift assays (EMSA) and antibody supershift assays were performed as previously described (35). For bacterially expressed E2F-4 protein was subjected to the same GST pull-down assay as in A. Lane 1, 1/100 of the E2F-4 protein input into the binding reaction. Lane 2, GST only. Lane 3, GST-cyclin A bound to glutathione beads. WB, Western blot.

Fig. 1. E2F-3B directly interacts with cyclin A. A. schematic comparison of the E2F-3A and E2F-3B gene products. aa, amino acids; NLS, nuclear localization signal. B. GST pull-down assays were performed on bacterially expressed E2F and GST proteins. Lanes 1 and 6, 1/10,000 (10 ng) of the E2F-3A and E2F-3B protein input used in the GST binding experiments. Lanes 2 and 4, GST only, ~1 µg, as a negative control. Lane 3 and 5, ~1 µg of GST-cyclin A was incubated with the glutathione beads and then incubated with E2F-3A or E2F-3B. C. bacterially expressed E2F-4 protein was subjected to the same GST pull-down assay as in A. Lane 1, 1/100 of the E2F-4 protein input into the binding reaction. Lane 2, GST only. Lane 3, GST-cyclin A bound to glutathione beads. WB, Western blot.

Experimental Procedures

Construction of Plasmids—Myc epitope-tagged E2F-3A and E2F-3B cDNAs were obtained as gifts from Dr. Gustavo Leone (Ohio State University) and subcloned into the pcDNA3 vector using HindIII and XhoI sites. The same sites were also used to subclone into the pET-23b vector for bacterial expression. To generate bacterial expression constructs of Myc-E2F-3A and Myc-E2F-3B, the corresponding inserts were cut out of the pcDNA3 vector using HindIII and XhoI sites, with XhoI sites filled by Klenow, and cloned into the HindIII and XhoI sites of the pET-23b vector, with the XhoI site filled with Klenow.

The DP-1/pET construct was a gift from Patrick Hearing (41). The cyclin A/pGEX fusion construct was a gift from Jack Pledger (H. Lee Moffitt Cancer Center). The cyclin A cDNA was subcloned from the GST1 vector into the pcDNA3 vector using BamHI and XhoI sites, with the XhoI sites filled by Klenow, and cloned into the HindIII and XhoI sites of the pET-23b vector, with the XhoI site filled with Klenow.

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Cell Culture—BALB/c-3T3 fibroblasts were obtained from Jack Pledger (H. Lee Moffitt Cancer Center) and were in Dulbecco’s modified Eagle’s medium supplemented with 5% calf serum. Cells were density-arrested and induced to reenter the cell cycle by the addition of platelet-derived growth factor to the medium as described previously (35). C-33A cells and T98G cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum.

Bacterial Expression and GST Pull-down Assays—Escherichia coli BL-21 cells were transformed with pGEX, pGEX-cyclin A, or the pET-E2F constructs described above. Protein expression was induced with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were pelleted and resuspended in STE buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1 mM EDTA supplemented with final concentrations of 5 mM dithiothreitol and 1.5% N-lauryl sarcosine). The mixture was sonicated 3 times for 10 s each, mixed with 3% Triton X-100, and then cleared by centrifugation at 13,000 x g for 10 min. For each binding assay, lysates containing 1 µg of GST or GST-cyclin A were incubated with 30 µl of glutathione-Sepharose beads (Amersham Biosciences) for 1 h at room temperature. Beads were washed 4 times in phosphate-buffered saline, 0.5% Nonidet P-40, and the lysates containing the E2F proteins were incubated with the beads in the same buffer for 1 h at room temperature. Beads were washed 6 times in phosphate-buffered saline, 0.5% Nonidet P-40 after the binding, and complexes bound to the beads were then subjected to SDS-PAGE followed by Western blotting. All experiments were repeated at least three times.

Immunoprecipitation (IP) and Western Blotting Analysis—Myc tagged E2Fs and E2F-4 in pcDNA3 were transfected into C-33A cells using the calcium phosphate method as described (42, 43). After transfection, C-33A cells were washed twice in phosphate-buffered saline and resuspended in lysis buffer containing 50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 5 mM EDTA, and 2% Nonidet P-40, supplemented with protease inhibitors (5 µg/ml each antipain, aprotinin, leupeptin, and soybean trypsin inhibitor and 0.5 µg/ml pepstatin), 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. Protein concentrations were determined by the Bradford assay following (Bio-Rad). For each IP, 40 µl of sheep anti-mouse IgG-conjugated magnetic beads (Dynal, Inc.) were used. Beads were washed three times with phosphate-buffered saline, 0.1% bovine serum albumin and incubated with 1 µg of the c-Myc monoclonal antibody (sc-40, Santa Cruz Biotechnology) or 1 µg of the E2F-4 polyclonal antibody (sc-866, Santa Cruz Biotechnology) for 30 min at 4 °C. Beads were washed 3 times with the same protein lysis buffer, incubated with 400 µg of protein extract for 2 h at 4 °C, and washed again. Proteins bound to the beads were subjected to SDS-PAGE followed by Western blotting. Antibodies used for Western blotting were E2F-3A and -3B C-18 antibody (sc-878, Santa Cruz Biotechnology), E2F-4 C-20 antibody (sc-866, Santa Cruz Biotechnology), cyclin A polyclonal antibody (BP643) (45), and cyclin A monoclonal antibody (BP643) (45).

Electromobility Shift Assays—For DNA binding assays, the E2F-1 expressing bacteria were lysed as previously described (41). Electrophoretic mobility shift assays (EMSA) and antibody supershift assays were performed as previously described (35). For bacterially ex-
pressed proteins, 1 μl of each of the E2F and DP-1 crude lysate was incubated with the dihydrofolate reductase probe at room temperature for 10 min. Relative amounts of the E2F proteins, DP-1, GST, and GST-cyclin A used in the binding reactions were determined by Coomassie staining. For the GST-cyclin A titration experiments, identical amounts of the recombinant Myc-E2F-3A and Myc-E2F-3B proteins were mixed and incubated with the probe, and different amounts of the GST-cyclin A protein were added into the binding reaction as indicated.

**RESULTS**

**E2F-3B Directly Interacts with Cyclin A in Vitro**—Based on the fact that a peptide with the sequence AKRRLELG abolishes the interaction of cyclin A with E2F (38), we predicted that E2F-3B would retain a functional cyclin A binding domain (see Fig. 1A). To test this, we performed GST-cyclin A pull-down assays on bacterially expressed E2Fs. In these experiments, E2F-3A served as a positive control, and E2F-4 served as a negative control. Fig. 1B shows that both Myc-E2F-3A and Myc-E2F-3B were pulled down by GST-cyclin A fusion protein but were not pulled down by the GST protein by itself. This result demonstrates that E2F-3B possesses a functional cyclin A binding domain. Bacterially expressed E2F-4, which does not have a cyclin A binding domain (46, 47), was subjected to the same assay. Fig. 1C shows that E2F-4 does not bind GST-cyclin A, as expected. The same results were observed in repeated experiments under a variety of conditions.

As an alternative method of detecting the E2F-3B/cyclin A interaction, we performed EMSAs using crude extracts of bacteria expressing either Myc-E2F-3A, Myc-E2F-3B, or combinations thereof in the presence of purified GST-cyclin A or GST only as a negative control. Bacterial extracts of the Myc-E2F and DP-1 as well as purified GST and GST-cyclin A fusion protein were subjected to SDS-PAGE and Coomassie staining to compare their protein levels. Fig. 2A shows that...
Myc-E2F-3A and Myc-E2F-3B proteins were equally expressed in crude extracts, whereas DP-1 was expressed about 5 times more efficiently (lanes 1, 2, and 3; note that 1/5 of DP-1 was loaded compared with the E2Fs). GST and GST-cyclin A, both purified with glutathione beads, were recovered at similar levels (lanes 4 and 5). For the EMSA experiments, identical amounts of the E2F proteins were added to the binding reactions. Fig. 2B reveals that the addition of either E2F-3A (lane 1) or E2F-3B (lane 5) alone results in a diffuse complex that represents the formation of homodimers. The addition of bacterially expressed DP1 protein together with the E2Fs results in the formation a stronger, more stable complex (Fig. 2B, lanes 2 and 6). DP-1 by itself had no DNA binding activity (lane 9). Heterodimeric complexes containing either Myc-E2F-3A or Myc-E2F-3B and the DP-1 partner were confirmed by antibody supershift experiments using the E2F-3 C-terminal antibody that recognizes both E2F-3A and E2F-3B (sc-878, data not shown). The addition of purified GST protein had no effect on the migration of the E2F-3A-E2F-3B complexes (Fig. 2B, lanes 3 and 7); however, the addition of GST-cyclin A to either E2F-3A-E2F-3B complex resulted in supershifted bands (lanes 4 and 8). This observation again demonstrates that cyclin A interacts with both E2F-3A and 3B.

To compare the cyclin A binding affinity of E2F-3A and E2F-3B in a more quantitative way, we combined the two E2Fs together in the same binding reaction with the presence of DP-1 so that the binding conditions for both E2F-3A and E2F-3B were identical. The same amount of E2F-3A and E2F-3B was used as in the previous experiment (Fig. 2B), and the total DNA binding activities of the two proteins were approximately the same (Fig. 2C, lane 2). Various amounts of GST-cyclin A protein were added to the fixed amount of E2F-3A-E2F-3B-DP-1 mixture. Representative results from several repeated experiments are shown in Fig. 2C. Lanes 6 and 7 in Fig. 2C reveal that part of the DNA binding activity of both E2F-3A and E2F-3B was supershifted by GST-cyclin A at 0.5- and 1-µg levels, with E2F-3B affected slightly less efficiently. When a sufficient amount of GST-cyclin A protein was present, E2F-3B complexes were supershifted as effectively as E2F-3A complexes (lanes 8 and 9). Thus, it appears that E2F-3A may be slightly more sensitive to cyclin A than is E2F-3B. This result is consistent with our hypothesis that E2F-3B can interact with cyclin A directly although with less efficiency than E2F-3A.

E2F-3B Associates with Cyclin A in Vivo—To determine whether the in vitro association of E2F-3B with cyclin A is of sufficient affinity to result in association in vivo, we transfected C-33A cells with the Myc-E2F-3A or Myc-E2F-3B constructs and used anti-Myc IP followed by anti-cyclin A Western blotting to detect E2F interaction with endogenous cyclin A. Fig. 3 shows the representative results from several such experiments that we performed. Consistent with the in vitro GST pull-down assays, both Myc-tagged proteins were found to be in complex with cyclin A (Fig. 3A, lanes 3 and 6). Incubation of the...
protein extracts with beads only did not pull down cyclin A, indicating that the interaction between E2F and cyclin A is specific. Overexpressed E2F-4 served as another negative control to demonstrate the specificity of the interaction (Fig. 3B). An E2F-4 polyclonal antibody was used to immunoprecipitate overexpressed E2F-4. Although E2F-4 was clearly immunoprecipitated by the E2F-4 antibody, cyclin A was not detected in the E2F-4 immune complex (lane 3).

Attempts to measure the endogenous E2F-3A and E2F-3B association with cyclin A under physiological conditions were not successful. We believe this is because the association of cyclin A with the various E2Fs is very transient and results in the degradation of the E2F proteins. Combined, these make it difficult to measure the interaction without the benefit of tagged proteins.

**Cyclin A Inhibits the Transcriptional Activity of E2F-3B**—If E2F-3B is a physiological cyclin A target, then we would predict that cyclin A would inhibit transcriptional activation by E2F-3B. To address this prediction, we co-transfected T98G cells with either Myc-tagged E2F-3A or E2F-3B with a luciferase reporter under the control of the AdE2 promoter, which is known to respond to E2F transcriptional activity (42, 48). Cells were also transfected with an RL-TK construct encoding a Renilla luciferase (internal control) and a plasmid expressing HA-DP-1 to increase the binding and transcriptional activity of E2Fs. Each combination of plasmids was transfected in triplicate, and so were the luciferase assays. As shown in Fig. 4, E2F-3A activated the reporter 40-fold above basal level, but its activity dropped dramatically to about 15-fold in the presence of cyclin A (a more than 60% reduction). Compared with E2F-3A, E2F-3B activated the reporter less efficiently, about 18-fold, as we previously reported (35). Co-transfection of cyclin A reduced E2F-3B activity more than 70%; only a 5-fold increase in reporter activity was seen in cells expressing both proteins. As a negative control, E2F-4 transcriptional activity was examined in the presence and absence of cyclin A. E2F-4 activated the reporter 10-fold, and cyclin A had no effect on its activity, as expected.

**Fig. 5.** E2F-3B protein expression is cell cycle-dependent. A, BALB/c-3T3 cells were density-arrested at G0 and stimulated with 10 ng/ml platelet-derived growth factor and fresh 5% calf serum. Cells at different times after induction of cell cycle were harvested. Percentages of the cells with a G0-G1, an S phase, and G2-M DNA contents, as determined by flow cytometry, are indicated. B, BALB/c-3T3 cells were harvested at the times indicated. Cell extracts (100 μg) were Western-blotted with E2F-3 antibody to detect both E2F-3A and E2F-3B. The same membrane was rebotted with cyclin A antibody BF683 (45).

**Down-regulation of E2F-3B Protein Coincides with Cyclin A Appearance in the Cell Cycle**—The E2F-3A promoter is silent in quiescent cells and becomes dramatically activated in late G1. In contrast, the E2F-3B promoter is constitutively active and E2F-3B mRNA levels do not change during the cell cycle (36). Thus, if the E2F-3B protein is subject to down-regulation by cyclin A in a cell cycle-dependent manner, we would expect that there would be a reciprocal relationship between the protein levels of E2F-3B and cyclin A. To explore this, density-arrested quiescent BALB/c-3T3 cells were treated with platelet-derived growth factor to induce synchronous progression from G1 into S phase. Fig. 5A shows the percentage of G0-G1, S, and G2-M cells from samples taken at three intervals following platelet-derived growth factor treatment. Based on this graph, the cells are all in G0-G1 until 9 h after stimulation, and start to pass G0/S boundary at 12–15 h. At 18 h most of the cells are in S phase, and after that point, cells start to move to G2-M and will go back to G0 eventually. This pattern of cell cycle progression after stimulation was seen in every such experiment. Samples harvested at same time points were Western blotted with antibody to cyclin A (Fig. 5B, top panel) or with an antibody that recognizes both E2F-3A and E2F-3B (bottom panel).

Consistent with its known expression pattern, cyclin A was expressed at low levels in G0 cells and early G1, but its protein level increased as cell entered DNA synthesis at 12 h and later fell again as cell exited S phase. Likewise, E2F-3A protein expression was potently activated as cells progressed from G1 phase into S phase (6–9 h after growth factor stimulation), and reached the highest level 12–15 h post stimulation, at the late G1–G2/S transition. E2F-3A protein levels then dropped dramatically afterward (18–21 h), consistent with E2F-3A down-regulation by cyclin A. In contrast to E2F-3A, the E2F-3B protein was highly expressed in G0 and early G1, with peak levels at 18 h post stimulation. E2F-3B dropped (as cyclin A appeared), rebounding again at 27 h (as cyclin A levels dropped). To eliminate the possible random effects in the experiments, the same experiments were repeated and we saw the same results. These results are con-
sistent with a role for cyclin A in the down-regulation of E2F-3A and E2F-3B.

**DISCUSSION**

The promoter of E2F-3A is regulated by Myc and E2F, and is activated in late G1, when cells start to progress into S-phase. E2F-3B, however, is transcribed from an alternative promoter, which is equally active throughout the cell cycle (49). Thus, the observed regulation of E2F-3B is apparently at the post-transcriptional level. E2F-3B protein level is the highest in G0, and it rapidly decreases as cells enter S phase. E2F-3A, however, is expressed only at the G1/S transition, and its level goes down after cells progress into S phase. We demonstrate here that cyclin A likely accounts for the observed down-regulation of E2F-3A during S phase. We note that the down-regulation of E2F-3A in S phase appears to lag behind the down-regulation of E2F-3B. We explain this observation in terms of competing synthesis and degradation steps. E2F-3B is synthesized constitutively throughout the cell cycle. Thus, its down-regulation is apparent immediately following up-regulation of cyclin A. In contrast, E2F-3A synthesis occurs in a surge only at the G1/S boundary. Thus, early in S phase, transcriptional up-regulation of E2F-3A temporarily overcomes post-transcriptional down-regulation of E2F-3A protein level is high and E2F-3A expression is no longer activated. When E2F-3A expression is no longer activated, two levels of down-regulation take over and a rapid decrease in E2F-3A activity is observed. E2F-3A and -3B have particularly important roles in the regulation of the G1/S transition. For example, E2F-3 null mice show that specific E2F-3A-responsive genes will not be activated. Two levels of down-regulation take over and a rapid decrease in E2F-3A activity is observed.

E2F-3A and -3B have particularly important roles in the regulation of the G1/S transition. For example, E2F-3 null mice are the only E2F knockout animals in which defects in cellular proliferation are observed (8,50). Likewise, microinjection of E2F-3A antibodies demonstrates that E2F-3A is the only member of the E2F family essential for S phase induction under conditions that are not efficiently recognized by other members of the E2F family.

Recent studies contribute to a model for E2F activity in which E2F-Rb complexes in G0 serve to actively repress transcription of the promoters to which they are bound (2,3). In late G1, the E2F-Rb complexes dissociate (due to Cdk phosphorylation), and E2F-regulated promoters are then bound by activating members of the E2F family. Within this general model it is clear that different members of the E2F family have unique roles (3,51). Promoter binding analysis has shown that distinct complexes of E2F and pRB family members mediate activation or repression throughout the cell cycle (52).

Based upon its G0 expression pattern and association with pRB, E2F-3B likely fits into this model primarily as a transcriptional repressor but could also contribute in the activation of genes, including E2F-1, -2, and -3A, in late G1. In contrast, E2F-3A likely serves exclusively as a potent transcriptional activator of genes essential for S phase entry during its brief appearance late in G1. Because E2F-3B has a DNA binding domain that is identical to E2F-3A, its major function may be to make sure that specific E2F-3A-responsive genes will not be active in G0 by bringing pRB to the promoter. If E2F-3B is a repressor in G0, inhibition of E2F-3B at G1/S transition will be as important as activation of E2F-3A for G1/S transition. Because both forms are transcriptional activators when bound by pRB (see Fig. 4), they must be destabilized and removed during S phase lest they induce apoptosis. Finally, once S phase is completed and cyclin A levels drop, E2F-3B levels rebound due to its constitutive synthesis (note the 27- and 30-h time points of Fig. 5). This rebounding of E2F-3B activity is likely necessary to serve as a tether for pRB as cells enter the next G1.
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