RESEARCH COMMUNICATION

G1 cyclin-dependent kinases are insufficient to reverse dE2F2-mediated repression

Maxim V. Frolov, Olivier Stevaux, Nam-Sung Moon, Dessislava Dimova, Eun-Jeong Kwon, Erick J. Morris, and Nicholas J. Dyson¹

Massachusetts General Hospital Cancer Center, Charlestown, Massachusetts 02129, USA

Here we show that the cell cycle defects of dE2F1-depleted cells depend on the cooperative effects of dE2F2 and DACAPO (DAP), an inhibitor of Cyclin E/cyclin-dependent kinase 2 (CycE/cdk2). The different properties of cells lacking dE2F1/dE2F2 and dE2F1/DAP lead to the surprising observation that dE2F2-mediated repression differs from retinoblastoma family protein 1 (RBF1) inhibition of dE2F1, and is resistant to both CycE/cdk2 and Cyclin D/cyclin-dependent kinase 4 (CycD/cdk4). This resistance occurs even though dE2F2/RBF1 complexes are disrupted by CycE/cdk2, and may explain why dE2F2 is so potent in the absence of *de2f1*. The implication of these results is that cells containing dE2F2 require dE2F1 to either prevent, or reverse, dE2F-mediated repression.

Received August 12, 2002; revised version accepted January 24, 2003.

The general view of mammalian E2F regulation has been simplified by recent studies suggesting that E2Fs can be broadly classified into two subgroups: a group of E2Fs that are potent transcriptional activators (E2F-1, E2F-2, and E2F-3), and a second group (E2F-4, E2F-5, and E2F-6) that appear to function primarily as repressors (Takahashi et al. 2000; Trimarchi and Lees 2002). This interpretation is supported by chromatin immunoprecipitation (ChIP) experiments showing that E2F-1, E2F-2, and E2F-3 replace E2F-4 or E2F-6 at E2F-regulated promoters as cells progress from G0/G1 toward S phase, a change that correlates with the induction of gene transcription (Takahashi et al. 2000; Ogawa et al. 2002; Rayman et al. 2002; Weinmann et al. 2002). The combined ablation of *E2f-1*, *E2f-2*, and *E2f-3* in primary mouse fibroblasts (MEFs) results in the repression of E2F target genes, reduced S-phase entry, and a block of cell proliferation (Wu et al. 2001).

The *Drosophila* genome contains two E2F genes, *de2f1* and *de2f2*, with properties similar to the two classes of mammalian E2Fs. dE2F1 is a potent activator of transcription. Its expression is tightly regulated and, during larval stages, it is induced at the G1-to-S transition (Asano et al. 1996). Overexpression of dE2F1 drives cells

into S phase and is opposed by retinoblastoma family protein 1 (RBF1), an RB-related protein (Du et al. 1996a). *de2f2* encodes a broadly expressed transcriptional repressor. dE2F2 blocks transcription from E2F-regulated promoters and antagonizes dE2F1-mediated activation (Sawado et al. 1998; Frolov et al. 2001). Mutation of *de2f1* gives phenotypes that are strikingly similar to the properties of *E2f-1*^{-/-}; *E2f-2*^{-/-}; *E2f-3*^{-/-} mutant MEFs. *de2f1* mutants show a dramatic reduction of E2F-dependent transcription and severely compromised cell proliferation (Duronio et al. 1995; Royzman et al. 1997). In contrast, *de2f2* mutant animals are semiviable, and patterns of cell proliferation and differentiation are relatively normal in embryos and larvae (Cayirlioglu et al. 2001; Frolov et al. 2001).

The idea that E2F regulation combines the antagonistic effects of activator and repressor E2Fs is supported by genetic interactions between *de2f1* and *de2f2*. Mutation of *de2f2* suppresses the cell cycle and cell proliferation defects of *de2f1* mutant larvae. In *de2f1*; *de2f2* double-mutant larvae, E2F-inducible genes such as *PCNA* and *Cyclin E* are expressed at surprisingly robust levels, but their patterns of expression are lost, or diminished, indicating that E2F is needed for the proper timing of gene expression. Thus, the early phenotypes of *de2f1* mutant animals depend on dE2F2. ChIP assays show that dE2F1 and dE2F2 are both normally present at E2F-regulated promoters and, as dE2F1 and dE2F2 have opposing effects on transcription, the simplest interpretation is that *de2f1* mutant phenotypes are caused by the unchecked activity of dE2F2 at sites that are normally serviced by dE2F1 (Frolov et al. 2001).

A curious characteristic of *de2f1* mutant embryos and *E2f-1*^{-/-}; *E2f-2*^{-/-}; *E2f-3*^{-/-} MEFs is that transcription of E2F-regulated genes is constitutively repressed in the absence of activator E2Fs. This observation is puzzling if one considers that E2F-regulated genes are expressed in *de2f1*; *de2f2* double mutants and that E2F-mediated repression is thought to be relieved by the ability of G1 cyclin-dependent kinases (cdks) to phosphorylate RB family members and disrupt E2F repressor complexes (for example, see Harbour et al. 1999). G1 cyclins are induced in response to a variety of developmental cues that are largely E2F independent (Sherr and Roberts 1999). The developmentally regulated induction of *Cyclin E* expression happens normally in *Drosophila de2f1* mutant embryos in several cell types, but E2F-regulated genes stay repressed, and cell proliferation remains compromised (Duronio and O'Farrell 1995). Similarly, serum-stimulated *E2f-1*^{-/-}; *E2f-2*^{-/-}; *E2f-3*^{-/-} MEFs have high levels of *Cyclin E* expression, but this fails to relieve the repression of E2F targets or to drive the cell cycle (Wu et al. 2001).

What is the explanation for this paradox? Wu et al. (2001) noted that the expression of p21 is up-regulated in *E2f-1*^{-/-}; *E2f-2*^{-/-}; *E2f-3*^{-/-} MEFs. An increase in cdk inhibitors might provide a buffer against cdk activation that allows repressor complexes to persist. As a result, a level of cyclin synthesis that is sufficient to drive S-phase entry in normal cells might be inadequate in cells lacking the activator E2Fs. Here we tested the model that was proposed by Wu et al. (2001) by inactivating either DAP, the *Drosophila* homolog of p21, or

[Keywords: E2F; G1 cyclins; *Drosophila*]

¹Corresponding author.

E-MAIL dyson@helix.mgh.harvard.edu; FAX (617) 726-7808.

Article and publication are at <http://www.genesdev.org/cgi/doi/10.1101/gad.1031803>.

dE2F2, the repressor E2F, in cells lacking dE2F1. The results of these and subsequent experiments suggest a different explanation for the *de2f1* mutant phenotype.

Results and Discussion

The depletion of dE2F1 by RNAi results in a G1 arrest

To facilitate the biochemical analysis of cells lacking E2F/RBF proteins, we used RNA interference (RNAi) to specifically deplete individual family members from *Drosophila* tissue culture cells. An example is shown in Figure 1A. Following the addition of dE2F2 double-strand RNA (dsRNA), the level of dE2F2 protein fell dramatically and was barely detectable by 4 d of treatment. This change was specific; similar results were observed using another dsRNA fragment of nonoverlapping -dE2F2 sequences. No change was seen when dsRNA fragments from other genes were used. In the same way, dE2F1, RBF1, DAP, or combinations of these proteins could be efficiently and specifically removed by RNAi treatment (Fig. 1B). Proteins were effectively depleted when cells were treated with a single dsRNA or with a mixture of two or three dsRNAs. The depletion of dE2F1 was effective when cells were treated with dE2F1 dsRNA alone or with a mixture of dE2F1 and dE2F2, or dE2F1, dE2F2, and DAP dsRNAs.

dE2F1-depleted cells were collected after 4 d of treatment with dsRNA, counted, and replated into fresh media. The properties of these cells were assessed after three further days of incubation. Western blot analysis confirmed that the depleted proteins did not reappear during this period. To assess effects on cell cycle distri-

Figure 1. Specific depletion of RBF/E2F proteins in *Drosophila* SL2 cells by RNAi. (A) Cells were incubated with dE2F2 dsRNA or non-specific (NS) dsRNA. Extracts prepared immediately or 1–4 d after treatment were analyzed on Western blots. (B) Cells treated with 50 μ g of nonspecific (NS), DACAPO (DAP), dE2F1 (E1), dE2F2 (E2), and RBF1 (R1) dsRNAs, either alone or in combination. The total amount of dsRNA applied to a single well was adjusted to 150 μ g with nonspecific dsRNA. Extracts were analyzed on Western blots using the appropriate antibodies. An antitubulin mouse monoclonal antibody provided a loading control.

Figure 2. dE2F1-deficient cells have cell cycle defects that can be rescued by the depletion of DAP and dE2F2. SL2 cells were treated with the dsRNAs indicated and analyzed. Means and standard error of triplicate samples are shown. (A) Two-dimensional FACS distribution of cells labeled with BrdU for 16 h prior to harvesting. The horizontal axis reflects DNA content, and the vertical axis shows cell numbers. The populations of cells with G1 and G2 DNA content are indicated. BrdU-positive cells are inferred to have been in S phase at some stage during the labeling period. Depletion of dE2F1 blocks BrdU incorporation, causing G1 accumulation. The appearance of BrdU-negative cells with intermediate DNA content in this population may represent cells stalled within S phase, or cells that are progressing very slowly through S phase. The depletion of dE2F2, RBF1, or DAP allows dE2F1-deficient cells to enter S phase but does not rescue the cell cycle phasing defects. The codepletion of both dE2F2 and DAP allowed dE2F1-deficient cells to return to a normal distribution. (B) The number of BrdU-positive cells in the populations analyzed in A. (C) Proliferation indexes (from day 4 to day 7 after RNAi treatment) of the depleted cultures. (D) Levels of DNA synthesis, assayed using [3 H]-thymidine incorporation, and normalized to number of cells per sample. (E) Cyclin A levels in dsRNAs-treated cells. Cyclin A accumulation in dE2F1/DAP- and dE2F1/dE2F2/DAP-depleted cells suggests that they progress further into the cycle than dE2F1/dE2F2-depleted cells.

bution, we labeled depleted cells with 5-Bromo-2'-deoxyuridine (BrdU) and analyzed them by fluorescence-activated cell sorting (FACS; Fig. 2A,B). Changes in total cell number were monitored (Fig. 2C) and the level of DNA synthesis was assessed using [3 H]-thymidine (Fig. 2D). Each assay was performed with triplicate samples. Cell cultures were also processed for Northern and Western blot analyses.

As expected, depletion of dE2F1 from SL2 cells gave a G1-phase cell cycle arrest. FACS analysis revealed a striking accumulation of cells with a G1 DNA content,

and a 20-fold reduction in the number of BrdU-positive cells (Fig. 2A,B). Cultures of dE2F1-deficient cells ceased to incorporate [³H]-thymidine and failed to proliferate (Fig. 2C,D). The expression of *PCNA*, *MCM3*, and *Cyclin E* (*CycE*), three E2F-regulated genes, was strongly reduced in the absence of dE2F1 (Fig. 3A).

Cell cycle arrest of dE2F1-depleted cells is partly relieved by the removal of dE2F2

Because mutation of *de2f2* extends the development of *de2f1* mutant animals, we examined the effects of depleting both dE2F1 and dE2F2. Removing dE2F2 alone had no effect on cell cycle distribution but partially suppressed the effects of dE2F1 loss. Depletion of dE2F2 allowed dE2F1-deficient cells to incorporate BrdU, giving 40% of the number of BrdU-positive cells seen with control cells (Fig. 2A,B). In keeping with this, the level of *PCNA* and *MCM3* mRNAs was higher in SL2 cells depleted for both dE2F1 and dE2F2 than in cells lacking dE2F1 alone (Fig. 3A). Codepleting RBF1 gave a similar, but smaller, effect. The difference between RBF1 and dE2F2 in these experiments is due to the presence of RBF2, an alternative partner for dE2F2 (data not shown).

Although the loss of dE2F2 enabled some dE2F1-deficient cells to enter S phase, these cells failed to proliferate (Fig. 2C). No increase in apoptosis was evident, suggesting that this failure is unlikely to be due to cell death. The proportion of G2-phase cells remained low, indicating that few of the BrdU-positive cells successfully complete S phase (Fig. 2A). In the control population, the majority of BrdU-positive cells gained a 4N DNA content during the labeling period, but far fewer dE2F1/dE2F2- or dE2F1/RBF1-depleted cells reached 4N DNA content. [³H] thymidine incorporation assays showed that the dramatic fall in the level of DNA synthesis seen when dE2F1 is removed was not reversed by the codepletion of either dE2F2 or RBF1 (Fig. 2D). Thus,

cells depleted of dE2F1 and dE2F2 are able to enter S phase but appear to have a defect in S-phase progression.

Removing DAP allows dE2F1-deficient cells to enter S phase, rescues the S-phase defects of cells depleted of dE2F1 and dE2F2, and extends the development of *de2f2*; *de2f1* double-mutant animals

No increase in DAP levels was detected in dE2F1-depleted cells (Fig. 1B). Nevertheless, as DAP is the only p21^{CIP1} homolog found in the *Drosophila* genome, we used RNAi to test the idea that this type of inhibitor plays an important role in dE2F1-deficient cells. Depletion of DAP had a minimal effect on the cell cycle distribution of SL2 cells but suppressed the G1 arrest in dE2F1-deficient cells. The number of BrdU-positive cells seen when DAP and dE2F1 were codepleted was higher than that seen when dE2F2 and dE2F1 were removed (Fig. 2A,B). dE2F1/DAP-depleted cells had reduced rates of DNA synthesis (Fig. 2D) but expressed cyclin A, suggesting that they progress further into the cell cycle than do dE2F1/dE2F2-depleted cells (Fig. 2E). Like dE2F1/dE2F2-depleted cells, dE2F1/DAP-depleted cells failed to proliferate (Fig. 2C).

The codepletion of DAP and dE2F2 substantially rescued the level of DNA synthesis in cells lacking dE2F1 (Fig. 2D). Strikingly, the FACS distribution of triple-depleted cells was similar to wild-type cells (Fig. 2A) and these cultures increased in cell number, albeit at a rate that was approximately half that of control cells (Fig. 2C). Thus, dE2F2 and DAP both contribute to the cell cycle arrest seen when dE2F1 is removed, and they act cooperatively. This agrees with the results of overexpression experiments showing that coexpression of E2F proteins enhances the *CycE*/cdk2-mediated induction of S phase in mammalian cells (Leone et al. 1999).

To understand why DAP and dE2F2 act cooperatively, we examined the changes that occur in the RNAi-treated cells. In dE2F1-depleted cells, cyclin E-associated kinase activity is dramatically reduced. Codepletion of DAP removes the physiological inhibitor of this kinase, and restores the kinase activity to wild-type levels (Fig. 3B). However, Northern blot analysis shows that E2F-regulated genes remain repressed when dE2F1 and DAP are removed; the expression of *PCNA* and *MCM3* was identical in cells depleted for dE2F1 and in cells depleted for both dE2F1 and DAP (Fig. 3A). Conversely, removing dE2F2 had only a minimal effect on the low level of cyclin E-associated kinase activity seen in dE2F1-depleted cells, but the levels of *PCNA* and *MCM3* expression were higher in dE2F1/dE2F2-depleted cells than in cells lacking dE2F1 alone. Thus, DAP or dE2F2 depletion appears to rescue S-phase entry in dE2F1-deficient cells in different ways; removing DAP restores cyclin E-associated kinase, whereas removing dE2F2 alleviates the repression of E2F targets. The synergism seen when both DAP and dE2F2 were depleted presumably reflects the need for both high levels of cyclin E-associated kinase and expression of E2F-regulated genes for efficient S-phase progression.

The cooperation between DAP and dE2F2 was readily apparent because dE2F2 RNAi gave a less effective rescue of dE2F1-depleted SL2 cells than the rescue evident in *de2f1*; *de2f2* double mutants. This is likely because the role of dE2F1 in *CycE* expression varies between cell

Figure 3. Effects of DAP depletion and dE2F2 depletion on E2F-dependent transcription and cyclin E-associated kinase activity in dE2F1-deficient cells. (A) Total RNA was isolated from cells treated as described in Figure 2 and analyzed on Northern blots with probes for *Cyclin E* (*CycE*), *MCM3*, and *PCNA*. β -tubulin was used as a loading control. The signals were measured and normalized to the loading control; the quantitation is shown above each lane. (B) *CycE*/cdk2 activity in dE2F1-deficient cells depleted of DAP and dE2F2. Cell lysates were normalized by protein content and associated *CycE*/cdk2 activity was immunoprecipitated using antibody specific for cyclin E. The kinase reactions were carried out for 20 min using histone H1A as a substrate. (C) Mutation of *dap* extends the development of *de2f1*; *de2f2* double mutants. The percentage of progeny that develop to early and late pupal stages are shown.

types (Duronio and O'Farrell 1995); in SL2 cells, expression of *CycE* was strongly dependent on dE2F1, whereas Northern blots of dissected imaginal discs revealed that the overall levels of *CycE* transcripts were relatively normal in *de2f1*; *de2f2* mutants (Frolov et al. 2001).

To test whether mutant alleles of *dap* can rescue the developmental defects of *de2f1* mutants, we generated *dap*; *de2f1* double-mutant animals. Two *de2f1* alleles (*de2f1*⁹¹ and *de2f1*^{rm729}) were recombined with three *dap* alleles (*dap*^{2x10}, *dap*⁴⁴⁵⁴, and *dap*²; de Nooij et al. 1996; Lane et al. 1996) in all possible permutations and assayed as heteroallelic combinations at both loci. *dap*; *de2f1* double mutants were delayed in larval growth, giving small sluggish larvae that were almost undistinguishable from *de2f1* mutants. Experiments with dominantly active forms of pRB have shown that cells are unable to proliferate normally when the E2F transcriptional program is permanently blocked, even when the resulting G1/S arrest is bypassed by the overexpression of cyclin E (Lukas et al. 1997; Knudsen et al. 1998). The failure of *dap* alleles to rescue the *de2f1* mutant developmental defects supports the idea that release of E2F repression is needed for sustained cell proliferation, and underscores the significance of the rescue provided by *de2f2* alleles. When mutant alleles of *dap*, *de2f2*, and *de2f1* were combined, significantly more triple mutant animals developed to late pupal stages than did *de2f2*; *de2f1* double mutants (Fig. 3C), but no viable adults were recovered. Thus, mutation of *dap* extends the development of *de2f2*; *de2f1* mutant animals but is insufficient to suppress the requirement for E2F function.

dE2F2-mediated repression is resistant to CycE/cdk2 and CycD/cdk4

The cooperation between DAP and dE2F2, and the observation that E2F targets are repressed in dE2F1-deficient cells that contain CycE/cdk2 activity, raises the possibility that dE2F2-mediated repression may be resistant to some cdks.

To investigate the mechanism of E2F-mediated repression, we first asked whether the two RB family members that interact with dE2F2 (Stevaux et al. 2002) are required for this activity. SL2 cells were depleted of RBF1 or RBF2 or both RBF1 and RBF2 by RNAi, and then transfected with a plasmid containing the promoter region of the *PCNA* gene fused to a luciferase reporter (Sawado et al. 1998) and a dE2F2 expression construct (Fig. 4A,B). In control-treated cells, dE2F2 repressed the *PCNA* promoter up to fivefold. This effect was unaffected by the depletion of RBF2 but was compromised by the removal of RBF1. Strikingly, dE2F2 was almost completely unable to repress the reporter in the absence of both RBF1 and RBF2. These results show that dE2F2-mediated repression is dependent on pocket proteins and suggest that, although there is partial redundancy between the two RBs, RBF1 is the more important component.

The ability of CycE/cdk2 to relieve transcriptional repression by E2F/RBF complexes was tested directly in transient transfection assays. As expected, dE2F1 activated transcription from the *PCNA* promoter. This effect was inhibited by coexpression of RBF1. Cotransfection of CycE/cdk2 relieved the effect of RBF1, restoring dE2F1-dependent activation (Fig. 4C).

Next, we cotransfected the same amount of RBF1-expression plasmid with a constant amount of dE2F2 and

Figure 4. dE2F2-mediated repression requires pocket proteins and is resistant to G1 cdks. E2F-dependent transcription was assayed in SL2 cells using a transfected *PCNA*-luciferase reporter. pE3-lacZ was used to normalize for transfection efficiency. The mean and standard deviation of three independent experiments are shown. (A) The depletion of RBF1 increases transcription from the *PCNA* promoter. Cells were treated with RBF1, RBF2, or control (white) dsRNA and transfected with the reporter construct 4 d later. (B) dE2F2-mediated repression is impaired by the depletion of pocket proteins. RNAi-treated cells were transfected as described in A with *PCNA*-luciferase reporter and 100 or 300 ng of dE2F2 expression construct (pIE4-dE2F2). (C) CycE/cdk2 relieves RBF1-mediated repression of dE2F1. One-hundred nanograms of pIE4-dE2F1 was cotransfected with pIE4-RBF1 and 0.25 or 1 μ g of each CycE and cdk2 expression construct. (D) dE2F2-mediated repression is resistant to CycE/cdk2. One-hundred nanograms of pIE4-dE2F2 was transfected with pIE4-RBF1 and with 0.25 or 1 μ g of each CycE and cdk2 expression constructs. (E) A titration of dE2F2. Quantities of CycE/cdk2 (0.25, 1, and 2 μ g of each construct) that inactivate RBF1 have no effect on dE2F2-mediated repression. (F) CycD/cdk4 (150 ng of each) relieves RBF1 inhibition of dE2F1 but has no effect on dE2F2-mediated repression.

asked whether CycE/cdk2 could relieve repression by E2F2/RBF1. Expression of dE2F2 repressed the reporter and coexpression of RBF1 and dE2F2 increased this effect (Fig. 4D). Coexpression of CycE/cdk2 had no effect on repression by dE2F2/RBF1, even though the same amount of CycE/cdk2 readily reversed the effect of RBF1 on dE2F1. To assess this further, we assayed CycE/cdk2 expression plasmids with progressively decreasing quantities of dE2F2 (Fig. 4E). The repressive effect of dE2F2 was unaffected by CycE/cdk2, even when dE2F2 gave only a twofold repression.

To test whether dE2F2-mediated repression is resistant to other G1 cdks, we performed similar experiments using CycD/cdk4 expression constructs. CycD/cdk4 reversed the ability of RBF1 to repress activation by dE2F1, but the same amount of CycD/cdk4 had no effect on the repression seen when dE2F2 was transfected alone or cotransfected with RBF1 (Fig. 4F).

These experiments demonstrate that dE2F2-mediated repression differs from RBF1-mediated inhibition of dE2F1 and is resistant to both *Drosophila* G1 cdks, CycE/cdk2 and CycD/cdk4. This effect occurs even though dE2F2/RBF1 complexes can be disrupted by cdks. Coexpression of dE2F2 and RBF1 in SL2 generated an E2F

complex that can be detected in E2F electrophoretic mobility shift assay (EMSA) and comigrated with endogenous dE2F2/dDP/RBF complexes. This complex was disrupted by cotransfection with CycE/cdk2 (Fig. 5A). To exclude the possibility that this was an artifact of non-physiologic levels of kinase, or non-DNA-bound complexes, we examined the properties of the endogenous proteins. ChIP assays were performed using antibodies specific for dE2F2 and RBF1 on control-treated SL2 cells, and SL2 cells depleted of DAP and dE2F1. dE2F2 and RBF1 were both detected at the *PCNA* and *DNA pol α* promoters in control SL2 cells and in asynchronous populations of DAP/dE2F1-depleted cells. However, in cells synchronized in S phase by hydroxyurea treatment, RBF1 was no longer detected at these promoters by ChIP (Fig. 5B) or coimmunoprecipitated with dE2F2 antibodies (Fig. 5C). This suggests that RBF1/dE2F2 complexes, and dE2F2 recruitment of RBF1 to the *PCNA* and *DNA pol α* promoters, are disrupted during S phase.

The finding that dE2F2-mediated repression is resistant to G1 cdk's suggests a simple and surprising explanation for the strong *de2f1* mutant phenotype: in *de2f1* mutant animals, E2F-regulated promoters are repressed via a dE2F2-dependent mechanism and this effect is potent because it cannot be relieved simply by the activation of G1 cdk's. As a result, E2F targets remain repressed even when cyclin E expression is generated by developmental signals, or from an inducible transgene (Duronio

and O'Farrell 1995). The implication of these results is that cells containing dE2F2 require dE2F1 to either prevent, or reverse, dE2F2-mediated repression.

In normal cells, the switch from repression to activation occurs when both dE2F1 and dE2F2 are present. We therefore asked how CycE/cdk2 affects the functional antagonism between dE2F1 and dE2F2. RBF2 was used for these experiments because it synergizes with dE2F2 to repress transcription but does not interact directly with dE2F1. As a result, the cotransfection of dE2F1, dE2F2, and RBF2 gives an intermediate level of transcription in which dE2F1-mediated activation is reduced by dE2F2/RBF2-mediated repression (Stevaux et al. 2002). Addition of CycE/cdk2 to this mix changes the balance in favor of activation (Fig. 5D), suggesting that G1 cdk's may normally serve to enable dE2F1 to override dE2F2-mediated repression.

Why is dE2F1 needed to relieve dE2F2-mediated repression? One simple possibility might be that the repression seen in dE2F1-deficient cells is caused by the inappropriate binding of dE2F2 to dE2F1-specific promoters. The difficulty with this model is that, at present, there is no evidence that such dE2F1-specific targets exist. ChIP assays show that dE2F1 and dE2F2 are both normally present at the well-studied E2F regulated promoters, including the *PCNA* promoter that was used for the transient transfection experiments (Frolov et al. 2001). Thus, the failure of G1 cdk's to reverse repression by dE2F2/RBF1 on the *PCNA* reporter is not because it is a dE2F1-specific target.

An alternative possibility, and one that we favor, is the idea that reversal of E2F-mediated repression is a two-step process requiring not only the disruption of the repressor complex by cdk phosphorylation, but also the action of activator E2Fs. In support of this model, we note that E2F-regulated promoters like B-myb that had previously been proposed to be regulated by repression and the release of repressor complexes (Zwicker et al. 1996) have more recently been shown to be transiently occupied by activator E2Fs during G1/S progression (Takahashi et al. 2000). We suggest that this binding is needed to reverse the effect of the repressor complexes and that, in essence, the action of the repressor E2F complex generates a requirement for activator E2Fs. Such an interpretation is consistent with studies in mammalian cells that show a tight correlation between E2F regulation and changes in histone modification (Brehm and Kouzarides 1999). E2F-induced changes in histone acetylation may be one example of this interplay between activator and repressor E2Fs. pRB family members interact with several different types of repressor complexes, and the effects of each may need to be reversed by activator E2Fs. Further studies are needed to delineate the mechanisms of dE2F2-mediated repression and to understand the ways that these mechanisms are reversed by dE2F1.

Materials and methods

The following mutant alleles were used in this study: *de2f1^{tm729}* and *de2f1⁹¹* (Duronio et al. 1995), *dap⁴⁴⁵⁴*, *dap²*, and *dap^{2x10}* (de Nooij et al. 1996; Lane et al. 1996); and *de2f2^{76Q1}* and *de2f2^{G5.1}* (Frolov et al. 2001).

RNAi, transient transfections in *Drosophila* SL2 cells, Northern and Western blot analyses, and ChIP were performed as previously described (Frolov et al. 2001; Stevaux et al. 2002). The following antibodies were used: mouse monoclonal anti-RBF1, anti-dDP (Du et al. 1996b), anti-DAP (de Nooij et al. 2000), and anti-cyclin A (Lehner and O'Farrell 1989),

Figure 5. Disruption of exogenous and endogenous dE2F2/RBF1 complexes. (A) E2F EMSA assays showing the E2F complexes present in SL2 extracts. Transfection of pIE4-dE2F2 and pIE4-RBF1 generates a dE2F2/RBF1 complex that is disrupted by the coexpression of CycE/cdk2. (B) ChIP assays showing the enrichment of E2F-regulated promoters (*PCNA* and *DNA pol α*) over a negative control (*rp49*) in dE2F2 or RBF immunoprecipitates that were prepared from extracts of control-treated cells, asynchronous dE2F1/DAP-depleted cells, or hydroxyurea-treated dE2F1/DAP-depleted cells. RBF1 is no longer detected at these promoters when cells are arrested in S phase with hydroxyurea. (C) Immunoprecipitation (IP)/Western analysis of the cells treated in B showing that RBF1 is present in the whole cell extracts (WCX) but no longer coprecipitates with dE2F2 in hydroxyurea-treated dE2F1- and DAP-depleted cells. RaM is a negative control polyclonal antibody. (D) Expression of CycE/cdk2 increases transcriptional activation by dE2F1 (50 ng pIE4-dE2F1) on the *PCNA* reporter in the presence of dE2F2 (250 ng pIE4-dE2F2) and RBF2 (4 μ g pIE4-RBF2). Transfections were as described in Figure 4.

guinea pig anti-dE2F1 and anti-cyclin E (generous gift by Drs. T. Orr-Weaver and G. Bosco), rabbit polyclonal anti-dE2F2 (Frolov et al. 2001), and anti-dE2F1 raised against full-length GST-dE2F1 protein.

Flow cytometry analysis was performed on a Becton Dickinson FACScan, collecting data from 20,000 cells per sample. For in vitro kinase assay, cells were lysed and treated as described (Lane et al. 1996; Stevaux et al. 2002). EMSA was performed with 5 µg of total extract from SL2 cells. ³²P-labeled double-stranded oligonucleotides containing E2F site (~10 pg) were added to extracts and incubated for 30 min at 4°C. Samples were loaded on a 4% polyacrylamide gel and visualized by autoradiography.

Acknowledgments

We thank Drs. Fred Dick and Marie Classon for help with FACS analysis, Dr. Terry Orr-Weaver for generously providing the dE2F1 and cyclin E antibodies, Lori Pile and David Wasserman for sharing the RNAi protocol, and Wei Du for CycE/cdk2 and CycD/cdk4 constructs. This work was supported by NIH postdoctoral fellowships F32 CA93045 to D.D. and F32 CA88474 to E.J.M., Rose and Jean Hogue foundation and BAEF fellowships to O.S., a Canadian CIHR fellowship 210853 to N.S.M., a Tosteson Postdoctoral Fellowship from MBRC to M.V.F., and by NIH grant GM53203 to N.D. M.V.F. is a Leukemia & Lymphoma Society Special Fellow.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

- Asano, M., Nevins, J.R., and Wharton, R.P. 1996. Ectopic E2F expression induces S phase and apoptosis in *Drosophila* imaginal discs. *Genes & Dev.* **10**: 1422–1432.
- Brehm, A. and Kouzarides, T. 1999. Retinoblastoma protein meets chromatin. *Trends Biochem. Sci.* **24**: 142–145.
- Cayirlioglu, P., Bonnette, P.C., Dickson, M.R., and Duronio, R.J. 2001. *Drosophila* E2f2 promotes the conversion from genomic DNA replication to gene amplification in ovarian follicle cells. *Development* **128**: 5085–5098.
- de Noij, J.C., Letendre, M.A., and Hariharan, I.K. 1996. A cyclin-dependent kinase inhibitor, Dacapo, is necessary for timely exit from the cell cycle during *Drosophila* embryogenesis. *Cell* **87**: 1237–1247.
- de Noij, J.C., Graber, K.H., and Hariharan, I.K. 2000. Expression of the cyclin-dependent kinase inhibitor Dacapo is regulated by cyclin E. *Mech. Dev.* **97**: 73–83.
- Du, W., Vidal, M., Xie, J.-E., and Dyson, N. 1996a. RBF, a novel RB-related gene that regulates E2F activity and interacts with cyclin E in *Drosophila*. *Genes & Dev.* **10**: 1206–1218.
- Du, W., Xie, J.-E., and Dyson, N. 1996b. Ectopic expression of dE2F and dDP induces cell proliferation and death in the *Drosophila* eye. *EMBO J.* **15**: 3684–3692.
- Duronio, R.J. and O'Farrell, P.H. 1995. Developmental control of the G1 to S transition in *Drosophila*; cyclin E is a limiting downstream target of E2F. *Genes & Dev.* **9**: 1456–1468.
- Duronio, R.J., O'Farrell, P.H., Xie, J.-E., Brook, A., and Dyson, N. 1995. The transcription factor E2F is required for S phase during *Drosophila* embryogenesis. *Genes & Dev.* **9**: 1445–1455.
- Frolov, M.V., Huen, D.S., Stevaux, O., Dimova, D., Balczarek-Strang, K., Elsdon, M., and Dyson, N.J. 2001. Functional antagonism between E2F family members. *Genes & Dev.* **15**: 2146–2160.
- Harbour, J., Luo, R., Dei Santi, A., Postigo, A., and Dean, D. 1999. Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. *Cell* **98**: 859–869.
- Knudsen, E.S., Buckmaster, C., Chen, T.-T., Feramisco, J.R., and Wang, J.Y.J. 1998. Inhibition of DNA synthesis by RB: Effects on G1/S transition and S-phase progression. *Genes & Dev.* **12**: 2278–2292.
- Lane, M.E., Sauer, K., Wallace, K., Jan, Y.N., Lehner, C.F., and Vaessin, H. 1996. Dacapo, a cyclin-dependent kinase inhibitor, stops cell proliferation during *Drosophila* development. *Cell* **87**: 1225–1235.
- Lehner, C.F. and O'Farrell, P.H. 1989. Expression and function of *Drosophila* cyclin A during embryonic cell cycle progression. *Cell* **56**: 957–968.
- Leone, G.J., DeGregori, J., Jakoi, L., Cook, J.G., and Nevins, J.R. 1999. Collaborative role of E2F transcriptional activity and G1 cyclin-dependent kinase activity in the induction of S phase. *Proc. Natl. Acad. Sci.* **96**: 6626–6631.
- Lukas, J., Herzinger, T., Hansen, K., Moroni, M.C., Resnitzky, D., Helin, K., Reed, S.I., and Bartek, J. 1997. Cyclin E-induced S phase without activation of the pRb/E2F pathway. *Genes & Dev.* **11**: 1479–1492.
- Ogawa, H., Ishiguro, K.-i., Gaubatz, S., Livigston, D.M., and Nakatani, Y. 2002. A complex with chromatin modifiers that occupies E2F- and Myc-responsive genes in G0 cells. *Science* **296**: 1132–1136.
- Rayman, J.B., Takahashi, Y., Indjeian, V.B., Dannenberg, J.-H., Cathpole, S., Watson, R.G., Riele, H., and Dynlacht, B.D. 2002. E2F mediates cell cycle-dependent transcriptional repression in vivo by recruitment of an HDAC1/mSin3B corepressor complex. *Genes & Dev.* **16**: 933–947.
- Royzman, L., Whittaker, A.J., and Orr-Weaver, T.L. 1997. Mutations in *Drosophila* DP and E2F distinguish G1-S progression from an associated transcriptional program. *Genes & Dev.* **11**: 1999–2011.
- Sawado, T., Yamaguchi, M., Nishimoto, Y., Ohno, K., Sakaguchi, K., and Matsukage, A. 1998. dE2F2, a novel E2F-family transcription factor in *Drosophila melanogaster*. *Biochem. Biophys. Res. Commun.* **251**: 409–415.
- Sherr, C. and Roberts, J. 1999. CDK inhibitors: Positive and negative regulators of G1-phase progression. *Genes & Dev.* **13**: 1501–1512.
- Stevaux, O., Dimova, D., Frolov, M.V., Taylor-Harding, B., Morris, E., and Dyson, N.J. 2002. Distinct mechanisms of E2F regulation by *Drosophila* RBF1 and RBF2. *EMBO J.* **21**: 4927–4937.
- Takahashi, Y., Rayman, J., and Dynlacht, B. 2000. Analysis of promoter binding by the E2F and pRB families in vivo: Distinct E2F proteins mediate activation and repression. *Genes & Dev.* **14**: 804–816.
- Trimarchi, J.M. and Lees, J.A. 2002. Sibling rivalry in the E2F family. *Nat. Rev. Mol. Cell Biol.* **3**: 11–20.
- Weinmann, A.S., Yan, P.S., Oberley, M.J., Huang, T.H., and Farnham, P.J. 2002. Isolating human transcriptional factor targets by coupling chromatin immunoprecipitation and CpG island microarray analysis. *Genes & Dev.* **15**: 235–244.
- Wu, L., Timmers, C., Maiti, B., Saavedra, H.I., Sang, L., Chong, G.T., Nuckolls, F., Giangrande, P., Wright, F.A., Field, S.J., et al. 2001. The E2F1-3 transcription factors are essential for cellular proliferation. *Nature* **414**: 457–462.
- Zwicker, J., Liu, N., Engeland, K., Lucibello, F.C., and Muller, R. 1996. Cell cycle regulation of E2F site occupation in vivo. *Science* **271**: 1595–1597.

G1 cyclin-dependent kinases are insufficient to reverse dE2F2-mediated repression

Maxim V. Frolov, Olivier Stevaux, Nam-Sung Moon, et al.

Genes Dev. 2003, **17**:

Access the most recent version at doi:[10.1101/gad.1031803](https://doi.org/10.1101/gad.1031803)

References

This article cites 25 articles, 15 of which can be accessed free at:
<http://genesdev.cshlp.org/content/17/6/723.full.html#ref-list-1>

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).