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A Dual Role for the dREAM/MMB Complex in the Regulation of Differentiation-Specific E2F/RB Target Genes

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E2F and RB proteins regulate the expression of genes involved in cell cycle progression, apoptosis, differentiation, and development. Recent studies indicate that they function as part of an evolutionarily conserved multiprotein complex termed dREAM/DREAM/LINC. Here we characterize the role of the *Drosophila* complex, dREAM, in the regulation of differentiation-specific E2F target genes in actively proliferating cells. These genes are regulated differently from cell cycle-related E2F targets, they do not depend on E2F activation, and E2F/RB repression is maintained throughout the cell cycle. In proliferating cells, their repression is dependent on dREAM. We find that dREAM plays a dual role in their regulation. First, it is required for the stability of the repressive dE2F2/RBF complexes at their promoters during S phase. Second, we find that dREAM is indispensable for both transcriptional repression mechanisms employed at these genes.

Both cell division and exit from active proliferation cycles are integral parts of the development of multicellular organisms and are orchestrated by transcription factors that regulate spatiotemporal expression of specific sets of target genes. The E2F and RB families of transcription factors are critical regulators of these processes, and E2F/RB activity is altered in many human tumors (5, 7, 9, 31, 40).

The E2F/RB pathway was first studied in the context of G_1/S control (6, 9, 37). E2F proteins can be subdivided into repressors and activators of transcription. RB family members, also known as pocket proteins, negatively regulate E2F activity in two ways. Binding to activator E2Fs inhibits E2F transactivation. When bound to repressor E2Fs, they recruit transcriptional corepressors such as histone deacetylases (HDACs) or histone methyltransferases (HMTs) to inhibit expression of genes required for cell cycle progression (11). Cyclin-dependent kinases (CDKs) phosphorylate pocket proteins during cell cycle progression, thereby disrupting E2F-RB interactions. This results in both the relief of E2F/RB-mediated repression and the release of transcriptionally active E2F that triggers entry into S phase.

It is now clear that E2F/RB functions extend beyond controlling S-phase entry. Biological activities for the pathway are inferred from both functional studies in mammals and other model organisms and through the identification of a vast network of target genes (2, 9, 19, 34, 39), including the control of the G_1/S and G_2/M transitions of the cell cycle, checkpoint control, DNA repair and recombination, apoptosis, differentiation, and development. How are all these diverse activities regulated? One answer may lie in the composite nature of the E2F/RB network; there are eight E2F factors in mammals, five of which can associate with three different pocket proteins. Individual E2F/RB complexes could perform distinct tasks and regulate different sets of genes in response to various signals. This idea is supported by several lines of evidence (9) and references therein.

Another answer might be provided by the recent discovery of native E2F-RB-containing complexes from several different organisms (14, 17, 20, 22, 24). These complexes, called dREAM (*Drosophila* RBF, dE2F2, and dMyb-interacting proteins) or MMB (Myb-Muv B) in flies, DREAM or LINC in humans, and DRM in worms, contain a repressive E2F, pocket proteins, the Myb transcription factor, three Myb-interacting proteins (Mip40, Mip120, and Mip130), and p55CAF1 (RbAp46/48). Interestingly, all of the components of the complex except Myb are related to the *Caenorhabditis elegans* synMuv class B genes that negatively regulate vulva development. The human complexes appear to be comprised of either Myb or E2F4, but not both, and are referred to as MMB (Myb) or DREAM (E2F4) (23, 38). The switch between Myb and E2F4 is a regulated event (23). While dMyb and dE2F2 were initially purified as part of the same complex in flies, they do regulate different sets of genes and behave in a mutually exclusive manner to position the dREAM complex at different promoters (15).

Accumulating evidence supports the idea that the dREAM/MMB complexes may have different compositions to perform distinct functions in different cellular contexts. The complex is required to repress differentiation-specific but not cell cycle-regulated E2F target genes in proliferating cells (15, 20, 22, 36; B. Taylor-Harding, D. K. Dimova, and N. J. Dyson, unpublished data); in quiescent cells, it represses cell cycle-specific targets, it is required for the activation of G_2/M-specific genes (probably the MMB-Myb-containing complex) (18, 26, 28, 30), and it regulates site-specific DNA replication in *Drosophila* follicle cells (1). Additionally, genome-wide studies have implicated dREAM/DRM in the regulation of a wide range of genes and complexes of different compositions that have been found at a large number of sites throughout the genome (15, 24, 35).

We have taken advantage of the relative simplicity of the *Drosophila* system to study gene regulation by E2F/RB and dREAM. Specifically, we have examined the regulation of a set of E2F tar-

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gets (group D/E genes) that have functions in differentiation and development and exhibit gender- and tissue type-specific expression patterns. These genes are repressed by dE2F2/RBF in proliferating cells, and the repression is dependent on dREAM (15, 20, 22, 36; Taylor-Harding et al., unpublished). Transcriptional regulation of these genes differs from that of classic, cell cycle E2F target genes in several ways. We have explored whether the dREAM complex is responsible for the distinct type of regulation at these genes. We find that dREAM is required to maintain two distinct types of repression mechanisms and to stabilize dE2F2/RBF complexes in S phase.

MATERIALS AND METHODS

Cell culture. *Drosophila melanogaster* SL2 cells were cultured at 24.5°C in Schneider's insect medium (Invitrogen/GIBCO) supplemented with 10% fetal bovine serum (FBS) (HyClone). RNA interference (RNAi) treatment was performed as previously described (32). Cell cycle synchronization was performed using a double hydroxyurea (HU)-aphidicolin (both manufactured by Sigma) block and release as previously described (10). For cell cycle synchronization of RBF1-expressing cell lines, RBF1 wild-type (WT) or R1A expression was induced for 24 h during the arrest and prior to harvesting the cells. To determine the number of cells in S phase, cell proliferation labeling reagent (bromodeoxyuridine [BrdU]; Amersham) was added to the media for 6 h. Cells were fixed and stained with anti-BrdU antibody (Becton Dickinson) and a fluorescein-labeled anti-mouse IgG secondary antibody (Vector Laboratories). Fluorescence-activated cell sorter (FACS) analysis was performed with the Beckman Coulter FC500 analyzer, and data were analyzed with CXP analysis software (Beckman Coulter).

RBF1 mutant cell lines. Previous work with RBF1 was done with a protein of 797 amino acids (aa), yet genomic sequencing predicts an additional 48 aa at the C terminus. To generate a clone containing the complete coding region, we obtained pOT2-RBF1 (cDNA clone LD45859) from Open Biosystems. A SacI/SpeI fragment from pOT2-RBF1 was ligated to a SacI/SpeI-digested pMK33-RBF1 (36). The resulting plasmid contained the full coding sequence of RBF1 tagged with the FLAG peptide at the N terminus under the control of the inducible metallothionein promoter and was used to generate stable cell lines expressing the wild-type RBF1 protein (R1WT). To generate mutations, full-length RBF1, a BamHI/SpeI fragment of pMK33-RBF1, was subcloned into pBluescript II SK+ (Stratagene). pBSK-RBF1 was mutagenized using the QuikChange multisite kit (Stratagene) in a two-step process. All mutations were confirmed by sequencing. Mutated RBF1 was reinserted into pMK33 as a BamHI/SpeI fragment to generate pMK33-RBF1A (phosphomutant) or pMK33-RBF1E (phosphomimic). Stable cell lines expressing either FLAG-tagged RBF1 wild-type (R1WT) or RBF1 mutant (R1A or R1E) proteins under the inducible metallothionein promoter were generated using Effectene (Qiagen). Cells were transfected by following the manufacturer's instructions, and stable transfectants were selected for 3 to 4 weeks in media containing 0.2 mg/ml hygromycin B (Roche). RBF1-expressing cell lines were incubated with copper sulfate (200 μM) for 24 h to induce expression.

Western blotting and immunoprecipitation. Western blotting was performed using standard techniques, and the following antibodies were used: dE2F2 (polyclonal rabbit), dDP (monoclonal mix Yun1-6), p55CAF1 (Abcam ab1766), Mip130/TWT (Abcam ab12131), and β-tubulin (monoclonal E7). For immunoprecipitation assays, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer and immunoprecipitated with either anti-p55/CAF1 (Abcam ab536161 and ab1766), dE2F2 (rabbit polyclonal), or nonspecific (anti-β-tubulin) antibodies. For visualization of phospho-RBF forms, whole-cell extracts were separated using homemade Novex NuPAGE (Invitrogen) gels (bis-Tris SDS-PAGE) and transferred using the NuPAGE transfer buffer system. Western blotting was performed using anti-RBF1 antibodies (monoclonal DX3 and DX2). Protein levels were quantified using Kodak molecular imaging software (Kodak) and internal standards.

ChIP assays. Chromatin immunoprecipitation (ChIP) was performed as previously described (10, 12), with the following modifications. Chromatin was sheared using the Bioruptor (Diagenode) to an average size of 300 bp, and immunoprecipitated DNA was analyzed by quantitative real-time PCR (LightCycler 1.5; Roche) using the standard curve method. Results are represented as the ratio of a specific sequence over a nonspecific (RP49 promoter or bxdPRE) sequence. Each immunoprecipitation was performed at least three times, and the standard deviation was calculated. Antibodies used for ChIP were as follows: anti-dE2F2 (rabbit polyclonal), anti-dE2F1 (rabbit polyclonal), anti-FLAG (Rockland, Sigma), anti-RBF1 (mouse monoclonal antibodies DX2 and DX5 and rabbit polyclonal gift from D. Arnosti), anti-H3K27me2 (Upstate 07-452), anti-acetyl H3 (Upstate 06-599), anti-H3K27ac (Abcam ab4729-25), anti-acetyl H4 (Upstate 06-866). Primers were designed to amplify between 100 and 150 bp of sequence; primer sequences are available upon request.

RNA isolation, RT-PCR, and Northern blotting. Total RNA was isolated using TRIzol (Invitrogen) reagent. Northern blotting using riboprobes was performed as previously described (10). A two-step reverse transcription-PCR (RT-PCR) using 0.3 μg of total RNA was performed as follows. cDNA was prepared by reverse transcription with random primers using the High Capacity cDNA kit (Applied Biosystems) by following the manufacturer's instructions. Analysis of cDNA was performed with PCR using GoTaq (Promega). Primer sets used were RP49 forward (5′-TCCAAGAAGCGCAAGGAG-3′) and reverse (5′-ATTCCGACCACGTTACAAGAA-3′) and MIP40 forward (5′ACAGCTGGATTCTTGGGTTG-3′) and reverse (5′-CCTACGTAATGCGCCTGTTT3′).

RESULTS

The dREAM complex is required for dE2F2 binding at differentiation-specific but not cell cycle-regulated E2F/RB target gene promoters. In actively proliferating cells the dREAM/MMB complex is required for the repression of differentiation-specific (group D/E) but not cell cycle E2F/RB target genes (15, 20, 22, 36; Taylor-Harding et al., unpublished). Several features set these two groups of target genes apart (10). Group D/E genes remain repressed during S phase. In contrast, dE2F2/RBF-mediated repression of cell cycle target genes is relieved during S phase and they are transcriptionally activated by dE2F1. Accordingly, dE2F2/RBF complexes are displaced from the cell cycle but not from group D/E gene promoters during S phase. Group D/E genes are not regulated by the activator dE2F1, and dE2F1 does not bind to their promoters. We have previously shown that dREAM/MMB plays a role in two different mechanisms of repression of group D/E genes: the deacetylation of upstream promoter regions and the dimethylation of histone H3 K27 at sequences that are downstream of the transcription start site (21). While the first mechanism of repression is shared with cell cycle-regulated genes, the second is unique for group D/E genes. We next wanted to ask whether dREAM/MMB is responsible for other unique features of E2F/RB regulation of group D/E genes. Specifically, we asked whether the differential binding of dE2F1 is due to dREAM/MMB.

dE2F1 does not bind to group D/E gene promoters even in the absence of dE2F2 (10), indicating that there is no competition between the E2Fs for binding. We reasoned that dREAM could function to prevent dE2F1 binding at these promoters. Alternatively, both E2Fs are unable to bind to these promoters on their own, and dREAM is required for the binding of dE2F2. To test these hypotheses, we disrupted the dREAM/MMB complex by depleting the Mip40 subunit via RNAi (Fig. 1C) and assayed the

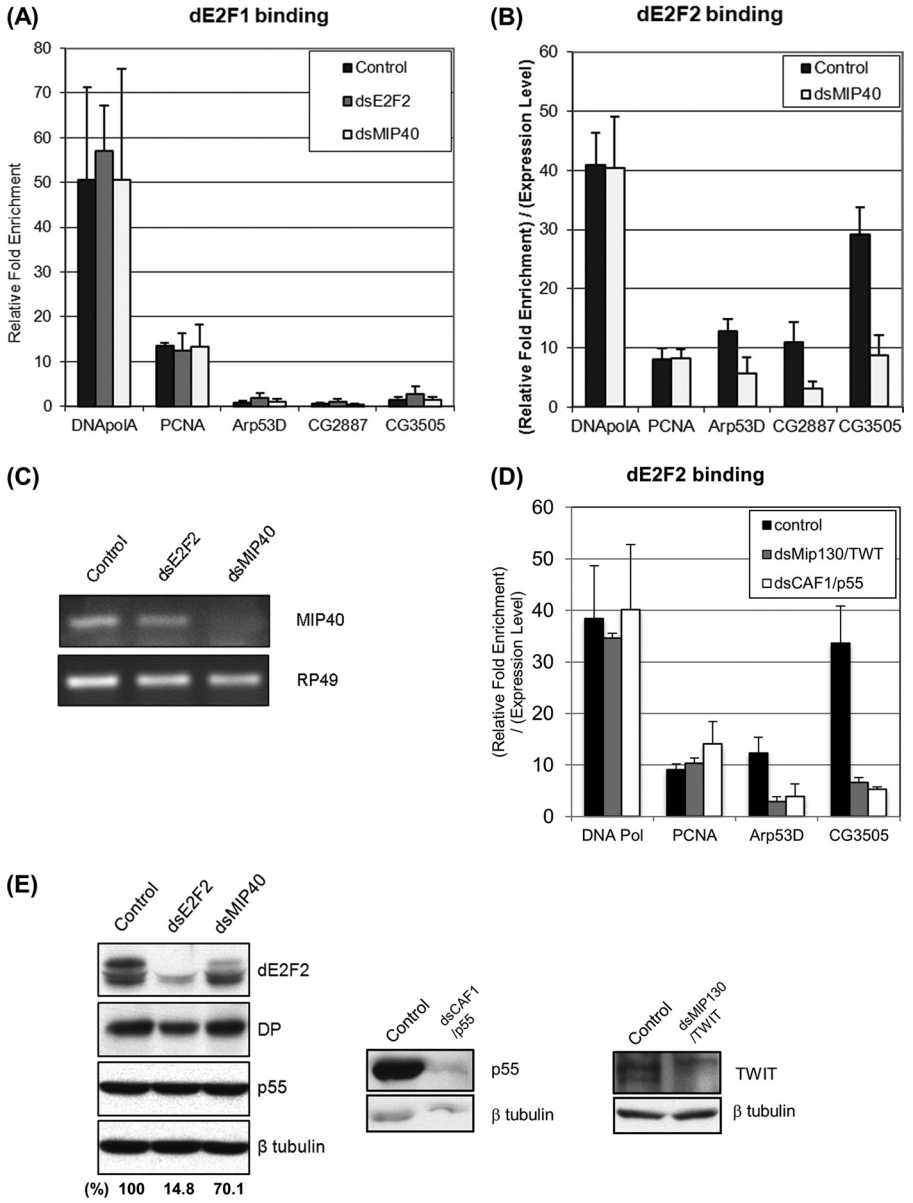

FIG 1 E2F binding at target gene promoters in dREAM-disrupted cells. (A and B) Chromatin immunoprecipitation (ChIP) performed with antibodies recognizing dE2F1 (A) or dE2F2 (B) in cells incubated with double-stranded RNA (dsRNA) targeting white (control), dE2F2, or Mip40. The amount of coprecipitated DNA was quantified by quantitative real-time PCR. Results are normalized to a nonspecific sequence (promoter of RP49) and represent the average of three independent experiments. Promoter sequences analyzed are DNA Pol α and PCNA (cell cycle-regulated genes) and Arp53D, CG2887, and CG3505 (group E genes). (C) RT-PCR analysis of mRNA levels from cells incubated with dsRNA targeting white (control), dE2F2, or Mip40. (D) ChIP assay performed with antibodies recognizing dE2F2 in cells incubated with dsRNA targeting white (control), Mip130/TWT, or p55/CAF1. (E) Western blot analysis of whole-cell extracts from SL2 cells incubated with dsRNA targeting white (control), dE2F2, Mip40, p55/CAF1, or Mip130/TWT. Numbers at the bottom represent quantified dE2F2 protein levels. ChIP results in panels B and D are corrected for total dE2F2 protein levels.

binding of both E2F proteins. We reasoned that if the first model were correct, we would observe dE2F1 binding at the location of the group D/E gene promoters in the absence of dREAM. However, if the second model were true, we would observe that dE2F2 binding is compromised.

dE2F1 was present at cell cycle-regulated gene promoters (Fig. 1A, DNA polymerase α [DNA Pol α] and PCNA) in both control and dREAM-depleted cells and absent at group E gene promoters (Fig. 1A, Arp53D, CG2887, and CG3505) under all conditions assayed. These results indicate that neither dE2F2 nor the dREAM complex is preventing dE2F1 from binding at group D/E gene promoters.

We next asked whether dREAM/MMB might be required to recruit dE2F2. Removal of Mip40 results in a slight reduction in dE2F2 levels (Fig. 1E). Therefore, we normalized the ChIP results to dE2F2 protein levels. We observed that the binding of dE2F2 was unchanged in Mip40-depleted cells at cell cycle-regulated promoters, DNA Pol α and PCNA, but impaired at group E gene promoters (Fig. 1B). The binding of dE2F2 was also compromised in cells lacking other subunits of dREAM, Mip120, Mip130, or

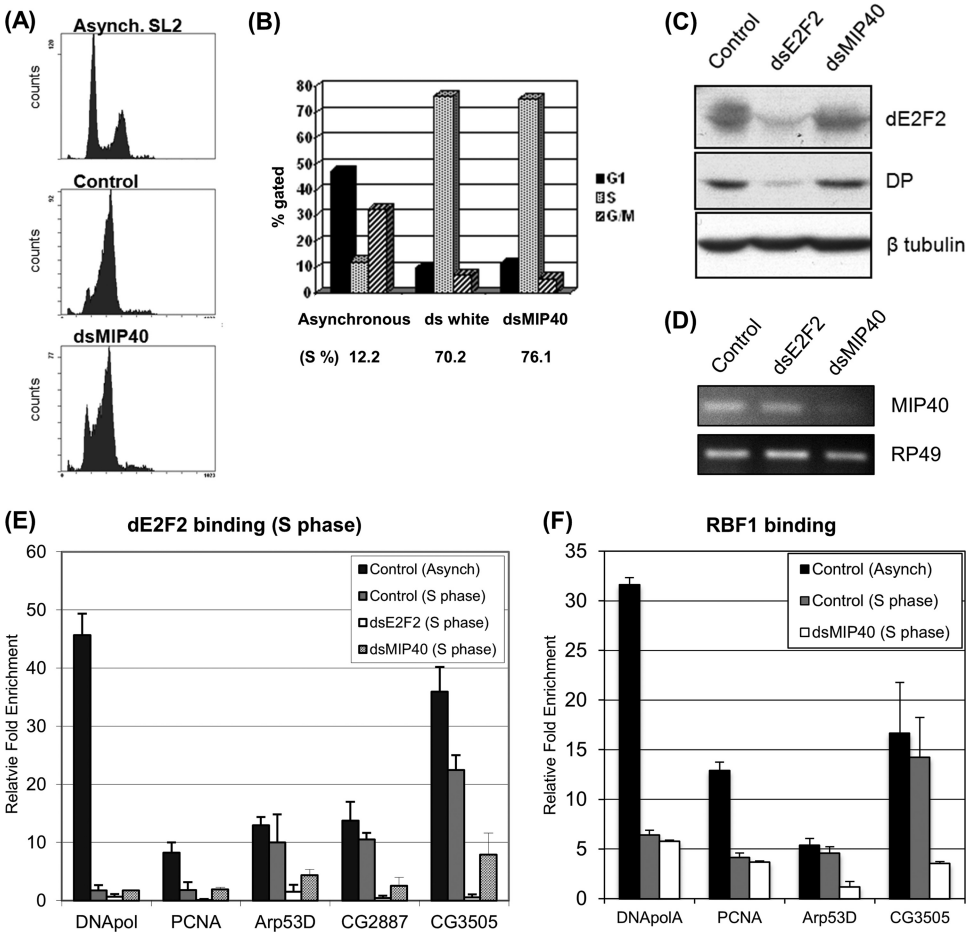

FIG 2 dREAM is required for dE2F2 binding at group E gene promoters during S phase. (A) Cell cycle profiles of asynchronously growing SL2 cells or cells synchronized in S phase and treated with dsRNA targeting either white (control) or Mip40. (B) Percentage of cells in G_1, S, and G_2/M phases of the cell cycle as determined by two-dimensional (2D) FACS analysis. Numbers under graph represent the percentage of S-phase cells. (C) Western blot analysis of whole-cell extracts from SL2 cells incubated with dsRNA targeting white (control), dE2F2, or Mip40. (D) RT-PCR analysis of mRNA levels from cells incubated with dsRNA targeting white (control), dE2F2, or Mip40. (E) ChIP assay performed with antibodies recognizing dE2F2 in asynchronously growing cells (black bars) and in cells synchronized in S phase and incubated with dsRNA targeting white (control), dE2F2, or Mip40. (F) ChIP assay performed with antibodies recognizing RBF1 in asynchronously growing cells (black bars) and in cells synchronized in S phase and incubated with dsRNA targeting white (control), dE2F2, or Mip40.

p55/CAF1 (Fig. 1D; also data not shown). Taken together, these results show that dREAM/MMB is required to recruit dE2F2 at differentiation-specific but not at cell cycle-regulated E2F target gene promoters.

dREAM complex is required for dE2F2/RBF binding only in S phase. Another feature that sets group D/E genes apart from traditional targets is the fact that the E2F/RB repressive complexes remain bound at the promoters during S phase. We wondered if dREAM/MMB played a role in this stability. We first asked whether dREAM/MMB was required for dE2F2/RBF binding during S phase. To obtain S-phase cells, we arrested cells using a double hydroxyurea (HU)-aphidicolin block and harvested them 3 h after release from the block. This resulted in approximately 75% BrdU-positive cells (Fig. 2A and B). We then examined the binding of dE2F2 in both control and Mip40-depleted cells. As previously observed (10), in S phase, dE2F2 is not present at cell cycle-regulated promoters such as PCNA and DNA Pol α but remains bound to group D/E gene promoters [Fig. 2E, compare control (Asynch) versus control (S phase)]. Similar to what we observed in asynchronously growing cells, the binding of dE2F2 was greatly reduced in cells lacking Mip40 (Fig. 2E). RBF1 is recruited to group D/E promoters by dE2F2 (33). As expected, its binding in S phase was also compromised in Mip40-depleted cells (Fig. 2F).

We next asked whether dREAM/MMB functioned specifically in S phase or whether it was required for the binding of dE2F2/RBF in general. To address this question, we obtained a population of cells devoid of S-phase cells. To this end, we depleted the activator dE2F1, which leads to a severe reduction of BrdU-positive cells and an accumulation of cells in G_1 phase (Fig. 3A and B) (10, 12, 13), and assayed dE2F2 and RBF1 binding in control and Mip40-depleted cells. Predictably, we observed increased binding of dE2F2 and RBF1 in dE2F1- and dE2F1+Mip40-depleted cells compared to asynchronously growing cells at cell cycle-regulated promoters (Fig. 3C and D). At the location of the group D/E gene promoters, we find that while dE2F2/RBF1 binding was reduced in cells lacking Mip40, it was unaffected in dE2F1+Mip40-depleted cells (Fig. 3C and D). These results indicate that dREAM plays no role in dE2F2 and RBF1 binding outside S phase and that the observed reduc-

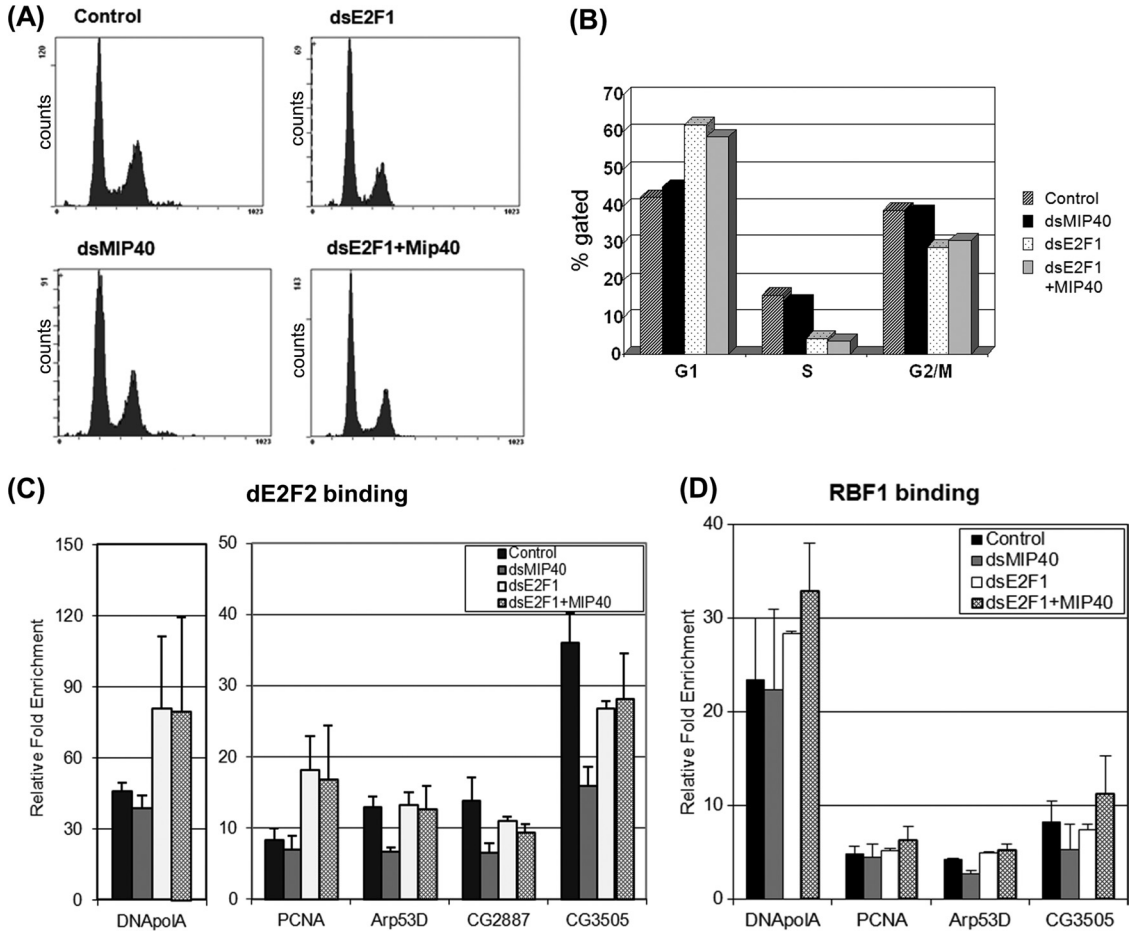

FIG 3 dREAM is not required for dE2F2 binding outside S phase. (A) Cell cycle profiles of cells incubated with dsRNA targeting white (control), Mip40, dE2F1, and dE2F1+Mip40 (cotreated). (B) Percentage of cells in G_1, S, and G_2/M phases of the cell cycle as determined by 2D FACS analysis. (C) ChIP assay performed with antibodies recognizing dE2F2 in cells incubated with dsRNA targeting white (control), Mip40, dE2F1, and dE2F1+Mip40 (cotreated). (D) ChIP assay performed with antibodies recognizing RBF1 in cells incubated with dsRNA targeting white (control), Mip40, dE2F1, and dE2F1+Mip40 (cotreated).

tion in asynchronous cells was due to impaired retention of the repressor complex during S phase.

Collectively, these results demonstrate that at group D/E gene promoters, dREAM/MMB is required for the stability of dE2F/RBF repressive complexes in S phase.

dREAM is required to counteract the activity of G_1 CDKs at differentiation-specific E2F/RB target gene promoters. The stability of E2F/RB complexes at group D/E genes in S phase suggests that RB proteins might be protected from CDK phosphorylation at these promoters. Alternatively, other factors may contribute to the stability of phosphorylated E2F/RB complexes. To begin to address these possibilities and, specifically, the role of the dREAM/MMB complex in this stability, we generated RBF1 mutants. We mutated the 7 putative CDK phosphorylation sites (Fig. 4A) to either alanine (S/T→A) to generate phosphomutant or to glutamate (S/T→E) to generate phosphomimic and created stable cell lines with inducible expression of wild-type (R1WT), phosphomutant (R1A), or phosphomimic (R1E) RBF1 proteins. We then asked whether phosphomimic RBF proteins could bind to E2F target gene promoters. As predicted, R1E proteins exhibited reduced binding at cell cycle-regulated gene promoters (Fig. 4C, left panels). Interestingly, binding was reduced at group D/E gene promoters as well (Fig. 4C, right panels). The reduced RBF1 binding was not due to lack of dE2F2 binding; dE2F2 binding was similar in R1WT and R1E cell lines (Fig. 4B). These results suggest that phosphorylation of RBF1 will disrupt binding at both types of target gene promoters and that dREAM/MMB may function to protect RB proteins from being phosphorylated at group D/E gene promoters.

If the dREAM/MMB complex functions to protect RB proteins from being phosphorylated by CDKs, we hypothesize that cells expressing a phosphomutant RBF1 that cannot be phosphorylated (R1A) will no longer require dREAM/MMB for stable binding of E2F/RB at group D/E gene promoters. Expression of an R1A mutant for a prolonged period of time results in accumulation of cells in G_1. We induced R1WT and R1A expression for 24 h to minimize the effects on the cell cycle and assayed dE2F2 binding in control and Mip40-depleted cells (Fig. 5A). In cells expressing R1WT, dE2F2 binding was reduced in cells lacking Mip40. The binding of dE2F2 in Mip40-depeleted cells was partially restored when the R1A mutant protein was expressed.

As the expression of unphosphorylatable RBF1 causes a reduc-

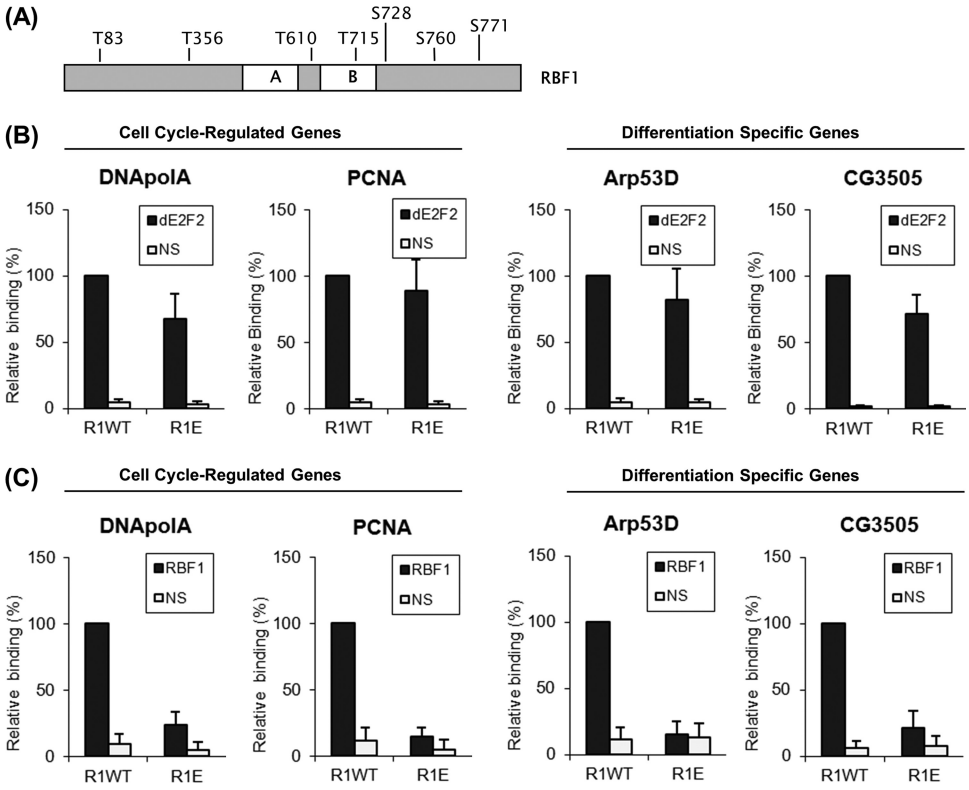

FIG 4 Phosphomimic RBF1 mutant cannot bind at both cell cycle-regulated and group E gene promoters. (A) Schematic representation of the 7 putative CDK phosphorylation sites of RBF1. (B) ChIP assay using antibodies that recognize dE2F2 or nonspecific antibodies in cell lines expressing either RBF1 WT (R1WT) or RBF1 S/T→E phosphomimic (R1E). Left panels, cell cycle-regulated gene promoters; right panels, group E gene promoters. (C) ChIP assay using antibodies that recognize RBF1 or nonspecific antibodies in cell lines expressing either RBF1 WT (R1WT) or RBF1 S/T→E phosphomimic (R1E). Left panels, cell cycle-regulated gene promoters; right panels, group E gene promoters.

tion in S-phase cells (Fig. 5B) and dREAM is only required for E2F/RB binding in S phase, it is possible that some of the observed effects are an indirect consequence of cell cycle stage. To address this, we synchronized cells in S phase as described above and induced expression of RBF1WT or R1A 24 h prior to collecting. This resulted in a population enriched in S-phase cells (34 to 48% versus asynchronously growing cells, ~10%). R1A-expressing cells exhibited only a slight reduction in S-phase population compared to R1WT cells (Fig. 6A). We assayed both dE2F2 and RBF1 binding and observed that while it was greatly reduced in Mip40-depleted R1WT cells, there was little change in Mip40-depleted R1A-expressing cells (Fig. 6B and C).

These results indicate that phosphorylation of RBF1 by CDKs imposes a requirement for dREAM at group D/E gene promoters. G_1 CDKs can disrupt dE2F2/RBF1 interactions (13). Consistent with this, we find that both dE2F2 and the dREAM subunit p55/CAF1 preferentially bring down the faster-migrating, hypophosphorylated form of RBF1. In asynchronously growing cells, which have few S-phase cells, the hypophosphorylated form is more abundant than the hyperphosphorylated form, yet phosphorylated RBF1 is still readily detectable (Fig. 6E, input lane). Both p55/CAF1 and dE2F2, which have been shown to robustly bind to RBF1 in multiple assays, fail to interact with the hyperphosphorylated form of RBF1 (Fig. 6E). We also used a bis-Tris SDS-PAGE system for better visualization of the hypo- and hyperphosphorylated forms of RBF1 in an S-phase enriched cell population (Fig. 6D). Interestingly, when dREAM was disrupted (Mip40-depleted cells), we observed a relative increase of the hyperphosphorylated form, suggesting that when part of dREAM, RBF1 is underphosphorylated.

Taken together, these results are consistent with the idea that a major means by which dREAM/MMB maintains the stability of dE2F2/RBF complexes in S phase at group D/E gene promoters is by protecting RB proteins from phosphorylation. However, we cannot exclude the possibility that dREAM also functions by stabilizing a partially phosphorylated dE2F2/RBF1 complex.

Repression is compromised in both wild-type and phosphomutant RBF1-expressing cells upon dREAM disruption. We have previously shown that dREAM/MMB is required to maintain repression of group D/E genes via two different mechanisms (21).

Our results described above raise the question whether dREAM inhibits their transcription indirectly, by maintaining the stability of a dE2F2/RBF repressor complex. To address this issue, we asked whether dE2F2/RBF1 could maintain repression of group D/E genes in the absence of dREAM/MMB. We depleted Mip40 from cells expressing either the wild-type or the phosphomutant RBF1 protein. If dREAM/MMB is not required for RB-mediated repression, we expected to see few or no changes even in the absence of Mip40, as binding of dE2F2/RBF is restored in R1A-expressing cells.

We examined both repressive mechanisms: histone deacetylation of promoter regions and histone H3 K27 dimethylation of

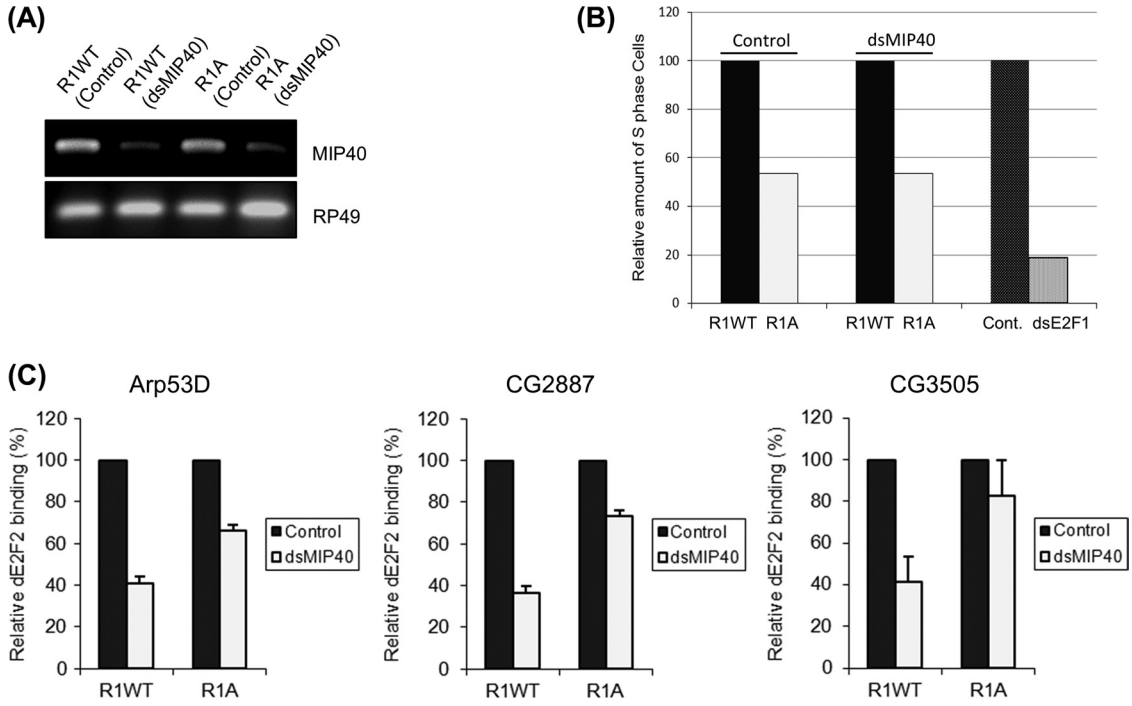

FIG 5 Phosphomutant RBF1 suppresses dE2F2 binding defects in dREAM disrupted cells. (A) RT-PCR an analysis of mRNA levels from cells expressing RBF1 WT (R1WT) or RBF1 phosphomutant (R1A) and incubated with dsRNA targeting white (control) or Mip40. (B) Percentage of cells in S phase of the cell cycle expressing RBF1 WT (R1WT) or RBF1 phosphomutant (R1A) and incubated with dsRNA targeting white (control) or Mip40; cells incubated with dsRNA targeting white (control) or dE2F1 are shown as comparison. (C) ChIP assay using antibodies that recognize dE2F2 in cells expressing RBF1 WT (R1WT) or RBF1 phosphomutant (R1A) and incubated with dsRNA targeting white (control) or Mip40.

sequences downstream of the transcription start site. As previously observed, histone H3 and H4 acetylation levels rose in cells lacking Mip40, but there was no difference between R1WT- and R1A-expressing cells (Fig. 7A and B). While the histone H3 K27 dimethylation levels varied in the RBF1-expressing cell lines, the reduction dimethylation observed in Mip40-depleted cells was similarly unaffected in R1A-expressing cells (Fig. 7C). Most importantly, mRNA levels of group E genes rose in Mip40-depleted cells in both cell lines (Fig. 7D). The genes were derepressed in cells lacking dREAM in both R1WT- and R1A-expressing cells. Taken together, these results show that dREAM/MMB is not dispensable for repression. Even when dE2F2/RBF1 complexes can be stably maintained at these promoters, the repression mechanisms are compromised in the absence of a functional complex.

DISCUSSION

The identification of native pocket protein-associated complexes in flies, worms, and humans called dREAM/MMB/DRM/LINC indicates that such complexes play important roles in RB functions. The mechanisms of action of the complex are not very well understood. The complex has been shown to regulate the expression of G_1/S, G_2/M, and differentiation-specific E2F target genes and to potentiate RB tumor-suppressive functions. It has been reported to repress as well as activate transcription and to regulate site-specific DNA replication, and it has been located at a large number of genomic sites. These observations indicate that dREAM/MMB/DRM/LINC plays a vital role in RB functions but also raise the question of how it can support all these diverse E2F/RB activities.

In this study, we have explored the means by which the *Drosophila* complex, dREAM, represses differentiation-specific E2F/RB targets in actively proliferating cells. Differentiation-specific target genes (group D/E) differ in their regulation from classic, cell cycle-regulated targets, and we find that the function of dREAM is required for some of the unique features of this gene regulation. Specifically, dREAM is not responsible for the target specificity of E2Fs. Instead, it is required for the stability of dE2F2/RBF complexes at group D/E gene promoters during S phase. Our results indicate that it may function, at least in part, by protecting RBF1 from phosphorylation. In addition, the complex is also essential to maintain both mechanisms of repression at these genes.

Lack of dE2F1 binding at the group E gene promoters. One of the distinctive features of group E gene regulation is the lack of dependence on E2F activation; the activator dE2F1 does not bind to their promoters or regulate their expression. This poses the question of how dE2F2 specificity is achieved at these promoters. While a comparison of E2F binding sites did not reveal any major differences between sites found at the group E and cell cycle gene promoters, dE2F1 is unable to bind to group E gene promoters even in the absence of dE2F2 (10). This finding suggests either that dE2F1 is inherently incapable of binding to these promoters or that some factor(s) other than dE2F2 prevents it from binding. It is also possible that dE2F2 is not capable of binding without the assistance of another factor. One candidate for such a factor is the dREAM complex. It could either assist dE2F2 or prevent dE2F1 from binding. We find that disruption of dREAM did not lead to dE2F1 binding but disrupted dE2F2 binding, suggesting that neither E2F is capable of binding to these promoters without assis-

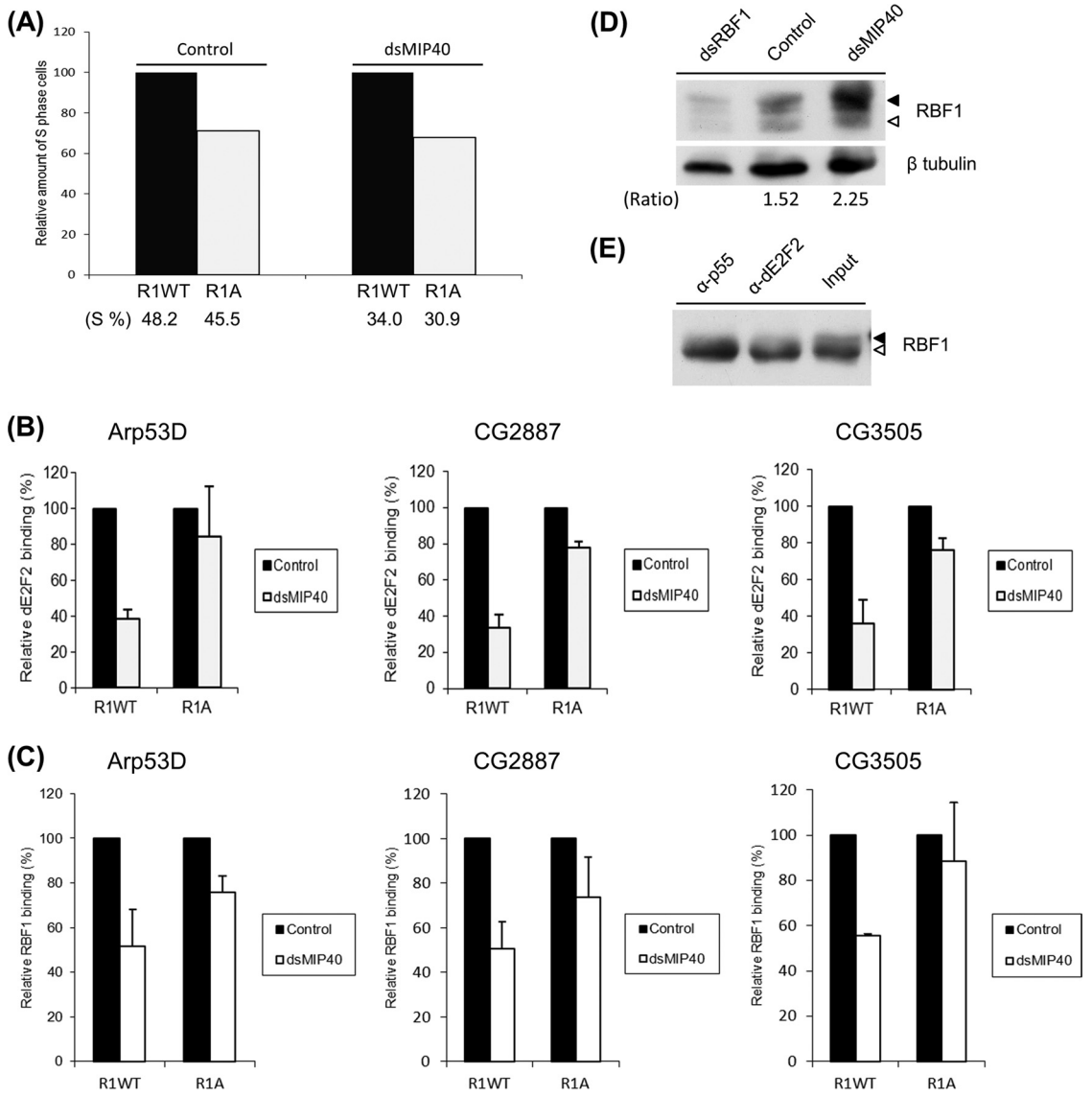

FIG 6 Phosphomutant RBF1 restores dE2F2 binding in S-phase enriched cells lacking dREAM. (A) Cells were treated with hydroxyurea-aphidicolin and collected 3 h after release. Expression of RBF1 WT or R1A mutant was induced 24 h prior to collection. Percentage of cells in S phase of cells expressing RBF1 WT (R1WT) or RBF1 phosphomutant (R1A) and incubated with dsRNA targeting white (control) or Mip40. (B) ChIP assay using antibodies that recognize dE2F2 in S-phase enriched cells, expressing RBF1 WT (R1WT) or RBF1 phosphomutant (R1A) and incubated with dsRNA targeting white (control) or Mip40. (C) ChIP assay using antibodies that recognize RBF1 in S-phase enriched cells, expressing RBF1 WT (R1WT) or RBF1 phosphomutant (R1A) and incubated with dsRNA targeting white (control) or Mip40. (D) Western blot analysis of whole-cell extracts from S-phase enriched cells incubated with dsRNA targeting white (control), RBF1, or Mip40; hyper- and hypophosphorylated forms of RBF1 (black and white arrowheads, respectively) were separated using a bis-Tris SDS-PAGE system; blots were probed with anti-RBF1 and anti-β-tubulin (loading control) antibodies. Numbers under blots represent the ratio of quantified levels of slower-migrating/faster-migrating forms of RBF1. (E) Coimmunoprecipitation of RBF1 with p55/CAF1 and dE2F2. Anti-dE2F2, anti-p55, or antibodies were used in immunoprecipitations from whole-cell extracts. Blots were probed with anti-RBF1 antibodies.

tance. However, upon close examination, we discovered that dREAM is dispensable for dE2F2 binding outside S phase, demonstrating that dE2F2 can bind to group E gene promoters without the assistance of dREAM. These results indicate that dE2F1 is not capable of binding at these promoters. It is possible that there are some subtle differences in the E2F binding sites; alternatively, the two E2F proteins may have different affinities for a particular chromatin landscape.

Stability of dE2F2/RBF complexes in S phase. In S phase, dE2F/RBF complexes are replaced by dE2F1 at cell cycle-regulated promoters yet remain bound and functional at the group E gene promoters. Several observations in mammals also indicate that E2F/pocket protein complexes exist irrespective of cell cycle stage and can function in a CDK-independent manner (references 4 and 9 and references therein; 41, 42, 45). It is well established that E2F/RB complexes are disrupted at cell cycle genes at the G_1/S transition (reviewed in references 6, 9, 34, 37). Are E2F/RB complexes then protected from CDK phosphorylation in some cases or is the regulation of E2F/pocket protein interactions even more complex? Structural studies of pRB have suggested that the C ter-

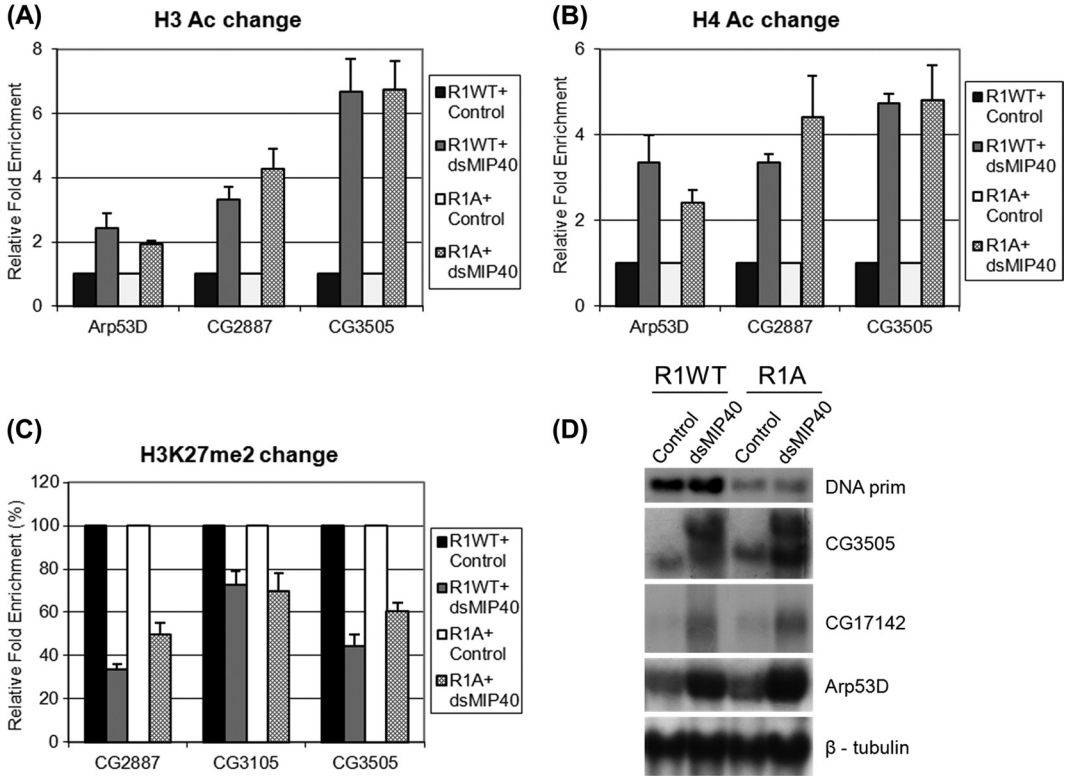

FIG 7 dREAM is required for the repression of group E genes in both R1WT- and R1A-expressing cells. (A and B) ChIP assay performed with antibodies recognizing pan-acetylated histone H3 (A) or pan-acetylated histone H4 (B) in cells expressing RBF1 WT (R1WT) or phosphomutant (R1A) and incubated with dsRNA targeting white (control) or Mip40. (C) ChIP assay performed with antibodies recognizing histone H2 K27 dimethylation (H3K27me2) in cells expressing RBF1 WT (R1WT) or phosphomutant (R1A) and incubated with dsRNA targeting white (control) or Mip40. (D) Northern blot analysis of total RNA isolated from cells expressing RBF1 WT (R1WT) or phosphomutant (R1A) and incubated with dsRNA targeting white (control) or Mip40.

minus of pRB functions as a molecular sensor that recognizes CDK-mediated phosphorylation (29, 43). When pRB is hypophosphorylated, its C-terminal region stabilizes the interaction with E2F1. Phosphorylation by CDKs causes a conformational change, and a series of intramolecular interactions between the C terminus and the pocket domain are thought to inhibit the binding between pRB and E2F1 (16, 29). However, it is not clear whether this is true for other RB family members or that all E2Fs interact in the same manner with pocket proteins. Furthermore, it has been shown that pRB has two distinct E2F binding sites, one being specific for E2F1 and linked to its ability to regulate E2F1-dependent apoptosis. This indicates that pRB interacts with individual E2F proteins in different ways and that regulation of distinct E2F functions is physically separable (8). Studies in *Drosophila* also indicate that G_1 CDKs are not sufficient to disrupt repressive E2F/RB complexes at cell cycle genes (13).

In the case of differentiation-specific target genes in flies, we find a simple explanation of the stability of E2F/RB complexes in S phase: dREAM. In the absence of a functional dREAM complex, dE2F2 and RBFs exhibit reduced binding in S phase. Our results show that expression of a phosphomimic RBF mutant results in impaired binding at both cell cycle-regulated and differentiation-specific (group E) gene promoters. Conversely, a mutant RBF1 that cannot be phosphorylated is sufficient to bypass the need for a functional dREAM at the group E gene promoters; E2F/RB complexes are stable in S phase even in the absence of dREAM. These findings indicate that CDK phosphorylation can disrupt dE2F2/RBF repressive complexes at the group E gene promoters and that this necessitates their assembly into the dREAM complex.

But how does assembly into dREAM ensure stability of E2F/RB complexes? The simplest model is that the phosphorylation sites are blocked, inaccessible to CDKs. Alternatively, dREAM could stabilize either fully or partially phosphorylated RBF protein. Previous studies have shown that CycE/cdk2 can phosphorylate RBF1 as part of the dREAM complex *in vitro* and that phosphorylation does not disrupt the association (22). However, RBF1 is regulated by both CycD/cdk4 and CycE/cdk2 (44) and it is likely that when assembled into dREAM *in vivo* not all phosphorylation sites are modified. In agreement with this idea, we find that in cells lacking functional dREAM, hyperphosphorylated RBF1 is increased. Thus, our findings suggest that the complex functions, at least in part, by protecting RBF1 from being phosphorylated by CDKs at these promoters.

dREAM, possibly in association with additional factors, may also modify RBF1. For instance, pRB is known to be acetylated during differentiation of monoblastoid cells and keratocytes (3, 25, 27). Additional posttranslational modifications of the protein could either prevent phosphorylation and/or induce a conformational change to promote stability of phosphorylated E2F/RB complexes. It will be interesting to investigate if RBF proteins bound in the dREAM complex have modifications other than phosphorylation.

What is the role of the dREAM complex in the regulation of differentiation-specific E2F/RB target genes? The initial identi-

fication and characterization of the dREAM/MMB complexes has led to the speculation that dREAM is a repression complex that functions by affecting chromatin structure (20, 22). The complex is not stably associated with any enzymatic activity, yet we have demonstrated that group D/E genes are repressed via two distinct mechanisms in a dREAM-dependent manner (21). Is the observed dependence on dREAM the result of its ability to affect chromatin structure or is it an indirect consequence of its role in dE2F2/RBF binding? We favor the idea that dREAM plays a direct role in the repression of group E genes for two reasons. Binding of dE2F2/RBF1 complexes does not require dREAM outside S phase. Only about 10% of asynchronously growing SL2 cells are in S phase, yet disruption of dREAM leads to the same level of derepression as the removal of dE2F2 or RBFs. Moreover, while the expression of phosphomutant RBF1 restores the binding of dE2F2/RBF1 in S phase, it does not restore the repression in cells lacking dREAM. A dual role for dREAM in the regulation of these genes is also more consistent with its reported involvement in other RB functions. It would suggest that the complex is capable of potentiating diverse RB activities.

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