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RBF Binding to both Canonical E2F Targets and Noncanonical Targets Depends on Functional dE2F/dDP Complexes

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The retinoblastoma (RB) family of proteins regulate transcription. These proteins lack intrinsic DNA-binding activity but are recruited to specific genomic locations through interactions with sequence-specific DNA-binding factors. The best-known target of RB protein (pRB) is the E2F transcription factor; however, many other chromatin-associated proteins have been described that may allow RB family members to act at additional sites. To gain a perspective on the scale of E2F-dependent and E2F-independent functions, we generated genome-wide binding profiles of RBF1 and dE2F proteins in *Drosophila* larvae. RBF1 and dE2F2 associate with a large number of binding sites at genes with diverse biological functions. In contrast, dE2F1 was detected at a smaller set of promoters, suggesting that it overrides repression by RBF1/dE2F2 at a specific subset of targets. Approximately 15% of RBF1-bound regions lacked consensus E2F-binding motifs. To test whether RBF1 action at these sites is E2F independent, we examined *dDP* mutant larvae that lack any functional dE2F/dDP heterodimers. As measured by chromatin immunoprecipitation-microarray analysis (ChIP-chip), ChIP-quantitative PCR (qPCR), and cell fractionation, the stable association of RBF1 with chromatin was eliminated in *dDP* mutants. This requirement for dDP was seen at classic E2F-regulated promoters and at promoters that lacked canonical E2F-binding sites. These results suggest that E2F/DP complexes are essential for all genomic targeting of RBF1.

The retinoblastoma protein (pRB) and two related proteins, p107 and p130, are critical regulators of cell proliferation. Analysis of mutant animals shows that the inactivation of these proteins causes defects in the control of cell proliferation and differentiation and alters the cellular sensitivity to apoptosis and senescence (9). In most cellular contexts the normal functions of RB family members suppress cell proliferation, potentially explaining why these proteins are inactivated or dysregulated in many types of cancer.

In the 25 years since the retinoblastoma susceptibility gene (*RB1*) was first identified (19), more than 200 cellular proteins have been found to physically associate with pRB. These encompass a diverse array of biochemical and cellular activities, but the majority of the reported pRB-associated proteins are transcription factors or chromatin-associated proteins (43). In general, pRB has been reported to inhibit the activity of factors that promote cell proliferation, but it can either enhance or repress gene expression programs that are associated with cell differentiation.

The best known target of pRB is the E2F transcription factor. The basic form of E2F is a heterodimer consisting of an E2F and a DP subunit. Activator E2F complexes drive the expression of genes that are crucial for cell cycle progression, allowing the expression of these genes to be coordinately induced during the G_1/S transition. This activity is counteracted by pRB family proteins, which not only bind and inhibit the activator E2Fs but also cooperate with repressor E2F proteins to suppress transcription from E2F target genes. The interplay between repressor and activator E2Fs is critical for proper cell cycle control.

Results from mouse models and *in vitro* differentiation systems have led to the suggestion that pRB also has numerous E2F-independent functions. The elimination of pRB in a variety of cellular contexts has been shown to alter the normal process of differentiation. Reports that pRB can interact with diverse transcription factors (e.g., Elf1 [66], Jun [45], MyoD [25], and Runx2 [62]) suggest that pRB is a versatile regulator that is used at many different types of targets. A naturally occurring mutant form of *RB1* (661W), that has a compromised ability to associate with E2F (59), retains activity in differentiation assays (57).

A key, unresolved issue for this area of research is the relative importance of E2F-dependent and E2F-independent activities in the functions of pRB family proteins. This subject has been difficult to resolve in mammalian cells because of several complicating issues. The fact that the mammalian pRB family contains three related proteins that have overlapping functions makes it difficult to perform a definitive structure/function analysis, and this is particularly true for a protein like pRB that has been proposed to interact with a very large number of cellular proteins. Biochemical approaches have also failed to answer this question because only a small fraction of the overall pool of pRB is found in association with any one of its potential partners. Antibodies specific for endogenous pRB have generally been found to give weak signals in chromatin immunoprecipitation (ChIP) assays, and there is relatively little information about the genome-wide distribution of pRB on chromatin, especially in primary tissues. Recent genome-wide binding studies for pRB proteins provided valuable insight into pRB binding on a global level (8, 39) but did not address the issue of E2F-dependent versus -independent recruitment to chromatin. Such studies often rely on the search for transcription factor-binding motifs, and a number of sequence motifs, apart from

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the E2F consensus motif, were found significantly enriched at binding sites (39).

To obtain a general perspective on the relationship between the pRB and E2F families of proteins, we have turned to the *Drosophila* model system. Flies have a streamlined version of the RB/E2F pathway, containing two E2F proteins (dE2F1 and dE2F2), one DP protein (dDP), and two pRB family members (RBF1 and RBF2) (65). dE2F1 is a potent activator of E2F targets, while dE2F2 is a repressor, and both dE2Fs act in heterodimers with dDP. RBF2 associates preferentially with dE2F2 and has a restricted pattern of expression (58), whereas RBF1 is broadly expressed and interacts with both dE2F proteins. Thus, in most cell types RBF1 represents the functional ortholog of the mammalian family of pRB-related proteins. As in mammalian cells, RBF1 is a transient and reversible inhibitor of dE2F1, and this interaction generates pulses of E2F-dependent gene expression that are associated with cell proliferation (11). In contrast to dE2F1, dE2F2 is a component of a stable multisubunit transcription repressor complex (dREAM/Myb-MuvB). These complexes also contain either RBF1 or RBF2 (33, 37), and the repressive activity of dREAM/Myb-MuvB complexes can be uncoupled from cell proliferation. While there are fewer reports of E2F-independent roles for pRB family proteins in *Drosophila* than in mammalian cells, recent work using neuroblast squashes from *rbf1* mutant larvae revealed an important role for RBF1 in chromatin condensation (40). This condensation defect was not seen in *dDP* mutants or *de2f de2f2* double mutant animals, suggesting that it may be E2F independent.

Here, we have used ChIP to examine the distribution of RBF1-binding sites in *Drosophila* larvae. As described below, the genome-wide distribution of RBF1-binding sites overlaps extensively with sites bound by the activator dE2F1 and the repressor dE2F2. In many regions, the RBF1-binding sites include sequences with potential E2F-binding motifs, consistent with the idea that E2F is an important target of RBF1 regulation. However, a significant subset of RBF1-bound regions lacks a motif corresponding to a consensus E2F-binding site. This could indicate that RBF1 can be recruited to chromatin in an E2F-independent manner. Alternatively, since E2F proteins have been shown to bind to noncanonical E2F motifs and since several different E2F-binding motifs have been described, this might reflect our limitations in predicting, or detecting, E2F-binding sites. To provide a definitive answer to this question, we have taken advantage of the relative simplicity of the *Drosophila* RBF-dE2F network. Mutation of the single *dDP* gene eliminates dE2F1 and dE2F2 DNA-binding activity in *Drosophila* (21). This mutant provides an opportunity to determine how much of the RBF1 that associates with chromatin is dependent on "classic" E2F/DP complexes. Because of the large number of E2F and DP genes, an equivalent null background would be extremely difficult to generate in mammalian cells. Our results show that mutation of *dDP* completely eliminates RBF1 association with chromatin and that this is the case even at RBF1-binding sites that lack consensus E2F-binding motifs. These results indicate that most (if not all) of the RBF1 protein that is stably associated with chromatin in wild-type larvae and detectable by ChIP is recruited via E2F/DP and that this is the case even when consensus E2F-binding motifs are absent.

MATERIALS AND METHODS

Antibodies and Western blotting. Anti-dE2F1 (1:500 for Western blotting) and anti-dE2F2 antibodies were generated by injecting rabbits with a peptide derived from the dE2F1 or dE2F2 sequence, respectively (Open Biosystems). Other primary antibodies included rabbit anti-dE2F1 (1:1,000 for Western blotting), which was a generous gift from P. Spierer (Geneva, Switzerland), and rabbit anti-heat shock factor (anti-HSF) and anti-Rpb3, which were gifts from J. Lis (Cornell University). Mouse anti-RBF1 (1:10 for Western blotting), anti-RBF2 (1:10 for Western blotting), and anti-dDP (1:10 for Western blotting) and rabbit anti-dDP and anti-dE2F2 antibodies were described previously (11, 14, 15, 58). Rabbit anti-H3 (Abcam) was used at a dilution of 1:5,000 for Western blotting. Mouse antitubulin antibody was obtained from the Developmental Studies Hybridoma Bank and used at a dilution of 1:500 for Western blotting. Western blotting was carried out using standard procedures (7).

Fly strains. *w1118* or *yw* flies were used as wild-type control flies. The following null alleles were used in this work: $rbf1^{\Delta 14}$ (13), $de2f1^{rm729}$ and $de2f1^{91}$ (16), $de2f2^{76Q1}$ and $de2f2^{c03344}$ (20) (Exelixis), and dDP^{a3} and dDP^{a4} (53). Extra lethal mutations were removed from the dDP^{a3} and dDP^{a4} chromosomes by homologous recombination (21). The hypomorphic $rbf1^{120a}$ allele was used in combination with $rbf1^{\Delta 14}$. All mutations were analyzed under transheterozygous conditions.

Preparation of larval extract and larval chromatin fractionation. For larval extracts, third-instar larvae were collected and washed in phosphate-buffered saline (PBS). After removal of PBS, larvae were snap-frozen in liquid nitrogen, resuspended in an equal volume of buffer HoB (25 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol [DTT], protease inhibitors) and homogenized using a motor-driven Mini-Douncer (Kontes Glass Co.). The extracts were cleared by centrifugation and loaded on a gel for Western blotting.

Larval chromatin fractionation was carried out as previously described (34), with minor modifications. Briefly, 300 third-instar larvae were rinsed with tap water and washed with 25 ml of larval wash buffer (0.7% NaCl, 0.1% Triton X-100) followed by 50 ml of MilliQ water. Larvae were weighed and resuspended in 2 ml of larval nuclear buffer I (LNB I; 15 mM HEPES [pH 7.6], 10 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 350 mM sucrose, DTT, protease inhibitors) per gram of larvae. The larvae were homogenized by three pulses (5 s at 5,000 rpm) with a Tissuemiser homogenizer (Fisher Scientific), followed by 10 strokes in a glass/Teflon potter (Kontes Glass Co.). The homogenate was filtered through a 64-μm-pore-size Nitex nylon membrane (Genesee Scientific). The debris in the nylon membrane was homogenized five more times in the glass/Teflon potter with 1 ml of LNB I per gram of larvae, filtered through the nylon membrane, and combined with the first filtrate. The resulting cell suspension was broken up by 10 strokes with a B pestle (Wheaton), and the nuclei were collected by centrifugation at 4,000 × g for 5 min at 4°C. The nuclei were successively extracted with 0.25 ml of larval nuclear buffer II (LNB II; 15 mM HEPES [pH 7.6], 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1% Tween, 10% glycerol, DTT, protease inhibitors) per gram of original larvae, containing 80, 150, or 600 mM KCl, to elute proteins from chromatin with increasing stringency. The first two extraction steps (80 plus 150 mM) were carried out for 90 min; the last step (600 mM) was performed overnight at 4°C. Extracts were collected after centrifugation for 10 min at 2,000 rpm and analyzed by Western blotting.

Chromatin immunoprecipitation and ChIP with microarray technology (ChIP-chip). ChIP experiments from *Drosophila* third-instar larvae were performed using a previously published method (46). Wild-type or mutant animals were homogenized, cross-linked, sonicated, and immunoprecipitated as described by Negre et al. (46). Specific primer pairs were used to directly amplify ChIP samples and input by quantitative reverse transcription-PCR (qRT-PCR). Alternatively, ChIP samples and input were amplified using ligation-mediated PCR (LM-PCR) for hybridization to *Drosophila* whole-genome tiling microarrays (2.1 M *Drosophila* Whole-Genome Tiling Arrays; NimbleGen). Briefly, single-strand overhangs were filled in with T4 DNA polymerase (1.5 units; New England BioLabs [NEB]) for 30 min at 12°C. Following phenol-chloroform extraction and ethanol precipitation, a previously annealed linker mix (linker 1, GCGGTGACCCGGGAGATCTGAATTC; linker 2, GAATTCAGATC; 2

μM final concentration) was ligated to the DNA fragments using T4 DNA Ligase (200 units; NEB) for 16 h at 16°C. The DNA was ethanol precipitated and amplified, using linker 1 (1 μM final concentration) and *Taq* polymerase (2.5 units; Promega), for 24 cycles (30 s at 95°C, 30 s at 60°C, and 1 min at 72°C). The amplified DNA was purified using a Qiagen PCR purification kit and processed for microarray hybridization according to the manufacturer's instructions (NimbleGen ChIP-chip user manual [48]).

ChIP-chip analysis. Each ChIP-chip experiment was carried out in duplicate, with preimmune serum or purified IgG as a control. Raw hybridization signal data were generated using NimbleScan software (NimbleGen). Peak detection was done using Ringo (63). Briefly, the measured $\log_2$ ratios were smoothed by a running median over chromosomal probe location. A peak was called in the smoothed curves if three or more probes in a row showed ratios higher than a cutoff. Cutoffs were determined using the preimmune serum or IgG antibody control to estimate the null distribution of peaks and then adjusted to give a false-discovery rate of 0.05. Only peaks called in both duplicates and not present in the preimmune serum or IgG control were considered real peaks and kept for further analysis.

Gene ontology analysis. Genes associated with the peaks were identified if a peak was within 1,000 bp upstream and 200 bp downstream of a transcription start site (TSS). Peaks located between two divergently paired genes were considered to be associated with both genes if both met the above-mentioned criteria. Gene ontology (GO) analysis of target genes was conducted by using the NIH Database for Annotation, Visualization, and Integrated Discovery (DAVID [http://david.abcc.ncifcrf.gov/]) (10).

Motif search. DNA sequences covered by peaks were extracted and analyzed for the presence of consensus E2F motifs using published position weight matrices (22, 31). The E2F consensus motifs derived from the published position weight matrices are TTGGCGCGCATTTT (*Drosophila*, biphasic motif), TTTSGCGC (human, E2F motif), and TTGGCGC (*Drosophila*, E2F motif). Position weight matrices were matched to bound regions using the method of Wasserman and Sandelin (67).

RESULTS

Genomic binding profiles for pRB and E2F proteins in *Drosophila* third-instar larvae. To identify sites in the *Drosophila* genome that are bound by RBF1 and dE2F proteins, we used antibodies specific for RBF1, dE2F1, and dE2F2 to perform ChIP on chromatin from wild-type (*w1118*) third-instar larvae. Probes generated from the immunoprecipitated DNA were hybridized to NimbleGen 2.1 M whole genome tiling arrays. To control for the specificity of the ChIP procedure, we performed ChIP using preimmune serum and control IgG antibodies and only considered peaks to be specific if they were absent from the control profiles.

Visual inspection of the data showed peaks of RBF1 and dE2F binding at many of the well-characterized E2F target genes with functions in cell cycle progression (Fig. 1A; also data not shown). Importantly, these peaks were absent from the preimmune serum and IgG control profiles. Binding sites for RBF1, dE2F1, and dE2F2 were strongly enriched around the transcription start site (TSS) of known genes (Fig. 1B). Eighty-two percent of dE2F1-, 64% of dE2F2-, and 79% of RBF1-binding sites were located within 1,000 bp upstream and 200 bp downstream of a transcription start site, a distribution that is in keeping with previous genome-wide maps of E2F-binding sites and consistent with the notion that dE2F and RBF proteins regulate gene expression. As expected, functional classification of the bound genes showed a strong enrichment for processes such as DNA replication, cell cycle regulation, DNA repair, and chromatin organization, processes that have all previously been intimately linked to the pRB-E2F pathway (Fig. 1C). Given that these binding profiles contained well-known hallmarks of pRB/E2F regulation, we used them as a framework for a more detailed analysis.

pRB and E2F proteins associate with thousands of sites in *Drosophila*. First, we compared the number and distribution of RBF1- and dE2F-binding sites. Using a false-discovery rate (FDR) of <0.05, we found RBF1 associated with 2,130 genomic regions, whereas dE2F2 was bound to 3,746 sites and dE2F1 associated with 278 regions (Fig. 2A; see also Data set S1 in the supplemental material). The majority of dE2F1-binding sites were also bound by RBF1, but the converse was not true, and RBF1 was found at many sites that lacked any detectable dE2F1 binding (Fig. 2A). As indicated by the high degree of overlap between RBF1- and dE2F2-binding sites (81%) (Fig. 2A), RBF1 is bound with dE2F2 at most of these sites. In agreement with this, we found that 74% of the genomic regions bound by RBF1 in third-instar larvae had previously been shown to be bound by dE2F2 in the *Drosophila* KC cell line (22). We randomly selected a set of genes bound by RBF1 in our array data and used quantitative PCR (qPCR) to test whether these regions were enriched in ChIP with antibodies against RBF1, dE2F1, and dE2F2. All of the promoter regions tested were bound by both RBF1 and dE2F2, but only a subset was bound by dE2F1 (Fig. 2B; see also Fig. 5). We conclude that RBF1 binds to many sites in the *Drosophila* genome. Although it can potentially be recruited to DNA by both dE2F1 and dE2F2, RBF1 colocalizes with dE2F1 at only a subset of sites and is more commonly found at sites occupied by dE2F2.

Next, we compared the RBF1-, dE2F1-, and dE2F2-bound promoters with the lists of genes that are deregulated following the depletion of these proteins from S2 tissue culture cells. RBF1 was detected at the promoters of 84% of the genes that were previously reported to show elevated expression in the absence of RBF1 or RBF2 (11), suggesting that the vast majority of these are directly regulated by RBF1 (Fig. 2C). Similarly, dE2F2 was bound to the promoter regions of 92% of genes that were previously shown to be upregulated in dE2F2-depleted cells. Unexpectedly, we found that dE2F1 was bound at only 38% of the dE2F1-dependent genes previously described. While this represents a strong enrichment above what would be expected by chance, it is far lower than the overlap seen with RBF1 and dE2F2. When we examined the bound promoters more closely, we noted that the functional classification of RBF1- and dE2F2-bound promoters showed a strong enrichment of genes with functions in mitosis, while this class of genes was largely missing in the dE2F1-bound data set (Fig. 1C). Our ChIP studies were carried out using chromatin from third-instar *Drosophila* larvae, a developmental stage characterized by widespread endoreplication. Endoreplication is an important strategy for *Drosophila* larvae to quickly increase their size and is characterized by successive rounds of DNA replication without intermediary rounds of mitosis (18). The absence of a mitotic program of gene expression in most larval tissues may explain why many of these targets were not bound by dE2F1. To test this idea further, we compared our genomic binding data for RBF1, dE2F2, and dE2F1 in larvae with ChIP-chip data for dE2F1 obtained from S2 cells (M. Korenjak and N. Dyson, unpublished data). In keeping with the interpretation, we found that most dE2F-dependent genes with functions in mitosis were bound by dE2F1 in S2 cells, which have a classical mitotic cell cycle (Table 1). In contrast, only RBF1 and dE2F2 were detected at the promoters of these M-phase genes in the larval chromatin. Together, these results suggest that

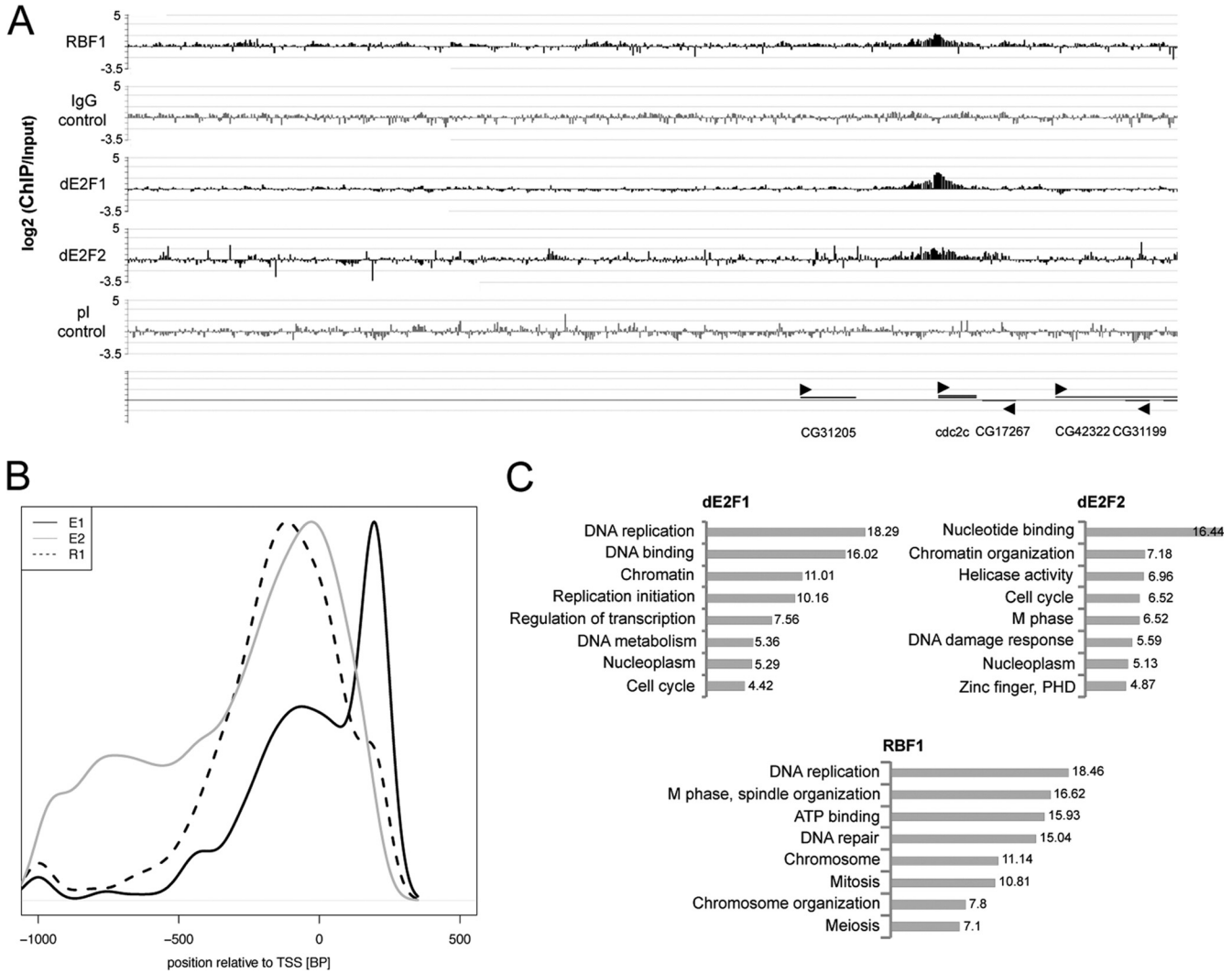

FIG 1 RBF1 and dE2F proteins bind to the promoter regions of many cell cycle genes. (A) RBF1, dE2F1, and dE2F2 colocalize at the cdc2c promoter. Specific RBF1, dE2F1, and dE2F2 binding is represented by prominent peaks in the promoter region of the cdc2c gene. Genes are represented by black bars in the bottom panel. The direction of transcription is indicated by arrows. pI, preimmune serum. (B) RBF1 and dE2F proteins bind around transcription start sites. The plots display the probability density of peak centers for RBF1 (R1), dE2F1 (E1), and dE2F2 (E2) peaks with respect to transcription start sites (TSS). 0, TSS; negative values, regions upstream. (C) RBF1 and dE2F proteins bind to the promoter region of cell cycle genes. Bound genes were functionally annotated using the DAVID database (http://david.abcc.ncifcrf.gov/). Values indicate enrichment scores.

there is a suite of potential dE2F1 targets that are repressed by RBF1 and dE2F2 but are generally not activated by dE2F1 in bulk larval tissue.

The E2F dependence of the RBF1 ChIP signal. Although E2F is the best-known target for pRB, pRB family proteins have also been reported to interact with many different chromatin-associated proteins. In addition, E2F-independent properties of pRB have been suggested by studies in mammalian cells and flies. We wanted to directly test how many of the RBF1 peaks represent sites where RBF1 was recruited to chromatin via its interaction with E2F and how many peaks reflect E2F-independent functions.

As an initial step, we asked whether the RBF1-bound regions contained putative E2F-binding sites. The DNA-binding properties of E2F transcription factors have been studied using multiple approaches, and several different variations of an E2F consensus motif have been described previously (31, 47, 50, 61). Using three different position weight matrices, we found that E2F consensus motifs were significantly enriched ($P < 0.05$) in RBF1-bound regions (Fig. 3A), but the percentages of the RBF1-bound genes that contained a putative E2F-binding element varied depending on the stringency of the consensus motif. A long, biphasic binding site identified from dE2F2-bound genes (*Drosophila*, biphasic) (22), was present in 26% of RBF1-bound regions. In contrast, shorter E2F motifs generated in mammalian cells (human, E2F) (31) and flies (*Drosophila*, E2F) (22) were found in 76% and 86% of the RBF1-bound regions, respectively.

These numbers suggest that much of the RBF1 detected on chromatin is bound via E2F, but we noted two concerns with this interpretation. First, although E2F-binding motifs display a statistically significant enrichment at RBF1-binding sites, E2F-binding motifs are relatively abundant in promoter regions, and a substantial number of potential E2F-binding sites were also seen when

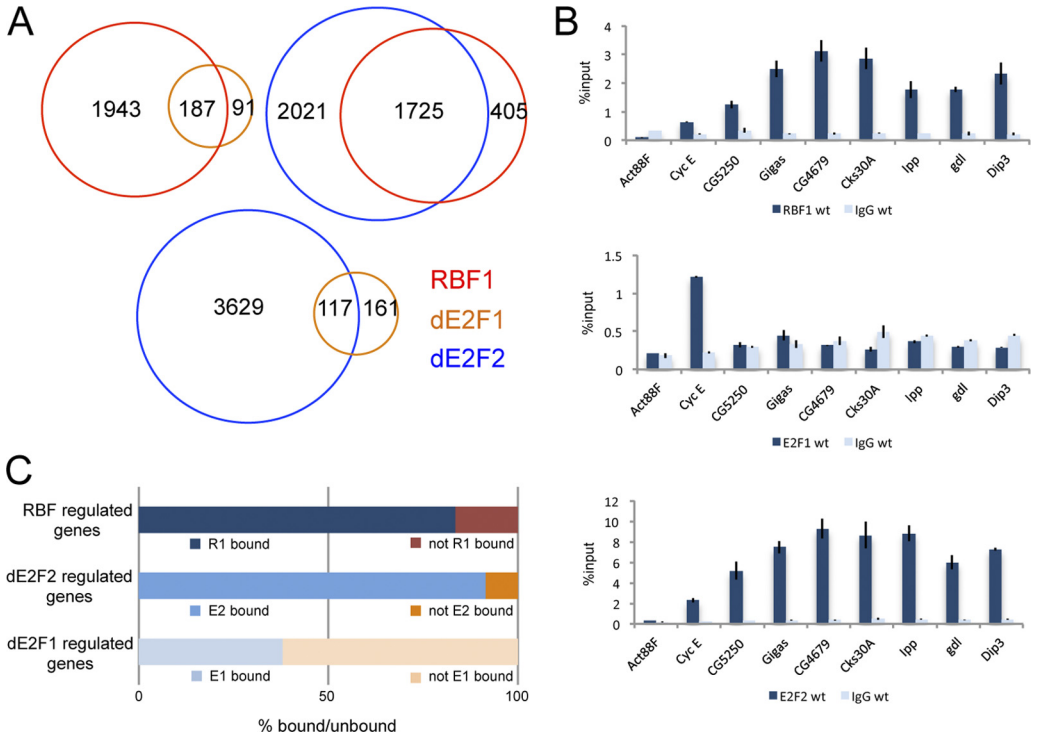

FIG 2 The RBF1-dE2F network associates with thousands of sites *in vivo* in flies. (A) RBF1 and dE2F2 bind many more sites than dE2F1. RBF1 and dE2F2 colocalize at a large number of binding sites. Most of the sites associated with dE2F1 were also bound by RBF1 and dE2F2, but, vice versa, only a small fraction of RBF1- and dE2F2-binding sites colocalized with dE2F1. The values represent the numbers of sites bound by RBF1, dE2F1, dE2F2, or a combinations thereof. (B) dE2F2 colocalizes extensively with RBF1. ChIP-qPCR was performed using antibodies against RBF1, dE2F1, and dE2F2 for a set of randomly chosen genes. Whereas RBF1 and dE2F1 colocalized only at one gene (CycE), RBF1 and dE2F2 were found at the promoter region of all tested genes. Act88F served as a negative control. The average of three independent experiments is shown. wt, wild type. (C) RBF1 and dE2F2 associate with the majority of RBF- and dE2F2-regulated genes. Percentages of RBF-repressed genes, which are bound by RBF1 (R1), dE2F2-repressed genes, which are occupied by dE2F2 (E2), and dE2F1-activated genes, which are bound by dE2F1 (E1) are shown. The lists of RBF- and dE2F-regulated genes were based on acute depletion of these proteins by RNAi in a *Drosophila* cell line (11).

similar sets of promoter regions were selected at random. Thus, when a putative E2F-binding motif is found in an RBF1-bound promoter region, it is uncertain whether the motif is truly important for the recruitment of RBF1 or whether it is simply present by chance. Second, even when the most permissive E2F consensus motifs were used, a significant subset of RBF1-bound regions still lacked a potential E2F-binding element. At these regions, it was unclear whether RBF1 associated with E2F complexes that were bound to a nonconventional site or whether RBF1 was recruited to chromatin via its interaction with a different factor. Previous studies have suggested that both possibilities might occur (24, 40, 43, 56, 64).

To distinguish between these possibilities, we took advantage of the relative simplicity of the pRB-E2F pathway in *Drosophila* and asked how the ability of RBF1 to associate with chromatin was altered by mutation of *dDP*. dDP is the sole DP ortholog present in *Drosophila* and is necessary for DNA binding of both dE2F1 and dE2F2 (21). A transheterozygous combination of dDP null alleles (dDP^{a3} and dDP^{a4}) was used to eliminate dE2F activity. Western blot analysis showed that the overall levels of the fly pRB family proteins RBF1 and RBF2 were unchanged in *dDP* mutant third-instar larvae, while dDP protein was undetectable (Fig. 3B).

First, we examined binding to the promoters of genes with functions in cell cycle control and differentiation that have previously been shown to be directly regulated by the fly pRB-E2F pathway (11, 22). ChIP experiments were carried out on chromatin from wild-type and *dDP* mutant third-instar larvae and analyzed by quantitative real-time PCR (qRT-PCR). dDP, RBF1, and dE2Fs were strongly enriched at known target genes, whereas they were not bound to the promoter of a control gene (Act88F) (Fig. 3C, dark blue bars). As expected, neither dDP nor dE2Fs were detected at these genes in *dDP* mutants, confirming that dE2F DNA binding was lost in these animals (Fig. 3C, red bars). RBF1 binding to these genes was eliminated in *dDP* mutant animals, confirming that the recruitment of RBF1 to known E2F targets is completely dependent on E2F complexes.

A potential caveat for this experiment is that dE2F/dDP heterodimers are known to associate with several chromatin-associated proteins and that their loss might cause whole-scale changes to chromatin structure. Indeed, polytene chromosomes of *dDP* mutant animals are abnormally thin and more fragile than wild-type controls (data not shown). To exclude the possibility that the lack of RBF1 ChIP signal was a nonspecific effect, we used chromatin from *dDP* mutants for ChIP with antibodies against a set of proteins involved in transcriptional regulation. Chromatin association of a transcription factor (heat shock factor [HSF]), a subunit of RNA polymerase II (Rpb3), and a chromatin protein (histone H3) were largely unaffected in *dDP* mutants compared to *w1118* controls (Fig. 3D; also data not shown). HSF was strongly enriched at two of its target gene promoters, those for Hsp70 and

TABLE 1 RBF1 and dE2F binding to M-phase genes in *Drosophila* larvae and S2 cells

M-phase gene	Binding by:[a]			
	RBF1 (larvae)	dE2F1 (larvae)	dE2F2 (larvae)	dE2F1 (S2 cells)
Bub1	−	−	+	+
SMC2	+	−	+	+
Nnf1a	−	−	+	+
msd1	+	−	+	+
Mad2	+	−	+	+
msd5	+	−	+	−
Cap-G	−	−	+	+
Hel25E	−	−	+	+
Klp67A	+	−	+	+
polo	+	−	+	+
Spc105R	−	−	+	−
Kmn1	+	−	+	−
Mod(mdg4)	+	−	−	+
neb	+	−	+	+
nod	+	−	+	+
ncd	+	−	+	+
pav	+	−	+	−
spd-2	+	−	+	+
stg	−	+	−	+
sti	+	+	+	+
cdc2c	+	+	+	+
Map60	−	−	−	−
Incenp	+	+	+	+
CG11788	+	−	+	+
mars	+	−	+	+
Sep5	+	−	+	−
FANCI	+	−	+	+

[a] Binding of RBF1, dE2F1, and dE2F2 to mitotic genes in larvae and S2 cells is based on ChIP-chip experiments (this paper; also Korenjak and Dyson, unpublished observations). The chromatin source is given in parentheses. +, binding; −, absence of binding.

HSP83, in *w1118* and *dDP* mutant animals but was undetectable at a control gene (the Act88F gene) and two dE2F target genes (the CG5250 and CycE genes). These results confirm that the lack of RBF1 binding is a specific defect of *dDP* mutant chromatin. We conclude that RBF1 association with dE2F target genes is dependent on the presence of functional dE2F/dDP complexes and is specifically lost in *dDP* mutants.

Second, to extend this analysis to all RBF1 target genes, we performed whole-genome ChIP analysis of RBF1 from *dDP* mutant third-instar larvae. Strikingly, all of the RBF1-binding sites detected in control animals were lost in *dDP* mutants (Fig. 4). This was evident upon visual inspection of the data. When we analyzed the *dDP* mutant binding data using the peak-calling criteria that we previously applied to the *w1118* animals, no RBF1-binding peaks were detected. Indeed, the binding profile for RBF1 on *dDP* mutant chromatin was indistinguishable from the profile generated using the IgG control (data not shown). To verify this result, we randomly picked a set of RBF1-bound promoters from the wild-type animals, performed ChIP using antibodies to RBF/dE2F/dDP proteins, and used qRT-PCR to assess the signal (Fig. 5). RBF1 was bound to all sites in the *w1118* background, but the binding was lost in *dDP* mutant animals (Fig. 5A), confirming the ChIP-chip results. In addition to RBF1, we also detected dE2F and dDP proteins at all tested promoters in *w1118* animals, but these signals were lost in the *dDP* mutants (Fig. 5B to D). Similarly, the binding of RBF2 to these promoters was impaired in *dDP* mutant animals (Fig. 5E).

The efficiency of the ChIP varied, with RBF1-bound genes that were also bound by dE2F1 showing lower levels of RBF1 binding than genes that were mainly associated with dE2F2. A simple explanation for this is that dE2F1-bound genes are likely to be actively transcribed, which means that RBF1 dissociates cyclically from these genes, whereas dE2F2-bound genes are stably repressed (11, 33, 37).

Fractionation experiments confirm that dDP is generally required for RBF1 to associate with chromatin. The results described above suggest that dDP is generally required for RBF1 to associate with chromatin. We sought to confirm this conclusion using a different experimental approach and turned to biochemical fractionation of *Drosophila* third-instar larvae (34). We modified the method by sequentially eluting the isolated nuclei with buffers with increasing stringency in order to fractionate proteins that are more or less tightly associated with chromatin. The fractions were tested for the presence of tubulin and histone H3 as examples of a cytoplasmic protein and a protein tightly associated with chromatin, respectively. Whereas tubulin was found exclusively in the cytoplasm and nuclear elution 1 (NE1), H3 was detected primarily in NE3 and the insoluble nuclear pellet (NP) (Fig. 6), showing that the larval fractionation experiment gave the anticipated results. The presence of tubulin in NE1 suggests that the isolated nuclei still contain a certain percentage of intact cells and that NE2 represents the first entirely nuclear fraction. Tubulin and histone H3 showed similar patterns of fractionation in extracts from *w1118* and *dDP* mutant larvae (Fig. 6, compare lanes 1 to 5 to lanes 6 to 10). dDP was mainly found in the nucleus in *w1118* animals although some protein was also detected in the cytoplasmic fraction (Fig. 6, lanes 1 to 5). As expected, no dDP protein was detected by Western blotting in *dDP* mutant larvae.

This fractionation protocol was used to compare the distribution of RBF1 in wild-type and *dDP* mutant larvae. Because RBF1 is relatively unstable, we used extracts prepared from rbf^{120a}/rbf^{14} transheterozygotes, a hypomorphic combination that expresses <10% of the normal levels of RBF1, as a control to identify authentic RBF1. In control animals we detected full-length RBF1 protein (Fig. 6, arrowhead) in the cytoplasm and in the nucleus, with the nuclear fraction accounting for a large portion of the protein (lanes 2 to 4). In addition we observed a faster-migrating band that resolved into two proteins: a cross-reactive nuclear protein (marked by an asterisk) that was still evident in extracts from *rbf1* mutant larvae and a primarily cytoplasmic protein form of RBF1 that was almost completely absent from the *rbf1* hypomorphs. Strikingly, full-length RBF1 protein was virtually undetectable in the nuclear fractions from *dDP* mutant animals (Fig. 6, NE2 and -3, lanes 8 and 9, respectively). In fact, the amount of nuclear full-length RBF1 in *dDP* mutant animals was well below the levels detected in the *rbf1* hypomorphs. Instead, the *dDP* mutants contain an increased amount of the shortened form of RBF1 that fractionated with cytoplasmic proteins. These observations are highly consistent with the results of the ChIP-chip analysis and suggest that there is little or no RBF1 protein tightly associated with chromatin in *dDP* mutant animals.

dDP is required for RBF1 to bind to promoters that lack a consensus E2F-binding site. About 300 RBF1 sites (14%) lack a recognizable E2F consensus motif (Fig. 3A), but since these rep-

A

	RBF1 sites
Drosophila, biphasic motif [22]	26% (540/2048)
Human, E2F motif [31]	76% (1551/2048)
Drosophila, E2F motif [22]	86% (1757/2048)

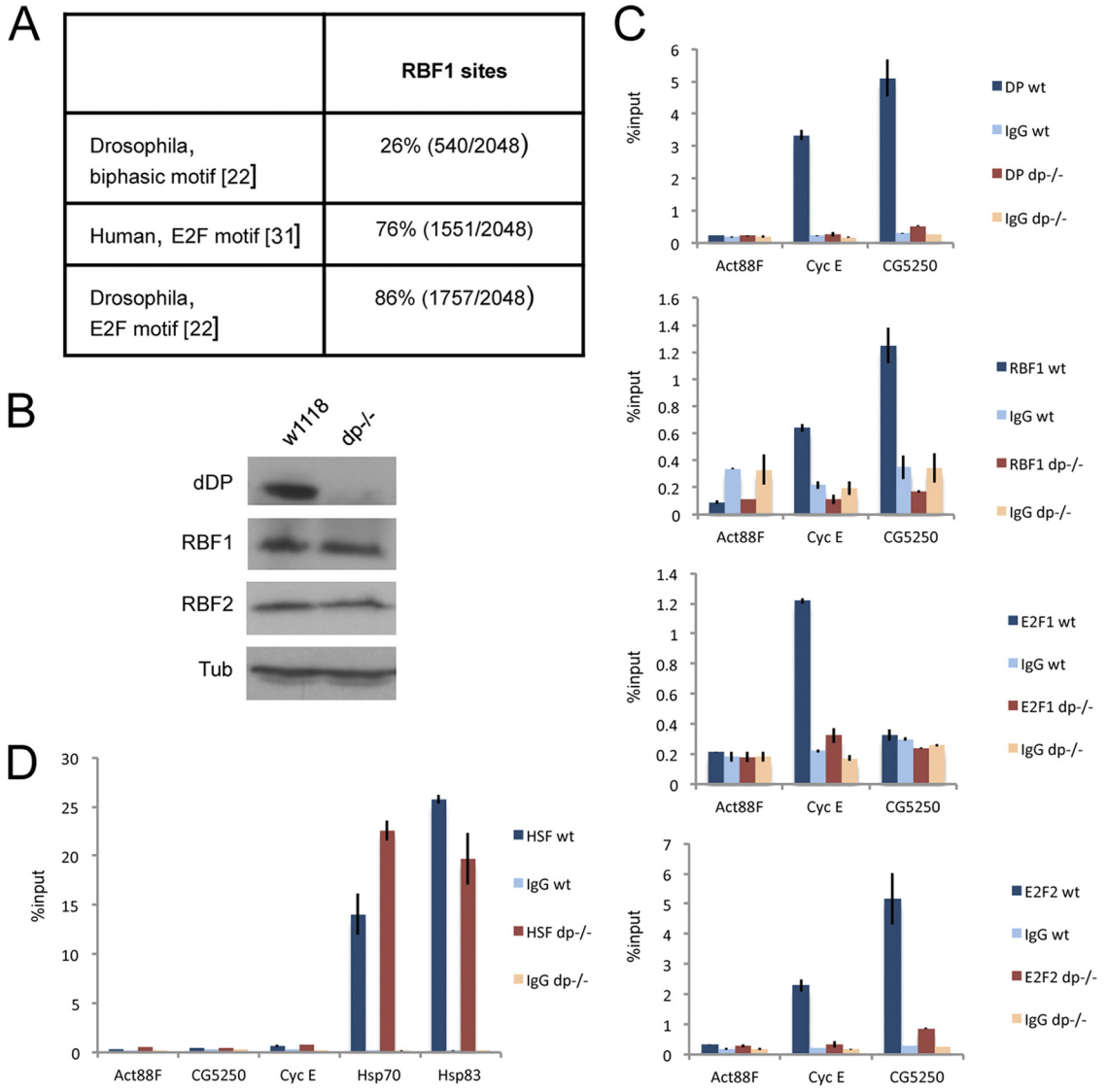

FIG 3 Mutation of *dDP* results in loss of RBF1 binding to dE2F target genes. (A) A subset of RBF1-binding sites lacks an E2F consensus motif. Position weight matrices for E2F consensus motifs with various stringencies were used to search RBF1-binding regions for the presence of these motifs (*Drosophila*, biphasic motif > human, E2F motif > *Drosophila*, E2F motif). Percentages of sites containing an E2F consensus motif are given for each stringency level. Numbers in brackets indicate references in which the corresponding motifs were identified. Values in parentheses indicate the numbers of sites containing a motif compared to all sites. (B) dDP protein is lost in *dDP* mutant animals. Western blotting from larval extract using the indicated antibodies confirmed the loss of dDP in *dDP* mutant animals, whereas the levels of RBF1 and RBF2 were unaffected. Tubulin (Tub) was used as a loading control. *w1118*, wild-type animals. (C) RBF1, dDP, and dE2F binding to E2F target genes is lost in *dDP* mutant animals. ChIP-qPCR was performed using antibodies against dDP, RBF1, and dE2F proteins. Association of all proteins with the promoters of the dE2F target CycE and CG5250 genes in *w1118* animals (wt) was lost in a *dDP* mutant background ($dp^{-/-}$). IgG was used for control ChIP in both backgrounds. Act88F served as a negative control. The average of three independent experiments is shown. (D) *dDP* mutation does not affect the chromatin binding of heat shock factor. ChIP-qPCR using an antibody against HSF revealed no change in binding to two of its target genes (the Hsp70 and Hsp83 genes) between *w1118* (HSF wt) and *dDP* mutant (HSF $dp^{-/-}$) animals. IgG was used for control ChIP in both backgrounds. HSF did not bind to CycE, CG5250, and Act88F. The average of three independent experiments is shown.

resent a minority of the binding sites, it is possible that the pool of associated RBF1 behaves differently from the bulk of RBF1. Such a small subpopulation of protein might easily be missed in fractionation experiments. It is further possible that the loss of dDP indirectly affects the binding of RBF1 to these sites by influencing other DNA-binding proteins. To test whether dDP is directly required for RBF1 to associate with these "non-E2F" sequences, we selected a random set of six genes that were bound by RBF1 in the array data but lacked an E2F-binding motif, and we performed ChIP followed by qRT-PCR on chromatin from *w1118* and *dDP* mutant larvae. Strikingly, dDP and dE2F2 bound at the promoters of the tested genes, and this binding was absent, along with binding of RBF1, in *dDP* mutant animals (Fig. 7A). These results suggest that dE2F/dDP/RBF complexes can get recruited to chromatin in the absence of a classical E2F motif. This might occur through either the direct binding of dE2F/dDP to nonclassical E2F sites or the indirect recruitment of these complexes through interaction with other DNA-binding proteins.

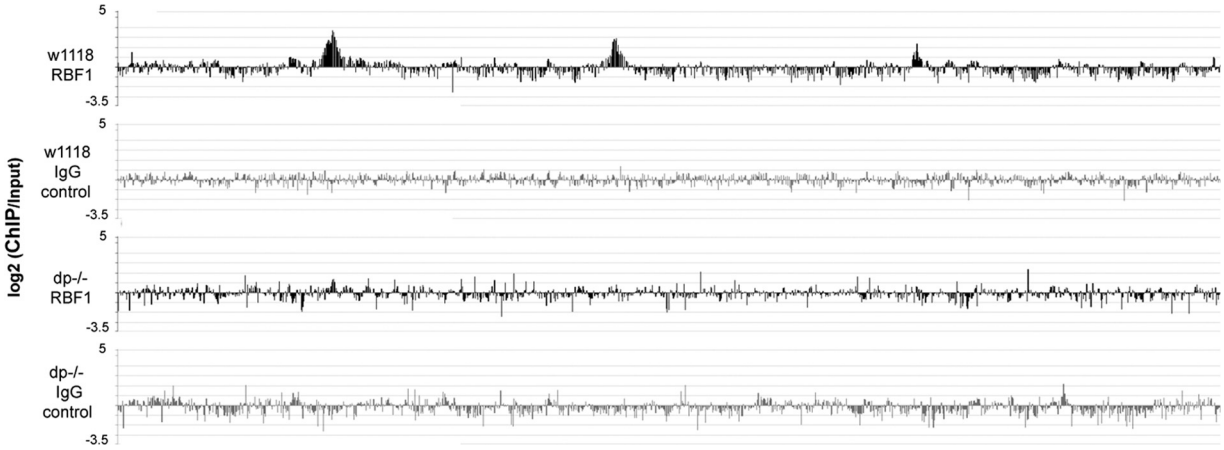

FIG 4 Genome-wide RBF1 chromatin association is abolished in *dDP* mutant animals. RBF1 binding to a region on chromosome 3L in *w1118* and *dDP* mutant animals. Specific RBF1 binding is represented by prominent peaks in *w1118* animals. This binding is abolished in the *dDP* mutant background. Very weak binding was sometimes retained in regions with the highest peaks in the wild-type animals (leftmost peak). The overall binding profile obtained from *dDP* mutant animals is indistinguishable from the profile obtained using IgG in the ChIP-chip experiments (compare $dp^{-/-}$ RBF1 with $dp^{-/-}$ IgG control).

These findings prompted us to search for sequence motifs that frequently cooccur with dE2F binding and that might serve as predictors for binding in the absence of E2F sites. AMADEUS motif-finding software (38) was used to search the promoters of RBF1-bound genes with or without a recognizable E2F consensus motif for enriched sequence patterns. As expected, the consensus E2F site was the most highly enriched sequence motif found in the set of genes that harbor E2F motifs (Fig. 7B, motif 1) but was not present in genes that lack an E2F motif. We identified two additional sequence motifs that strongly correlated with RBF1-binding sites. These motifs were highly similar to the DNA replication element (DRE) and the cell cycle gene homology region (CHR) (Fig. 7B, motifs 2 and 3). Intriguingly, the presence of these motifs correlated with RBF1 binding in both the presence and absence of recognizable consensus E2F sites (Fig. 7B and C), suggesting that factors that bind to these sites may help either to recruit or bind dE2F/dDP/RBF complexes. Regardless of whether the E2F/DP proteins are recruited directly to DNA or recruited by interaction with other DNA-binding proteins, at all of the RBF1-binding sites that we have detected in this study, the stable association of RBF1 with chromatin is dependent on dDP.

DISCUSSION

The *Drosophila* RBF and E2F families lack the complexity of their mammalian counterparts, and the distribution of RBF1- and dE2F-binding sites described here gives a snapshot of the RB-E2F network in an *in vivo* context. The results show that RBF1 acts at >2,000 sites in the *Drosophila* genome and, in accordance with the idea that RB family proteins regulate gene expression, that most of these binding sites are close to promoter regions.

RBF1-bound regions overlap extensively with binding sites for dE2F2. Additionally, RBF1 is a component of dREAM/Myb-MuvB complexes, and a large percentage (74%) of RBF1-bound regions correspond to dREAM/Myb-MuvB-binding sites (22). In contrast, only a relatively small proportion of the regions that were bound by RBF1 were also bound by dE2F1. These findings are consistent with the idea that dE2F2-RBF1 repressor complexes are present throughout the genome, with their effects being transiently reversed by dE2F1 at a small subset of these targets in proliferating cells (20). One of the implications of this model is that different subsets of RBF1-repressed genes may be activated in different cellular contexts. In support of this, we note that GO classification of RBF1-bound promoters showed enrichment for several functional categories that were absent from the lists of dE2F1-bound genes (Fig. 1C), and potentially these targets may represent groups of RBF1-repressed genes that may be activated by dE2F1 only under specific circumstances or that may be activated by factors that are different from dE2F1. Since E2F-mediated activation can result from the transient presence of dE2F1, a caveat to this interpretation is the likelihood that RBF1 proteins that are components of a stable repressor complex will be more easily detected by ChIP than a transiently bound dE2F1 activator, and it is probable that our list of dE2F1-bound promoters underestimates the overall number of dE2F1 targets. Interestingly, we found that dE2F1 target genes with functions in mitosis were conspicuously underrepresented in the lists of genes bound by dE2F1 in larvae, even though they were bound by RBF1 and dE2F2, and that they were bound by dE2F1 when chromatin prepared from S2 tissue culture cells was profiled. This difference is consistent with the fact that many larval cells grow via endocycles and do not need to express mitotic genes, but it also suggests that dE2F1 binds to different sets of promoters in mitotically dividing cells and endoreduplicating tissue. Although dE2F1 is thought to be a ubiquitous driver of cell proliferation, these results suggest that different programs of dE2F1-activated transcription are used in different cell types.

We have used this experimental system to address a key issue in E2F/RB research: how much of the RB-protein that is found on chromatin is bound via E2F, and how much is bound in an E2F-independent manner and might potentially have E2F-independent functions? This has been a long-standing question, in part because pRB has been shown to associate with many different chromatin-associated proteins and in part because mammalian cells express many different E2F complexes, with the result that it is difficult to dissect the relative contributions of these complexes on pRB chromatin association. The genome-wide binding studies for pRB family members that have been carried out in mammalian cells show an enrichment of E2F consensus sequences in the re-

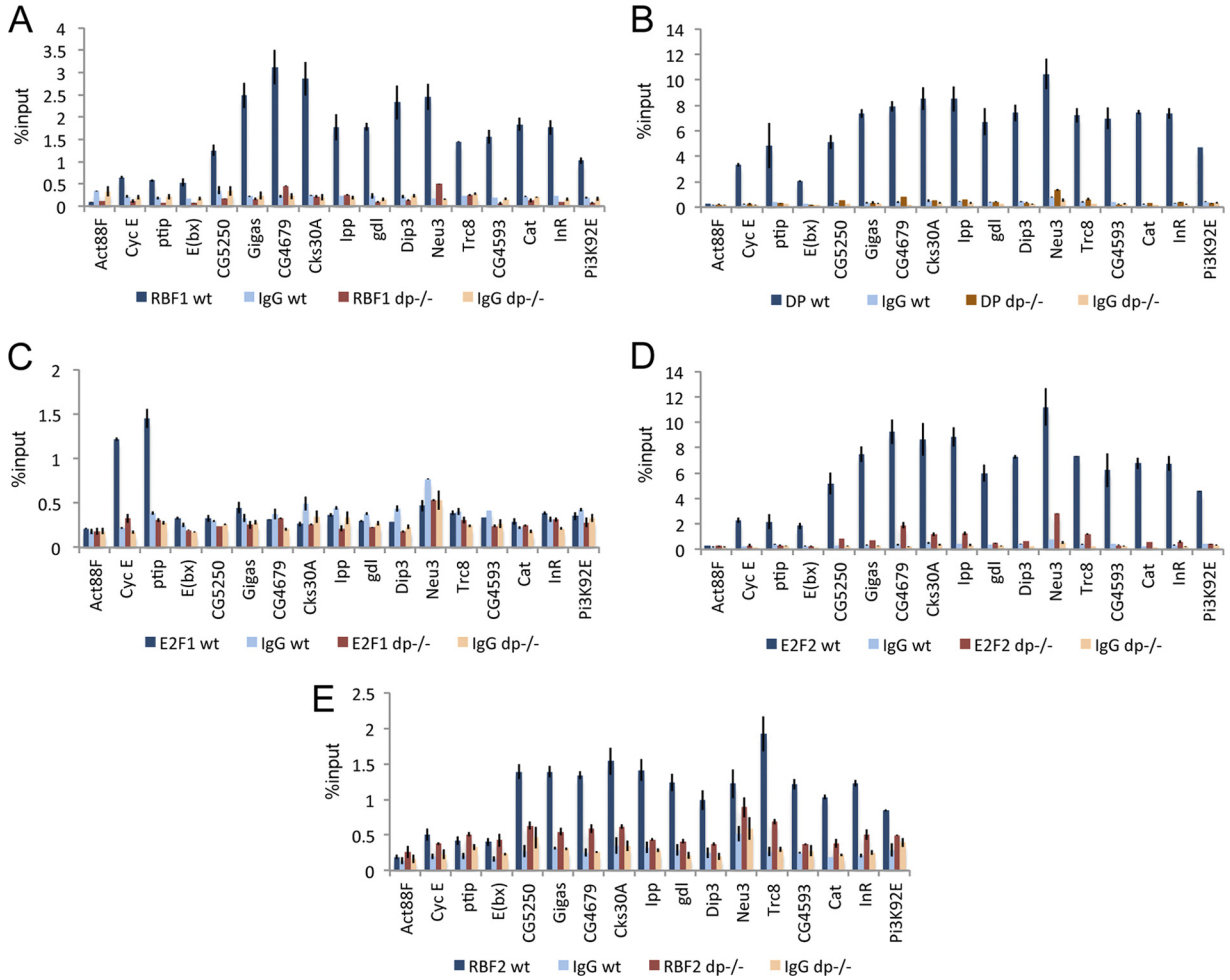

FIG 5 Binding of RBF-dE2F family proteins to target gene promoters is lost in *dDP* mutant animals. Chromatin binding of RBF, dDP, and dE2F proteins is abolished in *dDP* mutant animals. ChIP-qPCR using antibodies against RBF1 (A), dDP (B), dE2F1 (C), dE2F2 (D), and RBF2 (E) for a set of randomly chosen RBF1-binding sites revealed complete loss of binding in *dDP* mutants (red bars) compared to wild-type animals (dark blue bars). IgG was used for control ChIP in both backgrounds. The occasionally observed residual binding is in most cases less than 10% and might be due to cross-reactivity of the antibody. The average of three independent experiments is shown.

gions bound by pRB, p107, and p130 (2, 8, 39), but it has been unclear whether these motifs are essential for the pocket proteins to associate with chromatin and whether the E2F-binding site represents either the sole genomic location, the main location, or one of many potential locations for the set of pRB family proteins. Previous studies have examined the distribution of pRB family members in cultured cells, and it is unclear how accurately the distributions seen in cell lines reflect the distribution of these proteins in normal tissues.

In the experiments described here, our ability to detect chromatin-associated peaks of RBF1 by ChIP was absolutely dependent on dDP. Consistent with this, most RBF1-bound regions contained a sequence corresponding to a consensus E2F-binding site, but this dependency on dDP was also seen in regions that lacked an obvious E2F-binding motif. In agreement with the ChIP results, in cell fractionation experiments we failed to detect full-length RBF1 tightly associated with chromatin in nuclear extracts from *dDP* mutant larvae. The simplest interpretation of our data is that the stable association of RBF1 with chromatin in *Drosophila* larvae is completely dependent on its recruitment by dE2F/dDP complexes.

This finding is surprising given the number of reports that have proposed that RB family proteins have E2F-independent functions (1, 23, 40, 57, 59, 62). There are several potential explanations for this paradox. First, the experiments described here provide a picture of the general properties of RBF1 in larval chromatin but do not exclude the possibility that RBF1 has E2F-independent targets in specific subsets of cells or at other stages of animal development. We note that loss of RBF1 causes defects in chromosome compaction in neuroblasts that were not seen in *de2f1*, *de2f2*, or *dDP* mutants (40). This role might not require a stable association of RBF1 with chromatin, or alternatively there may be types of RBF1-DNA complexes that were not detected using our ChIP conditions and were disrupted by the fractionation procedure.

A second possibility is that E2F proteins play a larger role in the differentiation-specific functions of pRB than has been appreciated. For example, the fact that pRB and Runx2 colocalize at os-

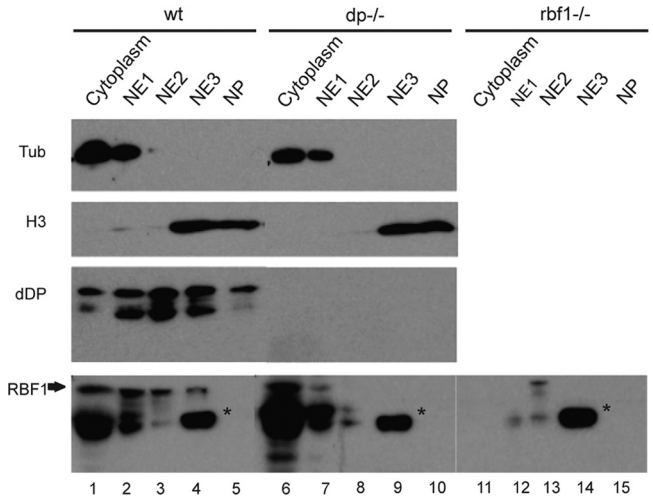

FIG 6 RBF1 localizes primarily to the cytoplasm in *dDP* mutant animals. *Drosophila* third-instar larvae were fractionated into cytoplasmic extract and nuclei. The isolated nuclei were successively extracted with buffers with different stringencies to consecutively wash off proteins with increasing chromatin affinity (NE1, NE2, and NE3). Finally, the insoluble pellet (NP) was resuspended in SDS-PAGE sample loading buffer. The fractionation experiments were carried out from wild-type (wt), *dDP* mutant ($dp^{-/-}$) and *rbf1* mutant ($rbf1^{-/-}$) larvae. The fractions were analyzed by Western blotting for the presence of the indicated proteins. The arrow indicates the band corresponding to full-length RBF1. The asterisks in the RBF1 panel indicate a cross-reacting band because it does not disappear in the *rbf1* mutant. Since the extraction profile of this protein does not change in *dDP* mutant animals, showing that it is still tightly associated with chromatin in the mutant, it is highly unlikely that it was picked up in the ChIP-chip analysis.

teoblast-specific promoters during osteogenic differentiation and the fact that Runx2 is required for pRB to be recruited to these promoters in Saos-2 cells (62) do not exclude the possibility that E2F proteins are also involved in this process. A recent study demonstrated that the osteogenesis defects resulting from the conditional deletion of murine *Rb* can be suppressed by the combined inactivation of E2F1 (4). An alternative possibility is that some of the differentiation defects seen when pRB family members are inactivated are due primarily to problems in establishing permanent cell cycle exit rather than to a direct role for pRB in differentiation.

A third scenario is suggested by the observation that pRB may act in some contexts by blocking the function of inhibitors of differentiation, such as EID-1, RBP2, and Id2 (3, 29, 30, 41, 42). Id2 is targeted for degradation by the anaphase-promoting complex/cyclosome (APC/C) upon cell cycle exit. Interestingly, during cell cycle exit pRB promotes a physical interaction between the APC/C and Skp2 that results in the targeted degradation of Skp2 (6). Although such activities are not E2F dependent, it is possible that they normally occur in the vicinity of E2F-recruited complexes. Alternatively, they might not occur in the context of chromatin at all.

Fourth, almost all of the E2F-independent functions of RB family proteins have been discovered using mammalian cells, and it is possible that there is a basic difference in the roles of these proteins between flies and mammals. Perhaps during evolution mammalian pRB has acquired an ability to interact with additional proteins, while the *Drosophila* orthologs have remained specific partners of dE2Fs.

Our results provide a cautionary note to the use of consensus E2F-binding motifs. Consensus E2F-binding sequences are widely used to predict sites of E2F regulation. However, human E2F proteins bind to a large number of promoter regions (2, 5, 32, 35, 39, 52, 68), and many of these regions do not harbor classical E2F consensus sequences (5, 51). Approximately 15% of RBF1-binding sites lacked a clear E2F-binding motif. Further analysis of examples of these promoters showed that dE2F/dDP proteins could be detected at these regions, and, moreover, analysis of *dDP* mutant larvae showed that these complexes were essential for the recruitment of RBF1.

These observations raise the question of whether additional sequence motifs (and binding proteins) mediate or assist the binding of E2F complexes to DNA. As a component of dREAM/Myb-MuvB complexes, RBF1 is associated with proteins such as dMyb and Mip120 that have known DNA-binding activities, and recent work suggests that several dREAM/Myb-MuvB subunits may assist the binding of dE2F2 and RBF1 to E2F target genes (36). In contrast to a previous study, which identified a consensus DNA-binding site for Myb in a small subset of dREAM/Myb-MuvB-binding sites (22), we failed to detect an enrichment of this motif in dE2F/RBF-bound regions (data not shown). However, a sequence motif with similarity to the CHR element was significantly enriched at dE2F/RBF-binding sites. CDE/CHR elements have recently been linked to the sequence-specific DNA binding of the human and *Caenorhabditis elegans* Lin54 proteins, which are the orthologs of Mip120 (55, 60). Moreover, human DREAM binds the *cyclin B2* promoter preferentially through the CHR but not the CDE element (44). Given that the majority of E2F binding in *Drosophila* appears to be in the context of dREAM/Myb-MuvB, CHR elements may play an important role in dE2F DNA binding and be useful in predicting functional E2F-binding sites.

Interestingly, dE2F/RBF-bound promoters that lack an E2F consensus motif show a strong enrichment for a sequence resembling the DNA replication element (DRE). DREs are often found in core promoter sequences in *Drosophila* and are bound by the DNA replication element-binding factor (DREF) (26, 49). dE2F and DREF regulate the expression of an overlapping set of genes involved in DNA replication, and both proteins are required for cell proliferation *in vivo* (17, 28, 54). DREF forms a complex with TATA box-binding protein-related factor 2 (TRF2) that regulates the expression of several E2F target genes, including the PCNA gene (27). In the PCNA promoter, the DRE was found to be required, in addition to an E2F-binding site, for dE2F1/dDP-dependent transcriptional activation. It seems likely, therefore that, at some promoters, DRE/DREF facilitates the binding and action of dE2F/RBF1 complexes.

Despite the presence of these additional motifs and their potential role in dE2F/dDP DNA binding, the complete lack of RBF1 binding to chromatin in *dDP* mutants shows that no additional proteins can compensate for the absence of dE2F/dDP in *Drosophila* larvae and allow the recruitment of RBF1 to chromatin. This suggests that RBF1 and dE2F/dDP functions are intimately linked in *Drosophila*. In support of this, larval lethality due to the homozygous inactivation of *rbf1* can be rescued by a mutation in dE2F1 that deletes sequences in the RBF1 binding and transactivation domains (12). The binding of Mip130, another dREAM/Myb-MuvB subunit, to polytene chromosomes was only marginally affected in *dDP* animals (data not shown), suggesting that submodules of dREAM/Myb-MuvB complexes are recruited to

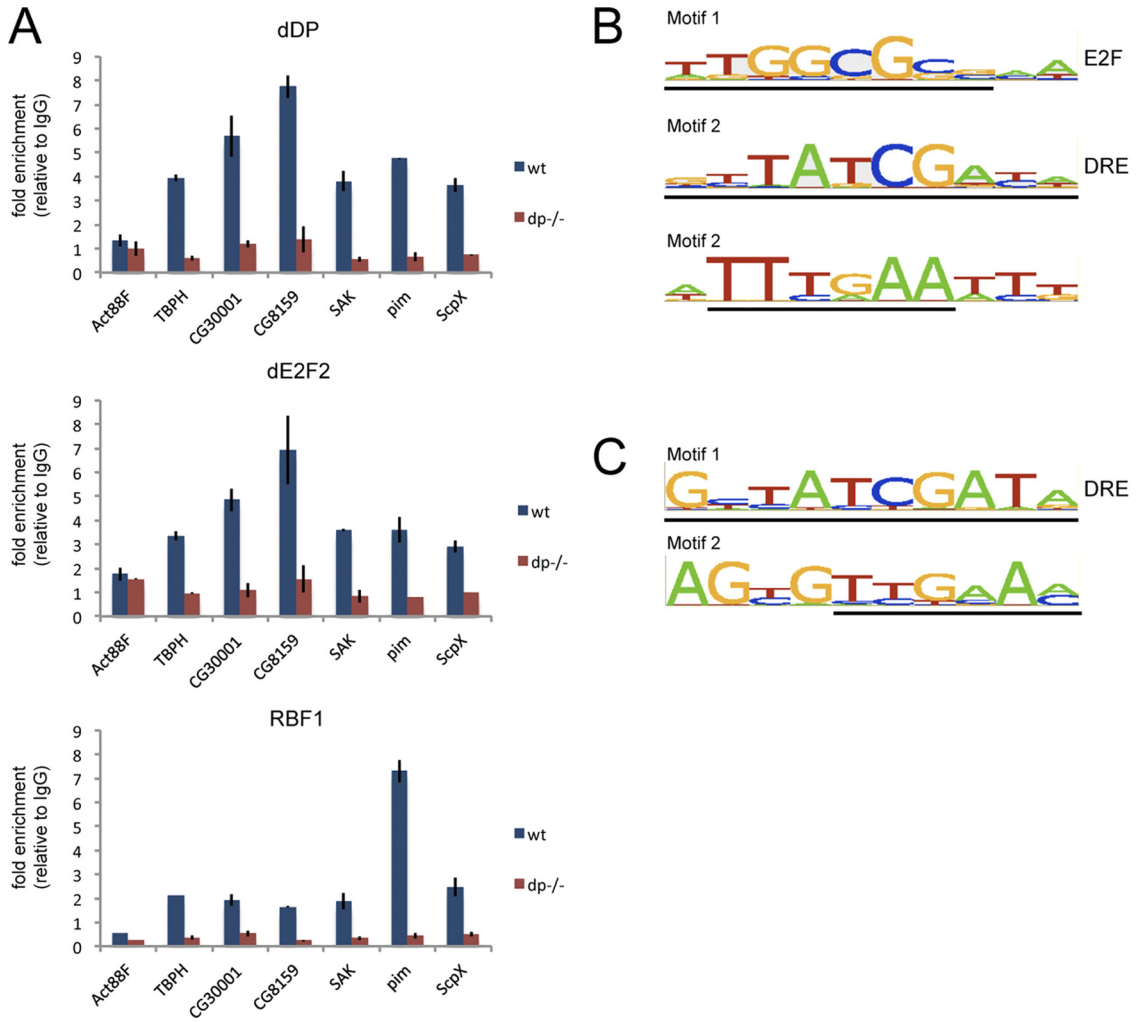

FIG 7 RBF1-binding sites lacking consensus E2F motifs are dDP dependent. (A) ChIP-qPCR using antibodies against dDP, dE2F2, and RBF1 for a set of randomly chosen RBF1-binding sites that lack consensus E2F motifs. ChIP was performed from wild-type and *dDP* mutant larvae. All tested genes were bound by dDP, dE2F2, and RBF1 in wild-type animals, whereas binding was completely lost in mutant animals. dE2F1 and RBF2 were not found to be enriched at these genes (data not shown). Act88F served as a negative control. The average of three independent experiments is shown. (B and C) The AMADEUS motif-finding software (38) was used to search the promoters of RBF1-bound genes for enriched sequence motifs. RBF1-binding sites were divided into two groups based on the presence (B) or absence (C) of a consensus E2F motif. Panel B shows enriched sequence motifs at RBF1 sites harboring consensus E2F motifs. Three highly enriched sequence motifs were identified. Motif 1 corresponds to the consensus E2F-binding motif (TTTSGCGC), motif 2 resembles the DNA replication element (DRE) (WATCGATW), and motif 3 displays strong similarity to the cell cycle gene homology region (CHR) element [TT(T)GAA]. S is G or C; W is A or T. The respective motifs are underlined. (C) Enriched sequence motifs at RBF1 sites lacking consensus E2F motifs. As expected, a consensus E2F motif was not found. DRE (motif 1) and CHR-like elements (motif 2) were highly enriched at this set of RBF1-binding sites.

chromatin independent of one another. Our results are consistent with the idea that protein-protein interactions with dREAM/Myb-MuvB subunits and DREF may help to recruit dE2F/dDP/RBF complexes to promoters, and this may be especially relevant at promoters that lack a consensus E2F motif; but even in this situation, the presence of RBF1 at these promoters is completely dependent on dE2F/dDP complexes. When it becomes possible to completely inactivate all of the different classes of E2F complexes in mammalian cells, it will be interesting to learn how this finding compares with the recruitment of pRB, p107, and p130 to chromatin in humans.

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