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A Novel Repressive E2F6 Complex Containing the Polycomb Group Protein, EPC1, That Interacts with EZH2 in a Proliferation-specific Manner*

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The transcriptional repressor E2F6 has been identified as a component of two distinct polycomb group protein (PcG)-containing complexes, suggesting a mechanism for the recruitment of repressive complexes to target sequences in DNA. Whereas one complex is involved in the repression of classic E2F target genes in G₀, a role for E2F6 within the cell cycle has yet to be defined. We searched for novel E2F6-binding proteins using a yeast two-hybrid screen and identified the PcG protein, EPC1. We showed that, both *in vitro* and *in vivo*, E2F6, DP1, and EPC1 form a stable core complex with repressive activity. Furthermore, we identified the proliferation-specific PcG, EZH2, as an EPC1-interacting protein. Using affinity purification, we showed that E2F6, DP1, EPC1, EZH2, and Sin3B co-elute, suggesting the identification of a novel E2F6 complex that exists *in vivo* in both normal and transformed human cell lines. EZH2 is required for cellular proliferation and consistent with this, EZH2 elutes with the E2F6-EPC1 complex only in proliferating cells. Thus we have identified a novel E2F6-PcG complex (E2F6-EPC1) that interacts with EZH2 and may regulate genes required for cell cycle progression.

The transcription factor E2F6 differs structurally and functionally from the other E2F family members (E2Fs 1–5), in that it lacks both the retinoblastoma protein binding and transcriptional activation domains, and is an active repressor of transcription (1–3). However the role of E2F6 in cell growth and proliferation remains unclear. Overexpression in U2OS cells leads to an accumulation of cells in S phase, either by inhibition of progression through, or inhibition of exit from, S phase (1). In contrast, asynchronously growing NIH 3T3 cells are unaffected by E2F6 overexpression, although in quiescent NIH 3T3 stimulated to exit G₀, exogenous E2F6 inhibits entry into S phase (4).

E2f6-null mice are healthy and viable, although they display

homeotic transformations of the axial skeleton indicating a requirement for E2F6 in developmental patterning (5). Such skeletal transformations are also seen in some polycomb group protein (PcG)¹-deficient mice (Bmi1, M33, MEL-18, and Ring1A) (6–11), consistent with E2F6 being identified as a component of PcG-containing complexes.

The PcG proteins are best known for their role in the stable repression of homeotic (Hox) genes during *Drosophila* development, and thus in correct body pattern formation. PcG mutants therefore exhibit posterior transformations caused by the de-repression of these loci (12). However, PcG proteins are also involved in the regulation of the cell cycle and in lymphopoiesis. Proliferation defects are seen in some cell types following the targeted disruption of *Bmi1*, *mel18*, and *mph1*, and *Ezh2* has now been shown to be essential for cellular proliferation (10, 11, 13–15).

How PcG complexes are targeted to specific DNA sequences remains unclear. In *Drosophila*, binding is mediated by DNA fragments termed polycomb response elements (PREs). However, PREs are several hundred base pairs in length, and thus far only one specific DNA binding sequence has been identified within a PRE (that of the *Drosophila* protein Pleiohomeotic (Pho), a homologue of the mammalian transcription factor YY1) so the precise mechanism of targeting remains uncertain (16). In higher eukaryotes, PcGs are assumed to associate with promoters through binding to sequence specific transcription factors, such as YY1 and E2F6 (17–19), although PcG proteins have not yet been demonstrated to associate with promoters regulated by these factors.

In both *Drosophila* and mammalian cells, two distinct PcG protein complexes have been described; the PRC2 complex, involved in the initiation of gene repression, and the PRC1 complex, thought to be required for the continued maintenance of repression once the correct gene expression patterns have been established (20). The PRC2 complex contains the PcG proteins EZH2 and EED (21–23), whereas the PRC1 complex contains BMI1, HPC, and HPH (9, 24).

E2F6 is known to associate with members of the PRC1 complex (19). This complex (E2F6-PRC1) contains some PRC-complex PcG proteins (Ring1, MEL-18 and mph1) as well as the Ring1- and YY1-binding protein (RYBP). Recently, another E2F6-PcG complex (E2F6-G₀) was described, containing sev-

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¹ The abbreviations used are: PcG, polycomb group protein; CAT, chloramphenicol acetyltransferase; PBS, phosphate-buffered saline; DTT, dithiothreitol; GST, glutathione S-transferase; HA, hemagglutinin; Y2H, yeast two-hybrid assay; EPC, enhancer of polycomb; AEBBSF, 4-(2-aminoethyl)-benzene sulfonyl fluoride hydrochloride.

eral novel PcG proteins (17). From this complex, E2F6, Max, and HP1 γ were found associated with E2F target promoters in G₀, but not following re-entry into the cell cycle, suggesting an involvement of this complex in gene repression in quiescent cells. However E2F6 is expressed in all stages of the cell cycle, implying a functional role beyond that in G₀.

Enhancer of polycomb (EPC) is an unusual member of the PcG family. While EPC mutations in *Drosophila* are homozygous embryonic lethal, heterozygous mutations do not by themselves result in a zygotic homeotic phenotype. Rather, mutations in EPC enhance mutations in the polycomb (*Pc*) gene (25, 26). To date, there are no reports of EPC in PcG complexes, although it has been identified as a component of the p400/NuA4 and Tip60 complexes, which possess histone acetyltransferase (HAT) activity (27, 28). Within these complexes, EPC1 is thought to be required for global acetylation (29), and while it does not have enzymatic activity, EPC1 has been reported to possess both activating and repressive activities. Consistent with this, human EPC1 is also known to interact with the transcriptional repressor RET finger protein, RFP (similar to RING1) (30).

Here we describe the identification of a novel E2F6 complex. We show that E2F6, DP1, and EPC1 form a complex that can associate with EZH2 via binding to EPC1, and that this occurs only in proliferating cells. This is consistent with the role of EZH2 in cellular proliferation and thus suggests a potential role for E2F6 within the cell cycle. Furthermore, we show that E2F6 and EPC1 repress activity from a reporter containing 4 E2F sites, and that a mutant of E2F6 that is unable to bind EPC1 loses this repressive activity. This study therefore shows that E2F6 can associate with members of both the PRC1 and PRC2 complexes, and suggests a role for E2F6 in regulating cell cycle progression via transcriptional repression.

MATERIALS AND METHODS

Yeast Two-hybrid Screen

Yeast strains, methods, and plasmids pPC97 (DNA binding domain) and pPC86 (activation domain) are described elsewhere (31). Briefly, full-length human E2F6 (1) was cloned into pPC97 and used as bait, and a mouse fibroblast library, cloned into pPC86, was used as prey. Approximately 1×10^6 clones were screened using two-step selections as previously described (32). From the resulting clones, plasmid DNA was prepared and sequenced.

Directed Yeast Two-hybrid (Y2H) Assay

For the directed Y2H assay, cDNAs coding for the open reading frames (ORFs), or deletion mutants thereof, of E2F6 and EPC1 were cloned into the pPC97 yeast expression vector (bait constructs), and the full-length ORFs of EED, EZH2, and EPC1 were cloned into the pPC86 vector (Prey). For EED, we used the shorter cDNA, giving rise to the two faster migrating forms, which are incorporated into both the PRC2 and PRC3 complexes (33, 34). To test for interaction, constructs were co-transformed into the yeast two-hybrid reporter strain Mav203 and selected using two-step selection (32).

Generation of Antibodies

Mouse monoclonal antibodies against E2F6, DP1, EPC1, EZH2, and EED, and rabbit polyclonal antibodies against E2F6, EPC1, and EZH2 were generated by standard procedures and are available on request.

Tissue Culture, Transient Transfections, and CAT Assays

Cell lines were grown according to standard procedures. U2OS cells were transiently transfected using the calcium phosphate co-precipitation method as described (35). Cells were washed twice in PBS and harvested into E1A lysis buffer (ELB: 50 mM HEPES, pH 7.5, 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 1 mM DTT, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin).

To assay for activity of the E2F-driven CAT reporter construct, cells were transfected with the reporter construct and combinations of pCMV-E2F6 (or mutants thereof), pCMV-mycEPC1 and pCMV-HAEZH2. Cells were also co-transfected with pCMV- β Gal to normalize

the reporter activity. 48 h after transfection, cells were washed three times in PBS and incubated in TEN buffer (40 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl) for 5 min at room temperature. Cells were scraped into microcentrifuge tubes and spun at 13,000 rpm for 1 min at 4 °C. The pellet was resuspended in 0.25 M Tris-HCl, pH 8.0, and subjected to 3 rapid freeze/thaw cycles, vortexing vigorously after each thaw cycle. After the final thaw cycle, lysates were split into two, and assayed either for CAT or β -galactosidase activity. For CAT assays, lysates were incubated with [¹⁴C]chloramphenicol, *N*-butyryl coenzyme A and 0.25 M Tris-HCl, pH 8.0 for 1 h, the reaction terminated by the addition of mixed xylenes, and the upper phase extracted by phase separation with 0.25 M Tris-HCl, pH 8.0. A fixed volume of the extracted phase was counted in a scintillation counter using an appropriate scintillation fluid. For β -galactosidase assays, lysate was incubated in Z buffer, pH 7.0 (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM Mg₂SO₄, 4 μ l/ml β -mercaptoethanol) with ONPG buffer (4 mg/ml *O*-nitrophenyl- β -D-galactopyranoside in 100 mM PO₄ buffer, pH 7.0) for 30 min at 30 °C. The reaction was terminated by the addition of 1 M Na₂CO₃ and the absorbance measured at 420 nm. Each CAT reporter assay reaction was normalized against the corresponding β -galactosidase assay to give the relative activity from each sample. Transfections were repeated in triplicate and the average of each is shown with standard deviations.

In Vitro Binding Experiments

In vitro translation was carried out using pBSK-EPC1 and the Promega TNT® quick-coupled transcription/translation systems according to the manufacturer's instructions. For *in vitro* binding assays, 10 μ g of GST alone, GST-E2F6, or GST-E2F6 mutants were incubated with 50 μ l of IVT EPC1 in ELB buffer. GST proteins were immunoprecipitated, washed six times in ELB buffer and separated by SDS-PAGE. Gels were stained, destained, soaked in Amplify (Amersham Biosciences), dried, and exposed to autoradiographic film.

In Vivo Labeling

For labeling with ³⁵S, U2OS cells transfected with combinations of pCMV-HAE2F6, pCMV-DP1, and pCMV-mycEPC1 were incubated in 4 ml of methionine/cysteine-free media, containing 0.5mCi of ³⁵S-ProMix per 10-cm plate, at 37 °C for 3 h prior to harvesting. Cells were washed in PBS, harvested, and lysed in ELB for immunoprecipitation. Immunoprecipitations were carried out as described below, washed three times in ELB buffer and loaded onto SDS gels for electrophoresis. Gels were fixed, incubated in Amplify and dried on a gel drier. Dried gels were exposed to autoradiographic film overnight, or as required, at -80 °C.

Immunoprecipitation and Immunoblotting

For U2OS and WI38, cells were resuspended in ELB and sonicated briefly prior to centrifugation. Lysates were quantified and used for immunoprecipitation or Western blotting. For exogenous protein, 250 μ g of whole cell lysate was used per immunoprecipitation. For endogenous proteins, 1 mg of lysate was used.

For 293T and TIG3 cells, 1 mg of nuclear extract was resuspended in Buffer A (50 mM HEPES pH 7.6, 250 mM NaCl, 10% glycerol, 0.5% Triton X-100, 1 mM EDTA, 1 mM DTT, 5 mM NaF, 1 mM AEBSF, and 10 μ g/ml aprotinin and leupeptin). Monoclonal antibodies used for immunoprecipitation and Western blotting were: 9E10 (α -Myc), 12CA5 (α -HA), TFE61 (α -E2F6), D19 (α -EPC), TFD10 (α -DP1), AC22 (α -EZH2), and AA19 (α -EED), and C2 (α -Actin, Santa Cruz Biotechnology). Polyclonal antisera used for immunoprecipitation and Western blotting were: M27/SI243 (α -E2F6), SI26 (α -EPC1), and SI246 (α -EZH2) plus AK12 (α -Sin3B); C20 (α -YY1); C17 (α -Max), and C19 (α -cyclin A) all from Santa Cruz Biotechnology, and K320 (α -ring1) a kind gift from A. Otte.

Fractionation

Superose 6 Chromatography—Isolated nuclei were lysed in Buffer A. Up to 1 mg of nuclear protein was fractionated on a Superose 6 HR 3.2 column (Amersham Biosciences) equilibrated with Buffer A. Fractions of 100 μ l were collected at a flow rate of 50 μ l/min. Fractions were precipitated with acetone (for Western blotting) or were immunoprecipitated with M27 (E2F6) antibody, separated by SDS-PAGE and analyzed by Western blotting. Size of nuclear complexes was determined by comparison to known gel-filtration grade molecular mass markers. Because of the lack of a suitable large size marker, the void volume and mass were estimated from the manufacturer's specifications.

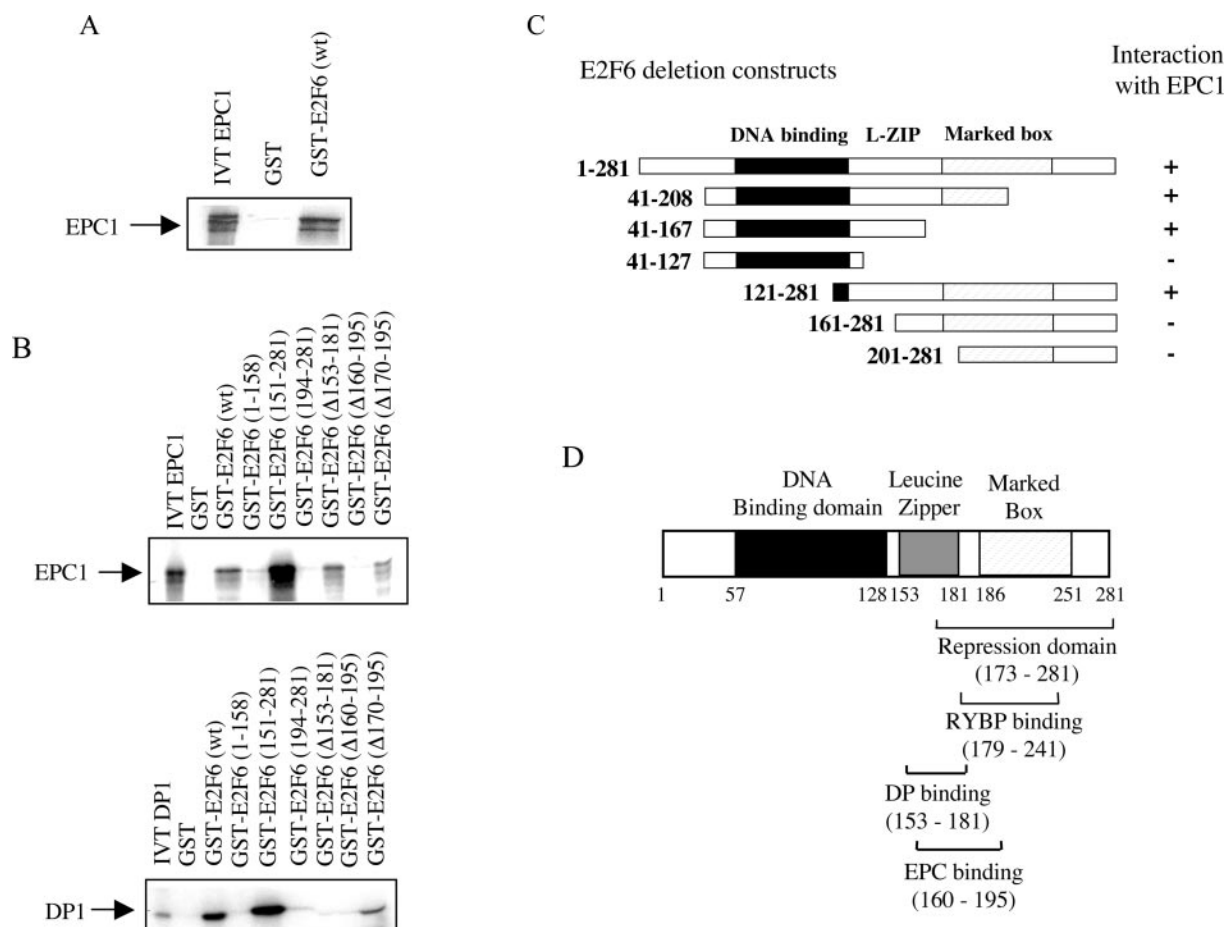


FIG. 1. **E2F6 binds EPC1 *in vitro*.** **A**, full-length GST-E2F6 binds *in vitro* translated EPC1. **B**, EPC1 and DP1 bind to E2F6 via similar but distinct domains *in vitro*. Amino acids 160–195 of E2F6 are required for binding to EPC1, and a similar region, amino acids 153–181, is required for E2F6 binding to DP1. **C**, E2F6-EPC1 binding as predicted by the directed Y2H assay. **D**, schematic representation of E2F6 binding domains.

Mono S Chromatography—250 μ g of nuclear protein was loaded onto a 0.1-ml Mono S PC 1.6/5 column (Amersham Biosciences) column equilibrated with Buffer A. Proteins were eluted with a 20 column-volume linear gradient from Buffer A to Buffer B (50 mM HEPES pH 7.6, 1 M NaCl, 10% glycerol, 0.5% Triton X-100, 1 mM EDTA, 1 mM DTT, 5 mM NaF, 1 mM AEBSF, 10 μ g/ml aprotinin, and leupeptin). 100- μ l fractions were collected, precipitated with acetone, and analyzed by Western blotting using the antibodies indicated.

Sequential Fractionation—5 mg of 293 nuclear extract were loaded onto a Superose 6 HR 10/30 column (Amersham Biosciences), and 1-ml fractions were collected at 0.40 ml/min flow in Buffer A. High molecular mass fractions (2–4 MDa) were pooled together and diluted to 10 ml with Buffer C (50 mM HEPES pH 7.6, 40 mM NaCl, 10% glycerol, 0.5% Triton X-100, 1 mM EDTA, 1 mM DTT, 5 mM NaF, 1 mM AEBSF, 10 μ g/ml aprotinin, and leupeptin). After 2 h of dialysis against Buffer C, sample was loaded onto a Buffer C-equilibrated Mono S PC 1.6/5 column (Amersham Biosciences) and fractionated in 50- μ l fractions with a 20 CV linear gradient from Buffer C to Buffer B at 100 μ l/min flow. 25 μ l from each fraction were analyzed by Western blotting using the indicated antibodies.

RESULTS

Identification of E2F6-interacting Proteins—E2Fs 1–5 are able to function as repressors of transcription by recruiting histone deacetylases via their pocket protein binding partners. E2F6 is unable to bind pocket proteins, yet functions as an active repressor of transcription possibly through the formation of large multimeric PcG-containing complexes (1, 4, 17, 19). We carried out a Y2H screen to identify novel E2F6-interacting proteins that may cooperate with E2F6 for its repressive activity. We screened a mouse fibroblast cDNA library using a full-length human E2F6 clone (1) as bait. From this screen we identified 6 clones, one of which contained a partial

sequence homologous to the PcG protein EPC1. We, therefore, cloned and sequenced the full-length mouse EPC1 cDNA according to GenBank™ (AF079765), to confirm this interaction.

E2F6 Interacts with the PcG Protein EPC1 *In Vitro* and *In Vivo*—We incubated *in vitro* translated EPC1 with full-length human GST-E2F6, and precipitated the GST fusion protein using glutathione beads. *In vitro* translated EPC1 associated with GST-E2F6 but not with GST alone (Fig. 1A). To delineate the E2F6 domain required for this interaction, we generated E2F6 mutants and incubated as before with *in vitro* translated EPC1 or with DP1 as a control for binding. As shown in Fig. 1B, the interaction domain for E2F6-EPC1 is similar to but distinct from that of E2F6-DP1. EPC1 requires amino acids 160–195 of E2F6 for binding, whereas DP1 binds residues 153–181. We also confirmed these interaction domains using a directed Y2H assay, which showed that the leucine zipper domain of E2F6 is required for EPC1 binding (Fig. 1C).

To confirm the interaction between E2F6 and EPC1 *in vivo*, we co-expressed Myc-tagged human E2F6 with HA-tagged mouse EPC1 in U2OS cells and showed that the two proteins associate (Fig. 2A). Immunoprecipitation of EPC1 by E2F6 frequently resulted in the presence of two bands for EPC1. These may represent different isoforms of EPC1, or modifications of the protein; however, we have not investigated this in further detail.

E2F6 Associates with Both DP1 and EPC1 *In Vivo*—Because of the similarity in binding domains of EPC1 and DP1 we wanted to establish whether binding of these proteins to E2F6 was mutually exclusive. Thus we carried out 35 S-labeled im-

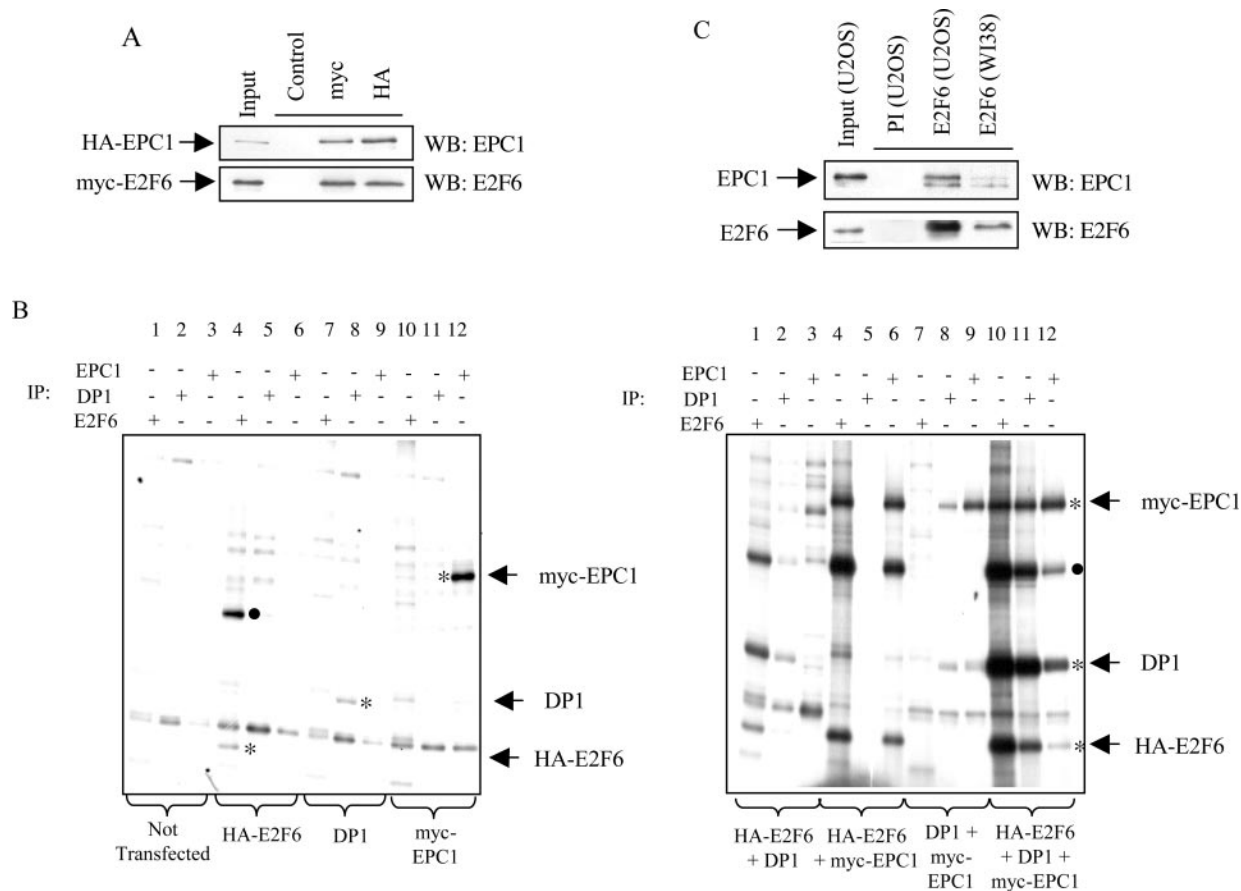


FIG. 2. **E2F6 binds DP1 and EPC1 *in vivo*.** A, E2F6 and EPC1 co-immunoprecipitate when overexpressed in U2OS cells. B, EPC1 and DP1 can bind to E2F6 simultaneously, stabilizing the proteins. Proteins were overexpressed, labeled with ^{35}S -ProMix, and immunoprecipitated using α -HA, α -DP1, or α -Myc antibodies. Labeled proteins were resolved on 8% SDS-polyacrylamide gels and visualized by autoradiography. Asterisk indicates specific bands; ● indicates a background band arising from a specific run-on product from the pCMV-HA-E2F6 construct. C, endogenous EPC1 co-immunoprecipitates with α -E2F6 (M27) antibody in U2OS and WI38 cells.

munoprecipitations on combinations of exogenously expressed proteins. We found that E2F6, DP1, and EPC1 can all interact independently with each other (Fig. 2B, right panel, lanes 1–9). However, the interaction between DP1 and EPC1 is stronger in the presence of exogenous E2F6 (Fig. 2B, right panel, lanes 7–9) and in a directed Y2H assay, no interaction was seen between DP1 and EPC1 (see Fig. 3A). This suggests the DP1-EPC1 interaction seen may be caused by the presence of endogenous E2F6 in the lysate. Importantly, when co-expressed, E2F6, DP1, and EPC1 associate more abundantly, indicating an increased stability of the proteins. (Fig. 2B, right panel, lanes 10–12). These data therefore suggest that E2F6, DP1, and EPC1 form a stable tripartite complex *in vivo*.

To confirm that E2F6 and EPC1 interact at the endogenous level, we immunoprecipitated E2F6 from both human tumor (U2OS) and normal diploid (WI38) cells, and found an association of EPC1 with E2F6 in both cell types (Fig. 2C). These results demonstrate that EPC1 and E2F6 associate *in vivo*. We also immunoprecipitated EPC1 from U2OS cells and were able to detect E2F6, albeit weakly (data not shown). We were not able to confirm the co-immunoprecipitation of DP1 at the endogenous level caused by masking of the specific DP1 band by the IgG heavy chain. However, considering the *in vivo* labeled co-immunoprecipitations we believe that E2F6, DP1, and EPC1 are part of the same complex *in vivo*.

Since the binding site for EPC1 in E2F6 is partially conserved among the E2F proteins, we tested whether EPC1 is able to interact with the other E2F family members. We found that endogenous E2F3, but not the other E2Fs, is able to bind

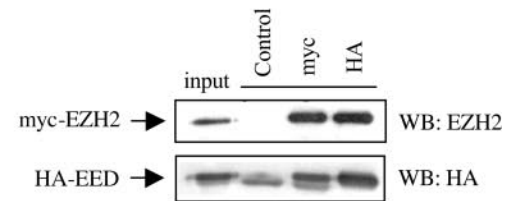
to EPC1 (data not shown). Though the binding is significantly reduced compared with E2F6, this raises the possibility that EPC1 may also form a complex with E2F3.

E2F6 and EPC1 Interact with Other PcG Proteins—In this study, we identified an endogenous *in vivo* interaction between the transcription factor E2F6 and the PcG protein, EPC1. Although *Drosophila* E(Pc) was originally identified as a PcG gene, to date, EPC1 has not been found associated with any other PcG proteins. To test whether EPC1 and E2F6 could associate with other PcG proteins, we carried out a directed Y2H assay. The directed Y2H assay indicated that EPC1 associates directly with EZH2 but not EED (Fig. 3A), and that E2F6 is unable to associate directly with either protein, binding only to EPC1 directly. We confirmed this *in vivo* showing that over-expressed EPC1 is able to immunoprecipitate EZH2 but not EED (Fig. 3B, top and middle panels), whereas EZH2 immunoprecipitates EED as expected (Fig. 3C). However, when expressed together all three proteins (EPC1, EZH2, and EED) can co-immunoprecipitate (Fig. 3B, bottom panel). These data are consistent with EZH2 and EED being binding partners (21–23) and suggest that EPC1 does not bind directly to EED but is able to co-immunoprecipitate it in the presence of EZH2. However, EPC1 is able to bind to both E2F6 and EZH2 directly, and we therefore wondered whether an indirect association, occurring via EPC1, could also exist between E2F6 and EZH2 *in vivo*. Indeed, we found that exogenous E2F6 and EZH2 do co-immunoprecipitate (Fig. 3D, top panel), although this interaction is significantly weaker than that seen between EPC1 and EZH2 (Fig. 3B, top panel), suggesting that endogenous

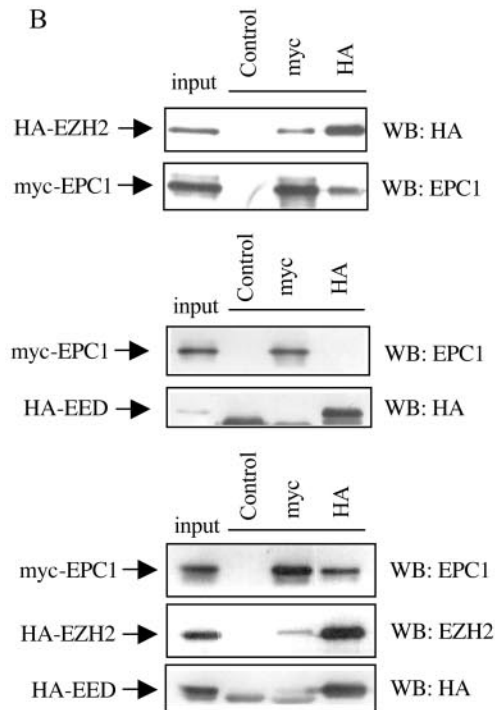
A

<u>E2F6 interactors</u>		<u>EPC1 interactors</u>	
E2F6 + DP1	+	EPC1 + DP1	-
E2F6 + EZH2	-	EPC1 + EZH2	+
E2F6 + EED	-	EPC1 + EED	-

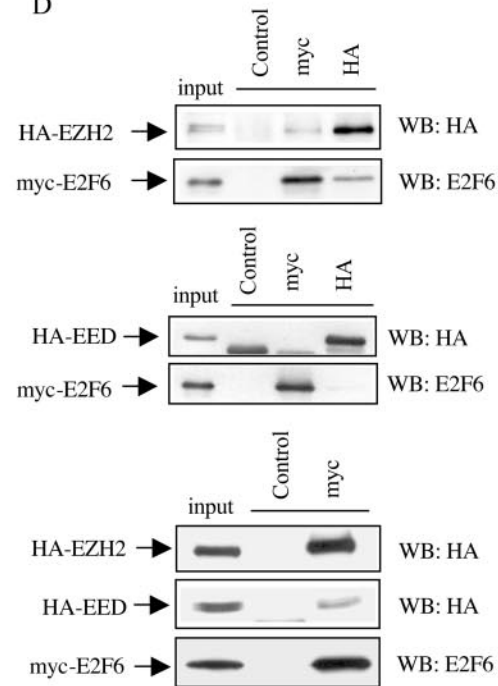
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B



D



E

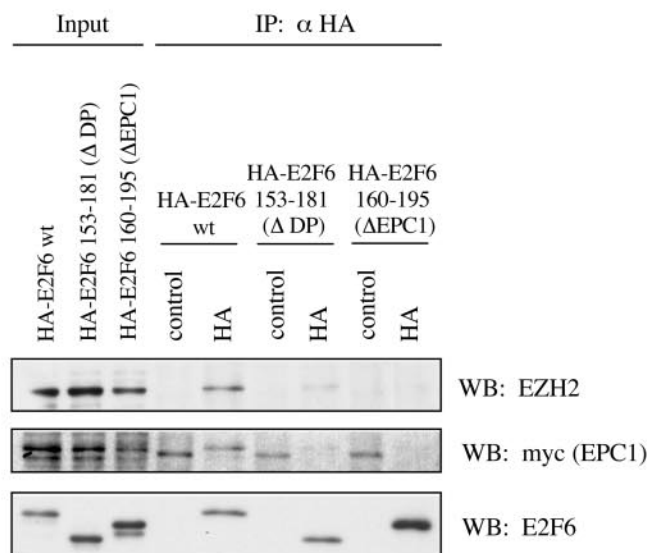


FIG. 3. **E2F6 binds to EZH2 via EPC1.** A, summary of directed Y2H assay. EPC1 interacts directly with EZH2 but not EED, while E2F6 does not interact with either PcG protein. DP1 interacts E2F6 but not EPC1. B, overexpressed EZH2 but not EED binds to EPC1. When expressed together, EPC1 associates with both EZH2 and EED. C, overexpressed EZH2 and EED co-immunoprecipitate. D, overexpressed EZH2 but not EED associates with E2F6, probably via endogenous EPC1. When expressed together, E2F6 can immunoprecipitate both EZH2 and EED. E, wild-type E2F6 associates with EPC1 and EZH2 while a mutant lacking the EPC1-binding domain does not. A DP1 binding mutant is also severely compromised in its ability to associate with EPC1 and EZH2.

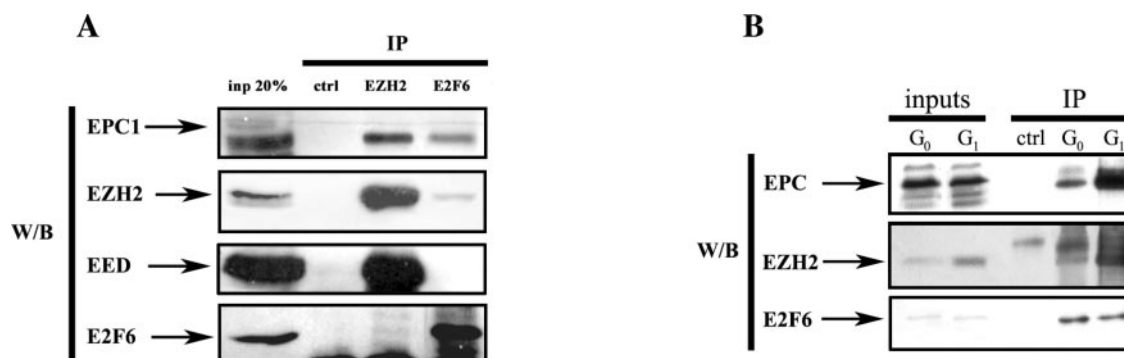


FIG. 4. *In vivo* association of E2F6, EPC1, and EZH2. A, nuclear extracts from 293T cells were immunoprecipitated with polyclonal α -E2F6 (M27), α -EZH2 (SI246), or preimmune serum (control), then immunoblotted with monoclonal antibodies against EPC1 (D19), EZH2 (AC22), EED (AA19), and E2F6 (TFE61). B, nuclear extracts from TIG3 cells synchronized either in G₀ or in G₁, were immunoprecipitated with α -E2F6 (M27) or with preimmune serum (control), then immunoblotted using the antibodies described in A. Whereas EPC1 interacts with E2F6 in both quiescent and proliferating cells, EZH2 interacts only upon re-entry into the cell cycle.

EPC1 is required and function as a bridge. Furthermore, in agreement with the directed Y2H assay, we failed to detect binding between E2F6 and EED (Fig. 3D, middle panels). However, as seen with EPC1, when overexpressed together with EZH2, EED is co-immunoprecipitated by E2F6 (Fig. 3D, bottom panel), again suggesting that this interaction is indirect and occurs only via EZH2.

We also tested these interactions with *in vivo* labeled immunoprecipitations. In agreement with the results described above, we saw that E2F6 is able to bind EZH2 only weakly in the absence of exogenous EPC1, and that EZH2 associates more strongly when co-expressed with EPC1 (data not shown). Taking into consideration the Y2H data, which showed that E2F6 is unable to bind to EZH2 directly, we propose that EPC1 mediates the interaction between EZH2 and E2F6. In order to test this, we transfected cells with E2F6, either wild-type or mutants lacking the DP1 or EPC1 binding domain, and EPC1 and EZH2. We then immunoprecipitated the exogenous E2F6 protein and looked for interactions with EPC1 and EZH2. As previously described, we found that both EPC1 and EZH2 immunoprecipitate with full-length E2F6 (Fig. 3E). In contrast, the EPC1 binding mutant (Δ EPC1) is no longer able to immunoprecipitate either EPC1 or, significantly, EZH2. In addition, we found that the DP1 binding mutant (Δ DP1) also shows a reduced ability to bind to EPC1, and hence to EZH2, which may suggest that DP1 is required for stabilization of the E2F6-EPC1 interaction, and supports the idea that EZH2 binds through its interaction with EPC1. In summary, our data indicate that E2F6, DP1, and EPC1 form a stable tri-molecular complex *in vivo* that is able to interact with EZH2 via EPC1.

E2F6 Associates with Endogenous EPC1 and EZH2—To show an interaction between E2F6, EPC1, and EZH2 at the endogenous level, we immunoprecipitated E2F6 and EZH2 from 293T cells. We found that E2F6 can immunoprecipitate both EPC1 and EZH2 but not EED, whereas EZH2 associates strongly with EED and weakly with EPC1 (and very weakly with E2F6) (Fig. 4A). To show that this interaction also occurs in non-transformed cells, we immunoprecipitated E2F6 from human diploid fibroblast TIG3 cells. We synchronized cells in G₀ by serum starvation and released into the cell cycle through the addition of serum. Cells were collected either in quiescence or upon re-entry into the cell cycle in G₁. Samples were immunoprecipitated for E2F6 and blotted for interaction with EPC1 and EZH2 (Fig. 4B). Whereas E2F6 and EPC1 proteins are present in all stages of the cell cycle (see Fig. 6C), EZH2 is a cell growth regulated gene, and the protein is present in only minor quantities in G₀ (15). Thus, in G₀ we were able to co-immunoprecipitate only E2F6 and EPC1, whereas in cells entering the

cell cycle (G₁) we saw co-immunoprecipitation of E2F6, EPC1, and EZH2. It is important to note that the relative proportions of E2F6, EPC1, and EZH2 that interact together are low, consistent with them all being members of other protein complexes. In particular the proportion of EZH2 that interacts with E2F6-EPC1 is very low and, furthermore, appears to be transient as it is consistently difficult to detect.

E2F6 Forms a Novel PcG-containing Complex *in Vivo*—To show that these interactions form part of a multi-protein complex, we fractionated nuclear extracts from 293T cells using gel filtration or cation exchange chromatography. Immunoblotting of fractions derived from a gel filtration column indicated that E2F6 elutes broadly, but in at least two peaks. The first elutes immediately after the void volume of the column (fractions 1–2) with an estimated molecular mass of 2–4 MDa, whereas the second peak elutes at a lower molecular mass of around 660 kDa. We found that, while also displaying broad elution profiles, a proportion of DP1, EPC1, EZH2, and EED all co-eluted in the high molecular mass fractions 1–2, suggesting a large multisubunit complex (Fig. 5A). Also eluted in these fractions were Sin3B, a known component of co-repressor complexes that associates with HDAC and SWI/SNF activity (36), plus a small amount of YY1 and Ring1. These high molecular mass fractions are distinct from that of the E2F6-G₀ complex as they contain neither Max (Fig. 5A) nor HP1 γ (data not shown) (17). Furthermore, Ring1 peaks in fractions 6–9 (co-eluting with E2F6 in fractions 6 and 7) suggesting that the E2F6-Bmi1 complex is also distinct (19). To show that these proteins not only co-elute but also interact in these fractions we immunoprecipitated E2F6 from each fraction and analyzed by Western blotting. This confirmed that EPC1 and Sin3B associate with E2F6 in fractions 1–2 and 1–4, respectively (Fig. 5B). We have not been able to co-immunoprecipitate EZH2 with E2F6 following fractionation, which may be due either to the stability or to the low proportion of EZH2 protein seen in fractions 1–2, and hence, the low abundance of this complex. As expected, Max also associates with E2F6 but peaks in fractions 4–6, representing the E2F6-G₀ complex (17). We did not observe YY1 or EED co-immunoprecipitating with E2F6 in any fraction (data not shown).

As further evidence for the existence of a novel E2F6 complex we performed an alternative fractionation, eluting nuclear proteins from an ion exchange column. To increase selectivity for E2F6-containing complexes, we used stringent binding conditions (250 mM NaCl). Again E2F6, EPC1, EZH2, and Sin3B co-eluted from the column (at 700 mM salt) providing further support for the identification of a novel E2F6-PcG complex (Fig. 5C).

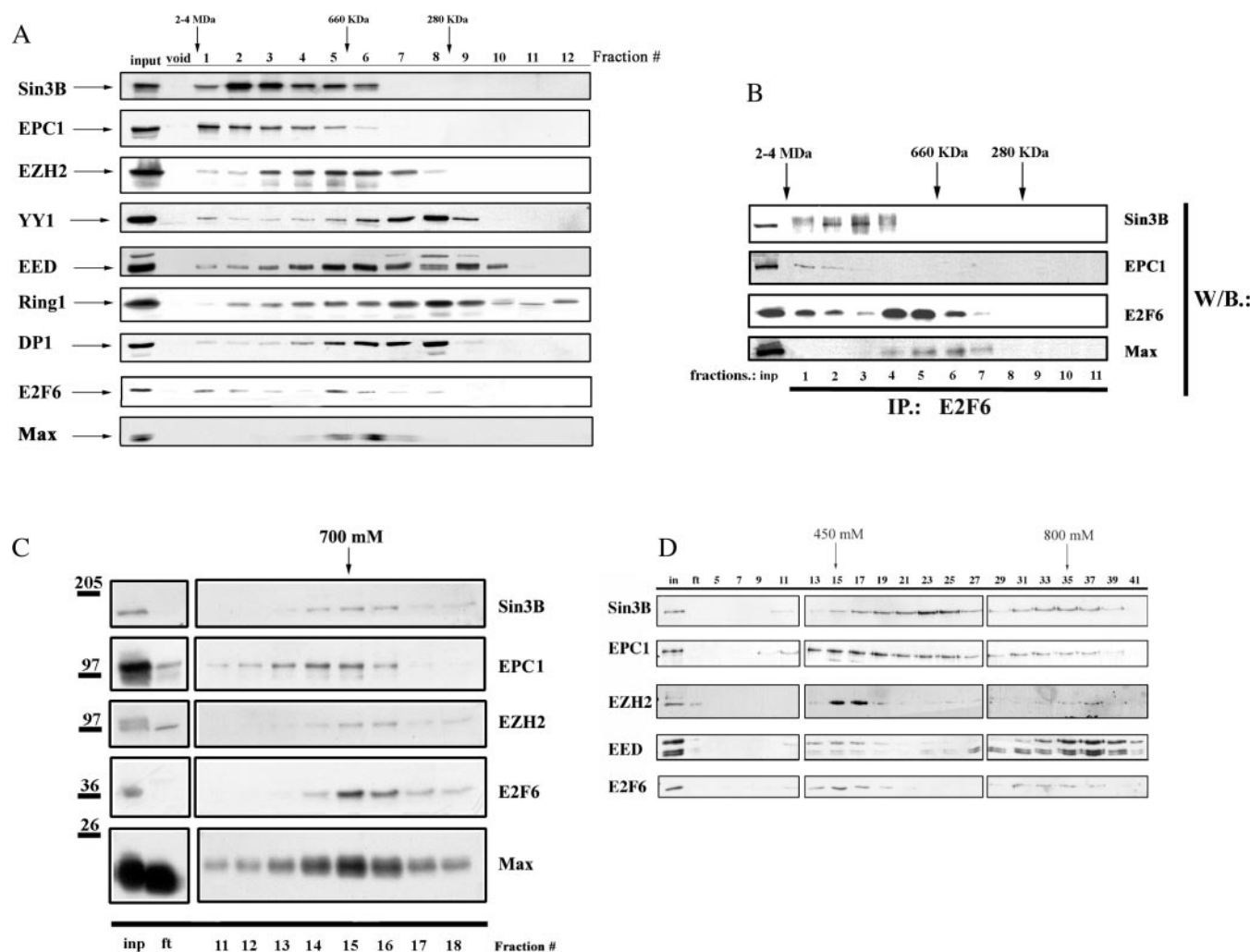


FIG. 5. **E2F6 co-elutes with DP1, EPC1, and EZH2.** *A*, 293T cells were fractionated on a gel filtration column and analyzed by Western blot with the indicated antibodies. Size standards are indicated above the lanes. *B*, immunoprecipitation of E2F6 from Superose 6 fractions. Fractions were immunoprecipitated with α -E2F6 (M27) or preimmune serum (data not shown) and probed with the indicated antibodies. *C*, Mono S fractionation of nuclear extracts. Extracts from each fraction were probed with the indicated antibodies. A fraction of the input (*inp*) and the flow-through (*ft*) of the column were loaded. *D*, sequential fractionation of the complex containing E2F6. Following Superose 6 fractionation, the high molecular mass fractions (fractions 1–3 in panel *A*) were pooled and loaded onto a Mono S column. E2F6, EPC1, EZH2, EED, and Sin3B co-elute.

To provide further confirmation for the identification of the complex, we performed sequential fractionations. Following an initial fractionation using the gel filtration column, we ran the high molecular mass fractions (fractions 1–3) on the ion exchange column. Following this sequential separation, we found that E2F6, EPC1, EZH2, EED, and Sin3B still co-elute (fractions 15 and 17), which, together with our previous results, strongly suggests the existence of a novel multiprotein complex (Fig. 5D).

The E2F6-EPC1-EZH2 Complex Is Proliferation-specific—Fractionation of asynchronously growing TIG3 fibroblasts revealed similar elution profiles, but with low or undetectable levels of EZH2 and EED in fractions 1 and 2 (Fig. 6A). As EZH2 and EED are cell growth regulated proteins and are known to be required for cellular proliferation (15), it is likely that this difference between normal and transformed cell lines is caused by the higher levels of EZH2 in transformed cells. We therefore examined the formation of the E2F6-EPC1-EZH2 complex during the cell growth cycle. Nuclear extracts of TIG3 fibroblasts were prepared following release from G_0 and fractionated on a gel filtration column. We confirmed cell cycle phases by Western blot and FACS analysis (Fig. 6C). In G_0 and early G_1 , E2F6 and EPC1 co-elute in these fractions as previously observed (Fig. 6B, G_0 and G_1). At this stage, EZH2 expression is very

low, and the protein is only detected in the lower molecular mass fractions as part of the previously reported 600-kDa PRC2 complex (37). We did not detect EED in the high molecular mass fractions of these cells at any stage. In late G_1 , EZH2 expression increases and the protein is now seen in fraction 1 as part of a higher molecular mass complex. Interestingly, the E2F6 elution profile changes dramatically at this time point, shifting to only the lower molecular mass complexes (Fig. 6B, late G_1). We are currently investigating the molecular mechanisms resulting in this shift. However, that EPC1 remains in the high molecular mass fractions at this point, may reflect its involvement in other large multiprotein complexes, as previously reported (27, 28, 38). Finally in S phase, E2F6 shifts back to the high molecular mass fractions and co-elutes with EPC1 and EZH2 in fraction 1 (Fig. 6B, S). This, therefore, suggests that E2F6 forms a high molecular mass complex with EPC1 and that this complex can also interact with the proliferation-specific PcG, EZH2, thus forming a novel proliferation-specific E2F6 complex.

E2F6 and EPC1 Cooperate to Repress the Activity of an E2F-responsive Promoter—To understand the potential function of this complex, we used a reporter construct that consists of a portion of the *ARF* promoter, which contains 4 E2F sites linked to a *CAT* promoter (39). We found that endogenous

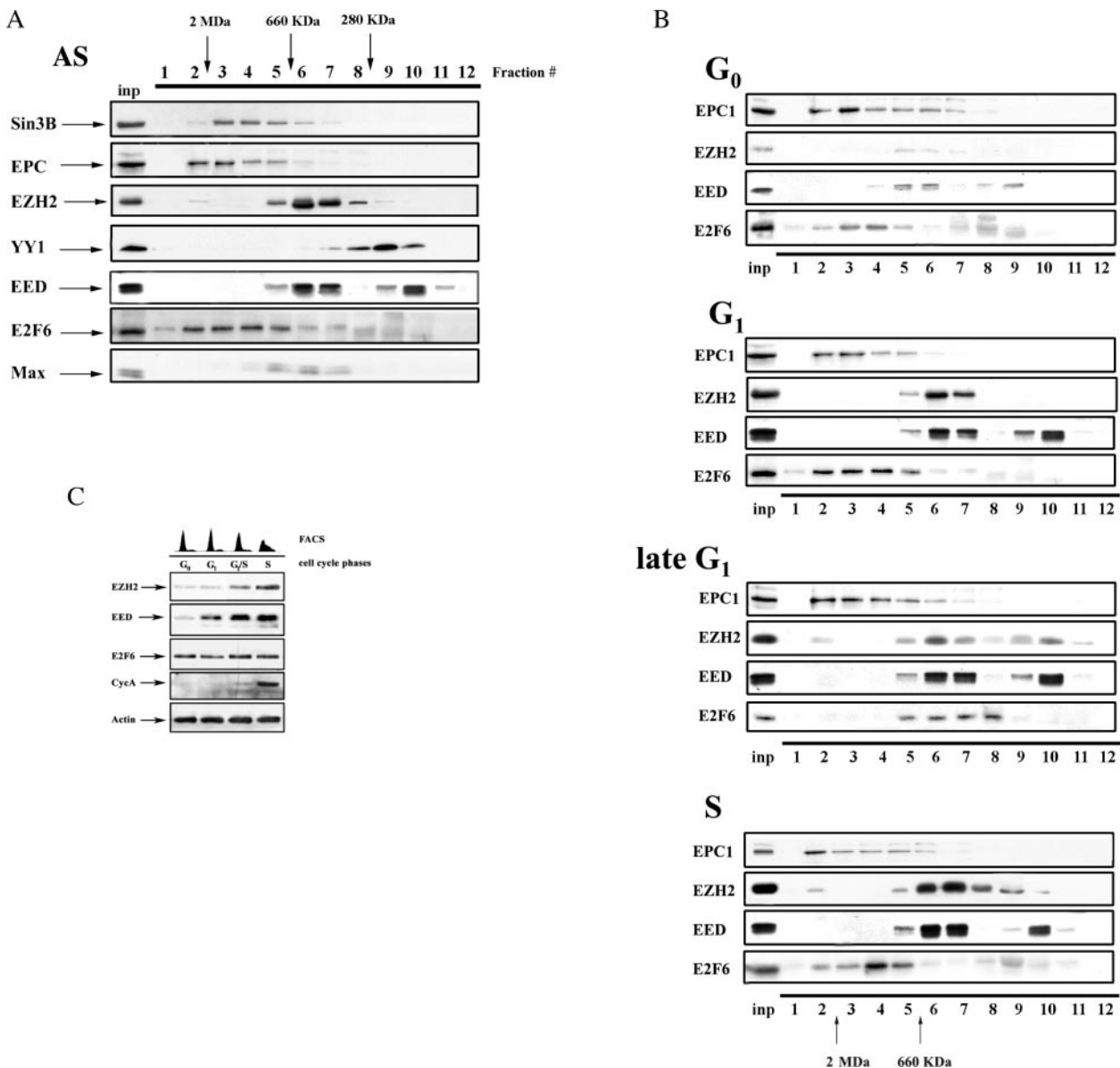


FIG. 6. Superose 6 elution profile in TIG3 cells. A, Western blotting analysis of asynchronous TIG3 nuclear extracts from Superose 6 fractionation. B, comparison of the elution profiles in TIG3 synchronized in G₀, G₁, late G₁, and S phase. C, cell growth profile of TIG3 fibroblasts by Western blot analysis. Actin and cyclin A were used to confirm equal loading and cell cycle phase, respectively.

activity of this promoter is activated by exogenous E2F1 (data not shown) and repressed by E2F6, consistent with E2F6 being a transcriptional repressor. Furthermore, we found that E2F6 mutants lacking either the DP1 binding domain (E2F6 Δ 153–181) or the EPC1 binding domain (E2F6 Δ 160–195) lose repressive activity (Fig. 7A). This is consistent with the observation that both of these mutants show reduced or abrogated EPC1 and EZH2 binding *in vivo* (see Fig. 3E). In addition, we found that expression of either E2F6 or EPC1 alone represses activity from the E2F responsive promoter, and that this repression is augmented by co-expression of the two proteins (Fig. 7B). Thus EPC1 cooperates with E2F6, augmenting its repressive activity. We did not see any additional effect of EZH2 on this repression, despite EZH2 alone having mild repressive activity (data not shown). However, these data indicate that E2F6 and EPC1 form a repressive core complex that may function together with EZH2 in proliferating cells.

DISCUSSION

We have identified a novel E2F6 complex that is distinct from the previously reported PcG-containing E2F6 complexes

(17, 19). This complex appears to contain a stable core of E2F6, DP1, and EPC1, and may also contain EZH2 and Sin3B. This is of particular importance for two reasons. First, it describes for the first time a direct functional link between the transcription factor, E2F6 and the polycomb proteins. Furthermore, we demonstrate a functional role for EPC1 in this transcriptional repression. Second, we describe the formation of a further proliferation-specific complex with EZH2, thus implicating E2F6 in an active role in the cell cycle. Since EZH2 is required for cellular proliferation and is involved in oncogenesis, the E2F6-EPC1-EZH2 complex is likely to play a role in proliferating cells (15, 40, 41). Supporting this, the complex appears to be present at higher levels in transformed cells indicating a possible role in the transformation process. It remains a formal possibility however, that the failure to detect this complex at the early stages of cell cycle re-entry may simply reflect an inability to detect the EZH2 protein, because of its very low levels. In addition, following the shift of E2F6 in G₁, E2F6 appears to co-elute with EZH2 in the lower molecular mass fractions. Since we have been unable to co-immunoprecipitate

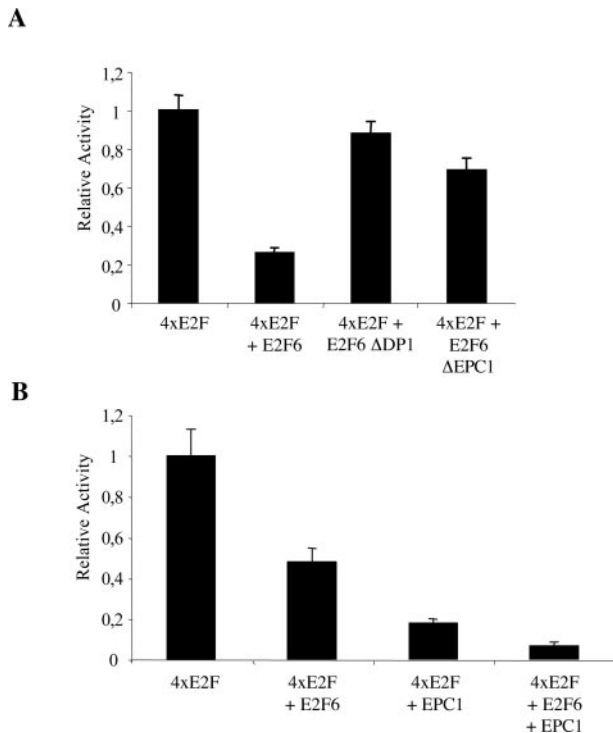


FIG. 7. E2F6 and EPC1 repress CAT activity driven by an E2F-responsive promoter. A, COS1 cells were transfected with an E2F reporter construct containing 4 E2F DNA binding sites and with an E2F6 expression vector, or mutants thereof. Wild-type E2F6 represses activity from the reporter, whereas E2F6 mutants lacking either the DP1 binding domain (E2F6 ΔDP1 (Δ153–181)) or the EPC1 binding domain (E2F6 ΔEPC1 (Δ160–195)) are impaired in their ability to repress. B, E2F-responsive promoter is repressed by E2F6 or EPC1 alone, and the two proteins cooperate to further enhance the repression. Co-expression of EZH2 does not alter the repressive activity of the E2F6-EPC1 complex (data not shown).

EZH2 with E2F6 following fractionation, we are unable to exclude that the two proteins associate in these fractions. However, the yeast two-hybrid data showing that EZH2 is able to bind to EPC1 but not to E2F6, combined with the fact that EPC1 only co-immunoprecipitates with E2F6 in fractions 1 and 2, argue against this.

Since EZH2 is known to possess histone H3 methyltransferase (HMTase) activity, we assayed immunoprecipitated E2F6 for associated activity, but were unable to detect any (data not shown). In contrast, both endogenous EZH2 and EED, and overexpressed E2F6 with $E\mu$ -HMTase showed histone H3 activity as expected. Since the E2F6- $E\mu$ -HMTase complex is specific to G_0 (17), it would not be detected at endogenous levels in proliferating cells. Therefore, lack of detection of endogenous E2F6 activity may simply be caused by the low abundance of the complexes in the cell type tested, or the lack of adequate immunological reagents. Indeed, assay of the total fractions following gel filtration, while not specific for E2F6, shows histone H3 specific activity in fractions 1–10, peaking in fractions 2 and 4–6 (data not shown).

We were not able to detect endogenous EED associated with E2F6, but cannot exclude that it interacts via EZH2. In *Drosophila*, EED is phosphorylated *in vivo* and this modified form of EED associates preferentially with EZH2 (37). One possible explanation, therefore, is that dephosphorylation of EED destabilizes its interaction with EZH2 and allows its dissociation and subsequent association with E2F6/DP1/EPC1 (although the phosphorylation of EED is not known to be required for its association with EZH2). Alternatively, EPC1 may recruit EZH2 directly, independently of EED, or EED may be present

in the E2F6-EPC1-EZH2 complex and we were simply unable to detect it. A final possibility is that the HDAC activity usually recruited by EED with EZH2 is replaced in this complex by the HDAC-associated protein, Sin3B.

EPC is highly conserved across species. While EPC is known to be important for PcG function (26), it has been identified to date only in the non-PcG complexes, TIP60 and p400/NuA4, which contain histone acetyltransferase activity (27, 28, 38). Consistent with this, EPC1 has been reported to possess both activating and repressive domains (30, 42). Interestingly, EZH2 is also thought to have dual roles, functioning both as a PcG protein and a trxG protein, and therefore having roles in both gene repression and in the maintenance of transcriptional activity (43). Furthermore, mutations in either *Drosophila* EPC or EZH2 were recently found to enhance the phenotype of trxG mutations (44). These findings strongly suggest that EPC1 and EZH2 may have additional activating roles beyond that of classic PcG proteins. EPC and EZH2 are also both predicted to encode heterochromatic proteins that also bind to euchromatin, so the identification of the mammalian homologues of these two proteins in a complex together is intriguing. In addition, EPC, EZH2, and EED are the only PcG proteins that are conserved in *Caenorhabditis elegans*, and only EPC and EZH2 have homologues in yeast (42). Based on this, Stankunas *et al.* (42) suggested that EPC may cooperate with EZH2 in yeast and with EZH2 and EED in *C. elegans* to regulate chromatin structure. The data presented here support this, showing that E2F6 forms a complex with DP1, EPC1, and that this complex interacts with EZH2 in proliferating cells. Furthermore, we demonstrate a role for EPC1 in the repression of gene activity with E2F6 and potentially also with EZH2.

The identification of target genes of this complex will be of particular importance. As EZH2 is required for cellular proliferation, E2F6-EPC1-EZH2 may be required for successful completion of the cell cycle. Alternatively, E2F6-EPC1 and E2F6-EPC1-EZH2 may have distinct roles. We have not been able to identify target genes for this complex because of the lack of functional ChIP antibodies against EPC1 and EZH2. However, a ChIP assay for E2F6 did not show any enrichment of the *ARF* promoter suggesting that repression in the CAT assay does not reflect the endogenous situation, but simply the presence of E2F sites within the promoter construct, and that E2F6 and EPC1 function as repressors.

It will be intriguing to establish whether the E2F6-EPC1-EZH2 and E2F6-PRC1 (BMI1) complexes function independently, or are involved in co-ordinate repression of target genes. In *Drosophila* development, the EZH2 complex is recruited first to target genes and is believed to be replaced by BMI1 for the maintenance of stable repression. It is therefore feasible that a similar mechanism for repression could exist in proliferating cells. Indeed both EZH2 and BMI1 are now known to play a role in cellular proliferation adding weight to such a model.

Our data indicate that the E2F6-EPC1 complex functions as a transcriptional repressor. However, recent evidence from our laboratory indicates a role for EZH2 in maintained gene activation throughout the cell cycle, functioning as an antagonist to BMI1 (15). This implies that E2F6-EPC1-EZH2 and E2F6-PRC1 may not function cooperatively. Alternatively, E2F6-EPC1-EZH2 may have a role in proliferating cells while E2F6-PRC1 may repress targets in differentiated cells, or E2F6-PRC1 may repress targets while E2F6-EPC1-EZH2 activates them. Detailed studies will be required to distinguish between these intriguing possibilities and target gene identification will be a crucial factor in elucidating the functions of the E2F6-PcG complexes.

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