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33. The same holds for asynchronous updating of the strategies. A related behavior occurs in the case of the prisoner's dilemma (with two-player interactions) if sites are allowed to go empty (*36*).
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A Complex with Chromatin Modifiers That Occupies E2F- and Myc-Responsive Genes in G_0 Cells

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E2F-6 contributes to gene silencing in a manner independent of retinoblastoma protein family members. To better elucidate the molecular mechanism of repression by E2F-6, we have purified the factor from cultured cells. E2F-6 is found in a multimeric protein complex that contains Mga and Max, and thus the complex can bind not only to the E2F-binding site but also to Myc- and Brachyury-binding sites. Moreover, the complex contains chromatin modifiers such as a novel histone methyltransferase that modifies lysine 9 of histone H3, HP1γ, and Polycomb group (PcG) proteins. The E2F-6 complex preferentially occupies target promoters in G_0 cells rather than in G_1 cells. These data suggest that these chromatin modifiers contribute to silencing of E2F- and Myc-responsive genes in quiescent cells.

Normal cells can exit the cell cycle and enter the G_0 stage, whereas malignant tumor cells have lost the ability to enter the G_0 stage. Although G_0 and G_1 are often viewed together as G_0/G_1, these stages are quite distinct. Whereas G_0 is a long-term quiescent stage, G_1 is a transient stage between the M and S phases of growing cells. Given that most cells in adult human are in G_0 and that defects in the ability to maintain the G_0 stage often lead to tumorigenesis, it would be a significant advance to elucidate mechanisms whereby normal cells maintain quiescence. Such mechanisms could include repression of E2F and Myc activities, which transactivate various genes required for mitotic stimulation, cell-cycle progression, and DNA replication (*1*–*3*). Retinoblastoma (RB) protein and other related proteins, such as p107 and p130, are known to be key players in repression of E2F-mediated transcription (*4*, *5*). Among RB family members, p130 has been proposed as responsible for repression in G_0, because the E2F-p130 complex accumulates in G_0 (*6*). However, our chromatin immunoprecipitation experiments show that p130 preferentially binds to E2F-responsive promoters in G_1 rather than in G_0 in human fibroblasts (this report), indicating that the amount of the E2F-p130 complex in cells does not simply reflect that bound to target promoters.

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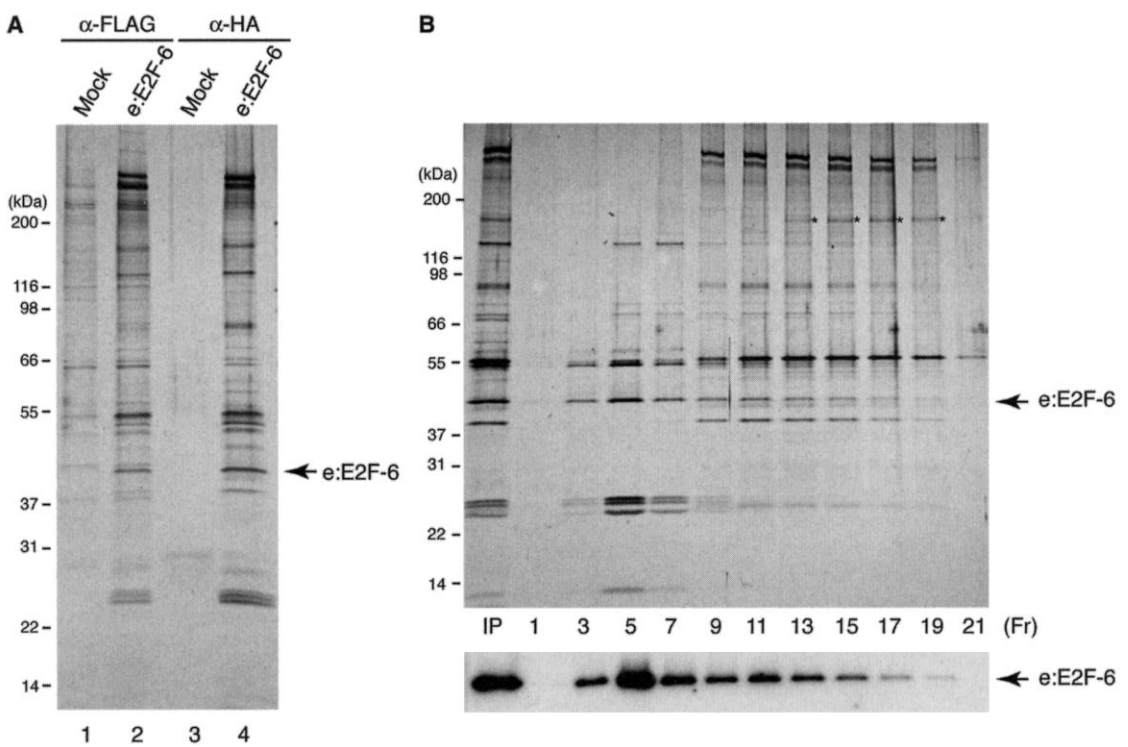

Fig. 1. Purification of the E2F-6–containing complexes. **(A)** FLAG-HA–epitope–tagged E2F-6 (e:E2F-6) was purified from HeLa cells expressing e:E2F-6 by immunoprecipitation with antibody specific for FLAG (lane 2), followed by antibody specific for HA (lane 4). As a control, mock purification was performed from nontransduced HeLa cells (lanes 1 and 3). **(B)** The E2F-6–containing complexes was separated on a 10 to 30% glycerol gradient by centrifugation. Input (IP) and fractions (the top to bottom) were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by silver staining (top) and immunoblot with HA-specific antibody (bottom).

To explore repressive mechanisms of E2F-responsive genes in quiescent cells, we investigated E2F-6, which differs from other E2F family members (E2F-1 to E2F-5) in that it lacks domains for binding to RB family proteins and for transactivation (*7–9*). E2F-6-containing complexes were purified from HeLa cells that stably express E2F-6 tagged with the FLAG–hemagglutinin (FLAG-HA) epitope (e:E2F-6) by immunoprecipitation with antibody against FLAG (Fig. 1A, lane 2) (*10*). The immunoprecipitated materials were eluted from the matrix with FLAG peptide and further purified by HA-specific antibody immunoprecipitation (lane 4). As a control, mock purification was performed from nontransduced HeLa cells (lanes 1 and 3). After a second immunoprecipitation, many polypeptides that associate with e:E2F-6 were identified by silver staining. In contrast, almost no polypeptides were detected in the mock-purified control.

To determine the homogeneity of the purified material, E2F6-containing complexes were separated on a 10 to 30% glycerol gradient by ultracentrifugation. Silver staining (Fig. 1B, top) and immunoblot (Fig. 1B, bottom) analyses revealed that e:E2F-6 appears in at least two complexes: a fast sedimenting form, referred to as E2F6.com-1 (peak at fractions at about 11 to 13), and a slowly sedimenting form, referred to as E2F6.com-2 (peak at fraction 5) (Fig. 1B). This paper reports the characterization of E2F6.com-1.

The polypeptides in E2F6.com-1 were identified by mass spectrometry. As expected, DP-1, a heteromeric partner of E2F family members, was found as a 50-kD band (Fig. 2A). However, the other heteromeric partner, DP-2, was not detected in E2F6.com-1 by mass spectrometric or immunoblot analyses, even though HeLa cells contain a substantial amount of DP-2 (*11*), indicating that E2F-6 preferentially binds to DP-1 in E2F6.com-1. Note that Mga and Max were identified in the 300- and 26-kD bands, respectively (Fig. 2A). Mga and Max are known to form a heterodimer and to bind specifically to E boxes such as the Myc-binding elements (*12*). Transformation of rat embryonic fibroblasts by ectopic expression of Ras and Myc is suppressed by coexpressing Mga (*12*), indicating that Mga acts antagonistically to Myc. Moreover, Mga has a second DNA binding motif known as the T box, originally found in the *Brachyury*, or T locus, gene product. Members of the T box gene family act as transcriptional activators or repressors, both of which play key roles in the regulation of embryonic devel-

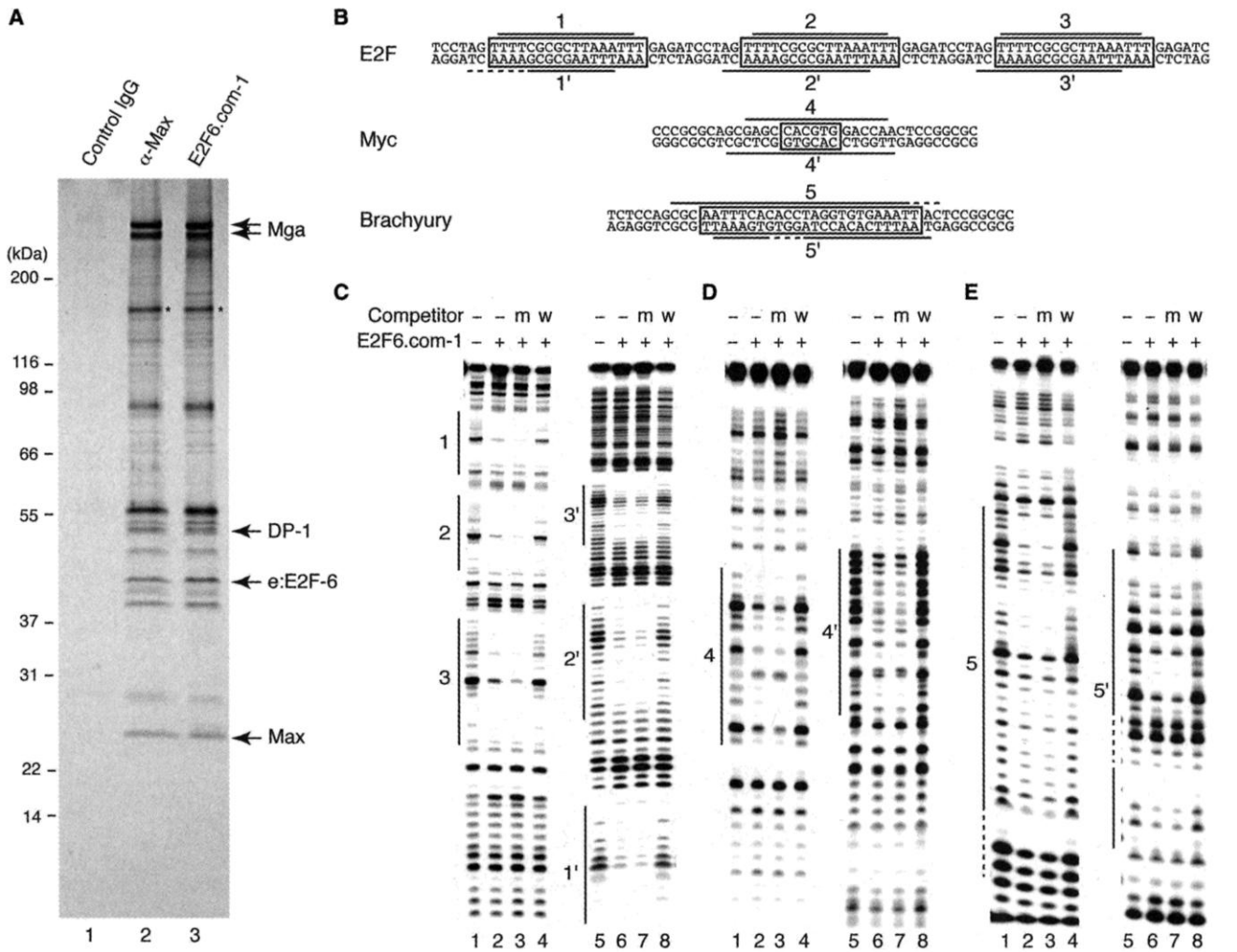

Fig. 2. Multi-DNA binding factors in E2F6.com-1. (**A**) The FLAG-purified material (corresponding to Fig. 1A, lane 2) was immunoprecipitated with control IgG (lane 1) and Max-specific antibody (lane 2). The HA-purified E2F6.com-1 was also analyzed (lane 3). (**B**) DNA sequences of probes for DNase I footprinting are shown. Consensus sequences for E2F- and Myc-binding sites are boxed; nucleotides protected by E2F6.com-1 are shown by lines. (**C**) Binding of E2F6.com-1 to the E2F-binding sites was examined by DNase I footprinting. The upper (lanes 1 to 4) and lower (lanes 5 to 8) strand labeled probes were incubated with buffer (lanes 1 and 5) or E2F6.com-1 (lanes 2, 3, 4, 6, 7, and 8) in the absence (lanes 2 and 6) or in the presence of the mutant competitor (lanes 3 and 7) or the wild-type competitor (lanes 4 and 8). (**D** and **E**) Binding of E2F6.com-1 to the Myc- (D) and Brachyury- (E) binding sites was demonstrated as indicated in (B).

opment and the assignment of cell fate (*13*).

A link between E2F-6/DP and Mga/Max was further confirmed by reciprocal immunoprecipitation. Max-specific antibody immunoprecipitated from the FLAG-purified materials (corresponding to Fig. 1A, lane 2) a set of polypeptides that are indistinguishable from those demonstrated in E2F6.com-1 by HA-antibody/glycerol gradient sedimentation (Fig. 2A). Thus, we conclude that E2F-6/DP and Mga/Max are present in the same complex and contribute to binding to multi-DNA sequences including E2F-, Myc-, and Brachyury-binding elements. To demonstrate such interactions, we performed in vitro DNase I footprinting. The double-stranded DNA containing three E2F-binding elements was labeled with ^{32}P at the 3′ end of either the upper or lower strand, and these were used as probes (Fig. 2B). In both probes, E2F6.com-1 protected E2F-binding sites from DNase I digestion (Fig. 2C, lanes 2 and 6). In both cases, protection was competitively inhibited by unlabeled competitor DNA containing a wild-type E2F-binding sequence (lanes 4 and 8), but not by that containing a mutated binding sequence (lanes 3 and 7). Similarly, E2F6.com-1 protected the Myc- and Brachyury-binding elements (Fig. 2D and 2E, respectively) from DNase I digestion. These results show that E2F6.com-1 binds to three distinct DNA elements: E2F-, Myc-, and Brachyury-binding elements.

To gain insight into the mechanisms by which E2F6.com-1 represses transcription, we measured histone-modifying activity in the complex. Although histone acetylase and deacetylase activities were not detected in E2F6.com-1 (*11*), methyltransferase activity was (Fig. 3A). E2F6.com-1 specifically methylates histone H3 in free core histones (Fig. 3A, top, lane 1) and in nucleosomes (lane 3). Amino acid sequencing of methylated histone H3 revealed that Lys^9 (K9) is the major target (Fig. 3A, bottom).

Histone methyltransferase assays of fractions from glycerol gradient sedimentation, corresponding to the samples in Fig. 1B, showed that most of the activity is found only in fractions 13 to 19 (Fig. 3B, top), whereas most subunits in E2F6.com-1 are detected in fractions 9 to 19 (Fig. 1B, top). Unlike the other subunits, the 150-kD polypeptide (Fig. 1B, top) cosediments with the methyltransferase activity (Fig. 3B, top). Mass spectrometric analysis identified two proteins containing a SET (Suvar3-9, Enhancer-of-zeste, Trithorax) domain in the 150-kD band: a novel protein, referred to as Eu-HMTase1 (euchromatic histone methyltransferase 1) (*14*), and the previously reported NG36/G9a histone methyltransferase (*15*, *16*). Immunoblot analysis of fractions from glycerol gradient sedimentation with antibodies against Eu-HMTase1 and NG36/G9a showed that the immunoreactive band (Fig. 3B, middle and bottom) corresponds to the 150-kD band detected by silver staining (Fig. 1B, top, marked with asterisks) in both sedimentation profile and molecular weight. Although only a portion of E2F6.com-1 has histone methyltransferases, the following data indicate that Eu-HMTase1 and NG36/G9a associates with all other subunits in the complex: Max-specific (see Fig. 2B) and HP1γ-specific (see Fig. 4A) antibodies immunoprecipitated from the FLAG-purified materials a set of polypeptides, including the 150-kD polypeptide (marked with asterisks), that are indistinguishable from those in E2F6.com-1.

Eu-HMTase1 and NG36/G9a both have

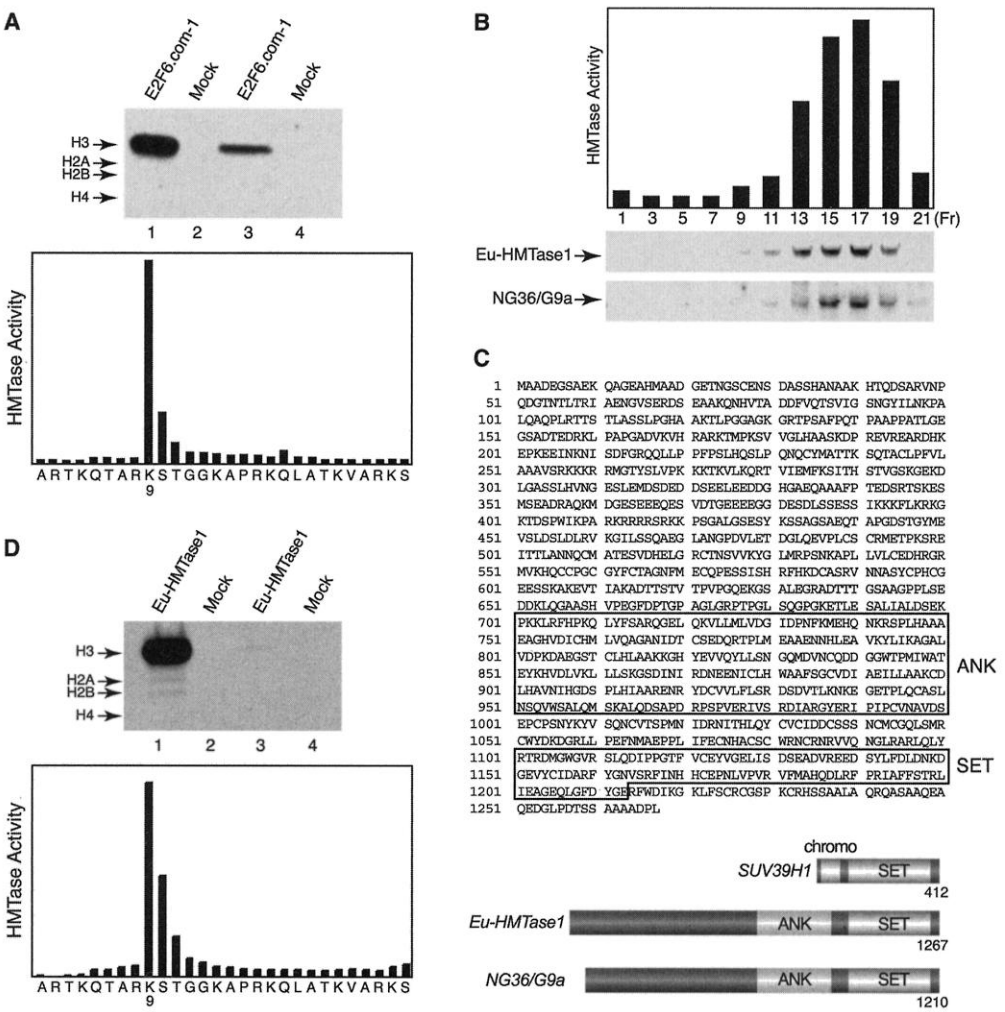

Fig. 3. Chromatin modifiers in E2F6.com-1. **(A)** (Top) Histone methyltransferase activity in E2F6.com-1 (lanes 1 and 3) and mock control (lanes 2 and 4) was measured using either free core histones (lanes 1 and 2) or mononucleosomes (lanes 3 and 4) as substrates. (Bottom) [^3H]methylated histone H3 with E2F6.com-1 was analyzed by microsequencing. **(B)** Relative methyltransferase activity (top) and immunoblot with antibodies against Eu-HMTase1 (middle) and NG36/G9a (bottom) in the fractions from glycerol gradient sedimentation (corresponding to Fig. 1B) is represented. **(C)** (Top) The encoded polypeptide sequence of Eu-HMTase1 (*14*). The ankyrin repeats and SET domain are boxed. (Bottom) Schematic diagram representing the structures of histone methyltransferases. **(D)** Histone methyltransferase activity in the recombinant Eu-HMTase1 was determined as described in (A).

ankyrin repeats and a SET domain, and they share 63% sequence similarity in the entire regions (Fig. 3C). Like NG36/G9a, recombinant Eu-HMTase1 exhibited histone methyltransferase activity with specificity for histone H3 in core histones (Fig. 3D, top, lane 1), whereas it hardly methylated nucleosomes (top, lane 3), suggesting that other subunits in E2F6.com-1 are required for modification of nucleosomal substrates. Moreover, amino acid sequencing of histone H3 methylated by the recombinant protein showed that Lys^9 is the target (Fig. 3D, bottom), as does E2F6.com-1.

Mass spectrometric analysis identified HP1γ in addition to Max in the 26 kD band (Fig. 4A). HP1α and β, on the other hand, could not be detected by mass spectrometric and immunoblot analyses (*11*). HP1γ-specific antibody immunoprecipitated a set of polypeptides that are indistinguishable from those in E2F6.com-1 (Fig. 4A), indicating that HP1γ is a bona fide subunit of E2F6.com-1. These data raise a possibility that E2F6.com-1 binds to Lys^9-methylated histone H3 via HP1γ (*17*, *18*). Biotinylated histone H3 peptides both with dimethylation at Lys^9 and without were incubated with E2F6.com-1 and were precipitated with streptavidin-conjugated matrix. Immunoblot analysis indicates that all E2F6.com-1 subunits analyzed preferentially bind to methylated histones (Fig. 4B).

In the human, three HP1 proteins (HP1α, β, and γ) have been isolated (*19*). Whereas HP1α and β are localized predominantly in pericentric heterochromatin, HP1γ is localized in euchromatin (*19*). Like HP1γ, Eu-HMTase1 and NG36/G9a are present in euchromatic locus (*11*). Thus, these histone methyltransferases and HP1γ appear to contribute to gene silencing in euchromatic locus, whereas HP1α (and possibly HP1β) and SUV39H1 could play a role in heterochromatin formation.

Mass spectrometric analyses identified several Polycomb group (PcG) proteins (or putative PcG proteins) in E2F6.com-1, including RING1, RING2, MBLR, h-l(3)mbt-like protein, and YAF2 (Fig. 4A). RING1 and RING2 are related proteins with a ring finger motif. These factors have sequence similarity to *Drosophila* DRING protein. MBLR is another ring finger protein, which has sequence similarity to *Drosophila* Psc (posterior sex combs). The h-l(3)mbt-like protein contains an MBT repeat. The *Drosophila* CG16975 gene product appears to be the closest counterpart of h-l(3)mbt-like protein. Using a yeast two-hybrid screen, YAF2 was originally identified as a YY1-interactive protein that is a homolog of PHO, the product of *Drosophila* PcG gene *pleiohomeotic* (*20*). While this study was in progress, another laboratory showed, also by a yeast two-hybrid screen, that RYBP, a protein closely related to YAF2 (Fig. 4C), is an E2F-6–interactive partner (*21*). However, we could not detect RYBP in E2F6.com-1 by mass spectrometric and immunoblot analyses (*11*).

We next tested promoter occupancies of E2F6.com-1 in G_0 and G_1 fibroblasts by chromatin immunoprecipitation (Fig. 5A). After immunoprecipitation with various antibodies, the presence of E2F-responsive promoters (*2*, *5*) was determined by quantitative polymerase chain reactions (PCR). E2F-6, HP1γ, and Max preferentially occupy these promoters in the G_0 stage in all E2F-responsive promoters tested. In contrast, E2F-1, E2F-4, and p130 preferentially occupy these promoters in the G_1 stage. These results suggest that p130 plays a role in the repression of E2F-responsive genes in G_1, whereas E2F6.com-1 plays a role in G_0.

Given that G_0 is the quiescent stage, stable repression mechanisms would be required in G_0 cells. In this regard, formation of transcriptionally inactive chromatin would be suitable for long-term repression. Our data suggest that E2F- and Myc-responsive genes are coregulated by E2F6.com-1 in quiescent cells. This appears to be reasonable because Myc and E2F (E2F-1 to E2F-5) share common functions such as mitotic responses, cell-cycle stimulation, and induction of apoptosis (*3*). Indeed, a variety of genes, including *CDC2*, *CDC25A*, *cyclin A*, *cyclin D1*, *cyclin D3*, *cyclin E*, *DNA polymerase*α, *E2F-1* to *E2F-3*, *MCM7*, *TK*, *uracil-DNA glycosylase*, and *telomerase reverse transcriptase* are known to contain both E2F- and Myc-binding sites, suggesting that E2F6.com-1 occupies both E2F- and Myc-binding site in such genes.

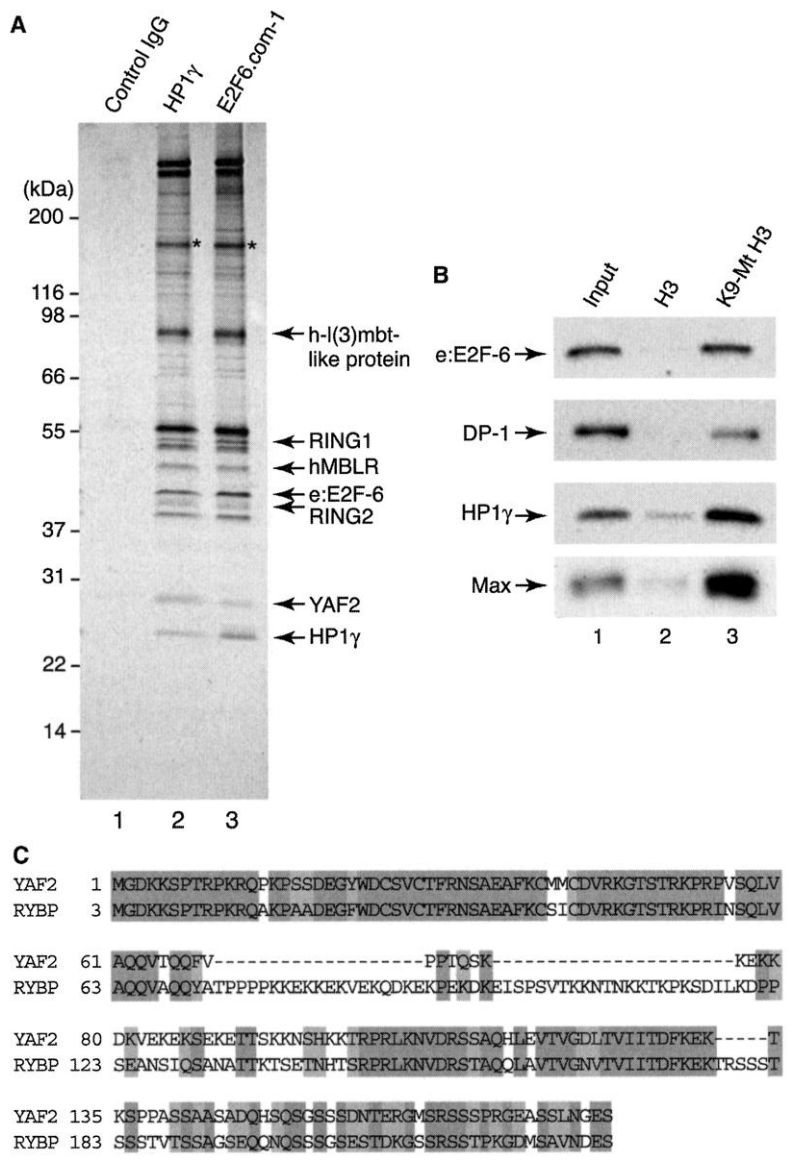

Fig. 4. HP1γ and PcG proteins in E2F6.com-1. **(A)** HP1γ and PcG proteins identified in E2F6.com-1 are shown. The FLAG-purified material (corresponding to Fig. 1A, lane 2) was immunoprecipitated with control IgG (lane 1) and antibody against HP1γ (lane 2). The HA-purified E2F6.com-1 (lane 3) was also analyzed. **(B)** E2F6.com-1 was incubated with biotinylated histone H3 tail with (lane 3) and without (lane 2) dimethylation at Lys^9. After precipitation with streptavidin-conjugated matrix, bound proteins were analyzed by immunoblot with indicated antibodies. 15% of input was also analyzed (lane 3). **(C)** Sequence similarity between YAF2 and RYBP.

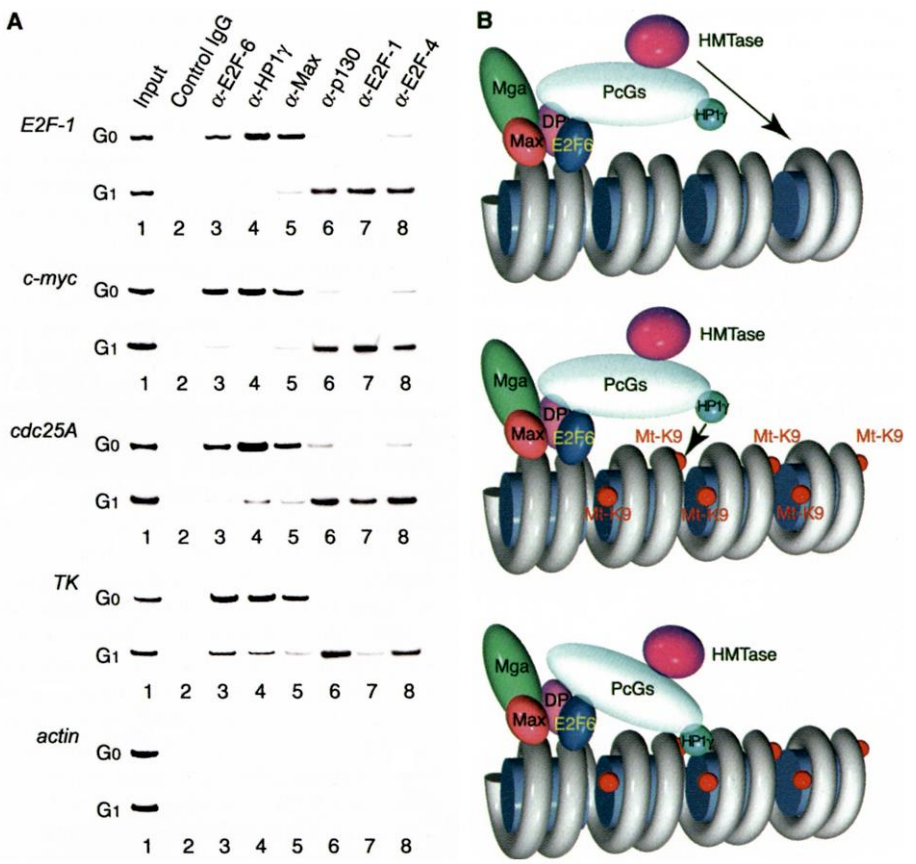

Fig. 5. (**A**) Binding of E2F6.com-1 to the target promoters in vivo. Chromatin immunoprecipitation was performed from G_0 (top) and G_1 (bottom) cells with the indicated antibodies. The presence of target genes in the immunoprecipitates was detected by PCR with primers that amplify *E2F-1*, *cdc25A*, c-*myc*, and *TK* promoters as well as β-*actin*-coding region. (**B**) Model of repression by E2F6.com-1 via formation of transcriptionally inactive chromatin.

As illustrated in Fig. 5B, E2F6.com-1 could be recruited on E2F- and Myc-responsive genes via sequence-specific DNA binding domains in the complex (top), and it could methylate proximate nucleosomes (middle) and load HP1γ and PcG proteins (bottom). This recruitment could form a "platform" that is required for nucleating PcG proteins and which thus contribute to the propagation of inactive chromatin, leading to entire repressed regions.

It is intriguing that E2F6.com-1 is present in HeLa cells that cannot enter the quiescent stage, even though it does not occupy target genes. Revealing the details of these processes would provide further insights into mechanisms whereby normal cells can enter quiescent stage, whereas malignant tumor cells cannot.

Note added in proof: In contrast to our data, Takahashi *et al. (22)* reported that p130 and E2F4 occupy the target promoters in G_0 and early G_1 stages. We believe that this discrepancy could be due to different cell types and conditions for G_0 induction. Takahashi *et al.* used T98G human glioblastoma cells, whereas we used human BJ-1 fibroblasts. Moreover, they arrested the cell cycle by serum starvation, whereas we first arrested the cycle by contact inhibition, then by serum starvation.

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Supporting Online Material

(www.sciencemag.org/cgi/content/full/296/5510/1132/DC1)
Materials and Methods
References

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Autosomal Dominant Mutations Affecting X Inactivation Choice in the Mouse

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X chromosome inactivation is the silencing mechanism eutherian mammals use to equalize the expression of X-linked genes between males and females early in embryonic development. In the mouse, genetic control of inactivation requires elements within the X inactivation center (*Xic*) on the X chromosome that influence the choice of which X chromosome is to be inactivated in individual cells. It has long been posited that unidentified autosomal factors are essential to the process. We have used chemical mutagenesis in the mouse to identify specific factors involved in X inactivation and report two genetically distinct autosomal mutations with dominant effects on X chromosome choice early in embryogenesis.

During early preimplantation development, female cells have two active X chromosomes. As these cells begin to differentiate, they undergo X chromosome inactivation, the epigenetic process that results in the stable silencing of a majority of genes on one X