E2F6, a member of the E2F-family of transcription factors, is a retinoblastoma protein-independent transcriptional repressor. E2F6 associates with polycomb group (PC-G) multiprotein complexes that contain histone H3 methyltransferases, suggesting that E2F6 represses genes by covalent histone modification. However, genes that are repressed by E2F6 via a mechanism that involves histone H3 methylation have not been identified. Using cDNA microarray experiments comparing wild-type and E2f6−/− mouse embryonic fibroblasts, we now found that E2F6 is required to silence the meiosis-specific genes SMC1β and STAG3 in somatic cells. Re-expression of E2F6 in E2f6−/− cells was sufficient to restore their repression. E2F6 binds in vivo to the promoters of these genes through a conserved binding site. Transcriptional repression of SMC1β and STAG3 by E2F6 involves multiple mechanisms, including methylation of histone H3 on lysine 9 and lysine 27. Our findings suggest a molecular mechanism for the stable transcriptional silencing of meiotic genes in somatic cells by E2F6.

A mechanism for transcriptional repression by E2F6 was suggested by the observation that E2F6 associates with polycomb and histone methyltransferases (11–13). A contribution of E2F6 to the biological function of polycomb proteins in vivo is supported by the phenotype of mice with a targeted deletion of E2F6, which present with homeotic transformations of the axial skeleton that are very similar to those identified in polycomb-deficient mice (14). However, evidence that E2F6 represses genes in vivo through covalent histone modification is still missing. Indeed, in a recent study it was found that E2F6 represses a set of genes, including the BRCA1 tumor suppressor gene, by competing for activating E2Fs (15). No evidence for a role of histone H3 methylation in repression of these genes was found on these promoters. Here we report a novel function of E2F6 to stably repress certain meiotic genes in somatic cells through multiple mechanisms including methylation of histone H3 on lysine 9 and lysine 27.

MATERIALS AND METHODS

Primary Mouse Embryonic Fibroblasts and Retroviral Infection—E2f6−/− mice and mouse embryonic fibroblasts (MEFs)2 have been described previously (14). Retroviral infection of MEFs was performed as described (16).

Reporter Assays—The SMC1β (structural maintenance of chromosomes 1β) promoter was amplified by PCR from genomic mouse DNA. Promoter fragments were generated by PCR and cloned into the luciferase reporter vector pGL2basic (Promega). Details are available upon request. Transfections were performed as described previously (6). Briefly, 0.5 × 10^5 NIH-3T3 cells or early passage MEFs (wild-type or E2f6−/−) were plated in 24-well culture dishes. 24 h later, 1 μg of luciferase reporter plasmid and expression plasmids were cotransfected with Lipofectamine 2000 (Invitrogen). Luciferase and β-galactosidase assays were performed as described (6). 0.5 μg of cytomegalovirus-β-galactosidase was cotransfected, and luciferase activity was normalized to β-galactosidase activity to account for differences in transfection efficiency. Error bars in Fig. 2 represent the standard deviation within a representative experiment. Each experiment has been repeated at least three times.

RT-PCR—RNA was isolated from MEFs with RNAeasy columns (Qiagen) and reverse transcribed with 200 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen). cDNA was amplified by PCR with 1 unit of Taq (Invitrogen). Primer sequences are available upon request.

Chromatin Immunoprecipitation (ChIP)—Chromatin immunoprecipitation was performed as described previously (17). Briefly, cross-linked chromatin was prepared from early passage MEFs, sonicated to an average length of 500–1500 nucleotides, and immunoprecipitated with the following antibodies: mouse E2F6 (14); E2F1, sc-193; E2F3, sc-879; E2F4, sc-1082 (Santa Cruz Biotechnology); H3-K9/K27 dimethyl, ab7312, H3-K9/K27 trimethyl, and ab8898 (Abcam), H3-K9 monomethyl, dimethyl, and trimethyl and H3-K27 monomethyl, dimethyl, and trimethyl (18) (kind gift from T. Jenuwein). Precipitated DNA was amplified by PCR. Primer sequences are available upon request.

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2 The abbreviations used are: MEF, mouse embryonic fibroblast; ChIP, chromatin immunoprecipitation; DAC, 5′-aza-deoxycytidine; DHFR, dihydrofolate reductase; RT, reverse transcription; SMC1β, structural maintenance of chromosome 1β; STAG3, stromal antigen 3; TSA, trichostatin A.
Gel Retardation Assays—Gel retardation assays were performed as described (6) with 5 μl of in vitro translated E2F proteins and E2F sites derived from the DHFR, SMC1β, and STAG3 (stromal antigen 3) promoters. Oligonucleotide sequences are available upon request. Competition experiments were performed with unlabeled, double-stranded oligonucleotides.

Immunoblotting—Primary mouse tissues (testis and thymus) or MEFs (wild-type or E2f6−/−) were lysed in radioimmune precipitation assay buffer (50 mM HEPES, pH 7.5, 140 mM NaCl, 1% Triton X-100, 0.1% deoxycholate, 1 mM EDTA, 1 mM dithiothreitol, and protease inhibitors (Sigma)). Equal amounts of protein (100 μg) were separated by SDS-gel electrophoresis and transferred to Immobilon-P (Millipore) membranes. STAG3 was detected with polyclonal antiserum D-20 (Santa Cruz Biotechnology catalog number sc-20341).

RESULTS

We have previously generated mice with a targeted deletion of E2F6 and reported normal cell cycle progression of fibroblasts isolated from E2f6−/− embryos (14). This finding suggests that E2F6 is not required for the regulation of typical E2F-regulated cell cycle genes. To identify genes that depend on E2F6, we performed cDNA microarray experiments with mRNA from E2f6+/+ and E2f6−/− MEFs. A relatively small number of genes was up-regulated in E2f6−/− cells when compared with wild-type control cells (data not shown). Activation of genes in E2f6−/− cells is consistent with the proposed role for E2F6 in transcriptional repression. In this study we focused on two genes that were significantly reduced expression of all three genes (Fig. 1A). To analyze the expression of these genes in different MEF preparations by RT-PCR. STAG3, SMC1β, and XM_196054 were undetectable in wild-type fibroblasts and strongly elevated in E2f6−/− MEFs, confirming the microarray data (Fig. 1A). Re-introduction of E2F6 into E2f6−/− cells significantly reduced expression of all three genes (Fig. 1B), suggesting that the expression of these genes in E2f6−/− cells is directly caused by the lack of E2F6 and is not due to secondary changes. We used immunoblotting with a STAG3-specific polyclonal antiserum and with lysates from E2f6−/− MEFs to test whether STAG3 is also increased on the protein level (Fig. 1C). A band of the expected size for STAG3 of 135-kDa was detected in E2f6−/− cells, but not in wild-type cells. The band detected in E2f6−/− cells co-migrates with STAG3 in testis lysates. We concluded that STAG3 is also induced on the protein level in cells that lack E2F6 (Fig. 1C). To analyze the normal expression pattern of XM_196054, we prepared RNA from a panel of different organs from an adult mouse and performed RT-PCR. XM_196054 expression was undetectable in most organs but was highly expressed in testis (Fig. 1E). Thus, it is possible that XM_196054 also has a function in meiosis. We conclude that the loss of E2f6 results in the abnormal expression of testis-specific genes in mitotic cells and that E2F6 is normally required to repress these genes in somatic cells.

It was reported previously (15) that E2F6 represses a set of genes, including BRCA1 and HP1α, in human tumor cell lines. Surprisingly, expression levels of these genes were equal in E2f6−/− and wild-type MEFs (Fig. 1A). Overexpression of E2F6 in MEFs had no effect on the expression of these genes (Fig. 1B), suggesting that E2F6 does not appear to regulate the same set of target genes as in human tumor cell lines. In addition, our microarray experiments do not support a role for E2F6 in the regulation of cell cycle genes in MEFs, because we did not find any of the typical E2F-regulated cell cycle genes among the E2F6-regulated genes (data not shown). These findings are consistent with our previous report of normal cell cycle progression of E2f6−/− MEFs (14). Taken together, our data suggest that E2F6 is specifically required for tissue-specific silencing of a subset of meiotic genes in non-mitotic tissues. Surprisingly, expression of STAG3 and SMC1β in E2f6−/− cells does not cause any obvious problems for the proliferation of these cells, perhaps because these proteins are unable to function as cohesins in mitotic cells.

To begin to analyze the mechanism of repression of meiotic genes by E2F6 we first performed reporter assays with the promoter of the SMC1β gene (Fig. 2A). A SMC1β promoter fragment of 941 nucleotides had higher activity in wild-type MEFs than the empty reporter plasmid. Thus, unlike the endogenous gene, the transiently expressed promoter...
is not completely silenced in wild-type cells. Nevertheless, the activity of the promoter was severalfold higher in E2f6−/− and E2f6+/− MEFs. As a control, the empty reporter plasmid pGL2-basic was used. E2F6 represses the SMC1β promoter. The indicated SMC1β reporter plasmids were cotransfected together with empty vector or with expression plasmids for mouse E2F6 (E2F6), human E2F6 (hE2F6), or a point mutant of human E2F6 deficient in DNA-binding (E68). ctrl, control. D, comparison of the putative E2F-binding site in the murine (mu) and human (hu) SMC1β and STAG3 promoters with a consensus E2F binding site (29). The sequence of the mutated reporter construct used in panels E and F is also shown. E, the activity of the mutated reporter construct (Mut) was compared in wild-type and E2f6−/− cells. As control, the unmodified −150 reporter was used. F, the indicated reporter plasmids were cotransfected (Transf) together with empty vector or with an expression plasmid for E2F6. Mut, mutated construct; ctrl, control.
E2F binding site (see Fig. 2D). To test whether this E2F site is required for repression of the SMC1β promoter by E2F6, we mutated the E2F element and analyzed the activity of the mutated reporter construct in E2f6+/+ and E2f6−/− cells (Fig. 2F). The mutated reporter gene had equal activity in E2f6+/+ and E2f6−/− cells, demonstrating that the E2F site is required for repression by E2F6. Consistent with this notion, overexpression of E2F6 repressed the wild-type but not the mutated reporter construct (Fig. 2F). We conclude that the E2F site of the SMC1β promoter is required for transcriptional repression by E2F6. Interestingly, identical elements are present in the human SMC1β promoter and in the murine and human STAG3 promoters (Fig. 2D). In addition, sequences flanking the E2F site are also highly conserved in the human and murine promoters. In particular, in each case the E2F-core binding site is followed by a poly(T) stretch (Fig. 2D). Whether the XM_196054 gene contains a similar binding site remains to be shown. Its promoter could not be analyzed, because the structure of the gene is unknown and the promoter could not be identified in the nucleotide data base.

Next, we wanted to test whether E2F6 binds directly to the predicted E2F-binding sites of the SMC1β and STAG3 promoters. To address this question we performed gel retardation experiments with in vitro translated E2F6 and DP2 and radiolabeled oligonucleotides corresponding to the E2F sites of the SMC1β and STAG3 promoters. Binding of E2F6 to these elements was compared with binding to the E2F-site of the DHFR promoter, a bona fide E2F-binding sequence (22). As shown in Fig. 3A, binding of E2F6 to the SMC1β site was readily detected, whereas under the same conditions E2F6 did not significantly bind to the DHFR sequence (compare lane 1 and lane 7 in Fig. 3A). Preferential binding of E2F6 to the SMC1β sites was confirmed by competition with unlabeled oligonucleotides. The SMC1β, but not the DHFR oligonucleotide, efficiently competed for the binding of E2F6 to the E2F element (Fig. 3A, lanes 2–4). We concluded that E2F6 binds with higher affinity to the SMC1β promoter than to the classical E2F binding site in the DHFR promoter. In contrast, endogenous E2F proteins present in the reticulocyte lysate preferentially associate with the DHFR element (Fig. 3A, lane 7, indicated by an asterisk). Consistent with these data, in vitro translated E2F1/DP2 bound only weakly to the SMC1β E2F-site when compared with E2F6-site of the DHFR promoter (Fig. 3B, compares lane 1 and 4). As described above, the STAG3 promoter contains an E2F site that is identical to the one in the SMC1β promoter (Fig. 2D), and in vitro translated E2F6 readily bound to the STAG3 element (Fig. 3C). Specific binding to the STAG3 E2F site was demonstrated by competition with unlabeled wild-type oligonucleotides (Fig. 3C, lanes 2 and 3), but not by oligonucleotides with an mutation in the E2F-binding site (lanes 4 and 5) or by DHFR oligonucleotides (lanes 6 and 7). Together, these obser-
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vations show that the STAG3 and SMC1β promoters contain E2F sites that are highly specific for E2F6.

To determine whether E2F6 associates with the STAG3 promoter in vivo, we used ChIP experiments. Chromatin prepared from wild-type and E2f6−/− MEFs was immunoprecipitated with a polyclonal antibody directed against mouse E2F6. Immunoprecipitates were analyzed by PCR with primers specific for the STAG3 promoter. Binding of E2F6 to the STAG3 promoter could readily be detected in E2f6−/− MEFs that were infected with a recombinant retrovirus directing the expression of E2F6 (Fig. 4A, lane 2). Importantly, no signal was detected in control infected E2f6−/− cells (Fig. 4A, lane 1). In addition, no binding of E2F6 to a control region 4 kb downstream of the STAG3 promoter was evident. Endogenous E2F6 associated with the STAG3 promoter in wild-type MEFs (Fig. 4A, lane 3), although, as expected, its binding was weaker than the binding of the overexpressed protein. Specific association of E2F6 with the STAG3 promoter was confirmed by quantitative real-time PCR with independent primer sets (Fig. 4, B and C). Although it was more difficult to PCR amplify the GC-rich SMC1β promoter, binding of E2F6 to this promoter could also be detected by ChIP (Fig. 4D). We concluded that E2F6 associates with the STAG3 and SMC1β promoters in vivo. These observations are consistent with the gel retardation experiments that demonstrated high affinity E2F6-binding sites in the STAG3 and SMC1β promoters.

It has been shown that can E2F6 represses a set of target genes in human cancer cell lines by competing for activating E2Fs (15). Consequently, RNA interference-mediated depletion of E2F6 results in the association of E2F1 with these E2F6-regulated promoters (15). To begin to analyze whether meiotic genes are repressed by a similar mechanism, we next analyzed whether activating E2Fs associate with the STAG3 promoter in E2f6−/− cells. As a positive control, we analyzed the p107 promoter. Binding of E2F proteins to this promoter has previously been demonstrated (23). As expected, we detected E2Fs at the p107 promoter, and, importantly, their binding was equal in wild-type and E2f6−/− cells. In contrast, E2F1 and E2F2 did not significantly bind to the STAG3 promoter in wild-type cells (Fig. 4E, lane 2), although, as expected, its binding was weaker than the binding of the overexpressed protein. Specific association of E2F6 with the STAG3 promoter was confirmed by quantitative real-time PCR with independent primer sets (Fig. 4, B and C). Although it was more difficult to PCR amplify the GC-rich SMC1β promoter, binding of E2F6 to this promoter could also be detected by ChIP (Fig. 4D). We concluded that E2F6 associates with the STAG3 and SMC1β promoters in vivo. These observations are consistent with the gel retardation experiments that demonstrated high affinity E2F6-binding sites in the STAG3 and SMC1β promoters.

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plexes that contain histone methyltransferases with specificity for histone H3, lysine 9, and lysine 27 (H3-Lys9 and H3-Lys27, respectively) (12–14). Methylation of histone H3-Lys9 and H3-Lys27 has been linked to gene repression (reviewed in Ref. 25). ChIP assays with modification-specific antibodies showed that histone H3 is dimethylated and trimethylated at Lys9 and/or Lys27 on the STAG3 and SMC1B promoters in wild-type cells (Fig. 5A). Interestingly, ChIP assays with chromatin isolated from E2f6+/+ MEFs indicate that both modifications depend on E2f6. (Fig. 5A). We concluded that E2f6 is required for methylation of H3-Lys9/H3-Lys27 at the STAG3 and SMC1B promoters. Because the antibodies we used in these initial ChIP experiments cannot discriminate between methylated Lys9 or Lys27 of histone H3, we next used a set of more specific antibodies developed in the Jenuwein laboratory that can distinguish between these modifications (18). ChIP assays with these antibodies demonstrated that E2f6 is required for histone H3-Lys9 dimethylation and H3-Lys27 trimethylation on the promoters of the meiotic cohesins STAG3 and SMC1B. However, because methylated histone H3 and H4 at the STAG3 and SMC1B promoters is significantly increased in E2f6−/− MEFS, the STAG3 and SMC1B promoters both contain CpG islands that may be subjected to methylation in vivo (not shown). Therefore, we next explored the possibility that cytosine methylation plays a role in the repression of SMC1B and STAG3 by E2f6. To test this possibility, cells were treated with the DNA demethylating agent 5′-aza-deoxycytidine (DAC), either alone or in combination with TSA. DAC alone had little effect on the expression of STAG3 and SMC1B. In contrast, sequential treatment with DAC and TSA resulted in reactivation of SMC1B. The combined use of DAC and TSA also resulted in a slightly stronger activation of STAG3 compared with the treatment with TSA alone. Together, these results confirm that histone deacetylation is involved in the repression of STAG3 and SMC1B in somatic cells. In addition, our data illustrate that DNA-methylation is required to maintain repression of SMC1B. However because TSA treatment alone was sufficient to activate STAG3, DNA methylation may not play a major role in the transcriptional silencing of this gene in non-meiotic cells.

**DISCUSSION**

In this study we describe a novel function for E2f6 in the transcriptional repression of tissue-specific genes. We demonstrate that E2f6 is required to repress the genes encoding for the meiotic cohesins STAG3 and SMC1B and for a third meiotic protein with unknown function. These genes normally show a highly restricted, testis-specific, expression pattern. In the absence of E2f6, however, they are de-repressed in non-meiotic cells. Thus, silencing of these genes in somatic cells is critically dependent on E2f6. This finding is consistent with a recent study by Pohlers et al. (27), who identified a role for E2f6 in the repression of testis-specific α-tubulin 3 and α-tubulin 7 genes. We could not identify these α-tubulin genes in our study because they were not represented on our cDNA microarray.
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We identified multiple histone modifications that correlate with the inactive state of the STAG3 and SMC1β promoters in E2f6+/+ cells. These modifications include histone H3-Lys9/H3-Lys27 methylation (Fig. 5) and histone H3 deacetylation (Fig. 6). In addition, our data suggest that DNA methylation is involved in the silencing of SMC1β, similar to what was described before for the E2F6-regulated α-tubulin 3 gene (27). Significantly, however, our data also suggest that DNA-methylation may not be a general mechanism for the silencing of E2F6-regulated genes. First, whereas the DNA demethylation agent DAC was sufficient to activate SMC1β, it did not reactivate STAG3 in somatic cells. Thus, cytosine methylation may only play a minor role in repression of STAG3 (Fig. 6B). Second, we found that E2F6 associates with the silenced STAG3 and SMC1β promoters in wild-type cells (Fig. 4). This finding implies that the silenced STAG3 and SMC1β promoters are accessible and does not support a model in which E2F6-mediated DNA methylation prevents the binding of transcription factors. Consistent with this notion, activating E2Fs were not enriched on the STAG3 promoter in E2f6−/− cells. Thus, although DNA methylation may be an indirect consequence of long-term silencing of a subset of E2F6-regulated meiotic promoters.

In light of these observations, we propose that E2F6 primarily acts as a sequence-specific DNA-binding subunit to recruit a complex with histone methyltransferase/deacetylase activity to the promoters of selected meiotic genes. E2F6 has been found previously in a complex with the histone methyltransferases G9a and Eu-HMTase1 (12). Although we failed to detect G9a and HMTase1 in CHIP assays at the STAG3 and SMC1β promoters (data not shown), it is possible that these methyltransferases act at an earlier time point during embryonic development and that they are not necessary to maintain repression of the STAG3 and SMC1β genes in fibroblasts. EZH2, the catalytic subunit of the PRC2polycomb complex, is another histone methyltransferase that has been found in association with E2F6 (Ref. 11).3 Because this enzyme is specific for histone H3-Lys9, it could be responsible for E2F6-dependent H3-Lys927 tri-methylation of the STAG3 and SMC1β promoters. Because of the lack of functional ChIP antibodies for EZH2, we were not able to analyze the recruitment of this protein to E2F6-regulated promoters. We speculate that silencing of the STAG3 and SMC1β promoters is maintained by proteins that recognize the methylated histone H3-Lys9 and H3-Lys27 residues. Candidates include the chromodomain proteins HP1 and M33/Cbx2. Further work will be necessary to identify the proteins that maintain stable repression of E2F6-regulated genes.

Interestingly, recent results from Drosophila demonstrate cell cycle-dependent regulation of some dE2F-regulated genes and, for other target genes, stable repression that is completely resistant to cell cycle progression (28). In Drosophila, repression of both types of genes is mediated by retinoblastoma-related proteins. It is possible that in the mammalian system E2F6 has evolved to silence tissue-specific genes independently from pocket proteins and to completely separate their regulation from the cell cycle-dependent repression and activation of the typical E2F-regulated genes.

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