Tbx2 Is Overexpressed and Plays an Important Role in Maintaining Proliferation and Suppression of Senescence in Melanomas

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Abstract
The INK4a and ARF genes found at the CDKN2A locus are key effectors of cellular senescence that is believed to act as a powerful anticancer mechanism. Accordingly, mutations in these genes are present in a wide variety of spontaneous human cancers and CDKN2A germ line mutations are found in familial melanoma. The Tbx2 gene encoding a key developmental transcription factor is amplified in pancreatic cancer cell lines and preferentially amplified and overexpressed in BRCA1 and BRCA2 mutated breast tumors. Overexpression of Tbx2 and the related factor Tbx3, which is also overexpressed in breast cancer and melanomas, can suppress senescence in defined experimental systems through repression of ARF expression. However, it is not known how Tbx2 mediates its repressive effect nor whether endogenous Tbx2 or Tbx3 perform a similar antisenescence function in transformed cells. This is a particularly important question because the loss of CDKN2A in many human cancers would, in principle, bypass the requirement for Tbx2/3-mediated repression of ARF in suppressing senescence. We show here that Tbx2 is overexpressed in melanoma cell lines and that Tbx2 targets histone deacetylase 1 to the p21(Cip1) (CDKN1A) initiator. Strikingly, expression of an inducible dominant-negative Tbx2 (dnTbx2) leads to displacement of histone deacetylase 1, up-regulation of p21(Cip1) expression, and the induction of replicative senescence in CDKN2A-null B16 melanoma cells. In human melanoma cells, expression of dnTbx2 leads to severely reduced growth and induction of senescence-associated heterochromatin foci. The results suggest that the activity of endogenous Tbx2 is critically required to maintain proliferation and suppress senescence in melanomas. (Cancer Res 2005; 65(6): 2260-8)

Introduction
The fact that oncogenic Ras can induce senescence in primary fibroblasts (1) has led to the notion that senescence represents a powerful defense against cancer (2, 3). Indeed, recent evidence indicates that senescence in human cells in response to oncogenic stress exemplified by activated Ras leads to the formation of senescence-associated heterochromatin foci (SAHF) in an Rb-dependent fashion (4). The CDKN2A locus encoding both the INK4A and ARF genes can act to promote senescence, and both genes have been established as important tumor suppressors in human cancer (5). Mutation or deletion of either, or frequently both, the INK4a and ARF genes is associated with melanoma (6–8), a highly dangerous form of skin cancer with an alarming increase in incidence. The key role of CDKN2A in the melanocyte lineage is underscored by the observation that in contrast to fibroblasts (9), a single copy of a deletion at this locus disrupts senescence in mouse melanocytes (10), and targeted expression of activated Ras to melanocytes in transgenic mice generates melanomas only if the INK4a gene is absent (11). Moreover, the INK4a gene is necessary for the normal senescence of primary human melanocytes that occurs in the absence of elevated p21(Cip1) (CDKN1A, hereafter termed p21) or p53 levels (12); human melanocytes deficient for INK4a have an extended life span but do eventually senesce under conditions where p21 expression is induced. Thus, the key role of the CDKN2A locus and the INK4a gene in particular in melanocyte senescence is likely to explain its loss or mutation in melanoma.

The T-box gene family (for reviews, see refs. 13–16) illustrates an important example of proteins that are involved both in controlling fundamental developmental decisions and are also misregulated in cancer. In addition to their role in development, increasing evidence suggests that Tbx2 and the highly related factor Tbx3 play a role in cancer progression. The Tbx2 gene maps to 17q23, a region that is frequently mutated in ovarian carcinomas (17, 18) and Tbx2 is also amplified in pancreatic cancer cell lines (19) and is preferentially amplified and overexpressed in BRCA1 and BRCA2 mutated breast tumors (20). Furthermore, Tbx3 is also overexpressed in breast cancer lines (21) and can cooperate with Ras and Myc to transform cells and disrupt pathways required for apoptosis (22). Significantly, in defined experimental systems using CDKN2A wild-type primary fibroblasts or striatal cells overexpression of either Tbx2 and Tbx3 can inhibit senescence, most likely through their ability to repress expression of the ARF and p21 promoters (23–27). However, it is not known how Tbx2 mediates its repressive effect nor whether endogenous Tbx2 or Tbx3 perform a similar antisenescence function in transformed cells. This is a particularly important question because the loss of CDKN2A in many human cancers would, in principle, bypass the requirement for Tbx2/3-mediated repression of ARF in suppressing senescence.

In this study, we show that activation of a dnTbx2 molecule induces senescence in CDKN2A-null B16 melanoma cells. Senescence is accompanied by an increase in the levels of the Tbx2 target gene p21 and displacement of histone deacetylase 1 (HDAC1) from the p21 promoter. In human melanoma cells, dnTbx2 induces the formation of SAHF and severely reduced proliferation. These results identify a molecular mechanism for transcriptional repression by Tbx2 and suggest that continued Tbx2 activity is required to prevent CDKN2A-independent senescence in transformed cells.
Materials and Methods

Plasmid Constructs. To generate the hemagglutinin (HA) epitope-tagged ER-dnTbx2 expression vector, Tbx2-1-301 deletion mutant was PCR cloned as a BamHI-SalI fragment into pBabe.HA-ER (26). Primers used were as follows: Tbx2 5'-AGACGGCATCCATGGCTTACCAACCGTTCCCA-3' and 5'-AGACGTGCTCAACATTGCTTCCATTACA-3'. To generate glutathione S-transferase (GST) expression vectors, Tbx2 was cloned as a BamHI fragment into pGEX-2TK. Tbx2(1-361) and Tbx2(361-701) were subcloned as BamHI-BglII and BglII-BamHI fragments, respectively, from pCMV-Tbx2 into pGEX-2TK. pCMV-Tbx2 contains a silent mutation introducing a BglII site at amino acid position 361.

Generation and Characterization of Cell Lines. All cell lines were grown in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin unless stated. To generate virus-producing cell lines, Psi2 cells were transfected with pBabe.HA-ER or pBabe.HA-ER-dnTbx2 expression vectors using FUGENE 6 (Roche Molecular Biologicals, Lewes, United Kingdom) according to the manufacturer's instructions. Two days later, 1 µg puromycin was added and pooled cell lines were expanded and maintained under selection conditions. HA-ER-dnTbx2 and HA-ER-expressing cell lines were generated by infecting B16 cells using viral supernatants from Psi2-HA-ER-dnTbx2 and Psi2-HA-ER cells. Retroviral infections were carried out as previously described (23). WM266-4 cells were transduced with the retroviral DNA. Three days after infection or transfection, 1 µg/mL puromycin was added. Individual clones were isolated and expanded under selection conditions and characterized for expression by indirect immunofluorescence and Western blotting. Estrogen receptor (ER) fusion proteins were activated by the addition 4-hydroxy tamoxifen (4-OHT) to a final concentration of 300 nmol/L.

Transfection. 1 Day after infection, cells were transfected with the retroviral DNA. Three days after infection or transfection, 1 µg/mL puromycin was added. Individual clones were isolated and expanded under selection conditions and characterized for expression by indirect immunofluorescence and Western blotting. Estrogen receptor (ER) fusion proteins were activated by the addition 4-hydroxy tamoxifen (4-OHT) to a final concentration of 300 nmol/L.

Senessence-Associated β-Galactosidase Assay. For the in situ assay, cells were washed twice with PBS, fixed for 5 minutes in 2% formaldehyde/0.2% glutaraldehyde, washed again with PBS, and then incubated for 16 hours at 37°C (no CO2) in fresh senescence-associated β-galactosidase (SA-β-gal) buffer [1 mg/mL X-Gal, 5 mM/L potassium ferrocyanide, 5 mM/L potassium ferricyanide, 2 mM/L MgCl2 in PBS (pH 6.0)]. Cytoplasmic SA-β-gal activity results in a cytoplasmic blue staining that can be visualized by light microscopy. For the liquid assay, B16 cells were transfected with anti-Tbx2 or anti-Mitf siRNAs or a control nonsilencing siRNA 1 day after plating, and then again 3 days later. After 7 days, the cells were washed with PBS and harvested in 0.25 ml/L Tris-HCl (pH 6.0). Equal amounts of protein were used to perform the liquid β-gal assay. Twenty milliliter cell extracts were mixed with 500 µL of Z buffer [50 mM/L Na2PO4, 40 mM/L NaHPO4, 10 mM/L KCl, 1 mM/L MgSO4, 50 mM/L β-mercaptoethanol (pH 6.0)] and 100 µL 0-nitrophenyl-β-galactoside at 4 mg/mL. The reaction was incubated overnight at 37°C and the absorbance was read at 420 nm against the control siRNA sample. The Tbx2 siRNAs used were siRNA 5′-GCGAGGCGUAUGGGCGUUU-3′, as described previously (23), and siRNA 5′-GUUCACCAUCUCGGCCGUGT-3′.

Immunofluorescence Microscopy. Cells grown on glass coverslips were washed twice with PBS and fixed with 4% paraformaldehyde for 15 minutes at room temperature before permeabilization using 0.2% Triton X-100 for 10 minutes at room temperature. Coverslips were incubated for 1 hour with 1:100 dilution anti-HA (Clone HA-7, Sigma, Poole, United Kingdom) mouse monoclonal antibody, washed thrice with PBS, and then incubated for 1 hour with 1:100 dilution anti-mouse secondary antibody coupled to FITC (Vector Laboratories, Peterborough, United Kingdom). Cells were washed again with PBS and mounted using Vectashield mounting medium with propidium iodide and examined by confocal microscopy.

For BrdUrd assays cells grown on coverslips were labeled with BrdUrd for 1 hour, washed twice with PBS, and then fixed and permeabilized as described above. Coverslips were then incubated with 2 mol/L HCl, washed thrice with PBS, and blocked using 5% fetal bovine serum before incubating with 1:100 dilution anti-BrdUrd (PharMingen, San Diego, CA) monoclonal antibody. Following processing for indirect immunofluorescence using FITC-coupled anti-mouse secondary antibody, percentage of cells positive for BrdUrd incorporation was determined by confocal microscopy.

Glutathione S-transferase Pull-Down Assays and Immunoprecipitations. GST fusion proteins were expressed in E. coli strain BL21(DE3) pLysS and purified as previously described (28). GST pull-down assays were done as described (29). For immunoprecipitations, extracts were prepared from 10 cm dishes of COS7 cells transiently transfected with 5 µg either pCMV5V5-Tbx2 or pCMV expression vectors. Cells were lysed in 1.5 mL radioimmunoprecipitation assay buffer [50 mM/L Tris (pH 8.0), 0.1% SDS, 0.01% sodium deoxycholate, 150 mM/L NaCl], 1% Triton X-100, protease inhibitor cocktail (Roche Molecular Biochemicals) for 15 minutes on ice and clarified by centrifugation. Extracts were precleared with 50 µL Protein A Sepharose for 2 hours at 4°C and the beads were then removed by centrifugation. Ten microliters anti-SV5 monoclonal antibody were added and incubated for 16 hours at 4°C with rotation. Fifty microliters Protein A Sepharose were then added and incubated for 3 hours at 4°C with rotation to capture the immune complexes. Beads were pelleted, washed five times with radioimmunoprecipitation assay buffer, and bound proteins eluted by heating in 20 µL SDS-PAGE loading buffer.

Western Blot Analysis. Whole cell extracts were prepared by lysing cells directly in SDS-PAGE loading buffer, sonicating, and boiling for 5 minutes. Proteins were resolved on 10% to 15% SDS-PAGE gels and then transferred onto Hybond C (Amersham, Amersham, United Kingdom) membranes. Membranes were probed with appropriate primary antibodies, detected using peroxidase-conjugated secondary antibodies, and visualized by enhanced chemiluminescence (Amersham). Primary antibodies used were anti-p21 (C-19) rabbit polyclonal, anti-lamin B (C-20) goat polyclonal (both from Santa Cruz Biotechnology, Santa Cruz, CA), anti-HDAC1 rabbit polyclonal (Upstate, Milton Keynes, United Kingdom), anti-SV5 mouse monoclonal (Serotec, Oxford, United Kingdom), and anti-Tbx2 monoclonal (23). Anti-3meK9H3 antibody was obtained from Abcam (Cambridge, United Kingdom), anti-heterochromatin-binding protein 1 (HP1α) from Euromedex (Mundolsheim France), and anti-HP1β antibodies from Chemicon International (Harrow, United Kingdom).

Histone Deacetylase Assay. One microgram GST and GST-Tbx2 proteins immobilized on beads was incubated with 1 mg HeLa nuclear extract in a pull-down assay. Associated HDAC activity was determined using 10 µL 3H-acetate-labeled chicken erythrocyte histones as a substrate as described (30).

Chromatin Immunoprecipitation. Chromatin immunoprecipitation assays were done as previously described (31) using either 5 µg anti-HDAC1 rabbit polyclonal antibody (Upstate) or 5 µg nonspecific IgG (Bio-Rad, Hemel Hempsted, United Kingdom). Primers were designed to PCR amplify the p21 promoter region for mouse Tbx2 (5′-CTAGGCTGCAAGGTCATA-3′ and 5′-CTAGGGTTTCTCTCTCTGACAA-3′).

Results

Tbx2 Is Overexpressed in Melanoma Cells. Tbx2 has been shown to be overexpressed in BRCAl and BRCAl2 mutant breast cancer cell lines. Although we have previously shown that the Tbx2 protein is expressed in murine B16 melanoma cells (32), its expression profile in human melanoma cells has not previously been examined or compared with its expression in untransformed melanocytes. Therefore, we examined Tbx2 expression levels in a series of human and mouse melanoma cell lines as well as primary human melanocytes and the mouse melanocyte cell line melan-c. The results (Fig. 1) obtained by Western blotting using a Tbx2-specific monoclonal antibody revealed that whereas Tbx2 was poorly expressed in both the primary human melanocytes and the mouse melanocyte cell line, it was overexpressed in all melanoma cell lines tested, with expression being particularly robust in 501mel, B16, and WM266-4 cells. This is consistent with evidence
from expression profiling using gene arrays, which suggests that Tbx2 is highly expressed in primary melanomas and renal cell carcinomas compared with many other cancers (33). The fact that Tbx2 is expressed at elevated levels in melanoma cells raised the possibility that it might play a key role in melanoma proliferation. Given that melanoma is a highly aggressive and increasingly common skin cancer and that metastatic melanoma is notoriously refractive to treatment, understanding the role of Tbx2 in these cells is a key issue.

**Tbx2 Associates with Histone Deacetylase 1 In vitro and In vivo.** Although Tbx2 has been characterized as a transcriptional repressor (34, 35), the mechanism by which Tbx2 represses expression of its target genes is unknown. Transcriptional repression is mediated in part by non-DNA-binding corepressors, such as the HDAC family. HDACs are components of large multiprotein complexes that repress transcription when targeted to a promoter by promoting a more compact local chromatin organization (for reviews, see refs. 36, 37). It has been proposed that the regulation of HDAC function is disrupted in cancer cells resulting in inappropriate gene expression and loss of checkpoint control (38, 39). Accordingly, inhibition of HDAC activity by compounds such as Trichostatin A, sodium butyrate, and trapoxin control (38, 39). Accordingly, inhibition of HDAC activity by compounds such as Trichostatin A, sodium butyrate, and trapoxin

> **Figure 1.** Tbx2 is overexpressed in melanoma cells. Western blot using anti-Tbx2 monoclonal antibody and extracts from the indicated cell lines. Melc is a mouse melanocyte cell line; B16 a mouse melanoma cell line; and 501, WM266-4, MeWo, HMB2, and A375P are all human melanoma cell lines. Lamin B2 was used as a loading control. The relative expression of Tbx2 normalized to lamin B2 is indicated.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Relative Expression</th>
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<tr>
<td>Melc</td>
<td>&lt; 5</td>
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<tr>
<td>B16</td>
<td>&lt; 5</td>
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<tr>
<td>501</td>
<td>27</td>
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<tr>
<td>WM266-4</td>
<td>100</td>
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<tr>
<td>MeWo</td>
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<td>HMB2</td>
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To identify which HDAC might interact with Tbx2, a series of GST pull-down assays was done using the transcriptional repression domain-containing carboxyl-terminal portion (amino acids 361-701) of Tbx2 fused to GST or GST alone together with in vitro translated HDAC1, a class I HDAC family member, or the class II HDAC, HDAC4. The results (Fig. 2B) indicated that GST-Tbx2(361-701) specifically bound HDAC1 but not HDAC4 and that no binding of either HDAC was observed using GST alone. In the converse experiment, in vitro translated Tbx2 was specifically retained on GST-HDAC1 beads but not on beads containing GST alone (Fig. 2C). Similar results were obtained if HeLa cell nuclear extract was used as a source of HDAC (Fig. 2D), with GST-Tbx2(361-701) associating with HDAC1 as detected by Western blotting of the bound proteins. Importantly, no binding was detected when GST or a fragment of Tbx2 containing the T-box but lacking the transcriptional repression domain [GST-Tbx2(1-361)] was used in the pull-down assay with nuclear extract. Finally, we were also able to show that SV5 epitope-tagged-Tbx2 expressed

> **Figure 2.** Tbx2 associates with histone deacetylase 1. A, GST and GST-Tbx2 fusion proteins were incubated with HeLa nuclear extract in a pull-down assay. Associated HDAC activity was determined using 10 μl [3H]acetate-labeled chicken erythrocyte histones as a substrate. Columns, HDAC activity released (cpm) relative to background (beads alone). B-E, Tbx2 interacts with HDAC1 in vitro and in vivo. The indicated GST fusion proteins were immobilized on beads and incubated with either 35S-labeled in vitro translated proteins (B, C) or HeLa nuclear extract (D). After extensive washing, bound proteins were separated by SDS-PAGE and visualized either by autoradiography (B, C) or Western blotting using anti-HDAC1 antibody (D). E, Tbx2 expressed in transiently transfected COS cells communoprecipitates with HDAC1. COS cells were transiently transfected with pCMV.SV5-Tbx2 or pCMV.SV5 expression vectors. Forty eight hours after transfection, lysates were prepared and immunoprecipitated using anti-SV5 antibody. Proteins in the complex were identified by Western blotting using anti-SV5 and anti-HDAC1 antibodies.

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[Image 98x609 to 197x649]

[Image 386x204 to 425x265]
of Tbx2 to repress expression of the mechanism proposed by Jacobs et al. (24) was through the ability of T antigen at the nonpermissive temperature (23, 24). The mouse striatal cells expressing a temperature-sensitive SV40 large fibroblasts deficient for the polycomb group protein Bmi1 and to bypass the premature senescence of both mouse embryo genes. Ectopically overexpressed Tbx2 has previously been shown that it may serve an important function in melanoma growth consistent up-regulation of Tbx2 in the melanoma cells suggest that the ER-dnTbx2 chimera was fully functional, we assessed its impact on the expression of the p21 gene, a known Tbx2 target (23). Thus, Western blot analysis using anti-p21 antibodies (Fig. 3D) revealed that in cells expressing HA-ER, p21 expression was barely detectable either in the presence or absence of 4-OHT. A low level of p21 expression was observed in clone 1 cells expressing HA-ER-dnTbx2 in the absence of 4-OHT, suggesting that a proportion of the dnTbx2 expressed may be 4-OHT-independent. In

Figure 3. dnTbx2 up-regulates p21 gene expression and displaces HDAC1 from the p21 promoter. A, schematic representation of full-length Tbx2, ER ligand–binding domain (LBD) and ER-dnTbx2 fusion proteins. The position of the DNA-binding domain (T-box) and repression domain in Tbx2 is indicated. B, immunofluorescence assays using anti-HA antibody (B) or Western blots using either anti-HA or anti-Tbx2 antibodies (C) of B16 cells expressing HA-ER and HA-ER-dnTbx2 fusion proteins grown in the absence or presence of 4-OHT for 24 hours as indicated. Results from two independent clones expressing dnTbx2 are shown. (D) ER-dnTbx2 induces p21 expression. The levels of p21, a known Tbx2 target gene, were analyzed by Western blotting using anti-p21 antibodies 4 days after activation of HA-ER or HA-ER-dnTbx2. E, chromatin immunoprecipitation assay using cross-linked chromatin sheared to ~500 bp from B16 ER-dnTbx2 cells grown in the absence or presence of 4-OHT for 4 days. Chromatin was immunoprecipitated using either anti-HDAC1 or nonspecific IgG antibodies, the captured immune complex was washed, the cross-links reversed, and the DNA was PCR amplified using primers specific to the p21 promoter region or p21 exon 2.

Following a transient transfection coimmunoprecipitates with endogenous HDAC1 from COS cells (Fig. 2E). No HDAC1 was immunoprecipitated from extracts of cells transfected with an empty vector. Taken together, these data suggest that Tbx2 is likely to use HDAC1 to repress transcription.

dnTbx2 Displaces Histone Deacetylase 1 from the p21 Promoter and Leads to Increased p21 Expression. The consistent up-regulation of Tbx2 in the melanoma cells suggest that it may serve an important function in melanoma growth control, presumably by targeting HDAC1 to repress Tbx2 target genes. Ectopically overexpressed Tbx2 has previously been shown to bypass the premature senescence of both mouse embryo fibroblasts deficient for the polycomb group protein Bmi1 and mouse striatal cells expressing a temperature-sensitive SV40 large T antigen at the nonpermissive temperature (23, 24). The mechanism proposed by Jacobs et al. (24) was through the ability of Tbx2 to repress expression of the ARF gene. However, because

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the CDKN2A locus is frequently mutated in cancers, how or even whether Tbx2 acts to suppress senescence in such cells is unknown. B16 melanoma cells express Tbx2 as a single full-length species (Fig. 1; ref. 23) and Tbx2 has been reported to be the only member of the T-box family expressed in these cells (32). Because B16 cells, like many human melanomas, bear a defined deletion at the CDKN2A locus (44), leading to loss of function of both the INK4A and ARF genes, they provide a good model system for examining the role of endogenous Tbx2 in transformed cells. To do this, we generated B16 melanoma cell lines expressing an inducible dnTbx2 protein. The dnTbx2 protein used (Fig. 3A) comprises amino acids 1 to 301 that contains the T-box DNA-binding domain but lacks the carboxyl-terminal residues necessary for transcriptional repression and interaction with HDAC1. This deletion mutant is able to bind DNA in vitro in a bandshift assay (data not shown) and is designed to compete with endogenous Tbx2 complexes for binding to target promoters. Deletion mutants generated in this way have been shown to function as dominant-negatives for several other T-box factors in a variety of tissues and organisms (27, 45). To circumvent any potential problems, such as cell cycle arrest or phenotypic selection that might arise as a result of disrupting endogenous Tbx2 function, the dnTbx2 molecule was made hormone inducible by fusing it to a modified version of the ER ligand–binding domain containing a G525R mutation that renders it transcriptionally inactive and unable to bind estrogen whereas retaining responsiveness to the synthetic ligand 4-OHT (46). Proteins fused to the modified ER ligand–binding domain are inactive in the absence of 4-OHT but are rapidly activated following addition of 4-OHT.

B16 melanoma cells were therefore infected with retroviruses expressing HA epitope–tagged ER and HA-ER-dnTbx2 fusion proteins. Following selection with puromycin, individual clones were isolated and expanded under selective conditions and tested for expression by indirect immunofluorescence. Figure 3B shows the results of an immunofluorescence assay using anti-HA antibodies of a B16 cell line expressing HA-ER and two clones expressing HA-ER-dnTbx2 fusion proteins in the absence and presence of 4-OHT. In the absence of ligand, the fusion proteins are expressed at low levels and are located predominantly in the cytoplasm, whereas within 24 hours following addition of 4-OHT the majority of the protein had translocated to the nucleus. Western blotting (Fig. 3C) using anti-HA and anti-Tbx2 antibodies (Fig. 3D) or Western blots using immunofluorescence assays using anti-HA antibody (Fig. 3E) shows the results of an immunofluorescence assay using anti-HA antibodies of a B16 cell line expressing HA-ER and two clones expressing HA-ER-dnTbx2 fusion proteins in the absence and presence of 4-OHT. In the absence of ligand, the fusion proteins are expressed at low levels and are located predominantly in the cytoplasm, whereas within 24 hours following addition of 4-OHT the majority of the protein had translocated to the nucleus. Western blotting (Fig. 3C) using anti-HA and anti-Tbx2 antibodies revealed that in the absence of 4-OHT the HA-ER and HA-ER-dnTbx2 proteins in clone 1 were poorly expressed, but that the addition of 4-OHT led to a large increase in the amount of ER protein detected compared with a tubulin loading control and a somewhat smaller increase in the levels of ER-Tbx2 expressed. The increased expression of ER fusion proteins in the presence of 4-OHT has been seen by us using a number of other genes (not shown) and most likely results from increased stability of the ER fusion proteins. The second clone expressed substantially higher amounts of the HA-ER-dnTbx2 protein even in the absence of 4-OHT.

To determine whether the ER-dnTbx2 chimera was fully functional, we assessed its impact on the expression of the p21 gene, a known Tbx2 target (23). Thus, Western blot analysis using anti-p21 antibodies (Fig. 3D) revealed that in cells expressing HA-ER, p21 expression was barely detectable either in the presence or absence of 4-OHT. A low level of p21 expression was observed in clone 1 cells expressing HA-ER-dnTbx2 in the absence of 4-OHT, suggesting that a proportion of the dnTbx2 expressed may be 4-OHT-independent. In
contrast, the addition of 4-OHT to the HA-ER-dnTbx2-expressing cells led to a dramatic increase in p21 expression consistent with it acting to displace the endogenous Tbx2 from the p21 promoter. In clone 2 that expresses a high level of HA-ER-dnTbx2, p21 levels were high compared with the control HA-ER-only cells even in the absence of tamoxifen, but were increased further on its addition. For both clone 1 and clone 2, the levels of p21 were elevated up to 20-fold in the presence of 4-OHT compared with that observed in the control ER-only cells under the same conditions.

Given the interaction between Tbx2 and HDAC1, the up-regulation of p21 by the expression of active dnTbx2 suggested that dnTbx2 would act to displace Tbx2-associated HDAC1 from the p21 initiator. To address this question, we did a chromatin immunoprecipitation analysis using anti-HDAC antibody on cross-linked chromatin prepared from HA-ER-dnTbx2-expressing cells grown in the absence or presence of 4-OHT. We used PCR primers specific for either the p21 initiator, where Tbx2 binds at the initiator (23), or, as a control, the p21 coding sequence spanning exon 2. The results (Fig. 3E) show that in the absence of 4-OHT, when the p21 promoter is repressed, the anti-HDAC antibody specifically immunoprecipitates HDAC1 at the p21 promoter. No signal above background was obtained using the nonspecific IgG control or using primers specific for the p21 coding region. In contrast, following activation of ER-dnTbx2 by treatment of cells with 4-OHT, HDAC1 was no longer detected at the p21 promoter by chromatin immunoprecipitation analysis using PCR primers that span the T-element at the p21 initiator.

The results confirm that Tbx2 acts to target HDAC1 to the p21 initiator and the dnTbx2 molecule acts to displace HDAC1 from the p21 promoter and to induce p21 expression.

**dnTbx2 Induces Senescence in B16 Melanoma Cells.** Although ectopic overexpression of Tbx2 has been reported to suppress senescence in CDKN2A wild-type fibroblasts (24) or striatal cells (23) engineered to undergo replicative senescence, the role of endogenous Tbx2 in transformed cells has not been previously examined. The availability of B16 cells inductively expressing dnTbx2 gave us an opportunity to determine whether Tbx2 functions as an antisenescent factor in melanoma cells that lack the CDKN2A locus that plays a key role in senescence in human and mouse melanocytes (10, 12). We therefore measured the levels of the SAβ-gal activity, a marker of senescent melanocyties (10), in HA-ER and the two clones of HA-ER-dnTbx2-expressing B16 cells grown in the presence or absence of 4-OHT for 6 days. Whereas only the occasional cell expressing the control HA-ER protein stained positive for SAβ-gal following addition of 4-OHT, activation of ER-dnTbx2 by 4-OHT induces a marked accumulation of cells staining positive for SAβ-gal activity. ~25% of clone 1 B16 HA-ER-dnTbx2-expressing cells and ~80% of the clone 2 cells (Fig. 4A and B). Closer examination of the SAβ-gal-positive cells indicated that they had adopted a flattened morphology characteristic of senescent cells (not shown). To determine whether the accumulation of cells expressing SAβ-gal was correlated with a decrease in population of cells undergoing replication, we measured the proportion of cells able to incorporate BrdUrd as determined by immunofluorescence using an anti-BrdUrd antibody. The results (Fig. 4C) indicate that whereas over 25% of the control ER-expressing cells were BrdUrd-positive following treatment with 4-OHT, the clones expressing dnTbx2 exhibited a significant reduction in BrdUrd incorporation that paralleled the numbers of cells expressing SAβ-gal (Fig. 4A) and p21 (Fig. 3D). This was reflected in the fact that in the presence of 4-OHT cells expressing dnTbx2 proliferated more slowly than those expressing ER only (Fig. 4D). Similarly, transfection of B16 cells with either of two Tbx2-specific siRNAs (23), but not a control siRNA, also induced SAβ-gal activity days later. The results were quantitated by counting four random fields under a brightfield microscope. C, BrdUrd incorporation following a 1-hour pulse was determined using an anti-BrdUrd antibody and cells scored positive or negative for BrdUrd incorporation. D, relative growth rates of B16 melanoma cells expressing ER or ER-dnTbx2 grown in the presence of 4-OHT. E, senescence in B16 cells is induced by Tbx2-specific siRNA. B16 cells were transfected with either a control siRNA specific for Mitf or two Tbx2-specific siRNAs (see Materials and Methods) as indicated and stained for SAβ-gal activity as described in Materials and Methods. B, the percentage of cells staining positive for β-gal was determined by counting four random fields under a brightfield microscope. C, BrdUrd incorporation following a 1-hour pulse was determined using an anti-BrdUrd antibody and cells scored positive or negative for BrdUrd incorporation. D, relative growth rates of B16 melanoma cells expressing ER or ER-dnTbx2 grown in the presence of 4-OHT. E, senescence in B16 cells is induced by Tbx2-specific siRNA. B16 cells were transfected with either a control siRNA specific for Mitf or two Tbx2-specific siRNAs (see Materials and Methods) as indicated and stained for SAβ-gal activity days later. The results were quantitated by performing a liquid assay for β-gal activity using extracts from cells treated with a control, nonsilencing siRNA as the blank. F, Tbx2 siRNAs suppress Tbx2 and elevate p21 expression. Western blot of B16 cells treated with the two Tbx2-specific siRNAs used in E probed with the indicated antibodies. Extracellular signal-regulated kinase 2 (ERK2) and tubulin were used as loading controls. The same blot was probed for Tbx2 and extracellular signal-regulated kinase 2, and the same samples analyzed in parallel on a second blot for p21 and tubulin.
activity (Fig. 4E), with both siRNAs efficiently down-regulating Tbx2 and consequently up-regulating p21 expression compared with the control siRNA (Fig. 4F). Thus, strikingly, endogenous Tbx2 functions to inhibit replicative senescence in a melanoma cell line lacking the CDKN2A locus, implying that Tbx2 must function in a pathway independent of its ability to repress ARF.

Note that prolonged culture of the dnTbx2-expressing cells eventually leads to selection of populations of cells expressing reduced levels of protein and consequently an increased growth rate. However, consistent with the senescence of the B16 cells induced by dnTbx2 being irreversible, withdrawal of 4-OHT from the cells failed to induce BrdUrd incorporation into the SAβ-gal-positive senescent population (not shown).

**dnTbx2 Impairs Growth and Induces Heterochromatin Foci in Human Melanoma Cells.** The results obtained thus far suggest that Tbx2 may act as an antisenescence and proproliferative factor in the B16 melanoma cell line. To extend these observations to human melanoma cells, we stably transfected the human WM266-4 melanoma cell line with the ER or ER-dnTbx2 expression vector. Following selection and expansion, two representative clones expressing dnTbx2 were chosen for further analysis. Western blotting revealed that clone 1 expressed a high level of dnTbx2, whereas clone 2 expressed a lower amount of protein (Fig. 5A). In both cases, the level of HA-ER-dnTbx2 was increased by treatment with 4-OHT as was the level of the HA-ER protein in the control cells. The analysis of clone 1 proved difficult owing to an extreme slow growth phenotype and sensitivity to trypsinization (not shown). Consequently, the majority of the subsequent analysis was restricted to clone 2. Although we were unable to assess the induction of SAβ-gal activity in these cells because human melanocytes and melanoma cells can constitutively express this marker (12), the proportion of cells in S-phase was substantially reduced in the WM266-4 (clone 2) cells expressing HA-ER-dnTbx2 in the presence of 4-OHT compared with the control cells or the HA-ER-dnTbx2-expressing cells in the absence of 4-OHT (Fig. 5B); these results are consistent with that obtained using B16 cells. In agreement with this, the proliferation of the HA-ER-dnTbx2-expressing cells was severely reduced in the presence of 4-OHT compared with the controls (Fig. 5C). However, the most striking phenotype of the HA-ER-dnTbx2 cells was revealed by extending the analysis of the intensely DAPI-staining foci in the dnTbx2-expressing cells.

**Figure 5.** dnTbx2 induces slow growth and heterochromatin foci in human melanoma cells. A, Western blot using anti-HA antibody of WM266-4 melanoma cells stably transformed with vectors expressing HA-ER or HA-ER-dnTbx2 and grown in the presence or absence of 4-OHT. The results for two independent clones are presented. Lamin B2 was used as a loading control. B, proportion of cells from the indicated clones in S-phase as determined using BrdUrd incorporation. C, growth curve of WM266-4 cell lines expressing ER of ER-dnTbx2 [clone 2 (cl. 2)] grown in the presence or absence of 4-OHT. D, immunofluorescence of ER and ER-dnTbx2–expressing WM266-4 cells in the presence or absence of 4-OHT. Note the appearance of the intensely stained foci in the dnTbx2-expressing cells.

**Figure 6.** dnTbx2 induces heterochromatin foci in WM266-4 cells. WM266-4 cells expressing ER-dnTbx2 and treated with 4-OHT to induce heterochromatin formation were stained with (A) anti-3meK9H3, (B) anti-3meK27H3, (C) anti-HP1α and HP1β, or (D) anti-3meK9H3 and human CREST serum.
immunofluorescence where large, intensely 4′,6-diamidino-2-phenylindole (DAPI)-staining foci were observed in the ER-dnTbx2-expressing cells but not in the parental cells (not shown) or those expressing ER only (Fig. 5D). For the slowly growing clone 1 that expressed a high level of dnTbx2 the foci were readily visible both in the presence and absence of 4-OHT, whereas in the clone 2 cell line that expresses lower levels of dnTbx2 the intensity of the DAPI-staining foci significantly increased in the presence of 4-OHT. The DAPI-staining foci induced by the expression of dnTbx2 resembled the SAHFs that are induced in an Rb1-dependent fashion by oncogenic stress (4). Note that SAHFs are readily apparent in human cells undergoing senescence but are not easily detected in murine cells, like the B16 melanoma cell line, against the background of the constitutive pericentric heterochromatin foci.

To further characterize the nature of the DAPI-staining foci induced by dnTbx2, we did immunofluorescence on the clone 2 cells grown in the presence of 4-OHT using antibodies raised against heterochromatin markers. Consistent with the intense DAPI-staining foci induced by dnTbx2 representing heterochromatin, the foci colocalized with histone H3 trimethylated on lysine 9 (3meK9H3; Fig. 6A), a well characterized heterochromatin mark (47), but not with trimethyl K27 (Fig. 6B). A similar pattern of colocalization was observed using antibodies specific for either HP1α or HP1β (Fig. 6C). Whereas SAHFs induced by oncogenic stress also colocalize with 3meK9H3, HP1α, and HP1β, they have been reported mostly not to overlap with centromeric markers (4). We therefore examined the localization of the heterochromatin foci induced by dnTbx2 using a human CREST serum that recognizes components of centromeres (48) together with the 3meK9H3 antibody. Staining using the CREST serum (Fig. 6D) revealed a pattern of small discreet foci that overlapped somewhat with the heterochromatin foci induced by dnTbx2, but which were nevertheless consistently localized to the edge of the 3meK9H3-positive foci. This pattern of staining seems to be identical to that reported for the Rb1-dependent SAHFs induced by oncogenic stress (4). Therefore, we view it as likely that the SAHFs represent an expansion of the pericentric heterochromatin although we do not rule out the possibility that dnTbx2 induces the formation of heterochromatin domains at other loci.

**Discussion**

The fact that oncogenic stress (e.g., signaling by activated Ras) can lead to replicative senescence has led to the notion that senescence may represent a major defense mechanism operating to prevent malignant transformation. Therefore, it follows that the activation of machinery designed to bypass senescence must represent a cornerstone of the transformation process.

Previous studies have established that ectopic overexpression of Tbx2, or the related repressor Tbx3, can act to bypass senescence in mouse fibroblasts or striatal cells in defined experimental systems where the CDKN2A locus is intact (21, 23–27). However, whether the endogenous T-box factors perform an antisenescent function in transformed cells, particularly those lacking a functional CDKN2A locus, has not been addressed. This is an especially important issue because the expression of Tbx2 is up-regulated in a number of cancers, including as we show here in melanoma cells, and in many neoplasms, particularly melanoma, the CDKN2A locus is mutated or deleted (6, 7, 49). In such cells, the absence of ARF expression arising from mutation or deletion of the locus would potentially abrogate any requirement for T-box factors in suppressing senescence. Significantly, however, the expression of dnTbx2 led to senescence in the B16 cells and the formation of SAHFs in the human melanoma cell line. The results indicate, for the first time, that endogenous Tbx2 is required to maintain proliferation and suppress a CDKN2a-independent senescence pathway in melanomas.

The mechanism by which dnTbx2 can induce senescence in the CDKN2A-negative B16 cells is most likely related to its ability to derepress p21 expression through displacement of a Tbx2-HDAC1 complex from the p21 promoter. In a key study by Svederskaya et al. (12), normal human melanocytes in culture underwent senescence after a maximum of 10 population doublings and did not exhibit elevated p21 expression. In contrast, melanocytes lacking a functional INK4A gene failed to execute the normal program of senescence and continued to proliferate. Eventually, after 45 to 55 population doublings, the INK4A null melanocytes did undergo senescence characterized by elevated p21 levels. The ability of dnTbx2 to promote a rapid onset of senescence in CDKN2A-null B16 melanoma cells suggests that one role for the endogenous Tbx2 protein is to block the p21-associated, CDKN2a-independent senescence pathway. Thus, strategies designed to interfere with Tbx2 function should be an effective means of inhibiting proliferation in cancers where loss of CDKN2A has bypassed the normal program of senescence that would be activated by oncogenic stress, such as the presence of activated Ras or BRAF. This is especially relevant for melanoma, which is frequently characterized by both deletion of the CDKN2A locus (50) and the presence of activating BRAF mutations (51).

HDAC inhibitors have recently come to attention as promising anticancer agents. The results from several studies suggest that the antiproliferative effects of HDAC inhibitors are caused by the targeted activation of a subset of genes involved in cell cycle control, such as p21 (43, 52, 53) and myc (54). Indeed, up-regulation of p21 is required for G1 arrest mediated by Trichostatin A, trapoxin, and butyrate (52, 55). Although reporter gene assays using p21 promoter deletion mutants have suggested that the increase in p21 promoter activity in response to HDAC inhibitors is p53-independent and is mediated by an SP3 binding site ~100 bp upstream from the transcription start site (56, 57), the contribution of the SP3 sites to transcriptional repression of the chromosomal p21 gene was not established. It is possible, therefore, that the requirement for SP3 in Trichostatin A–mediated derepression of the p21 promoter observed in the transient transfection assays used reflects more the ability of SP3 to overcome the barrier to transcription represented by the loading of nucleosomes onto transcribed plasmid DNA rather than their participation in targeting HDACs to the endogenous chromosomal gene. Indeed, a more recent study has suggested that p21 core promoter elements are potential mediators of the response to HDAC inhibitors (58), although, in this case, how HDAC activity might be targeted to the p21 initiator remained an unresolved question. Here we show that expression of a dnTbx2 that contains the amino-terminal DNA-binding domain, but which lacks the carboxyl-terminal transcriptional repression domain, leads to up-regulation of p21 expression and concomitant loss of HDAC1 at the endogenous chromosomal p21 promoter. Because we also show that the carboxyl-terminal domain of Tbx2 can recruit HDAC activity and bind HDAC1, the data are consistent with a model in which endogenous Tbx2
represses p21 expression by targeting HDAC1 to the AGGTGTTGA T-element motif close to the p21 initiator (23) and underscores the role of Tbx2 as a key regulator of p21 expression. The results therefore define for the first time a molecular mechanism for transcriptional repression by Tbx2 and reveal a link between HDACs and T-box factors, two families of proteins implicated in cell cycle control. Indeed, Tbx2-mediated targeting of HDAC1 to the p21 promoter may play an important role in the control of cell cycle exit and differentiation as studies using transformed cell lines have shown that inhibition of HDAC activity can arrest cells in both the G1 and G2 phases of the cell cycle and induce differentiation or apoptosis (41–43). Although HDAC1 is likely to be recruited to a range of promoters by many different transcription factors, the link between HDAC1 and Tbx2 established here may be particularly significant as HDAC1-deficient embryos and embryonic stem cells show decreased proliferation rates and a corresponding increase in expression of the p21 and p27GIP1 cyclin-dependent kinase inhibitors (59), indicating an essential function of HDAC1 in cell cycle control. Moreover, mice in which both HDAC1 alleles have been disrupted die before E10.5 because of severe proliferation and developmental defects (59).

Finally, whether Tbx2 is the only T-box factor able to regulate p21 expression is unclear. Although Tbx3, also a transcriptional repressor, can also suppress senescence, it has yet to be shown to target the p21 promoter. However, Tbx3 has been reported to be strongly up-regulated in some melanoma cell lines (60) and its activity can potentially also be inhibited by the dnTbx2 used in this study. Other T-box factors, such as Tbx5 (61, 62) and Brachury (63), act as transcriptional activators and might therefore be expected to inhibit cell proliferation or promote differentiation or senescence if they too can target the p21 promoter. Consistent with this, Tbx5 has been shown to inhibit cardiomyocyte proliferation during heart development (64). However, whether members of the T-box family other than Tbx2 are indeed able to regulate p21 expression will depend on the ability of these proteins to interact with dimerization partners that facilitate cooperative binding to the p21 initiator. Although the identity of such factors is currently unknown, it is an intriguing possibility that the relative expression levels and activity of different T-box factors during development and in cellular transformation will be a key feature of p21-mediated control of the cell cycle, differentiation, and senescence.

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References


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