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The Retinoblastoma Protein Modulates Tbx2 Functional Specificity

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Tbx2 is a member of a large family of transcription factors defined by homology to the T-box DNA-binding domain. Tbx2 plays a key role in embryonic development, and in cancer through its capacity to suppress senescence and promote invasiveness. Despite its importance, little is known of how Tbx2 is regulated or how it achieves target gene specificity. Here we show that Tbx2 specifically associates with active hypophosphorylated retinoblastoma protein (Rb1), a known regulator of many transcription factors involved in cell cycle progression and cellular differentiation, but not with the Rb1-related proteins p107 or p130. The interaction with Rb1 maps to a domain immediately carboxy-terminal to the T-box and enhances Tbx2 DNA binding and transcriptional repression. Microarray analysis of melanoma cells expressing inducible dominant-negative Tbx2, comprising the T-box and either an intact or mutated Rb1 interaction domain, shows that Tbx2 regulates the expression of many genes involved in cell cycle control and that a mutation which disrupts the Rb1-Tbx2 interaction also affects Tbx2 target gene selectivity. Taken together, the data show that Rb1 is an important determinant of Tbx2 functional specificity.

INTRODUCTION

Members of the T-box family of transcription factors play important roles in the regulation of cell fate decisions and morphogenesis during development. For example, the prototypical T-box factor, brachyury, is essential for mesoderm induction (Herrmann *et al.*, 1990), Tbx4 and Tbx5 determine limb identity (Rodriguez-Esteban *et al.*, 1999; Takeuchi *et al.*, 1999), T-pit is a specifier of pituitary fate (Pulichino *et al.*, 2003), and T-bet controls commitment to the T helper type 1 lineage (Szabo *et al.*, 2000). In addition, Tbx2 determines ventral tubero-mamillary (vt-m) hypothalamic identity, is essential for outflow tract morphogenesis and atrioventricular canal patterning during cardiogenesis and is involved in specifying posterior digit identity (Harrelson *et al.*, 2004).

Several T-box genes are also involved in the progression of cancer. Tbx2 and the highly related Tbx3 are amplified and/or overexpressed in a subset of breast cancers and in melanoma (Sinclair *et al.*, 2002; Hoek *et al.*, 2004; Jonsson *et al.*, 2007). Tbx2 is additionally overexpressed in pancreatic cancer cell lines (Mahlamaki *et al.*, 2002), whereas Tbx3 is implicated in ovarian and uterine cervical cancer (Lomnytska *et al.*, 2006; Lyng *et al.*, 2006). These proteins have been proposed to promote cancer progression through several different mechanisms. Tbx2 and Tbx3 have been shown to cooperate with active Ras to promote anchorage-independent growth and transform mouse embryo fibroblasts (Carlson *et al.*, 2002; Vormer *et al.*, 2008), whereas Tbx3 can function to enhance melanoma invasiveness by down-regulating expression of the E-cadherin cell–cell adhesion protein (Rodriguez *et al.*, 2008). Tbx2 is also required to maintain proliferation and suppress senescence in melanomas by repressing expression of the $p21^{CIP1}$ cyclin-dependent kinase (cdk) inhibitor (Vance *et al.*, 2005), and Tbx2 and Tbx3 can block premature senescence of mouse striatal cells in culture by either directly repressing $p19^{ARF}$ (Jacobs *et al.*, 2000; Lingbeek *et al.*, 2002) or $p21^{CIP1}$ (Prince *et al.*, 2004).

Despite the discovery that T-box transcription factors are important regulators of development and disease the molecular mechanisms of transcriptional control by these proteins are relatively unknown. Identifying interacting proteins and associated activities is therefore essential to understand T-box factor mode of action. To date, several lines of evidence suggest that T-box factors can function through epigenetic mechanisms. T-bet is able to recruit histone H3K4 methyltransferase and H3K27 demethylase activities to regulate transcription (Lewis *et al.*, 2007; Miller *et al.*, 2008). This interaction is mediated by conserved amino acids in the T-box and may be a general feature of the family. Accordingly, the distantly related Tbx6 and Brachyury proteins can induce H3K4me2 at target genes and Eomes, Brachyury, and Tbx5 also interact with H3K27 demethylase activity. In addition, the pituitary specific T-box factor Tpit recruits the SRC/p160 histone acetyltransferase to regulate hormone dependent transcription from the proopiomelanocortin (POMC)

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promoter (Maira *et al.*, 2003). Furthermore, Tbx2 specifically binds the histone H3 amino-terminal tail (Demay *et al.*, 2007) and interacts with histone deacetylase (HDAC) activity to repress transcription and can recruit HDAC1 to target promoters (Vance *et al.*, 2005). The function of T-box family members can also be regulated by their associated cofactors. The Ripply family of proteins interact with the zebrafish Brachyury and Tbx24 orthologues (Kawamura *et al.*, 2008) and the *Xenopus* Tbx1 and Tbx6 proteins (Hitachi *et al.*, 2009). Ripply binding recruits a Groucho/TLE corepressor complex containing HDAC activity, which functions to convert these transcription factors from activators into repressors.

The retinoblastoma tumor suppressor protein (Rb1) associates with a wide range of transcription factors to control cell cycle progression, cellular senescence, apoptosis, and differentiation. Rb1 mediates these effects by modulating the function of its interacting transcription factors. The best characterized role for Rb1 is in the control of E2F1 activity to negatively regulate cellular proliferation. Rb1 exerts this function by interfering with the ability of E2F1 to communicate with the basal transcription apparatus and/or recruiting chromatin modifying enzymes to block the activation of E2F responsive genes (Brehm and Kouzarides, 1999). In this context Rb1 has been shown to target HDACs (Brehm *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998), histone and DNA methyltransferases (Robertson *et al.*, 2000; Nielsen *et al.*, 2001), and components of ATP-dependent chromatin remodeling complexes (Dunaief *et al.*, 1994; Trouche *et al.*, 1997) to actively repress E2F transcription. In addition to its role in E2F regulation, Rb1 has been shown to interact with transcription factors that function in cellular differentiation such as C/EBP, NFIL-6, and c-jun (Chen *et al.*, 1996a,b; Nead *et al.*, 1998). Rb1 enhances the transcriptional activity of these factors by stimulating DNA binding. Furthermore, Rb1 interacts with $p120^{E4F}$ (E4F), a GLI-Kruppel–related transcription factor involved in cell cycle control, increasing both E4F binding to DNA and E4F-mediated transcriptional repression (Fajas *et al.*, 2000). Taken together these studies show that Rb1 has the ability to inhibit or enhance the transcriptional activity of its interacting proteins by a variety of mechanisms.

Here we show that Rb1 modulates the function of the Tbx2 transcription factor. The results provide mechanistic insights into Tbx2 transcriptional control and reveal a molecular link between two families of proteins involved in normal development and dysregulated in cancer.

MATERIALS AND METHODS

Plasmid Constructs

pGEX2TK-Tbx2(1-361) and pGEX2TK-Tbx2(361-701) vectors have been described previously (Vance *et al.*, 2005). Further glutathione *S*-transferase (GST) Tbx2 expression vectors were generated by PCR cloning Tbx2 amino acids 84-366, 84-316, 84-301, 84-287, and 84-262 from pCMV-Tbx2 (Prince *et al.*, 2004) as BamHI-BglII fragments into pGEX2TK. pGEX-Rb(379-928) and the RB1 expression vector were kind gifts from S. Mittnacht (Institute of Cancer Research, London). To generate pIVEX-Rb(379-928) Rb amino acids 379-928 were subcloned as a BamHI fragment from pGEX-Rb(379-928) into pIVEX. The $p21^{CIP1}$-luciferase reporter contains a 2.2-kb SacI-HindIII fragment of the human $p21^{CIP1}$ promoter described in Prince *et al.* (2004) inserted upstream of the luciferase gene in pGL3 (Promega, Madison, WI). pCMV-Tbx2L294AL296A, pGEX2TK-Tbx2L294A,L296A, pGEX2TK-Tbx2(84-301)L294A,L296A, and pBabeHAER-Tbx2(1-301L294A,L296A) mutant constructs were generated with the QuikChangeII Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions using pCMV-Tbx2 (Prince *et al.*, 2004), pGEX2TK-Tbx2, pGEX2TK-Tbx2(84-301), and pBabeHAER-Tbx2(1-301) (Vance *et al.*, 2005) as templates, respectively.

Pulldown Assays and Immunoprecipitations

GST fusion proteins were expressed in *Escherichia coli* strain BL21(DE3) pLysS as described in Aksan and Goding (1998). His-tagged proteins were expressed using the cell-free Rapid Translation System (Roche, Indianapolis, IN) and purified under native conditions using the Ni-NTA Spin Kit (Qiagen, Chatsworth, CA) both according to the manufacturer's instructions. GST pulldown assays were performed as shown in Yavuzer *et al.* (1995), and immunoprecipitation experiments were carried out as described (Carreira *et al.*, 2005) using 10 μl mouse anti-Tbx2 (Prince *et al.*, 2004) or anti-SV5 monoclonal (Serotec, Oxford, United Kingdom) antibodies and 50 μl protein G Sepharose to capture the immune complexes.

Western Blotting

Cell extractions using cytoskeleton (CSK) buffer (10 mM Pipes-KOH, pH 7.0, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$) plus 0.5% Triton X-100 were as described (Martini *et al.*, 1998). Alternatively, cells were lysed directly in SDS-PAGE loading buffer, sonicated, and boiled for 5 min. Proteins were resolved on SDS-PAGE gels and then transferred onto Hybond C (Amersham, Piscataway, NJ) membranes. To separate the different phosphorylated forms of Rb we used a polyacrylamide/bisacrylamide ratio of 200:1. Membranes were probed with appropriate primary antibodies, detected using peroxidase-conjugated secondary antibodies, and visualized by enhanced chemiluminescence (Amersham). Antibodies used were anti-human Rb1 mouse monoclonal (PharMingen, San Diego, CA), anti-HA (Clone HA-7, Sigma, St. Louis, MO), anti-p107, anti-p130, anti-tubulin, anti-extracellular signal-regulated kinase (ERK)-2 rabbit polyclonals and anti-GST mouse monoclonal (all Santa Cruz Biotechnology, Santa Cruz, CA).

Immunofluorescence Microscopy

To characterize the B16 HAER-Tbx2–expressing cell lines immunofluorescence staining was performed using an anti-hemagglutinin (HA; Clone HA-7, Sigma) mouse mAb as described in Vance *et al.* (2005). For the colocalization studies, cells grown on coverslips were washed with PBS+ (phosphate-buffered saline containing 0.5 mM $MgCl_2$ and 0.5 mM $CaCl_2$) and then with CSK buffer to extract soluble proteins. Cells were then incubated in CSK buffer supplemented with 0.5% Triton X-100 and protease inhibitor cocktail (Roche) for 5 min at room temperature. After two washes in CSK buffer, the cells were fixed in 4% paraformaldehyde for 10 min at room temperature before permeabilization using 0.5% Triton X-100 for 6 min at room temperature. Coverslips were then incubated with anti-Tbx2 mouse monoclonal and anti-Rb1 rabbit polyclonal (Santa Cruz) antibodies, washed three times with PBS, and then incubated with both anti-mouse Texas Red and anti-rabbit FITC secondary antibodies (Vector Laboratories, Burlingame, CA). Cells were washed again with PBS and mounted using Vectashield mounting medium. We imaged a single optical section using a Zeiss Axiovert 135 microscope with a PlanApoChromat 63× 1.40 NA oil objective (Thornwood, NY).

Electrophoretic Mobility Shift Assays

Binding reactions were performed with purified GST-Tbx2 fusion proteins and ^{32}P-labeled T-element oligonucleotide probes and resolved on a 6% polyacrylamide gel as described previously (Carreira *et al.*, 1998). The following sequence of the double-stranded consensus T-element oligonucleotide was used: 5′-ctagaGGGAATTTCACACCTAGGTGTGAAATTCCCt-3′ (underlined sequences designate a palindromic T-box binding site). For the supershift experiment purified proteins were preincubated with anti-Rb1 rabbit polyclonal antibody (C-15, Santa Cruz) for 1 h at room temperature before addition of the probe.

Cell Culture and Luciferase Assays

B16, Phoenix, SAOS-2, and HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum and 1% penicillin streptomycin. B16 cells expressing the estrogen receptor (ER) fusion proteins were generated and characterized as described in Vance *et al.* (2005). ER fusion proteins were activated by the addition of 4-hydroxy tamoxifen (4-OHT) to a final concentration of 300 nmol/l. For transcription assays 2.5×10^4 cells were seeded per well in a 24-well plate. The next day cells were transfected with reporter constructs and expression vectors using FuGENE 6 (Roche) according to the manufacturer's instructions. pCMV-B-gal expression vector (25 ng) was also included to normalize for transfection efficiency. We used 50 ng pGL3-$p21^{CIP1}$pro, 25 ng and 50 ng of either pCMV-Tbx2 or pCMV-Tbx2L294AL296A plasmids, and 100 ng Rb expression vector. The total amount of DNA was made equal in each case by the addition of empty pCMV vector. Forty-eight hours after transfection lysates were prepared and assayed for luciferase and β-galactosidase activity. All transfections were repeated at least three times in duplicate.

Microarrays

Total RNA was isolated from ER-Tbx2(1-301) and ER-Tbx2(1-301mt) cells grown in the absence or presence of ligand for 24 h using the Qiagen Mini RNeasy kit. The quality of the RNA was assessed on a Bioanalyzer (Agilent Technologies, Wilmington, DE) . Total RNA (150 ng) was labeled using the GeneChip 3′ IVT Express Kit (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions. Fragmented cRNA (15 μg) was then hybridized to the mouse Affymetrix MOE430A expression array. The hybridization mix was preserved and subsequently hybridized to the MOE430B array. Chips were washed, stained,

and scanned using the GeneChip Scanner 3000 7G (Affymetrix). Microarrays were performed in triplicate for each experimental condition.

Microarray Analysis

We used the updated chip definition files provided on the brainarray and NuGO Web pages to correct for updated gene annotations that were not available when the microarrays were designed (custom-CDFs and annotations for moe430 version 12.0.0; http://brainarray.mbni.med.umich.edu/Brainarray/Database/CustomCDF/12.0.0/entrezg.asp and http://nugo-r.bioinformatics.nl/Packages_Prior_to_R2.10.html). Use of updated annotations has been shown to make a significant improvement to the accuracy of microarray data analysis (Dai *et al.*, 2005). A tool called Harshlight (http://www.bioconductor.org/packages/2.2/bioc/html/Harshlight.html) was applied to remove the effects of spatial blemishes on the arrays (Suarez-Farinas *et al.*, 2005). We used GeneChip Robust Multiarray Average (GCRMA; http://www.bioconductor.org/packages/2.2/bioc/html/gcrma.html) to do background correction because this method removes the bias introduced by nucleotide content of probes (Naef and Magnasco, 2003). Harshlight, GCRMA, quality controls, principal component analysis, differential gene expression, GO (gene ontology)-term analyses, and heat maps were done by developing small R functions based on the tools of the Bioconductor package (http://www.bioconductor.org/; Gentleman *et al.*, 2004).

RESULTS

Tbx2 Binds the Retinoblastoma Tumor Suppressor Protein

The Tbx2 transcription factor plays an important role in embryonic development and is mis-expressed in several cancers (Jacobs *et al.*, 2000; Harrelson *et al.*, 2004; Vance *et al.*, 2005). However, the molecular mechanisms underlying the function of Tbx2 in transcriptional control remain poorly understood. Because the Rb-family of transcriptional cofactors, comprising Rb1, p107, and p130, are known to regulate the activity of many different transcription factors involved in controlling cell cycle progression and differentiation, we sought to determine whether they can associate with Tbx2 and modulate its activity.

To do this, a series of GST pulldown experiments were first carried out to determine if Tbx2 can interact with Rb1. Figure 1A shows that in vitro–translated Tbx2 interacts with the Rb1 large pocket domain (Rb379-928) but not with GST alone. This region of Rb1 mediates its tumor suppressor function and is responsible for the interaction with the majority of its cellular partners (for a review see Morris and Dyson, 2001). In addition, the T-box–containing amino-terminal region of Tbx2 (amino acids 1-361) when fused to GST purifies Rb1 from HeLa nuclear extract in a pulldown assay (Figure 1B). No binding was detected when a carboxy-terminal fragment of Tbx2 (amino acids 361-701) containing the transcriptional repression and HDAC1-interaction domain or GST alone were used as a bait. We next assessed the specificity of Tbx2 to target different Rb1-related pocket proteins. The results of this analysis are presented in Figure 1B and show that Tbx2 specifically interacts with Rb1 and does not purify p130 and p107 from HeLa nuclear extract. We further characterized the interaction by separating the phosphorylated forms of Rb1 in complex with Tbx2. This showed that Tbx2 binds a fast-migrating hypophosphorylated form of Rb1 and that slower migrating phosphorylated forms of Rb1 were still detected in the unbound extract (Figure 1C). This is consistent with the work from different laboratories showing that cell cycle–dependent phosphorylation inactivates Rb1 and disrupts the interaction with many of its protein partners (see Mittnacht, 1998 for a review). Finally, experiments using purified proteins (Figure 1D) indicate that Tbx2 binds directly to Rb1 as His-Rb(379-928) interacts with GST-Tbx2(1-361) and not GST alone. Taken together, these data show that the Tbx2 transcriptional factor specifically associates with active hypophosphorylated Rb1 in vitro.

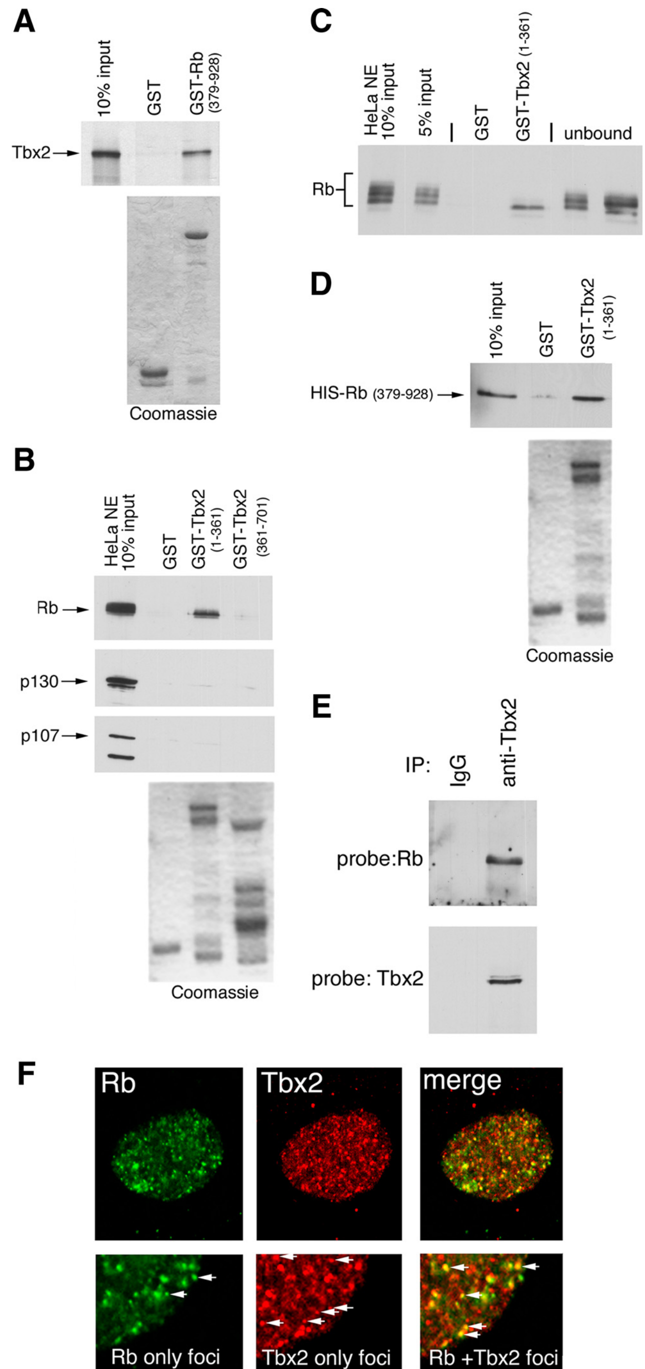

Figure 1. Tbx2 directly interacts with hypophosphorylated Rb1. The indicated GST fusion proteins were immobilized on glutathione beads and incubated with either ^{35}S-labeled IVT Tbx2 (A), HeLa nuclear extract (B and C), or purified HisRb(379-928) protein (D). After extensive washing, bound proteins were separated by SDS-PAGE and visualized either by autoradiography (A) or Western blotting (B–D). Tbx2 associates with Rb1 in B16 melanoma cells (E). Lysates from B16 cells were prepared and immunoprecipitated using either anti-Tbx2 or an unrelated control mAb. Proteins in the complex were identified by Western blotting using anti-Rb1 (top) or anti-Tbx2 antibody (bottom). (F) Immunofluorescence staining of B16 cells using anti-Tbx2 and anti-Rb1 antibodies after salt extraction of soluble proteins.

Tbx2 Interacts with Rb1 in Melanoma Cells

Tbx2 is overexpressed in melanomas when compared with primary melanocytes and melanocyte cell lines (Vance *et al.*, 2005), whereas Rb1 is necessary for both postnatal melanocyte survival (Yu *et al.*, 2003; Carreira *et al.*, 2005) and senes-

cence in melanocytes in culture and melanocytic nevi in vivo (Bandyopadhyay and Medrano, 2000; Michaloglou *et al.*, 2005). To expand on our in vitro binding data and determine whether endogenous Tbx2 and Rb1 interact in cells in culture an anti-Tbx2 mAb was used to immunoprecipitate protein complexes from B16 mouse melanoma cells. The results of this analysis are shown in Figure 1E and reveal that Tbx2 coimmunoprecipitates with Rb1, whereas no association was detected using a control antibody. Indirect immunofluorescence staining was also carried out to provide additional evidence that Tbx2 and Rb1 interact in the melanocyte lineage and showed that Tbx2 and Rb1 partially colocalize in distinct nuclear foci in B16 melanoma cells after extraction of soluble proteins (Figure 1F). Previous analysis of Tbx2 subcellular localization demonstrating that Tbx2 protein is associated with DNA in subnuclear foci and excluded from pericentric heterochromatin is consistent with these results (Bilican and Goding, 2006). Taken together these data show that Tbx2 and Rb1 interact in vitro and in living cells and suggest that Rb1 may be able to modulate the function of Tbx2.

Tbx2 Binds Rb1 in a LXCXE-independent Manner

Rb1 has been shown to interact with many of its associated proteins via an LXCXE motif (reviewed in Morris and Dyson, 2001). However, examination of the Tbx2 amino acid sequence revealed that this motif is not present in Tbx2. We therefore performed GST pulldown assays using HeLa nuclear extract and a panel of GST-Tbx2 deletion mutants to map the Rb1 interaction domain (Figure 2A). Removal of the amino-terminal 83 amino acids of Tbx2 had no effect on Rb1 binding. However, successive carboxy-terminal truncations of the Tbx2(84-301) protein revealed that deletion of Tbx2 amino acids 301-287 immediately C-terminal to the T-box greatly reduces the interaction between Tbx2 and Rb1. Analysis of the Tbx2 amino acid sequence from 287 to 301 identified two hydrophobic leucine residues at positions 294 and 296 that could potentially be involved in forming protein–protein interactions. We therefore generated a mutated GST fusion protein containing leucine-to-alanine changes at positions 294 and 296 within the smallest fragment of Tbx2 (amino acids 84-301) that can bind Rb1. The resultant GST-Tbx2(84-301)L294AL296A protein failed to interact with Rb1 from HeLa nuclear extract in a pull-down experiment (Figure 2B) but retained the ability to bind DNA (Figure 2C) in a bandshift assay in which either purified wild-type or mutated GST-Tbx2(83-301) fusion proteins were incubated with a consensus T-element probe. Taken together, these results show that the L294AL296A mutation specifically disrupts the interaction between Tbx2 and Rb1 without affecting overall Tbx2 protein conformation.

Rb1 Enhances the Ability of Tbx2 to Repress Transcription

The Rb1 protein can function as a transcriptional corepressor. To determine whether Rb1 plays a role in Tbx2-mediated transcriptional control, we first assayed the ability of the wild-type Tbx2 and Tbx2 L294AL296A proteins to regulate p21^{CIP1}. Tbx2 is known to directly repress p21^{CIP1} expression through a T-element half site located near the p21^{CIP1} initiator region (Prince *et al.*, 2004). To do this, we cotransfected Phoenix cells, a derivative of HEK-293 cells, which do not express Tbx2, with a human p21^{CIP1} promoter-luciferase reporter and increasing concentrations of either Tbx2 or Tbx2L294AL296A expression vector. The results are shown in Figure 3A and reveal that wild-type Tbx2 repressed p21^{CIP1} promoter activity by up to threefold. The

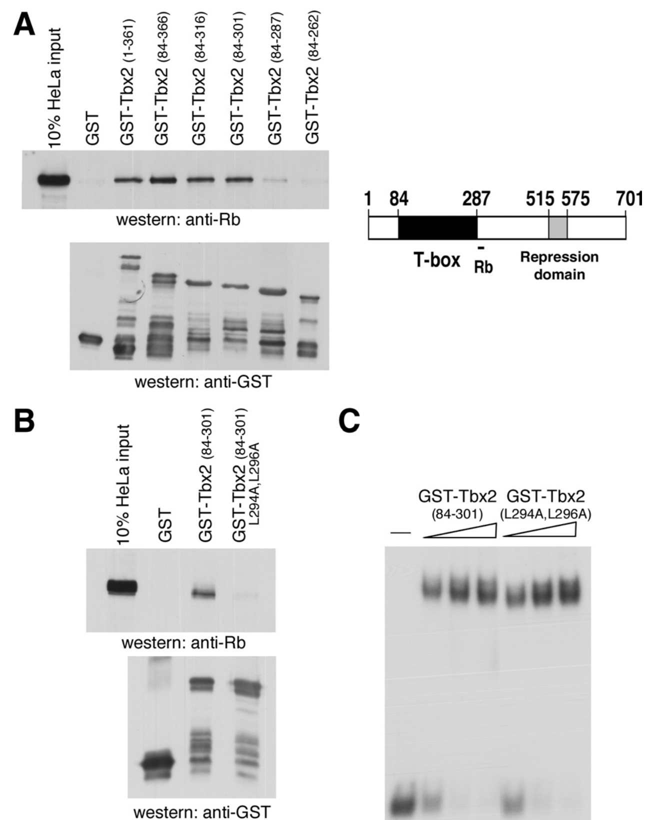

Figure 2. Mapping the Rb1 interaction domain in Tbx2. (A) Deletion of Tbx2 amino acids 301-287 greatly reduces binding to Rb1. (B) Identification of a Tbx2 mutant protein that fails to bind Rb1. The indicated GST-Tbx2 deletion mutants immobilized on glutathione beads were incubated with HeLa nuclear extract in a pulldown assay. Proteins were detected by Western blotting using anti-Rb1 and anti-GST antibodies. Tbx2 L294AL296A mutant protein is able to bind DNA (C). Equal amounts of purified GST-Tbx2(84-301) and GST-Tbx2(L294A,L296A) fusion proteins were incubated with radiolabeled T-element as a probe in a band-shift assay.

non-Rb1 binding Tbx2 mutant protein has a much reduced ability to regulate p21^{CIP1} and only repressed p21^{CIP1} expression up to a maximum of 1.4-fold. Western blotting showed that the wild-type and mutant Tbx2 proteins were expressed to similar levels. Additional evidence of a role for Rb1 in the modulation of Tbx2 function is provided in Figure 3B. This showed that cotransfection of an Rb1 expression vector further increases Tbx2-dependent repression of the p21^{CIP1} promoter in Phoenix cells. Although Tbx2 alone repressed p21^{CIP1} activity about fourfold in this set of experiments p21^{CIP1} levels were repressed ninefold in the presence of Rb1. The addition of Rb1 did not stimulate the ability of the Tbx2L294AL296A mutant protein to repress p21^{CIP1}, and Rb1 alone did not regulate p21^{CIP1}activity. Western blotting additionally showed that Rb1 overexpression does not affect the levels of the transfected Tbx2 proteins. This demonstrates that the ability of Tbx2 to repress expression of a known target gene is sensitive to the levels of Rb1. Although we cannot rule out that an interaction between Tbx2 and other factors, in addition to Rb1, is abolished by the L294AL296A mutation, the results show that Tbx2-mediated transcriptional repression of p21^{CIP1} expression correlates with its capacity to interact with Rb1.

To provide further proof that Rb1 functions as a transcriptional corepressor for Tbx2, we assayed the ability of the

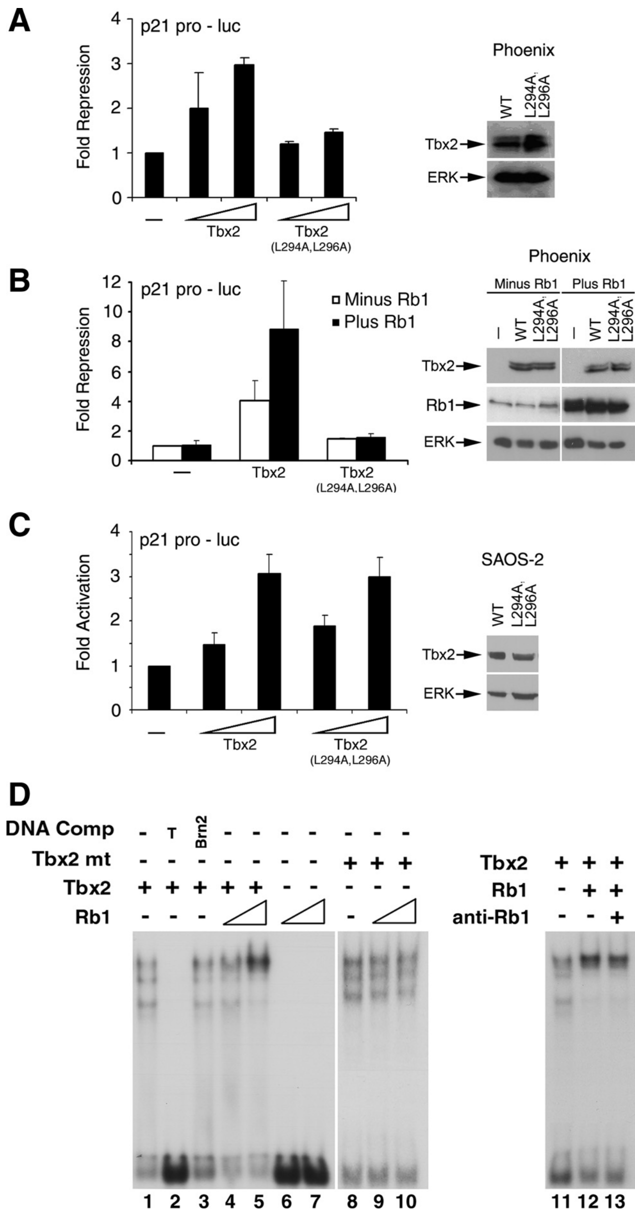

Figure 3. Tbx2 protein that does not bind Rb1 has an impaired transcriptional repression function (A). Phoenix cells were transfected with 50 ng p21^{CIP1} promoter luciferase reporter and either 25 ng or 50 ng of wild-type Tbx2 or Tbx2 L29AL296A expression vectors. Cotransfection of Rb1 enhances Tbx2-mediated transcriptional repression (B). Phoenix cells were cotransfected with 50 ng p21^{CIP1} promoter reporter construct along with the indicated combinations of either Tbx2 (50 ng), Tbx2mt (50 ng), and/or Rb1 (100 ng) expression vectors. Tbx2 activates transcription in Rb1 negative cells (C). SAOS-2 cells were transfected with 50 ng p21^{CIP1} promoter luciferase reporter and either 25 ng or 50 ng of wild-type Tbx2 or Tbx2 L29AL296A expression vectors. For these reporter experiments cells were assayed for luciferase and β-galactosidase activity 48 h after transfection. pCMV-B-gal (25 ng) was used as a transfection control and empty pCMV vector was added to make the total amount of transfected DNA equal in each case. Western blotting using anti-Tbx2, ant-Rb1, and anti-ERK antibodies was used to determine the levels of the transfected proteins. Rb1 increases the ability of Tbx2 to bind DNA (D). The indicated combinations of purified GST-Tbx2, GST-Tbx2(L294A,L296A), and His-Rb(379-928) proteins were incubated in a band-shift assay using radiolabeled T-element as a probe. Excess unlabeled T-element and Brn-2 oligos were used to assess the specificity of binding. For the supershift experiment purified proteins were incubated with 1 μl anti-Rb1 rabbit polyclonal antibody before addition of the probe.

wild-type and L294AL296A Tbx2 proteins to regulate p21^{CIP1} reporter activity in Rb1-negative SAOS-2 cells. Strikingly, the results revealed that Tbx2 functions to enhance p21^{CIP1} promoter activity in cells lacking Rb1 with both the wild-type and non-Rb1–binding mutant Tbx2 proteins activating p21^{CIP1} expression about threefold at high concentrations of expression vector (Figure 3C). Although previous deletion mapping experiments have suggested that Tbx2 contains a transcriptional activation domain (Paxton *et al.*, 2002), this is the first time that Tbx2 has been shown to activate expression of a known target gene. Additionally, the ability of the L294AL296A mutation to specifically disrupt the interaction with Rb1 is supported by the finding that there is no difference in the capacity of the wild-type and mutant Tbx2 proteins to regulate p21^{CIP1} expression in these cells. Although the result could add strength to our data, suggesting that Rb1 is involved in Tbx2-mediated repression, it is difficult to draw this conclusion given that other aspects of the cell background are so different. Moreover, our attempts to convert Tbx2-mediated activation of p21 to repression by reintroduction of Rb1 into these cells were confounded by the observation that in these cells, Rb1 alone showed a strong nonspecific activation of the p21^{CIP1} promoter in the absence of exogenous Tbx2 (data not shown).

Rb1 Increases the DNA-binding Activity of Tbx2

Because Rb1 can modulate the activity of its interacting transcription factors in a number of different ways, we tested the ability of Rb1 to regulate the DNA-binding function of Tbx2 in a bandshift assay. The results of this experiment are displayed in Figure 3D. Lane 1 shows the binding of suboptimal levels of purified GST-Tbx2 protein to the T-element probe. Binding is specific, because the retarded complexes could be competed with excess of a nonlabeled T-element competitor (lane 2) but not with an unrelated oligonucleotide (lane 3). Adding increasing levels of purified His-Rb(379-928) to the bandshift reaction enhanced the binding of Tbx2 to its consensus site (lanes 4 and 5), whereas Rb1 alone did not bind DNA (lanes 6 and 7). This effect is specific as it is abolished by the L294AL296A mutation as Rb1 did not stimulate the DNA-binding activity of a GST-Tbx2L294AL296A fusion protein (lanes 8–10) and is not due to nonspecific mass action of increased protein in the reaction (Supplemental Figure 1). In addition, lanes 11–13 show that we were unable to supershift the specific T-element DNA protein complex using an anti-Rb1 antibody. A similar inability to obtain a Rb1 supershift has been described before in studies of Rb1-dependent stimulation of DNA binding by c-jun, C/EBP, NF-IL6, and E4F (Chen *et al.,* 1996a,b; Nead *et al.,* 1998; Fajas *et al.,* 2000). It is therefore possible that Rb1 increases the DNA-binding activity of these transcription factors by a common mechanism involving a transient interaction that facilitates or stabilizes the interaction with the DNA.

These biochemical data are consistent with the results of our reporter assays showing that the non-Rb1 binding Tbx2 mutant has an impaired transcriptional repression function. Taken together, they suggest that Rb1 can enhance the function of Tbx2 as a transcriptional repressor by increasing its ability to interact with its cognate DNA recognition sequence.

Rb1 Modulates Tbx2 Target Gene Recognition

We previously showed that an ER-Tbx2(1-301) fusion protein containing amino acids 1-301, encompassing the T-box (amino acids 84-287) and adjacent sequence, fused to the ligand-binding domain of the estrogen receptor is able to

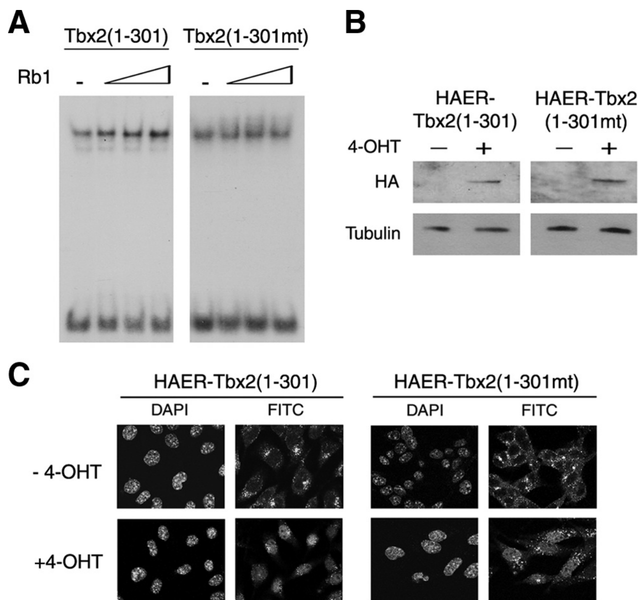

Figure 4. Generation and characterization of B16 cell lines expressing ER-Tbx2(1-301) and ER-Tbx2(1-301mt) proteins. (A) Rb1 enhances the DNA-binding activity of a Tbx2(1-301) but not Tbx2(1-301mt) protein. Bandshift assay in which suboptimal levels of purified GST-Tbx2(1-301) and GST-Tbx2(1-301mt) fusion proteins were incubated with a consensus T-element probe and increasing concentrations of purified His-Rb(379-928). Western blotting (B) using anti-HA or anti-tubulin antibodies and immunofluorescence staining (C) with an anti-HA antibody showing the levels and subcellular localization of ER-Tbx2(1-301) and ER-Tbx2(1-301mt) proteins grown in the absence or presence of 4-OHT for 24 h.

bind DNA and displace endogenous Tbx2 complexes from their target promoters in mouse and human melanoma cells (Vance *et al.,* 2005). Our biochemical analysis mapping the Rb1 interaction domain to amino acids immediately C-terminal to the T-box coupled with the finding that Rb1 increases the ability of Tbx2 to bind DNA in a bandshift reaction suggests that Rb1 may play a role regulating Tbx2 target gene recognition and specificity.

To explore this possibility, we generated B16 cell lines expressing either the ER-Tbx2(1-301) protein or an ER-Tbx2(1-301) mutant containing the non-Rb1 binding L294AL296A substitution. In contrast to the wild-type Tbx2(1-301) fragment the DNA-binding activity of the mutant protein on the consensus T-element is not enhanced by Rb1 when tested in a bandshift assay (Figure 4A). The cell lines we established were screened by Western blotting and indirect immunofluorescence to determine the expression levels and the subcellular localization of the fusion proteins. Figure 4B shows that we were able to generate two clonal lines expressing the ER-Tbx2(1-301) and ER-Tbx2(1-301mt) proteins to similar levels after 4-hydrotamoxifen (4-OHT) induction. The ER fusion proteins have a cytoplasmic localization in the absence of ligand but translocate to the nucleus within 24 h after 4-OHT addition, demonstrating that their activity can be tightly regulated (Figure 4C).

We next carried out expression profiling to first identify genes whose expression changes after induction of ER-Tbx2(1-301) and subsequently to identify targets whose misregulation is dependent on the ability of Tbx2 to bind Rb1. To do this, RNA was extracted and labeled from ER-Tbx2(1-301) and ER-Tbx2(1-301mt) cells grown in the absence or presence of ligand for 24 h and hybridized to Affymetrix mouse MOE430 expression arrays. Statistical analysis iden-

Table 1. List of known Rb1-repressed genes identified as potential Tbx2 targets

DNA replication/repair	Mitosis	Chromatin/epigenetic regulation
Lig1	CyclinB1	**Dnmt1**
Mcm2	**Cdc2a**	**Np95***
Mcm6	**Plk**	Ezh2
Mcm7	**Prc1**	Hmgb2
CyclinA2	**Bub1***	Asf1b
Top2a		
PCNA		
Rrm1		

Boldface indicates genes that are targeted by ER-Tbx2(1-301) but not by the ER-Tbx2(1-301mt) protein, which does not bind Rb1. Asterisks mark genes that are not regulated by E2F.

tified 431 genes whose expression are significantly different (tt = 0.0001) after ER-Tbx2(1-301) induction. One hundred sixty-six of these genes were up-regulated, and 265 genes were down-regulated in response to ER-Tbx2(1-301) activation (see Supplemental Figure 2 for the full list). These included previously identified Tbx2 targets such as connexin43, whose expression is directly regulated through two Tbx2-binding sites in its proximal promoter (Chen *et al.,* 2004), the helix loop helix transcription factor ID2, the Wnt target ENC1, and the cdc2a and MAD2 cell cycle regulators, which have been identified in previous microarray analyses (Chen *et al.,* 2001; Butz *et al.,* 2004). Additionally, this analysis identified 18 potential Tbx2 target genes that have previously been shown to be repressed by Rb1 (Table 1; Markey *et al.,* 2002). This is consistent with the finding that Rb1 can associate with and regulate Tbx2 function.

Comparison of the gene expression signatures of ER-Tbx2(1-301) and ER-Tbx2(1-301mt) cells grown in the presence of ligand for 24 h identified a total of 415 genes whose expression changes after ER-Tbx2(1-301) induction relative to ER-Tbx2(1-301mt) (Supplemental Figure 3 for full gene list). Although other cofactors in addition to Rb1 may play a role, the regulation of these genes correlates with the capacity of Tbx2(1-301) to bind Rb1. Principal component analysis (PCA) of ER-Tbx2(1-301)– and ER-Tbx2(1-301mt)–expressing cells revealed that the three replicates from each cell line cluster together and that there large differences between the transcriptomes of ER-Tbx2(1-301) and ER-Tbx2(1-301mt) cells (Figure 5A). These microarray experiments cannot discriminate between direct and indirect target genes but clearly demonstrate differences in expression programs between cells expressing the activated ER-Tbx2(1-301) and ER-Tbx2(1-301mt) proteins, suggesting that Rb1 is able to modulate Tbx2 target gene selection.

The ER-Tbx2(1-301) responsive gene list was sorted based on biological function using GO annotation and was found to be highly enriched for regulators of DNA replication (p = 1.15E-13) and cell division (p = 1.55E-06). These include Chaf1b, Cdt1, Cdkn2c, and components of the Mcm complex, as well as Smc2, Prc1, Bub1, and the Aurkb and Plk1 kinases. Figure 5B shows a heat map displaying the relative expression levels of the 38 identified cell cycle control genes (GO:0007049) in our dataset in each of the three replicates for each state. This revealed that the expression of many positive regulators of cell cycle was decreased and that the levels of several cell cycle inhibitors increased after induction of the Tbx2(1-

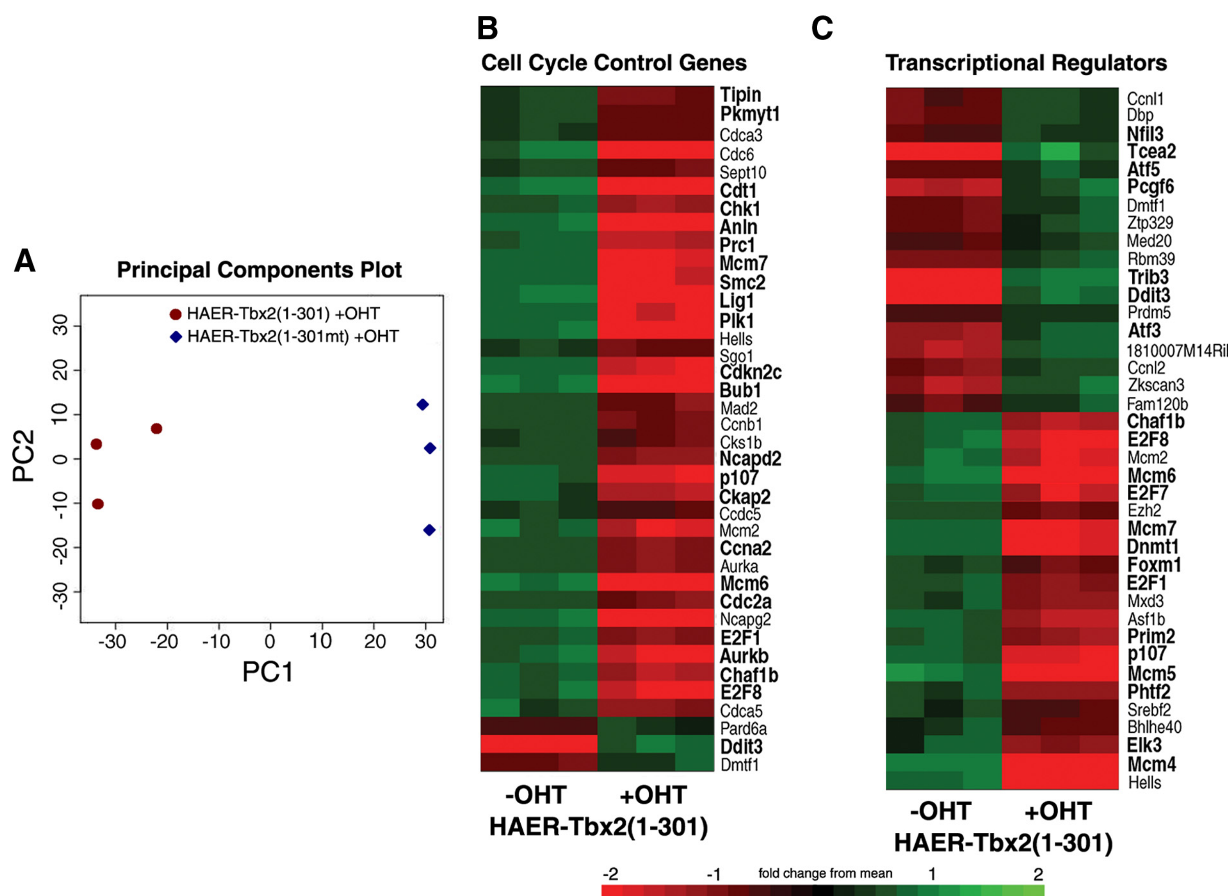

Figure 5. Rb1 modulates Tbx2(1-301) target gene specificity. (A) PCA analysis reveals large transcriptomic differences between ER-Tbx2(1-301)– and ER-Tbx2(1-301mt)–induced cells. (B and C) Heat map displaying gene expression changes of cell cycle (GO:0007049) and transcription factor (GO:0006350) genes after ER-Tbx2(1-301) induction. Green-to-red shift indicates repressed and red-to-green shift marks up-regulated genes. Some targets are present in both categories. Genes indicated in bold are differentially expressed between ER-Tbx2(1-301)– and ER-Tbx2(1-301mt)–induced cells.

301) molecule, consistent with the previously identified function of Tbx2 as an antisenescence factor (Vance *et al.*, 2005). Importantly, 24 of these cell cycle genes (Figure 5B, shown in bold) were differentially expressed between the ER-Tbx2(1-301) and ER-Tbx2(1-301mt) cell lines grown in the presence of ligand, suggesting that their regulation was dependent on the ability of Tbx2 to interact with Rb1. GO annotation (GO:0006350) was also used to gain a further understanding of the role of Tbx2 in transcription control (Figure 5C). ER-Tbx2(1-301) induction increased the expression of 18 transcriptional regulators and down-regulated a further 21. These included genes that have been implicated in the control of cellular proliferation, differentiation, and cancer, such as members of the E2F family, the Elk3 ETS domain factor, ATF-3 and -5, the forkhead factor FoxM1, the Ezh2 polycomb protein, and the DNA methyltranfserase Dnmt1. Consistent with a role for Rb1 in modulating Tbx2 target specificity and function, 21 genes in this cluster were also differentially regulated by ER-Tbx2(1-301) induction but not by ER-Tbx2(1-301mt). Taken together these results lead to the large-scale identification of novel downstream Tbx2 effectors and show that a mutation that disrupts the Rb1-Tbx2 interaction leads to altered recognition and regulation of target genes.

DISCUSSION

The T-box family of transcription factors is defined by homology to the DNA-binding domain or T-box. However, individual T-box family members can recognize and regulate different sets of target genes. Identification of the molecular mechanisms controlling target gene selectivity and transcriptional control is therefore essential to understand the function of this family of transcription factors in development and disease. The functional specificity of T-box transcription factors is determined by a number of factors. The prototypical T-box region from the brachyury protein can bind the palindromic consensus T-element as a dimer and can interact with individual half sites as a monomer. However, x-ray crystallographic studies have indicated that the T-box domain only makes two base-specific contacts per half site (Muller and Herrmann, 1997), suggesting that additional mechanisms may play a role in determining specificity. In fact, various family members have been reported to display differing preferences for the number, position, and spacing of T-element half sites within the regulatory regions of potential target genes (Sinha *et al.*, 2000; Conlon *et al.*, 2001). Such differential target recognition could feasibly be regulated through protein–protein interactions with DNA-binding proteins and cofactors such as Rb1.

Interactions between T-box factors and other transcription factors have been shown to contribute to target gene regulation. Indeed, the T-box can bind the homeodomain from a number of different homeobox factors. Tbx5 and Nkx2-5 interact and synergistically activate cardiac gene expression (Hiroi *et al.*, 2001) and Tbx2 binds and enhances Msx1-mediated repression of the Cx43 promoter (Boogerd *et al.*, 2008), whereas Tpit activates POMC expression in cooperation with Pitx1 (Lamolet *et al.*, 2001). Factors contributing to DNA specificity also reside outside the T-box. For example, the *Xenopus* brachyury protein (Xbra), via its amino-terminus, interacts with Smad1 to up-regulate expression of the Xom homeobox gene (Messenger *et al.*, 2005). Although the Xom promoter contains a consensus T-element, mutated variants of Xbra that are unable to bind Smad1 but have an intact T-box do not induce Xom expression, suggesting that the DNA-binding activity of the T-box can be modulated by additional regulatory domains within the protein. This is consistent with our findings that Rb1 interacts with Tbx2 via a domain immediately C-terminal to the T-box and that a mutation which disrupts the Rb1-Tbx2 interaction also affects Tbx2 target gene specificity. The organization and modification status of the chromatin at target gene loci may also contribute to specificity as several T-box factors have been shown to bind condensed chromatin. T-bet can bind to a T-element half site within the interferon-γ promoter even when this gene is epigenetically silenced by DNA methylation (Tong *et al.*, 2005), and Tbx2 has been shown to colocalize with pericentric heterochromatin in the developing mouse brain (Demay *et al.*, 2007). The discovery that Rb1 interacts with and modulates Tbx2 target gene selectivity therefore provides an important insight into the molecular mechanisms controlling T-box functional specificity.

The transcription program regulated by Tbx2 is poorly understood. Our microarray analysis extends the repertoire of potential Tbx2 targets and enhances our understanding Tbx2 function. We identify 38 cell cycle control genes and 39 transcription factors whose expression changes after induction of a ER-Tbx2(1-301) fusion protein containing the Tbx2 T-box and the surrounding sequence. The differential regulation of 24 cell cycle and 21 transcription factor genes in this set was shown to be dependent on the ability of Tbx2(1-301) to interact with Rb1. Several studies have demonstrated that Tbx2 directly represses $p19^{ARF}$ and $p21^{CIP1}$ expression to induce senescence in a variety of cell types (Jacobs *et al.*, 2000; Lingbeek *et al.*, 2002; Prince *et al.*, 2004; Vance *et al.*, 2005). Although our microarray analysis identified several known Tbx2 targets such as connexin43, the expression of $p19^{ARF}$ and $p21^{CIP1}$ did not change in this experiment. We did not expect to find changes in $p19^{ARF}$ expression because the CDKN2a locus is deleted in B16 cells (Melnikova *et al.*, 2004). In the case of $p21^{CIP1}$, a known Tbx2 target in B16 cells, it is important to note that we carried out our microarray profiling of ER-Tbx2(1-301)–expressing cells after 24 h of 4-OHT induction in contrast to our previous analysis where we measured changes in $p21^{CIP1}$ expression after inducing ER-Tbx2(1-301) for 4 d (Vance *et al.*, 2005). We detected an increase in $p21^{CIP1}$ proteins levels by Western blotting after 2 d of ER-Tbx2(1-301) induction using the clonal cell line described in this article (data not shown), which is consistent with the finding that Tbx2 represses $p21^{CIP1}$ expression. Our results here also suggest that Tbx2 can regulate the expression of the $p18^{INK4c}$ cyclin-dependent inhibitor and the A2 and B1 type cyclins as well other cell cycle control kinases such as bub1, plk1, chk1, pkmyt1, and aurkb, potentially targeting the cell cycle machinery at additional points. We also identified 18 previously known targets of Rb1-mediated transcriptional repression in our microarray analysis whose expression specifically changes after ER-Tbx2(1-301) induction. These genes can be grouped into important regulators of DNA replication/repair and the G2-M phase transition and factors that target chromatin to control gene expression. The majority these genes are bound by E2F family members (Ishida *et al.*, 2001; Muller *et al.*, 2001; Polager *et al.*, 2002; Ren *et al.*, 2002), suggesting that Tbx2 and E2F may coregulate the expression of specific sets of genes. However, the Bub1 and Np95 genes are repressed by Rb1 but are not E2F targets, suggesting that Rb1 has additional functions in transcriptional control independent of E2F that may involve Tbx2.

The interaction between Tbx2 and Rb1 is likely to be tightly regulated during cellular proliferation and differentiation in response to growth signals and external stimuli. It is well described that the activity of Rb1 is controlled by cell cycle–dependent phosphorylation (for a review see Mittnacht, 1998) and our results suggest that Tbx2 associates with the active hypophosphorylated form of Rb1 that is present in early to midG1 phase of the cell cycle and in late mitosis. However, Tbx2 proteins levels are regulated during cell cycle progression (Bilican and Goding, 2006). In B16 melanoma cells, MCF-7 breast cancer cells, and MRC-5 lung fibroblasts, Tbx2 levels are low in G1 and rise in mid-S phase with a peak of expression in G2 before being rapidly degraded at the onset of mitosis. It has also been suggested that, in some cancers, Tbx2 protein is not degraded after G2, resulting in mitotic defects and polyploidy (Davis *et al.*, 2007). We therefore speculate that Tbx2 and Rb1 may have other functions in addition to the control of cellular proliferation. Tbx2 and Rb1 may function in a checkpoint control pathway. Consistent with this we find that ER-Tbx2(1-301) expression misregulates the expression of the Chk1, E2F1, E2F 8, Ddit3, Aurkb, Tipin, and Lig1 genes, which play a role mediating the response to DNA-damaging agents and/or apoptotic stimuli. Various studies have also described the accumulation of hypophosphorylated Rb1 in response to cellular stress. Specifically, hypophosphorylated Rb1 has been shown to accumulate in late G1 and S phase in response to γ-irradiation (Linke *et al.*, 1997) and is induced in S and G2 by hypoxia (Amellem *et al.*, 1996). This possibility is supported by the observation that Tbx2 function can be regulated by stress-induced signaling pathways. Tbx2 is phosphorylated by the p38 mitogen-activated protein kinase in response to UV irradiation in breast cancer cell lines. Phosphorylation stabilizes Tbx2 protein levels and increases its transcriptional repressor function (Abrahams *et al.*, 2008).

It is also likely that Tbx2 and Rb1 may cooperate to control cellular growth and differentiation. Studies in a number of different cell types have shown that hypophosphorylated Rb1 accumulates upon differentiation and interacts with different transcription factors to promote cell cycle exit, induce tissue specific gene expression, and play a role in the maintenance of the differentiated state (reviewed in Lipinski and Jacks, 1999). Rb1 may modulate Tbx2 function during development and differentiation in a manner analogous to its regulation of the HMG box transcriptional repressor HBP1 (Lavender *et al.*, 1997; Tevosian *et al.*, 1997). HBP1 has a dual role in the control of proliferation and differentiation and can transform cells in culture. HBP1 and E2F both regulate the expression of the important cell cycle regulator N-Myc but have opposing effects (Tevosian *et al.*, 1997). HBP1-mediated repression of N-myc is enhanced by Rb1 association, whereas Rb1 blocks E2F-mediated activation of N-myc expression. In C2C12 myoblasts HBP1 overexpression inhibits the expression of N-myc and induces cell cycle

exit but also blocks differentiation by repressing the expression of specific myogenic regulatory factors (Tevosian *et al.*, 1997; Shih *et al.*, 1998). It is of interest to note that in addition to functioning in cell cycle control Tbx2 has been shown to regulate the expression of the TRP-1 pigmentation gene, which is involved in melanocyte differentiation (Carreira *et al.*, 1998). Thus, the balance between the levels of active Rb1 and Tbx2 during development or in disease may control Tbx2 target gene recognition and regulation and lead to distinct downstream effects.

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