The T-box Repressors TBX2 and TBX3 Specifically Regulate the Tumor Suppressor Gene p14ARF via a Variant T-site in the Initiator*

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The murine tumor suppressor p19ARF (p14ARF in humans) is thought to fulfill an important protective role in preventing primary cells from oncogenic transformation via its action in the p53 pathway. Several disease-implicated regulators of p19ARF are known to date, among which are the T-box genes TBX2, which resides on an amplicon in primary breast tumors, and TBX3, which is mutated in the human developmental disorder Ulnar-Mammary syndrome. Here we identify a variant T-site, matching 13 of 20 nucleotides of a consensus T-site, as the essential TBX2/TBX3-binding element in the human p14ARF promoter. Mutant analysis indicates that both the consensus T-box and a C-terminal conserved repression domain are essential for p14ARF repression. Whereas the core nucleotides required for interaction of the archetypal T-box protein Brachyury with a consensus T-site are conserved in the variant site, additional flanking nucleotides contribute to the specificity of TBX2 binding. This is illustrated by the inability of TBX1A or Xbra to activate via the variant p14ARF T-site. Importantly, this suggests a hitherto unsuspected level of specificity associated with T-box factors and corresponding recognition sites in regulating their target genes in vivo.

T-box genes are a relatively recently discovered gene family characterized by a 180–200-amino acid DNA-binding T-box domain (reviewed in Refs. 1–3). Genetic studies in species ranging from Xenopus to human point to a crucial role for T-box genes in controlling development in a gene dose-dependent manner. This is clearly illustrated by two human syndromes that lead to multiple developmental abnormalities and that are characterized by both patterning defects and hypoplasia: Holt-Oram syndrome and Ulnar-Mammary syndrome. These syndromes are associated with haplo-insufficiency of TBX5 and TBX3, respectively (4, 5). Although data on the functions of the steadily growing family of T-box genes are now emerging, relatively little is known about T-box gene targets and the molecular mechanism underlying specific gene regulation by individual T-box genes. In vitro site selection experiments with Brachyury, the founder of the T-box gene family, revealed the preference for a palindromic sequence (6) and the crystal structure showed that the Brachyury T-box binds this so-called T-site as a dimer (7). Of all T-box genes only three, TBX2, TBX3 and Xenopus ET, are currently known to be transcriptional repressors, probably acting via a putative repression domain (8, 9). TBX2 was shown to act on the melanocyte-specific TRP-1 promoter, not by a regular T-site but by two T-half-sites located 200 bp apart (8). Also for other in vivo T-box targets a picture is now emerging in which they are controlled by separate T-half-sites.

We recently found TBX2 in a genetic senescence bypass screen to potently down-regulate the p19ARF tumor suppressor, thereby causing efficient immortalization of primary fibroblasts (10). Additionally, in an independent screen, TBX3 was found to have that same activity (11). This placed T-box gene family members for the first time in the regulation of cell proliferation. P19ARF in mice or p14ARF in humans is the alternative transcript encoded by the unusual INK4a/ARF locus coding also for p16INK4a (reviewed in Ref. 12). Both of these cell cycle inhibitors and tumor suppressors are implicated in cancer-relevant pathways; p16INK4a acts to inhibit CDK4 and CDK6, thereby preventing inactivating phosphorylation on the Rb tumor suppressor protein (13, 14), whereas p19ARF acts in the p53 pathway (reviewed in Refs. 15 and 16). Induction of p19ARF upon serial passaging of rodent cells leads via MDM2 to a stable and transcriptionally active p53, which by activating its targets can lead to a growth arrest called replicative senescence. P19ARF is also induced upon hyperproliferative signaling by oncogenes such as c-myc and adenoviruses E1A, E2F-1, and RasV12 (17–20). Therefore, p19ARF activation is thought to work as an important fail-safe mechanism, because efficient oncogenic transformation by these factors can occur only when the ARF-Mdm2-p53 pathway is inactivated (15, 16).

Several regulators of the p19ARF promoter are known to date. Of these a number are most likely indirect regulators, such as c-Myc (17), Twist (21), JunD (22), DAP kinase (23), c-Abl (24), E1A (18), and RasV12 (20). However, some of them potentially are direct regulators, for example DMP-1 (25, 26), BMI-1 (27), p53 (28), and E2F1 (19). In accordance with the important cancer-preventing role of p19ARF, deregulation of many of these p19ARF transcriptional regulators has been shown to play a role in tumorigenesis. For instance overexpression of Bmi-1, a member of the Polycomb group of repressor proteins, results in lymphomagenesis and likely contributes to several human malignancies (29, 30). For TBX2 a potential role in breast cancer development has been suggested because the locus resides on an amplicon present in a subset of primary breast cancers (10). Here we have studied the mechanism and cis-requirements for repression of p14ARF/p19ARF as a bona fide feature of TBX2 and TBX3 target and found that TBX2 and TBX3 are direct and specific regulators acting via a variant T-site that is present close to the p14ARF transcriptional start site.

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EXPERIMENTAL PROCEDURES

Cell Culture, Growth Curves, and Retroviral Infection—We performed cell culturing of primary mouse embryo fibroblasts (MEFs), retroviral infections, growth curves, and 3T3 schedules as described (10, 27, 31) using the following retroviruses: LZRS-iresGFP (control), LZRS-TBX2-iresGFP, LZRS-TBX2RD-iresGFP, MSCV (control), MSCV-TBX2, MSCV-TBX2RD, MSCV-TBX2T(R122E,R123E), and MSCV-TBX2T(A272E). The latter two constructs, the T-box mutants, were generated by PCR and verified by sequencing.

Western Blot Analysis—For protein expression analysis, cell lysates were separated on 9% (for TBX2 levels) or 13% (for p14ARF levels) SDS-PAGE and blotted on nitrocellulose or Immobilon-P membranes (Amer sham Biosciences). Analysis was done according to standard methods using enhanced chemiluminescence (Amersham Biosciences). Primary antibodies were R562 (Abcam) for p14ARF, 12CA5 for HA-tagged TBX1A, and rabbit polyclonal or mouse monoclonal α-TBX2 antibodies (10).

Repression Assays— COS-7 cells were co-transfected at 40% confluence by calcium phosphate precipitation with 20 μg of reporter plasmid and 0–1 μg of expression plasmid. The TBX2 constructs, TBX2, TBX2RD, TBX2T(R122E, R123E), and TBX2T(A272E), were expressed from pCDNA3.1 (Invitrogen), TBX1A-HA from pCMV, Xbra from pEGFP-C1 (32), and E2F1 from pCMV. For the p14ARF promoter, the original CAT reporter constructs described by Robertson and Jones (28) were used. In addition we used the pGL3-basic vector (Promega) in the original CAT reporter constructs described (32). Into the SmaI site of this construct the same double-stranded oligos as used for electrophoretic mobility shift assays (EMSA)1 (see below) were cloned, resulting in a construct with two Brachyury consensus binding sites (B), two variant T-sites (+9/+29), two mutant variant T-sites (+9/+29mut), or one variant T-site including flanking sequences (−14/+35). All transfections contained equal amounts of pCDNA3.1 and included 1 μg of pSv β-galactosidase plasmid (Promega) as an internal transfection control. CAT, luciferase, and β-galactosidase activities were measured 48 h after transfection by standard methods. All transfections were performed in duplicate, and at least two independent experiments were done to confirm reproducibility.

Electrophoretic Mobility Shift Assays—Whole cell extracts were prepared from COS-7 cells transfected with expression vectors (pCDNA3.1) for TBX2, TBX2RD, TBX2T(R122E, R123E), TBX2T(A272E), TBX3, L143P TBX3, or 1498S TBX2 or from normal MEFs infected with LZRS-iresGFP, LZRS-TBX2-iresGFP, or LZRS-TBX2RD-iresGFP retroviruses as described (33). Binding reactions were performed with double-stranded 32P-labeled oligonucleotide probes, cell extracts, antibodies, and competitor oligos as described (8) and resolved on a 4% native polyacrylamide gel. Oligonucleotides used (complementary strand not shown) were: +9/+29, 5’-CTGCTCACCCTGTTGCGCACA-3’; +9/+29mut, 5’-CTGCTGATCTACTGTTGCGCACA-3’; −14/+35, 5’-AATGAAAGGCGGAGGAGTCGCGACTTGGTTGCGCACAAGGCGGCGCGCTCTTTGGCAGC-3’; B, 5’-GGAGAACACTTACACAGGTTGGAATTTCCCTCCT-3’; and E2F, 5’-CAATAGAGCTGCCTTGGCAGCCCTTGGCAGGCAACAA-3’ and 5’-CTTCGCTATGTTGCGCACA-3’. Site mutations for probe −14/+35 are described in Fig. 2B. Probe −19/+54 was generated by PCR with primers 5’-GAAGCTTGAGGCGCTGCGCGCCCTTTGGCCAGC-3’ and 5’-CTTCGCTATGTTGCGCACA-3’.

RESULTS

Both T-box Mutations and Deletion of the Repression Domain of TBX2 Abrogate p14ARF Repression—To investigate the role of the T-box domain of TBX2 in mediating p14ARF repression, we made two point mutants, TBX2T(R122E,R123E) and TBX2T(A272E). These mutations were designed based on the crystal structure of the T-box domain of Brachyury in complex with a consensus T-site (7) and were predicted to disrupt DNA binding. In previous work we showed that TBX2 rescues MEFs from senescence and leads to their immortalization (10). In addition, the high p14ARF levels in Bmi−/− MEFs due to abrogation of Polycomb repression of the INK4a/ARF locus is efficiently reduced by even low levels of TBX2, resulting in immortalization of these prematurely senescent fibroblasts (27). In contrast, mock-infected wild-type and Bmi−/− MEFs as well as cells infected with either one of the T-box mutants enter senescence at the same passage (Fig. 1A). Western blot analysis revealed that both T-box mutants are expressed but show the same high levels of p14ARF as senescent cells, in sharp contrast to the severely down-regulated p14ARF levels in TBX2-overexpressing cells (Fig. 1B). Mutants of the T-box of TBX3 were also incapable of rescuing MEFs from senescence, as opposed to the readily immortalizing wild-type protein (11). This indicates an essential role for the conserved T-box domain in p14ARF repression.

Deletion constructs of the human p14ARF promoter transfected to COS-7 cells revealed that TBX2 and TBX3 act as strong dose-dependent repressors through an element located in the region of −19 to +54 (10, 11). This repression was found to be dependent on the repression domain of TBX2 (10). In agreement with the data described above, TBX2T(R122E,R123E) is incapable of and TBX2T(A272E) is severely impaired in repressing p14ARF, whereas both proteins could be detected readily by Western blot (Fig. 1, E and F). Although both the repression domain and the T-box domain are involved in mediating p14ARF or p14ARF repression, neither of the mutants appears to work as a dominant-negative in transient repression assays, i.e. we observed no competition with wild-type TBX2 or TBX3 for p14ARF promoter repression (data not shown).

A Newly Identified Variant T-site in the p14ARF Promoter Is Bound by TBX2 and TBX3—To test whether the inactivating mutations of the T-box reflect impaired binding to the p14ARF promoter we performed electrophoretic mobility shift assays. Incubation of probe −14/+35, spanning part of the repressed region, with either TBX2- or TBX3-overexpressing COS-7 cell extracts resulted in a shifted complex that was not observed using mock-transfected extracts (Fig. 2A, lanes 1, 2, 15, and 16). This complex does contain TBX2 because it is not affected by pre-immune serum but can be supershifted by rabbit polyclonal α-TBX2 antibody to a complex barely capable of entering the gel (Fig. 2A, lanes 3 and 4). The same supershifts were observed with an independently generated mouse monoclonal α-TBX2 antibody (data not shown and Fig. 2E). The presence of such large aggregates upon antibody addition was noted before in case of TBX2 (32). The complex is specific because cold −14/+35 probe is able to compete the complex, whereas an unrelated E2F binding site oligo cannot do so (Fig. 2A, lanes 5 and 7). A probe containing a Brachyury consensus binding site that has been described to bind TBX2 (Ref. 8 and our own unpublished observations), is also able to compete the complex (Fig. 2A, lane 6). When overexpressing the repression domain mutant the complex with probe −14/+35 could still be formed and supershifted with α-TBX2 antibody, although less efficiently (Fig. 2A, lanes 8–10). In contrast, both T-box mutants of

1 The abbreviations used are: EMSA, electrophoretic mobility shift assay; MEF, mouse embryo fibroblast; HA, hemagglutinin; CAT, chloramphenicol acetyltransferase; HSV, herpes simplex virus; tk, thymidine kinase; GFP, green fluorescent protein; MSCV, mouse stem cell virus; Luc, luciferase; oligo, oligonucleotide.
Both T-box point mutants and a repression domain mutant of TBX2 are impaired in repressing p19ARF/p14ARF and incapable of rescuing MEFs from senescence. A, growth curve of passage 7 wild-type MEFs infected at passage 1 with empty control, TBX2, TBX2RD, TBX2TB(R122E, R123E), or TBX2TB(A272E) MSCV retroviruses. B, Western blot analysis of TBX2 (FL, full-length protein; RD, repression domain deletion mutant) and p19ARF levels of the cells mentioned in panel A at passage 7. Tubulin levels serve as loading control. The asterisk indicates a band that appears because of non-specific antibody recognition. C, proliferation of Bmi−/− MEFS infected with control, TBX2, and TBX2RD LZRS-iresGFP retroviruses on a 3T3 schedule. Note that TBX2RD is capable of extending MEF life span although it will not immortalize cells as TBX2 can do. D, Western blot analysis of TBX2 and p19ARF levels of Bmi−/− MEFS at passage 4. Note that TBX2RD is less effective in down-regulating p19ARF than TBX2, despite its higher expression levels. E, dose-dependent repression of the (−19/+54) p14ARF promoter CAT reporter by TBX2 is impaired in the case of TBX2RD, TBX2TB(R122E,R123E), and TBX2TB(A272E) mutants. F, expression of TBX2 wild-type and mutant proteins in COS-7 cells.
TBX2 and TBX3 were unable to form a complex with probe −14/+35 either in the presence or absence of antibody (Fig. 2A, lanes 11–14 and 17–18). For TBX2R(B122E, R123E) this is in agreement with the inability of a TBX2R122A mutant to bind a consensus T-site (32).

The only elements present in the small promoter fragment of p14ARF that is still repressed by TBX2 are an initiator element and an inverse E2F site (28). Although no consensus T-site is present, after a closer inspection of region −19 to +54 we identified a variant T-site matching 13 of 20 of the nucleotides of a consensus palindromic T-site (Fig. 2B). In this variant T-site the T-half-sites are spaced one nucleotide apart but are orientated similarly as in the original consensus site. The possibility that this site is a functional T-site is strengthened by the fact that all four nucleotides marked as important DNA specificity determinants in the crystal structure of Brachury in complex with a T-site are retained (Ref. 7, Fig. 2B), as are most of the nucleotides (9 of 12) that are selected in 85% of the cases of in vitro binding site selection experiments with Brachury (Ref. 6, Fig. 2B).

Using EMSA, a TBX2-specific complex could be seen with probe +9/+29 (spanning this variant T-site) that could be supershifted with the rabbit polyclonal α-TBX2 antibody but was absent from mock-transfected lysates (Fig. 2C, lanes 1–4). The complex with probe +9/+29 appears to be formed less efficiently than with probe −14/+35, indicating that sequences outside of the +9/+29 probe might also contribute to DNA binding. The addition of α-TBX2 antibody appears to stabilize the complex (Fig. 2C, lane 4). Others have seen such a T-protein-DNA complex stabilization by antibodies as well in the case of Brachury and Xbra (6, 32). In contrast to probe −14/+35, the supershifted complex with probe +9/+29 migrates faster and yields a sharper band. The complex could be competed with cold +9/+29 oligo and a Brachury consensus binding site but not with +9/+29mut in which critical nucleotides were mutated (Fig. 2C, lanes 5–7). Moreover, when +9/+29mut was used (as probe), no complex in TBX2 overexpression extracts could be seen either in the presence or absence of α-TBX2 antibody, indicating that the mutated nucleotides are indeed involved in DNA binding (data not shown). The repression domain mutant was able to bind +9/+29 in the presence of α-TBX2 antibody, again indicating that DNA binding ability of this mutant apparently is weaker and that α-TBX2 antibody stabilizes this complex (Fig. 2C, lanes 8 and 9). As expected, the T-box mutant proteins are incapable of binding the variant T-site (Fig. 2C, lanes 10–13).

To get a better insight into the requirements for DNA binding by TBX2, we performed a more detailed mutagenesis of the variant T-site. We made a set of four mutants: two (14/−35CC and −14/+35GG) in which we mutated two nucleotides in either T-half-site, thereby leaving the other T-half-site intact; and two (−14/+35CCGG and −14/+35mut) in which we mutated both T-half-sites, respectively, by a four- or seven-nucleotide change (Fig. 2B). When introduced into probe +9/+29 all mutations including those that disrupt only one half-site disrupted binding (data not shown). However when introduced into the larger −14/+35 probe, only mutations disrupting both T-half-sites completely abolished DNA binding (Fig. 2D). In agreement with TBX2 being able to bind consensus T-half-sites (32), we could still detect binding of TBX2 to mutants of either T-half-site, albeit with a lower efficiency. Moreover mutating the left T-half-site appears to disrupt DNA binding more severely than mutating the right T-half-site. Together, these results indicate that the core structure of T-sites is functionally conserved in the variant T-site and that additional sequences in probe −14/+35 contribute to the stability of the complex.

In extracts of MEFs infected with TBX2 we were unable to detect a complex with p14ARF promoter probes (Fig. 2E, lanes 1 and 2, 4 and 5). Similar TBX2 binding detection problems have been observed previously (Ref. 8). The detection problems are most likely due to masking by the multiple complexes that apparently are formed with the probe in these extracts, as in strong support of direct binding we could detect a large supershifted complex in TBX2-infected MEF cell extracts with both the rabbit polyclonal and mouse monoclonal α-TBX2 antibody (Fig. 2E, lane 3). Likewise, a somewhat faster migrating complex was observed with the repression domain mutant (Fig. 2E, lane 6). The complex was absent when a probe containing the incomplete T-site was used (probe −12/+23, data not shown) or when non-TBX2-overexpressing MEFs were used (Fig. 2E, lanes 9 and 10). In conclusion these EMSA experiments prove that TBX2 and TBX3 can directly bind p14ARF promoter oligos that span a variant T-site.

The Variant T-site of p14ARF Is Required for p14ARF Promoter Repression—To prove that the variant T-site is the relevant site via which TBX2 and TBX3 mediate repression we mutated the site in (−19/+54) p14ARF promoter-reporter constructs at exactly the same positions as those used in EMSAs. Because these mutations reside in the core promoter region, it was not surprising that substantial and progressive activity loss was observed. However, in agreement with the binding data obtained in EMSA, T-half-site mutants (−19/+54CC and −19/+54GG) could still be repressed by TBX2, whereas mutants in which both T-half-sites were disrupted (−19/+54CCGG and −19/+54mut) were insensitive to TBX2 even at a very high dose (Fig. 3A). From this finding we not only conclude that mutating the variant T-site abolishes the repressive effect of TBX2, thereby proving that this is the relevant site, but also that TBX2 is able to bind and repress when only one half-site is intact.

To further test whether TBX2 can mediate repression through a variant T-site, we made chimeric promoters consisting of the HSV tk promoter driving the luciferase gene, upstream, of which we cloned both the consensus and variant T-sites (Fig. 3B). These constructs have been described previously as only being responsive to TBX2 when four T-half-sites are present (32). However, in our hands the sole HSV tk promoter was repressed 4-fold by TBX2 (Fig. 3C). This might be explained by the cell type used (COS-7 and Phoenix cells by us versus 293 cells in the other study). Nevertheless, when either two consensus T-sites, two variant T-sites of the p14ARF promoter (sequence +9/+29) or a somewhat larger part of the p14ARF promoter (sequence −14/+35), are upstream of the HSV tk promoter, these constructs were repressed up to 10-fold by TBX2 (Fig. 3C). In contrast, when the mutant T-site (+9/+29mut), which was incapable of binding TBX2 in EMSAs, was cloned upstream, the level of repression was comparable with that of HSV tk alone (−4-fold, Fig. 3C). In conclusion, these results demonstrate that TBX2 is able to mediate repression through the variant T-site in the p14ARF promoter.

TBX1A and Xbra Are Not Able to Act on the p14ARF Promoter—To test whether the variant T-site in the p14ARF promoter can also potentially be regulated by other T-box factors or might be specific to TBX2 and TBX3, we performed co-transfection assays in COS-7 cells with the p14ARF reporter constructs and two T-box-containing transcriptional activators, TBX1A and Xbra. Xbra had no effect on the small (−19/+54) p14ARF promoter (Fig. 4A) and could only activate the long (−2465/+54) p14ARF promoter 2-fold at high dose (Fig. 4B). Expression of the protein could clearly be detected by its eGFP tag (data not shown). In agreement with previous results, we could potentially activate the HSV tk promoter with two up-
FIG. 2. **TBX2 and TBX3 bind to a variant T-site present in the p14ARF promoter.**

**A**. EMSA with probe −14/+35 and extracts of COS-7 cells overexpressing TBX2, TBX2RD, TBX2TB(R122E, R123E), TBX2TB(A272E), TBX3, TBX3 L143P, or TBX3 Y149S. Competitor oligos (−14/+35, Brachyury consensus binding site B, and E2F binding site) and antibodies (pr, pre-immune serum; r, rabbit polyclonal α-TBX2 antibody) are added as indicated. Note that TBX2, TBX2RD, and TBX3 can bind probe −14/+35 in both the absence and presence of α-TBX2 antibody as opposed to the DNA binding inability of T-box mutants of TBX2 and TBX3.

**B**. Schematic outline of sequences −19 to +54 of the p14ARF promoter with the TBX2 and TBX3 Repress p14 ARF via a Variant T-site.

**C**. EMSA with probe +9/+29 and extracts of COS-7 cells overexpressing TBX2, TBX2RD, TBX2TB(R122E, R123E), TBX2TB(A272E), TBX3, TBX3 L143P, or TBX3 Y149S. Competitor oligos (+9/+29, Brachyury consensus binding site B, and E2F binding site) and antibodies (pr, pre-immune serum; r, rabbit polyclonal α-TBX2 antibody) are added as indicated.
specificity determinants in the crystal structure of Brachyury in complex with a T-site are shown in
in vitro
complex is detected, upon addition of antibody, that migrates slightly faster for TBX2RD compared with wild-type TBX2.

...TBX1A and Xbra are not able to act via the variant
other TBX1A-responsive promoters have been described to our
...factors TBX1A and Xbra are not able to act via the variant
...activity of the protein because TBX1A is not active on the HSV
tk promoter with the two upstream T-sites (Ref. 32) and no
activity of the protein because TBX1A is not active on the HSV
...see an effect of TBX1A (Fig. 4
...DISCUSSION
In this report we demonstrate that TBX2 and TBX3 bind a
variant T-site in the p14ARF promoter, thereby down-regulating its gene expression. TBX2/TBX3 can bind this variant T-site, whereas point mutants of the DNA-binding T-box domain, both at the C-terminal and N-terminal parts, can not do so. The point mutants are to a variable extent impaired in repressing p14ARF in transient repression assays, i.e. TBX2TB(R122E,R123E) is completely inactive and TBX2TB(A272E) weakly active at high concentrations. Nevertheless, both mutants are incapable of down-regulating endogenous p19ARF levels in MEFs and because of this are incapable of bypassing senescence arrest. Although in vitro site selection experiments with Brachury in the past (6) and with Xbra, VegT, and eomesodermin more recently (34) appear to select for a repeat (palindrome) of T-half-sites. Some of these promoters contain multiple, separate T-half-sites, such as in the case of Ci-trop regulation by Ci-Bra in Ciona intestinalis (35), Bix4 by VegT (36) or eFGF by Xbra in Xenopus (37), orthopedia regulation by Brachyteron (Byn) in Drosophila (38), and the melanocyte-specific TRP-1 promoter by Tbx2 (8). Interestingly, for Bix4 and TRP-1, the T-half-sites map in the vicinity of the transcriptional start site or even within the initiator element (TRP-1), in analogy to the here described variant T-site in the p14ARF

...variant T-site. A, dose-dependent repression of the (−19/+54) p14ARF promoter CAT reporter by TBX2 but not by TBX1A and Xbra. The activity level of the promoter in the absence of TBX2 is set at 100%. B, dose-depend-ent repression of the (−2465/+54) p14ARF promoter-CAT reporter by TBX2 but not by TBX1A and Xbra. The activity level of the promoter in absence of TBX2 is set at 100%. C, activity of Xbra on the HSV tk Luc constructs depicted in Fig. 3B. Note that Xbra is able to activate via consensus T-sites but not via the variant T-sites. D, Western blot analysis of HA-tagged TBX1A in COS-7 cells.

FIG. 3. The variant T-site is necessary for the repression of the p14ARF promoter by TBX2. A, dose-dependent repression of wild-type and T-half-site mutant (−19/+54) p14ARF promoter-Luc reporters (p14ARF wt, p14ARF (CC), and p14ARF (GG)) by TBX2 but not of (−19/+54) p14ARF promoter-Luc reporter constructs in which both T-half-sites are disrupted (p14ARF (CCCG) and p14ARF (mut)). B, schematic representation of the chimeric HSV tk constructs (labeled a–e) made with the upstream consensus T-site (b), the variant T-site (+9/+29 and −14/+35), and the mutated variant T-site (+9/+29 mut). C, fold repression of the constructs depicted in panel B by the presence of 1 μg of TBX2. Note that the constructs harboring consensus or variant T-sites (columns b, c, and e) are relatively more strongly repressed (up to 10-fold) than the sole HSV tk promoter (column a) or the construct harboring the mutant variant T-site (column d) (both are repressed 4-fold).

...stream consensus T-sites with Xbra (Fig. 4C and Ref. 32). In contrast, Xbra did not activate the HSV tk promoter with two upstream variant T-sites (Fig. 4C). Both experiments show that Xbra is not able to regulate via the p14ARF variant T-site.

...TBX1A can reduce p14ARF promoter activity of the short (−19/+54) construct when added at very high concentrations, but in comparison, TBX2 is much more active in repressing, also at much lower concentrations (Fig. 4A). Moreover, on the long and more active (−2465/+54) p14ARF promoter, we did not see an effect of TBX1A (Fig. 4B). Although we could clearly detect expression of TBX1A (Fig. 4D), we could not check the activity of the protein because TBX1A is not active on the HSV tk promoter with the two upstream T-sites (Ref. 32) and no other TBX1A-responsive promoters have been described to our knowledge. In conclusion, we show that at least the T-box factors TBX1A and Xbra are not able to act via the variant T-site located in the p14ARF promoter, suggesting a level of specificity for T-box family proteins in target gene recognition.

initiator (inv) element and the inverse E2F site underlined. An alignment is made with a consensus T-site. Nucleotides marked as important DNA specificity determinants in the crystal structure of Brachury in complex with a T-site are shown in capital letters (7), and nucleotides that are selected in 85% of the cases of in vitro binding site selection experiments with Brachury are underlined in bold (6). Underneath the p14ARF promoter sequence, the probes used in the EMSAs are depicted. For variant T-site mutations the differences with wild-type are indicated in bold. C, EMSA with probe +9/+29 and extracts of COS-7 cells. In analogy to panel A, TBX2 and TBX2RD are able to bind probe +9/+29, spanning the variant T-site in p14ARF. D, EMSA with both wild-type and mutant −14/+35 probes demonstrating the TBX2 is able to bind when one of the T-half-sites is disrupted but not when both T-half-sites are disrupted. Extracts used are from mock-transfected (−) or TBX2-overexpressing (T) COS-7 cells. E, EMSA with probe −19/+54 and extracts of ARF−/− MEFs noninfected (−), TBX2-infected (T), or TBX2RD-infected (RD). Pre-immune serum (pm) and mouse polyclonal α-TBX2 antibody (m) were added as indicated. In strong support of direct binding, a supershifted complex is detected, upon addition of antibody, that migrates slightly faster for TBX2RD compared with wild-type TBX2.
TBX2 and TBX3 Repress p14^{ARF} via a Variant T-site

initiator. Other T-box targets are regulated by cooperation of a T-box protein and a cofactor that bind to contiguous sites within the same regulatory element. Good examples are Tbx5 and Nkx2–5, which synergistically activate the cardiac-specific Nppa, ANF, and cx40 promoters. Moreover, these two proteins bind these promoters in tandem, on an element containing both binding sites, but can also interact in the absence of DNA (39, 40). In addition, TBX2 and Nkx2–5 simultaneously interact on a double site of the ANF promoter, leading to its repression (41). In pituitary cells, Tpit or TBX19 and Pitx cooperate to activate POMC (pro-opiomelanocortin) gene transcription also by binding such a double site (42, 43). Notwithstanding these recent discoveries, relatively little is known about the sequence requirements that determine individual target gene specificity for the large family of T-box domain-containing transcription factors.

Here, we demonstrate for the first time that an important T-box target, the p14^{ARF} tumor suppressor gene, is regulated by a variant palindromic T-site. Whereas the core CACCNNNGGGTTG nucleotides of the variant site, which form an imperfect palindrome, are well conserved in the “consensus” T-site, flanking nucleotides are divergent (Fig. 2B). The imperfect palindromic site is reminiscent of the preference for a repeat of T-half-sites for Brachyury, Xbra, VegT, and eomesoderm found in in vitro binding studies (6, 34). In addition, our mutational analysis of the conserved “core” sequence clearly demonstrates its requirement for the binding of TBX2 and TBX3 to the variant site, likely reflecting the highly conserved structure of the T-box domain (7). Although TBX2 in vitro has previously been shown able to bind to T-half-sites, a preference for (palindromic) T-sites appears to exist (32). In addition, it was shown that TBX2 can bind variants of a consensus T-half-site that were generated by mutagenesis of the consens T-site (8). However such sequences are not present in the small –19/+54 p14^{ARF} promoter fragment, and their biological relevance for in vivo target gene regulation remains to be demonstrated. Interestingly, it has just been reported that orthopedia is regulated by Byn via 15 binding sites, which all differ in at least two nucleotides from a consensus T-half-site (38). Therefore, in agreement with our data, Byn can regulate a promoter through variants of a T-half-site. However, the orthopedia promoter does not contain a palindromic T-site as seen for the p14^{ARF} promoter. Close inspection of more upstream sequence of the p14^{ARF} promoter revealed no other variant T-half sites. Presumably the p14^{ARF} palindromic variant T-site is a high affinity site in vivo and might therefore be capable of conferring repression on its own. Importantly, the divergent flanking sequences of the variant p14^{ARF} T-site contribute to determining the specificity for binding to TBX2 and TBX3, as the related T-box-containing proteins TBX1A and Xbra are not able to activate transcription of reporter constructs harboring this variant site. In contrast, the orthopedia promoter could also be activated by mouse and Xenopus Brachyury, suggesting that these proteins can also recognize Byn sites (38). However the activation by Brachyury via the multiple Byn sites is much lower than in case of Byn, which could reflect intrinsic Byn preference/specificity associated with these sites. The occurrence of variant T-sites in relevant target genes clearly illustrates the in vivo selected specificity in target gene recognition associated with individual T-box protein family members.

A further indication of such divergence in target gene recognition is illustrated by the fact that TBX2 alone can potently repress p14^{ARF}, in contrast to the situation for TBX5 and its co-factor Nkx2–5, which need to bind and act in cooperation to strongly activate their target genes (39, 40). This fact and the apparent inability of the C-terminally deleted TBX2 mutant to compete with wild type TBX2 for p14^{ARF} binding suggest that the existence of a co-factor for TBX2 may not be a prerequisite. However, our EMSAs do point to a role for flanking sequences outside of the variant T-site to promote stable complex formation, which in turn could point to the requirement for binding of such a co-factor, although the sequences of the p14^{ARF} promoter, aside from an imperfect E2F site, do not indicate the presence of consensus binding sites for established or potential co-factors such as Pitx or Nkx2–5.

In addition to the T-box we also found a C-terminal conserved domain of TBX2 to be essential for repression. A deletion mutant of this repression domain could still bind the variant T-site, although somewhat less efficiently. As the affinity of the repression domain mutant for the variant T-site is impaired, this might explain why this mutant could not compete with wild-type TBX2 for repression. In contrast, previously others (32) have demonstrated comparable DNA binding activity of wild-type and repression domain mutant proteins; however, in these studies a probe was used that contained four T-half-sites, which may well explain the different outcome. The repression domain was first mapped within Xenopus ET and its human ortholog, TBX3, and has subsequently been found to be highly conserved in TBX2 (amino acids 535–629 in TBX2, Ref. 9). Others (32) claim the existence of an additional repression domain in TBX2 at position 407–561, although they do not acknowledge the small overlap between these two regions. Our inactivating deletion encompassed amino acids 501–618, thus affecting both proposed domains. Although the repression domain deletion mutant is incapable of fully rescuing MEFs from senescence, the protein does appear to have some residual ability to down-regulate p19^{ARF}, contributing to an extended MEF life span. Likewise, others (44) have recently noticed a low efficiency of MEF immortalization by TBX3 repression domain deletion mutants as well. This may well be explained by the fact that the repression domain mutants retain the ability to bind to the variant T-site, which is embedded in the initiator sequence. Conceivably, such binding may interfere to some extent with promoter function by obstruction. Nevertheless, our results clearly indicate that active repression by TBX2 via the repression domain is required for p14^{ARF} down-regulation. We hypothesize that the repression domain is involved in recruiting other proteins, such as co-repressors, to the p14^{ARF} promoter, thereby mediating repression and creating the observed slow migrating EMSA complexes. Remarkably, so far only one protein, in addition to the two above described transcription co-factors, has been shown to bind a T-box protein family member. This factor is CASK (calcium/calmodulin-dependent serine protein kinase), a membrane-associated guanylate kinase and component of cell junctions, which binds Tbr-1 and then translocates to the nucleus (45). In vitro this complex can bind T-sites and activate transcription of the T-site-containing reelin promoter. Whether this reflects a brain-specific or more general mechanism remains to be seen.

In conclusion we identified a variant T-site, composed of two inverted, imperfect T-half-sites, as the essential TBX2/TBX3 binding element in an important in vivo relevant TBX2/TBX3 target, the p14^{ARF} tumor suppressor promoter. The core structure of T-box/T-site is conserved, as disrupting mutations could be made in either the binding site DNA or in critical T-box amino acids, based on knowledge of the complex of Brachyury and a consensus T-site. The existence of a variant palindromic T-site described herein is, to our knowledge, unprecedented and offers a possible explanation for the selection of a repeat of T-half-sites in the in vitro binding site selection experiments performed with T-box proteins. Importantly, our study points to a hitherto unsuspected level of specificity for individual
T-box factors in recognizing their respective target genes, which opens new avenues of research in the further exploration of T-box target gene regulation.

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The T-box Repressors \textit{TBX2} and \textit{TBX3} Specifically Regulate the Tumor Suppressor Gene \textit{p14\textsuperscript{ARF}} via a Variant T-site in the Initiator

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