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TBX-3, the Gene Mutated in Ulnar-Mammary Syndrome, Is a Negative Regulator of p19^{ARF} and Inhibits Senescence*

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Prolonged culturing of rodent cells *in vitro* activates p19^{ARF} (named p14^{ARF} in man), resulting in a p53-dependent proliferation arrest known as senescence. The p19^{ARF}-Mdm2-p53 pathway also serves to protect primary cells against oncogenic transformation. We have used a genetic screen in mouse neuronal cells, conditionally immortalized by a temperature-sensitive mutant of SV40 large T antigen, to identify genes that allow bypass of senescence. Using retroviral cDNA expression libraries, we have identified *TBX-3* as a potent inhibitor of senescence. *TBX-3* is a T-box gene, which is found mutated in the human developmental disorder Ulnar-Mammary Syndrome. We have shown that TBX-3 potently represses expression of both mouse p19^{ARF} and human p14^{ARF}. We have also shown here that point mutants of *TBX-3*, which are found in Ulnar-Mammary Syndrome, have lost the ability to inhibit senescence and fail to repress mouse p19^{ARF} and human p14^{ARF} expression. These data suggest that the hypoproliferative features of this genetic disorder may be caused, at least in part, by deregulated expression of p14^{ARF}.

The high frequency of *p53* mutation found in different types of human cancers suggests a central role for *p53* in suppression of tumorigenesis. The p53 tumor suppressor protein functions as a transcription factor that regulates genes involved in a variety of cellular processes, including induction of cell cycle arrest and apoptosis. The regulation of p53 is complex and involves post-translational mechanisms such as protein degradation, phosphorylation, and acetylation (1). In a normal cell the levels of p53 are low because the protein is prone to rapid proteasomal degradation. p53 activation can be induced by a variety of stimuli and results in stabilization of the protein and regulation of its target genes. Activation of p53 can be the result of cellular stresses, such as DNA damage, hypoxia, or ribonucleotide deprivation (2). p53 appears to play a central part in the cellular response to stress. Cells deficient for p53 continue to proliferate after exposure to genotoxic stress, whereas cells expressing wild-type p53 arrest in order to repair the DNA damage or undergo apoptosis (3). The ability of p53 to respond to DNA damage either by induction of cell cycle arrest or activation of a suicide program might explain why loss of p53 function allows the survival of cells with mutated DNA, leading to the rapid accumulation of multiple oncogenic mutations.

p53 is also involved in limiting the proliferative capacity of primary rodent cells. Rodent fibroblasts lacking p53 proliferate indefinitely in tissue culture without undergoing replicative senescence (4), a program that arrests wild-type cells after a limited number of divisions *in vitro*. When cells are cultured *in vitro* they accumulate increasing levels of p19^{ARF}, p53, p21^{CIP1} and p16^{INK4A} over time and undergo a permanent arrest, which is dependent on the presence of the retinoblastoma family of pocket proteins (5–7). Activation of p19^{ARF} appears to be critical for the induction of senescence, as cells lacking p19^{ARF} have an indefinite lifespan *in vitro* (referred to as "immortality") (8). The human ortholog of p19^{ARF} is p14^{ARF}. We here use the term *ARF* when we refer to both the human and mouse genes. The ARF protein interacts with Mdm2 (9, 10) and inhibits the activities of Mdm2 to shuttle p53 to the cytoplasm (11). In addition, ARF binding to Mdm2 inhibits the ubiquitin-ligase activity of Mdm2 toward p53 and inhibits p53 loading with ubiquitin molecules (12). The result of activation of p19^{ARF} is stabilization of transcriptionally active p53, which in turn can activate target genes like p21^{CIP1}. Indeed, p19^{ARF} expression in cells containing wild-type p53 is able to induce a senescence-like arrest, whereas expression of p19^{ARF} in cells lacking p53 does not inhibit proliferation (8).

The p19^{ARF}-Mdm2-p53 pathway is also required to protect primary cells against oncogenic transformation. Aberrant mitogenic signaling induced by activation of oncogenes like c-*myc*, adenovirus *E1A*, *E2F-1,* and *ras*^{V12} causes increased p19^{ARF} levels, leading to stabilization and activation of p53 (13–16). Activation of p53 by Ras^{V12} or E2F-1 is known to cause a senescence response (17, 18), whereas high levels of c-Myc or the viral oncogene E1A render the cell susceptible to undergo p53-dependent apoptosis (13, 14). Which factors influence the suicidal tendencies of the p19^{ARF}-p53 pathway is not yet clear. One possible explanation could be that anti-apoptotic activities of Ras^{V12} promote senescence rather than apoptosis, while the promitogenic functions of c-Myc and E1A may bypass the senescence arrest making a cell susceptible to undergo apoptosis. Especially for E1A this is a likely scenario as its ability to bind and inactivate the retinoblastoma family proteins explains a bypass of senescence, as cells lacking these proteins are immortal and unresponsive to high levels of p19^{ARF} (6, 7).

The relevance of the p19^{ARF}-p53-mediated antiproliferative response in tumorigenesis is best documented for the *ras*^{V12} and c-*myc* oncogenes. In contrast to wild-type cells, cells deficient for either p19^{ARF} or *p53* continue to proliferate in the presence of oncogenic *ras*^{V12} and are fully transformed (8). For c-*myc* a dramatic increase in tumorigenesis is observed when

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mice overexpressing c-*myc* lack one allele of p19^{ARF} (19–21). It is likely that the function of the p19^{ARF}-Mdm2-p53 pathway to restrict the proliferative response following oncogene activation is as important to prevent tumorigenesis as the ability of p53 to prevent the accumulation of viable cells with damaged DNA.

Given the central role of the p19^{ARF}-Mdm2-p53 pathway in proliferation and senescence, we have performed a genetic screen to find new genes, which when overexpressed can modify the activity of the components of this pathway. We have used a mouse embryonic striatum cell line, conditionally immortalized by expression of a temperature-sensitive allele of the SV40 T antigen (22) and retroviral cDNA expression library screens to identify genes that mediate escape from senescence. This strategy allowed the isolation of *TBX-3* as a gene that results in a bypass of senescence by down-regulation of p19^{ARF} expression. *TBX-3* is found to be mutated in the human Ulnar-Mammary Syndrome, a genetic developmental disorder accompanied by hypoproliferation of cells in a number of tissues, including the breast (23). We show that point mutants in *TBX-3*, which are found in Ulnar-Mammary Syndrome (24), have lost the ability to repress the human p14^{ARF} promoter. We propose that the developmental defects seen in Ulnar-Mammary Syndrome are caused, at least in part, by deregulated expression of human p14^{ARF}.

EXPERIMENTAL PROCEDURES

Cell Lines, Culture Conditions and Growth Curves, and Retroviral Infections—All cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Ecotropic retroviral supernatants were produced by transfection of phoenix packaging cells by calcium-phosphate precipitation. Forty-eight hours post-transfection, the tissue culture medium was filtered through a 0.45-μm filter, and the viral supernatant was used for infection of cells after addition of 4 μg/ml polybrene. Cells were infected for at least 6 h and allowed to recover for 48 h with fresh medium. ST.*Hdh*^{Q111} mouse striatum cells express a mutant version of the huntingtin protein with an expanded polyglutamine repeat from a knock-in *Hdh* allele and a temperature-sensitive mutant of SV40 T antigen, introduced by retroviral transduction. ST.*Hdh*^{Q111} mouse striatum cells were cultured at 32 °C and were shifted to the non-permissive temperature (39 °C) when indicated. For growth curves, 1.5×10^4 cells were plated in 12-well plates, and relative cell numbers were measured as described (17). To measure immortalization of wild-type mouse embryo fibroblasts (MEFs),[1] primary MEFs of FVB genetic background at passage 5 were infected with retroviral supernatants and cultured for one passage. At passage 7 cells were used for growth curves.

Retroviral Library Screen—High titer retroviral library supernatants derived from human placenta tissue or whole mouse embryo libraries (CLONTECH) were used to infect 2×10^6 ST.*Hdh*^{Q111} striatal cells. Twenty-four hours after infection, cells were plated at a density of 0.8×10^5 cells per 10-cm dish and 48 h after infection the cells were shifted to 39 °C (the non-permissive temperature for the transforming SV40 T antigen). Colonies of cells appeared only in the library-infected populations. These colonies were picked and expanded. To analyze whether the rescue was indeed due to expression of a retroviral library-derived cDNA, a second round screen was performed, in which cells were infected with wild-type replication competent Moloney virus (MoMuLV) to mobilize the integrated proviruses. Supernatant containing the mobilized proviruses was used to infect fresh ST.*Hdh*^{Q111} striatal cells at 32 °C. Retroviral cDNA inserts were PCR-amplified using specific retroviral primers. Specific TBX-3 primers were used to isolate the TBX-3 coding sequence and to clone it into a pBABE-puro retroviral vector. L143P and Y149S mutants of TBX-3 were generated by PCR and sequence verified and cloned into a HA tag containing pBSK vector. Finally, HA-tagged versions of mutant and wild-type TBX-3 were cloned into the pLIB retroviral vector or into pcDNA 3.1.

Western Blot Analysis and Immune Fluorescence—Western blots were carried out on whole cell extracts, separated on 10–12% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Millipore). Western blots were developed using enhanced chemiluminescence (Amersham Biosciences, Inc.) following the instructions of the manufacturer. The p19^{ARF} (R562) antibody was obtained from Abcam, Y-11 (Santa Cruz Biotechnology) was used to detect HA-tagged versions of TBX-3, and rabbit polyclonal serum was used to detect TBX-2 (25). For immunofluorescence the cells were fixed in 4% paraformaldehyde and stained. Second antibodies used were TEXAS-RED goat anti-rabbit (1:75, Vector Laboratories) and fluorescein isothiocyanate-conjugated goat anti-mouse (1:75, DAKO).

Repression Assays—COS-7 cells were transfected at 40% confluency by calcium-phosphate precipitation with the human p14^{ARF} (−19 to +54) promoter CAT construct in combination with 0–5 μg of pcDNA 3.1 TBX-2 or TBX-3 expression vector. All transfections contained equal amounts of pCDNA3 vector. Transfection efficiencies were measured by co-transfection of 1 μg of pSV-βgal (Promega). CAT and β-galactosidase activities were analyzed 48 h after transfection by standard methods.

RESULTS

Retroviral cDNA Library Screen for Inhibition of Senescence—As a system to isolate cDNAs that prevent the induction of replicative senescence in rodent cells, we used ST.*Hdh*^{Q111} cells: primary mouse striatum cells derived from knock-in mice expressing a mutant huntingtin protein with an expanded glutamine repeat, which were conditionally immortalized *in vitro* by retroviral transduction of a temperature-sensitive mutant (tsA58) of SV40 large T antigen (22). Immortalization by ts-LT transduction occurred at a much lower frequency in primary striatum cells expressing the expanded huntingtin repeat, and these cells show lower spontaneous escape frequencies from the senescence response after shift to the non-permissive temperature than their wild-type counterparts. The stress induced by the expression of the mutant Hdh protein might explain the more robust senescence response after shift to the non-permissive temperature (22).

ST.*Hdh*^{Q111} cells proliferate rapidly at the permissive temperature (32 °C) but enter into a synchronous senescence-like arrest when shifted to the non-permissive temperature (39 °C, Fig. 1*A*). We favored the use of ST.*Hdh*^{Q111} cells for the experiments described below, because they have a lower spontaneous immortalization frequency compared with both ts-LT-immortalized wild-type striatum cells and ts-LT-immortalized wild-type MEFs (data not shown). The low spontaneous immortalization frequency greatly facilitates the detection of a rare retrovirus-mediated immortalization event. To identify cDNAs, which when overexpressed inhibit this robust senescence response, we infected these cells at the permissive temperature (32 °C) with a whole mouse embryo retroviral cDNA library, a human placenta cDNA library or control green fluorescent protein retrovirus. After a shift to the non-permissive temperature the vast majority of cells were arrested and rare non-senescent proliferating colonies were observed only in the cDNA library-infected populations.

To distinguish the colonies that were truly rescued by a retrovirally encoded cDNA from those that escaped senescence spontaneously, the proliferating colonies were expanded and infected with wild-type MoMuLV. This leads to packaging of library-derived retroviruses through the production of retroviral coat proteins by the wild-type MoMuLV. This supernatant was then used in a second round to infect fresh ST.*Hdh*^{Q111} cells. After temperature shift, infected cells were again tested for rescue of senescence. For each cDNA library, several independent colonies were identified of which the integrated provirus was able to inhibit senescence of ST.*Hdh*^{Q111} cells in second round infection (Fig. 1*B*).

TBX-3 Inhibits Senescence—Because the T-box protein TBX-2 efficiently inhibits senescence in primary murine fibroblasts (25), we used immunofluorescence to discard the colonies, which were rescued by retrovirally encoded TBX-2. Indeed all three colonies obtained following infection with the

[1] The abbreviations used are: MEF, mouse embryo fibroblast; HA, hemagglutinin; CAT, chloramphenicol acetyltransferase.

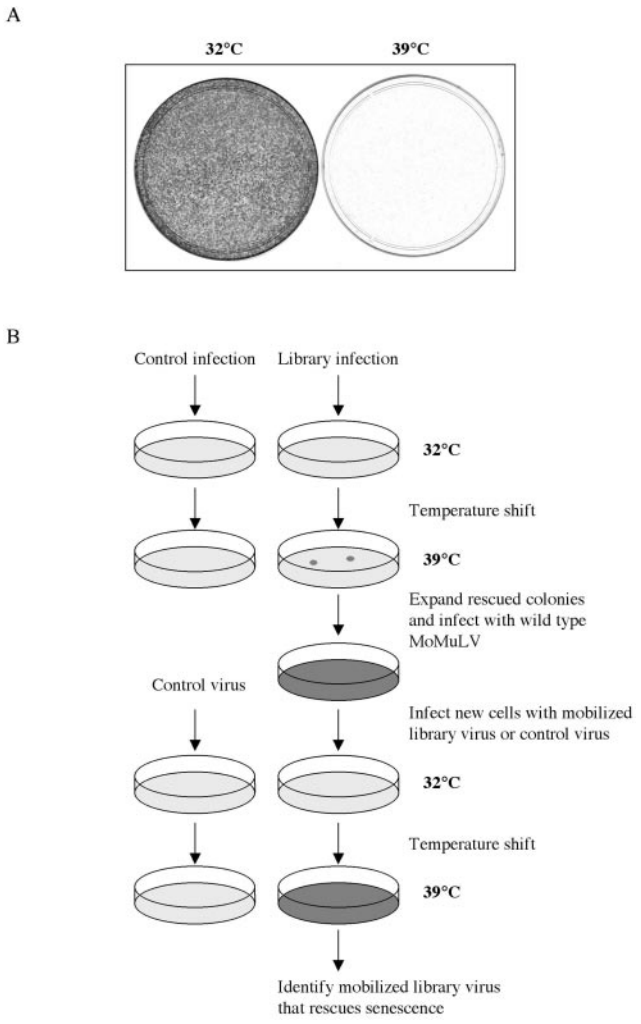

FIG. 1. **Senescence rescue screen in conditionally immortalized mouse striatum cell line.** *A,* conditionally immortalized mouse striatum cells expressing a mutant huntingtin repeat (ST.Hdh^{Q111} cells) proliferate at 32 °C but enter a senescence-like arrest after shift to the non-permissive temperature (39 °C). Cells were fixed and stained after incubation at the indicated temperature for 7 days. *B,* schematic outline of a library screen to bypass senescence in conditionally immortalized ST.Hdh^{Q111} cells.

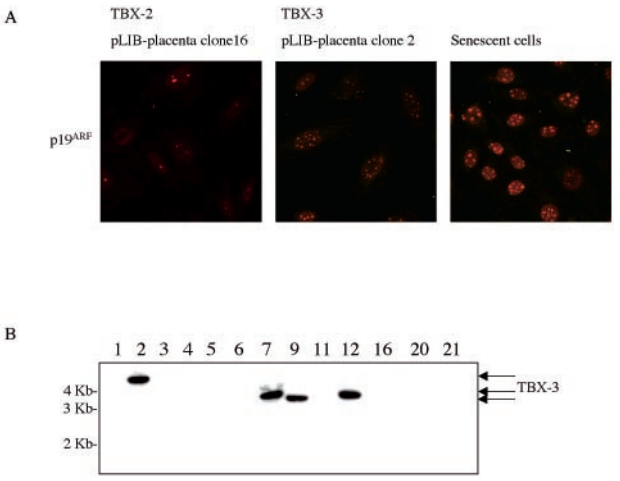

FIG. 2. **Rescued clones express reduced levels of $p19^{ARF}$ and contain TBX-3.** *A,* rescued colonies and senescent ST.Hdh^{Q111} cells were analyzed for $p19^{ARF}$ expression (*red*) by indirect immunofluorescence. *B,* PCR products of rescued colonies were hybridized with a TBX-3-specific probe. Multiple independently isolated cDNAs were identified as being TBX-3.

whole mouse embryo cDNA library overexpressed TBX-2. From the human placenta cDNA library five out of the ten colonies that rescued senescence in second round selection overexpressed TBX-2, even though all colonies that were rescued by a retroviral cDNA displayed a similar cellular morphology (Fig. 2*A*). Colonies that did not show TBX-2 expression were used to recover the retrovirally encoded cDNA by PCR. One of these clones appeared to contain a ~3-kb cDNA insert, which after sequence identification proved to be *TBX-3*, a member of the T-box family, which is found mutated in the human Ulnar-Mammary Syndrome (23). Consistent with its identification from a human placenta cDNA library, *TBX-3* is highly expressed in placental tissue (24). Hybridization of the cDNAs present in other rescued colonies with a *TBX-3* probe shows that *TBX-3* was isolated in four independent colonies (Fig. 2*B*). In total, we isolated nine cDNAs that were active in second round selection; five of these encoded TBX-2 and the remaining four TBX-3. To verify that *TBX-3* alone is able to rescue senescence, the open reading frame of *TBX-3* was cloned in a retroviral vector. Fig. 3*A* shows that *TBX-2* and *TBX-3* were both very active in preventing senescence of ST.Hdh^{Q111} cells at the non-permissive temperature.

To ask whether *TBX-3* could also inhibit senescence in primary MEFs we introduced *TBX-3* in wild-type MEFs and monitored the ability of these MEFs to become immortal. Fig. 3*B* shows that both *TBX-2* and *TBX-3* prevent the induction of senescence in primary MEFs. TBX-3-expressing MEFs can be cultured indefinitely without showing any signs of senescence (data not shown). Co-introduction of *TBX-3* and a ras^{V12} oncogene in primary MEFs results in escape from Ras^{V12}-induced senescence, without causing complete oncogenic transformation as *TBX-3* and ras^{V12} co-expressing cells were unable to grow in soft agar (data not shown). Similar observations have been made recently for *TBX-2* and ras^{V12} co-expressing cells (25).

TBX-3 Down-regulates $p19^{ARF}$—In primary MEFs senescence induction requires elevation of $p19^{ARF}$ expression, which occurs when the cells are cultured *in vitro*. MEFs lacking the transcription factor DMP1 (which activates the $p19^{ARF}$ promoter) or MEFs deficient for $p19^{ARF}$ will escape the senescence response (8, 26). Since TBX-3 is a transcriptional repressor (27) and because TBX-2 has been shown to act as a repressor of $p19^{ARF}$ expression, we analyzed the expression of $p19^{ARF}$ in the *TBX-3*-immortalized clones. As shown by immunofluorescence, the $p19^{ARF}$ signal in a *TBX-3* immortalized clone is lower than in the senescent ST.Hdh^{Q111} cells grown at the non-permissive temperature, even though the subcellular localization of $p19^{ARF}$ was not affected (Fig. 2*A*). Western blot of ST.Hdh^{Q111} cells, 48 h after infection with a *TBX-3* or *TBX-2* retrovirus at the permissive temperature showed that the reduction of $p19^{ARF}$ protein is an early event (Fig. 3*C*).

Point Mutants of TBX-3 Are Defective in $p19^{ARF}$ Repression and Immortalization—Haplo-insufficiency for *TBX-3* results in the developmental Ulnar-Mammary Syndrome (23). Mutant alleles of *TBX-3* found to cause the Ulnar-Mammary Syndrome often give rise to truncated protein products, but point mutants in the T-box have also been found (24). To examine the activity of these point mutants of TBX-3 we constructed retroviral vectors expressing HA-tagged versions of wild type, [L143P]TBX-3 or [Y149S]TBX-3. Expression of wild type, but not the L143P or Y149S mutant forms of TBX-3, resulted in bypass of senescence in ST.Hdh^{Q111} cells after shift to the non-permissive temperature (Fig. 4*A*), even though the wild-type and mutant TBX-3 proteins were expressed at comparable levels in these cells (Fig. 4*B*).

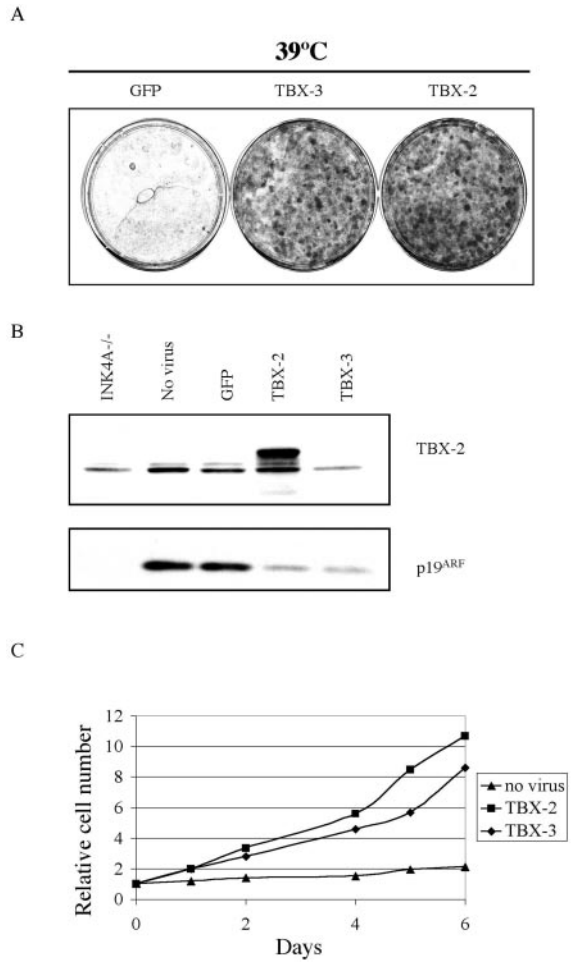

FIG. 3. **TBX-3 down-regulates $p19^{ARF}$ in ST. Hdh^{Q111} cells and immortalizes wild-type MEFs.** *A,* retroviral transduction of TBX-2 or TBX-3 results in a rescue of senescence in conditionally immortalized ST.Hdh^{Q111} cells after shift to the non-permissive temperature. Cells were fixed and stained after 10 days. *B,* growth curve of passage 7 wild-type MEFs infected at passage 5 with TBX-2-or TBX-3-expressing retroviruses. *C,* ST.Hdh^{Q111} cells 48 h after infection with TBX-2- or TBX-3-expressing retroviruses show low levels of $p19^{ARF}$ at the permissive temperature as compared with ST.Hdh^{Q111} cells infected with control green fluorescent protein retrovirus. No virus control sample was derived from MEFs at passage 7.

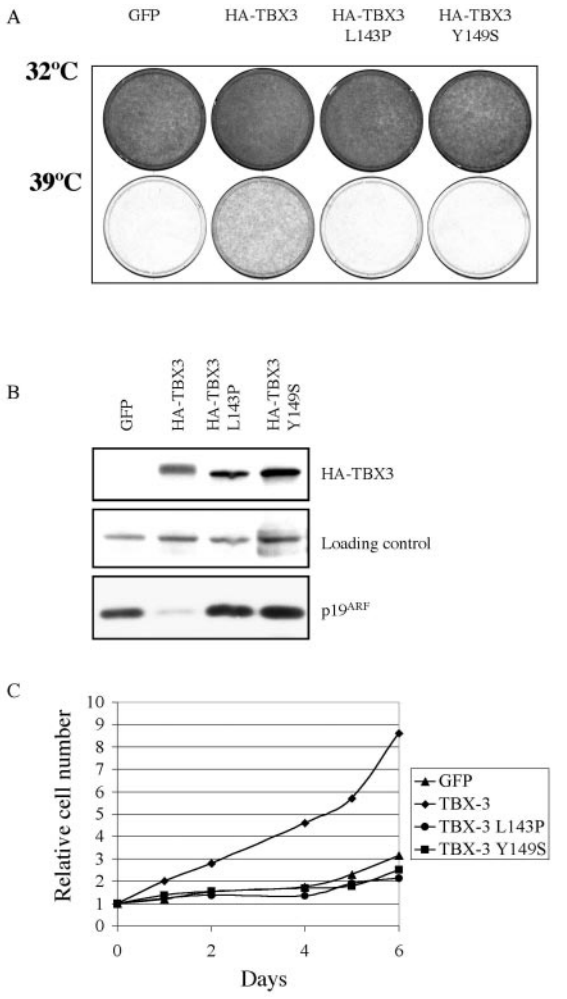

FIG. 4. **Point mutants of TBX-3 found in the Ulnar-Mammary Syndrome do not bypass senescence or downregulate $p19^{ARF}$.** *A,* expression of wild-type TBX-3 induces proliferation in conditionally immortalized ST.Hdh^{Q111} cells at the non-permissive temperature, whereas TBX-3 mutant alleles are unable to override the senescence response. Cells were fixed and stained 8 days after incubation at the indicated temperatures. *B,* retrovirally transduced wild-type TBX-3 is able to down-regulate $p19^{ARF}$ protein levels in ST.Hdh^{Q111} cells at the permissive temperature, but the point mutants L143P and Y149S are not able to cause a decrease in $p19^{ARF}$ protein levels. The mutant forms of TBX-3 were expressed at comparable levels (*top panel*). *C,* primary MEFs infected with wild-type TBX-3 do not undergo senescence, whereas MEFs infected with mutant forms of TBX-3 do undergo senescence. Shown are growth curves of primary MEFs infected at passage 5 and growth curves were made at passage 7.

Infection of primary MEFs at passage 5 with wild-type or mutant forms of TBX-3 showed that the mutant proteins are inactive in inhibiting senescence in fibroblasts (Fig. 4*C*). Analysis of $p19^{ARF}$ expression levels shows that the mutant TBX-3-infected populations fail to show $p19^{ARF}$ down-regulation at the protein level (Fig. 4*B*). To determine whether the observed down-regulation of $p19^{ARF}$ expression by wild-type TBX-3 was transcriptional, we performed a reporter assay using the human $p14^{ARF}$ promoter linked to a CAT reporter gene. Fig. 5*A* shows that TBX-2 and TBX-3 are able to mediate transcriptional repression of a $p14^{ARF}$ promoter region spanning from −19 to +54 in a dose-dependent manner. This promoter region does not contain a consensus T-box site (GTGGTA) (28), suggesting that T-box proteins bind to a non-consensus T-box site in the $p14^{ARF}$ promoter or that T-box proteins interact indirectly with the $p14^{ARF}$ promoter. Importantly, the point mutants of TBX-3, which were not able to immortalize the ST.Hdh^{Q111} cells or the primary MEFs, were severely compromised in their ability to repress the $p14^{ARF}$ promoter (Fig. 5*A*) while they were expressed at similar levels compared with wild-type TBX-3 (Fig. 5*B*).

DISCUSSION

In this study we have described a genetic screen to identify novel regulators of the $p19^{ARF}$-Mdm2-p53 pathway. We have used a striatal cell line that expresses a mutated form of the huntingtin protein and was conditionally immortalized by overexpression of a ts SV40 large T allele. Upon inactivation of the ts large T oncogene (by a temperature shift to 39 °C) p53 is no longer inactivated, resulting in the induction of p53 target genes like $p21^{CIP}$. The result of this conditional p53 response is a homogeneous cell cycle arrest that resembles replicative senescence. Indeed introduction of HPV-16 E6, which degrades p53, results in a bypass of this antiproliferative arrest (data not shown). However, the precise nature of the senescence response downstream of p53 is not clear since $p21^{CIP1}$-deficient MEFs do undergo both spontaneous and ras^{V12}-induced senescence (29). Moreover there is currently little insight in how the $p19^{ARF}$-p53 pathway is activated upon prolonged culturing of

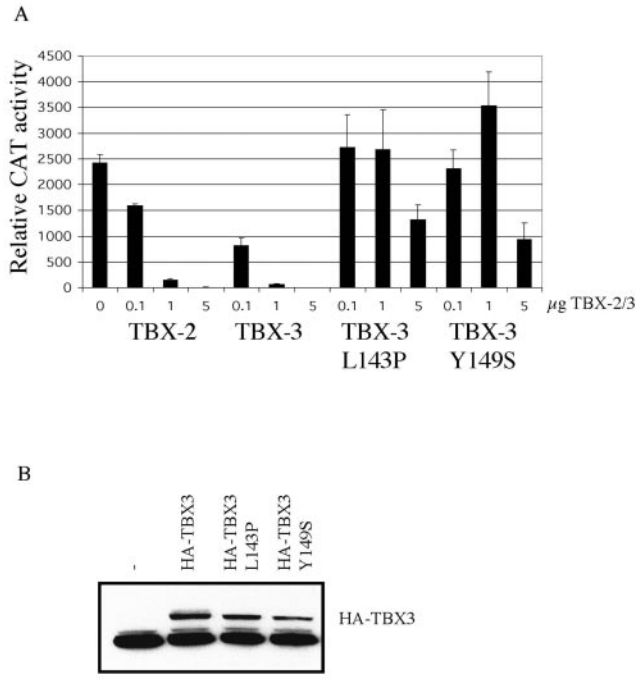

FIG. 5. **Wild-type, but not mutant, TBX-3 represses the p14^{ARF} promoter.** *A,* dose-dependent repression of the p14^{ARF} (−19 to +54) promoter region by TBX-2 and TBX-3 in COS-7 cells. Note that the TBX-3 mutants L143P and Y149S are impaired in their ability to down-regulate p14^{ARF} transcription. COS-7 cells were transiently transfected with the p14^{ARF}-CAT reporter plasmid and increasing amounts of TBX expression vector. *B,* Western blot showing comparable levels of protein expression of wild-type and mutant forms of TBX-3 in COS-7 cells.

primary cells *in vitro*, or by stress evoked by oncogenes like *c-myc* and *ras*^{V12} (13, 16). Given the high frequency of mutations in p14^{ARF} and *p53* in human tumors, genes involved in the regulation of *ARF* expression or execution of the senescence response downstream of p53, potentially encode cancer-relevant genes.

The highly stringent senescence response of our conditionally immortalized ST.*Hdh*^{Q111} cell line allowed a genetic approach to identify genes that mediate bypass of this response. This resulted in the isolation of multiple independently cloned *TBX-3* cDNAs from a placental cDNA library. The T-box family member TBX-3 is not only able to immortalize the ST.*Hdh*^{Q111} cell line, but also efficiently inhibits senescence in wild-type MEFs. This validates the ST.*Hdh*^{Q111} cell system used to identify inhibitors of senescence as it demonstrates that the gene identified in the ST.*Hdh*^{Q111} cells also prevents spontaneous immortalization in primary fibroblasts that do not harbor a mutant huntingtin gene. *TBX-3* induces immortalization by repression of both murine and human *ARF* expression. The repression of *ARF* appears to be transcriptional as a reporter construct containing the −19 to +54 sequences of the human p14^{ARF} promoter was repressed by TBX-3. These results add TBX-3 to a growing list of proteins that affect the activity of the p19^{ARF} promoter. Of these, TBX-2 (25), TBX-3, DMP-1 (30), and E2F1 (15) have been shown to interact with promoter-proximal elements in the *ARF* promoter, whereas no binding sites have been identified in the *ARF* promoter for factors like c-Myc (13), Twist (31), JunD (32), DAP kinase (33), c-Abl (34), E1A (14), Bmi-1 (35), and Ras^{V12} (16). *ARF* activity seems to be regulated mainly at the level of transcription, and a strong selective pressure for loss of *ARF* expression is observed during tumorigenesis, often resulting from mutation or methylation of the *ARF* locus. Therefore it is not surprising that many of the genes that affect *ARF* promoter activity either by a direct or indirect mechanism also play a role in tumorigenesis. Loss of the transcripional activator DMP-1 for instance results in a tumor prone phenotype in mice (26), and overexpression of the transcriptional repressor BMI-1 results in lymphomagenesis (36). At this time we do not know whether TBX-3 is overexpressed in human cancer, but our data clearly warrant a detailed investigation for its role in cancer development.

TBX-3 is found mutated in the Ulnar-Mammary Syndrome, where haplo-insufficiency for *TBX-3* appears to be the cause of the developmental defects in this disease (23). In patients suffering from the Ulnar-Mammary Syndrome, limb, dental, external genitalia, apocrine-gland, and hair abnormalities are observed. In agreement with this *TBX-3* is widely expressed in adult tissues (24). Breast hypoplasia seen in Ulnar-Mammary Syndrome patients, suggest a role for *TBX-3* in regulation of proliferation. Consistent with a role for *TBX-3* in proliferation, we have cloned *TBX-3* due to its ability to drive cell proliferation in our genetic senescence bypass screen. This could suggest that the ability of *TBX-3* to drive proliferation resulting in a senescence bypass is the same activity, which upon loss in the Ulnar-Mammary Syndrome results in tissue hypoplasia. Indeed point mutations in *TBX-3* that result in the Ulnar-Mammary Syndrome in humans (24) have lost the potential to bypass senescence in our system. This is correlated with a defect in repression of the human p14^{ARF} promoter, making deregulated p14^{ARF} levels a likely cause for the hypoproliferation seen in these patients. Coupling deregulated *ARF* expression to developmental defects might appear at odds with the absence of any developmental defects in p19^{ARF}-deficient mice. However, mice deficient for the p19^{ARF}-repressor *Bmi-1* manifest cerebellar defects, which can be rescued by crossing *Bmi-1*-deficient mice to *INK4a*$^{-/-}$ mice (35). This suggests strongly that elevated p19^{ARF} expression is causally related to the observed brain pathology in the *Bmi-1*$^{-/-}$ mice. By analogy, haplo-insufficiency for *TBX-3* might lead to supraphysiological levels of p14^{ARF} expression, which interfere with normal proliferation of the tissues that are affected in Ulnar-Mammary Syndrome.

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