Prolonged culturing of rodent cells in vitro activates p19ARF (named p14ARF in man), resulting in a p53-dependent proliferation arrest known as senescence. The p19ARF-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 p19ARF and human p14ARF. 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 p19ARF and human p14ARF expression. These data suggest that the hypoproliferative features of this genetic disorder may be caused, at least in part, by deregulated expression of p14ARF.

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 degrada-
dation, 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 p19ARF, p53, p21CIP1 and p16INK4A over time and undergo a permanent arrest, which is dependent on the presence of the retinoblastoma family of pocket proteins (5–7). Activation of p19ARF appears to be critical for the induction of senescence, as cells lacking p19ARF have an indefinite lifespan in vitro (referred to as “immortality”) (8). The human ortholog of p19ARF is p14ARF. 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 p19ARF is stabilization of transcriptionally active p53, which in turn can activate target genes like p21CIP1. Indeed, p19ARF expression in cells containing wild-type p53 is able to induce a senescence-like arrest, whereas expression of p19ARF in cells lacking p53 does not inhibit proliferation (8).

The p19ARF-Mdm2-p53 pathway is also required to protect primary cells against oncogenic transformation. Ablerrant mitogenic signaling induced by activation of oncogenes like c-myc, adenovirus E1A, E2F-1, and RasV12 causes increased p19ARF levels, leading to stabilization and activation of p53 (13–16). Activation of p19ARF by RasV12 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-independent apoptosis (13, 14). Which factors influence the suicidal tendencies of the p19ARF-p53 pathway is not yet clear. One possible explanation could be that anti-apoptotic activities of RasV12 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 p19ARF (6, 7).

The relevance of the p19ARF-p53-mediated antiproliferative response in tumorigenesis is best documented for the rasV12 and c-myc oncogenes. In contrast to wild-type cells, cells deficient for either p19ARF or p53 continue to proliferate in the presence of oncogenic rasV12 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 p19ARF (19–21). It is likely that the function of the p19ARF-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 p19ARF-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 p19ARF 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 p14ARF promoter. We propose that the developmental defects seen in Ulnar-Mammary Syndrome are caused, at least in part, by deregulated expression of human p14ARF.

**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. Ectopic 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.HdhQ111 mouse striatum cells express a mutant version of the huntingtin protein with an expanded polyglutamine repeat from a knock-in mouse (22) and retroviral cDNA expression library screens to identify genes that mediate escape from senescence. 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 p19ARF 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 p14ARF promoter. We propose that the developmental defects seen in Ulnar-Mammary Syndrome are caused, at least in part, by deregulated expression of human p14ARF.

**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.HdhQ111 cells: primary mouse striatum cells derived from knock-in mice expressing a mutant huntingtin protein with an expanded polyglutamine 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.HdhQ111 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. 1A). We favored the use of ST.HdhQ111 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 embryonic 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.HdhQ111 cells. After temperature shift, infected cells were again tested for rescue of senescence. For each cDNA library, several independent clones were identified of which the integrated provirus was able to inhibit senescence of ST.HdhQ111 cells in second round infection (Fig. 1B).

**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

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1 The abbreviations used are: MEF, mouse embryonic fibroblast; HA, hemagglutinin; CAT, chloramphenicol acetyltransferase.
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**Fig. 1. Senescence rescue screen in conditionally immortalized mouse striatum cell line.** A, conditionally immortalized mouse striatum cells expressing a mutant huntingtin repeat (ST.HdhQ111 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 mouse striatum cell line. Hdh striatum cells expressing a mutant huntingtin repeat (ST.HdhQ111) were infected with a retrovirus at the permissive temperature and then cultured indefinitely without showing any signs of senescence (data not shown). Co-introduction of TBX-3 and a rasV12 oncogene into primary MEFs results in escape from RasV12-induced senescence, without causing complete oncogenic transformation as TBX-3 and rasV12 co-expressing cells were unable to grow in soft agar (data not shown). Similar observations have been made recently for TBX-2 and rasV12 co-expressing cells

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. 3B 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 rasV12 oncogene in primary MEFs results in escape from RasV12-induced senescence, without causing complete oncogenic transformation as TBX-3 and rasV12 co-expressing cells were unable to grow in soft agar (data not shown). Similar observations have been made recently for TBX-2 and rasV12 co-expressing cells

**TBX-3 Down-regulates p19ARF**—In primary MEFs senescence induction requires elevation of p19ARF expression, which occurs when the cells are cultured *in vitro*. MEFs lacking the transcription factor DMP1 (which activates the p19ARF promoter) or MEFs deficient for p19ARF 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 p19ARF expression, we analyzed the expression of p19ARF in the TBX-3-immortalized clones. As shown by immunofluorescence, the p19ARF signal in a TBX-3 immortalized clone is lower than in the senescent ST.HdhQ111 cells grown at the non-permissive temperature, even though the subcellular localization of p19ARF was not affected (Fig. 2A). Western blot of ST.HdhQ111 cells, 48 h after infection with a TBX-3 or TBX-2 retrovirus at the permissive temperature showed that the reduction of p19ARF protein is an early event (Fig. 3C).

**Point Mutants of TBX-3 Are Defective in p19ARF Repression and Immortalization**—Haplo-insufficiency for TBX-3 results in the development of 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.HdhQ111 cells after shift to the non-permissive temperature (Fig. 4A), even though the wild-type and mutant TBX-3 proteins were expressed at comparable levels in these cells (Fig. 4B).
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. 4C). Analysis of p19ARF expression levels shows that the mutant TBX-3-infected populations fail to show p19ARF down-regulation at the protein level (Fig. 4B). To determine whether the observed down-regulation of p19ARF expression by wild-type TBX-3 was transcriptional, we performed a reporter assay using the human p14ARF promoter linked to a CAT reporter gene. Fig. 5 shows that TBX-2 and TBX-3 are able to mediate transcriptional repression of a p14ARF 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 p14ARF promoter or that T-box proteins interact indirectly with the p14ARF promoter. Importantly, the point mutants of TBX-3, which were not able to immortalize the ST.HdhQ111 cells or the primary MEFs, were severely compromised in their ability to repress the p14ARF promoter (Fig. 5A) while they were expressed at similar levels compared with wild-type TBX-3 (Fig. 5B).

**DISCUSSION**

In this study we have described a genetic screen to identify novel regulators of the p19ARF-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 p21CIP1. 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 p21CIP1-deficient MEFs do undergo both spontaneous and rasV12-induced senescence (29). Moreover there is currently little insight in how the p19ARF-p53 pathway is activated upon prolonged culturing of
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primary cells in vitro, or by stress evoked by oncogenes like c-myc and rasV12 (13, 16). Given the high frequency of mutations in p14ARF 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.HdhQ111 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. 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.

Bypass of the senescence response by TBX-3 is dependent on its ability to down-regulate p14ARF transcription. 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. 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.

TBX-3 is found 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 rapidly 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 p14ARF promoter, making deregulated p14ARF 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 p19ARF-deficient mice. However, mice deficient for the p19ARF-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 p19ARF expression is causally related to the observed brain pathology in the Bmi-1−/− mice. By analogy, haplo-insufficiency for TBX-3 might lead to supra-physiological levels of p14ARF expression, which interfere with normal proliferation of the tissues that are affected in Ulnar-Mammary Syndrome.

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