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p53 E3 ubiquitin protein ligase homolog regulates p53 in vivo in the adult mouse eye lens

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Purpose: p53 is a transcription factor that plays an important role in preventing cancer development. p53 participates in relevant aspects of cell biology, including apoptosis and cell cycle control and must be strictly regulated to maintain normal tissue homeostasis. p53 E3 ubiquitin protein ligase homolog (Mdm2) is an important negative regulator of p53. The purpose of this study was to determine if Mdm2 regulates p53 in vivo in the adult lens.
Methods: We analyzed mice expressing human *p53* transgene (Tg*p53*) selectively in the lens in the presence or absence of *Mdm2*. Mice with the required genotypes were obtained by crossing transgenic, *mdm2*$^{+/-}$, and *p53*$^{-/-}$ mice. Eye phenotype and lens histology and ultrastructure were analyzed in adult mice.
Results: In a wild-type genetic background (*mdm2*$^{+/+}$), lens damage and microphthalmia were observed only in mice homozygous for Tg*p53* ($^{t/t}$). However, in an *mdm2* null background, just one allele of Tg*p53* (*mdm2*$^{-/-}$/Tg*p53*$^{t/0}$ mice) was sufficient to cause lens damage and microphthalmia. Furthermore, *Mdm2* in only one allele was sufficient to rescue these deleterious effects, since the *mdm2*$^{+/-}$/Tg*p53*$^{t/0}$ mice had eye size and lens morphology similar to the control mice.
Conclusions: Mdm2 regulates p53 in the adult lens in vivo. This information may have relevance for analyzing normal and pathological conditions of the lens, and designing cancer therapies targeting Mdm2–p53 interaction.

p53 is a transcription factor that plays an important role in preventing cancer development by inducing apoptosis or inhibiting the proliferation of cells undergoing cellular stress [1]. About 50% of all human cancers contain mutations within the *p53* gene, rendering it the most frequently mutated single gene in human cancer known thus far [2]. In addition to p53's role in apoptosis and cell cycle control, p53 activity in important processes such as metabolism, differentiation, and cellular reprogramming has been observed [3].

p53 affects the transcription of many target genes and interacts with key cellular proteins. Therefore, p53 function and stability must be subject to stringent multilevel regulation under most physiologic conditions [4,5]. The strong antiproliferative activity of p53 may have detrimental effects in normal cells if it is activated inappropriately. Mdm2 is a key negative regulator of p53 activity. The *Mdm2* gene was originally identified as an amplified and overexpressed gene in a spontaneously transformed mouse BALB/c cell line [6]. *Mdm2* is an oncogene and is amplified in 10% of all human cancers and in approximately 20% of soft tissue sarcomas and osteosarcomas [7].

Mdm2 binds directly to the transcriptional activation domain of p53 and blocks p53-dependent transcription. In addition, Mdm2 promotes the nuclear export of p53 and its proteasomal degradation [8]. The importance of Mdm2 in negatively regulating p53 is best illustrated by the finding that mice lacking *Mdm2* are embryonic lethal and die before implantation; this phenotype is completely rescued by concomitant deletion of *p53*, indicating that embryo lethality was due to active p53 [9,10].

The lethality of *mdm2*$^{-/-}$ mice before implantation makes it impossible to analyze Mdm2 interaction with p53 in vivo at specific times of development, at postnatal/adult stages, or in specific cell types. This problem has been circumvented with the use of several knockout, knock-in, and overexpressing transgenic mouse models. These models have provided invaluable insight into the importance of Mdm2 as a negative regulator of p53 activity in specific tissues or specific cell types, such as in the spleen, thymus, bone marrow, small and large intestines, heart, bone, lung, kidney, liver, testis, smooth muscle, and brain (for reviews with appropriate references, see references 3 and 11).

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The eye lens is composed of a single layer of epithelial cells on the anterior surface that divide and differentiate into quiescent and structurally highly differentiated fiber cells characterized by changes in cell shape, expression of crystallins, and degradation of cellular organelles, thus ensuring lens transparency [12]. The unique spatial organization makes the lens a valuable model in which to study the mechanisms that control the switch between cell proliferation and withdrawal from the cell cycle during terminal differentiation. Furthermore, since apoptosis has been implicated in lens fiber cell differentiation and organelle loss [13], the lens provides a suitable model for studying the apoptosis pathway. Given the primary relevance of the p53 pathway in cell proliferation, differentiation, and apoptosis, the lens has been used to analyze p53 interaction with proteins that may also be important in these processes, such the retinoblastoma protein (pRb) [14], bcl-2 [15], CREB-2 [16], OREBP [17], Nbs1 [18], BMP [19], Eaf2 [20], c-Maf, Prox-1 [21], and Bak [22].

Few studies have investigated the interaction of p53 and Mdm2 in the lens, except several indirect analyses. Pomerantz et al. [23] found that p19 (Arf) neutralizes Mdm2 in the embryonic mouse lens, thus enhancing p53-mediated apoptosis in this model. Geatrell et al. [24] found that expression of *Mdm2* and *p53* was spatiotemporally regulated in various compartments of the lens in mice and chicks, including lens epithelial and fiber cells, indicating potential roles for these factors in regulating lens epithelial cell proliferation and/or fiber cell differentiation. To gain insight into the in vivo significance of Mdm2 in regulating p53 in the postnatal lens, we analyzed mice expressing the human *p53* gene selectively in the lens in the presence or absence of *Mdm2*.

METHODS

Transgenic mice expressing human *p53* (Tg*p53*) in the lens under regulation of the αA-crystalline promoter were provided by T. Nakamura [25]. Mice harboring a heterozygous mutation at the *Mdm2* locus ($mdm2^{+/-}$) and a homozygous mutation at the *p53* locus ($p53^{-/-}$) have been described previously [10]. These mice were maintained in a 129/Sv and C57BL/6J mixed background and were crossed to establish colonies with the required genotypes (see below). $p53^{-/-}$ mice were used in crosses aimed to obtain $mdm2^{-/-}$ animals and thus avoid the embryonic lethality of *mdm2* null mice in a $p53^{+/+}$ genetic background.

Transgenic animals were genotyped with PCR analysis using primers specific to gene regions of human *p53*. A PCR assay was designed to genotype $mdm2^{+/-}$ animals using mice *Mdm2*-specific primers in combination with a neo-specific primer to detect wild-type and mutant alleles simultaneously. Similarly, *p53* mutated animals were genotyped with mice *p53*-specific primers in combination with a neo-specific primer (Table 1) [10].

The same PCR program was used to amplify the three genes and consisted of an initial denaturation step of 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 3 min, and a final extension step of 72 °C for 7 min. Amplification products were visualized after electrophoresis on 1% agarose gels under ultraviolet illumination in the presence of ethidium bromide. The DNA for these assays was obtained by tail biopsy and a phenol-chloroform extraction method. Transgenic mice were identified by the presence of a 500 bp band. Wild-type and mutated alleles of *Mdm2* yielded fragments of 415 bp and 284 bp, respectively. The *p53* wild-type allele yielded a fragment of 450 bp, whereas the mutated allele was identified by a 650 bp band.

Mice were maintained on a 12 h:12 h light-dark cycle with food and water ad libitum and were handled according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Visual Research. Newborn, 1-month-old, and 2-month-old mice were analyzed. Mice were sacrificed by cervical dislocation, the eyes enucleated, and the lenses removed through a posterior incision in the eyeballs under a stereo microscope. The material was immediately fixed in Karnovsky and post-fixed with 1% osmium tetroxide. After block-staining in 1% uranyl acetate, the specimens were dehydrated in a graded series of ethanols, and finally embedded in soft Spurr resin. Semithin sections were obtained and mounted on glass slides. Mounted sections were stained with 1% toluidine blue, and then examined with a light microscope. Ultrathin sections were obtained and examined with a transmission electron microscope EM 109 (Carl Zeiss, Oberkochen, Germany) after double-staining with 5% uranyl acetate and 0.4% lead citrate. All the reagents for microscopy analysis were purchased from Pelco International (Redding, CA).

RESULTS

The transgenic founder mice showed eyes with a normal phenotype. According to previous data [25], these mice were probably heterozygous for Tg*p53* ($^{t/0}$). As the first step in our study, these mice were mated to wild-type mice, producing in the first generation (F1) wild-type and transgenic mice. The F1 transgenic mice showed a normal eye phenotype. When we mated transgenic mice from F1 with each other, we obtained wild-type mice (1/4) and transgenic mice (3/4) as expected (F2). However, contrary to the offspring in F1, in F2 two kinds of phenotypes in mice positive for Tg*p53* were observed: normal eye and microphthalmia. When the

Table 1. Primer sequences used to amplify human *p53* transgene (Tg*p53*), and wild-type and mutant alleles of p53 E3 ubiquitin protein ligase homolog (*Mdm2*) and *p53* from DNA samples of the mice analyzed in this study.

Gene	Primer name	Sequence (5'-3')
Tg*p53*	P3	ATTTGATGCTGTCCCCGGACGATATTGAAC
	GE5R	TCCAAATACTCCACACGCAA
Mdm2	mdm20	TGTGGCTGGAGCATGGGTATTG
	mdm4	ATCTGAGAGCTCGTGCCCTTCG
	Neo3'd	GGCGGAAAGAACCAGCTGGGGC
p53	X6	AGCGTGGTGGTACCTTATGAGC
	X7	GGATGGTGGTATACTCAGAGCC
	Neo19	GCTATCAGGACATAGCGTTGGC

transgenic mice with microphthalmia were mated with each other, all the offspring were positive for Tg*p53* and showed microphthalmia. Based on these results, we conclude that the transgenic mice that showed microphthalmia are homozygous for Tg*p53* ($^{t/t}$).

If the mice with microphthalmia are Tg*p53*$^{t/t}$, by mating them with wild-type mice (Tg*p53*$^{0/0}$), all their offspring should possess one locus of the Tg*p53* ($^{t/0}$) and therefore should not show microphthalmia. However, by mating Tg*p53*$^{t/0}$ with Tg*p53*$^{0/0}$ mice, 50% of the offspring should have Tg*p53*, but no microphthalmia, and the other 50% should not show either Tg*p53* or microphthalmia. Last, all the offspring of the mating between two mice with microphthalmia (Tg*p53*$^{t/t}$) should be positive for the transgene and have microphthalmia. These inferences were confirmed by analyzing the offspring from the crosses between transgenic and wild-type mice (Table 2).

At birth, the eye phenotype of the Tg*p53*$^{t/t}$ mice closely resembled that of the wild-type mice. Microphthalmia developed in the weeks after weaning, and by adulthood, the eyelids were completely closed. In fact, Nakamura et al. [25] demonstrated, with terminal deoxynucleotidyl transferase-mediated uridine 5′-triphosphate-biotin nick end labeling (TUNEL) assay, apoptosis in the lens of transgenic mice. In this study, we analyzed the lens with light and electron microscopy to characterize the morphological damage due to Tg*p53*.

Microscopic analysis of lenses from 2-month-old wild-type and Tg*p53*$^{t/t}$ mice is shown in Figure 1. In the light microscopy examination, the wild-type lens showed an extracellular collagenous capsule, and a single layer of epithelial cells lined the front surface of the organ. At the equatorial region, these cells transformed into highly elongated, ribbon-like fiber cells (Figure 1A). In the Tg*p53*$^{t/t}$ mice (Figure 1B), the collagenous capsule was not visible, the nuclei of apoptotic cells formed clusters, and the interior of the organ showed a highly disordered structure and nuclei with condensed chromatin. In the ultrastructural analysis, the capsule that borders the wild-type lens was clearly visible, as well as epithelial cells with nuclei with normal morphology and fiber cells forming highly organized layers (Figure 1C). In contrast, the Tg*p53*$^{t/t}$ mouse lens appeared disorganized, and apoptotic bodies with condensed chromatin were visible (Figure 1D).

Since the αA-crystalline gene begins expression in the mouse lens cup at E10.0–E10.5 [26], and the promoter of this gene drives the expression of Tg*p53* in this model, lens damage probably exists at mouse birth. Figure 2 shows microscopic analysis of lenses from newborn and 1-month-old wild-type and Tg*p53*$^{t/t}$ mice. Newborn wild-type mice had an external capsule; surface epithelial cells and elongated fiber cells in the interior of the organ were also observed (Figure 2A). In the newborn Tg*p53*$^{t/t}$ mice, the capsule and the epithelial cells were also visible, but some structural disorganization was observed in the interior (Figure 2B). In 1-month-old wild-type mice, the capsule and epithelial cells

Table 2. Eye phenotype of mice expressing human p53 transgene (*Tgp53*) in the lens, in presence or absence of *Mdm2*.

Mouse genotype		Eye phenotype	
mdm2	*Tgp53*	Normal	Microphthalmia
+/+	0/0	√	
+/+	t/0	√	
+/+	t/t		√
–/–	0/0	√	
–/–	t/0		√
+/–	t/0	√	

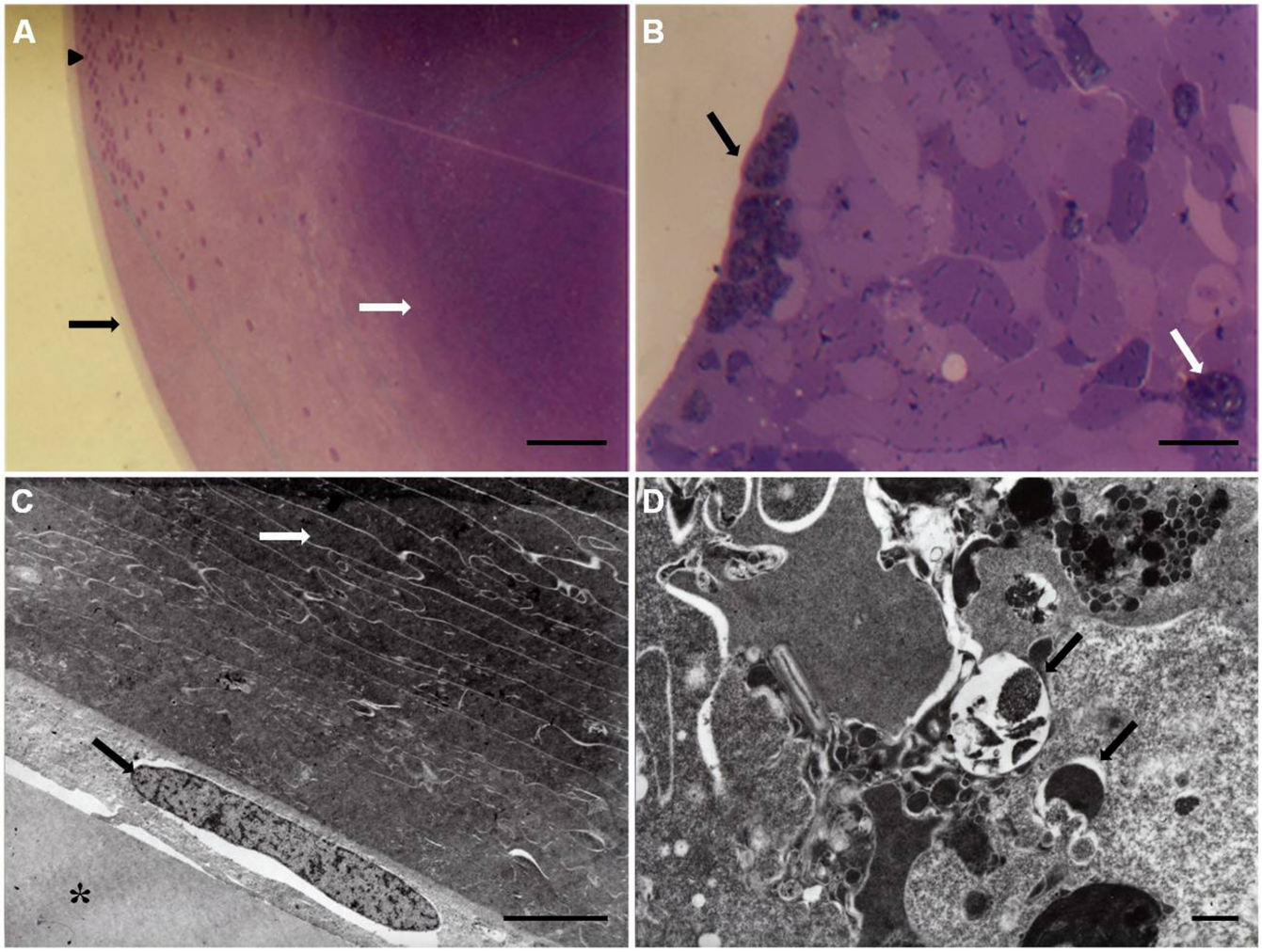

Figure 1. Light and electron microscopy of lenses from 2-month-old wild-type and human *p53* homozygous transgenic (Tg*p53*$^{t/t}$) mice. **A**: Wild-type mice show by light microscopy an extracellular collagenous capsule (black arrow), a surface monolayer of epithelial cells (arrow head), and elongated fiber cells (white arrow). Bar: 20 µm. **B**: Analysis of Tg*p53*$^{t/t}$ mice by light microscopy demonstrate that the external capsule is not visible, the nuclei of the apoptotic cells form superficial clusters (black arrow), and the interior of the organ has a highly disordered structure and nuclei with condensed chromatin (white arrow). Bar: 20 µm. **C**: Wild-type mice show by electron microscopy a collagenous capsule (asterisk), epithelial cells with nuclei with normal morphology (black arrow), and fiber cells organized in layers (white arrow). Bar: 5 µm. **D**: In the electron microscopy analysis of lenses of Tg*p53*$^{t/t}$ mice, the interior does not show a defined order or evidence of normal fiber cells. Apoptotic bodies (arrows) are visible. Bar: 1 µm.

and fiber cells organized in layers were observed (Figure 2C). In 1-month-old Tg*p53*$^{t/t}$ mice, the capsule was still visible, but the epithelial cells were not present, and disorganization increased in the interior of the organ (Figure 2D). Thus, damage in the lens increased with age and was in parallel with microphthalmia.

These experiments indicated that in a wild-type genetic background, Tg*p53* must be present in a homozygous state ($^{t/t}$) to cause microphthalmia, and that its presence in only one allele ($^{t/0}$) can be regulated by Mdm2. To further analyze the interaction between Mdm2 and p53 in this model, we performed mice crosses to obtain mice that expressed Tg*p53* in the absence ($^{-/-}$) or presence ($^{+/-}$) of *Mdm2*. The control line *mdm2*$^{-/-}$/Tg*p53*$^{0/0}$ mice had eye size and lens morphology similar to wild-type mice (Figure 1A,C and Figure 3, Table 2). The presence of Tg*p53* in only one allele ($^{t/0}$) in an *mdm2*$^{-/-}$ background was sufficient to cause the microphthalmia (Figure 3, Table 2) and lens damage previously described in the microscopic analysis (Figure 1B,D). Last, we observed that the presence of *Mdm2* in only one allele was sufficient to rescue these deleterious effects, since the *mdm2*$^{+/-}$/Tg*p53*$^{t/0}$ mice showed eye size and lens morphology similar to that of the *mdm2*$^{-/-}$/Tg*p53*$^{0/0}$ mice (Figure 1A,C and Figure 3, Table

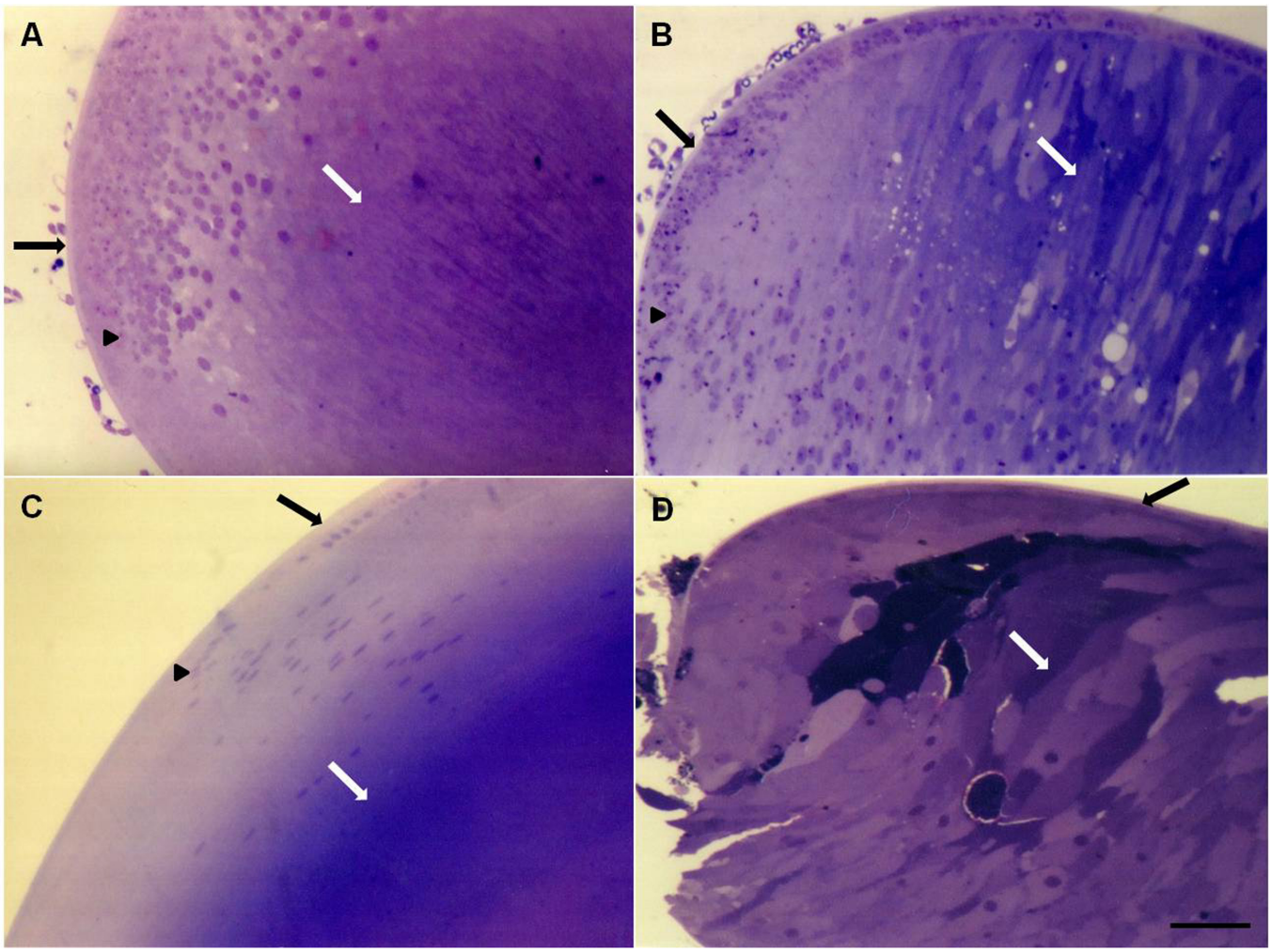

Figure 2. Light microscopy of lenses of newborn and 1-month-old wild-type and human *p53* homozygous transgenic (Tg*p53*$^{t/t}$) mice. **A**: Newborn wild-type mice show an external capsule (black arrow); epithelial cells line the front surface of the organ and at the equatorial region (arrow head) transform into highly elongated, ribbon-like fiber cells (white arrow). **B**: In newborn Tg*p53*$^{t/t}$ mice, the capsule (black arrow) and the superficial epithelial cells (arrow head) are also visible, but some structural disorganization can already be observed in the interior (white arrow). **C**: In 1-month-old wild-type mice, the capsule (black arrow), the single layer of epithelial cells at the front surface (arrow head), and the fiber cells organized in layers (white arrow) are observed. **D**: In 1-month-old Tg*p53*$^{t/t}$ mice, the capsule (black arrow) is still visible, but the superficial epithelial cells are not present, and disorganization is increased in the interior of the organ (white arrow). Bar: 20 µm.

2), demonstrating that Mdm2 regulates p53 in vivo in the adult lens.

DISCUSSION

The involvement of p53 in pathologic states has been well established. However, information about its function in normal adult tissues is more limited. To understand the physiologic role of p53, the effects of its expression and the proteins interacting with it in those tissues must be characterized.

The ocular lens represents an ideal model system for studying the molecular mechanisms controlling cell proliferation, differentiation, and death. Furthermore, the eye is easily visualized, thus facilitating phenotypic analysis. We used the adult lens in transgenic and/or knockout mice as a model system to examine in vivo the interaction between p53 and Mdm2.

Our findings and those of others [25] show that overexpression of human p53 causes apoptosis of lens cells that results in microphthalmia in adult transgenic mice. In a wild-type genetic background, Tg*p53* must be present in a homozygous state ($^{t/t}$) to cause microphthalmia. These results

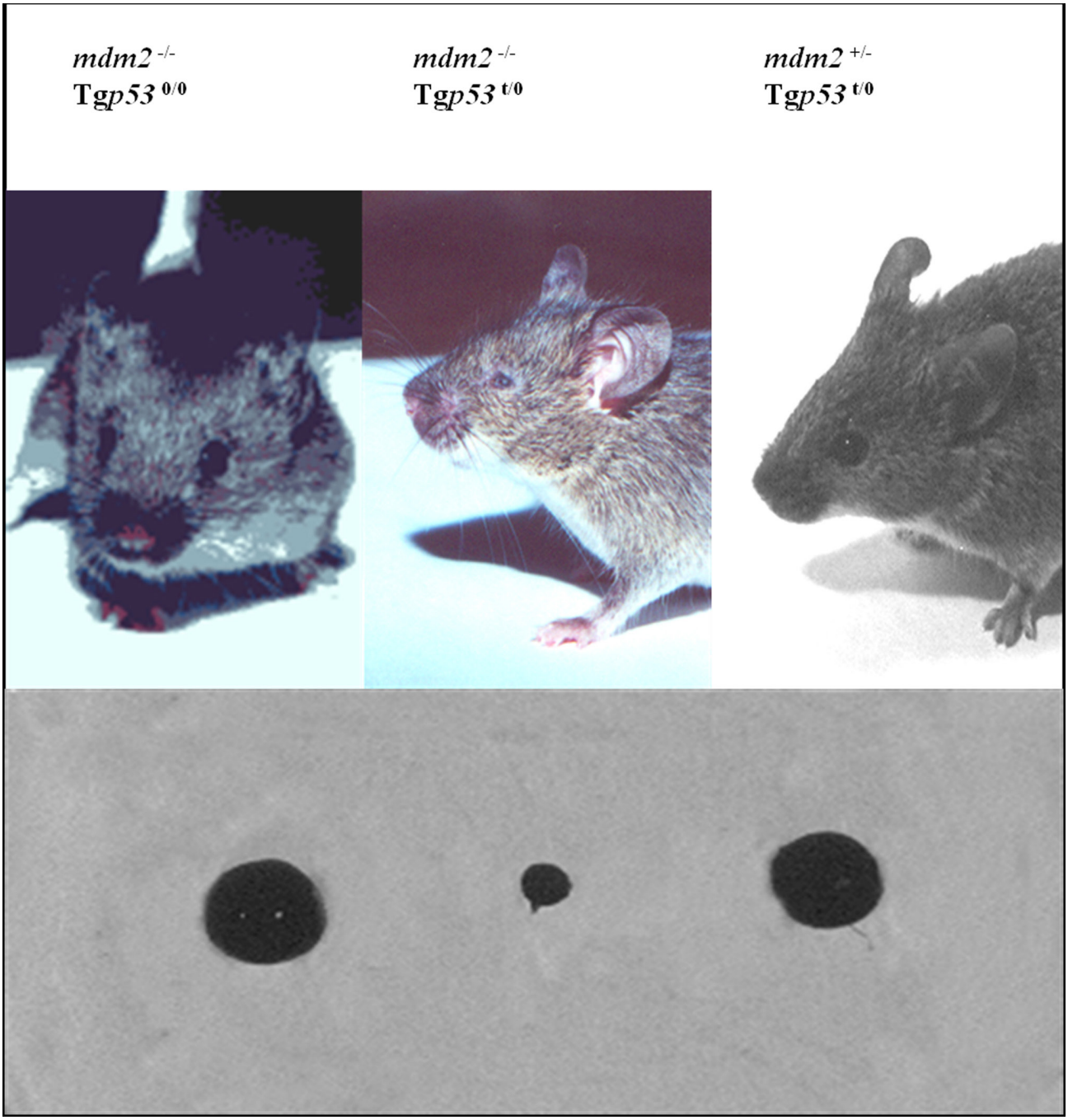

Figure 3. Phenotypic analysis of eyes from 2-month-old mice expressing human *p53* transgene (Tg*p53*) in the lens, in the presence or absence of p53 E3 ubiquitin protein ligase homolog. Non-transgenic mice harboring a homozygous mutation at the p53 E3 ubiquitin protein ligase homolog (*Mdm2*) locus *(mdm2*$^{-/-}$/Tg*p53*$^{0/0}$) show a normal eye size. In contrast, the *mdm2*$^{-/-}$/Tg*p53*$^{t/0}$ mice have microphthalmia and closed eyelids. Microphthalmia is rescued by the concomitant presence of an *Mdm2* allele (*mdm2*$^{+/-}$/Tg*p53*$^{t/0}$). For comparison, the enucleated eyes are shown in the bottom panel.

indicate that Mdm2 can regulate p53 to a certain extent, but p53 overexpression can overcome inhibition by Mdm2.

Since *Mdm2*-deficient mice die prenatally due to *p53*-dependent apoptosis [9,10], we inactivated *p53* in *mdm2* null mice to examine the effect of Tg*p53* in the absence of *Mdm2*. The presence of Tg*p53* in only one allele ($^{t/0}$) in an *mdm2*$^{-/-}$ background was sufficient to cause microphthalmia, further supporting a role of Mdm2 in regulating p53 in this

model. The fact that this ocular phenotype was rescued in the presence of one *Mdm2* allele (*mdm2*$^{+/-}$/Tg*p53*$^{t/0}$ mice) demonstrates that Mdm2 regulates p53 in vivo in the adult lens.

Under most physiologic conditions, p53 activity and stability are subject to stringent multilevel regulation by an abundance of modulators [27]. One of these, Mdm4, also known as Mdmx, is an Mdm2 homolog that also negatively regulates p53. Due to this, when overexpressed, Mdm4 acts as an oncogene [28]. Similarly to *Mdm2*, loss of *Mdm4* leads to embryonic lethality that can be completely rescued in a *p53* null background [29]. The role of mdm4 in regulating p53 in specific cell types in the adult stage has been analyzed with several mouse models (for reviews, see references 30 and 31).

Mdm2 and mdm4 seem to contribute to regulating p53 in vivo to different extents in different tissues. However, most studies suggested that Mdm2 is the major inhibitor of p53 function, while mdm4 may be required to regulate p53 only in certain cell types at specific time points [3,32-35].

Our findings indicate that in the in vivo mouse lens, Mdm2 acts as a regulator of p53, as demonstrated for pRb [14], CREB-2 [16], and BMP [19]. However, whether other regulators of p53, such as Mdm4, function in the mouse lens remains to be determined.

In addition, more research is necessary to assess the spatial and temporal pattern of p53 inhibition by Mdm2 in the lens. Lens development occurs throughout the lifetime of the individual and involves the proliferation of epithelial cells that differentiate into quiescent fiber cells. In the lens of adult mice, p53 immunoreactivity was found in the epithelial cells of the central and preequatorial zones, as well as in the fiber cells at the bow region [36]. Geatrell et al. [24] demonstrated that *Mdm2* is also expressed in the lens of adult mice, although the cellular localization of this expression was not described. However, since Mdm2 regulates p53 activity in proliferative and terminally differentiated stages of the same cell type [37,38], and this regulation has been demonstrated even in quiescent cells [33], Mdm2 probably inhibits p53 in any spatial/functional compartment of the lens in which they may interact.

p53 is activated in response to various stresses that may arise in the organism. Similarly, large doses of ultraviolet radiation or genotoxic stress increase the content of the p53 protein in lens cells; this excess of p53 in the lens results in serious defects, as we and others [20,39,40] have observed. However, p53 protects the lens against the formation of certain types of cataracts by suppressing the proliferation of fiber cells and promoting death by apoptosis of any fiber cell that fails to withdraw from the cell cycle [19]. Thus, understanding the molecular mechanisms involved in p53 regulation in the lens has implications for gaining further insights into the normal functioning of the lens and its alterations in disease.

The disruption of the interaction between Mdm2 and p53 could be a potential therapeutic strategy against cancer in patients who retain wild-type p53. Several Mdm2 inhibitors have been published and are undergoing clinical trials [41-43]. However, data from this and other studies [44,45] indicate that activating p53 could induce deleterious consequences in normal tissues. This information is important for designing anticancer strategies targeting the p53 pathway, and suggests that Mdm2-inhibitory drugs must be used with great caution and that local treatment is better for cancer patients to avoid any damage caused by these inhibitors.

In conclusion, our data demonstrate that Mdm2 regulates p53 in vivo in the adult lens. This information may be relevant in analyzing normal and pathological conditions of the lens, and in designing cancer therapies targeting Mdm2–p53 interaction.

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