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Genetics

Lens complementation system for the genetic analysis of growth, differentiation, and apoptosis *in vivo*

(chimera/homologous recombination/aphakia)

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ABSTRACT **A genetic approach has been established that combines the advantages of blastocyst complementation with the experimental attributes of the developing lens for the functional analysis of genes governing cellular proliferation, terminal differentiation, and apoptosis. This lens complementation system (LCS) makes use of a mutant mouse strain, aphakia (*ak*), homozygotes of which fail to develop an ocular lens. We demonstrate that microinjection of wild-type embryonic stem (ES) cells into *ak/ak* blastocysts produces chimeras with normal ES-cell-derived lenses and that microinjection of *Rb−/−* ES cells generates an aberrant lens phenotype identical to that obtained through conventional gene targeting methodology. Our determination that a cell autonomous defect underlies the aphakia condition assures that lenses generated through LCS are necessarily ES-cell-derived. LCS provides for the rapid phenotypic analysis of loss-of-function mutations, circumvents the need for germ-line transmission of null alleles, and, most significantly, facilitates the study of essential genes whose inactivation is associated with early lethal phenotypes.**

The capacity to manipulate the genetic composition of the mouse has allowed for significant advances in the study of developmental and cancer biology. Much of this progress is the result of methods that permit the precise structural alteration of genes through homologous recombination in embryonic stem (ES) cells followed by germ-line transmission of these targeted alleles through chimera formation (1). Successful implementation of gene targeting requires maintenance of ES-cell pluripotency to passage the mutant allele through the germ line. A potential impediment to the analysis of loss-of-function mutations generated through the classical knockout approach is attendant embryonic lethality. This outcome is particularly problematic in cancer-relevant models wherein early lethality contracts the time available for the emergence of additional genetic lesions that cooperate to bring about a fully transformed state. Procedural modifications developed to circumvent some of these obstacles include the generation of chimeras from homozygous null ES cells (2) and the use of a modified Cre–LoxP approach for the production of a cell-type-specific nullizygous condition (3). However, the former approach requires a detailed *in situ* assessment of whether tissues of the chimera are ES- or host blastocyst-derived, and the latter can be complicated by mosaicism caused by inefficient Cre-mediated deletion (A. Nagy, personal communication).

To overcome these problems, Chen *et al.* (4) developed a method for evaluating gene function in the immune system that makes use of host blastocysts derived from RAG-2-deficient mice, which are incapable of variable-diversity-joining (VDJ) recombination and thus lack mature B and T lymphocytes. Injection of normal or genetically modified ES cells into these blastocysts can lead to chimeras with mature B and T cells derived from the injected ES cells. The feasibility of this complementation approach rests on the fact that immune-deficient homozygous null *rag-2* mice are reproductively competent and capable of producing donor blastocysts. The *rag-2* blastocyst complementation system has proven to be highly effective for assessing the impact of targeted gene mutations upon immunocyte function and development (5). In addition, it permits the study of mutations that would have otherwise compromised the viability of the organism. The obvious potential of this genetic approach prompted us to develop an equivalent system for the ocular lens, a model system uniquely suited for the genetic dissection of mechanisms governing growth, differentiation, and apoptosis (6–10).

The lens comprises a single well-characterized cell type whose growth, differentiation, and occasional apoptosis occur in regionally distinct compartments. This anatomical organization greatly facilitates the phenotypic evaluation of the effects of genetic manipulations upon these processes. The fully formed lens consists of postmitotic differentiated fiber cells that are covered anteriorly by a layer of proliferating immature epithelial cells that occasionally undergo apoptosis. With respect to charting a clear sequence of pathogenetic events, the lens is a nonrenewing organ structure with cells persisting throughout life and, thus, serving as permanent records of developmental events. The utility of the lens is further enhanced by an abundance of stage-specific differentiation markers and the availability of several crystallin promoters that have proven successful in directing lens-specific transgene expression (11–14). Finally, experimental manipulations that perturb lens homeostasis do not affect viability or fertility of the mouse and the lens itself is readily accessible to observation through slit lamp examination (6).

Our objective was to test the utility of a lens complementation system (LCS) that makes use of blastocysts derived from a mutant mouse strain that fails to develop an ocular lens. The microinjection of normal ES cells harboring an integrated DNA marker allowed for the determination of whether resultant lenses were consistently ES-cell-derived. In this study, normal or *Rb*-deficient ES-cell lines were microinjected into the mutant blastocysts and assessed morphologically and molecularly for the potential to generate either normal lenses or lenses exhibiting the abnormal patterns of growth, differentiation, and apoptosis that typify the null Rb phenotype.

MATERIALS AND METHODS

Maintenance of ES Cell Lines and Generation and Microinjection of Blastocysts. Ten to 15 WW6 ES cells, maintained on γ-irradiated SNL STO feeders and LIF, were microinjected into *ak/ak* blastocysts by established protocols (15). The WW6 ES cell line was derived from a transgenic mouse line harbor-

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Abbreviations: ES, embryonic stem; TUNEL, terminal deoxynucleotidyltransferase-mediated biotinylated dUTP nick-end-labeling; E, embryonic day(s); LCS, lens complementation system.

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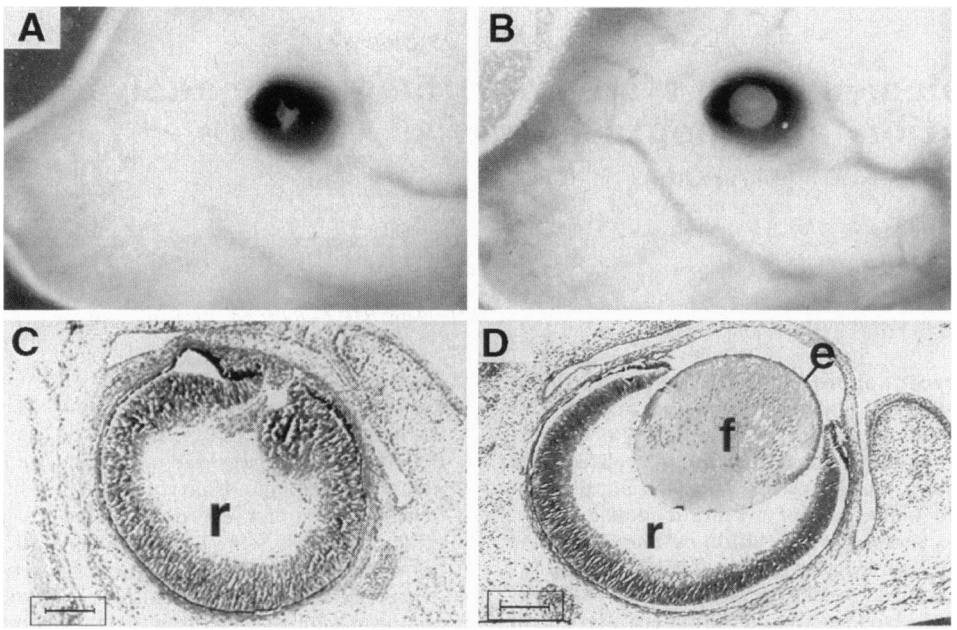

FIG. 1. Gross and histological comparisons of embryos derived from aphakia homozygous (*ak/ak*) intercrosses (*A* and *C*) or derived from the microinjection of wild-type ES cells into *ak/ak* blastocysts (*B* and *D*). The *ak/ak* adult presents with closed eyelids, resulting from abortive lens development evident grossly in E14.5 embryos and thereafter (*A*) and absence of a lens structure with collapse of the pigmented epithelium in E16.5 embryos (*C*). After microinjection of WW6 ES cells into *ak/ak* blastocysts, a significant proportion of the chimeric mice possessed grossly normal lenses at adulthood and on E14.5 (*B*) and, upon histological analysis, E16.5 lenses generated in these studies (*D*) were found to be morphologically indistinguishable from age-matched wild-type controls (data not shown). f, Lens fiber cells; e, epithelial layer; r, retina. (Bars = 100 μm.)

ing a nonexpressed β-globin/pBR322 plasmid transgene (16); the utility of this ES cell line in chimera studies has been described (17). The homozygous null *Rb* ES cell lines used in the LCS studies were provided by Anton Burns and Hein te Riele (The Netherlands Cancer Institute, University of Amsterdam) (18) and by Tyler Jacks (Whitehead Institute, Massachusetts Institute of Technology, Cambridge, MA) (2). Female blastocyst donors were derived from intercrosses between *ak/ak* females and *ak/ak* males; the original male was derived from an *Ak/ak* mating pair (C57BL/6 × C57BLKS *ak*,

Table 1. Lens formation using LCS with WW6 or *Rb* null ES cell lines

Exp.	No. of plugged females	No. of blastocysts retrieved	No. of blastocysts injected	Age of analysis	No. of animals	No. with eyes	% with eyes
A	7†	48	48	Adult	13	7	54
	8†	47	47	E14.5	36	1	2.8
	5†	103	103	E14.5	28	7	25
	7†	46	46	E14.5	26	7	27
	10†	11	11	E15.5	4	2	50
	9‡	42	42	E16.5	17	7	41
	12‡	44	44	E14.5	17	9	53
	8‡	22	22	E16.5	20	4	20
B	14†	36	22	E16.5	14	3	21
	9†	119	77¶	E14.5	28	1	3.5
	12†	50	50	E16.5	ND	7	ND
	9‡	16	16	E16.5	12	4	33
	12‡	35	35	Adult	13	10§	73
	10‡	55	40	E16.5	8	2	25
	8‡	25	22	E16.5	8	6	75

In experimental group A, LCS experiments were performed with the WW6 ES cell line. Individual experiments yielded the indicated number of mice or embryos and percentage with eyes. *ak/ak* female blastocyst donors were derived from *Ak/ak* (†) or *ak/ak* (‡) intercrosses. In a small number of samples, WW6-derived lenses were found to be ruptured along the posterior capsule, this possibly arising from minor contributions from developmentally compromised *ak/ak* lens cells that had involuted. Such an anomaly did not interfere with phenotypic characterization. Rupture was observed only when *ak/ak* blastocysts were obtained from female blastocyst donors derived from *Ak/ak* rather than *ak/ak* intercrosses. In experimental group B, LCS experiments were conducted with homozygous null *Rb* ES cell lines (2, 18). Individual experiments yielded the indicated number of mice and percentage with eyes. *ak/ak* female blastocyst donors were derived from either *Ak/ak* (†) or *ak/ak* (‡) intercrosses. The § indicates that these 10 adult chimeras developed 19 lenses. Although the *ak* mutation on the CD1 background resulted in an increase in the number of blastocysts (¶), very poor ES-cell contribution to the lens was observed in chimeras derived from these host blastocysts in a limited series of microinjections. ND indicates that for that particular LCS experiment, seven embryos with eyes were harvested while the total number of embryos in the litter was not determined.

The Jackson Laboratory) and additional *ak/ak* males were obtained through crosses onto a C57BL/6 background.

Immunohistochemical Assays for Growth, Differentiation, and Apoptosis. Embryos were fixed, embedded, and stained with hematoxylin/eosin. Differentiation marker (α- and γ-crystallin antibodies; a gift from Sam Zigler, Laboratory of Vision Research, National Eye Institute) and 5-bromodeoxyuridine (BrdU) incorporation (S-phase detection) assays were performed as described (8). The terminal deoxynucleotidyltransferase-mediated biotinylated dUTP nick-end-labeling (TUNEL) assay was performed as described (7) except that 11-biotinylated dUTP and GIBCO terminal deoxynucleotidyltransferase were used.

***In Situ* Detection of β-Globin Transgene DNA.** To detect the β-globin transgene *in situ*, we followed the published protocol (16) except that lenses were fixed, embedded, and sectioned as described (8).

RESULTS AND DISCUSSION

To establish a complementation system for the lens, we tested whether a naturally occurring mutant mouse strain, aphakia (*ak*), could serve as a source for donor blastocysts. Aphakia is an autosomal recessive disorder in which adult homozygotes (*ak/ak*) have closed eyelids resulting from aberrant embryonic lens development (19). The defect produces a malformed lens vesicle that fails to develop beyond embryonic day (E) 11.5 and by E14.5 involutes into a clump of crystallin-negative cells (20). Midgestational homozygotes are identified grossly as those with an inward collapse of the pigmented epithelium (uneven pupil) and an ocular globe that is approximately half the normal diameter (Fig. 1*A*) (14).

In the first series of experiments, wild-type WW6 ES cells were microinjected into *ak/ak* blastocysts and assayed for their ability to generate normal fully formed lens structures. Upon gross inspection and histological examination, microinjected embryos harvested at midgestation could be classified easily into two distinct groups—embryos presenting with the classical aphakia phenotype and those possessing at least one fully formed lens (Fig. 1 *A* and *C* vs. *B* and *D*). Moreover, lens-positive adult chimeras appeared to respond to visual stimuli and most lenses were free of cataracts or other obvious imperfections, as assessed by slit-lamp examination (data not shown). On average, one-third of the WW6-microinjected embryos possessed one or both lenses (Table 1, experiment A), and these complemented embryos were easily discernible on gross inspection, thus allowing one to focus exclusively on these samples. This screen provides a distinct advantage over conventional chimeric approaches in which the documentation of ES-cell contribution to somatic tissues requires a rigorous examination at the cellular level for each embryo.

Lenses generated in the WW6 microinjection studies were subjected to a detailed analysis of embryonic patterns of cellular growth, differentiation, and apoptosis (7, 8). Morphologically, both age-matched wild-type and WW6-derived lenses possessed a highly organized parallel array of elongated fiber cells covered anteriorly by a single layer of immature cuboidal epithelial cells (Fig. 2*B*, WW6-derived lens; wild-type lens, Fig. 2*A*). *In situ* BrdU incorporation assays demonstrated that progression through the cell cycle was appropriately restricted to the anterior epithelial cell compartment in both WW6-derived and wild-type lenses (Fig. 2*D* and *C*). The α- and β-crystallins represent early-stage lens fiber cell differentiation markers, with α-crystallin expressed earliest and distributed throughout the epithelial and fiber cell compartments and β-crystallin expressed initially in equatorial cells and in lens fibers subsequently (11–14). Expression of the γ-crystallins (11–14) is restricted to the central lens fiber cells. Since normal lens cell differentiation is dependent upon the proper temporal and spatial expression of the α-, β-, and γ-crystallins (6, 8, 11–14), indirect immunofluorescence studies were performed to examine each of these stage-specific markers. These studies confirmed that each crystallin exhibited normal levels, distribution, and developmental patterns of expression in control and experimental samples [only α-crystallin (Fig. 2 *E* and *F*) and γ-crystallin (Fig. 2 *G* and *H*) immunostains are shown]. Lastly, the TUNEL assay (7) demonstrated that WW6-derived lenses exhibited a pattern identical to that of wild type (7), with a very occasional apoptotic cell detected solely in the anterior epithelial cell compartment (data not shown).

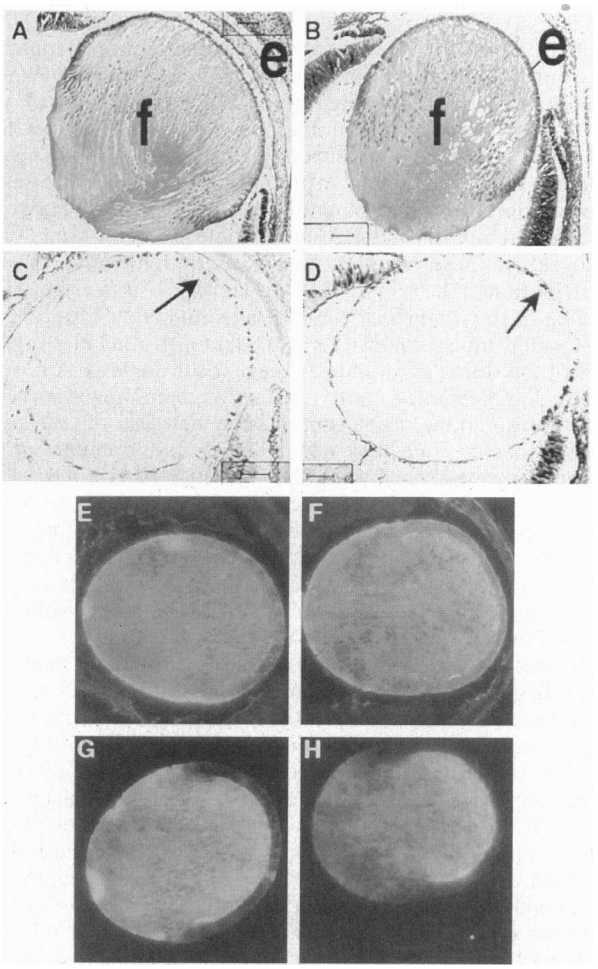

FIG. 2. Proliferation and differentiation in age-matched wild-type (*A, C, E,* and *G*), and WW6-derived (*B, D, F,* and *H*) lenses on E16.5 (*A–D*) and E14.5 (*E–H*). BrdU incorporation studies demonstrate that in each case only the anterior epithelial layer progresses through S phase; arrows denote BrdU-positive nuclei (*C* and *D*). Indirect immunofluorescence studies of the distribution and level of α (*E* and *F*)- and γ (*G* and *H*)-crystallin expression employing the corresponding primary antisera. The lenses are oriented with the anterior epithelial cell layer facing the upper right corner in *A–D* and facing right in *E–H*. (Bars: *A–D*, 50 μm; *E–H*, magnification is reduced by 20%.)

A fundamental requirement for the LCS strategy is that abortive lens development in the host blastocyst strain results from a cell autonomous rather than inductive defect. In the inductive scenario, somatic contribution by ES cells could conceivably lead to formation of the lens without their populating the lens proper. Although previous reports indicated that a single gene mutation causes a lens-specific cell autonomous defect in the aphakia mouse (19), we sought to verify that lenses derived through LCS were indeed ES-cell-derived. An amplified inert β-globin transgene, present in the WW6 ES cell line, served as an invariant molecular tag detectable by DNA *in situ* hybridization methods (16). Lens sections derived from age-matched wild-type and WW6-microinjected embryos

were assayed for hybridization to a biotinylated β-globin DNA probe followed by its detection by streptavidin-phosphatase blue precipitation. Sections through the lenses of 16 WW6-microinjected embryos were examined at either E14.5 or E16.5 for nuclear-associated staining. By microscopic inspection, wild-type lenses were consistently negative (Fig. 3 *A* and *B*), whereas lenses generated in the WW6 microinjection series showed intense blue punctate staining readily apparent throughout all low- and high-power fields (Fig. 3 *C* and *D*). In a subset of these samples, there was minimal or no WW6 contribution to the retina (data not shown). This is a significant finding in the context of classical lens induction experiments performed by Spemann (21, 22), demonstrating the crucial role of the retina as an inductive agent for the lens. A normal lens in complemented embryos exhibiting minimal or no WW6 contribution to the retina supports the view that the *ak* defect is lens-specific and that normal lens development in the chimeras does not result from the correction of a retinal defect. These β-globin *in situ* studies confirm that, in LCS, all lenses are composed of the microinjected ES cells and are consistent with the view that a cell-autonomous defect underlies the *ak/ak* condition. However, these studies do not rule out the possibility that a small proportion of lens cells are aphakia-derived and are rescued by WW6-derived cells.

To test the utility of LCS as a rapid and simple means of assaying gene function *in vivo*, homozygous null *Rb* ES cells (2) were tested therein for their ability to recapitulate the pathological sequelae observed in *Rb*-deficient lenses resulting from classical heterozygous intercrosses (7). The retinoblastoma tumor suppressor gene was selected as a test case because the loss of Rb function is associated with a very dramatic phenotype characterized by unchecked proliferation, impaired expression of late-stage differentiation markers, and inappropriate apoptosis in lens fiber cells (7, 10). In addition, *Rb*-deficient embryos die between E12.5 and E14.5 (23–25). The microinjection of homozygous null *Rb* ES cells (2) into *ak/ak* blastocysts yielded an obvious lens structure (Fig. 4*A*) in approximately one-third of the embryos harvested on E16.5 (Table 1, experiment B). Histological analysis of these lenses revealed a significant increase in the number of nuclei as well as occasional mitotic figures in the lens-fiber-cell compartment (Fig. 4*B*). Additionally, the highly organized parallel and extended array of normally differentiated lens fiber cells was replaced by a disorderly arrangement (Fig. 4*B*; compare to Fig. 1*D*). BrdU incorporation assays showed nuclear-associated staining in the lens fiber cell region, confirming that the loss of Rb function is clearly associated with inappropriate progression through S phase (Fig. 4*C*, compare to Fig. 2 *C* or *D*). Immunohistochemistry was also used to demonstrate that, similar to earlier *Rb* studies (7), the regional distribution and level of the interme-

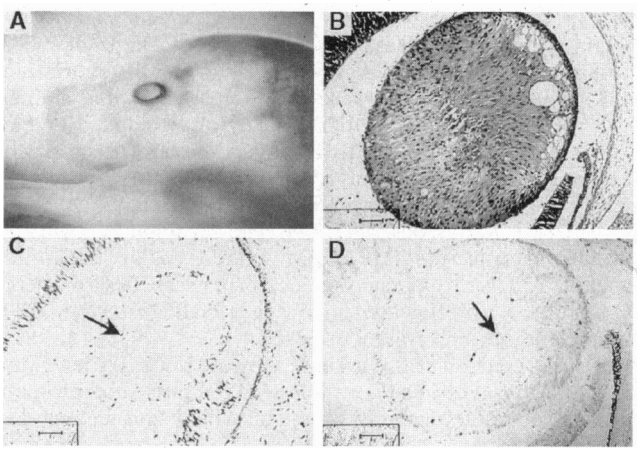

FIG. 4. Rb-deficient phenotype in E16.5 lenses obtained through LCS. (*A*) Gross appearance under a dissecting microscope. (*B*) Hematoxylin/eosin-stained lens sections exhibiting a hypercellular lens fiber region. (*C*) BrdU incorporation studies confirming that many lens fiber cells are progressing through S phase. (*D*) TUNEL assay demonstrating that lens fiber cells are undergoing apoptosis. Arrows in *C* and *D* indicate nuclei positive for DNA synthesis and apoptosis, respectively. (Bars = 50 μm.)

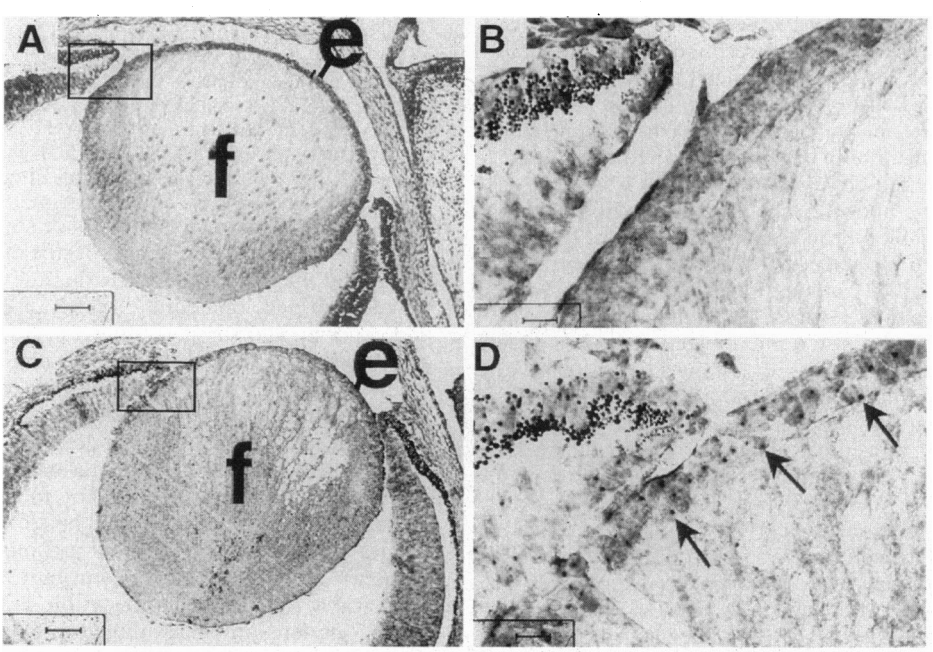

FIG. 3. Documentation that lenses are derived from the WW6 ES cell line. Wild-type (*A* and *B*) and WW6-derived (*C* and *D*) lens sections were subjected to a DNA *in situ* hybridization protocol designed to detect an inert β-globin transgene present in many copies in the WW6 genome (16). A nuclear-associated blue precipitate (arrows in *D*) denotes the presence of the marker as assayed by DNA *in situ* detection of hybridized biotinylated β-globin probe and subsequent streptavidin–phosphatase and NBT/BCIP reaction. The black granules of the pigmented epithelial layer can be seen in the upper left corner of *B* and *D* and do not represent positive staining in this assay. *B* and *D* are higher magnifications of the boxed regions in *A* and *C*. Notably, the omission of the β-globin probe from hybridizations to these lenses generated from the WW6 microinjections yielded no signal as expected (data not shown). (Bars: *A* and *C*, 50 μm; *B* and *D*, 10 μm.)

diate and late-stage markers, β- and γ-crystallin, were more severely affected than the early-stage differentiation marker α-crystallin (data not shown). Finally, the TUNEL assay confirmed that many fragmented and pyknotic nuclei in the lens fiber region reflected a high degree of apoptosis (Fig. 4*D*). Overall, the spectrum of pathophysiological findings obtained through LCS was identical to that obtained through the traditional knockout strategy (7).

Aside from faithfully recapitulating the phenotype of a known knockout in the lens, LCS offers several advantages over the conventional approach. These include substantial reductions in the time necessary to generate and evaluate potential phenotypes resulting from homozygous inactivation of new genes, lack of a requirement for germ-line transmission of the mutant allele, and elimination of the need to genotype each sample prior to analysis and to document ES-cell contribution to the organ under study. Most significantly, LCS allows for increased longevity of a homozygous null condition by virtue of host blastocyst contribution to other organ systems of the chimera. Survival to much later stages of development permits the detailed study of cancer-relevant genes whose loss of function results in early embryonic lethality. In the case of *Rb*, LCS has resulted in a high percentage of viable adult chimeras with lenses (Table 1, experiment B). For *Rb* and other essential genes, the extended postnatal survival of chimeras coupled with the ease of introducing crystallin-driven expression constructs into available mutant ES cell lines provide an opportunity to answer structure–function questions *in vivo* and to explore how candidate modifier genes impact upon the homozygous null condition.

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