Neither MafA/L-Maf nor MafB is essential for lens development in mice

Takashi Takeuchi[1,2], Takashi Kudo[1], Kiyohito Ogata[1], Michito Hamada[1], Megumi Nakamura[1], Katsumi Kito[2], Yasuhito Abe[2], Norifumi Ueda[2], Masayuki Yamamoto[3], James Douglas Engel[4] and Satoru Takahashi[1]*

[1]*Department of Anatomy and Embryology, Doctoral Program in Life System Medical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan*
[2]*Division of Molecular Pathology, Ehime University Postgraduate School of Medicine, Toh-on, Ehime 791-0295, Japan*
[3]*Department of Medical Biochemistry, Tohoku University Graduate School of Medicine, 2-1 Seiryo-cho, Aoba-ku, Sendai 980-8587, Japan*
[4]*University of Michigan Medical School, Ann Arbor, Michigan 48109 0616, USA*

The importance of the large Maf transcription factor family has been investigated in lens development in the chick, *Xenopus* and mammals. Previously we reported that *c-maf*-deficient mice exhibit severe defects in lens fibre cells. Here, we report the roles of other large Mafs, MafA/L-Maf and MafB, during mouse lens development. MafA/L-Maf and MafB were expressed in lens epithelial cells and fibre cells at E12.5 but had largely disappeared from the lens at E18.5. The lens of *mafA*-, *mafB*-deficient and *mafA::mafB* double-deficient mice developed normally. In *c-maf*-deficient mice, the pattern of expression of MafA and MafB differed from their expression in wild-type mice. Moreover, the expression of crystallin genes was unchanged in *mafA*-, *mafB*- and *mafA::mafB* double-deficient lens. These results indicate that c-Maf alone is essential for lens development, and that MafA/L-Maf and MafB are dispensable in mice.

Introduction

The optic lens has been used as a model to study the molecular mechanisms of cellular differentiation. In the rodent, lens morphogenesis starts by embryonic day (E) 9.5 (McAvoy 1980). At E9.5, the mouse optic vesicle bulges out from the prospective forebrain and makes contact with the head ectoderm. The head ectoderm thickens and forms the lens placode. Invagination of the lens placode occurs by E11.5 to form the lens vesicle. Posterior cells of the lens vesicle elongate and differentiate into the primary lens fibre cells. By E13.5, the lumen of the lens vesicle is almost completely filled with primary lens fibre cells and the anterior cells of the lens vesicle form the lens epithelial cells. Lens epithelial cells proliferate in the equatorial region and differentiate into the secondary fibre cells at this stage. Thereafter, lens fibre cells are produced continuously in this position and are maintained throughout life (Piatigorsky 1981).

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**Correspondence*: satoruta@md.tsukuba.ac.jp

The Maf transcription factor family was originally identified following the discovery of the *v-maf* oncogene within the genome of the avian musculoaponeurotic fibrosarcoma virus, AS42 (Nishizawa *et al.* 1989). Maf family proteins share a conserved basic region and leucine zipper (bZip) motif that mediates dimer formation and DNA binding to the Maf recognition element (MARE) (Kataoka *et al.* 1994). Maf family proteins can be divided into large and small Mafs. Large Maf proteins, MafA/L-Maf/SMaf, MafB, c-Maf and NRL contain an acidic domain to enable transcriptional activation and play key roles in cellular differentiation (Nishizawa *et al.* 1989; Swaroop *et al.* 1992; Kataoka *et al.* 1994; Ogino & Yasuda 1998; Kajihara *et al.* 2001).

Several studies have demonstrated a relationship between large Maf proteins and lens development. Ogino *et al.* reported that MafA/L-Maf (a lens-specific member of the Maf family) is sufficient to induce lens-specific crystallin expression in the head ectoderm of the chick and that L-Maf expression is first detected within the lens placode and is maintained specifically within lens cells (Ogino & Yasuda 1998; Yoshida &

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Yasuda 2002; Shimada *et al.* 2003). On the other hand, disruption of the *c-maf* gene has been shown to prevent elongation of mouse lens fibre cells and to decrease expression of crystallins (Kawauchi *et al.* 1999; Kim *et al.* 1999; Ring *et al.* 2000). *Ofl* (opaque flecks in lens) mutant mice have an R291Q missense mutation in the DNA-binding domain of the *c-maf* gene (Lyon *et al.* 2003). In addition to these observations, Jamieson *et al.* (2002) reported mutation of the human *c-maf* gene in the pedigrees of individuals exhibiting ocular developmental abnormalities. These results suggest that c-Maf is the major large Maf factor driving lens development in mammals in contrast to the situation in chick and *Xenopus*. However, the precise demonstration of this role for c-Maf is lacking due to the absence of *mafA*- or *mafB*-deficient mice.

To elucidate the role of each large Maf transcription factor in mouse lens development, we analyzed *mafA*-, *mafB*-, *c-maf*-, and *mafA::mafB* double-knockout mice, as we have recently generated *mafA*- and *mafB*-knockout mice (Zhang *et al.* 2005; Moriguchi *et al.* 2006) and it has been reported that the remaining large Maf factor, Nrl, does not contribute to lens development (Mears *et al.* 2001). In this article, we demonstrate that c-Maf, but neither MafA nor MafB, is the major large Maf transcription factor driving mouse lens development.

Results

Expression of large Maf family members

We first examined the expression of MafA, MafB and c-Maf proteins in the lens, as Nrl has been reported not to be involved in lens development in the mouse. We performed immunohistochemistry using polyclonal anti-Maf antibodies to monitor the expression of MafA, MafB and c-Maf in the developing lens. At E12.5, MafA, MafB and c-Maf were detected in both lens epithelial cells and primary lens fibre cells of wild-type mice (Fig. 1a–c). MafA was more strongly expressed in fibre cells than epithelial cells at E12.5 (Fig. 1a), but was not detected in lens cells at E18.5 (Fig. 1e). Strong MafB expression was detected in lens epithelial cells relative to its expression in fibre cells at E12.5 (Fig. 1b), and only weak expression of MafB was detected in epithelial cells at E18.5 (Fig. 1f). On the other hand, c-Maf protein was mainly expressed in the lens fibre cells at E12.5 (Fig. 1c). c-Maf was also expressed exclusively in lens fibre cells, but not in the epithelial cells at E18.5 (Fig. 1g). At E10.5, c-Maf was expressed in the lens placode, whereas neither MafA nor MafB was detected (data not shown).

Expression of maf transcript in the lens and transcript activities of large Maf

The levels of expression of *mafA*, *mafB* and *c-maf* transcripts in the lens at E12.5 were determined by quantitative real-time PCR. As shown in Fig. 2a, *c-maf* transcripts were much more abundant relative to the other *maf* transcripts.

To assess the contribution of large Maf proteins to the transactivation of the chick αA crystallin gene promoter by the αCE2 enhancer (Matsuo & Yasuda 1992), we analyzed the activities of MafA, MafB, and c-Maf on an αCE2 reporter plasmid in a co-transfection/

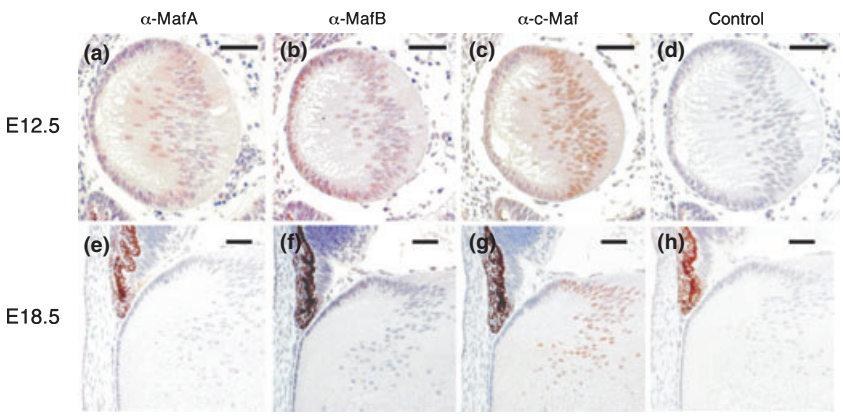

Figure 1 Immunohistochemical analysis of large Maf proteins in wild-type lens at E12.5 (a–d) and E18.5 (e–h). The lens of wild-type mice (WT) were stained with anti-MafA (a,e), anti-MafB (b,f) or anti-c-Maf (c,g) antibody, and negative control (d,h). Scale bars: 100 μm.

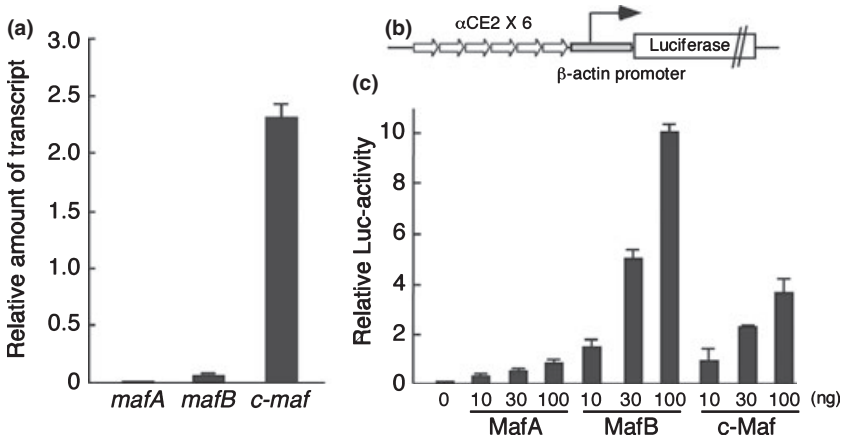

Figure 2 Expression in lens and transcriptional activity of large Maf transcription factors. (a) Quantitative analysis of the expression of large Maf genes in wild-type lens at E12.5 using real-time PCR. The level of expression of each *maf* gene was normalized to the level of the *hprt* transcript. (b) Schematic representation of the αCE2 reporter construct. (c) Transactivation activity of large Maf proteins. αCE2 reporter plasmid was transfected into NIH3T3 cells with the indicated quantities of Maf expression plasmids.

transactivation assay in NIH3T3 fibroblast cells (Kajihara *et al.* 2003). Recombinant Maf genes were prepared that encoded a FLAG tag at the N-termini. The reporter constructs comprised a β-actin promoter downstream of six tandem copies of αCE2 driving expression of a luciferase gene (Fig. 2b). The level of reporter gene expression increased proportionally with increasing amounts of each co-transfected Maf construct (Fig. 2c). Reporter gene transactivation by MafB and c-Maf was 15- and sixfold greater, respectively, than that observed by MafA (Fig. 2c).

Lens morphology of large maf knockout mice

To evaluate the contribution of each large Maf factor to mouse lens development, histological analyses were conducted on lenses from wild-type mice and mice null for each large Maf family member using paraffin-embedded tissues that were sectioned and stained with hematoxylin and eosin. At E12.5, lens vesicles of wild-type (Fig. 3a), *mafA*- (Fig. 3b), *mafB*- (Fig. 3c) and *mafA::mafB* double-knockout mice (Fig. 3e) were partially filled with elongated primary lens fibre cells. In *c-maf*- (Fig. 3d), *mafA::c-maf* double- (Fig. 3f) and *mafB::c-maf* double- (Fig. 3g) knockout mice the posterior cells of the lens vesicle, which normally correspond to the primary lens fibre cells, did not show any cellular elongation. At E18.5, lens development was complete in wild-type mice (Fig. 3h). At this stage, the lenses of *mafA*- (Fig. 3i), *mafB*- (Fig. 3j) and *mafA::mafB* double- (Fig. 3l) knockout mice had also developed normally and were similar in size. In *c-maf*- (Fig. 3k) and *mafA::c-maf* double- (Fig. 3m) deficient mice, the elongation of the posterior lens cells was incomplete and the lens vesicle was still present. We did not observe the lens of *mafB::c-maf* double-deficient mice at E18.5, because these mice were embryonic lethal around E13.5.

Expression of knock-in genes in large maf-deficient mice

We inactivated the mouse *c-maf* and *mafA* genes by inserting into these loci marker genes encoding either a normal or nls-tagged *β-galactosidase* (*lacZ*), respectively, and we inactivated the *mafB* gene by inserting into that locus a marker gene encoding *gfp*. Therefore, the expression of these marker genes could serve as a surrogate for normal expression of the *maf* genes in the mutant mice. In *mafA*-deficient mice, we detected strong anti-LacZ staining in the nuclei of lens fibre cells at E12.5 (Fig. 4a,c). Strong expression of green fluorescent protein (GFP) was detected in the epithelium of MafB null lens (Fig. 4b,d). These results indicate that the pattern of expression of *mafA* or *mafB* in $mafA^{-/-}$or $mafB^{-/-}$ mouse was similar to that in wild-type mice (Figs 1a,b and 4a,b). On the other hand, in *c-maf*-deficient mice, the expression of MafA and MafB proteins was similar in both anterior and posterior cells of the lens vesicle (Fig. 4e,f), whereas the expression of LacZ was abundant in the posterior cells (Fig. 4g) as was the expression of c-Maf in wild-type mice (Fig. 1c). These results suggest that c-Maf acts upstream of MafA and MafB.

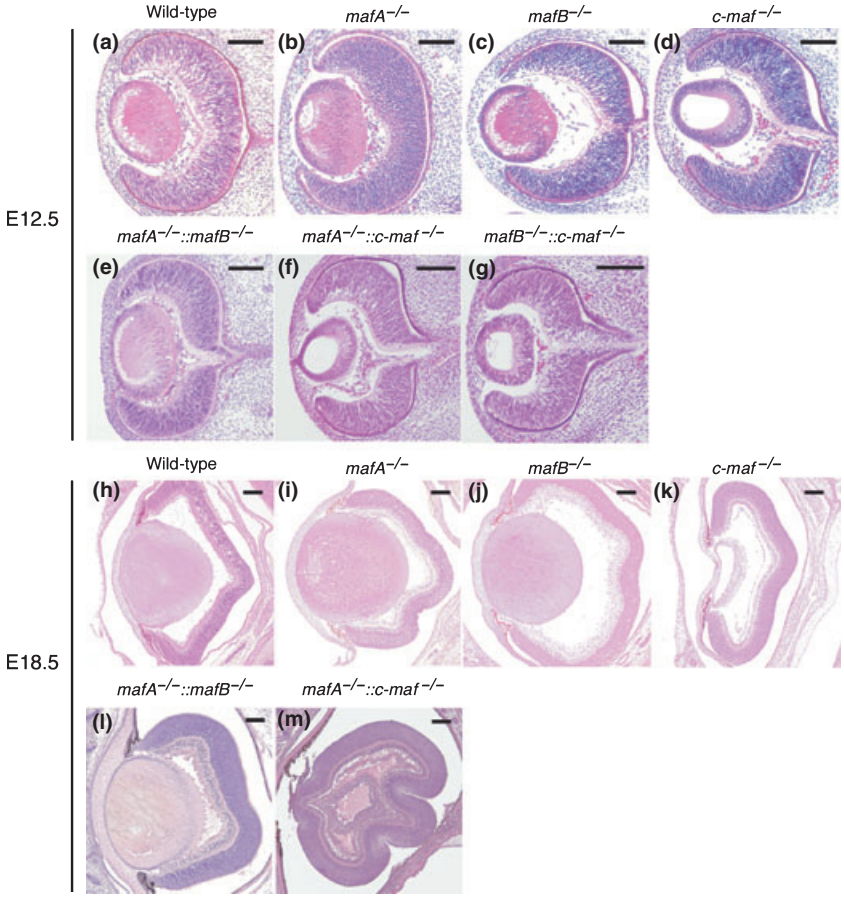

Figure 3 Lens morphology of *large maf*-deficient mice. Lens of wild-type (a), *mafA*$^{-/-}$ (b), *mafB*$^{-/-}$ (c), *c-maf*$^{-/-}$ (d), *mafA*$^{-/-}$::*mafB*$^{-/-}$ (e), *mafA*$^{-/-}$::*c-maf*$^{-/-}$ (f) and *mafB*$^{-/-}$::*c-maf*$^{-/-}$ (g) mice at E12.5. Lens of wild-type (h), *mafA*$^{-/-}$ (i), *mafB*$^{-/-}$ (j), *c-maf*$^{-/-}$ (k), *mafA*$^{-/-}$::*mafB*$^{-/-}$ (l) and *mafA*$^{-/-}$::*c-maf*$^{-/-}$ (m) mice at E18.5. Hematoxylin and eosin staining. Scale bars: 100 μm.

Transcription level of crystallin genes in large maf null mice

As crystallins are the most abundant proteins in lens fibre cells and critical for proper development of the lens (Kawauchi *et al.* 1999; Kim *et al.* 1999; Ring *et al.* 2000), we used quantitative RT-PCR to determine whether MafA or MafB is required for transcriptional activation of crystallin genes *in vivo*. Total RNA was obtained from the heads of E18.5 embryos, and primers for *hprt* were used as an internal control. We found that the expression of crystallin genes in *mafA*$^{-/-}$, *maB*$^{-/-}$ and *mafA*$^{-/-}$::*mafB*$^{-/-}$ mice was similar to that in wild-type mice (Table 1). In *c-maf*$^{-/-}$ lens, the expression of αA-, γC-, γE- and γF-crystallin was reduced as has been previously reported (Kawauchi *et al.* 1999; Kim *et al.* 1999; Ring *et al.* 2000).

Discussion

In the present study, we analyzed the expression of MafA, MafB and c-Maf during normal lens development and compared the phenotypes of mice deficient for these factors. The MafA and MafB proteins and transcripts were expressed in the lens at E12.5; however, the levels of those transcripts were much lower than that of the *c-maf* transcript. Moreover, lens development and the expression of crystallin genes were normal in *mafA*-, *mafB*- and *mafA::mafB* double-knockout mice. Only *c-maf*-deficient mice exhibited abnormal lens development at both E12.5 and E18.5, whereas MafA and MafB mutant mice showed normal development. This is the first *in vivo* demonstration that MafA is not necessary for lens development in the mouse. In addition, the expression of MafA and MafB was altered by the absence of c-Maf.

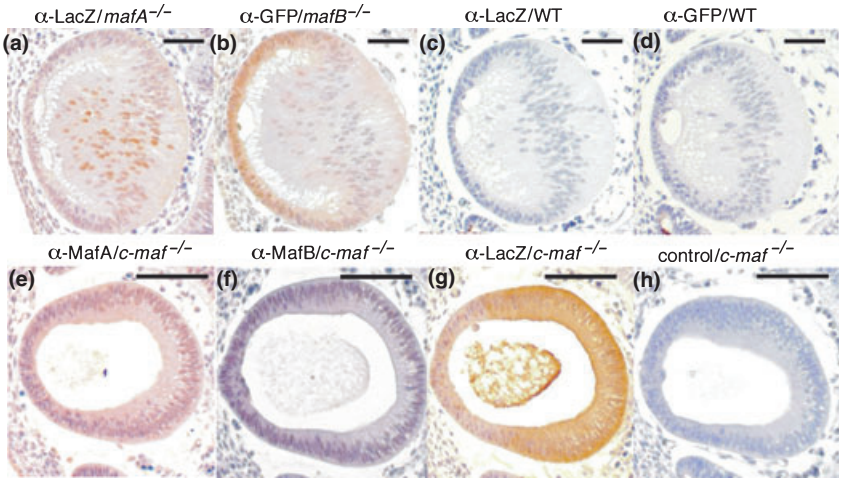

Figure 4 The expression of large Maf transcription factor in each *maf*-deficient mouse at E12.5. Lens of *mafA*$^{-/-}$ mouse (a) was stained with anti-β-galactosidase antibody (α-LacZ) and lens of *mafB*$^{-/-}$ mouse (b) with anti-GFP antibody (α-GFP). Negative controls using α-LacZ or α-GFP are shown in (c) and (d) respectively. The lens of *c-maf*$^{-/-}$ mice were stained with anti-MafA (e), anti-MafB (f) or anti-LacZ (g) antibody, and negative control (h). Scale bars: 100 μm.

Table 1 Relative mRNA level of crystallin genes at E18.5 in each Maf-deficient head

	Percentage of wild-type			
	mafA$^{-/-}$	*mafB*$^{-/-}$	*mafA*$^{-/-}$::*mafB*$^{-/-}$	*c-maf*$^{-/-}$
αA-crystallin	113.0 ± 20.1	98.1 ± 22.6	117.5 ± 12.6	4.8 ± 1.0★
αB-crystallin	115.7 ± 24.1	102.2 ± 12.2	106.2 ± 22.0	84.3 ± 18.6
γC-crystallin	134.7 ± 21.3	75.3 ± 19.3	95.3 ± 9.6	2.0 ± 1.3★
γE-crystallin	101.5 ± 22.3	95.8 ± 12.4	92.5 ± 16.8	7.9 ± 1.1★
γF-crystallin	95.1 ± 43.3	116.9 ± 16.9	86.9 ± 10.7	4.1 ± 1.7★

$n = 3$ or 4.
★$P < 0.01$.

These results indicate that c-Maf acts upstream of MafA and MafB in the mouse.

Members of the large Maf family are differentially expressed in the lens during morphogenesis (Yoshida & Yasuda 2002; Lecoin *et al.* 2004). The role of large Maf family members during lens development appears to differ between species. In the chick, MafA/L-Maf expression is first detected in the head ectoderm, which corresponds to the lens placode and the expression continues into the later developmental stages. Its expression has also been observed in both lens epithelial and fibre cells (Yoshida & Yasuda 2002). On the other hand, the expression of c-Maf is observed in the lens placode of the mouse and its expression is sustained in the fibre cells until E18.5 (Kawauchi *et al.* 1999; Kim *et al.* 1999; Ring *et al.* 2000). Moreover, *mafB* is expressed at early stages in the presumptive lens ectoderm of *Xenopus* and zebrafish (Coolen *et al.* 2005). In this study, we demonstrated that c-Maf acts upstream of MafA and MafB during mouse lens development. In contrast, in the chick, the ectopic expression of MafA/L-Maf induces the expression of several transcription factors that are important for lens development or differentiation, including other members of the large Maf family (Reza *et al.* 2002). These results also indicate that the order of the appearance of factors in the transcriptional activation cascade differs between mouse and chick.

The difference in the pattern of expression of large Maf family members in the lens among species is very specific, because it is well established that MafA is the dominant large Maf in the somite while MafB is the major factor in the hindbrain of chick, mouse, *Xenopus* and zebrafish. It is not currently clear why the expression of members of the large Maf family differs in the lens of different species. It may reflect differences in eye development in these species. Further analysis will

be needed for a more comprehensive understanding of the role of large Maf proteins during lens development.

Experimental procedures

Mice

mafA-, *mafB*- and *c-maf*-knockout mice were generated using standard gene-targeting techniques described previously (Kawauchi *et al.* 1999; Zhang *et al.* 2005; Moriguchi *et al.* 2006). The *gfp* or *lacZ* gene was inserted into each *maf* gene locus to monitor the expression of the knockout gene. Mice were maintained in specific pathogen-free conditions in a Laboratory Animal Resource Center. All experiments were performed according to the Guide for the Care and Use of Laboratory Animals at the University of Tsukuba.

Immunohistochemical analysis

Embryos were fixed with phosphate-buffered 4% paraformaldehyde for 2 h at 4 °C and embedded in paraffin according to the routine procedure. Serial horizontal head sections of 3 μm thickness through both eyes were prepared. After antigen retrieval [20 min of autoclaving in 10 mM buffered citric acid (pH 6.0)], sections were rinsed in PBS and treated with 10% H_2O_2 in methanol for 10 min. These sections were washed in PBS and incubated with 10% normal rabbit serum albumin for 1 h at room temperature. Sections were then incubated with anti-MafA (BL1069; 1 : 500), MafB (BL658; 1 : 500), c-Maf (BL662; 1 : 500) rabbit polyclonal antibodies (Bethyl Laboratories Inc., Montgomery, TX), anti-GFP rabbit polyclonal antibody (1 : 1000; Molecular Probes, Eugene, OR) or anti-β-galactosidase (β-gal) rabbit polyclonal antibody (AB616; 1 : 1000; Abcam K. K., Tokyo, Japan) for 2 h at room temperature. Anti-MafB antibody (BL658) cross-reacted weakly with MafA protein. The sections were washed in PBS and incubated with Envition+peroxidase-linked anti-rabbit Ig (DAKO, Glostrup, Denmark) for 1 h at room temperature. They were washed in PBS, and visualized with simple stain DAB solution (Nichirei, Tokyo, Japan) and counterstained in haematoxylin, dehydrated in ethanol and xylene, and mounted. To evaluate the specificity of the primary antibodies, the sections of each *maf* mutant mouse were also used as a control.

Histology

Sections were prepared and stained with hematoxylin and eosin, then observed by light microscopy.

Cell culture and transient transfection assay

Mouse MafA, MafB, c-Maf cDNAs were subcloned into the pEFX3-FLAG eukaryotic expression plasmid (Kajihara *et al.* 2003). The reporter plasmid comprised the αCE2x6/β-actin promoter fragment (Matsuo & Yasuda 1992) cloned into the pGL2-Basic vector (Promega, Madison, WI). NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum. The cells were transfected using Lipofectamine 2000 Reagent (Invitrogen, Groningen, Netherlands) and then harvested at 48 h post-transfection. The luciferase assay was performed according to the supplier's protocol using the Dual-Luciferase Reporter Assay System (Promega). Transfection efficiencies were routinely normalized by the co-expressed *Renilla reniformis* luciferase activity, which was expressed from the pRL-tk plasmid.

Quantitative analysis of the expression of large maf and crystallin genes by real-time RT-PCR

Mouse lens and heads were dissected from E12.5 and E18.5 embryos, respectively, and total RNA was isolated using the Qiagen RNeasy Kit (QIAGEN, Valencia, CA). First-strand cDNAs were synthesized by QuantiTect Reverse Transcription (QIAGEN). The PCR was carried out using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) as the reporter or using the Thermal Cycler Dice Real Time System, TP850 (Takara) with SYBR Premix Ex Taq II (Takara, Shiga, Japan). The expression plasmids, pEFX3-FLAG-mafA, -mafB, -c-maf, were used as standard DNAs. Oligonucleotide primers of the amplified products for different genes assayed were as follows. *mafA*, 5′-CACTGGCCATCGAGTACGTCA-3′ and 5′-CTTCACCTCGAACTTCATCAGGTC-3′; *mafB*, 5′-TGAGCATGGGGCAAGAGCTG-3′ and 5′-CCATCCAGTACAGGTCCTCG-3′; *c-maf*, 5′-CTGCCGCTTCAAGAGGGTGCAGC-3′ and 5′-GATCTCCTGCTTGAGGTGGTC-3′; *αA-crystallin*, 5′-ACAACGAGAGGCAGGATGAC-3′ and 5′-AGGGGACAACCAAGGTGAG-3′; *αB-crystallin*, 5′-GCGGTGAGCTGGGATAATAA-3′ and 5′-GCTTCACGTCCAGATTCACA-3′; *γC-crystallin*, 5′-TGCTGCCTCATCCCCCAACA-3′ and 5′-TCGCCTAAAGAGCCAACTT-3′; *γE-crystallin*, 5′-ACCCTGACTACCAGCAGTGG-3′ and 5′-GTCCAGATGGAGAAAATGGT-3′; *γF-crystallin*, 5′-GTGGCTGCTGGATGCTCTAT-3′ and 5′-GCCTATACTCCCCTGGCCTC-3′; *hprt*, 5′-CAAACTTTGCTTTCCCTGGT-3′ and 5′-CAAGGGCATATCCAACAACA-3′. The relative amount of each *maf* or *crystallin* gene was normalized to the amount of *hprt* transcript in the same cDNA.

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