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c-Maf deletion in adult C57BL/6J mice induces cataract formation and abnormal differentiation of lens fiber cells

Running title

ADULT C-MAF CKO MICE DEVELOP CATARACTS

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Abstract

The transcription factor c-Maf is a member of the large Maf family, members of which possess transactivation and bZIP domains. c-Maf plays an important role in lens formation, T-lymphocyte differentiation, hypertrophic chondrocyte differentiation, and kidney development in mouse embryos. However, because homozygous deletion of c-Maf in C57BL/6J mice causes embryonic lethality, the functions of c-Maf in adult mice remain largely uninvestigated. To address this issue, we generated c-Maf floxed (c-Maf^{fl/fl}) C57BL/6J mice and established tamoxifen-inducible c-Maf knockout mice (c-Maf^{fl/fl}; CAG-Cre-ER^{TM} mice, c-Maf^{ΔTAM}). After tamoxifen injection, adult c-Maf^{ΔTAM} mice showed successful deletion of c-Maf protein and developed severe cataracts; cataracts are also seen in human patients who have mutations in the c-MAF DNA binding domain. Furthermore, adult c-Maf^{ΔTAM} mice exhibited abnormal lens structure and impaired differentiation of lens fiber cells. In summary, we established c-Maf^{fl/fl} and c-Maf^{ΔTAM} C57BL/6J mice, which can be useful animal models for the investigation of c-Maf function in various developmental stages and can also be used as a disease model for cataracts.
Introduction

The transcription factor c-Maf belongs to the large Maf family, all members of which have a basic region and a leucine zipper domain. The leucine zipper domain mediates dimer formation, and the basic region allows the protein to bind DNA to Maf recognition elements (MARE). In addition to these domains, Maf family members are characterized by having an acidic transactivation domain in their N-terminal region [1].

Previous studies have reported that mutations in the DNA binding and transactivation domains of human c-MAF are associated with familial juvenile cataracts [4, 9, 18, 26]. Moreover, c-Maf knockout embryos showed impaired lens fiber cell differentiation and lens formation due to dysregulation of crystallin gene expression during the differentiation of primary posterior lens fibers [10, 11]. These results indicate that c-Maf is important for lens maintenance. However, the postnatal functions of c-Maf have remained largely unknown because c-Maf null mice on a C57BL/6J background exhibit embryonic lethality due to impaired definitive erythropoiesis in fetal liver cells [13].

The lens is composed of lens epithelial cells at the anterior pole and lens fiber cells at the posterior pole. Lens epithelial cells differentiate into lens fiber cells in a transitional region that
is known as a germinative zone; lens fiber cells migrate toward the core of lenses and form lens
nuclear cells. Crystallins are soluble proteins found in lenses, and there are three types: α, β and
γ-crystallin. α-Crystallin is distributed in lens epithelial cells and lens fiber cells; in contrast, β- and γ-crystallin are only found in lens fiber cells. Crystallins are involved in lens development
and maintenance, and they control lens cell proliferation and differentiation [3, 22, 24].

Moreover, c-Maf is one of the key transcription factors that regulates crystallin expression [10,
11, 21].

Herein, for the purpose of investigating c-Maf functions in adults, we generated c-Maf^{fl/fl} mice
on a C57BL6/J background and tamoxifen-inducible c-Maf conditional knockout mice (c-
Maf^{fl/fl} and CAG-Cre-ER^{TM} offspring were named c-Maf^{ΔTAM}) using CRISPR/Cas9 technology.

Our analysis revealed that the c-Maf^{ΔTAM} mice had disrupted lens structures, abnormal
differentiation of epithelial cells and cataracts, which recapitulate the human phenotype of c-
MAF mutations. These data reveal that these model mice can be used for investigating c-Maf
function in adults.


Materials and Methods

Animals

Mice were kept under specific pathogen-free conditions in the Laboratory Animal Resource Center of the University of Tsukuba. All experiments in this study were performed in compliance with the guidelines of the Care and Use of Laboratory Animal Resource Center at the University of Tsukuba, and they were approved by the Institutional Review Board of the university. Male and female C57BL/6J mice (8 weeks old) were purchased from SLC Japan (Tokyo, Japan). Mice with the floxed allele (c-Maf^{fl/fl}) were bred with Ayu1-Cre mice [20] (c-Maf^{+/fl}; Ayu1-Cre) to generate mice lacking the c-Maf gene (c-Maf^{fl/fl}; Ayu1-Cre). CAG-Cre-ERTM mice [5] were used to generate c-Maf^{ΔTAM} mice. CAG-Cre-ERTM transgenic mice were purchased from the Jackson Laboratory (Bar Harbor, Maine).

To activate the Cre recombination system, 8-week-old c-Maf^{ΔTAM} and control mice were injected intraperitoneally with 75 mg/kg tamoxifen for 5 consecutive days. Tamoxifen (TAM, Sigma-Aldrich, T5648) was dissolved in ethanol and then mixed with corn oil as described...
previously [16]. Four mice from each group were sacrificed 7 months after TAM injection for histopathological and qPCR analyses of the lens.

Genotyping of mice

Genomic DNA was isolated from mouse tails, and PCR was performed to identify the floxed allele, *Ayu1-Cre* and the *c-Maf* deletion; the primers used are listed in Table S1A. The wild-type PCR product was 431 bp in length, the floxed PCR product was 493 bp in length, the *Ayu1-Cre* PCR product was 269 bp in length, and the *c-Maf* deletion PCR product was 993 bp in length.

Histopathological and immunohistochemical analyses

For IHC staining, lenses were extracted from *c-Maf<sup>+/+</sup>*/*, *c-Maf<sup>fl/fl</sup>* and *c-Maf<sup>fl/fl</sup>*; *Ayu1-Cre* embryos at E14.5 and were fixed in 4% PFA at 4 °C for 24 hours; then, The lenses were embedded in paraffin, and 5 μm sections were cut and placed on slides. The paraffin sections were subjected to paraffin removal and then were washed with PBS and distilled water. Then, these sections were permeabilized with 0.3% Triton X-100/PBS before undergoing antigen retrieval for 10 minutes at 121 °C, which was followed by incubation on ice for 1 hour and
blocking for 1 hour in PBS with 5% BSA and 10% donkey serum. Then, these sections were incubated overnight at 4 °C with the following primary antibodies: rabbit anti-c-Maf (1:200; A300-613A; Bethyl) and mouse anti-E-cadherin (1:200; 610181; BD Transduction Laboratories). Antigens were visualized using a secondary antibody conjugated to Alexa Fluor 594 (1:500; Life Technologies) for c-Maf and Alexa Fluor 488 for E-cadherin; the nuclei were labeled with Hoechst 33342 (Molecular Probes). The sections were mounted with Fluoromount (Diagnostic BioSystems). All images were captured with a fluorescence microscope (Biorevo BZ-9000; Keyence).

Quantitative (q)PCR analysis

Total RNA was extracted from the eye of c-MafΔTAM and c-Maffl/fl mice using ISOGEN Reagent (NIPPON GENE) 7 months after TAM injection. cDNA was synthesized in accordance with the protocol of a QuantiTect reverse transcription kit (Qiagen). qPCR was performed in duplicate with a Thermal Cycler Dice real-time system (TaKaRa) using THUNDERBIRD SYBR qPCR Mix (Toyobo). The primers that were used for amplification and detection are listed in Table S1B. The expression of target genes was normalized to the expression of Hprt. Each sample was tested in duplicate.
Statistical analysis

All data were presented as the mean and the standard error of the mean (SEM). To assess whether differences between $c\text{-}Maf^{\Delta TAM}$ and control mice were statistically significant, a minimum of three biological replicates were analyzed using Welch's t test, and a $P$-value $< 0.05$ was considered significant.
Results

Generation of $c$-$Maf^{ΔTAM}$ mice

We applied CRISPR/Cas9 methods to generate double-stranded DNA with a floxed $c$-$Maf$ allele (Figure 1A). The targeting vector contained a $c$-$Maf$ protein-coding sequence and two $loxP$ sites. To confirm the insertion of the floxed allele ($c$-$Maf^{fl/fl}$) and the Cre-dependent excision of the floxed allele, $c$-$Maf^{fl/fl}$; $Ayu1$-$Cre$ mice were generated by mating $c$-$Maf^{fl/fl}$ and $c$-$Maf^{+/fl}$; $Ayu1$-$Cre$ mice [20]. The $loxP$ insertion at the $c$-$Maf$ locus, the $Cre$ insertion in the $Ayu1$-$Cre$ mice and the $c$-$Maf$ deletion were all verified by genotyping PCR (Figure 1B).

To confirm the $c$-$Maf$ deletion at the protein and phenotype levels, we investigated the lenses of $c$-$Maf^{fl/fl}$; $Ayu1$-$Cre$ mice because a previous study reported that $c$-$Maf$ is expressed in lens fiber cells and that $c$-$Maf$ null embryos show aplasia of the lens at E18.5 [10]. Hematoxylin and eosin (HE) staining revealed an abnormal lens structure in the homozygous knockout mice, which is similar previously reported HE staining in $c$-$Maf$ null mouse embryos (Figure 1C). The IHC results showed that $c$-$Maf$ expression was completely depleted in lens fiber cells (Figure 1D). These results demonstrated that we successfully achieved the integration of the floxed allele into the $c$-$Maf$ target locus.
Next, for the purpose of investigating c-Maf functions in adult mice, we mated $c$-$Maf^{fl/fl}$ and $CAG$-$Cre$-$ER^TM$ transgenic mice [5]. Genotyping PCR confirmed that tamoxifen-inducible $c$-$Maf$ knockout mice ($c$-$Maf^{ΔTAM}$) were successfully generated (Figure 1E).

$c$-$Maf^{ΔTAM}$ mice displayed abnormal lens structure

All of the four $c$-$Maf^{ΔTAM}$ mice, which were analyzed 7 months after tamoxifen injection, exhibited an abnormal lens appearance when compared with that of the control mice. The white dot on lenses represents opacification of the lenses (Figure 2A). Next, to histologically examine abnormal appearances in the $c$-$Maf^{ΔTAM}$ mice, we conducted HE staining and analyzed the lens structure. Red squares show an anterior pole, a posterior pole and a germinative zone in the lens (Figure 2B). Around the lenses of the $c$-$Maf^{ΔTAM}$ mice, lens epithelial-like cells were present and produced multiple layers, which was different from the control mice in which lens epithelial cells were in a monolayer and existed from the anterior pole to the germinative zone. In the germinative zone of the $c$-$Maf^{ΔTAM}$ mice, differentiation was not maintained, and migration did not occur either. Moreover, lens epithelial cells were greatly amplified in the anterior pole; it
was possible that the amplification led to a multilayered structure in the germinative zone and posterior pole instead of differentiation.

c-Maf<sup>ΔTAM</sup> mice showed impaired differentiation and migration, and lens epithelial cells were observed in the posterior pole

We next performed IHC staining to identify which cells emerged on all sides of the lenses and what cells were present in the multiple layers around the lenses in the c-Maf<sup>ΔTAM</sup> mice. Because E-cadherin is known as a marker of lens epithelial cells, we used antibodies against E-cadherin to detect lens epithelial cells. In the control mice, E-cadherin-positive lens epithelial cells (green) appeared to transition from the anterior pole to the germinative zone (Figure 3B and C), and the c-Maf-positive lens fiber cells (pink) migrated from the germinative zone into the lens nuclear cells. (Figure 3C). However, there were no epithelial cells at the posterior pole (Figure 3D) of the control mice. In the c-Maf<sup>ΔTAM</sup> mice, lens epithelial cells appeared in the three regions (Figure 3B, C and D); in contrast, no lens fiber cells appeared in the germinative zone (Figure 3B). In addition, lens epithelial cells (E-cadherin-positive) formed multiple layers around the lenses, which was unlike the single layers observed in the control mice (Figure 3B, C and D).
Moreover, the cells were arranged in a disordered row and displayed a disrupted structure in the anterior pole (Figure 3B).

c-MafΔTAM mice showed reduced crystallin genes expression

We next used qPCR to examine the expression of crystallin genes, *Cryaa, Cryba and Crygd*, in the whole eye of c-MafΔTAM and control mice. The results clearly indicated significant decreases in *Cryaa* (P-value = 0.0011) and *Cryba* (P-value = 0.0095) gene expression levels (Figure 4A and B). There was no notable difference in the expression of *Crygd* (P-value = 0.164); however, there was almost no expression of *Crygd* in the c-MafΔTAM mice (Figure 4C). Taken together, c-Maf deletion leads to decreased gene expression of the lens crystallin genes in c-Maf in adult mice.
The transcription factor c-Maf is associated with the differentiation of various cells and tissues, such as lens fiber cells [10, 11], subsets of T-lymphocytes [2, 6], hypertrophic chondrocytes during the development of endochondral bone [15, 19], and embryonic kidney and liver cells [8]. However, the physiological roles of c-Maf in adults have not been well studied because the homozygous deletion of c-Maf in mice on a C57BL/6J background causes embryonic lethality [13]. Here, we generated c-Maf\textsuperscript{fl/fl} mice and tamoxifen-inducible c-Maf conditional knockout mice (c-Maf\textsuperscript{ΔTAM}). The c-Maf\textsuperscript{fl/fl}; Ayu1-Cre mice developed severe cataracts and lens anomalies, which are consistent with previous studies on c-Maf homozygous null embryos [10, 11]. These results indicated that we successfully generated c-Maf\textsuperscript{fl/fl} and c-Maf conditional knockout mice. In previous studies, c-Maf was found to regulate crystallin gene expression, and c-Maf deficiency induced aberrant lens structures. For example, c-Maf was shown to be essential for Cryaa expression and the induction of the differentiation of lens fibers [21]. c-Maf deficiency resulted in a noticeable decrease in γ- gene expression and defective lens formation [11]. c-Maf knockout embryos showed dysfunctional differentiation of lens epithelial cells into lens fiber
cells, and they exhibited disrupted lens formation due to changes in Cryaa, Cryba and Crygd gene expression [10]. However, most previous studies focused on the fetal and postnatal stages of lens development and the associated abnormalities; one of the major reasons for this focus is the embryonic lethality observed in transgenic mice that was previously mentioned. In this study, we focused on the adult stage and defined c-Maf as the key factor in maintaining lens physiological structures.

First, crystallin gene expression was significantly decreased in the c-MafΔTAM mice. Previous studies using knockout mice showed the importance of crystallin gene expression for the maintenance of lens structures. For example, Cryaa is essential for the maintenance of lens transparency [3], Cryba is associated with age-related cataracts [22], and a Crygd deficiency in mice causes cataracts that are similar to human Coppock cataracts [24]. Our qPCR results showed a dramatic decrease in Cryaa, Cryba and Crygd gene expression in the c-MafΔTAM adult mice. Therefore, we demonstrate that c-Maf is related to crystallin expression in adults. The reason why the expression levels of Crygd are different in each mouse is unknown, but according to Huang Y, et al (Mol Vis. 2010 Mar 3;16:341-52.) [7], the expression level of Crygd
is very low compared with $\alpha$- and $\beta$-crystallin in the rat lens. This low expression level might be the cause of the large variation.

Second, lens structures in $c$-$Maf^{\Delta TAM}$ mice were dramatically different from control lenses. Previous studies showed that abnormal lens structures were caused by disorganization or by a lack of differentiation to produce lens fiber cells. For example, mice with a $Lim2$ mutation show vacuolated lens structures and disorganized arrangement of fiber cells [25]; further, multiple layers of fiber-like cells in the central epithelium emerged in transgenic mice that overexpress TGF-$\beta$ in the lens [14]. Mice with a mutation in the $Ercc2$ gene demonstrate a delayed differentiation in the production of primary and secondary lens fiber cells [12]. Our HE and IHC staining results showed arrested differentiation and multiple layers of lens epithelial cells around the lenses due to a deficiency in c-Maf. Interestingly, there has been no evidence that the multiple layers are composed of lens epithelial cells. Since c-Maf can induce crystallin expression, c-Maf acts as a differentiation switch in lens epithelial cells. Without c-Maf, lens epithelial cells cannot differentiate, so they do not stop proliferating. As a result, the epithelial cells in $c$-$Maf^{\Delta TAM}$ mice covered the whole lens and established multiple layers. Therefore, these results indicate that c-Maf is necessary for maintaining the polarity of the lens in adults.
Finally, opacification of lenses appeared in the \textit{c-Maf}^{\Delta TAM} mice. Opacification is one of the major phenotypes used to detect cataracts [17, 23, 27]. All three \textit{c-Maf}^{\Delta TAM} mice failed to maintain transparency in their lenses. In addition, our preliminary study demonstrated that the \textit{c-Maf}^{\Delta TAM} mice 3 months after TAM treatment showed opacification and multiple layers of lens epithelial cells (n=1). Compared with the mice 3 months after TAM injection, the layers in the 7-month-old mice were spread more widely. Therefore, the results indicate that the stratification of the lens epithelial cells can be a time-dependent change. Moreover, there are many papers showing that \textit{Cryaa} is related to age-related cataracts. Considering these results and the observation that \textit{c-Maf}^{\Delta TAM} mice clearly had cataracts, it is possible that c-Maf is related to age-related cataracts.

In conclusion, we applied CRISPR/Cas9 technology to generate \textit{c-Maf}^{fl/fl} and \textit{c-Maf} conditional knockout mice. Given that c-Maf is ubiquitously expressed in adults, c-Maf can play functional roles in various tissues. One example is that the \textit{c-Maf}^{\Delta TAM} mice had cataracts, and c-Maf was found to play an important role of c-Maf in the maintenance of lens physiological structures.
Therefore, our \textit{c-Maf}^{0/0} \textit{mice} could provide a useful model for a more detailed investigation of the physiological functions of c-Maf in each organ in adult mice.
Acknowledgements

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Conflict of interest

The authors declare no competing financial interests.
References


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Figures

Figure 1. Generation of c-Maf conditional knockout mice.

(A) Schematic targeting strategy for the generation of floxed alleles in the c-Maf locus and the detected genotyping bands for each allele. KI, knock-in; L-HA, left homology arm; R-HA, right homology arm. (B) Genotyping PCR screening of floxed and deleted alleles, including floxed c-Maf (493 bp), Ayu1-Cre (269 bp) and c-Maf deletion (993 bp). (C) HE staining of lens sections from control (c-Maf^fl/fl) and c-Maf^fl/fl; Ayu1-Cre mice at E14.5. The control mice were c-Maf^fl/fl. (D) Immunostaining for c-Maf in lens fiber cells of control (c-Maf^+/+) and c-Maf^fl/fl, Ayu1-Cre mice at E14.5. (E) Genotyping PCR signals for deletion confirmed that c-Maf knockout was successfully induced by tamoxifen injection. All scale bars = 100 μm.
Figure 2. *c-Maf*\[^{ATAM}\] mice showed abnormal appearance of eye and lens structures.

(A) Eyes of control and *c-Maf*\[^{ATAM}\] mice. (B) HE staining for the anterior and posterior poles and germinative zone in the lenses of control and *c-Maf*\[^{ATAM}\] mice. N= 4 each (1 male, 3 females). Scale bars = 100 μm.
Figure 3. IHC revealed the expression of c-Maf and E-cadherin in the lenses of control and $c-Maf^{\Delta TAM}$ mice.
(A) Bright-field and IHC staining images of the lens. IHC staining for (B) germinative zone, (C) posterior and (D) anterior pole of the lens in the \( c-Maf^{\Delta TAM} \) and control mice. IHC staining images are merged: E-Cadherin in green, c-Maf in red and DAPI in blue. \( N=4 \) each (1 male, 3 females). Scale bars = 100 μm.
Figure 4. c-MafΔTAM mice had decreased gene expression of Cryaa, Cryba and Crygd in the whole eye.

Gene expression levels for Cryaa (A), Cryba (B), and Crygd (C) in the eyes of control and c-MafΔTAM mice 7 months after TAM injection. N= 3 each (1 male, 2 females). Scale bars = 100 μm.

Table 1. The primer sequence for mice genotyping and qPCR analysis
### Supplementary Table 1A

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<td>B</td>
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