Transcription Factor GATA-3 Is Essential for Lens Development

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During vertebrate lens development, the anterior, ectoderm-derived lens vesicle cells differentiate into a monolayer of epithelial cells that retain proliferative potential. Subsequently, they exit the cell cycle and give rise to posterior lens fiber cells that form the lens body. In the present study, we demonstrate that the transcription factor GATA-3 is expressed in the posterior lens fiber cells during embryogenesis, and that GATA-3 deficiency impairs lens development. Interestingly, expression of E-cadherin, a premature lens vesicle marker, is abnormally prolonged in the posterior region of Gata3 homozygous mutant lenses. Furthermore, expression of γ-crystallin, a differentiation marker for fiber cells, is reduced. This suppressed differentiation is accompanied by an abnormal cellular proliferation, as well as with diminished levels of the cell-cycle inhibitors Cdkn1b/p27 and Cdkn1c/p57 and increased Ccnd2/cyclin D2 abundance. Thus, these observations suggest that GATA-3 is essential for lens cells differentiation and proper cell cycle control. Developmental Dynamics 238:2280–2291, 2009. © 2009 Wiley-Liss, Inc.

Key words: GATA-3; crystallin; lens fiber; differentiation; cell cycle; apoptosis

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INTRODUCTION

During vertebrate lens development, a group of head ectoderm cells thickens and forms the lens placode in response to inductive signals from the underlying optic vesicle at embryonic day (e) 9.5 of mouse embryogenesis (Muthukkaruppan, 1965; McAvoy, 1980; Lovicu and McAvoy, 2005; Medina-Martinez and Jamerich, 2007). By e11.5, the lens placode, through invagination, develops into a lens vesicle in which the primary lens fiber cells in the posterior region eventually exit the cell cycle to elongate toward the anterior wall. Three days later, this elongation is complete, and the fully differentiated fiber cells come into contact with the monolayer of cuboidal lens epithelial cells at the anterior of the eye. Throughout most of life, cell proliferation occurs preferentially in a subset of the epithelial cells located near the equatorial zone. After undergoing cell division, they withdraw from the cell cycle and move posteriorly to differentiate into secondary lens fiber cells (McAvoy, 1980; Piatigorsky, 1981; Lovicu and McAvoy, 2005; Medina-Martinez and Jamerich, 2007). As fiber cells differentiate, they rapidly increase in length and volume and accumulate high levels of crystallins, the proteins that account for the transparency and high refractive index of the lens (Piatigorsky, 1981; Lovicu and McAvoy, 2005; Andley, 2007). After completing elongation, fiber cells partially fuse with their neighbors and degrade all membrane bound organelles, including the nuclei.

The cessation of cell proliferation requires the expression of cyclin-dependent kinase (CDK) inhibitors

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(CKIs). Two families of CKIs have been identified. The Cip/Kip family contains Cdkn1a/p21, Cdkn1b/p27, and Cdkn1c/p57, which inhibit all kinases involved in the G1/S transition. The INK4a family, composed of Cdkn2b/p15, Cdkn2a/p16, Cdkn2c/p18, and Cdkn2d/p19, specifically inhibit Cdk4 and Cdk6, blocking entry into the cell cycle (Harper and Elledge, 1996; Sherr and Roberts, 1995; Nakayama and Nakayama, 1998). Cdkn1b/p27 and Cdkn1c/p57 are coexpressed during murine lens development, especially in the equatorial zone of the fetal lens (Zhang et al., 1998; Nagahama et al., 2001). The withdrawal of lens fiber cells from the cell cycle largely depends on the expression of Cdkn1b/p27 and Cdkn1c/p57 because in mice that lack these genes, fiber cells continue to proliferate and cause incomplete lens fiber elongation. Eventually, these fiber cells undergo apoptosis in Cdkn1b/p27−/− and Cdkn1c/p57−/− mutants (m, denoting maternal active Cdkn1c/p57 allele) compound mutant mice (Zhang et al., 1998). The other cell cycle regulators involved in lens differentiation are the D-type cyclins. All three D-type cyclins are expressed during lens differentiation, with Ccnd2/Cyclin D2 being the most highly expressed cyclin in the posterior region (Zhang et al., 1998). Down-regulation of Ccnd2/Cyclin D2 in the postmitotic lens fiber cell is required for the maintenance of the postmitotic state (Gomez et al., 1999).

Several genes have been identified that play important roles in the development of the lens. Gata3 encodes a transcription factor containing two steroid hormone receptor-like zinc fingers that serve as a DNA binding domain, a motif that is highly conserved amongst all six members (GATA-1 to -6) (Patient and McGhee, 2002). These zinc fingers bind most avidly to the consensus motif AGATCTTA (Ko and Engel, 1993). The physiological roles of GATA-3 have been revealed through the analysis of GATA-3−/− mouse lenses (Oosterwegel et al., 1992; Lakshmanan et al., 1999), although the physiological significance of this observation is unknown. In the present study, we examined the consequences of a GATA-3 loss-of-function mutation in lens development of TgAPDH-G3−/− rescued Gata3 null mutants. We demonstrate that Gata3 inactivation lead to abnormal development of the posterior lens fiber cells, which exhibit reduced levels of the differentiation marker γ-crystallin, sustained expression of lens vesicle marker E-cadherin and the increased signal of proliferation markers, i.e., BrdU incorporation and Ki67 immunoreactivity. The abnormal proliferation of the lens fiber cells in TgAPDH-G3−/− rescued Gata3 null mutant lenses correlates with reduced levels of Cdkn1b/p27 and Cdkn1c/p57 CKIs as well as increased Ccnd2/Cyclin D2 abundance. Subsequently, these cells succumbed to apoptotic cell death. The molecular pathway that regulates lens differentiation is intimately intertwined with normal cell cycle control, and GATA-3 plays an important role in cellular differentiation of lens fiber cells by inducing cell cycle exit as a part of its regulatory functions.

**RESULTS**

**TgAPDH-G3-Rescued Gata3 Null Mutants Displayed Defective Lens Fiber Cell Differentiation**

We previously reported that GATA-3 is expressed in lens fiber cells at e12.5, although its ontogeny in the mammalian lens has not been well described (Lakshmanan et al., 1999). To determine the precise temporal and spatial expression profiles of GATA-3 in the lens, we performed GATA-3 immunofluorescence analysis and whole-mount X-gal staining by examining Gata3lacZ knock-in heterozygous embryos (van Doorninck et al., 1999). In the developing embryonic lens, lacZ expression was first weakly observed at e10.5, then became stronger and was clearly detected in the optic vesicle at e11.5 (Fig. 1A–C). Consistently, GATA-3 immunoreactivity was first specifically observed in the nuclei of e11.5 posterior primary fiber cells (Fig. 1D). By e12.5, when posterior lens fiber cells have normally begun to elongate toward the anterior wall, GATA-3 immunoreactivity was observed in the nuclei of elongating primary lens fiber cells, most prominently in the equatorial zone where fiber cell differentiation first initiates (Fig. 1E,F). GATA-3 immunoreactivity was consistently observed in the fiber cell nuclei along the equatorial zone of e14.5 embryos (Fig. 1G,H), although GATA-3 expression got decreased from e16.5 onward and was hardly observed after birth (data not shown). Importantly, GATA-3 immunoreactivity was observed in differentiated lens fiber cells, but not in proliferating lens epithelial cells of the anterior wall. Hence, GATA-3 expression is strictly confined to the differentiating lens fiber cells of the embryonic eye.

Next, we examined the biological consequences, if any, of Gata3 loss-of-function mutation on lens development, using TgAPDH-G3−/− rescued Gata3 null mutant mice (Moriguchi et al., 2006). Intriguingly, lenses dissected from e16.5 TgAPDH-G3−/− rescued Gata3 null mutant mice were smaller and...
appeared densely opaque when compared with the lenses of wild-type littermates (Fig. 2A). Although the initial formation of the lens vesicle was not affected at e11.5 (Fig. 2B,C), histological analyses of e12.5–e18.5 Tg\(^{\text{TghDBH-G3}}\)-rescued Gata3 null mutants showed that lens development was disrupted later in development. In the mutants, the lens fiber cells appeared as shortened spindle-shaped cells that failed to extend to the anterior of the lens (Fig. 2E,G). By e18.5, a lumen remained visible in the embryonic eye (Fig. 2E,G, I). And moreover, the degradation of fiber cell nuclei, a marker for terminal differentiation of secondary fiber cells, does not take place in the mutants at E18.5 (Fig. 2H,I). This contrasted starkly with the normal lens development in the control embryo, in which the lumen at e12.5 gradually disappeared by e14.5. By e18.5 the secondary lens fiber cells elongated to fill the cavity and properly degraded their nuclei (Fig. 2D,F,H). In keeping with the spatial expression pattern of GATA-3, the an-
terior epithelial cell layer was essentially unaffected in the Tg^{DBRH-G3}-rescued *Gata3* null mutant lens. Hence, these data indicate that GATA-3 is essential for the differentiation of the lens fiber cells from e12.5 onward.

**Crystallin Expression in Tg^{DBRH-G3}-Rescued Gata3 Null Mutant Lens**

The opacity of the GATA-3-deficient lens led us to examine crystallin expression using pan anti-α, pan anti-β, or pan anti-γ-crystallin monoclonal antibodies (Sawada et al., 1993). Unexpectedly, the anti-crystallin staining in the *Gata3*-deficient lens hardly differed from that of wild-type lens (Fig. 3A–F). However, DAPI nuclear staining showed that there was increased nuclear density and disorganized alignment of the mutant lens fiber cells (Fig. 3A–F).

We then examined the mRNA level of each crystallin subtype in e16.5 embryonic lenses using quantitative real-time reverse transcriptase polymerase chain reaction assay (qRT-PCR). α-Crystallins are normally expressed in both lens epithelial and fiber cells, whereas members of the β- and γ-crystallin families are expressed more abundantly in the fiber cells (McAvoy, 1978; Murer-Orlando et al., 1987; Andley, 2007). As anticipated, crystallin mRNA accumulation was moderately to severely reduced in the *Gata3* mutant lenses (Fig. 3G). Of interest, γ-crystallin (γ-A, C, and D) expression, which is most abundantly expressed in fiber cells (Murer-Orlando et al., 1987; Goring et al., 1992; Andley, 2007), was more significantly affected than the other types of crystallins (Fig. 3G). Hence, the presence of GATA-3 activity is essential for normal γ-crystallin expression.

**Aberrant Proliferation and Apoptosis in Tg^{DBRH-G3} rescued Gata3 Null Mutant Lens**

In normal lens development, a precise transition from actively proliferating epithelial cells to terminally differentiating, nonproliferating fiber cells occurs in the equatorial zone of the lens. Our analyses indicated that GATA-3-deficient fiber cells lacked characteristics of fully differentiated lens fiber cells, hence we examined several markers that are indicative of cell proliferation.

E-cadherin is a marker first expressed in lens vesicle cells in early eye development, which then becomes associated with proliferating epithelial cells from e12.5 onward (Wigle et al., 1999; Pontoriero et al., 2009). Although E-cadherin was expressed throughout the entire vesicle of e11.5 wild-type and *Gata3* mutant lenses, the expression pattern was different after initiation of fiber cell elongation (Fig. 4A,B). At e12.5, E-cadherin expression in wild-type lens was restricted to the lens epithelium, whereas in *Gata3* mutant lenses, we observed that the E-cadherin immunoreactivity was still present throughout the lens with stronger staining in the anterior lens epithelium (Fig. 4C,D). The prolonged E-cadherin expression in the posterior lens of e12.5 *Gata3* mutants was quenched 2 days later, as anti-E-cadherin staining was similar in e14.5 wild-type and *Gata3*-deficient lenses, although the size of the mutant lens never caught up to wild-type control (Fig. 4E,F). These observations suggest that the *Gata3*-deficient posterior lens vesicle cells failed to fully differentiate into fiber cells, so that the posterior half of the lens vesicle retained epithelial cell properties longer than their e12.5 wild-type counterparts.

During later lens development, Ki67- or bromodeoxyuridine (BrdU) -positive proliferating cells were exclusively observed in the epithelial layer of e12.5 and e16.5 wild-type embryos (arrowheads in Fig. 5A,C,E,G). However, e12.5 and e16.5 *Gata3*^-/-:Tg^{DBRH-G3} lenses had a substantially greater number of Ki67- or BrdU-immunopositive nuclei in the posterior fiber cell zone (arrowheads in Fig. 5B,D,F,H,I). Concomitantly, we also detected increased number of programmed cell death in the posterior region of lenses from e12.5 and e16.5 *Gata3*^-/-:Tg^{DBRH-G3} embryos, whereas TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) -positive nuclei were only rarely detected in the lenses of wild-type control embryos (Fig. 6A–E). This conclusion was further substantiated by the flow cytometric analysis using Annexin-V as an apoptotic cell marker (van Engeland et al., 1998). Lenses from e18.5 *Gata3*^-/-:Tg^{DBRH-G3} or wild-type embryos were dispersed by trypsin treatment, and then single cell suspensions were stained with Annexin-V and 7-Amino Actinomycin D (7-AAD; nucleic acid dye) before being analyzed by flow cytometry. Early apoptotic cells resided in the Annexin-V-single positive fraction, viable cells were negative for both Annexin-V and 7-AAD, and late apoptotic and necrotic cells stained positively for both (Lecoeur et al., 1997; van Engeland et al., 1998; Rasola and Geuna, 2001). As shown in Figure 6F,G, the Annexin-V-single positive fraction (early apoptotic cells) was dramatically increased in the *Gata3*^-/-:Tg^{DBRH-G3} lens (10.85 ± 0.23%; n = 5) in comparison to wild-type lens (3.12 ± 1.23%; n = 5). Late apoptotic and necrotic cells (positive for both 7-AAD and Annexin-V) also significantly increased in the *Gata3*^-/-:Tg^{DBRH-G3} lens (3.09 ± 0.06%; n = 5) compared with the wild-type lens (1.06 ± 0.23%; n = 5; Fig. 6F,G). Hence, the *Gata3*-deficient lens fiber cells display epithelial cell property as well as abnormally high proliferative and apoptotic indices.

**Elevated Ccnd2/Cyclin D2, Diminished Cdkn1b/p27 and Cdkn1c/p57 Levels in Gata3^-/-: Tg^{DBRH-G3} Lens Fiber Cells**

Given the abnormal accumulation of proliferative or apoptotic lens fiber cells, we next examined the expression of the cell cycle regulators Cdkn1b/p27, Cdkn1c/p57, and Ccnd2/cyclin D2 in wild-type and *Gata3*^-/-: Tg^{DBRH-G3} lenses. At e12.5, posterior lens vesicle cells begin to express Cdkn1b/p27 and Cdkn1c/p57 in the wild-type lenses (Fig. 7A,E). In e16.5 wild-type embryos, Cdkn1b/p27 and Cdkn1c/p57 were expressed predominantly in the equatorial zone where the epithelial cells exit cell cycle to differentiate into lens fiber cells (Fig. 7C,G). However, both Cdkn1b/p27 and Cdkn1c/p57 expression was conspicuously reduced in e12.5 and e16.5 *Gata3*^-/-:Tg^{DBRH-G3} lenses (Fig. 7B,D,F,H). Meanwhile, anti-Ccnd2/Cyclin D2 labeled nuclei were observed in the equatorial zone of the e16.5 wild-type lens (Fig. 7I), but the fiber cells in the equatorial zone of the e16.5 *Gata3*^-/-:Tg^{DBRH-G3} lens dis-
played significantly more abundant Ccnd2/Cyclin D2 immunoreactivity (Fig. 7J). Indeed, mRNA quantification of isolated e16.5 embryonic lenses demonstrated that both Cdknb1/p27 and Cdk1c/p57 mRNA expression was suppressed and that Ccnd2/Cyclin D2 mRNA expression was activated, consistent with the immunohistochemical observations (Fig. 7K).

Thus, these data indicate that, in the absence of GATA-3, the lens fiber cells exhibited impaired terminal differentiation as evidenced by the abnormal lens morphology, misexpression of epithelial cell characteristics and reduced γ-crystallin levels. Instead, they remained Cdknb1/p27-negative, Cdk1c/p57-negative, and Ccnd2/Cyclin D2-positive and failed to properly exit the cell cycle, probably undergoing apoptotic cell death.

**Elevated GATA-3 Expression in c-Maf Knock-out Mice**

To address possible genetic programs in which GATA-3 might participate during lens development, we examined the expression of several transcription factors that were previously implicated in the regulation of lens development. Prox1 is a homeobox protein that is essential for fiber cell differentiation. Prox1 deficiency in mice leads to aberrant fiber cell proliferation accompanied by suppression of Cdknb1/p27, Cdk1c/p57, and γ-crystallins, an alteration in expression that is similar to what is observed in the GATA3-deficient lens (Wigle et al., 1999). Indeed, immunohistochemical analysis of Prox1 demonstrated a quite similar expression pattern in the e14.5 lens to that of GATA-3, except for the epithelial expression (compare Fig. 1F and Fig. 8A). Quantitative RT-PCR performed on GATA-3–deficient e16.5 lenses showed that Prox1 mRNA level was only modestly suppressed in comparison to wild-type controls (Fig. 8C), and Prox1 immunoreactivity was virtually identical in the e14.5 GATA-3-deficient and wild-type lens (Fig. 8A,B). Additionally, we examined the mRNA expression of Sox1, Pax6, and Foxe3, all of which are required for normal lens development and crystalline gene expression. However, the abundance of those transcription factor mRNAs was essentially unchanged in the Gata3 mutant lens (Hogan et al., 1986; Hill et al., 1991; Matsuo et al., 1993; Nishiguchi et al., 1998; Medina-Martínez et al., 2005; Supp. Fig. S1, which is available online).

c-Maf is a basic leucine zipper transcription factor that is expressed specifically in lens fiber cells of the equatorial zone, and is essential for early lens morphogenesis as well as for crystalline gene activation (Kim et al., 1999; Kawauchi et al., 1999; Ring et al., 2000). c-Maf mRNA levels, as was the case with the previously examined factors, were unchanged in the GATA-3–deficient lens (Fig. S1). However, given the coincident expression pattern of c-Maf and GATA-3 in lens fiber cells, we also examined GATA-3 expression in the c-Maf–deficient embryonic lens, assuming a possible regulatory interaction between those genes in the lens fiber hierarchy. Of interest, we observed an almost eightfold increase of GATA-3 mRNA in c-Maf-deficient e16.5 lens (Fig. 9C). In concert with the elevation in GATA-3 mRNA levels in the c-Maf mutants, increased GATA-3 immunoreactivity was recorded in all vesicle cells of the c-Maf–deficient dysplastic remnant lens at e14.5, demonstrating that GATA-3 expression is either directly or indirectly negatively regulated by c-Maf during normal lens fiber cell development (Fig. 9A,B).

**DISCUSSION**

In the present study, we demonstrated that GATA-3 expression begins in the developing lens vesicle at mid-embryogenesis (around e11.5) and continues to be expressed in fiber cells throughout embryonic lens development. Its expression is specifically restricted to fiber cells during lens morphogenesis. Consistent with its spatiotemporal expression in the developing lens, the absence of GATA-3 led to interrupted differentiation of posterior lens fiber cells from e12.5 onward, as evidenced by the diminished γ-crystallin levels and prolonged E-cadherin expression in primary lens fiber cells. There was also an increase of mitotic (BrdU- or Ki67-immunopositive) and apoptotic fiber cells in the GATA-3–depleted lens.

**Cell Cycle Regulation by GATA Factors Has Been Reported in a Variety of Different Tissues**

Recently, it was reported that Gata2-deficient mouse embryonic neuroepithelial cells exhibited aberrant proliferation and that GATA-2 overexpression induced neural differentiation by inhibiting the proliferation of neuronal progenitors by means of activation of Cdknb1/p27 expression (El Wakil et al., 2006). In erythroid cell differentiation, GATA-1 was reported to induce erythro-megakaryocytic differentiation by suppressing the active cell cycle of hematopoietic progenitor cells by means of induction of Cdknb2/p16 expression (Pan et al., 2005). Although it is still unclear if GATA-2 or GATA-1 directly regulates Cdknb1/p27 or Cdknb2/p16 expression, respectively, these reports as well as the present observations suggest that the potential cell cycle regulatory function for GATA factors in the normal differentiation process acts by activating expression of CKIs. GATA-3
Altered cellular proliferation in the lenses of Gata3+/+ embryos.

A–H: In wild-type embryos, Ki67- or bromodeoxyuridine (BrdU) -immunoreactive cells were observed exclusively in the anterior lens epithelium (arrowheads in A,C,E,G), whereas an increased number of mitotic cells were present in the lens fiber cells located in the lens posterior (arrowheads in B,D,F,H) in e12.5 and e16.5 Gata3+/+ embryos. le, lens epithelium; lf, lens fiber; re, retina.

I: Quantification of Ki67-positive cells in e12.5 and e16.5 embryonic lenses epithelial and fiber cells from wild-type (n = 6) and Gata3+/+ (n = 6) embryos (*P < 0.05; Student’s t-test). Six lenses from six different embryos of each genotype were analyzed. Data are presented as means ± SEM. The statistical significance of the differences between Gata3+/+ and Gata3+/+ are indicated by (*P < 0.05; Student’s t-test). Scale bars = 100 μm.

Fig. 3

Fig. 4

Fig. 5.
has been reported to suppress abnormal proliferation of mesonephric cells as well as mammary epithelial cells, although the molecular basis for these phenomena remains elusive (Grote et al., 2006; Kourous-Mehr et al., 2006). More recently, transcriptome analysis of GATA-3 conditional deletion in hair follicles indicated that multiple cell cycle regulatory genes were altered in expression (Kurek et al., 2007). Further studies will be necessary to determine how GATA-3 functionally coordinates cell cycle regulation with normal differentiation in a variety of GATA-3–expressing tissues, including lens fiber cells.

Although the mechanistic details of how the loss of GATA-3 results in lens fiber differentiation failure remains to be elucidated, cell cycle regulators may be the potential key molecules underlying the abnormal increase of proliferating cells. In the wild-type lens, the epithelial cells near the equatorial zone exit the cell cycle to give rise to fiber cells, and in the process, they initiate the expression of Cdkn1b/p27 and Cdkn1c/p57. Cdkn1b/p27 and Cdkn1c/p57 cooperatively control the cell cycle exit and the subsequent differentiation of lens fiber cells. Cdkn1b/p27 is normally dispensable for lens development due to its redundancy with Cdkn1c/p57, whereas Cdkn1b/p27–/– and Cdkn1c/p57–/–, like the Gata3–deficient mice, exhibit significant deficiencies in cell cycle withdrawal and in the subsequent differentiation of lens fiber cells (Zhang et al., 1998; Nagahama et al., 2001). In the Gata3–deficient lens, we demonstrated that Cdkn1b/p27 and Cdkn1c/p57 are either not direct target genes of GATA-3 or that GATA-3 regulates those genes through enhancers that lie outside of the promoter boundaries.

We examined Sox1, Foxe3, Prox1, c-Maf, and Pax6 mRNA expression in e16.5 GATA-3–deficient lenses to examine potential genetic interactions between GATA-3 and each of those other known lens developmental regulators. Sox1 expression initiates in the lens vesicle at around e10 and continues to be expressed in lens fiber cells at e15.5 (Nishiguchi et al., 1998). Foxe3, Prox1, and c-Maf expression is first detected at around e9.0 to e9.5 over the lens placode (Wigle et al., 1999; Kawauchi et al., 1999; Medina-Martinez et al., 2005). Foxe3 expression later becomes restricted to the anterior lens epithelium, while Prox1 and c-Maf expression are maintained in the lens fiber cells (Wigle et al., 1999; Kawauchi et al., 1999; Medina-Martinez et al., 2005). Pax6 expression is observed much earlier (in head neural ectoderm) including in the optic pit at e8.0, although from e13.5

![Fig. 6. Increased cell death in lens fiber cells of Gata3–/–:TgDBH-G3 mutant embryos. A–D: TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) assays detected an increase in the number of apoptotic cells in the posterior chamber of embryonic day (e) 12.5 and e16.5 Gata3–/–:TgDBH-G3 lenses (arrowheads). If, lens fiber; re, retina. E: Quantification of TUNEL-positive cells in e12.5 and e16.5 embryonic lenses of wild-type (n = 6) and Gata3–/–:TgDBH-G3 (n = 6) embryos. Six lenses from six different embryos of each genotype were analyzed. Data are presented as means ± SEM. The statistical significance of the differences between Gata3–/–:TgDBH-G3 and Gata3–/– is indicated by *(P < 0.05; Student’s t-test). F: Representative flow cytometric profiles of single-cell suspensions that were dissociated from lenses of e18.5 wild-type or Gata3–/–:TgDBH-G3 mutant wild-type and e15.5 GATA-3–deficient lenses, stained with PE-conjugated Annexin-V antibody (horizontal axis) and 7-AAD (vertical axis). The percentage of cells in each quadrant is indicated. G: In the lens fiber cells from e18.5 Gata3–/–:TgDBH-G3 embryos, the Annexin-V–single positive (SP) population, representing early apoptotic cells, increased by more than three-fold (10.85 ± 0.23% in Gata3 mutant [n = 5], 3.09 ± 1.23% in wild-type control [n = 5]). The 7-AAD– and Annexin-V–double positive (DP) cell population (representing late apoptotic and necrotic cells) also increased by more than two-fold (1.06 ± 0.23% in Gata3 mutant [n = 5], 3.09 ± 0.06% in wild-type control [n = 5]). Scale bars = 100 μm.]

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onward, Pax6 expression is down-regulated in lens fiber cells (Grindley et al., 1995; Donner et al., 2007). Given those spatiotemporal expression patterns and the similarities in lens deficiencies encountered in various mutant mice, we initially expected to establish a genetic regulatory relationship between GATA-3 and Sox1 or Prox1 expression in the developing lens fiber cells. However, all of those transcriptional regulators are in general only modestly, if at all, changed in the GATA-3 deficient lens. Given the later appearance of GATA-3 expression in the e10.5 lens vesicle as well as the relatively mild lens deficiency in $Gata3$ mutant mice, we assumed that GATA-3 might be located at a lower position in the hierarchy, but upstream of $\gamma$-crystallin and both CKIs (Cdkn1b/p27 and Cdkn1c/p57) in the genetic program of lens development. Of interest, GATA-3 expression is strongly activated in the remnants of the c-Maf–deficient lens. This observation clearly demonstrates that GATA-3 expression is directly or indirectly negatively controlled by c-Maf in normal developing lens fiber cells, so that a c-Maf deficiency derepresses GATA-3 expression, possibly to compensate for the suppressed crystallin gene activation. Precise mapping of lens-specific $Gata3$ gene regulatory sequences, which are presumably located within a 2-kbp region lying 5’ to the gene (George et al., 1994; Lieuw et al., 1997), will provide additional insight into the identities of upstream regulators of GATA-3 expression in lens fiber cells.

During differentiation, mature lens fiber cells produce abundant $\alpha$- and $\gamma$-crystallins (McAvoy, 1978). Of the crystalline subtypes, $\alpha$-crystallins are normally expressed in both lens epithelial and fiber cells, and are first expressed at the lens vesicle stage (McAvoy, 1978; Murer-Orland et al., 1987; Goring et al., 1992; Horwitz, 2003). $\gamma$-Crystallin expression, which begins at e11 in the mouse embryo, serves as an early marker of fiber cell differentiation, whereas $\gamma$-crystallin gene activation initiates around e12.5 (Goring et al., 1992; Nishiguchi et al., 1998; Ring et al., 2000). We showed here that $\gamma A$, $\gamma C$ and $\gamma D$-crystallin expression, which are normally restricted in expression to terminally differentiated lens fiber cells, is markedly increased in the $Gata3$ deficient lens. It appears that GATA-3 is involved in repression of the expression of these genes in the developing lens, as a c-Maf deficiency derepresses GATA-3 expression, which in turn might be involved in the activation of the $\gamma$-crystallin gene family. Precise mapping of lens-specific $Gata3$ gene regulatory sequences, which are presumably located within a 2-kbp region lying 5’ to the gene (George et al., 1994; Lieuw et al., 1997), will provide additional insight into the identities of upstream regulators of GATA-3 expression in lens fiber cells.
differentiated fiber cells, were more diminished than αA- and β1-crystallin in Gata3−/−:TgDBH-G3 lenses. In the Gata3 mutant lens, the fiber cell differentiation failure is associated with aberrant accumulation of mitotic posterior cells. There are two possible explanations for this observation. One is that GATA-3 primarily promotes fiber cell differentiation, i.e., activation of γ-crystallin genes as well as suppression of E-cadherin expression, so that a GATA-3 deficiency would primarily induce a fiber cell deficiency which in turn would lead to the accumulation of premature epithelial cell-like posterior cells. The other possibility is that the GATA-3 deficiency primarily but indirectly leads to transcriptional suppression of Cdkn1b/p27 and Cdkn1c/p57, which then cause the failure of cell cycle cessation during epithelial to fiber cell transition. Consequently, the posterior lens fiber cells do not fully differentiate and eventually apoptosis. Although these two explanations could both be partially correct, cell differentiation and cell cycle cessation are probably tightly interwoven. It will, therefore, be of great interest to further define how GATA-3 functions during induction of cell differentiation as well as how it regulates cell cycle suppression during lens development.

In conclusion, we demonstrated here that GATA-3 is essential for terminal differentiation of lens fiber cells. It will be intriguing to clarify the underlying mechanisms by which the

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**Fig. 8.** Expression of Prox1 in Gata3 mutant lenses. **A,B:** Prox1 immunoreactivity was approximately equal in embryonic day (e) 16.5 Gata3−/−:TgDBH-G3 and Gata3+/+ littermate embryonic lenses. **C:** e16.5 Gata3−/−:TgDBH-G3 (n = 7) embryonic lenses also had statistically equal levels of Prox1 mRNA in comparison with wild-type control embryos (n = 6). Data are presented as mean ± SEM (normalized to Hprt mRNA).

**Fig. 9.** GATA-3 expression is de-repressed in c-Maf mutant lenses. **A,B:** The embryonic day (e) 14.5 c-Maf mutant lenses had significantly greater GATA-3 immunoreactivity than wild-type littermates. **C:** Real-time RT-PCR quantification of GATA-3 mRNA in e16.5 wild-type and c-Maf mutant lenses (normalized to Hprt mRNA). Data are presented as mean ± SEM. The statistical significance of the differences between c-Maf+/+ (n = 5) and c-Maf−/− (n = 6) are indicated (**P < 0.01; Student’s t-test).
expression of CKIs and CDKs are controlled by GATA factors, and to identify other cell cycle/apoptosis-related factors which might be responsible for the increased cell death observed in the Gata3-deficient lens fiber cells. We conclude, from the data presented here, that the Gata3 mutant mouse lens may serve as another useful model for elucidating the general principles of cell cycle regulation by GATA transcription factors.

**EXPERIMENTAL PROCEDURES**

**Mice**

Generation of c-Maf knock-out mice, Gata3lacZ knock-in mice, Gata3 knock-out mice (Gata3+/−), SA lineage-specific GATA-3–expressing transgenic mice (TghDBH-G3) and Gata3+/−: TghDBH-G3 compound heterozygotes were reported previously (Pandolfi et al., 1995; Kawauschi et al., 1999; van Doorninck et al., 1999; Moriguchi et al., 2006). Animals were genotyped by PCR and/or Southern blotting as previously reported (Moriguchi et al., 2006). Primers used to detect the hDBH-GATA-3 transgene are shown in Table 1. All experiments were performed according to the Guide for the Care and Use of Laboratory Animals at the University of Michigan and the University of Tsukuba.

**qRT-PCR**

Total RNA was extracted from isolated lens tissues of e16.5 wild-type or mutant embryos using TRIZOL (Invitrogen Corp, Carlsbad, CA). First-strand cDNA was synthesized starting with 0.5 μg of total RNA using Superscript III (Invitrogen). qRT-PCR was performed using an ABI PRISM 7700 sequence detector (PE-Applied Biosystems, Foster City, CA) with a 2X SYBR Green PCR master mix (Invitrogen), reverse transcribed cDNA and gene-specific primers as previously described (Moriguchi et al., 2006). The sequences of the primers are listed in Table 1. The data were recorded as means ± standard error of the mean. The statistical significance of differences among means of several groups was determined by Student’s t-test.

**Histological Analysis, Immunofluorescence, and TUNEL Assays**

Embryos (e12.5–e18.5) were fixed overnight in 4% paraformaldehyde at 4°C and then processed for paraffin or frozen sections. Paraffin sections (3 μm) were cut with a microtome and stained with mouse anti-BrdU (Becton Dickinson, San Jose, CA), rabbit anti–E-cadherin (Chemicon, CA), rabbit anti–E-cadherin (Taken Biotech, Tokyo, Japan), rabbit antibody anti–Cdkn1b/p27, goat anti–Cdkn1c/p57, and rabbit anti-Cyclin D2 (all from Santa Cruz Biotechnology, Santa Cruz, CA). For immunofluorescence staining, Alexa Fluor 488-conjugated donkey anti-goat and goat anti-rabbit IgG (Molecular Probes, Eugene, OR) or fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (Zymed, San Francisco, CA) secondary antibodies were used. Whole-mount X-gal staining was performed as previously described (Lakshmanan et al., 1999).

To analyze 5-bromo-2’-deoxyuridine (BrdU) uptake, pregnant females were administered BrdU (100 μg/gram of body weight) by intraperitoneal injection. After 2 hr, embryos were collected and fixed overnight in 4% PFA. Sections were collected and fixed overnight in 4% PFA. Sections were collected and fixed overnight in 4% FPA. Sections were then stained with mouse anti–BrdU (Becton Dickinson, San Jose, CA). Ki67 is a nuclear protein expressed in all proliferating cells during late G1, S, M, and G2 phases of the cell cycle (Gerdes et al., 1984, 1991). Rabbit anti-Ki67 (Novocea Laboratories Ltd, UK) was used for detection.

TUNEL assays were performed using the In Situ Apoptosis Detection Kit (Takara BIOTECH) according to

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**TABLE 1. Sequence of Primers Used in Quantitative RT-PCR Analyses and Genotyping**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense primer</th>
<th>Antisense primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>hDBH-GATA-3</td>
<td>AGT GAC CAG CTA CAG TCG GA</td>
<td>GGA GAG GGG TCG TTT AAT GG</td>
</tr>
<tr>
<td>GATA-3</td>
<td>GGT GGA CTT ACT TTT TTA CAT CGA</td>
<td>CCC TGA CGG AGT TTC CGT AG</td>
</tr>
<tr>
<td>Crystallin αA</td>
<td>ACA AGC AGA GCC AGG ATC GC</td>
<td>AGG GGA CAA CCA AGG TGA G</td>
</tr>
<tr>
<td>Crystallin β1</td>
<td>AAC TTC CAG GGC AAG AGG AT</td>
<td>AGA TGG GTC GGA AGG ACA T</td>
</tr>
<tr>
<td>Crystallin γA</td>
<td>CTC GTG GTA GCG CCT GTA GT</td>
<td>GTC GTG GTA GCG CCT GTA GT</td>
</tr>
<tr>
<td>Crystallin γC</td>
<td>TGG TCC CTC ATC CCC CAA CA</td>
<td>TCC CTT AAA AGA GCC AAC TT</td>
</tr>
<tr>
<td>Crystallin γD</td>
<td>CTG CTG GAT GCT CTA TGA GC</td>
<td>TTC CGT GAA CTC TAT CAC TTG GC</td>
</tr>
<tr>
<td>Cdkn1p57</td>
<td>GAG GAC CAG AAC CGC TGG GAC CT</td>
<td>ACT CGC TGT CCA CCT TCA TCA A</td>
</tr>
<tr>
<td>Cdkn1p27</td>
<td>CGC CAT TAG CGC AAC TGA</td>
<td>CGG CTG CAG AGA TTA GGG</td>
</tr>
<tr>
<td>Ccdc1/Cyclin D1</td>
<td>TCT ATC CCG CCC GAG</td>
<td>CAG CCT GTT CAC CAG AGG CAG</td>
</tr>
<tr>
<td>Ccdc2/Cyclin D2</td>
<td>ACT GTG TGG ATT GTTGCTCAAGGCT</td>
<td>CCA CGC GGC ACA ATA GCA ACTACG</td>
</tr>
<tr>
<td>Ccdc3/Cyclin D3</td>
<td>GCC TAT GAA CTA CCT GGA TCG CTA</td>
<td>GTC CCT AGA AGC TGC AAT TG</td>
</tr>
<tr>
<td>Prox1</td>
<td>GCT CCA ACA TGC TGA AGA CC</td>
<td>TCA TGG ATG TGA CGC GC</td>
</tr>
<tr>
<td>Pdx6</td>
<td>GGA GAG AAC ACC AAC TCC AT</td>
<td>TCT GGA TAA TGG GTC TTC TC</td>
</tr>
<tr>
<td>Foxe3</td>
<td>AGT GGC AGA AGA GCA TCC GC</td>
<td>TCG AGC GTC CAG TAG TTG CC</td>
</tr>
<tr>
<td>Sox1</td>
<td>AAG ATG CAC AAT CCG AGA TAC ATG</td>
<td>TGT AAT CCG GGT CCT TCA T</td>
</tr>
<tr>
<td>c-Maf</td>
<td>CTG CCG CTT CAA GAG GGT GAC GA GC</td>
<td>TCG GCT GTC ACA CTC TCA TG</td>
</tr>
<tr>
<td>HPRT</td>
<td>CAA ACT TTG TT CTC CTG GT</td>
<td>CAA GGG CAT ATC CAA CAA CA</td>
</tr>
</tbody>
</table>
the manufacturer's instructions. For quantification, three transverse sections extending from the center of the lens of each e12.5 or e16.5 embryo were examined by TUNEL or anti-Ki67 antibody (Novocastra Laboratories Ltd, UK). The numbers of TUNEL-positive fiber cells and Ki67-immunoreactive epithelial or fiber cells on the sections was individually determined for each embryo.

Flow Cytometric Analysis of Apoptosis in Lens

Single cell suspension was prepared from e18.5 lens of each mouse genotype by treatment with 0.05% trypsin and 0.53 mM ethylenediaminetetraacetic acid (GIBCO BRL, Gaithersburg, MD) at 37°C for 30 min, and cells were dissociated using fine-tipped pipettes. After the cells were filtered through a 35-μm nylon mesh screen, they were resuspended in phosphate buffered saline containing 4% fluorescence cell sorting. Apoptotic cell analysis was performed using Annexin-V:PE Apoptosis Detection Kit I (BD-Biosciences, San Jose, CA) according to the manufacturer's instructions. Apoptotic cells were stained with Annexin-V, while necrotic cells were distinguished by staining with 4% fluorescence cell sorting. Apoptotic peripheral human lymphocytes were also stained with the FACS LSR and 7-AAD (Herault et al., 1999). FACS analysis was performed according to the manufacturer's instructions. Apoptotic cells were stained with Annexin-V, while necrotic cells were distinguished by staining with both Annexin-V and 7-AAD (Herault et al., 1999). FACS analysis was performed with the FACS LSR and CellQuest software (BD-Biosciences).

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