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Regulation of Mouse Lens Maturation and Gene Expression by Krüppel-Like Factor 4

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Abstract

Conditional disruption of *Klf4* in the surface ectoderm-derived tissues of the eye results in defective cornea, conjunctiva and the lens. This report describes the effects of disruption of *Klf4* in the lens in greater detail. Expression of *Klf4*, first detected in the embryonic day-12 (E12) mouse lens, peaked at E16 and was decreased in later stages. Early embryonic disruption of *Klf4* resulted in a smaller lens with cortical vacuolation and nuclear opacity. Microarray comparison of *Klf4*CN and WT lens transcriptomes revealed fewer changes in the E16.5 (59 increases, 20 decreases of >1.5-fold) than the PN56 *Klf4*CN lens (239 increases, 182 decreases of >2-fold). *Klf4*-target genes in the lens were distinct from those previously identified in the cornea, suggesting disparate functions for *Klf4* in these functionally related tissues. Transcripts encoding different crystallins were down-regulated in the *Klf4*CN lens. *Shsp/aB-crystallin* promoter activity was stimulated upon co-transfection with pCI-*Klf4*. Mitochondrial density was significantly higher in the *Klf4*CN lens epithelial cells, consistent with mitochondrial dysfunction being the most significantly affected pathway within the PN56 *Klf4*CN lens. The *Klf4*CN lens contained elevated levels of *Alox12* and *Alox15* transcripts, less reduced glutathione (GSH) and more oxidized glutathione (GSSG) than the WT, suggesting that it is oxidatively stressed. Although the expression of 2087 genes was modulated during WT lens maturation, transcripts encoding crystallins were abundant at E16.5 and remained stable at PN56. Among the 1065 genes whose expression increased during WT lens maturation, there were 104 *Klf4*-target genes (9.8%) with decreased expression in the PN56 *Klf4*CN lens. Taken together, these results demonstrate that *Klf4* expression is developmentally regulated in the mouse lens, where it controls the expression of genes associated with lens maturation and redox homeostasis.

Keywords

Lens; Development; Maturation; Gene Expression; KLF4

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Introduction

A complex network of transcription factors regulates the well-orchestrated events in embryonic development and postnatal maturation of the ocular lens (Chow and Lang, 2001; Cvekl and Duncan, 2007; Cvekl and Piatigorsky, 1996). Chief among them is the paired box-homeobox factor Pax6 required for lens progenitor cell formation in the pre-placodal region of the mouse embryo, where it controls the expression of other transcription factors such as *FoxE3*, *c-Maf*, *Sox1*, *Sox2*, *Pitx3*, and *Prox1* necessary for lens morphogenesis (Blixt et al., 2007; Bremond-Gignac et al., 2010; Chauhan et al., 2002a; Chauhan et al., 2002b; Kondoh et al., 2004; Shaham et al., 2009; Xie and Cvekl, 2011; Yang et al., 2004; Yang and Cvekl, 2005; Yang et al., 2006). In the differentiating fiber cells, Pax6 regulates the expression of *crystallins* (Cvekl and Duncan, 2007; Cvekl and Piatigorsky, 1996) and $\alpha 5\beta 1$ integrin complex (Donner et al., 2007; Duncan et al., 2000). ChIP-chip and microarray analysis of the *Pax6* heterozygote mouse lens identified the Pax6-target genes in the lens (Chauhan et al., 2002b; Xie et al., 2013). Several other transcription factors, such as HSF4 (Bu et al., 2002; Fujimoto et al., 2004; Somasundaram and Bhat, 2004), AP-2 α (West-Mays et al., 1999), Cited2 (Chen et al., 2008b), Meis (Zhang et al., 2002), and Msx2 (Zhao et al., 2012) play critical roles during lens morphogenesis. Additional transcription factors regulating mammalian lens development and/or maturation are expected to be uncovered in the near future as the methods to generate genetically modified mouse strains improve (Wang et al., 2013).

Krüppel-like factor 4 (Klf4) is a key regulator of epithelial cell development in several tissues (Adam et al., 2000; Black et al., 2001; Chen et al., 2002; Higaki et al., 2002; Jenkins et al., 1998; Miller et al., 2001; Shields and Yang, 1998; Swamynathan, 2010). Expression of *Klf4* begins around embryonic day 10 in mesenchymal cells extending from the forelimb bud to the developing eye (Ehlermann et al., 2003), and is retained in differentiated skin and gastrointestinal epithelial cells (Garrett-Sinha et al., 1996; Shields et al., 1996) and cornea (Norman et al., 2004). *Klf4*-null mice die within 15 h after birth due to late-stage defects in skin development (Segre et al., 1999). Conditional disruption of *Klf4* in the head surface ectoderm derived tissues results in corneal epithelial fragility, stromal edema, loss of conjunctival goblet cells and a small lens with extensive vacuolation in the anterior corticular region (Swamynathan et al., 2011; Swamynathan et al., 2007). Microarray comparison of the wild type (WT) and *Klf4*-conditional null (*Klf4*CN) corneal gene expression suggested that Klf4 contributes to corneal homeostasis by regulating the expression of genes involved in cell cycle progression, cell-cell adhesion, epithelial barrier formation, expression of corneal crystallins, and maintenance of corneal hydration (Swamynathan et al., 2008). Similar comparison in the conjunctival fornix identified the conjunctival Klf4-target genes that differed significantly from the corneal Klf4-target genes, suggesting tissue-dependent functions for Klf4 (Gupta et al., 2011). The *Klf4*CN lens was small with anterior and equatorial corticular vacuolation (Swamynathan et al., 2007).

In this report, we present the results of comparison of the embryonic day-16.5 (E16.5) and post-natal day 56 (PN56) WT and *Klf4*CN lens transcriptomes to catalogue the (i) changes in gene expression accompanying the WT lens maturation, and (ii) Klf4-target genes that are responsible for the *Klf4*CN lens phenotype. We demonstrate that (i) *Klf4* expression is developmentally regulated in the lens, (ii) Klf4 regulates a unique set of target genes in the lens that is distinct from its target genes in the other parts of the eye, and that (iii) Klf4 contributes to lens maturation and maintenance of redox homeostasis by regulating the expression of genes associated with mitochondrial functions, arachidonic acid metabolism, aryl hydrocarbon receptor signaling, and glutathione metabolism.

Materials and Methods

Conditional Deletion of *Klf4* Gene

Klf4^{ΔCN} mice were generated by mating *Klf4*^{loxP/loxP}, Le-Cre^{-/-} mice with *Klf4*^{loxP/loxP} mice and genotyped as described earlier (Ashery-Padan et al., 2000; Katz et al., 2002; Swamynathan et al., 2007). Lenses from E12, E16, PN1, PN13, PN21 and 8-week-old mice were used for quantification of *Klf4* transcripts in the lens, while rest of the experiments were done using E16.5 and/or 8-week-old mice. Dissected lenses were immersed in PBS (pH 7.4) for photography, in 4% paraformaldehyde in PBS for histology, and in RNA Later (Applied Biosystems/Ambion, Austin, TX) for RNA isolation. All procedures described herein were approved by the University of Pittsburgh Institutional Animal Care and Use committee and conformed to the ARVO statement on use of animals in research.

Lens Measurements and Histology

Lenses from 3 WT and *Klf4*^{ΔCN} mice each were photographed immediately after dissection by using Leica MZFL III Microscope (Leica Microsystems GmbH, Wetzlar, Germany) and QICAM Fast 1394 cooled mono 12 bit imaging system (Qimaging Surrey, BC, Canada). Light micrographs generated by focusing at the equatorial plane of the mouse lens using 1.6X objective and transmitted light were used for measuring the diameter and intensity of scattered light at the center of the lens using Metamorph 7.5.6 software (Molecular Devices, Sunnyvale, CA). Histology was studied by light microscopy (Nikon Eclipse TS100 microscope) of hematoxylin and eosin stained sections from 4% paraformaldehyde fixed eyes embedded in glycol methacrylate (Polysciences, Warrington, PA) (Swamynathan et al., 2007). Transmission electron microscopy (TEM) of ultrathin sections collected on 300-mesh grids was performed using JEM-100CX electron microscope (JEOL USA, Inc., Peabody, MA) (Swamynathan et al., 2007). Seven different TEM images were used to quantify the mean density of mitochondria in the WT and *Klf4*^{ΔCN} lens epithelial cytoplasm.

Lens RNA Isolation and Quantification of *Klf4* Transcripts

Total RNA was isolated from dissected lenses from E12, E16, PN1, PN13, PN21 and 8 week-old WT mice using RNeasy micro kit (Qiagen, Valencia, CA) and quantified using Nanodrop Spectrophotometer ND1000 (Nanodrop Technologies Inc. Wilmington, DE). Superscript® VILO™ cDNA Synthesis kit (Invitrogen, Carlsbad, CA) was used to prepare cDNA using 40 ng RNA from different developmental stages. Standard curve method was used to calculate the number of *Klf4* transcripts in the lens at different developmental stages (Zhang et al., 2001) using a standard curve generated with linearized plasmid pCI-*Klf4* as template following published protocols (Whelan et al., 2003). The reagents and the equipment used for quantitative PCR (QPCR) were from Applied Biosystems Inc., Foster City, CA.

In Situ Hybridization

In situ hybridization was performed as earlier (Gupta et al., 2011; Norman et al., 2004) using 12 μm cryosections cut from OCT-embedded eye tissues flash-frozen in liquid nitrogen. Freshly cut sections were fixed in 4% paraformaldehyde, treated with proteinase K (0.2 μg/mL PBS) for 5 minutes, and processed as described earlier (Gupta et al., 2011; Norman et al., 2004). Riboprobes were synthesized using digoxigenin (DIG) RNA labeling kit (Sp6/T7; Roche Molecular Biochemicals, Indianapolis, IN) with linearized plasmid cDNA templates for respective genes. Color development reaction was allowed to proceed for 60 minutes, after which the reactions for both the sense and antisense riboprobes were terminated at the same time. Images were acquired by light microscopy (Nikon Eclipse TS100 microscope) as above.

Microarray Analysis

Total RNA was isolated from WT or *Klf4*CN lens at E16.5 (three independent preparations, each pooled from 5 embryos) or at PN56 (two independent preparations, each pooled from 3 mice). RNA of good quality (RNA integrity number > 5.0) was subjected to microarray analysis using Mouse 430 2.0 arrays (Affymetrix, Santa Clara, CA). Raw data were processed using Affymetrix GCOS v1.4 software with default settings, and then examined in the Excel spreadsheet program (Microsoft, Richmond, WA). The mean unscaled signals for E16.5 lenses were 311 ± 27 and 329 ± 8 for WT and *Klf4*CN samples respectively (mean $\pm$ SD, $n = 3$). The equivalent signals for the PN56 lenses were 95 and 84 (mean 90) for WT samples; and 127 and 142 (mean 135) for *Klf4*CN samples. Differences among the means of different groups dictated the use of different thresholds for gene selection. For the E16.5 data (*Klf4*CN / WT = $329 / 311 = 1.06$), a 1.5 fold threshold was used; for the PN56 data (*Klf4*CN / WT = $135 / 90 = 1.5$) a 2-fold threshold; and for maturation data (all PN56 / all E16.5 = 2.8) a 3-fold threshold.

GCOS v1.4 was used to evaluate all pairwise comparisons. Gene expression was considered significantly changed if 1) the mean expression was increased by the appropriate value (1.5-, 2- or 3-fold); 2) there was no overlap in values between the two sets; 3) the higher expressing set had at least two detectable signals (i.e. called Present); and 4) the number of significant pairwise differences between sets was at least 4, 5, or 6 for 2 vs. 2, 2 vs. 3, and 3 vs. 3 comparisons, respectively. Significantly affected genes were further analyzed by Ingenuity pathway analysis software to identify the networks, molecular function pathways and canonical pathways affected in the *Klf4*CN lenses (Ingenuity Systems, www.ingenuity.com) (Dozmorov et al., 2009). MIAME-compliant microarray data is available under accession number GSE47694 in NCBI GEO database (<http://www.ncbi.nlm.nih.gov/geo>).

Validation of Microarray Results

Superscript® VILO™ cDNA Synthesis kit (Invitrogen, Carlsbad, CA) was used to synthesize cDNA using 1 µg of total RNA from E16.5 and PN56 WT and *Klf4*CN lens. QPCR assays were performed with SYBR green reagent (Super-Array Biosystems, Frederick, MD) using pre-standardized gene-specific primers in ABI StepOne Plus Real-Time PCR machine with GAPDH as endogenous control and were analyzed using StepOne software v2.1. The sequence of primers used for QPCR is provided in Supplemental Table 1.

Reporter Vectors, Cell Culture and Promoter Activities

The influence of *Klf4* on *Shsp/aB-crystallin* promoter activity was quantified using the -959/+42 bp *Shsp/aB-crystallin-luciferase* (*paBcry-L*) reporter construct described earlier (Swamynathan and Piatigorsky, 2002), or its derivatives with progressive 5' deletion. Mouse lens epithelial α TN4 cells grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin, and streptomycin at 37°C in a humidified chamber containing 5% CO₂ were transfected with 400 ng of *paBcry-L* along with 10 ng pRL-uc SV40 for normalization of transfection efficiency and 400 ng of pCI or pCI-*Klf4*, using 2.4 µL Fugene-6 (Roche Molecular Biochemicals, Indianapolis, IN). After 2 days of transfection, the cells were washed with PBS and lysed with 200 µL passive lysis buffer for dual luciferase assay (Promega, Madison, WI). The luminescence was measured using Synergy-II microplate reader (BioTek® Instruments, Inc. Winooski, VT), was integrated over 10 seconds with a delay of 2 seconds. Transfection efficiency was normalized using the SV40 promoter-driven *Renilla* luciferase activity. Relative promoter activity was determined by dividing the mean promoter activity in the presence of the plasmid pCI-*Klf4* by the mean promoter activity in the presence of the plasmid pCI-control.

Results from three independent experiments were used to obtain mean promoter activities and standard deviation values presented here.

Measurement of Reduced (GSH) and Oxidized (GSSG) Glutathione Levels

GSH and GSSG levels were determined by O-phthalaldehyde (OPT) fluorimetric method with standard curves generated using known amounts of GSH and GSSG (Hissin and Hilf, 1976). Freshly dissected lenses were homogenized in 0.1M phosphate buffer (pH 8.0) containing 5 mM EDTA and 25% HPO₃, and centrifuged at 10,000g for 30 min. GSH was quantified by mixing 100 µl of the supernatant with 1.8 ml phosphate buffer and 100 µl of OPT, incubating at room temperature for 15 min, and measuring fluorescence with excitation at 350 nm and emission at 420 nm using Synergy-II microplate Fluorimeter (BioTek® Instruments, Inc. Winooski, VT). For quantifying GSSG in the lens lysate, GSH was first quenched by mixing 250 µl of the supernatant with 100 µl 0.04M N-ethylmaleimide and incubating at room temperature for 30 min. 140 µl of the GSH-quenched lens lysate was then mixed with 1.76 ml of 0.1M NaOH and 100 µl of OPT, incubated for 15 min at room temperature, and fluorescence measured with excitation at 350 nm and emission at 420 nm.

Results

Expression of *Klf4* is Developmentally Regulated in the Mouse Lens

Our previous study suggested a role for *Klf4* in development and/or maturation of the mouse lens (Swamynathan et al., 2007). In order to characterize the developmental expression pattern of *Klf4* in the mouse lens, we quantified the *Klf4* transcripts at embryonic day-12 (E12), E16, postnatal day-1 (PN1), PN13 and PN21, by real time QPCR using a standard curve generated with different amounts of linearized pCI-*Klf4* template. The average number of *Klf4* transcripts in the E12, E16, PN1, PN13, PN21 and 8 week-old mouse lens was 8.5, 33.5, 6.2, 2.6, 2.4, and 2.2 per ng total RNA, respectively (Fig. 1A). Thus, *Klf4* expression is developmentally regulated in the mouse lens, peaking at E16 and gradually decreasing in the postnatal stages. Consistent with this, in situ hybridization of E15.5 mouse eye cryosection with *Klf4*-specific anti-sense strand probe detected high levels of *Klf4* expression in the developing lens epithelium and equatorial region as well as the eyelid, cornea, and the retina (Fig. 1B).

Defects in *Klf4*CN Lens Morphology and Histology

In order to further characterize the functions of *Klf4* in the lens, we examined the *Klf4*CN lens in greater detail. Deletion of *Klf4* in the *Klf4*CN lenses was confirmed by QPCR using total RNA isolated from WT and *Klf4*CN lenses (Fig. 2A). Examination of the dissected lenses revealed that the *Klf4*CN lens is smaller (diameter $\pm$ standard error = 0.14 ± 0.0052 cm) with occasional central opacity, unlike the larger (diameter $\pm$ standard error = 0.24 ± 0.0011 cm), transparent WT lens (Fig. 2B). The average diameter of the 8-week-old *Klf4*CN lens was 57% of the age-matched WT lens and its opacity was 1.41 times higher in the center compared to the WT (Fig. 2C). Examination of the E17.5 and PN56 lens histology revealed cortical vacuolation and disorganized arrangement of nuclei in the differentiating *Klf4*CN lens equatorial region, compared with the WT (Fig. 3). Transmission electron microscopy (TEM) of the PN56 lens sections revealed varying electron densities in the irregularly arranged *Klf4*CN lens fiber cells, unlike the uniform electron density in the WT lenses (Fig. 4). TEM also demonstrated that the *Klf4*CN anterior lens cortical fiber cells are relatively larger than those in the WT. While the *Klf4*CN lens capsule was normal and comparable to that in the WT lens, the *Klf4*CN lens epithelial cells were densely packed with mitochondria (Fig. 4). Further examination revealed that the mean mitochondrial density was roughly doubled in the *Klf4*CN lens epithelial cell cytoplasm (6.8 mitochondria

per 10 μm^2 cross sectional area; SD=1.8; n=7) compared with that in the WT (3.7 mitochondria per 10 μm^2 cross sectional area; SD=1.0; n=7) (Fig. 4).

Comparison of WT and *Klf4*CN Lens Transcriptomes

We compared the WT and *Klf4*CN lens transcriptomes at E16.5 and PN56 using microarrays; these data were validated by QPCR analysis of the expression of a few arbitrarily chosen genes using independent samples of freshly isolated total RNA (Fig 5). Next, we tested the consistency of our data by calculating the correlation coefficients (r^2) between members of the four experimental groups. The three E16.5 WT samples correlated well together (0.988 ± 0.003 , mean $\pm$ SD, n=3), as did the three E16.5 *Klf4*CN samples (0.988 ± 0.003 , mean $\pm$ SD; n=3). Correlation between all E16.5 WT and all E16.5 *Klf4*CN samples was nearly as good (0.986 ± 0.004 , mean $\pm$ SD, n=9), consistent with the small number of genes changed by *Klf4*CN at this age. The PN56 WT samples correlated at 0.983, while the PN56 *Klf4*CN samples were less well correlated (0.974). The decreased correlation between the PN56 WT and *Klf4*CN samples (0.921 ± 0.030 , mean $\pm$ SD, n=4) was consistent with the larger number of transcripts altered in the *Klf4*CN lens at PN56 relative to E16.5. The lowest values of r^2 were between the E16.5 and PN56 lenses (0.671 ± 0.010 mean $\pm$ SD, n=6 for the WT, and 0.745 ± 0.012 mean $\pm$ SD, n=6 for the *Klf4*CN), consistent with major changes in gene expression accompanying lens maturation between E16.5 and PN56.

Developmental Stage- and Tissue-Dependent Differences in *Klf4*-Target Genes

Ablation of *Klf4* affected larger number of genes in the PN56 lens (239 increases and 182 decreases) compared with the E16.5 lens (59 increases and 20 decreases) (Table 1). The smaller number of changes in the E16.5 lens is consistent with the relatively normal appearance of the embryonic *Klf4*CN lens, unlike the severely defective PN56 *Klf4*CN lens. The two developmental stages shared 7 concordant increases (*Cdkn1a*, *Ceacam1/Ceacam2*, *Dusp4*, *Igf2r*, *Lsm12*, *Phlda3* and *Gpnm3*; see Table 1), one concordant decrease (*Car8*) and two discordant changes (*Abcb1b* and *Rgs13*). Canonical pathways significantly ($p < 0.05$) enriched in the genes modulated in E16.5 or PN56 *Klf4*CN lens are listed in Tables 2 and 3, respectively. Only two pathways (p53 Signaling and Aryl Hydrocarbon Receptor Signaling) fulfilled our significance requirements for both E16.5 and PN56 lenses, suggesting that *Klf4* serves different functions in the E16.5 and PN56 lens.

Next, we compared *Klf4*-target genes in the PN56 mouse lens (see above) with those previously identified in the PN56 cornea (Swamynathan et al., 2008). This comparison yielded a surprisingly small list of common *Klf4*-target genes in lens and cornea (25 up-regulated and 15 down-regulated genes; Table 4). Thus, *Klf4* regulates disparate functions in the developmentally and functionally related cornea and the lens.

Significantly Affected Biological Functions in the *Klf4*CN Lens

Mitochondrial Dysfunction is the most significantly affected pathway in the PN56 *Klf4*CN lens (Table 3), consistent with the increased density of mitochondria in these lenses (Fig. 4). Many upregulated genes in this pathway (e.g., NADH dehydrogenase (ubiquinone) 1 alpha subcomplex (*Ndufa*) components 1, 2, 3, 5, 7 and b1; *Ndufb* component 2; ubiquinol-cytochrome c reductase, complex III (*Uqc*) components 10, 11 and Q; cytochrome c oxidase subunit (*Cox*) components 6c and 7c; mitochondrial ATPase complex V components 5e, 5j and 5l) encode mitochondrial components, consistent with a more prominent mitochondrial presence in the PN56 *Klf4*CN lens. Enzymatic antioxidants such as superoxide dismutases, catalases, glutathione peroxidases, glutathione reductase, and aldehyde dehydrogenases are essential for safeguarding the eye against oxidative stress (Marchitti et al., 2011). Arachidonic acid metabolism, glutathione metabolism, and aryl

hydrocarbon receptor signaling were among the canonical pathways affected in the PN56 *Klf4*CN lens (Table 3), suggesting that it is oxidatively stressed. Consistently, the expression of glutathione peroxidases *Gpx1* and *Gpx4*, and glutathione-S-transferase *Gstk1* was increased in the PN56 *Klf4*CN lens. Further evidence for elevated oxidative stress in the *Klf4*CN lens was provided by biochemical estimate of GSH and GSSG levels, which revealed that it contains lower levels of reduced glutathione (1.86 μ g/mg compared with 2.65 μ g/mg in the WT) and elevated levels of oxidized glutathione (0.29 μ g/mg compared with 0.22 μ g/mg in the WT) (Fig. 6). Taken together, these data indicate that *Klf4* plays an important role in regulating redox status in the lens.

Maturation-Associated Changes in Lens Gene Expression

During maturation of the WT and *Klf4*CN lens respectively, the expression of 1065 and 860 genes increased, and that of 1022 and 983 genes decreased, with 732 concordant increases, 687 concordant decreases and 1 discordant change (Table 1). Comparison of the top 210 highly expressed genes ranked according to mean expression in the WT embryonic and adult lens identified 163 genes abundantly expressed in both age groups (fractional overlap = 0.78) (Table 6), suggesting that the levels of the abundantly expressed genes remained relatively stable during maturation of the WT lens. Thus, although there are many changes between the E16.5 and PN56 WT lens transcriptomes, transcripts encoding abundant proteins in the lens are already highly expressed in the embryonic stage.

Our data revealed maturation-associated changes in expression of many genes which play critical roles in the lens. For example, expression of glutathione metabolism related genes such as glutathione-S-transferases *Gstm1*, *Gstm2*, *Gsto1*, *Gsta4*, and glutathione peroxidase *Gpx3* (Ganea and Harding, 2006; Marchitti et al., 2011) increased substantially (3.5- to 13.9-fold) during lens maturation (Table 6). Expression of *Bfsp1* and *Bfsp2*, which encode lens fiber cell-specific intermediate filaments filensin and phakinin respectively (Oka et al., 2008), also increased during lens maturation (3.2 and 22-fold respectively). Similarly, expression of *Tdrd7*, an RNA binding protein expressed in lens fiber cells, and required for the posttranscriptional control of mRNAs critical to normal lens development (Lachke et al., 2011) also increased during lens maturation.

Maturation-Associated *Klf4*-Target Genes in the Lens

Among the 1065 genes that displayed maturation-associated increase in expression in the WT lens, there were 104 *Klf4*-target genes whose expression decreased in the PN56 *Klf4*CN lens relative to WT (Table 1). This group of maturation-associated *Klf4*-target genes includes many genes with important functions in the lens, such as aquaporin-1 (Ruiz-Ederra and Verkman, 2006) and -4, connexin-43 (Gao and Spray, 1998), aldehyde dehydrogenases (*Aldh3a1* and *Aldh2*) (Chen et al., 2013), and cell adhesion molecule 2 (*Cadm2*), a member of the nectin family (Pellissier et al., 2007).

The aldehyde dehydrogenase (*Aldh*) family plays a major role in protecting both lens and cornea from UV-induced oxidative damage (Chen et al., 2013; Marchitti et al., 2011). *Aldh3a1* and *Aldh2* are undetectable in the E16.5 lens and robustly expressed in the PN56 WT, with a roughly 2-fold decrease in the PN56 *Klf4*CN lens. Ablation of *Klf4* caused a 2-fold decrease in *Aldh1a7* that is robustly expressed at E16.5, and is down-regulated 3.4-fold during maturation. Interestingly, the lens expression of *Aldh1a1* is ~2.5-fold higher than that of the highest *Aldh3a1* value, and is essentially unaffected by either maturation or *Klf4* ablation. Thus, individual members of the *Aldh* family display different developmental expression patterns in the mouse lens, where they are independently regulated by *Klf4*.

Influence of Klf4 on *Shsp/αB-crystallin* Promoter Activity

Crystallins, the major water-soluble proteins of the lens, play essential roles ensuring the refractive and transparent properties of the lens (Piatigorsky, 1981). Ablation of *Klf4* caused a modest (1.5-fold) decrease in the expression of αB-crystallin. In order to determine if *Klf4* has a direct effect on *Shsp/αB-crystallin* promoter, we performed *in vitro* co-transfection experiments in mouse lens epithelial αTN4 cells. The -959/+42bp *Shsp/αB-crystallin* promoter activity was up-regulated by 4-fold upon co-transfection with pCI-*Klf4*, confirming that *Shsp/αB-crystallin* promoter activity is regulated by *Klf4* in the lens (Fig. 7B). Upon progressive 5' terminal deletion, *Klf4*-response gradually decreased to about 2-fold in -645/+42 bp fragment (Fig. 7C), suggesting that *Klf4*-responsive elements are distributed throughout the -950/+42 bp *Shsp/αB-crystallin* promoter fragment.

Transcription Factors Downstream of Klf4

We employed IPA software to compare the present data with the known downstream target pools of different transcription factors to identify the other transcription factors acting downstream of *Klf4* in the lens (Table 7) or during WT lens maturation (Table 8). *Egr1* was the only transcription factor fulfilling the 1.5-fold requirement, being up-regulated in both E16.5 and PN56 *Klf4*CN lens (Table 7). *Egr1* knockout mice show a myopic shift, with possible changes in lens characteristics (Schippert et al., 2007). *Atf4*, essential for lens fiber cell differentiation (Tanaka et al., 1998), and its downstream targets were upregulated in the PN56 (but not E16.5) *Klf4*CN lens. *Atf4*-deficient lens displays a normal gross structure in early development until E14.5, but thereafter undergoes apoptotic degeneration without the formation of secondary fiber cells (Tanaka et al., 1998). Ablation of *Klf4* therefore appears to trigger compensatory up-regulation in transcription factors *Egr1* and *Atf4*, both of which contribute to orderly lens development. *Hif1a* is among the transcription factors down-regulated in the PN56 *Klf4*CN lens, with a 48% decrease just missing the 2-fold threshold. This is accompanied by a 2-fold increase in *Hif1an* (encoding the Hif1α-inhibitor). Therefore, decreased Hif1α activity may also contribute to the defective *Klf4*CN lens maturation.

Transcription Factors Modulated during Lens Maturation

We performed a similar IPA analysis as above for groups of genes modulated between E16.5 and PN56 WT lenses to identify the other transcription factors essential for WT lens maturation. This analysis revealed the up-regulation of *Ctnnb1* and *Hif1*, and down-regulation of *E2f1*, *Myb* and *Myc*, coupled with corresponding changes in their respective downstream target genes (Table 8).

Discussion

Previously, we demonstrated that *Klf4* contributes to the ocular surface homeostasis by regulating the expression of a large number of genes in the cornea and the conjunctiva (Gupta et al., 2011; Swamynathan et al., 2011; Swamynathan et al., 2008; Swamynathan et al., 2007). The present report shows that *Klf4*, whose expression is developmentally regulated in the lens, plays an important role in lens maturation. We also demonstrate that *Klf4* regulates a unique set of target genes in the lens distinct from those in the cornea, conjunctiva, gastrointestinal tract and the skin (Gupta et al., 2011; Katz et al., 2002; Segre, 2003; Swamynathan et al., 2011; Swamynathan et al., 2008; Swamynathan et al., 2007). Interestingly, *Klf4* regulates fewer genes in the lens than in the cornea, conjunctiva, colon and skin. The molecular basis for the lower number of *Klf4*-target genes in the lens compared with that in the cornea and other tissues remains to be identified. This report adds *Klf4* to the ranks of other transcription factors (*Pax6*, *AP2α* and *Cited2* to name a few) used

for both lens and corneal maturation (Chen et al., 2008a; Chen et al., 2009; Cvekl et al., 2004; Pontoriero et al., 2008).

The data presented in this manuscript are expected to serve as a useful reference resource for maturation-associated changes in WT lens gene expression. Our data revealed that *Ctnnb1* and *Hif1* are up-regulated, and *E2f1*, *Myb* and *Myc* are down-regulated during WT lens maturation. As conditional deletion of *Ctnnb1* from the E13.5 mouse lens results in increased progression of epithelial cells through the cell cycle with delayed fiber cell differentiation (Martinez et al., 2009), orderly maturation of the lens appears to depend on increasing levels of *Ctnnb1*. Though *Ctnnb1* is a direct target of *Klf4* (Tiware et al., 2013), it is unaffected in the *Klf4*CN lens unlike *Egr1* and *Atf4*. The robust maturation-associated decrease in E2F1 is to be expected during normal development, as over-expression of E2F1 or E2F2 in post-mitotic lens fiber cells induces re-entry into the cell cycle, and p53-dependent apoptosis (Chen et al., 2000). Substantial decreases in *Myb* and *Myc* during maturation also are likely concomitant with the post-mitotic state of the lens fiber cells. Finally, increased expression of *Hif1a* is to be expected in the maturing avascular lens, where it is likely to suppress lens growth (Shui et al., 2008).

Being avascular and exposed to light, the lens is prone to oxidative stress-induced damages. The lipoxygenase (LOX) pathway is responsible for the synthesis of bioactive metabolites of arachidonic acid such as hydroperoxyeicosatetraenoic acids (HETEs) that influence cell proliferation, ion transport, renal and pulmonary functions, vascular tone, inflammatory and growth responses (Kenchegowda and Bazan, 2010; van Leyen, 1998; Zelenka and Arpitha, 2008). Arachidonate lipoxygenase-15 (*Alox15*) is required for regulating organelle degradation during lens fiber cell differentiation (Grulich et al., 2001; van Leyen et al., 1998). The current and previous microarray analyses showed that *Alox12*, *Alox12e* and *Alox15* are significantly up-regulated in the *Klf4*CN lens and cornea, suggesting that these tissues are oxidatively stressed (Swamynathan et al., 2008). Consistent with this, the expression of connexin-43 (*Gja1*), a component of the gap junctions required for maintenance of optimal redox conditions (Berthoud and Beyer, 2009; Lou, 2003) is decreased in the *Klf4*CN lens. Moreover, both arachidonic acid metabolism and aryl hydrocarbon receptor signaling pathways that play important roles in protecting the lens from oxidative stress (Lou, 2003) are significantly affected in the *Klf4*CN lens. Finally, glutathione peroxidase-1 and -4 are up-regulated in the *Klf4*CN lens, while the amount of reduced glutathione (GSH) is decreased and the oxidized glutathione (GSSG) is increased, confirming that the *Klf4*CN lens is oxidatively stressed. Taken together, these results identify *Klf4* as a key regulator of different components of the diverse pathways that safeguard the lens against oxidative stress.

Decreased expression of different crystallins in the *Klf4*CN lens is consistent with the ability of *Klf4* to regulate the expression of functionally related subsets of genes, first identified in the cornea (Swamynathan et al., 2011; Swamynathan et al., 2008; Swamynathan et al., 2007). Different transcription factors including Pax6, retinoic acid/retinoid receptors, MyoD, and Hsf4 contribute towards high expression of *Shsp/aB-crystallin* in the lens, moderate expression in the heart and skeletal muscle, and low expression in several other tissues. The results presented here add *Klf4* to the list of transcription factors contributing to *Shsp/aB-crystallin* expression in the lens and probably other tissues as well. Though transient transfection assays with progressively shorter promoter constructs indicated that the *Klf4*-responsive elements are dispersed within the -950/+42 bp promoter region of the *Shsp/aB-crystallin* promoter, their precise location remains to be identified by chromatin immunoprecipitation and electrophoretic mobility shift assays, and their function validated by mutagenesis studies.

Several transcription factors are affected in the *Klf4*CN lens, suggesting that a number of the identified *Klf4*-target genes are indirect targets. For example, *Egr1*, *Myb*, *Id2*, *ATF4*, and *ATF5* are up-regulated and *Max*, *Mcm2*, *Rab2* and *Hmgn2* are down-regulated, and may contribute indirectly to the identified changes in *Klf4*CN lens gene expression. The expression of *FoxC2*, with established roles in lens development also is affected in the *Klf4*CN lens, lending further support to the possibility that many of the changes in *Klf4*CN lens gene expression reflect indirect effects. Further studies are necessary to distinguish the direct and indirect *Klf4*-target genes, and to establish the hierarchical relationship between these transcription factors.

Though this study provides useful insights into lens gene expression and its regulation by *Klf4*, a few limitations need to be considered. First, as Cre recombinase can potentially be toxic to mammalian cells (Schmidt-Suprian and Rajewsky, 2007), it is possible that at least some of the changes in the *Klf4*CN lens gene expression are due to the presence of Cre recombinase, and not the absence of *Klf4*. Thus, despite the widespread use of Le-Cre validating it as a useful tool to study gene functions in the ocular surface ectoderm-derived tissues (Ashery-Padan et al., 2000; Chen et al., 2009; Dwivedi et al., 2005; Garcia et al., 2011; Huang et al., 2011; Kenchegowda et al., 2011; Rajagopal et al., 2009; Shaham et al., 2009; Swamynathan et al., 2007; Wiley et al., 2011), one needs to be cautious in interpreting these results. Second, though microarrays are widely used to document changes in gene expression, these changes may not accurately represent the changes in proteome as reported in the yeast (Gygi et al., 1999). Thus, although our demonstration that the *Klf4*CN lens is oxidatively stressed corroborates our microarray results, further validation by additional proteomics analyses is necessary.

In summary, our study identifies *Klf4* as an important component in the genetic network of transcription factors regulating gene expression in the lens. Taken together with the previously established roles of *Klf4* in maturation and maintenance of corneal epithelial barrier function, corneal stromal hydration, conjunctival goblet cells, and regenerative capacities of the retinal nerve cells (Gupta et al., 2011; Moore et al., 2009; Swamynathan et al., 2011; Swamynathan et al., 2008; Swamynathan et al., 2007), this study demonstrates that *Klf4* plays a major role throughout the eye. Identification of the upstream regulatory factors that control the expression of *Klf4* in different parts of the eye during ocular development remains a worthwhile challenge.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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- *Klf4* expression in the lens is developmentally regulated
- Absence of *Klf4* results in a smaller, malformed lens
- *Klf4* regulates a unique set of target genes in the lens
- *Klf4* is essential for lens redox homeostasis

Figure 1. Expression of *Klf4* in the mouse lens

(A) Expression level of *Klf4* in the E12, E16, PN1, PN13, PN21 and 8-week-old lenses, expressed as number of transcripts per ng total RNA. Error bars are standard errors. (B) In situ hybridization with *Klf4* sense (negative control; left panel) and anti-sense strand (right panel) probes using E15.5 mouse eye. *Klf4* expression is detected in the eyelid (E), cornea (C), lens epithelium (L; arrow), lens equator (L; arrowheads) and the retina (R).

Figure 2. Effect of conditional deletion of *Klf4* on lens morphology and transparency

(A) Confirmation of deletion of *Klf4* in E16 lens. Relative levels of *Klf4* expression in the wild type and *Klf4*^Δ lens, estimated by real time QPCR are shown. (B) *Klf4*^Δ lens is smaller and displays opacity in the center. Light micrographs were generated using 1.6 X objective and transmitted light mode. The objective focal point was set at the equatorial plane of the lens (C) Center of the *Klf4*^Δ lens scatters 1.41 fold more light on average compared to the WT, suggesting nuclear opacity. Error bars represent standard error of mean (SEM).

Figure 3. Effect of conditional deletion of *Klf4* on lens histology

H & E-stained sections of the WT and *Klf4CN* lens sections at E17.5 (A) and PN56 (B) are shown, with the indicated equatorial region from the PN56 lenses enlarged in (C) to display cortical vacuolation and disorganized nuclei. Scale bar, 100 μm.

Figure 4. Transmission electron microscopy of anterior corticular region of the WT and *Klf4*CN lenses

Note that the *Klf4*CN lens fiber cells display varying electron densities, unlike the WT fiber cells. While there is no difference between the WT and *Klf4*CN lens capsules, *Klf4*CN epithelial cells contain a significantly larger number of mitochondria. Boxed-in areas from panels (i) and (ii) are shown enlarged in panels (iii) and (iv), respectively. Arrows indicate mitochondria. C, capsule; E, epithelial cell; EN, epithelial cell nucleus; F, fiber cell. Original magnification: 5,000X. Scale bar, 2 μ m.

Figure 5. QPCR validation of the microarray data

Expression levels of randomly selected genes were tested by QPCR using equal amounts of total RNA isolated from WT and *Klf4*CN E16.5 or PN56 lenses (red bars). For comparison, expression level of each gene as detected by microarray is shown in blue.

Figure 6. Effect of conditional deletion of *Klf4* on lens glutathione levels

Mean levels of reduced (GSH) and oxidized (GSSG) glutathione in freshly dissected lenses from five age-matched WT and *Klf4*CN mice are shown. Error bars represent standard error of mean (SEM).

Figure 7. Influence of Klf4 on *Shsp/αB-crystallin* promoter activity

Effect of Klf4 on *Shsp/αB-crystallin* promoter activity was tested by transient co-transfection assays in mouse lens epithelial cell line αTN4. (A) Schematic representation of the reporter constructs tested. (B) Relative *Shsp/αB-crystallin* promoter activities in lens epithelial αTN4 cells upon co-transfection with increasing amounts of pCI-Klf4. (C) Relative activities of different deletion constructs of *Shsp/αB-crystallin* promoter in lens epithelial αTN4 cells upon co-transfection with pCI-Klf4. Error bars represent SEM.

Table 1

Numbers of unique characterized genes differentially expressed in E16.5 and/or PN56 *Klf4*CN lens compared with the WT.

	During WT Development, expression is	Expression in PN56 <i>Klf4</i> CN lens		
		Increased Total = 239	Decreased Total = 182	No Change
Expression in E16.5 <i>Klf4</i> CN lens	Increased	2 (1)	1 (0)	11 (14)
	Decreased	3 (2)	0 (0)	6 (11)
	No change	3 (5)	0 (1)	33 (26)
	Increased	0 (0)	1 (0)	5 (2)
	Decreased	1 (1)	0 (0)	5 (6)
	No change	0	0 (1)	8 (10)
	Increased	27 (10)	102 (18)	916 (1030)
	Decreased	73 (69)	3 (41)	931 (934)
	No change	130 (153)	75 (120)	17233

Numbers of unique characterized genes differentially expressed in E16.5 and/or PN56 *Klf4*CN compared with the corresponding WT lens. Rows are broken down according to developmental changes, i.e., differences between WT PN56 and WT E16.5 lenses, as designated in the shaded column. Number of genes which show valid >2-fold changes in PN56 *Klf4*CN cornea are shown in parentheses. Note that for both the lens and cornea, changes during development (**bold values** in bottom right corner) exceed the number of changes due to *Klf4* ablation.

Table 2Canonical pathways enriched in the E16.5 *Klf4*CN lens

Canonical Pathways changed by <i>Klf4</i> ablation at E16.5	-log (p value)	Genes increased, decreased, total
p53 Signaling	3.9	4, 1, 96
Hepatic Fibrosis / Hepatic Stellate Cell Activation	3.07	4, 1, 146
VDR/RXR Activation	2.08	2, 1, 81
Bladder Cancer Signaling	2	1, 2, 91
Glycine Degradation (Creatine Biosynthesis)	1.95	1, 0, 9
Glioma Signaling	1.84	3, 0, 112
NRF2-mediated Oxidative Stress Response	1.83	3, 1, 192
Amyotrophic Lateral Sclerosis Signaling	1.81	3, 0, 117
ILK Signaling	1.8	3, 1, 192
Pancreatic Adenocarcinoma Signaling	1.7	3, 0, 120
Melanoma Signaling	1.66	1, 1, 46
Heme Degradation	1.66	1, 0, 11
PTEN Signaling	1.58	3, 0, 133
Aryl Hydrocarbon Receptor Signaling	1.45	2, 1, 161
Phospholipases	1.44	1, 1, 66
Remodeling of Epithelial Adherens Junctions	1.32	1, 1, 67
Prostanoid Biosynthesis	1.31	1, 0, 15

The canonical pathways were populated by 48 changes of 23 unique genes from the 1.5-fold group. Seventeen genes (74%) appeared in only one or two pathways. The most prolific gene (*Cdkn1a*) appeared in 8 pathways. *Cdkn1a* is also one of the most prolific genes modulated in the *Klf4*CN lens at PN56 (see Table 3). Pathways in **bold** appear in Table 3 also.

Table 3

Canonical pathways enriched in the PN56 *Klf4*CN lens.

Canonical Pathways changed by <i>Klf4</i> ablation at PN56	-log (p value)	Genes increased, decreased, total
Mitochondrial Dysfunction	8.72	16, 3, 186
GADD45 Signaling	3	4, 0, 22
EIF2 Signaling	2.14	6, 4, 200
Glutathione Redox Reactions I	2.13	3, 0, 24
Putrescine Degradation III	2.13	1, 2, 30
eNOS Signaling	1.99	4, 4, 151
Cell Cycle: G1/S Checkpoint Regulation	1.81	4, 1, 66
Aryl Hydrocarbon Receptor Signaling	1.72	5, 3, 161
Regulation of eIF4 and p70S6K Signaling	1.72	4, 4, 175
S-methyl-5'-thioadenosine Degradation II	1.59	0, 1, 6
Asparagine Biosynthesis I	1.59	1, 0, 8
p53 Signaling	1.59	5, 1, 96
Ketogenesis	1.59	0, 2, 21
Superpathway of Cholesterol Biosynthesis	1.5	2, 1, 86
Mevalonate Pathway I	1.43	1, 1, 28
ERK/MAPK Signaling	1.37	8, 1, 206
Histamine Degradation	1.37	0, 2, 29
Molecular Mechanisms of Cancer	1.36	9, 5, 378
Inhibition of Angiogenesis by TSP1	1.31	2, 1, 39
Fatty Acid β -oxidation	1.31	0, 2, 21

The canonical pathways (column 1) were populated by (column 3) 108 occurrences of 70 unique genes from the 1.5-fold group. 63 genes (90%) appeared in only one or two pathways. The most prolific genes (*Ccnd1*, *Ccnd2* and *Cdkn1a*) appeared in 5 pathways. Pathways in **bold** appear in Table 2 also.

Table 4Genes modulated by *Klf4*CN in both lens and cornea at PN56

	Increased expression in <i>Klf4</i> CN lens	Decreased expression in <i>Klf4</i> CN lens
Increased expression in <i>Klf4</i> CN cornea	<i>Alox15</i> <i>B2m</i> <i>Car2</i> <i>Ccdc109b</i> <i>Ccnd2</i> <i>Ceacam1</i> <i>Ceacam1 / Ceacam2</i> <i>Fxyd5</i> <i>Igf2r</i> <i>Krt15</i> <i>Mmd</i> <i>Msn</i> <i>Myo5a</i> <i>Olfm1</i> <i>Pmaip1</i> <i>S100a4</i> <i>Serpina3n</i> <i>Stk39</i> <i>Stxbp6</i> <i>Tgfb2</i> <i>Vwc2</i> <i>Xist</i>	<i>Abcb1b</i> <i>Hba-a1 / Hba-a2</i> <i>Igk-V28</i> <i>LOC100505062 / Ywhaz</i> <i>Nus1</i> <i>Rbp1</i>
Decreased expression in <i>Klf4</i> CN cornea	<i>AI413582</i> <i>Dnaja4</i> <i>Gstk1</i> <i>Id2</i> <i>Malat1</i>	<i>Arhgef4</i> <i>Btf3l4</i> <i>Car3</i> <i>Chchd7</i> <i>Ddx3y</i> <i>Eif2s3y</i> <i>Ephx2</i> <i>Fam176a</i> <i>Naprt1</i> <i>Necab1</i> <i>Necap2</i> <i>Ngef</i> <i>Txndc12</i>

Complete annotation of Igk-V28 (upper right quadrant) is Igk-V28 /// Igkc /// Igkj1 /// Igkv4-53 /// Igkv6-23 /// Igkv8-30

Table 5

Canonical pathways analysis of developmental changes between E16.5 and PN56, made separately for WT and Klf4CN lens.

Pathway	All changes in WT	Changes exclusive to WT	Concordant changes WT and Klf4 CN	Changes exclusive to Klf4 CN	All changes in Klf4CN
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	2.69		2.9	3.55	6.27
Cell Cycle Control of Chromosomal Replication	4.19		4.83	2.25	4.27
Inhibition of Angiogenesis by TSP1	4.62	2.01	3.22		4.57
p53 Signaling	3.98		3.84		3.35
Mitotic Roles of Polo-Like Kinase			2.13	2.76	4.59
eNOS Signaling	4.04		3.16		2.55
Glioma Signaling	3.66	2.83	2.27		
Mouse Embryonic Stem Cell Pluripotency	3.52	2.25	2.58		
Angiopoietin Signaling	3.18		2.41		2.4
Aryl Hydrocarbon Receptor Signaling	3.16	2.99			2.13
Thyroid Cancer Signaling	2.37		3.07		2.79
Phototransduction Pathway				3.96	2.76
Role of Tissue Factor in Cancer	2.8	3.95			
UVB-Induced MAPK Signaling	2.02	3.72			
Molecular Mechanisms of Cancer	2.47	3.27			
Nitric Oxide Signaling in the Cardiovascular System	3.23		2.59		
G Beta Gamma Signaling	2.1				3.1
Regulation of Cellular Mechanics by Calpain Protease	3.09		2.55		

Values are $-\log(p)$ for significant enrichment of pathway members. Pathways shown contain at least one extremely significant group ($p < 0.001$, $-\log(p) > 3$), and values are shown only for $-\log(p) > 2$. Pathways in **bold** are also in Tables 2 and 3.

Table 6

Most highly expressed genes common between E16.5 and PN56 lens

Gene Title	Gene Symbol	PN56		E16.5 rank	PN56 rank	Fold Change in <i>Klf4</i> CN	
		E16.5	PN56			E16.5	PN56
4930583H14Rik	(as title)	1.84		157	117	0.88	0.93
actin, beta	<i>Actb</i>	2.65		28	29	1.01	0.77
actin, gamma, cytoplasmic 1	<i>Actg1</i>	1.72		136	121	0.96	1.01
aldo-keto reductase family 1, member A4 (aldehyde reductase)	<i>Akr1a4</i>	1.93		90	75	0.94	0.68
aldehyde dehydrogenase family 1, subfamily A1	<i>Aldh1a1</i>	1.24		175	195	0.98	0.82
ATPase, H ⁺ -transporting, lysosomal V0 subunit C	<i>Atp6v0c</i>	0.97		125	182	0.99	0.88
ataxin 10	<i>Atn10</i>	0.93		164	172	0.95	1.10
beaded filament structural protein 1, in lens-CP94	<i>Bfsp1</i>	3.22		66	11	0.90	0.60
CD24a antigen	<i>Cd24a</i>	2.07		4	14	0.91	0.83
coiled-coil-helix-coiled-coil-helix domain containing2	<i>Clchd2</i>	1.70		77	90	0.94	0.96
clusterin	<i>Clu</i>	4.76		207	25	0.89	0.63
calponin 3, acidic	<i>Cnn3</i>	3.27		190	63	1.08	1.08
collagen, type IV, alpha 1	<i>Col4a1</i>	1.56		142	209	0.89	0.67
cytochrome c oxidase, subunit Va	<i>Cox5a</i>	1.27		155	177	0.98	0.94
cytochrome c oxidase, subunit VI a, polypeptide 1	<i>Cox6a1</i>	1.37		122	150	0.89	1.00
cytochrome c oxidase, subunit VIIa 2	<i>Cox7a2</i>	0.97		115	207	0.94	1.50
crystallin, alpha A	<i>Cryaa</i>	4.33		195	20	0.81	0.66
crystallin, alpha B	<i>Cryab</i>	3.02		2	4	0.87	0.65
crystallin, beta A1	<i>Cryba1</i>	3.78		91	6	0.82	0.68
crystallin, beta A2	<i>Cryba2</i>	3.53		113	13	0.86	0.66
crystallin, beta A4	<i>Cryba4</i>	2.47		27	23	0.91	0.71
crystallin, beta B1	<i>Crybb1</i>	4.83		210	12	0.83	0.71
crystallin, beta B3	<i>Crybb3</i>	3.73		139	15	0.87	0.73
crystallin, gamma A	<i>Cryga</i>	1.24		130	168	0.85	0.90
crystallin, gamma B	<i>Crygb</i>	2.98		15	7	0.84	0.73
crystallin, gamma B / crystallin, gamma C	<i>Crygb / c</i>	4.12		96	3	0.86	0.67

Gene Title	Gene Symbol	PN56		E16.5 rank	PN56 rank	Fold Change in <i>Klf4</i> CN	
		E16.5	PN56			E16.5	PN56
crystallin, gamma D	<i>Crygd</i>	3.03	14	5	0.87	0.70	
crystallin, gamma F	<i>Crygf</i>	1.87	6	38	0.87	0.49	
crystallin, gamma S	<i>Crygs</i>	2.94	13	8	0.88	0.73	
catenin (cadherin associated protein), beta 1	<i>Ctnnb1</i>	1.39	159	163	0.93	0.86	
eukaryotic translation elongation factor 1 alpha 1	<i>Eef1a1</i>	2.60	16	16	0.89	0.79	
eukaryotic translation elongation factor 1 beta 2	<i>Eef1b2</i>	1.45	188	169	0.93	1.00	
eukaryotic translation elongation factor 1 gamma	<i>Eef1g</i>	1.97	170	112	0.88	0.85	
eukaryotic translation initiation factor 1	<i>Eif1</i>	1.07	135	200	1.01	1.40	
eukaryotic translation initiation factor 4, gamma 2	<i>Eif4g2</i>	0.67	59	174	0.97	1.13	
fatty acid binding protein 5, epidermal	<i>Fabp5</i>	4.68	173	9	0.89	0.79	
RFad1, flavin adenine dinucleotide synthetase, homolog (yeast) / lens epithelial protein	<i>Ffad1 / Lenep</i>	3.31	169	18	1.12	0.77	
ferritin heavy chain 1	<i>Fth1</i>	6.55	176	126	0.92	1.29	
ferritin light chain 1	<i>Ftl1</i>	1.84	184	189	0.97	1.40	
glyceraldehyde-3-phosphate dehydrogenase	<i>Gapdh</i>	1.32	41	42	0.90	0.87	
growth arrest specific 5	<i>Gas5</i>	1.87	181	153	0.90	1.22	
guanosine diphosphate (GDP) dissociation inhibitor 2	<i>Gdi2</i>	1.59	117	118	1.04	0.81	
protein tyrosine phosphatase 4a1-like / protein tyrosine phosphatase 4a1	<i>Gml3363 / Ptp4a1</i>	1.53	167	70	0.93	0.86	
predicted gene 13430 / SMT3 suppressor of mif two 3 homolog 2 (yeast)	<i>Gml3430 / Sumo2</i>	2.50	197	170	0.97	0.99	
ubiquitin pseudogene / ubiquitin B	<i>Gml821 / Ubb</i>	1.51	65	22	0.94	0.80	
GNAS (guanine nucleotide binding protein, α stimulating) complex locus	<i>Gnas</i>	2.44	186	190	0.91	1.18	
guanine nucleotide binding protein (G protein), beta polypeptide 2 like 1	<i>Gnb2l1</i>	1.12	83	139	0.91	0.95	
guanine nucleotide binding protein (G protein), gamma 5	<i>Gng5</i>	1.33	129	158	0.90	1.25	
glutathione S-transferase, pi 1	<i>Gstp1</i>	1.32	148	194	1.02	1.66	
H3 histone, family 3A / hypothetical LOC100503721	<i>H333a / LOC100503721</i>	1.30	82	149	0.95	0.90	
high mobility group nucleosomal binding domain 3	<i>Hmg3</i>	1.13	127	92	1.09	0.74	
heat shock protein 90, alpha (cytosolic), class A member 1	<i>Hsp90aal</i>	1.25	154	203	1.14	1.26	
heat shock protein 90 alpha (cytosolic), class B member 1	<i>Hsp90ab1</i>	2.30	161	176	0.93	1.02	
heat shock protein 8	<i>Hspa8</i>	2.06	119	32	1.05	0.88	

Gene Title	Gene Symbol	PN56		E16.5 rank	PN56 rank	Fold Change in <i>Klf4</i> CN	
		E16.5	PN56			E16.5	PN56
insulin-like growth factor binding protein 7	<i>Igfbp7</i>	1.32		98	140	0.88	0.83
lens intrinsic membrane protein 2	<i>Lim2</i>	1.48		78	55	0.87	0.83
major intrinsic protein of eye lens fiber (Aqp0)	<i>Mip</i>	1.36		94	73	0.90	0.85
mortality factor 4 like 1	<i>Morf4l1</i>	2.19		189	113	0.93	1.25
nascent polypeptide-associated complex alpha polypeptide	<i>Naca</i>	2.26		104	175	0.94	1.23
nucleosome assembly protein 1-like 1	<i>Nap1l1</i>	1.63		102	144	0.95	1.16
poly(A) binding protein, cytoplasmic 1	<i>Pabpc1</i>	0.87		36	115	0.93	0.88
phosphoribosylaminimidazole carboxylase, phosphoribosylaminoribosylaminimidazole, succinocarboxamide synthetase	<i>Paics</i>	0.33		153	109	0.93	0.89
phosphatidylethanolamine binding protein 1	<i>Pebp1</i>	1.32		114	122	0.90	1.13
peptidylprolyl isomerase A	<i>Ppia</i>	1.86		55	49	0.89	0.98
peroxiredoxin 1	<i>Prdx1</i>	1.42		192	107	0.99	0.69
stearoyl-Coenzyme A desaturase 2	<i>Scd2</i>	2.16		57	62	0.95	0.85
serpinel mRNA binding protein 1	<i>Serbp1</i>	2.43		123	178	0.97	0.73
solute carrier family 25 (mitochondrial carrier, adenine nucleotide translocator), member 4	<i>Slc25a4</i>	2.00		206	148	0.98	1.06
secreted acidic cysteine rich glycoprotein	<i>Sparc</i>	1.61		146	83	0.92	0.81
tudor domain containing 7	<i>Tdrd7</i>	1.68		166	124	0.92	0.63
transketolase	<i>Tkt</i>	2.13		138	35	0.89	0.55
thymosin, beta 10	<i>Tmsb10</i>	1.87		80	156	1.09	2.70
translocase of outer mitochondrial membrane 20 homolog (yeast)	<i>Tomm20</i>	2.86		187	161	1.01	1.00
tumor protein, translationally-controlled 1	<i>Tpt1</i>	0.30		37	17	0.88	0.79
tubulin, alpha 1A	<i>Tubal1a</i>	1.51		22	44	0.92	0.78
tubulin, alpha 1B	<i>Tubal1b</i>	2.76		162	57	0.89	0.77
ubiquitin A-52 residue ribosomal protein fusion product 1	<i>Uba52</i>	2.05		126	154	0.91	1.22
ubiquinol-cytochrome c reductase binding protein	<i>Uqcrb</i>	2.75		34	197	0.99	1.81
vimentin	<i>Vim</i>	1.36		72	45	0.93	0.84

Genes were ranked by mean expression (highest expression = 1) within each wild-type age group. Iterative analysis showed that the top 210 PN56 genes contained 163 of the top 210 E16.5 genes (fractional overlap = 0.78). Of these 163 genes, 79 encoded ribosomal proteins and are omitted from the Table; of the remaining 84, four (*Fabp5*, *Gapdh*, *Morf4l1* and *Uba52*) appeared also in panels with multiple annotations, which were omitted. In the fourth data column genes which show a modest (1.5-fold) change due to *Klf4* ablation at PN56 are shown in **bold**. At E16.5, there were no such changes in the *Klf4*/CN lens.

Table 7

Transcription factor downstream target pools which are significantly enriched by *Klf4* ablation.

Transcription factor	Fold change at E16.5 (<i>Klf4</i> CN / WT)	-log(p) for downstream targets	Fold change in PN56 (<i>Klf4</i> CN / WT)	-log(p) for downstream targets WT
<i>Atf4</i>		1.88	2.39	3.10
<i>Trp73</i>		3.78		
<i>Elf4</i>		2.11		2.39
<i>Nr1i3</i>		2.98		1.61
<i>Smad3</i>		6.92		3.08
<i>Gli1</i>		2.17		1.87
<i>Mtpn</i>				1.83
<i>Egr1</i>	1.89	7.53	1.98	3.72
<i>Cebpa</i>		3.01		1.90
<i>Ets1</i>		3.51		1.39
<i>Ppargc1b</i>				2.75
<i>Pprc1</i>				1.79
<i>Rela</i>		4.25		

Data columns 1 and 3 show the valid fold change for each transcription factor: blank cells indicate no valid change. Data columns 2 and 4 show -log(p) values for enrichment of transcription factor targets, and are in **bold** if the associated |z-value| is ≥ 2, confirming that the direction of modulation of target pool members is consistent.

Table 8

Transcription factors with significant downstream target pools during development

Transcription factor	Fold change in WT	Fold change in <i>Klf4</i> CN	All changes in WT 1,884 genes modulated	Exclusive to WT 451 genes modulated	Concordant changes 1,429 genes modulated	Exclusive to <i>Klf4</i> CN 689 genes modulated	All changes in <i>Klf4</i> CN 2,113 genes modulated
<i>Ahr</i>			5.3	3.7	3.7	4.1	6.9
<i>Arf4</i>	-1.36	1.6	5.9	4.3	3.1		2.9
<i>Cnnb1</i>	3.7	4.5	7.1	3.3	4.1	1.4	3.9
<i>E2f1</i>	-17.1	-5.1	13.5	3.4	10.9	2.4	10.1
<i>E2f2</i>			9.7	2.3	8.7	2.4	7.6
<i>E2f3</i>	-1.8	-1.9	7.9	1.9	7.3	2.4	6.6
<i>Erg</i>			2.2		1.9		1.8
<i>Foxm1</i>			10.8	2.7	9.1	1.7	7.8
<i>Foxo1</i>			3.1		2.6		3.2
<i>Hhex</i>			4.4		4.3		3.0
<i>Hif1a</i>	35.7	50.3	5.6	1.8	4.6	1.9	4.6
<i>Kdm5a</i>		-3.2	2.4		3.1		2.2
<i>Max</i>	2.3	-1.5	4.3		4.8		4.4
<i>Mnt</i>	-1.6	-1.3			2.0		2.2
<i>Mxi1</i>	-1.3	10.8	3.5		3.6		2.3
<i>Myb</i>	-5.4	-3.8	3.3		3.3		3.0
<i>Myc</i>	-34.6	-12.9	18.4	5.1	15.1	4.9	14.8
<i>Nfya</i>		7.4				2.7	1.4
<i>Nkx2-1</i>			2.5		2.4	2.3	3.2
<i>Nkx2-3</i>			3.0	1.5	2.4	3.0	5.0
<i>Sm3a</i>					1.9	2.3	4.3
<i>Snad7</i>	-2.5	-3.7	4.9	3.5	2.5	1.6	3.4
<i>Sp3</i>		-2.6	3.2	1.9	1.8	1.4	2.7
<i>Tbx2</i>			16.2	3.7	12.1	4.4	13.2
<i>Tfdp1</i>	-2.3	-2.3	4.9		4.8		3.5
<i>Trp73</i>			6.1	4.8	2.1		2.3

Transcription factor downstream target pools which are significantly enriched during development. Values are $-\log(p)$, and are in **bold** if the associated $|z\text{-value}|$ is ≥ 2 , confirming that the direction of modulation of target pool members is consistent.