Regulation of α A-crystallin via Pax6, c-Maf, CREB and a broad domain of lens-specific chromatin

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Pax6 and c-Maf regulate multiple stages of mammalian lens development. Here, we identified novel distal control regions (DCRs) of the α A-crystallin gene, a marker of lens fiber cell differentiation induced by FGF-signaling. DCR1 stimulated reporter gene expression in primary lens explants treated with FGF2 linking FGF-signaling with α A-crystallin synthesis. A DCR1/ α A-crystallin promoter (including DCR2) coupled with EGFP virtually recapitulated the expression pattern of α A-crystallin in lens epithelium and fibers. In contrast, the DCR3/ α A/EGFP reporter was expressed only in 'late' lens fibers. Chromatin immunoprecipitations showed binding of Pax6 to DCR1 and the α A-crystallin promoter in lens chromatin and demonstrated that high levels of α A-crystallin expression correlate with increased binding of c-Maf and CREB to the promoter and of CREB to DCR3, a broad domain of histone H3K9-hyperacetylation extending from DCR1 to DCR3, and increased abundance of chromatin remodeling enzymes *Brg1* and *Snf2h* at the α A-crystallin locus. Our data demonstrate a novel mechanism of Pax6, c-Maf and CREB function, through regulation of chromatin-remodeling enzymes, and suggest a multistage model for the activation of α A-crystallin during lens differentiation.

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Introduction

Multicellular organisms achieve cellular differentiation through precisely regulated gene expression. The specification of individual cell lineages occurs through a myriad of operations including cell-to-cell interactions, and extra-cellular signaling that result in the activation and repression of specific genes. The lens provides an excellent system to study this process at the molecular level, because the expression of any gene in the lens can be temporally and spatially characterized as a consequence of the lens' unique morphology (Lovicu and McAvoy, 2005).

Crystallins are the most abundant proteins in terminally differentiated lens fiber cells. Their high level of expression in lens fibers together with their distinct spatial distribution is essential for lens transparency and its refractive properties. α A-crystallin represents as much as 17% of all newborn mouse lens water-soluble proteins, while all crystallins represent about 90% of total lens proteins (Bloemendal *et al*, 2004). Loss of expression of α A-crystallin or expression of its mutants causes lens opacification and formation of cataracts (Bloemendal *et al*, 2004).

Lens differentiation commences as soon as the lens vesicle, comprised of a single layer of cuboidal epithelial cells, pinches off from the surface ectoderm. The lens vesicle is polarized with its anterior cells retaining their epithelial morphology and proliferative capacity, whereas the posterior lens precursor cells initiate terminal differentiation forming the primary lens fibers. Lens differentiation is initially regulated by secreted growth factors including members of the FGF family originating from the prospective retina (Lovicu and McAvoy, 2005). Secreted FGFs interact with four types of tyrosine kinase receptors, FGFR1–4, all of them expressed in lens. The mechanism by which FGFs regulate lens fiber cell differentiation is not known. One possibility is that FGF-signaling regulates abundance and activity of members of Ets and large Maf families of transcription factors (Barolo and Posakony, 2002). Recent genetic studies also suggested roles of Wnt pathway (Stump *et al*, 2003; Lyu and Joo, 2004) and TGF β -signaling (de Longh *et al*, 2001) in 'late' stages of lens fiber cell differentiation.

α A-crystallin mRNA appears first in the invaginating lens placode followed by uniform expression in the lens vesicle (Robinson and Overbeek, 1996). Dramatic upregulation of α A-crystallin expression is associated with primary lens fiber cell differentiation. Previous studies have shown that a –366 to +46 α A-crystallin promoter fragment is active in primary lens fibers with positional and copy-number effects in transgenic mice (Cvekl *et al*, 2004). In addition, this promoter supported levels of expression two to three orders of magnitude lower than that of the endogenous α A-crystallin gene (Duncan *et al*, 1996), suggesting that additional regulatory regions are necessary.

Three distinct DNA-binding transcription factors, Pax6, c-Maf, and CREB, were implicated as α A-crystallin regulatory

factors (Cvekl *et al*, 1995; Kawauchi *et al*, 1999; Ashery-Padan *et al*, 2000; Ring *et al*, 2000; Yang and Cvekl, 2005). Pax6 is critical in establishing the lens lineage from surface ectoderm (Grindley *et al*, 1995; Ashery-Padan *et al*, 2000). Multiple Pax6 binding sites were identified in the lens-specific promoter of the mouse α A-crystallin gene (Yang and Cvekl, 2005). However, expression of α A-crystallin requires additional factors as Pax6 is expressed in other tissues, including brain, olfactory epithelium and pancreas not expressing α A-crystallin (Walther and Gruss, 1991). Genetic studies of the bZIP protein, c-Maf, established its essential role in the transcription of many crystallin genes during lens fiber cell differentiation (Kawauchi *et al*, 1999; Ring *et al*, 2000). In c-Maf null lenses, expression of α A-crystallin is reduced to about 1% of its normal level (Ring *et al*, 2000). Like Pax6, c-Maf can interact with multiple regions of the α A-crystallin promoter (Yang and Cvekl, 2005) and CREB interactions with a region of $-110/-103$ was shown *in vitro* (Cvekl *et al*, 1995). Regardless of the established roles of Pax6, c-Maf and CREB in various developmental contexts, the molecular basis of their activities during differentiation and in chromatin structure is unclear.

Herein, we examined mechanisms that regulate mouse α A-crystallin gene expression *in vivo*. We identified novel distal regulatory regions of this locus and determined their activities in transgenic mice. We also characterized interactions of specific DNA-binding transcription factors and ATP-dependent chromatin remodeling enzymes in a 16 kb region in cells/tissues exhibiting high, moderate or no expression of this gene.

Results

Identification of three distal control region (DCRs) in the mouse α A-crystallin locus

To identify putative DCRs, we aligned the mouse and human α A-crystallin loci using the VISTA algorithm for comparative genomics to identify evolutionary conserved noncoding regions as described in Supplementary data. We found three such regions termed DCR1, DCR2 and DCR3, as schematically shown in Figure 1A. To test their possible enhancer-like activities, we generated a series of reporter constructs containing the lens-specific α A-crystallin promoter fragment -111 to $+46$ driving luciferase expression combined with individual DCRs (see Figure 1B). It has been shown previously that treatment of explanted lens epithelia with FGF2 can induce endogenous α A-crystallin expression (Leenders *et al*, 1997). To assess whether any of these DCRs could confer induction of reporter activities in the presence of FGF2, we performed a series of transfections in primary lens epithelial explants and evaluated their promoter activities in the presence or absence of 100 ng/ml of FGF2. In the absence of FGF2, DCR1 activated the promoter 1.6-fold (Figure 1C). In contrast, both DCR2 and 3 reduced this promoter activity approximately two-fold. In the presence of FGF2, DCR1 increased the promoter activity approximately seven-fold compared to that of the promoter alone (Figure 1D). In contrast, the promoter activities of constructs containing DCR2 and DCR3 were similar to the activity of the promoter alone. Thus, DCR1 is likely to play a role in α A-crystallin gene regulation downstream of FGF-signaling.

Figure 1 Schematic representation of the mouse α A-crystallin locus and initial characterization of DCRs. (A) Genomic organization of the α A-crystallin gene showing the presence of evolutionary conserved noncoding DCR1 (-7706 to -7492), DCR2 (-1900 to -1670), and DCR3 ($+3650$ to $+3856$). Three exons (Ex1, Ex2 and Ex3) and a rodent specific exon (Ins) are shown. Positions of adjacent genes are shown in Supplementary data. (B) A schematic diagram of four reporter constructs used to test the activities of individual DCRs. Analysis of relative promoter activities in transfected rat lens explants in the absence (C) and presence (100 ng/ml) of FGF2 (D). The relative promoter activities from two independent experiments performed in triplicate were calculated by setting the activity of the -111 to $+46$ promoter fragment as 1. The transfection data were normalized using the CMV *Renilla* luciferase. Note that data shown in (C) and (D) are not directly comparable as the presence of FGF2 influences the differentiation state of the transfected cells.

DCR1 and DCR3 confer different temporal and spatial patterns of expression in lens vesicle, lens epithelium and primary lens fibers

To assess the roles of DCR1 and DCR3 in α A-crystallin gene regulation *in vivo*, we generated transgenic mice expressing EGFP under the control of DCR1, DCR3 and DCR1/DCR3 in combination with a 1.9 kb genomic fragment harboring DCR2 and the promoter (see Figure 1A and Supplementary data). We rationalized that the integrity of the genomic region including the promoter and its proximal DCR2 would be more comparable to their endogenous functions. Modest

expression of EGFP, driven by the 1.9 kb genomic region of α A-crystallin and assessed by direct fluorescence, was observed in lens fibers from E13.5 (see Figure 2). In contrast, DCR1, DCR3 and DCR1/DCR3 strongly activated this promoter in four, two and four transgenic lines, respectively (Figure 2). Analysis of RNAs and proteins from transgenic lenses confirmed the differences in fluorescence intensities (data not shown). No 'leaky' expression of EGFP was found in the brain, kidney and muscle in any of the mouse lines (data not shown). We thus concluded that both DCR1 and DCR3 act in combination with the 1.9 kb α A-crystallin promoter as potent lens-specific enhancers.

Analysis of the temporal and spatial expression of an EGFP reporter gene driven by DCR1 and the 1.9 kb α A-crystallin promoter during eye development revealed expression of EGFP in the lens vesicle of E11.5 embryos (Figure 2A) and expression upregulated in primary fibers at E12.5, while EGFP expression was maintained in the anterior lens epithelium (see Figure 2B). In contrast, expression driven by DCR3 and the 1.9 kb α A-crystallin promoter was delayed approximately 24 h in relation to the onset of EGFP expression driven by DCR1 (Figure 2). However, this expression preceded the expression driven by the 1.9 kb promoter alone. A combination of both DCR1 and DCR3 yielded expression pattern of EGFP comparable to the DCR1/1.9 kb promoter. Our data therefore suggest that DCR1 and DCR3 play distinct temporal roles in the regulation of the α A-crystallin gene *in vivo*: DCR1 directs both early expression in the lens vesicle and the initial increase during primary fiber cell differentiation. DCR3 contributes to this differentiation-associated expression pattern, although expression directed by DCR3 in mature lens fiber cells is delayed compared with that directed by DCR1. Our data do not formally exclude the possibility that DCR3 is also capable of supporting expression in lens epithelium, albeit below the detection limit of the EGFP assay.

Histone H3 acetylation and methylation mark the mouse α A-crystallin locus

Next, it was of interest to study the distribution of specific DNA-binding proteins and other regulators of chromatin structure and transcription at the α A-crystallin locus to understand the molecular mechanisms regulating α A-crystallin expression *in vivo*. However, the small amount of lens tissue available at the developmental stages of interest made ChIP experiments impractical in this system. In lieu of this, we used three cell and/or tissue models exhibiting high, moderate, and no expression of the α A-crystallin gene. One-day old mouse lens (P1) represents a tissue with high levels of expression. Cultured mouse lens α TN4-1 epithelial cells express moderate levels of α A-crystallin expression. In contrast, mouse L929 fibroblasts do not express α A-crystallin (Kantorow *et al*, 1993). Using quantitative RT-PCR, we found approximately 19-fold higher expression of α A-crystallin in the P1 mouse lens compared to α TN4-1 cells (Table 1 in Supplementary data). Therefore, these materials can provide nuclei for study of α A-crystallin chromatin *in vivo* using ChIPs.

Since high level histone H3 lysine 9 acetylation is a hallmark of transcriptionally active genes (Litt *et al*, 2001), we first examined its distribution at the α A-crystallin locus (Figure 3A). In fibroblasts, as expected, we detected a marginal presence of acetylated H3 K9 (Figure 3B). In lens

epithelial cells, the highest levels of H3 K9 acetylation were observed in the promoter region with moderately increased levels of H3 K9 acetylation at positions of -9 kb, DCR1 and $+4$ kb. In contrast, in mouse lens, we found that the highest H3 K9 acetylation occurred in DCR1 and the lowest was detected at $+4$ kb (Figure 3B). Thus, moderate levels of α A-crystallin expression are associated with a narrow region of H3 K9 acetylation in the promoter and coding regions of this gene. This H3 K9 acetylation domain was expanded in mouse lens chromatin and included both DCR1 and DCR3. The highest local level of H3 K9 acetylation in DCR1 correlates with its functional significance in temporal and spatial regulation of the α A-crystallin gene *in vivo*.

To further characterize epigenetic regulation of the α A-crystallin gene, we analyzed distribution of trimethylated lysine 4 histone H3 (H3K4Me3), since it is also associated with transcriptionally active genes (Kim *et al*, 2005). The localization of H3K4Me3 at the α A-crystallin locus was predominantly in the promoter and in its downstream region (see Figure 3C). As with H3 K9 acetylation, the intensity of this modification in the promoter region of cultured lens cells was lower compared to lens chromatin. The most likely reason for this finding is a depletion of histone H3, and, hence entire nucleosomes, from the promoter region in lens chromatin (Carey, 2005; Mito *et al*, 2005).

Binding of Pax6, c-Maf and CREB in the mouse α A-crystallin locus

Previous studies suggested Pax6, c-Maf and CREB as specific DNA-binding regulatory factors of the mouse α A-crystallin gene (Cvekl *et al*, 2004). To determine binding of Pax6, c-Maf, CREB and TBP in the mouse α A-crystallin locus, we prepared chromatin from fibroblasts, lens epithelial cells and 1-day old lenses, and evaluated *in vivo* binding of these factors in a region from -10 kb to $+6$ kb of the mouse α A-crystallin locus extending from DCR1 to DCR3. We started our analysis with Pax6, a key regulator of lens development (Ashery-Padan *et al*, 2000). We did not find Pax6 in chromatin from mouse fibroblasts (data not shown), in agreement with lack of Pax6 expression in these cells (Cvekl *et al*, 1995). In lens, epithelial cells, we found Pax6 at DCR1, DCR2 and the promoter at comparable levels (Table I and Supplementary data). We also found Pax6 at DCR3, albeit at lower levels. In contrast, in mouse lens chromatin, we detected the presence of Pax6 only at DCR1 and at the promoter. These data established that Pax6 associates with important regions of the mouse α A-crystallin locus *in vivo*.

We next determined the occupancy of c-Maf in the 16 kb α A-crystallin locus. We did not find any c-Maf at this locus in fibroblasts (data not shown). In the chromatin of lens epithelial cells, we detected higher binding of c-Maf at DCR1, DCR2, the promoter and exon 2 compared to a slightly reduced binding at DCR3 (Table I and Supplementary data). In the mouse lens, we detected approximately 20-fold increased abundance of c-Maf in the promoter while the amount of c-Maf in DCR1 remained almost unchanged from its levels in lens epithelial cells. No evidence of occupancy of MafA, MafB and NRL at the α A-crystallin locus was obtained (data not shown). These data demonstrate that the increased levels of c-Maf in the promoter correlate with strong transcriptional activity of the α A-crystallin gene in P1 lens compared to lens epithelial cells.

Figure 2 Expression of EGFP driven by four transgenic constructs, promoter, DCR1–promoter, promoter–DCR3 and DCR1–promoter–DCR3 in embryonic eye. **(A)** Transverse sections of the developing eye at E10.5, E11.5, E12.5, E13.5 and E14.5. The signals are EGFP (green), DNA (blue) and phalloidin (red). **(B)** Higher magnifications showing expression of EGFP in the lens epithelium.

Figure 3 ChIPs of the distribution of histone H3 lysine 9 acetylation and lysine 4 trimethylation over the 16 kb mouse α A-crystallin locus. (A) Schematic diagram of the α A-crystallin locus showing positions of PCR amplicons. (B) Distribution of H3 K9 acetylation. (C) Distribution of H3 K4 trimethylation. The relative enrichments are shown as 1% of the input.

Previously, we have shown CREB binding to the distal promoter region of the mouse α A-crystallin promoter (Cvekl *et al*, 1995). Although CREB proteins were detected in L929 nuclear extracts (Cvekl *et al*, 1995), we did not find evidence for *in vivo* interactions between CREB and the α A-crystallin promoter *in vivo* in mouse fibroblasts (data not shown). However, in cultured lens epithelial cells, we detected CREB in the promoter (Table I and Supplementary data). In the lens, we found increased abundance of CREB both in the promoter, exon 2 and DCR3. These data established that strong enrichments of CREB in both the promoter and DCR3 in the lens correlated with higher transcriptional activity of α A-crystallin in lens compared to lens epithelial cells.

Expression of EGFP directed by DCR1-promoter is reduced in Pax6 heterozygous lens

To demonstrate whether Pax6 regulates early embryonic expression of the α A-crystallin gene, we crossed mice harboring the DCR1-promoter-EGFP construct with Pax6 hetero-

zygous mice, and determined both the temporal-spatial and quantitative levels of transgenic EGFP and endogenous α A-crystallin genes. Pax6 haploinsufficiency delayed the process of lens placode formation. As a result, smaller lens vesicles are produced (van Raamsdonk and Tilghman, 2000). Our data show that primary lens fiber cell differentiation was delayed in DCR1-promoter $\times$ Pax6 +/– eye at E12.5 (see Figure 4) compared to more elongated fibers found at the DCR1-promoter-EGFP eye (see Figure 2A). Next, we analyzed the transcript levels for EGFP driven by the DCR1- α A-promoter and the endogenous α A-crystallin genes in newborn transgenic and Pax6 +/– lenses and found that Pax6 +/– animals had lower transcript levels for both of these genes (see Table II), demonstrating that transgenic expression of the entire DCR1-promoter-EGFP is sensitive to Pax6 gene dosage.

Pax6 and c-Maf activate transcription via DCR1

Given the function of DCR1 in the above developmental studies and its association with Pax6 and c-Maf *in vivo*, we

Table I Distribution of Pax6, c-Maf, CREB, TBP, RNAP, Brg1 and *Snf2h* over the 16 kb of mouse α A-crystallin locus

		−10 kb	−9 kb	−8 kb (DCR1)	−7 kb	−6 kb	−4 kb	−2 kb (DCR2)	Promoter	+ 2 kb (Exon2)	+ 3 kb (DCR3)	+ 4 kb	+ 6 kb
Pax6 ^a	α TN4-1	0.00	0.00	1.61	0.00	0.04	0.02*	1.19	1.02	0.00	0.28	0.00	0.00
	P1 lens	0.08	0.07	2.66	0.02	0.08	0.09*	0.00	1.44*	0.07*	0.14*	0.09*	0.09*
c-Maf ^a	α TN4-1	0.00	0.00	1.63	0.01	0.09	0.05*	0.99	1.16	0.00	0.43	0.00	0.00
	P1 lens	0.33*	0.04*	0.46	0.21*	0.37	0.21	0.00	19.53*	3.78	0.28	0.83	0.00
CREB ^a	α TN4-1	0.00	0.00	0.01	0.00	0.00	0.01	0.03	0.42	0.00	0.04	0.01	0.01
	P1 lens	0.15	0.07	0.00	0.24	0.54*	0.00	0.00	11.38*	4.22	61.26	0.15	0.04
TBP	α TN4-1	0.00	0.00	3.61	0.06*	0.11	0.03	1.03	0.62	0.00	0.56	0.00	0.02
	P1 lens	0.02	0.04	0.73	0.11	0.04	0.07	0.00	0.97	0.04	0.10	0.06	0.04*
RNAP	α TN4-1	0.00	0.00	0.26	0.02	0.04*	0.04*	0.22	0.16	0.00	0.17	0.00	0.01
	P1 lens	0.06	0.03	0.93*	0.16	0.04	0.03*	0.00	1.23	0.08	0.15	0.16*	0.09
Brg1	α TN4-1	0.00	0.00	0.06	0.00	0.10	0.00	0.07*	0.13	0.12	0.08	0.17	0.06
	P1 lens	3.62	2.62	0.76	3.44	0.93	3.27	0.17	1.61	0.78	1.30	2.72	1.88
<i>Snf2h</i>	α TN4 -1	0.00	0.00	0.05*	0.00	0.09	0.00	0.06	2.01	0.04*	0.06	0.03	0.12
	P1 lens	2.09	2.49	0.41	1.69	0.49	1.52	0.36	0.72	0.53	0.69	1.87	1.07*

The values are relative enrichments shown as 1% of the input.

Standard deviations (SDs) of the majority of qChIP data were less than 20% of the mean values. The higher SDs (20–30% of the mean value) are indicated by asterisk.

^aBar diagrams of relative enrichments of these three factors including SDs are shown in the Supplementary data.

Figure 4 Temporal and spatial analysis of EGFP expression in DCR1-promoter-EGFP $\times$ Pax6 $\pm$ mice. EGFP expression at E11.5 (A, E), 12.5 (B, F), 13.5 (C, G) and 14.5 (D, H).

Table II Expression levels of α A-crystallin and EGFP in transgenic mouse lenses

Transgenic model	CRYAA (%)	EGFP (%)
DCR1	100	100
DCR1 $\times$ Pax6 $\pm$	36.9	33.7

analyzed its sequence for such binding sites. The results show the presence of two candidate Pax6- and three Maf-binding sites (MAREs) in DCR1 (see Figure 5A). To test if DCR1 mediates transcriptional activation in the presence of Pax6 and c-Maf, we prepared a reporter containing DCR1 cloned 5' of the heterologous β^A -globin minimal promoter (see Figure 5A) that is known to be un-responsive to

these factors (Czerny and Busslinger, 1995). Pax6 activated this reporter seven-fold and c-Maf activated this reporter 41-fold as shown in Figure 5B. These data further support the idea that DCR1 acts as a distal enhancer of the α A-crystallin locus, and at least a part of its function can be mediated by interactions with Pax6 and c-Maf. In summary, our data suggest interactions of key DNA-binding transcription factors with novel regulatory regions of the α A-crystallin gene, and show notable differences in interactions of c-Maf and CREB with the mouse α A-crystallin locus *in vivo*. The function of DCR1 in the lens vesicle and lens epithelium is consistent with binding of Pax6 and c-Maf *in vivo* and its ability to be activated by these factors in reporter assays. In contrast, DCR3 function correlated with strong binding of CREB, supporting the idea that DCR1 and DCR3 play distinct roles as shown above.

Figure 5 Activation of DCR1 by c-Maf and Pax6 in cultured cells. **(A)** Diagrammatic representation of DCR1. The positions and sequences of two Pax6- and three Maf-binding sites are shown. **(B)** 293T cells were transiently transfected with 2 μ g of reporter plasmid, 200 ng of each expression plasmid encoding Pax6, c-Maf or empty vector (pKW10), and 0.5 ng of *Renilla*-CMV. Each transfection was performed twice in triplicates. Relative promoter activities were calculated using the 'empty vector' value set as 1.

Recruitment of RNA polymerase II to the α A-crystallin locus

Identification of regions harboring RNA polymerase II provides information regarding the recruitment of this enzyme to the α A-crystallin locus. We first assessed binding of TBP/TFIID to the α A-crystallin promoter and locus. In ChIP assays of lens epithelial cells, we detected the majority of TBP in DCR1 (Table I) although DCR1 does not contain any obvious TATA-box. We also found smaller amounts of TBP in DCR2, the TATA-containing promoter and DCR3. In contrast, in mouse lens, we found a substantial fraction of TBP in the promoter region with appreciable levels also in DCR1. In lens epithelial cells, we detected enrichments of RNA polymerase II in DCR1, DCR2, the promoter and DCR3 (Table I). This pattern of enrichment correlated with enrichments of TBP. In mouse lens, we found increased abundance of RNA polymerase II in two regions, the promoter and DCR1. This profile was almost identical to the profile of TBP in the same chromatin. In addition, novel enrichments of RNA polymerase II were found in exon 2, and regions +2 and +4 kb downstream from the promoter. These enrichments could signal an active transcriptional process.

An alternate explanation for the presence of TBP in DCR1 is that this region harbors a cryptic promoter. To address this possibility, we performed 5'-RACE mapping and conducted amplification of a series of regions between DCR1 and the promoter using total RNAs from mouse E12.5 embryos, newborn lenses and cultured lens epithelial cells (see Supplementary data). We did not obtain any evidence for transcription in this region supported by analysis of ESTs in various databases.

Differential recruitment of Brg1 and Snf2h to the α A-crystallin locus mark its transcriptional status

Condensed chromatin characterized by a regular nucleosomal organization represents a major structural barrier for transcription that interferes with many phases of the transcriptional process (Khorasanizadeh, 2004). It has been shown that active chromatin remodeling mediated by SNF/SWI and ISWI ATP-dependent complexes is required for transcription; however, different genes and their variable levels of expression are likely to function in a broad continuum of remodeling activities. Herein, we examined the presence and distribution of Brg1, a catalytic subunit of SWI/SNF, and Snf2h,

a similar subunit of the ISWI complex in the α A-crystallin locus (Table I). In lens epithelial cells, we found low levels of *Brg1* at the majority of sites (8 of 12) along the α A-crystallin locus, particularly between DCR2 to +6 kb. In contrast, in mouse lens chromatin, we observed significantly higher amounts of *Brg1* across the entire 16 kb of the analyzed region. The amounts of *Brg1* were on average 30 times higher in lens compared to lens epithelial cells. In both systems, *Brg1* was present at DCR1, DCR2, the promoter and DCR3, which we have shown are all occupied by Pax6 and c-Maf. However, we also found high levels of *Brg1* at regions -10, -9, -7, -4 and +4 kb in lens chromatin. These regions did not associate with Pax6, c-Maf, CREB or TBP (see Table I). These results suggest that high levels of α A-crystallin expression are associated with an abundance of *Brg1*, and by inference the SWI/SNF chromatin remodeling complexes, throughout the 16 kb of the locus.

As *Brg1* and *Snf2h* are thought to play nonredundant roles in chromatin remodeling during development (Bultman *et al*, 2000; Stopka and Skoultschi, 2003; Fan *et al*, 2005), we next examined the distribution patterns of *Snf2h*. In the chromatin of lens epithelial cells, we found *Snf2h* at a single region, the promoter (see Table I). In contrast, in lens chromatin, we found a broad region of *Snf2h* covering positions -10 to +6 kb of the α A-crystallin locus. Enrichment of *Snf2h* dropped nearly three-fold in the promoter region in lens epithelial cells compared to the lens chromatin. Limited abundance of *Snf2h* to the α A-crystallin promoter in lens epithelial cells raised the possibility that *Snf2h* is needed for a distinct step in chromatin remodeling in the promoter to achieve the moderate levels of α A-crystallin expression in epithelial cells prior to broad recruitment of SWI/SNF and ISWI complexes to the whole α A-crystallin locus in lens fiber cells. It was shown earlier that SWI/SNF and ISWI complexes use different mechanisms for chromatin remodeling (Fan *et al*, 2005). To further test this model, we determined which DNA-binding factors recruit *Brg1* and *Snf2h*. Using co-immunoprecipitation assays, we found that Pax6 bound *Brg1* (Figure 6A) and c-Maf bound *Snf2h* (Figure 6B). In addition, CREB interacted with both *Brg1* and *Snf2h* (Figure 6C and D). In contrast, no interactions between Pax6 and *Snf2h*, and c-Maf and *Brg1* were detected (data not shown). Inclusion of ethidium bromide to disrupt protein-DNA interactions did not prevent formation of *Brg1*/Pax6 complexes but *Snf2h*/c-Maf complexes were disrupted (data not shown). These data raise the possibility that c-Maf and CREB directly recruit *Snf2h* and Pax6 lures *Brg1* to the α A-crystallin locus. However, it is not known if these complexes are direct or mediated by other proteins.

Discussion

DCR1 and DCR3 temporally and spatially control α A-crystallin gene expression

The transcription of tissue-restricted genes during development often depends on the function of a promoter in combination with multiple DCRs. This modular organization of regulatory regions within the locus allows various external signals to control the expression of specific genes. Here, we identified two such regions DCR1 and DCR3 that regulate the expression of the mouse α A-crystallin gene in the lens. We show that DCR1 is sufficient for EGFP reporter gene expression in combination with the 1.9 kb α A-crystallin pro-

Figure 6 Protein-protein interactions between *Brg1*, *Snf2h*, Pax6, c-Maf and CREB. (A) 293T cells were cotransfected with 3xFlag-Pax6 and *Brg1* and immunoprecipitated with either anti-Flag antibodies (left) or *Brg1* (right), and the interacting partner was detected by its antibody and Western blot analysis (lower panels). (B) 293T cells were cotransfected with Flag-*Snf2h* and c-Maf and immunoprecipitated with either anti-c-Maf (left) or anti-Flag antibodies (right) and the interacting partner was detected as described above. (C) 293T cells were cotransfected with Flag-*Snf2h* and CREB, followed by immunoprecipitation with Flag antibody and Western analysis using the anti-Flag and anti-CREB antibody. (D) 293T cells were cotransfected with *Brg1* and CREB, followed by immunoprecipitation with CREB antibody and Western analysis using anti-CREB and anti-*Brg1* antibody.

motor in lens vesicle and lens epithelium of transgenic mice. Both expression patterns directed by DCR1- and DCR1/DCR3-transgenes virtually recapitulated the spatial and temporal pattern of expression of the endogenous α A-crystallin gene (Robinson and Overbeek, 1996). The early activity of DCR1 in primary lens fibers in transgenic mice was consistent with the roles of FGFs as key lens differentiation signals (Faber *et al*, 2001) and its capacity to mediate this signaling in primary lens explants (Leenders *et al*, 1997). In contrast, DCR3/transgenes were activated 24 h later compared to DCR1/transgenes, and their expression was restricted to more elongated lens primary fiber cells. In transfected primary lens cells, DCR3 did not increase the *luc* reporter gene activity in the presence of FGF2. Thus, DCR3 could be a downstream target for Wnt- (Stump *et al*, 2003; Lyu and Joo, 2004; Smith *et al*, 2005) and TGF β - (de Jongh *et al*, 2001) signaling operating after FGF-triggered lens fiber cell differentiation (Lovicu and McAvoy, 2005).

Changes in binding of Pax6, c-Maf and CREB to the α A-crystallin locus

Analysis of positions and quantities of transcription factors in the 16 kb region of α A-crystallin locus in chromatin with no, moderate or high levels of expression of α A-crystallin provided novel insights into the mechanism of up-regulation of this gene during lens fiber cell differentiation. Although each DCR and the promoter interacted with Pax6, a redistribution of Pax6 was evident in chromatin expressing moderate and high levels of α A-crystallin. Stable association of Pax6

proteins with the α A-crystallin promoter and DCR1 in the lens chromatin is in agreement with Pax6's dual roles in the establishment of lens lineage and direct regulation of the α A-crystallin gene.

The high level of α A-crystallin expression in the lens correlated with a nearly 20-fold higher focal abundance of c-Maf in the promoter in lens compared to its amount detected in the promoter in lens epithelial cells. This finding agrees both with the upregulation of c-Maf in primary lens fibers (Kawauchi *et al*, 1999; Ring *et al*, 2000), dramatic reduction of α A-crystallin expression in c-Maf null lenses and higher expression of α A-crystallin in the lens compared to lens epithelial cells. Our data show that c-Maf associates with the same regions of the α A-crystallin locus as Pax6; however, we found nearly 20-fold higher binding of c-Maf in the promoter in the lens compared to lens epithelial cells. Since DCRs occupied by c-Maf do not undergo this dramatic change, some selective mechanism recruiting c-Maf to the promoter is involved.

In spite of the ability of chicken L-Maf/MafA to induce transdifferentiation of neuroretina to lens and its activation of the chicken α A-crystallin promoter (–244 to +89) in cultured cells (Ogino and Yasuda, 1998), post-translational regulation of quail MafA via FGF signaling (Benkhelifa *et al*, 2001) and expression of MafB and NRL in lens (Coolen *et al*, 2005), we could not establish interactions of MafA/L-Maf, MafB and NRL with the mouse α A-crystallin locus using ChIPs. Furthermore, variable expression patterns of large Maf genes in mouse, chicken, frog and zebrafish lenses, termed 'expression domain shuffling' (Coolen *et al*, 2005) suggest that c-Maf and L-Maf/MafA play distinct roles during mouse and chick lens differentiation.

Similar to c-Maf, an increased abundance of CREB was also found in the α A-crystallin promoter in lens chromatin. Owing to sequence similarity between Maf- and CREB-binding sites and their ability to form heterodimers (Vinson *et al*, 2002), it is possible that either homodimers and/or heterodimers of these proteins are present in the α A-crystallin promoter *in vivo*. The only molecular marker of the active DCR3 was a 26-fold higher enrichment of CREB in the lens compared to lens epithelium. Therefore, our data suggest CREB and its upstream signaling as one of the possible mechanisms of DCR3 function in 'late' lens primary fibers (Figure 2). Notably, TGF β and Wnt signaling also regulate lens fiber cell differentiation (de Jongh *et al*, 2001; Stump *et al*, 2003), and both these pathways can operate via protein kinase A, an activator of CREB (Schiller *et al*, 2003; Chen *et al*, 2005). Our data provide a possible downstream target region for TGF β and Wnt signaling pathways, DCR3, for future studies of lens fiber cell differentiation.

The pattern of TBP binding to DCR1, 2 and 3 correlates with that of Pax6 in chromatin of lens epithelial cells and in lens. We have shown earlier that Pax6 could interact with TBP/TFIID in lens nuclear extracts (Cvekl *et al*, 1999). Other studies showed interactions between large Maf proteins, including c-Maf, with TBP (Friedman *et al*, 2004), and, thus, both Pax6 and c-Maf may recruit TBP/TFIID to these DCRs. In lens chromatin, Pax6 and TBP co-localized in the promoter and DCR1, and RNA polymerase II co-localized with TBP/Pax6. Identification of RNA polymerase II enrichments in DCR1 requires further explanation: one possibility is that a DNA loop formed between DCR1 and the α A-crystallin

promoter (Wijgerde *et al*, 1995; Carter *et al*, 2002) or recruitment of RNA polymerase II via Pax6/c-Maf/TBP to DCR1 serve as an 'intermediate' storage compartment for the enzyme. Additional studies of these nonmutually exclusive mechanisms are necessary.

Distribution of modified histones and chromatin remodeling enzymes Brg1 and Snf2h in the α A-crystallin locus

Epigenetic regulation of gene expression ultimately changes nucleosomal organization of individual genes by chromatin remodeling (Narlikar *et al*, 2002). Chromatin remodeling can be divided into covalent histone modifications (Berger, 2002) and ATP-dependent movement of nucleosomes (Kingston and Narlikar, 1999). Herein we found a 13 kb domain of acetylated histone H3 K9 in lens chromatin marked at its 5'/3' borders by DCR1 and DCR3. In contrast, we detected a less than 5 kb long H3 K9 hyperacetylated domain in lens epithelial cells (see Figure 7). Methylated histone H3 (H3K4Me3) was mostly found in the α A-crystallin locus at the promoter with asymmetric extension into the transcribed region in chromatin from both lens epithelial cells and whole lens. Interpretation of local intensities of H3 K9 acetylation and K4 trimethylation along the α A-crystallin locus requires identification of positions of individual nucleosomes and enzymes catalyzing post-translational modifications of histones. It is likely that reduced amounts of H3 K9 acetylated and K4 methylated histones in the promoter region of α A-crystallin in whole lens chromatin are caused by the removal of multiple nucleosomes from this region (Carey, 2005; Mito *et al*, 2005).

Removal of nucleosomes is performed by structurally and functionally distinct SWI/SNF and ISWI chromatin remodeling complexes (Fan *et al*, 2005). Our data show localized recruitment of Snf2h to the α A-crystallin promoter (see Figure 7) in combination with a broad and low presence of Brg1 in chromatin from lens epithelial cells. In contrast, in whole lens chromatin, high abundance of both Brg1 and Snf2h was found through the entire 16 kb region of the α A-crystallin locus. There is a great demand for crystallins in lens fibers as they accumulate to an average concentration of 450 mg/ml of total protein (Horwitz, 2003). As α A-crystallin is a single-copy gene, its high level expression in lens fibers is likely to require elimination of all structural barriers to transcription. The sustained recruitment of both SWI/SNF and ISWI complexes to the entire α A-crystallin locus may be required to fulfill this need.

A multistage model of α A-crystallin gene activation

The molecular mechanisms that regulate expression levels of differentiation markers are still poorly characterized. Our data are most consistent with a multistage model, whereby the lens progenitor cells forming the lens placode do not express the α A-crystallin gene (Robinson and Overbeek, 1996). The transcriptionally inactive α A-crystallin locus is then converted into an 'open' chromatin domain suitable for transcription. Recent studies of early development and initiation of tissue-specific transcription showed that specific 'decisive/pioneer' transcription factors initiate chromatin opening (Cosma, 2002; Lomvardas and Thanos, 2002). Genetic studies of Pax6 and its *Drosophila* homologue *eyeless* established Pax6 as an early developmental 'stage-selector'

Figure 7 Diagrammatic summary of ChIP results. In lens epithelial cells, enrichments of Pax6, c-Maf, CREB, TBP and *Snf2h* are shown. A limited H3 K9 acetylation domain and low abundance of *Brg1* are shown as a horizontal bracket, and as dotted ovals, respectively. Localized recruitment of *Snf2h* to the promoter results in moderate transcriptional activity. In lens fiber cells, increased enrichments of c-Maf and CREB to the promoter, and CREB to DCR3 are shown by their enlarged shapes. An extended H3 K9 acetylation domain is shown as a horizontal bracket. Recruitment of both *Brg1* and *Snf2h* into the locus results in high expression of α A-crystallin.

gene (Graw, 2003). We hypothesize that some stage-selector proteins including Pax6 aid chromatin remodeling of their target genes.

High levels of Pax6 and *Brg1* expression in the lens placode (Chauhan *et al*, 2002) prior to the expression of c-Maf (Ring *et al*, 2000) indirectly suggests an early role for Pax6 via recruitment of *Brg1* to initiate chromatin remodeling of the α A-crystallin locus in the lens placode. In addition, recent studies have implicated Pax6 in the regulation of c-Maf (Sakai *et al*, 2001) and *Brg1* (Chauhan *et al*, 2002) expression. α A-crystallin expression would then begin as *Snf2h*/ISWI complexes complete the chromatin remodeling necessary for its transcriptional initiation in the invaginating lens pit. Nevertheless, our data cannot exclude the alternate possibility that c-Maf/*Snf2h*/ISWI localize to the α A-crystallin first during the invagination of the lens placode facilitating binding of Pax6 to the locus. We hypothesize that remodeling of the locus requires DCR1 as the promoter alone is inactive in transgenic mice at this stage.

Our data show that the increase of α A-crystallin expression in response to FGF-signaling is mediated by both DCR1 and the α A-crystallin promoter. We propose that FGF-signaling directly or indirectly increases the selective abundance of c-Maf in the promoter and boosts promoter strength in primary lens fibers. While c-Maf abundance at DCR1 did not increase in the whole lens as compared to lens epithelial cells, the presence of two consensus Ets-binding sites in this element suggest that Ets factors, nuclear executors of FGF-signaling (Barolo and Posakony, 2002), are responsible for FGF mediated activation of DCR1. Recruitment of *Snf2h* via c-Maf and *Brg1* via Pax6 may then increase the abundance of chromatin remodeling enzymes in the α A-crystallin locus. The highest transcriptional state of the α A-crystallin gene may be reached by CREB activation triggered by activated Wnt- and TGF β -cooperating with FGF-signaling in 'late' primary lens fibers and recruitment of CREB to both DCR3 and its promoter. Additional studies will be needed to probe various aspects of this model.

Materials and methods

Materials

Antibodies used for ChIPs: anti-Pax6, anti-c-Maf, anti-TBP and anti-RNA polymerase II were purchased from Santa Cruz Biotechnology; anti-CREB-1 from ActiveMotif, anti-acetylated H3 K9 and anti-trimethylated H3 K4 from Upstate Biotechnology and rabbit IgG from Oncogene. Recombinant FGF2 was from Sigma, phalloidin and DAPI were from Molecular Probes.

Reporter plasmids and transient transfections

DCRs generated by PCR were subcloned 5' of the mouse α A-crystallin promoter (−111 to +46) in pGL2 (Yang and Cvekl, 2005). Primary lens explants were obtained from 3-day-old rat lenses, 2 μ g of reporter and 50 ng of CMV *Renilla* reference plasmids were transfected with Effecten system (Qiagen) as described elsewhere (Golestaneh *et al*, 2004). Transfections of 293T cells by Lipofectamine Plus (Invitrogen) and their analyses were described earlier (Yang *et al*, 2004). Dual-Luciferase Reporter Assay System (Promega) was used according to the manufacturer's instructions.

Transgenic mice production and analysis of EGFP expression

Four reporter plasmids were generated in pEGFP-1 (Clontech) as diagrammatically shown in Supplementary Figure 2A. The reporter cassettes were released from the plasmids by *Mlu*I and *Afl*III digestion to generate transgenic mice by pronuclear injection of fertilized eggs at the AECOM Transgenic Core Facility. Pax6 heterozygous mice (Pax6 +/−lacZ) were obtained from P Gruss (St-Onge *et al*, 1997). EGFP fluorescence was directly visualized using a Leica AOBs confocal microscope. DNA/nuclei and cell morphology were visualized by DAPI and phalloidin (Beebe *et al*, 2001) counter-staining. For qRT-PCR analysis, the following primers were used: α A-crystallin (5'-GAGATTACACGGCAAACACAA and 5'-ACATTGGAAGGCAGACGTA) and EGFP (5'-ACACGCGCACTACAAGACC and 5'-GTCTCTCTGAAGTCGATGC).

qChIP

Formaldehyde crosslinked chromatin was obtained from two different cultivations of mouse lens epithelial (α TN4-1) and fibroblast (L929) cells, and from two groups of pooled 400 1-day-old lenses as described earlier (Yang and Cvekl, 2005). The sheared chromatin (average size 600 bp) was generated by sonication (Stopka *et al*, 2005). Aliquots of chromatin prepared from 2.5×10^6 cells and 20 lenses were incubated with 1–2 μ g of antibody bound to 8 μ l of a 1:1 mixture of protein G- and protein A-Sepharose beads (Sigma). The immunoprecipitates were washed three times

and resuspended in a buffer containing 10 mM Tris-HCl pH 8.0, 100 mM NaCl, 25 mM EDTA supplemented with 0.1 mg/ml RNaseA and 0.2 mg/ml proteinase K. After the 2 h incubation at 55°C, the crosslinks were reversed by overnight incubation at 65°C. Genomic DNA was eluted into 250 μ l of water using QIAquick Spin Gel Purification kit (Qiagen).

The amounts of each specific DNA fragment in immunoprecipitates were determined by quantitative PCR reactions using a standard curve generated for each primer set with 0.05, 0.2, 0.5 and 1% input DNA samples (Stopka *et al*, 2005). Using a standard curve, we transformed C_t values into DNA copy numbers. The copy number of a specific DNA fragment in each assay was compared to the copy number of that fragment before immunoprecipitation ('input DNA'). The values obtained with control antibody (IgG) were subtracted from that corresponding value.

Primers were designed using Primer 3 (<http://frodo.wi.mit.edu/cgi-bin/primer3>) and their sequences are given in Supplementary data. The PCR products were between 60 and 130 bp. The reactions were analyzed using a 7900 ABI PRISM PCR Instrument and a 2xSYBR mix (ABI). The parameters were: 95°C/10 min followed by 45 cycles of (94°C/10 s, 60°C/20 s and 72°C/30 s) (Stopka *et al*, 2005).

Co-immunoprecipitations

Co-immunoprecipitations were performed using 3xFlag tagged Pax6, c-Maf and CREB proteins (Yang and Cvekl, 2005), Flag tagged *Snf2h* (provided by Dr GJ Narlikar) and *Brg1* co-expressed in 293T cells as described elsewhere (Wu *et al*, 2003). Briefly, the cells grown on 10 cm dishes were transfected with 10 μ g of each plasmid DNA using calcium phosphate precipitates. The transfected

cells were cultured for 48 h and lysed in a buffer containing 20 mM Tris-HCl pH 7.5, 140 mM NaCl, supplemented with 1% Triton X-100, and proteinase inhibitor mixture (Roche). In total, 500 μ g of total protein was incubated with anti-FLAG M2 (Sigma), anti-*Brg1*, anti-Pax6, anti-c-Maf and anti-CREB antibody followed by incubation with protein G-Sepharose beads. The beads were washed five times with the above buffer containing 0.5% Tween-20. The precipitated proteins were released by boiling for 5 min in a buffer containing 100 mM Tris, 10 mM DTT and 4% SDS. The proteins were subjected to SDS-gel electrophoresis in 6% precast gels. Specific experiments included 100 μ g/ml ethidium bromide to disrupt the protein-DNA interactions (Lai and Herr, 1992).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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References

- Ashery-Padan R, Marquardt T, Zhou X, Gruss P (2000) Pax6 activity in the lens primordium is required for lens formation and for correct placement of a single retina in the eye. *Genes Dev* **14**: 2701–2711
- Barolo S, Posakony JW (2002) Three habits of highly effective signaling pathways: principles of transcriptional control by developmental cell signaling. *Genes Dev* **16**: 1167–1181
- Beebe DC, Vasiliev O, Guo J, Shui YB, Bassnett S (2001) Changes in adhesion complexes define stages in the differentiation of lens fiber cells. *Invest Ophthalmol Vis Sci* **42**: 727–734
- Benkhelifa S, Provot S, Nabais E, Eychene A, Calothy G, Felder-Schmittbuhl MP (2001) Phosphorylation of MafA is essential for its transcriptional and biological properties. *Mol Cell Biol* **21**: 4441–4452
- Berger SL (2002) Histone modifications in transcriptional regulation. *Curr Opin Genet Dev* **12**: 142–148
- Bloemendal H, de Jong W, Jaenicke R, Lubsen NH, Slingsby C, Tardieu A (2004) Ageing and vision: structure, stability and function of lens crystallins. *Prog Biophys Mol Biol* **86**: 407–485
- Bultman S, Gebuhr T, Yee D, La Mantia C, Nicholson J, Gilliam A, Randazzo F, Metzger D, Chambon P, Crabtree G, Magnuson T (2000) A *Brg1* null mutation in the mouse reveals functional differences among mammalian SWI/SNF complexes. *Mol Cell* **6**: 1287–1295
- Carey M (2005) Chromatin marks and machines, the missing nucleosome is a theme: gene regulation up and downstream. *Mol Cell* **17**: 323–330
- Carter D, Chakalova L, Osborne CS, Dai YF, Fraser P (2002) Long-range chromatin regulatory interactions *in vivo*. *Nat Genet* **32**: 623–626
- Chauhan BK, Reed NA, Yang Y, Cermak L, Reneker L, Duncan MK, Cvekl A (2002) A comparative cDNA microarray analysis reveals a spectrum of genes regulated by Pax6 in mouse lens. *Genes Cells* **7**: 1267–1283
- Chen AE, Ginty DD, Fan CM (2005) Protein kinase A signalling via CREB controls myogenesis induced by Wnt proteins. *Nature* **433**: 317–322
- Coolen M, Sii-Felice K, Bronchain O, Mazabraud A, Bourrat F, Retaux S, Felder-Schmittbuhl MP, Mazan S, Plouhinec JL (2005) Phylogenomic analysis and expression patterns of large Maf genes in *Xenopus tropicalis* provide new insights into the functional evolution of the gene family in osteichthyans. *Dev Genes Evol* **215**: 327–339
- Cosma MP (2002) Ordered recruitment: gene-specific mechanism of transcription activation. *Mol Cell* **10**: 227–236
- Cvekl A, Kashanchi F, Brady JN, Piatigorsky J (1999) Pax-6 interactions with TATA-box-binding protein and retinoblastoma protein. *Invest Ophthalmol Vis Sci* **40**: 1343–1350
- Cvekl A, Kashanchi F, Sax CM, Brady JN, Piatigorsky J (1995) Transcriptional regulation of the mouse alpha A-crystallin gene: activation dependent on a cyclic AMP-responsive element (DE1/CRE) and a Pax-6-binding site. *Mol Cell Biol* **15**: 653–660
- Cvekl A, Yang Y, Chauhan BK, Cveklava K (2004) Regulation of gene expression by Pax6 in ocular cells: a case of tissue-preferred expression of crystallins in lens. *Int J Dev Biol* **48**: 829–844
- Czerny T, Busslinger M (1995) DNA-binding and transactivation properties of Pax-6: three amino acids in the paired domain are responsible for the different sequence recognition of Pax-6 and BSAP (Pax-5). *Mol Cell Biol* **15**: 2858–2871
- de Jongh RU, Lovicu FJ, Overbeek PA, Schneider MD, Joya J, Hardeman ED, McAvoy JW (2001) Requirement for TGFbeta receptor signaling during terminal lens fiber differentiation. *Development* **128**: 3995–4010
- Duncan MK, Li X, Ogino H, Yasuda K, Piatigorsky J (1996) Developmental regulation of the chicken beta B1-crystallin promoter in transgenic mice. *Mech Dev* **57**: 79–89
- Faber SC, Dimanlig P, Makarenkova HP, Shirke S, Ko K, Lang RA (2001) Fgf receptor signaling plays a role in lens induction. *Development* **128**: 4425–4438
- Fan HY, Trotter KW, Archer TK, Kingston RE (2005) Swapping function of two chromatin remodeling complexes. *Mol Cell* **17**: 805–815
- Friedman JS, Khanna H, Swain PK, Denicola R, Cheng H, Mitton KP, Weber CH, Hicks D, Swaroop A (2004) The minimal transactivation domain of the basic motif-leucine zipper transcription factor NRL interacts with TATA-binding protein. *J Biol Chem* **279**: 47233–47241
- Golestaneh N, Fan J, Fariss RN, Lo WK, Zelenka PS, Chepelinsky AB (2004) Lens major intrinsic protein (MIP)/aquaporin 0 expression in rat lens epithelia explants requires fibroblast growth factor-induced ERK and JNK signaling. *J Biol Chem* **279**: 31813–31822
- Graw J (2003) The genetic and molecular basis of congenital eye defects. *Nat Rev Genet* **4**: 876–888
- Grindley JC, Davidson DR, Hill RE (1995) The role of Pax-6 in eye and nasal development. *Development* **121**: 1433–1442
- Horwitz J (2003) Alpha-crystallin. *Exp Eye Res* **76**: 145–153

- Kantorow M, Cvekl A, Sax CM, Piatigorsky J (1993) Protein-DNA interactions of the mouse alpha A-crystallin control regions. Differences between expressing and non-expressing cells. *J Mol Biol* **230**: 425-435
- Kawauchi S, Takahashi S, Nakajima O, Ogino H, Morita M, Nishizawa M, Yasuda K, Yamamoto M (1999) Regulation of lens fiber cell differentiation by transcription factor c-Maf. *J Biol Chem* **274**: 19254-19260
- Khorasanizadeh S (2004) The nucleosome: from genomic organization to genomic regulation. *Cell* **116**: 259-272
- Kim TH, Barrera LO, Zheng M, Qu C, Singer MA, Richmond TA, Wu Y, Green RD, Ren B (2005) A high-resolution map of active promoters in the human genome. *Nature* **436**: 876-880
- Kingston RE, Narlikar GJ (1999) ATP-dependent remodeling and acetylation as regulators of chromatin fluidity. *Genes Dev* **13**: 2339-2352
- Lai JS, Herr W (1992) Ethidium bromide provides a simple tool for identifying genuine DNA-independent protein associations. *Proc Natl Acad Sci USA* **89**: 6958-6962
- Leenders WP, van Genesen ST, Schoenmakers JG, van Zoelen EJ, Lubsen NH (1997) Synergism between temporally distinct growth factors: bFGF, insulin and lens cell differentiation. *Mech Dev* **67**: 193-201
- Litt MD, Simpson M, Recillas-Targa F, Prioleau MN, Felsenfeld G (2001) Transitions in histone acetylation reveal boundaries of three separately regulated neighboring loci. *EMBO J* **20**: 2224-2235
- Lomvardas S, Thanos D (2002) Modifying gene expression programs by altering core promoter chromatin architecture. *Cell* **110**: 261-271
- Lovicu FJ, McAvoy JW (2005) Growth factor regulation of lens development. *Dev Biol* **280**: 1-14
- Lyu J, Joo CK (2004) Wnt signaling enhances FGF2-triggered lens fiber cell differentiation. *Development* **131**: 1813-1824
- Mito Y, Henikoff JG, Henikoff S (2005) Genome-scale profiling of histone H3.3 replacement patterns. *Nat Genet* **37**: 1090-1097
- Narlikar GJ, Fan HY, Kingston RE (2002) Cooperation between complexes that regulate chromatin structure and transcription. *Cell* **108**: 475-487
- Ogino H, Yasuda K (1998) Induction of lens differentiation by activation of a bZIP transcription factor, L-Maf. *Science* **280**: 115-118
- Ring BZ, Cordes SP, Overbeek PA, Barsh GS (2000) Regulation of mouse lens fiber cell development and differentiation by the Maf gene. *Development* **127**: 307-317
- Robinson ML, Overbeek PA (1996) Differential expression of alpha A- and alpha B-crystallin during murine ocular development. *Invest Ophthalmol Vis Sci* **37**: 2276-2284
- Sakai M, Serria MS, Ikeda H, Yoshida K, Imaki J, Nishi S (2001) Regulation of c-maf gene expression by Pax6 in cultured cells. *Nucleic Acids Res* **29**: 1228-1237
- Schiller M, Verrecchia F, Mauviel A (2003) Cyclic adenosine 3',5'-monophosphate-elevating agents inhibit transforming growth factor-beta-induced SMAD3/4-dependent transcription via a protein kinase A-dependent mechanism. *Oncogene* **22**: 8881-8890
- Smith AN, Miller LA, Song N, Taketo MM, Lang RA (2005) The duality of beta-catenin function: a requirement in lens morphogenesis and signaling suppression of lens fate in pericocular ectoderm. *Dev Biol* **285**: 477-489
- St-Onge L, Sosa-Pineda B, Chowdhury K, Mansouri A, Gruss P (1997) Pax6 is required for differentiation of glucagon-producing alpha-cells in mouse pancreas. *Nature* **387**: 406-409
- Stopka T, Amanatullah DF, Papetti M, Skoultschi AI (2005) PU.1 inhibits the erythroid program by binding to GATA-1 on DNA and creating a repressive chromatin structure. *EMBO J* **24**: 3712-3723
- Stopka T, Skoultschi AI (2003) The ISWI ATPase Snf2h is required for early mouse development. *Proc Natl Acad Sci USA* **100**: 14097-14102
- Stump RJ, Ang S, Chen Y, von Bahr T, Lovicu FJ, Pinson K, de Jongh RU, Yamaguchi TP, Sassoon DA, McAvoy JW (2003) A role for Wnt/beta-catenin signaling in lens epithelial differentiation. *Dev Biol* **259**: 48-61
- van Raamsdonk CD, Tilghman SM (2000) Dosage requirement and allelic expression of PAX6 during lens placode formation. *Development* **127**: 5439-5448
- Vinson C, Myakishev M, Acharya A, Mir AA, Moll JR, Bonovich M (2002) Classification of human B-ZIP proteins based on dimerization properties. *Mol Cell Biol* **22**: 6321-6335
- Walther C, Gruss P (1991) Pax-6, a murine paired box gene, is expressed in the developing CNS. *Development* **113**: 1435-1449
- Wijgerde M, Grosveld F, Fraser P (1995) Transcription complex stability and chromatin dynamics *in vivo*. *Nature* **377**: 209-213
- Wu K, Yang Y, Wang C, Davoli MA, D'Amico M, Li A, Cveklava K, Kozmik Z, Lisanti MP, Russell RG, Cvekl A, Pestell RG (2003) DACH1 inhibits transforming growth factor-beta signaling through binding Smad4. *J Biol Chem* **278**: 51673-51684
- Yang Y, Chauhan BK, Cveklava K, Cvekl A (2004) Transcriptional regulation of mouse alphaB- and gammaF-crystallin genes in lens: opposite promoter-specific interactions between Pax6 and large Maf transcription factors. *J Mol Biol* **344**: 351-368
- Yang Y, Cvekl A (2005) Tissue-specific regulation of the mouse alphaA-crystallin gene in lens via recruitment of Pax6 and c-Maf to its promoter. *J Mol Biol* **351**: 453-469