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Pitx3 directly regulates *Foxe3* during early lens development

NAFEES AHMAD[1,$], MUHAMMAD ASLAM[2], DORIS MUENSTER[1,#], MARION HORSCH[3], MUHAMMAD A. KHAN[1,§], PETER CARLSSON[4], JOHANNES BECKERS[3,5] and JOCHEN GRAW*[,1]

Helmholtz Center Munich, [1]Institute of Developmental Genetics and [3]Institute of Experimental Genetics, Neuherberg, Germany, [2]German Center for Neurodegenerative Diseases, Bonn, Germany, [4]Department of Chemistry and Molecular Biology, University of Gothenburg, Gothenburg, Sweden and [5]Chair of Experimental Genetics, Technical University Munich, Freising-Weihenstephan, Germany.

ABSTRACT *Pitx3* is a *bicoid*-related homeodomain transcription factor critical for the development of the ocular lens, mesencephalic dopaminergic neurons and skeletal muscle. In humans, mutations in *PITX3* are responsible for cataracts and anterior segment abnormalities of varying degree; polymorphisms are associated with Parkinson's disease. In *aphakia (ak)* mice, two deletions in the promoter region of *Pitx3* cause abnormal lens development. Here, we investigated systematically the role of Pitx3 in lens development including its molecular targets responsible for the *ak* phenotype. We have shown that *ak* lenses exhibit reduced proliferation and aberrant fiber cell differentiation. This was associated with loss of *Foxe3* expression, complete absence of *Prox1* expression, reduced expression of ε-tubulin and earlier expression of γ-crystallin during lens development. Using EMSA and ChIP assays, we demonstrated that Pitx3 binds to an evolutionary conserved *bicoid*-binding site on the 5′-upstream region of *Foxe3*. Finally, Pitx3 binding to 5′-upstream region of *Foxe3* increased transcriptional activity significantly in a cell-based reporter assay. Identification of *Foxe3* as a transcriptional target of Pitx3 explains at least in part some of the phenotypic similarities of the *ak* and *dyl* mice (*dysgenic lens,* a *Foxe3* allele). These findings enhance our understanding of the molecular cascades which subserve lens development.

KEY WORDS: *Pitx3, aphakia, lens development, Prox1, Foxe3*

Introduction

Lens development is a complex process, starting with the thickening of surface ectoderm i.e., lens placode that invaginates to form the lens vesicle containing an anterior proliferative lens epithelium and a posterior layer differentiating into the elongated primary fiber cells. Later, in a life-long ongoing process, newborn cells from the germinative zone in the anterior epithelium migrate towards the lens equator and differentiate into elongated secondary fiber cells, and superpose the primary fiber cells in an onion-like manner (Bassnett *et al.*, 2009, Graw, 2003).

Over the past few decades mouse geneticists have focussed on several spontaneous mutants exhibiting ocular defects to identify genes with potential role in eye development. Most important of these are: *Small eye* (gene symbol: *Sey*; Roberts, 1967), later characterized as a *Pax6* allele (Hill *et al.,* 1991), *aphakia* (gene symbol *ak*; Varnum and Stevens, 1968), later characterized as an allele of *Pitx3* (Semina *et al.,* 2000), and *dysgenic lens* (gene symbol *dyl*; Sanyal and Hawkins, 1979), later characterized as an allele of *Foxe3* (Brownell *et al.,* 2000). A detailed analysis of these mutants revealed basic molecular cascade involved in precise spatiotemporal control of the lens epithelial cell proliferation and their differentiation into lens fiber cells; two cellular events key to the regular lens development.

Pitx3 is a *bicoid*-related homeodomain transcription factor and is expressed in the developing lens (Semina *et al.,* 2000), but also in the midbrain dopaminergic neurons (Smidt *et al.,* 1997) and skeletal muscle cells (Coulon *et al.,* 2007). In humans, mutations

Abbreviations used in this paper: ak, aphakia; dyl, dysgenic lens; sey, small eye.

***Address correspondence to:** Jochen Graw. Helmholtz Centre Munich – German Research Center for Environmental Health, Institute of Developmental Genetics, Ingolstädter Landstr. 1, D-85764 Neuherberg, Germany. Tel: +49-89/3187-2610. E-mail: graw@helmholtz-muenchen.de

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Present addresses: [#] Horses And More, Hirvimäentie 401, 41800 Korpilahti, Finland. [$] Institute of Biomedical and Genetic Engineering (IBGE), Islamabad, Pakistan and [§]German Cancer Research Center, Normal and Neoplastic CNS stem cells (A240), Heidelberg, Germany.

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in *PITX3* are responsible for varying degree of ocular phenotypes including cataract, anterior segment mesenchymal dysgenesis and microphthalmia (Berry *et al.*, 2004, Bidinost *et al.*, 2006, Burdon *et al.*, 2006, Semina *et al.*, 1998), and various polymorphisms have been linked to Parkinson's disease (Bergman *et al.*, 2010, Fuchs *et al.*, 2009, Gui *et al.*, 2012, Guo *et al.*, 2011, Haubenberger *et al.*, 2011, Liu *et al.*, 2011).

The phenotype of two *Pitx3* mouse mutant alleles, *ak* and *eyeless* (gene symbol *eyl;* Rosemann *et al.,* 2010) is rather identical and characterized by a severe microphthalmia without any lens. The *ak* mutant is characterized by two deletions in the promoter region of the *Pitx3* (Rieger *et al.*, 2001, Semina *et al.*, 2000), whereas *eyl* is characterized by an amino-acid exchange in the Pitx3 protein (Rosemann *et al.*, 2010). Investigation of lens development in the *ak* mutant revealed morphological changes at embryonic day 10.5-11.0 (Grimm *et al.*, 1998, Varnum and Stevens, 1968). The lens vesicle remains attached with the overlaying ectoderm showing a persistent lens stalk and does not form an anterior chamber. These rudimentary lenses are degraded later during development resulting in aphakic eyes, i.e. they lack lenses, and finally, the entire eyeball is filled with retinal layers. As a preface of this pathologic process, the lumen of the lens vesicle is filled with aggregated cells, which are contrary to the primary fiber cells formed in wild-type mice that remain posterior to the proliferating anterior epithelium. Malorientation of the mitotic figures in early lens rudiment in *ak* mice has been suggested as a possible explanation for the aggregated cells in *ak* lens vesicles (Zwaan and Kirkland, 1975), although molecular basis of maloriented spindles in developing *ak* lens is not clear as yet. Moreover, despite striking pathologic similarities observed between *Pitx3* and *Foxe3* (*dyl*) mutant mice (Brownell *et al.,* 2000), namely persistent lens stalk and subsequent degeneration of developing lens, no genetic interaction could be established between these factors. It was suggested that Pitx3 plays a role in the maintenance but not in the induction of *Foxe3* expression in lens epithelial cells (Medina-Martinez *et al.*, 2009). This is however contrary to the studies on zebrafish model for lens development (Shi *et al.*, 2006). To date, the only direct target gene of Pitx3 in lens development is *Mip* (encoding Aquaporin 0; Sorokina *et al.*, 2011).

In this study, we systematically analyzed morphological and molecular changes in *ak* lenses. We focused on the spatiotemporal expression of key lens defining factors, such as Pax6, Sox2, Foxe3, Prox1 and AP-2α in an attempt to investigate their potential interactions with Pitx3. Further, we analyzed the expression of ε-tubulin (gene symbol: *Tube1*), a potential determinant of mitotic spindle fiber orientation and centriol duplication (Chang *et al.*, 2003) in *ak* lenses during development. Our results are in line with several previous findings; additionally, we present novel evidence that Pitx3 directly regulates *Foxe3* as downstream transcriptional target, which explains at least in part some of the similarities between *ak* and *dyl* mutant mice with respect to lens development.

Results

Temporal expression of lens-defining transcription factors in aphakia

Morphological and cellular changes during lens development are associated with distinct functions of many transcription factors. To systematically analyze the expression of key developmental regulators involved in particular morphological events such as lens vesicle separation, proliferation and differentiation, we used specific antibodies for Pax6, Sox2, Ap-2α, Foxe3 and Prox1 at different developmental stages (E10.5 - E12.5) that distinguish *ak* phenotype from wild-type developing lenses.

We observed no gross difference in immunolabeling of Pax6; in *ak* lenses at E10.5 as compared to wild-type lenses. However, at E11.5 and later developmental stages ectopic expression of *Pax6* in *ak* lens was observed (Fig. 1). Dual immunolabeling of Pax6 with Pitx3 revealed overlapping expression pattern in the wild-type lens at both developmental stages studied indicating that these two factors act concurrently during lens formation (Fig. 1).

Immunolabeling for Sox2 (which cooperatively acts with Pax6 in lens placode thickening and lens pit invagination) revealed at E10.5 that it is expressed in the whole *ak* lens (Fig. 2A); this is contrary to the wild-type lens where its expression was restricted to the posterior half of the lens and extended towards the anterior half as the development proceeds. This indicates a loss of spatial control on Sox2 expression in early *ak* lenses. At E11.5, Sox2

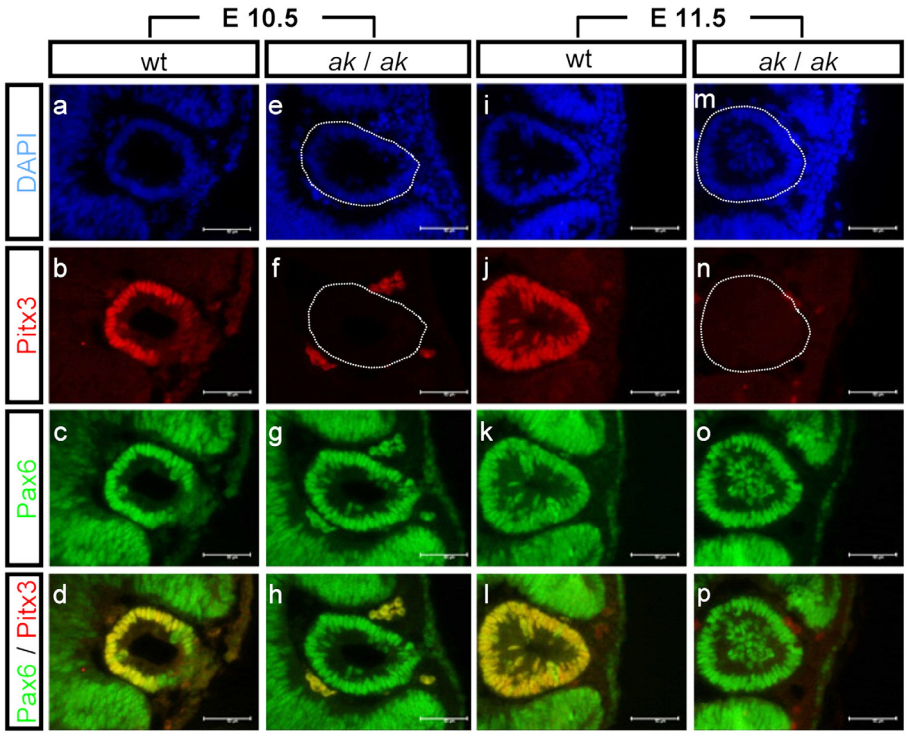

Fig. 1. Spatiotemporal expression of *Pax6* in *aphakia* during early development. *Expression of Pax6 is spread slightly anterior in the lens pit in* aphakia *at E10.5* **(g,h)** *compared to the wild-type samples* **(c,d)**. *At E11.5* **(i-p)**, *its expression is persistent in all the cells forming lens vesicle including those filling the vesicle in* aphakia *(o,p). Immunofluoresence staining was performed on 8 μm thick, PFA fixed paraffin sections. Scale bars, 50 μm.*

expression in wild-type developing lens was more obvious in the cells lining the anterior half of the lens and seemed to diminish in cells present in the posterior half. In *ak* lens vesicle ectopic expression of Sox2 was observed. No difference in immunolabeling for Sox2 was observed in the retina of the *ak* and wild-type eye (data not shown).

Immunolabeling for Ap-2α, a factor involved in lens vesicle separation (Pontoriero *et al.*, 2008), revealed expression in the epithelial cells and in the lens vesicle cells at E10.5 for both wild-type and *ak* lens (Fig. 2B). However, from E11.5 expression pattern of Ap-2α was similar to Pax6 and Sox2. Contrary to this, its expression in the wild-type lens was restricted to the anterior lens epithelium from E11.5; it is not present in the posterior part of the lens (Fig. 4B).

The most remarkable difference in immunolabeling pattern between wild-type and *ak* lens was observed for Foxe3, which was characterized previously as the lens epithelium maintaining factor (Blixt *et al.*, 2000, Ho *et al.*, 2009). Specific reduction in Foxe3 expression in *ak* lens was even more evident at E12.5, when only very few Foxe3 positive cells were detected (Fig. 3 r,x). At E12.5, Foxe3 expression was restricted to the anterior lens epithelium in the wild type (Fig. 3 q,w), but in *ak* lenses, Foxe3 positive cells were present just arbitrarily. These results suggest that Pitx3 might influence *Foxe3* expression at the observed

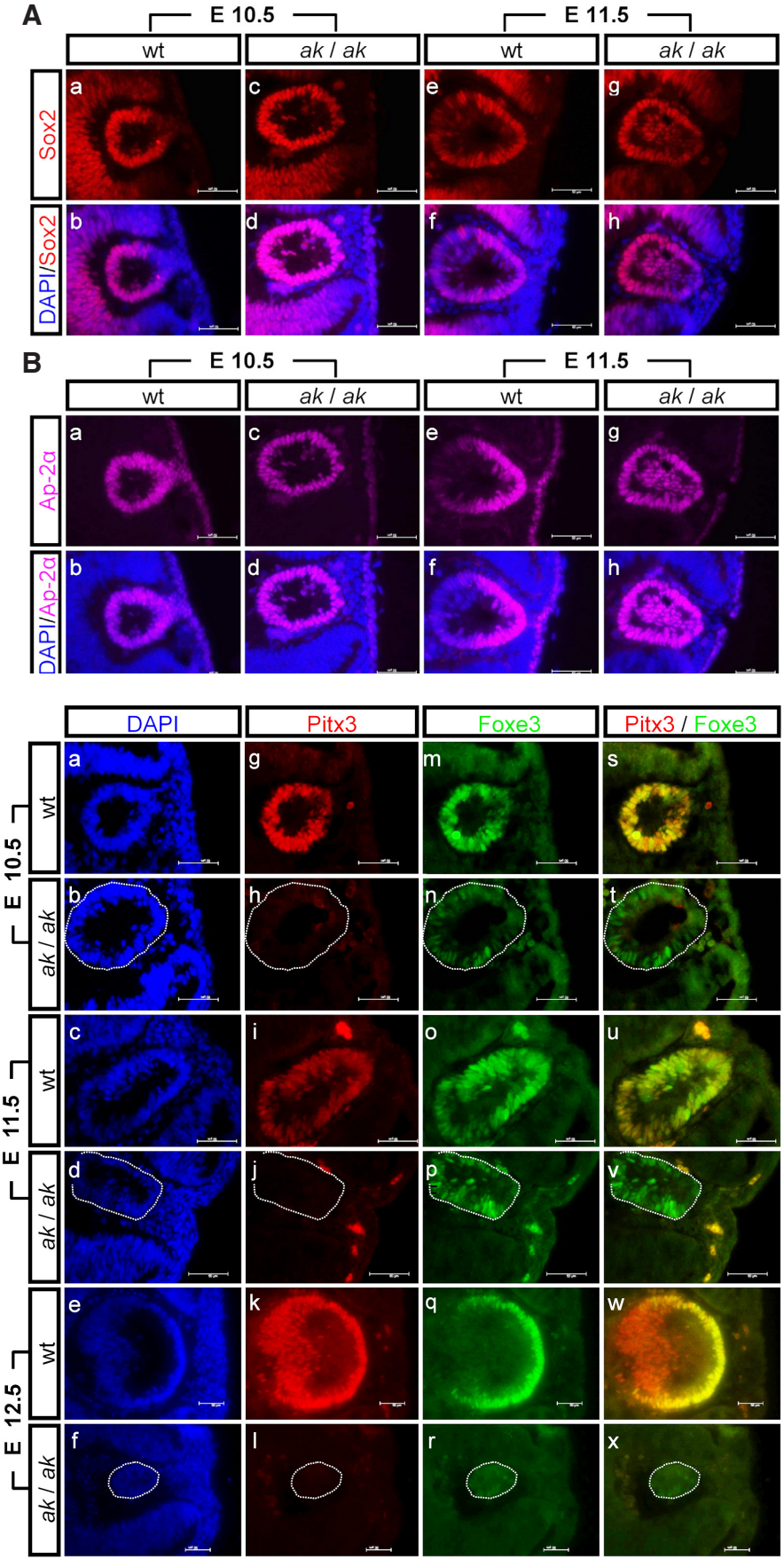

Fig. 2 (top panels). Altered expression of Sox2 in *aphakia*. (A) *Expression of Sox2 is altered at E10.5 (c,d) and E11.5 (g,h). At E10.5 its expression is not reached till the most anterior part of the lens in wild type (a,b) contrary to the* aphakia, *while at E11.5 all cells in the lens vesicle express Sox2.* **(B)** *At E10.5 (a-d) no apparent change in expression of Ap-2α is present in* aphakia *(c,d) compared to the wild-type lens (a,b). At E11.5 (e-h) it is expressed in all lens vesicle cells in* aphakia *(g,h); in the wild-type lens (e,f) its expression is persistent in the anterior half of the lens vesicle but not present in the posterior half. Immunofluoresence staining was performed on 8 μm thick, PFA fixed paraffin sections. Scale bars, 50 μm.*

Fig. 3 (bottom panels). Diminished Foxe3 expression in *aphakia*. *At E10.5, Foxe3 expression is low in* aphakia **(n,t)** *compared to wild-type lens* **(m,s)**, *but all the cells in the lens vesicle express Foxe3 at E11.5 in* aphakia **(p,v)**. *However, at E12.5 only few Foxe3 expressing cells are present in* aphakia **(r,x)**. *Co-staining of Pitx3 and Foxe3 has shown that their expression almost completely overlaps at both of these stages in wild-type embryos* **(s,u,w)**. *Immunofluoresence staining was performed on 12 μm thick, PFA fixed frozen sections. Scale bars, 50 μm.*

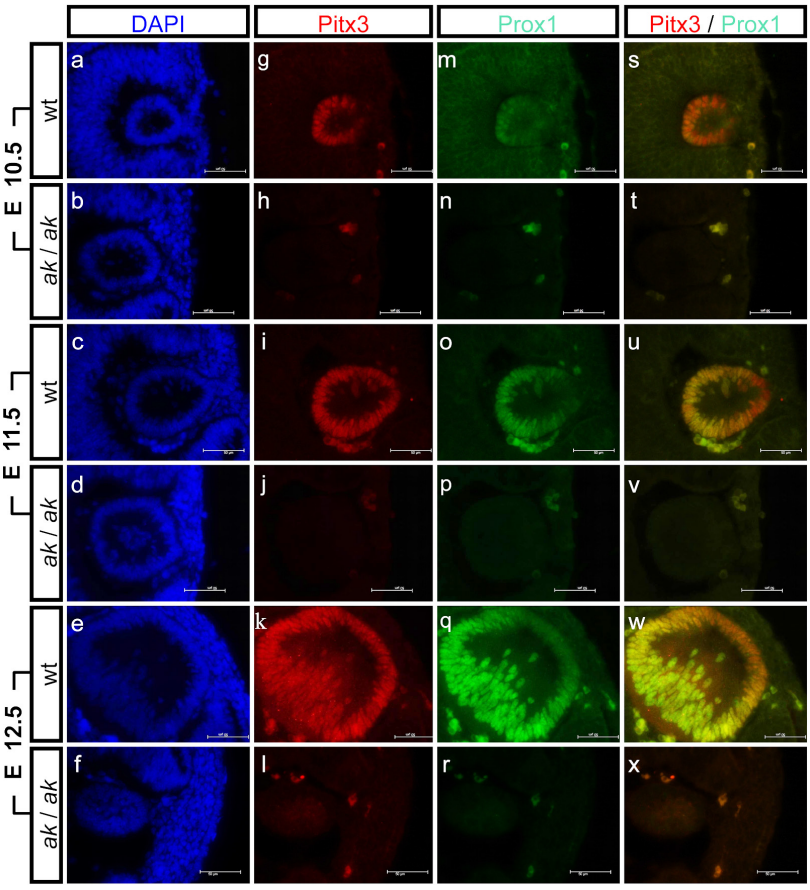

Fig. 4. Abolished expression of Prox1 in *aphakia*. *At E10.5, very few cells express Prox1 in the wild-type lens* **(m)**, *but it increases one day later* **(o)** *and spreads in the whole lens* **(q)**. *No expression of Prox1 in the* aphakia *lens was observed at all the stages studied* **(n, t, p, v, r, x)**. *An almost complete overlap of Pitx3 and Prox1 is evident in wild-type lenses at all these stages* **(s, u, w)**. *Immunofluoresence staining was performed on 8 μm thick, PFA fixed paraffin sections. Scale bars, 50 μm.*

Pitx3 and Prox1 in the developing lens of *Foxe3* deficient mice at E11.5. Expression of both Pitx3 and Prox1 was detected in *Foxe3*-deficient lens (Supplementary Fig. S1), indicating that the expression of *Pitx3* and *Prox1* is not dependent on Foxe3. However, expression of Prox1 was observed in the very anterior part of the lens at E11.5 in the *Foxe3*-mutant lens contrary to the wild-type lens (Fig. 4o) indicating that Foxe3 might exert a spatial control over its expression in developing lens epithelium.

Abnormal lens proliferation and differentiation in aphakia

Previous studies have implicated Foxe3 and Prox1 in proliferation and lens fiber differentiation (Blixt *et al.*, 2000, Wigle *et al.*, 1999). Diminished expression of these factors in *ak* lens provoked us to investigate cellular and morphological abnormalities in *ak* lens formation.

To observe the proliferation in developing *ak* lens, dividing cells were labeled with BrdU at E11.5. Staining with anti-BrdU antibody revealed defective proliferation in the *ak* lens with markedly reduced BrdU staining compared to the littermate wild-type controls (Fig. 5); these results confirm previous findings by Medina-Martinez et al. (2009) using phosphohistone H3 as a proliferation marker. In the wild-type lens vesicle, the anterior cells were actively dividing; in contrast, in the *ak* lens vesicle, no dividing cells could be observed. These results were further confirmed by *E4f1* expression, which was reduced at E11.5 (RT-qPCR) in *ak* lens compared to the littermate wild-type controls (Supplementary Fig. S2A). *E4f1* is a ubiquitously expressed transcription factor considered to be crucial for mitotic activity (Le Cam *et al.*, 2004). BrdU co-labeling with anti-Prox1 antibody revealed that cells with low Prox1 expression in the anterior lens epithelium are most active in proliferation as compared to the posterior part of the lens that comprises differentiating cells (Fig. 5 e,f). In the wild-type lens, we observed Pitx3 expression throughout the lens epithelium at this stage (Fig. 5g).

To further investigate the consequences of the reduced proliferation in the anterior epithelial cells of the lens vesicle, we studied epithelial patterning during lens development. As marker, we used E-Cadherin, a transmembrane protein involved in cell-cell adhesion (Shapiro *et al.*, 1995, Takeichi, 1988). E-cadherin is a member of the cadherin family that is expressed in the epithelial cells; in the lens, its expression is restricted to the anterior lens epithelium (Xu *et al.*, 2002). E-cadherin is a direct downstream target of Ap-2α (Faraldo *et al.*, 1997, West-Mays *et al.*, 2002, West-Mays *et al.*, 2003) that showed ectopic expression in *ak* lenses at E11.5 (Fig. 4B). Spatiotemporal analysis of E-cadherin expression in *ak* mice showed expanded immunolabeling as early as E10.5 as compared to the wild type, where its expression was found nicely organized in the cells forming the lens vesicle (Fig. 6 m,n). In the surface epithelium, broad E-cadherin expression

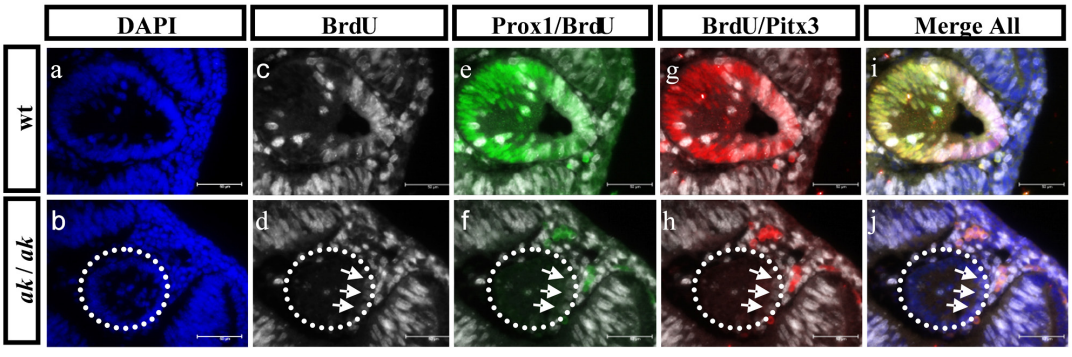

Fig. 5. Reduced proliferation in *aphakia* lens at E11.5. BrdU was injected in the pregnant female at E11.5, two hours before sacrifice. *Immunofluoresence staining using anti-BrdU showed very few positive cells in the* aphakia *lens* **(d)** *compared to the littermate wild-type controls* **(c)**. *Co-staining of BrdU with Prox1 revealed a higher expression in elongating primary fiber cells than in anterior epithelial cells* **(e)**.

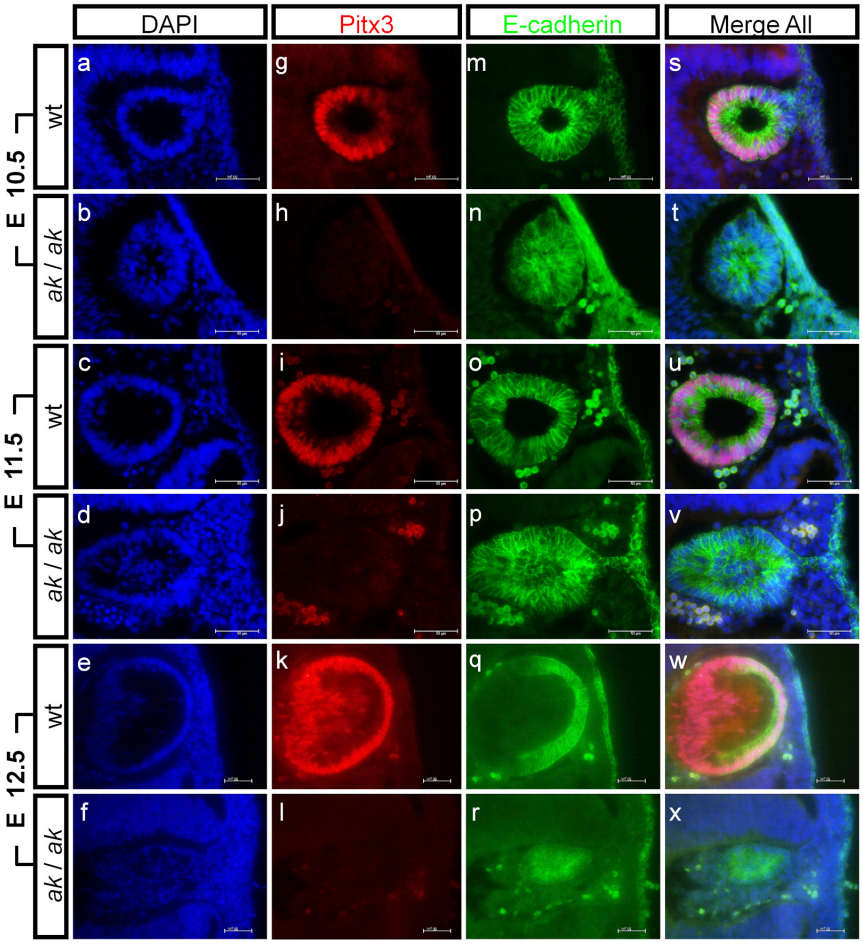

Fig. 6. Disorganized expression of E-Cadherin in *aphakia*. *Expression of E-cadherin is present in* aphakia *at all stages studied (from E10.5 to E12.5); see* **(n,t,p,v,r,x)** *although highly disorganized. Immunofluoresence staining was performed on 12 μm thick, PFA fixed frozen sections. Scale bars, 50 μm.*

was identified in *ak* lenses even at E10.5 (Fig. 6n), whereas it is expressed in the wild type lens a day later (E11.5; Fig. 5o). Additionally, in the *ak* lenses, the cell membranes of the cells inside the rudimentary lens vesicle were also E-cadherin positive at E11.5 (Fig. 6p). One day later (E12.5), E-cadherin is restricted in *ak* eyes to the cells inside the rudimentary lens vesicle (Fig. 6r); in the wild-type lens it is present in the anterior epithelial cells (Fig. 6q). These results indicate that cells in the rudimentary lens vesicle maintain the identity of epithelial cells.

To address the question, how *ak* lens cells fill the lumen of lens vesicle, we considered previous findings of maloriented mitotic spindles in developing lenses of *ak* mice (Zwaan, 1975). Since the relationship of this malorientation with underlying molecular changes and the *Pitx3* mutation is not clear, we tested whether ε-tubulin (gene symbol; *Tube1*) is involved in the malorientation of spindle fibres. ε-tubulin is considered as a marker of centriole duplication and microtubules assembly (Chang *et al.*, 2003), and its expression was detected in the anterior lens epithelium in wild-type lens; whereas *ak* lenses were devoid of this anterior epithelial expression (Fig. 7, arrow heads). We further analyzed the *Tube1* transcript level at E12.5 using RNA from the head of littermate samples. A significant reduction of its expression was observed in *ak* mice (~30%, $p < 0.05$; Supplementary Fig. S2B). These results strongly suggest that lack of Pitx3 disrupts directly or indirectly *Tube1* expression in the lens vesicle of *ak* mice. Therefore, it is tempting to speculate that this lack of *Tube1* expression in lens epithelial cells may result in spindle malorientation and aggregation of epithelial cells in the lumen of the lens vesicle in *ak* mutant mice.

Finally, we investigated the expression of γ-crystallins. They are considered to be specific for differentiated lens fiber cells; previous studies on this topic showed seemingly conflicting results: Malinina and Koniukhov (1981) could not find any immunoreactivity for γ-crystallins during the embryonic development of *ak* mice, whereas Medina-Martinez *et al.* (2009) demonstrated the presence of γ-crystallins in the lens rudiment at E12.5. Therefore, we re-investigated the γ-crystallin expression in developing *ak* lens between E10.5 and E14.5 (Fig. 8). In the wild type, γ-crystallin expression could be observed in the whole lens starting from E11.5 which gradually becomes restricted to differentiated lens fiber cells from stage E12.5 onwards. This particular suppression of γ-crystallin expression involves the anterior lens epithelium including the proliferative zone and the equatorial zone, where elongation of the fiber cells starts. Strikingly, the rudimentary lens in *ak* mice presented a completely different labeling pattern throughout the embryonic stages studied. Contrary to wild-type lens, γ-crystallin expression could be observed in *ak* lens cells as early as E10.5 including the anterior epithelial cells (Fig. 8i). This very early expression of γ-crystallin indicates an earlier initiation of differentiation in *ak* lens cells compared to the wild-type

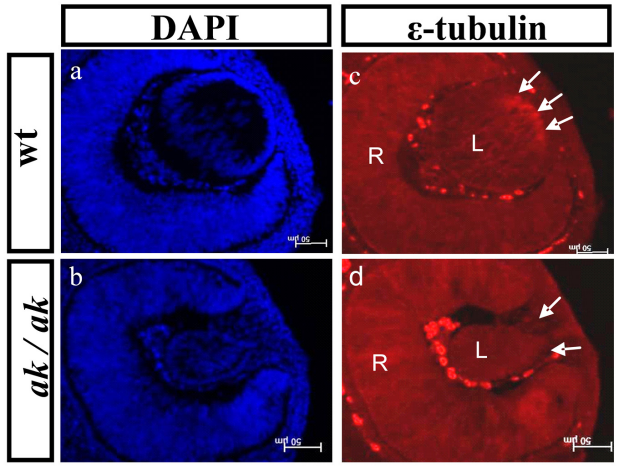

Fig. 7. Immunofluoresence staining at E12.5 *showed a concentrated crescentric expression of* **ε***-tubulin in the anterior lens epithelium in the wild type, white arrows in* **(c)**, *which is absent in the* aphakia *lens, white arrows in* **(d)**. *The red fluorescent dots in* **(c,d)** *indicate disrupted mesenchymal cells that give autofluorescence. Staining was performed on 8 μm thick, PFA fixed paraffin sections. Scale bars, 50 μm. Abbreviations: L, lens; R, retina.*

lens; however, these γ-crystallin positive cells fail to elongate and to form primary lens fibres; correspondingly, at E14.5 γ-crystallin expression is almost gone.

Overall, these results indicate a comprehensive investigation of the morphological events and their molecular correlates during a defined period of lens development in *ak* mice. It allows us to frame and test the hypothesis that *Foxe3* might be directly dependent on Pitx3 activity.

Foxe3 *as a direct downstream target of Pitx3*

As demonstrated in Fig. 3n, p, r, *Foxe3* was severely down-regulated in *ak* mice. To further confirm these results we carried out quantification of *Foxe3* mRNA expression by real-time PCR. We observed 50% ($p < 0.05$) reduction of *Foxe3* mRNA at E10.5 (Supplementary Fig. S3A) and up to 65% ($p < 0.05$) at E11.5 in *ak* mice as compared to corresponding wild-type controls (Supplementary Fig. S3B). We also observed diminished *Prox1* and *Tube1* mRNA expression in developing *ak* lens (Figs. 4 and 7). This indicates that all these lens-related factors may be either direct or indirect downstream targets of Pitx3. To test this possibility we analyzed 5'-upstream regulatory regions of these genes for Pitx3 binding (see methods).

Sequence conservation analysis was carried out in the 4 kb upstream region of *Foxe3* gene using mouse genome assembly GRCm38 as reference genome. Fig. 9A shows conservation profile of this region across all the mammalian species tested. We found an evolutionary signature element of 654 bp starting at - 2,916 bp relative to *Foxe3* gene. A search for *bicoid* binding sites within this signature element revealed a consensus *bicoid* site TAATCC (Dave *et al.*, 2000) starting at - 3,388 bp relative to *Foxe3* gene and a reverse complementary *bicoid* site GGATTA (starting at - 3,157 bp relative to *Foxe3* gene; Fig. 9B). The *bicoid* site TAATCC is similar to the regulatory element of tyrosine hydroxylase (*Th*) gene which is needed for Pitx3-dependent transcriptional activation of *Th* in dopaminergic neurons (Lebel *et al.*, 2001, Maxwell *et al.*, 2005) and *Mip* in the ocular lens (Sorokina *et al.*, 2011). Furthermore, the *bicoid* consensus site 'TAATCC' is required for transcriptional regulation of target promoters by other Pitx family members (Lamonerie *et al.*, 1996, Tremblay *et al.*, 1998).

No *bicoid* site was found in the 4 kb upstream region of *Prox1* gene. However, extending the search to 11 kb upstream region revealed a *bicoid* site (GGATTA; 10,036 bp upstream of *Prox1* start site) located in a 272 bp evolutionary signature element. No conserved *bicoid* site for putative Pitx3 binding was observed in the upstream region of the *Tube1* gene. Nevertheless, we considered two *bicoid*-like motifs (TAAGCC), known to bind Pitx family members *in vitro* albeit showing less affinity than consensus *bicoid* sites (Lamba *et al.*, 2008); starting -1,293 bp and -1,856 bp upstream of *Tube1* gene.

To test whether oligos derived from upstream regions of *Foxe3, Tube1* and *Prox1* genes containing *bicoid* (or *bicoid*-like) elements can interact with Pitx3 *in vitro*, we performed electrophoretic mobility shift assay (EMSA). Using biotin-labeled oligonucleotides and nuclear extracts over-expressing mouse Pitx3 (see methods) we observed strong binding with the oligonucleotide probe (*Foxe3*-1-EMSA; Supplementary Table 1) derived from the *Foxe3* upstream region (- 3,377 / - 3,415) containing the bicoid site TAATCC. The specificity of this binding was confirmed by a supershift assay using an anti-Pitx3 antibody and competition assay with unlabelled DNA containing the same *bicoid* site (Fig. 10A). These findings suggest that Pitx3 binds to a potential regulatory region of *Foxe3*. In contrast, no gel shift band was observed for the EMSA probes containing the *bicoid* sites GGATTA located upstream of *Foxe3* or the probes containing *bicoid* sites located upstream of *Tube1* and *Prox1* (data not shown). This however does not exclude the possibility of an indirect regulatory interaction of Pitx3 with *Tube1* and *Prox1*.

To test the validity of direct Pitx3 interaction with the consensus *bicoid*-binding site located upstream of *Foxe3* gene *in vivo*, we performed chromatin immunoprecipitation assay (ChIP) on the developing ocular tissue of wild-type mice (embryos

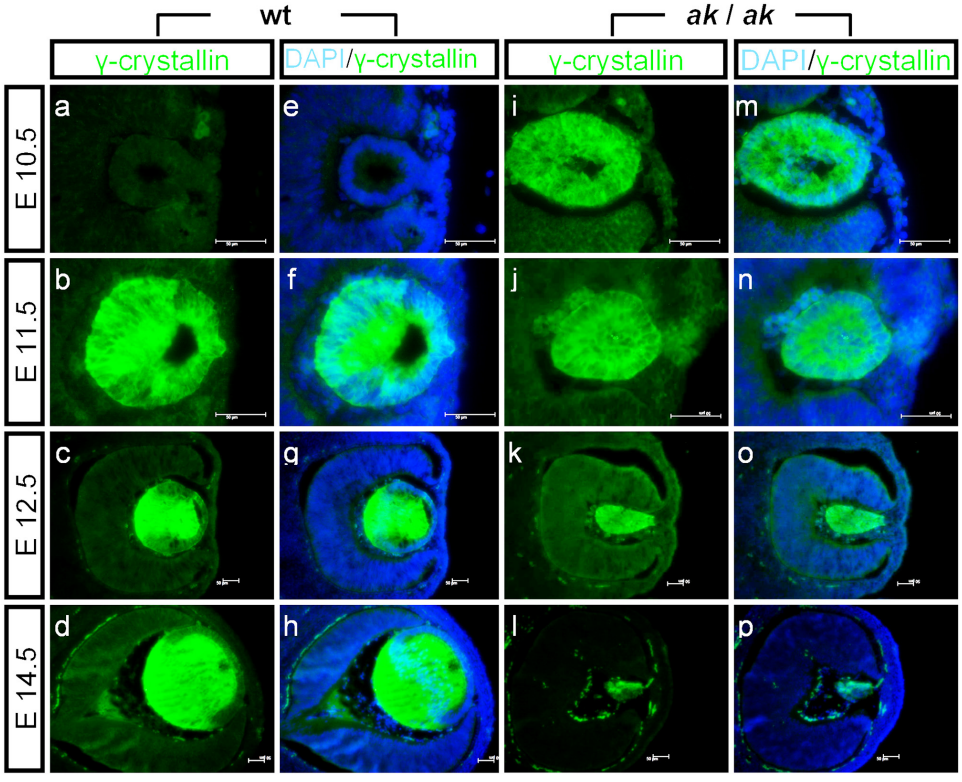

Fig. 8. Notice the earlier and persistent expression of γ-crystallin in *aphakia*. *Immunofluoresence staining of wild type and* aphakia *for γ-crystallin during different developmental stages (E10.5, E11.5, E12.5, and E14.5) revealed that it is expressed earlier in* aphakia **(c,d)** *compared to the wild-type lens* **(a,b)**. *From E12.5, a clear demarcation of lens epithelium and differentiating lens fiber cells can be seen that express γ-crystallin in wild-type embryos* **(i,j)** *and is more apparent at E14.5* **(m,n)**, *while no such demarcation is present in* aphakia, *and γ-crystallins are expressed throughout the lens* **(k,l)** *and rudimentary lens* **(o,p)**. *Immunofluorescence staining was performed either on PFA fixed 8 μm thick paraffin sections (E14.5) or 12 μm thick frozen sections (E10.5, E11.5, E12.5). Scale bars, 50 μm.*

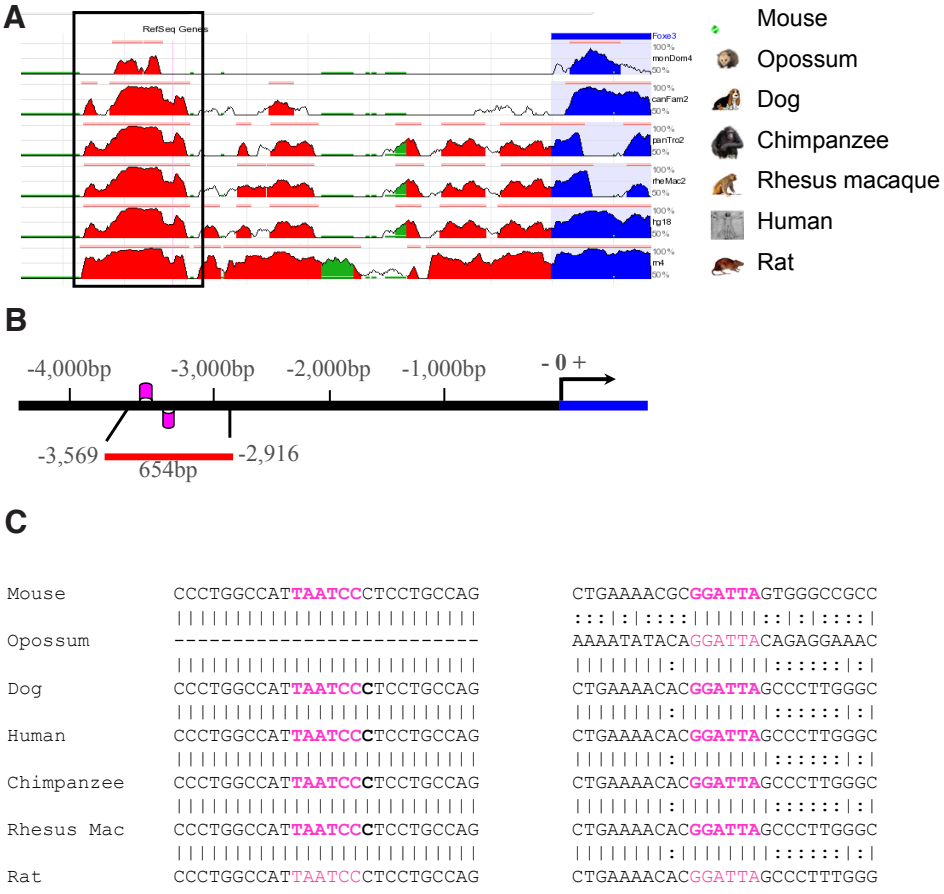

Fig. 9. An evolutionary sequence conservation generated by ECR browser in the 5′-upstream region of of Foxe3 using mouse as the base genome. *An element (654 bp located between - 2,916 / - 3,569) was detected* **(A)** *that contains conserved* bicoid *(Pitx3) binding sites (TAATCC), shown as pink boxes on opposite DNA strands* **(B)**. *These putative Pitx3-binding sites are conserved across mammalian species* **(C)**.

at E11.5; at this developmental stage we found a reduced *Foxe3* expression in Pitx3-deficient *ak* mice). Chromatin from mid-brain tissue of the same embryos was also precipitated with the Pitx3 antibody; it was used as a negative control to test the specificity of the assay, since *Pitx3* and *Foxe3* are not co-expressed in this area of the developing brain (Blixt *et al*., 2007, Medina-Martinez, 2012). PCR amplification in ChIP samples with Pitx3 antibody in comparison to the control antibody (goat-IgG) demonstrated a direct interaction of Pitx3 with the *Foxe3* regulatory region *in vivo* (Fig. 10B). No interaction was observed from the mid-brain immunoprecipitated-DNA indicating the specifity of the binding.

Finally, we performed a luciferase reporter assay to test the biological significance of Pitx3 binding to the upstream *bicoid* sites of *Foxe3* gene. A 401 bp region harbouring both *bicoid* sites TAATCC and GGATTA, located between - 3,117 / - 3,517 upstream to the *Foxe3* gene was cloned (Fig. 10C; *Foxe3*-wt) in pGL3-basic plasmid to test its effect on the expression of reporter luciferase gene in the presence of *Pitx3* expression. We observed a strong Pitx3 dose-dependent activation of the luciferase gene (Fig. 10C). Deletion of the site, 'TAATCC' (cloned sequence *Foxe3*-mt1; Fig. 10C) resulted in a significantly diminished luciferase activity ($p \leq 0.005$) compared to the undeleted sequence. Interestingly, deletion of both sites (TAATCC and GGATTA; cloned sequence *Foxe3*-mt2) lead to a similar decrease of luciferase activity as the single deletion, indicating that both sites might affect transcription of the target gene with different efficiencies. A significant difference in the luciferase activity was observed between *Foxe3*-mt1 and *Foxe3*-mt2 elements when a higher amount of *Pitx3* plasmid was cotransfected indicating that the 'GGATTA' site might interact weakly with Pitx3 to fine-tune the regulatory effect of the TAATCC site. Such a weak interaction between the GGATTA site and *Pitx3* was not observed in our EMSA or ChIP experiments.

Collectively, these results demonstrate for the first time that loss of *Foxe3* expression in *Pitx3*-deficient *ak* lens epithelial cells is due to the abrogation of a direct *Pitx3*-dependent cis-regulatory interaction in the 5'-upstream region of the *Foxe3* gene.

Discussion

The *bicoid*-like homeobox transcription factor *Pitx3* plays an important role in the development of the eye and of mesencephalic dopaminergic (meDA) neurons. Most of the studies focused on its role in the context of dopaminergic neurons found that Pitx3 exerts its developmental impact by directly regulating the expression of a cascade of downstream genes in these neurons including *Th* (Lebel *et al*., 2001), *Vmat2*, *Dat* (Hwang *et al*., 2009), *Bdnf* and *Gdnf* (Peng *et al*., 2011, Peng *et al*., 2007). However, less is known regarding the role of Pitx3 in the development of the ocular lens. Further, the transcriptional targets of Pitx3 in dopaminergic neurons are not relevant in lens formation. Particularly, *Nurr1*, which is considered as a co-factor of Pitx3 in the developing midbrain, is also not expressed in the lens (Medina-Martinez, 2012). In the present study, we investigated the role of Pitx3 and its downstream targets in the developing lens.

The main result reported here is the direct regulation of *Foxe3* by Pitx3; we showed by EMSA and ChIP that Pitx3 binds to a conserved *bicoid* site, upstream of *Foxe3*. Moreover, in reporter gene assays we provide a conclusive evidence that this upstream *bicoid* site is indeed functionally active and could drive luciferase expression in a Pitx3-dependent manner *in vivo* (Fig. 10). Ours is the first report of a direct interaction between Pitx3 and Foxe3 during ocular lens development that contrasts at least one previous study (Medina-Martinez *et al*., 2009).

This finding of a Pitx3-responsive *bicoid* element in the upstream regulatory region of *Foxe3* explains several features reported here from the *ak* and *Foxe3*-knockout mouse as well as from others. The first indication is that Foxe3 is dramatically reduced in *ak* lenses (Fig. 3 and Medina-Martinez *et al*., 2009). However, Pitx3 expression could be observed in *Foxe3* mutants (Supplementary

Fig. S1 and Medina-Martinez *et al.*, 2009). Similar results have been reported in the zebrafish (Shi *et al.*, 2006), where *Foxe3* morpholinos showed expression of Pitx3, but Foxe3 expression was not detected using *Pitx3* morpholinos. Therefore, *Foxe3* is placed downstream to Pitx3 in the signaling cascade that controls lens formation and is highly conserved during evolution between such distant species as zebrafish and mice. It is obvious from the data shown here that Pitx3 is not the only transcription factor interacting with *Foxe3* regulatory elements. Recently, Zhao *et al.* (2012) reported that loss of Msx2 function also downregulates *Foxe3* expression resulting in anterior segment dysgenesis resembling Peter's anomaly.

In contrast to the finding in Foxe3, we were not able to demonstrate a direct interaction of Pitx3 in the regulation of *Prox1*, although Prox1 expression is completely lost in *ak* mutant mice and the expression patterns of Prox1 and Pitx3 mainly overlap (Fig. 4). An indirect mechanism involving Pitx3 for the regulation of Prox1 expression can not be ruled out based on this study. Prox1 was shown previously to regulate directly the γ-crystallin expression (Lengler *et al.*, 2001); however, the authors of the study did not demonstrate that γ-crystallin expression is also inhibited by Six3, which was shown to be expressed in the *ak* lens remnants (Grimm *et al.*, 1998). Similarly, γ-crystallin expression is repressed by Pax6, but activated by several other transcription factors including Sox2 (Yang *et al.*, 2004). It can therefore be stated based on our data and the literature cited that the earlier expression of γ-crystallin in *ak* lenses between E10.5 and E12.5 (Fig. 8) can not be explained and needs further investigation. Nevertheless, this observation confirms and extends a previous report (Ho *et al.*, 2009), where expression of γ-crystallin (at E11.5) was detected in GFP-tagged Pitx3 deficient embryonic stem cells during lens formation. In general, based on our results it can be hypothesized that many lens-related genes are in the same regulatory loop for spatiotemporal expression, which involves many transcription factors and their targets. In *ak* mice, in which the master regulater of this regulatory loop i.e Pitx3 is missing, the spatiotemporal expression of many genes is affectd either directly or indirectly. For some target genes, e.g *Foxe3*, a direct regulatory interaction with Pitx3 could be observed. However, the regulatory influence of Pitx3 on many other target genes e.g *Prox1* and *Tube1*, is vague and difficult to establish presently and need further investigation to validate this hypothesis. Further evidence in support of a still unclear (direct or indirect) regulation of Pitx3 on the spatiotemporal expression of lens related genes is provided by an earlier and persistently ectopic expression of AP-2α (Fig. S4B), and E-cadherin (Fig. 6) in the epithelial cells located at the posterior half of developing Pitx3-deficient *ak* lenses. These cells do not elongate and differentiate into primary lens fibers as seen in the wild-type lenses. E-cadherin is an epithelial marker (Li *et al.*, 2011, Pontoriero *et al.*, 2009, Zeisberg and Neilson, 2009) whose expression is suppressed in the differentiating epithelial cells in wild-type lenses. Such persistent ectopic expression of E-cadherin and AP-2α has also been observed in *Prox1* knockout mice (Wigle *et al.*, 1999). Further studies will help to find out whether the interactions of Pitx3 with these lens-related genes is rather direct or indirect.

The reduced expression of *Tube1* (ε-tubulin) places this gene as a novel downstream effector of Pitx3. It has an important role in determining the orientation of spindle fibers, the symmetry of cell division and the organization of dividing cells and is a prerequisite for mitotic activity (Chang *et al.*, 2003, Dupuis-Williams *et al.*, 2002). This might be linked to our observation that proliferation of lens cells is severely impaired in *ak* lenses as shown by the BrdU incorporation assay (Fig. 5). Further, reduced expression of ε-tubulin may also have a role in another contrasting morphologi-

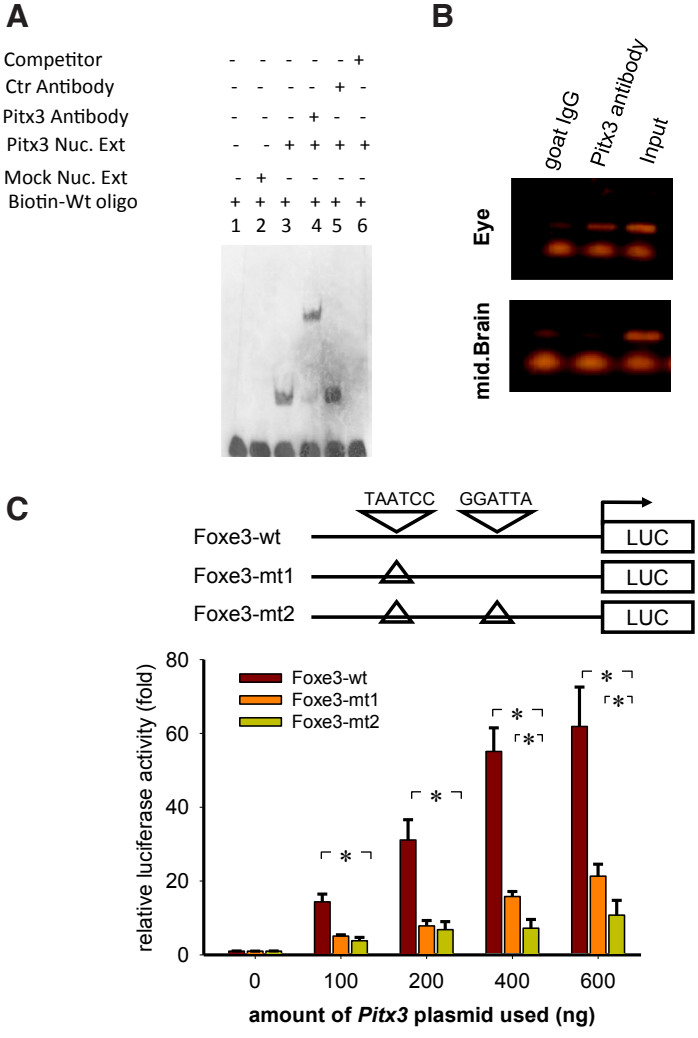

Fig. 10. Oligonucleotides derived from a *Foxe3* 5′-upstream regulatory region containing *bicoid* binding sites were tested for binding to Pitx3-overexpressing nuclear lysates (lane 3). *The specificity of the shifted band was examined by Pitx3 antibody (lane 4), mock nuclear extract (lane 5) or a competitor DNA containing the same* bicoid *site in 20 fold access (lane 6)* **(A).** *Eyes and midbrain tissue was collected from 7 wild-type C57BL/6J embryos at E11.5 and used for chromatin immunoprecipitation with anti-Pitx3 and control antibodies (Goat IgG). ChIP-DNA was used to amplify the 5′-upstream region of* Foxe3 *in a standard PCR reaction* **(B)**. *A 401 bp conserved sequence derived from* Foxe3 *gene (*Foxe3*-wt; - 3,117 /- 3,517) containting Pitx3 binding* bicoid *sites, 'TAATCC' and 'GGATTA' (*Foxe3*-wt), and a same sequence lacking either 'TAATCC' (*Foxe3*-mt1) or 'GGATTA' (*Foxe3*-mt2) were cloned into the pGL3 vector and transiently cotransfected in HEK293 cells along with a* Pitx3 *expression plasmid. The* Foxe3*-wt construct increased the luciferase activity in a Pitx3 dose-dependent manner* **(C)**. *Values are shown as firefly to renilla luciferase activity and normalized to values without Pitx3-expression plasmid. Data represents means ± SD from six experiments performed in triplicate. Statistical significance was determined with the Student t-test; * p=< 0.05.*

cal feature of developing *ak* lenses where the lumen of the lens is filled with epithelial cells. However, *Tube1* has not been implicated so far in any pathological phenotype. Moreover, due to the lack of a *Tube1* mutant, its role in organogenesis and morphological features could not be studied.

In this study, we have addressed several questions related to development of the ocular lens. One particular outcome of this study defines *Foxe3* as a direct target gene of Pitx3 explaining some of the similarities of the classical eye mutants *ak* and *dyl*. Moreover, *Tube1* (ε-tubulin), has been identified as a novel indirect downstream target of Pitx3. Its reduced expression in the *ak* lens remnants may be related to the loss of mitotic activity in these cells and therefore, one of the initial events in the inhibited terminal differentiation of lens fiber cells. Both aspects reported here are important contributions towards a better understanding of the role of Pitx3 in eye development and corresponding congenital diseases.

Materials and Methods

Animal and tissue preparation

All animals analyzed in this study; C57BL/6J wild-type, *ak* and *Foxe3-/-* (Blixt *et al*., 2007), were kept in the mouse facility of the Helmholtz Center Munich. Breeding and treatment of the animals were performed according to the German law for animal protection and institutional regulations.

To get the embryos, respective animals were bred and the vaginal plug was used to detect the pregnancy. The noon of positive plug day was used as post coitum day 0.5 and the females were sacrificed in a CO_2 chamber around noon of the respective post coitum days to collect the embryos. Once collected, embryos were fixed in 4% PFA overnight and processed for cryosection or paraffin embedding and sectioned For cryosectioning, embryos were cryoprotected in 30% sucrose solution in PBS, embedded in OCT compound (Sakura, Torrance, USA), and sectioned at 12 μm. For paraffin embedding, embryos were first dehydrated in serial dilution of methanol, followed by bleaching in 3% H_2O_2 for 1 hour, washed twice in absolute methanol for 10 minutes each, embedded in paraffin and sectioned at 8 μm by RM 2065-microtome (Leica, Wetzlar, Germany),.

Immunohistochemistry

Embryonic paraffin sections were washed in PBS and deparaffinized in Roti-Histol (Roth, Karlsruhe, Germany) followed by rehydration in descending ethanol series. For antigen retrieval, sections were boiled in 0.01 M sodium citrate buffer (pH 6.4) and cooled slowly by adding MilliQ water. Cryo-sections were processed without an antigen retrieval step. Tissue sections were treated with 3% H_2O_2 for 5 minutes, 3% normal donkey serum in PBS containing 0.25% Triton X100 (for blocking), and incubated with respective primary antibody(ies) at 4°C for overnight. After washing in PBS sections were incubated with respective secondary antibody(ies) for 90 minutes, counterstained with DAPI, mounted using polymount (Polysciences, Eppelheim, Germany). Images were obtained with a DFC 350 FX camera connected with DMI6000B fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Primary antibodies used: goat anti-Pitx3, 1:1000 (Santa Cruz, Heidelberg, Germany, sc-19307X); rabbit anti-Prox1 (1:1000; Millipore, AB5475); rabbit anti-Foxe3 (1:200; Santa Cruz, sc-134536); rat anti-BrdU (1:500, AbD Serotec, Puchheim, Germany; OBT0030CX); rabbit anti-γ-crystallin 1:100 (Santa Cruz, sc-22746); rabbit anti-Pax6, 1:500 (Chemicon, Darmstadt, Germany; PRB-278P); goat anti-Sox2, 1:500 (Santa Cruz, sc-17320); mouse anti-Ap-2α, 1:500 (Santa Cruz, sc-12726); anti-E-cadherin, 1:200 (Sigma, Taufkirchen, Germany; U3254); ε-tubulin, 1:200 (Sigma, T1323) and fluorescent-conjugated secondary antibodies were anti-goat Cy3, 1:250 (Jackson immuno, Bar Harbor, USA); anti-rat Cy3, 1:250 (Jackson immuno); anti-mouse Cy5, 1:250 (Jackson immuno); anti-rabbit Alexa Fluor® 488, 1:250 (Invitrogen, Darmstadt, Germany).

BrdU labeling

To label the dividing cells with the thymidine analog, 5-bromo-2'-deoxyuridine (BrdU), pregnant mice were injected peritoneally with BrdU solution at a concentration of 0.05 mg/g body weight on the required embryonic day. Two hours following BrdU injection, mice were sacrificed and embryos were collected, formalin-fixed and embedded in paraffin. Tail tips were collected to genotype the embryos. BrdU was detected by immunofluoresence staining (AbD Serotec; Puchheim, Germany; OBT0030CX).

Genotyping and RT-qPCR

Genomic DNA from tail tissues was used for genotyping. Primers spanning the *ak* distal promoter deletion were designed using primer 3 software (Rozen and Skaletsky, 2000); Supplementary Table S2), and used to amplify the DNA in a standard PCR reaction, which were then resolved on 2 % agarose gel. Genotypes were assigned on the basis of appropriate band size.

Head tissues from littermates were homogenized using Qia-shredder column and total RNA was extracted using RNeasy mini Kit (Qiagen, Hilden, Germany). DNA was eliminated by using 'on column DNase I' kit (Qiagen) and RNA yield and purity was measured using NanoDrop ND-1000 (PEQLAB, Erlangen, Germany). One microgram of total RNA was used in 33 μl of reaction volume for cDNA synthesis using Ready-To-Go T-primed first-strand kit (GE Health Care, Freiburg, Germany). For PCR, 1 μl of 10-fold diluted cDNA was used as template. Realtime qPCR was performed on ABI StepOne cycler using EvaGreen qPCR mix (Solis BioDyne, Tartu, Estonia). We used α-tubulin *(Tuba)* as endogenous reference gene for all qPCR experiments and analyzed them using SigmaPlot v 11.0.

All the RT-qPCR primers from the primer database or self-designed (Supplementary Table S3) were optimized using the standard curve method. Only those primers with an amplification efficiency of 90 to 110 % were used in the expression analysis.

Preparation of nuclear extract and electrophoretic mobility shift assay (EMSA)

To prepare nuclear extracts expressing Pitx3, 1 x 10^6 HEK293 cells were seeded in 6-well plates and transiently transfected with 3 μg of murine Pitx3 expressing pcDNA3.1 construct using lipofectamine 2000 reagent (Invitrogen). Cells were collected 24 hours post-transfection, washed with ice-cold PBS and resuspended in hypotonic buffer [10 mM HEPES, 10 mM KCl, 0.5 mM DTT and protease inhibitor cocktail (Sigma)] for 15 min on ice and vortexed for 10 sec. The nuclei were pelleted by centrifugation at 15,000 g for 1 min. The pellet containing nuclei was resuspended in hypertonic buffer [20 mM HEPES, pH 7.6, 25% glycerol, 0.4 M NaCl 1.5 mM MgCl2, 4 mM EDTA, 0.05 mM DTT and protease inhibitor cocktail] for 30 min on ice. The supernatant containing the nuclear extract was collected by centrifugation at 15,000 g for 2 min and stored at -80°C. As control, nuclear extract was prepared from untransfected HEK293 cells.

For EMSA, oligonucleotides (Supplementary Table S1) were biotin labeled at the 5′-end (Sigma). Oligonucleotides were annealed with complementary sequences in 1 X oligo annealing buffer [10 mM Tris (pH 7.5), 50 mM NaCl, and 1 mM EDTA]. Oligonucleotides (10 ng) were mixed with nuclear extracts (5 μg) in 10 μl of binding buffer containing 1 μg of poly (dI-dc), 15 mM HEPES, pH 7.6, 80 mM NaCl, 1 mM EDTA, 1 mM DTT, and 10% glycerol. Binding reaction was carried out at 30°C for 25 min. For competition, unlabeled DNA sequence containing the respective *Foxe3 bicoid* site) was used. DNA-protein complexes were separated from free DNA on a 6% DNA retardation gel (Invitrogen) and were blotted onto a nylon membrane. After crosslinking, the blot was incubated with blocking buffer [125 μM NaCl, 20 μM Na_2HPO_4, 10 μM NaH_2PO_4 and 175 μM SDS in distilled water] for 30 minutes. The blot was then incubated with a HRP-conjugated streptavidin solution in blocking buffer for 30 minutes. The protein-bound and free oligonucleotides were detected with Novex ECL chemiluminescent substrate reagent kit (Invitrogen). For super-shift assay 2 μg rabbit anti-mouse Pitx3 or control antibodies (Santa Cruz Biotechnology, Inc.) were added to the EMSA binding reaction.

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation was performed using EZ-ChIP (Millipore) according to the manufacturer's instructions. Briefly, eyes and midbrain from seven embryos at E11.5 were removed under microscope and crosslinked with 37% formalin for 10 minutes at room temperature. Glycine was then added to quench unreacted formaldehyde. Samples were centrifuged at 700 x g at 4°C and the pellet was resuspended in SDS lysis buffer containing protease inhibitor. Crosslinked samples were sheared using sonicator for 15 min with 30 sec on/off. The resulting chromatin fragments of 200-1000 bp were column-purified. Immunoprecipitation was performed with 1μg of Pitx3 (Pitx3-20N; Santa Cruz) and goat-IgG antibody (as control) for overnight at 4°C. After washing, the DNA-protein complexes were reverse crosslinked; DNA was precipitated and used as template in a PCR reaction using promoter-specific primers. Primer sequences are given in table S4 (Supplementary Table S4). The experiment was repeated with two independent chromatin preparations.

Cloning and reporter gene assays

PCR was performed with the wild-type mouse genomic DNA to amplify DNA sequences of interest using primers listed in table S4 (Supplementary Table S4). PCR products were cloned in pCR II Topo vector (Invitrogen) and inserts were confirmed by sequencing. DNA fragments were then subcloned in pGL3 basic luciferase plasmid (Promega) using appropriate restriction enzymes (*Xho*I, *Kpn*I, *Hind*III; Fermentas). The mutant plasmids with deletions of the consensus *bicoid* sites were prepared by deletion-specific primers (Supplementary Table S5) using QuickChange II site directed mutagenenesis kit (Agilent, Waldbronn, Germany).

For transient transfection, 3-5 x 10^4 HEK293 cells were platted overnight in DMEM media without any antibiotic in each well of 24-well plates at 37°C with 5% CO_2. All transfections were carried out using polyfect transfection reagent (Qiagen) according to manufacturer's instructions. Briefly, a total of 1.05 μg plasmid-DNA mixed with polyfect reagent was transfected containing 0.2 μg pGL3 basic plasmid construct, 0.2 - 0.8 μg effector pasmid, [e.g., Pitx3-pcDNA3.1 (Lebel *et al.*, 2001) or one of the other transcription factors, or parental plasmid pcDNA3.1 (Invitrogen) as negative control], and 0.05μg pRL-SV40 as an internal transfection control. Total DNA amount was adjusted with empty pcDNA3.1 plasmid wherever needed. Cells were harvested 48 hours post-transfection and subjected for lysis using passive lysis buffer (Promega, Mannheim, Germany). 15-20 μl of cellular extracts were assayed with the Dual-Luciferase Reporter Assay System (Promega) or P.J.K luciferase reagents (P.J.K, Kleinblittersdorf, Germany). Results were expressed as means ± standard deviation from at least three independent experiments performed in triplicate. Student's t-Test was used to evaluate statistical significance.

Bioinformatics and statistics

All the nucleotide sequences were retrieved from and numbered according to Ensembl genome browser (www.ensembl.org) using the mouse genome assembly GRCm38. Promoter sequences were retrieved and analyzed for the transcription factor binding using MatInspector (Genomatix, Munich, Germany). Evolutionary conserved elements were detected using the ECR browser (http://ecrbrowser.dcode.org) and analyzed by default parameters to find the conserved transcription factor binding sites using rVista 2.0 (http://rvista.dcode.org). Data analysis was carried out using SigmaPlot software (SigmaPlot v11.0).

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