Regulation of ocular lens development by Smad-interacting protein 1 involving *Foxe3* activation

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Summary

Sip1, a Smad-binding zinc-finger homeodomain transcription factor, has essential functions in embryonic development, but its role in individual tissues and the significance of its interaction with Smad proteins have not been fully characterized. In the lens lineage, Sip1 expression is activated after lens placode induction, and as the lens develops, the expression is localized in the lens epithelium and bow region where immature lens fibers reside. The lens-lineage-specific inactivation of the *Sip1* gene was performed using mice homozygous for floxed *Sip1* that carry a lens-specific Cre recombinase gene. This caused the development of a small hollow lens connected to the surface ectoderm, identifying two Sip1-dependent steps in lens development. The persistence of the lens stalk resembles a defect in *Foxe3* mutant mice, and *Sip1*-defective lenses lose *Foxe3* expression, placing *Foxe3* downstream of Sip1. In the *Sip1*-defective lens, β-crystallin-expressing immature lens fiber cells were produced, but γ-crystallin-expressing mature fiber cells were absent, indicating the requirement for Sip1 activity in lens fiber maturation. A 6.2 kb *Foxe3* promoter region controlled *lacZ* transgene expression in the developing lens, where major and minor lens elements were identified upstream of –1.26 kb. Using transfection assays, the *Foxe3* promoter was activated by Sip1 and this activation is further augmented by Smad8 in the manner dependent on the Smad-binding domain of Sip1. This Sip1-dependent activation and its augmentation by Smad8 occur using the proximal 1.26 kb promoter, and are separate from lens-specific regulation. This is the first demonstration of the significance of Smad interaction in modulating Sip1 activity.

Key words: ZFHX1, Sip1, Lens, Foxe3, Smad8

Introduction

ZFHX1 family transcription factors, comprising δEF1 and Sip1 in higher vertebrates, contain bipartite zinc-finger clusters and a homeodomain-like motif, named after the *Drosophila* counterpart Zfh-1 (Fortini et al., 1991). δEF1 was identified by its binding to a CACCT sequence of a *δ-crystallin* enhancer (Funahashi et al., 1991; Funahashi et al., 1993; Kamachi and Kondoh, 1993), while Sip1 (Smad-interacting protein 1) was identified by its binding to Smad proteins mediated by the Smad-binding domain (SBD) of Sip1 (Verschueren et al., 1999).

Sip1 and δEF1 are very similar in structure and share DNA-binding sequence specificity to the E2-box-like motif CACCT(G) in vitro; their activity as a transcriptional repressor has been demonstrated using several reporter constructs (Comijn et al., 2001; Kamachi et al., 1995; Postigo and Dean, 1997; Remacle et al., 1999; Sekido et al., 1994; Sekido et al., 1997; van Grunsven et al., 2001), suggesting a shared regulatory function. However, δEF1 lacks the SBD sequence and does not bind Smad proteins in vitro (van Grunsven et al., 2003), suggesting a unique Smad-dependent regulation exerted by Sip1. Expression patterns in the mouse embryo partly overlap but are generally diversified between two protein genes, e.g. only Sip1 is expressed in the lens and null mutant mouse phenotypes produced by targeted gene inactivation are distinct (Takagi et al., 1998; Van de Putte et al., 2003).

Although the augmentation of the Sip1 binding of Smads by Alk receptor-mediated phosphorylation through their MH2 domain has been demonstrated (Verschueren et al., 1999), whether or how the binding of a Smad protein affects transcriptional regulation by Sip1 has not been elucidated. Although all experiments using full-length Sip1 and CACCT(G)-containing target sequences indicate a repression activity, supported by its binding to the co-repressor CtBP (van Grunsven et al., 2003) as for δEF1 (Furusawa et al., 1999), it may still be only one of the functions of this multi-faceted protein.

During the embryonic development of mice, early *Sip1* expression in gastrula (e.g. E8.0) is observed primarily in the neural plate, neural crest and paraxial mesoderm (Van de Putte et al., 2003); however, late *Sip1* expression occurs in various tissues (T. Miyoshi, M. Maruhashi, T. Van de Putte, H.K., D. Huylebroeck and Y.H., unpublished). The *Sip1*-null knockout mouse embryos die around E9.5 after heart dysfunction and embryo turning failure (Van de Putte et al., 2003). Thus, the floxed (flanked by loxP sites) *Sip1* allele was generated (Higashi et al., 2002), which enables cell-lineage-specific *Sip1* inactivation.

As demonstrated in this study, in the lens lineage, *Sip1* is

expressed after lens placode induction. The lens is a simple tissue and is one of the best characterized in terms of transcriptional regulation (Kondoh, 1999; Kondoh, 2002). The lens-lineage-specific ablation of the floxed *Sip1* gene can be achieved by using the lens enhancer of *Pax6* (Kammandel et al., 1999; Williams et al., 1998) in controlling Cre recombinase. Therefore, the consequence of lens-specific *Sip1* inactivation was investigated, and two steps in lens development dependent on Sip1 activity were characterized: (1) the separation of the lens epithelium and surface ectoderm by the removal of the connecting lens stalk; and (2) the progression of lens fiber precursors in the bow region into γ-crystallin-expressing mature fiber cells. In this study *Foxe3* activation, which is involved in the first step, was demonstrated to be dependent on Sip1 activity. The *Foxe3* promoter was activated by Sip1 and this activation was augmented by the specific interaction of Sip1 with Smad8 in a transfection assay. This study is the first clear demonstration that Smad-Sip1 interactions are significant in transcriptional regulation.

Given evidence of the involvement of Sip1 in many important processes of embryogenesis, not limited to the lens (Eisaki et al., 2000; Papin et al., 2002; Sheng et al., 2003; Van de Putte et al., 2003; van Grunsven et al., 2000), this study provides new insight into the regulatory functions of this interesting transcription factor.

Materials and methods

Mouse lines

The mouse line carrying the floxed *Sip1* allele has been described (Higashi et al., 2002). The Pax6(LP)-Cre transgenic line [Lens-Cre (see Ashery-Padan et al., 2000)] was provided by Drs A Mansouri and P. Gruss through Dr S. Watanabe of the University of Tokyo. The Pax6(Lens)-Cre transgene was constructed by ligating the 340 bp lens-specific enhancer of *Pax6* (Kammandel et al., 1999; Williams et al., 1998) to the *Pax6* P0 promoter and Cre-encoding sequence. From seven founder lines, the one showing the highest Cre activity, as determined by crossing with the R26R mouse (Soriano, 1999) (obtained from the Jackson Laboratory) was employed. The *dyl* mutant mouse (Blixt et al., 2000; Brownell et al., 2000) was also from the Jackson Laboratory. The Pax6(LP)- or Pax6(Lens)-Cre transgene in a male mouse was introduced into homozygous floxed *Sip1* background by mating for two generations with floxed *Sip1* homozygotes, and embryos derived from the mating of a floxed *Sip1*-homozygous female and a Cre-carrying homozygous male were used in this study. In some cases, the R26R transgene was also introduced in experimental embryos to monitor Cre recombinase activity. X-gal staining for β-galactosidase activity in the embryos was carried out as previously described (Muta et al., 2002). The presence of the Cre transgene in mice was determined by detecting a 235 bp PCR product of DNA extracted from an ear punch, using the primers 5′AGGTTCGTTCACTCATGGA3′ (forward) and 5′TCGACCAGTTTAGTTACCC3′ (reverse), and the *lacZ* sequence by detecting a 478 bp product, using the primers 5′TTGCCGTCTGAATTTGACCTG3′ (forward) and 5′TCTGCTTCAATCAGCGTGCC3′ (reverse). Normal and floxed *Sip1* alleles were distinguished analogously (Higashi et al., 2002). Mice were maintained in C57B6/C3H mixed background.

Immunohistology

Anti-crystallin antibodies were raised in rabbit by injecting the following peptides ligated to keyhole limpet hemocyanin. Anti-α-crystallins (recognizing both αA and αB), CVSREEKPSSAPSS; anti-βA-crystallins without cross-reaction to the βB class, CHAQTSQIQSIRRIQQ; and anti-γ-crystallins, GKITFYEDRSFQGRC. The embryos were fixed with 4% paraformaldehyde in phosphate-buffered saline at 4°C overnight, dehydrated, embedded in paraffin and cut into 6 μm serial sections. The sections were treated with anti-crystallin primary antibodies and Alexafluor568-conjugated anti-rabbit IgG (Molecular Probe) antibodies, stained for nuclei with DAPI and mounted in Permafluor anti-fade reagent (Immunotech).

In situ hybridization

Embryo sections were hybridized with specific probes as described previously (Uchikawa et al., 1999). The full-length *Sip1* cDNA probe (Van de Putte et al., 2003), *Pax6* 3′ UTR probe (Xu et al., 1999), *Sox1* 3′ UTR probe (*Xho*I-*Stu*I fragment), and probes for *Foxe3*, *Maf,* γ-crystallins and *Pdgfra* (Yamada et al., 2003) were used.

TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling) assay

Apoptotic cells in histological sections were detected by the TUNEL technique using an Apo-Alert DNA Fragmentation Assay kit (Clontech). TUNEL-positive nuclei among DAPI (4′,6-diamidino-2-phenylindole)-stained nuclei in the anterior and posterior lens halves were counted in meridian sections through lenses, and data of individual embryo specimens were combined.

Transfection

Lens epithelial cells were prepared from E14 chicken embryos and cultured for transfection as previously described (Muta et al., 2002). Collagen-coated 24-well plates were inoculated with one-fifth of the epithelial cells derived from one lens per well. Similarly, E10 gizzard cells were inoculated at 4×10^4 cells per well. A 1.5 μg plasmid-DNA mixture for transfection, typically containing 100-200 ng of a *Foxe3* promoter-ligated GL3 firefly luciferase gene (Promega), 1-500 ng of effector plasmids and 10 ng of phRG-TK Renilla luciferase expression plasmid, was transfected into cells in a well using 3 μg of Fugene6 (Roche). Luciferase activity was measured after 48 hours using a Dual Luciferase Reporter Assay kit (Promega) and an LB940 Mithras Multilabel Reader (Berthold Technologies), normalizing firefly luciferase activity using Renilla luciferase. Transfection was carried out at least in triplicate. The activity of Smad expression vectors (provided by Drs M. Kawabata and K. Miyazono) driven by the elongation factor I enhancer/promoter was confirmed by the activation of p3GC2-Lux (Smad1, 5 and 8) or p3TP-Lux (Smad 2 and 3) (Ishida et al., 2000) in lens epithelial cells.

Results

Expression of *Sip1* during lens development

Sip1 expression in early eye development was examined by the in situ hybridization of transcripts. *Sip1* is expressed widely in embryonic tissues, and in both lens and retinal components of the eye, but its expression pattern in the lens dynamically changes with developmental stage (Fig. 1). At E9.0, before the occurrence of lens induction, *Sip1* is not expressed in the surface ectoderm (Fig. 1A), but in parallel with lens placode induction at E9.5, *Sip1* expression is initiated (Fig. 1B). As the lens vesicle is formed (E10.5-E11.5), *Sip1* is expressed in the entire vesicle (Fig. 1C,D). After E12, primary lens fiber cells differentiate in the posterior lens, where the *Sip1* expression level is very low, while a high level of *Sip1* expression continues in the lens epithelium and immature lens fibers in the equatorial bow region (Fig. 1E).

Lens-specific inactivation of *Sip1* gene

To clarify the intrinsic functions of Sip1 in lens cells, the *Sip1* gene was ablated in a lens-lineage-specific fashion, using

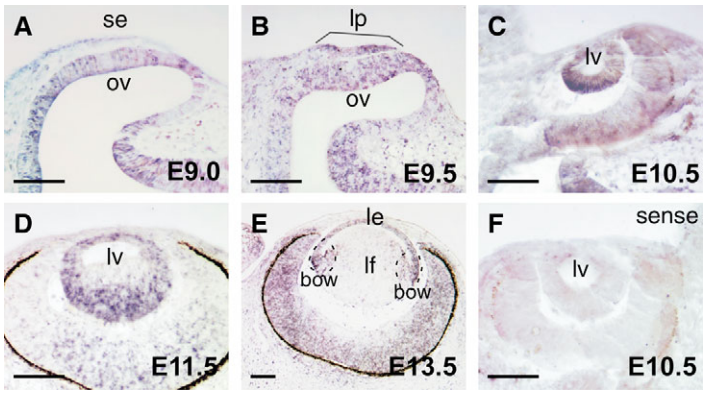

Fig. 1. *Sip1* expression in early lens development. Distribution of *Sip1* transcript detected by in situ hybridization of frontal sections through eye. Dorsal side is towards the right. (A) At E9.0 before lens induction, surface ectoderm does not express a significant level of *Sip1*. (B) At E9.5 soon after lens induction, *Sip1* expression initiates in the lens placode. (C) At E10.5 in ectoderm derivatives, *Sip1* expression is maintained only in invaginating lens cells but not in the adjacent surface ectoderm (future cornea). (D) At E11.5, all cells in the lens vesicle show *Sip1* expression. (E) At E13.5, once mature lens fibers differentiate, they loose *Sip1* expression, while immature lens fibers in the bow region and the lens epithelium show strong *Sip1* expression. (F) Section of E10.5 embryo hybridized with sense control probe. se, surface ectoderm; ov, optic vesicle; lp, lens placode; lv, lens vesicle; le, lens epithelium; lf, mature lens fibers. Bow regions are encircled by broken lines. Scale bars: 100 μm.

embryos homozygous for the floxed *Sip1* allele, in which the action of Cre recombinase causes the loss of detectable Sip1 protein (Higashi et al., 2002) (Fig. 2A).

A lens-lineage-specific Cre-expressing mouse line developed by Ashery-Padan et al. (Ashery-Padan et al., 2000) and another line established in this study were used; both lines use the *Pax6*-lens enhancer. The *Pax6* lens enhancer of 340 bp located 3.7 kb upstream of the P0 promoter gains its activity in the surface ectoderm of the prospective eye area, starting at E9.0 and its activity is maintained in its derivatives, lens epithelium and cornea in later development (Kammandel et al., 1999; Williams et al., 1998). The mouse line Pax6(LP)-Cre (Ashery-Padan et al., 2000) carries a large *Pax6* upstream region of 6.5 kb, including a pancreas enhancer (Fig. 2B). By crossing the mouse with an R26R reporter mouse (Soriano, 1999), Cre recombinase activity in the surface ectoderm, not confined to the eye area but extending to the olfactory epithelium area, was demonstrated (Fig. 2C, parts a,c). By contrast, the second mouse line developed in this study, carrying only the minimal lens enhancer, Pax6(Lens)-Cre (Fig. 2B), showed a strong activity in the lens and surface ectoderm confined to the eye-proximal region (Fig. 2C, parts b,d). Using histological sections, the specificity of Cre recombinase action in the lens and ectoderm was confirmed (Fig. 2C, parts e-h).

When these Cre transgenes were introduced into homozygous floxed *Sip1* embryos, they developed defective eyes; otherwise, they exhibited normal growth and fertility. In the homozygous floxed *Sip1* mouse population, the Cre transgenes were transmitted according to the Mendelian ratio. The lens defect was identical between the two Cre transgenic lines, Pax6(LP)-Cre and Pax6(Lens)-Cre; therefore, data using these two Cre lines were combined and used in the following analysis.

Two major defects of lens development in the absence of Sip1 activity

The consequence of the loss of Sip1 activity in the lens lineage was investigated at the histological level using various markers. Expression of Cre did not interfere with lens placode development (E9.5) (data not shown) or invagination (E10.5) (Fig. 3A,D); however, the first marked defect was observed at E11.5 (Fig. 3B,E) when the lens vesicle was normally separated from the surface ectoderm by tissue reorganization and local apoptosis involving the connecting lens stalk (van Raamsdonk and Tilghman, 2000). The Cre recombinase action in the lens lineage was confirmed using R26R mouse background. In the *Sip1*-defective lens, a thick stalk connecting the vesicle and ectoderm was persistent, and the vesicle was smaller (Fig. 3E). The lens vesicle that developed in the floxed *Sip1* embryo lacked *Sip1* expression in the surface ectoderm, stalk and anterior region of the vesicle, but had some residual *Sip1* expression in the posterior half, as determined by in situ hybridization (Fig. 3F). This is in contrast to the uniform expression of *Sip1* in the lens vesicle in the normal embryo (Fig. 3C). Thus, in the absence of *Sip1* activity, the cells positioned in the lens stalk persist.

At E14.5 and even at newborn (P0) stages the lens stalk still remains when *Sip1* is inactivated in the lens lineage (Fig. 3I,J), and a defect in lens mass development is evident. A stalk-persistent lens produced in *dyl/dyl* (*Foxe3* mutant) lens is shown in Fig. 3K for comparison.

When apoptotic cell distribution was measured using the TUNEL method from E10.5 to E12.5, apoptotic cells were mainly distributed in the anterior half of both normal and *Sip1*-defective lenses. In *Sip1*-defective lenses, the apoptotic cell population increased significantly, but the increment in apoptosis rate relative to that observed in the normal lens was comparable between the anterior and posterior halves at E10.5 and E11.5 without any specific suppression of apoptosis in the stalk region, and higher in the anterior half at E12.5 (Fig. 3L,M). Therefore, the increased apoptosis rate in *Sip1*-defective lenses accounts for the smaller lens size, but does not appear to contribute to either the persistence of lens stalk, or the specific loss of mature lens fibers in the posterior lens to be described below.

To clarify the cellular and molecular bases of lens development defects, various lens markers were examined at the histological level. At E12.5, anti-αA/B-crystallin antibodies stained all cells of normal and *Sip1*-defective lenses (Fig. 4A,F). βA-crystallins are expressed at a low level in immature fiber cells in the bow region where the *Sip1* expression level is high (Fig. 1E), and at a high level in mature lens cells (Fig. 4B). In *Sip1*-defective lenses, only a low βA-crystallin expression level was observed in the posterior-most side of the lens vesicle, which may correspond to the bow region of the normal lens. Mature lens fiber cells are marked by γ-crystallins in normal lenses (Fig. 4C). The most distinct characteristic of a *Sip1*-defective lens is the total absence of γ-crystallin expression (Fig. 4H). This absence of γ-crystallin expression was confirmed using in situ hybridization (data not shown).

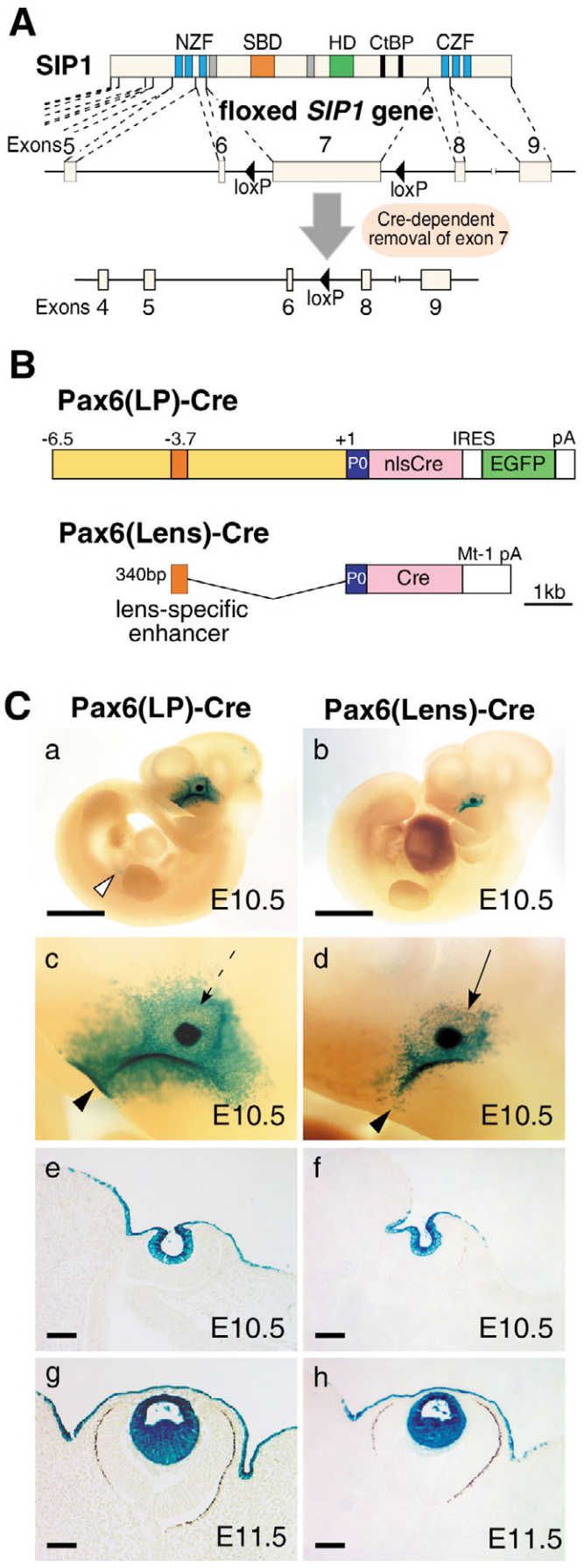

Fig. 2. Lens lineage-specific Cre recombinase system in ablation of *Sip1* gene. (A) Scheme of Sip1 protein organization (top) compared with that of floxed allele of *Sip1* (middle). NZF, N-terminal zinc fingers; CZF, C-terminal zinc fingers; SBD, Smad-binding domain; HD, homeodomain; CtBP, CtBP-binding sites. LoxP recombination sites flank major exon 7, and by the action of Cre recombinase, not only the deletion of this exon sequence occurs accompanying stop codon-generating splicing of exons 6 and 8, but detectable Sip1 protein synthesis is lost (Higashi et al., 2002). (B) Organization of two Pax6-Cre transgenes used in this study. Pax6(Lens)-Cre carries only 340 bp lens/cornea enhancer, while Pax6(LP)-Cre carries the entire 6.5 kb upstream sequence, including the pancreas enhancer in addition to the lens/cornea enhancer, both using the *Pax6* P0 promoter. (C) Assessment of Cre recombinase activity by *lacZ* expression with R26R background. (a,c) Activity of Pax6(LP)-Cre. At E10.5, recombinase activity includes presumptive lens ectoderm forming a pit and neighboring region (mainly future cornea), but extends anteriorly to include the olfactory epithelium area (arrowhead in c). In addition, activity was detectable in the pancreas primordium (arrowhead in a), as reported previously (Kammandel et al., 1999; Williams et al., 1998). (b,d) Activity of Pax6(Lens)-Cre. Using Pax6(Lens)-Cre, the Cre activity domain is narrower and grossly confined to the future lens and cornea, without extending to the olfactory epithelium area (arrowhead in d). (e,f) Sections of the same embryos through plane indicated by arrows in c,d. Cre recombinase activity is evident in the lens pit and extends to surrounding ectoderm area, but the extension is limited in f. (g,h) Sections through eye at E11.5, demonstrating Cre activity in the lens and overlying cornea, and showing limited extraocular *lacZ* staining using Pax6(Lens)-Cre. Scale bars: 1 mm for a,b; 100 μm for e-h.

The above observations indicate that the *Sip1*-defective lens lacks the region of a mature lens with γ-crystallin expression, located between the arcs indicated in Fig. 4C. As the *Sip1*-defective lens contains β-crystallin-positive regions (Fig. 4G), which presumably correspond to those outside arcs in normal lenses (Fig. 4B), the data are consistent with the model of a strong *Sip1* expression in the bow region promoting the maturation of fiber cells; in the absence of *Sip1* expression in the bow region, cells arrest at the premature stage.

γ-Crystallin genes are regulated by Sox1 and Maf, which synergistically function in the activation of their lens-specific promoters (Kamachi et al., 1995; Kawauchi et al., 1999; Kim et al., 1999; Nishiguchi et al., 1998; Ring et al., 2000), but the inactivation of *Sip1* does not affect expression of these transcription factor genes, as indicated by in situ hybridization (Fig. 4D,E,I,J).

Thus, two major defects were observed in the *Sip1*-defective lens: (1) persistent lens stalk; and (2) the arrest of lens fiber cell maturation at the bow-region stage.

Loss of *Foxe3* activation in *Sip1*-defective lens

A persistent lens stalk is called Peter's anomaly in human congenital diseases, and accompanies the inactivation of the transcription factor Foxe3 (Blixt et al., 2000; Brownell et al., 2000). Homozygous *dyl* (dysgenetic lens) mice with a mutation in the *Foxe3* gene (Blixt et al., 2000; Brownell et al., 2000) or a low Pax6 activity affecting *Foxe3* expression (Brownell et al., 2000; Dimanlig et al., 2001) are documented examples. This prompted us to examine the possibility that *Foxe3* activity is affected in *Sip1*-defective lenses (Fig. 5). In addition to *Foxe3* (Fig. 5A,E), *Pdgfra* gene under Foxe3 regulation (Blixt et al., 2000) (Fig. 5B,F), and an upstream gene *Pax6* required for

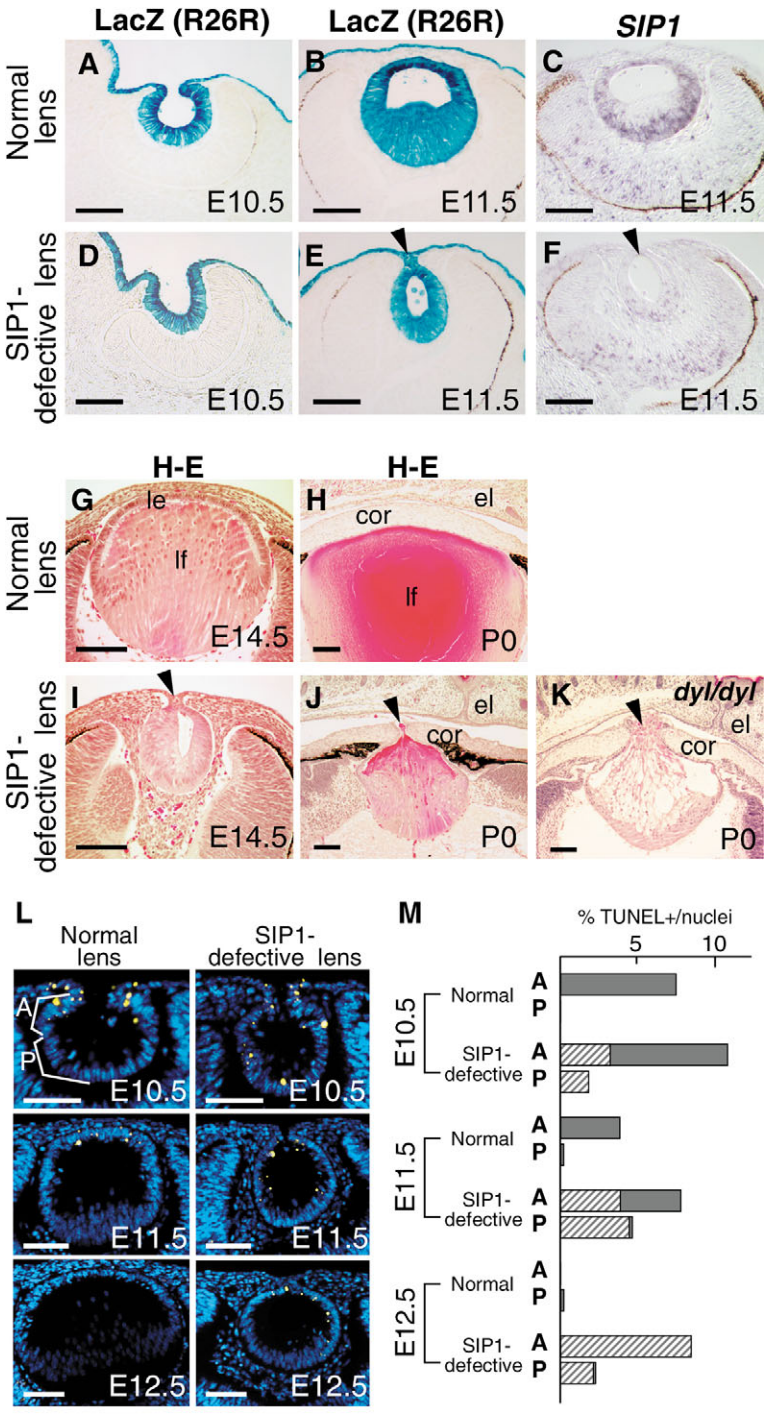

Fig. 3. Defect in lens development caused by lens lineage-specific ablation of *Sip1* gene. (A-F) Comparison of normal (A-C) and *Sip1*-defective (D-F) lenses. (B,E) After E11.5 *Sip1*-defective lens remains attached to surface ectoderm through persistent stalk (arrowhead). (C,F) In situ hybridization of E11.5 lens specimens analogous to (B,E) for *Sip1* transcripts. Normal lens shows *Sip1* expression throughout the lens vesicle (C), while the *Sip1*-defective vesicle shows no *Sip1* transcripts in the stalk and anterior half of the vesicle, and residual low-level *Sip1* transcripts in the posterior half (F). (G,I) Hematoxylin and Eosin (H-E) staining of E14.5 lenses. Normal lens shows full development of mature lens fiber cells (G), while *Sip1*-defective lens is still attached to the cornea through the stalk (arrowhead), and shows no development of mature lens fiber cells (I). (H,J) H-E staining of P0 lenses. *Sip1*-defective lens still attached to the cornea (arrowhead) as a small cell mass. (K) Homozygous *dyl* (*Foxe3*-defective) mouse lens at P0, showing morphological resemblance to *Sip1*-defective lens in J. (L) Distribution of apoptotic cells in the normal and *Sip1*-defective lens vesicles at E10.5, E11.5 and E12.5, where TUNEL-positive nuclei (yellow) among DAPI-stained nuclei (blue) are shown. Scale bars: 100 μm. (M) Statistics of apoptosis measured in meridian lens sections in their anterior and posterior halves. The fraction of TUNEL-positive nuclei in DAPI-stained nuclei is shown using data of two (E12.5) to six (other stages) lens specimens. Net increment of TUNEL+ apoptotic cell population under the *Sip1*-defective condition is indicated by hatched graph bars. le, lens epithelium; lf, mature lens fibers; cor, cornea; el, eyelid. Scale bars: 100 μm.

Foxe3 expression (Brownell et al., 2000) (Fig. 5C,G) were analyzed for their expression using in situ hybridization.

Foxe3 expression in the normal lens was divided into two domains: a strong expression in the anterior domain, including the lens epithelium and bow region; and a weak expression in the posterior domain (Fig. 5A). The *Sip1*-defective lens lacked the strong *Foxe3* expression in the anterior domain (Fig. 5E), accounting for the persistence of the stalk analogous to the *Foxe3* mutant. However, a weak *Foxe3* expression in the posterior domain of lens vesicle remained. This may reflect a low-level *Sip1* transcript remaining in the posterior half of the *Sip1*-ablated lens (Fig. 3F) or, alternatively, *Foxe3* expression in posterior lens cells may be regulated by a different mechanism. *Pdgfra* is regulated by Foxe3 and its expression pattern in the normal lens mirrors that of *Foxe3* (Blixt et al., 2000) (Fig. 5B). In the *Sip1*-defective lens, *Pdgfra* expression was downregulated in the anterior domain, paralleling the loss of *Foxe3* expression (Fig. 5F). These observations place *Sip1* upstream of *Foxe3*. Consistently, *Sip1* is expressed throughout the lens at a normal level in *Foxe3*-mutant *dyl* mice (Fig. 5D,H).

Pax6 expression was basically unaffected in the *Sip1*-defective lens (Fig. 5C,G). In contrast to *Pax6* expression in the normal lens, which is downregulated in the posterior region, *Pax6* expression prevails throughout the lens in *Sip1*-defective lens, but this difference is accounted for by the lack of a mature fiber compartment in the latter. Thus, Sip1 and Pax6 are both assigned as upstream regulator of *Foxe3*. As *Pax6*-null embryos develop no lens structure (Hill et al., 1991; Hogan et al., 1986), it was not determined whether *Sip1* itself is regulated by Pax6.

Activation of *Foxe3* promoter by Sip1 thorough interaction with Smad8

The 6.2 kb 5′ flanking region upstream of the *Sma*I site has promoter activity sufficient for controlling the expression of a *lacZ* transgene in the mouse lens (Brownell et al., 2000). Whether the *Foxe3* promoter is regulated by Sip1 and whether this regulation depends on interaction with Smads were investigated. The 6.2-kb promoter sequence was ligated to a

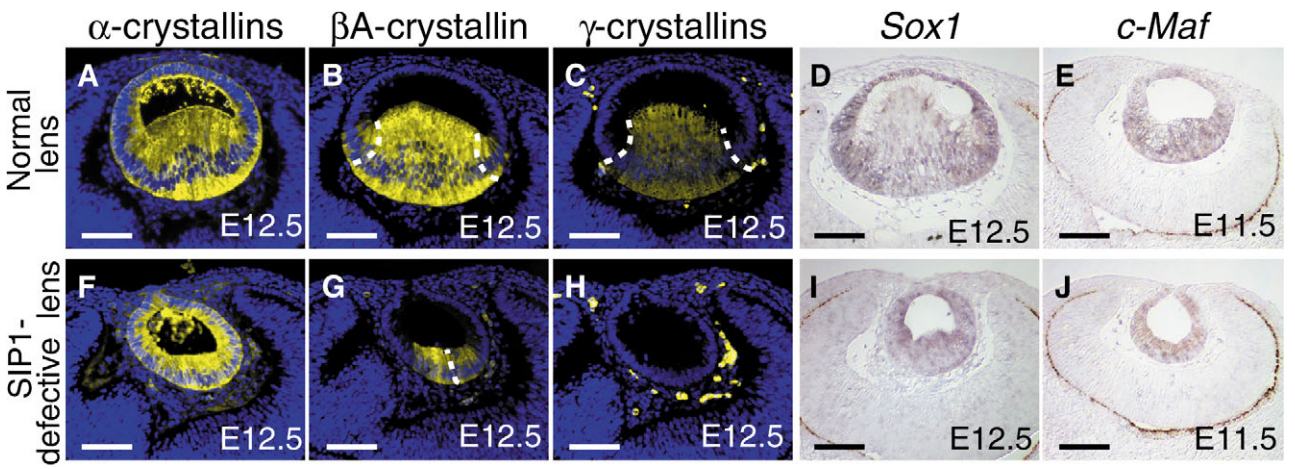

Fig. 4. Crystallin expression and its regulation in *Sip1*-defective lenses. (A-E) Normal lenses and (F-J) *Sip1*-defective lenses, subjected to immunostaining (A-C,F-H) and in situ hybridization (D,E,I,J). (A,F) At E12.5, α-crystallins are expressed throughout the lens under either normal (A) or *Sip1*-defective (F) conditions. βA-crystallins are normally expressed widely in nonepithelial domains through immature to mature lens fibers (B), but in *Sip1*-defective lens, its expression was confined to the innermost domain (G). γ-Crystallin expression marking mature lens fiber cells in normal lens (C) is totally missing in *Sip1*-defective lens (H), clearly indicating a defect in lens fiber maturation. Broken lines indicate the boundaries of the bow region. However, two known key transcription factor genes for γ-crystallin regulation, *Sox1* (Kamachi et al., 1995; Nishiguchi et al., 1998) and *Maf* (Kawauchi et al., 1999; Kim et al., 1999; Ring et al., 2000), are expressed in normal (D,E) and *Sip1*-defective (I,J) lenses. Scale bars: 100 μm.

luciferase reporter gene (Fig. 6A), then transfected with a Sip1 expression vector into primary-cultured lens epithelial and gizzard (smooth muscle) cells of chicken embryos. Results using these cells were similar and data for the lens epithelial cells are shown in Fig. 6.

The activity of the 6.2 kb promoter was progressively augmented by the exogenous expression of Sip1, sevenfold activation by 100 ng of expression vector and 20-fold activation by 500 ng of expression vector (Fig. 6B). The same increase was observed using a mutant Sip1 lacking the Smad-binding domain (SBD) (Fig. 6B), indicating that this activation occurs without interaction with a Smad protein.

Under the same transfection conditions, various Smad proteins were expressed by co-transfection (Fig. 6C). Smad1, Smad5 and Smad8, mediating BMP signals, and Smad2 and Smad3, mediating TGFβ signals, bind Sip1 in vitro through the SBD (Verschueren et al., 1999). Of these Smads, only Smad8 caused a significant Sip1-dependent activation of the *Foxe3* promoter, threefold more activation than Sip1 alone, while Smad8 by itself had no effect on the *Foxe3* promoter (Fig. 6C). However, using SBD-deleted Sip1, exogenous Smad8 did not augment Sip1-dependent activation (Fig. 6C). Under the same transfection condition, Smad1, Smad5 and Smad8 exhibited comparable levels of transactivation of the 3GC2-luciferase reporter gene (Fig. 6D). It was rather unexpected that Smad1 or Smad5 had no significant effect

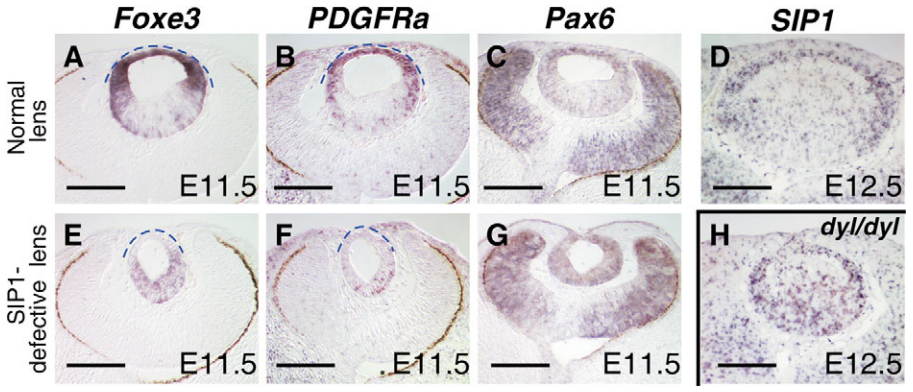

Fig. 5. Loss of *Foxe3* expression in the epithelial compartment of *Sip1*-defective lenses. (A-D) Normal lenses, (E-G) *Sip1*-defective lenses and (H) *dyl* (*Foxe3*-defective) mutant lens. *Foxe3* is expressed strongly in the anterior compartment (indicated by the broken line) of the lens vesicle that later contributes to lens epithelium (A). In *Sip1*-defective lens, however, *Foxe3* expression in the corresponding anterior compartment is missing (E) (a section adjacent to Fig. 3F). Accordingly, *Pdgfra* downstream of Foxe3 (Blixt et al., 2000) (B) is attenuated in the corresponding compartment of the *Sip1*-defective lens (F). However, *Pax6*, which is known to be involved in activation of *Foxe3* (Brownell et al., 2000), is expressed at similar levels in normal (C) and *Sip1*-defective lenses (G). Expression of *Sip1* in normal lens (D) is maintained in *dyl* (*Foxe3*) homozygous lenses (H). Scale bars: 100 μm.

under the transfection condition, although they are generally considered to act analogously to Smad8 (ten Dijke and Hill, 2004). When an inhibitory Smad, Smad6 or Smad7, was co-expressed with Smad8, the effect of Smad8 was cancelled and only the activation level attainable by Sip1 alone remains (Fig. 6E). The effect of Smad8 was saturated at approximately threefold the activation level attained by Sip1 alone, regardless of whether the original activation level by Sip1 alone was sevenfold (Fig. 6F) or nearly 20-fold (Fig. 6F).

These observations indicate that augmentation by Smad8 of Sip1-dependent activation of the *Foxe3* promoter is through SBD-mediated direct interaction between Sip1 and Smad8. They also indicate a two-step mechanism for the activation of the *Foxe3* promoter by Sip1, with moderate activation without the assistance of Smad8, and high-level activation with the Sip1-Smad8 complex.

The 6.2 kb promoter region was divided into four blocks, A to D, from the proximal side, and the block responsible for activation by Sip1 and Smad8 was then investigated. The removal of the upstream blocks B to D did not greatly affect the activation of the *Foxe3* promoter by Sip1 or by Sip1 plus Smad8, and the 1.26 kb block A promoter region is sufficient (Fig. 6G). As shown below using transgenic mouse embryos, block A is not involved in the lens-specific regulation of the *Foxe3* promoter. The Sip1-dependent activation of the *Foxe3* promoter and its augmentation by Smad8 is also observed in gizzard cells (see Fig. S1 in the supplementary material), confirming their tissue non-specific effect.

The shortening of the promoter sequence to 287 bp maintains the capacity for Sip1-dependent activation and further augmentation by Smad8 (Fig. 6G). Further shortening of the promoter to 127 bp resulted in the loss of response to Sip1. The unrelated δ-crystallin promoter was not affected by either Sip1 or Sip1 plus Smad8 (Fig. 6G). This observation suggests that the activation of the *Foxe3* promoter by Sip1 and Smad8 involves the proximal region.

Lens-specific regulation of *Foxe3* promoter

When the 6.2 kb *Foxe3* promoter was ligated to a *lacZ* transgene construct and primary transgenic mouse embryos were produced, lens- and brain-restricted *lacZ* expression was observed at E12.5 (Fig. 7A), confirming a previous report (Brownell et al., 2000). To investigate tissue-specific regulation, the effect of deleting various blocks was examined (Fig. 7A). The removal of the most distal block D, resulting in the promoter blocks A+B+C, did not have any appreciable effect, but further deletion of block C leaving promoter blocks A+B caused a large decrease in the expression level in the lens and the loss of expression in the brain. When block B was removed from A+B+C blocks, leaving A+C blocks of the promoter region, transgene expression in the lens and brain was indistinguishable from that using the full 6.2 kb sequence. By contrast, with only the most proximal block A, transgene expression was not observed. These results indicate that block C includes the major lens-specific element and a brain element, and block B contains a minor lens element, and that the combination of the activity of these blocks with the Sip1-dependent, cell type nonspecific activity of block A elicits *Foxe3* expression in embryonic lenses, as summarized in Fig. 7B.

Discussion

Two steps of lens development involving Sip1 activity

This study clarified the roles of the Sip1 transcription factor in ocular lens development, using a lens-lineage-specific gene ablation strategy (Fig. 2A). Two lens-specific Cre transgenic lines were used, both taking advantage of the lens-specific enhancer of the *Pax6* gene. Pax6(LP)-Cre (Ashery-Padan et al., 2000) carries a large *Pax6* upstream region driving a gene in the head ectoderm and pancreas, in addition to the lens lineage, and Pax6(Lens)-Cre carries only the lens/cornea enhancer developed in this study (Fig. 2B). These two *Pax6*-enhancer-dependent Cre lines gave identical results when crossed with the floxed *Sip1* mouse. Given the narrower tissue restriction of Cre recombinase action (Fig. 2C), the Pax6(Lens)-Cre transgenic mouse is useful for investigating a gene function in the lens-restricted lineage.

In lens development, *Sip1* is first activated in the lens placode, then after the lens vesicle is formed, *Sip1* expression is confined to the vesicle without detectable expression in the surface ectoderm. After mature lens fibers develop, strong *Sip1* expression is confined to the lens epithelium and bow region, and the expression is very low in the mature lens fibers (Fig. 1). The consequence of the lens-lineage-specific ablation of *Sip1* revealed two major Sip1-dependent steps in lens development (Fig. 3), consistent with the *Sip1* expression pattern.

The first significant defect is a persistent stalk connecting the surface ectoderm and lens epithelium (Fig. 3). This defect, called Peter's anomaly as a congenital disease in humans, is shared by defects in the transcription factors Pax6 or Foxe3, and a common denominator is the loss of functional Foxe3 (Blixt et al., 2000; Brownell et al., 2000; Dimanlig et al., 2001). A Sip1 defect also causes the downregulation of *Foxe3* in the lens epithelium (Fig. 5). This observation indicates that the *Foxe3* gene is downstream of *Sip1* in the regulatory pathway.

The second defect of the *Sip1*-defective lens is lack of mature lens fibers expressing γ-crystallins (Figs 3, 4). *Sip1* is strongly expressed in the bow region in the normal lens where βA-crystallins are already expressed, signifying the initiation step of fiber differentiation. The bow region has been thought of as merely a zone of transition between the epithelial fiber precursor and mature lens fibers. However, the arrest of lens fiber differentiation in the βA-crystallin-positive γ-crystallin-negative bow region state strongly suggests that *Sip1* expression in immature fiber cells promotes lens fiber maturation.

Regulation of *Foxe3* promoter

The possible involvement of the *Foxe3* promoter in the Sip1-dependent activation of *Foxe3* was examined, using cell transfection. Up to 20-fold activation of the 6.2 kb *Foxe3* promoter was observed from exogenous Sip1 (Fig. 6A,B). For this activation, a 1.2 kb promoter sequence was sufficient (Fig. 6F).

The activation of the *Foxe3* promoter by exogenous Sip1 allowed examination of the effect of exogenous Smads on its regulation. Of the Smads that bind Sip1 in vitro (Verschueren et al., 1999), only Smad8 further augmented Sip1-mediated *Foxe3* promoter activation by threefold (Fig. 6). However, this

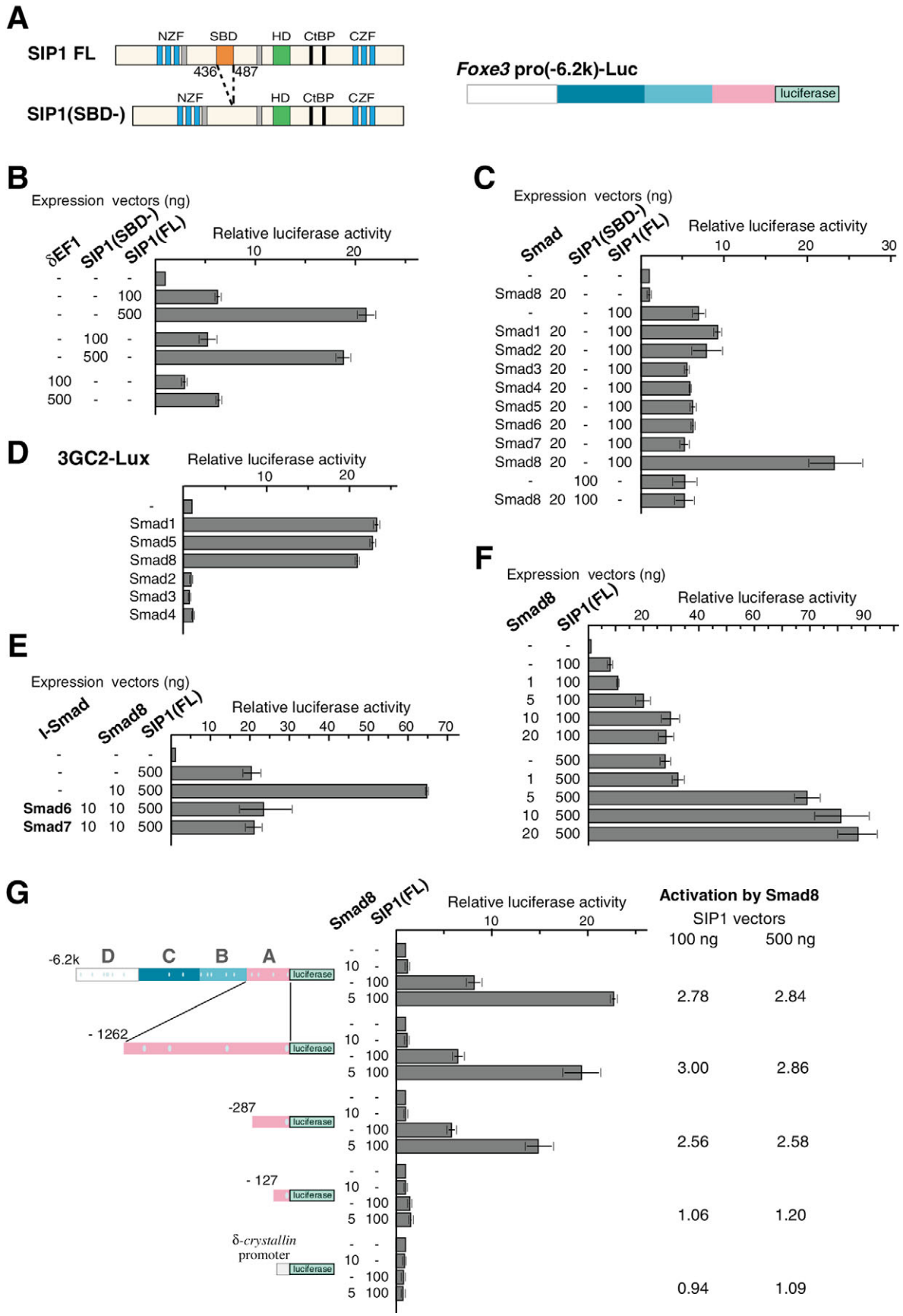

Fig. 6. See next page for legend.

Fig. 6. Activation of *Foxe3* promoter by Sip1 and Smad8. (A) Scheme of full-length Sip1 (FL) and SBD-deleted Sip1 (SBD-) (left), and *Foxe3* pro (–6.2k)-luciferase reporter construct used for transfection (right). (B) Exogenous Sip1, either full-length or SBD-deleted form, activates the promoter in a dose-dependent manner in transfected lens cells. Typically, 100 ng of Sip1 expression vector causes sevenfold activation and 500 ng of the vector elicits 20-fold activation. Using the δEF1 expression vector, only a marginal activation effect was observed. (C) Effect of exogenous Smads on Sip1-dependent activation of *Foxe3* promoter in transfected lens cells. Any of the Smads, alone, has no effect on promoter activity, as exemplified by Smad8. Of the eight Smads examined, only Smad8 augmented Sip1-dependent activation level. This effect was absent when Sip1 lacked SBD. (D) Activation of 3GC2-Luciferase reporter gene by expression vectors for Smad1, Smad5 and Smad8 in transfected lens cells. (E) Effect of inhibitory Smads on Smad8-mediated augmentation of Sip1-dependent promoter activation. Smad6 or Smad7 individually cancelled Smad8 effect. (F) Amount-dependent augmenting effect of exogenous Smad8 on Sip1-dependent activation of *Foxe3* promoter. Regardless of the initial activation level by exogenous Sip1, the effect of Smad8 saturates at approximately threefold augmentation level. (G) Effect of length of *Foxe3* promoter sequence on activation by Sip1 and augmentation by Smad8. In the scheme for the 6.2 kb promoter sequence, CACCT sequences are indicated by dots for comparison. A 1.26 kb promoter sequence was sufficient to show Sip1-dependent activation and Smad8-dependent augmentation. A 287 bp sequence still showed significant response to Sip1 and Smad8, but the shortening of the promoter to 127 bp resulted in loss of the responses. Data using 100 ng of Sip1 expression vector is shown, but basically the same promoter-length-dependent effect was observed using 500 ng of Sip1 expression vector as tabulated in the right panel.

Smad8 effect was not observed using SBD (Smad-binding domain)-deleted Sip1, demonstrating the involvement of a direct Sip1-Smad8 interaction. Amino acid sequence comparison of Smad8, Smad1 and Smad5 indicates that Smad8 has a considerably shorter and diversified linker sequence between MH1 and MH2 domains than the other two (see Fig. S2 in the supplementary material). However, given the demonstration of similar activities of Smad1, Smad5 and Smad8 in various assays (Moustakas et al., 2001; ten Dijke and Hill, 2004), it is possible that Smad1 and Smad5 also contribute to Sip1-dependent gene regulation in different contexts. In any case, this is the first clear demonstration that Smad interaction affects the regulatory potential of the Sip1 protein.

As previously demonstrated, the 6.2 kb *Foxe3* promoter controls the expression of a *lacZ* reporter gene in the lens and mid-forebrain region of transgenic mouse embryo (Brownell et al., 2000) (Fig. 7A). By the deletion of the promoter region using blocks A to D from the proximal side, a major lens element in block C and a minor lens element in block B were identified (Fig. 7A). Thus, lens-specific regulatory elements are separable from those involved in Sip1-dependent activation assigned to block A (Fig. 7B). Indeed, the Sip1- and Smad8-dependent activation of the *Foxe3* promoter was observed in non-lens cells (see Fig. S1 in the supplementary material), showing that this activation is not specific to lens cells. Block A of the *Foxe3* promoter is sufficient for this activation to occur in transfection assay (Fig. 6G), but by itself does not allow transgene expression in mouse embryos (Fig. 7A). Therefore, the combined action of lens element (blocks B and C) and Sip1-dependent (block A) promoter element appears to be required for *Foxe3* expression in embryonic lenses.

The block C sequence has a region strongly conserved between mouse *Foxe3* and human *FOXE3*, and with the aid of this sequence conservation, Grainger's group has independently identified the corresponding region in *Xenopus* as the lens element of the *Foxe3* promoter (H. Ogino and R. Grainger, personal communication).

Gene activation involving Sip1 activity

Gene activation by the action of Sip1 shown in this study expands the horizon of gene regulation involving ZFHX1 family transcription factors. Sip1 and δEF1 bind almost identical sets of sequences, owing to their highly conserved Krueppel-type zinc finger sequences (Funahashi et al., 1993; Verschueren et al., 1999). In addition, the bipartite zinc-finger clusters each bind to a similar set of sequences with a consensus of CACCT(G) (Remacle et al., 1999; Sekido et al., 1997), it has been postulated that ZFHX1 proteins bind a pair of CACCT(G) sequences in a two-footed fashion (Remacle et al., 1999). Under such conditions full-length Sip1 or δEF1 clearly exhibited the repression of gene transcription (Comijn et al., 2001; Funahashi et al., 1993; Kamachi and Kondoh, 1993; Papin et al., 2002; Remacle et al., 1999; Sekido et al., 1994; Sekido et al., 1997).

However, several lines of evidence support the view that the gene repression thorough a CACCT(G) pair is just one of many modes of regulatory function associated with ZFHX1 proteins.

(1) With a knockout allele of δEF1 lacking C-terminal zinc fingers, only a minor nonlethal phenotype develops in homozygous mouse (Higashi et al., 1997), in contrast to more severe lethal defects with a null allele (Takagi et al., 1998). This indicates that N-terminal zinc fingers are sufficient for DNA binding and exerting a regulatory function.

(2) The binding consensus CACCT(G) of N- and C-terminal zinc fingers was determined using oligonucleotide sequence pools preferentially binding to respective zinc finger clusters (Sekido et al., 1997). Re-examination of these sequences indicated that N-terminal zinc fingers bind DNA with a more relaxed specificity, including, for example, CACANNT.

(3) The 6.2 kb promoter sequence of *Foxe3* contains frequent recurrent CACCT sequences, many located in blocks B, C and D, but the removal of these upstream blocks did not significantly affect the response to exogenous Sip1 (Fig. 6G).

It has not been determined whether the Sip1 protein directly binds to the *Foxe3* promoter DNA, but further analysis of *Foxe3* promoter activation will reveal how Sip1 is involved in gene activation and how interaction with Smad affects its regulatory potential.

Smad-Sip1 interaction in transcriptional regulation

Since the discovery of Sip1 as a Smad-binding protein, Smads have been implicated in Sip1-mediated transcriptional regulation (Verschueren et al., 1999), but this study provides the first definitive evidence that Smad-Sip1 interaction has an impact on Sip1-dependent gene regulation.

The mechanism of augmenting the Sip1-dependent activation of the *Foxe3* promoter from interaction with Smad8 is not clear, but this interaction is not required for basal activation by Sip1 (up to 20-fold activation of the *Foxe3* promoter), as the same activation level is achieved using SBD-deleted Sip1 (Fig. 6B). A possible model would be that Sip1 itself possesses an activation domain that is exposed upon

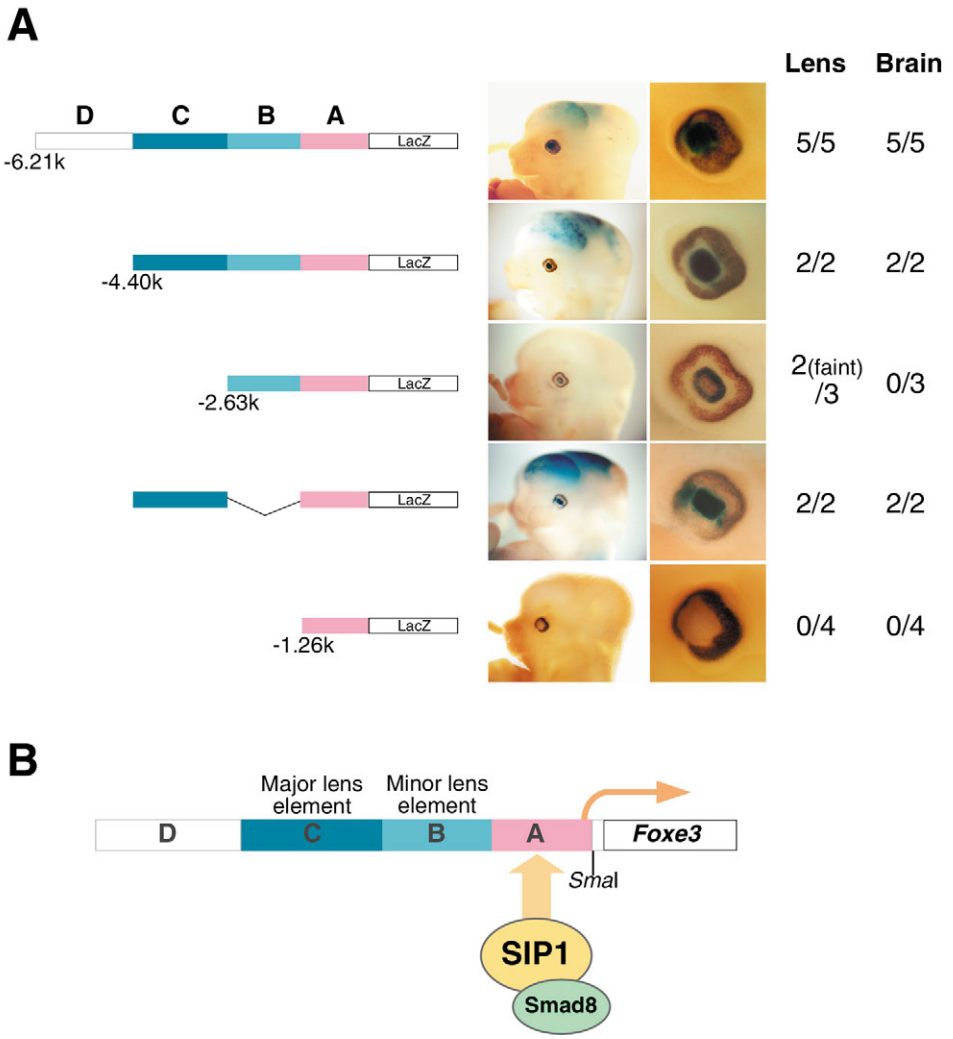

Fig. 7. Lens-specific regulation of *Foxe3* promoter in transgenic mouse embryos. (A) The 6.2 kb promoter sequence upstream of the *Sma*I site (Brownell et al., 2000) was divided into four blocks, A-D, from the proximal side, and in combination ligated to a *lacZ* expression cassette, and primary transgenic embryos were produced. Panels in the middle show transgene expression in the head (lens and brain) under low magnification (left) and in the lens under high magnification (right). The numbers in the right panel indicate cases of transgene expression in transgene (PCR)-positive embryos. (B) Schematic presentation of Sip1/Smad8-responsive region, and major and minor lens elements in *Foxe3* promoter.

binding to a proper sequence, and Smad8 bound to Sip1 provides an additional activation domain.

Many BMP signals are implicated in lens development (Faber et al., 2002). It has been demonstrated that BMP4 is required for the activation of *Sox2* in the prelens ectoderm (Furuta and Hogan, 1998), while BMP7 deficiency causes variable defects in later lens development from the absence of a lens to a slightly smaller lens (Jena et al., 1997; Wawersik et al., 1999). Developmental lens defect caused by lens lineage-specific *Sip1* inactivation and the involvement of Smad-Sip1 interaction in *Foxe3* promoter regulation underscore the importance of the BMP-Smad-Sip1 signaling pathway in lens development.

Sip1 gene activity is implicated in many steps of embryogenesis: gastrulation in chickens (Sheng et al., 2003), mesodermal development in *Xenopus* (Papin et al., 2002) and neural crest development in mice (Van de Putte et al., 2003). This study revealed important aspects of gene regulation mediated by Sip1 leading to gene activation. Most importantly, interaction with Smad proteins, at least with Smad8, modifies the transcriptional regulation mediated by Sip1. These new features of Sip1-mediated transcriptional regulation should help understanding of processes involving Sip1.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/20/4437/DC1

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