Cited2 is required for the proper formation of the hyaloid vasculature and for lens morphogenesis

Yu Chen1, Yong-qiu Doughman2, Shi Gu1, Andrew Jarrell1, Shin-ichi Aota3, Ales Cvekl4, Michiko Watanabe2, Sally L. Dunwoodie5, Randall S. Johnson6, Veronica van Heyningen7, Dirk A. Kleinjan7, David C. Beebe8 and Yu-Chung Yang1,*

Cited2 is a transcriptional modulator with pivotal roles in different biological processes. Cited2-deficient mouse embryos manifested two major defects in the developing eye. An abnormal corneal-lenticular stalk was characteristic of Cited2+/+ developing eyes, a feature reminiscent of Peters’ anomaly, which can be rescued by increased Pax6 gene dosage in Cited2+/+ embryonic eyes. In addition, the hyaloid vascular system showed hyaloid hypercellularity consisting of aberrant vasculature, which might be correlated with increased VEGF expression in the lens. Deletion of Hif1α (which encodes HIF-1α) in Cited2+/+ lens specifically eliminated the excessive accumulation of cellular mass and aberrant vasculature in the developing vitreous without affecting the corneal-lenticular stalk phenotype. These in vivo data demonstrate for the first time dual functions for Cited2: one upstream of, or together with, Pax6 in lens morphogenesis; and another in the normal formation of the hyaloid vasculature through its negative modulation of HIF-1 signaling. Taken together, our study provides novel mechanistic revelation for lens morphogenesis and hyaloid vasculature formation and hence might offer new insights into the etiology of Peters’ anomaly and ocular hypertelorpy.

KEY WORDS: Cited2, Hyaloid vasculature, Lens development, Mouse

INTRODUCTION

Cited2 [Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2] is one of the founding members of a family of transcriptional modulators (Shioda et al., 1997; Sun et al., 1998; Dunwoodie et al., 1998; Leung et al., 1999). Cited2 was previously named melanocyte-specific gene (MSG) related gene 1 (Mrg1; p35srj) (Shioda et al., 1997; Dunwoodie et al., 1998; Sun et al., 1998; Bhattacharya et al., 1999). It binds directly with high affinity to the first cysteine-histidine-rich (CH1) region of the transcription co-factors p300 and CBP. As a CBP/p300-dependent affinity to the first cysteine-histidine-rich (CH1) region of the transcription co-factors p300 and CBP. As a CBP/p300-dependent transcription factor, Cited2 functions as a negative regulator of hypoxia inducible factor 1 (HIF-1)-mediated signaling by competing with HIF-1α for binding to CBP/p300 (Bhattacharya et al., 1999). Cited2 physically interacts with several nuclear receptors and transcription factors, including PPAR (Ppara – Mouse Genome Informatics) (Tien et al., 2004), Hnf4α (Qu et al., 2007), Lhx2 (Glenn and Maurer, 1999), AP2 (Tcfap2) transcription factors (Bamforth et al., 2004) and Smad3/2 (Chou et al., 2006). Cited2 is also induced by many biological stimuli such as cytokines, serum and lipopolysaccharide in different cell types (Sun et al., 1998). Overexpression of Cited2 in Rat1 cells results in loss of cell contact inhibition, anchorage-independent growth and tumor formation in nude mice, demonstrating that Cited2 is a transforming gene (Sun et al., 1998). These initial in vitro studies underscore the potential roles of Cited2 in different biological processes.

Deletion of Cited2 results in embryonic lethality in mid- to late gestation, with embryos displaying cardiac malformations, neural tube defects, adrenal agenesis (Barbera et al., 2002; Bamforth et al., 2001; Yin et al., 2002; Val et al., 2007), left-right patterning defects (Weninger et al., 2005; Bamforth et al., 2004), placental defects (Withington et al., 2006), liver developmental defects (Qu et al., 2007) and defective fetal hematopoiesis (Chen et al., 2007). Further mechanistic studies have provided evidence that Cited2 plays pivotal roles in these processes through its transcriptional modulator functions for HIF-1 (Yin et al., 2002; Xu et al., 2007), AP2α (Tcfap2α – Mouse Genome Informatics) signaling (Bamforth et al., 2001; Bamforth et al., 2004), Hnf4α (Qu et al., 2007) and through other, as yet unknown, mechanisms.

The potential involvement of Cited2 in eye development was suggested by irregularly shaped pupils typical of Cited2-deficient embryos at 13.5 days post-coitum (dpc) (Yin et al., 2002). In this report, we show for the first time that Cited2 deficiency results in abnormal corneal-lenticular stalk formation and vitreous hypercellularity consisting of aberrant vasculature in the developing eye. We further demonstrate that Cited2 is an upstream positive regulator of Pax6 expression in the lens; this regulation is the mechanism that underlies the corneal-lenticular stalk formation resulting from Cited2 deficiency. In addition, our study also shows that genetic interaction of Cited2 with HIF-1 signaling contributes to the appropriate formation of the hyaloid vascular system (HVS) during development.

MATERIALS AND METHODS

Mouse lines and preparation of mouse embryos

Cited2−/+ (Yin et al., 2002) and Cited2flox/flox (Preis et al., 2006) mouse lines were maintained on the C57BL/6 background. Cited2flox/mice were mated with Le-Cre mice to generate Cited2flox/flox;Le-Cre and Cited2flox/flox;Le-Cre mice.
mice. Hif1a<sup>flox/flox</sup> (Cramer et al., 2003), Le-Cre<sup>+</sup> (Ashery-Padan et al., 2000) mice were mated with Cited2<sup>–/–</sup> mice to generate embryos with the following genotypes: Cited2<sup>–/–</sup>-Hif1a<sup>flox/flox</sup>-Le-Cre<sup>+</sup>, Cited2<sup>–/–</sup>-Hif1a<sup>flox/flox</sup>-Le-Cre<sup>+</sup>, Cited2<sup>–/–</sup>-Hif1a<sup>flox/flox</sup>-Le-Cre<sup>+</sup>, Cited2<sup>–/–</sup>-Hif1a<sup>flox/flox</sup>-Le-Cre<sup>+</sup>, Cited2<sup>–/–</sup>-Hif1a<sup>flox/flox</sup>-Le-Cre<sup>+</sup>, or Cited2<sup>–/–</sup>-Hif1a<sup>flox/flox</sup>-Le-Cre<sup>+</sup>. Primers for genotyping were: HIF-1α-flox: antisense (a), 5′-ATATGCTTTAGATAGGGCCCTATGGAGGC-3′ and sense (s), 5′-GATTCCTTCCGAGGACCTGTTCAATTCCC-3′; Le-Cre (a), 5′-GCATTACCCCCTGATGGAACGAGTGGGCAATGAGCGGAAAGAT-3′ and (s), 5′-GAGTGAACGAACTCGTGCGAAATCATGTCG-3′. PAX77 transgenic mice overexpressing the human Pax6 gene (Schedl et al., 1996) were mated with Cited2<sup>–/–</sup> mice to produce Cited2<sup>–/–</sup> mice carrying the Pax6 transgene. These compound mice were then mated with Cited2<sup>+/–</sup> mice to obtain Cited2<sup>+/–</sup> embryos with and without the transgene at 14.5 dpc. PAX77 transgenic mice were genotyped as described previously (Kleinjan et al., 2006). All animal husbandry and experiments were conducted in accordance with institutional guidelines of Case Western Reserve University. Timed pregnancy of Cited2<sup>+/–</sup> females was determined as 0.5 dpc if vaginal plug was found after overnight mating. Embryos were harvested by a caesarean-driven method.

**Histology**

Embryos at 10.5, 11.5, 12.5, 13.5, 15.5 and 18.5 dpc were fixed in 10% formalin, dehydrated, embedded in paraffin and processed with 7 μm transverse sectioning. Histology of the eyes was examined by light microscopy after Hematoxylin and Eosin staining.

**Immunohistochemistry and X-Gal staining for eye sections**

For immunohistochemistry, embryonic tissues were fixed in 1%-4% paraformaldehyde, equilibrated in 12%, 15% and 20% sucrose, embedded in OCT and processed with 10 μm cryosectioning. Immunostaining employed antibodies against Cited2 (Santa Cruz), E-cadherin (cadherin 1) (BD Pharmingen) and α-smooth muscle actin (α-SMA) (Sigma) and antibody staining was visualized with Alexa594-conjugated anti-mouse secondary antibody (Molecular Probes, Invitrogen). Phosphorylated histone H3 immunostaining was performed with anti-phospho-H3 antibody (Cell Signaling) and the staining was visualized with Alexa488-conjugated anti-rabbit secondary antibody (Molecular Probes, Invitrogen). Antibodies against CD31 (Pecam1 – Mouse Genome Informatics) (BD Pharmingen) and VEGFR2 (Flk1; Kdr) (BD Pharmingen) were visualized with 3,3′-diaminobenzidine (Sigma). Pax6 and AP2α antibodies were obtained from Developmental Studies Hybriodna Bank at University of Iowa and the staining was visualized by Alexa594-conjugated anti-mouse secondary antibody and 3,3′-diaminobenzidine, respectively. lacZ expression was detected by X-Gal (Roche) staining and was performed on 1% paraformaldehyde-fixed eye sections according to standard methods.

**TUNEL assay**

Cryosections were collected from 10.5 dpc embryos after fixation in 4% paraformaldehyde and processed for the TUNEL assay according to the manufacturer’s instruction (Chemicon).

**Real-time RT-PCR**

Total RNA was extracted using RNA Trizol (Invitrogen) and was reverse transcribed into cDNA using the SuperScript First-Strand Synthesis System for RT-PCR Kit (Invitrogen). PCR primers for detecting Pax6 expression in Cited2<sup>–/–</sup> and Cited2<sup>+/–</sup> embryonic lens were: antisense (a), 5′-CTACCAGCCTATCCCAGCAGC-3′ and sense (s), 5′-TTCGGGCCCAACTATGGAAC-3′. Primers for detecting Pax6 expression in lenses collected from Cited2<sup>–/–</sup>;PAX77 and Cited2<sup>+/–</sup> littermate control embryos were: (a), 5′-ATGTTGCGGATGTAGGCTGGAAGGAGCT-3′ and (s), 5′-GCGAGCCCTAGCCTCTGCTCA-3′. VEGF (Vegfa – Mouse Genome Informatics) and Hif1a mRNA expression was analyzed as described previously (Xu et al., 2007). The real-time PCR was performed in triplicate for each sample on MyiQ (BioRad). Ct value was recorded to perform data analysis.

**Luciferase assay**

α-TN4-1, NMuMG and HEK293 cells were seeded in 12-well plates for transfection of 0.15 ng pRLSV40, 270 ng of LE9-P0, LE0-P0 or P0 firefly luciferase reporter, various amounts of Cited2 expression plasmid, 75 ng of Pax6 expression plasmid, and control plasmid so that the total amount of DNA was 1 μg/well. Fugene6 transfection reagent (Roche) was used for the transfection. Firefly and Renilla luciferase activities were measured 24 hours after transfection using Dual-Luciferase Assay Reagents (Promega) on a luminometer. Relative luciferase activity was calculated by dividing firefly luciferase activity by Renilla luciferase activity.

**Chromatin immunoprecipitation**

Chromatin immunoprecipitation (ChIP) was performed in α-TN4-1 cells according to protocols previously described (Yang and Cvekl, 2005; Yang et al., 2006) using antibodies against Cited2 and Pax6 (Santa Cruz). The immunoprecipitated DNAs were amplified by PCR and analyzed by agarose gel electrophoresis. Primers for the ChIP assay spanning the Pax6 LE9 region and the Pax6 P0 promoter region were: LE9 region (a), 5′-TGGCCAATGAGCGGAAGATG-3′ and (s), 5′-TGTGGTGCACATTGAGAGG-3′; P0 region (a), 5′-CGAGGGTGGGTGGTGCACATGGTG-3′ and (s), 5′-GCCTTTGAGAAGTGTGGG-3′. Another pair of primers covering the region between LE9 and the P0 promoter was chosen as a negative control (NC): (a), 5′-TCAAGGAACATCTGGCTCGC-3′ and (s), 5′-GATGGGGCTCCACCAATCCTCA-3′.

**RESULTS**

**Expression of Cited2 in the developing lens**

Expression of Cited2 in the developing lens was examined by immunostaining of embryonic eye sections. Cited2 expression was detected in the surface ectoderm at 9.5 dpc (Fig. 1A,E), in the invaginating lens placode at 10.5 dpc (Fig. 1B,F), and in lens epithelial cells at later stages such as 15.5 dpc (Fig. 1C,G). The expression of Cited2 was not detected in differentiated lens fiber cells during lens development (Fig. 1C,G). The expression of Cited2 in the developing lens suggests its potential involvement in lens biology.
morphogenesis. In addition to the lens, Cited2 was also expressed in the other developing ocular components, such as the cornea, the non-pigmented layer of the ciliary epithelium and retina (Fig. 1C,G).

**Abnormal corneal-lenticular stalk formation in Cited2−/− developing eyes**

Gross morphological examination revealed several prominent abnormalities with full penetrance in all the Cited2−/− eyes examined (Table 1). In the anterior part of Cited2−/− eyes, abnormal corneal-lenticular stalk formation was consistently observed, which resembles Peters’ anomaly, a congenital defect with persistent central adhesion between the lens and the cornea (Yoon, 2001; Smith and Velzeboer, 1975; Myles et al., 1992; Kenyon, 1975). In contrast to wild-type littermate controls (Fig. 2A,C,E), Cited2−/− lens vesicles failed to separate from the surface ectoderm at 11.5 dpc (Fig. 2B), resulting in a persistent corneal-lenticular stalk as shown by representative pictures from 15.5 dpc (Fig. 2D) and 18.5 dpc (Fig. 2F). The corneal-lenticular stalk was further examined by immunostaining of E-cadherin. Abnormal corneal-lenticular stalk in Cited2−/− eyes (Fig. 2H), which was absent from the wild-type control (Fig. 2G), was positively stained for E-cadherin, validating the epithelial characteristics of the corneal-lenticular stalk. In addition, a distinctive endothelial layer on the posterior side of the cornea developed in wild-type eyes by 15.5 dpc (see Fig. S1A in the supplementary material), whereas it was absent in Cited2−/− eyes (see Fig. S1B in the supplementary material). Cited2−/− developing lenses were also smaller than in the wild type. Decreased lens size was first noted as early as 10.5 dpc in Cited2−/− embryos as smaller invaginating lens placodes (see Fig. S2B in the supplementary material) than in wild-type littermate controls (see Fig. S2A in the supplementary material). Smaller lens vesicles were thus formed in the developing Cited2−/− eyes at 11.5 dpc as shown above (Fig. 2B), and the Cited2−/− lenses remained small throughout the subsequent stages until 18.5 dpc (see Fig. S2D in the supplementary material), as compared with wild-type littermate controls (see Fig. S2C in the supplementary material). Retinal folding was also invariably detected in Cited2−/− eyes at 18.5 dpc (see Fig. S2D in the supplementary material), which might, in part, result from abnormal lens formation (Chow and Lang, 2001).

**Proliferation and cell death rate in early Cited2−/− developing lens**

To further explore the mechanisms responsible for small lens size in Cited2−/− eyes, proliferation of the lens cells was examined by the expression of a mitosis marker, phosphorylated histone H3, in the developing lens at 10.5 dpc. As shown in Fig. 3, no significant difference in mitosis was revealed when Cited2−/− lens (n=5) (Fig. 3B) was compared with Cited2+/+ littermate controls (n=3) (Fig. 3A) [14.3±1% in Cited2+/+ versus 12.5±1.6% in Cited2−/− lens, P>0.05 (Fig. 3C)]. Cell death in the developing lens at 10.5 dpc was also examined in parallel by the TUNEL assay. Compared with the Cited2+/+ littermate controls (n=3) (Fig. 3D), increased

| Table 1. Summary of ocular phenotypes in Cited2-deficient embryos |
|-----------------|---------------------|----------------------|----------------------|
| Stage dpc*     | Irregular pupil†    | Small lens pit/lens† | Lens stalk†          | Aberrant vasculature† |
| 10.5 (4)       | N/A                 | 4                    | N/A                  | N/A                  |
| 11.5 (4)       | N/A                 | 4                    | 4                    | 4                    |
| 12.5 (10)      | N/A                 | 10                   | 10                   | 10                   |
| 13.5 (16)      | 16                  | 16                   | 16                   | 16                   |
| 14.5 (10)      | 10                  | 10                   | 10                   | 10                   |
| 15.5 (10)      | 10                  | 10                   | 10                   | 10                   |
| 18.5 (12)      | 12                  | 12                   | 12                   | 12                   |

*The number of embryos analyzed is indicated in parentheses.
†The number of embryos identified with the indicated defect.
N/A, not applicable.
Aberrant vitreous hypercellularity consisting of aberrant vasculization in Cited2−/− developing eyes

In the posterior part of Cited2−/− eyes, hypercellularity of the hyaloid vasculature was observed. Fig. 4 shows representative pictures of Cited2−/− eyes from 15.5 (Fig. 4B) and 18.5 dpc (Fig. 4D) and wild-type littermate controls from corresponding stages (Fig. 4A,C). Further immunohistochemical examination for vasculature was performed with antibodies against CD31, a molecular marker for vascular endothelial cells, and vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2), which is expressed by angioblast cells and vascular endothelial cells (Ash and Overbeek, 2000). In wild-type littermate controls at 15.5 dpc, CD31 (Fig. 4E) and VEGFR2 expression (Fig. 4G) was detected in pupillary membrane vessels, tunica vasculosa lentis and vasculature overlying the developing retina. However, Cited2−/− eyes were predominantly featured by excessive and disorganized vasculature in the developing vitreous that was positive for expression CD31 (Fig. 4F) and VEGFR2 (Fig. 4H).

Cited2 is required for the regression of the hyaloid vascular system through modulating HIF-1 signaling during eye development

Angiogenic factor VEGF is expressed in different components of the developing eye, including the lens, the cornea and the retina, suggesting that VEGF might be one of the growth factors that initiate intraocular angiogenesis (Flamme et al., 1995). Overproduction of VEGF in the lens results in excessive accumulation of angioblasts and endothelial cells, indicating that the expression level of VEGF in the lens is critical for the maturation of the HVS (Ash and Overbeek, 2000; Mitchell et al., 2006; Rutland et al., 2007). VEGF is a direct target of HIF-1 (Liu et al., 1995; Shweiki et al., 1992) and, importantly, Cited2 has been shown to be a negative regulator for HIF-1 signaling through its competitive binding to the CH1 domain of CBP/p300 with higher affinity than does HIF-1α (Bhattacharya et al., 1999). In Cited2-deficient mouse heart, HIF-1 signaling is deregulated, as evidenced by increased expression of HIF-1-inducible genes, including Vegf (Yin et al., 2002). HIF-1α haploinsufficiency decreases VEGF expression and rescues the heart defects in Cited2-deficient embryos (Xu et al., 2007), indicating that upregulated HIF-1 signaling is in part responsible for defective cardiac morphogenesis resulting from Cited2 deficiency. Altered expression of VEGF as a result of upregulated HIF-1 signaling is of significance considering the role of VEGF in the hyaloid vasculature. Interestingly, a 4.5-fold increase in the Vegf mRNA level was detected in Cited2−/− lens compared with the wild-type littermate control (Fig. 4I), suggesting that upregulated HIF-1 signaling as a result of Cited2 deficiency could be responsible for the elevated VEGF expression and hyaloid hypercellularity and aberrant vasculization in Cited2−/− eyes. We tested this hypothesis by introducing Le-Cre (Ashery-Padan et al., 2000) mediated lens-specific deletion of Hif1α in Cited2-deficient eyes. Cited2−/−;Hif1afllox/fllox;Le-Cre eyes reproducibly displayed the persistence of lens stalk and hyaloid hypercellularity with aberrant vasculature at 15.5 (Fig. 5A) and 17.5 dpc (Fig. 5C) (n=3), which contrasted with the normal littermate control at 15.5 dpc (see Fig. S3A,B in the supplementary material) and 17.5 dpc (see Fig. S3C,D in the supplementary material) (n=2). Furthermore, compared with Cited2−/−;Hif1afllox/fllox;Le-Cre eyes, hyaloid hypercellularity with aberrant vasculature was not detected in Cited2−/−;Hif1afllox/fllox;Le-Cre eyes from the same litters at the corresponding stages; however, the corneal-lenticular stalk was still present in these eyes (n=3) (Fig. 5B,D). The efficiency of Le-Cre transgene-mediated deletion of Hif1α in Cited2−/− eyes was also assessed by analyzing Hif1α mRNA expression in the lens at 14.5 dpc. The result showed a 5-fold reduction of Hif1α mRNA expression in Cited2−/−;Hif1afllox/fllox;Le-Cre lens (n=3) compared with that in Cited2−/−;Hif1afllox/fllox;Le-Cre littermate controls (n=3) (Fig. 5E). Furthermore, there was a 3-fold reduction in the Vegf mRNA level in Cited2−/−;Hif1afllox/fllox;Le-Cre lens (n=3) compared with Cited2−/−;Hif1afllox/fllox;Le-Cre littermate controls (n=4) (Fig. 5F). Thus, our results support the hypothesis that upregulated HIF-1 signaling as a result of Cited2 deficiency is indeed responsible for the aberrant vitreous hypercellularity and disorganized hyaloid vasculature, as deletion of Hif1α in the lens can specifically rescue this phenotype in Cited2-deficient eyes.
Cited2 positively regulates Pax6 expression in the lens

Transcription factor Pax6 is a highly conserved master regulator for eye development (Grindley et al., 1995; Chow and Lang, 2001) and Pax6 gene dosage exerts a critical influence on lens morphogenesis. Haploinsufficiency of Pax6 results in abnormal lens morphogenesis highlighted by corneal-lenticular stalk formation (Dimanlig et al., 2001; Davis-Silberman et al., 2005), which shares striking similarities with the corneal-lenticular stalk phenotype observed in Cited2–/– eyes. Moreover, the expression of Cited2 in lens epithelial cells overlaps with that of Pax6 in the developing eye (Grindley et al., 1995; Walther and Gruss, 1991). We thus hypothesized that Cited2 might affect the level of Pax6 expression in developing lens and that decreased Pax6 expression in Cited2-deficient eyes might lead to corneal-lenticular stalk formation. To explore this possibility, immunostaining of Pax6 was performed. An appreciable level of Pax6 expression in Cited2–/– lens epithelial cells was detected at 13.5 dpc (Fig. 6B) as compared with the wild-type littermate control (Fig. 6A). Since a quantitative comparison of Pax6 expression levels was hard to achieve by immunostaining, we compared mRNA expression of Pax6 in wild-type and Cited2-deficient lens at 14.5 dpc by real-time PCR. We observed a 2.5-fold reduction in Pax6 mRNA expression in Cited2–/– lens (n=4) as compared with wild-type littermate controls (n=4) (Fig. 6C).

Pax6 expression in the lens is regulated through a series of cis-regulatory elements in addition to its promoter for correct spatiotemporal and quantitative expression (Xu et al., 1999; Kammandel et al., 1999; Kleinjan et al., 2006). Transgenic studies in conjunction with gene-targeting strategy and in vitro studies have established that the head surface ectoderm enhancer is one of the major regulators for Pax6 expression in the lens (Kammandel et al., 1999). Disruption of the ectoderm enhancer causes lens defects including small lens and fusion of the lens to the surface ectoderm (Dimanlig et al., 2001). In vitro studies have identified a Pax6-responsive element in the ectoderm enhancer and established a model in which Pax6 protein regulates its own expression through a direct interaction of Pax6 with the surface ectoderm enhancer (Aota et al., 2003). To further examine the potential involvement of Cited2 in regulating Pax6 expression, reporter constructs containing the Pax6 promoter (P0) and two enhancer fragments, LE9 (LE9-P0) and LE0 (LE0-P0) (Aota et al., 2003), responsible for autoregulated expression of Pax6, were tested in a lens epithelial cell line, α-TN4-1, which expresses a high endogenous level of Pax6 (Yang and Cvekl, 2005), and in a normal mouse mammary gland epithelial cell line, NMuMG, which does not express Pax6. In α-TN4-1 cells, Cited2 overexpression significantly increased LE9-P0 reporter activity (Fig. 6D,E) in a dose-dependent manner (data not shown). In addition, LE0-P0 and P0 reporter activity was enhanced when Cited2 was overexpressed (Fig. 6D). However, in NMuMG cells in which Pax6 is absent, Cited2 expression had no effect on the LE9-P0 reporter activity. When Cited2 was co-expressed with Pax6 in NMuMG cells, the reporter activity was significantly increased as compared with Pax6 alone (Fig. 6E). Similar data were obtained in human embryonic kidney (HEK293) cells (Fig. 6E). Chromatin immunoprecipitation (ChIP) was then carried out to test whether Cited2 is physically present on the Pax6 ectoderm enhancer and the P0 promoter region using chromatin prepared from α-TN4-1 cells. The results showed that Cited2 is present on the genomic region covering the LE9 sequence and the Pax6 P0 promoter (Fig. 6Fa,b), but absent in other regions upstream of the Pax6 P0 promoter (Fig. 6Fc). This is consistent with the transfection results (Fig. 6D) and the previous finding that Pax6 binds the LE9 enhancer and the P0 promoter of the Pax6 gene (Aota et al., 2003). These data strongly suggest that Cited2 is a potential positive regulator for Pax6 expression in the lens.

Fig. 4. Hyaloid hypercellularity and aberrant vasculature in Cited2–/– eyes. (A–D) Histological examination was performed after H&E staining of serial paraffin sections of mouse embryo heads. The analysis revealed hyaloid hypercellularity and aberrant vasculature in Cited2–/– eyes at 15.5 (arrow in B) and 18.5 (arrow in D) dpc, in comparison to normal intraocular vasculature in wild-type littermate controls at 15.5 (A) and 18.5 (C) dpc. (E–H) The abnormal vasculature was further confirmed by immunostaining Cited2–/– eye sections at 15.5 dpc for endothelial cells by CD31 (arrow in F) and endothelial and angioblast cells by VEGFR2 (arrow in H), as compared with normal expression patterns for CD31 (E) and VEGFR2 (G) in wild-type controls. (I) Vegf mRNA expression was increased in Cited2–/– lens (n=4) compared with the wild-type littermate control (n=4), as quantified by real-time PCR analysis.
To definitively demonstrate that Cited2 is a positive regulator of Pax6 in vivo, we generated compound embryos by crossing the Pax6 transgenic mouse line (Schedl et al., 1996) onto a Cited2 knockout background to test whether the Cited2-deficient lens phenotype could be rescued by increasing Pax6 gene dosage. As shown in Fig. 6E, Cited2−/−;Pax6 mice embryos reproducibly displayed corneal-lenticular stalk formation (n=2) (Fig. 6G), whereas the abnormal corneal-lenticular stalk was never detected in any of the Cited2−/−;Pax6 embryos analyzed (n=3) (Fig. 6H). Quantitative analysis revealed a 2.3-fold increase of Pax6 mRNA expression in Cited2−/−;Pax6 mouse lens (n=3) as compared with that from Cited2−/−;Pax6 littermate controls (n=3) (data not shown). Our data thus indicate that increased Pax6 expression in Cited2-deficient embryos is able to correct abnormal corneal-lenticular stalk formation. These data provide direct evidence that decreased Pax6 expression is indeed responsible for the corneal-lenticular stalk formation in Cited2-deficient embryos. Therefore, Cited2 is essential for lens morphogenesis by functioning upstream of, and/or together with, Pax6, as the latter increases its own expression. Taken together, our in vitro and in vivo data demonstrate that Cited2 is a novel regulator for Pax6 expression in the lens.

### Cited2 is required for lens morphogenesis and proper regression of HVS

Since targeted deletion of Cited2 is embryonic lethal, to further address the role of Cited2 in lens morphogenesis and HVS formation and regression in adult mice, lens-specific deletion of Cited2 was generated by crossing Cited2flox/flox mice with Le-Cre transgenic mice. It is worth noting that the construct strategy allows lacZ to be expressed under the control of the Cited2 promoter when the floxed Cited2 allele is recombined (Preis et al., 2006). Therefore, we could assess the Le-Cre transgene-mediated recombination of floxed Cited2 alleles by X-Gal staining. As shown in Fig. 7, lacZ expression was readily detected in Cited2flox/flox;Le-Cre+ eyes during early lens development, including lens placode at 9.5 dpc (Fig. 7A), invaginating lens pit at 10.5 dpc (Fig. 7B) and lens vesicle at 11.5 dpc (Fig. 7C), whereas no lacZ staining was detected in Cited2flox/flox;Le-Cre− control eyes (Fig. 7D). Additionally, failed separation of the lens from the surface ectoderm at 11.5 dpc was recapitulated in the Cited2flox/flox;Le-Cre+ eye (Fig. 7C), as was shown in Cited2−/−;Pax6 eyes at the same developmental stage (Fig. 2B). These results indicate that (1) the Le-Cre transgene mediates successful deletion of Cited2 in the developing lens and (2) the Le-Cre transgene is active in developing lens starting from the lens placode stage, which is consistent with what was reported previously (Ashery-Padan et al., 2000).

Moreover, morphological examination revealed that compared with the Cited2flox/flox;Le-Cre− littermate control at 6 weeks of age (Fig. 7E), the Cited2flox/flox;Le-Cre− eye was smaller and displayed failed formation of the anterior chamber (Fig. 7F). This was further supported by histological analysis, which showed failed separation of the lens from the cornea and defective anterior chamber formation (Fig. 7H) as compared with the normal histological feature exhibited by the Cited2flox/flox;Le-Cre− littermate control (Fig. 7G). In addition, abnormal retrolental tissue was invariably noted in Cited2flox/flox;Le-Cre− eyes (Fig. 7J) compared with the Cited2flox/flox;Le-Cre− littermate control (Fig. 7I). Higher magnification revealed that the retrolental mass consists of melanocytes and blood vessels (Fig. 7K), and the latter was confirmed by immunostaining for α smooth muscle actin, which labels the pericytes that stabilize the vessels (Fig. 7L). These results indicate that Cited2 is required for lens morphogenesis and that Cited2 deficiency is associated with abnormal HVS regression in the eye.

### DISCUSSION

The current study is the first evaluation of the role of Cited2 in eye morphogenesis. Our results show that Cited2 is expressed in the developing eye and that deletion of Cited2 disturbs normal eye development, causing fusion of the lens to the cornea and hyaloid hypervascularity. Genetic interaction of Cited2 with HIF-1 signaling is specifically involved in the process of HVS formation. In addition, Cited2 functions upstream of, and/or together with, Pax6, a key transcription factor in lens morphogenesis.
Fig. 6. Cited2 is a positive regulator for Pax6 expression. (A, B) Expression of Pax6 in Cited2+/– lens epithelial cells. Immunostaining revealed an appreciable level of Pax6 expression in Cited2+/– lens epithelial cells at 13.5 dpc (B) compared with that of wild-type littermate controls (A). (C) Pax6 mRNA expression in developing lens at 14.5 dpc was analyzed by real-time PCR, which revealed a 2.5-fold reduction of Pax6 expression in Cited2+/– lens (n=4) as compared with wild-type littermate controls (n=4). (D, E) Effect of Cited2 on Pax6 autoregulation. Transcriptional activation of reporters containing Pax6 enhancer and promoter fragments, LE9-P0, LE0-P0 and P0, in Cited2- or Pax6-overexpressing cells was measured by reporter assays. α-TN4-1 mouse lens epithelial cells were transfected with a reporter plasmid containing the indicated fragment (270 ng) with different combinations of Cited2 (225 ng) and Pax6 (75 ng) expression plasmids. Cited2 overexpression in Pax6-expressing α-TN4-1 cells significantly increased the activity of LE9-P0, LE0-P0 and P0 reporters (D). This effect was Pax6-dependent because Cited2 overexpression had no effect on LE9-P0 reporter activity in NMuMG and HEK293 cells, which do not express Pax6, but co-expression of Pax6 and Cited2 significantly increased the reporter activity (E). (F) Cited2 is present on the Pax6 promoter. ChIP assays were performed using 2×10^6 α-TN4-1 cells and antibodies against Cited2 and Pax6. The precipitated DNA was analyzed by PCR with primers covering the LE9 ectoderm enhancer and the P0 promoter region. Normal mouse IgG and PCR amplifying the sequence between LE9 and P0 were also included as negative controls. Input was 10% of the chromatin for immunoprecipitation. Representative pictures show occupancy of Cited2 on LE9 (a) and P0 region (b) as compared with the negative control (NC) (c). (G, H) Histological examination was performed after H&E staining of paraffin-embedded eye serial sections collected from Cited2–/–;PAX77+/– and Cited2–/–;PAX77+/– littermates at 14.5 dpc. Abnormal lens stalk formation was consistently detected in Cited2–/–;PAX77+/– embryonic eyes (n=2) (arrowhead in G). However, no lens stalk was detected in any of the serial sections collected from Cited2–/–;PAX77+/– mouse embryos (n=3) (H).

Function of Cited2 in regulating the fetal vasculature

Fetal hyaloid vasculature is required to provide nutrients to various compartments of the developing eye. However, the hyaloid vasculature is a transient blood supply system in that a gradual loss followed by a nearly complete regression is achieved during postnatal ocular development in mammals (Ito and Yoshioka, 1999). Abnormalities in the process of intraocular vascularization during embryogenesis are linked to several disorders, including Persistent fetal vasculature (PFV) with vision impairment (Pollard, 1997; Haddad et al., 1978; Goldberg, 1997). The mechanisms responsible for the fetal hyaloid vasculogenesis and subsequent regression have not been clearly defined.

Our current study provides evidence that Cited2-HIF-1 genetic interaction plays an important role in this process. HIF-1 signaling is the major machinery that responds to low oxygen level in various tissue types. Under hypoxic condition, HIF-1α, the subunit of HIF-1 whose expression is controlled by oxygen levels, accumulates, binds with its heterodimeric partner, HIF-1β, translocates to the nucleus, binds to hypoxia-response elements and recruits p300 via the CH1 domain, thus activating the transcription of its target genes, including Vegf (Pugh and Ratcliffe, 2003; Arany et al., 1996). Cited2 is a hypoxia-inducible gene that modulates HIF-1 signaling by binding p300 with higher affinity than does HIF-1α. This competitive binding reduces hypoxia-activated transcription (Freedman et al., 2003; Bhattacharya et al., 1999). Therefore, as a negative regulator for HIF-1 signaling, Cited2 plays an important role in controlling the HIF-1α-mediated hypoxia response. Our previous studies have shown that in Cited2–/– mouse hearts, HIF-1 signaling is upregulated, as measured by increased expression of HIF-1 target genes, including Vegf (Yin et al., 2002). Furthermore, HIF-1α haploinsufficiency partially rescues heart morphogenic phenotypes, providing evidence that Cited2 deficiency causes deregulated HIF-1 signaling under hypoxic conditions and that the latter is partially responsible for the heart malformations in Cited2–/– embryos (Xu et al., 2007).

The lens exists in a hypoxic environment (Bassnett and McNulty, 2003; Shui et al., 2003; Shui et al., 2006). The role of VEGF in intraocular vascularization has been studied extensively. Preliminary reports suggest that deletion of VEGF in the lens prevents the formation of the capillary network on the posterior of the lens without disturbing lens formation or the establishment of hyaloid vasculature...
By contrast, increased expression of VEGF in the lens invariably leads to the production of excess, aberrant hyaloid vascularization (Ash and Overbeek, 2000; Mitchell et al., 2006; Rutland et al., 2007). Based on these findings and the data presented in this work, increased VEGF levels resulting from deregulated Cited2-HIF-1 could be considered as one of the factors involved in aberrant hyaloid vascularization in Cited2–/– eyes. However, our results do not exclude the possibility that other mechanisms might also be involved. Specific deletion of Vegf in Cited2–/– lens will help to clarify the role of Cited2-HIF-1 interaction in the control of lenticular VEGF production during eye development and the participation of other growth factors in the process. Since mutants that form smaller lenses often have more mesenchyme in the vitreous (Beebe et al., 2004), it is possible that the smaller lens resulting from deletion of Cited2 might allow more mesenchymal cells to migrate into the developing vitreous, directly or indirectly promoting the formation of an increased vascular supply. Alternatively, lower levels of anti-angiogenic factors could be produced by the mutant lens, which might perturb the necessary balance between angiogenic and anti-angiogenic factors during HVS development.

Since HIF-1 signaling is also involved in regulating cell death and proliferation (Carmeliet et al., 1998), it might control the size of the lens through these mechanisms and, in turn, affect hyaloid vascularization. Our preliminary data showed that Cited2-deficient lens appeared to have increased levels of apoptotic cells at 10.5 dpc, which was only partially corrected by deleting Hif1a in Cited2-deficient eyes (data not shown). Proliferation assays did not reveal any obvious changes in Cited2-deficient embryos compared with their wild-type littermate controls. This is consistent with the conclusion that HIF-1 is not the decisive factor controlling lens growth in younger animals (Beebe, 2008). Further analysis will be required to define the relative contributions of lens size, VEGF expression, hyaloid vascularization and migration of mesenchymal cells to the formation of the HVS. Conditional deletion of both Cited2 and Hif1a might further clarify whether Cited2-HIF-1 interaction contributes to the regression of the HVS postnatally.

**Function of Cited2 in lens morphogenesis**

Our data have shown that the formation of a corneal-lenticular stalk in Cited2–/– eyes is mediated by mechanisms independent of HIF-1, as lens-specific deletion of Hif1a did not rescue this defect. Pax6 is a key regulator for various eye developmental events including lens morphogenesis (Hill et al., 1991; Hanson et al., 1994; Kaufman et al., 1995; Quiring et al., 1994). Pax6 gene dosage is critical for lens morphogenesis, which is supported by independent studies from Sey heterozygous mice (van Raamsdonk and Tilghman, 2000; Hill et al., 1991), Pax6 head surface ectoderm enhancer null mice (Dimanlig et al., 2001) and Pax6 single allele knockout mice (Davis-Silberman et al., 1998).
et al., 2005). For example, Pax6 single allele deletion in the lens results in a 34% reduction in the Pax6 level in Pax6\textsuperscript{loxP}\textsuperscript{\textminus}Le-Cre\textsuperscript{\textplus} mouse lens epithelium, which is sufficient to cause corneal-lenticular stalk formation in the eye (Davis-Silberman et al., 2005). In human, heterozygous Pax6 mutations are associated with Peters’ anomaly (Hanson et al., 1994; Smith and Velzeboer, 1975; Myles et al., 1992; Kenyon, 1975), in which the patients are characterized by fusion of the lens to the cornea. Owing to its gene dosage effect on lens morphogenesis, transcriptional control of Pax6 gene expression has been the subject of a number of studies, which have identified a series of cis-regulatory elements upstream of Pax6, within the introns of Pax6, downstream of Pax6 or within the introns of an adjacent gene (Williams et al., 1998; Xu et al., 1999; Kammandel et al., 1999; Kleinjan et al., 2001; Kleinjan et al., 2002; Kleinjan et al., 2006). Cited2 deficiency resulted in corneal-lenticular stalk formation and decreased Pax6 expression, suggesting that Cited2 might control Pax6 expression. Our in vitro data also suggested that Cited2 could be a positive regulator for Pax6 autoregulation. In vivo data from Cited2\textsuperscript{-/-};Pax6\textsuperscript{-/-} mouse embryonic eyes showed that increasing Pax6 gene dosage in Cited2\textsuperscript{-/-} eyes corrected the corneal-lenticular stalk phenotype, providing direct genetic evidence that Cited2 is required for normal levels of Pax6 expression in the lens. Although we found that Cited2 regulated Pax6 expression through its action on regions within the upstream head surface ectoderm enhancer and the P0 promoter, we cannot exclude the possible involvement of downstream regulatory elements (Kleinjan et al., 2006) in Cited2-mediated expression of Pax6. Further studies are necessary to determine how Cited2 interacts with these cis elements to control Pax6 expression. Additionally, although a direct physical interaction between Pax6 and Cited2 was not detected by co-immunoprecipitation after overexpressing Pax6 and Cited2 in HEK293 cells (data not shown), it is possible that Cited2, Pax6 and other proteins, such as Sox2 (Aota et al., 2003) and Oct1 (Pou2f1 – necessary to determine how Cited2 interacts with these cis elements 2006) in Cited2-mediated expression of Pax6. Further studies are necessary to determine how Cited2 interacts with these cis elements to control Pax6 expression. Additionally, although a direct physical interaction between Pax6 and Cited2 was not detected by co-immunoprecipitation after overexpressing Pax6 and Cited2 in HEK293 cells (data not shown), it is possible that Cited2, Pax6 and other proteins, such as Sox2 (Aota et al., 2003) and Oct1 (Pou2f1 – Mouse Genome Informatics) (Donner et al., 2007), are present in a multi-protein complex. Biochemical characterization of this multi-protein complex will be necessary to provide a molecular basis for Cited2 involvement in the autoregulation of Pax6 expression. It is also worth noting that although Cited2 activates Pax6 upstream regulatory elements in vitro (Fig. 6D), Cited2 might not be the only molecule controlling Pax6 expression in the developing lens. This is in part supported by the observation that Cited2 homozygous deletion only results in a 2.5-fold reduction of the Pax6 mRNA level in the developing lens (Fig. 6C). In addition, the Le-Cre transgene driven by the Pax6 upstream ectoderm enhancer and the P0 promoter in the Cited2-null background efficiently deletes floxed Hif1a (Fig. 5), suggesting that other factors might also contribute to the transcriptional activity of the Pax6 upstream ectoderm enhancer and P0 promoter.

Although we have provided experimental evidence that Cited2 plays important roles in lens morphogenesis in part through regulating Pax6 expression in the developing lens, other mechanisms, involving, for example, AP2\alpha cannot be ruled out at the present time. Cited2 and AP2\alpha physically interact to impact cardiac morphogenesis, left-right patterning and neural tube formation (Bamforth et al., 2001; Bamforth et al., 2004). Ap2a-null mice display eye developmental defects, such as corneal-lenticular adhesion, demonstrating that AP2\alpha is required for lens development (West-Mays et al., 1999). We detected appreciable level of AP2\alpha in Cited2\textsuperscript{-/-} lens epithelial cells (see Fig. S4B in the supplementary material) as compared with wild-type littermate controls (see Fig. S4A in the supplementary material). Cited2 could function as a co-activator of AP2\alpha to regulate the expression of its as-yet-unidentified target genes, which in turn would contribute to the lens phenotypes observed in Cited2\textsuperscript{-/-} eyes.

As a transcriptional modulator, Cited2 has been shown to be involved in the development of several organs and tissues. However, the role of Cited2 in eye development has not been explored previously. The current study has uncovered a novel function of Cited2 in lens morphogenesis and hyaloid vascular development, and offers mechanistic views of how Cited2 functions in these processes. This information might shed new light on the etiology of, and potential therapeutic strategies for, eye disorders such as Peters’ anomaly and PFV.

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Supplementary material
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References


