Human p32 Is a Novel FOXC1-Interacting Protein That Regulates FOXC1 Transcriptional Activity in Ocular Cells

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PURPOSE. Mutations in the human forkhead box C1 gene (*FOXC1*) cause Axenfeld-Rieger (AR) malformations, often leading to glaucoma. Understanding the function of FOXC1 necessitates characterizing the proteins that interact with FOXC1. This study was undertaken to isolate FOXC1-interacting proteins and determine their effects on FOXC1.

METHODS. To identify FOXC1-interacting proteins, a human trabecular meshwork (HTM) yeast two-hybrid (Y2H) cDNA library was screened. The interaction and colocalization between FOXC1 and its putative protein partner were confirmed by Ni^{2+} pull-down assays, immunoprecipitation, and immunofluorescence, respectively. The electrophoretic mobility shift assay (EMSA) was used to study the effect of the interacting protein on FOXC1 DNA-binding ability. Dual luciferase assays using FOXC1 reporter plasmids in HTM cells were performed to determine the effect of the interaction on FOXC1 transcription activity.

RESULTS. The human p32 protein was isolated as a putative FOXC1-interacting protein from a Y2H screen. The interaction of FOXC1 with p32 was confirmed by Ni-pull-down assays and immunoprecipitation. Although p32 is predominantly cytoplasmic, the portion of p32 that is within the nucleus colocalizes with FOXC1. The FOXC1 forkhead domain (FHD) was identified as the p32 interaction domain. p32 significantly inhibited FOXC1-mediated transcription activation in a dose-dependent manner but did not affect FOXC1 DNA-binding ability. Of interest, a FOXC1 mutation F112S displayed an impaired interaction with p32.

CONCLUSIONS. In the study, the human p32 protein as a novel regulator of FOXC1-mediated transcription activation. Failure of p32 to interact with FOXC1 containing the disease-causing F112S mutation indicates that impaired protein interaction may be a disease mechanism for AR malformations. (*Invest Ophthalmol Vis Sci.* 2008;49:5243–5249) DOI:10.1167/iovs.07-1625

The forkhead family of transcription factors is characterized by a 110-amino-acid DNA-binding domain termed the forkhead domain (FHD).[1] This DNA-binding domain was first identified as a region of homology between the *Drosophila mela-nogaster* forkhead protein[2] and the rat hepatocyte nuclear factor 3 protein.[3] The FHD is highly conserved in a variety of species, from yeast to humans. The FHD forms a winged-helix-turn-helix structure consisting of a three-α helix bundle and two large loops that form winglike structures.[4] Members of the forkhead family are necessary for a wide range of cellular and developmental processes, including cell migration and differentiation, organogenesis, and tumorigenesis.[5,6]

FOXC1 is a member of the forkhead transcription factor family and plays an important role in eye organogenesis. Mutations in *FOXC1* cause Axenfeld-Rieger (AR) malformations, an autosomal dominant developmental disorder.[7,8] The ocular defects of patients with AR malformations consist of anomalies in the structure of the anterior chamber angle, including trabecular meshwork defects, adhesions of the iris and cornea, iris hypoplasia, corectopia, and posterior embryotoxon. The anterior angle malformation in patients with AR syndrome can lead to an increased intraocular pressure (IOP) and consequently, glaucoma. More than half of patients with AR malformations have early-onset secondary glaucoma. Systemic manifestations may also present in some patients, such as dental dysgenesis and redundant periumbilical skin.[9] In rare cases, patients have congenital cardiac defects or hearing loss.[10,11] $Foxc1^{+/-}$ heterozygous mice have ocular defects similar to those found in patients with AR malformations.[12]

Several different mechanisms underlie the impairments to FOXC1 function caused by the disease-causing mutations, including reduction in protein stability, alteration in DNA-binding specificity, alteration in nuclear localization, and defects in DNA-binding capacity and transactivation.[13–17] Since mutations that lead to reduced FOXC1 activity and chromosome duplications that result in an extra copy of *FOXC1* both cause ocular defects,[18,19] it appears that there are strict requirements for upper and lower thresholds of FOXC1 activity for normal eye development and function.

The genetic pathways in which FOXC1 is involved in ocular developmental events are not fully understood. In the eye, recent work suggests that retinoic acid (RA) signaling may be upstream of *FOXC1*, as the expression of *Foxc1* in the periocular mesenchyme cells is reduced in mouse embryos with both RA-synthesizing retinaldehyde dehydrogenases (*Raldh1/3*) knocked out.[20] Moreover, the *Raldh1/3* knockout mouse displays ocular malformations that affect the anterior chamber. Recent experiments have also revealed potential genes downstream of *FOXC1* in ocular genetic pathways.[21–23] These genes implicate FOXC1 in a variety of processes, including IOP regulation and eye organogenesis.

Studying protein–protein interactions is also necessary to understand genetic pathways due to their important roles in regulation of the activities of transcription factors. In previous work in our laboratory, we found that the actin-binding protein filamin A (FLNA) interacts with FOXC1 and directs FOXC1 into HP1α heterochromatin-rich regions of the nucleus, resulting in the downregulation of FOXC1 transactivation.[24] Mutations in *PITX2*, encoding a homeodomain transcription factor also

cause AR malformations.[25] Very recent results indicate that FOXC1 and PITX2 can physically interact with each other, tying both proteins into a common pathway. PITX2 is a negative regulator of FOXC1, and PITX2 loss-of-function mutants lose their ability to inhibit FOXC1.[26] These studies indicate that FOXC1 activity is stringently controlled by protein–protein interactions.

In this study, we identified human p32 as a binding partner for FOXC1 through a yeast two-hybrid (Y2H) experiment. The FHD of FOXC1 was identified as the p32 interaction domain. The proportion of p32 that resides in the nucleus colocalizes with FOXC1. Of interest, the interaction with p32 results in reduced FOXC1 transactivation ability. Failure of p32 to interact with FOXC1 containing the disease-causing F112S mutation indicates that impaired protein interaction may be a disease mechanism underlying AR malformations and further demonstrates that correct regulation of FOXC1 is necessary for normal ocular development and functions.

MATERIALS AND METHODS

Plasmids and Reagents

The FOXC1 bacterial expression vector pET28-FOXC1 is described elsewhere,[24] as are the FOXC1 mammalian expression vectors pcDNA4-FOXC1 and pEGFP-FOXC1.[13] FOXC1 was amplified by PCR from pcDNA4-FOXC1 and subcloned into pDEST32 in-frame to the GAL4DBD or into the pcDNA3.1/nV5-DEST vector by gateway technology. pCMV-p32 was purchased from Open Biosystems (HudsonAlpha Institute, Huntsville, AL). p32 was generated by PCR amplification and subcloned into the pcDNA3.1/nV5-DEST by gateway technology or pET28b vector in-frame to the 6XHIS tag. All vectors were sequenced to confirm that no mutations were introduced into the cDNAs. FOXC1 mutants in pcDNA4 were also amplified by PCR, subcloned into pcDNA3.1/nV5-DEST vector and resequenced. Details of primer sequences can be provided on request. The goat polyclonal p32 antibody was kindly provided by Tom Hobman (University of Alberta).

Y2H Screen

A human trabecular meshwork cDNA library fused to the GAL4AD of pEXP-AD502 (Invitrogen) was screened for proteins that interact with human FOXC1 (ProQuest Two-Hybrid System; Invitrogen, Carlsbad, CA). Briefly, the pEXP-AD502 library plasmid and the pDEST32-FOXC1 bait plasmid were cotransformed into MaV203 yeast cells. Transformed yeast cells were plated on medium lacking histidine or uracil or on medium containing 5-fluoroorotic acid (5FOA). The transformed yeast cells were also plated on YPAD plates to conduct additional β-galactosidase assays. A total of $1.9 \times 10e6$ library clones were screened for growth on selective media and assayed for β-galactosidase activity. pEXP-AD502 cDNA plasmids were recovered by bacterial transformation of DNA isolated from positive yeast colonies. The candidate pEXP-AD502 cDNA plasmids were retransformed into yeast cells with the empty pDEST32 vector or pDEST-FOXC1 or pDEST32 plasmid encoding irrelevant bait to exclude false positives. Inserts of true-positive pEXP-AD502 cDNA clones were characterized by sequence analysis.

Mammalian Cell Culture and Transfection

HeLa cells, COS-7 cells, and HTM cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum at 37°C. The cells were transfected (Fugene6; Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol. Transfected cells were subjected to immunofluorescence on the next day of the transfection. Dual-luciferase assays (Promega, Madison, WI) were performed 48 hours after transfection.

Ni^{2+}-Agarose Pull-Down Assays

6XHIS-tagged proteins were produced from pET28-based constructs in *Escherichia coli* and were purified by the addition of Ni-NTA agarose beads (Qiagen, Valencia, CA). Whole HeLa cell lysates or lysates containing various FOXC1 or p32 constructs were obtained by lysing cells in lysis buffer (20 mM HEPES [pH 7.6], 0.5 M NaCl, 1.5 mM MgCl$_2$, 1 mM DTT, 0.1% Triton X-100, and 20% glycerol). Ni^{2+}-agarose pull-down assays were performed by incubating the cell lysates with 6XHIS-tagged proteins bound on the Ni^{2+}-agarose beads for 1 hour at 4°C, followed by four washes with wash buffer (46.6 mM Na$_2$HPO$_4$, 3.4 mM NaH$_2$PO$_4$, 300 mM NaCl, 50 mM imidazole [pH 6.0], and 0.05% Tween 20). Protein complexes captured on the beads were eluted in SDS-PAGE loading buffer, separated by 10% SDS-PAGE gels, and subjected to immunoblot analysis with antibody against the mammalian-expressed proteins.

Immunoprecipitation

HTM cells were transfected with FOXC1-expressing vector or the empty vector. Forty-eight hours after the transfection, the cells were lysed in the lysis buffer (50 mM Tris-Cl [pH 8.0], 150 mM NaCl, and 0.5% Igepal). Protein lysates (100 μL) were added into extra buffer (400 μL; 12.5 mM Tris-Cl [pH 8.0], and 87.5 mM NaCl, 0.125% Igepal) containing protein G-agarose (Sigma-Aldrich) and precleared for 2 hours at 4°C. The precleared cell lysates were incubated with 3 μg of anti-V5 antibody overnight at 4°C with rotation. Protein G-agarose (25 μL) blotted by BSA in the wash buffer (20 mM Tris-Cl [pH 8.0], 100 mM NaCl, 0.2% Igepal) were added and incubated for 4 hours at 4°C. Then, the protein G-agarose were collected and washed four times with the wash buffer. Western blots were then probed with anti-p32 antibody. The input fraction represented 5% of the protein extracts used in the immunoprecipitation.

Immunofluorescence

After 2 days of transfection, HeLa cells plated onto coverslips were subjected to immunofluorescence. HTM cells were fixed 2 days after the cells were seeded on the coverslips. The cells were fixed for 10 minutes in 2% wt/vol paraformaldehyde and then permeated in PBSX (PBS+0.05% Triton X-100) twice for 5 minutes, followed by blocking in PBSX containing 5% (wt/vol) BSA for 15 minutes. Coverslips were incubated for 1 hour at room temperature with the primary antibodies to Xpress epitope or p32 (diluted 1:500 in PBSX containing 5% BSA) or incubated overnight with the primary antibody to FOXC1 (diluted 1:100 in PBSX containing 5% BSA), washed twice with PBSX, and incubated for another 1 hour with the fluorescence dye–conjugated secondary antibody (diluted 1:500 in PBSX containing 5% BSA). The images were collected on a confocal microscope (LSM510; Carl Zeiss Meditec, Inc., Dublin, CA) or an immunofluorescence microscope (DMR; Leica Microsystems, Bannockburn, IL). Pixel intensities were obtained using the confocal imaging system software to create line scan plots for Xp-FOXC1 and p32 immunofluorescence.

Transactivation Assays

Subcultured HTM cells on 24-well tissue culture plates were transfected with FOXC1 expression vectors, or p32 expression vectors, or FOXC1 expression vectors and increasing amounts of p32 expression vectors along with FGF19RE-luc reporter, which is a 354-bp region of FGF19 promoter region containing a FOXC1 binding site[23] and pRL-CMV. The ratios of the doses of FOXC1 expression vectors to p32 expression vectors used in transfections are 1:1, 1:2, and 1:3. The total amount of transfected DNA was equalized with empty pcDNA4. Cells were harvested and assayed for luciferase activity according to the manufacturer's protocol (Promega). The luciferase assays were performed in triplicate and repeated at least three times.

Electrophoretic Mobility Shift Assays (EMSA)

COS-7 cell lysates containing recombinant FOXC1 and increasing amounts of p32 were equalized for amounts of recombinant FOXC1

using untransfected COS-7 cell lysate. Cell lysates were incubated in EMSA binding buffer[17] with 80,000 cpm of [^{32}P]-dCTP-labeled DNA probe containing the FOXC1-binding site.[17] The products of the reactions were separated in 6% polyacrylamide Tris-glycine-EDTA gels.

Mutation Screen of the p32 Gene in Patients with AR Malformations

This research adhered to the tenets of the Declaration of Helsinki. Six primer sets were designed to sequence the *p32* coding region and intron–exon boundaries in a panel of 50 patients with AR malformations. Details of primer sequences can be provided on request. PCR products amplified by these six primer sets were subjected to direct DNA sequencing to detect nucleotide alterations.

Real-Time qPCR

We used a gene expression PCR assay (*Taq*Man; Applied Biosystems, Inc., [ABI], Foster City, CA) to quantitate the copy number of the *p32* gene in patients with AR malformations. PCR primers and probe were designed in the region of exon 2 of the *p32* gene. Each 15 μL PCR reaction contained 14 μm of forward and reverse primers (GCATA-AAACCCTCCCTAAGATGTC; ATTTCGCTTCTGTCCCATTCA), 3.8 μm of a dual-labeled PCR (*Taq*Man; ABI) probe (VIC-AGGTTGGGAGCTG-GAA-TAMRA) and 75 ng of DNA sample as well. A commercial mixture for quantification of human CX40 locus was also included as an endogenous normalization control. Each sample was amplified in triplicate. The PCR reactions were amplified on a thermocycler (9700HT; ABI). Each 384-well plate included triplicate reactions of two unrelated normal samples and one DNA-free control. The data were analyzed by the comparative CT method. Samples with a $\Delta\Delta$CT within 1 ± 0.15 were considered to have two copies of the *p32* gene.

RESULTS

Isolation of p32 as a FOXC1-Interacting Protein by Y2H Screening

To identify proteins that interact with FOXC1, we screened a trabecular meshwork (TM) cDNA yeast two hybrid library that was created by using mRNA extracted from human TM primary cell culture. The TM cDNA library inserts were cloned into the plasmid pEXP-AD502 with the open reading frames (ORFs) fused to the GAL4 activation domain (GAL4AD). Full-length FOXC1 was cloned into the vector pDEST-32 fused to the GAL4 DNA binding domain (GAL4DB). Yeast cells containing three reporter genes (*HIS3, URA3,* and *lacZ*) were cotransformed with the GAL4AD-cDNA library and the GAL4DB-FOXC1 plasmid. A standard Y2H screen procedure was performed (Invitrogen). Approximately 1.9×10^6 transformants were subjected to the selection. Seventeen independent clones that fulfilled the criteria for interaction of the gene products were obtained (Supplementary Fig. S1; Supplementary Figures are online at http://www.iovs.org/cgi/content/full/49/12/5243/DC1). cDNAs were extracted from those clones and partially sequenced. All 17 cDNAs were found to match the published sequence of human p32, which had been copurified with the pre-mRNA splicing factor from a HeLa cell extract.[27] p32 protein is initially translated as a precursor that is proteolytically processed to produce the mature p32 by removing the N-terminal 73 amino acids.[28] All the cDNAs contained the full-length sequence of the mature protein and part of the sequence of the leader peptide. The specificity of the interaction between p32 and FOXC1 in the Y2H system was confirmed by retransformation of the positive cDNA clone into yeast cells, together with vectors expressing GAL4 DNA-binding domain alone or with GAL4DB-FOXC1 or the GAL4 DNA binding domain fused with a different protein, PITX2. Only the yeast cells that contained the positive cDNA plasmid and the GAL4DB-FOXC1
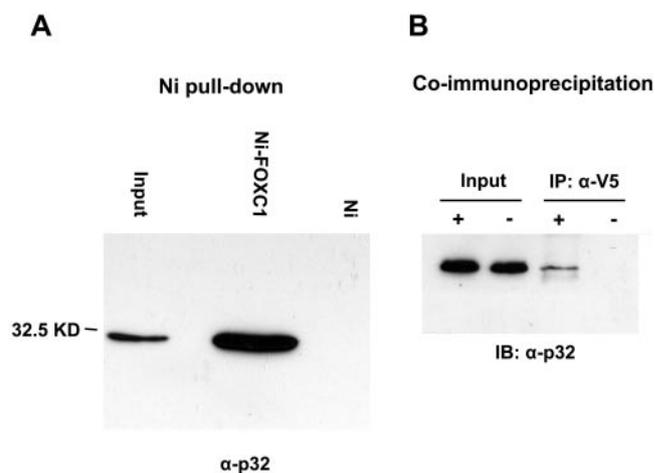

FIGURE 1. Confirmation of the interaction between FOXC1 and p32. (**A**) HeLa cell lysates were subjected to Ni pull-down assays with the 6XHIS tagged FOXC1 bound to Ni^{2+}-NTA beads (Ni-FOXC1) or empty beads (Ni). Bound proteins were analyzed by SDS-PAGE followed by Western blot analysis with an anti-p32 antibody. (**B**) Coimmunoprecipitation of FOXC1 and p32 in HTM cells. V5-FOXC1 (+) or empty (−) plasmid was transfected into HTM cells. The cell lysates were immunoprecipitated with an anti-V5 antibody and immunoblotted with an anti-p32 antibody.

plasmid displayed an interaction phenotype (Supplementary Fig. S2).

Confirmation of the Interaction between FOXC1 and p32

Ni^{2+} pull-down assays were performed to confirm whether the interaction could also be observed in other experimental systems. The full-length wild-type FOXC1 was cloned into a bacterial expression vector (pET28), allowing expression of FOXC1 as a 6XHIS-tagged fusion protein. Whole cell lyses prepared from HeLa cells transfected with V5-tagged full-length p32 or V5-tagged mature p32 were incubated with 6XHIS-FOXC1 bound to Ni^{2+}-agarose beads or with Ni^{2+}-agarose beads alone. Western blot analysis with an anti-V5 antibody showed that FOXC1 can interact with the precursor or mature p32 protein (data not shown). The endogenous p32 in the cell lysate with the molecular weight of the mature form[29,30] is also able to interact with FOXC1 (Fig. 1A).

We further confirmed this interaction between FOXC1 and p32 by immunoprecipitation. HTM cells were transfected with pcDNA3.1/nV5-DEST FOXC1 or empty pcDNA3.1/nV5-DEST constructs. Both cell lysates were incubated with anti-V5 antibody overnight. Western blot analysis with an anti-p32 antibody showed that FOXC1 was able to immunoprecipitate endogenous p32 in HTM cells (Fig. 1B).

Colocalization of FOXC1 and p32

Immunofluorescence was used to study the cellular distribution of FOXC1 and p32 and to determine the compartment in which they colocalize. The vector expressing Xpress epitope–tagged FOXC1 (Xp-FOXC1) was transfected into HeLa cells. The Xp-FOXC1 was detected by an anti-Xpress antibody and an anti-mouse IgG coupled to Alexa Fluor 488. Endogenous p32 was detected by an anti-p32 antibody and an anti-goat IgG coupled to Alexa Fluor 594. FOXC1 is predominantly in the nucleus (Fig. 2A), as expected.[17] p32 is localized predominantly in the cytoplasm (Fig. 2A) in a pattern consistent with a mitochondria location.[30,31] However, a proportion of the p32 signal can be observed in the nucleus and colocalized with
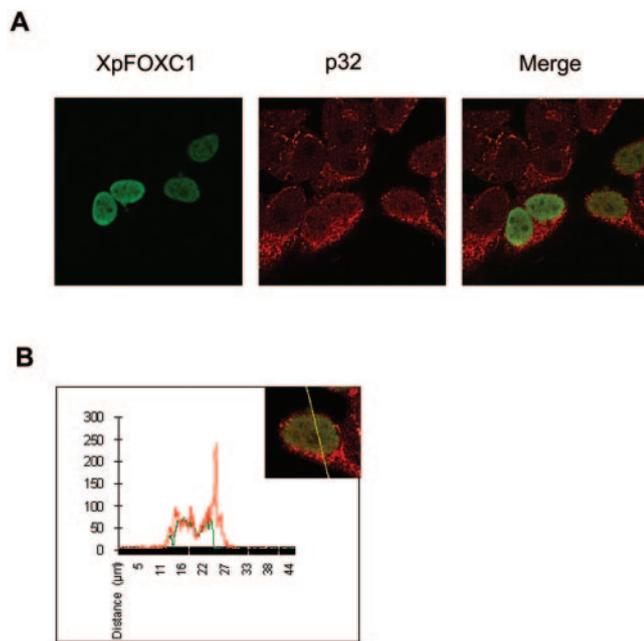
FIGURE 2. Subcellular localization of FOXC1 and p32. (**A**) HeLa cells were transfected with Xp-FOXC1 expression vector. Endogenous p32 was stained with goat polyclonal anti-p32 antibody followed by Alexa Fluor 594-conjugated anti-goat secondary antibody. Xp-FOXC1 was visualized with mouse monoclonal anti-Xp antibody followed by Alexa Fluor 488-conjugated mouse secondary antibody. *Red*: p32 protein; *green*: Xp-FOXC1. Merged image shows an overlay of *red* and *green* staining. (**B**) Line scan plot shows the intensity of each fluorescence emission along the *yellow* line across the cell.

FOXC1 (Fig. 2A). Line scan data graphically represented the distribution of Xp-FOXC1 and p32 immunoreactivity throughout the cell and indicated the extent of colocalization between FOXC1 and p32 (Fig. 2B). Because both our anti-FOXC1 and anti-p32 antibodies were raised in goat, it was not possible to use coimmunofluorescence to show the colocalization of endogenous FOXC1 and p32 in the nucleus. However, both endogenous FOXC1 and endogenous p32 are localized in similar nuclear regions of the HTM cells (Supplementary Fig. S3).

Role of the FOXC1 Forkhead Domain and Intact p32 in the Interaction between FOXC1 and p32

Ni^{2+} pull-down assays were performed to map the domains within FOXC1 and p32 that are involved in their interaction. Wild-type FOXC1 and a series of FOXC1 deletions (Fig. 3A) were cloned into the plasmid pcDNA3.1/nV5-DEST fused to an N-terminal V5 epitope. These deletion constructs were expressed in HeLa cells, and the cell lysates were incubated with Ni^{2+}-agarose beads alone or beads bound with p32 fused to a 6XHIS tag. As shown in Figure 3B, all the FOXC1 deletion constructs interacted with p32 except for FOXC1ΔFHD a FOXC1 construct lacking the forkhead domain. These results indicate that FOXC1 interacts with p32 through the FOXC1 forkhead domain.

Similar experiments were preformed to map the domain within p32 that is required for the interaction with FOXC1. p32 does not have any known functional domains. The crystal structure of the protein molecule exhibits a β-sheet core, flanked by its N- and C-terminal α-helices that interact in a coiled–coil fashion.[32] We designed two p32 deletion constructs by separating the β-sheet core from the N- and C-terminal α-helices. Neither of the deletion constructs of p32 interacted with FOXC1 (data not shown). It appears that the

intact p32 structure is necessary for the interaction between p32 and FOXC1.

Mutation Screen and Detection of Copy Number Variation of the *p32* Gene in Patients with AR Malformations

Approximately 60% of patients with AR syndrome do not have mutations in the two known AR malformation genes, *FOXC1* and *PITX2* (Mirzayans F, Walter MA, unpublished data, 2005). Proteins that interact with FOXC1 may be involved in the same genetic pathways as FOXC1. We hypothesized that FOXC1-interacting proteins may also be involved in AR syndrome and that mutations of the protein-coding genes may contribute to AR malformations. Therefore a mutation screen of *p32* was conducted in a panel of 50 patients with AR malformations, in whom no *FOXC1* and *PITX2* mutations were found. No nucleotide alterations were found in the exon or splice site regions of the *p32* gene in these patients. However, a 14-bp deletion, from −44 to −31 upstream of exon 3, was detected in both patients with AR malformations and unaffected control subjects. This alteration is therefore unlikely to be pathogenic for AR malformations. We also detected the copy number of *p32* gene in patients with AR malformations using real-time QPCR (*Taq*Man; ABI) and did not found any deletion or duplication of the *p32* gene in the patients. Our results therefore indicate that *p32* mutations are unlikely to be a direct cause of AR malformations.

p32 Inhibition of FOXC1-Mediated Transactivation

The role of p32 in regulating FOXC1 transactivation was studied by dual-luciferase assay. We used a luciferase reporter containing an *FGF19* promoter element that we have demonstrated to be regulated by FOXC1.[23] HTM cells were cotransfected with this luciferase reporter construct and FOXC1 ex-
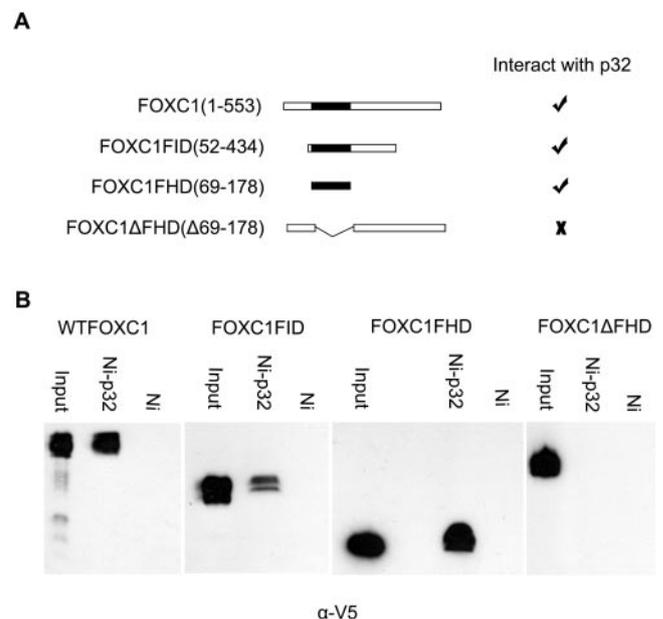

FIGURE 3. The FOXC1 forkhead domain was essential for binding to p32. (**A**) FOXC1 constructs used in the study. (**B**) HeLa cells were transiently transfected with plasmids expressing the series of FOXC1 deletion constructs (shown in **A**) tagged with the V5 epitope. The cell lysates were then used for Ni^{2+} pull-down assays with the 6XHIS tagged p32 bound to Ni^{2+}-agarose beads (Ni-p32) or empty beads (Ni). Bound FOXC1 deletions constructs were detected by Western blot analysis using an anti-V5 antibody.
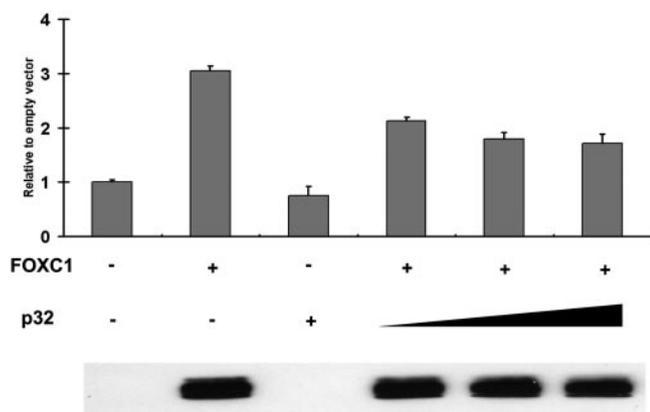
FIGURE 4. p32 impaired FOXC1-mediated transactivation. *Top:* HTM cells were transfected with the FGF19RE luciferase reporter along with the empty pcDNA4 vector; or FOXC1 expression vector, and increasing amounts of p32 expression vector. The mass ratios of the doses of FOXC1 expression vectors to p32 expression vectors used in transfections are 1:1, 1:2, and 1:3. The total amount of transfected DNA was equalized with empty pcDNA4. *Bottom:* Western blot showed that equal levels of FOXC1 were expressed.

pression vector along with an increasing amount of p32 expression vector (Fig. 4). Equivalent levels of FOXC1 were expressed in the cotransfections (Fig. 4, bottom). In this experiment, expression of p32 impaired transactivation by FOXC1 in a dose-dependent manner.

Effect of p32 on FOXC1 DNA Binding Ability

Given that p32 impaired FOXC1 transactivity (Fig. 4), we determined whether p32 alters FOXC1-DNA binding. EMSAs were performed to analyze FOXC1 DNA binding ability in the presence of p32. COS-7 cells were transfected with pcDNA3.1/nV5-DEST empty vector, FOXC1 expression vector, or p32 expression vector separately or cotransfected with the FOXC1 expression vector along with increasing amounts of p32 expression vector. Each cell lysate was incubated with a radiolabeled oligomer containing the FOXC1 consensus binding sequence (5'-GTAAATAAA-3'). FOXC1 showed consistent levels of DNA binding in the presence or absence of p32 (Fig. 5). This result demonstrated that the inhibition of FOXC1 transactivation by p32 was not due to impaired DNA binding.

Impaired Interaction with p32 in FOXC1 Carrying the Mutation F112S

To date, all published missense AR syndrome mutations identified in FOXC1 are located in the FHD. Previous work from our
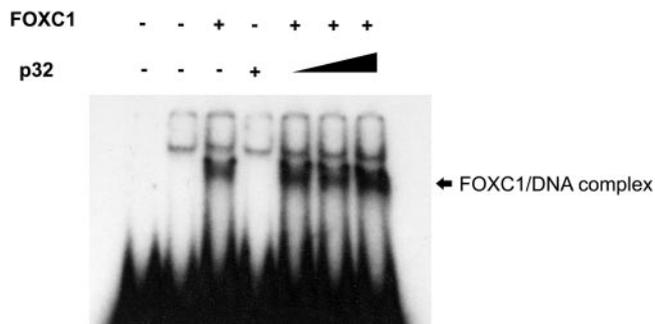

FIGURE 5. p32 did not affect FOXC1 DNA binding ability. A ^{32}P-labeled FOXC1 binding site probe was incubated with COS-7 cell lysates transfected with FOXC1 expression vector and lysates transfected with or without p32 expression vector and used in an EMSA.
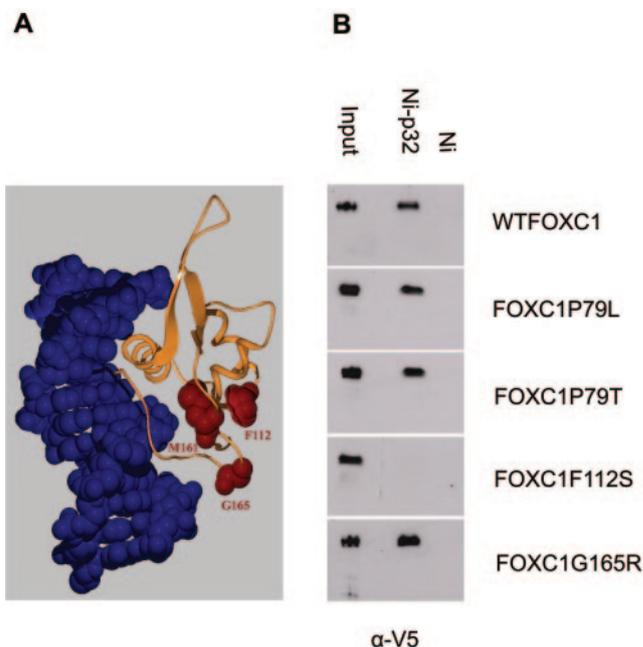

FIGURE 6. The FOXC1 F112S mutant was not able to interact with p32. (**A**) Molecular model of the FOXC1 FHD (based on FOXA3) shows the predicted outward orientation of the side chains of the amino acids of P79, F112, and G165 that were previously shown to decrease FOXC1 transactivation activity without affecting DNA binding or nuclear localization.[13,15,17] (**B**) The FOXC1 F112S mutant displayed an impaired interaction with p32 in Ni^{2+} pull-down assay. The other three FOXC1 mutants, FOXC1 P79T, FOXC1 P79L, and FOXC1 G165R, interacted with p32 similar to wild-type FOXC1. HeLa cells were transiently transfected with plasmids expressing V5 tagged WT FOXC1, FOXC1 P79T, FOXC1 P79L, FOXC1 F112S, or FOXC1 G165R. The cell lysates were then used for Ni pull-down assays with the 6XHIS tagged p32 bound to Ni^{2+}-agarose beads (Ni-p32) or empty beads (Ni). Proteins bound to the beads were detected by Western blot analysis with an anti-V5 antibody.

laboratory showed that mutations in the three residues P79, F112, and G165 impair FOXC1 transactivation, yet have near-normal DNA-binding ability.[13,15,17] Moreover, molecular modeling of the FOXC1 FHD predicts that the side chains of these three residues point away from the DNA and face opposite to the DNA-binding interface, suggesting the possibility that these three residues are not involved in DNA binding but rather in protein–protein interactions (Fig. 6A). The fact that p32 interacts with FOXC1 through the FHD yet does not interfere with DNA-binding led us to test the interaction of p32 with FOXC1 mutants in these three residues. Four recombinant FOXC1 proteins, harboring four patient mutations (P79L, P79T, F112S, and G165R), respectively, were analyzed by Ni^{2+} pull-down assays for their ability to interact with p32. FOXC1 carrying the P79L, P79T, or G165R mutations were able to interact with p32 (Fig. 6B). However, the FOXC1-F112S mutant was not able to interact with p32 (Fig. 6B), indicating that this FOXC1 mutation impairs the protein–protein interaction capacity of FOXC1.

DISCUSSION

In this study, we sought to isolate proteins that interact with the transcription factor FOXC1 to elucidate the molecular mechanisms involved in FOXC1-mediated gene regulation. Human p32 protein was identified as a novel FOXC1 interacting protein by Y2H screening of a human trabecular meshwork cDNA library. p32 is expressed in a variety of human tissues

including the brain, ear, heart, liver, and eye (http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Hs.555866). In the eye, p32 is found in the iris, lens, cornea, and retina (http://neibank.nei.nih.gov, National Eye Institute, Bethesda, MD). The specific interaction between p32 and FOXC1 was confirmed by in vitro analyses and was observed in mammalian cells. Human p32 is a multicompartmental and multifunctional protein that is able to interact with a variety of cellular proteins in the mitochondrion and nucleus and with exogenous virus proteins under infectious conditions. Some of the interactions between p32 and its interacting partners have been shown to regulate gene expression. For example, p32 interacts with a splicing factor ASF/SF2 and inhibits its function.[27] CDC2L5, a protein having a role in pre-mRNA splicing, is able to interact with p32.[33] p32 is also shown to be involved in transcription, as p32 was copurified in a cellular transcription factor CBF complex from HeLa cell extracts and was found to specifically repress CBF-mediated transcription in an in vitro transcription reaction.[34]

In our functional analyses using transactivation assays, the interaction with p32 negatively regulated FOXC1 transactivation, which is consistent with the previously established role of p32 in regulating gene expression. Others have suggested that p32 might act as a corepressor and/or a molecular barrier for the recruitment of transcription factors necessary for transactivation.[27] However, the impaired interaction between p32 and the FOXC1 F112S mutant, which is able to bind DNA but not transactivate genes, is consistent with our hypothesis that p32 is not simply a corepressor and/or a molecular barrier for the recruitment of FOXC1. There is growing evidence that several transcription coregulator complexes are able to serve as either corepressors or coactivators, depending on the gene being regulated, or cell-signaling switches.[35-38] p32 could be a component in such a transcription coregulator complex, helping the complex to be recruited to the promoter bound by FOXC1 to regulate FOXC1 transcription activity. The complex would either inhibit or promote FOXC1 transcription activation in different cell contexts. In our studies, overexpression of p32 results in inhibition of FOXC1 transactivation. We hypothesize that mutations of FOXC1, such as F112S, that result in impaired protein–protein interaction with proteins such as p32 lose both negative and positive regulation by these interacting proteins and that this deregulation deficiency underlies the transactivation deficits of these FOXC1 mutations. The FOXC1 F112S mutant, which cannot interact with regulatory proteins such as p32, thus loses both the positive and negative regulation by such molecules and is unable to transactivate the reporter genes in transactivation assays. Alternatively, p32 may serve as a tether protein such as SKIP, to recruit both corepressor and coactivator complex to bind to FOXC1 and help the switch of FOXC1 between transcription repression and activation.[39] Another possibility that cannot be completely ruled out is that the F112 residue plays an important role in recruitment of both the positive and negative regulatory complex for FOXC1.

Of interest, clinical study of family members with a FOXC1 F112S mutation found that these patients present with severe ocular manifestations, including iris processes, iris hypoplasia, corectopia, and posterior embryotoxon. In addition, some F112S patients also have systemic findings including cardiac, facial, and dental anomalies, which usually are not found in patients with FOXC1 mutations.[40] The severe eye phenotype and the wide spectrum of clinical defects found in patients with FOXC1 F112S mutations could be due to the lack of both positive and negative regulation of FOXC1 mediated by proteins such as p32, but the small number of patients with F112S mutations precluded unequivocal phenotype–genotype analyses.

Mutations in the genes that are part of the same genetic pathway may cause the same genetic disease or similar syndromes. Since p32 interacts with FOXC1, it is possible that p32 may also play a role in eye development and therefore mutations in *p32* could also result in ocular disease such as AR malformations. However, we did not find any nucleotide changes in *p32* except for a deletion within an intron in a panel of 50 patients with AR malformations. We observed a similar frequency of the intronic deletion in the patients with AR malformations and the unaffected control subjects. We also did not observe any copy number alterations of the *p32* gene in patients with AR malformations. Although these results indicate that mutations in the *p32* gene are not a direct cause of AR malformations, impaired FOXC1/p32 interaction, due to FOXC1 mutation, may have a role in the pathogenesis of AR malformations.

Previous work on our FOXC1 molecular model, which is based on FOX FHD structures and an analysis of FOXC1 mutants,[13-15,17] has suggested that this FOXC1 model has prediction value.[41] Our current work strongly supports this suggestion. Mutation of one of the residues (F112S) previously predicted to be involved in protein–protein interaction based on this model (Fig 6A) does result in failure of FOXC1 F112S to interact with p32. This result provides further evidence that the FOXC1 molecular model has the capacity to predict effects of mutations on FOXC1 function. Last, our data suggest that altered protein–protein interaction may be a disease-causing mechanism for AR malformations. Understanding the factors that regulate the activity of FOXC1 may allow modulation of the effects of FOXC1 mutations in cells.

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