

The establishment of a predictive mutational model of the forkhead domain through the analyses of *FOXC2* missense mutations identified in patients with hereditary lymphedema with distichiasis

Fred B. Berry^{1,*}, Yahya Tamimi², Michelle V. Carle¹, Ordan J. Lehmann^{1,2}
and Michael A. Walter^{1,2}

¹Department of Ophthalmology and ²Department of Medical Genetics, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

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The *FOX* family of transcription factor genes is an evolutionary conserved, yet functionally diverse class of transcription factors that are important for regulation of energy homeostasis, development and oncogenesis. The proteins encoded by *FOX* genes are characterized by a conserved DNA-binding domain known as the forkhead domain (FHD). To date, disease-causing mutations have been identified in eight human *FOX* genes. Many of these mutations result in single amino acid substitutions in the FHD. We analyzed the molecular consequences of two disease-causing missense mutations (R121H and S125L) occurring in the FHD of the *FOXC2* gene that were identified in patients with hereditary lymphedema with distichiasis (LD) to test the predictive capacity of a FHD structure/function model. On the basis of the *FOXC2* solution structure, both *FOXC2* missense mutations are located on the DNA-recognition helix of the FHD. A mutation model based on the paralogous *FOXC1* protein predicts that these *FOXC2* missense mutations will impair the DNA-binding and transcriptional activation ability of the *FOXC2* protein. When these mutations were analyzed biochemically, we found that both mutations did indeed reduce the DNA binding and transcriptional capacity. In addition, the R121H mutation affected nuclear localization of *FOXC2*. Together, these data indicate that these *FOXC2* missense mutations are functional nulls and that *FOXC2* haploinsufficiency underlies hereditary LD and validates the predictive ability of the *FOXC1*-based FHD mutational model.

INTRODUCTION

The forkhead domain (FHD) is a conserved 100 amino acid DNA-binding motif that is present in eukaryotic organisms ranging from yeast to humans (1–4). The structure of the FHD from several forkhead box (*FOX*) proteins has been solved by X-ray crystallography and NMR spectroscopy (5–7). These data reveal that the FHD is a variant of the helix–turn–helix motif, consisting of three major α -helices, a smaller fourth α -helix and three anti-parallel β -sheets. The third α -helix aligns along the major groove of DNA and

residues in this region make a number of contacts with the DNA molecule. Thus, Helix 3 is referred to as the DNA-recognition helix. The region between the two β -sheets, which lies C-terminal to the helical bundle, form two loops that wrap around the DNA molecule and stabilize the protein–DNA interaction. It is the presence of these loop regions that gives the FHD domain its characteristic winged-like appearance and lends the FHD its winged-helix moniker.

To date, at least 43 *FOX* transcription factor genes have been identified in humans (4). Many of these proteins regulate diverse cellular processes, including cell growth and

*To whom correspondence should be addressed at: 832 Medical Sciences Building, University of Alberta, Edmonton, Alberta, Canada T6G 2H7. Tel: +1 7804923028; Fax: +1 7804926934; Email: fberry@ualberta.ca

Table 1. Summary of disease-causing mutations in *FOX* genes

Gene	Disorder (OMIM)	Mutation type							
		Missense in FHD	Missense outside FHD	Nonsense	Splice site	Deletions	Insertions	Indels	Rearrangements (including gene duplications)
<i>FOXC1</i>	Axenfeld–Rieger (601090)	13	0	3	0	6	2	0	5
<i>FOXC2</i>	Lymphedema with distichiasis (602402)	3	1	8	7	22	22	1	1
<i>FOXE1</i>	Thyroid agenesis (602617)	2	0	0	0	0	0	0	0
<i>FOXE3</i>	Anterior segment ocular dysgenesis and cataracts (601094)	0	0	0	0	0	1	0	0
<i>FOXL2</i>	Blepharophimosis, Ptosis and Epicanthus Inversus (605597)	4	7	10	0	13	10	3	2
<i>FOXN1</i>	Congenital alopecia, nail dystrophy and T-cell immunodeficiency (600838)	0	0	1	1	0	0	0	0
<i>FOXP2</i>	Developmental verbal dyspraxia (605317)	1	1	1	0	0	0	0	0
<i>FOXP3</i>	X-linked immunodysregulation, polyendocrinopathy enteropathy (300292)	4	0	0	1	3	0	1	0

differentiation, oncogenesis and metabolic homeostasis (2,3,8). Furthermore, disease-causing mutations have been identified in eight human *FOX* genes (2,3). Mutations in *FOXC1* result in ocular malformations and glaucoma associated with Axenfeld–Rieger syndrome (9–11). Hereditary lymphedema with distichiasis (LD) is attributed to *FOXC2* mutations (12–15). *FOXE1* mutations underlie thyroid agenesis (16). Mutations in *FOXE3* cause anterior segment ocular dysgenesis and cataracts (17). Blepharophimosis–ptosis–epicanthus inversus syndrome and premature ovarian failure are caused by *FOXL2* mutations (18). Mutations in the *FOXN1* gene result in T-cell immunodeficiency, congenital alopecia and nail dystrophy (19). Speech acquisition disorders have been attributed to *FOXP2* mutations (20). The X-linked syndrome of immunodysregulation, polyendocrinopathy and enteropathy is caused by mutations in *FOXP3* (21,22). A summary of disease-causing mutations in *FOX* genes is presented in Table 1.

LD is a rare developmental disorder that affects the formation of the lymphatic vasculature system (23,24). Affected individuals typically exhibit distichiasis, an accessory row of eyelashes. These superfluous lashes often abrade the cornea resulting in corneal epithelial defects and opacification. Additional features of *FOXC2* mutations may include cardiac defects, cleft palate and extradural cysts (12). The reported mutations primarily comprise of nonsense mutations, insertions or deletions that introduce a premature termination codon and are predicted to form a truncated *FOXC2* protein (12–15,25,26). Three nucleotide missense mutations that cause amino acid substitutions (W116R, R121H and S125L) in the *FOXC2* coding region have been reported in LD patients (26,34). However, the molecular consequences of these *FOXC2* mutations have not been assessed.

The analyses of disease-causing missense mutations provide valuable information regarding structure–function relationships. To date, the molecular consequence of 13 disease-causing mutations in the *FOXC1* FHD have been analyzed

in our laboratory (27–30). Mutations in residues that lie N-terminal to and in Helix 1 reduce DNA binding, impede nuclear localization and impair transactivation. Helix 2 amino acid mutations can impair transcriptional activation; however, do not affect nuclear localization or DNA binding. Mutations to residues in Helix 3 affect nuclear localization and grossly impair DNA binding and specificity. Mutations to residues in Wing 2 impair DNA binding and transactivation. These analyses provide a framework for a predictive model for the functional consequence of disease-causing missense mutations in the FHD of other *FOX* genes. In this report, we test the predictive ability of our FHD mutational model through the molecular analyses of the two known missense mutations in the *FOXC2* FHD identified in patients with LD (26). Two *FOXC2* mutations, R121H and S125L, occur at paralogous residues to *FOXC1*. On the basis of the location of these residues in Helix 3 of the FHD, we predict that both of the *FOXC2* missense mutations would impede DNA binding and subsequent transcriptional activation. As an initial assessment of our predictive FHD mutational model, we analyzed the molecular consequences of these *FOXC2* missense mutations.

RESULTS

The FHD of *FOXC1* and *FOXC2* share 98% sequence identity differing at only two positions. *FOXC2* contains glutamic acid residues at positions 90 and 110, whereas *FOXC1* contains aspartic acid residues at the equivalent position in the FHD (Fig. 1A). Outside of the FHD, *FOXC1* and *FOXC2* share only about a 36% amino acid sequence identity. Similar disease-causing mutations to R121H and S125L occur at paralogous positions in *FOXC1* (Fig. 1A) and lie in the third α -helix of the FHD (Fig. 1B). Because this helix is involved in contacts with DNA and previous molecular analysis of mutations in Helix 3 is critical for DNA binding and transcriptional activation (27,28,31), we predicted that the *FOXC2*

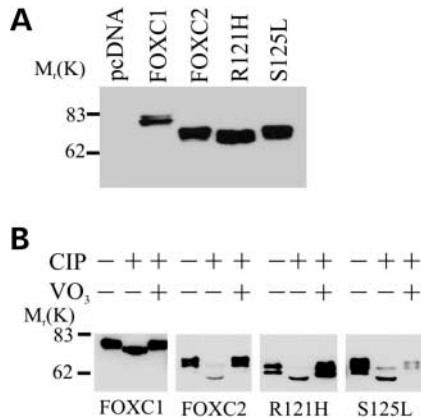


Figure 2. Expression analysis of WT and mutant FOXC2 cDNAs transfected into COS-7 cells. (A) FOXC1, WT and mutant FOXC2 recombinant proteins were detected by immunoblotting with an α -Xpress antibody. (B) FOXC2 is a phosphoprotein. COS-7 extracts transfected with WT FOXC1, WT FOXC2, R121H or S125L were incubated with CIP and the phosphatase inhibitor sodium vanadate (VO_3) as indicated. FOXC1 and FOXC2 proteins were detected by immunoblotting with an α -Xpress antibody.

lower molecular weight than FOXC1, consistent with the lower predicted molecular weight of FOXC2 compared with FOXC1. Both the R121H and S125L proteins were expressed at equal levels as WT FOXC2 indicating that these missense mutations do not affect protein stability. Multiple immunoreactive bands were detected in WT and mutant FOXC2 transfected COS-7 extracts, suggesting FOXC2 is subject to post-translational modifications, such as protein phosphorylation. Interestingly, the R121H displayed a faster migration than either WT FOXC2 or S125L suggesting that R121H may not be modified to the full extent as that of WT FOXC2 or S125L.

To test whether protein phosphorylation contributed to the post-translational modifications of FOXC2, COS-7 extracts transfected with WT FOXC1, WT FOXC2, R121H or S125L were incubated with calf intestinal alkaline phosphatase (CIP). As indicated in Figure 2B, the addition of CIP increased the mobility of WT FOXC1 which is consistent with previous observations that FOXC1 is phosphorylated (32). The mobility of WT FOXC2, R121H and S125L was also increased when cell extracts were incubated with CIP. Remarkably, CIP-treated R121H extracts exhibited an equal mobility as that of WT FOXC2 and S125L suggesting that an incomplete phosphorylation pattern accounts for the reduced mobility of R121H observed in untreated extracts.

Next, the nuclear localization of FOXC2 missense mutations was examined. COS-7 cells were cotransfected with Xpress-tagged FOXC2 cDNAs along with FOXC1-EGFP. As indicated in Figure 3, WT FOXC2 was localized exclusively to the nucleus and displayed a similar subnuclear distribution as FOXC1-EGFP. In contrast, R121H displays a gross defect in nuclear localization. Much of the R121H immunofluorescence signal was detected in the cytoplasm. FOXC1-EGFP was still localized to the nucleus, indicating that the deficit in R121H nuclear localization was not the result of a disruption in the nuclear integrity of the cells transfected with the R121H expression vector. Immunoreactivity

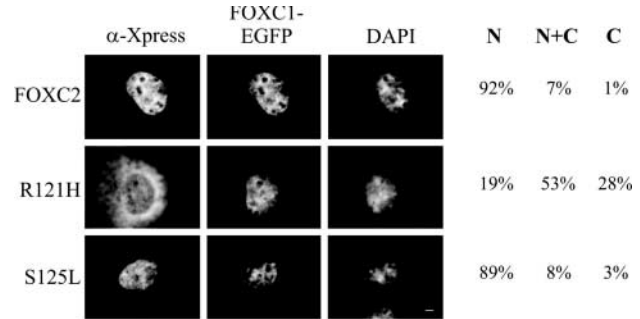


Figure 3. The R121H mutation disrupts efficient nuclear localization of FOXC2. COS-7 cells were cotransfected with Xpress-tagged FOXC2 (WT or mutant) along with FOXC1-EGFP. The localization of FOXC2 was detected by indirect immunofluorescence with anti-Xpress antibodies. The cells were scored for the presence of WT FOXC2, R121H or S125L in the nucleus only (N), in the nucleus and cytoplasm (N + C) or in the cytoplasm only (C). More than 200 cells from two independent immunofluorescence experiments were scored.

for S125L was localized mainly to the nucleus; however, a small proportion of signal could be detected in the cytoplasm. These results indicate that impaired nuclear localization accounts for a portion of the molecular deficit exhibited by these FOXC2 mutations and indicate a role for residues in Helix 3 in nuclear localization.

To assess the impact of the FOXC2 mutations on the DNA-binding capacity of the protein, electrophoretic mobility shift assays (EMSA) were performed. Whole cell COS-7 extracts transfected with WT FOXC1, WT FOXC2 or mutant FOXC2 expression vectors were incubated with an *in vitro* derived FOXC1 consensus site (5'-GTAAATAAA-3') to test for DNA binding (33). As indicated in Figure 4, both WT FOXC1 and FOXC2 bound to the FOXC1 target site. The enhanced DNA binding observed with FOXC2 when compared with FOXC1 is likely due to increased FOXC2 expression levels (Fig. 2). Minimal binding to the DNA probe was observed when the mutations R121H or S125L were introduced into FOXC2. Increasing the levels of protein extract failed to improve the binding of either R121H or S125L. These results indicate that these mutations to the Helix 3 grossly impaired FOXC2 binding to its DNA-target sequence.

Finally, the ability of WT and mutant FOXC2 to activate a luciferase reporter containing six tandem copies of the FOXC1 consensus site was examined in HeLa cells. WT FOXC2 activated this luciferase reporter by at least 40-fold compared with the empty expression vector, indicating that FOXC2 is a potent transcriptional activator (Fig. 5). Neither R121H nor S125L was able to activate the luciferase reporter above background levels, indicating that these mutations rendered FOXC2 transcriptionally inactive.

DISCUSSION

Analyses of the FOXC2 disease-causing missense mutations

In this report, we demonstrate that the disease-causing FOXC2 mutations in the FHD grossly impair its DNA-binding ability

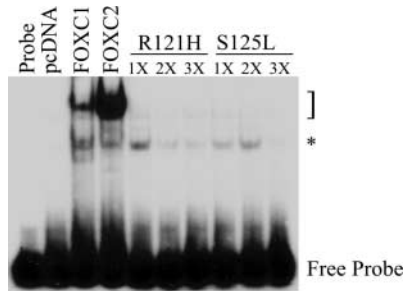


Figure 4. FOXC2 missense mutations abolish DNA-binding activity. Whole cell extracts from COS-7 cells transfected with FOXC1 or FOXC2 expression vectors were incubated with the *in vitro* derived FOXC1/2 consensus oligonucleotide probe (5'-GTAAATAAA-3'). FOXC1 or FOXC2 DNA-protein complexes are indicated with a square bracket. A non-specific DNA-protein complex was observed and indicated with an asterisk. Both R121H and S125L failed to bind to this sequence even when a 3-fold excess of protein was tested.

and subsequent transcriptional activation of a luciferase reporter gene. Furthermore, our findings indicate that the R121H mutation largely disrupts the proper nuclear localization of FOXC2. On the basis of these observations of greatly reduced functional activity, these mutations are essentially null mutations. In addition to these missense mutations, LD can result from *FOXC2* nonsense mutations, as well as small insertions and deletions, all of which are predicted to produce a truncated FOXC2 protein (12–15,34). The expression of such truncated proteins does indeed reduce FOXC2 transcriptional activation (24). Thus, the hypothesis that FOXC2 haploinsufficiency underlies LD (12) is completely supported by the lack of functional activity exhibited by FOXC2 R121H and S125L missense mutations.

Although the FOXC2 missense mutations behaved similar to the paralogous FOXC1 mutations, these studies revealed a difference between FOXC2 and FOXC1 in their genetic dosage requirements. Both FOXC2 missense mutations resulted in a complete loss of FOXC2 protein function. This is in contrast to FOXC1 disease-causing missense mutations in the FHD that retain residual DNA-binding and transcriptional regulatory activity (27–29). In this situation, the underlying genetic defect is not simply FOXC1 haploinsufficiency *per se*, rather, a threshold of FOXC1 activity must be achieved for the proper development of the anterior segment of the eye (27).

The targeted disruption of a single murine *Foxc1* or *Foxc2* allele can result in anterior segment dysgenesis, including iris hypoplasia, small or absent Schlemm's canal, aberrantly developed trabecular meshwork, eccentric pupils and displaced Schwalbe's line reminiscent of Axenfeld–Rieger malformations caused by *FOXC1* mutations in humans (35). LD patients with *FOXC2* mutations do not typically present with severe anterior segment findings and glaucoma; however, the *FOXC2* missense mutations analyzed in this report or mutations that introduce a stop codon in the FHD are associated with milder anterior segment abnormalities (36). These findings suggest that an intact FOXC2 FHD is required for the development of the anterior segment of the eye. *FOXC2* mutations that truncate the protein after the FHD exhibit a loss of transcriptional regulatory activity (24). In the eye, however, these truncating mutations could

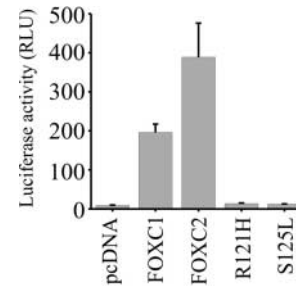


Figure 5. Impaired transcriptional activation by R121H and S125L mutations. Transactivation assays were performed in HeLa cells transfected with a 6× FOXC1/2 BS-Luc reporter along with FOXC1 or FOXC2 cDNAs. Luciferase values were normalized to *Renilla* luciferase. Mean luciferase values from a representative experiment transfected in triplicate are presented. Error bars correspond to the standard error of the mean.

exhibit sufficient transcriptional activity for normal development to proceed. Alternatively, there may be a novel functional requirement for an intact FHD. This hypothesis suggests that a similar, although lower, minimal threshold for FOXC2 activity may be required in the development of the anterior segment of the eye as is required for FOXC1.

The examination of FOXC2 missense mutations revealed that both FOXC2 missense mutations exhibited a markedly impaired DNA-binding activity. The solution structure of FOXC2 indicates that the two affected residues lie within Helix 3 and their side chains are orientated away from this helix and toward the surface of DNA (Fig. 2B) (6). As the arginine and serine residues at positions 121 and 125 are conserved in all known 43 human *FOX* genes (Fig. 6) (4), it suggests a fundamental role for these residues in FHD function. X-ray crystallographic analysis of the related FOXA3 FHD reveals that the FOXA3 serine residue that is equivalent to FOXC2 serine 125 makes a phosphate contact with DNA backbone (5) indicating a similar role for FOXC2 serine 125 consistent with our observations that the S125L mutation has serious ramifications for DNA-binding activity. Although the arginine residue at position 121 in FOXC2 is not predicted to make a DNA contact, mutation of the equivalent arginine residues in other FOX proteins is predicted to eliminate the positively charged electrostatic surface potential of Helix 3 that is thought to be crucial for binding specificity of the FHD (28,31). In addition, this arginine residue is adjacent to a histidine residue that contacts the nitrogen base of the DNA backbone in the FOXA3 crystal structure (5). This mutation of a lysine to a histidine at position 121 may alter the protein conformation and impair this base contact. Thus, the FOXC2 missense mutations in Helix 3 impair DNA-binding ability which ultimately reduces FOXC2 transcriptional activity.

These analyses also reveal the requirement for R121 in correct nuclear localization of FOXC2. This function may be conserved among other FOX proteins because the paralogous R127H mutation in FOXC1 also displays impaired nuclear localization (28). Although the nuclear localization signal (NLS) for FOXC2 has not been identified, work from our laboratory defined the NLS of FOXC1 to two regions in the

permit assignment of functional subdomains to regions of the FHD (Fig. 6A) and indicate that the FHD possesses conserved activities that extend beyond DNA binding. The fact that the known FOXC2 FHD mutations behave in a similar manner to paralogous FOXC1 mutations supports our assignment of these functional subdomains to the FHD and is an important initial step in verifying the predictive value of our FHD mutational model.

Disease-causing mutations have been identified in at least eight human *FOX* genes, and missense mutations occurring in the FHD have been identified in six *FOX* genes (9,10,17,20–22,39,40). Naturally occurring and chemically induced mutations occur in the FHD of two mouse *Fox* genes (41,42). These missense mutations are distributed along the entire length of the FHD and indicate regions of functional importance (Fig. 6B). In addition, missense mutations are detected at common amino acid residues in many *FOX* genes and several residues harbor mutations that are present in at least three different genes. It is of particular interest that three of the FHD mutation clusters illustrated in Figure 6B result in C → T or G → A transversions at CpG dinucleotides indicating that numerous mutational hotspots are present in *FOX* genes.

Although the two FOXC2 missense mutations analyzed in this study grossly disrupted protein function, all FHD missense mutations may not result in a null allele, as illustrated by many FOXC1 missense mutations retaining residual DNA-binding and transcriptional activation activity (27–31). Mutations to the FHD can have profound effects on FOX protein function, altering protein stability, nuclear localization, DNA binding and transcriptional activation (27–30). Moreover, in this study, individual missense mutations in the FHD can exert multiple functional consequences (27,28). Therefore, an optimal, predictive mutational model of the FHD will draw from both structural and functional information. To ascertain the functional consequence of an individual amino acid substitution, one must consider its localization in the three-dimensional structure of the FHD and its localization to a defined functional subdomain. It is this comparative analysis that will expand the predictive nature of the FHD mutation model to allow better genotype–phenotype correlations and to improve the prediction of the genetic mechanisms underlying diseases attributed to *FOX* genes. Our analyses of FOXC1 and FOXC2 missense mutations are the first step towards the development of truly predictive FHD mutational models.

MATERIALS AND METHODS

Plasmids and mutagenesis

Because the human *FOXC2* gene is encoded by a single exon, the full-length FOXC2 cDNA was PCR-amplified from genomic DNA using GC-Rich PCR kit (Roche). The primer pairs used for amplification are as follows: FOXC2F 5'-GGATCCATGCAGGCGCGCTACTCC-3'; FOXC2R 5'-TCTAGATCAGTATTCGTGCAGTG-3'. The PCR amplification generated a *Bam*H1 and *Xba*I sites into the 5' and 3' end of the FOXC2 cDNA, respectively. The FOXC2 cDNA was subcloned into a pcDNA4C-HIS MAX expression vector (Invitrogen) in which the *Apa*I site in the multiple

cloning site was abolished, to create Xpress-FOXC2. For mutagenesis reactions, a portion of the FOXC2 cDNA containing the FHD and flanking the *Apa*I and *Bsu*36I restriction sites was amplified and subcloned into pGEMT-Easy to create a mutagenesis template. Site-directed mutagenesis was performed on this template as described previously (27). The mutagenic primers used were as follows: R121H 5'-CAGAA-CAGCATCCACCACAACCTCTCG-3'; S125L 5'-CGCCA-CAACCTCTTGCTCAACGAGTGC-3'.

The mutated FHD regions were subcloned back into the Xpress-FOXC2 expression vector. All expression constructs were sequenced to verify that no additional mutations were introduced.

Cell culture and transfections

COS-7 and HeLa cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. For transient transfections, cells were plated at a density of 5×10^5 cells in a 60 mm plate. The next day, cells were transfected with 2 μ g of either Xpress-FOXC1 or Xpress FOXC2 using 6 μ l of FUGENE6 reagent. Cells were harvested 48 h following transfection and processed for immunoblotting as described previously (5).

Phosphatase treatment of cell extracts

Whole cell extracts were treated with CIP as described previously (32). Cell extracts were incubated in NEB Restriction Enzyme buffer 3 (New England Biolabs) along with 15 U of CIP (New England Biolabs). NaVO_3 (11 μM) was added to inhibit CIP activity in some reactions. Extracts were incubated for 1 h at 37°C. An equal volume of 2 \times SDS–PAGE loading buffer was added and reactions were loaded onto a 10% polyacrylamide gel.

Immunofluorescence

COS-7 cells were plated onto sterile cover slips at a density of 10^5 cells per cover slip. Cells were transfected with either WT, R121H or S125L Xpress-FOXC2 (500 ng) along with FOXC1-EGFP (500 ng) using FUGENE6. Eighteen hours after transfection, cells were fixed with 2% paraformaldehyde for 15 min and processed for indirect immunofluorescence. Briefly, cells were permeabilized with phosphate-buffered saline containing 0.1% (v/v) Triton X-100 (PBS-X), blocked with 5% (w/v) bovine serum albumin in PBS-X and incubated with α -Xpress antibodies (1:500) for 1 h at room temperature. After extensive washes in PBS-X, the cells were incubated with a Cy3-conjugated α -mouse IgG secondary antibody at dilution of 1:500 (Jackson Immuno-labs). Cells were stained with DAPI and mounted onto slides with Prolong mounting medium (Molecular Probes).

Electrophoretic mobility shift assays

EMSA were performed using COS-7 whole cell extract transfected with either Xpress-FOXC1 or Xpress-FOXC2 (WT or mutant) as described previously (27). Cell extracts were equalized for expression of the transfected expression vectors.

Dual luciferase assays

FOXC1 luciferase reporter assays were performed in 24-well plates as described previously (43). All transfections were performed in triplicate and each experiment was performed three times.

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Conflict of Interest statement. None declared.

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