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MUTATION IN BRIEF

Differential Functional Effects of Novel Mutations of the Transcription Factor FOXL2 in BPES Patients

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Mutations of the transcription factor FOXL2, involved in cranio-facial and ovarian development lead to the Blepharophimosis-Ptosis-Epicanthus Inversus Syndrome (BPES) in human. Here, we describe nine mutations in the open reading frame of *FOXL2*. Six of them are novel: c.292T>A (p.Trp98Arg), c.323T>C (p.Leu108Pro), c.650C>G (p.Ser217Cys) and three frameshifts). We have performed localization and functional studies for three of them. We have observed a strong cytoplasmic mislocalization induced by the missense mutation p.Leu108Pro located in the forkhead (FKH) domain of FOXL2. In line with this, transcriptional activity assays confirmed the loss-of-function induced by this variant. Interestingly, the novel mutation p.Ser217Cys, mapping between the FKH and the polyalanine domain of FOXL2 and producing a mild eyelid phenotype, led to normal localization and transactivation. We have also modeled the structure of the FKH domain to explore the potential structural impact of the mutations reported here and other previously reported ones. This analysis shows that mutants can be sorted into two classes: those that potentially alter protein-protein interactions and those that might disrupt the interactions with DNA. Our findings reveal new insights into the molecular effects of FOXL2 mutations, especially those affecting the FKH binding domain. © 2008 Wiley-Liss, Inc.

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INTRODUCTION

The Blepharophimosis Ptosis Epicanthus Inversus Syndrome (BPES; MIM# 110100) is a genetic disease leading to craniofacial, mainly eyelid, malformations. Two clinical forms of the syndrome have been described: in type I BPES eyelid and craniofacial malformations are associated with Premature Ovarian Failure (POF), whereas in type II BPES, the craniofacial phenotype appears isolated (Zlotogora et al., 1983). *FOXL2* is a single-exon gene encoding a transcription factor whose mutations are responsible for BPES (Crisponi et al., 2001). FOXL2 contains a characteristic forkhead (FKH) DNA binding domain and a polyalanine tract (PolyAla) of unknown function. The coding sequence of FOXL2 is highly conserved in vertebrates and the number of alanine residues in the polyAla tract is also conserved among mammals (Cocquet et al., 2002). FOXL2 is a nuclear protein present in peri-occular and ovarian cells, a localization which is compatible with the BPES phenotype and with a role in transcription (Cocquet et al., 2003). In mammals, FOXL2 is one of the earliest known markers of ovarian differentiation and its expression is maintained in the adult ovary (Cocquet et al., 2002). In the knockout models, infertility is produced after a premature massive follicular activation leading to an accelerated depletion of the follicular pool (Uda et al., 2004, Schmidt et al., 2004). Thus, FOXL2 may play a role in early ovarian development, folliculogenesis but also throughout female fertile life (Cocquet et al., 2002; Schmidt et al., 2004). In the ovarian context, FOXL2 has been implicated in the regulation of steroid metabolism, reactive oxygen species detoxification, apoptosis and inflammation (Batista et al., 2007 and references therein).

Up to now, a large spectrum of *FOXL2* mutations has been detected (details in the human *FOXL2* mutation database at http://medgen.ugent.be/foxl2). Among them, intragenic mutations are present in 70% of BPES patients (De Baere et al., 2001; De Baere et al., 2003). Expansions of the FOXL2 polyAla tract from 14 to 24 residues account for 30 % of the mutations reported in the open reading frame (ORF) (De Baere et al., 2001; De Baere et al., 2003). In previous studies we have demonstrated that polyAla expansions induce the formation of intranuclear aggregates and a mislocalization of the protein to the cytoplasm (Caburet et al., 2004, Nallathambi et al., 2007). We have recently shown that aggregation and mislocalization induce a perturbation of the transactivation capacity of FOXL2 (Moumné et al., 2008a). However, no studies dealing with the impact of other FOXL2 mutations on its localization and transcriptional activity have been described. It is known that missense mutations in the FKH domains of FOXC1, FOXC2 and FOXP2 lead to defective localization of the protein and to changes in transactivation (Berry et al., 2005; Saleem et al., 2004; Saleem et al., 2003; Vernes et al., 2006). Thus, other types of FOXL2 mutations could also perturb protein folding, subcellular localization and transcription activity due to a lower availability of soluble factor and/or to an impaired binding to the promoter region of its target genes.

In this study, we have identified nine mutations in the ORF of *FOXL2* in BPES patients. We have also performed localization and functional studies in COS-7 cells and in the KGN human granulosa-like cell line (Nishi et al., 2001). We report a strong protein mislocalization for a missense mutation lying in the FKH domain (i.e. p.Leu108Pro)**.** In line with this, we observed a loss of transcriptional activity. We have observed a differential pattern of protein aggregation and mislocalization between both cellular models. We have also modeled the structure of the FKH domain to explore the potential impact of this and other previously reported mutants on its structure. The other two mutations analyzed (p.Ser217Cys and an out-of-frame insertion p.Ala253fs, did not show any significant difference with respect to the wild-type (WT) protein. Interestingly, p.Ser217Cys appears in an individual with a mild form of BPES. Our findings reveal new insights into the molecular effects of FOXL2 mutations.

SUBJECTS AND METHODS

Subjects

We analyzed nine patients affected with BPES, aged from 1 to 9 years old from unrelated families and diagnosed at the Aravind Eye Hospital, Madurai, India. An informed consent was established following the guidelines of the Helsinki Declaration.

DNA extraction, sequencing and expression constructs

After genomic DNA extraction from whole blood samples *FOXL2* was PCR-amplified (Pfx polymerase from Invitrogen, Carlsbad, CA, USA, Crisponi et al., 2001, De Baere et al., 2001., De Baere et al., 2002). We directly sequenced the entire ORF using an ABI 3100 sequencer (Applied Biosystems, Foster City, CA, USA). The *FOXL2* mutations encoding the p.Leu108Pro, p.Ser217Cys and p.Ala253fs mutant proteins were cloned into the pcDNA3.1/CT-GFP topoTA cloning vector (Invitrogen, Carlsbad, CA, USA), to produce recombinant fusion proteins in frame with the Green Fluorescent Protein (GFP). As positive control of transfection, we used the WT-FOXL2 ORF (Caburet et al., 2004). All constructs were sequenced to confirm the fusion with the GFP and to exclude the presence of PCR-induced mutations.

Cell culture, protein subcellular localization and luciferase assays

COS-7 cells (African green monkey) were maintained in DMEM medium supplemented with 10 % of foetal bovine serum and 1% of penicillin/streptomycin. Cell culture and transfection of COS-7 cells with FOXL2 constructs was carried out as previously described (Caburet et al., 2004). KGN cells (Nishi et al., 2001) were seeded at 50 000 cells per well in 24-well culture dishes, in DMEM-F12 medium supplemented with 10 % of foetal bovine serum and 1 % of penicillin/streptomycin.

To assess subcellular localization/aggregation, cells were transfected in three independent experiments, using the calcium phosphate method (Sambrook and Russel, 2001). For KGN cells, searching to increase transfection efficiency, this step was repeated 24 h after the first transfection (tandem transfection). The protein subcellular localization and aggregation were observed and scored using standard (Nikon E600) and confocal fluorescence microscopy (Leica TCS SP2, Wetzlar, Germany). At least 300 GFP-positive cells were counted from three independent experiments. Statistical analysis was performed as previously described (Caburet et al., 2004).

The transcriptional activity of three FOXL2 mutants (p.Leu108Pro, p.Ser217Cys and p.Ala253fs) was assessed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). As luciferase reporter we used a previously described construct comprising 1055 bp of the promoter of *FOXL2* cloned upstream of the luciferase gene (DK3 promoter), that we have recently shown to be activated by FOXL2 itself (Moumné et al., 2008a). A total of five constructs (the empty vector, FOXL-WT, and the FOXL2 mutants described above) were studied by transfection in KGN cells. Each experiment was performed in quadruplicate. To monitor transfection efficiency a Renilla luciferase vector (Promega, Madison, WI, USA) was co-transfected in all experiments. All luciferase results are reported as relative light units (RLU). For each replicate, the firefly activity observed was divided by the activity recorded from Renilla luciferase vector. Statistical significance was estimated with a Student’s t-Test.

In silico 3D modeling of FOXL2 FKH mutations

The FKH domain of FOXL2 was modeled using the FoxP2 FKH domain crystal structure (PDB: 2A07) as template. Homology module of Insight 2000 Accelrys software on SGI-O2 was used for modeling. The mutations (p.Ile63Thr, p.Ala66Val, p.Glu69Lys, p.Leu77Pro, p.Ile80Thr, p.Trp98Arg, p.Arg103Cys, p.His104Arg, p.Asn105Ser, p.Leu106Phe, p.Leu108Pro, p.Phe112Ile) were placed within the FOXL2 model using standard rotamer geometry as in the biopolymer module of the Accelrys software. The association of six molecules of FOXL2 FKH with two DNA molecules seen in the FoxP2 FKH domain crystal structure was used to model the association of the native and mutated FOXL2 FKH, in order to understand the possible effects of these mutants.

RESULTS

In the present study, we detected a total of 9 mutations in the ORF FOXL2 in patients with BPES (Table 1). Six of them are novel: c.292T>A (p.Trp98Arg), c.323 T>C (p.Leu108Pro), c.650C>G (p.Ser217Cys), c.915_934dupGCCGCCTGCCCCACCGCACC (p.His312fs), c.757_758insAGGCG (p.Ala253fs) and c.216delG (p.Glu72fs). Among these, three are sporadic cases and two are familial (Table 1). Furthermore, we found 3 previously reported mutations, including a polyAla expansion. Mutation nomenclature is given with respect to the numbering of the Genbank AI: NM_023067 at the cDNA level and to NP_075555 at the protein level.

Table 1 Summary of *FOXL2* mutations identified in this study

Family ID	Age/sex	DNA sequence variation	Amino acid change	Location	Inheritance
3-1	9yrs/F	c.292T>A	p.Trp98Arg*	FKH	Familial
5-1	2yrs/M	c.915_934dupGCCGC CTGCCCCACCGCACC	p.His312fs*	After PolyAla	Sporadic
13-1	1yr/M	c.757_758insAGGCG	p.Ala253fs*	After PolyAla	Sporadic
15-1	8yrs/F	c.650C>G	p.Ser217Cys*	Between FKH and PolyAla	Familial
16-1	4yrs/F	c.644A>G	p.Tyr215Cys	Between FKH and PolyAla	Sporadic
17-1	2yrs/F	c.536C>G	p.Ala179Gly	Between FKH and PolyAla	Familial
19-1	2yrs/F	c.672_701dup30	A224_A234dup10	PolyAla	Familial
20-1	1yr/F	c.323T>C	p.Leu108Pro*	FKH	Familial
21-1	1yr/M	c.216delG	p.Glu72fs*	FKH	Sporadic

Mutation nomenclature is given with respect to the numbering of the Genbank AI: NM_023067.2 (cDNA) and NP_075555 (protein).
***** Novel mutations

We also studied the subcellular localization and potential aggregation of three mutant FOXL2 proteins, namely p.Leu108Pro, p.Ser217Cys, p.Ala253fs. We focused on these representative mutants because p.Leu108Pro is located in the FKH domain, while the others are outside the DNA-binding domain and flank the polyAla tract of FOXL2. These studies were performed in COS-7 (a well-known model) and KGN cell lines. The latter cells are a model of granulosa (ovarian) cells as they respond to FSH, possess a relatively high aromatase activity and are able to produce pregnenolone and secrete steroid hormones (Nishi et al., 2002). The FKH p.Leu108Pro mutation induced a significant increase in the nuclear aggregation when compared with the WT FOXL2 in both cellular models (COS-7: $p< 0.05$, KGN: $p< 0.001$, Figure 1). Moreover, we observed a dramatic increase of nuclear aggregation in the KGN cells with respect to that observed in COS-7 cells ($p<0.001$). In addition, this mutation showed increased cytoplasmatic mislocalization in both COS-7 and KGN cells. The cytoplasmatic mislocalization was also significantly increased in KGN compared to COS-7 cells ($p< 0.001$). The p.Ser217Cys and p.Ala253fs mutations induced no significant differences of nuclear aggregation and cytoplasmatic retention in our cellular models when compared with the FOXL2-WT.

In order to assess the transactivation capacity of the three mutants (p.Leu108Pro, p.Ser217Cys and p.Ala253fs) we performed a Reporter Assay using a construct where a fragment of the promoter of goat FoxL2 (DK3 promoter in Pannetier et al. 2005) drives transcription of the firefly luciferase gene (Figure 2). As previously mentioned, we have shown that Foxl2 is able to regulate its own promoter (Moumné et al. 2008a). This experiment revealed that the mutant p.Leu108Pro was virtually inactive, since the relative luminescence values dropped to the same level as the empty vector and showed a significant decrease of transactivation with respect to the WT protein. Conversely, we failed to detect any significant difference in transactivation capacity for p.Ser217Cys and p.Ala253fs mutants with respect to the WT.

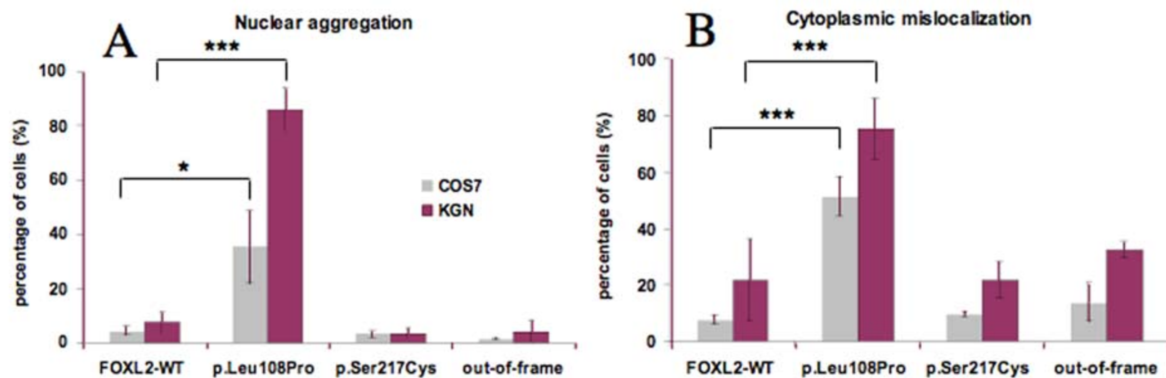

Figure 1. Subcellular localization studies involving three novel mutations of FOXL2 (p.Leu108Pro, p.Ser217Cys and p.Ala253fs/out-of-frame). Statistically significant differences with respect to the FOXL2-WT are represented by (*) for p<0.05 and (***) for p<0,001 (mean and standard deviations for n=4).

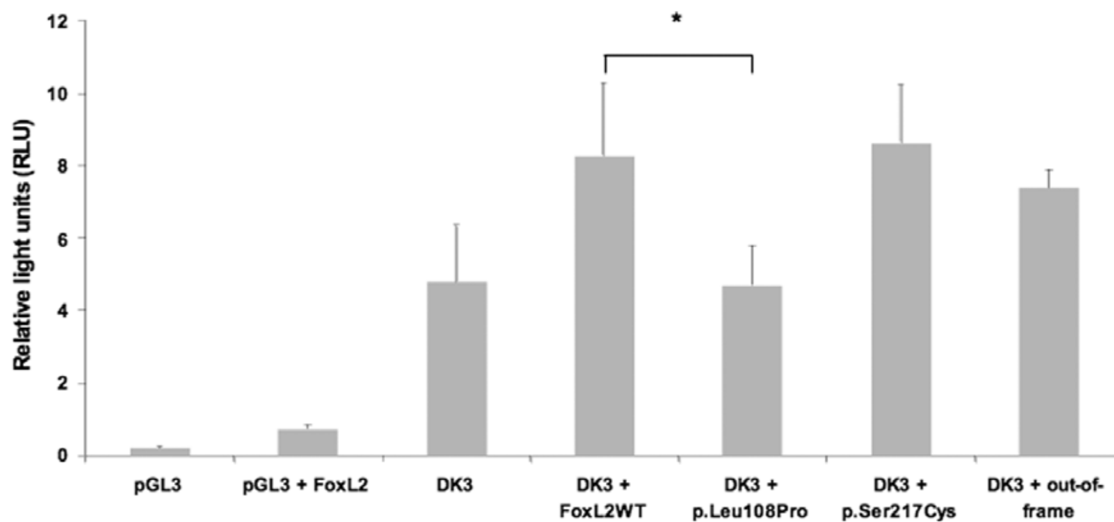

Figure 2. Differential impairment of transactivation capacity induced by three novel mutations of FOXL2 (p.Leu108Pro, p.Ser217Cys and p.Ala253fs/out-of-frame). Statistically significant differences with respect to the activity of wild-type FOXL2 is represented by (*) for p<0.05.

The FOXL2 FKH domain can be structurally accommodated into the same tertiary and quaternary association as reported for the FoxP2 FKH domain (Stroud et al., 2006). We have performed a preliminary model building in order to explore the possible effects of the mutants according to their structural positioning in the model. Mutants p.Leu77Pro, p.Arg103Cys, p.His104Arg, p.Asn105Ser and p.Leu108Pro (described here) are likely to primarily disrupt protein–DNA interactions, as these residues are supposed to interact with DNA (Figure 3). On the contrary, the previously reported mutants p.Ile63Thr, p.Ala66Val, p.Glu69Lys, p.Ile80Thr, p.Trp98Arg, p.Leu106Phe, p.Phe112Ile possibly alter intra- or intermolecular protein–protein interactions. These residues do not come into contact with the DNA but interact within and between the FKH domains, according to the FoxP2 structure used to model the FKH domain of FOXL2.

Figure 3. Structural model of the FKH of FOXL2 showing the positions of mutations found here and previously reported ones (numbering according to human FOXL2,Genbank AI: NP_075555). Upper panel: The main helices of a typical FKH domain are approximately displayed along with several point mutations (highlighted in bold). The lower panel, left, shows the mutants Leu77Pro, Arg103Cys, His104Arg, Asn105Ser and Leu108Pro, which are likely to disrupt protein–DNA interactions (the normal residues seem to interact with DNA). The protein is displayed as a grey ribbon and the DNA is shown in gold color as a stick model. Mutation Leu108Pro, identified in the present study, is shown in red. Lower panel, right: representation of mutants (Ile63Thr, Ala66Val, Glu69Lys, Ile80Thr, Trp98Arg, Leu106Phe, Phe112Ile), which are likely to disrupt protein–protein interactions. These residues do not contact the DNA but interact within or between FKH domain(s). The orientation of the FOXL2 molecule is different from that shown in the left panel. The mutation (Trp98Arg) identified in the present study is shown in red. The reported mutations were extracted from the human FOXL2 mutation database at http://medgen.ugent.be/foxl2.

DISCUSSION

In this paper we report 9 *FOXL2* mutations, including six novel ones, detected in BPES patients. In order to determine the potential defects in subcellular localization and transcriptional activity, we have performed functional assays in two different cellular systems for three of the novel mutations described.

Interestingly, p.Leu108Pro, located in the FKH domain, showed in both KGN and COS-7 cellular systems strong cytoplasmic mislocalisation and nuclear aggregation. These findings suggest that the impaired protein is unable to accomplish a normal function due to partial cytoplasmic sequestration. Furthermore, the protein fraction properly located in the nucleus is highly aggregated. Cytoplasmic mislocalisation of the mutant cannot be explained by a saturation of the transporters because such a phenomenon has never been observed for the WT protein. Several non exclusive hypotheses can be proposed to explain the cytoplasmic retention of the mutant FOXL2. The missense mutation might induce a protein misfolding that buries the nuclear localization signal (NLS) or disturbs interactions with nuclear transporters. FOXL2 contains two NLSs located at the C-term of the FKH (Moumné et al., 2008b). In addition, cytoplasmic oligomerisation or aggregation might lead to the production of structures whose size is not compatible with nuclear import. Finally, misfolded FOXL2 might strongly interact with cytoplasmic proteins that provoke some retention. Mislocalization and aggregation has been reported for mutations in the transcription factor FOXC1, suggesting this is a common mechanism due to mutations in FKH-containing proteins (Saleem et al., 2003; Saleem et al., 2004).

As mentioned above, KGN cells display a percentage of nuclear aggregation that is more than two times higher than that observed for COS-7 cells. This trend also appears for the cytoplasmatic mislocalization. This differential behavior is not due to a higher expression of FOXL2 variants in KGN cells. Indeed, we have estimated the expression levels of FOXL2 for each cell line by determining the minimum exposure time under which the cell fluorescence becomes imperceptible using epifluorescence microscopy (averaged over 150 cells). Using this proxi of protein expression, KGN cells are expected to express FOXL2 about three times less strongly than COS-7 cells. Thus, we suggest that the differences between the cell lines might be due to different proteomic environments (different concentrations of aggregation partners, proteasome, chaperons, etc). In our opinion, the existence of a species-specific effect on protein aggregation is unlikely as both cell lines come from primates (KGN/*Homo sapiens* vs COS-7/*Cercopithecus aethiops*).

We have corroborated the impairment of p.Leu108Pro at the functional level, as luciferase assays demonstrate that the transcriptional activity of this mutant on the FoxL2/DK3 promoter was completely abolished. The study of the p.Leu108Pro mutation constitutes the first report of a FOXL2-FKH missense mutation leading to aggregation and a severe transactivation defect.

The pAla253fs produces a truncated protein, which does not seem to alter the structure of the FKH and the polyAla domains. This is also the case of p.Ser217Cys. This seems to be compatible with a proper localization and transactivation on the FoxL2 promoter. To explain the BPES phenotype in these patients, we must suppose that these variants have a reduced activity on a specific set of target promoters important for eyelid development. Theory predicts that target promoters will have different sensitivities to decreased amounts of available/soluble FOXL2. For instance, when two promoters contain the same number of binding sites, the one having binding sites with the highest affinity for FOXL2 will be fully activated with a lower concentration of this factor (less sensitive to aggregation). Otherwise, when two promoters contain sites with similar affinity for FOXL2, the one having the highest number of binding sites will be less sensitive to a decrease of available FOXL2. The theoretical foundations of these predictions are discussed elsewhere (Veitia, 2003). However, it is interesting to note that the variant p.Ser217Cys, mapping between the FKH and the polyalanine domain of FOXL2 leads to an unusually mild BPES phenotype (Figure 4), suggesting that point mutations outside the FKH might alter protein function in a rather subtle way. Unfortunately, we do not have enough clinical information about the reproductive characteristics (and endocrinological data) of this familial case to classify the BPES.

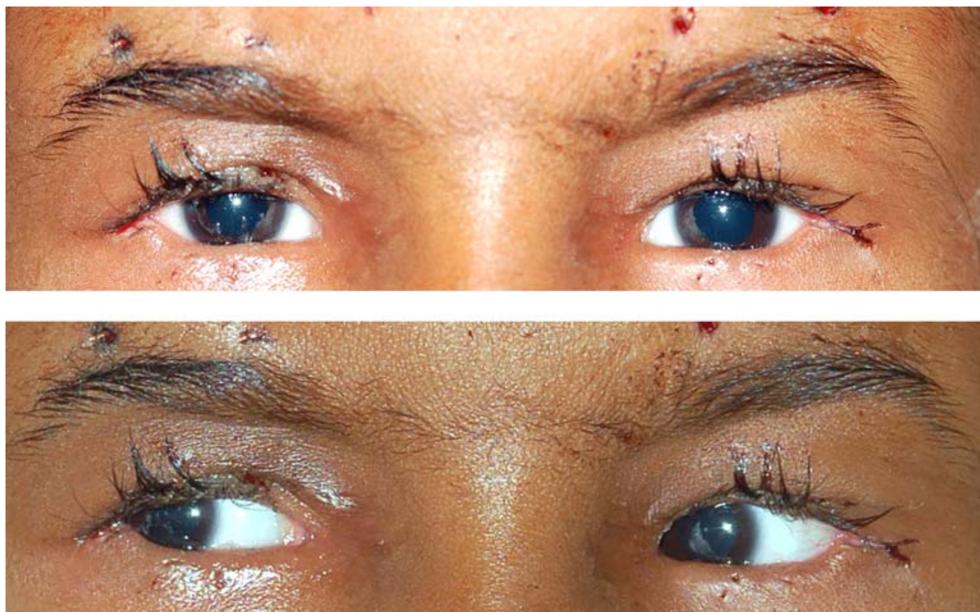

Figure 4. Post-surgery pictures of the patient carrying the substitution p.Ser217Cys.

The molecular modeling analysis shows that it is possible to sort the mutants into two classes: those that potentially alter protein interactions within and between FOXL2 molecules and those that disrupt the FOXL2-DNA interactions. Among the mutants identified in this study and falling in the FKH domain, p.Leu108Pro is

likely to change the interaction with DNA whereas p.Trp98Arg mutant might modify interactions within the protein domains. Based on the model we can also suggest that in the absence of binding to DNA, due to a change in the interaction surface, the unbound region can be available for protein-protein interactions potentially leading to aggregation or cytoplasmic retention. Although tempting, further studies are required to corroborate these ideas.

In conclusion, we provide evidence showing the extreme sensitivity of the conformation of FOXL2 since a point mutation in the FKH domain induces strong protein mislocalization, aggregation and loss of function. Our exploration of the FKH domain with a molecular model reveals new insights into the molecular effect of FOXL2 mutations.

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