

Missense mutations in the forkhead domain of *FOXL2* lead to subcellular mislocalization, protein aggregation and impaired transactivation

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Mutations of the *FOXL2* gene have been shown to cause blepharophimosis syndrome (BPES), characterized by an eyelid malformation associated with premature ovarian failure or not. Recently, polyalanine expansions and truncating *FOXL2* mutations have been shown to lead to protein mislocalization, aggregation and altered transactivation. Here, we study the molecular consequences of 17 naturally occurring *FOXL2* missense mutations. Most of them map to the conserved DNA-binding forkhead domain (FHD). The subcellular localization and aggregation pattern of the mutant *FOXL2* proteins in COS-7 cells was variable and ranged from a diffuse nuclear distribution like the wild-type to extensive nuclear aggregation often in combination with cytoplasmic mislocalization and aggregation. We also studied the transactivation capacity of the mutants in *FOXL2* expressing granulosa-like cells (KGN). Several mutants led to a loss-of-function, while others are suspected to induce a dominant negative effect. Interestingly, one mutant that is located outside the FHD (S217F), appeared to be hypermorphic and had no effect on intracellular protein distribution. This mutation gives rise to a mild BPES phenotype. In general, missense mutations located in the FHD lead to classical BPES and cannot be correlated with expression of the ovarian phenotype. However, a potential predictive value of localization and transactivation assays in the making of genotype–phenotype correlations is proposed. This is the first study to demonstrate that a significant number of missense mutations in the FHD of *FOXL2* lead to mislocalization, protein aggregation and altered transactivation, and to provide insights into the pathogenesis associated with missense mutations of *FOXL2* in human disease.

INTRODUCTION

Forkhead/winged-helix family of transcription factors regulates gene expression to control a variety of processes, such as embryogenesis, tumorigenesis and maintenance of differentiated cell states (1). These transcription factors share a highly conserved DNA-binding forkhead domain (FHD) of ~110 amino acids, consisting of three α helices and two large loops that form ‘wing’ structures, hence the name

‘winged-helix’. To date, ~40 human forkhead proteins have been identified and eight of them have been shown to be implicated in human developmental disorders, of which an important fraction has ocular implications (1). Within this family, the *FOXL2* gene has been shown to be mutated in the blepharophimosis syndrome (BPES, MIM 110100), an essentially autosomal dominant disorder affecting the eyelids and the ovary (2). In BPES type I, the eyelid malformation is associated with ovarian dysfunction leading to premature ovarian failure,

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whereas in BPES type II the eyelid defect occurs isolated (3). *FOXL2* is a single-exon gene encoding a protein of 376 amino acids. Apart from the characteristic FHD, *FOXL2* also contains a polyalanine tract (poly-Ala) of 14 residues. To date, different kinds of genetic defects have been identified including mutations in the coding sequence, and deletions of the gene or deletions located outside the transcription unit (overview in Human *FOXL2* Mutation Database, <http://medgen.ugent.be/foxl2>) (4–6). We have also demonstrated the existence of two mutational hotspots in *FOXL2* and intra- and interfamilial variability of the ovarian phenotype (4,5).

An expansion of the polyAla tract from 14 to 24 residues (Ala24) accounts for one of the mutational hotspots in BPES and involves 30% of patients (4,5). We have also reported the first homozygous *FOXL2* mutation leading to a polyAla expansion of 19 residues (Ala 19) in an Indian family (7). We have shown that the wild-type (WT) *FOXL2* protein (Ala14) exclusively localizes in the nucleus in a rather diffuse manner, whereas Ala24 leads to a mislocalization and cytoplasmic and nuclear aggregation in COS-7 cells. In turn, the Ala19 mutant only leads to cytoplasmic staining in a minority of transfected cells and not to detectable aggregation (7). More recently, we have shown that polyAla expansions lead to protein mislocalization, aggregation and altered intranuclear mobility in a length-dependent manner (8). Luciferase assays (9) and real time RT-PCR of several target genes showed that various polyAla expansions induce different losses of activity according to the target promoters analysed (8).

Several artificial nonsense mutations have been shown to lead to the production of N-terminally truncated proteins by re-initiation of translation downstream of the premature stop codon. They display strong nuclear aggregation, and partial mislocalization to the cytoplasm (10). For both Ala24 and truncating mutant proteins, it was shown that they retain a fraction of the WT protein, suggesting a dominant negative effect (10). Luciferase assays with natural nonsense mutations demonstrated the importance of the entire alanine/proline-rich carboxyl terminus of *FOXL2* for transcriptional repression of the StAR (steroidogenic acute regulatory gene) promoter. Furthermore, it was also demonstrated that these mutations produce a protein with a weak dominant negative effect (11).

These observations are an interesting starting point to motivate the study of the molecular consequences of other types of *FOXL2* alterations such as disease-causing missense mutations. Most of them are located in the FHD, in agreement with mutations in other forkhead transcription factor genes, such as *FOXC1*, *FOXC2*, *FOXE1*, *FOXP2* and *FOXP3* (12–17). To date, 24 unique missense mutations have been found in the FHD of *FOXL2*, being the largest number reported so far in a human FHD-containing transcription factor. Localization studies of missense mutations in the FHD of *FOXC1*, *FOXC2* and *FOXP2* revealed that these mutations lead to defective localization of the protein. Functional studies have shown changes in DNA-binding capacity and transactivation (12–16).

Here, we study the molecular consequences of a comprehensive series of 17 missense mutations of *FOXL2* predominantly located in the FHD, through the analysis of protein localization and function in cellular systems. We demonstrate

that some of the mutant proteins lead to mislocalization, protein aggregation and altered transactivation, and thus provide insights into the pathogenesis associated with missense mutations in the FHD of *FOXL2*. Moreover, we present experimental data that shed light on the pathogenesis of a missense mutation outside the FHD associated with a mild form of BPES.

RESULTS AND DISCUSSION

In this study, we have examined the molecular consequences on intracellular localization and function of 17 disease-causing missense mutations of *FOXL2* (S58L, I63T, A66V, E69K, S70I, I80T, I84N, F90S, W98G, S101R, I102T, R103C, H104R, L106F, L106P, N109K and S217F). The pathogenicity of these mutations, of which 16 are located in the FHD, has been discussed previously (4,5,18). The change S217F, located outside the FHD was considered to be a *bona fide* disease-causing mutation because: (i) it co-segregates with BPES in an affected father and his two affected daughters (showing compatibility with linkage but not a significant proof of linkage); (ii) it was not found in more than 200 control chromosomes; (iii) the S-residue itself and surrounding amino acids are highly conserved among *FOXL2* orthologues and (iv) Polyphen predictions and a Grantham score of 155 are suggestive of an effect on protein function (possibly damaging). The locations of the missense mutations in the FHD are represented in a 3D structure model of the FHD of *FOXL2* in Supplementary Material, Figure S1 (<http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml>) (19). In Figure 1 an alignment is provided of the FHD of *FOXL2* and other human transcription factors (*FOXC1*, *FOXC2* and *FOXP2*) in which missense mutations of the FHD have been reported. A summary of the *FOXL2* mutations, their positions within the FHD, the subcellular distribution and transactivation capacities of the respective mutant proteins, and some 'paralogous' missense substitutions is given in Table 1.

Missense mutations in the FHD impair subcellular localization and induce aggregation

Localization studies of the fusion protein *FOXL2*-GFP (green fluorescent protein) in COS-7 cells demonstrated a predominant nuclear localization of WT *FOXL2*, with a diffuse distribution, in agreement with our previous observations (10). The cellular system used (COS-7) is a well-established model to study *FOXL2* aggregation (8). Subcellular localization and aggregation pattern of the mutant *FOXL2* proteins was found to be highly variable, displaying: (i) a diffuse nuclear distribution identical to the WT *FOXL2* for H104R, N109K and S217F; (ii) granular and massive aggregates in the nucleus for E69K and S101R; (iii) extensive nuclear protein aggregation often in combination with a striking pattern of granular cytoplasmic staining, located more prominently at one side of the nucleus (capping) for S58L, I63T, A66V, I80T, I84N, F90S, W98G, I102T, R103C, L106F and L106P; and (iv) isolated massive cytoplasmic aggregates located at one side of the nucleus were seen in S70I (Fig. 2

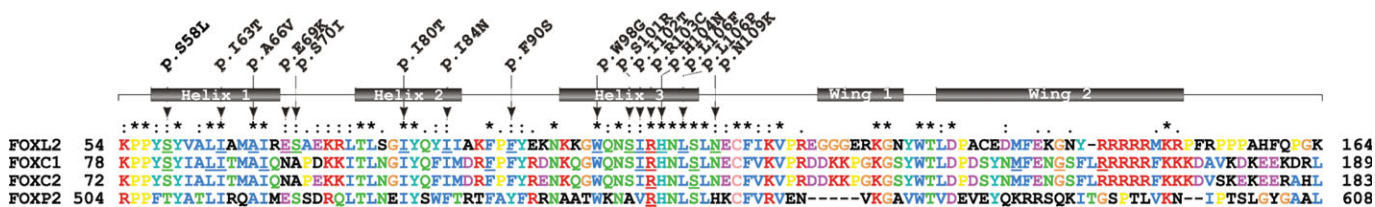


Figure 1. Multiple sequence alignment of the forkhead domain of human FOXL2 and related FOX proteins. The amino acid sequences of the forkhead domain (FHD) of human FOXL2, FOXC1, FOXC2 and FOXP2 were aligned with the ClustalX program. The use of paralogues for the alignment increases its informativity on conservation (since the FKH of proven FOXL2 orthologues is extremely well conserved), and allows comparison with natural ‘paralogous’ mutations. Only those human FOX proteins for which localization studies of missense mutants of the FHD have been performed, were included in the alignment. A consensus line at the top indicates conservation of the amino acid residues: an asterisk (*) indicates fully conserved sites, a colon (:) points to a conserved substitution and a point (.) a semi-conserved substitution. Amino acids are coloured according to the consensus ClustalX colour scheme. The position of predicted ‘helix’ and ‘wing’ segments are represented on top of the alignment. The locations of mutated residues that were analysed in this study are indicated with an arrow above the sequence. Missense mutations for which localization studies have been performed, are underlined.

and Supplementary Material, Fig. S2). A possible artefact of mislocalization and aggregation induced by the GFP in the fusion proteins has been ruled out in previous studies (8,10). In forkhead-containing proteins in general, several mechanisms for nuclear targeting have been described, including nuclear localization signals (NLS) within the FHD and interaction with transporter proteins (20,21). Investigations of FOXC1, FOXO1, FOXP2 and FoxP3 have illustrated that the FHD is important for a correct nuclear localization (22–25). Moreover, it has been suggested that mislocalization of FOXC1 and FOXC2 is one of the pathogenetic mechanisms of missense mutations in the FHD (15). Several hypotheses might explain the cytoplasmic mislocalization of mutant FOXL2. The missense mutation might induce a misfolding that hides the NLS or disturbs interactions with nuclear transporters. In FOXL2, a classical NLS containing multiple positively charged amino acid residues is located at the C-term of the FHD (i.e. RRRRRMKR) (10). Moreover, another non-canonical sequence with partial NLS activity has been mapped to the segment between residues 125 and 144 (i.e. KGNWTLDPACEDMFEKGN) (10). A similar mechanism of mislocalization by interference with nuclear import has been suggested for FOXC1 and FOXC2 (12,15). Moreover, cytoplasmic oligomerization or aggregation might lead to the presence of structures whose size is incompatible with the import through the nuclear pores. Finally, there might be cytoplasmic components that interact with misfolded FOXL2 leading to its retention. However, even if we do not know the details of FOXL2 nuclear import, cytoplasmic mislocalization of the mutant cannot be explained by a saturation of the transporters because it has never been observed for the WT protein (8).

Effects of missense mutations on transcriptional activity of FOXL2

To better understand the mechanisms of pathogenicity of missense mutations, we also studied their impact on transcriptional activity. For this, we used the previously described DK3-Luciferase (DK3-Luc) reporter construct. This construct corresponds to 1055 bp of the *FoxL2* promoter of the goat upstream of the firefly luciferase gene (10). This promoter is well-conserved with respect to the human one and responds similarly to both human or goat FOXL2 proteins (data not

shown). It was shown previously that the degree of loss of activity of FOXL2 mutants in the KGN-cellular model is dependent on the promoters tested. This can be explained by the existence of different sensitivities of target promoters to a decreased availability of FOXL2 due to mislocalization or aggregation. In comparison with another FOXL2-responsive promoter GRAS-Luc, the DK3-Luc promoter appeared the more sensitive (i.e. responding to a lower concentration of active FOXL2) (8). Thus, we used the latter reporter construct for our functional tests. We performed the luciferase assays in the granulosa-like KGN cells, that naturally express FOXL2 and that have been shown to be a suitable cellular model to study its activity (8). Moreover, KGN cells display a good correlation with COS-7 cells with respect to aggregation (8). The cells were co-transfected with the DK3-Luc construct and each one of the constructs coding for a FOXL2-GFP missense mutant or the empty GFP vector (baseline control). The WT FOXL2 induced a statistically significant increase in luciferase activity when compared with the GFP empty vector (Fig. 3). Interestingly, the various mutants showed different behaviours. Mutants E69K, R103C and H104R displayed activities not different from that of the WT. However, other mutants, such as S58L, I84N, F90S, W98G, S101R, L106F and N109K showed a luciferase activity significantly lower than that of the WT and not different from the control vector. In addition, I63T, A66V, S70I, I80T, I102T and L106P displayed activities statistically lower than that of both the WT protein and the control vector. This is suggestive of an interference of these mutants with endogenous FOXL2 that might drive—at least partially—the basal expression of the reporter. This evokes the existence of a potential dominant negative effect (i.e. with respect to the endogenous WT version), probably weak because it is driven here by the overexpression of mutant FOXL2. We suggest that the total loss of transcriptional activity of these mutants is due to their extensive nuclear and cytoplasmic aggregation. Moreover, soluble molecules bearing a misfolded FHD might be unable to recognize their binding sites in the target promoter. For the moment, it is difficult to disentangle the contribution of both effects, especially when the mutation lies within the recognition helix. Finally, the variant S217F was found to be hyperactive on the DK3 promoter. In this context it is worth mentioning that both haploinsufficiency and overexpression of FOXC1 lead to similar phenotypes (1). In spite of the limitations of

Table 1. Summary of clinical data, subcellular distribution, aggregation pattern and transactivation capacities of FOXL2 missense mutations

FOXL2 mutation	Secondary structure involvement	BPES type ^a	Clinical data ^b	Subcellular distribution and transactivation properties of mutant FOXL2-GFP	Human forkhead proteins with paralogous mutations	Subcellular distribution and transcriptional activity of paralogous mutant proteins, involved in human disease	References
S58L	Helix 1	S	BPES in 1-year old female, <i>de novo</i>	Nuclear and cytoplasmic aggregation Impaired transactivation function	FOXC1-S82T	Diffuse nuclear localization Impaired transactivation.	(13)
I63T	Helix 1	F	Two-generation BPES family	Nuclear and cytoplasmic aggregation Impaired transactivation (suggestive of weak dominant negative effect)	FOXC1-I91T FOXC1-I91S	Moderate mislocalization (50%) Impaired transactivation Severe mislocalization (85%) Impaired transactivation	(15)
A66V	Helix 1	F; S	BPES in 3-year old female, <i>de novo</i>	Nuclear and cytoplasmic aggregation Impaired transactivation (suggestive of weak dominant negative effect)			
E69K		S	BPES in 5-y old male, <i>de novo</i> . Bilateral vocal cord nodules and VSD	Nuclear aggregation Normal transcriptional activation			
S70I		S	BPES in 3-year old boy	Cytoplasmic aggregation Impaired transactivation (suggestive of weak dominant negative effect)			
I80T	Helix 2	F1	Three-generation BPES family, type 1	Nuclear and cytoplasmic aggregation Impaired transactivation (suggestive of weak dominant negative effect)			
I84N	Helix 2	S	BPES in 13-year old girl	Nuclear and cytoplasmic aggregation Impaired transactivation function			
F90S		S	BPES in 2-year old female, <i>de novo</i>	Nuclear and cytoplasmic aggregation Impaired transactivation function			
W98G	Helix 3	S	BPES in 1-y old male, <i>de novo</i>	Nuclear and cytoplasmic aggregation Impaired transactivation function			
S101R	Helix 3	F	Two-generation BPES family	Nuclear aggregation Impaired transactivation function			
I102T	Helix 3	S	BPES in 17-y old male with laterally protruding ears, cleft lip and mild syndactyly	Nuclear and cytoplasmic aggregation Impaired transactivation (suggestive of weak dominant negative effect)	FOXC1-I126M	Diffuse nuclear localization Impaired transactivation	(13)
R103C	Helix 3	S	BPES in 1-year old female	Nuclear and cytoplasmic aggregation Normal transcriptional activation	FOXC1-R127H FOXC2-R121H FOXP2-R553H	Nuclear and cytoplasmic localization Impaired transactivation function Cytoplasmic localization Impaired transactivation function Cytoplasmic localization Impaired transrepression	(12,15,16,24)
H104R	Helix 3	S	BPES in a male child	Diffuse nuclear localization Normal transcriptional activation			
L106F	Helix 3	S	BPES in 1-year old male, <i>de novo</i>	Nuclear and cytoplasmic aggregation Impaired transactivation function			
L106P	Helix 3	F	Three-generation BPES pedigree	Nuclear and cytoplasmic aggregation Impaired transactivation (suggestive of weak dominant negative effect)			
N109K		F2	Five-generation BPES type 2 pedigree	Diffuse nuclear localization Impaired transactivation function			

Continued

Table 1. Continued

FOXL2 mutation	Secondary structure involvement	BPES type ^a	Clinical data ^b	Subcellular distribution and transactivation properties of mutant FOXL2-GFP	Human forkhead proteins with paralogous mutations	Subcellular distribution and transcriptional activity of paralogous mutant proteins, involved in human disease	References
S217F	Outside FHD ^a	F	Mild BPES phenotype in father and two prepubertal daughters	Diffuse nuclear localization Increased transcriptional activation (suggesting hypermorphism)			
None (wild-type)	NA	NA	NA	Diffuse nuclear localization Normal transcriptional activation			

^aFHD, forkhead domain; F, familial; S, sporadic; F1, familial type 1; F2, familial type 2; -, not available; NA, not applicable.
^bVSD, ventricular septal defect.

transactivation studies using transient transfection, our study shows differential effects of the mutations on the capacity of FOXL2 to activate its own promoter. Although quantitative details can be different, the trends observed here are expected to be similar with other promoters, as we have shown previously (8).

Testing of a predictive model of the molecular consequences of missense mutations in the FHD

Recently, a predictive mutational model for the effect of missense mutations found in the FHD of FOXC1 and FOXC2 genes was proposed (12): (i) substitutions of residues of the N-terminal sequence and Helix 1 were predicted to reduce DNA-binding, perturb nuclear localization and impair transactivation, (ii) mutations in Helix 2 were assumed to affect transcriptional activation but not nuclear localization or DNA-binding, (iii) mutations in Helix 3 hinder nuclear localization, DNA-binding and specificity. Indeed, FOXL2 missense mutations located in Helix 1 showed significant changes in nuclear localization and impaired transactivation (summarized in Table 1). However, two disease-causing mutations I80T and I84N located in Helix 2 of FOXL2, were shown to affect transactivation but also to lead to nuclear and cytoplasmic aggregation, the latter not being in agreement with the predictive model. Similarly, mutation H104R, located in Helix 3, displays normal diffuse nuclear localization, as opposed to the model assuming impaired nuclear localization. Taken together, our data suggest that the predictive model for missense mutations in the FHD of FOXC1 and FOXC2 cannot be automatically applied to other FOX proteins.

Interestingly, several of the disease-causing FOXL2 mutations lie at paralogous positions mutated in FOXC1, FOXC2 or FOXP2 genes, as illustrated in Figure 1 and Table 1. FOXC1 missense mutations S82T and I126M did not alter nuclear localization of the protein (13,15), whereas corresponding FOXL2 mutations S58L and I102T induce nuclear and cytoplasmic aggregation. Transactivation properties are impaired by FOXC1 S82T and I102T, and by FOXL2 S58L; they are even suggestive of a dominant negative effect for FOXL2 I102T. FOXC1 mutations I91T and I91S display substantial mislocalization, and both have severely reduced transactivation capacities. This is in agreement with the nuclear and cytoplasmic aggregation of FOXL2 I63T, and with its severely impaired transactivation (possibly causing a dominant negative effect). Missense mutations R127H in FOXC1, R121H in FOXC2, and R553H in FOXP2, were shown to cause mislocalization but not aggregation of the respective fusion proteins (12,15,16), whereas the corresponding FOXL2 mutation R103C leads to both mislocalization and aggregation. In addition, transactivation assays show different effects of R127H, R121H, and R553H (loss of transactivation) compared with R103C (normal transactivation). These differences in localization and aggregation can be explained by different cellular proteomes of tissues expressing the respective FOX proteins (different expression levels of proteins involved in nuclear transport, folding, modulators of aggregation process such as heat shock proteins, ubiquitin conjugation or proteasomes) or by differences in the chemical nature of the substituted residues (i.e. case for R127H, R121H, R553H versus R103C; S82T

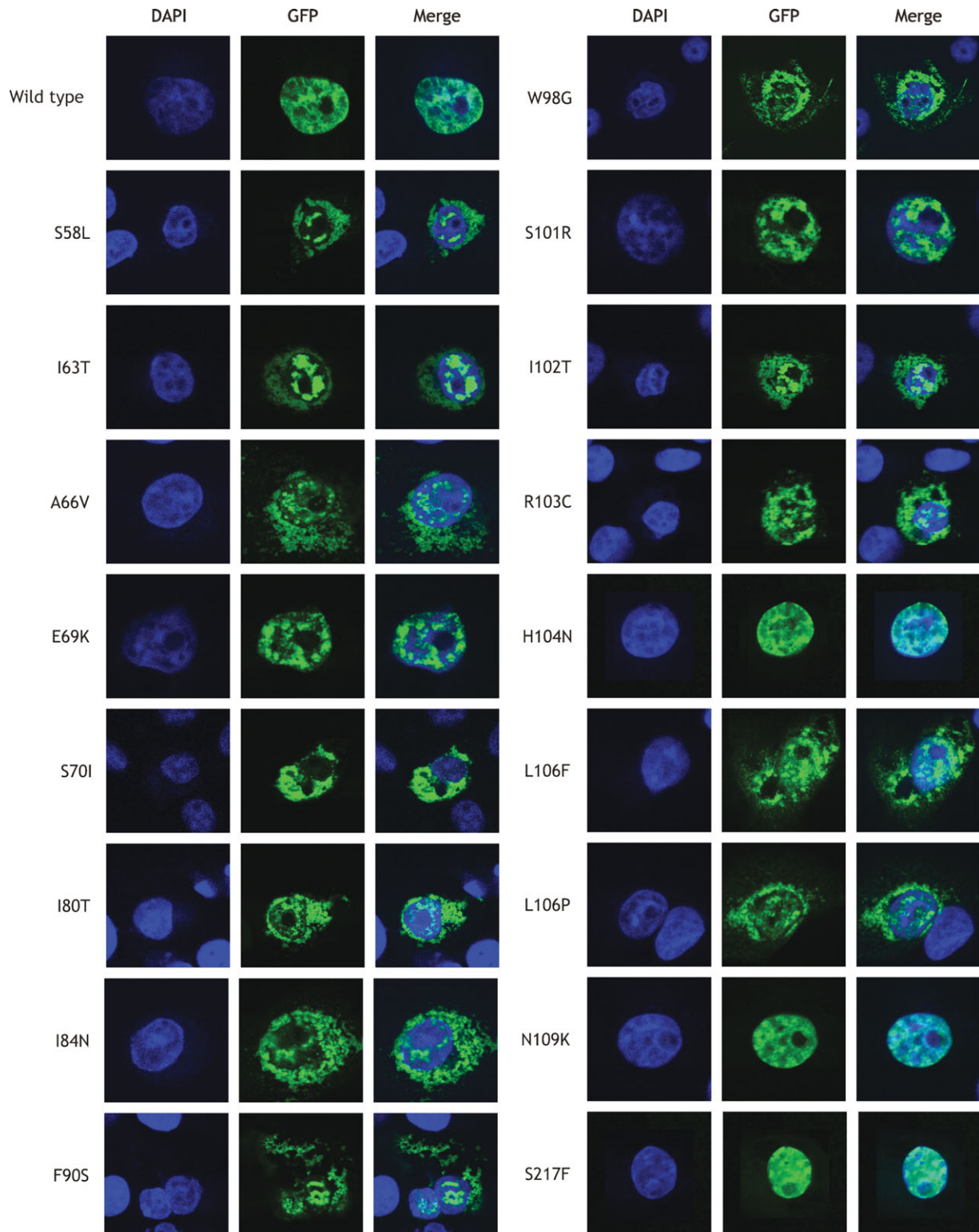


Figure 2. Subcellular localization of wild-type and mutant FOXL2 proteins. DAPI (4',6-Diamidino-2-phenylindole) counterstain (blue) in the left panels shows the localization of the nucleus. The middle panel corresponds to the most representative subcellular localization of the FOXL2 ORF as a fusion protein with GFP (green fluorescent protein). The right panel is a merge of the previous ones. The mutations are ordered according to their amino acid positions. Four predominant localization patterns could be distinguished: (i) normal localization and distribution (H104R, N109K, S217F); (ii) intranuclear aggregation (E69K, S101R); (iii) nuclear and cytoplasmic aggregation (S58L, I63T, A66V, I80T, I84N, F90S, W98G, I102T, R103C, L106F, L106P); (iv) cytoplasmic aggregation (S70I).

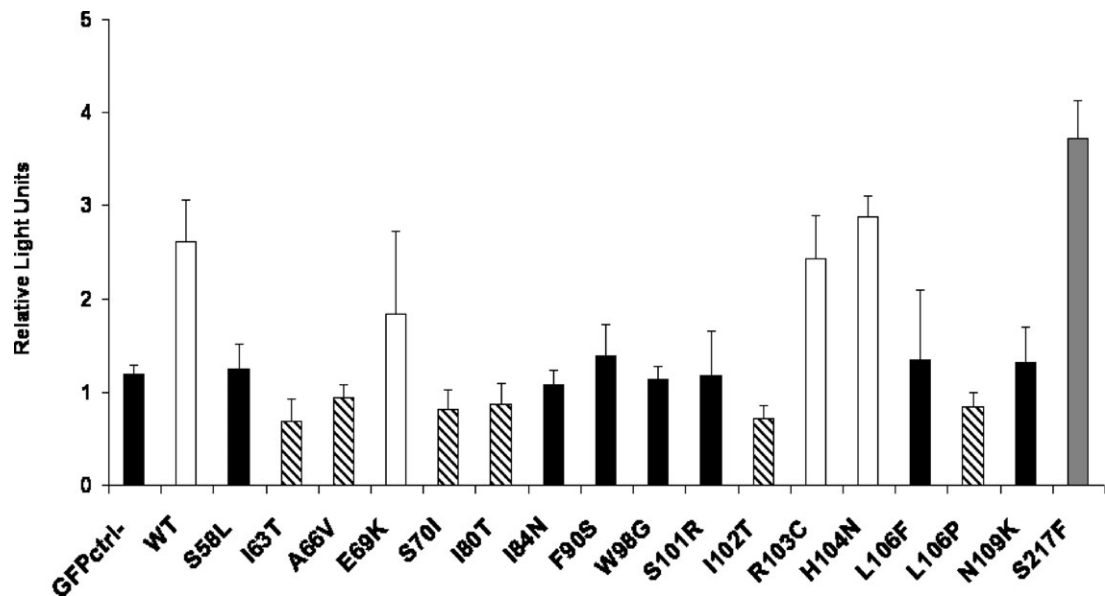


Figure 3. Transcriptional activities of FOXL2 variants studied by luciferase assays. Relative luciferase activity corresponds to the activity of the luciferase reporter DK3 construct in the presence of FOXL2 variants [or the GFP (green fluorescent protein) alone] over the activity of the Renilla (internal control for transfection efficiency). White bar: normal transcriptional activity of the wild-type protein and of constructs not displaying statistically significant differences in transactivation with respect to the wild-type protein. Black bars: constructs displaying statistically lower activities than the wild-type protein (at least $P < 0.05$), compatible with loss of transactivation function. Striped bars: constructs displaying statistically lower activities than both the wild-type protein and the control DK3 promoter (in absence of exogenous FOXL2, at least $P < 0.05$), compatible with a weak dominant negative effect. Grey bar: S217F showing a higher activity than wild-type FOXL2 ($P = 0.003$), suggestive of hypermorphism.

versus S58L; I126M versus I102T). The differences in transactivation function between paralogous mutations might also be explained by the aforementioned reasons, and by cell-specificity and a different sensitivity of the promoters tested. Again, these results indicate that molecular consequences of missense mutations in *FOX* genes cannot be necessarily transposed to the paralogous mutations in other forkhead transcription factors and that the nature of the substitutions or the cellular context has an impact on the molecular consequences and cellular phenotype induced by the mutations.

Potential predictive value of intracellular distribution, aggregation pattern and function of missense mutations on genotype–phenotype correlations

The missense change S217F is located outside the FHD of FOXL2, 5' to the polyAla tract. It was shown to have no effect on subcellular localization of the FOXL2 protein and to increase its transactivation capacity. As discussed before, S217F is considered to be a disease-causing mutation. However, this mutation leads to a mild BPES phenotype in the affected individuals of a two-generation family of Belgian origin (Fig. 4). Interestingly, this mutation S217F was found to be hyperactive on the DK3 promoter. As mentioned, both haploinsufficiency of *FOXC1* and increased gene dosage lead to similar albeit not identical phenotypes (1). This concept appears to have its equivalent in the context of *FOXL2*, as our data suggest that S217F, which does not impair protein distribution and increases its activity, might be better tolerated and would give rise to a less severe BPES phenotype. This is supported by the occurrence of an equally mild BPES phenotype resulting from a different

mutation in the same residue (S217C) in another family of Indian origin (26).

In general, for *FOXL2* missense mutations in the FHD, no genotype–phenotype correlations can be made with respect to the ovarian phenotype. Here, the BPES type could be assessed in two out of 18 families (Table 1) (4,5). In a five-generation BPES type II family, mutation N109K was found displaying a normal subcellular localization and decreased transactivation to a level compatible with loss-of-function. On the other hand, I80T was identified in a three-generation BPES type I family, causing nuclear and cytoplasmic aggregation and severely impairing transactivation, which is suggestive of a weak dominant negative effect. Although the sample of families with a clearly defined BPES type is small, and both genetic and environmental factors can influence expression of the ovarian phenotype, the assays described here might offer some predictive value with regard to the development of an ovarian phenotype. Hence missense mutations in the FHD leading to mislocalization and aggregation, and thus severely impairing transactivation, tend to lead to a more severe ovarian phenotype than missense mutations not significantly affecting protein localization and function. However, this preliminary hypothesis that is based on a small sample size, needs to be further refined by additional localization studies and functional assays in families with a well-defined type of BPES.

General conclusion

In conclusion, this is the first comprehensive study to demonstrate that missense mutations in the FHD of FOXL2 can lead to mislocalization, nuclear and cytoplasmic aggregation of the



Figure 4. Facial pictures of siblings carrying missense mutation S217F. The eyelid phenotype caused by S217F (i.e. blepharophimosis and telecanthus, but no ptosis and epicanthus inversus) is very mild in comparison with the classical BPES phenotype (i.e. blepharophimosis, ptosis, epicanthus inversus, telecanthus) that was observed in all patients carrying a missense mutation in the forkhead domain.

mutant protein as well as impaired transactivation. This clearly shows that the conformation and 'solubility' of FOXL2 is extremely sensitive to changes in the primary sequence. Other properties that are influenced by the primary sequence of the FHD such as DNA-binding and protein mobility will be subject to further study. Our data altogether suggest that the pathogenesis of a major subset of FOXL2 missense mutations in human disease results from failure of the mutant protein to properly localize to the nucleus, and to reach its target genes.

MATERIALS AND METHODS

Subjects

Genomic patient DNA was used that was available from previously approved mutation studies (4,5,18). This research followed the tenets of the Declaration of Helsinki.

Plasmid constructs

Since *FOXL2* is a single-exon gene, the full-length open reading frame was amplified by PCR from human control and mutant genomic DNA (except for construct I63T) (iProof polymerase, Bio-Rad). The primer pairs used for amplification are FOXL2- α 5'-ATGATGGCCAGCTACCCGAG-3' and FOXL2- Ω -GFP 5'-GATCGAGGCGCGAATGCAGC-3'. The *FOXL2* ORF was subsequently cloned into a pcDNA3.1/CT-GFP-TOPO TA vector (Invitrogen), which allows expression of fusion proteins with the GFP at the C-terminus of *FOXL2*. Construct I63T was obtained by junction-PCR using overlapping mismatched primers: 188F 5'-CGTGGCGCTCACC GCCATGGCGATC-3' and 188R 5'-GATCGCCATGGCGGTGAGCGCCACG-3' to induce mutations. First, PCR products from separate amplifications with primer combinations FOXL2- α and 188R, and FOXL2- Ω -GFP and 188F were gel-purified. Both PCR fragments were mixed and used as templates for a secondary amplification of the combined sequences with primers FOXL2- α and FOXL2- Ω -GFP, allowing both fragments to 'recombine' because of the sequence overlap. Next, the resulting PCR fragment was cloned into the pcDNA3.1/CT-GFP-TOPO TA vector. All expression constructs were sequenced to confirm the presence of the desired mutations and to exclude PCR-induced mutations.

Cell culture and transfection

African green monkey kidney COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-Invitrogen, CA, USA), KGN cells were maintained in

DMEM-F12 (Gibco-Invitrogen) and both media were supplemented with 10% fetal calf serum (FCS; Gibco-Invitrogen) and 1% penicillin/streptomycin, at 37°C in the presence of 5% CO₂. Twelve hours before transfection, cells were seeded in 24-well plates containing sterile cover slips at a concentration of 30 000 cells per well. Transfections were performed using the calcium phosphate method with 500 ng of DNA per well. At 48 h after transfection, cells were washed with phosphate buffered saline solution (PBS) and fixed with 4% paraformaldehyde in PBS at room temperature for 15 min. Nuclei were stained with DAPI (4',6-Diamidino-2-phenylindole) (1/5000) or Hoechst (1/500) and coverslips were mounted on slides with Vectashield mounting medium (Vector Laboratories). Aggregation of FOXL2 fusion proteins was assessed visually using epifluorescence (Nikon E600) and confocal microscopy (Radiance 2100, Bio-Rad). All transfections were performed in triplicate. From each transfection, at least 100 cells were counted by two independent researchers. Digital images were further processed with Image J software (National Institutes of Health, USA). For KGN cells, which exhibit a low-transfection efficiency (<30%), a second transfection was performed for all constructs 24 h after the first one (tandem transfection). We have checked that in terms of morphology there is no change after a tandem with respect to a normal transfection.

Luciferase assays

Biological activity of the different FOXL2 missense mutants and the GFP empty vector (negative control) on target reporter constructs was assessed by the Dual-Luciferase Reporter Assay System (Promega). Any potential interference of the GFP with the luminescence quantification was excluded (i.e. similar results obtained with FOXL2 alone or FOXL2-GFP) (data not shown), which is in agreement with our previous results and of others (8,27). As luciferase reporter construct we used DK3-Luc, which corresponds to the FOXL2 promoter, coupled to the firefly luciferase reporter gene (9). KGN cells were transfected as described above. A Renilla luciferase vector (Promega) was co-transfected in all experiments to monitor transfection efficiency. All luciferase results are reported as relative light units. For each replicate (five for each experiment), the firefly activity observed was divided by the activity recorded from Renilla luciferase vector, and the mean value and standard deviation of the five replicates were calculated. Luminescence was measured using Berthold Lumat LB 9507 luminometer (EG&G Berthold). Statistical significances were evaluated using Student's *t*-test.

Sequence alignment

The amino acid sequences of the FHD of human FOXL2, FOXC1, FOXC2 and FOXP2 were aligned with the ClustalX program.

(<http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html>).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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