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Towards a functional classification of pathogenic FOXL2 mutations using transactivation reporter systems

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Mutations of *FOXL2* are responsible for the Blepharophimosis–Ptotsis–Epicantus-inversus Syndrome (BPES), involving complex eyelid malformations often associated with premature ovarian failure (POF). Loss-of-function mutations are expected to lead to BPES associated with POF, whereas hypomorphic mutations would lead to BPES without ovarian dysfunction. However, multiple exceptions to the genotype–phenotype correlation have been described and missense mutations in the forkhead domain can lead to either type of BPES. This renders almost impossible the prediction of a POF condition from a given genotype. Moreover, no clear-cut correlation between nuclear and/or cytoplasmic aggregation or cytoplasmic retention of mutant FOXL2 forms and the BPES type has been established thus far. Here, we dissect the molecular and functional effects of 10 FOXL2 mutants, known to induce BPES associated with POF or not. We found a correlation between the transcriptional activity of FOXL2 variants on two different reporter promoters and the type of BPES. We used this functional classification framework to explore the behavior of 18 missense mutations leading to BPES of unknown type. The reporters used enabled us to assess the risk of POF associated with these mutations. Moreover, we document a previously overlooked correlation between subcellular mislocalization and aggregation of mutant FOXL2 and the type of BPES, known or predicted using our reporter assays. Thus, intranuclear aggregation and cytoplasmic mislocalization of mutant FOXL2 may be considered as loose predictors of ovarian dysfunction. The functional classification tool described here is a first step towards circumventing the lack of a clear-cut genotype–phenotype correlation in BPES.

INTRODUCTION

FOXL2 is a single-exon gene encoding a transcription factor that contains a typical DNA-binding (forkhead/fkh) domain (1). This domain is composed of three alpha α-helices and two large wing-like loops (2). The protein FOXL2 also contains a polyAlanine (polyAla) tract of 14 residues, which is strictly conserved in eutherian mammals (3). FOXL2 is expressed in peri-ocular tissues, as well as in the somatic compartment (i.e. granulosa cells) of fetal and adult ovaries (1,3,4). FOXL2 has been shown to play a crucial role in normal ovarian development and function (5–7). Recent studies have uncovered a series of potential FOXL2 target genes involved in inflammation, apoptosis regulation, transcription, oxygen free-radical and cholesterol metabolism (8 and references therein). Moreover, it is well established that aromatase, which is crucial for estrogen production, is a target of FOXL2 (9). Mutations of *FOXL2* have been shown to be responsible for the Blepharophimosis–Ptotsis–Epicantus-inversus Syndrome (BPES; MIM 110100) (1). BPES is a genetic disorder which generally displays an autosomal dominant inheritance pattern (1,5,10). Two types of BPES have been identified: in BPES of

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type I, complex eyelid malformations are associated with premature ovarian failure (POF), whereas in type II BPES, there is no ovarian dysfunction (11).

More than one hundred heterozygous *FOXL2* mutations have been reported within and outside the coding region of the gene (*FOXL2* Mutation Database: http://users.ugent.be/~dbeysen/foxl2/) (5,12,13). About two-thirds of the alterations described in BPES cases are intragenic mutations of various kinds. The expansion of the polyAla tract of FOXL2 from 14 to 24 residues accounts for 30% of intragenic mutations and leads mainly, but not exclusively, to isolated palpebral defects. Finally, ~70% of missense mutations described in BPES patients map to the fkh domain.

Previous *FOXL2* mutation screenings have shown a loose genotype–phenotype correlation (14). Indeed, it seemed that loss-of-function mutations, such as early truncating mutations, led to type I BPES (i.e. with a POF), whereas hypomorphic mutations (i.e. ensuring partial function) were expected to lead to type II BPES (no ovarian dysfunction). However, exceptions to the genotype–phenotype correlation have been described since then. This is the case of nonsense mutants, such as p.Y274X, which has been reported as causing both types of BPES in different family members (10). Moreover, missense mutations in the fkh domain can lead to either BPES I (i.e. p.I80T and p.I84S) or II (i.e. p.N109K or p.N105S), which makes it difficult to predict their phenotypic consequences. In our previous studies, the subcellular localization and transactivation ability of 18 of FOXL2 mutant proteins on the promoter of FOXL2 itself were analyzed (5,12,15). Most mutations were found to provoke cytoplasmic retention and intranuclear and/or cytoplasmic aggregation but no clear-cut correlation with the BPES type emerged.

POF is defined by amenorrhea before 40 years of age associated with high plasma levels of gonadotropins (FSH and LH) and low levels of steroid hormones (estradiol and progesterone) (16,17). In the case of BPES, the severity of POF is variable. Patients can present either with primary or secondary amenorrhea (5,18,19). The lack of a clear-cut genotype–phenotype correlation in BPES renders almost impossible the prediction of a future POF condition from a given genotype. Unfortunately, POF raises two major problems in women: the devastating effects of long-term estrogenic hormone deficiency and infertility. Estrogen deficiency increases the risk of osteoporosis, cardiovascular diseases, as well as neurodegenerative processes (5,20,21). At present, hormonal replacement therapy has been used to compensate for the deficiency of estrogens and its adverse effects, like osteoporosis (5,22–24).

By using a combinatorial approach, we have recently identified a high-affinity FOXL2 Response Element (FLRE), which seems to be highly specific (25). In that study, we built an artificial reporter promoter, hereafter referred to as 4 × FLRE-luc, containing four high-affinity FLRE upstream of a minimal CMV promoter driving the expression of the luciferase gene. This promoter was designed to be highly sensitive to the concentration of active FOXL2, and will be used in the present study. A recent analysis has shown that FOXL2 is an important actor of the cellular response to oxidative stress (26). As part of this response, FOXL2 is able to directly upregulate the activity of the promoter of the gene encoding SIRT1, a deacetylase which is in turn able to decrease FOXL2 transactivation ability. Our SIRT1-luc reporter is the most sensitive natural promoter we have ever tested (26) and will also be used here.

In the present study, we dissected the molecular and functional effects of ten FOXL2 mutants, known to induce type I or type II BPES. Our main goal was to identify a potential correlation between the type of BPES and the subcellular localization of the protein and/or an impairment of the transactivation capacity of the mutant proteins. Indeed, we found a clear correlation between the transcriptional activity of FOXL2 on our two different reporter promoters (i.e. 4 × FLRE-luc and SIRT1-luc) and the type of BPES. Moreover, we were able to show that 18 mutations leading to BPES of unknown type could be clustered with either type I or II mutations based on their transactivation abilities. Finally, we also found a loose correlation between the subcellular mislocalization and aggregation of mutant FOXL2 and the type of BPES (known or predicted using our reporter assays). The functional correlations detected here suggest that a mutant completely lacking activity on our reporter promoters is likely to lead to a BPES with POF. This information can be useful in the management of young female BPES patients.

RESULTS

FOXL2 mutations of both type I and type II BPES can induce protein mislocalization and aggregation

In order to better assess a potential connection between FOXL2 mislocalization and aggregation and the BPES type induced by its mutations, we generated a series of constructs driving the expression of mutant FOXL2 forms known to lead to either type I (p.I80T, p.I84S, p.F167X, p.W204X and p.Q219X) or type II BPES (p.N109K, p.N105S, p.K193S, p.Y215C and A224_A234dup10, referred to as Ala24). Our constructs express FOXL2 in fusion with the green fluorescent protein (GFP). These constructs were transiently transfected in COS-7 to evaluate the percentage of cells displaying cytoplasmic staining and/or aggregation, as well as intranuclear aggregation, by direct visualization using fluorescence microscopy, as we have previously described (27).

As expected, FOXL2-WT (wild-type) displayed a nuclear and rather diffuse staining (27) (Fig. 1). However, we found that the subcellular localization and aggregation patterns of the mutant forms were highly variable, irrespective of the BPES type. For instance, a diffuse nuclear distribution was observed for p.F167X (type I BPES), p.W204X (I), p.N109K (II) and p.N105S (II). In contrast, granular or massive aggregates were found in the nuclei of cells transfected with p.Y215C (II) or p.Q219X (I) (Fig. 1). Expression of the other constructs, p.I80T (I), p.I84S (I) and Ala24 (type II), led to nuclear aggregation combined with cytoplasmic retention and granular staining, again irrespective of BPES type. Thus, in this limited data set, it is impossible to detect any correlation between the type of BPES and a perturbation of the physical state or the subcellular localization of the mutant FOXL2 forms.

BPES type correlates with differences of transactivation capacity on two different luciferase reporter systems

Next, we explored a potential connection between the BPES type and the transactivation ability using two different

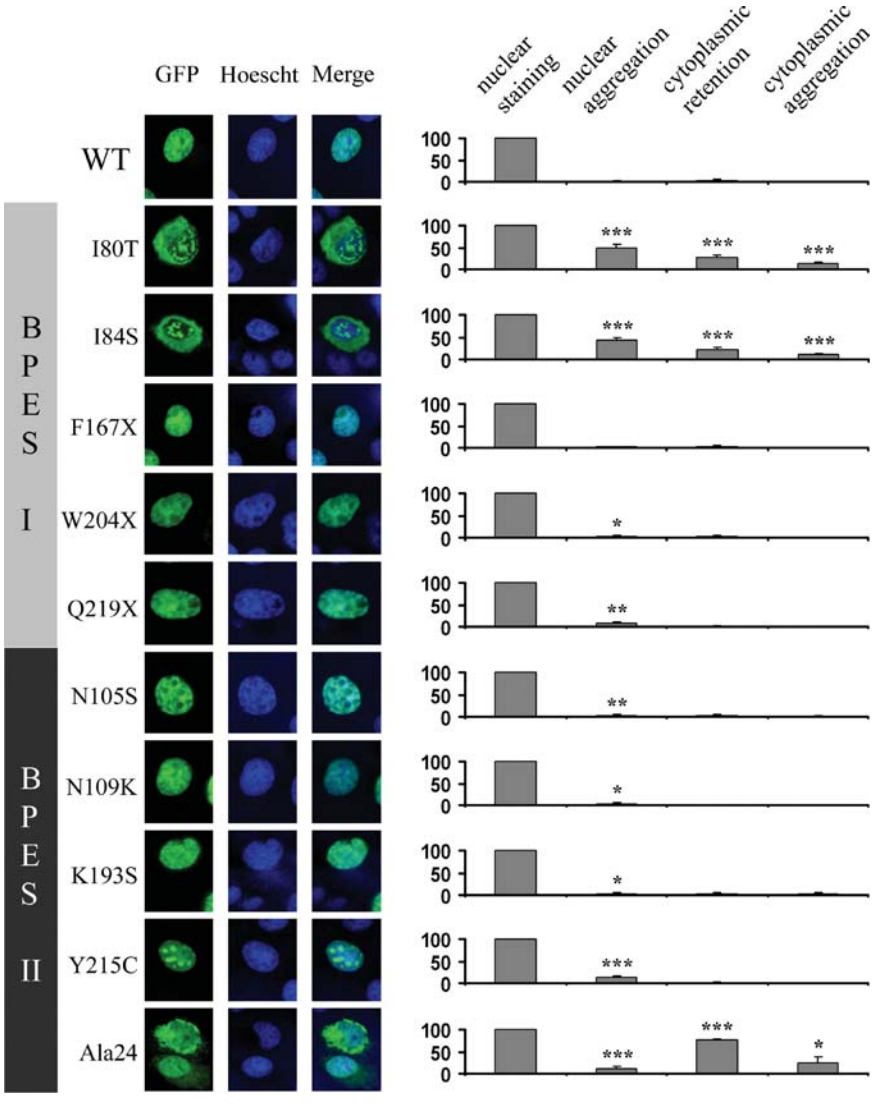

Figure 1. Subcellular localization of wild-type and mutant FOXL2 proteins. The left panel corresponds to the most representative subcellular localization of the FOXL2 as a fusion protein with GFP (green fluorescent protein), in transiently transfected COS-7 cells. Hoechst 33342 stain (blue) in the middle panel shows the localization of the nucleus. The right panel is a merge of the two previous ones. Mutations are ordered according to the type of BPES and the relevant amino acid positions. Three predominant localization patterns could be distinguished: (i) normal localization and distribution (p.F167X, p.W204X, p.N105S, p.N109K and p.K193S); (ii) intranuclear aggregation (p.Y215C); (iii) nuclear and cytoplasmic aggregation (p.I80T, p.I84S and Ala24). The rightmost graphs display the quantitative representation of subcelullar localization of FOXL2-GFP variants. Percentages are representative of 200 transfected COS-7 cell. Asterisks stand for statistically significant differences with respect to the WT protein ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$).

luciferase reporter systems. We decided to use two different reporter constructs, which we had shown to be very sensitive to even small amounts of active FOXL2. The first one was the 4 × FLRE-luc reporter, on which the FOXL2-Ala24 mutant retained some transcriptional activity (25). The second one was the SIRT1-luc reporter (26). Indeed, SIRT1 is a direct target of FOXL2, and our previous studies have shown that its promoter is very sensitive to FOXL2 (26).

We performed the transfections for functional studies in KGN cells (28) that naturally express FOXL2 and that constitute a suitable cellular model to study its activity (8). Specifically, KGN cells were transfected with the 4 × FLRE-luc or SIRT1-luc construct and one of the constructs driving the expression of a FOXL2 variant (along with the internal normalizing plasmid pRL-RSV). As expected for 4 × FLRE-luc, FOXL2−WT induced an important increase of luciferase activity when compared with the control (empty pcDNA-GFP vector, Fig. 2A and B). Interestingly, FOXL2 variants inducing type I BPES (p.I80T, p.I84S, p.F167X, p.W204X and p.Q219X) displayed activity levels significantly reduced when compared with the WT and not different from that of the empty vector (Fig. 2A). On the contrary, the constructs containing the mutations inducing type II BPES (p.N105S, p.N109K, p.K193S and p.Y215C) displayed activities not different from that of the WT (Fig. 2A). Interestingly, FOXL2-Ala24, which has been associated with both types of BPES, displayed an intermediate activity (i.e. different from that of both the empty vector and WT FOXL2 in a three-way

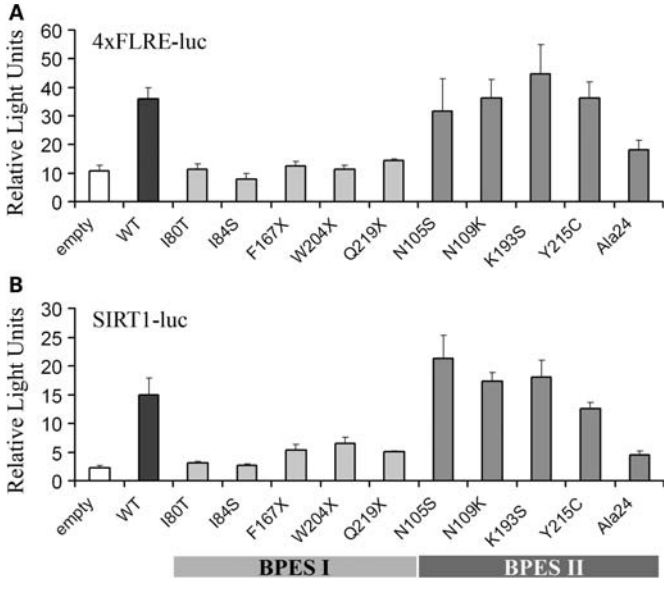

Figure 2. Transcriptional activities of FOXL2 mutant variants with known BPES type, studied through luciferase assays in KGN cells. Relative light units (RLU) correspond to the ratio of the activity of the firefly luciferase reporter over the levels of the renilla reporter (internal control of transfection efficiency). The transcriptional activity was tested on two different promoters: (**A**) activities on the 4 × FLRE-luc reporter. The white bar shows the basal activity of the promoter in absence of FOXL2. The black bar represents the transcriptional activity of FOXL2-WT. Light grey bars represent the type I BPES mutants and dark grey bars the type II BPES mutants. According to a Tukey–Kramer Minimum Significant Difference test, the type I BPES mutants displayed an activity significantly reduced compared with the WT ($P < 0.05$) and not different from that of the empty vector. The constructs containing the mutations inducing type II BPES displayed activities similar to that of the WT. FOXL2-Ala24 displayed an intermediate activity (i.e. different from that of both the empty vector and WT FOXL2 in a three-way Tukey–Kramer MSD test, but not significantly different from the activity of the empty vector in a multiple comparison with the whole set of mutants). (**B**) Activities on the SIRT1-luc reporter. The trends are similar as above. However, p.W204X displayed an intermediate activity between that of pcDNA3.1 and WT (according to a Tukey–Kramer MSD test).

Tukey–Kramer Minimum Significant Difference test/MSD). When the reporter SIRT1-luc was analyzed, the trends were similar (Fig. 2B). However, p.W204X displayed an intermediate activity between that of pcDNA3.1 and WT (according to a Tukey–Kramer MSD test), whereas Ala24 was found to be different from the WT but not from the empty vector.

Both reporter promoters displayed correlated transcriptional responses for the various FOXL2 variants. Indeed, the Pearson's correlation coefficient R of the log-transformed average relative light units (RLU) was 0.92 ($P < 10^{-5}$, $n = 12$, including pcDNA3.1 and WT). Our results, taken together, show that there is a good correlation between the transcriptional activity of mutated FOXL2 proteins on both 4 × FLRE-luc and SIRT1-luc reporters and the type of BPES they lead to.

Functional exploration of 18 missense mutations leading to an unknown type of BPES

We also examined the activity of 18 disease-causing missense mutations (p.S58L, p.I63T, p.A66V, p.E69K, p.S70I, p.I84N, p.F90S, p.W98G, p.S101R, p.I102T, p.R103C, p.H104N, p.L106P, p.L106F, p.L108P, p.S217F, p.S217C and p.A253fs) using our reporter systems. The pathogenic character of these mutations has been well documented, but the type of BPES they lead to is still unknown because they were detected in sporadic pre-pubertal female patients or in male patients (10,14,15,29). We performed the luciferase reporter analysis as described earlier (Fig. 3). Again, as shown in Figure 4A, the transcriptional responses of the 18 FOXL2 variants on both reporter promoters were correlated (Pearson's $R = 0.86$, $P < 10^{-6}$, $n = 18$ or $R = 0.88$, $P < 10^{-11}$, $n = 30$, including the type I and II mutants, pcDNA3.1 and FOXL2-WT). Interestingly, two well-defined clouds of points enriched in type I and type II mutants can be easily spotted in Figure 4A, which might in principle allow the classification of variants associated with unknown BPES type. In order to further explore this possibility more objectively, we conducted an unsupervised hierachical clustering capturing simultaneously the information of both reporters and arranging the mutants in groups according to the degree of similarity of their transcriptional activities. This analysis showed that p.S58L, p.A66V, p.S70I, p.I84N, p.W98G, p.S101R, p.I102T, p.R103C, p.L106P, p.L106F, p.L108P and p.A253fs clustered with the type I mutations. Not surprisingly, Ala24 was clustered with type I mutants. On the contrary, p.I63T, p.E69K, p.F90S, p.H104N, p.S217F and p.S217C were clustered with type II mutations (Fig. 4B). We have previously studied the transcriptional activity of some of these mutants on the promoter of FOXL2 itself (12). Thus, we asked whether taking into account this information (corresponding to Fig. 3 of reference 12) confirms or modifies the results of the hierarchical clustering. As shown in Supplementary Material, Figure S1, the combined data from the three reporter promoters supports the functional segregation of mutants observed in Figure 4B, in spite of the fact the pFOXL2-luc reporter alone does not discriminate the mutations very well because of its lower sensitivity to active FOXL2.

Combined analysis of subcellular localization, aggregation and functional data

The small number of type I and II mutations studied above prevented us from assessing the existence of a correlation between BPES type and a potential perturbation of the physical state or the subcellular localization of the mutant FOXL2. We re-examine this question here, by combining the subcellular localization with the functional data. First of all, we calculated the Pearson correlation coefficient between the percentage of transfected cells displaying intranuclear or cytoplasmic staining and/or aggregation and the activity of FOXL2 mutant forms on the 4 × FLRE-luc and SIRT1-luc reporters (re-analysis of data from the present paper and previous ones (12,15,29)). Interestingly, we found a significant negative correlation between transcriptional activity on 4 × FLRE-luc (or SIRT1-luc) and cytoplasmic retention or intranuclear aggregation (Fig. 5A and B, respectively). This means that, as intuitively expected, protein mislocalization and aggregation has a negative impact on FOXL2 transcriptional activity.

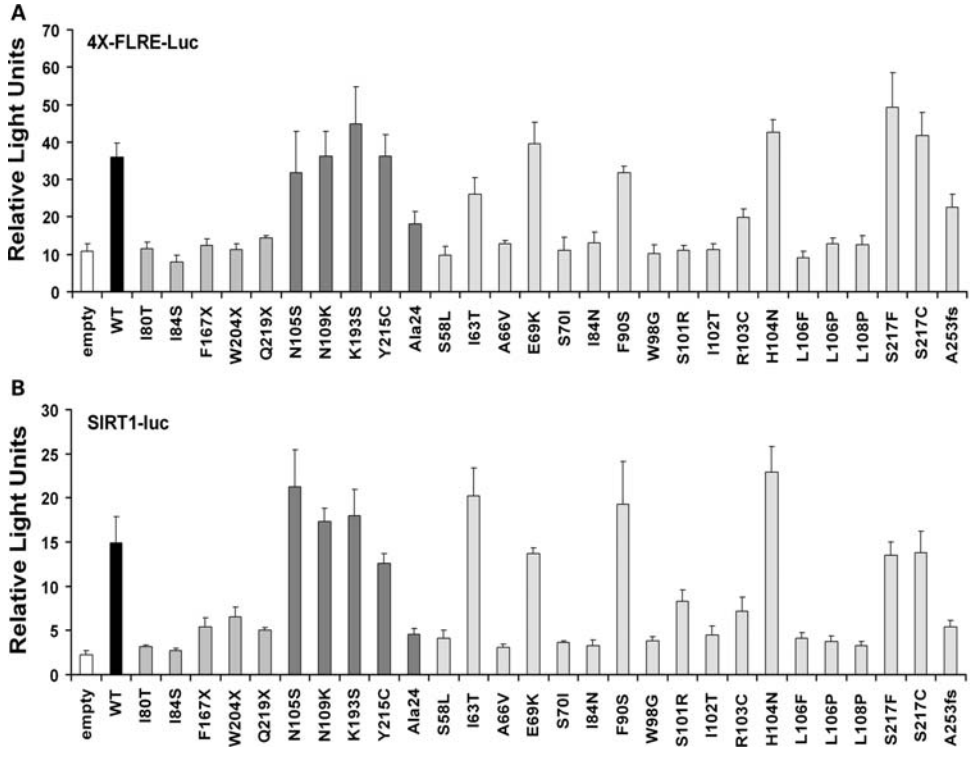

Figure 3. Transcriptional activities of FOXL2 mutated variants using two different reporter promoters. (**A**) Relative luciferase activity on 4 × FLRE-luc reporter, (**B**) relative luciferase activity on the SIRT1-luc reporter. The white bar shows the basal activity of the promoter in cells in absence of FOXL2 overexpression. The black bar is the transcriptional activity of FOXL2-WT. Light grey bars represent the type I BPES mutants and dark grey bars the type II BPES mutants (known/training set). Shaded bars represent the BPES mutants of unknown type. Data concerning the training set are the same as in Figure 2 and were obtained in the same experiments (to make the data sets directly comparable).

Next, we explored whether there was a link between the type of BPES (known or predicted) and the subcellular localization and/or the physical state of the mutant proteins. Specifically, we calculated the average percentage of cells displaying intranuclear aggregation and/or cytoplasmic staining for the missense mutants, known or predicted by the reporter systems to lead to either type of BPES. We found that type I BPES (known or predicted) missense mutations lead to significantly higher aggregation or mislocalization than type II mutants (Fig. 6).

A simplified classification framework of FOXL2 mutants

Finally, we have also sought to simplify the functional analysis by trying a classification focusing only on the 4 × FLRE-luc reporter, because as an artificial promoter it is expected to detect variations of FOXL2 transcriptional activity with minimal background effects from other cellular components, which is more difficult with the stress-responsive SIRT1 promoter. First, we estimated the probability of having type II BPES given the reporter activity using a logistic regression (Fig. 7A and B). As shown in Figure 7A, the *x*-threshold value (log transformed average RLU), corresponding to a $P = 0.5$, separating mutations of type I and type II, was estimated at 1.21. As the activities displayed by the two classes of mutants do not overlap, the slope of the sigmoid was quite steep and all mutations had a binary probability (i.e. either 0 or 1) of leading to BPES type II. This logistic regression approach classified p.S58L, p.A66V, p.S70I, p.I84N, p.W98G, p.S101R, p.I102T, p.L106F, p.L106P and p.L108P as potentially leading to type I BPES, whereas p.I63T, p.E69K, p.F90S, p.R103C, p.H104N, p.S217F, p.S217C and p.A253fs were classified as potentially leading to type II BPES. Most of the mutations (apart from p.R103C, p.A253fs and Ala24) were classified in agreement with the hierarchical clustering. However, this logistic approach classified Ala24 as type II (i.e. in agreement with the most common ovarian phenotype induced by this mutation). To simplify the analysis even more, we used only the WT construct (standing for type II mutations) and the empty vector pcDNA (standing for the type I mutations), as a reference framework for classification. Then, we compared the output of each mutant construct with that of the WT and the empty vector, using a simple Student's *t*-test (Fig. 7C). We found that the classification using this minimal reference system and the one obtained when using the full set of (type I and II) mutants on the 4 × FLRE-luc reporter or the hierarchical approach where highly consistent. Nevertheless, we noticed that p.Q219X (type I) and Ala24 (type II) displayed a borderline behavior (different from both pcDNA and WT). This is not surprising, given that the activity of p.Q219X is rather important for a type I mutant on this promoter and that, on the contrary, the activity of Ala24 is rather low for a type II mutation. Interestingly, those mutations for which the 4 × FLRE-luc system (including the training set of well-classified mutations) disagreed with the hierarchical clustering using the compound variable could not be classified using the simplified framework.

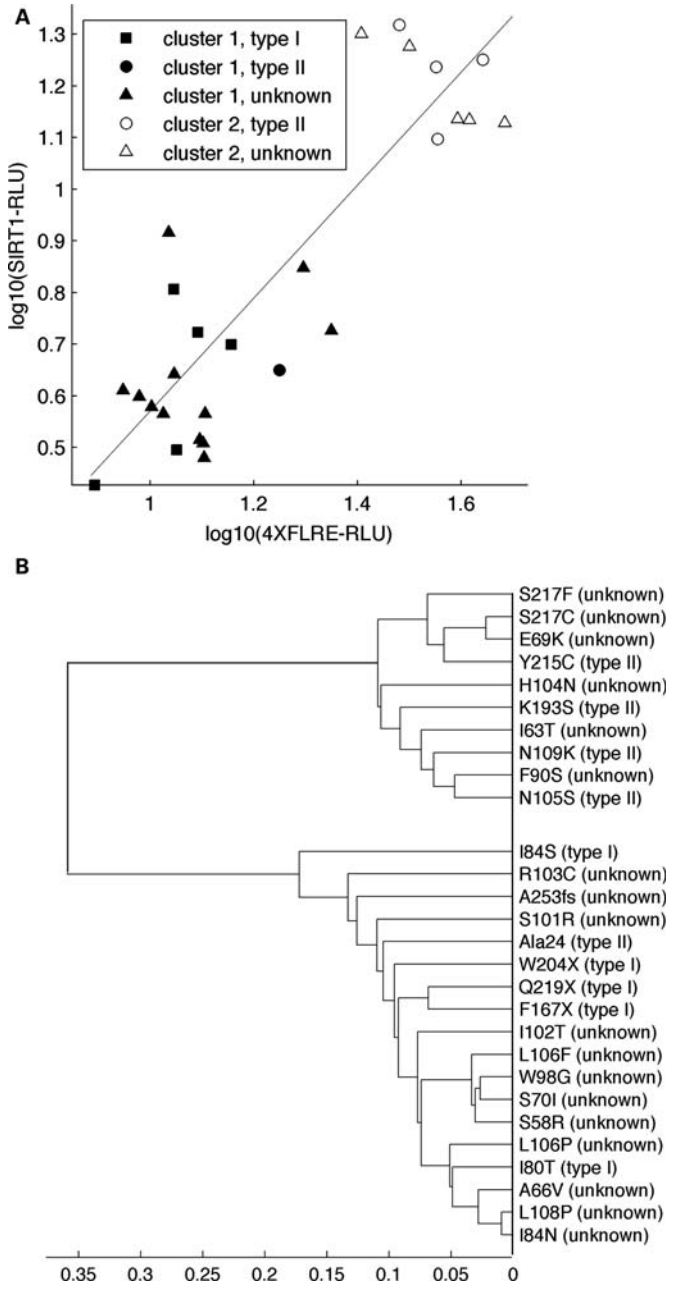

Figure 4. (**A**) Correlation between the transactivation abilities of FOXL2 mutants on 4 × FLRE-luc and SIRT1-luc reporters (decimal logarithms of average RLU). Notice the 'clustering' of the various FOXL2 variants into two well-separated clouds enriched in type I and II mutations. The regression line is represented. (**B**) Hierarchical classification of the FOXL2 variants according to their transactivation capacities on both 4 × FLRE-luc and SIRT1-luc reporters. There is a perfect agreement between this classification and the clusters of (A).

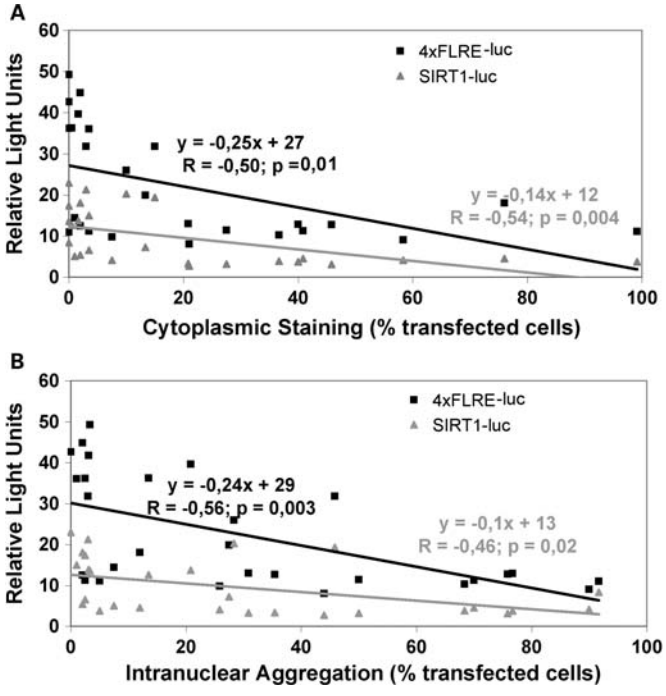

Figure 5. Negative correlation between cytoplasmic mislocalisation of mutant FOXL2 (**A**) or intranuclear aggregation (**B**) and transactivation capacity on the two reporter promoters tested. Notice that the relationship is clearly nonlinear (but a linear regression is shown for simplicity).

DISCUSSION

Molecular behavior of type I and type II BPES-causing FOXL2 mutations

Our results concerning the subcellular localization of a limited set of FOXL2 mutant forms leading to type I or II BPES show that there is no obvious correlation between the subcellular localization of the protein or its aggregation and the type of BPES induced by the corresponding mutations. Indeed, p.I80T and p.I84S lead to type I BPES and induce cytoplasmic retention and nuclear and/or cytoplasmic aggregation. A seemingly similar cellular phenotype is observed for p.Y215C, which induces a type BPES II. However, the existence of ultra-structural differences in the aggregates, undetectable at the light microscope level, cannot be ruled out. This apparent lack of correlation can also simply be due to a sampling effect because, unfortunately, the BPES type is not known for a large number of FOXL2 mutations, which prevents realistic statistical comparisons. Indeed, when we consider the combined data of known and predicted type I and type II missense mutations, we found that, as intuitively expected, the former lead to a higher percentage of cells displaying intranuclear aggregation and cytoplasmic staining than the latter. This suggests that (i) the molecular defects induced by type I mutations should be much more damaging than the structural lesions leading to type II BPES and that (ii) intracellular protein aggregation/mislocalization is a loose predictor of ovarian dysfunction associated with FOXL2 mutations. It is also worth noting that none of the mutations studied here affect the NLS sequences of FOXL2, which could have explained their cytoplasmic retention (30,31). However, we cannot exclude that cytoplasmic oligomerization or aggregation might lead to the formation of structures whose size would be incompatible with nuclear import, or that might bury the NLS inside the multimers.

To test the functional impact of the mutant versions of FOXL2, we chose two reporter systems displaying a high

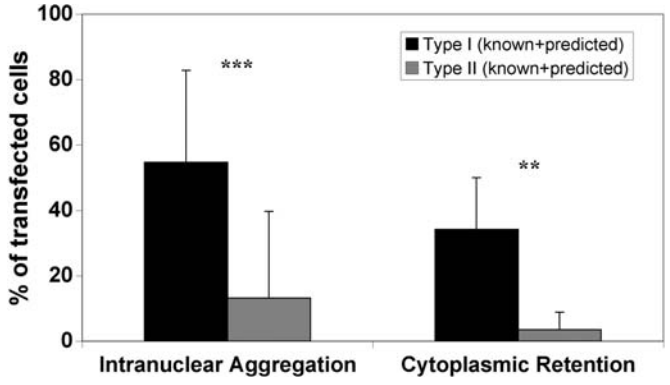

Figure 6. Correlation between the known or predicted BPES type and nuclear aggregation or cytoplasmic retention induced by FOXL2 mutations.

degree of sensitivity to FOXL2. Our functional results show that there is a correlation between the transactivation ability of FOXL2 mutant forms on both 4 × FLRE-luc and SIRT1-luc, and the type of BPES. In fact, the BPES I mutants seem to have a strongly decreased transactivation activity on these promoters, whereas the BPES II mutants are still (at least partially) active. The mutant Ala24 was difficult to classify, but this is not completely surprising, because patients bearing this mutation may have ovarian phenotypes ranging from normal to POF (29). Moreover, this mutation seems to be sensitive to the cellular context. Indeed, we have previously shown that the proportion of cells with nuclear aggregation and cytoplasmic retention of FOXL2-Ala24 is proteome-dependent (32). The type of BPES induced by polyAla expansions might depend on environmental effects and potential polymorphisms in modifier genes, such as chaperones. The intermediate (or even conflicting) behavior of Ala24 on the two promoters used here is a good representation of the phenotypic variability induced by this mutation (and perhaps others) and shows that these predictions although potentially helpful should be taken with caution.

Interestingly, there is no obvious correlation between the type of BPES (known or predicted) and the localization of the mutation within the protein. For instance, p.S58L and p.A66V seem to lead to BPES I, whereas p.I63T and p.E69K are predicted to lead to BPES II, even if they all map to the first helix of the fkh domain. Mutations p.I80T, p.I84S and p.I84N affect residues in the second α-helix of the fkh domain and are known or predicted to lead to type I BPES. However, with only two examples, no solid conclusion can be drawn. Finally, mutations in the third α-helix of the fkh lead also to phenotypic heterogeneity, depending on the particular residue affected (and the nature of the mutation). The study of the structural and functional impact of mutations of FOXC1 and C2, two other forkhead factors, allowed the establishment of a predictive model (33). These authors concluded that mutations in the first α-helix of the fkh destabilize protein–DNA interactions, interfere with the transactivation activity and affect subcellular localization. On the contrary, mutations in the second α-helix of the fkh do not affect subcellular localization, but decrease transcriptional activity. Finally, mutations in the third α-helix have consequences on nuclear localization and DNA binding, by destabilizing the recognition helix and altering binding specificity (33). Our previous studies indicate that this model is not directly applicable to FOXL2 (12,29). Here, we confirm these previous findings. Moreover, we provide statistical evidence indicating the existence of a previously overlooked relationship between protein mislocalization and/or aggregation and the BPES type they lead to. However, this link, although intuitively expected, is ill-defined and partialy relies on the analysis of mutations for which BPES type was predicted.

The distinct molecular behaviors of type I or type II FOXL2 mutations on two high-affinity target promoters further supports a hypothesis that we have previously formulated (25,32). Ovarian function is generally less sensitive to the effects of FOXL2 mutations than other FOXL2-regulated functions, such as eyelid development (i.e. all BPES patients present with craniofacial defects, whereas only a subset also develop POF). Thus, we have previously hypothesized that the absence of ovarian phenotype in BPES type II patients would result from the ability of some FOXL2 mutants to properly regulate crucial high-affinity ovarian targets (25,32). This may be linked to the important role of FOXL2 in reproduction. Selection favoring high-affinity targets might counteract the negative effects of at least some FOXL2 mutations on ovarian function and thus, reproduction. Our observation that FOXL2 mutants leading to BPES type II are generally able to properly regulate SIRT1 transcription, whereas type I mutants are not, suggests that SIRT1 should be one of these crucial FOXL2 ovarian targets. This may have important consequences for ovarian biology and function if we consider that SIRT1 and its orthologs are crucial regulators of aging across evolution through their ability to protect against cellular stress and to promote longevity (5,34–36). This and other still-open questions will be addressed in the future. Namely, a series of knock-in human cell lines for some FOXL2 mutations described earlier will be prepared. In-depth genome-wide analyses of such cellular models (i.e. transcriptomics and ChIP-on-Chip) will allow their exhaustive classification and hopefully provide insights into the molecular basis of BPES.

A predictive tool of BPES type? Promises and challenges

A mutation classification tool should take into consideration as much information as possible: functional data from several reporter promoters, protein aggregation and mislocalization, structural impact of the mutation, etc. However, this amount of data is often difficult to obtain. Here we show that testing the activity of a mutant FOXL2 with respect to pcDNA3.1 and FOXL2-WT on 4 × FLRE-luc provides a reasonable classification of the BPES type induced by the relevant mutation. Indeed, a loss-of-function allele, whose corresponding protein is unable to activate the highly sensitive 4 × FLRE-luc promoter, will very likely lead to POF. In such cases it is necessary, and recommended a close follow-up, to monitor the onset of ovarian dysfunction, which can be highly variable (i.e. between puberty to 40 years of age). In the case of steadily increasing FSH levels, new therapeutic options exist at present. For instance, human ovarian tissue

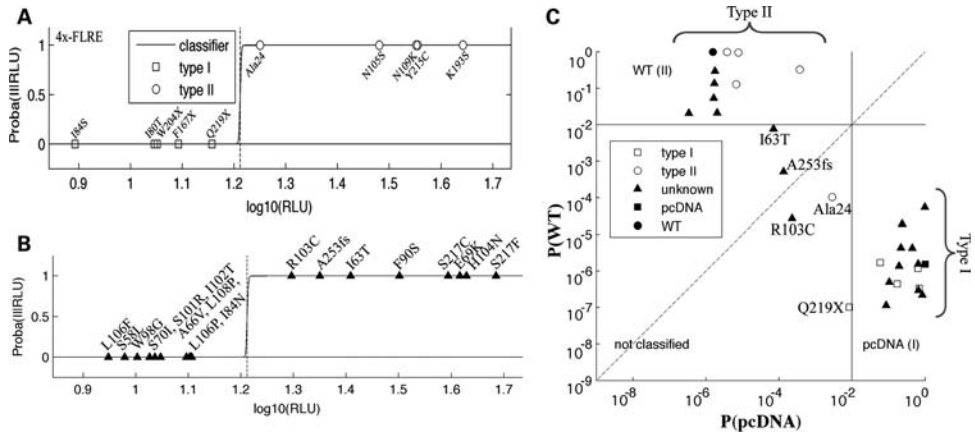

Figure 7. (**A**) Logistic classifier obtained using the 4 × FLRE-luc reporter system. X-axis: decimal logarithm of the average RLU values for each mutant form. Y-axis: estimated probability of developing a type II BPES given the transactivation activity of the mutant (log RLU). The threshold of decision, separating type I and II mutations, is 1.21 (obtained from the analysis of type I and II mutations). (**B**) Logistic classification of mutants of unknown BPES type (represented by full triangles). The sigmoidal function is that obtained from the analysis of the type I and II mutations (training set). (**C**) Comparison of the activities of mutant FOXL2 with respect to pcDNA and WT using 4 × FLRE-luc reporter data. The axes represent the *P*-values obtained in Student's *t*-tests for comparisons involving transactivation data of a particular mutant and either pcDNA (empty vector, x-axis) or the FOXL2-WT protein (y-axis). Most mutations leading to known BPES type (empty squares, type I and empty circles, type II) were successfully classified except Ala24 and p.Q219X, which displayed a borderline behavior. The continuous lines represent the first species risk used (1%). Thus, 'not classified' mutants had activities statistically different from those of the empty vector and WT FOXL2 with $P < 10^{-2}$. Most other mutants were classified in agreement with the results of other classifiers discussed in the text.

has been successfully cryopreserved, with good auto-graft survival and function after re-implantation, leading to restored ovarian function and several healthy live births (34–37). Hopefully, in the future, new therapies able to delay the onset of POF will also be available for young BPES patients.

MATERIALS AND METHODS

Plasmid constructs

The constructs expressing FOXL2-WT-(wild-type)-GFP and FOXL2-Ala24-GFP are those previously described (27,32). The FOXL2 constructs with the I80T and N109K mutations and those with mutations leading to (yet) unclassified BPES are those we have previously reported (12,15,29). The mutations I84S, Y215S, N105S and K193S were introduced by junction-PCR (38) using the FOXL2-WT-GFP plasmid as template. The truncated mutants p.F167X, p.Q219X and p.W204X were obtained by classical PCR using the FOXL2-WT-GFP plasmid as template. All the PCR fragments were cloned into the pCDNA3.1-CT-GFP-topoTA cloning vector (Invitrogen, CA, USA) in fusion with the GFP ORF. Constructs were screened by colony-PCR for properly oriented inserts and sequenced to exclude the existence of PCR-induced mutations. The artificial FOXL2-responsive promoter (4 × FLRE-luc) driving the expression of luciferase has previously been described (25). In short, it is based on a pGL3-Basic firefly luciferase reporter (Promega, Madison, WI, USA), driven by an artificial promoter containing four high-affinity FLRE upstream of a minimal CMV promoter. The SIRT1-luc construct contains about 1 kb of the human SIRT1 promoter, driving luciferase expression in the pGL3-Basic vector (Promega, Madison, WI, USA) and was also previously described (26).

Cell culture and transfections

COS-7 cells (African green monkey kidney cell line) were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-Invitrogen, CA, USA), KGN cells (28) were maintained in DMEM-F12 (Gibco-Invitrogen), both media were supplemented with 10% fetal calf serum (Gibco-Invitrogen) and 1% penicillin/streptomycin. Cells were seeded 24 h before transfection at 30% (COS-7) or 50% (KGN) of confluence. To analyze subcellular localization and potential aggregation, cells were seeded in 24-well plates containing sterile coverslips for direct FOXL2-GFP visualization. The cells were transfected with 1 μg of DNA per well, using the calcium phosphate method, and rinsed 24 h after transfection.

Fluorescence microscopy

Forty-eight hours after transfection, cells transfected over coverslips were washed with phosphate-buffered saline solution, and fixed for 15 min with 4% paraformaldehyde. Nuclei were stained with the Hoechst 33342 dye (Invitrogen, CA, USA), and coverslips were mounted on slides using fluorescence mounting medium (DAKO, CA, USA). Transfected cells were visualized by epifluorescence microscopy (Nikon E600). Nuclear and cytoplasmic staining/aggregation were scored as previously described (27). For each construct, 200 GFP-positive COS-7 cells were analyzed from three different transfection experiments.

Luciferase assays

The biological activity of the various FOXL2-GFP mutants and the GFP empty vector (negative control) on two reporter constructs (SIRT1-luc and 4 × FLRE-luc) was assessed with

the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) as previously described (30). Briefly, KGN cells were transfected as described earlier with a total of 1 μg per well [i.e. 570 ng of reporter, 400 ng of GFP constructs and 30 ng of the internal renilla luciferase control vector pRL-RSV (Promega, Madison, WI, USA)]. Luciferase results are reported as RLUs. For each replicate (five for each experiment), the observed firefly activity was divided by the activity recorded from renilla luciferase internal control vector, and the average and standard deviation of the five replicates were calculated. Luminescence was measured using an EG-G Berthold Lumat LB 9507 luminometer.

Statistical analysis

The existence of statistical differences in the percentages of cells displaying cytoplasmic staining and/or aggregation, as well as nuclear aggregation, were assessed as we have previously described (27).

In order to set up the logistic classification tool, we assumed Gaussian distributions for the log 10-transformed activities of the luciferase reporter outputs (RLU), and similar variances. The posterior probabilities of mutations of being of type II, given the reporter activity (x), were adjusted to follow a logistic function:

$$\mathrm{p(type\ II|x)} = 1/[1 + \exp(-(a_0 x + b_0))]$$

where a_0 and b_0 are two parameters with unknown value and which can be estimated by a maximum likelihood approach (39). Hierachical clustering of the transactivation activity of FOXL2 on the two reporter promoters was performed as described in Hastie *et al*. (39), using the log 10-transformed average activities of the luciferase outputs (RLU) and Euclidean distances as distance metrics.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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