

# The identification and characterization of a FOXL2 response element provides insights into the pathogenesis of mutant alleles

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**The Forkhead transcription factor FOXL2 plays a crucial role in ovarian development and maintenance. In humans, its mutations lead to craniofacial abnormalities, isolated or associated with ovarian dysfunction. Using a combinatorial approach, we identified and characterized a FoxL2 response element (FLRE) and showed that it is highly specific and that it diverges from that of other Forkhead transcription factors. This specificity should prevent aberrant regulation of FOXL2 targets by other members of the family and should prevent ectopic activation of the ovarian differentiation program in testes. We provide evidence that the FLRE is used in naturally occurring promoters. We show that polyAlanine expansions of FOXL2, which are the most frequent pathogenic mutations, induce a length-dependent loss of response on different artificial promoter reporters depending on the number and sequence of the FLREs that they contain. Thus, we provide clear mechanistic evidence explaining how the architecture of promoters influences their sensitivity to decreased transcription factor availability. Furthermore, we speculate that the generally absent ovarian phenotype of patients carrying the most frequent polyAlanine expansion should come from its ability to properly regulate high-affinity ovarian targets. The existence of critical high-affinity ovarian targets would be compatible with the role of FOXL2 in reproduction and ensure developmental and functional robustness. Taken together, our results give mechanistic insights on the molecular pathogenesis of FOXL2 polyAlanine expansions.**

## INTRODUCTION

The superfamily of Forkhead Box (Fox) transcription factors comprises more than 100 different members, found in evolutionary distant species, ranging from yeast to humans. Their main common feature consists of a highly conserved DNA-binding motif, known as the Forkhead box or ‘Winged Helix’ domain. The Forkhead domain, typically about a 100 amino acids long, folds into a 3D structure containing three N-terminal  $\alpha$ -helices, three  $\beta$ -strands and two loop regions

located at the C-terminus of the domain (1). In opposition to the high conservation of their DNA-binding domain, Fox proteins are highly divergent in other portions of their sequences.

A phylogenetic analysis has allowed the classification of Forkhead transcription factors into subfamilies (2). As of today, 19 forkhead proteins subfamilies, labeled A to S, have been identified. Forkhead transcription factors display a very wide range of expression patterns, regulation, and physiological functions. They have been found to operate in processes ranging from eye organogenesis (FoxC1–2) to

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**Table 1.** Described high-affinity binding sites of various Forkhead transcription factors

FOX factor name	Name in original description	High-affinity binding site described									Original description
FOXF2	FREAC-2	G/A	T	A	A	A	C/T	A	A		(9)
FOXC1	FREAC-3	G	T	A	A	A	C/T	A	A		(9)
FOXD1	FREAC-4	G/A	T	A/C	A	A	C	A	N		(9)
FOXL1	FREAC-7	G/A	T/C	A/C	A	A	C/T	A	N		(9)
FOXQ1	HFH-1	A/C	T	A	A	A	C	A	A/T		(10)
FOXD3	HFH-2	A/T	T	A	A	A	C	A	A/T		(10)
FOXA3	HNF-3	G/A	T/C	A/C	A	A	C/T	A	A/T		(10)
FOXK2	ILF-1	G	T	A	A	A	C	A	A		(11)
FOXO1	FKHR	G	T	A	A	A	C	A	A		(12)
FOXO3A	FKHRL1	G	T	A	A	A	C	A	A		(12)
FOXO4	AFX	G	T	A	A	A	C	A	A		(12)
FOXP1	n/a	A	T/C	A	A	A	C	A	A		(13)
FKH	General consensus	G/A	T/C	A/C	A	A	C/T	A	N		
FOXL2	n/a	G	T	C	A	A	G	G	T/C		Present study

Sites were aligned following the central 'AA' and reverse complemented when needed for reading convenience.

language acquisition [FoxP2 (3)], including stress response, ageing regulation and tumor suppression [FoxO (4)], as well as ovarian determination and female fertility [FoxL2 (5–7)]. Some have been found to be key players in cancer development (8). Consequently, mutations in several FOX proteins have been associated with various human pathologies (3).

To this day, the consensus binding sites for 12 distinct mammalian Forkhead proteins have been identified (Table 1), and a 'general forkhead consensus binding site' has been established as 5'-(G/A)(T/C)(A/C)AA(C/T)A-3' (9–14). Despite the high conservation of the Forkhead domain, the diversity of biological processes that are under the regulation of FOX factors suggests that some degree of specificity should exist to allow different members to achieve distinct functions. This can result from three non-exclusive mechanisms: (i) differences in expression patterns [for instance, the high peri-ocular expression of FOXE3 (15), or the high hepatic expression of FOXA3 (16)], (ii) some specificity in target sequence recognition (which, considering the high homology of described high-affinity binding sites, might seem limited; Table 1) and/or (iii) existence of different co-factors (either because they are tissue-specific, or because Forkhead factors contain different protein/protein interaction domains).

FOXL2 is mutated in the blepharophimosis ptosis–epicanthus–inversus syndrome (BPES), a genetic disorder characterized by craniofacial and palpebral defects, occurring in association with premature ovarian failure (type I BPES), or isolated (type II BPES (5,17)). It is mostly expressed in periocular and ovarian follicular granulosa cells (18,19), but its pattern of expression could be wider (20). FOXL2 possesses a polyAlanine domain of 14 residues, conserved among mammals (19). Interestingly, 30% of described intragenic FOXL2 mutations consist of expansions of this domain (21,22). Expansions to 24 or 26 alanines induce a dominant phenotype (FOXL2-Ala24/Ala26), whereas expansion to 19 leads to a recessive form [FOXL2-Ala19 (23)]. We have previously shown that the availability and mobility of FOXL2 decreases with the size of the polyAlanine domain, which translates in a size-dependent loss of its transactivation ability (24). In *Foxl2* knockout mice, correct primary follicles fail to develop, and, two weeks after birth, follicular activation

in the presence of defective granulosa leads to massive atresia and premature follicular depletion (6,7). This indicates a crucial role of FOXL2 in female fertility and ovarian maintenance. Moreover, recent data shows that forced *Foxl2* expression induces a partial impairment of testis tubule differentiation in XY transgenic mice (25).

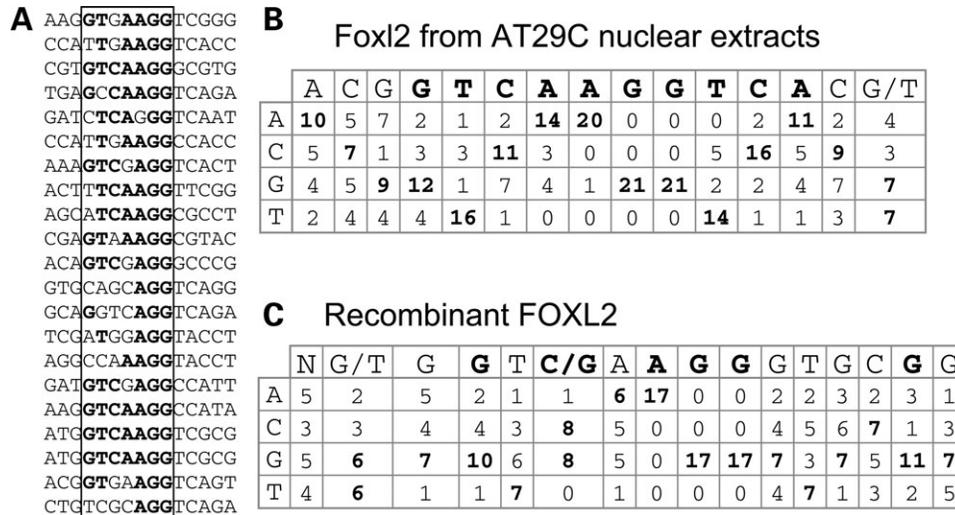
The gonad is unique among all organ primordia because of its bipotential nature (26). A single primordium will develop into one of two organs, a testis or an ovary. Given the critical role of FOXL2 in ovarian differentiation, it is expected to regulate very specifically the ovarian developmental program. One of the ways to achieve this kind of specificity, even when other members of the Forkhead superfamily might be synexpressed, would be to regulate gene expression through a rather unique high-affinity binding site. Moreover, a further layer of specificity could be provided by the interaction between FOXL2 and specific developmentally-regulated partners.

Here, we identify a high-affinity binding site of FOXL2. This site displays significant divergence from the usually admitted Forkhead consensus binding sequence. We show that it functions as a specific FoxL2 response element (FLRE) both *in vitro* and closer to *in vivo* situations. Moreover, using our polyAlanine-expanded FOXL2 allelic series (24), we validate the theoretical predictions according to which distinct target promoters of a transcription factor will display distinct sensitivities to its availability, according to the number and/or affinity of the binding sites they contain. This provides insights into the differential pathogenesis induced by various polyAlanine-expanded alleles. Finally, we provide evidence of the existence of a promoter-dependent interference exerted by the frequent FOXL2-Ala24 mutant on the activity of normal FOXL2.

## RESULTS AND DISCUSSION

### The FLRE diverges from the general Forkhead consensus

Supposing that all Forkhead transcription factors share very similar high-affinity binding sites (Table 1), the presence of many FOX factors in a single cell would be problematic to achieve specific functions. The crucial involvement of



**Figure 1.** Determination of FOXL2 binding site consensus sequence. (A) PCR-selection output using Foxl2 from AT29C nuclear extracts; the site is boxed, letters from the site that correspond to the consensus are in bold. (B) Consensus matrix established using the PCR-selection sequences obtained with Foxl2 from AT29C nuclear extracts. (C) Consensus matrix established using the PCR-selection sequences obtained with recombinant FOXL2.

FOXL2 in ovarian development, and presumably in the repression of testis differentiation (27), suggests a need for high target specificity when compared with more widely expressed and more ‘common purpose’ Forkhead factors, like members of the O subfamily. This problem is obvious in the human granulosa-like KGN cell line (28), which endogenously expresses FOXL2 (29). Indeed, an analysis of its transcriptome shows that at least 18 other FOX transcription factors out of the 40 reported in humans, from 11 distinct subfamilies, are expressed above background (ArrayExpress accession number E-MEXP-985, 29; Supplementary Material, Fig. S1).

To explore how FoxL2 can achieve its specific role in female reproduction, we sought to determine a high-affinity binding site of FoxL2. We performed PCR selection using a library of double-stranded DNA fragments with a central degenerate sequence and nuclear extracts from the murine granulosa cell line AT29C (30), which expresses Foxl2 endogenously at rather high levels. As a control for specificity of our precipitating anti-FoxL2 antibodies, we also used purified recombinant FOXL2 expressed in bacteria. Sequences bound to FoxL2 were in each case precipitated using anti-FoxL2 antibodies (18), and controls were performed using pre-immune serum to check the specificity of target sequence amplification. After eight (AT29C extracts) and five (recombinant FOXL2) selection rounds, both assays allowed us to identify a common 7 bp core binding site for FOXL2, i.e. 5'-GT(C/G)AAGG-3', or its reverse complement in each clone of the anti-FoxL2 precipitated sequences (Fig. 1), whereas sequences selected using the pre-immune serum displayed no significant consensus. We have shown previously that FOXL2 is highly post-translationally modified *in vivo* (31), thus observed variations around the core binding site between sequences obtained using recombinant or ‘native’ (from AT29C extracts) FOXL2 could be accounted for by the absence/presence of modifications and/or co-factors. However, we could observe that the same central sequence was selected independently using both sources of FoxL2.

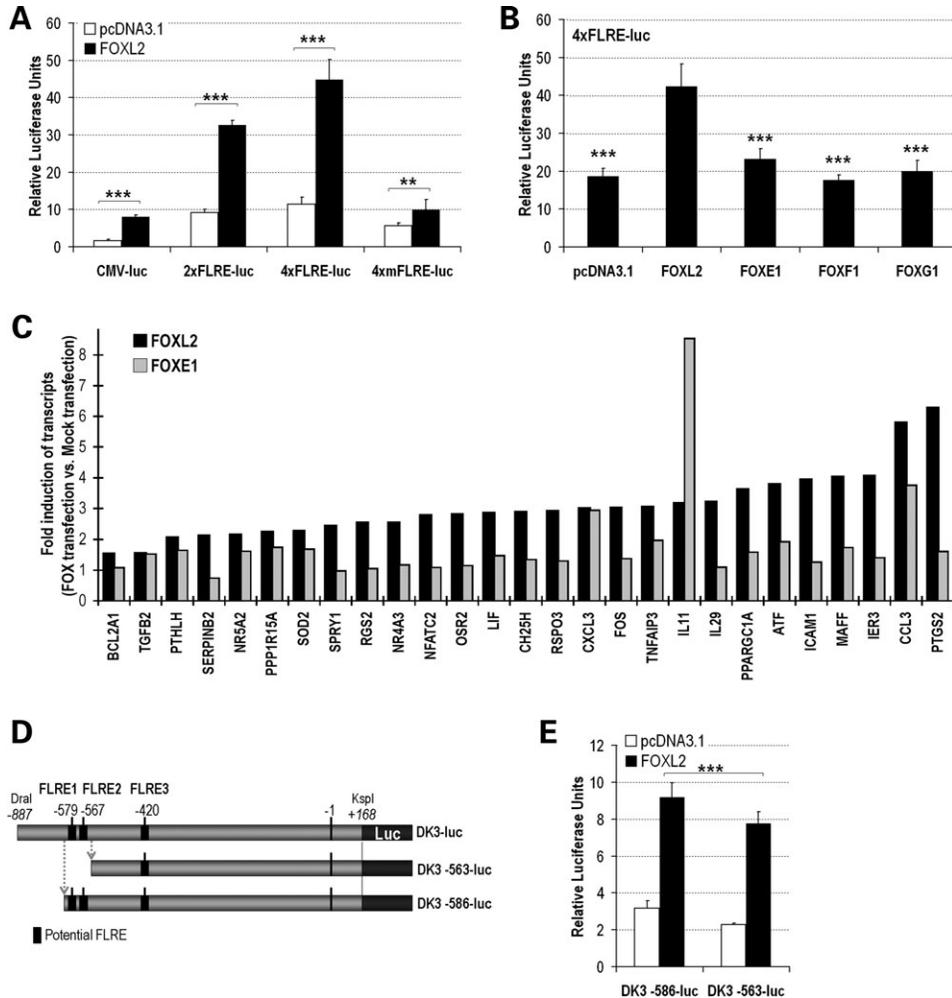
This indicates a similar binding specificity, irrespective of potential post-translational modifications or of potential molecular partners.

The final G doublet of the core sequence is particularly noteworthy, because this is the first case where G's are described at these positions in a FOX factor high-affinity binding site (Table 1). The only documented instance of a minor usage of a G in one of those positions is for FOXL1/FREAC-7, although this variation was found in only 4% of bound sequences (9). It is also interesting to note that the high-affinity binding site of FOXL1 (Table 1) significantly differs from that of FOXL2, whereas members of the FOXO subfamily share a single high-affinity binding site. This could be explained by the fact that there is a 30% divergence in the sequences of the DNA-binding domains of the human members of the L subfamily, whereas there is only an average 12% divergence among human members of the O subfamily (statistics from a sequence alignment, data not shown).

### The FLRE is specifically responsive to FOXL2

Next, we aimed at verifying that the consensus sequence bound by Foxl2 *in vitro* was able to function as a response element in living cells. Thus, we generated artificial luciferase promoter reporters: CMV-luc (minimal CMV promoter), 2×FLRE-luc (two FLRE sequences upstream of the minimal CMV promoter) or 4×FLRE-luc (four FLRE sequences upstream of the minimal CMV promoter). We also generated a construct with four copies of a mutant version of the FLRE (mFLRE), 4×mFLRE-luc, where the ‘mandatory’ G's were replaced by T's, which should act as a binding site of weaker affinity. We transfected these constructs in KGN cells along with a FOXL2 expression vector or an empty control vector for luciferase activity quantification.

First, we observed that basal activity levels of the reporters were significantly increased with the number of consensus FLREs present upstream of the minimal CMV promoter



**Figure 2.** The FLRE site is specifically activated by FOXL2. (A) Influence of the number and sequence of FLRE sites in luciferase reporters on amplitude of activation by FOXL2. (B) Specificity of response of the 4×FLRE-luc reporter to FOXL2 transactivation. Significant differences with respect to FOXL2 are represented. (C) Activation of potential transcriptional targets of FOXL2 (29) by FOXL2 and FOXE1 in KGN cells. Values represent levels in FOX-transfected cells over levels in mock-transfected cells. A value of 1 means that the transfection had no consequences on the transcript levels. From these results, it is obvious that IL11 might be a common target of both FOXL2 and FOXE1. (D) DK-3-luc promoter analysis for FLRE sequences, and schematic representation of deletion constructs. (E) Luciferase activities and sensitivities to FOXL2 transactivation of DK3-luc deletion constructs. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

(Fig. 2A): the 2×FLRE-luc reporter basal activity was much stronger than that of CMV-luc (about 5.5-fold,  $P = 10^{-7}$ ), but the 4×FLRE-luc basal activity was even stronger than that of 2×FLRE-luc (about 1.3-fold,  $P = 0.03$ ). The increased basal activities of 2×FLRE-luc and 4×FLRE-luc when compared with CMV-luc are expected to result from endogenous FOXL2 activity. When FOXL2 was overexpressed, even if a significant activation of CMV-luc was detected, luciferase activity induction was much stronger when FLREs were present upstream of the minimal CMV reporter (Fig. 2A). Indeed, FOXL2 was able to activate 2×FLRE-luc about 3.5-fold and 4×FLRE-luc about 4-fold ( $P < 10^{-6}$ ). Moreover, the relative level of luciferase activity attained when overexpressing FOXL2 was significantly higher when four FLREs were present in the artificial promoter than when there were only two sites ( $P = 0.001$ ). This clearly shows that the FLRE is able to function as a FLRE in living cells and that the number of sites in the promoter modulates

the amplitude of the response, as previously proposed on theoretical grounds (32,33). This behaviour is predicted to arise from cooperative and synergistic effects during the recognition of the promoter by the transcription factor (in this case, FOXL2) and the RNA polymerase. More specifically, cooperativity implies that the binding of one molecule of transcription factor facilitates subsequent binding of other ones. Once this step has been achieved, the DNA-bound transcription factors can interact in concert with the transcriptional machinery [synergy (32)].

Interestingly, the 4×mFLRE construct, with the four mutated FLREs, was significantly more active than the CMV-luc control ( $P = 2 \times 10^{-6}$ ) and was also able to respond, though weakly, to FOXL2 overexpression, by a 1.8-fold induction when compared with its basal activity. This represents a much lower level of induction than that of the consensus 4×FLRE-luc construct and even the 2×FLRE-luc construct ( $P < 0.0001$ ; Fig. 2A). This has been predicted previously (32,33). Namely, a decrease

of the affinity between the transcription factor and its binding sites in a promoter (likely the case here, due to the mutations in the FLRE) is expected to lead to a weaker transcriptional output. Our results with 4×mFLRE-luc also suggest that FOXL2 binding to the consensus FLRE is highly preferred to degenerate FLREs. However, this shows that degenerate FLRE sequences can respond weakly to FOXL2. The interplay between the number and affinity of FLREs in target promoters should account for the diversity of amplitude of their response in terms of activation/repression (29).

We decided to assess the specificity of FLRE sequence recognition by FOXL2. We therefore tested the ability of other FOX factors (i.e. FOXE1, F1 and G1) to activate the 4×FLRE-luc reporter system, the most sensitive of those that we have generated (Fig. 2B). Interestingly, we found that overexpression of FOXE1 induced a marginal increase of activity (1.2-fold;  $P = 0.02$ ) of the reporter in comparison to the empty control GFP vector. FOXF1 and FOXG1 overexpression did not induce any significant activation of the reporter. These observations indicate that the strong functional specificity of FOXL2 when compared with other Forkhead factors should stem from the recognition of a divergent binding site.

Since FOXE1 displayed some activity on the 4×FLRE-luc reporter, we decided to further analyze its ability to modulate the expression of a set of 27 FOXL2-responsive genes (29), when overexpressed in KGN cells. In most instances, FOXE1 failed to activate FOXL2-responsive genes (Fig. 2C), and this was associated with a lack of correlation between expression changes induced by the two factors (Pearson's correlation coefficient  $R = 0.21$ ; n.s.). This strengthens our observations on FOXL2 specificity.

Five complexes of FOX-DNA have already been crystallized and analyzed (1,34–37 and reviewed in 38). From these structures, it has been determined that the Forkhead domain folds into three  $\alpha$ -helices, three  $\beta$ -strands and two wing-like loop regions with variable conformations. The third helix is the main DNA recognition structure, and other regions of the Forkhead domain, in particular, the variable regions between the second and the third helices and the two wings, are important for modulating the DNA-binding specificity (38). It appears that the specificity of FOXL2 DNA-binding relies both on highly conserved residues in the recognition helix displaying precise conserved interactions, and on more variable surrounding regions, expected to enable the specific interactions between FOXL2 and DNA (Supplementary Material, Fig. S2). The latter are expected to explain the divergent binding specificity of FOXL2.

Next, we wanted to test whether naturally occurring FLREs were actually used by FOXL2. For this purpose, we used the caprine promoter of FoxL2, which is activated by FOXL2 (24). The sequence cloned in pFoxL2-luc/DK3-luc reporter (39) contains three good potential FLREs compatible with our consensus matrix in (Fig. 1). The first two, FLRE1-2, were located in tandem positions at  $-579$  (5'-GCAAAGGGCA-3') and  $-567$  (5'-GCAAAGGAGC-3') with respect to the mapped transcription start site. The third one, FLRE3, was found isolated at position  $-420$  (5'-TTCAAGGGCT-3'; Fig. 2D). As theory predicts that contiguous binding sites, potentially ensuring cooperativity, should drive the trans-

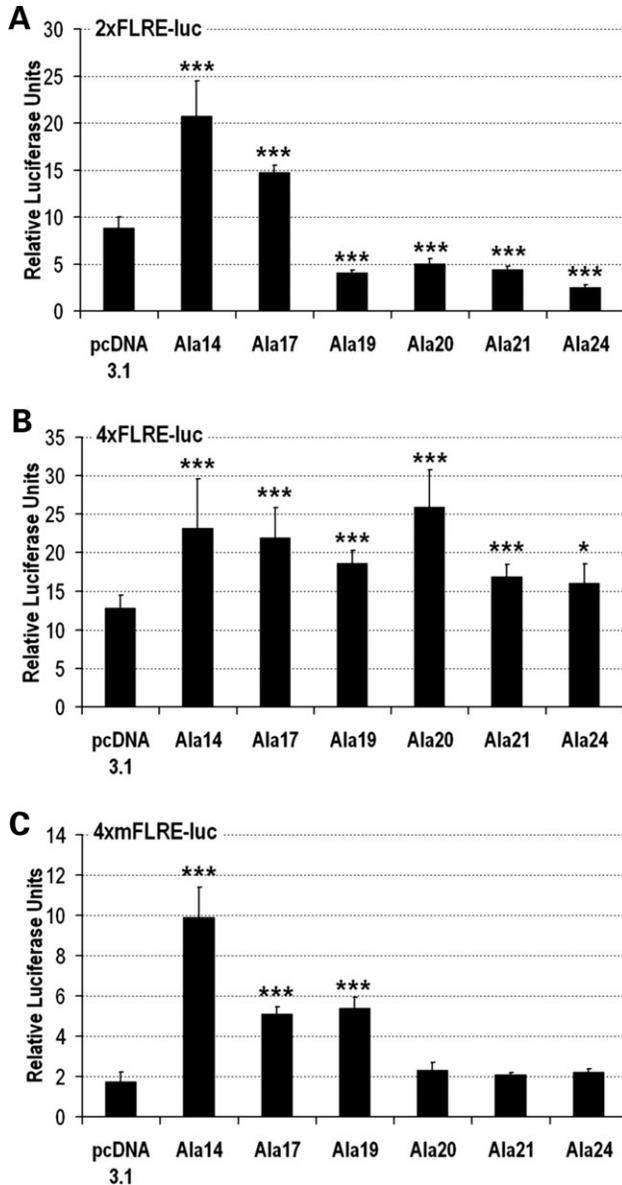
criptional response of a promoter (32,33) we focused on FLRE1-2. Thus, we generated deletion constructs of the luciferase reporter immediately upstream and downstream of FLRE1-2 (DK3  $-586$ -luc and DK3  $-563$ -luc; Fig. 2D), and tested their responsiveness to FOXL2 (Fig. 2E). Tandem sites FLRE1-2 seemed important for the self-activation of FoxL2 because their obliteration induced a 15% activity loss in the context of FOXL2 overexpression ( $P = 0.007$ ). This clearly indicates that: (i) using the matrix that we obtained from PCR-selection experiments allows identification of real FOXL2 responsive sites, (ii) imperfect sites are used by FOXL2 (FLRE1-2) and (iii) other sequence elements must be of importance to explain why their deletion did not abolish promoter recognition by FOXL2. The fact that FOXL2 is still able to transactivate the DK3 lacking the two best FLRE candidates suggests that more divergent sites must also be used by FOXL2, or that there might also be some indirect contribution of FOXL2 to its activation [for instance, in a feed-forward mechanism (40)].

We have previously described potential direct and indirect transcriptional targets of FOXL2 in the human granulosa-like KGN cell line (29). Since we found that the FLRE sequence is used by FOXL2 in a known natural target promoter of FOXL2, we tested whether promoter sequences of the genes known to respond to FOXL2 (29), were enriched in high-affinity FLREs versus all other promoter sequences in the genome (i.e. genomic background). When considering in our analysis only the 20% best-scoring sites, we found that their number is significantly higher in promoter sequences of FOXL2-responsive genes ( $P = 0.024$ ) when compared with the background, despite the fact that this list includes indirect targets. Interestingly, considering only the best-scoring site in each promoter sequence is not sufficient to detect a significant difference between transcriptional targets and random gene promoters ( $P = 0.51$ ). This highlights the importance of considering both the sequence of the sites (score, and presumably affinity) and their number in the detection of target promoters.

### **PolyAlanine expansions of FOXL2 induce a length-dependent loss of response of different target promoters depending on the number and sequence of FLREs**

We have previously shown that mobility and solubility of FOXL2 decrease with increasing sizes of the polyAlanine domain, due to intracellular aggregation and cytoplasmic mislocalization (24). We also showed that this was correlated with a progressive loss of function of mutant alleles on two reporter systems: the promoter of FoxL2 (pFoxL2-luc/DK3-Luc) and the artificial 3×GnRHR Activating Sequence promoter reporter (3×GRAS-luc). PolyAlanine expanded alleles behaved differently on these promoters (24).

The identification of the FLRE sequence gives us the opportunity to test experimentally how the number and sequence of sites affects promoter response to FOXL2 variants. Specifically, we have assessed potential differences in transactivation of polyAlanine-expanded FOXL2 mutants using our luciferase promoter reporters, containing various numbers of FOXL2 binding sites (two or four) and with various affinities (FLRE



**Figure 3.** Transcriptional activities of polyAlanine-expanded FOXL2 variants on artificial FLRE-luc reporters. (A) Activation of 2×FLRE-luc by FOXL2 variants. Ala14 and Ala17 activate significantly the reporter, and other variants induce significant decrease of the luciferase readout. (B) Activation of 4×FLRE-luc by FOXL2 variants. All variants induce significant activation of the reporter. (C) Activation of 4×mFLRE-luc by FOXL2 variants. Ala14, Ala17 and Ala19 activate significantly the reporter, and other variants have no effect. Statistically significant differences with respect to empty control pcDNA3.1 are represented. \* $P < 0.05$ ; \*\*\* $P < 0.001$ .

versus mFLRE). These constructs should be insensitive to regulatory interferences compared with DK3-luc and 3×GRAS-luc. We transfected the reporter constructs in KGN cells together with wild-type FOXL2, FOXL2 variants containing 17, 19, 20, 21 or 24 alanines (named Ala17, Ala19, etc.), or with the empty control vector.

First, we tested the activity of the different variants on 2×FLRE-luc (Fig. 3A). Although Ala17 retained significant transactivation ability (1.7-fold when compared with empty

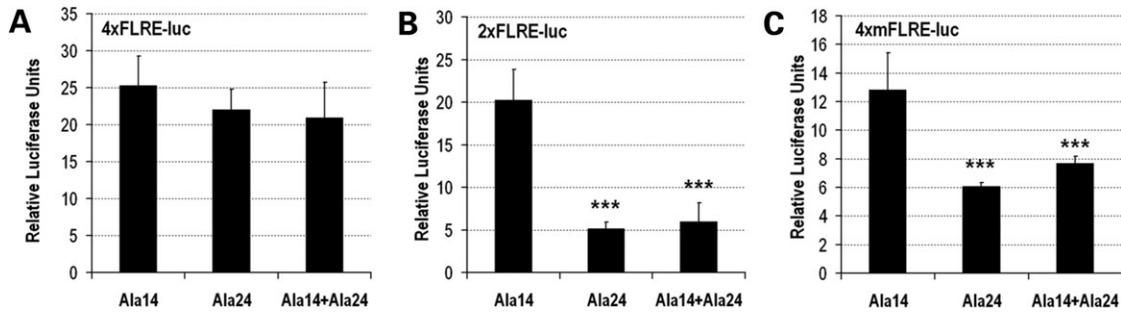
vector;  $P = 10^{-5}$ ), it displayed a significant loss of activity with respect to wild-type FOXL2 (1.4-fold loss;  $P = 0.008$ ). Interestingly, other polyAlanine-expanded mutants showed a significant decrease of activity with respect to the basal level of 2×FLRE-luc, suggesting a potential dominant negative effect over endogenous wild-type FOXL2 ( $P < 0.0002$ ; Fig. 3A). Then, we tested the allelic series on the 4×FLRE-luc reporter. On this promoter, all tested variants, even Ala24, displayed transactivation (1.3-fold;  $P = 0.03$ ; Fig. 3B). This clearly indicates that increasing the number of FLREs in a promoter enhances its capacity to be activated by lower levels (i.e. more aggregation-prone forms) of FOXL2 and confirms what we have previously proposed (24,32). Finally, we tested the impact of expansions on the ability of FOXL2 to activate the low affinity 4×mFLRE-luc promoter (Fig. 3C). As expected, wild-type FOXL2 activated the reporter (5.6-fold;  $P = 3 \times 10^{-6}$ ). Interestingly, on this promoter, both Ala17 and Ala19 variants retained partial transactivation (about 3-fold;  $P < 10^{-5}$ ), which was significantly lower than that of Ala14 ( $P < 0.0002$ ). All the other tested variants showed no significant activity when compared with the basal activity of 4×mFLRE-luc.

In brief, we were able to show that not only the number, but also presumably the affinity of FLRE sites influences the response of target promoters to decreased availability of a transcription factor. In fact, some variants, such as Ala17, have the ability to activate all target promoters, though to a lesser extent than the wild-type form; others, such as Ala19, retain sufficient availability to induce the activation of 4×mFLRE-luc and 4×FLRE-luc, but not of 2×FLRE-luc. Finally, other variants are only active when four high-affinity sites are present in the reporter. These differential activities of polyAlanine-expanded FOXL2 mutants are presumably at work *in vivo* depending on the composition of target promoters (quantity and affinity of FLREs) and explain why some expanded variants induce a more severe phenotype than others (i.e. at the heterozygous state, the Ala19 variant does not lead to BPES while the Ala24 mutant does).

### The frequent FOXL2-Ala24 mutant interferes with the wild-type product in a promoter-dependent fashion

The FOXL2-Ala24 variant mainly induces a dominant BPES of type II (5,21,22). The cause of the dominance of the phenotype remains elusive: (i) aggregation leading to loss of function, (ii) toxic gain of function through aggregation or (iii) dominant-negative effect.

A potential dominant negative effect of FOXL2-Ala24 was proposed previously (41). Indeed, we showed that FOXL2-Ala24 interacted with the wild-type protein in intranuclear aggregates, thus potentially decreasing the availability of the latter. However, at that time, we could not assess the impact of this interaction on the activity of wild-type FOXL2. As shown above, FOXL2-Ala24 induces a significant decrease in activity with respect to the basal activity of 2×FLRE-luc, which we interpreted as evidence of an interference with the activity of endogenous wild-type FOXL2 (Fig. 3). Therefore, we tested whether FOXL2-Ala24 could also interfere with the activity of the wild-type protein when expressed from constructs transfected in equimolar pro-



**Figure 4.** FOXL2-Ala24 acts as a promoter-specific dominant negative. KGN cells were co-transfected with the 4×FLRE-luc (A), 2×FLRE-luc (B) or 4×mFLRE-luc (C) and one dose of either Ala14 or Ala24 or with one dose of each. Statistically significant differences with respect to Ala14 alone are represented. \*\*\* $P < 0.001$ .

portions. We simulated situations of wild-type (Ala14) and mutant (Ala24) hemizygous states (transfections with a half dose of FOXL2 variant expression vector completed with the GFP empty vector) and of a heterozygous state (transfection with equal doses of both expression vectors; Fig. 4). As FOXL2-Ala24 is able to activate the 4×FLRE-luc reporter, both hemizygous and heterozygous contexts led to a similar activation of the reporter. Thus, on the highly sensitive 4×FLRE promoter, FOXL2-Ala24 acts much like the wild-type variant (Fig. 4A). This is not unprecedented, as we have previously found that a few of FOXL2 potential transcriptional targets were equally transactivated by both the wild-type and the Ala24 proteins (24). In the light of our results, these targets could be under the regulation of numerous high-affinity FLRE sequences.

A very different behavior was observed when using the 2×FLRE-luc reporter. Indeed, on this reporter, Ala14 induced a luciferase activity about four times higher than that of the Ala24 variant ( $P = 2 \times 10^{-5}$ ), which means that the Ala24 allele cannot induce correct activation of this reporter (Fig. 4B). Moreover, co-transfection of both variants resulted in an activity similar to that of Ala24 alone (Fig. 4B). This suggests the existence of a dominant negative effect at the molecular level. When a similar experiment was performed using 4×mFLRE-luc, on which Ala24 expression resulted in two times less luciferase activity than Ala14 ( $P = 0.0004$ ), co-transfection of both alleles also resulted in an activity similar to that of Ala24 alone (Fig. 4C). Again, on this reporter, co-expression of wild-type and mutant proteins in similar amounts results in a complete loss of activity.

These results indicate that, in addition to a plain loss of function, the dominant pathogenesis induced by the Ala24 variant might also result from a promoter-specific dominant negative effect. Indeed, whereas the small fraction of Ala24 which remains available for transactivation may be enough to function properly on a subset of high-affinity promoters, lower affinity promoters would not be correctly regulated.

Although a negative dominant allele is generally believed to induce more severe phenotypes than heterozygous null alleles, in the case of FOXL2, there is no apparent difference in the ocular phenotype resulting from heterozygous loss of function mutations and polyAlanine expansions (41). This is compatible with our results if: (i) a heterozygous null allele already leads to the most severe eyelid phenotype or (ii) the dominant

negative effect is exerted on a subset of promoters not responsible for the generation of the most obvious BPES stigmata. In most cases, a FOXL2-Ala24 mutation induces a type II BPES (21,22), with only palpebral defects, whereas premature stop mutations, such as Q53X [no protein (42)], induce a type I BPES (also displaying premature ovarian failure). The lack of a severe ovarian phenotype suggests that the crucial ovarian targets of FOXL2 have high-affinity promoters.

## CONCLUSION

In this study, we identify the sequence of a high-affinity FLRE and find that it diverges significantly from the general Forkhead consensus binding sequence. The strong target specificity that can be achieved through the FLRE is not unexpected, as FOXL2 is a key regulator of ovarian formation and maintenance. Indeed, ovaries and testes are the only organs stemming from a single bipotential primordium (26). To ensure proper testicular development and prevent unscheduled ovarian differentiation, it seems mandatory to avoid aberrant cross-talk of other FOX transcription factors through the FLRE. On evolutionary grounds, it is therefore understandable that the binding site of FOXL2 should have diverged from that of other members of the family.

Additionally, we present mechanistic evidence showing how promoter architecture influences promoter sensitivity to decreased transcription factor availability. Moreover, our artificial promoters containing numerous sites close to the consensus can be activated by strongly aggregation-prone variants, like Ala24. We were able to detect a molecular promoter-dependent dominant negative effect of FOXL2-Ala24 only on 'lower-affinity' reporters. We speculate that the generally absent ovarian phenotype of Ala24 patients comes from its ability to regulate properly high-affinity ovarian targets. The existence of those crucial high-affinity ovarian targets would be compatible with the role of FOXL2 in reproduction and the need for a developmental failsafe.

From a practical perspective, artificial promoters containing various combinations and versions of the FLRE identified here can be powerful diagnostic tools to discriminate FOXL2 mutations leading to BPES associated or not with premature ovarian failure, which is a main concern in genetic counseling.

## MATERIALS AND METHODS

### PCR-selection amplification for FOXL2 DNA-binding site

We used the PCR-selection amplification protocols already described in (43,44) to determine FOXL2 binding site, modified as described below. We prepared a dsDNA library of 76mers oligonucleotides (Invitrogen), containing constant predefined 5' and 3' sequences (for PCR primer annealing) and a variable 26 bases central portion (N<sub>26</sub>, for binding site selection),

CAGGTCAGTTCAGCGGATCCTGTCC-N<sub>26</sub>-GAGGCG AATTCAGTGC AACTGCAGC. This library was diluted to 1 ng/μl. We performed two independent PCR-Selection assays, using either an endogenous Foxl2 from nuclear extracts or a recombinant FOXL2. Nuclear extracts from transformed murine granulosa AT29C cells, which express Foxl2 endogenously at the protein level (data not shown), were prepared as described in (45). Recombinant HA-tagged FOXL2 was expressed in a bacterial system and purified as described previously (18). We performed several rounds of the following selection/amplification procedure. A first mix was obtained with 2 ng of oligonucleotides from the library, poly(dI-dC) (Sigma; to reduce non-specific DNA binding) and FOXL2 (either from extract or recombinant) and incubated on ice for 30 min, to allow for the binding of FOXL2 to specific oligonucleotides. In a second mix, ProteinA-Dynabeads (Invitrogen/Dynal) were incubated 30 min at RT with a blend of our two anti-FoxL2 antibodies [against the FoxL2 N-terminal and C-terminal peptides, characterized by Cocquet *et al.* (18)] or an equivalent amount of rabbit pre-immune serum, as a control for specificity. The magnetic beads were then collected on magnets, washed in PBS, resuspended in the FOXL2/oligo mix and then incubated for 1 h at 4°C. Bound oligonucleotides were eluted from the beads in 50 μl sterile water by incubation for 10 min at 99°C. Then 5 μl of eluted oligonucleotides were amplified by PCR for 25 cycles (or only 20 cycles for the last rounds, in order to avoid concatemerization of oligonucleotides) using primers annealing on the constant sequences of the 76mers and verified on a 1% agarose gel. One-tenth of the PCR-amplified DNA was then used in the following round of PCR-selection amplification. PCR products obtained after the eighth (AT29C nuclear extracts) or fifth (bacterial recombinant FOXL2) rounds of PCR-select were cloned in the PCR4-TOPO vector (Invitrogen). Faint PCR products obtained when using rabbit pre-immune serum instead of FOXL2 antibodies were also cloned. For each experiment, 40 distinct clones were amplified by colony-PCR and analyzed by automated sequencing. The sequences were inspected manually to detect recurrent motifs and aligned to produce a consensus sequence matrix. When constructing the matrix, we excluded sites overlapping between the variable and the constant sequences of the oligonucleotides.

### Plasmids and expression vectors

FOXL2-GFP expression vectors, for wild-type and extended polyAlanine-tract versions, are pcDNA3.1 vectors (Invitrogen) and have been described previously (24). FOXE1-GFP, FOXF1-GFP and FOXG1-GFP contain the human ORFs of

those genes fused C-terminally to the GFP in the pcDNA3.1 vector. Artificial FOXL2-responsive promoters were generated by amplification of a minimal CMV promoter (100 bp, containing the CAAT and TATA boxes) from pcDNA3.1, using synthetic long overhang primers containing desired sequence/number of sites and restriction enzymes sites (sequences in Supplementary Material). We included in the constructions the 'core' sequence of the FLRE, TCAAGG in its highest affinity form (FLRE) and TCAATT in a lower affinity form (mFLRE). PCR products were digested with the *XhoI* and *HindIII* endonucleases (Boehringer-Mannheim) and cloned in pGL3-Basic (Promega) to generate luciferase reporters. Truncations of caprine FoxL2 promoter DK3, starting at -586 and -563 relatively to the mapped transcription start site, were amplified by PCR on the pFoxL2-Luc/DK3-luc plasmid (39) using appropriate primers and cloned upstream of the firefly luciferase gene in pGL3-Basic, using primer introduced *XhoI* and *HindIII* restriction sites.

### Cell culture and transient transfections

Granulosa-like KGN (28) and AT29C (30) cells were grown in DMEM-F12 medium, supplemented with 10% FBS and 1% penicillin/streptomycin (Invitrogen). For transfections, KGN cells were seeded 12 h prior to transfection at a density of  $4 \times 10^4$  cells/cm<sup>2</sup> and transfected using the calcium-phosphate method (45).

### Luciferase assays

KGN cells were seeded in 24-well plates. Assays were performed as described in Moumne *et al.* (24). Relative luciferase units represent mean values obtained from five biologically independent replicates and are the ratio of Firefly luciferase activity over Renilla luciferase activity in the samples. Statistical significance was estimated by a Student's *t*-test. Errors bars represent standard deviation between replicates.

### RNA extraction and cDNA synthesis

KGN cells were transfected with FOXL2-GFP, FOXE1-GFP or the empty GFP control vector pcDNA3.1-GFP. Total RNA pools from transfected KGN cells were extracted using the TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Total cDNA synthesis was performed using the SuperScript II reverse transcription kit (Invitrogen) and oligo-dT primers.

### Quantitative real-time PCR

Quantitative real-time PCR experiments were performed as described in Batista *et al.* (29). Primer ordered from Eurogentec.

## SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* Online.

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