RAPID COMMUNICATION

The post-translational modification profile of the forkhead transcription factor FOXL2 suggests the existence of parallel processive/concerted modification pathways

Bérénice A. Benayoun[1, 2, 3, 4, 5], *Jana Auer*[1, 2, 3], *Sandrine Caburet*[1, 2, 3, 4] *and Reiner A. Veitia*[1, 2, 3, 4]

[1] INSERM U567, Team21, Genetics and Development Department, Institut Cochin, Paris, France
[2] CNRS UMR8104, Institut Cochin, Paris, France
[3] Université Paris Descartes, Faculté de Médecine Cochin-Port-Royal, Paris, France
[4] Université Denis Diderot, Paris VII, Paris, France
[5] École Normale Supérieure, Paris, France

The transcription factor Forkhead box L subfamily member 2 (FOXL2) is involved in craniofacial development and ovarian function. Using 2-DE and immunoblotting, we show that it is highly modified post-translationally. The most outstanding feature of its migration profile is the presence of two distinct modification "trains" and the absence of intermediates. A theoretical analysis of the modification profile of FOXL2 suggests that it undergoes parallel processive/concerted modifications. The absence of intermediates is compatible with the recruitment of poorly modified FOXL2 into a post-translational "modification factory."

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Mutations in the Forkhead box L subfamily member 2 (FOXL2) transcription factor have been shown to be responsible for the blepharophimosis ptosis epicantus inversus syndrome (BPES; MIM 110100) in humans [1]. BPES is a genetic disorder characterized by eyelid and craniofacial abnormalities, which can be associated or not with premature ovarian failure [2]. Our immunohistochemical studies have shown that FOXL2 is expressed in both periocular and ovarian follicular (mostly granulosa) cells, where it localizes to the nucleus. This is compatible with the function of FOXL2 as a transcription factor [3, 4]. A recent study has revealed that, in addition to the expected steroid metabolism genes (like aromatase), regulators of inflammation, of apoptosis and of oxygen free radical metabolism are also potential transcriptional targets of FOXL2 [5].

In order to characterize the PTMs of FOXL2, we have analyzed total extracts from granulosa-like KGN cells by 2-DE followed by anti-FOXL2 immunoblotting (Fig. 1A; *see* Supporting Information). We have chosen the granulosa-like KGN cell line [6] to conduct this biochemical analysis because these cells express FOXL2 robustly at the protein level [5].

The theoretical p*I* of FOXL2 as predicted from its primary protein sequence is 9.26 (http://expasy.org/cgi-bin/pi_tool). Indeed, in KGN cell extracts, a very faint "basic modification train" can be detected, close to the expected p*I*. This train is composed of four distinct spots whose p*I*s range between 8.9 and 9.2 (Fig. 1A). However, the vast majority of FOXL2 forms in the steady-state are concentrated between p*I* = 7.8 and 8.2, within what we call the "acidic train" of modifications, composed of six distinct spots. Thus, the observed general tendency is to an acidification of the protein through its PTM.

Correspondence: Professor Reiner A. Veitia, Faculté de Médecine Cochin-Port-Royal, Equipe 21, Institut Cochin, 24 rue du Faubourg Saint-Jacques, 75014 Paris, France
E-mail: veitia@cochin.inserm.fr
Fax: +33-1-44-41-23-02

Abbreviations: BPES, blepharophimosis ptosis epicantus inversus syndrome; **FOXL2,** forkhead box L subfamily member 2; **HDAC,** histone deacetylase; **SIRT1,** silent mating-type information regulation 2 homolog 1

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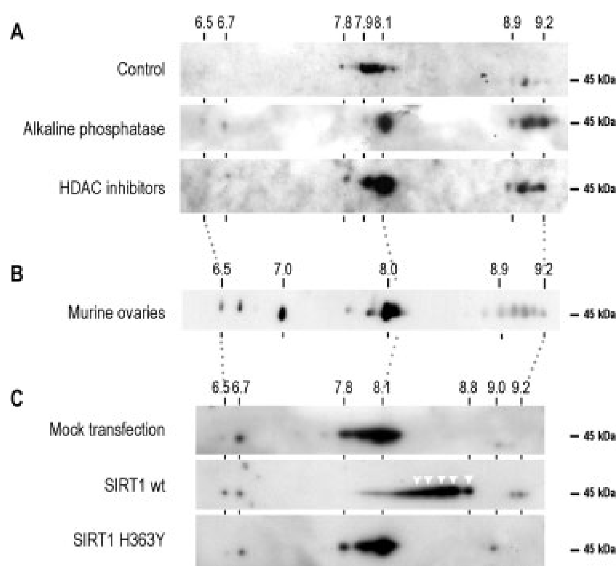

Figure 1. Western blot analysis of protein extracts after 2-D electrophoretic separation of FOXL2. Separation was carried out on Immobiline Drystrips pH 6–11 (first dimension) followed by SDS-PAGE (second dimension). Detection was carried out with the anti-C-terminus FOXL2 antibody [3]. The observed spots are the result of distinct combinations of PTMs. Indicated p*I* values were calculated from the nonlinear abacus provided by the manufacturer (Amersham/GE Healthcare), who warrants over 99% accuracy and reproducibility. (A) Untreated (control), alkaline phosphatase, and HDAC inhibitor-treated KGN cell extracts. (B) Murine ovary extracts (detail of the blot around 45 kDa). (C) Transfected KGN cells extracts (empty vector/mock, wild-type SIRT1, and noncatalytic mutant H363Y). Arrowheads indicate the presence of resolved spots on the Western blot profile that were present only under SIRT1 overexpression.

The shift toward more acidic p*I*s can result, for instance, from two nonexclusive types of modifications: addition of negative charges to the protein (such as phosphorylation), or masking of positive charges (such as acetylation, or more generally acylation, of K residues). In line with this, several FOXL2 mutations in BPES patients involve K, S, and Y residues, which are potential PTM sites (namely, S58L, S70I, S101R, K193C, Y215C, S217F, and Y258N; for details *see* the FOXL2 mutation database at: http://users.ugent.be/~dbeysen/foxl2/Tables/tables.htm). We have very recently studied the molecular consequences of four BPES-causing missense mutations involving serine residues (*i.e.*, S58L, S70I, S101R, and S217F) on intracellular localization and function [7]. Localization studies in COS-7 cells have shown that wild-type FOXL2 displays a diffuse nuclear pattern [8]. The fluorescent signal of the mutant protein S217F was consistently nuclear and indistinguishable from the wild type. However, a presence of predominantly intranuclear aggregates was observed for S101R, whereas S58L induced extensive nuclear protein aggregation. Finally, massive cytoplasmic aggregates were observed for S70I. The functional impact of these mutations on FOXL2 transactivation was assessed in luciferase reporter assays [7]. S58L and S101R mutants showed no significant activity, suggesting a complete loss of function, as expected from their strong nuclear aggregation and cytoplasmic mislocalization. Surprisingly, S70I mutant displayed an activity even lower than basal reporter level, suggesting a potential dominant negative effect on endogenous FOXL2 activity. In contrast, the S217F variant displayed enhanced activity. These findings suggest that the integrity of these phosphorylatable residues might be important for correct cellular localization, solubility, and functionality of FOXL2.

The most outstanding feature of the migration profile of FOXL2 is the existence of a “p*I* leap” observed between the most acidic spot of the basic train (p$I = 8.9$) and the most basic spot of the acidic train (p$I = 8.2$), which shows that intermediate modification forms of FOXL2 are virtually nonexistent in KGN cells under control culture conditions. The existence of a similar FOXL2 modification pattern *in vivo* was confirmed using extracts from murine ovaries (Fig. 1B).

We have further explored the nature of FOXL2 modifications in KGN cells by treating protein extracts with alkaline phosphatase to remove accessible phosphate groups (Supporting Information). These extracts displayed a migration profile which was very different from untreated ones (Fig. 1A). Indeed, the acidic train was reduced to two spots. Assuming that dephosphorylation was complete, FOXL2 in these spots should contain modifications other than phosphorylation. In addition, the basic train is highly strengthened in this condition, which likely results from a transfer of protein from the acidic train to this one, similarly to a communicating vessels phenomenon. This finding also suggests that the basic train contains non (or poorly) phosphorylated FOXL2, and that multiple phosphorylation events should be necessary to transfer poorly modified FOXL2 (basic train) to the highly modified more acidic forms.

Next, we exposed KGN cells to Class I and II histone deacetylases; (HDAC) inhibitors for 24 h, which are supposed to induce cellular hyperacetylation (Supporting Information). The migration profile of those extracts is quite similar to the migration profile of phosphatase-treated ones (Fig. 1A). Again, the signal from the basic train is highly strengthened, and the acidic train is concentrated in fewer spots. The similarity between the effects of dephosphorylation and hyperacetylation suggests that FOXL2 hyperacetylation might somehow compete with its phosphorylation. If this is true, the remaining spot in the basic train after alkaline phosphatase treatment should be hyperacetylated FOXL2 (*see* below). However, at this point we cannot exclude an indirect effect of HDAC inhibitors on the modification profile (*i.e.*, a kinase involved in FOXL2 regulation might be inhibited by hyperacetylation).

Puzzled by the p*I* leap observed in the FOXL2 steady-state modification pattern in KGN cells, we sought a way to reveal modification intermediates. Since cellular hyperacetylation affects the migration profile of FOXL2, we decided to test the effect of overexpression of the class III HDAC silent mating-type information regulation 2 homolog 1

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(SIRT1). For this, we transfected KGN cells with a wild-type and noncatalytic mutant versions of SIRT1 (Fig. 1C; *see* Supporting Information). The profile obtained after expression of the noncatalytic mutant was basically identical to the usual migration profile. However, in the presence of wild-type SIRT1, we observed the appearance of at least five previously undetected FOXL2 intermediate modification forms.

As seen in Fig. 1, two extremely modified FOXL2 forms, found in faint spots, can also be observed after another p*I* leap of about 1 pH unit (between 7.8 and 6.7). These spots were observed in extracts from both KGN cells and murine ovaries. The only situation where we failed to detect these spots was in the first control blot, where relative protein load was less important. However, they were detected consistently in all our other 2-D Western blots for FOXL2.

Next, we performed a theoretical analysis to explore the PTMs of FOXL2 that might explain its migration profile. Specifically, using p*I* calculations according to two different sources (http://scansite.mit.edu/calc_mw_pi.html and http://www.nihilnovus.com/Palabra.html#duh4), we estimated potential p*I* changes induced by the addition of phosphate groups (pK_{a1} = 2.12, pK_{a2} = 7.21) or by mimicking lysine acetylation (*i.e.* substituting lysines by nonionic residues in the primary sequence). All p*I* variations induced by those modifications are indicated in Fig. 2. We named spots with letters. The basic train contains up to six spots (A, B, C, D, E, and X), and the acidic train contains up to six spots as well (G, H, I, J, K, and L). The two most acidic spots observed after the second p*I* leap were labeled M and N. Moreover, the five additional spots, which appear under SIRT1 overexpression, were labeled F0–F4. For simplicity, we considered that the observed effects were direct (*i.e.*, dephosphorylation by alkaline phosphatase treatment and deacetylation by SIRT1 overexpression), but also that SIRT1

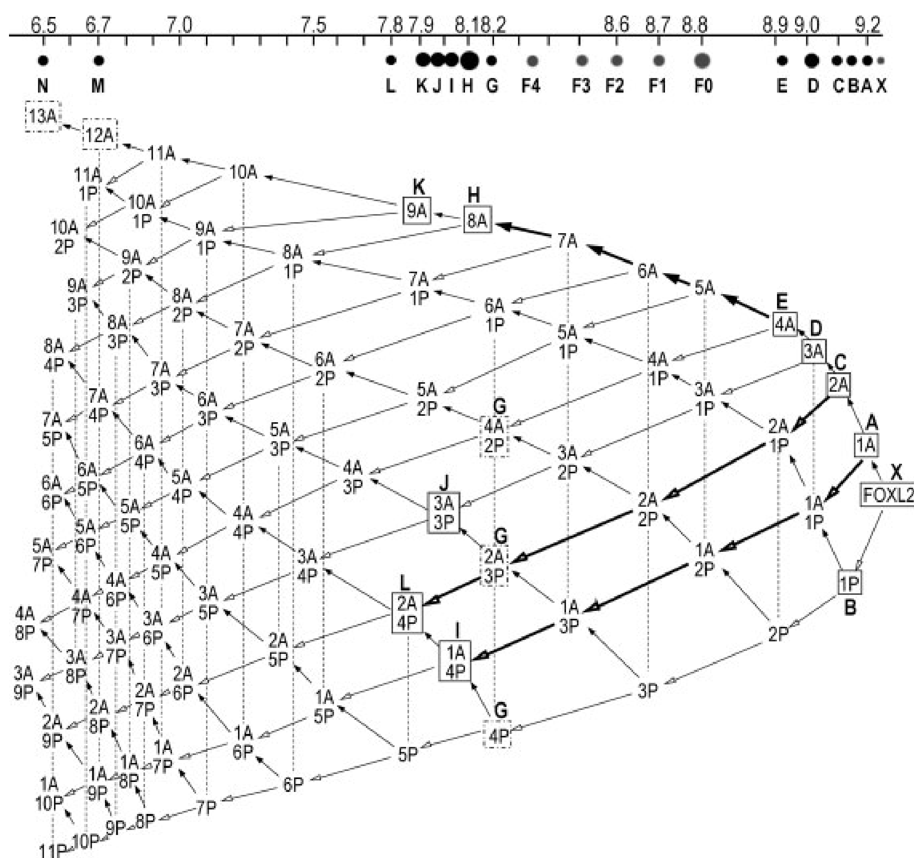

Figure 2. Theoretical analysis of p*I* variations induced by PTMs of transcription factor FOXL2. (Top) the diagram shows the various spots observed in 2-DE (black spots for those observed in normal conditions; gray spots for additional ones observed only after the various treatments). Letters correspond to the name attributed to the spots. The represented p*I* scale is derived from experiments (Fig. 1). (Bottom) The various forms of FOXL2 are located along the p*I* scale according to their predicted p*I*. Each form is named with the number of putatively acetylated residues (nA) and the number of phosphorylated ones (nP). The changes from one form to another are depicted as up-pointing filled arrows for acetylation and down-pointing open arrows for phosphorylation. Full-boxed names correspond to forms for which several lines of evidence allow identification (cited spot above), and dashed boxes indicate spots/forms with only a putative identification (see text for details). Vertical dashed lines link forms with predicted p*I* within 0.05 U, undistinguishable with this method, and that would therefore comigrate in a single spot. Thick arrows show the possible parallel modifications pathways that would explain observed p*I* leaps.

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effects were not always complete. We are of course aware that an indirect action of SIRT1 cannot be excluded.

The most basic spot we can observe for FOXL2 is spot X. It is only detectable after alkaline phosphatase treatment or SIRT1 overexpression and its observed p*I* is between 9.3 and 9.2. According to our p*I* calculations (Fig. 2), this indicates that spot X contains "naked" FOXL2 (at p*I* = 9.26).

Spot A, the most basic spot under control conditions, has an observed p*I* of 9.2, and this fits the theoretical p*I* of a monoacetylated (*i.e.*, acylated, in general) form of FOXL2 (hereafter named [1A]).

Spot B is barely detectable in control conditions and is clearly visible under HDAC inhibitors treatment. However, it disappears under alkaline phosphatase treatment, but shows no sensitivity to cellular acetylation levels. Moreover, its disappearance correlates with the reappearance of "naked" FOXL2. This strongly suggests, considering its observed p*I* between 9.2 and 9.1, that it is a monophosphorylated form (*i.e.*, [1P]).

Spot C, at an observed p*I* of about 9.1, is always detectable, except under SIRT1 overexpression, which suggests it corresponds to a diacetylated form, [2A].

Spot D is the main one in the basic train in control conditions, and is very strong under both alkaline phosphatase treatment and HDAC inhibitors exposition. However, it disappears completely under SIRT1 overexpression. At the observed p*I* of 9.0, the only possible form that would satisfy all these criteria is a triacetylated form of FOXL2, [3A].

Spot E is present, though faint, under control conditions, and alkaline phosphatase treatment. It is strengthened under HDAC inhibitors treatment and disappears totally under SIRT1 overexpression. At a p*I* of 8.9, these data suggest it contains a tetraacetylated form, [4A].

Spots F0–F4, appearing only after SIRT1 overexpression, are in a zone where many potential forms could comigrate, and information is insufficient to attribute modification combinations to the observed spots. The most acidic spot of this train actually corresponds to spot G.

Spot G, present in all controls at observed p*I* = 8.2, is undetectable under alkaline phosphatase and HDAC inhibitors treatment, but is still detectable under SIRT1 overexpression. It cannot be the [6A + 1P] form, because alkaline phosphatase treatment would then produce a spot at p*I* = 8.66, which we failed to detect. Thus, it could be any, or a blend of three forms: [4A + 2P], [2A + 3P], and/or [4P]. Indeed, under alkaline phosphatase treatment, the first form would release the [4A] form, which is observed as spot E; the second form would release the [2A] form, which is also observed as spot C; and the third form would release "naked" FOXL2, which is indeed observed (spot X). However, if SIRT1 effect was complete on all acetylated lysines, spot G should be the [4P] form (resistant to deacetylation but not to alkaline phosphatase).

Spot H is always detectable except under SIRT1 overexpression, and is especially strengthened under HDAC inhibitors treatment. Its observed p*I* of 8.1 suggests that it should contain an octa-acetylated form of FOXL2, [8A].

Spot I (p*I* ~ 8.0), which is sensitive to alkaline phosphatase treatment, should correspond to the [1A + 4P] form. Indeed, if dephosphorylated, this form yields a monoacetylated form, at p*I* = 9.2 (spot A); this is compatible with the fact that spot A is highly strengthened under alkaline phosphatase treatment.

In the same manner, spot J should contain a [3A + 3P] form, according to its calculated p*I* value of 7.95. Indeed, if dephosphorylated, this form yields a triacetylated form at p*I* = 9.0 (spot D), which is compatible with the fact that spot D is very strong under alkaline phosphatase treatment.

Spot K (p*I* = 7.9) is clearly visible in control conditions, faint under alkaline phosphatase treatment, but absent under SIRT1 overexpression, which suggests a high acetylation status. At this p*I*, the only alkaline phosphatase-resistant form would be a nona-acetylated FOXL2, [9A]. However, since spot K is much fainter after dephosphorylation, it is possible that distinct forms could comigrate in this spot in control conditions, one of which would be [9A], along with other unidentified ones (*i.e.*, involving other types of modifications).

At p*I* = 7.8, we can observe spot L, which disappears under both alkaline phosphatase treatment and SIRT1 overexpression, indicating that it contains both acetylation and phosphorylation. The only form that would make sense is [2A + 4P]. Indeed, after dephosphorylation, it should yield a diacetylated form (spot C), observed after alkaline phosphatase treatment. This is also consistent with the fact that complete deacetylation by SIRT1 would yield a [4P] form (spot G).

Finally, spots M and N, respectively at p*I* = 6.7 and 6.5, are resistant to alkaline phosphatase treatment. This suggests that these spots correspond to the dodeca- and trideca-acetylated forms of FOXL2 ([12A] and [13A], respectively). It is difficult to imagine that such acidic forms still retain the wild-type ability to bind DNA, and their biological relevance is unclear (inactive storage forms or bound to degradation?). We cannot exclude the existence of even more acidic forms of FOXL2. However the pH range of the strips does not allow us to detect them and their biological role would be even more doubtful.

According to this analysis and to our modification map (Fig. 2), the first p*I* leap we observe can result from any of four parallel series of successive modifications: (i) leap from A to I, four phosphorylations; (ii) leap from C to L, four phosphorylations; (iii) leap from D to J, three phosphorylations; (iv) leap from E to H, four ac(et)ylations. The second p*I* leap, according to our previsions, might result from the modification of spots K–M, by three ac(et)ylations.

A potential explanation for the existence of p*I* leaps (*i.e.*, absence of modification intermediates) can be processive/concerted modification (for review, *see* [9]). Processive phosphorylation by a single kinase has been reported in the case

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of proteins containing multiple consensus repeats. For instance, the carboxy-terminal repeated domain of RNA polymerase II can be phosphorylated on 52 of its tyrosines by the Abl kinase [10] and the alternate splicing factor/splicing factor 2 (ASF/SF2), which contains numerous SR repeats, is phosphorylated processively on 10 serines by the SFRS protein kinase 1 (SRPK1) [11]. This mechanism does not apply to FOXL2 since it does not contain phosphorylation consensus repeats. An apparently concerted mechanism of modification has previously been proposed for Glycogen Synthase and β-catenin by CKII or CKI (priming the phosphorylation sequence) and the glycogen synthesase kinase 3 (GSK3) (4 subsequent phosphorylations; [12, 13]). A similar phenomenon, of smaller amplitude, has been reported for a forkhead factor, the O subfamily member 1 (FOXO1): upon phosphorylation of a serine residue by protein kinase B (PKB), a CKI consensus is created, which, after phosphorylation, creates in turn another CKI consensus [14]. However, when more than one kinase is involved in the multiple modifications of these proteins, whether intermediates are detectable or not has not been investigated yet. Indeed, the modifications could be purely concerted, sequential, or somehow processive. Processive/concerted acetylation of a single protein substrate, as is likely the case for FOXL2 (for instance, the leap from E to H, four acetylations), is not well documented. According to our observations in the case of FOXL2, the first p*I* leap should involve three to four phosphorylations or four ac(et)ylations, depending on the forms, and it could occur through parallel modification pathways in the steady state.

The absence of intermediates is compatible with the recruitment of poorly modified FOXL2 into a "modification factory" (by analogy with replication and transcription factories: [15, 16]). This idea is in line with the fact that major modification enzymes, like the acetyl transferase p300, have been shown to colocalize with RNA polymerase II in discrete regions of variable size throughout the nucleus [17]. Our observations suggest that the process of FOXL2 acetylation could involve a series of slow reaction steps, as illustrated by the basic train, that prepare FOXL2 to be addressed into the modification factory before emerging in its highly modified form. This factory could be either a multisubunit enzymatic complex, or a subcellular compartment where modifying enzymes are concentrated. Indeed, in both cases high local enzyme concentration reduces the slow-down effect that diffusion might have on FOXL2 sequential modification. The mechanism hypothesized here is reminiscent of the concept of metabolic channeling, where a multienzyme complex sequentially processes a micromolecular substrate until the final product emerges.

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The authors declare no conflict of interest.

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