

# Discovery of novel protein partners of the transcription factor FOXL2 provides insights into its physiopathological roles

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**FOXL2 transcription factor is responsible for the *Blepharophimosis Ptosis Epicantus inversus Syndrome* (BPES), a genetic disease involving craniofacial malformations often associated with ovarian failure. Recently, a somatic FOXL2 mutation (p.C134W) has been reported in >95% of adult-type granulosa cell tumors. Here, we have identified 10 novel FOXL2 partners by yeast-two-hybrid screening and co-immunoprecipitation. Most BPES-inducing mutated FOXL2 proteins display aggregation in cultured cells. Here, we show that two of the partners (NR2C1 and GMEB1) can be sequestered in such aggregates. This co-aggregation can contribute to the pathogenesis of FOXL2 mutations. We have also measured the effects of FOXL2 interactants on the transcriptional regulation of a series of target promoters. Some of the partners (CXXC4, CXXC5, BANF1) were able to repress FOXL2 activity indistinctively of the promoter. Interestingly, CREM- $\tau 2\alpha$ , which acted as a repressor on most promoters, increased wild-type (WT) FOXL2 activity on two promoters (*PTGS2* and *CYP19A1*), but was unable to increase the activity of the oncogenic mutant p.C134W. Conversely, GMEB1, which also acted as a repressor on most promoters and increased WT FOXL2 activity on the *Per2* promoter, increased to a greater extent the activity of the p.C134W variant. Interestingly, partners with intrinsic pro-apoptotic effect were able to increase apoptosis induction by WT FOXL2, but not by the p.C134W mutant, whereas partners with an anti-apoptotic effect decreased apoptosis induction by both FOXL2 versions. Altogether, these results suggest that the p.C134W mutated form fails to integrate signals through protein–protein interactions to regulate target promoter subsets and in particular to induce cell death.**

## INTRODUCTION

FOXL2 belongs to the large family of winged-helix forkhead transcription factors. It is expressed at high levels in the eyelids, the adrenal and the pituitary glands and in ovarian granulosa cells (1). Mutations of *FOXL2* are responsible for the *Blepharophimosis Ptosis Epicantus inversus Syndrome* (BPES), a condition involving palpebral abnormalities, along with ovarian failure (type I BPES) or isolated (type II BPES). Some of FOXL2-mutated proteins display a cytoplasmic mislocalization and cytoplasmic and/or nuclear aggregation

when overexpressed. This is the case of a mutated protein involving an expansion of the polyAlanine domain of FOXL2 from 14 to 24 residues (referred to as 'Ala24') but aggregation has also been described for variants bearing single amino acid substitutions (2). Recently, a somatic FOXL2 mutation (p.C134W) has been reported in >95% of adult-type granulosa cell tumors (GCTs) (3), suggesting a role for FOXL2 in oncogenesis. The p.C134W FOXL2 version displays lower pro-apoptotic activity than wild-type (WT) FOXL2 (4). However, the molecular bases of the oncogenic effect of this mutation remain unclear.

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Constitutive mouse knock-out (KO) models of *Foxl2* display ovarian abnormalities such as early follicular depletion and a subsequent ovarian failure (5,6). A conditional KO in murine adult granulosa cells leads to their reprogramming into Sertoli-like cells, with a significant expression of testis-specific genes, such as *Sox9* (7). These mouse models of human pathology emphasize the important role of *Foxl2* in the development of the ovary and the maintenance of its identity and function.

Like other members of this family (such as FOXO proteins), FOXL2 has been implicated in numerous cellular processes, such as apoptosis, cell cycle control, steroidogenesis and reactive oxygen species (ROS) detoxification (8). However, little is known about its regulation and in particular, about the mechanisms by which FOXL2 regulates different target gene subsets depending on developmental stages and/or other signals. Such target specification may be achieved via direct changes of the interaction of FOXL2 with DNA through post-translational modifications and/or through context-dependent interactions with protein partners affecting the specificity of promoter recognition (9). In line with this, FOXL2 is known to undergo phosphorylation, acetylation and sumoylation (10), which modify its stability, subcellular localization and transactivation capacity.

Eight protein partners of FOXL2 have been described thus far and might help understand the regulation of specific gene subsets/pathways (reviewed in 11,12). For example, the interaction between the DEAD box protein 20 (DDX20) and FOXL2 in granulosa cells enhances the pro-apoptotic function of the latter (13). *Foxl2* interacts with the Steroidogenesis Factor 1 (SF-1/NR5A1) to inhibit its binding to the promoter of the *cyp17* gene in granulosa cells (14). *Foxl2* binding to estrogen receptor 1 (Esr1/ER- $\alpha$ ) inhibits, in a DNA-independent way, tamoxifen-induced activation of the promoter of the prostaglandin-endoperoxide synthase 2 gene (*cox2* or *ptgs2*) in heterologous human embryonic kidney 293FT cells (15). Moreover, *Foxl2* and *Esr1* have been described to synergistically repress *Sox9* expression *in vitro* (7). Finally, *Foxl2* has been shown to interact with Smad3 (mothers against decapentaplegic homolog 3) in a mouse gonadotrope cell model to regulate the *GnrhR* promoter via a composite DNA element involving *Foxl2*-Smad3-API-binding sequences (16). Smad3 and *Foxl2* have also been reported to co-regulate the expression of *Fsh- $\beta$*  (17) and *follicle-stimulating* genes (18). SUMO-conjugating enzyme UBC9 and E3 SUMO-protein ligase PIAS1, which also interact with FOXL2 in the ovary, are involved in its SUMOylation. This modification increases FOXL2 stability and transcriptional activity while promoting its recruitment to promyelocytic leukemia (PML) nuclear bodies (NBs) (10). Finally, in an ovarian cell model, the kinase LATS1, involved in FOXL2 phosphorylation, has been reported as enhancing its repression of the promoter of the *Star* gene (19).

Here, we have further explored the FOXL2 interactome and uncovered its interaction with CXXC4 (IDAX), CXXC5 (RINF or WID), CREM, GMEB1 (P96PIF), NR2C1 (TR2), SP100, RPLP1, BANF1 (BAF), XRCC6 (KU70) and SIRT1. We then investigated the potential modulation of the FOXL2 transactivation ability by such partners on several promoters in luciferase assays and demonstrate that CXXC4/5,

CREM- $\tau$ 2 $\alpha$ , GMEB1 and BANF1 modulate FOXL2 transactivation on a large panel of promoters. We have also explored the impact of these partners on the pro-apoptotic function of FOXL2 and found that the cancer-related mutant p.C134W was less affected than WT FOXL2 by the pro-apoptotic enhancing effect of some of the partners.

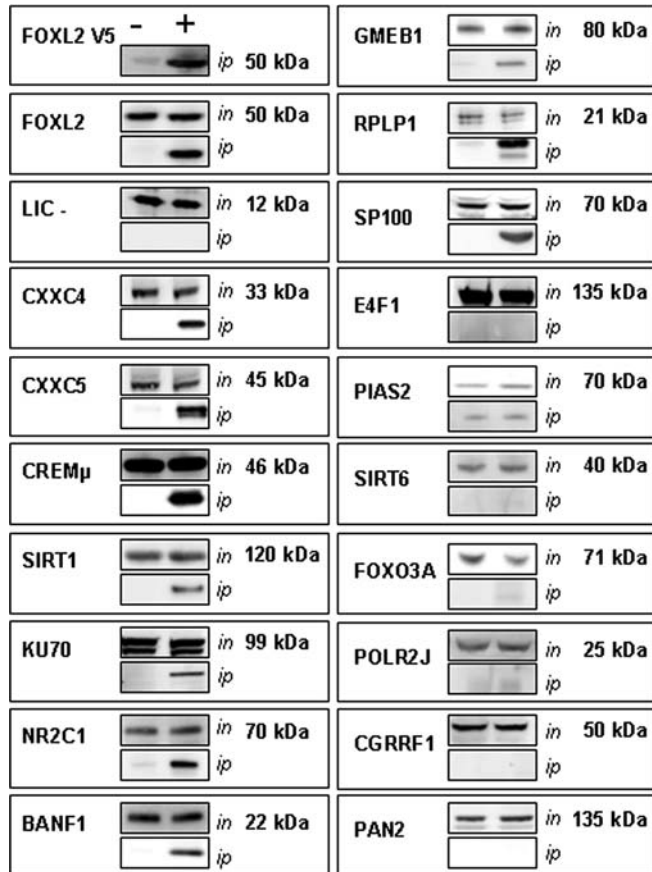
## RESULTS

### Identification and validation of FOXL2 partners

In order to discover novel FOXL2 protein partners, we have performed a yeast-two-hybrid (Y2H) screening using a mouse ovary cDNA library and the FOXL2 mutant p.H104N as the bait. This mutant is hypomorphic in the ovarian context and was used to avoid the toxicity that we have observed when expressing WT FOXL2 in yeast. We were able to identify 24 potential partners that are listed in the Supplementary Material, Table S1. In order to validate the partners found in the Y2H screening, we performed co-immunoprecipitation experiments in easily transfectable COS-7 cells for the most pertinent gene products. We excluded mitochondrial and most ribosomal proteins from further analyses because FOXL2 is clearly localized in the nucleus. However, we retained RPLP1, which was the most frequently found interactor. We also tested potential FOXL2 candidate partners, such as FOXO3, SIRT1 and SIRT6. Thus, we systematically co-overexpressed, by transfection of COS-7 cells, the tagged versions of the proteins identified by Y2H and either the human V5-tagged WT FOXL2 (i.e. FOXL2-V5) or the FOXL2-C134W-V5 cancer-related mutant. A complete list of the cloned partners and their tags appears in the Supplementary Material, Table S2. We could confirm interactions of FOXL2 with CXXC4, CXXC5, CREM- $\tau$ 2 $\alpha$ , GMEB1, NR2C1, SP100, RPLP1, BANF1, KU70 and SIRT1 (Fig. 1). All partners were co-precipitated with C134W FOXL2, with no visible difference with respect to WT FOXL2, suggesting that the mutation does not affect these interactions in an obvious manner in our experimental conditions. However, in more physiological conditions or using systems allowing lower expression levels of WT or mutant FOXL2, it might be possible that subtle differences in their interaction with the partners might be revealed. A brief description of the various *bona fide* partners can be found in Table 1. We failed to immunoprecipitate PIAS2, PAN2, E4F1, POLR2J, CGRRF1 and GNAI2 in three independent attempts. They might be either false positives or their interaction with FOXL2 may be of low affinity or take place in tissue- or (less likely) species-specific contexts. It is also possible but not likely that these partners might display a higher affinity for p.H104N FOXL2 mutant used in 2YH experiment than for WT FOXL2, used in co-immunoprecipitation experiments.

### Foxl2 and its partners are co-expressed in mouse follicular cells and co-localize within the cell

To confirm the expected expression overlap of the validated partners and *Foxl2*, we isolated follicles from adult mouse ovaries and cultured their granulosa cells. Granulosa-cell



**Figure 1.** FOXL2 interaction with its partners revealed by co-immunoprecipitation. COS-7 cells were co-transfected with human WT V5-tagged FOXL2 (or NLS-V5 as a control) along with the tagged versions of the potential partners of FOXL2 cloned in mammalian expression vectors (or LIC-expression vector as a control). Forty-eight hours post-transfection, FOXL2 was immunoprecipitated using an anti-V5 agarose-conjugated antibody, and partners were immunodetected through their respective tags (see vector list). We found no interaction between FOXL2-V5 or V5 beads with HSV tag alone (LIC-). As a positive control, we co-immunoprecipitated a FLAG-tagged version of FOXL2 with FOXL2-V5. For each protein, the upper panel represents the detection of a specific band at the indicated molecular weight on the western blot of cell lysates (i.e. input, referred to as *in*) and the lower panel shows the same detection on the western blot of separated eluates (i.e. immunoprecipitated material, referred to as *ip*). Direct physical interaction in COS-7 with WT FOXL2 was confirmed for CXXC4, CXXC5, CREM- $\tau$ 2 $\alpha$ , GMEB1, NR2C1, SP100, RPLP1, BANF1, KU70 and SIRT1. No interactions with POLR2J, PIAS2, GNAI2, PAN2, CGRRF1, SIRT6 and FOXO3A protein were revealed. At least three different experiments were performed for each potential partner.

enrichment in our preparation was checked by real-time polymerase chain reaction for several marker genes. We confirmed strong expression of *Foxl2* itself, *Inha*, *Inhba*, *Inhbb* and *Kitl* genes (markers of granulosa cells) and the absence of *Pou5f1* expression (marker of oocyte contamination). High expression of *Foxl2* and its partners in granulosa cell primary cultures or AT29C immortalized granulosa cells was confirmed for all partners except *Crem- $\tau$ 2 $\alpha$*  and *Sp100* (Supplementary Material, Fig. S1). *Crem- $\tau$ 2 $\alpha$*  was detectable at low levels, but other isoforms of the gene may be present. *Sp100* expression seemed very low in this experiment. However, transcriptomic data suggest that *Sp100* is significantly

expressed in human granulosa-like KGN cells (array-express E-MEXP-985).

We then assessed a potential co-localization of FOXL2 and its partners at the sub-cellular level. WT FOXL2-GFP and its HSV-tagged partners were co-overexpressed in COS-7 cells. Immunolabeling showed that all FOXL2 partners, except RPLP1, displayed a clear nuclear localization overlapping at least partially the FOXL2-GFP signal (Fig. 2A). NR2C1 perfectly co-localized with FOXL2 following the same intranuclear pattern (Fig. 2A). We have previously shown that FOXL2 and SP100 colocalize in PML NBs (10).

The details of the molecular dysfunction of FOXL2 leading to BPES are poorly known. A majority of FOXL2 BPES-inducing mutated proteins display aggregation when overexpressed in cultured cells (20). The expansion of the polyAlanine region of FOXL2 (i.e. Ala24) is the canonical case (21). In principle, protein aggregates can sequester FOXL2 partners and this might explain, at least in part, their pathogenic effects. Thus, we tested the potential co-aggregation of FOXL2 partners with the Ala24 FOXL2 mutant in COS-7 cells (Fig. 2B and Supplementary Material, Fig. S2). We found that the NR2C1 nuclear receptor perfectly co-localized with the aggregates of mutant FOXL2, suggesting that it can be sequestered in such structures (Fig. 2B). To a lower extent, GMEB1 also concentrated in FOXL2 aggregates but still displayed its original distribution pattern when overexpressed (i.e. both nuclear and cytoplasmic). The other tested partners did not seem to be 'trapped' within the aggregates and conserved their normal localizations.

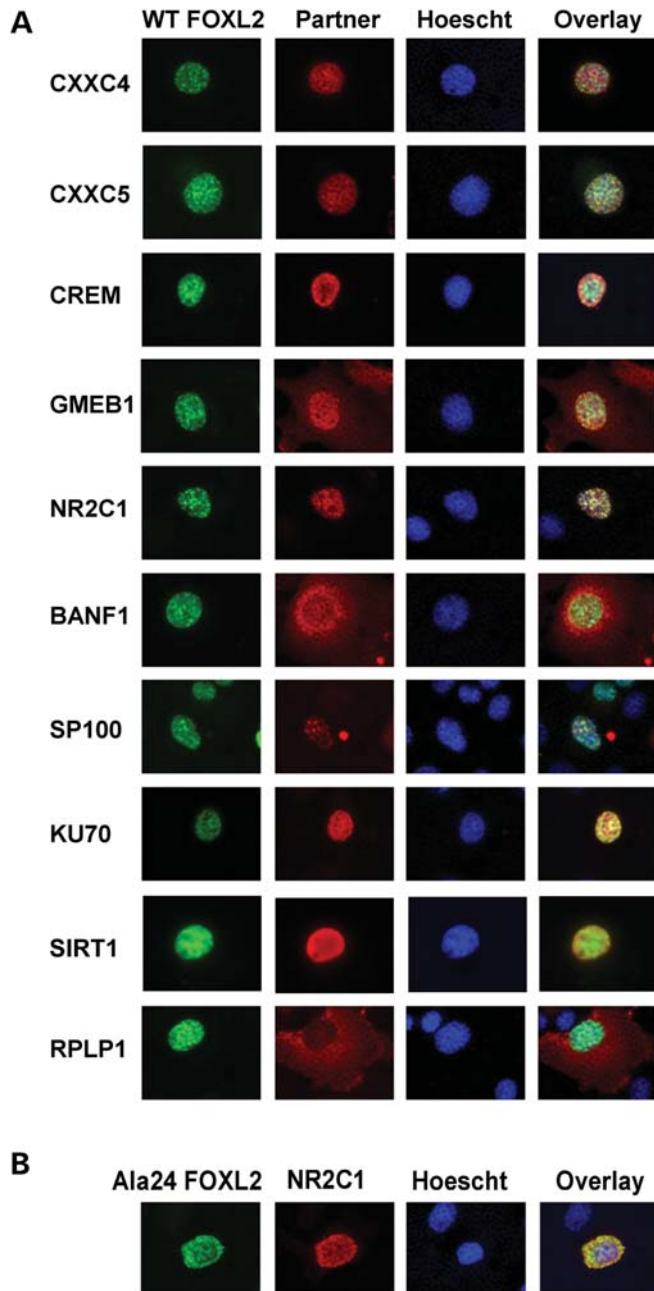
### Modulation of FOXL2 transactivation ability by its partners

We next assessed the influence of FOXL2 partners on its transactivation capacity. Specifically, we tested their effects in the human KGN granulosa-like cell model on 15 different promoters (i.e. luciferase assays), some of which belong to genes involved in the various processes modulated by FOXL2 (Supplementary Material, Table S3). Figure 3 displays a heat map representation of the modulation of FOXL2 transactivation by its partners on the promoters tested. We observed a systematic and reproducible repression of FOXL2 activity by CXXC4, CXXC5, CREM- $\tau$ 2 $\alpha$ , GMEB1 and BANF1 proteins. However, interesting exceptions were noticed. For instance, FOXL2 transactivation ability was increased on the human *PTGS2* and *CYP19A1* proximal promoters by CREM- $\tau$ 2 $\alpha$  (Fig. 4). These two promoters are known FOXL2 targets and drive the expression of genes encoding enzymes implicated in ovulation and oestradiol production, respectively (15,22). Moreover, CREM- $\tau$ 2 $\alpha$  failed to stimulate to the same extent C134W FOXL2 on the *PTGS2* promoter (-20% compared with WT FOXL2) (Fig. 4A). We also confirmed that C134W FOXL2 was more active than WT FOXL2 on the *CYP19A1* promoter. Interestingly, CREM- $\tau$ 2 $\alpha$  increased WT FOXL2 activity to the same level as p.C134W mutant alone, but failed to increase C134W FOXL2 activity on this promoter (Fig. 4B). We have previously shown that *Per2*, a gene modulating cell proliferation (23), is a FOXL2 target (24). FOXL2 transactivation of the *Per2* promoter was increased by GMEB1 and C134W FOXL2 was more stimulated than WT

**Table 1.** Overview of the function of the new FOXL2 partners

Protein symbol	Description	Uniprot accession	Pathway from biosystem	Main function	Comments
CXXC4	CXXC-type zinc finger protein 4	Q9H2H0	Wnt signaling pathway	DNA-binding and signal transduction	Inhibitor of the Wnt/ $\beta$ -catenin pathway (30)
CXXC5	CXXC-type zinc finger protein 5	Q7LFL8	Wnt signaling pathway	DNA-binding and signal transduction	Inhibitor of the Wnt/ $\beta$ -catenin pathway (35)
CREM	cAMP responsive element modulator	Q03060	Multiple	DNA-binding and signal transduction	Transcription factor: activators and/or repressors according to relevant splicing isoform (55)
GMEB1	Glucocorticoid modulatory element-binding protein 1	Q9Y692	Unknown	DNA-binding and signal transduction	Trans-acting factor binding GME (Glucocorticoid Modulatory Element) DNA sequences Modulator of the glucocorticoid receptor-mediated response (56)
NR2C1	Nuclear receptor 2 subfamily 2, group C, member 1	P13056	Nuclear receptor transcription pathway	DNA-binding and signal transduction	Orphan nuclear hormone receptor Transcription factor that binds to hormone response elements DNA sequences Regulate Oct4 and RAR- $\beta$ promoters (reviewed in 57)
SP100	SP100 nuclear antigen	P23497	Interferon signaling and immune system	Transcription factor binding	Major component of the PML (Promyelocytic Leukemia Nuclear) NBs Potential role as tumor suppressor
BANF1	Barrier to autointegration factor 1	O75531	Integration of provirus	Transcription factor binding	Component of the nuclear envelope Binds to dsDNA, chromatin, nuclear lamina proteins, histones and various transcription factors (29) Required for many cellular functions, such as chromatin organization and transcription regulation (58)
XRCC6 (KU70)	X-ray repair complementing defective repair in Chinese hamster cells 6	P12956	Non-homologous end-joining and double-strand break repair	DNA and transcription factor binding	Involved in double-strand DNA breaks repair (59) Transcription regulation activity (60) Interacts with numerous proteins such as Hox and FoxO transcription factors (61,62)
SIRT1	NAD-dependent deacetylase sirtuin 1	Q96EB6	FoxO family signaling, etc.	Transcription factor binding and acetylation	Deacetylase enzyme implicated in the control of numerous cellular functions such as DNA repair, cell aging and regulation of cell growth (63)
RPLP1	60S acidic ribosomal protein P1	P05386	Ribosome	Translation	Member of the P group of ribosomal proteins that form the ribosomal stalk Potential role in cell proliferation and aging (64)

The first three columns indicate the protein symbol, description and accession number in the UniprotKB/Swiss-prot database (For XRCC6, KU70 is the most commonly used symbol and is used in the rest of the manuscript). The fourth column indicates protein pathway annotation according to the NCBI Biosystems database. Fifth and sixth columns indicate the main protein functions and features based on bibliography.



**Figure 2.** Subcellular localization of WT FOXL2 and its partners. (A) COS-7 cells were transfected with WT FOXL2-GFP and its HSV-tagged partners, or FOXL2-mCherry and KU70-GFP. Forty-eight hours after transfection, cells were fixed and FOXL2 partners were identified by anti HSV immunofluorescence. Nuclei were stained with Hoechst 33342. Observations were performed with an epifluorescence microscope equipped with an apotome module. Each row shows a typical cell with the localization of FOXL2 (first micrograph), the relevant partner (second micrograph), Hoechst/nuclear staining (third micrograph) and an overlay of three signals (fourth micrograph, pseudo-colors). CXXC4 displays its described nuclear localization (some cells presented a variable level of diffuse cytoplasmic signal). FOXL2 and CXXC4 localized in the same subcellular compartment, but the two proteins do not display systematic overlap in the nucleus, especially in the NBs where FOXL2 is present. CXXC5 displays a similar cellular localization as CXXC4 (mainly nuclear). FOXL2 and CXXC5 localized in the same subcellular compartment, and the two proteins present a similar chromatin localization pattern. CREM- $\tau 2\alpha$ , a transcription repressor displays a diffuse intra-nuclear pattern. This distribution partially overlaps that of FOXL2. GMEB1 displays a nuclear and

cytoplasmic localization (some cells presented nuclear-only localization). In the nucleus, GMEB1 distribution pattern followed that of chromatin. Only partial overlap with FOXL2 distribution can be seen. NR2C1 is solely localized in the nucleus. Strikingly, its distribution perfectly overlaps with that of FOXL2, namely, in the same NBs (when they are apparent in the cell). F) BANF1 was localized as described in the literature: a clear staining at the nuclear membrane can be observed, with a weaker cytoplasmic and nuclear diffuse distribution. Co-localization between FOXL2 and BANF1 can be observed mainly in the chromatin near the nuclear envelope. SP100 is mainly localized in discrete nuclear dots, the PML bodies. As we already described, FOXL2 and SP100 co-localized in PML bodies. KU70, a DNA repair and transcription modulating factor, is localized solely in the nucleus. KU70 and FOXL2 display a close but not identical localization on the chromatin. SIRT1 displays its described nuclear localization (some cells presented with a variable level of diffuse cytoplasmic signal). This distribution partially overlaps that of FOXL2. RPLP1 is a ribosomal protein and its distribution is strictly cytoplasmic. FOXL2 and RPLP1 have exclusive subcellular localization, which suggests that their interaction, if confirmed in a physiological context, is transient. (B) Same experiment as above, conducted using Ala24 FOXL2-GFP aggregation-prone mutant. When co-transfected with FOXL2 mutant, NR2C1 localizes in nuclear and cytoplasmic FOXL2 aggregates.

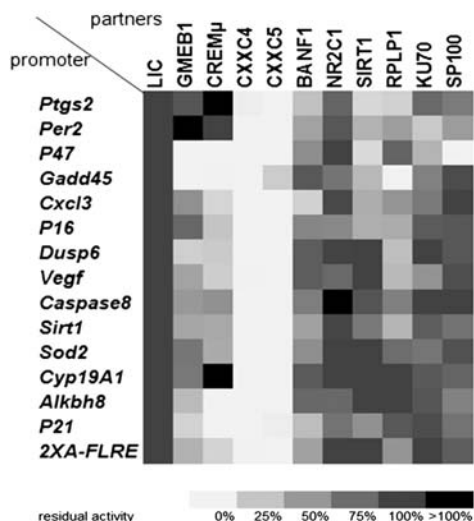
### Modulation of the pro-apoptotic function of FOXL2 by its partners

One of the known outputs of FOXL2 activity is an increase in apoptosis. We previously showed that C134W FOXL2 has a decreased pro-apoptotic ability compared with WT FOXL2 (4), which may explain in part the role of this mutation in GCT formation/progression. However, the molecular mechanisms underlying this observation are still unknown. We therefore tested the modulation of WT FOXL2 or C134W mutant pro-apoptotic activity by the partner proteins uncovered here. The level of apoptosis was estimated by measuring cell viability after over-expression of WT or C134W FOXL2 and the partner proteins in KGN cells (Fig. 5). WT FOXL2 and the p.C134W mutant expressed alone decreased cell viability by  $\sim 60$  and 40%, respectively. Some of the partners (such as CXXC4, CXXC5, KU70, BANF1 and SIRT1) displayed a moderate intrinsic pro-apoptotic (i.e. viability-decreasing) ability when overexpressed. When WT or mutant FOXL2 was co-overexpressed with the partners, three classes of responses were observed:

- (i) (i) KU70, BANF1, SIRT1 and RPLP1 decreased FOXL2 WT or C134W pro-apoptotic activity to the same extent.
- (ii) CXXC5, GMEB1, NR2C1 and SP100 increased apoptosis specifically in the presence of WT FOXL2, but not when C134W FOXL2 was expressed.
- (iii) Finally, CREM- $\tau 2\alpha$  and CXXC4 did not significantly modify either WT or mutant FOXL2 pro-apoptotic ability.

These results suggest that the C134W-mutated form fails to integrate pro-apoptotic signals to induce cell death.

cytoplasmic localization (some cells presented nuclear-only localization). In the nucleus, GMEB1 distribution pattern followed that of chromatin. Only partial overlap with FOXL2 distribution can be seen. NR2C1 is solely localized in the nucleus. Strikingly, its distribution perfectly overlaps with that of FOXL2, namely, in the same NBs (when they are apparent in the cell). F) BANF1 was localized as described in the literature: a clear staining at the nuclear membrane can be observed, with a weaker cytoplasmic and nuclear diffuse distribution. Co-localization between FOXL2 and BANF1 can be observed mainly in the chromatin near the nuclear envelope. SP100 is mainly localized in discrete nuclear dots, the PML bodies. As we already described, FOXL2 and SP100 co-localized in PML bodies. KU70, a DNA repair and transcription modulating factor, is localized solely in the nucleus. KU70 and FOXL2 display a close but not identical localization on the chromatin. SIRT1 displays its described nuclear localization (some cells presented with a variable level of diffuse cytoplasmic signal). This distribution partially overlaps that of FOXL2. RPLP1 is a ribosomal protein and its distribution is strictly cytoplasmic. FOXL2 and RPLP1 have exclusive subcellular localization, which suggests that their interaction, if confirmed in a physiological context, is transient. (B) Same experiment as above, conducted using Ala24 FOXL2-GFP aggregation-prone mutant. When co-transfected with FOXL2 mutant, NR2C1 localizes in nuclear and cytoplasmic FOXL2 aggregates.



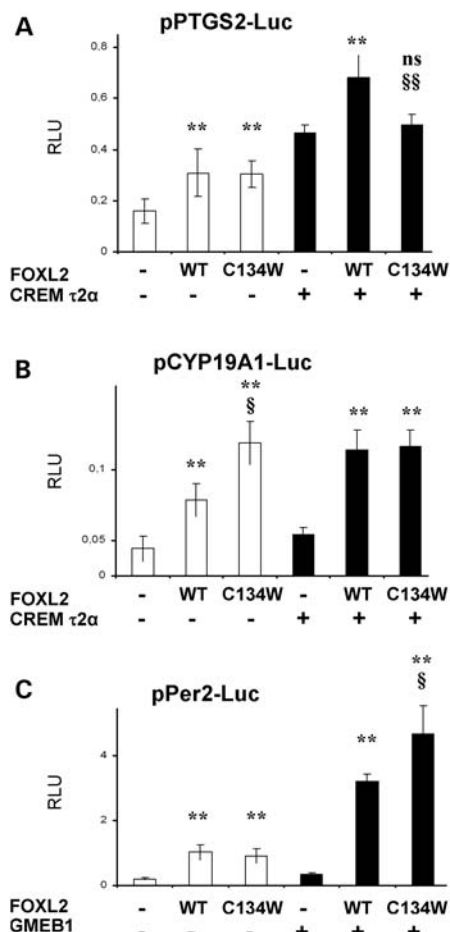
**Figure 3.** Modulation of FOXL2 transactivation by its partners on a panel of target promoters. KGN cells were transfected with firefly luciferase promoter reporters, Renilla luciferase control, FOXL2 (or NLS control) and FOXL2 partners (or LIC control). Transcriptional activity was tested on 15 different promoters, already known to be FOXL2 targets. Here is shown a heat map representing the modulation of FOXL2 transactivation activity by its partners on the promoters tested. Each square represents the average ratio of luciferase activity with FOXL2 overexpression on luciferase activity without FOXL2 overexpression in two experiments with four replicates each. The scale running from white to black is proportional to FOXL2 transactivation relative to the conditions without partner. For instance, dark grey squares of the 'FOXL2 + LIC' column represent normal FOXL2 activity on each promoter (and set the 100% of the scale), darker/lighter squares represent increased/decreased FOXL2 activity when the partner is present. Means, standard deviations and tests of statistical significance are given in Supplementary Material, Table S4.

## DISCUSSION

As already mentioned, FOXL2 is involved in the etiology of premature ovarian failure and of GCTs. In KGN cells, FOXL2 binds to the promoters of a wide variety of genes, modulating processes such as cell cycle, ROS detoxification and apoptosis (reviewed in 11). Overexpression of FOXL2 in this cell model represses progression through cell cycle and promotes apoptosis. An emerging idea in the field of gene regulation is that combinations of different transcription factors are the key to specify an adequate transcriptional response to a particular stimulus (10). Thus, to gain insights into how FOXL2 is able to regulate diverse processes, we searched for new FOXL2 partners potentially able to modulate its activity.

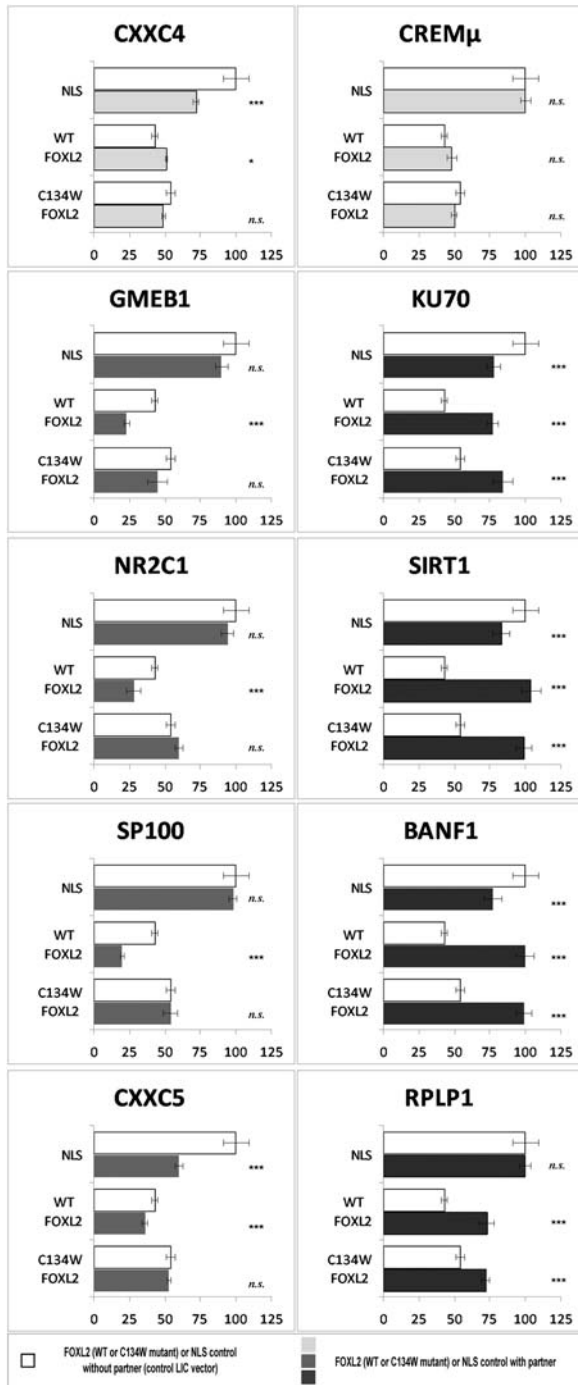
We demonstrate here that FOXL2 interacts with the SIRT1 deacetylase, completing our previous work showing that SIRT1 is able to modulate FOXL2 activity (24). We also show that SIRT1 represses the pro-apoptotic activity of FOXL2, in the same way as it has been described for *FOXO* genes (25).

Consistently with known FOXL2 functions, it interacts with CREM- $\tau$ 2 $\alpha$ . This CREM isoform can be an activating or an inhibitory transcription factor according to the promoter context (26). *CREM* gene products modulate several cellular processes such as apoptosis, cells cycle, extra-cellular matrix remodeling or hormone and prostaglandin production. In the



**Figure 4.** Modulation of FOXL2 (WT and C134W) transactivation by CREM- $\tau$ 2 $\alpha$  or GMEB1 on *PTGS2*, *CYP19A1* or *Per2* promoters. KGN cells were transfected with firefly luciferase promoter reporters, Renilla luciferase control, FOXL2 (WT and C134W) or control and FOXL2 partners (or control). Relative lights units (RLU) correspond to the ratio of the activity of the firefly luciferase reporter over that of the Renilla reporter (internal control of transfection efficiency). Each value is the average of six biological replicates, and each experiment shown is representative of at least four independent experiments. (A) WT and C134W FOXL2 activated *PTGS2* promoter to the same extent and WT FOXL2 transactivation ability was increased on the *PTGS2* by CREM- $\tau$ 2 $\alpha$ . CREM- $\tau$ 2 $\alpha$  failed to stimulate to the same extent C134W FOXL2 on the *PTGS2* promoter ( $-20\%$  compared with WT FOXL2,  $P = 0.02$ ). (B) WT FOXL2-activated *CYP19A1* promoter and C134W FOXL2 mutant overactivated this promoter when compared with WT. FOXL2 transactivation ability was increased on the *CYP19A1* by CREM- $\tau$ 2 $\alpha$  but it failed to stimulate C134W FOXL2 on this promoter. (C) WT and C134W FOXL2 activated *Per2* promoter to the same extent. FOXL2 transactivation capacity was increased on the *Per2* promoter by GMEB1 and C134W FOXL2 was more stimulated than WT FOXL2 by this partner on the *Per2* promoter ( $+45\%$ ,  $P = 0.01$ ). Statistical significance according to Student's *t*-tests versus pcDNA (control condition). n.s., non-significant.  $**P < 0.01$ . When relevant, statistical significance in Student's *t*-tests FOXL2 WT versus C134W.  $^{\$}P < 0.05$ ;  $^{\$\$}P < 0.01$  (for C134W when nothing is indicated, not-significant).

rat ovary, *Crem* participates in the control of *Cyp19A1* expression (27), a gene also modulated by FOXL2. The peptide segment of CREM interacting with FOXL2 is present in several protein isoforms, such as CREM- $\alpha$ , which is a strong transcriptional repressor. Thus, interaction with



**Figure 5.** Modulation of FOXL2-induced apoptosis by its partners. KGN cells were transfected with WT or mutants FOXL2-V5 (or NLS-V5 control) and FOXL2 partners (or control). Cell viability was assessed as previously described and taken as a proxy to assess apoptosis (4). Briefly, KGN cells were transfected with a total of 0.33  $\mu$ g plasmid DNA, including 0.15  $\mu$ g pcDNA3.1 expression construct (control, WT FOXL2 or C134W FOXL2), 0.15  $\mu$ g of pTriex4 expression construct (partners) and 0.03  $\mu$ g of the pCMV- $\beta$ -gal reporter. The number of  $\beta$ -gal-expressing cells was determined at 24 h after transfection. White bars represent FOXL2-V5 (or NLS-V5 control) transfected alone, grey bars represent FOXL2-V5 (or NLS-V5 control) transfected with the indicated partner. Data (means  $\pm$  SEM) are from at least two different experiments in triplicate, normalized to percentage of control, and presented as cell viability. Statistical significance according to Student's *t*-test NLS, WT FOXL2 or C134W FOXL2 with control plasmid versus the same condition, respectively, with a partner.

different CREM isoforms might lead FOXL2 to promote either activation or repression depending on the cellular/signaling context. Interestingly, WT FOXL2 activation of the human *CYP19A1* promoter was further stimulated by CREM- $\tau$ 2 $\alpha$ , whereas C134W FOXL2, which is hypermorphic on this promoter, was insensitive to CREM- $\tau$ 2 $\alpha$ . Similar observations were made on the human *PTGS2* promoter. These findings suggest that the C134W mutation can attenuate/abolish the regulatory effects of FOXL2 partners.

BANF1 is a nuclear protein implicated in nuclear and chromatin assembly and cell cycle progression through the S phase in mammalian cells (28). It interacts with transcription factors such as the retinal cone-rod homeobox factor (Crx) and represses CRX-dependent reporter activity *in vivo* (29). We show that FOXL2 also interacts with BANF1 and that this interaction leads to a mild inhibition of the transactivation capacity of both WT and p.C134W FOXL2 on most of the reporter promoters tested. These results are suggestive of a role of BANF1 on the control of FOXL2 global activity. Consistently with these data, BANF1 abrogated FOXL2 pro-apoptotic function. Thus, the expression level of BANF1 may indirectly impact granulosa cell differentiation and apoptosis.

CXXC4 (IDAX) is a tumor suppressor (30) able to inhibit Wnt/ $\beta$ catenin pathway by directly binding to the PDZ domain of the Dvl protein (31). CXXC5 (RINF or WID) is closely related to CXXC4 and acts in a similar fashion on the Wnt/ $\beta$ catenin pathway. We show here that FOXL2 interacts with both CXXC4 and CXXC5. Luciferase assays indicated a strong inhibition of FOXL2 activity by both CXXC4 and CXXC5 on all the promoters tested, suggesting either the existence of a direct repressive effect on FOXL2 or that active Wnt/ $\beta$ catenin is required for FOXL2 activity. An interaction between FOXL2 and the  $\beta$ -catenin pathway has already been proposed based on the study of sex-reversed *Foxl2-Wnt4* (32) and *Foxl2-Rspo1* KO mice (33). A potential pro-male/anti-female role of CXXC5 is compatible with the sex dimorphic expression of the mouse ortholog during gonadal sex determination (data from [www.germonline.org](http://www.germonline.org)). Indeed, *Cxxc5* is more expressed in testicular supporting cells than in the ovary and its expression increases during testis development and decreases during ovarian development. This potential inhibition of FOXL2 by CXXC5 might be at work through follicular maturation during which FOXL2 is down-regulated (6). Several studies suggest that *Cxxc5* may act as a tumor suppressor. For instance, *Cxxc5* is silenced in mouse mammary gland tumors (34) and may have a role in tumoral myelopoiesis (35,36). Moreover, CXXC5 has been described to be implicated in p53 signaling in response to DNA damage and apoptosis (37). In line with this, we show here that CXXC5 increases WT FOXL2 pro-apoptotic ability but not that of p.C134W FOXL2. The interaction between

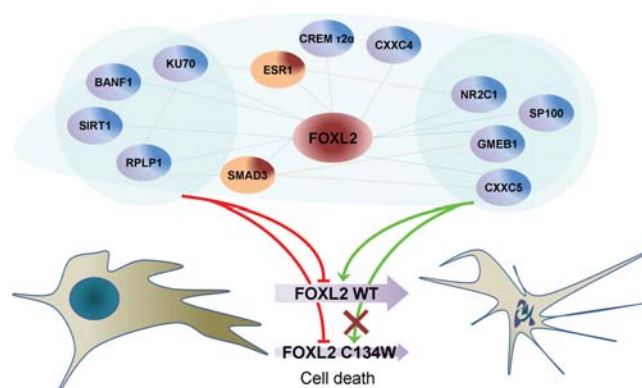
n.s., not-significant. \* $P < 0.05$ . \*\*\* $P = 0.001$ . When WT or mutant FOXL2 was co-overexpressed with its partners, three classes of behaviors could be observed: KU70, BANF1, SIRT1 and RPLP1 decreased FOXL2 WT or C134W pro-apoptotic activity to the same extent (dark grey bars), CXXC5, GMEB1, NR2C1 and SP100 increased apoptosis specifically in the presence of WT FOXL2, but not when C134W FOXL2 was overexpressed (medium grey bars), Finally, CREM- $\tau$ 2 $\alpha$  and CXXC4 did not significantly modify either WT or mutant FOXL2 pro-apoptotic ability (light grey bars).

C134W FOXL2 and CXXC5 deserves further study as CXXC5 might be implicated in ovarian pathologies (38). Genome-wide analyses such as transcriptomics and ChIP-on-Chip/Seq may also help explain why CXXC5 inhibits FOXL2 in luciferase assays and yet it stimulates the pro-apoptotic capacity of the WT form.

We find that GMEB1 partially inhibits FOXL2 activity on all the promoters tested (with a complete inhibition for the *P47*, *DUSP6* or *GADD45* promoters). Conversely, GMEB1 seems to cooperate with FOXL2 to activate the *Per2* promoter, to activate more strongly p.C134W than WT FOXL2. The *Per2* gene has been described to be an anti-oncogene and a perturbation of its expression through the cell cycle has been reported in breast cancer (23). Thus, it is conceivable that p.C134W mutant might lead to a misregulation of the *PER2* gene through an altered interaction with GMEB1 (and potentially other factors). GMEB1 has been described to be an apoptosis repressor through inhibition of pro-caspase 2 auto-proteolytic activation, especially under oxidative stress conditions (39). Here, we show that GMEB1 increases WT FOXL2 pro-apoptotic activity but not that of p.C134W mutant. Considering that the pro-apoptotic capacity of FOXL2 was shown to be mediated by caspases 3 and 8 (4), the anti-apoptotic effect of GMEB1 may not interfere with FOXL2 pro-apoptotic ability. It is therefore possible that FOXL2 recruits GMEB1 as a co-activator of pro-apoptotic target genes. Interestingly, GMEB1 over-activates C134W FOXL2 on the *Per2* promoter. However, this over-activation does not translate into an enhancement of the pro-apoptotic capacity of p.C134W FOXL2 by GMEB1. This suggests that the effect of GMEB1 on *Per2* [known to induce apoptosis when up-regulated (40)] is not essential for p.C134W-induced oncogenesis.

The nuclear receptor NR2C1 is involved in various cellular processes, including differentiation (41), proliferation (42,43) and apoptosis (44). Nr2c1 interacts with Esr1 and inhibits its transactivation capacity (42). Interestingly, Foxl2 also interacts with Esr1 to inhibit its function (7). Here, we show that FOXL2 and NR2C1 interact and perfectly co-localize. We have previously shown that FOXL2 localizes to PML NBs. Interestingly, NR2C1 is also recruited to PML NBs where it undergoes SUMOylation (43). Considering that (i) FOXL2 and NR2C1 physically interact, (ii) that they have common partners (such as Esr1), and (iii) that they co-localize, one might suspect that FOXL2 and NR2C1 can be involved in an oligomeric structures. NR2C1 has been described to be a pro-apoptotic factor in embryo-derived teratocarcinoma mouse p19 cells (44). Here, in KGN cells, we did not observe a pro-apoptotic effect of NR2C1 alone, but it increased apoptosis induced by WT FOXL2. As described for GMEB1, the p.C134W mutant pro-apoptotic capacity seemed unaffected by NR2C1. NR2C1 expression is regulated by retinoic acid (RA) and is probably implicated in RA-induced apoptosis (44). RA is involved in the regulation of granulosa cell proliferation (45), which might be mediated, at least in part, by the interaction between NR2C1 and FOXL2.

KU70, a product of the *XRCC6* gene, is implicated in double-stranded DNA repair (46) and telomeric stability (47). Not surprisingly, a potential role in cancer susceptibility has been suggested (48). Interestingly, KU70 has also been



**Figure 6.** Differential modulation of WT and oncogenic FOXL2 mutant pro-apoptotic function by its protein partners. Upper panel: network of previously known (brown) and newly discovered (blue) FOXL2-interacting proteins. Dashed lines represent old (brown) and new (blue) protein-protein interactions. Lower panel: WT FOXL2-induced apoptosis in a human granulosa cell model. As described previously, the p.C134W mutant displays less apoptotic potential. Newly described FOXL2 partners can be distinguished in three classes: (i) inhibitors of FOXL2 WT or C134W pro-apoptotic activity (red blunt arrow), (ii) enhancers of WT FOXL2 induced apoptosis (green arrow), these do not enhance C134W FOXL2 apoptotic activity (crossed arrow), (iii) partners that did not significantly modify either WT or mutant FOXL2 pro-apoptotic ability. These results suggest that C134W mutation may be unable to integrate pro-apoptotic signals to induce cell death.

shown or suggested to interact with three FOXL2 interactants: ESR1 (49), SMAD3 (50) and RPLP1 (51). We show that the KU70-FOXL2 interaction leads to an inhibition of the FOXL2 transactivation capacity on the *Per2* and *VEGF* promoters, suggesting a potential interplay of FOXL2 and KU70 in the etiology of GCTs. Moreover, KU70 has been related to apoptosis regulation in several studies. Up-regulation of KU70 has been described to promote oncogenic phenotypes, such as resistance to apoptosis (52). In agreement with this, we observe that KU70 is able to strongly inhibit FOXL2-induced apoptosis. Thus, KU70 might be implicated in the C134W FOXL2 oncogenic effect by further decreasing its pro-apoptotic capacity in physiological contexts.

To summarize, we describe 10 novel FOXL2 protein partners in the ovarian context. Interestingly, some of these partners also interact with known FOXL2 partners (such as NR2C1 and ESR1 or KU70 with either ESR1, RPLP1 or SMAD3). Thus, it is possible that FOXL2 acts in heteromeric and/or alternative complexes with different target specificities, depending on their compositions. At the cellular level, FOXL2 and its partners regulate common functions (cell cycle, apoptosis and cellular stress response). From a pathological point of view, FOXL2 mutations might affect proper formation or stability of such complexes, the perturbations of which will eventually lead to BPES or GCTs. This deserves a more detailed exploration. Several results of our work point towards a partial defect in the regulation of the apoptotic activity of C134W FOXL2 (Fig. 6). Indeed, partners that increase the pro-apoptotic capacity of WT FOXL2 failed to increase the pro-apoptotic ability of C134W FOXL2. Moreover, partners that abolish this function in the context of the WT protein, do it to the same extent for the mutant one. The physiological/integrated output of this would be that



cells bearing the p.C134W mutation would not be able to trigger FOXL2-mediated apoptosis which might constitute a first step towards oncogenesis.

## MATERIALS AND METHODS

### Mouse strains

Ovaries were retrieved from mouse females from C57BL6 genetics background. The animals were housed in a controlled environment in Monod Institute's animal facility. Mice were sacrificed at the age of 5–10 weeks. All the experimental procedures were conducted in accordance with the policies of the Guidelines for Biomedical Research Involving Animals.

### RNA extraction and cDNA synthesis

Total RNA from mouse ovaries or mouse granulosa cells was 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 random hexamers.

### Yeast two-hybrid screening

The yeast two-hybrid screen using the FOXL2 bait was carried out by Dualsystems Biotech AG, Zurich, Switzerland as described in reference (10).

### Mouse granulosa cell primary culture

Mouse granulosa cells were retrieved as followed: briefly, ovaries were digested in collagenase I DNase supplemented medium. Follicles were retrieved under magnifying glasses, dispersed by trypsination and grown in the DMEM-F12 medium, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen-Gibco).

### Cell culture and transient transfections

Granulosa-like KGN cells were grown in the DMEM-F12 medium and supplemented with 10% FBS and 1% penicillin/streptomycin (Invitrogen-Gibco). COS-7 cells were grown in the DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were seeded 16 h prior to transfection at a density of  $4 \times 10^4$  cells  $\text{cm}^{-2}$  (KGN cells) or  $2.5 \times 10^4$  cells  $\text{cm}^{-2}$  (COS-7 cells), and transfected using the calcium-phosphate method (COS-7) or Lipofectamine 2000 reagent (KGN) (Invitrogen).

### Mammalian cell expression and reporter vectors

The FOXL2-V5 expression vectors (WT and C134W mutant) were described previously (10). CXXC4, CXXC5, GMEB1, NR2C1, SP100, RPLP1, PIAS2, PAN2, POLR2J, CGRRF1 and GNAI2 were amplified from pooled cDNAs from human cultured cells (HeLa, Jurkat, K562, KGN and COV434); CREM- $\tau 2\alpha$  and BANF1 (generously given by Gellersen (53) and Haraguchi (54), respectively) were amplified from expression vectors and inserted in frame with an HSV

tag into the pTriex4 Ek/LIC vector (Novagen), according to the manufacturer's instructions. LIC- (expressing a fusion protein His-Tag:S-Tag:HSV-Tag:His-Tag) was created by inserting a short DNA sequence (10 bp) in frame with the tags of pTriex-4 Ek/LIC vector. KU70-GFP and E4F1-eGFP vector were generously given by Dr Lecam. SIRT1-myc vector was generously given by Dr Kouzarides. SIRT6 and FOXO3A vector were retrieved from Addgen (nos 13817 and 1787). Reporter vectors for *SIRT1*, *Per2*, *P21*, *P16*, *P47*, *GADD45*, *ALKBH8*, *VEGF*, *SOD2*, *DUSP6*, *CXCL3*, *CASPASE8* and *PTGS2* have been described elsewhere.

### Luciferase assays

Luciferase assays were performed in KGN cells as previously described (20). Twenty-four hours after transfection, cells were lysed and analyzed with the Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's instruction. Relative luciferase units given for all experiments are the means of values obtained from eight biologically independent replicates, and represent the ratio of firefly luciferase activity over Renilla luciferase activity in the samples. Statistical significance was estimated by Student *t*-tests. Error bars represent the standard deviation.

### Cell viability assays

KGN cells were transfected with a WT or mutant FOXL2-V5 expression vector (or NLS-V5 control) and an expression vector for FOXL2 partners (or control). Cell viability was assessed as previously described (4). Briefly, KGN cells were transfected with a total of 0.33  $\mu\text{g}$  plasmid DNA including 0.15  $\mu\text{g}$  pcDNA3.1 expression construct (NLS, WT FOXL2 or C134W FOXL2), 0.15  $\mu\text{g}$  of pTriex4 expression construct (partners) and 0.03  $\mu\text{g}$  of the pCMV- $\beta$ -gal reporter. The number of  $\beta$ -gal-expressing cells was determined at 24 h after transfection.  $\beta$ -gal units given for all experiments are the means of values obtained from eight biologically independent replicates. Statistical significance was estimated by Student's *t*-tests. Error bars represent the standard deviation.

### Immunoprecipitation and antibodies

COS-7 cells were lysed 48 h after transfection in lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, pH 7.6) supplemented with protease inhibitors (PMSF 0.1 mM and complete mini EDTA-free cocktail, Roche) and phosphatase inhibitors (PhosSTOP, Roche). Clarified lysates were immunoprecipitated using anti-V5 M2 beads (Sigma) according to the manufacturer's instructions. Precipitated proteins were eluted in SDS-Laemmli buffer. Eluates were then separated by SDS-PAGE using NuPage Bis-Tris 4–12% gels and MOPS-SDS Running Buffer (Invitrogen) and proteins were electrotransferred onto PVDF membranes (Hybond-P, GE Healthcare). Partners were revealed using polyclonal rabbit anti-HSV (Abcam) antibody, mouse polyclonal anti-myc (Invitrogen), mouse polyclonal anti-GFP (Roche) or mouse monoclonal anti-HA (Sigma) according to their tag. FOXL2 was detected using an anti-C terminal anti-FOXL2 antibody available in the lab.

## Immunocytochemistry

Forty-eight hours after transfection, COS-7 cells were washed with PBS, fixed for 15 min with Histofix (Trend Scientific, Inc), permeabilized for 15 min with PBS/1% Triton-X100/10% FBS and then blocked with 5% non-fat milk and 2% FBS in PBS/0.1% Tween-20. Cells were incubated overnight in the anti-HSV antibody (1/1000). Cells were then washed three times in PBS, and incubated for 1 h in an anti-rabbit Cy3 secondary antibody (diluted 1/500). The cover slips were mounted on glass slides with DakoCytomaton (Dako). Cells were visualized by epifluorescence microscopy using a ZEISS Axiovert 200 microscope with ZEISS apotome system under  $\times 63$  magnification.

## SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

*Conflict of Interest statement.* None declared.

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