Human Molecular Genetics, 2011, Vol. 20, No. 9 ***1673–1686***
doi:10.1093/hmg/ddr042
Advance Access published on February 2, 2011

Transcription factor FOXL2 protects granulosa cells from stress and delays cell cycle: role of its regulation by the SIRT1 deacetylase

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Received January 13, 2011; Revised January 13, 2011; Accepted January 28, 2011

FOXL2 is a transcription factor that is essential for ovarian function and maintenance, the germline mutations of which are responsible for the Blepharophimosis Ptosis Epicanthus-inversus Syndrome (BPES), often associated with premature ovarian failure. Recent evidence has linked FOXL2 downregulation or somatic mutation (p.Cys134Trp) to cancer, although underlying molecular mechanisms remain unclear. Using a functional genomic approach, we find that FOXL2 modulates cell-cycle regulators in a way which tends to induce G1 arrest. Indeed, FOXL2 upregulation promotes cell accumulation in G1 phase and protects cells from oxidative damage, notably by promoting oxidized DNA repair and by increasing the amounts of anti-oxidant agent glutathione. In agreement with clinical observations, we find that FOXL2-mutated versions leading to BPES along with ovarian dysfunction mostly fail to transactivate cell-cycle and DNA repair targets, whereas mutations leading to isolated craniofacial defects (and normal ovarian function) activate them correctly. Interestingly, these assays revealed a mild promoter-specific hypomorphy of the tumor-associated mutation (p.Cys134Trp). Finally, the SIRT1 deacetylase suppresses FOXL2 activity on targets linked to cell-cycle and DNA repair in a dose-dependent manner. Accordingly, we find that SIRT1 inhibition by nicotinamide limits proliferation, notably by increasing endogenous FOXL2 amount/activity. The body of evidence presented here supports the idea that FOXL2 plays a key role in granulosa cell homeostasis, the failure of which is central to ovarian ageing and tumorigenesis. As granulosa cell tumors respond poorly to conventional chemotherapy, our findings on the deacetylase inhibitor nicotinamide provide an interesting option for targeted therapy.

INTRODUCTION

Members of the Forkhead box superfamily of transcription factors regulate a number of biological processes such as development, differentiation and proliferation (1). Mutations in Forkhead factor genes have been associated with human genetic diseases, and a growing body of evidence links their misregulation to cancer progression (2,3). Germline mutations of Forkhead factor *FOXL2* are responsible for the Blepharophimosis Ptosis Epicanthus-inversus Syndrome (BPES; 4). This disorder is characterized by craniofacial abnormalities, either isolated (BPES type II) or associated with premature ovarian failure (POF; BPES type I; 5). Interestingly, mutations of FOXL2 have also been identified in isolated POF cases

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(6–8). Two independent *Foxl2*$^{-/-}$ knock-out mice models have been generated and have confirmed the key role of *Foxl2* in granulosa cell physiology. Although perinatal lethality is high in both models (50–95% according to the genetic background) and remains unexplained, the phenotype of survivors supports the role of *Foxl2* in ovarian determination and granulosa cell differentiation (9,10). Moreover, an adult ovarian *Foxl2* knock-out has confirmed that its expression is crucial to maintain the identity and differentiation of the ovary and to suppress the testis differentiation program (11).

FOXL2 is expressed in ovarian granulosa cells throughout female life (12). Its few known targets have been identified mainly by a candidate approach and are mostly involved in the hypothalamus–pituitary–gonadal function (13–20). A transcriptome study in the KGN granulosa cell model has extended and/or confirmed the spectrum of FOXL2-regulated processes to inflammation, apoptosis, reactive oxygen species (ROS) metabolism and cholesterol homeostasis (21). Oxidative stress is a topical issue in the ovary, as massive amounts of ROSs are produced during ovulation and luteinization (22). Supporting its role in granulosa cell homeostasis, FOXL2 can promote apoptosis (23). Moreover, FOXL2 activity can be inhibited by SIRT1, which modulates its ability to regulate stress-related genes (24).

FOXL2 misregulation has been linked to cancer. First, inactivation of the *FOXL2* locus by somatic hypermethylation was found in colorectal cancer (25). Second, extinction/decrease of *FOXL2* expression was reported in aggressive, highly proliferating juvenile ovarian granulosa cell tumors (OGCTs; 26). Third, a recurrent somatic FOXL2 mutation, p.Cys134Trp, was uncovered in >95% of adult OGCTs and confirmed to be specific to this tumor type (27–31). Interestingly, a recent study has revealed that the mutated variant was defective in its ability to induce apoptosis (32). OGCTs are endocrine malignancies, divided into juvenile and adult types, accounting for 2–5% of ovarian cancers. Recurrent metastatic disease can arise up to 40 years after primary tumor resection, leading to decreased survival of patients (33). Traditional chemotherapy is poorly effective, which highlights the need for novel therapeutics (33).

In this study, we use functional genomics to explore the FOXL2 regulation network and find that cell-cycle genes are enriched among targets. We show that FOXL2 interferes with proliferation by slowing down the cell cycle at the G1/S transition. We also find that FOXL2 protects cells from oxidative stress damage, notably by promoting oxidized DNA repair. These findings support the idea that FOXL2 plays a crucial role in the granulosa cell homeostasis, the failure of which plays a central role in ovarian ageing and cancer progression. We show that SIRT1 negatively regulates FOXL2 in targets linked to cell-cycle and DNA repair, and limits FOXL2 expression/activity in a dose-dependent manner. Consistently, we find that SIRT1 inhibition by nicotinamide reduces proliferation of granulosa tumor cells (both OGCT-derived cell lines and primary explanted OGCT cells) by increasing the amount/activity of endogenous FOXL2, which provides a therapeutic lead.

RESULTS AND DISCUSSION

FOXL2 modulates the expression of cell-cycle regulators

To explore the FOXL2-regulated gene network, we performed genomic studies of its targets in the human KGN granulosa cell model (34). For this purpose, we carried out ChIP-on-Chip experiments using immunoprecipitated material from native KGN cells, from a KGN subclone stably overexpressing wild-type (WT) FOXL2 and from whole mouse ovaries. To identify FOXL2 direct targets, we combined data from ChIP-on-Chip experiments and our previous transcriptomic study of KGN cells transiently overexpressing FOXL2 (21). We have previously shown that a rapid increase in *FOXL2* expression follows cell exposure to stress (24). Thus, analysis of FOXL2 targets modulated upon mild transient overexpression (<2-fold) should provide insights into the effects of their sudden upregulation, as if in response to stress. Because we combined several lines of evidence, we lowered the threshold for potentially significant variations to a 1.4-fold activation or inhibition of transcript levels and filtered this list to keep only direct FOXL2 targets found in the human ChIP-on-Chip assays (respectively 1117 activated and 703 inhibited genes; see Supplementary Material, Table S1).

To gain insights into FOXL2-regulated processes, we performed functional annotations of the target gene lists with three softwares: DAVID (Database for Annotation, Visualization and Integrated Discovery; 35), GSEA (Gene Set Enrichment Analysis; 36) and Genecodis2.0 (37; see Supplementary Material, Table S2). This analysis showed that FOXL2 direct targets were significantly enriched for annotations linked to apoptosis, stress response and ovulation/inflammation, as found previously (21). Interestingly, regulation of the cell cycle and proliferation was also significantly represented, which had been overlooked by previous analyses. Indeed, the gene ontology (GO) annotation *cell cycle arrest* (0007050) was significantly enriched in genes that are directly activated by FOXL2 (DAVID: $P = 1.36 \times 10^{-3}$; Genecodis2.0: $P = 6.95 \times 10^{-3}$). Among inhibited genes, the annotations *cell cycle* (0007049; DAVID: $P = 3.3 \times 10^{-3}$; Genocodis2.0: $P = 8.85 \times 10^{-4}$), *cell cycle process* (0022402; DAVID: $P = 3.82 \times 10^{-3}$; GSEA: $P = 1.25 \times 10^{-4}$), *mitosis* (00070067; DAVID: $P = 1.26 \times 10^{-4}$; Genecodis2.0: $P = 1.42 \times 10^{-2}$; GSEA: $P = 7.93 \times 10^{-4}$) and *mitotic cell cycle* (0000278; DAVID: $P = 5.62 \times 10^{-4}$; GSEA: $P = 2.66 \times 10^{-4}$) were significantly enriched. Inhibited genes were also significantly found in the KEGG Cell Cycle pathway (DAVID: $P = 9.68 \times 10^{-3}$; Genecodis2.0: $P = 9.5 \times 10^{-3}$). Furthermore, a functional re-annotation of Foxl2 targets identified in murine KK1 granulosa cells (as listed in 'Additional file 5'; 38) is in agreement with our findings in KGN cells. Indeed, identified targets were significantly associated with GO terms *Cell cycle* (0007049) and *mitosis* (00070067), and with the KEGG Cell Cycle pathway. A summary of functional analyses on FOXL2 targets in KGN and KK1 cells can be found in Supplementary Material, Table S2.

To confirm the accuracy of our ChIP-on-Chip results, we assessed the presence of FOXL2 at relevant chromatin regions by ChIP-qRT-PCR and confirmed significant occupancy in 42 out of 48 potential target promoters involved in

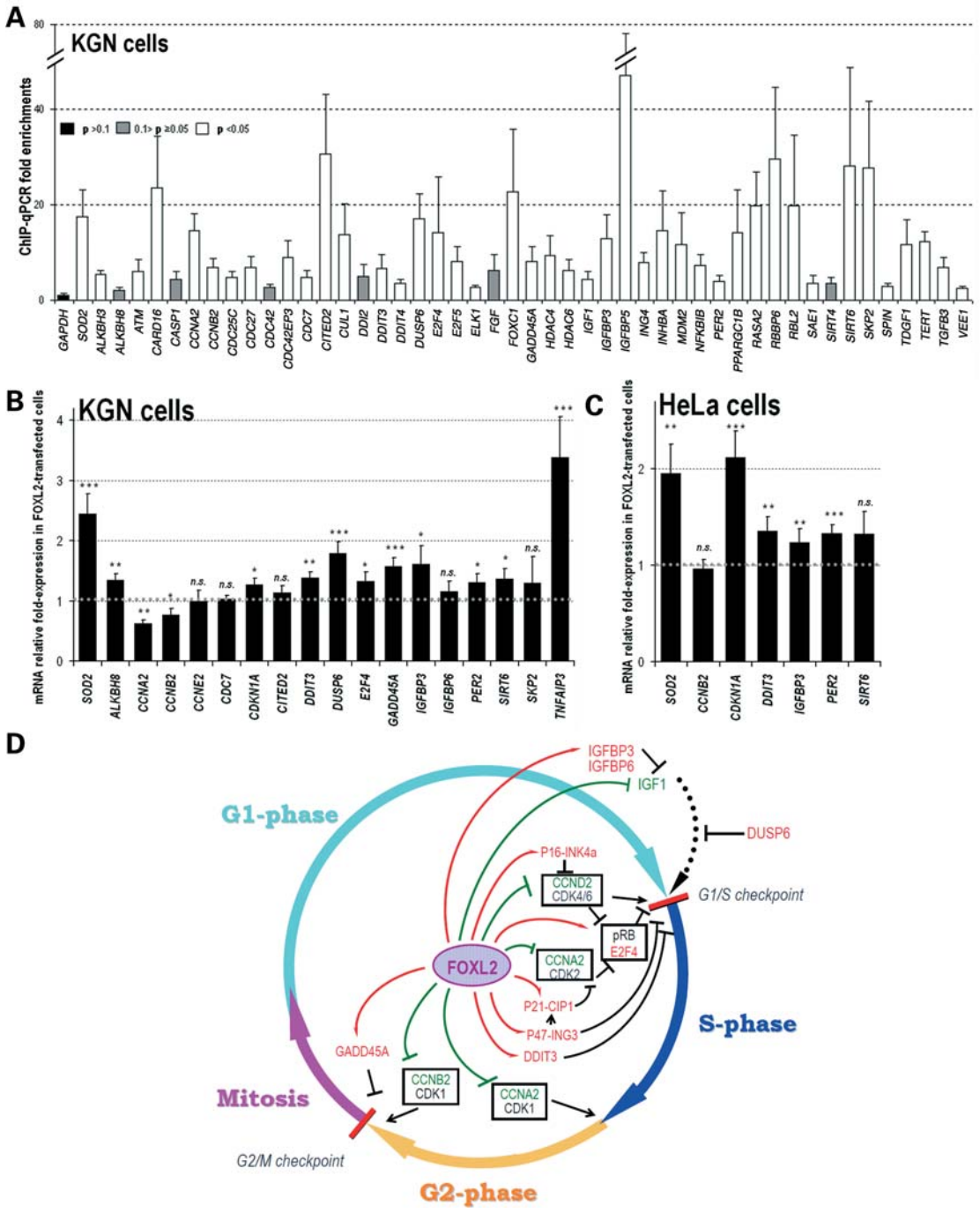


Figure 1. FOXL2 regulates the proliferation gene network consistently with growth inhibition and G1 arrest. (**A**) Results from four ChIP-qRT-PCR experiments in KGN cells. FOXL2 was significantly enriched at 42 promoters, and no enrichment was detected at the GAPDH promoter (negative control). Results are displayed $\pm$SEM. Statistical significance in one-sample Student's *t*-tests. Black bars: $P > 0.1$, grey bars: $0.1 < P \leq 0.05$ and white bars $P < 0.05$. qRT-PCR was performed on RNA from three pcDNA-transfected and FOXL2-transfected samples in KGN (**B**) or HeLa (**C**) cells. Indicated levels were obtained by normalizing FOXL2 versus pcDNA samples. Results represent the average values of three experiments. Statistical significance was estimated using one-sample Student's *t*-tests. n.s., non-significant. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Results are $\pm$SEM. (**D**) Schematic representation of FOXL2 action on confirmed targets and their reported action on the cell cycle. All targets are direct, with the exception of *P16/INK4a* (described here) and *CCND2* (described in 65). Red arrows/text: activation by FOXL2 and green blunt ended lines/text: inhibition by FOXL2. Notice that FOXL2 activation tends to promote arrest at the G1/S checkpoint.

cell-cycle and DNA repair (Fig. 1A; Supplementary Material, Table S3). Furthermore, *in vivo* mouse ovary ChIP data were consistent with KGN results (Supplementary Material, Table S3). Transcriptional regulation of targets by FOXL2 was confirmed in KGN and HeLa cells through transient transfection followed by qRT-PCR (Fig. 1B and C). Regulation of some other transcriptional targets [*ALKBH8*, *GADD45A*, *IGFBP3*, *IGFBP6*, *P21/CIP1* (*CDKN1A*), *P16/INK4A*

(*CDKN2A*), *P47*/*ING3* and *PER2*] was confirmed using luciferase assays, as described below.

Interestingly, growth factor signaling (necessary for G1 exit and S-phase entry) should be inhibited by FOXL2, notably by upregulating IGFBP, which can interfere with IGF signaling (39) and by upregulating *DUSP6*, which encodes a phosphatase counteracting the pro-mitogenic action of ERK1/2 (40). FOXL2 should promote decreased activity of CDK/cyclin complexes promoting G1 exit, by activating inhibitors P16/INK4A and P21/CIP1, and down-regulating *CyclinA2* expression (41). Finally, FOXL2 also activates inhibitors of the G1/S transition, such as *P47/ING3* and *DDIT3*/*GADD153* (42,43). In addition, FOXL2 upregulation of *GADD45A* and downregulation of *Cyclin B2* suggest that FOXL2 may inhibit the G2/M transition (44). Thus, the action of FOXL2 on confirmed targets suggests that it interferes with progression through the cell cycle (see Fig. 1D; Supplementary Material, Table S3).

FOXL2 upregulation delays cell-cycle progression and protects cells from oxidative damage

To confirm the predicted action of FOXL2, we assessed the effect of its overexpression on proliferation of KGN and HeLa cells. FOXL2 was able to inhibit colony formation to a similar extent as p53 (Fig. 2A and B). Moreover, increased FOXL2 expression significantly inhibited proliferation (Fig. 2C). Transfection of the FOXL2 paralog FOXM1B had the opposite effect (45), which suggests that the effect of FOXL2 is not merely the result of Forkhead protein toxicity. Unfortunately, depletion of FOXL2 by RNAi could not be used to study this phenomenon in KGN cells, since *SOX9* expression was re-activated as early as 24 h after transfection (data not shown). According to a recent study, such a re-activation in granulosa cells should translate into a change of cellular identity through transdifferentiation into Sertoli-like cells, which should have different cycling properties (11,46).

To better understand the effect of FOXL2 on cell proliferation, we generated KGN (and HeLa) clones stably transfected with a *FOXL2* transgene. Obtaining FOXL2 overexpressing clones was difficult, even when conditioning resistance gene expression to that of FOXL2 (through expression of a bicistronic transcript). Two WT-FOXL2 stable clones (KIRWT4/KIRWT5) were obtained this way. Four matched control clones were also selected (KIREM1/KIREM4/KIREM5/KIREM6). Stably transfected clones displayed a ~10-fold increase in *FOXL2* transcripts, which is similar to endogenous induction levels upon stress (24; Supplementary Material, Fig. S1).

We assessed the ability of stably transfected clones to enter S-phase using deoxythymidine analog EdU. Interestingly, *FOXL2* stably overexpressing cells entered S-phase less efficiently (Fig. 2D). We also assessed cell-cycle profiles of exponentially growing KGN clones by flow cytometry. Consistent with expectations, the G0/G1 population was increased at the expense of the G2/M population in *FOXL2*-overexpressing cells (Fig. 2E and F). Similar results were obtained using stably transfected HeLa cells (Supplementary Material, Fig. S1). This is consistent with S-phase delay and a tendency to G1-arrest, as predicted above (Fig. 1D).

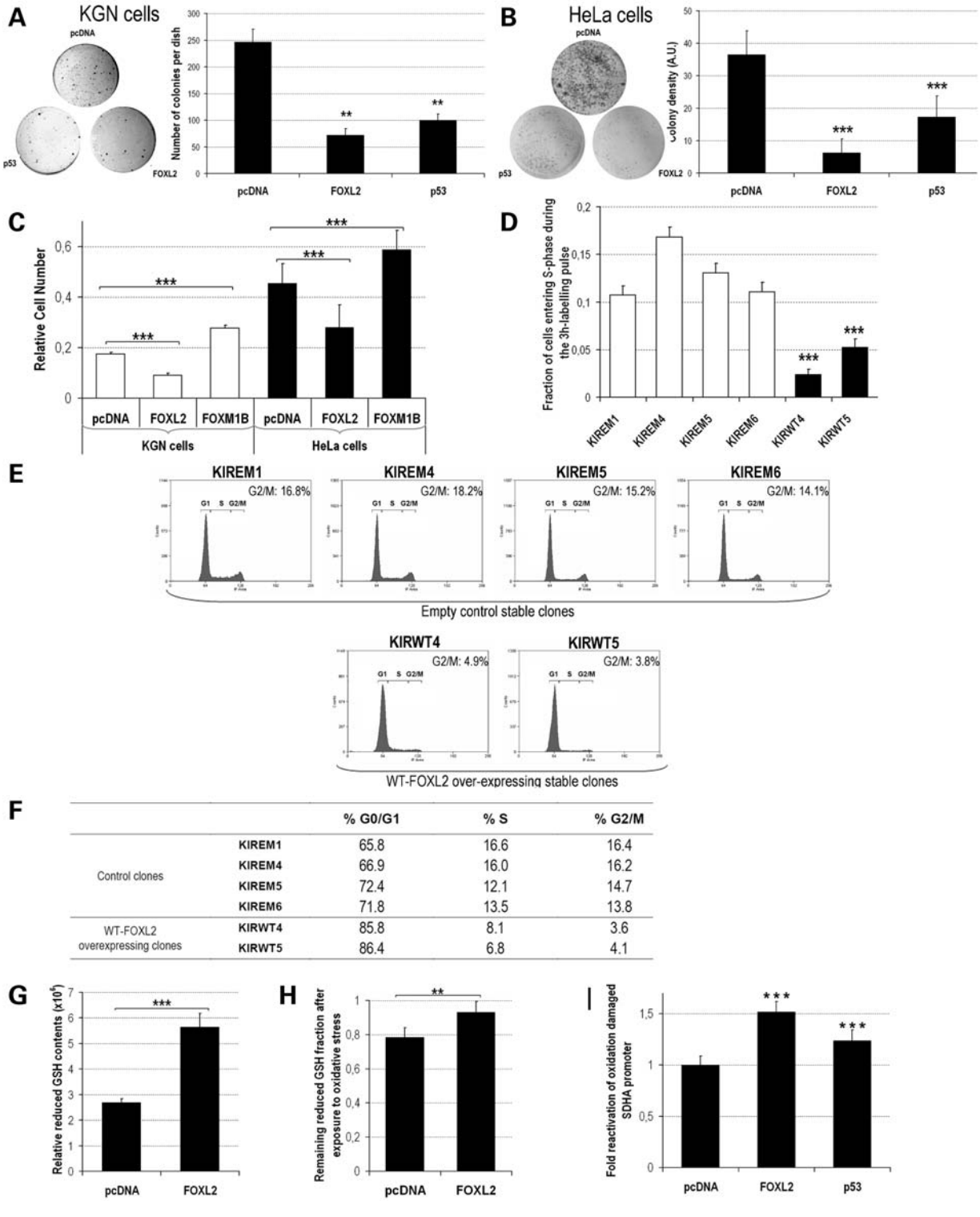
The effect of FOXL2 is thus consistent with *in vivo* observations, indicating that high FOXL2 expression is incompatible with sustained proliferation: (i) *Foxl2* levels decline in increasingly proliferating granulosa cells of late-stage follicles (9), (ii) Foxl2 is never found in actively dividing pituitary cells (16), (iii) downregulated FOXL2 expression correlates with higher mitotic activity in juvenile OGCTs (26) and (iv) *FOXL2* locus is silenced in colorectal cancer (25).

We have previously shown that FOXL2 is an actor of the cellular response to oxidative stress (24). Thus, we assessed the result of its action on cellular stress levels and found that FOXL2-overexpressing cells exhibit increased levels of reduced glutathione (GSH; Fig. 2G), indicating lower levels of 'chronic' oxidative stress. Moreover, when we measured GSH consumption in response to oxidative stress (i.e. a 150 μM H_2O_2 treatment), we found that FOXL2-overexpressing cells used less GSH, again indicating an improved competence to maintain homeostasis (Fig. 2H). Finally, we found that FOXL2 was able to induce significant re-activation of an oxidation-damaged *SDHA* (a housekeeping gene and not a FOXL2 target) promoter in host cell re-activation assays (Fig. 2I). This effect of increased FOXL2 expression is consistent with the fact that FOXL2 directly activates the expression of DNA damage repair genes *GADD45A* and *ALKBH8* (Figs 1 and 3). Taken altogether, our results indicate a key role for FOXL2 in the control of granulosa cell proliferation and stress response.

Regulation of cell-cycle and DNA repair target genes by disease-causing FOXL2 variants

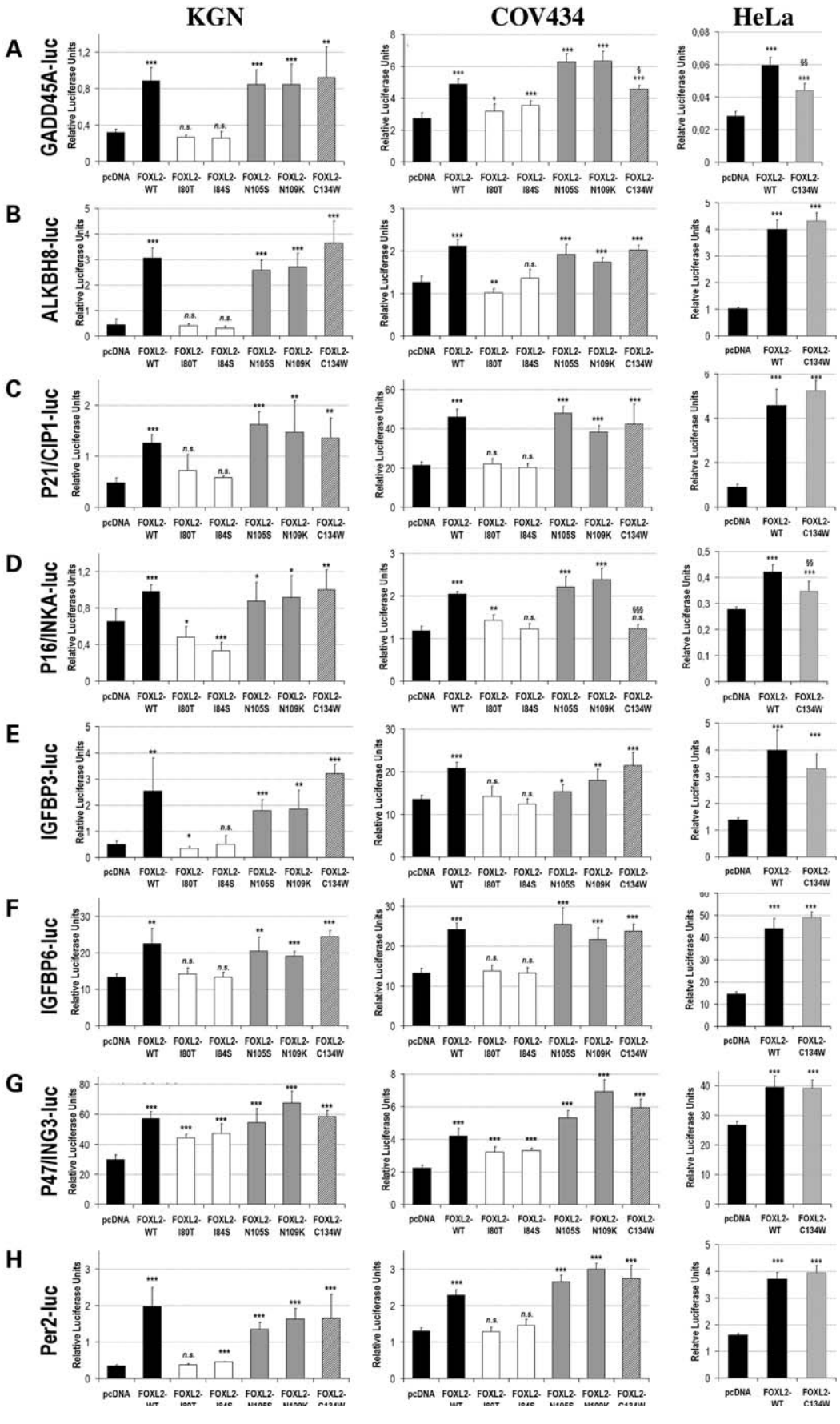
As stated above, type II BPES-associated *FOXL2* mutations do not lead to ovarian dysfunction or unscheduled proliferation, whereas type I-associated mutations lead to pathological ovarian function. This fact suggests that type II-causing BPES mutants are still functional on crucial targets involved in the maintenance of granulosa cells. To test this hypothesis, we selected missense FOXL2 mutants with a clear association with type I (I80T/I84S) or type II (N105S/N109K) BPES (47), and generated or obtained luciferase reporters for *ALKBH8*, *GADD45A*, *IGFBP3*, *IGFBP6*, *P21/CIP1*, *P16/INK4A*, *P47*/*ING3* and *Per2*. All these genes have been linked to cell-cycle or DNA repair and were identified by our genomic approach as FOXL2 targets. ALKBH8 and GADD45A are involved in the repair of genotoxic lesions, which are crucial in preventing genomic instability (44,48). IGFBP3/IGFBP6 are IGF binding proteins, whose main action is to counteract pro-proliferative IGF signaling (39). Cell-cycle inhibitors P21/CIP1 and P16/INK4A inhibit progression into the S-phase (41), and *P16/INK4A* is often downregulated in adult OGCT (49). P47/ING3 can interfere with proliferation and promote apoptosis in response to DNA damage (42). Finally, circadian clock transcription factor *PER2* can act as a powerful tumor suppressor (50). We also took advantage of these experimental tools to test potential effects of the adult OGCT-associated C134W mutant on this type of FOXL2 target genes.

We assessed the transactivation ability of *FOXL2* variants on our reporters in human OGCT-derived cell lines KGN



		% G0/G1	% S	% G2/M
Control clones	KIREM1	65.8	16.6	16.4
	KIREM4	66.9	16.0	16.2
	KIREM5	72.4	12.1	14.7
	KIREM6	71.8	13.5	13.8
WT-FOXL2 overexpressing clones	KIRWT4	85.8	8.1	3.6
	KIRWT5	86.4	6.8	4.1

Figure 2. FOXL2 delays cell-cycle progression and protects cells from oxidative injury. (**A**, **B**) Colony formation assays in KGN/HeLa cells. Representative wells are shown next to graphs. Results averaged from six experiments. Error bars: SD. Statistical significance in Student's *t*-tests versus pcDNA. $^{**}P < 0.01$; $^{***}P < 0.001$. (**C**) Cell titer assays in transiently transfected KGN/HeLa cells (details in the Materials and Methods section). (**D**) Fractions of stable KGN clones entering S-phase during a 3-h EdU-labeling pulse (estimated on 30 groups of 50 cells per cell line). Statistical significance in Student's *t*-tests. $^{***}P < 0.001$ versus all control clones. (**E**, **F**) Cell-cycle profiles of stably transfected KGN clones. Representative profiles and mean values from three experiments are shown. (**G**) Relative cellular reduced GSH contents of pcDNA and FOXL2 transiently transfected KGN cells. (**H**) Remaining GSH fraction in transiently transfected KGN cells after a 150 μM H_2O_2 1 h stress. (**I**) Host cell re-activation assays in HeLa cells, assessing FOXL2/p53 ability to induce oxidative damage repair to an *SDHA* reporter. Data from six independent assays. Errors bars: SD. Statistical significance in Student's *t*-tests versus pcDNA. $^{**}P < 0.01$; $^{***}P < 0.001$.



(with strong endogenous *FOXL2* expression) and COV434 (without substantial endogenous *FOXL2* expression; 31). Reporter transactivation by FOXL2 was also tested in HeLa cells, where FOXL2 is functional (28). As expected, FOXL2 activated these promoters in all cell lines (Fig. 3). Our assays indicate that mutants leading to ovarian dysfunction (type I BPES) mostly fail to transactivate cell-cycle and DNA repair targets, whereas mutations leading to isolated craniofacial defects (type II BPES) activate them correctly (Fig. 3). In the context of type I BPES, as no functional mature granulosa cells can differentiate, constitutional FOXL2 malfunction should not lead to OGCT formation. On the other hand, the fact that type II BPES-inducing mutations behave normally on cell-cycle and DNA repair targets may explain why BPES patients are not more prone to develop OGCTs. This is in contrast with the situation in patients with Li-Fraumeni Syndrome or retinoblastoma, with germline heterozygous mutations of tumor suppressors *p53* or *pRB*, predisposing them to develop tumors at a young age (51,52).

The p.Cys134Trp mutant generally behaved like the WT protein (Fig. 3). This behavior is consistent with the maintenance of a granulosa cell identity in OGCT and is reminiscent of our previous observations on eight reporter promoters related to other cellular functions (28). However, an exception was observed on the P16/INK4A-luc reporter in COV434 cells, where this mutant displayed no transactivation, which is in contrast to WT transactivation levels in KGN cells. Interestingly, the variant was also significantly hypomorphic in HeLa cells (Fig. 3). We also found a mild (but significant) hypomorphic behavior of the p.Cys134Trp mutant on the GADD45A-luc reporter in COV434 and HeLa cells (Fig. 3). This suggests that the ability of FOXL2 to upregulate *P16/INK4A* and *GADD45A* depends on the proteomic background and may be influenced by modifier factors. As KGN cells carry the p.Cys134Trp mutation, we cannot exclude that the lack of hypomorphy may also reflect an adaptation of the FOXL2 interacting network to the endogenous mutation.

Furthermore, we had also obtained two clones stably overexpressing the p.Cys134Trp FOXL2 variant (KIRC134W3/KIRC134W6), in parallel to control and WT-FOXL2 overexpressing clones presented above. However, *FOXL2* expression in these clones was >6 times higher than in our WT-FOXL2-overexpressing clones (76.6 ± 9.7 versus 12.6 ± 0.8 mean fold increase compared with endogenous *FOXL2* transcript levels, as assessed by qPCR; $P = 7.8 \times 10^{-4}$ in Student's *t*-test). Thus, such a difference forbids direct comparison between WT and p.Cys134Trp stably transfected clones. Interestingly, in spite of this dissimilarity in expression levels, cell-cycle profiles were rather similar (Supplementary Material, Fig. S2). The fact that cells are able to withstand higher expression levels of mutant *FOXL2* pleads in favor of it being hypomorphic (i.e. a defective tumor suppressor) rather than a gain of function mutation (i.e. an oncogenic mutation). This is consistent with our results on the *P16/INK4A* and *GADD45A* promoter reporters. So far, our results do not support the existence of a general defect of the p.Cys134Trp variant on cell-cycle FOXL2 targets, but rather a mild hypomorphy on specific targets. Moreover, a recent study has revealed that the p.Cys134Trp mutant is strongly defective in its ability to promote apoptosis, which has been proposed to account, at least, in part, for its implication in OGCT tumorigenesis (32).

SIRT1 inhibits FOXL2 transactivation and expression in granulosa cells

SIRT1 is an NAD-dependent deacetylase, which can deacetylate FOXL2 in KGN cells (53,54). Nicotinamide, the active form of vitamin B3, is a non-competitive sirtuin inhibitor (53). SIRT1 deacetylation inhibits FOXL2 on stress-response targets and its ability of self-activation (24). Considering this, we investigated whether SIRT1 had a similar effect on FOXL2 transactivation of cell-cycle and DNA repair targets. As expected, SIRT1 inhibited FOXL2 on most targets (Fig. 4), with the exception of *IGFBP6*, on which SIRT1 had no impact on FOXL2 transactivation. This behavior is reminiscent of what we previously observed on the *SIRT1* promoter itself, where deacetylation did not inhibit FOXL2 (24).

Interestingly, we had previously observed that nicotinamide supplementation might upregulate FOXL2 expression and activity (24). Here, we found that a luciferase reporter driven by the *FoxL2* promoter (Fig. 5A and B) is fine-tuned by SIRT1 activity levels, with a dose-dependent inhibition by SIRT1 overexpression ($P < 0.0001$) and a dose-dependent activation by nicotinamide supplementation ($P < 0.0001$). The same trend was observed for endogenous FOXL2 transactivation (i.e. without overexpression), as assessed using the specific FOXL2-responsive 2xFLRE-luc reporter (55; Fig. 5C and D). Quantifications confirmed that nicotinamide supplementation upregulated FOXL2 expression in KGN cells at the protein level (Fig. 5E), even in the context of the heterozygous p.Cys134Trp mutation (28,29). Increased endogenous *Foxl2* expression upon nicotinamide treatment was also observed in the murine AT29C granulosa cell line (56), which harbors WT *Foxl2* alleles (Fig. 5F). Further evidence suggests that the effect of nicotinamide on FOXL2 is mainly mediated by SIRT1 and not by the other sirtuin deacetylases (Supplementary Material, Fig. S3).

The juvenile OGCT-derived COV434 cells have WT *FOXL2* alleles but no significant *FOXL2* expression (31). Interestingly, neither SIRT1 overexpression nor nicotinamide supplementation regulates *FoxL2* transcription in COV434 cells (Supplementary Material, Fig. S4). This may be explained by the fact that SIRT1 inhibits *FOXL2* expression

Figure 3. Transactivation ability of WT FOXL2 and disease-causing mutations on cancer-relevant target promoters. Transactivation ability of FOXL2 measured by luciferase assays in KGN, COV434 and HeLa cells. Cells were transfected with (**A**) GADD45A-luc, (**B**) ALKBH8-luc, (**C**) P21/CIP1-luc, (**D**) P16/INK4-luc, (**E**) IGFBP3-luc, (**F**) IGFBP6-luc, (**G**) P47/ING3-luc and (**H**) Per2-luc reporters (calcium phosphate method). Transfections were performed in the presence of FOXL2 variants (I80T and I84S, both causing type I BPES; N105S and N109K, both causing type II BPES; C134W, associated with adult OGCTs) or empty control vector as indicated, to assess transactivation and its modulation by mutations. Error bars: SD. Results of six independent replicates. Statistical significance in Student's *t*-tests versus pcDNA. n.s., non-significant. $^{*}P < 0.05$, $^{**}P < 0.01$; $^{***}P < 0.001$. When relevant, statistical significance in Student's *t*-tests FOXL2 WT versus C134W. $^{\S}P < 0.05$; $^{\S\S}P < 0.01$; $^{\S\S\S}P < 0.001$ (when nothing is indicated, non-significant).

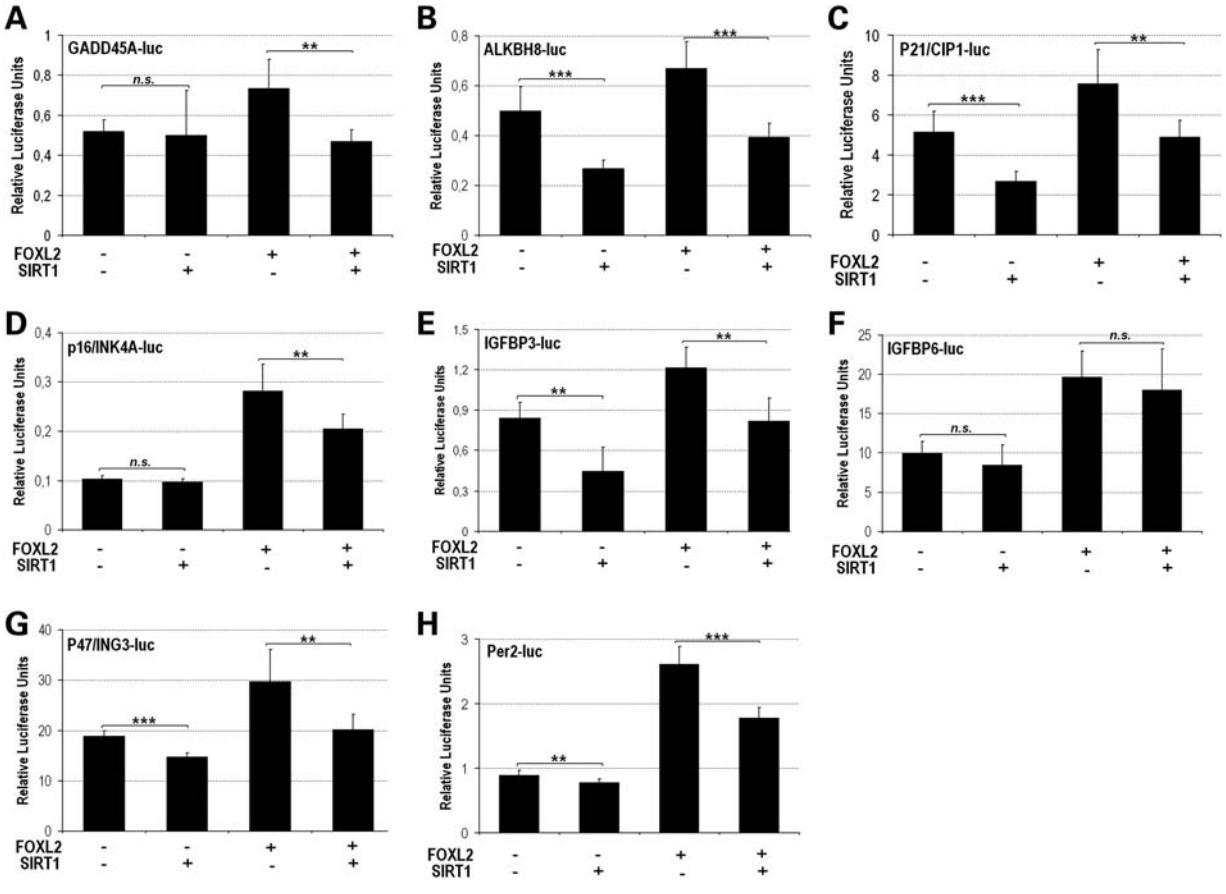


Figure 4. Repression of FOXL2 transactivation on cancer-relevant target promoters by the SIRT1 deacetylase. Luciferase assays in KGN cells transfected with (**A**) GADD45A-luc, (**B**) ALKBH8-luc, (**C**) P21/CIP1-luc, (**D**) P16/INK4-luc, (**E**) IGFBP3-luc, (**F**) IGFBP6-luc, (**G**) P47/ING3-luc or (**H**) Per2-luc reporters. Transfections with or without FOXL2 and/or SIRT1 overexpression. Error bars: SD. Statistical significance in Student's *t*-tests. n.s., non-significant. $^{**}P < 0.01$; $^{***}P < 0.001$.

by inducing decreased FOXL2 transactivation on the *FOXL2* promoter (24). Thus, our findings suggest that SIRT1 decreases FOXL2's ability of promoting cell-cycle arrest and that its inhibition leads to increased *FoxL2* expression in a dose-dependent manner, as long as the *FoxL2* locus is active.

SIRT1 inhibition in granulosa cells leads to impaired cell-cycle progression

To further understand the impact of FOXL2 regulation by SIRT1, we assessed whether upregulating *FoxL2* through sirtuin inhibition by nicotinamide had any effect on proliferation of KGN and AT29C cells, which both have significant endogenous *FoxL2* expression. As KGN cells are slow-cycling ones, we synchronized a cell culture at the G1-phase (Supplementary Material, Fig. S5) to make effects more easily detectable. Twenty-four hours after release, we analyzed cell-cycle profiles with or without nicotinamide supplementation. Interestingly, nicotinamide treatment altered cell-cycle progression in a dose-dependent manner. Indeed, for doses >5 mM, the proportion of proliferating cells was significantly reduced (Fig. 6A). When returned to normal medium, cells resumed normal cycling, indicating that nicotinamide effects are reversible and non-toxic. We confirmed the proliferation-inhibitory effect of the 15 mM nicotinamide dose on asynchronous KGN and AT29C cells (Fig. 6B–E). Although AT29C cells are not transfectable, increased *Foxl2* expression following nicotinamide treatment correlates with upregulation of FoxL2 cell-cycle-related targets (Supplementary Material, Fig. S6). Nicotinamide treatment had no effect on the proliferation of COV434 cells, which no longer express *FOXL2* at substantial levels and thus cannot upregulate its expression (Supplementary Material, Fig. S4).

To confirm that modifications of flow cytometry profiles by nicotinamide treatment result from decreased proliferation, we performed complementary proliferation assays. We found a dose-dependent decrease in proliferation following nicotinamide treatment in KGN cells (Fig. 6F). Although less dramatically, treatment of AT29C cells also led to decreased cell proliferation (Fig. 6G). Closer to *in vivo* conditions, reduced cell proliferation was also observed after nicotinamide treatment of a primary culture of explanted OGCT-derived cells, confirmed to carry the recurrent heterozygous p.Cys134Trp mutation (Fig. 6H). Considering this effect and taking into account that both KGN cells and the primary OGCT sample are heterozygous for the p.Cys134Trp mutation, we believe that mutant protein upregulation does not prevent the proliferation-inhibitory effects of the WT protein. Again,

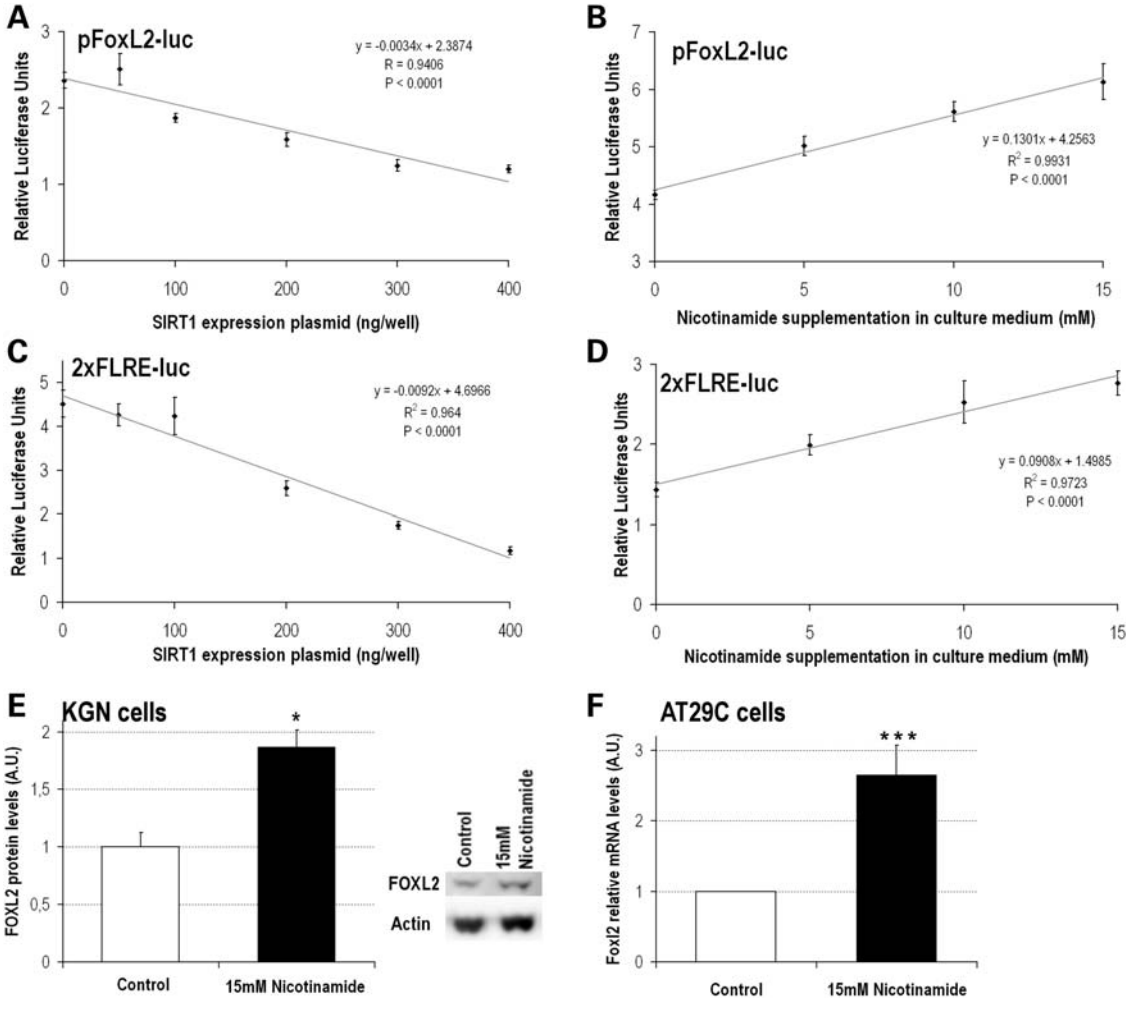


Figure 5. SIRT1 and its inhibitor nicotinamide regulate FOXL2 expression and activity in a dose-dependent manner. Luciferase assays in KGN cells transfected with the pFoxL2-luc reporter with increasing amounts of SIRT1 plasmid (**A**) or with increasing nicotinamide concentrations in culture medium (**B**). Luciferase assays in KGN cells transfected with the FOXL2-responsive 2xFLRE-luc reporter with increasing amounts of SIRT1 plasmid (**C**) or with increasing nicotinamide concentrations in culture medium (**D**). (A–D) Linear regressions and *P*-value of correlation significance test are shown on graphs. (**E**) FOXL2 protein levels in KGN cells before and after a 24 h 15 mM nicotinamide treatment, with actin as loading control. Statistical significance in a Student's *t*-test, $^{*}P < 0.05$. Representative lanes shown on the side. (**F**) *Foxl2* transcript levels in AT29C cells by qRT-PCR after a 15 mM nicotinamide treatment. Statistical significance in a one-sample Student's *t*-test. $^{***}P < 0.001$. (A–F) Error bars (SD) from six independent replicates.

these results suggest that the p.Cys134Trp mutant is most likely hypomorphic, thus reinforcing our hypothesis that FOXL2 is a tumor suppressor (57).

Consistent with flow cytometry data, the deficit in cycling KGN cells upon nicotinamide treatment results from a decreased capacity of cells to exit (10 mM nicotinamide dose) or to enter (15 mM nicotinamide dose) S-phase (Fig. 6I). Thus, nicotinamide seems to promote G1 arrest, at least partly through upregulation of *FoxL2* expression. Moreover, our evidence suggests that nicotinamide supplementation can slow down *ex vivo* OGCT cell proliferation. Consistently, the SIRT1-specific inhibitor Sirtinol (58), which has different chemical properties than nicotinamide, also leads to decreased proliferation of KGN cells (Fig. 6J). As SIRT1 inhibition is the common molecular action of both nicotinamide and Sirtinol, these results suggest that decreased proliferation of OGCT-derived cells is the result of decreased SIRT1 activity. Given the low toxicity of nicotinamide in humans even at high doses during long time periods (59), nicotinamide supplementation of OGCT patients may have beneficial effects to slow down tumor cell proliferation.

DISCUSSION

Forkhead factors are involved in a wide spectrum of processes, with key roles during development and cell differentiation (1). A direct consequence is that their misregulation can severely alter cell fate and impair differentiation, thereby promoting malignant transformation. Indeed, Forkhead misregulation is common in cancer, with factors acting as oncogenes or defective tumor suppressors (3). *FOXL2* germline mutations lead to BPES (4) its somatic perturbation has been linked to cancer progression (25–27). In both instances, the molecular mechanisms leading to the pathologies are still poorly understood.

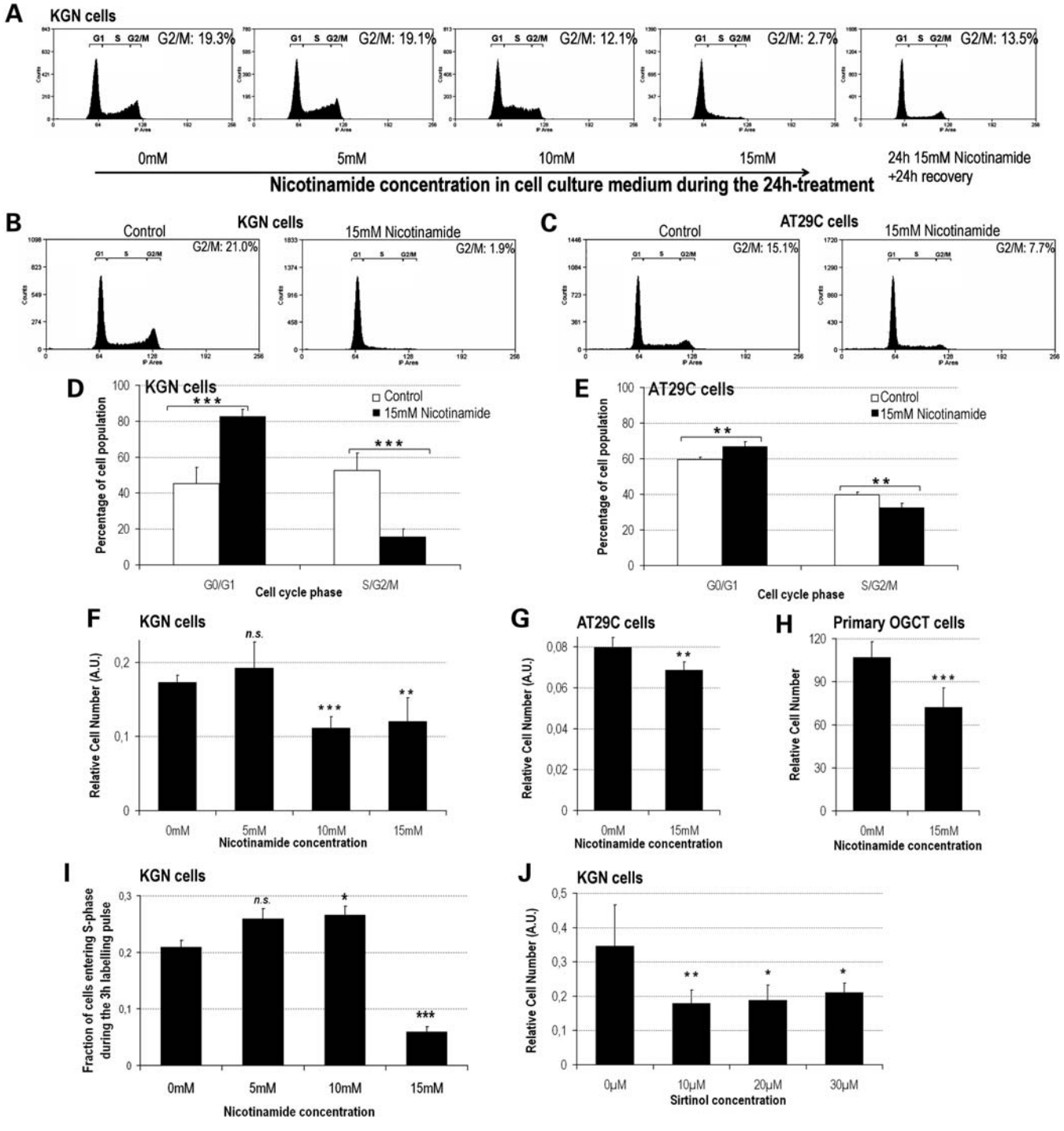


Figure 6. Nicotinamide inhibits proliferation of human OGCT-derived KGN cells, murine granulosa AT29C cells and primary human OGCT cells. (**A**) G1-synchronized cells were released in medium supplemented with increasing doses of nicotinamide, and flow cytometry profiles were measured after 24 h. A control sample was also switched back to control medium for 24 h to assess the reversibility of effects. Results reflect two experiments. Asynchronous KGN (**B**, **D**) or AT29C cells (**C**, **E**) were grown for 24 h in medium with or without 15 mM nicotinamide, and cell-cycle profiles were estimated by flow cytometry on four samples. KGN (**F**) or AT29C (**G**) cells were seeded in complete medium supplemented with nicotinamide as indicated and proliferation was measured after 24 h. Results for six independent replicates. (**H**) Relative number of primary human OGCT cells treated with 15 mM nicotinamide for 96 h. Results are the means of 12 groups of 10 fields of Hoechst-stained cells with SD. Data from cells collected from one patient. (**I**) Fractions of KGN cells (with or without nicotinamide treatment), entering S-phase during a 3 h EdU-labeling pulse (estimation from 30 groups of 50 cells per cell line). Statistical significances in Student's t-tests versus growth in control medium. n.s., non-significant. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Errors bars: SD. (**J**) KGN cells were seeded in complete medium supplemented with Sirtinol as indicated and proliferation was measured after a 24-h treatment. Results for six independent replicates.

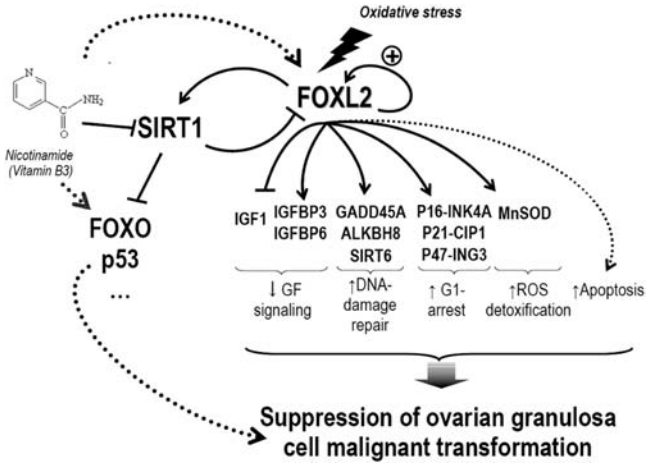


Figure 7. Model of FOXL2 action in granulosa cell homeostasis. FOXL2 expression and activity levels are regulated by positive and negative feedback signaling (24). Upon stress, a spike in FOXL2 expression would increase regulation of targets known to promote decreased growth factor (GF) signaling, increased genomic stability, G1 arrest and ROS detoxification. FOXL2 itself promotes cell-cycle arrest and, in some cases, apoptosis (23). These abilities are under the control of FOXL2 inhibitor SIRT1 and can be enhanced upon its inhibition by nicotinamide. Nicotinamide treatment has also been described to alleviate SIRT1 inhibition of FOXO factors and p53 (53). Cooperation of FOXL2 with other tumor suppressors should prevent malignant transformation of granulosa cells.

In this study, we find that FOXL2 negatively regulates cell-cycle progression and could thus act as a tumor suppressor, as previously proposed (57). Indeed, FOXL2 regulates genes involved in cell-cycle progression, with a tendency for G1-arrest promotion. Accordingly, FOXL2-overexpressing cells exhibit decreased proliferation rates and accumulate at the G1/S checkpoint. We found that mutations causing type II-BPES (i.e. with normal ovarian function) display WT-like transactivation on cell-cycle and DNA repair targets, which is consistent with clinical data showing that type II BPES female patients have no increased OGCT risk. In the case of mutations causing type I BPES (i.e. with ovarian dysfunction), FOXL2 variants are either amorphic or strongly hypomorphic. As constitutional *FOXL2* haploinsufficiency during development has been shown to impair differentiation and maturation of granulosa cells (9,10), such a defect should prevent unscheduled proliferation because undifferentiated potential OGCT precursors would be unable to undergo malignant transformation. Finally, FOXL2-negative regulation by the SIRT1 deacetylase seems to play a key role in regulating its ability to induce cell-cycle arrest. Indeed, inhibition of SIRT1 with nicotinamide upregulates *FOXL2* in a dose-dependent manner, correlating with a decreasing ability of cells to progress through the cell cycle.

As tumor suppressors FOXO3a and p53 are also SIRT1 targets (53,60), we cannot exclude their contribution to the effects of nicotinamide supplementation. A model of the FOXL2 action on granulosa cell homeostasis, as well as its regulation by SIRT1 and nicotinamide, is shown in Fig. 7.

Ten years ago, six 'hallmarks of cancers' were delineated, i.e. key events believed to be crucial to allow/promote malignant transformation: evasion of apoptosis, self-sufficiency in growth signals, insensitivity to anti-growth signals, increased invasive abilities, ability to sustain angiogenesis and a limitless replicative potential (61). Therefore, an implication of FOXL2 in these processes would reveal its contribution towards thwarting cancer progression. According to current knowledge, FOXL2 may thus work against cancer on several battlefronts: (i) by promoting apoptosis (23,32), (ii) by promoting DNA repair and genomic stability (i.e. by upregulation of *GADD45A*, *ALKBH8*, *SIRT1* and *SIRT6*) and (iii) by interfering with growth factor signaling (i.e. by downregulation of IGF expression and upregulation of antagonists IGFBP3/6). A deficiency in any of these roles, such as shown for apoptosis in the case of the recurrent p.Cys134Trp mutation (32), could thus participate in the malignant transformation process.

Because of ovulation, the ovary undergoes local genotoxic effects on a regular basis (the so-called 'ovulatory genotoxicity'; 22). Indeed, high ROS levels linked with ovulation and luteinization favor the formation of DNA adducts in nuclei of somatic cells (and thus mutations). FOXL2 action in granulosa cells should limit unscheduled proliferation and increase stress resistance, thus ensuring an improved protection of both somatic and germ cells in the ovary. Taken together, our study implicates FOXL2 as a novel regulator of cell-cycle progression and homeostasis in granulosa cells, substantiating its candidacy as a tumor suppressor. In the context of OGCTs, whose treatment strategies as a whole remain a challenge, modulation of FOXL2 expression and activity through SIRT inhibition (e.g. with nicotinamide) represents an interesting lead and warrants further exploration.

MATERIAL AND METHODS

PCR primers and plasmids

PCR primer sequences are available upon request. Plasmid constructs used/generated for this study are detailed in Supplementary Material.

Cell culture, transfections, stress, nicotinamide/Sirtinol treatment and luciferase assays

Granulosa cell lines KGN (34), COV434 (62) and AT29C (56) were grown in DMEM-F12, supplemented with 10% FBS and 1% penicillin/streptomycin (Invitrogen/Gibco). HeLa cells were grown in supplemented DMEM. KGN, COV434 and HeLa cells were plated 12 h prior to transfection and transfected using the calcium phosphate method (63). Stable transfections are detailed in Supplementary Material. Nicotinamide treatment and oxidative stress and luciferase assays were performed as previously (24,28). Sirtinol (Sigma) was dissolved in dimethyl sulfoxide carrier, and treatment was performed similarly to published work (58). Relative luciferase units correspond to the ratio of Firefly over Renilla luciferase activity from at least six independent replicates.

Anti-FoxL2 chromatin immunoprecipitation (ChIP) assays and ChIP-on-Chip experiments

ChIP was performed as previously, on KGN and KF3 cells (21,24). ChIP was also performed on whole mice ovaries (details in Supplementary Material). Hundred nanograms of

ChIPed/input DNA were linearly amplified using the Whole Genome Amplification Kit (Sigma-Aldrich). DNA was sent to the NimbleGen ChIP-on-Chip platform for competitive hybridization on the HG18-deluxe human promoter array, containing 2.1×10^6 probes tiling promoters. Mouse ovaries ChIP-DNA was hybridized on the MM8-deluxe array. DNA end-labeling, hybridization, scanning and data normalization were performed at NimbleGen, which provided normalized data files. To confirm the enrichment sites, sequences centered on microarray enrichment peaks were chosen as qPCR targets. For ChIP-qPCR, each site was assessed using at least four ChIP samples using an amplicon in the *TBP* promoter for normalization. Microarray data have been submitted to the ArrayExpress Repository (E-MTAB-399 and E-MTAB-400).

Quantitative real-time-PCR (qRT-PCR)

qRT-PCR was performed as previously described (24). Statistical significance was computed on normalized crossing point deltas prior to exponentiation using one-sample Student's *t*-tests.

RNA extraction and cDNA synthesis

RNA was extracted using the TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. cDNA synthesis was performed using SuperScriptII (Invitrogen) and random hexamers. For qRT-PCR, human *ACTB* (or mouse *actb*) amplicons were used for normalization.

SDS–PAGE and western blot analysis

Protein separation was performed on NuPage Bis–Tris 4–12% pre-cast gels with 3-(N-morpholino)propanesulfonic acid running buffer (Invitrogen). Proteins were electrotransferred onto polyvinylidene fluoride membranes (GE Healthcare). Western blotting was conducted as previously, with an anti-FoxL2 C-terminus polyclonal serum (12) or a monoclonal anti-actin antibody (Abcam). Band intensities were quantified with ImageJ.

S-phase entry study

Assays were performed using the Click-iT EdU Alexa Fluor-657 Kit (Invitrogen). Cells were seeded at 40% confluency on coverslips. EdU was added to 10 μM to the medium for 3 h, and cells were fixed using Histofix (Panreac). Click-iT reaction was performed according to the manufacturer's instructions, and nuclei were stained with Hoechst 33342. Coverslips were mounted on slides using Dako (Dakocytomation). Ratios of nuclei stained by EdU over nuclei stained by Hoechst were obtained from at least 1500 cells.

Proliferation assays and cell-cycle analysis

The CellTiter-96 AQueous One Solution Cell Proliferation Assay (Promega) was used according to the manufacturer's instructions. Cells were passaged 24–48 h before measure and seeded at 3000–5000 per well of a 96-well plate. All presented results result from at least six independent replicates. Colony formation assays were performed similarly as in (25), and details are provided in Supplementary Material. For flow cytometry study of the cell cycle, $\sim 10^6$ cells were harvested and fixed with 70% Ethanol at −20°C. DNA was labeled by propidium iodide (Invitrogen). DNA contents of cells were measured with a CyanADP cytometer and the Summit software (Dakocytomation). Results reflect at least three experiments.

Primary OGCT cell studies

Following OGCT diagnosis, tumor tissue was processed as previously (64). Recovered cells were plated on 4-well glass slides at low confluency and cultured for 48 h in supplemented DMEM-F12. Cells were supplemented with 0 or 15 mM nicotinamide for 96 h, with a medium change after 48 h. Subsequently, nuclei were stained with 4′,6′-diamidino-2-phenylindole, and cells were counted per fields of view (fov). Means of 10 randomized fovs were analyzed in 12 replicates of control or treated wells. The p.Cys134Trp mutation status of *FOXL2* was analyzed as described (31). The ethical committee of Helsinki University Central Hospital and the National Supervisory Authority for Welfare and Health in Finland approved the OGCT study as a whole, with informed consent from all the patients enrolled in the study.

Oxidative stress levels and host cell re-activation assays

The redox status of control/stressed cells was assessed using the GSH-Glo Glutathione Assay (Promega) according to the manufacturer's instructions. Cells were seeded in 24-well plates and transfected with either empty control pcDNA vector, or a FOXL2-V5 expression vector. Twenty-four hours after transfections, monolayers were rinsed. Twenty-four hours later, transfected cells were trypsinized and were counted with a hemocytometer. Five thousand cells were reseeded per well of a 96-well plate. For stressed cells, H_2O_2 was added to the medium at 150 μM final concentration for 1 h before lysis. Normalizations were as follows: each well's luminescence value was normalized by Cell Titer Aqueous measurements of a replicate plate. Relative GSH consummation consequently to stress was then obtained by normalizing reduced GSH contents of stressed cells over contents of control cells. Host cells' re-activation assays were performed on a luciferase reporter driven by the housekeeping *SDHA* promoter, subjected to Fenton reaction oxidation damage, and transfection in HeLa cells. Details are provided in Supplementary Material.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

ACKNOWLEDGEMENTS

The authors thank Nicole Bogetto from the Flow Cytometry platform of the Institut Jacques Monod for her assistance; Dr Arun, Dr Deal, Dr Tejpar, Dr Yao and Dr Schibler for

plasmids; Dr Unkila-Kallio, Dr Bützow, and Prof. Heikinheimo for help and support on the human OGCT studies.

Conflict of Interest statement. None declared.

FUNDING

B.A.B. and A.B.G. were funded by AMN/Université Paris Diderot-Paris 7 fellowships. D.L., A.D., A.L.T., S.C. and C.B. were funded by Université Paris Diderot-Paris 7. R.A.V. was funded by Université Paris Diderot-Paris 7, Centre National pour la Recherche Scientifique and Institut Universitaire de France. This work was supported by grants from Association pour la Recherche Contre le Cancer (1140), from Fondation pour la Recherche Médicale (FRM) and from Academy of Finland, Sigrid Juselius Foundation and Helsinki University Central Hospital.

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