Positive and negative feedback regulates the transcription factor FOXL2 in response to cell stress: evidence for a regulatory imbalance induced by disease-causing mutations

Bérénice A. Benayoun[1,2,3,4,5], **Frank Batista**[1,2,3,4,†], **Jana Auer**[1,2,3,†], **Aurélie Dipietromaria**[1,2,3,6,†], **David L'Hôte**[1,2,3,7], **Elfride De Baere**[8] and **Reiner A. Veitia**[1,2,3,4,*]

[1]INSERM U567, Team 21, Department of Genetics and Development and [2]CNRS UMR8104, Institut Cochin, 75014 Paris, France, [3]Faculté de Médecine Cochin-Port-Royal, Université Paris Descartes, Paris 75014, France, [4]Université Denis Diderot, Paris VII, 75013 Paris, France, [5]École Normale Supérieure, 75005 Paris, France, [6]Paris-Sud, Paris XI, 91400 Orsay, France, [7]Unité de Génétique Moléculaire Animale, INRA/Université de Limoges, UMR 1061, Université de Limoges, Limoges, France and [8]Center for Medical Genetics, Ghent University Hospital, 9000 Ghent, Belgium

FOXL2 is a forkhead transcription factor, essential for ovarian function, whose mutations are responsible for the blepharophimosis syndrome, characterized by craniofacial defects, often associated with premature ovarian failure. Here, we show that cell stress upregulates FOXL2 expression in an ovarian granulosa cell model. Increased FOXL2 transcription might be mediated at least partly by self-activation. Moreover, using 2D-western blot, we show that the response of FOXL2 to stress correlates with a dramatic remodeling of its post-translational modification profile. Upon oxidative stress, we observe an increased recruitment of FOXL2 to several stress-response promoters, notably that of the mitochondrial manganese superoxide dis-mutase (*MnSOD*). Using several reporter systems, we show that FOXL2 transactivation is enhanced in this context. Models predict that gene upregulation in response to a signal should eventually be counterbalanced to restore the initial steady state. In line with this, we find that FOXL2 activity is repressed by the SIRT1 dea-cetylase. Interestingly, we demonstrate that SIRT1 transcription is, in turn, directly upregulated by FOXL2, which closes a negative-feedback loop. The regulatory relationship between FOXL2 and SIRT1 prompted us the test action of nicotinamide, an inhibitor of sirtuins, on FoxL2 expression/activity. According to our expectations, nicotinamide treatment increases FoxL2 transcription. Finally, we show that 11 disease-causing mutations in the ORF of *FOXL2* induce aberrant regulation of FOXL2 and/or regulation of the FOXL2 stress-response target gene *MnSOD*. Taken together, our results establish that FOXL2 is an actor of the stress response and provide new insights into the pathogenic consequences of *FOXL2* mutations.

INTRODUCTION

Mutations in the forkhead transcription factor FOXL2 are responsible for the blepharophimosis ptosis epicanthus inversus syndrome (BPES; MIM 110100; 1), a genetic disorder characterized by craniofacial defects, in association with premature ovarian failure (POF) or isolated (type I or II BPES, respectively; 2). A number of BPES type I cases (with POF) seem to result from defects in FOXL2 expression levels through haplo-insufficiency, namely heterozygous FOXL2 partial or total locus deletions (3), early nonsense mutations such as Q53X (no protein; 4) and other heterozygous null mutations. Interestingly, mutations in FOXL2 that do not induce a BPES have been identified in patients presenting with POF (5,6).

Our previous studies have shown that FOXL2 is expressed in periocular and ovarian follicular (mostly granulosa) cells at both fetal and adult stages, where it localizes exclusively

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*To whom correspondence should be addressed at: INSERM E21-GDPM, Institut Cochin, 24, Rue du Faubourg Saint-Jacques, 75014 Paris, France. Tel: +33 144412301; Fax: +33 144412302; Email: reiner.veitia@inserm.fr
†The authors wish it to be known that, in their opinion, the second, third and fourth authors contributed equally to this work.

to the nucleus (7,8). FOXL2 is one of the earliest known markers of ovarian differentiation in mammals, and its expression is maintained through adulthood (7). This suggests that it plays a role throughout female fertile life in follicular maturation and/or maintenance. In *Foxl2* knockout (KO) mice, eyelids do not form correctly and granulosa cells do not mature properly, leading to the absence of primary follicles. Two weeks after birth, follicular activation in the presence of a defective granulosa leads to massive atresia and premature follicular depletion (9,10). Interestingly, the KO mouse model of the member of the forkhead family *Foxo3a*, a more widely expressed forkhead factor, also displays precocious follicular activation, leading to an early depletion of the follicular pool and infertility (11). FOXO factors generally function as tumor suppressors and regulate apoptosis and cell stress response (12). In particular, they enhance reactive oxygen species (ROS) detoxification through the upregulation of ROS-scavenging enzymes like the mitochondrial manganese superoxide dismutase (MnSOD) and catalase (13,14). The MnSOD is a crucial actor of ROS metabolism and oxidative stress response through its antioxidant enzymatic activity, the dismutation of the superoxide anion $O_2^{\bullet-}$ into O_2 and H_2O_2 (15), whereas catalase converts H_2O_2 into H_2O and O_2. An interesting feature of FOXO transcription factors is their conserved ability to increase lifespan by delaying the onset of senescence (16), and there is accumulating evidence that this property is dependent on their ability to induce stress resistance (12,17,18). Oxidative stress and oxidative damages to cellular components through ROS production increase along with aging. Theories of aging propose that oxidative damage is an important part of the aging process at the cellular level, even if it may not be its primary cause, and that control of the ROS production and detoxification is mandatory for cell survival and protection against age-related diseases, such as cancer (19). The issue of ROS detoxification is particularly relevant in the ovary, as ROS are produced massively during the ovulation process (20).

A recent study in human granulosa-like KGN cells (21) revealed that potential targets of FOXL2 include key cell stress response genes (22). Indeed, among FOXL2-responsive genes were found key players of ROS metabolism, for instance the MnSOD and the immediate early response 3 protein (IER3). FOXL2 appeared to have the ability to modulate apoptosis (positively or negatively) through the regulation of the BCL2-related protein A1 (BCL2A1), the activating transcription factor 3 (ATF3), the tumor necrosis factor alpha-induced protein 3 (TNFAIP3), the cholesterol 25-hydroxylase (CH25H), as well as IER3 (22 and references therein). Further evidence for a role of FOXL2 in apoptosis regulation can be found in the work of Lee *et al*. (23).

In a previous study, we have shown that the overexpression of the SIRT1 deacetylase in KGN cells induced demodification of highly post-translationally modified forms of FOXL2 (24). SIRT1 belongs to the class III NAD-dependent histone deacetylase family, also known as 'sirtuins', and is the closest homolog of *Saccharomyces cerevisiae* protein Sir2p (25,26). In yeasts, *SIR2* works as a major regulator of replicative lifespan (27). Since its discovery, many homologs have been identified, and they are involved in dietary restriction-induced lifespan extension in metazoans, be it in the nematode *Caenorhabditis elegans* (28), in the fruitfly *Drosophila melanogaster* (29) or even in mice (30). SIRT1 was found to have a crucial role in the cell stress response and the regulation of physiological processes, which contribute to aging in mammals. Indeed, its targets include key stress response actors and apoptosis regulators (such as p53, Ku70, NF-κB and FOXO factors; 25). *Sirt1* KO mice display a palpebral phenotype (i.e. *Sirt1*$^{-/-}$ individuals fail to open one or both eyelids), a low fertility/fecundity phenotype (anovulation), as well as growth retardation and are subject to high perinatal lethality (31). The organs and functions affected by *Sirt1* gene inactivation are reminiscent of those affected in *Foxl2* KO mice (9,10). In adult mice, Sirt1 is particularly abundant in granulosa cells (31), where Foxl2 displays its highest detected expression level. This, as well as our previous results (24), points toward a potential regulatory relationship between these proteins.

In this study, we show that FOXL2 expression levels increase upon both oxidative stress and heat shock. FOXL2 increased levels are associated with a complex reorganization of its post-translational modification profile, which presumably includes a hyperacetylation of FOXL2 upon oxidative stress. We observe an increased recruitment of FOXL2 to target genes promoters involved in the stress response after oxidative stress and we find that FOXL2 transactivation ability is enhanced in this context, notably leading to a stress dose-dependent upregulation of *MnSOD* transcription. We show that FOXL2 transactivation is repressed by SIRT1 on target promoters, including the promoter of *FoxL2* itself and that of the *MnSOD*. We also show that FOXL2 is able to directly activate SIRT1 transcription, suggesting the existence of a negative-feedback loop, which should contribute to the fine-tuning of the cellular FOXL2 activity level. Taken together, our results indicate that FOXL2 is an actor of the granulosa cell stress response, in cooperation with known key players, such as SIRT1. We provide evidence that nicotinamide, the active form of the B3 vitamin and a noncompetitive inhibitor of sirtuins (32), is able to induce an increase in FoxL2 expression/activity. Moreover, we also find that 11 BPES-causing mutations of FOXL2 have a tendency to upset the (positive and negative) feedback regulation balance, thus presumably inducing aberrant FOXL2 expression and activity levels, often in association with an impaired activation of the *MnSOD* gene.

RESULTS AND DISCUSSION

FOXL2 is transcriptionally upregulated in response to stress

As a first approach to explore a potential role of FOXL2 in cell stress response, we investigated the effect of oxidative stress (exposure to 150 μM H_2O_2 for 2 h) and heat shock (exposure to 42°C for 2 h) on the activity of the FOXL2 promoter. These stress conditions are likely to occur in the ovary during the course of reproductive life: oxidative stress occurs through mitochondrial activity, and more particularly during ovulation (20), and heat shock can occur during fevers.

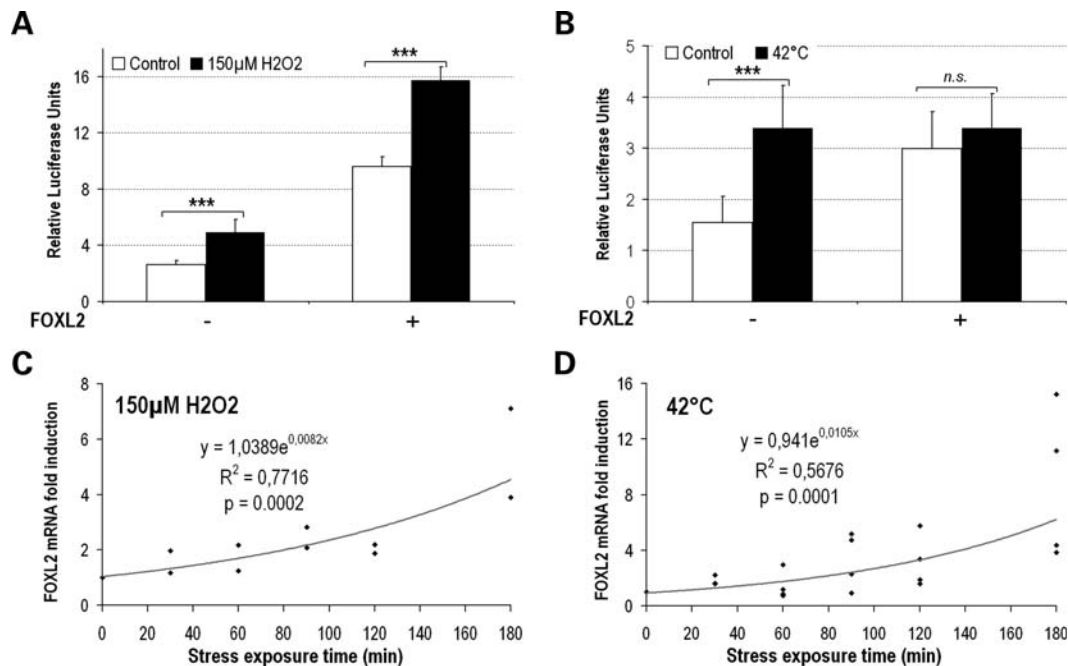First, we used a luciferase reporter driven by the caprine promoter of FoxL2, pFoxL2-luc (33), for transfection in

Figure 1. Transcriptional upregulation of FoxL2 during oxidative and heat stress. (**A** and **B**) Luciferase assays in KGN cells transfected with the FoxL2 caprine luciferase reporter pFoxL2-luc, with or without FOXL2 overexpression, to assess the effects of oxidative (A) or heat stress (B). Values are the means of five biological replicates, and error bars are the SD. *n.s.*, non-significant; ***P < 0.001 in a Student's *t*-test. (**C** and **D**) qPCR quantifications of FOXL2 mRNA levels in KGN cells during oxidative (C) or heat (D) stress over 180 min. Results of exponential regressions on the data and of the correlation coefficient significance test are shown on the graphs.

KGN cells, which express FOXL2 endogenously (22). Transfected cells were stressed for 2 h before luciferase activity quantification. The activity of the reporter was increased under both oxidative stress (~1.8-fold; Fig. 1A) and heat shock (~2.3-fold; Fig. 1B), suggesting that there is indeed an increased promoter activity upon stress. We sought to confirm the activation of FOXL2 transcription during stress by quantifying endogenous FOXL2 mRNA levels in KGN cells exposed to oxidative stress or heat shock through real time RT–PCR in a time-course experiment (over 180 min). Our data show that endogenous FOXL2 mRNA levels increase exponentially with time during cell stress, up to ~5-fold during oxidative stress or up to ~8-fold during heat shock (Fig. 1C–D). This increase in FOXL2 transcript levels could come either from (i) transcript stabilization during cell stress, and/or (ii) stress-induced increase in transcription (which is supported by our luciferase assays).

We have recently shown that FOXL2 is able to activate its own promoter (34). Our findings are consistent with the observation that transcription from the *Foxl2* promoter was decreased significantly to very low levels up to 16 weeks of life in homozygous $Foxl2^{-/-}$ mutant ovaries, where the promoter of *Foxl2* drives the expression the beta-galactosidase (9). It was thus conceivable that such a positive feedback loop could contribute to the increase of FOXL2 levels in response to stress in our granulosa model. Under FOXL2 overexpression, we indeed observed a further increase (i.e. extra-activation) of pFoxL2-luc activity level in response to oxidative stress (1.6-fold extra induction) when compared with overexpression without stress exposure (Fig. 1A). This suggests that, in the case of oxidative stress, the stress-induced

increase in FOXL2 transcription levels can be mediated at least partly by FOXL2 itself. Interestingly, we failed to detect any further activation of pFoxL2-luc under the overexpression of FOXL2 upon heat stress (Fig. 1B). This difference between the effects of oxidative stress and heat shock could result from the use of distinct transduction pathways for these two types of stress signals.

FOXL2 post-translational modification profile and protein level vary upon cell stress

Next, we analyzed total protein extracts from stressed and non-stressed KGN cells using 2D-electrophoresis followed by anti-FOXL2 immunoblotting (2D-western) to resolve FOXL2 post-translational modification isoforms and explore their potential reorganization after various stimuli (Fig. 2A). We tested two conditions on KGN cells for each stress: 150 and 300 μM H_2O_2 for 2 h and 42°C for 1 or 2 h. As reported previously, in control conditions, FOXL2 2D-western migration profile is formed of two main modification trains, designated as the 'basic' (pI ~9.2) and the more 'acidic' trains (pI ~8.1; 24). Interestingly, all tested stress conditions induced a significant remodeling of FOXL2 2D-western profile (Fig. 2A). Indeed, after stress exposure, both oxidative stress and heat shock induced strengthening of the less modified basic train. The post-stress increase of the basic train, which is hardly detectable in the steady state, is expected to result from FOXL2 stress-induced neosynthesis, as a consequence of the observed increase in transcription (see confirmation in what follows).

Further remodeling can be seen on the 150 μM H_2O_2 and 2 h, 42°C 2D-western profiles. Indeed, in these conditions,
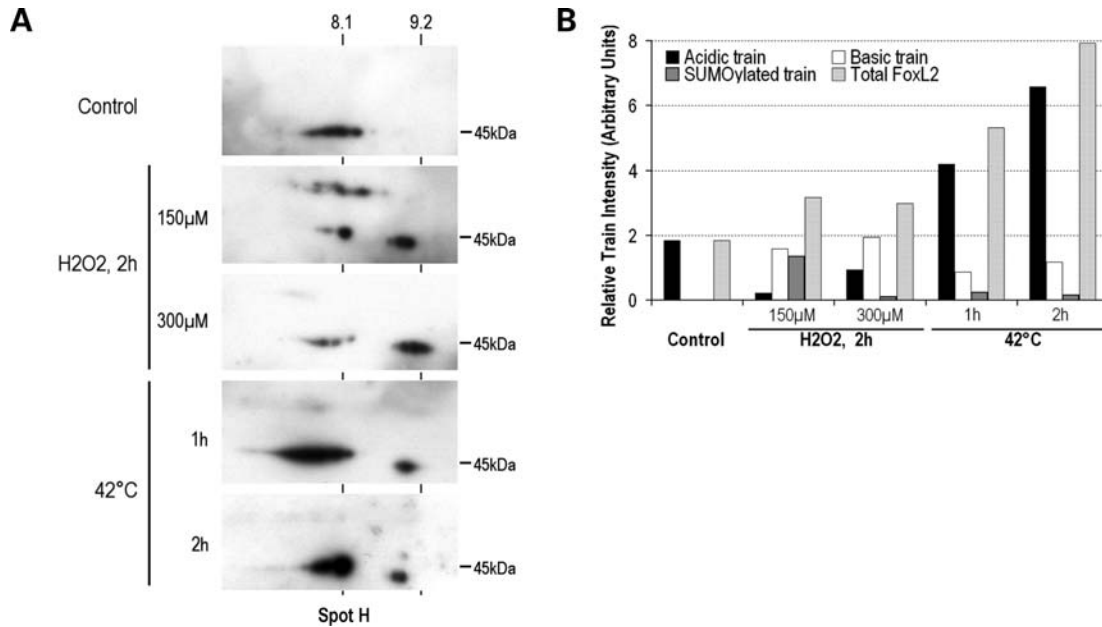
Figure 2. Post-translational profile remodeling of FOXL2 upon cell stress. (**A**) Two-dimensional western profiles of FOXL2 in KGN cell extracts subjected to various treatments, revealed with the anti-C-terminus FoxL2 antibody (7) and shown after same film exposure time. pIs and molecular weights were calculated as indicated in Materials and Methods. The spot H, predicted to contain octa-acetylated FOXL2, from Benayoun *et al.* (24), is indicated at pI 8.1. (**B**) Quantification of FOXL2, involved in the various modification trains, as well as total FOXL2, normalized by total protein load (silver stained membranes, Supplementary Material, Fig. S2). Results are representative of two experiments, only one of which is shown.

we observe a concentration of the acidic train in two distinctive spots, the major one at pI 8.1 (Fig. 2A; spot H). Our previous findings indicate that this is presumably hyperacetylated FOXL2 (24). Interestingly, this concentration in two spots is not clear after 1 h heat shock (Fig. 2A), and thus seems to be a time-dependent process reflecting the impact of the duration of the stress signal on FOXL2 modification profile. Increased H_2O_2 dosage in the culture medium (300 μM) also induced a concentration of the acidic train, though an extra spot (at pI 8.2) was also strengthened.

In addition, on the 2D-western of cells treated with 150 μM H_2O_2, we also observe the appearance of a third train of modifications, comprising isoforms migrating $\sim$15–17 kDa higher than native FOXL2 isoforms. This shift toward higher molecular weights suggests FOXL2 mono-conjugation to a small regulatory protein of the ubiquitin-like family. Note that this train can also be detected, though much more faintly, in all other FOXL2 2D-westerns of stressed cells extracts where increased FOXL2 neosynthesis is occurring, as we show in what follows. However, it is not detectable through this method on unstressed cell extracts. This train contains SUMO-conjugated forms of FOXL2 (Supplementary Material, Fig. S1), whose function is under investigation (manuscript in preparation), and we will refer to it thereafter as the SUMOylation train.

We used the 2D-western experiments to estimate variations in FOXL2 protein levels after stress exposure, as we had observed an increase in its transcription. Thus, we estimated FOXL2 signal, normalized by the protein load (explained in Supplementary Material, Fig. S2). FOXL2 expression levels in all conditions, for total FOXL2 as well as specific modification trains, are reported in Fig. 2B (results representative of two independent experiments). In these experiments, total FOXL2 increased $\sim$2-fold after 2 h oxidative stress (150 or 300 μM H_2O_2), $\sim$3-fold after 1 h of 42°C heat shock and $\sim$4.5-fold after 2 h of heat shock. This indicates that the increase in FOXL2 transcription detected previously upon cell stress is associated with an increase in protein production in the four conditions that we tested.

The high speed of FOXL2 translational response upon cell stress is worth noting, since it is observed only after 1–2 h exposure to cell stress. This increase in FOXL2 synthesis goes against a general cellular tendency, as, from yeast to mammals, global protein synthesis rates are downregulated in response to stress (35,36). Genes able to escape the global translational slowdown are usually involved in the stress response, which is compatible with our data implicating FOXL2 as an actor of the stress response.

FOXL2 is increasingly recruited to target promoters upon oxidative stress

Next, we analyzed whether the observed stress-induced changes concerning FOXL2, both quantitatively and qualitatively, were associated with alterations of its interaction with target promoters. For this purpose, we carried out chromatin immunoprecipitation (ChIP) experiments using KGN cells treated with 150 μM H_2O_2 and control cells. We assessed FOXL2 recruitment at the promoters of a subset of potential target genes (22), involved in stress response, ROS metabolism or apoptosis regulation (i.e. *IER3, CH25H, TNFAIP3, BCL2A1, ATF3* and *MnSOD*). As a negative control for the recruitment of FOXL2 at stress-response promoters, we selected the promoter of a glutathione peroxidase, the *GPx7*,
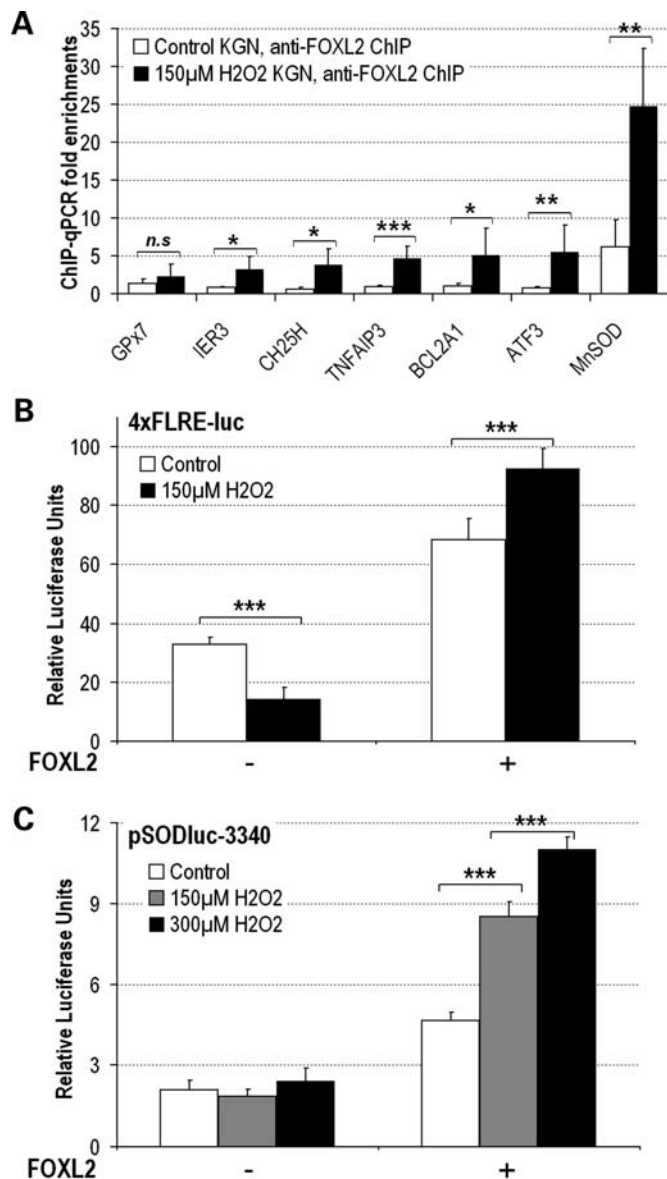
Figure 3. Enhancement of FOXL2 binding and transactivation of target promoters upon oxidative stress. (**A**) Anti-FoxL2 chromatin IP, followed by qPCR, normalized against a non-target promoter sequence, on chromatin extracted from control or stressed KGN cells. Results are the means of at least 10 independent assays, and errors bars are corrected SEMs. (**B**) Luciferase assays using the artificial FOXL2 target promoter reporter 4xFLRE-luc (four high-affinity FLREs), with or without FOXL2 overexpression, to assess the effect of oxidative stress on FOXL2 transactivation. (**C**) Luciferase assays using the MnSOD promoter reporter pSODluc-3340, with or without FOXL2 overexpression, to assess the effect of two doses of H_2O_2 on FOXL2 transactivation. Statistical tests for (A) and (B): Student's t-tests and (C): one-way ANOVA followed by Tukey HSD tests. *n.s.*, non-significant; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

another stress-response enzyme, which is well expressed in KGN cells, but whose transcription was unaffected by FOXL2 overexpression (22; Fig. 3A). Relative enrichments of FOXL2 at the *GPx7* promoter in control and oxidative stress conditions were compatible with it not being a target of FOXL2. Interestingly, we observed that the promoters of the six targets were significantly (over-)enriched in the

immunoprecipitated DNA pool of stressed cells when compared with that of control cells. Thus, upon oxidative stress, FOXL2 can be over-recruited to the promoters of target genes involved in ROS metabolism and apoptosis regulation. This shows that increased FOXL2 expression, along with the stress-induced remodeling of its post-translational modification profile, correlates with increased FOXL2 occupancy at specific target gene promoters (Fig. 3A).

Oxidative stress induces enhanced FOXL2 transactivation ability

Since endogenous FOXL2 can be increasingly recruited to target promoters of genes involved in stress response after exposure to oxidative stress, we sought to determine whether this was associated with changes in transactivation. We aimed at detecting variations of FOXL2 transcriptional activity with minimal background from other cellular components, which is difficult with naturally occurring promoters. Therefore, we used a luciferase reporter driven by an artificial promoter containing a minimal CMV promoter downstream of four high-affinity FOXL2 response elements (FLREs), which we have recently described (37), the 4xFLRE-luc reporter. This reporter provides us with a sensor of cellular FOXL2 activity/expression levels.

We studied potential changes in FOXL2 activity through the transfection of the 4xFLRE-luc construct in KGN cells, with or without the overexpression of FOXL2, in control conditions and after exposure to 150 μM H_2O_2 for 2 h. In the absence of FOXL2 overexpression, the reporter activity decreases upon oxidative stress, which is compatible with a general transcriptional slowdown for genes whose promoters are not responsive to cell stress (as should be the case of our artificial promoter; Fig. 3B; 38), but not with a loss of activity of FOXL2 (which would be contradictory with its upregulation upon stress, as described earlier). Since the small quantity of endogenous FOXL2 is expected to be specifically recruited to stress-response target genes upon stress, the 4xFLRE-luc construct should not respond to stress without FOXL2 overexpression. However, under FOXL2 overexpression, the activity of the reporter increased significantly upon oxidative stress (Fig. 3B). These results suggest that oxidative stress enhances the global transcriptional activity of FOXL2. Indeed, the duration of the experiment should only be enough to allow qualitative changes of the cellular FOXL2 pool (post-translational modification), but not substantial neosynthesis since the endogenous FOXL2 promoter activity, even stress-stimulated, is negligible when compared with the stress-insensitive CMV promoter driving the expression vector.

Our previous data on the ability of FOXL2 to 'extra-activate' pFoxL2-luc upon oxidative stress suggest a similar effect: again, increased activity of FOXL2 endogenous promoter cannot account for the increase in FOXL2 transactivation during the duration of the experiment, and suggest enhanced transactivation ability of FOXL2 in response to oxidative stress (Fig. 1A).

We also tested whether FOXL2 displayed a similar behavior on a classical stress response promoter, that of the *MnSOD*, to which FOXL2 is over-recruited upon oxidative stress (Fig. 3C). As already mentioned, MnSOD is a critical actor
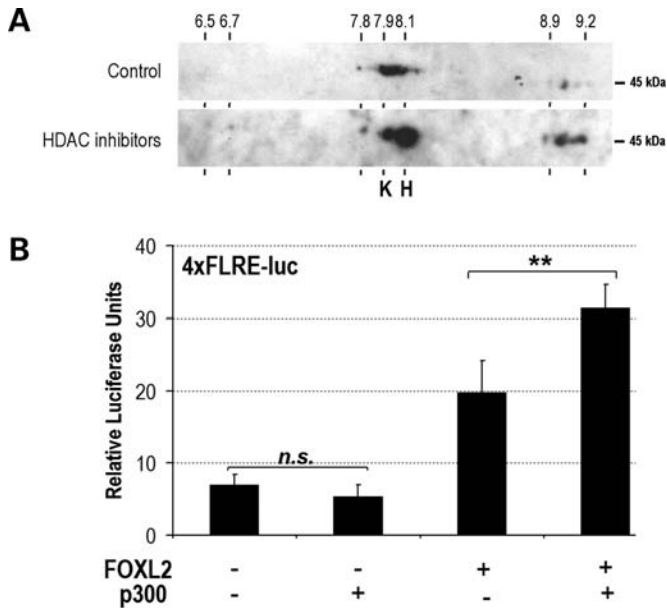
Figure 4. Hyperacetylation of FOXL2 induces increased transactivation in KGN cells. (**A**) Two-dimensional western profile of FOXL2 in control KGN cells and KGN cells treated with histone deacetylases (HDAC) inhibitors. Note the strengthening of the acidic train and of hyperacetylated spots H and K (24). (The post-translational modification profile of the forkhead transcription factor FOXL2 suggests the existence of parallel processive/concerted modification pathways. Proteomics, 2008, 8, 3118-3123. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced and modified with permission.). (**B**) Luciferase assays in KGN cells transfected with the 4xFLRE-luc construct, with or without FOXL2 and p300 acetyl-transferase overexpression. Statistical significance in Student's *t*-tests. *n.s.*, non-significant; **$P < 0.01$.

of oxidative stress response, and its importance is underlined by the severity of KO mice, which display severe cardiomyopathy, neurodegeneration and perinatal death (39). Using the pSODluc-3340 reporter (40), which contains >3 kb of the human *MnSOD* promoter sequence, we tested the effect of two doses of H_2O_2 (150 and 300 μM) in the presence or absence of FOXL2 overexpression. The reporter activity increases upon FOXL2 overexpression ($P = 5.10^{-6}$), which confirms the *MnSOD* status as a direct target of FOXL2. More interestingly, we were able to detect a significant enhancement of FOXL2 transactivation on the reporter, depending on the stress dose ($P < 0.001$; one-way ANOVA test and *post hoc* Tukey HSD test; Fig. 3C). These results indicate that not only is FOXL2 over-recruited to the promoter of the MnSOD during stress, but also that it induces a dose-dependent upregulation of its expression.

We have seen previously that oxidative stress induced an important remodeling of the post-translational modification profile of FOXL2 in KGN cells (Fig. 2). Interestingly, the spots strengthened under oxidative stress [spots H and K according to our previous analysis (24)] are also strengthened when cellular hyperacetylation is promoted in KGN cells using HDAC inhibitors (Fig. 4A). Moreover, luciferase assays using the 4xFLRE-luc reporter, to assess the effect of the generalist p300 acetyl-transferase on FOXL2 transactivation, clearly shows that FOXL2 activity is enhanced under hyperacetylation (Fig. 4B). Taken together, our results indicate that highly modified isoforms

of FOXL2 favored under oxidative stress (hyperacetylated forms of FOXL2) have increased transactivation ability in comparison with other isoforms.

FOXL2 transcriptional activity and expression are repressed by the NAD-dependent deacetylase SIRT1

We have observed that FOXL2 tends to be hyperacetylated in response to oxidative stress, which correlates with a significant increase of its recruitment to stress response promoters and of its transactivation ability. This includes an increase of its self-activation capacity that should contribute to the increase of FOXL2 cellular levels. In this context, we sought to identify a regulator that could counteract the increasing FOXL2 activity occurring upon oxidative stress, and more specifically a regulator that could counteract FOXL2 hyperacetylation. Indeed, opposing regulations are commonplace in dynamic systems, as they allow swift and fine-tuned responses to environmental cues (41).

The SIRT1 deacetylase has been shown to modulate the activity of FoxO transcription factors through control of their acetylation upon cell stress, and this interaction generally repressed their pro-apoptotic activity and enhanced their ability to induce stress resistance and cell survival (42,43). Moreover, (i) the existence of similarities in organs affected in the KO mice for Sirt1 and Foxl2 (9,10,31), (ii) the fact that both genes are highly expressed in granulosa and KGN cells (data not shown) and (iii) the fact that overexpression of SIRT1 in KGN cells induced a demodification of FOXL2 (disappearance of highly acetylated isoforms, notably isoforms from spots K and H; Fig. 5A; 24) led us to investigate a potential regulatory link between these proteins. Therefore, we measured potential variations of FOXL2 activity in response to SIRT1 overexpression on our reporters.

First, on the 4xFLRE-luc construct, SIRT1 overexpression was able to inhibit the FOXL2-induced upregulation by ~25% (Fig. 5B). A basal activity repression of similar amplitude was also observed without the overexpression of FOXL2 in the context of SIRT1 overexpression, which is likely the result of the demodification of endogenous FOXL2 by SIRT1. We also used the pFoxL2-luc and the pSODluc-3340 constructs to assess FOXL2 transactivation ability in the context of naturally occurring target gene promoters, with or without SIRT1 overexpression. In this case, we found that SIRT1 repressed dramatically FOXL2 transactivation on stress-related targets. Indeed, FOXL2 transactivation was diminished by ~40% on pFoxL2-luc and completely abolished on pSODluc-3340 (Fig. 5C–D). Interestingly, the effect of SIRT1 overexpression on pFoxL2-luc also suggests that it can tune down FOXL2 promoter activity, and thus FOXL2 expression itself (Fig. 4C). Our observations further support the idea that hypermodified/hyperacetylated forms of FOXL2, which increase during cell stress, are the most active FOXL2 isoforms. This also demonstrates the ability of SIRT1 to inhibit FOXL2-mediated increase in target genes transcription.

SIRT1, and the sirtuin family of deacetylases in general, are peculiar among histone deacetylases (HDACs) because they use NAD as a co-factor for catalysis, therefore yielding as products the deacetylated substrate protein, nicotinamide (a form
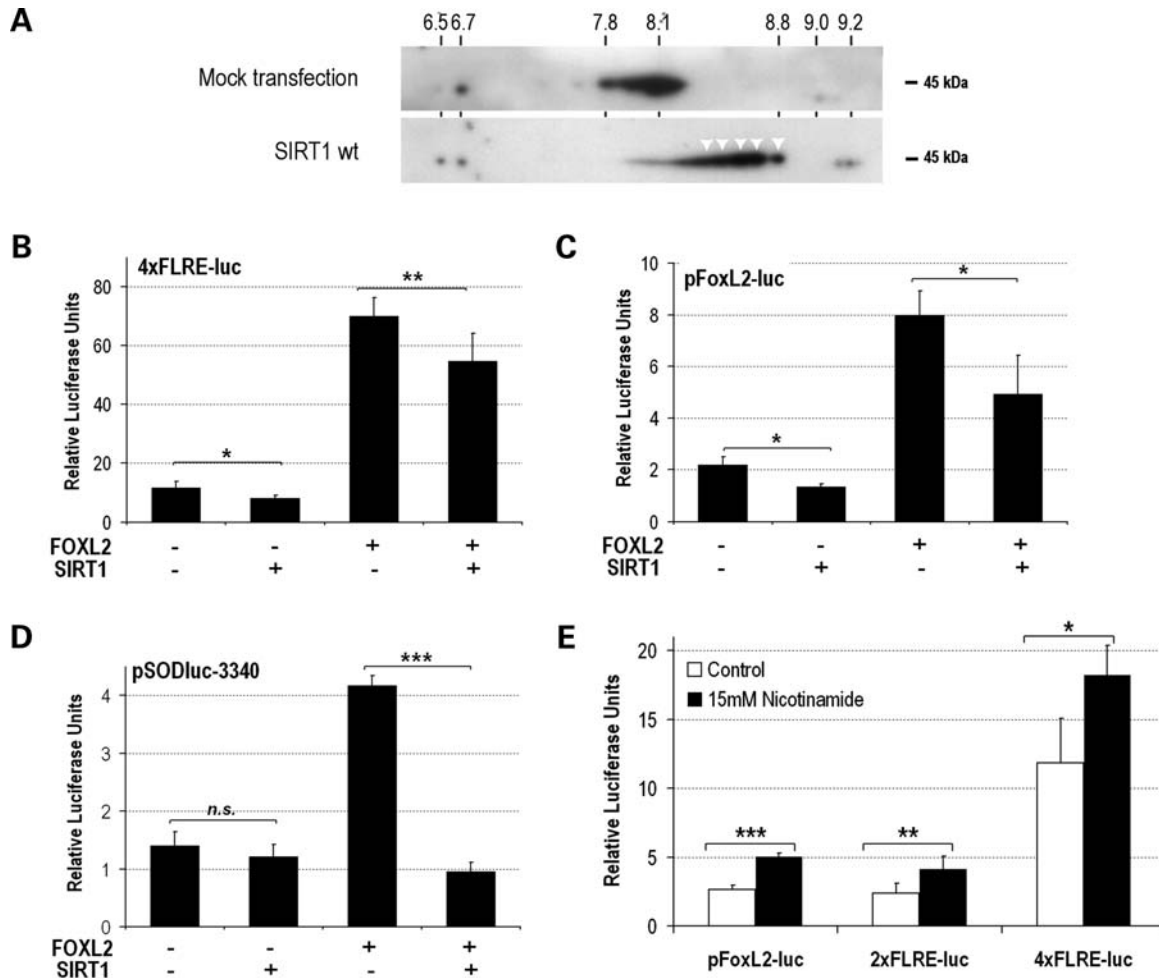
Figure 5. Repression of FOXL2 transactivation on target promoters by SIRT1. (**A**) Two-dimensional western profile of FOXL2 in KGN cells mock-transfected or transfected with SIRT1. Note the disparition of the acidic train and of hyperacetylated spots H and K (24). (The post-translational modification profile of the forkhead transcription factor FOXL2 suggests the existence of parallel processive/concerted modification pathways. Proteomics, 2008, 8, 3118-3123. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced and modified with permission.). Luciferase assays in KGN cells transfected with (**B**) the 4xFLRE-luc construct, (**C**) the pFoxL2-luc construct or (**D**) the pSODluc-3340 construct, with or without FOXL2 and SIRT1 overexpression, to assess SIRT1 effect on FOXL2 transactivation. (**E**) Luciferase assays in KGN cells transfected with the pFoxL2-luc, the 2xFLRE-luc or the 4xFLRE-luc constructs, with or without addition of 15 mM nicotinamide, the sirtuin inhibitor, in the cell culture medium 24 h before luciferase activity quantification. Activity of the FoxL2 promoter and FOXL2 activity in the cell increases significantly in response to the addition of nicotinamide to the medium. Statistical significance in Student's *t*-tests.

of niacin/vitamin B3) and *O*-acetyl-ADP-ribose (26,44). It has been shown that, at physiological concentrations, nicotinamide is a potent non-competitive inhibitor of SIRT1 and other sirtuins, by preventing the hydrolysis of NAD necessary for the deacetylation reaction (32,44,45). Thus, it has been proposed that nicotinamide could regulate SIRT1 activity levels *in vivo* (32,46).

Since we have observed that SIRT1 was able to inhibit FOXL2 transactivation and, eventually, its expression, we assessed the effect of nicotinamide on FOXL2 activity and expression. We therefore performed luciferase assays in control KGN cells, or KGN cells treated with 15 mM nicotinamide for 24 h before luciferase activities quantification (Fig. 5E), using the pFoxL2-luc reporter (to assess FoxL2 promoter activity) and the 2xFLRE-luc and 4xFLRE-luc reporters (to assess the endogenous FOXL2 activity in KGN cells). Interestingly, we observed that the addition of nicotinamide to the cell culture medium induced a significant activation of

FoxL2 promoter (~1.9-fold), which indicates that, presumably through quenching SIRT1 (and other sirtuins) activity, nicotinamide upregulates FoxL2 transcription (Fig. 5E). In these conditions, we also observe an increase of the basal activities of the 2xFLRE-luc (~1.8-fold) and 4xFLRE-luc (~1.5-fold) reporters (Fig.5E), which are specific sensors of FOXL2 cellular concentration and activity levels (37). Thus, the enhanced FLRE-luc reporter activity indicates that there is an increase in FOXL2 concentration/activity in KGN cells upon nicotinamide treatment. Interestingly, preliminary evidence suggests that the treatment of C57/B6 mice with nicotinamide can also lead to an upregulation of Foxl2 expression *in vivo* (Supplementary Material, Fig. S3). As BPES in association with POF often results from heterozygous null mutations, finding a way to increase FOXL2 expression from the functional allele is of therapeutic relevance. In humans, nicotinamide toxicity has been found to be low even at daily doses as high as 1260 mg over 2 years (about

a thousand times higher than the recommended daily intake; 47). Therefore, a potential upregulation of ovarian FOXL2 expression/activity by nicotinamide supplementation deserves further exploration *in vivo*.

FOXL2 can directly activate the transcription of SIRT1

The stress-induced activation of FOXL2, if maintained for long, would lead to a considerable amplification of the response to stress and would be difficult to halt after the end of the signal. Since SIRT1 is able to counteract FOXL2 trans-activation, it is an ideal candidate to be involved in a negative feedback loop. Thus, to test whether FOXL2 is able to activate the expression of its inhibitor SIRT1, we generated a lucifer-ase reporter driven by SIRT1 promoter sequences (SIRT1-luc). Interestingly, FOXL2 induced a strong activation of the reporter ($\sim$7.4-fold), which indicates that SIRT1 is a very sensitive target of FOXL2 (Fig. 6A). Moreover, exposure to 150 μM of H_2O_2 also led to significant activation of the reporter ($\sim$2.6-fold), which confirms the status of SIRT1 as a stress-response gene in the context of oxidative stress. This stress-induced increase of SIRT1 transcription is also observed at the endogenous level in KGN cells (Supplementary Material, Fig. S4). Interestingly, we also observed a significant over-activation of SIRT1-luc under FOXL2 overexpression upon stress (i.e. reporter activation was further enhanced under oxidative stress by a 1.6-fold). This suggests that SIRT1 transcriptional upregulation in response to oxi-dative stress can be mediated at least partly by FOXL2.

Next, we performed ChIP experiments on chromatin from KGN cells (Fig. 6B) to determine whether the regulation of SIRT1 transcription by FOXL2 was direct or not. FOXL2 was found to be significantly recruited to the promoter of SIRT1, which indicates indeed that the effect of FOXL2 on SIRT1 expression comes through a direct regulation. This closes a negative feedback loop of FOXL2 through the action of SIRT1 and provides the first demonstrated instance of such a mechanism involving SIRT1 and a forkhead tran-scription factor. Interestingly, this negative feedback loop of FOXL2 is observed at first post-translationally, which allows a rapid decrease of FOXL2 activity. However, since SIRT1 overexpression decreases pFoxL2-luc activity (Fig. 5C), upre-gulation of SIRT1 expression is also expected to induce a decrease in FOXL2 expression *in vivo*.

Finally, we tested the molecular consequences of this feed-back loop on FOXL2-mediated activation of the SIRT1-luc reporter. Surprisingly, we found that the demodifying action of SIRT1 on FOXL2 (24) induced increased FOXL2 transac-tivation on the promoter of SIRT1 (2.2-fold over-activation when compared with the effect of FOXL2 expressed alone), in contrast to the results that we had previously observed on the 4xFLRE-luc, pFoxl2-luc and pSODluc-3340 promoter reporters (Fig. 5). These data suggest that, in a context of cell stress where FOXL2 activation increases dramatically, amplified activation of SIRT1 by SIRT1-demodified forms of FOXL2 should accelerate the tuning down of the FOXL2 response and maybe favor the return to cellular homeostasis. Our findings also show that demodification of FOXL2 by SIRT1 can have different consequences on distinct target promoters, possibly switching the action of FOXL2 toward
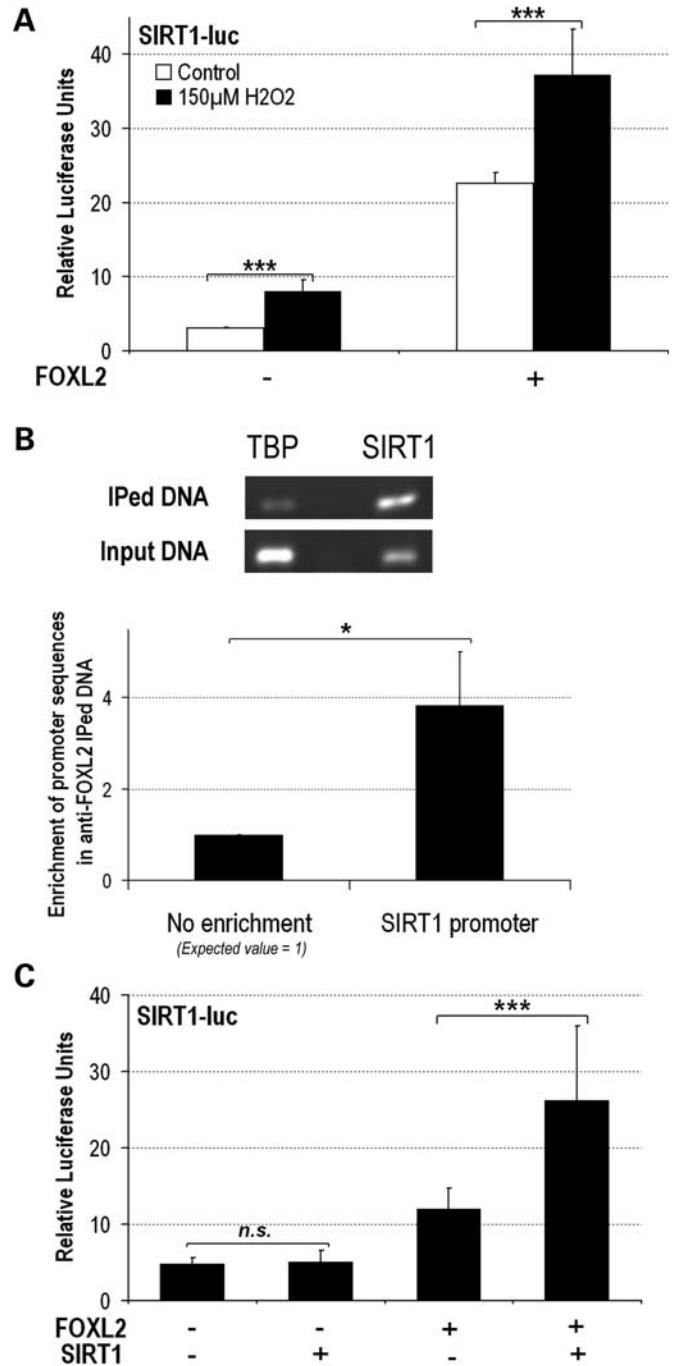

Figure 6. Direct activation of SIRT1 transcription by oxidative stress and by FOXL2, reinforced by SIRT1 itself. (**A**) Luciferase assays in KGN cells trans-fected with the SIRT1-luc construct, with or without FOXL2 overexpression, to assess the potential effect of oxidative stress on FOXL2 transactivation on SIRT1 transcription. (**B**) Anti-FoxL2 ChIP-PCR on control KGN cell chroma-tin, SIRT1 promoter PCR. Quantification of PCR band intensities was per-formed through ImageJ. Top: ethidium bromide-stained 1% agarose gel, representative of our 10 independent ChIP experiments. Bottom: means of quantified fold-enrichment values in immunoprecipitated samples versus total input DNA in 10 ChIP-PCR experiments when compared with a value of 1 (expected if there is no recruitment of FOXL2 to SIRT1 promoter). (**C**) Luciferase assays in KGN cells transfected with the SIRT1-luc construct, with or without FOXL2 and SIRT1 overexpression, to assess SIRT1 effect on FOXL2 transactivation on SIRT1 own promoter. Statistical significance in Student's *t*-tests. *n.s.*, non-significant; *$P < 0.05$; ***$P < 0.001$.

Table 1. Influence of various disease-causing mutations on the ability of FOXL2 to induce positive and negative feedback or to upregulate the expression of a stress-response target gene, the *MnSOD*

	Ability to induce positive feedback	Ability to induce negative feedback	Ability to upregulate a stress response target gene, *MnSOD*	Predicted expression levels of FOXL2 for heterozygous mutations carriers	Predicted outcome
FOXL2-WT	++	++	++	WT	Reference WT allele
FOXL2-I84N	−	−	−	?	Null allele, presumably leading to haploinsufficiency.
FOXL2-R103C	++	++	+	WT	Correct cellular activity levels, but possibly impaired stress response
FOXL2-H104N	++	+++	+	<WT	Imbalance of feedback signaling in favor of negative feedback; intracellular FOXL2 level will have a tendency to decrease abnormally; in all cases but one (N109K), association with an impaired activation of stress-response gene, *MnSOD*
FOXL2-N109K	−	++	++	<WT	
FOXL2-S58L	−	+	+	<WT	
FOXL2-S101R	−	+	+	<WT	
FOXL2-W98G	−	+	−	<WT	
FOXL2-Ala24	−	+	− −	<WT	
FOXL2-E69K	++	+	++	>WT	Imbalance of feedback signaling in favor of positive feedback; intracellular FOXL2 level will have a tendency to increase abnormally; possibly impaired stress-response
FOXL2-Ala19	++	+	−	>WT	
FOXL2-S217F	+++	++	++	>WT	

The variants' reported ability to induce positive feedback corresponds to the activity on the pFoxL2-luc construct, and was determined previously in Beysen *et al*. (48) for point mutations and in Moumne *et al*. (34) for expansions of the polyalanine domain. The ability to induce negative feedback signaling is measured by the activity of the variants on the SIRT1-luc reporter (Fig. 7A).

distinct possible molecular outcomes as a function of the cellular signaling input.

Disease-causing FOXL2 variants display imbalanced feedback regulation and/or impaired stress-response target gene activation

We decided to assess the effect of several disease-causing mutations of FOXL2 [nine missense mutants; (48) and two pathogenic polyalanine-expanded variants (34; Table 1)] on its ability to induce correct feedback regulation and to regulate the expression of the *MnSOD*.

The ability to induce proper positive feedback regulation, i.e. the transactivation capacity of these variants on the FoxL2 promoter reporter pFoxL2-luc (also known as DK3-luc), was investigated previously (34,48), and a summary is included in Table 1. The ability to induce proper negative feedback regulation, as measured by its transactivation ability on the SIRT1-luc reporter, and the ability to regulate the expression of the stress-response target gene *MnSOD* are reported in Figure 7 and summarized in Table 1.

Interestingly, according to our luciferase assays, the I84N variant seems to be a null mutant, since it does not display any activity on any of the tested reporters (Fig. 7; Table 1). Also interestingly, the H104N variant behaves in a hypermorphic manner on the SIRT1-luc reporter, thus presumably inducing an aberrantly strong negative feedback signaling (Fig. 7A). The Ala24 variant displays a weak dominant-negative effect on the endogenous FOXL2 on the pSODluc-3340 reporter, which is compatible with our previous results (37; Fig.7B).

The confrontation of the transactivation abilities of these 12 mutants on pFoxL2-luc, SIRT1-luc and pSODluc-3340 allows us to classify mutants in four main categories (Table 1): (i) null mutations, (ii) mutants with presumably correct expression levels but defective stress-response target activation, (iii) mutants having an imbalanced feedback regulation ability when compared with the wild-type, potentially leading to pathogenic decreased cellular FOXL2 activity and (iv) mutants having an imbalanced feedback regulation ability, potentially leading to pathogenic increased cellular FOXL2 activity. It is important to note that potentially increased FOXL2 expression levels, from both the wild-type and the pathogenic allele, do not necessarily result in higher FOXL2 cellular activity on all of its targets, especially when the mutant protein is known to aggregate (Ala19 or E69K; 34,48). In any case, altered dosage of FOXL2 activity levels (whether increased or decreased) might be pathogenic, as was reported for other forkhead transcription factors, such as FOXC1-2 (49). According to our findings, abnormal regulation of FOXL2 expression and activity, as well as defective regulation of stress-response target gene(s), could contribute to the pathogenesis of *FOXL2* mutations.

GENERAL DISCUSSION

Ever since *daf-16*, the Foxo transcription factor of *C. elegans*, was found to be involved in the genetic modulation of longevity (50), there has been a growing interest in the molecular pathways that regulate aging. There is accumulating evidence suggesting that the molecular regulation of aging is much more complex in mammals than in invertebrates. Moreover, it seems that forkhead factors, more generally, play a crucial role in the regulation of this process at different levels, in different organs and in different environmental conditions.
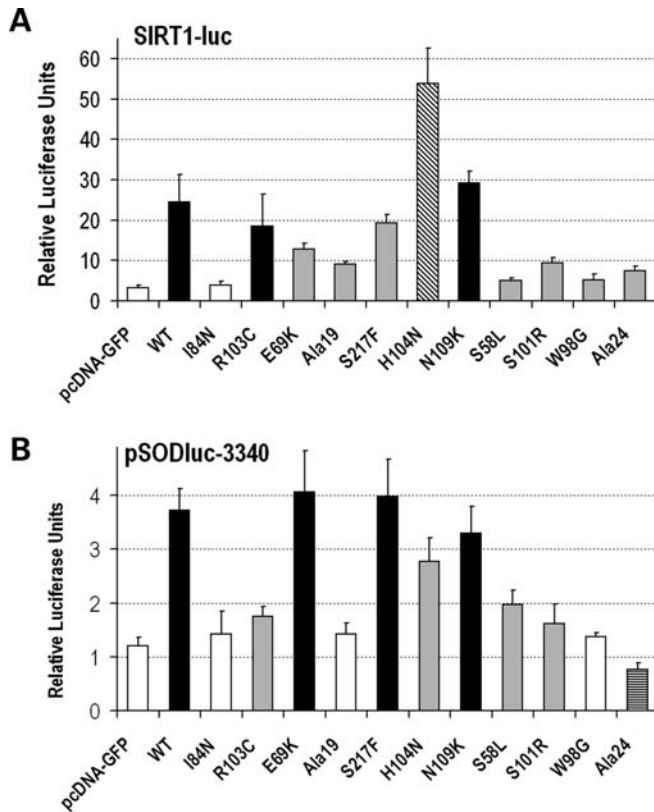
Figure 7. Influence of various disease-causing mutations on the ability of FOXL2 to regulate the expression of SIRT1 and MnSOD. Luciferase assays in KGN cells transfected with (**A**) the SIRT1-luc construct or (**B**) the pSODluc-3340 construct, in the presence of FOXL2 variants (34,48) or the empty control vector, to assess the effect of disease-causing mutations. White bars: basal transcriptional activity of the reporter promoters, and variants not displaying statistically significant differences when compared with the empty control vector, compatible with a loss of function on the promoter studied. Black bars: wild-type protein transcriptional activity, and variants not displaying statistically significant differences when compared with the wild-type protein ('isomorphic' mutations). Gray bars: variants displaying an intermediate transcriptional activity level when compared with both the empty control vector and the wild-type protein, statistically different with both. Inclined stripped bar: H104N variant, displaying a statistically higher activity level than the wild-type protein on the SIRT1-luc reporter. Horizontal stripped bar: Ala24 variant, displaying a statistically lower activity level than the empty control vector on the pSODluc-3340 reporter, suggestive of a weak dominant-negative effect. Statistical significances in Student's *t*-tests at least such as *P* < 0.05 even with the Bonferroni correction for multiple testing.

Indeed, *Pha-4* (Foxa) was shown to play a crucial role in longevity regulation in *C. elegans* (51). Furthermore, deregulation of several FOX factors seems critical for cancer progression, which increases with aging (52). Here, we show that FOXL2 is an actor of the ovarian oxidative stress response. This suggests that FOXL2 may be a part of the complex networks that orchestrate the regulation of aging in mammals, at least at the ovarian level.

We show that FOXL2 mRNA and protein levels increase after exposure of cells to oxidative stress or heat shock, which can be amplified through self-activation. However, to explain the rapid switch between a steady state where FOXL2 maintains a stable expression and a situation involving

dramatical increase of protein production, other ingredients are needed. Part of the answer resides in the post-translational modification remodeling potential of FOXL2. Indeed, post-translational modification remodeling correlates with the modulation of FOXL2 activity on specific target promoters. The stress-induced increase of FOXL2 expression could also result from FOXL2 cooperating with another transcription factor, for instance a FOXO or a heat-shock factor, which are both actively relocalized to the nucleus upon stress (42,53). Stress-induced alterations in FOXL2 post-translational modification profile seem to induce an enhancement of its activity, as our luciferase assays indicated, which would allow the steady state to be shifted toward an increase of FOXL2 cellular concentration and activity.

FOXL2 is not just a passive bystander of the granulosa cell stress response: it is increasingly recruited to specific target promoters upon oxidative stress and, in particular, to the promoter of the crucial ROS-scavenging enzyme *MnSOD*. This correlates with a detectable enhancement of its transactivation. FOXL2 thus seems to have the ability to respond actively to stress in granulosa cells, by further regulating the expression of stress response target genes.

We also found that the key stress-response SIRT1 deacetylase repressed FOXL2 transactivation ability, whereas, in turn, FOXL2 directly increased SIRT1 transcription. This indicates that, in addition to its self-activation, FOXL2 is also able to induce the repression of its own activity, through upregulation of SIRT1. These two feedback loops, which might seem contradictory at first, as one of them leads to increased levels of FOXL2 and the other to decreased FOXL2 activity, are not expected to be simultaneous.

We propose that FOXL2 acts as a molecular sensor of granulosa cell stress, influenced by the action of its regulator SIRT1. Since we observed that SIRT1 affects differentially the transactivation capacity of FOXL2 on different target promoters, its action may change the subset of FOXL2 targets recognized/regulated at any given time. This could potentially affect granulosa cell fate in response to environmental signals. We propose a model of consequences of stress stimuli on FOXL2 activity in the granulosa cells (Fig. 8; Supplementary Material, Fig. S5).

According to our results, the potential dysregulation of the feedback loops involving FOXL2 and/or of stress-response target genes in granulosa cells induced by mutations in *FOXL2* might result in a defective stress-response regulation. This could contribute to explain how follicular depletion and premature menopause can arise in *Foxl2* KO mice models, type I BPES patients and isolated POF cases with *FOXL2* mutations. The weakening of the ovarian stress-response along with aging might explain the physiological onset of menopause. Though the most obvious consequence of menopause is the onset of infertility, it has other serious repercussions on women's health [increased prevalence of osteoporosis (54), cardio-vascular diseases (55) and neurodegenerative diseases (56,57)]. It is therefore crucial to elucidate the molecular pathways that regulate ovarian aging, in which FOXL2 is likely involved, so as to eventually have the ability to modulate its onset and decrease the impact of the serious consequences that menopause causes in women.
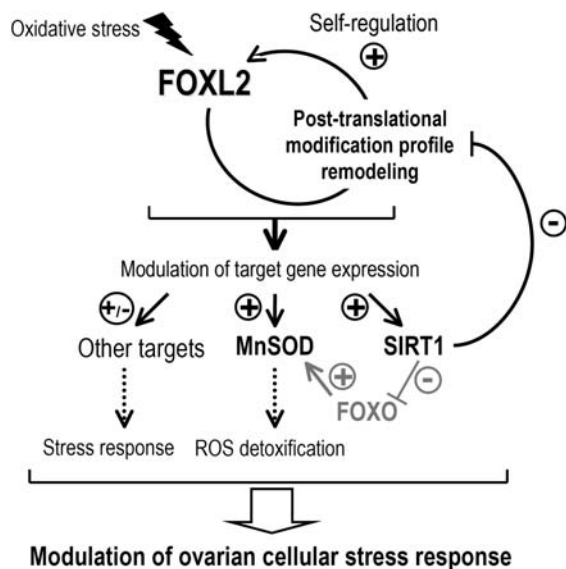
Figure 8. Modulation of ovarian stress response through FOXL2. Increase in FOXL2 production and modification triggers the modulation of stress-related targets, among which the ROS-scavenging MnSOD enzyme and SIRT1. FOXO factors have previously been shown to also participate in a stress-induced increase of MnSOD expression (13), which indicates a potential cooperation. At some point during stress, the increase of SIRT1 levels, partly induced by FOXL2, will limit the activity increase of FOXL2 (and FOXO; 42,43).

MATERIALS AND METHODS

PCR primers and antibodies

PCR primers were ordered from Eurogentec; sequences are available upon request. The anti-FoxL2 polyclonal rabbit antibodies were described previously (7).

Plasmids

pFoxL2-Luc and pSODluc-3340 have been described previously (33,40). 2xFLRE-luc (4xFLRE-luc) is a pGL3-basic firefly luciferase reporter (Promega) driven by an artificial promoter containing two (four) high-affinity FOXL2 responsive elements (FLRE) upstream of a minimal CMV promoter, and has been described previously (37). To generate the SIRT1-luc construct, we amplified 1053 bp of the human SIRT1 promoter, from -1000 to $+53$, from human genomic DNA, with PCR primers introduced restriction endonucleases sites *Xho*I and *Hin*dIII. The PCR product was digested with both *Xho*I and *Hin*dIII (Boerhinger Mannheim) and ligated in pGL3-Basic (Promega). Sequences were verified by automated sequencing. The FOXL2-GFP expression vector and its polyalanine expanded versions FOXL2-Ala19-GFP and FOXL2-Ala24-GFP were described previously (34), and the expression vectors for point mutant versions of FOXL2-GFP used in this work were described previously (48). Wild-type human myc-SIRT1 expression vector was also described previously (58). The p300 expression vector used here was described previously (59).

Cell culture, transfections and luciferase assays

KGN cells (21) were cultured in DMEM-F12 medium, supplemented with 10% FBS and 1% penicillin/streptomycin (Invitrogen-Gibco). KGN cells were plated 12 h prior to transfection at a density of 4×10^4 cells cm^{-2} and transfected using the calcium phosphate method (60). For luciferase assays, KGN cells were seeded in 24-well plates, and pFoxL2-luc, pSODluc-3340, SIRT1-luc, 2xFLRE-luc and 4xFLRE-luc were used to assess FOXL2 activity. A Renilla luciferase reporter driven by the RSV promoter (Promega) was included as an internal control of transfection efficiency. Luciferase activity quantification was performed as described previously (34). Relative luciferase units are the ratio of firefly over Renilla luciferase activity and come from at least five biologically independent replicates. Statistical significance was estimated by Student's *t*-tests.

Exposure of cultured cells to stress and treatment with nicotinamide

To induce oxidative stress, H_2O_2 was added to culture media to 150 or 300 μM for 2 h. To induce heat shock, cells were placed at 42°C for indicated times. Stress exposure was conducted for 2 h and treatment with 15 mM nicotinamide for 24 h before luciferase activities quantifications.

SDS–PAGE and western blot protein analysis

Samples were supplemented with a protease inhibitor cocktail (Sigma), 25 μM PMSF, phosphatase inhibitors (100 μM NaF, 500 μM Na$_3$VO$_4$, 10 μM NaMo, 3 mM NaP$_2$O$_7$) and 10 mM iodoacetamide to prevent ubiquitin-like protein deconjugation. When needed, the protein concentrations were estimated using the Bradford method (Bio-Rad). Molecular weights were estimated using the Precision Plus Kaleidoscope Protein Standards (Bio-Rad). For 1D-western blot analysis, samples were electrophoresed and electrotransfered onto nitrocellulose membranes (Hybond-C; GE Healthcare). Western blot was conducted as in Cocquet *et al*. (7). Two-dimensional sample analysis was conducted as previously (24). Immobiline Dry-strip, pH 6–11, 7 cm strips (GE Healthcare) were used for first dimension separation, and 40 μg of proteins were applied to the strips.

Protein band/spot intensity quantification

Relative amounts of protein signal were quantified (spot intensities versus background noise) using the ImageJ software (http://rsb.info.nih.gov/ij).

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) experiments were performed as in Batista *et al*. (22). Statistical significance was computed on normalized crossing point deltas (treated versus controls), prior to exponentiation, using a one-sample Student's *t*-test.

RNA extraction and cDNA synthesis

Total RNA 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. For stress-related RT–qPCR experiments, an amplicon in the succinate dehydrogenase (SDHA) was used as a normalizing amplicon.

Anti-FoxL2 ChIP assay

We performed ChIP assays as described before (22), using an iso-volumic blend of anti-N and anti-C FoxL2 antibodies. Fold enrichments were estimated by qPCR, by comparing immuno-precipitated material and DNA from fixed, sheared and depro-teinized chromatin (input DNA). Sequences in the promoters of TBP and PPP1R15A were used for sample normalization (details in Supplementary Material). Fold-enrichments represent the geometric mean of at least 10 independent ChIP assays. Errors are represented as SEM values.

Statistics and data analysis

Regression correlation coefficients were estimated using Microsoft Excel. Two-sample Student's t-tests, one-way ANOVA test and the correlation coefficient significance test were performed using the VassarStats website for statistical computation (http://faculty.vassar.edu/lowry/VassarStats.html), and one-sample Student's t-tests were performed using the Graphpad Quickcalcs software (http://www.graphpad.com/quickcalcs/index.cfm). Differences were considered statistically significant when $P < 0.05$.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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