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

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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 dismutase (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 deacetylase. 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 periciliar and ovarian follicular (mostly granulosa) cells at both fetal and adult stages, where it localizes exclusively...
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$_2^-$ into O$_2$ and H$_2$O$_2$ (15), whereas catalase converts H$_2$O$_2$ into H$_2$O 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-kB and FOXO factors; 25). Sirt1 KO 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 non-competitive 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$_2$O$_2$ 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...
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 Foxl22/2 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. Indeed, after stress exposure, both oxidative stress and heat shock induced strengthening 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 H2O2 and 2 h, 42°C 2D-western profiles. Indeed, in these conditions,

![Figure 1. Transcriptional upregulation of FoxL2 during oxidative and heat stress.](image-url)

(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.
we observe a concentration of the acidic train in two main 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 H2O2 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 H2O2, we also observe the appearance of a third train of modifications, comprising isoforms migrating 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 ~2-fold after 2 h oxidative stress (150 or 300 μM H2O2), ~3-fold after 1 h of 42°C heat shock and ~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 H2O2 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.
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 H2O2 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
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$_2$O$_2$ (150 and 300 µM) in the presence or absence of FOXL2 overexpression. The reporter activity increases upon FOXL2 overexpression ($P = 5.10^{-4}$), 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 acetyltransferase on FOXL2 transactivation, clearly shows that FOXL2 activity is enhanced under hyperacetylation (Fig. 4B).

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.

FOX11, 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 of FOXL2 favored under oxidative stress (hyperacetylated forms of FOXL2) have increased transactivation ability in comparison with other isoforms.

**FOX11 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.

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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...
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 activity quantification (Fig. 5E), using the pFoxL2-luc construct, (C) 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.

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.
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 transactivation, 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 luciferase reporter driven by SIRT1 promoter sequences (SIRT1-luc). Interestingly, FOXL2 induced a strong activation of the reporter (~7.4-fold), which indicates that SIRT1 is a very sensitive target of FOXL2 (Fig. 6A). Moreover, exposure to 150 μM of H2O2 also led to significant activation of the reporter (~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 oxidative 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 transcription 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), upregulation of SIRT1 expression is also expected to induce a decrease in FOXL2 expression in vivo.

Finally, we tested the molecular consequences of this feedback 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 transactivation 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 FOXL2 is expected to 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...
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).

### 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 forkhed 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.

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<table>
<thead>
<tr>
<th>FOXL2-WT</th>
<th>++</th>
<th>++</th>
<th>++</th>
<th>WT</th>
<th>Reference WT allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOXL2-I84N</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>?</td>
<td>Null allele, presumably leading to haploinsufficiency.</td>
</tr>
<tr>
<td>FOXL2-R103C</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>WT</td>
<td>Correct cellular activity levels, but possibly impaired stress response</td>
</tr>
<tr>
<td>FOXL2-H104N</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>&lt;WT</td>
<td>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</td>
</tr>
<tr>
<td>FOXL2-N109K</td>
<td>–</td>
<td>++</td>
<td>++</td>
<td>&lt;WT</td>
<td>Impairment of feedback signaling in favor of negative feedback; intracellular FOXL2 level will have a tendency to decrease abnormally; MnSOD</td>
</tr>
<tr>
<td>FOXL2-S58L</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>&lt;WT</td>
<td>Null allele, presumably leading to haploinsufficiency.</td>
</tr>
<tr>
<td>FOXL2-S101R</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>&lt;WT</td>
<td>Null allele, presumably leading to haploinsufficiency.</td>
</tr>
<tr>
<td>FOXL2-W98G</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>&lt;WT</td>
<td>Null allele, presumably leading to haploinsufficiency.</td>
</tr>
<tr>
<td>FOXL2-Ala24</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>&lt;WT</td>
<td>Null allele, presumably leading to haploinsufficiency.</td>
</tr>
<tr>
<td>FOXL2-E69K</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>&gt;WT</td>
<td>Imbalance of feedback signaling in favor of positive feedback; intracellular FOXL2 level will have a tendency to increase abnormally; possibly impaired stress-response</td>
</tr>
<tr>
<td>FOXL2-Ala19</td>
<td>++</td>
<td>+</td>
<td>–</td>
<td>&gt;WT</td>
<td>Impairment of feedback signaling in favor of negative feedback; intracellular FOXL2 level will have a tendency to decrease abnormally; MnSOD</td>
</tr>
<tr>
<td>FOXL2-S217F</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>&gt;WT</td>
<td>Null allele, presumably leading to haploinsufficiency.</td>
</tr>
</tbody>
</table>

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.
Indeed, in KGN cells transfected with (FOXL2 to regulate the expression of SIRT1 and MnSOD. Luciferase assays Figure 7. Influence of various disease-causing mutations on the ability of evity regulation in (FOXL2 is an actor of the ovarian oxidative stress response. which can be amplified through self-activation. However, to explain the rapid switch between a steady state where after exposure of cells to oxidative stress or heat shock, at least at the ovarian level. 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 transactivation. However, to explain the rapid switch between a steady state where FOXL2 maintains a stable expression and a situation involving dramatrical 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 relocated 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.
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 \( 2^{1000} + 53 \) from human genomic DNA, with PCR primers introduced restriction endonucleases sites XhoI and HindIII. The PCR product was digested with both XhoI and HindIII (Boehringer 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 \( 4 \times 10^4 \) 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 \( \mu M \) for 2 h. To induce heat shock, cells were placed at 42 \( ^\circ C \) for indicated times. Stress exposure was conducted for 2 h and treatment with 15 \( \mu M \) 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 \( \mu M \) PMSF, phosphatase inhibitors (100 \( \mu M \) NaF, 500 \( \mu M \) Na\(_3\)VO\(_4\), 10 \( \mu M \) NaMo, 3 mM NaP\(_2\)O\(_7\)) and 10 \( \mu M \) 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 electrotransferred onto nitrocellulose membranes (Hybond-C; GE Healthcare). Western blot was conducted as in Cocquet \textit{et al.} (7). Two-dimensional sample analysis was conducted as previously (24). Immobiline Drystrip, pH 6–11, 7 cm strips (GE Healthcare) were used for first dimension separation, and 40 \( \mu g \) 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 \textit{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.

![Figure 8. Modulation of ovarian cellular 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).](http://rsb.info.nih.gov/ij)
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 ampiclon 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 deproteinated 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 analysis/normalization. We performed two-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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Conflict of Interest statement. None declared.

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