Dear Editor,

Early ovarian expression of Newborn oogenesis homebox (NOBOX), a transcription factor belonging to the homeoprotein class, is decisive for the progression of primordial follicles to the primary follicular stage as demonstrated by the sterility of NObox−/− female mice (Rajkovic et al., 2004). Consequently, the loss of the NObox gene causes primary ovarian insufficiency (POI) (Rajkovic et al., 2004) and we have shown that 6.2% of POI cases harbor mutations in the NOBOX gene (Qin et al., 2007; Bouilly et al., 2011). Such a high prevalence of mutations demonstrates that NOBOX is a major autosomal gene involved in POI (Bouilly et al., 2011). However, the exact role of this transcription factor during folliculogenesis is unclear.

The forkhead transcription factor 2 (Fox2) is a central element in ovarian development and follicular growth and maturation. FOXL2 mutations are responsible for blepharophimosis-ptosis-epicanthus inversus syndrome (BPES), often associated with POI (Moumne et al., 2008; Beysen et al., 2009). Lack of Fox2 during development leads to the absence of the secondary follicles and no differentiation of squamous granulosa cells (FGCs) into cuboidal cell (Schmidt et al., 2004; Uda et al., 2004). However, little is known about the mechanism by which Fox2 regulates different target gene subsets depending on developmental stages and/or other signals. Such target specification may be achieved through interactions with protein partners modulating promoter recognition.

Homeoproteins and Forkhead transcription factors are protein families characterized by conserved DNA-binding domains. Interestingly, it has been documented that homeoproteins can interact with Forkhead box transcription factors giving rise to generic ‘inter-family’ interactions (Foucher et al., 2003). In this study, we show that NOBOX, initially described as expressed in the oocyte (Rajkovic et al., 2004), is also expressed in FGCs. Besides, we identify NOBOX as a key FOXL2 partner and report the existence of the first ovarian homeoprotein/forkhead box complex to regulate pivotal ovarian genes involved in folliculogenesis.

Although NOBOX is undoubtedly involved in the etiology of POI, only limited information regarding its regulators is available. To further explore the localization of the NOBOX protein, we have performed an immunohistological examination of ovarian sections from human fetuses revealing the presence of a strong staining of oogonia and also future follicular cells (Figure 1A and B). A similar analysis performed on ovarian sections of a 14-year girl showed that NOBOX was present in oocytes of primordial and primary follicles grouped in nests in the ovarian cortex (Figure 1C).

Unexpectedly, primordial, primary, and secondary follicles also showed NOBOX labeling in most FGCs (Figure 1C and D), which was confirmed by RT–PCR in human FGCs (Figure 1E), suggesting a new ovarian role for this protein. Taking into account the fact that both NOBOX and FOXL2 are co-expressed, and that homeodomain/forkhead generic interactions can take place (Foucher et al., 2003), we hypothesized that they could be partners in the regulation of transcriptional targets.

To validate this hypothesis, we showed that in transfected cells, NOBOX binds to FOXL2 to form a stable complex resisting co-immunoprecipitation (Figure 1F). To confirm the NOBOX–FOXL2 interaction, we used a mammalian two-hybrid system where the readout is a luciferase activity. In agreement with our co-immunoprecipitation data, we detected a significant increase of luciferase activity after co-overexpression of both proteins, confirming the interaction between NOBOX and FOXL2 (Supplementary Figure S1A). Based on the suggestion that forkhead domains and homeoboxes can foster generic interactions, we performed a two-hybrid assay using the relevant domains. A significantly increased luciferase activity demonstrated that NOBOX and FOXL2 interact via their DNA-binding domains (Supplementary Figure S1B).

FOXL2 has been shown to activate its own promoter (hereafter named DK3 promoter) (Benayoun et al., 2008), containing three FOXL2 response elements (FLRE). This promoter also contains two NOBOX binding elements (NBE) as determined in silico. To assess the impact of the interaction of NOBOX and FOXL2 on their function, we tested the activity of NOBOX and FOXL2 on the DK3 promoter. Overexpression of either NOBOX or FOXL2 alone led to a significant transactivation of the promoter (P < 0.01). However, the co-overexpression of both transcription factors led to a significantly decreased activity with respect to the levels observed when each factor was overexpressed alone (Figure 1G). We confirmed these results with another reporter. Indeed, the OCT4 promoter, although ‘artificial’ because OCT4 is not expressed in FGCs, proved to be informative in molecular terms (Choi and Rajkovic, 2006) (Supplementary Figure S2A). Thus, we also examined whether the presence of the NOBOX and FOXL2 binding sites in the OCT4 promoter were involved in mediating their effects on transcription. NOBOX was, as expected, unable to activate the OCT4 promoter bearing a mutated NBE (Supplementary Figure S2B). Similarly, FOXL2 was unable to activate the promoter mutated for the FLRE (Supplementary Figure S2C). Surprisingly, the co-transfection of both factors represses the transcriptional activity of the mutated versions of OCT4 (Supplementary Figure S2B).
suggested that the reciprocal inhibitory effects of NOBOX and FOXL2 are independent of their binding to the specific DNA elements.

We have also tested the FOXL2-binding capacity of a truncated naturally occurring NOBOX mutation (c.907C>T/p.R303X) previously detected in a POI case (Bouilly et al., 2011). Mutant NOBOX-R303X with a partial homeodomain deletion lost its interaction with FOXL2 (Supplementary Figure S3A and B). Coherently, we observed a loss of transcriptional repressive activity of NOBOX-R303X on the DK3 (Figure 1H) and OCT4 promoters (Supplementary Figure S3C). As expected, the transcriptional activity of FOXL2 was not inhibited by the overexpression of the p.R303X NOBOX mutant, indicating the inability of this mutant to interact with and modulate the activity of FOXL2. These results suggest that the truncated NOBOX mutation found in women with POI may disrupt the NOBOX–FOX2L interaction and lead to a progressive ovarian insufficiency.

In sum, both NOBOX and FOXL2 are expressed in human FGCS and their interaction plays an inhibitory role in the transcriptional response of these promoters. The in vivo relevance of the physical and functional interactions between NOBOX and FOXL2 is strengthened by the similarity of the phenotypes of the knockout models. Indeed, mice lacking Nobox or Foxl2 mimic the ovarian failure observed in women bearing mutations in these genes (Rajkovic et al., 2004; Schmidt et al., 2004; Uda et al., 2004). These coherent phenotypes add to other lines of evidence, such as (i) their co-expression in
FGCs and (ii) the presence of neighboring specific binding sites for both factors in cognate target promoters. From a pathological viewpoint, NOBOX mutations might affect proper formation or stability of the complex with FOXL2, eventually leading to POI. The question remains open of how both proteins interact during the formation of primordial follicles and their subsequent growth. In short, our study reveals that NOBOX is a new FOXL2 interactor and lends credence to the homeodomain-forkhead generic inter-family interactions.

[Supplementary material is available at Journal of Molecular Cell Biology online.]

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References


