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Materials and Methods

Targeting construct, genotyping, colony generation and analysis.

We have isolated \textit{Nobox} genomic clones from a 129S6/SvEv genomic library and generated a targeting construct (Fig. S1A). The \textit{Nobox} targeting construct was electroporated into AB2.2 ES cells to mutate the wild type \textit{Nobox} locus by homologous recombination as previously described\cite{S1,S 2}. The mutant \textit{Nobox} allele replaces exons 2-8 with the \textit{Pgkl}-HPRT cassette. We screened 192 ES clones by Southern blot analysis and identified 19 ES cells that carry a targeted mutant allele by Southern blot analysis. The \textit{Nobox} \textasciitilde:\textasciitilde ES cells were injected into blastocysts, and chimeras were generated from three independent ES cell lines. The \textit{Nobox} \textasciitilde:\textasciitilde mice generated from chimeras were bred to produce homozygous null \textit{Nobox} \textasciitilde:\textasciitilde mice, which were identified by Southern blot analysis of tail DNA (Fig. S1, B, C and D). Mice were genotyped by Southern blot analysis and multiplex PCR (primers and conditions are available from A.R. on request).
Breeding, Histology, Histomorphometric analysis, Immunohistochemistry, and TUNEL

All murine experiments were carried out on C57BL/6/129S6/SvEv hybrid background. Litters were weaned at 3 weeks and breeding pairs set up at 6 weeks of age. One mating pair was placed per cage and inspected every morning for presence of litters. For histological analysis, ovaries were freed from their capsule, immediately placed in 10% buffered formalin, embedded in paraffin, serially sectioned (5 µm) and stained with hematoxylin and eosin or with Periodic Acid Schiff (PAS). At least five pairs of ovaries of each genotype were subjected to gross and microscopic analysis for each time point (Fig.1 and S3). For histomorphometric analysis, every 5th section was photographed, and oocytes counted. Germ cell cysts were defined as two or more oocytes that were not individually separated by stromal cells. Primordial follicles were defined as small oocytes (<20 µm) surrounded by flat epithelial cells. Primary follicles were defined as having larger oocytes (>20 µm) surrounded by a single layer of cuboidal granulosa cells while secondary follicles were defined as larger oocytes surrounded by two or more layers of granulosa cells.

For immunohistochemistry, we used antibodies against GCNA1, MSY2 and NOBOX proteins. GCNA1 rat monoclonal antibody (S3) was kindly provided by Dr. George C. Enders (University of Kansas, KS), and MSY2 rabbit immunoaffinity-purified antibody (S4) was kindly provided by Dr. Richard Schultz (University of Pennsylvania, Philadelphia, PA). Polyclonal goat
antibodies against NOBOX (COOH terminus, amino acids 281-504) were generated by expressing a partial NOBOX protein in the pET-23b vector and immunizing goats at Cocalico (Lancaster, PA). The anti-NOBOX antibodies were immunoaffinity purified over Affi-Gel 10 (Bio-Rad, CA) and used in immunohistochemistry as previously described (S5).

We studied apoptosis in Nobox heterozygous and null ovaries (Fig. S5) by terminal deoxynucleotidyl transferase nick end labeling (TUNEL) using the ApopTag Peroxidase In situ apoptosis detection kit (Intergen, NY).

**RNA Isolation and RT-PCR**

Timed matings were set up to collect ovaries at various time points and total RNA was isolated using RNA STAT-60 as described by the manufacturer (Leedo Medical Laboratories, Houston, TX) (S6). Five micrograms of total RNA were initially treated with RNase-free DNase to remove contaminating DNA and then used to synthesize first strand cDNA using the Superscript system (Life Technologies, Rockville Maryland). Oligonucleotides, corresponding to Nobox, Oct4, Gdf9 and genes analyzed in Fig. 2 and S4, were selected using Primer 3 software (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) to generate an approximately 500 nucleotide fragment that is interrupted by an intron within the mouse genome. The sequences of all these primers are available on request from A.R. Mouse actin-specific primers were used to verify cDNA synthesis from RNA isolated from each tissue. Polymerase chain reaction was carried out for 28 cycles. For each cycle the template was
denatured at 94°C for 30 seconds, annealed at 55°C for 30 seconds and extended at 72°C for 30 seconds. PCR products were resolved on 2% agarose gels and visualized by ethidium bromide staining. RT-PCR results were verified on three independently collected pools of newborn ovaries.

**In situ hybridization**

Mouse cDNA fragments corresponding to *Nobox, Rfpl4, Gdf9, AW554400, Kit* and *Kitl* were subcloned into pGEM-T Easy vectors (Promega, Madison, WI) and used to generate anti-sense and sense strands by labeling with [α-35S] UTP using the Riboprobe T7/SP6 Combination System (Promega, Madison, WI) (S7). Hybridization was carried out at 55°C with 5 X 10^5 cpm of each riboprobe per slide overnight in 50% deionized formamide/0.3 M NaCl/20mM Tris-HCl (pH 8.0)/5 mM EDTA/10 mM NaPO4 (pH 8.0)/10% Dextran sulfate/1xDenhardt’s/0.5mg/mL yeast RNA. High-stringency washes were carried out in 2XSSC/50% formamide and 0.1XSSC at 65°C. Dehydrated sections were dipped in NTB-2 emulsion (Eastman Kodak, Rochester, NY) and exposed for 4 to 7 days at 4°C. After the slides were developed and fixed, they were stained with hematoxylin and mounted for photography. In situ hybridizations were performed on ovarian sections derived from two different animals.
Fig. S1. *Nobox* targeting strategy.  

A. We cloned a 22 kb region encompassing the mouse 129S6/SvEv *Nobox* gene. Based on Southern blot analysis, sequencing, and comparison to the cDNA sequence, we determined that *Nobox* is composed of 8 exons. The targeting vector was designed to delete a portion of exon 2 and exons 3-8 that encode the homeodomain and polyadenylation signal. *Pgk1*-HPRT and MC1-Tk cassettes were included in the targeting vector for positive and negative selection, respectively. K-KpnI, S-SmaI, X-XhoI, E-EcoRI, B-BamHI,
H-HindIII. B, C, D. Southern blot analysis of tail DNA extracted from wild type (wt), Nobox+/-(+/-) and Nobox-/-(-/-) mice. We used two probes external to the targeting vector to distinguish the Nobox wild-type (15 kb) and null alleles (7 kb for the 5' probe and 7 kb for the 3' probe). The DNA isolated from tails was digested with HindIII and hybridized with either the 5', 3' or internal probe.
Fig. S2. Morphologic and histological analysis of adult reproductive tracts. (A) Morphology of wild type (wt), Nobox^{+/−} and Nobox^{−/−} 6 week old uteri and ovaries are shown. (B) Histology of six week old Nobox^{+/−} ovary is shown. Antral follicles (AnF), and primary follicles (PrF) are present. (C) Histology of Nobox^{−/−} six week old ovary is shown. Notice the lack of discernible
follicular structures and oocytes. Scale bar=100µm.

Fig. S3. Histomorphometric analysis. (A,C,E) Oocyte counts comparing five pairs of Nobox+/− and Nobox−/− ovaries in newborn (Day 0), and postnatal days 7 and 14. Numbers represent total counts of every fifth section from serially sectioned ovaries. (B,D,F) Nobox+/− and Nobox−/− oocytes within germ cell cysts (GC), primordial, primary and secondary follicles were counted
and compared. Data are represented as mean values with error bars representing the SEM.

Fisher’s exact $t$ test was used to calculate $p$ values.

**Fig. S4. RNA expression of selective genes in Nobox$^{+/-}$ and Nobox$^{-/-}$ ovaries.** Equal amounts of Nobox$^{+/-}$ and Nobox$^{-/-}$ newborn cDNA were amplified with oligonucleotides specific for particular RNA sequences. The oligonucleotides spanned at least one intron to eliminate possibility of genomic DNA contamination, in addition to DNase treatment of RNA prior to reverse transcription.
Fig. S5. TUNEL analysis of *Nobox* heterozygous and null ovaries. *Nobox*+/− seven day old (A), and three day old (C), and *Nobox*−/− seven day old (B), and three day old (D) ovaries were subjected to TUNEL to study apoptosis. Very few somatic cells and oocytes labeled with TUNEL in *Nobox*+/− and *Nobox*−/− ovaries.
Fig. S6. Kit and Kitl in situ hybridizations. A, C. Dark field views of newborn Nobox<sup>-/-</sup> and Nobox<sup>+/+</sup> newborn ovaries hybridized to 35S radio-labeled anti-sense Kit RNA. The signal is located in oocytes within germ cell cysts (GC) and primordial follicles (PF) in Nobox<sup>-/-</sup> and Nobox<sup>+/+</sup> ovaries. B, D panels show bright field views. E, G. Dark field views of newborn Nobox<sup>-/-</sup> and Nobox<sup>+/+</sup> newborn ovaries hybridized to 35S radio-labeled anti-sense Kitl RNA. The signal is located in the stroma of the newborn ovaries in Nobox<sup>-/-</sup> and Nobox<sup>+/+</sup> ovaries. F, H. panels
show brightfield views.

Fig. S7. Brightfield views for in situ hybridization Figure 3. Panels A-D represent brightfield views of Nobox +/- ovaries, while panels E-H represent brightfield views of Nobox-/- ovaries. PF-primordial follicles, GC-germ cell cysts

Supporting References


