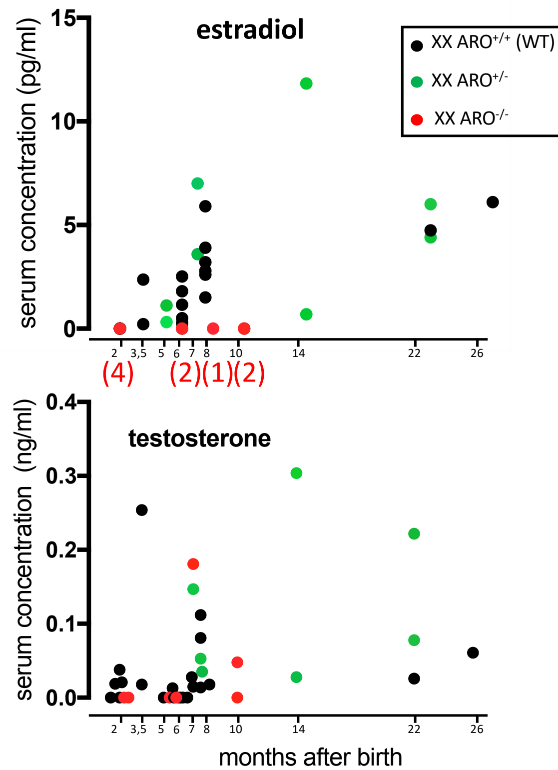
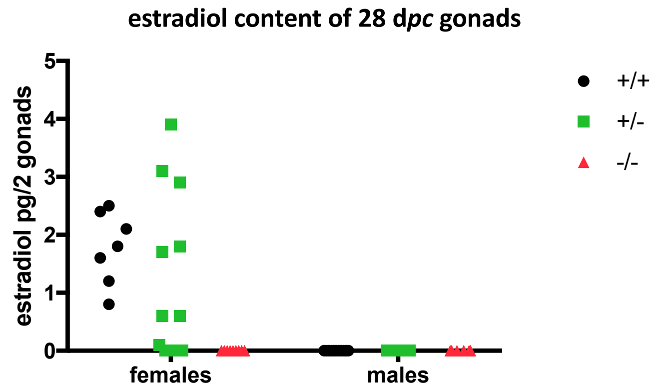
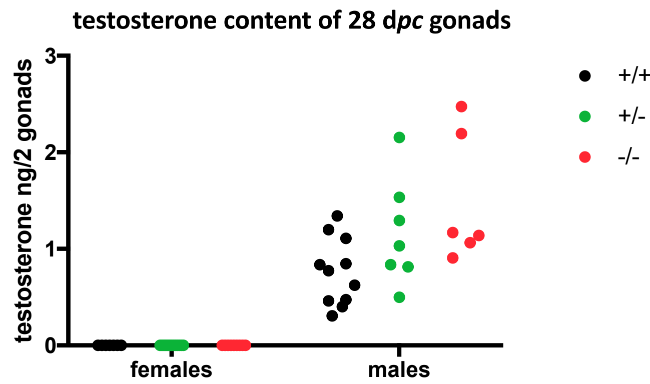
Supplementary information



Supplementary Figure 1: estradiol and testosterone serum concentrations in female rabbits collected from 2 to 26 months after birth.

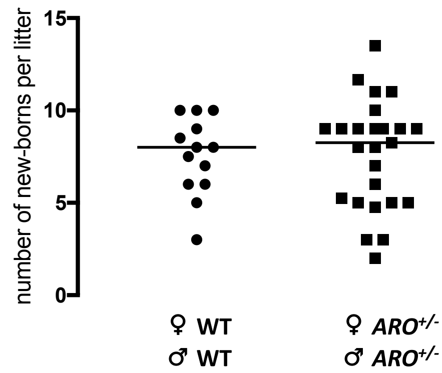
Supplementary Figure 1: **estradiol and testosterone serum concentrations in female rabbits collected from 2 to 26 months after birth.** Steroid concentrations were measured by GC/MS in serum of wild type XX rabbits (black points), $ARO^{+/-}$ rabbits (green points) and $ARO^{-/-}$ rabbits (red points). The number of analyzed sera samples from $ARO^{-/-}$ females is given in red between brackets. The same sera samples were used for assay of testosterone and estradiol concentrations.





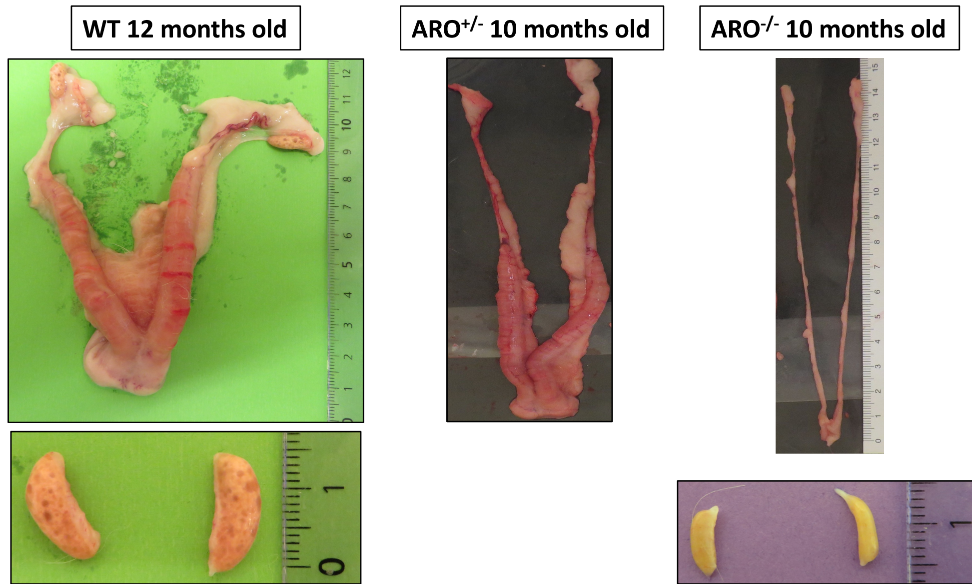
Supplementary Figure 2: estradiol and testosterone content of 28 d*pc* gonads.

Supplementary Figure 2: estradiol and testosterone content of 28 d*pc* gonads. Gonads of fetuses were collected 28 days after mating. Pairs of gonads were immediately frozen at -80°C. Steroid amounts were assayed by GC/MS. Each value is the amount of steroid in the pool of both gonads of one animal.



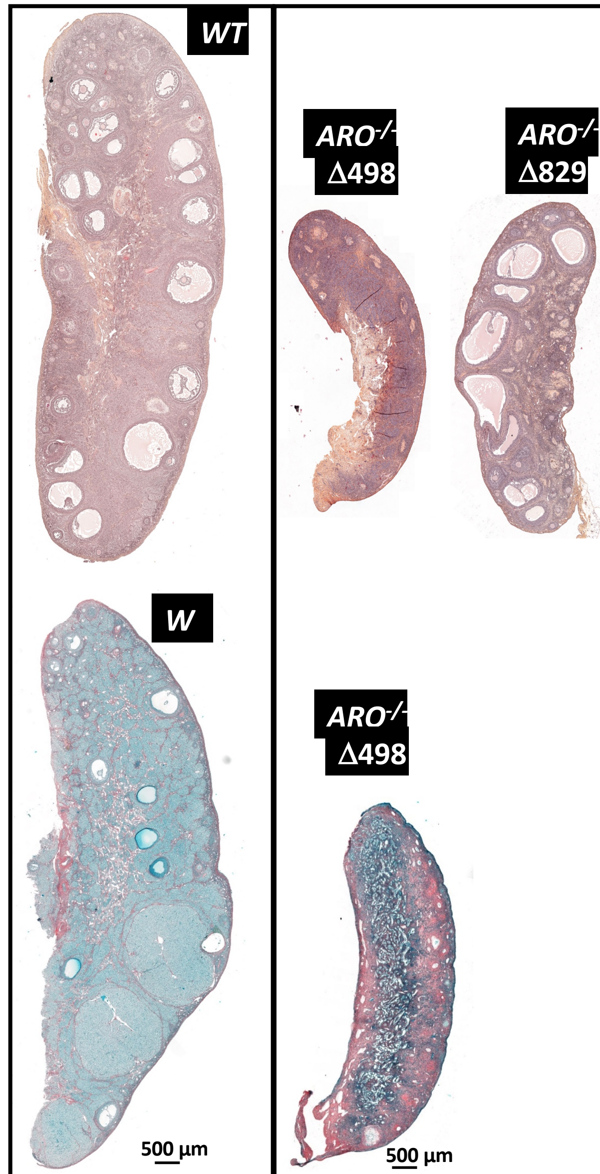
Supplementary Figure 3: number of neonates per litter after WT or *ARO*$^{+/-}$ mating

Supplementary Figure 3: number of neonates per litter in WT or *ARO*$^{+/-}$ mating. Mating were performed between *ARO*$^{+/-}$ males and *ARO*$^{+/-}$ females of the same mutant line, or between wild type males and females from the New-Zealand strain from the facility. Each point represents the mean size of litters from each female, each female having being mated by different males. When one female was mated several times by the same male, the graph reports the mean of the different litter sizes. The horizontal bars represent the medians.



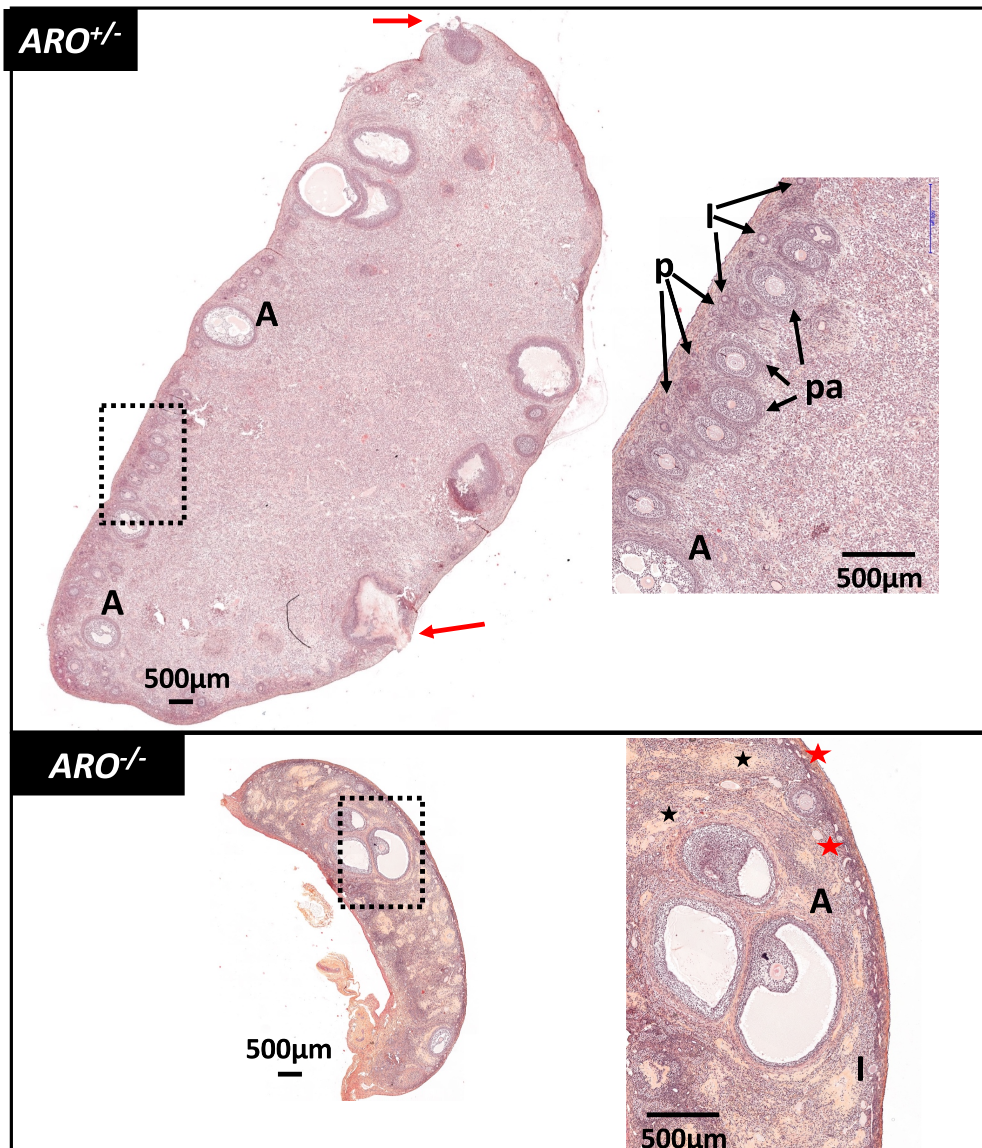
Supplementary Figure 4: genital tracts and ovaries of adult females from wild type (WT), heterozygous *ARO*$^{+/-}$ and homozygous *ARO*$^{-/-}$ animals (D339 strain).

Supplementary Figure 4: genital tracts of adult females from wild type (WT), heterozygous *ARO*$^{+/-}$ and homozygous *ARO*$^{-/-}$ animals (Δ339 strain). The pictures below show the ovaries from the same females after dissection.



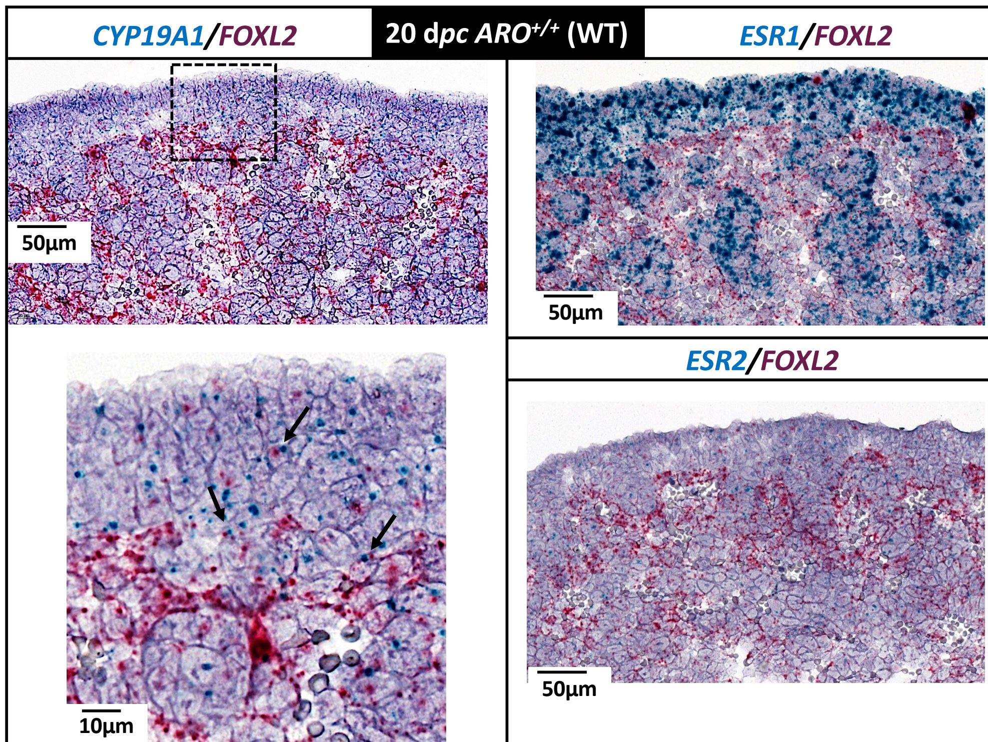
Supplementary Figure 5: ovaries of wild type (WT) and *ARO*$^{-/-}$ rabbits from lines D498 and D829. Representative longitudinal sections of ovaries of 5-6 months old XX rabbits are shown. The diagram reports the AMH serum levels.

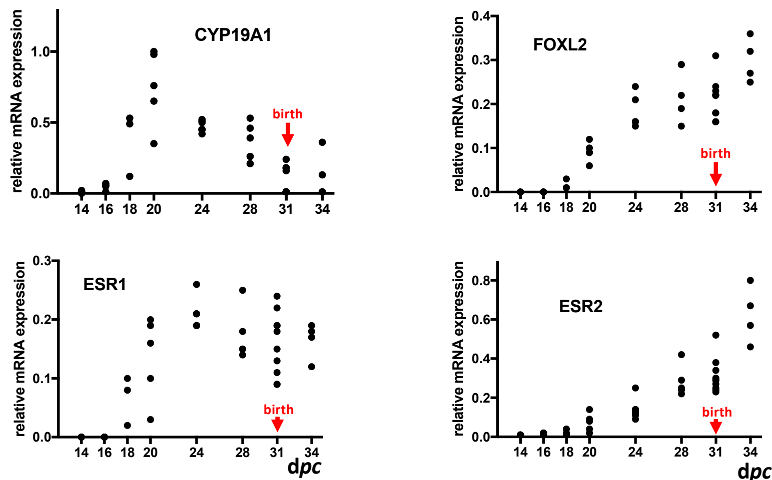
Supplementary Figure 5: structure of ovaries of wild type (WT) and *ARO*$^{-/-}$ rabbits from lines Δ498 and Δ829. The figure shows representative longitudinal sections of ovaries of 5-6 months old XX rabbits. Homozygous *ARO*$^{-/-}$ XX mutants are issued from Δ498 and Δ829 lines. Organs were fixed with PAF then HES or Sirius red/fast green colored. With the Sirius-red/fast-green coloration (lower panel), collagen fibers were red colored. Cytoplasm was light-green colored and nuclei were dark-green. An intense red/collagen coloration was detected throughout the *ARO*$^{-/-}$ ovary, with a strong staining in the cortical part. By contrast in the wild type ovary, collagen fibers were detected mostly in the medulla part. The Δ829 *ARO*$^{-/-}$ ovary was filled with numerous large antrum follicles. However, the follicular reserve was almost null as in other *ARO*$^{-/-}$ strains.



Supplementary Figure 6. Ovaries of 10 months old rabbits after hormonal treatment to induce superovulation.

Supplementary Figure 6. Ovaries of 10 months old rabbits after hormonal treatment to induce superovulation. Rabbits were hormonally treated for induction of ovulation, mated and sacrificed the following morning. Ovaries were collected and fixed (PAF) for histological HES colored observation. Follicular rupture (red arrow) occurred in *ARO*$^{+/-}$ ovaries, but not in *ARO*$^{-/-}$ ones. Antral (A), pre-antral (pa), primary (I) and primordial (p) follicles are pointed in enlargements. Black stars: accumulation of fibrous tissue.



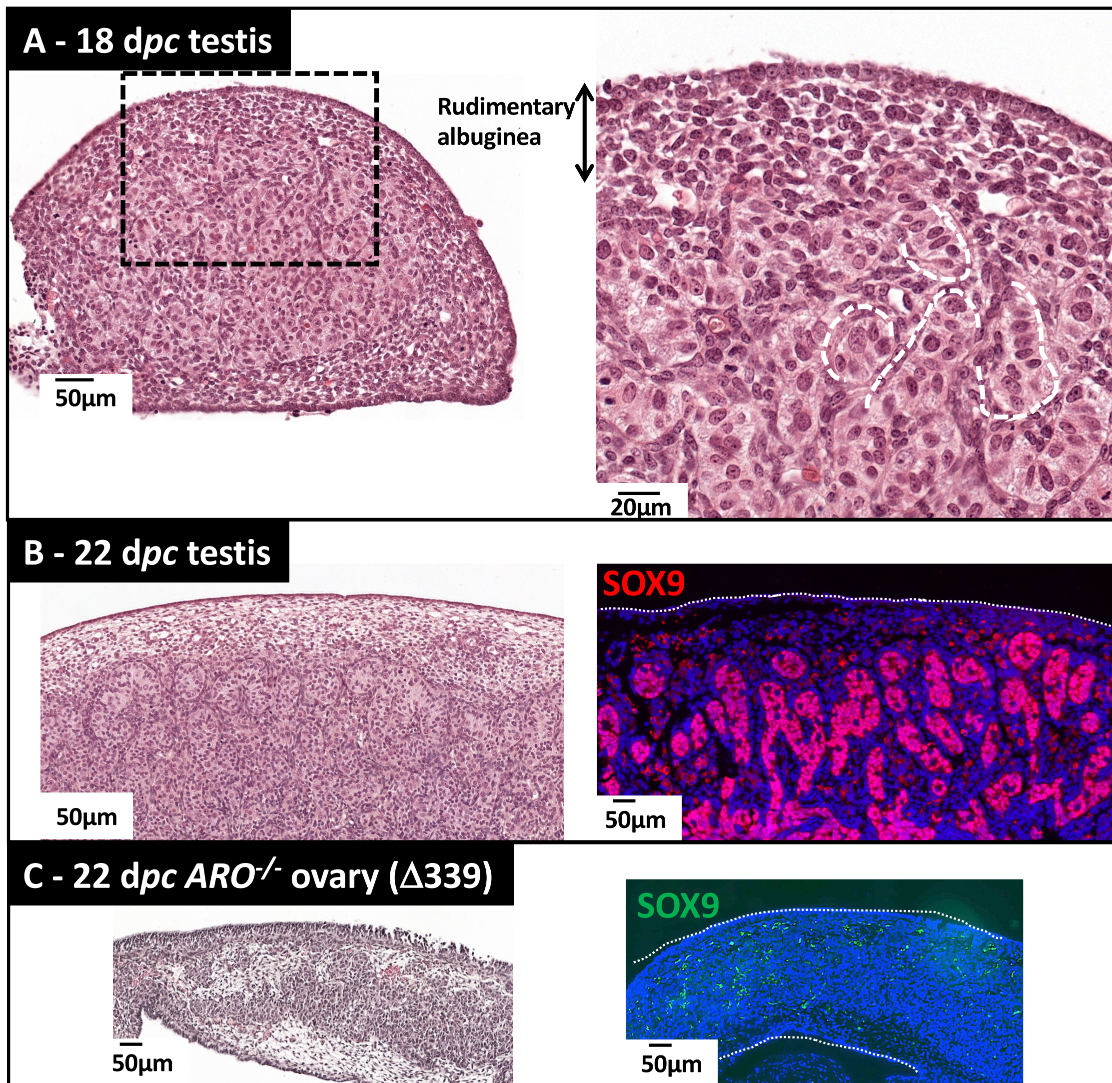


Supplementary Figure 7: *CYP19A1, FOXL2, estrogen receptor* genes in wild type 20 d*pc* fetal ovaries.

Supplementary Figure 7: *CYP19A1, FOXL2, estrogen receptor* genes in wild type 20 d*pc* fetal ovaries.

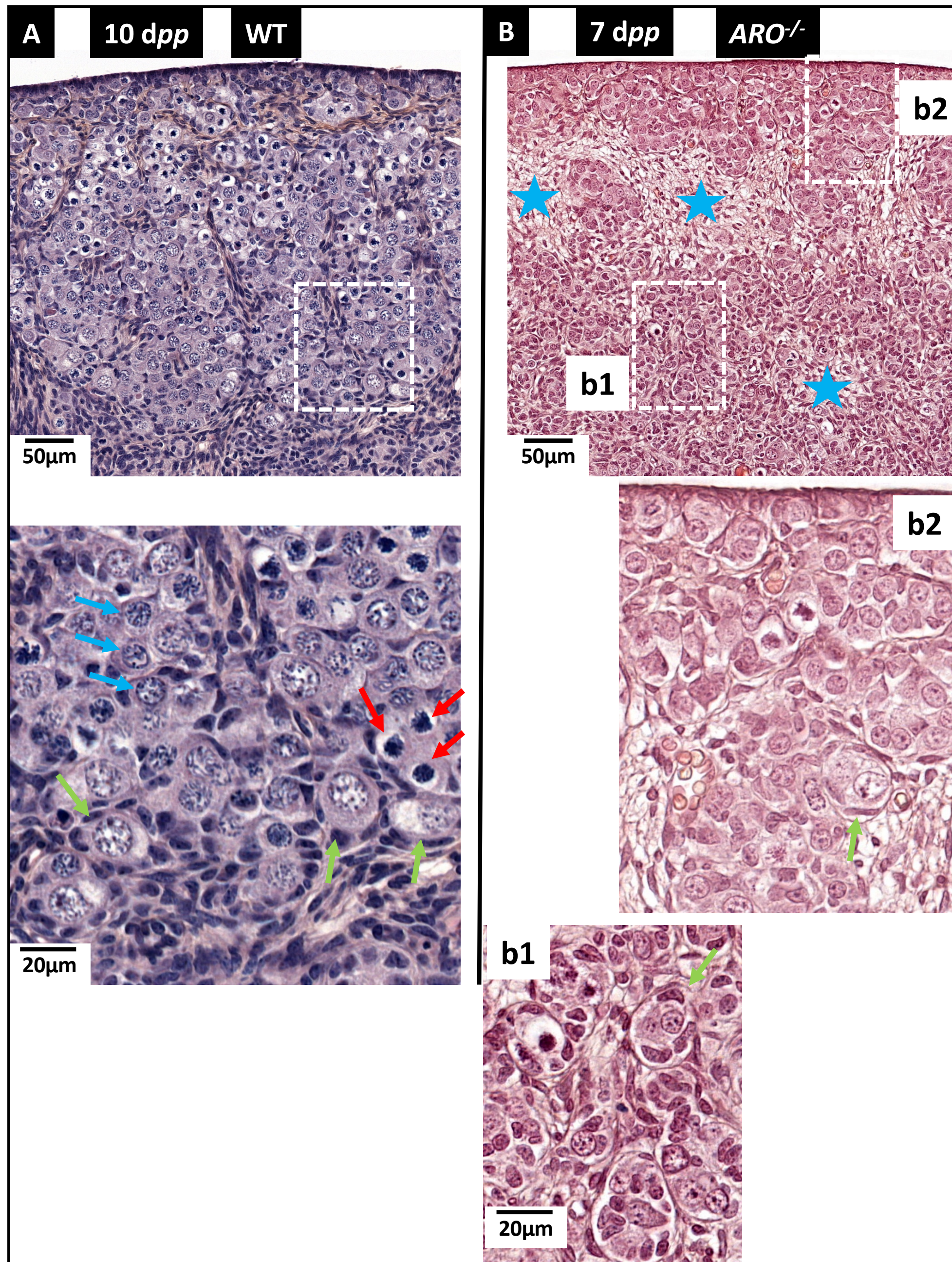
The cellular localization of gene expression was determined using dual ISH with blue (*CYP19A1, ESR1, ESR2*) or red (*FOXL2*) labelling. Each picture represents the epithelium surface of the ovary and the tissue underneath. The black arrows point some cells with both blue and red signals in the enlarged picture.

The diagrams represent the relative mRNA expression levels. Each point represents one RNA sample extracted from both gonads of one animal at the indicated developmental stage. The same RNA samples were analyzed in the 4 diagrams. The vertical red arrow points the birth that occurs 31 days after conception in the rabbit species. Data concerning *CYP19A1* and *FOXL2* mRNA expression levels are those already published in Daniel-Carlier et al, 2013.



Supplementary Figure 8: structure of 18 and 22 d*pc* old fetus testes. SOX9 positive cells were detected by immunodetection.

Supplementary Figure 8: structure of 18 and 22 d*pc* old fetus testes. Gonads of rabbit fetuses from normal XY genotype were collected at 18 (**A**) and 22 d*pc* (**B, C**) then fixed in Bouin's. The picture in (**A**) shows a HES colored slice with an enlargement. The 18 d*pc* gonad developed as a testis, with differentiating seminiferous tubes (white dotted lines) wrapped in a rudimentary albuginea. The lower pictures (**B**, **C**) show HE staining (left panel) and fluorescence after labelling using an antibody directed against the SOX9 transcription factor (right panel). A wild type testis (**B**) and an $ARO^{-/-}$ ovary (**C**) were collected at 22 d*pc* then fixed in 4% PAF before immunohistological analysis. The SOX9 antibody (red fluorescence) labelled the nucleus of somatic Sertoli cells of the testis (**B**). No specific SOX9 labelling was detected in the $ARO^{-/-}$ ovary; the green fluorescent points correspond to non-specific labelling of red blood cells (**C**).



Supplementary Figure 9: a few numbers of oocytes undergo the diplotene stage in *ARO$^{-/-}$* ovaries seven days after birth. Ovaries were collected 10 days (WT) or 7 days (*ARO$^{-/-}$*) after birth.

Supplementary Figure 9: a few numbers of oocytes undergo the diplotene stage in *ARO$^{-/-}$* ovaries seven days after birth. Ovaries were collected 10 days (WT) or 7 days (*ARO$^{-/-}$*) after birth, treated by Bouin's fixative then paraffin embedded. Sections were HES colored. Blue stars point the connective tissue in *ARO$^{-/-}$* ovaries. In wild type ovaries, red, blue and green arrows point the zygotene, pachytene and diplotene stages respectively. Somatic flat cells were found around some oocytes in both wild type (diplotene stage) and *ARO$^{-/-}$* (green arrows, meiotic stage difficult to determine) ovaries.

Genomic location in the rabbit genome	overlapping gene	length of homology	% of homology	score
sequence of the targeted region TGCTTCATCTGAAGCCAAAGAGCACACGATGGTTTTGGAAATACTGAACCCA (52nt)				
17:21282661-21282712	CYP19A1	52	100	6,00E-21
1:41363894-41363914		21	100	0.019
10:11913651-11913671		21	100	0.019
19:31257617-31257637		21	100	0.019
11:46433289-46433311		23	95,65	0.29
14:61084103-61084121		19	100	0.29
15:106972249-106972274		26	92,31	1.2
16:27461959-27461980	RYR2	22	95,45	1.2
2:70265022-70265039	TUSC3	18	100	1.2
1:149838996-149839012	TUB	17	100	4.6
1:159223429-159223445		17	100	4.6
1:171088368-171088384	ELP4	17	100	4.6
1:75427837-75427853		17	100	4.6
11:67725913-67725929		17	100	4.6
12:55382223-55382239		17	100	4.6
14:161219422-161219438		17	100	4.6
14:78017712-78017728		17	100	4.6
15:47467930-47467946	SLC39A8	17	100	4.6
18:60262016-60262032	VTI1A	17	100	4.6
2:105241067-105241083		17	100	4.6
2:172155957-172155973		17	100	4.6
20:28226256-28226272		17	100	4.6
20:31338072-31338088	DNAL1	17	100	4.6
7:110853285-110853301		17	100	4.6
7:117990691-117990707	OSBPL6	17	100	4.6
7:168493075-168493091		17	100	4.6
7:87316358-87316374		17	100	4.6
7:91302331-91302347		17	100	4.6
8:34242276-34242292	M6PR	17	100	4.6
GL018700:7687914-7687930		17	100	4.6
GL018776:319476-319492		17	100	4.6
GL018909:412658-412674		17	100	4.6
X:34152874-34152890	CLCN5	17	100	4.6
X:52742450-52742466		17	100	4.6

Supplementary Table 1: Off-target sequences found by BLAST analysis as provided by the Ensembl genome browser. The searched 52nt long sequence is given at the top of the table. The table reports the length of the homology sequence with the percentage and the final score. The best score (smallest number) was got, as expected, with the *CYP19A1* rabbit gene.

gene name	sense primers	antisense primers	position of the amplified fragment in the rabbit genome or transcript
CYP19A1 (detection of mutants)	F0 5'-TGAATTCAACAGACAGCCTAATGG-3'	R0 5'-CCATTAGTGGTAGAATGGGAGG-3'	chromosome 17; -622, +864 relative to the translation initiation codon ATG of the *CYP19A1* gene (ENSOCUT00000012304)
CYP19A1 (routine genotyping)	F1 5'-TCTTCAATTTTTCTGCCCCTTTA-3'	R1 5'-TGCATTGGGTTCAGTATTTCCA-3'	chromosome 17; -71, +28 relative to the translation initiation codon ATG of the *CYP19A1* gene (ENSOCUT00000012304)
rabbit β *GLOBIN*	5'-GCCCCTCTGCTAACCATGTTC-3'	5'-TTGCCAAAATGATGAGACAGCAC-3'	chromosome 1; +16,109; +16,200 relative to the translation initiation codon ATG of the β *GLOBIN* gene (ENSOCUG00000000568)
SRY	5'-GTTCGGAGCACTGTACAGCG-3'	5'-GCGTTCATGGGTCGCTTGAC-3'	Y chromosome; +14, +158 relative to the translation initiation codon ATG of the *SRY* gene (Gene ID =100328958)
ESR1 = oestrogen receptor alpha	5'-GCACCCAGGGAAGCTTCTAT-3'	5'-AGCCAGCAACATGTCAAAGATTT-3'	chromosome 12; +1199;+1299 relative to the translation initiation codon ATG of the ESR1 cDNA (ENSOCUT00000004827.4)
ESR2 = oestrogen receptor beta	5'-CTCACCAAGCTGGCTGACAA-3'	5'-AGAGGCGCACTTGGTCCAA-3'	chomosome 20; +882;+982 relative to the translation initiation codon ATG of the ESR2 cDNA (ENSOCUT00000005082.4)

Supplementary Table 2 : Sequences of the primers used for genotyping

The position of the amplicon is given relative to the translation initiation codon ATG of each gene. All primers match exactly with the sequence of the gene (wild type) and amplify a 100-140bp-long DNA fragment. Other primers are those used in the previously published paper (Daniel-Carlier *et al.* 2013).

gene name	RNAScope probe catalogue number	transcript accession number (total length of cDNA in bp from ATG to STOP codon)	Position of probe versus ATG and STOP position
CYP19A1 (AROMATASE)	440821	NM_001170921.2 (1512bp)	ATG 1 CYP19A1 cDNA STOP 1512 CYP19A1 probe
DDX4	402341	XM_002714 040.3 (2190bp)	ATG 1 DDX4 cDNA STOP 2190 DDX4 probe
ESR1	865461	XM_002714947.3 (1797bp)	ATG 1 ESR1 cDNA STOP 1797 ESR1 probe
ESR2	803171	ENSOCUT00000 005082.3 (1572bp)	ATG 1 ESR2 cDNA STOP 1572 ESR2 probe
FOXL2 FOXL2-C2	488221 488221-C2	3'UTR of a putative rabbit FOXL2 gene (985bp)	FOXL2 3'UTR FOXL2 probe
OCT4 OCT4-C2	513271 513271-C2	NM_001099 957.1 (1083bp)	ATG 1 OCT4 cDNA STOP 1083 OCT4 probe
RSPO2	803161	ENSOCUT0000 0000779 (732bp)	ATG 1 RSPO2 cDNA STOP 732 RSPO2 probe
SPO11	563501	ENSOCUG0000 0000378 (1188bp)	ATG 1 SPO11 cDNA STOP 1188 SPO11 probe
STRA8	549411	XM_008258 146.2 (1101bp)	ATG 1 STRA8 cDNA STOP 1101 STRA8 probe

Supplementary Table 3. In Situ Hybridization probes. For each gene, the accession number of the transcript, the targeted region within the transcript and the catalogue number of the probe are given. The “C2” name refers to the probes conjugated to alkaline phosphatase; other probes are conjugated to horseradish peroxidase.

protein	source	type	reference	working concentration
AMH	mouse	monoclonal (B-11 clone)	Santa Cruz sc-166752	1:50
AROMATASE	mouse	monoclonal (H4 clone) (C-term)	ABD Serotec MCA2077S	1:200
γH2AX	mouse	monoclonal (D7T2V clone)	Cell Signaling	1:200
KI67	rabbit	monoclonal (SP6 clone)	Thermo Fisher	1:200
OCT3/4	goat	polyclonal	Santa Cruz Sc-8628	1:100
SOX9	rabbit	polyclonal	gift from Francis Poulat (IGH, Montpellier)	1:200

supplementary table 4 : antibodies and dilutions

Supplementary material and methods:

Talen assembly

The TALEN kit used for TALE assembly was a gift from the Keith Joung laboratory (Addgene kit # 1000000017). The left TALEN was constructed by assembling units of the kit in the following order: 9, 12, 20, 25, 27, 11, 20, 22, 30, 14, 16, 21, 29, 12, 17 and 21 (by groups of four units). The right TALEN was constructed by assembling units of the kit in the following order: 9, 14, 19, 25, 30, 12, 16, 24, 30, 11, 20, 25, 30, 12, 17 and 21. Each right and left insert was subcloned into the *Bsmb1* restriction site in JDS70 plasmid. Sequencing of the cloned DNA fragments was performed to check for the identity with what expected.

TALEN mRNAs were prepared from the TALEN-JDS70 plasmids using the ARCA T7 capRNA pol kit (Cellscript, TEBUbio France), polyadenylated with the polyA polymerase tailing kit (Epicentre Biotechnologies) then purified using the Qiagen RNEasy minikit (Qiagen France).

Measurement of estradiol level in serum sample or gonads by Gas Chromatography / Mass Spectrometry (GC/MS)

Sample extraction and purification: Briefly, samples (1 ml of serums, or the 2 gonads of one foetus, calibration standards, quality controls, and blank matrix) were collected in 8 ml borosilicate tubes. A spiking solution of deuterated steroid internal standard (IS) (50 µl containing 10 pg of E2-d4, except for blank matrix) (CDN Isotopes, Inc., Point-Claire, Canada), and 3 ml of 1-chlorobutane were added to each sample. Gonads were grinded using a small glass piston. After mixing (Vortex) and rapid centrifugation, the upper organic phase was collected and layered on conditioned Hypersep SI 500mg SPE minicolumn (Thermo Scientific, Rockwood, USA). The column and adsorbed material were then washed with ethyl acetate / hexane (6 ml; 1/9, v/v). The second fraction containing oestradiol was eluted using ethyl acetate / hexane (4 ml; 1/1, v/v), then evaporated at 60°C to dryness.

Derivatization reaction and determination of rabbit plasma E2 level: Estradiol was derivatized with pentafluorobenzoyl chloride (PFBC) (103772-1G, Sigma-Aldrich, Steinheim, Germany). Final extracts were reconstituted in isooctane, then transferred into conical vials for injection into the GC system (GC-2010 Plus, Shimadzu, Japan) using a 50% phenylmethylpolysiloxane VF-17MS capillary column (20m x 0.15mm, internal diameter, 0.15µm film thickness) (Agilent Technologies, Les Ulis, France). A TQ8050 (Shimadzu, Japan)

triple quadrupole mass spectrometer equipped with a chemical ionization source and operating in Q3 single ion monitoring mode was used for detection. The reagent gas for the NCI detection was methane. The GC was performed in pulsed splitless mode with a 1 min pulsed splitless-time. The oven temperature was initially 150°C for 0.50 min, further increased to 305°C at 20°C/min and held at 305°C for 3.60 min, and then to 335°C at 30°C/min and held at 335°C for 1.7 min. The injection port and transfer line temperatures were respectively 290 and 280°C. The flow-rate of helium (carrier gas) was maintained constant at 0.96 ml/min. The mass spectrometer CI source temperature was 220°C. The linearity of steroid measurement was confirmed by plotting the ratio of the steroid peak response / internal standard (IS) peak response to the concentration of E2 for each calibration standard. Accuracy, target ions, corresponding deuterated internal control, range of detection, low limit of quantification (LLOQ), and intra & inter assay CVs of the quality control are given in the supplementary Table 5.

Accuracy (%)	**Analyte**	**Target ion analyte / IS** (amu)	**Range** (pg/ml) **R^2**	**Mean (17 runs)** ***Intra- & Inter assay CVs (%)***			
				LLOQ Mean (pg/ml) Intra- & Inter assay CVs (%)	**Low QC Mean (pg/ml) Intra- & Inter assay CVs (%)**	**Middle QC Mean (pg/ml) Intra- & Inter assay CVs (%)**	**High QC Mean (pg/ml) Intra- & Inter assay CVs (%)**
94 - 107	E2	660 / 664	0.2 - 56.0	0.22	2.87	6.07	12.88
			0.9994	*17.3 - 19.6*	*2.9 - 4.2*	*2.6 - 3.7*	*2.3 - 3.3*

Supplementary Table 5: GC/MS plasma analytical control validation.
LLOQ : low limit of quantification
QC : quality control