SUPPORTING ONLINE MATERIAL

Materials and Methods

EST database analysis.

An expressed sequence tag database (S1) was constructed by 5' sequencing of 19,000 clones from a randomly primed, non-normalized cDNA library made from fully grown, zona-free, prophase-arrested mouse oocytes (Eppig/Hampl library; I.M.A.G.E. Consortium library I.D. #1182). The oocytes were obtained from 22-day old C57BL/6J X SJL/J F_1 mice (Jackson Laboratory, Bar Harbor, ME) that had been primed 46 hours previously by injection of 5 I.U. equine chorionic gonadotropin (eCG). The database was assembled and annotated using methods similar to those described by Evsikov et al. (S2), and was searched for predicted 7-transmembrane proteins; the 15 receptors that were identified by this search are listed in Table S1.

In situ hybridization.

An ovary from a 22-day old, eCG-primed C57BL/6J X SJL/J F_1 mouse was fixed with 4% paraformaldehyde overnight, dehydrated, and embedded in paraffin. 4 μm sections were processed for in situ hybridization using a ^{33}P-labelled antisense riboprobe, as previously described (S3). The riboprobe (537 bp) was made by subcloning the KpnI-XbaI fragment of mouse *Gpr3* (nucleotides 498-1679 of D21062) into pBluescript II SK+, linearizing with BglII, and transcribing with T3 RNA polymerase.

The sections were imaged using a Zeiss Pascal confocal microscope, with a 20X, 0.75 N.A. objective. The scanning transmission mode was used to record follicle morphology, and the reflected light mode was used to record the silver grain distribution in the autoradiographic emulsion overlying the sections. A sense control showed only background levels of silver grains over all cell types.

RT-PCR to determine the relative amounts of *Gpr3* RNA in oocytes and somatic cells.

Antral follicles were dissected from 23 day old C57BL/6J X SJL/J F_1 mice that had been injected 40-46 hours previously with 5 I.U. eCG. Cumulus-oocyte complexes were then isolated from the follicles, and the cumulus cells were removed from the oocytes. The cumulus cells were combined with the mural granulosa and theca cells comprising the follicle wall; this pool is referred to as somatic cells. The separation of oocytes and somatic cells was carried out in the presence of 250 μM dbcAMP, in order to prevent the spontaneous resumption of meiosis. Oocytes that were obtained from the follicle dissections were combined with additional oocytes that were released from ovaries by puncturing follicles with a 30 gauge needle.

RNA was extracted from 150-1000 oocytes, or from the somatic cells from 34-50 follicles, using TRIzol reagent (Invitrogen, Carlsbad, CA) and chloroform. It was then precipitated with isopropyl alcohol overnight, and treated with DNAase to remove genomic DNA (Turbo DNA-free kit, Ambion). After the DNAase treatment, the concentration of RNA was determined by measuring absorbance at 260 nm; the yield was 0.5-1.5 ng per oocyte and 11-16 ng from the somatic cells of one follicle. cDNA was reverse transcribed from 180-370 ng of RNA, using oligo dT as a primer. RT-PCR was

performed either as shown in Fig. 1B (4 experiments), or by real time analysis (2 experiments). Similar results were obtained with both methods.

For experiments like those shown in Fig. 1B, the *Gpr3* forward and reverse primers were as listed in Table S2, and cDNA derived from 0.2 -100 ng of total RNA from oocytes or somatic cells was used. The thermocycler parameters were as follows: denaturation (96°C, 10 sec), annealing (60°C, 30 sec), extension (68°C, 90 sec), 35 cycles. Gels were stained with SybrGold (Molecular Probes, Eugene, OR). A single 506 bp product was amplified; the DNA extracted from this band was cloned and sequenced and shown to be *Gpr3*. No bands were seen in mock reactions in which reverse transcriptase had been omitted. Photographs of the SybrGold stained gels were scanned, and band intensities were measured using NIH Image. For each set of RT-PCR samples, the expression level of *Gpr3* RNA in somatic cells, relative to that in oocytes, was determined by comparison with a semilog plot of *Gpr3* band intensity as a function of the amount of total oocyte RNA used per reaction.

Real time RT-PCR analysis was carried out using the ABI PRISM 7900 sequence detection system (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The sequences of the PCR primers as well as the Taqman probe were selected using the Primer Express software. The Taqman probe for *Gpr3* (5'-VIC-TGTTCCAGATGGTCAGGGTCCCACTC-TAMRA-3') was labeled with VIC as the reporter and TAMRA (6-carboxy-4, 7, 2, 7'-tetramethylrhodamine) as a quencher. The forward and reverse PCR primers for *Gpr3* were 5' -CTGACCGCGTGGCTCTAGA-3' and 5'- CACTTGGGCTGTGAGACATTTC-3', respectively. PCR was performed in 96-well microtiter plates using 12.5 μl of TaqMan Universal PCR master mixture (Applied Biosystems), each of the primers at a final concentration of 900 nM, 200 nM TaqMan probe, and a cDNA sample derived from 5 ng total RNA in a total volume of 25 μl. After a 2-min incubation at 50°C followed by 10-min incubation at 95°C, the samples were subjected to 40 cycles of amplification (95°C for 15 s, 60°C for 1 min). The fluorescent signal from each well was normalized by using an internal passive reference. The cycle threshold (C_T) values obtained from each sample were compared using the comparative C_T method as described in the user manual (User Bulletin #2 for ABI Prism 7700; Applied Biosystems).

<u>*Gpr3* knockout mice</u>.

Gpr3 knockout mice were generated by Deltagen, Inc. (San Carlos, CA). *Gpr3* is a single exon gene (http://www.ncbi.nlm.nih.gov/sutils/evv.cgi?org=mouse&contig=NT_039267.2&gene=Gpr3&lid=14748&from=640800&to=643291). A 983 bp fragment (513-1495 of D21062), from the 993 bp protein-coding region of *Gpr3*, was replaced by a 6.9 kb IRES-lacZ reporter and neomycin resistance casette. The prepubertal mice used in our studies had been backcrossed 8 times with C57BL/6J mice; the adult mice had been backcrossed 5 times.

$Gpr3^{-/-}$ mice were obtained by breeding +/- males and females, or by breeding -/- males and +/- females. $Gpr3^{+/+}$ mice were obtained by breeding +/- males and +/- females. Mice were genotyped from tail or ear DNA, using a multiplex PCR designed to simultaneously detect the wildtype and mutant alleles. The 3 primers used are listed in Table S2. The *Gpr3* forward and reverse primers were used to amplify a 506 bp segment

of the wildtype allele, and the Neo forward and *Gpr3* reverse primers were used to amplify a 358 bp region of the mutant allele. Thermocycler parameters were as described above.

Histological analysis of ovaries.

Ovaries were collected from 22-24 day old $Gpr3^{-/-}$ and $Gpr3^{+/+}$ prepubertal mice (4 animals each) that had been injected 41-44 hours earlier with 5 I.U. equine chorionic gonadotropin (eCG) (National Hormone and Peptide Program, Torrance, CA). eCG activates the same receptor as follicle stimulating hormone, and was used to stimulate the formation of antral follicles. (Eight ovaries from $Gpr3^{-/-}$ mice that were not injected with eCG also showed spontaneous resumption of meiosis, but these were not analyzed in detail.) Two additional ovaries were collected from adult mice ($Gpr3^{-/-}$, 4 months old; $Gpr3^{+/+}$, 3 months old). The adult mice were selected without reference to their stage during the estrous cycle, and were not injected with eCG. For demonstration of cumulus expansion, ovaries were collected from a 22 day old C57BL/6J mouse that had been injected with eCG, followed 48 hours later by 5 I.U. human chorionic gonadotropin (hCG) (Sigma, St. Louis, MO), 9 hours prior to fixation. The ovaries were fixed overnight at 4ºC in Bouin's solution (Sigma), dehydrated in EtOH and toluene, and embedded in paraffin. Meiotic resumption was scored by examining serial 5 μm sections, stained with periodic acid/Schiff reagent and Lillie-Mayer hematoxylin. Photographs were taken using a Kodak DC4800 or a Nikon CoolPix 3100 camera.

"Antral" refers to follicles in which a single antral space separates the mural granulosa cells from the cumulus cells surrounding the oocyte. "Early antral" refers to follicles with 4 or more layers of granulosa cells, in which the antrum is in the process of forming, and consists of multiple small pockets of space between the granulosa cells; a distinct cumulus mass has not yet formed. "Preantral" refers to follicles with 1-3 layers of granulosa cells surrounding a fully grown oocyte. Occasional follicles with 3 layers of granulosa cells showed the beginnings of antral formation, but were classified as preantral if there were ≤3 granulosa cell layers.

Oocytes were considered to be in prophase if an intact nucleus (germinal vesicle) and nucleolus were observed. They were considered to have resumed meiosis if the germinal vesicle and nucleolus were no longer present, and condensed chromosomes were visible. Figs 3D and S2 show counts of all healthy antral follicles in which the oocyte was intact and in normal contact with the granulosa cells. ~50 randomly selected preantral follicles from each ovary were also counted.

For the prepubertal ovaries, ~50 randomly selected early antral follicles were counted per ovary; 89% of these follicles had diameters ranging from 130 to 250 μm, and 11% had diameters ranging from 260 to 350 μm. In both size ranges, almost all of these early antral follicles had a normal morphology.

However, in the two adult ovaries that we studied (one $Gpr3^{+/+}$ and one $Gpr3^{-/-}$), some of the early antral follicles in the smaller size class appeared to be undergoing degenerative changes. While almost all of the early antral follicles that were ≥250 μm in diameter had a well defined theca layer surrounding the granulosa cells, some of the follicle-like structures < 250 μm in diameter consisted of abnormal masses of somatic cells, >3 cell layers thick, which were not surrounded by a defined layer of theca cells. These may have been atretic follicles that were in the process of being transformed into

secondary interstitial tissue (S4). In the adult "large early antral" category (≥250 μm), all of the *Gpr3*$^{+/+}$ oocytes were prophase arrested, but in adult follicles that were <250 μm in diameter, some of the *Gpr3*$^{+/+}$ oocytes showed metaphase chromosomes. For this reason, we restricted our analysis of early antral follicles in the adult ovary to those ≥250 μm in diameter (Fig. S2), and used prepubertal ovaries for all other studies.

Determination of meiotic competence of oocytes dissected from *Gpr3*$^{-/-}$ antral and early antral follicles.

Meiotic competence of oocytes within follicles of various diameters was determined as described by Erickson and Sorenson (S5). Ovaries were collected from 22-24 day old *Gpr3*$^{-/-}$ mice that had been injected 40-43 hours earlier with 5 I.U. eCG. Follicles with diameters ≥140 μm were dissected in a dish containing HEPES-buffered MEM (S6), using 30 gauge hypodermic needles. The follicles were grouped into 2 size classes, 140-250 μm, and >250 μm. The 140-250 μm size group was classified as "early antral", because it corresponded to the range of diameters of most of the early antral follicles identified in histological sections of ovaries from 22-24 day old mice. The >250 μm size group was classified as "antral".

Oocytes were then removed from each group of follicles by nicking the follicle wall with a 30 gauge needle, squeezing out the cumulus-oocyte complex, and pipetting the cumulus-oocyte complex repeatedly through a small bore micropipet to remove cumulus cells from the oocyte. The isolated oocytes were scored for the presence of a prophase nucleus and nucleolus, then cultured for 5 hours in a 200 μl drop of HEPES-buffered MEM at 37ºC, then rescored to determine whether meiosis had resumed in the oocytes that were prophase-arrested at the time of removal from the follicle.

RT-PCR to determine the relative amounts of *Csp2* RNA in ovaries from hCG stimulated vs unstimulated mice, and from *Gpr3*$^{-/-}$ vs *Gpr3*$^{+/+}$ mice.

Ovaries were collected from 22-23 day old *Gpr3*$^{+/+}$ and *Gpr3*$^{-/-}$ mice that had been injected 41-43 hours earlier with 5 I.U. eCG. One *Gpr3*$^{+/+}$ mouse was also injected with 5 I.U. hCG, 4 hours prior to ovary collection. Ovary RNA and cDNA were prepared as described above. RT-PCR for *Csp2* was performed using primers as previously described (S7). The thermocycler parameters were as follows: denaturation (94ºC, 30 sec), annealing (55ºC, 30 sec), extension (72ºC, 30 sec), 30 cycles. A single 628 bp product was amplified.

In vitro transcription of RNA encoding GPR3 and mRFP1.

To make *Gpr3* RNA for microinjection into oocytes, the KpnI-XbaI fragment of D21062 (498-1679), which includes the coding region (499-1491) and some 3'UTR of the *Gpr3* cDNA (S8), was cloned into the pHGCX vector (S9). The plasmid was linearized with PacI, and RNA was transcribed using T7 polymerase (mMessage mMachine T7, Ambion Inc., Austin TX).

RNA encoding monomeric red fluorescent protein 1 (mRFP1; S10) was used as a control. PRSET-B-mRFP1 was kindly provided by Roger Tsien. The plasmid was linearized with ScaI, and RNA was transcribed using T7 polymerase.

Injection and culture of follicle-enclosed oocytes.

Preantral (or very early antral) follicles, 140-180 μm in diameter, and containing prophase-arrested oocytes, were dissected from ovaries of 20-23 day old unprimed *Gpr3*$^{-/-}$ mice. The follicles were divided into two sets, one to be injected with 40 pg of RNA encoding GPR3 or mRFP1 before culturing, and one to be cultured without injection. The follicle-enclosed oocytes were microinjected by compressing the follicles between coverslips separated by a single layer of double stick tape, forming a 100 μm spacer. Four to five follicles were put into the injection chamber at a time, and were kept in the chamber at ~22ºC for ~10-20 min; the injection was done as previously described (S11, S12, S13).

Follicles were cultured on Millicell culture plate inserts (PICMORG50, Millipore Corp., Bedford, MA), as previously described (S14). The medium was MEMα (#12000-022, Invitrogen) supplemented with 25 mM $NaHCO_3$, 75 μg/ml penicillin G, 50 μg/ml streptomycin sulfate, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium (all from Sigma), 5% mouse serum (Abcam Inc., Cambridge, MA), and 10 ng/ml ovine follicle stimulating hormone (National Hormone and Peptide Program). Four to five follicles were placed on each 25 mm diameter membrane, with 1.6 ml of medium under the membrane (Fig. S3A). The cultures were maintained at 37ºC with 5% CO_2 in humidified air, and the medium was exchanged after 2 days. After 4 days, the follicles were opened and scored for the presence or absence of a prophase nucleus. At this point, the follicles were ~220-450 μm in diameter, as measured on the Millicell membrane, and cumulus and antrum formation were seen in many (Fig. S3B). Control follicles that had been injected with mRFP1 RNA were observed with a fluorescence microscope; this confirmed that the red fluorescent protein was synthesized within a few hours, and was still present after the 4 day culture period (Fig. S3C).

Supplementary figures and tables

Fig. S1. Low magnification views of ovary sections. A. Ovary from a *Gpr3*$^{-/-}$, 22 day old (prepubertal) mouse. B. Ovary from a *Gpr3*$^{+/+}$, 22 day old (prepubertal) mouse. C. Ovary from a *Gpr3*$^{-/-}$, 4 month old (adult) mouse. D. Ovary from a *Gpr3*$^{+/+}$, 3 month old (adult) mouse. Scale bar = 1 mm.

Fig. S2. Spontaneous resumption of meiosis in oocytes within *Gpr3*$^{-/-}$ antral follicles (adult mouse). A. Percentages of oocytes that had resumed meiosis, counted in sections of adult *Gpr3*$^{-/-}$ and *Gpr3*$^{+/+}$ ovaries. The graph shows data from one 4 month old *Gpr3*$^{-/-}$ mouse, and one 3 month old *Gpr3*$^{+/+}$ mouse. Numbers above each bar indicate the total number of follicles counted. Note that the "large early antral" category for these adult mice differed from that for the prepubertal mice used in Fig. 3D; see Materials and Methods. The 58% meiotic resumption in the *Gpr3*$^{-/-}$ antral follicles of the adult ovary may be an underestimate, because there were 6 additional follicles that contained fragmenting oocytes in contact with a complete or partial cumulus mass. These appeared to be oocytes that resumed meiosis and then fragmented or underwent some abnormal mitotic divisions; all contained chromosomes and/or nuclei, but no well defined blastomeres and no differentiation (S15) were seen. Counting these fragmented oocytes, 72% of oocytes in *Gpr3*$^{-/-}$ antral follicles had resumed meiosis. No fragmenting oocytes in contact with a cumulus mass within an antral follicle were observed in the *Gpr3*$^{+/+}$ adult ovary, or within antral follicles of *Gpr3*$^{-/-}$ prepubertal ovaries. B. An example of a fragmenting oocyte within an antral follicle of an adult *Gpr3*$^{-/-}$ mouse. Scale bar = 100 μm.

Fig. S3. Injection of RNA into oocytes within preantral follicles, followed by culture to the antral stage. A. A preantral follicle soon after injection, photographed on a Millicell culture membrane, showing the nucleolus and an oil drop introduced by injection. Diameter = 180 μm. B. An antral follicle, injected 3 days previously and then cultured and photographed on a Millicell membrane. The oocyte image is obscured by overlying follicle cells, but the oil drop can be seen (a different follicle than shown in A). Diameter = 320 μm. C. Fluorescence image of the same follicle shown in B, demonstrating expression of red fluorescent protein (RFP) as a result of injection of RFP RNA. Scale bar for all panels = 100 μm.

Table S1. Genes in a mouse oocyte EST database that encode 7-transmembrane proteins.

Gene name(s)	Alias(es)	GenBank accession ID	# of ESTs in library	G-protein coupling	Ligands
Gpr3	GPCR21, ACCA	D21062	1	G_s, G_i (S16-S18)*	Sphingosine-1-phosphate (S17)*
Celsr1	ME2, FMI2, CDHF9, HFMI2	AF031572	1	?	?
Gpr1	—	BC032934	3	?	?
Gpr19	—	U46923	2	G_i (S18)	?
Gpr56	TM7LN4, TM7XN1, serpentine receptor	AF166382	4	$G_{q/11}$? (S19)	?
Olfr701	4933433E02Rik	AY073025, AK016560	1	?	?
Edg3	LPb3, S1P3	AB028143	1	G_i, G_q, $G_{12/13}$ (S20)	Sphingosine-1-phosphate (S20)
Adrb3	BETA3AR	X72862	1	G_s, G_i (S21)†	Isoproterenol (S22)†
Cmklr1	DEZ, ChemR23	U79525	1	G_i (S23)	Chemerin (S24)
Opn3	ERO, ECPN, NMO-1, PANOPSIN	AF140241	1	?	?
Gpr35	—	AF200349	3	?	?
3830613O22Rik	GPR125, PGR21, TEM5L	BC052391	1	?	?
Olfr288	—	AY317381	5	?	?
Olfr976	—	AY073726	2	?	?
Olfr1514	—	AY318733	1	?	?

*GPR3 has been demonstrated to constitutively elevate cAMP in a variety of cultured cell lines (S16-S18), implying that it constitutively activates G_s. One study has indicated that GPR3 can also couple to G_i, and that G_i coupling is increased by sphingosine-1-phosphate (S17). Sphingosine-1-phosphate has been reported to have a slight (15%) stimulatory effect on GPR3 coupling to G_s, although it was not definitively established that the sphingosine-1-phosphate effect was due to GPR3 vs another endogenous sphingosine-1-phosphate-sensitive receptor (S17).

† ADRB3, the beta 3 adrenergic receptor, activates G_s, and therefore could possibly function in maintaining meiotic prophase arrest in mouse oocytes. However, this seems unlikely since 10 μM isoproterenol does not prevent the spontaneous resumption of meiosis in isolated oocytes (S25).

Table S2. Primers used for PCR amplification of *Gpr3* wildtype and mutant alleles. Superscripts show primer position according to *Gpr3* nucleotide sequence accession number D21062.

Allele	Primer name	Amplicon size (bp)	Sequence
wildtype	*Gpr3* F *Gpr3* R	506	^{1060}TATCCACTCTCCAAGAACCATCTGG^{1084} ^{1564}GGAATTAAGCCCTGGTGGACCTAAC^{1540}
mutant	Neo F *Gpr3* R	358	GGGCCAGCTCATTCCTCCCACTCAT ^{1564}GGAATTAAGCCCTGGTGGACCTAAC^{1540}

Table S3. The percentage of oocytes resuming meiosis within early antral follicles of *Gpr3*$^{-/-}$ ovaries is greater in larger follicles. Measurements were made from histological sections of 2 ovaries from 22-23 day old eCG-primed mice; they were made in the sections containing the nucleus or chromosomes, and were taken as the largest dimension for irregularly shaped follicles. The % meiotic resumption was significantly different in 140-180 μm follicles compared with 190-250 μm follicles (Fisher's exact test, $p = 0.001$).

Follicle diameter (μm)	% Meiotic resumption
140-180	22% (n=45)
190-250	59% (n=41)

Table S4. Diameters of *Gpr3*$^{-/-}$ and *Gpr3*$^{+/+}$ antral follicles are the same, and diameters of *Gpr3*$^{-/-}$ antral follicles containing oocytes at metaphase or prophase are the same. Measurements were made from histological sections of ovaries from 22-24 day old eCG-primed mice (3 *Gpr3*$^{+/+}$ ovaries, 2 *Gpr3*$^{-/-}$ ovaries). They were made in the sections containing the nucleus or chromosomes, and were taken as the largest dimension for irregularly shaped follicles. The diameters did not differ for *Gpr3*$^{-/-}$ and *Gpr3*$^{+/+}$ antral follicles, or for *Gpr3*$^{-/-}$ antral follicles with metaphase or prophase oocytes (mean ± S.D., unpaired t test, $p > 0.2$).

Follicle genotype	Follicle diameter (μm) (mean ± SD)
Gpr3$^{+/+}$	364 ± 47 (n = 51)
Gpr3$^{-/-}$	350 ± 54 (n = 39)
Gpr3$^{-/-}$ (metaphase only)	346 ± 48 (n = 33)
Gpr3$^{-/-}$ (prophase only)	375 ± 78 (n = 6)

Table S5. Within *Gpr3*$^{-/-}$ early antral follicles, there is no correlation between meiotic resumption and oocyte size. Measurements were made from histological sections of an ovary from a 22 day old eCG-primed *Gpr3*$^{-/-}$ mouse. They were made in the sections containing the largest diameter cross-section of each oocyte, and were taken as the largest dimension for irregularly shaped oocytes. Because some oocytes were not spherical, and because of possible changes in dimensions during histological processing, the values listed should be considered relevant only to the other values in the table, and not to maturational competence of the oocytes. No signficant differences were found between oocytes that had or had not resumed meiosis (mean ± S.D., unpaired t test, $p > 0.5$).

Meiotic stage	Oocyte diameter (μm)
Prophase	66±5 (n=25)
Metaphase or prometaphase	67±4 (n=15)

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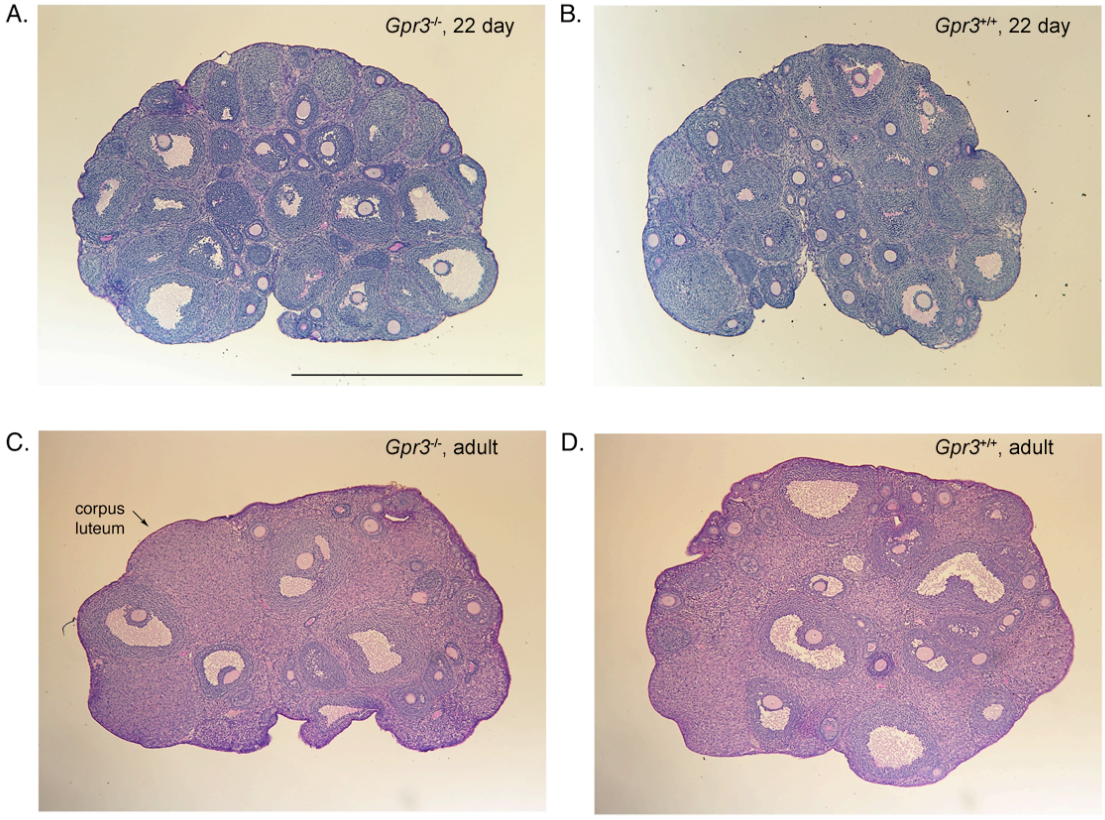
A.
Gpr3-/-, 22 day
B.
Gpr3+/+, 22 day
C.
Gpr3-/-, adult
corpus luteum
D.
Gpr3+/+, adult

Fig. S1

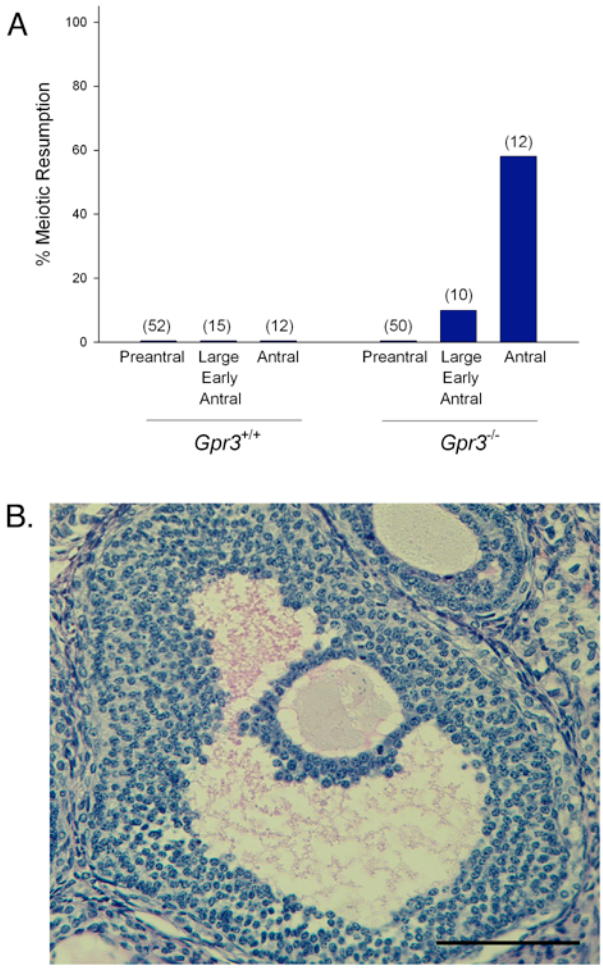
A
% Meiotic Resumption
100
80
60
40
20
0
(52)
(15)
(12)
(50)
(10)
(12)
Preantral
Large Early Antral
Antral
Preantral
Large Early Antral
Antral
Gpr3+/+
Gpr3-/-
B.

Fig. S2

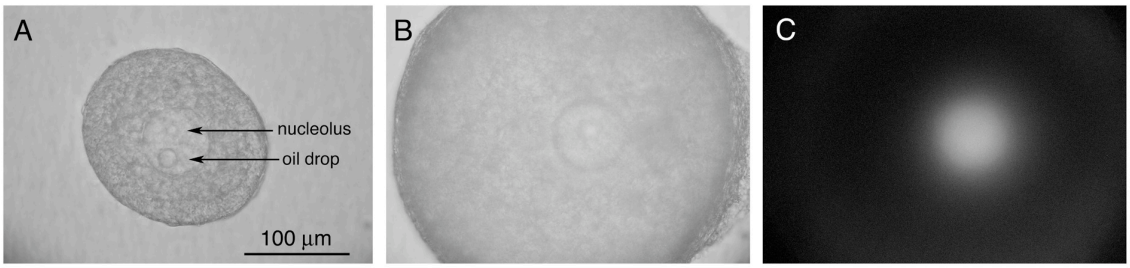

Fig. S3