
# Loss of *Ntrk2/Kiss1r* Signaling in Oocytes Causes Premature Ovarian Failure

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Neurotrophins (NTs), once believed to be neural-specific trophic factors, are now known to also provide developmental cues to non-neural cells. In the ovary, NTs contribute to both the formation and development of follicles. Here we show that oocyte-specific deletion of the *Ntrk2* gene that encodes the NTRK2 receptor (NTRK2) for neurotrophin-4/5 and brain-derived neurotrophic factor (BDNF) results in post-pubertal oocyte death, loss of follicular organization, and early adulthood infertility. Oocytes lacking NTRK2 do not respond to gonadotropins with activation of phosphatidylinositol 3-kinase (PI3K)-AKT-mediated signaling. Before puberty, oocytes only express a truncated NTRK2 form (NTRK2.T1), but at puberty full-length (NTRK2.FL) receptors are rapidly induced by the preovulatory gonadotropin surge. A cell line expressing both NTRK2.T1 and the kisspeptin receptor (KISS1R) responds to BDNF stimulation with activation of *Ntrk2* expression only if kisspeptin is present. This suggests that BDNF and kisspeptin that are produced by granulosa cells (GCs) of periovulatory follicles act in concert to mediate the effect of gonadotropins on *Ntrk2* expression in oocytes. In keeping with this finding, the oocytes of NTRK2-intact mice fail to respond to gonadotropins with increased *Ntrk2* expression in the absence of KISS1R. Our results demonstrate that the preovulatory gonadotropin surge promotes oocyte survival at the onset of reproductive cyclicity by inducing oocyte expression of NTRK2.FL receptors that set in motion an AKT-mediated survival pathway. They also suggest that gonadotropins activate NTRK2.FL expression via a dual communication pathway involving BDNF and kisspeptin produced in GCs and their respective receptors NTRK2.T1 and KISS1R expressed in oocytes. **(*Endocrinology* 155: 3098–3111, 2014)**

Acquisition and maintenance of female reproductive capacity requires the extrusion of a viable oocyte from the ovary at ovulation. Oocytes grow surrounded by somatic cells of epithelial (granulosa cells, GCs) and mesenchymal (thecal cells) origin. Together, these three cell types form follicles, the structural and functional unit of the ovary (1).

Integrity of ovarian follicles requires a healthy oocyte; in its absence, follicular structure disintegrates (2). It is, therefore, not surprising that in recent years a major focus of attention in the field has been the identification of regulatory pathways that, operating within the ovarian microenvironment, contribute to maintaining oocyte integ-

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Abbreviations: BDNF, brain-derived neurotrophic factor; GC, granulosa cell; GV, germinal vesicle; hCG, human chorionic gonadotropin; LCM, laser-capture microdissection; NT, neurotrophin; NTRK2, NTRK2 receptor; PI3K, phosphatidylinositol 3-kinase; PMSG, pregnant mare serum gonadotropin; POF, premature ovarian failure; WT, wild type.

rity during the reproductive lifespan (3). Identifying the factors required for oocyte survival during the reproductive lifespan is an important endeavor because knowledge of the underlying pathways may provide significant new insights into the pathology of premature ovarian failure (POF), a disorder that affects 1% of women of reproductive age (4) and is associated with premature loss of viable oocytes (5).

Within this framework, one of our laboratories has developed the concept that neurotrophins (NTs) and their NTRK receptors, long thought to be exclusively required for the development of the nervous system, are also involved in the control of ovarian function (6). Studies using conventional *Ntrk2*-null mice identified tropomyosin-related kinase receptor B (TrkB, now termed NTRK2), the high-affinity receptor for neurotropin-4/5 and brain-derived neurotrophic factor (BDNF), as a molecule required for follicular assembly and early follicular growth (7, 8). One of these studies (7) also provided results suggesting that NTRK2 may be essential for oocyte survival in antral follicles, and therefore, is required for the structural integrity of these follicles. This notion stems from the finding that the ovaries from conventional 4- to 5-day-old *Ntrk2*-null mice grafted under the kidney capsule of ovariectomized wild-type (WT) adult mice fail to thrive and show widespread oocyte death, in addition to loss of follicular structure, despite elevated gonadotropin levels seen in the host animals.

The first indication that NTs could play a role in promoting postovulatory oocyte development was provided in 2002 by Seifer and colleagues (9), who showed that cumulus cells secrete BDNF and that BDNF promotes maturation of cultured mouse oocytes. Three years later, Kawamura et al (10) showed that BDNF synthesis increases in GCs after the preovulatory surge of gonadotropins, and corroborated the ability of BDNF to facilitate oocyte development. Kawamura et al also demonstrated that this effect is mediated by high-affinity NTRK2 receptors expressed in the oocyte, and that ligand-induced activation of these receptors promotes the in vitro development of zygotes into preimplantation embryos (10, 11) by activating a PI3 kinase signaling pathway (11). A remarkable finding made in this study was that blockade of NTRK receptor tyrosine kinase activity suppressed by more than 70% the capacity of zygotes to develop into blastocyst-stage embryos, and reduced by 50% the number of cells present in these blastocysts. Subsequent studies by other authors (12) demonstrated that, as in the mouse, BDNF promotes cytoplasmic competence of bovine oocytes and supports embryo development. A similar conclusion was reached by investigators working with porcine oocytes (13); these authors showed that exposing zygotes to a combination of epidermal growth factor (EGF) and BDNF significantly improved preimplantation development. Importantly, inhibition of NTRK signaling reduced the number of blastocysts reaching the expanded stage, and increased the rate of cell death in developing blastocysts (11). Altogether, these findings provide strong evidence supporting the concept that ligand-dependent activation of NTRK2 receptors plays an important role in promoting postovulatory oocyte development.

The present study was undertaken to determine if NTRK2 receptors contribute to sustaining oocyte integrity after completion of folliculogenesis. With this purpose, we used mutant mice in which the *Ntrk2* gene was selectively deleted from either oocytes or GCs. Because BDNF promotes oocyte competence (11) and activates truncated NTRK2 (NTRK2.T1) receptors (14) that are the predominant form of the receptor expressed in oocytes of developing ovaries (7, 8), we performed experiments to determine if BDNF could induce *Ntrk2* expression by activating NTRK2.T1-mediated signaling. We also considered the potential involvement of the kisspeptin-KISS1R system (15) for three reasons: a) both kisspeptin and its receptor KISS1R are expressed in the ovary, b) kisspeptin production is increased by the preovulatory surge of gonadotropins (16), and c) both NTRK2.T1 and KISS1R-mediated signaling converge intracellularly to activate PLC/inositol-1,4,5-triphosphate-dependent calcium mobilization (14, 17).

Our findings document the existence of a functional connection between the NTRK2 and KISS1R signaling systems in the ovary and show that both systems are required for oocyte survival and follicular integrity in the adult ovary. In the accompanying paper (18) we demonstrate that ovarian KISS1R signaling is required for normal adult ovarian function and that its partial deficiency results in a reproductive phenotype of POF, similar to that caused by the lack of NTRK2 in oocytes.

## Materials and Methods

### Mutant mice

All mice were maintained on a 12-hour light, 12-hour dark cycle (lights off at 7:00 PM) with food and water available ad libitum. The breeders were fed with LabDiet 5001, PMI Nutrition International Brentwood. The use of mice was duly approved by the Oregon National Primate Research Center Animal Care and Use Committee, in accordance with the guidelines provided by the National Institutes of Health Guide and Use of Laboratory Animals. Experimental protocols using *Kiss1r*$^{-/-}$ mice were approved by the Córdoba University Ethical Committee of animal experimentation and conducted in accordance with the European Union guidelines for use of experimental animals.

The *Ntrk2*$^{loxP/loxP}$ mice in a BALB/c; 129S genomic background (19) were kindly provided by Dr Louis Reichardt (University of California San Francisco, San Francisco, California) and were backcrossed to C57BL/6J mice for at least 10 generations. Transgenic mice carrying a growth differentiation factor 9 (*Gdf-9*) promoter-*Cre* recombinase construct (20) (a generous gift from Dr Austin Cooney, Baylor College of Medicine, Houston, Texas), were backcrossed to C57BL/6J mice for 6 generations. This transgene begins to be expressed in oocytes of primordial follicles and continues to be expressed throughout the life of the oocyte (20, 21). After two rounds of crossing, we generated mutant female mice that had either an *Ntrk2*$^{loxP/loxP}$/*Gdf9Cre*$^{+}$ (*OoNtrk2*$^{-/-}$) or *Ntrk2*$^{loxP/loxP}$/*Gdf9Cre*$^{-}$ (WT control) genotype.

*Amhr2-Cre* transgenic mice (22) were the generous gift of Dr. Richard Behringer (Baylor College of Medicine, Houston, Texas). Mice carrying a null *Ntrk2* gene in GCs were obtained by crossing *Amhr2Cre*$^{+}$ males with *Ntrk2*$^{loxP/loxP}$ females. Because all males carrying the *Amhr2Cre* transgene in their genome have germ line recombination of any loxP-flanked gene (22), males from this cross that are *Amhr2Cre*$^{+}$/*Ntrk2*$^{loxP/-}$ will produce offspring with one *Ntrk2* deleted (null) allele in every cell of the body. Consequently, crossing *Amhr2Cre*$^{+}$/*Ntrk2*$^{loxP-}$ males with *Ntrk2*$^{loxP/loxP}$ females yields 50% of females that are *Amhr2Cre*$^{+}$/*Ntrk2*$^{floxed/-}$ (ie, conditional *Ntrk2* KO in GCs; *GCNtrk2*$^{-/-}$) and 50% *Amhr2Cre*$^{-}$/*Ntrk2*$^{loxP/-}$ (control mice). B6.129(Cg)-*Gt(ROSA)26Sor*$^{tm4(ACTB-tdTomato,-EGFP)Luo}$/J mice (*mT/mG*) were obtained from Jackson Laboratory and used as a reporter mice to monitor CRE activity in the ovaries of *Gdf9Cre* mice.

Mice carrying one deleted *Kiss1r* allele on a C57BL/6J background were obtained by crossing *Kiss1r*$^{loxP/loxP}$ mice (23) with transgenic animals expressing *Cre* under the control of the Protamine1 promoter (*Prm1Cre*$^{+}$) that is expressed in male germ cells (24). To generate mice lacking both *Kiss1r alleles* (*Kiss1r*$^{-/-}$) we crossed F1 *Kiss1r*$^{+/-}$ males and female mice as described in detail elsewhere (23).

## Genotyping

*OoNtrk2*$^{-/-}$ animals were genotyped by PCR using tail genomic DNA and two sets of specific oligodeoxynucleotide primers. The first set amplifies a DNA segment within exon 2 in the *Ntrk2* gene that is either WT or flanked by loxP sequences (P1: 5′-ATG TCG CCC TGG CTG AAG TG-3′; P2: 5′-ACT GAC ATC CGT AAG CCA GT-3′) (Supplemental Figure 1A and upper gel in B). The second set amplifies a segment of the improved *Cre* (i*Cre*) transgene (20) using the primers iCre forward (5′-TCT GAT GAA GTC AGG AAG AAC C-3′) and iCre rev (5′-GAG ATG TCC TTC ACT CTG ATT C-3′) (Supplemental Figure 1B, bottom gel). Ovarian gDNA was analyzed using primers P1, P2, and P3 (5′-TGG GCA CTC AGA CAT TCA TTC-3′). The latter in combination with P2 detects the deleted *Ntrk2* allele (Supplemental Figure 1, A and C).

*Amhr2Cre/Ntrk2* floxed mice were genotyped using the same set of primers described above (Supplemental Figure 1, D and E). The *Amhr2Cre* transgene was detected with the primers Cre3: 5′-TTC AAT TTA CTG ACC GTA CAC C-3′; Cre4: 5′-CGT TTT CTT TTC GGA TCC-3′ (Supplemental Figure 1F).

*Kiss1r* KOs were genotyped as previously described (23), using the primer pair 5′- AGC GCA AGG CTC TGA AGC GGC-3′ and 5′-CAA TGT CGC CTC GGT GGC CAT-3′ to detect the WT allele, and the primer pair 5′-AGC GCA AGG CTC TGA AGC GGC-3′ and 5′-AAC AAC CCG TCG GAT TCT CCG-3′ to detect the mutant allele.

## Gdf9Cre-mediated deletion of the *Ntrk2* gene from oocytes

A reporter mouse (*mT/mG;*GT(ROSA)*26Sor*$^{tm4(ACTB-tdTomato,-EGFP)Luo}$/J) that has a loxP-flanked sequence encoding a red fluorescent protein (mT, tomato) located upstream from a cDNA encoding green fluorescent protein (mG, green) was used to verify that *Gdf9* promoter-driven CRE recombination occurs specifically in oocytes. The mT/mG construct consists of a chicken β-actin core promoter with a cytomegalovirus enhancer (pCA) driving a loxP flanked sequence that encodes a membrane-targeted tandem dimer red fluorescence protein with membrane localization. After CRE-mediated recombination, the mT sequence is excised allowing the pCA promoter to drive expression of a membrane-targeted enhanced green fluorescent protein (mG) (25).

## Amhr2Cre-mediated deletion of the *Ntrk2* gene from GCs

To verify the loss of *Ntrk2* expression in GCs of animals born to *Ntrk2* floxed mice bred to *Amhr2Cre* animals, we measured *Ntrk2* mRNA in GCs captured by laser capture microdissection. The laser-capture microdissection (LCM) procedure used was modified from Bonet et al (26). The ovaries were quickly removed after decapitation, embedded in OCT on a plastic cryomold (VWR International) and frozen by dropping the cryomold into isopentane-dry ice bath. Eight-micrometer serial frozen sections were cut on a cryostat at −20°C, mounted on cooled (4°C) noncharged sterile microscope glass slides and stored in a clean box inside the cryostat chamber for few minutes. The sections were fixed by immersing the slides for 30 seconds in a 50-mL plastic tube filled with 75% ethanol and stored at −85°C until the day of LCM.

To perform the microdissection, the sections were removed from the freezer and immediately stained with Cresyl Violet as follows: 50% ethanol for 20 seconds, 0.5% cresyl violet for 20 to 30 seconds, 50% ethanol for 20 seconds, 75% ethanol for 30 seconds, 95% ethanol for 40 seconds twice, 100% ethanol for 1 minute, twice (new bottle each day), and xylene for 5 minutes, twice. The stained, dehydrated sections were kept under vacuum for at least 45 minutes before starting the microdissection of GCs. This procedure was carried out under 10x magnification using an Arcturus XT apparatus (Arcturus, Applied Biosystems).

After microdissecting samples for 1 hour, the collecting cap was removed, inserted into an RNase-free microcentrifuge tube containing 70 μL of lysis buffer from the Micro RNeasy Isolation kit (Qiagen), vortexed for 30 seconds upside down, and frozen at −80°C until RNA extraction.

## Oocyte collection

To measure *Ntrk2.fl* and *Ntrk2.t1* mRNAs, 12-day-old *Ntrk2*$^{loxP/loxP}$/*Cre*$^{-}$ and *OoNtrk2*$^{-/-}$ mice were quickly euthanized, and the ovaries were immediately placed in 3 mL Tyrode Lactate-Hepes media supplemented with 3 mg/mL bovine serum albumin (Millipore); they were dissected free of fat and connective tissue using a stereomicroscope. The ovaries were transferred to a new petri dish and the cumulus-oocyte complexes

were obtained by puncturing individual follicles visible in the cortical region of the ovary; the oocytes were then denuded by repeat pipetting using a Flexipet Micro-Manipulation Pipette (COOK Medical Inc), and classified by germinal vesicle (GV) stage. Oocytes with GV breakdown or undergoing atresia were discarded, and oocytes with intact GV were immediately placed on lysis buffer for RNA extraction (Micro RNeasy Kit, Qiagen).

For Western blot analysis, 23-day-old *Ntrk2*$^{loxP/loxP}$/*Cre*$^{-}$ and *OoNtrk2*$^{-/-}$ mice were injected ip with 7.5 IU of pregnant mare serum gonadotropin (PMSG), followed 48 hours later by a single ip injection (5 IU) of human chorionic gonadotropin (hCG). Eight hours after hCG, the mice were euthanized, the ovaries were dissected, and antral follicles were punctured using a stereomicroscope. The oocytes were isolated as described by Reddy et al (27). The protein concentrations were measured using the Pierce 660 nm Protein Assay Reagent and equal amounts of proteins were used for Western blots.

### Granulosa cells isolation

GCs were collected as described previously (28). Briefly, ovaries were punctured with a 25G needle in 200 $\mu$L of DMEM under a stereomicroscope, and the cell suspension was carefully aspirated with a pipette and transferred to a 1.5-mL plastic tube. The cells were pelleted by centrifugation at 250 x g for 3 minutes, washed three times with PBS and stored at −80°C until RNA extraction.

### Quantification of ovarian follicles and histological analysis

Ovaries from 4-, 6-, 8-, and 22-week-old mice, were fixed in Kahle's fixative, embedded in paraffin, serially sectioned at 6 $\mu$m, stained with Weigert's iron hematoxylin, and counterstained with picric acid–methyl blue, as reported (7, 29, 30). Ovarian follicles were counted in all sections of an ovary, and were classified in different developmental stages according to the criterion proposed by Pedersen and Peters (31). The total number of follicles, either preantral or antral, per ovary was counted. In addition, changes in the ovarian follicle population were evaluated by assessing oocyte loss, as defined by signs of oocyte atresia at any stage of follicle development. Degenerating oocytes were recognized by the presence of morphological alterations such as shrinkage and/or fragmentation (32, 33).

### Measurement of serum gonadotropins

LH and FSH were measured by RIA using reagents provided by the National Institutes of Health National Pituitary Agency. The binding in the LH assay was 30% and the sensitivity was 0.7 ng/mL. The standard curve had a correlation coefficient ($r^2$) of 0.999. The FSH assay had a binding of 21%, a sensitivity of 0.4 ng/ml, and an $r^2 = 0.995$.

### Cell culture

The rat glial cell line, C6, was obtained from ATCC; the cells were maintained in DMEM supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% $CO_2$ at 37°C. The cells were seeded in 12-well plates at a density of $2.5 \times 10^5$ cells per well in serum-free medium. Twenty-four hours later, the cells were exposed for 4 hours to BDNF (100 ng/ml; Promega), kisspeptin (100 nM, Phoenix Pharmaceutical, Inc), or left untreated. Other cultures were treated with BDNF and kisspeptin together in the presence or absence of 1 mM EGTA. At the end of these treatments, the cells were washed with PBS and stored at −80°C until RNA extraction.

### Statistical analysis

Differences among several groups were analyzed (GraphPad Prism) by 1-way ANOVA followed by the Neuman-Keuls multiple comparison test. Differences between 2 groups were analyzed using Student's *t* test. Values were considered significantly different if $P < .05$.

### Supplemental information

A description of the procedures employed for RNA extraction, end-point and real-time PCR, primers used, Western blotting, and immunohistofluorescence, in addition to 4 supplementary figures are provided as Supplemental Information.

## Results

### Conditional knockout mice

The strategy used to identify animals in which the *Ntrk2* gene is conditionally deleted using Cre-loxP technology (*OoNtrkt2*$^{-/-}$ mice) from either oocytes or GCs is shown in Supplemental Figure 1, A–F. Animals born to *Gdf9Cre* mice crossed with the reporter mouse *mT/mG* that carry a loxP-flanked sequence encoding red fluorescent protein (mT) located upstream from a cDNA encoding green fluorescent protein (mG) (Figure 1A) demonstrated that *Gdf9* promoter-driven CRE recombination occurs specifically in oocytes (Figure 1, B and C). To determine the effectiveness of *Amhr2Cre* to reduce *Ntrk2* expression in GCs, we measured *Ntrk2* mRNA in GCs obtained with laser capture microdissection (Figure 1D), and found that *Ntrk2* mRNA transcripts were essentially absent in GCs of *GCNtrk2*$^{-/-}$ mice as compared with their WT littermates (Figure 1E). Jeyasuria et al (34), using a ROSA26-galactosidase *Cre* reporter allele, previously showed that GCs were the only ovarian cells expressing *Cre* in *Amhr2Cre* mice.

### Oocytes only express truncated NTRK2 receptors and these are absent in *OoNtrkt2*$^{-/-}$ mice

Alternative splicing of *Ntrk2* pre-mRNA generates a full-length (FL) receptor that uses an intracellular tyrosine kinase domain for signaling, and truncated isoforms (known as T1 and T2) lacking the intracellular kinase domain (35). Prepubertal mouse oocytes show an abundance of *Ntrk2.T1* mRNA and negligible levels of *Ntrk2.FL* transcripts (7, 8). In keeping with these findings, only *Ntrk2.T1* mRNA was detected in isolated *Ntrk2*$^{loxP/loxP}$/*Cre*$^{-}$ oocytes and expression of this transcript was lost in *OoNtrk2*$^{-/-}$ mice (Figure 1, F and G). Likewise, NTRK2.T1 receptor immunoreactivity was abundant in

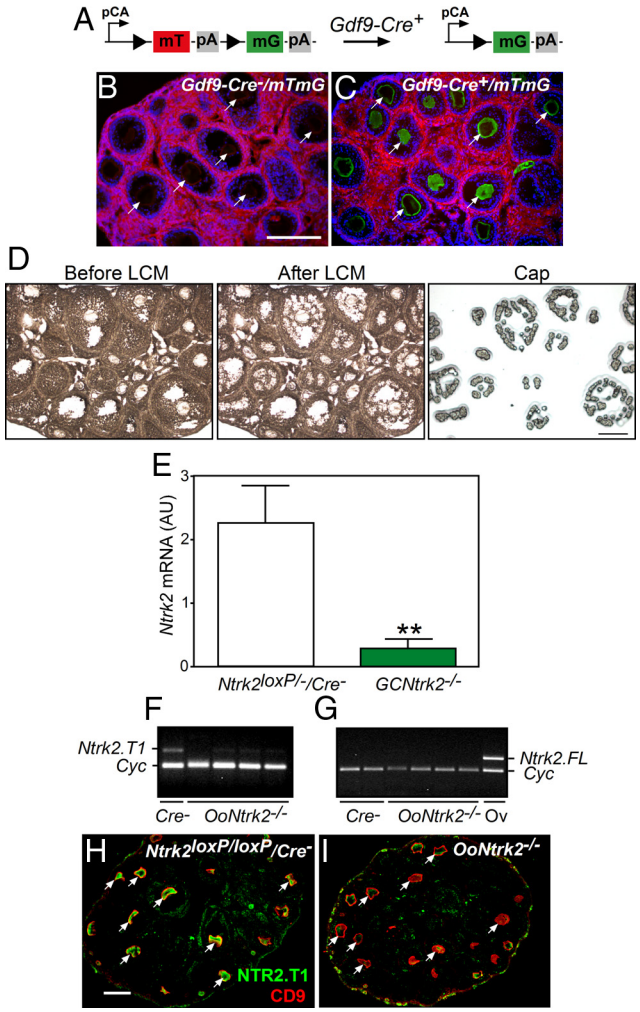


**Figure 1.** Cre-loxP mediated deletion of *Ntrk2* from oocytes or GCs. A, The reporter mT/mG construct before and after Cre-mediated recombination. Arrows indicate direction of transcription; triangles indicate loxP sites; pA indicates polyadenylation sequences. B, Ovary of a 28-day-old *Gdf9Cre*$^{-}$*/mTmG* mouse showing that most mT expression (*red*) is detected in thecal interstitial cells and oocytes (*arrows*). C, Ovary from a *Gdf9-Cre*$^{+}$*/mTmG* mouse showing that only oocytes (*arrows*) are mG-positive (*green*). D, Representative section from an *Amhr2Cre-Ntrk2*$^{-/-}$ ovary before and after laser-capture microdissection (LCM). E, Loss of *Ntrk2* mRNA in GCs by *Amhr2Cre*-mediated *Ntrk2*$^{loxP}$ recombination. **, $P < .01$ *GCNtrk2*$^{-/-}$ mice (n = 5) versus *Ntrk2*$^{loxP/-}$*/Cre*$^{-}$ mice (n = 4). F, *Ntrk2.t1* and G, *Ntrk2.fl* mRNA in isolated oocytes from *Ntrk2*$^{loxP/loxP}$*/Cre*$^{-}$ and *OoNtrk2*$^{-/-}$ mice. H and I, Lack of NTKR2 immunoreactivity in oocytes of a 12-day-old *OoNtrk2*$^{-/-}$ mouse ovary, as determined by double immunohistofluorescence using antibodies against the oocyte membrane-specific protein CD9 (*red*) and NTRK2.T1 (*green*). Scale bars, 200 $\mu$m.

*Ntrk2*$^{loxP/loxP}$*/Cre*$^{-}$ oocytes and absent from most *OoNtrk2*$^{-/-}$oocytes (Figure 1, H and I).

## Oocyte-specific deletion of the *Ntrk2* gene causes infertility

*OoNtrk2*$^{-/-}$ mice had a normal age at puberty, as determined by the age at vaginal opening and the age at first estrus (Supplemental Figure 2A). After mating, the number of pups born in the first and second litter was similar in *OoNtrk2*$^{-/-}$ and *Ntrk2*$^{loxP/loxP}$*/Cre*$^{-}$ mice (Supplemental Figure 2B). However, only 4 of 7 *OoNtrk2*$^{-/-}$ females delivered a second litter (Supplemental Figure 2B) and all of the dams became infertile thereafter (Figure 2A). In contrast, reproductive performance was normal in *GCNtrk2*$^{-/-}$mice (Figure 2B). Circulating FSH and LH levels were mildly, but not significantly, elevated in adult *OoNtrk2*$^{-/-}$ mice (26 weeks of age) as compared with *Ntrk2*$^{loxP/loxP}$*/Cre*$^{-}$ mice (Figure 2C).

## Oocyte-specific *Ntrk2* deletion results in oocyte death and disintegration of follicular structure

We found no apparent difference in ovarian follicular morphology on either postnatal week 4 (before puberty) or week 6 (time at which at least 2 estrous cycles had occurred) between *OoNtrk2*$^{-/-}$ and *Ntrk2*$^{loxP/loxP}$*/Cre*$^{-}$ mice (Figure 2, D, F, G, and I). However, at this later time the ovaries of *OoNtrk2*$^{-/-}$ mice showed a 2-fold increase in the number of degenerating oocytes (Figure 2, H and I). By 8 weeks of age, the *OoNtrk2*$^{-/-}$ ovaries no longer had a normal morphology (Figure 2, J and K), and exhibited significant loss of antral follicles and healthy oocytes (Figure 2, K and L). Surprisingly, neither the number of primordial follicles nor the number of growing preantral follicles (primary, secondary) was consistently altered at this time, suggesting that antral follicles are the follicle population directly affected by the loss of NTRK2 receptors in oocytes. In half of the animals (5 of 10), follicle depletion was complete by 22 weeks of age, the time at which a dramatic reduction in the number of primordial, primary, secondary, and antral follicles, in addition to oocytes was evident (Figure 2, M–O). This result suggests that the loss of preantral follicles is secondary to the initial loss of antral follicles caused by the absence of NTRK2 in oocytes.

Instead of follicles, the bulk of the ovarian parenchyma at postnatal week 22 was occupied by interstitial tissue with luteal cell-like aspect (Figure 2N). Large, single follicle cysts were also observed (not shown). The cells of the abnormally expanded interstitial tissue were rich in the androgen producing enzyme 17$\alpha$-hydroxylase (Supplemental Figure 3) indicating that they were steroidogenically active. We also observed that the disintegration of follicular structure that occurs in *OoNtrk2*$^{-/-}$ovaries is accompanied by disorganization of androgen-producing cells of the theca interna that appear to become part of the interstitial compartment (Supplementary Figure 3B).

These changes appeared to progress at a slower pace in the other half of *OoNtrk2*$^{-/-}$ mice, as at 22 weeks the ovaries of these animals still had some antral follicles despite widespread oocyte death (Figure 3, A and B). The antral follicles still present were in different stages of de-

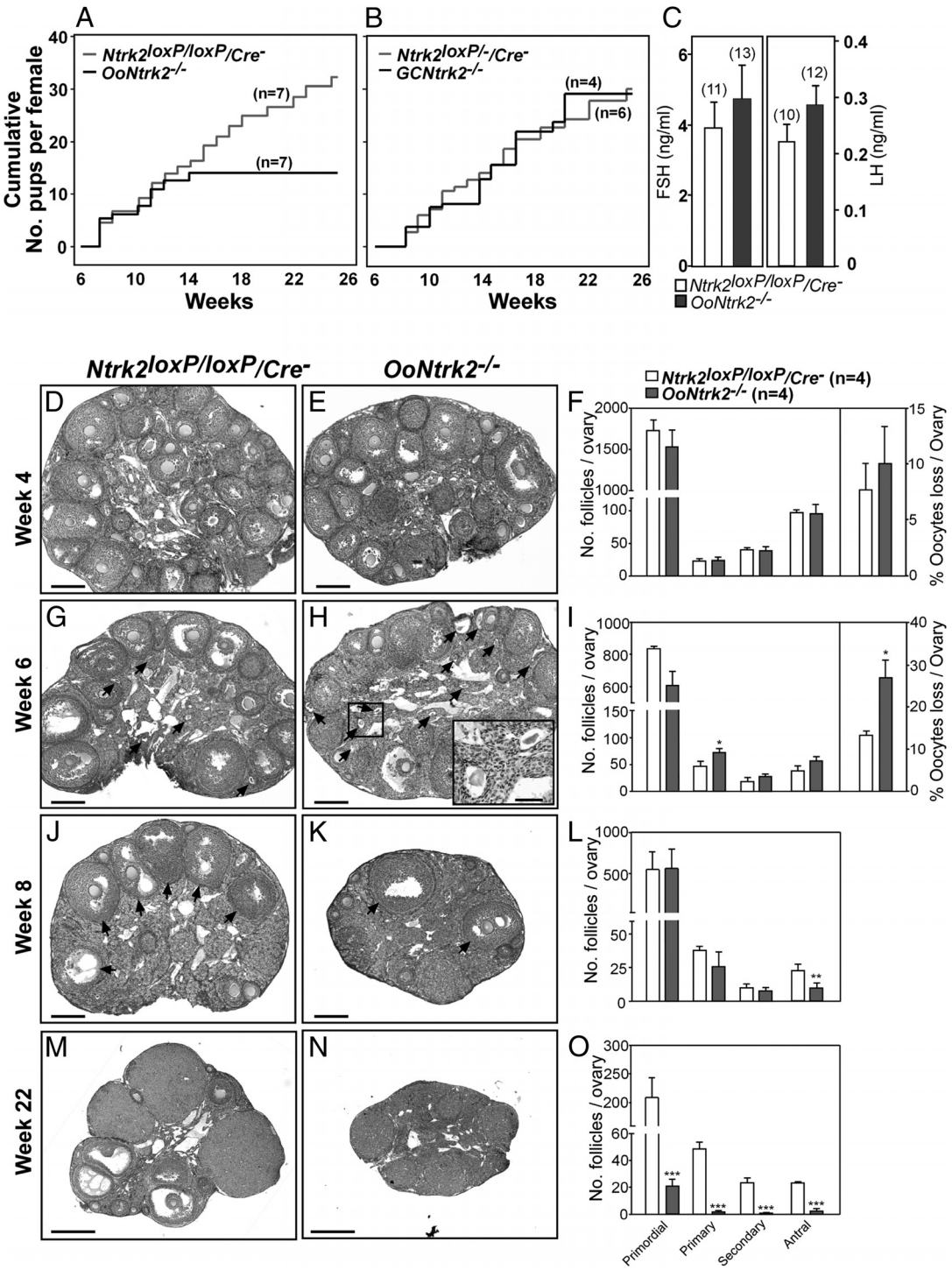


**Figure 2.** Postpubertal ovarian failure in *OoNtrk2*$^{-/-}$ mice. A, Cumulative numbers of pups per *Ntrk2*$^{loxP/loxP}$/*Cre*$^{-}$ (*gray line*) and *OoNtrk2*$^{-/-}$ (*black line*) dam. B, Similar representation for GC*Ntrk2*$^{-/-}$ dams. C, Serum FSH and LH in *Ntrk2*$^{loxP/loxP}$/*Cre*$^{-}$ and *OoNtrk2*$^{-/-}$ mice. Bars are means ± SEM. Numbers of animals per group are in parentheses. D, E, G, H, J, K, M, and N, Ovarian morphology of *Ntrk2*$^{loxP/loxP}$/*Cre*$^{-}$ and *OoNtrk2*$^{-/-}$ mice at different postnatal ages. D and E, Four weeks. G and H, Six weeks. Inset in H shows atretic oocytes. J and K, Eight weeks. M and N, Twenty-two weeks. Scale bar in D, E, G, H, J, and K, 200 $\mu$m; M and N, 500 $\mu$m; inset in H, 50 $\mu$m. F, I, L, and O, Number of primordial, primary, secondary (more than two layers of GCs) and antral follicles detected at different postnatal ages in serial 6 $\mu$m sections of ovaries from *Ntrk2*$^{loxP/loxP}$/*Cre*$^{-}$ and *OoNtrk2*$^{-/-}$ mice. F, Week 4: There are no differences in the number of follicles at any stage of development between genotypes at this age, ie, before puberty. I, Week 6: The number of primordial follicles appears to decrease and the number of primary to increase in *OoNtrk2*$^{-/-}$ mice. At this time, both *Ntrk2*$^{loxP/loxP}$/*Cre*$^{-}$ and *OoNtrk2*$^{-/-}$ mice had undergone two ovulatory cycles. L, Week 8: The changes in the number of primordial and primary follicles observed at 6 weeks are not maintained at this age, but a significant ($P < .01$) loss of antral follicles in becomes evident in *OoNtrk2*$^{-/-}$ mice. O, Substantial loss of all types of follicles occurs at this age. Bars are means ± SEM of at least 3 animals per group. Bars represent mean and vertical bars are ± SEM. *, $P < .05$; **, $P < .01$; ***, $P < .001$ versus *Ntrk2*$^{loxP/loxP}$/*Cre*$^{-}$ group.

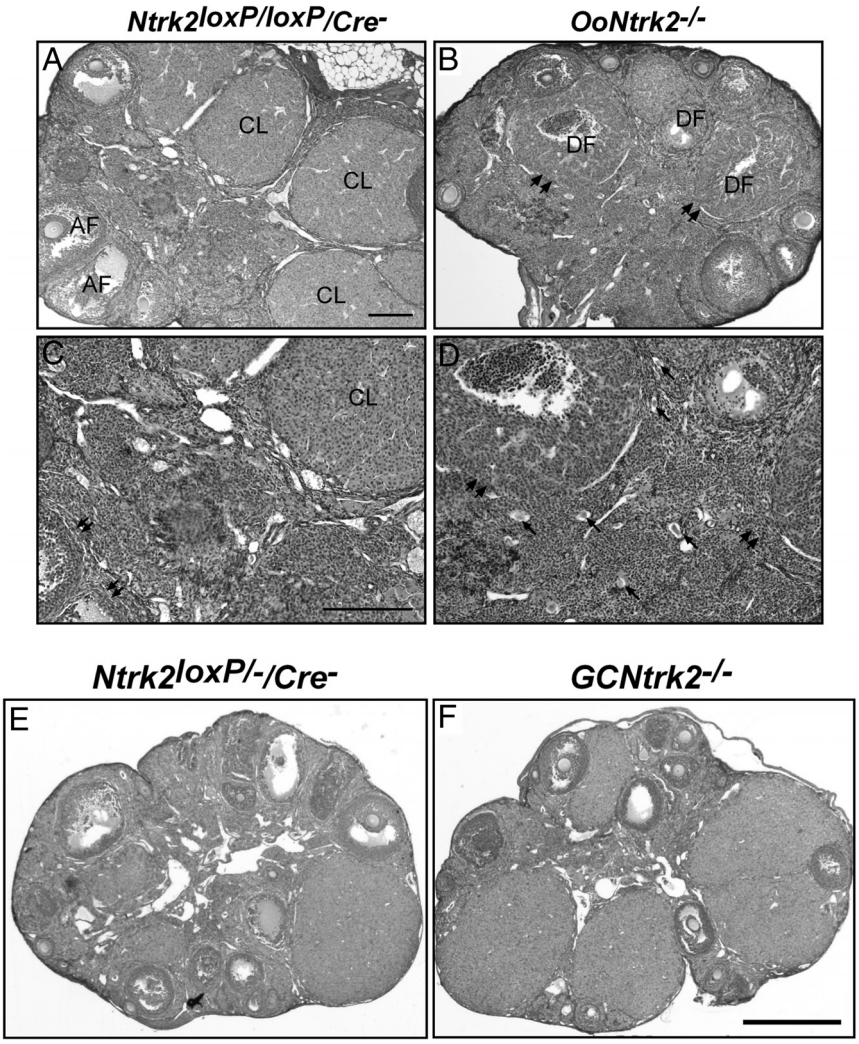


**Figure 3.** Delayed progression of the ovarian *OoNtrk2*$^{-/-}$ phenotype and lack of effect on ovarian morphology of deleting the *Ntrk2* gene from GCs. A, Representative ovarian section from an *Ntrk2*$^{loxP/loxP}$*/Cre*- control mouse. B, Representative ovarian section from an *OoNtrk2*$^{-/-}$ mouse. Notice the abundance of corpora lutea (CL) in (A), and the abundance of degenerating follicles (DF) accompanied by loss of follicle-interstitial tissue boundaries (examples denoted by double arrows) in (B). C, Higher magnification view of the *Ntrk2*$^{loxP/loxP}$*/Cre*- control ovary depicted in (A) illustrating the presence of CL and the well-defined thecal separation of follicles from the interstitial tissue (*examples denote by double arrows*). D, A similar view of the *OoNtrk2*$^{-/-}$ ovary highlighting an abundance of dead oocytes (single black arrows) and the disintegration of follicle-interstitial boundaries (double arrows). E, Morphological aspect of the ovary from an *Ntrk2*$^{loxP-}$*/Cre*-control mouse. F, *GCNtrk2*$^{-/-}$ mouse demonstrating that neither follicular development nor successful ovulation (assessed by the presence of corpora lutea) are compromised in *GCNtrk2*$^{-/-}$ mice. The sections shown are representative of 3 mice per group. The ovaries were collected when the animals were 22 weeks of age. Scale bars, 250 $\mu$m in A and B; 500 $\mu$m in C–F.

generation, displaying loss of thecal boundaries separating them from the interstitial cell compartment (Figure 3, B and D). The latter was abundant and composed of luteal-like cells. No corpora lutea were detected. In contrast to *OoNtrk2*$^{-/-}$ mice, the ovarian morphology of *GCNtrk2*$^{-/-}$ mutants examined at week 22 was indistinguishable from that of WT littermates (Figure 3, E and F). Thus, oocytes devoid of all isoforms of NTRK2 receptors cannot survive after the initiation of estrous cyclicity, resulting in follicle loss and premature ovarian failure.

### The preovulatory gonadotropin surge induces formation of full-length NTRK2 receptors in oocytes and this change is prevented by oocyte-specific *Ntrk2* deletion

Oocytes of immature ovaries express only NTRK2.T1 receptors, and yet they are healthy in *OoNtrk2*$^{-/-}$ mice until at least the time of first ovulation, suggesting that their death may be related to events initiated by the first preovulatory surge of gonadotropins. Mimicking this surge by treating juvenile WT mice with PMSG, followed 48 hours later by hCG, did not alter NTRK2.T1 abundance (Figure 4, A–E), but resulted in the appearance of immunoreactive NTRK2.FL protein in oocytes as early as 4 hours after hCG (Figure 4, F–H). These high levels were maintained for at least 8 hours after hCG and were lower by 24 hours (Figure 4, I and J). Based on these results, we selected the interval of 6 hours after hCG to study the NTRK2.FL response to hCG. Whereas NTRK2.FL immunoreactivity increased markedly at this time in *Ntrk2*$^{loxP/loxP}$*/Cre*$^{-}$ oocytes (Figure 5, A1–A3), no detectable NTRK2.FL protein was found in the oocytes of *OoNtrk2*$^{-/-}$ mice (Figure 5, B1–B3). Western blot analysis of NTRK2 proteins extracted from oocytes isolated 6 hours after hCG confirmed this finding (Figure 5, A4 and B4).

### Lack of full-length NTRK2 receptors in oocytes prevents gonadotropin-induced activation of an AKT-dependent survival pathway in oocytes

The phosphoinositide-3-kinase (PI3K)/AKT signaling pathway (Supplemental Figure 4) is required for primordial follicle activation (27, 36). In neural cells, NTRK2.FL receptors support cell survival by activating this pathway (37), and ligand-dependent stimulation of NTRK2 activates AKT in oocytes (38). *Ntrk2*$^{loxP/loxP}$*/Cre*$^{-}$ oocytes,

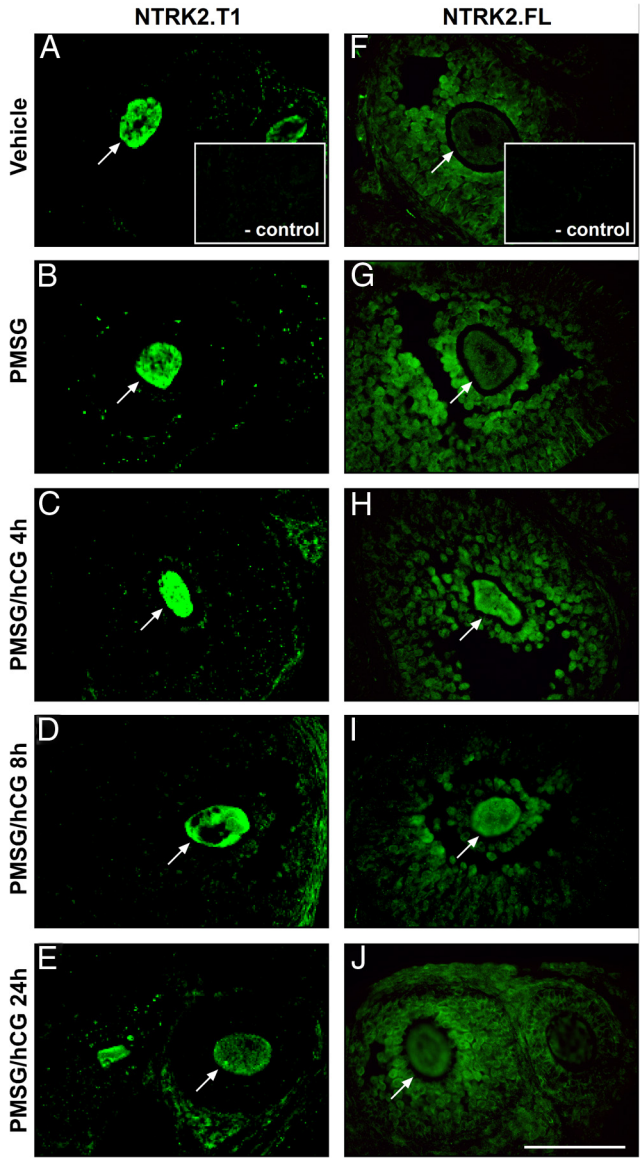


**Figure 4.** Preovulatory levels of gonadotropins induce expression of NTRK2.FL in mouse oocytes. Immunohistofluorescence detection of NTRK2.T1 and NTRK2.FL was performed in ovarian sections from 26-day-old WT mice injected ip on postnatal day 24 with saline (A and F), 7.5 IU of PMSG (B and G), or 7.5 IU PMSG followed 48 hours later by 5 IU of hCG (C–E, H–J ). No changes in the intensity of NTRK2.T1 immunostaining were detected in oocytes after gonadotropin stimulation at any interval examined (A–E). NTRK2.FL immunoreactivity did not change in oocytes after PMSG treatment alone (G), but increased noticeably 4 hours after hCG injection (H). This change was maintained 8h after hCG (I), but subsided by 24 hours (J). Inset in figures A and F are negative controls for the immunohistofluorescence reaction (sections incubated without first antibody). Images are representative from at least 6 sections per ovary and 3 animals per treatment. Scale bar, 200 $\mu$m.

but not *OoNtrk2*$^{-/-}$ oocytes, responded to PMSG/hCG stimulation with an increase in phosphorylated (Ser 473) AKT immunoreactivity 6 hours after hCG (Figure 5, C1–C3 versus Figure 3, D1–D3). Western blot of phosphorylated AKT in oocytes isolated 6 hours after hCG confirmed this finding (Figure 5C4). Phosphoinositide-dependent kinase 1 (PDK1) and AKT phosphorylate the ribosomal protein RPS6. Immunoreactive Ser235/236 phosphorylated RPS6 was abundant in oocytes of both *Ntrk2*$^{loxP/loxP}$/*Cre*$^{-}$ and *OoNtrk2*$^{-/-}$ mice treated with saline (Figure 5, E1 and F1). It remained elevated in *Ntrk2*$^{loxP/loxP}$/*Cre*$^{-}$ oocytes (Figure 5, E2 and E3) 6 hours after hCG, but was reduced in *OoNtrk2*$^{-/-}$ oocytes, as determined by both immunohistofluorescence (Figure 5, F2 and F3) and Western blot analysis (Figure 5E4). Non-phosphorylated RPS6 was also decreased in *OoNtrk2*$^{-/-}$ oocytes (Figure 5E4). The loss of phosphorylated RP6 was oocyte-specific, because phosphorylated RPS6 immunoreactivity increased at this time in the cumulus GCs of both groups (Figure 5, E3 and F3). In contrast to these AKT-related deficiencies, gonadotropins increased p42/44 MAPK (Erk1/2) phosphorylation equally well in *Ntrk2*$^{loxP/loxP}$/*Cre*$^{-}$ and *OoNtrk2*$^{-/-}$ oocytes as assessed by both immunohistofluorescence (Figure 5, G1–G3 versus H1–H3) and Western blot (Figure 5G4). Thus, the gonadotropin-dependent activation of AKT and RPS6, one of the downstream components of the PI3K/AKT signaling pathway, does not occur in the absence of NTRK2 receptors in oocytes.

We also examined three substrates negatively regulated by AKT: GSK3$\beta$ (glycogen synthase kinase beta), FOXO3a, and BAD. When these proteins are phosphorylated by p-AKT, apoptotic cell death is reduced (36). Immunoreactive phosphorylated GSK3$\beta$ increased in oocytes of *Ntrk2*$^{loxP/loxP}$/*Cre*$^{-}$, but not *OoNtrk2*$^{-/-}$ mice, 6 hours after hCG (Figure 6A1–A3; B1–B3), a difference that was also apparent in Western blots of oocyte proteins extracted at this time (Figure 6A4). As was the case of RPS6, nonphosphorylated GSK3$\beta$ content was also reduced in oocytes lacking NTRK2 receptors (Figure 6A4). BAD phosphorylation that suppresses BAD proapoptotic activity, also failed to increase after hCG in *OoNtrk2*$^{-/-}$ oocytes as compared with oocytes from *Ntrk2*$^{loxP/loxP}$/*Cre*$^{-}$ animals, as detected by immunohistofluorescence (Figure 6, C1–C3 vs D1–D3). Unexpectedly, the phosphorylated form of FOXO3a that lacks proapoptotic activity appeared to increase more in oocytes from *OoNtrk2*$^{-/-}$ mice than *Ntrk2*$^{loxP/loxP}$/*Cre*$^{-}$ controls after gonadotropins stimulation (Figure 6, E1–E3 versus F1–F3; Western blot data in E4). Together, these findings suggest that the preovulatory surge of gonadotropins activates an AKT/RPS6/GSK3$\beta$-mediated survival pathway in oocytes via NTRK2 receptors, but these receptors are not required for gonadotropins to activate p42/44 MAPK-mediated signaling in these cells.

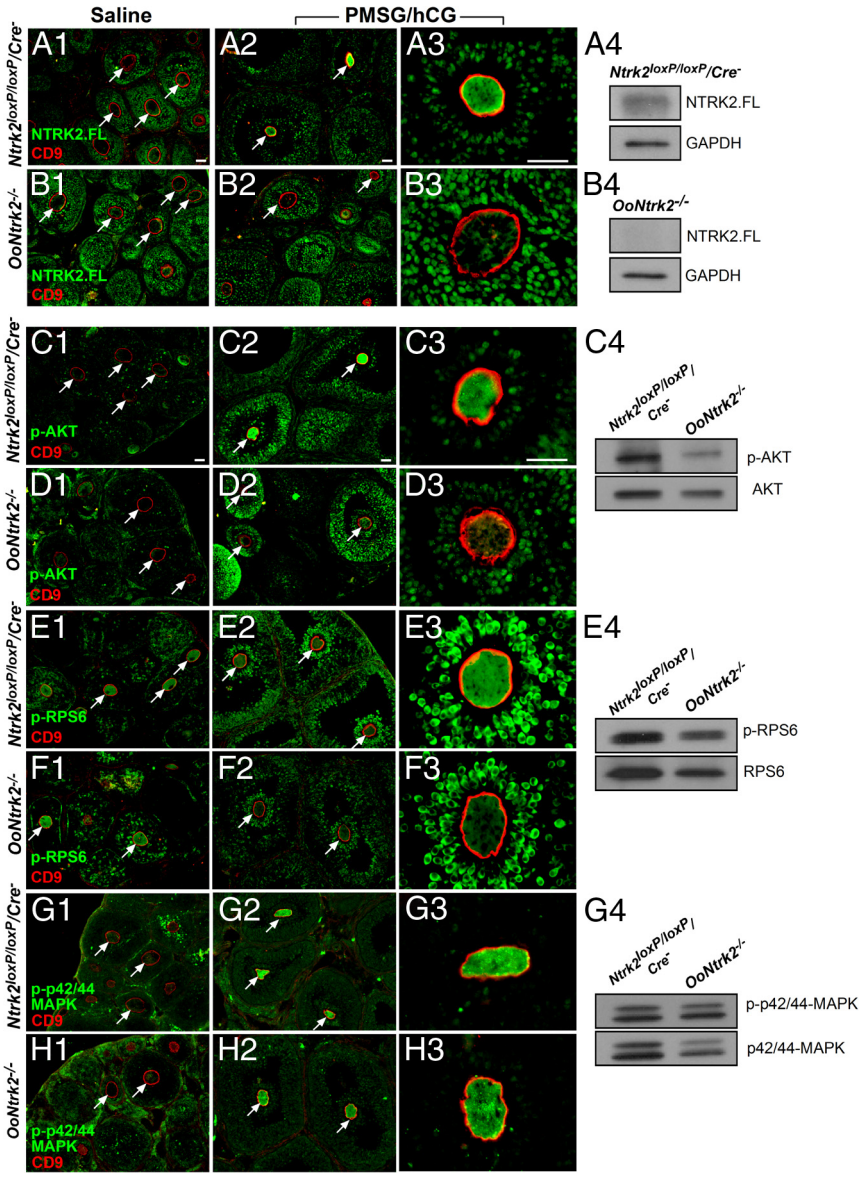


**Figure 5.** Gonadotropin-dependent activation of PI3K/AKT signaling in oocytes is mediated by NTRK2.FL receptors. The animals received PMSG on postnatal day 23 and hCG 48 hours later. The ovaries were collected 6 hours after hCG. (A1–A3) NTRK2.FL (*green*) in *Ntrk2*$^{loxP/loxP}$/*Cre*$^{-}$ oocytes (*red*). B1–B3, Absence of NTKR2.FL in *OoNtrk2*$^{-/-}$ oocytes. (A4) and (B4) Immunoblot detection of NTRK2.FL in isolated (A4) *Ntrk2*$^{loxP/loxP}$/*Cre*$^{-}$ oocytes and (B4) *OoNtrk2*$^{-/-}$ oocytes. C1–C3 and D1–D3, pAKT abundance increases in oocytes from *Ntrk2*$^{loxP/loxP}$/*Cre*$^{-}$ mice in response to gonadotropins, but not in *OoNtrk2*$^{-/-}$ oocytes. C4, Immunoblotting of pAKT in isolated oocytes. E1–E3 and F1–F3, P-RPS6 content also increases in *Ntrk2*$^{loxP/loxP}$/*Cre* oocytes, but not *OoNtrk2*$^{-/-}$ oocytes, in response to gonadotropins. E4, Immunoblotting of p-RPS6 in isolated oocytes. G1–G3 and H1–H2, Gonadotropins activate p42/44 MAPK phosphorylation in both *Ntrk2*$^{loxP/loxP}$/*Cre*$^{-}$ oocytes and *OoNtrk2*$^{-/-}$ oocytes. G4, p-p42/44 MAPK detected by immunoblotting. Images are representative of at least 6 sections per ovary and 3 animals per treatment. Scale bars, 50 $\mu$m.

## Ligand-dependent activation of truncated NTRK2 receptors and KISS1R is required for gonadotropin-dependent induction of full-length NTRK2 receptors in oocytes

We next determined if GC-derived substances are involved in the process by which gonadotropins induce NTRK2.FL expression in oocytes. We considered BDNF because: a) BDNF production by GCs is increased by preovulatory gonadotropin levels (10); b) BDNF binding to NTRK2.T1 receptors activates PLC/inositol-1,4,5-triphosphate-dependent calcium entry from extracellular sources (14); and c) elevated intracellular calcium levels increase *Ntrk2.FL* expression (39). We considered kisspeptin for similar reasons: kisspeptin is produced in GCs (40), the preovulatory LH surge increases ovarian kisspeptin production (16), and kisspeptin binding to KISS1R receptors results in PLC/ inositol-1,4,5-triphosphate-dependentcalcium mobilization (17). To test these peptides in a controlled system we used a cell line (C6) that, like oocytes (7, 8), expresses *Ntrk2.T1* mRNA abundantly and negligible amounts of *Ntrk2.FL mRNA* (Figure 7A, top gel images and bar graph), in addition to *Kiss1r* mRNA (Figure 7A, bottom gel image). BDNF or kisspeptin alone did not alter *Ntrk2.FL* mRNA abundance, but increased it in a calcium-dependent manner when added together (Figure 7B).

As shown by others (10, 40), GCs of antral follicles contain both BDNF and kisspeptin immunoreactive material (Figure 7C). The abundance of both peptides increases in luminal GCs and cells of the cumulus oophorus 6 hours after hCG (Figure 7C). Although *Bdnf* mRNA content increased marginally in GCs 6 hours after hCG (Figure 7D), *Kiss1* mRNA abundance increased significantly in GCs of both *Ntrk2*$^{loxP/loxP}$/*Cre*$^{-}$ and *OoNtrk2*$^{-/-}$ ovaries (Figure 7E). Oocytes from both groups of mice express *Kiss1r* mRNA (Figure 7F). NTRK2.FL immunoreactivity became detectable in *Ntrk2*$^{loxP/loxP}$/*Cre*$^{-}$ oocytes after PMSG/hCG, but not in oocytes of *Kiss1r* knockout mice that otherwise had intact NTRK2 receptors (Figure 7G), indicating that KISS1R signaling in oocytes is required for gonadotropins to induce NTRK2.FL expression in these cells.

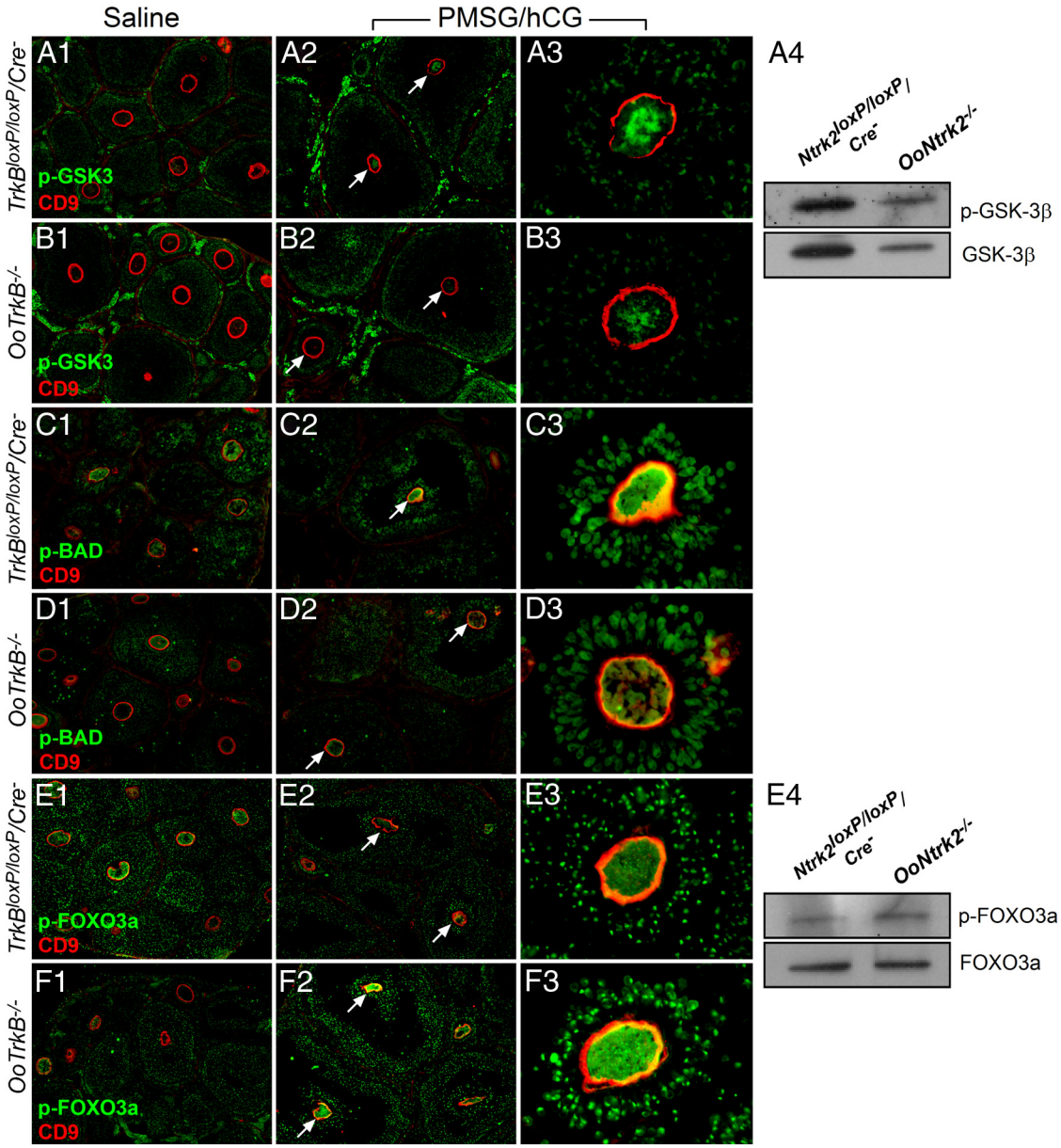


**Figure 6.** Gonadotropin-dependent activation of the AKT substrates GSK3$\beta$ and BAD in oocytes requires the presence of NTRK2 receptors. Oocytes were detected by staining with antibodies against the oocyte-specific membrane protein CD9 (*red*) in combination with antibodies to p-GSK3 (A1–B3; *green*), p-BAD (C1–D3; *green*) and p-FOXO3a (E1–F3; *green*). The immunoreaction was carried out using ovary sections from 26-day-old $Ntrk2^{loxP/loxP}/Cre^{-}$ and $OoNtrk2^{-/-}$ mice treated with saline (A1–F1) or 7.5 IU PMSG on day 24 followed 48 hours later by 5 IU of hCG (A2 and A3; B2 and B3; C2 and C3; D2 and D3, E2 and E3; F2 and F3). The last image of each row depicts a higher magnification view of representative oocytes after gonadotropin stimulation. The changes in protein abundance detected by immunohistofluorescence were verified by immunoblot. (A4) p-GSK-3$\beta$ and nonphosphorylated GSK-3$\beta$; (C4) p-BAD; (E4) p-FOXO3a and nonphosphorylated FOXO3a. Due to technical difficulties with the antibody used to detect nonphosphorylated BAD this blot is unavailable. The abundance of phosphorylated (p) GSK3$\beta$ and p-BAD increases in oocytes from $Ntrk2^{loxP/loxP}/Cre^{-}$ mice (A1–A3 and C1–C3, respectively), but not in oocytes of $OoNtrk2^{-/-}$ mice after PMSG/hCG treatment (B1–B3 and D1–D3, respectively). However, phosphorylation of FOXO3a, a canonical AKT substrate, increases more in oocytes of $OoNtrk2^{-/-}$ (F1–F3) than $Ntrk2^{loxP/loxP}/Cre^{-}$ (E1–E3) mice after gonadotropins stimulation. Images are representative of at least 6 sections per ovary and 3 animals per treatment. Scale bars, 50 $\mu$m.

## Discussion

The present study shows that gonadotropin levels of ovulatory strength provide a survival signal to the oocytes that remain in antral follicles of the ovary after extrusion of the oocytes selected for ovulation. This survival signal is mediated by NTRK2.FL receptors, whose expression is rapidly induced in oocytes (within 4 hours) by ovulatory levels of hCG. Activation of NTRK2.FL expression appears to require the concerted action of a novel, dual somatic cell-oocyte communication system operating collaboratively within the preovulatory follicle (Figure 8). Our in

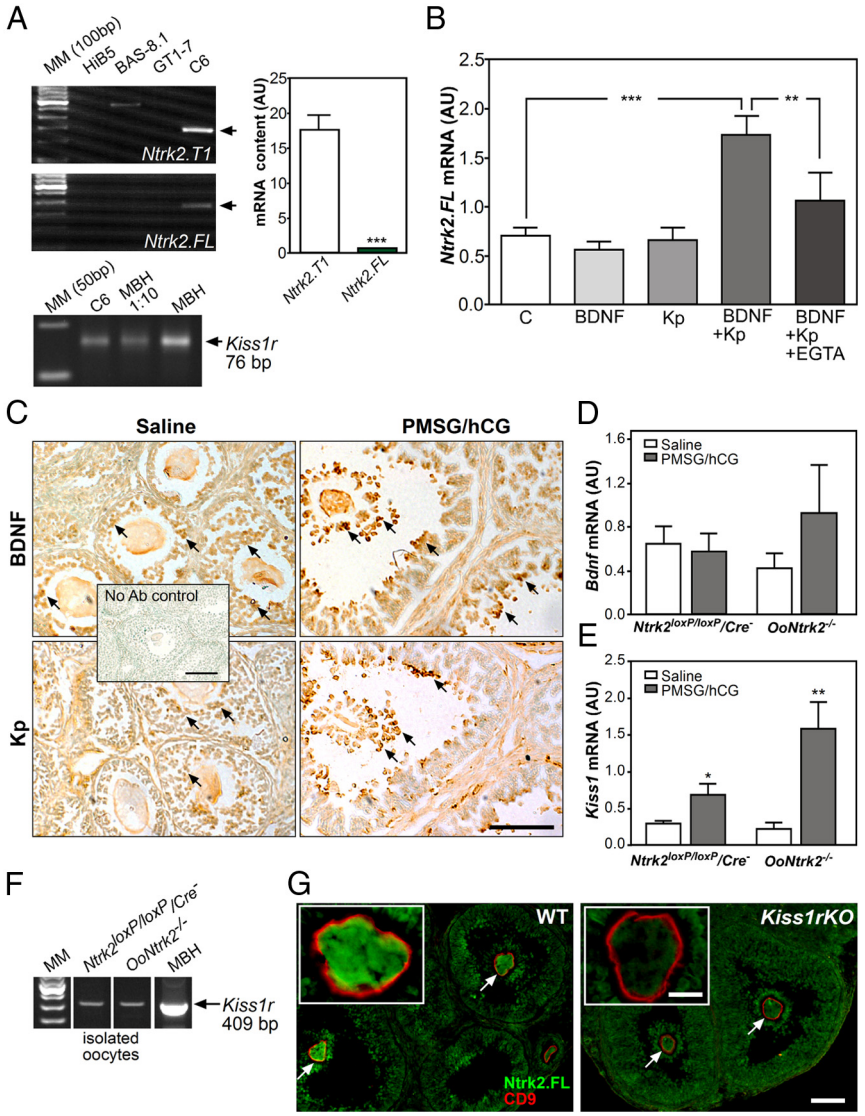


**Figure 7.** BDNF and kisspeptin (Kp) increase NTRK2.FL expression in C6 cells and KISS1R is necessary for gonadotropins to increase NTRK2 abundance in oocytes. A, *Top gel*, Detection of *Ntrk2.T1* and *Ntrk2.FL* mRNA by RT-PCR in C6 cells, but not in hippocampal progenitor HiB5 cells, astrocytic BAS-8.1 cells, and GnRH-producing GT1–7 cells. Bar graph: Relative abundance of *Ntrk2.FL* mRNA and *Ntrk2.T1* mRNA in C6 cells. *Bottom gel,* C6 cells contain *Kiss1r* mRNA; MBH, medial basal hypothalamus. B, BDNF and Kp together induce *Ntrk2.FL* mRNA expression; C, control, no treatment. Panel C, BDNF and Kp immunoreactivity in ovary sections from prepubertal mice treated with PMSG/hCG or saline. The ovaries were collected 6h after hCG. *Bdnf* (D) and *Kiss1* (E) mRNA levels in GCs isolated from *Ntrk2*$^{loxP/loxP}$/*Cre*$^{-}$ and *OoNtrk2*$^{-/-}$ mice treated with PMSG/hCG. F, *Kiss1r* mRNA in isolated oocytes; MBH, positive control. G, Detection of NTRK2.FL (*green*) in oocytes (CD9, *red*) of wild-type and *Kiss1r* null mice treated with PMSG/hCG. Insets: higher magnification image of oocytes. *, $P < .05$; **, $P < .01$; ***, $P < .001$. Scale bars, 50 $\mu$m in C and G; Scale bar, 25 $\mu$m (inset in G).

vitro results using a cell line that expresses both NTRK2.FL and NTRK2.T1 at levels similar to those seen in oocytes suggest that one arm of this system employs BDNF and the receptor NTRK2.T1 for signaling, and the other involves the neuroendocrine peptide kisspeptin and its receptor KISS1R. The physiological importance of this communication system is supported by several observations including the presence of both NTRK2.T1 and KISS1R in oocytes, the production of both BDNF and kisspeptin by GCs, the increase in *Kiss1* mRNA expression and kisspeptin content observed in GCs 6 hours after hCG, and the inability of gonadotropins to induce NTRK2.FL receptors in oocytes lacking either *Ntrk2* or *Kiss1r*. The importance of KISS1R signaling for ovulatory competence is underscored by the findings reported in a companion paper (18) showing that mice haplo-insufficient for *Kiss1r* develop a POF phenotype. This study also demonstrates that *Kiss1r* null mice fail to ovulate a normal number of oocytes in response to gonadotropins even after application of a protocol of intense ovarian stimulation consisting of GnRH priming followed by administration of PMSG and an ovulatory dose of hCG.

The lack of NTRK2 receptors in oocytes result in a reproductive phenotype similar to that caused by the premature activation of primordial follicles (27, 41). However, the underlying mechanism appears to be different. Whereas the premature activation of oocyte growth in primordial follicles results in infertility due to progressive depletion of the follicle pool (27, 41), the oocytes of *OoNtrk2*$^{-/-}$ mice grow normally before puberty, without alterations in the number of either primordial follicles or follicles entering the proliferative pool. Strikingly, loss of preantral (primordial, primary, and secondary) follicles was detected only after the loss of antral follicles was well under way, suggesting that the loss of preantral follicles and their oocytes is an event secondary to the demise of antral follicles. We have no firm explanation for this sequence of events, but speculate that antral follicles might provide a nurturing/permissive environment that helps maintain the structural integrity of primordial and primary follicles.

Remarkably, signaling via catalytically active NTRK2.FL is initiated in earnest only after the first preovulatory gonadotropin surge. Before this time expression of

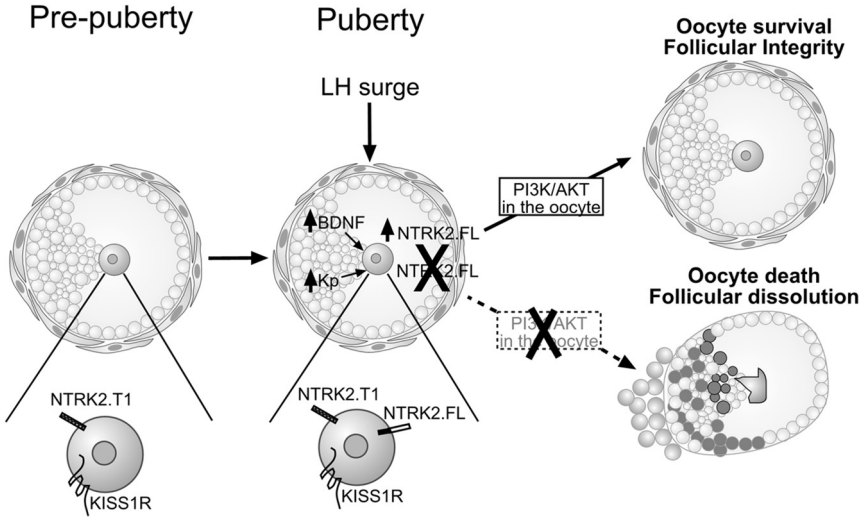


**Figure 8.** A GC-oocyte communication pathway postulated to promote oocyte viability in postpubertal ovaries after the first ovulation. According to this model, oocytes before puberty only express NTRK2.T1 receptors; they might, however, express KISS1R. BDNF and kisspeptin (Kp) production by GCs is increased by the preovulatory surge of gonadotropins; each ligand then binds to its respective receptor expressed in oocytes (NTKR2.T1 and KISS1R, respectively) to induce the formation of full-length catalytically active NTRK2 receptors (NTKR2.FL) in oocytes. Upon ligand-dependent activation NTRK2 receptors set in motion a PI3K-AKT-mediated survival pathway that maintains oocyte viability and ensures recurrent follicular development. In the absence of NTRK2.FL this does not occur, oocytes die and follicles disintegrate surrounded by an abnormal expanded interstitial tissue composed of steroidogenically active, luteal-like cells.

NTRK2.FL is nearly absent in oocytes. The postpubertal manifestation of a POF phenotype in *OoNtrk2*$^{-/-}$ is remarkably similar to that observed in *Kiss1r* haplo-insufficient mice (18). Whereas the morphological features, progression, and completion of the ovarian phenotype are identical in both cases, the time course is different with ovarian function declining much more rapidly in *OoNtrk2*$^{-/-}$ than *Kiss1r*$^{+/-}$ mice. This is likely due to a difference in severity of the underlying deficiency as in the former NTRK2.FL-mediated signaling fails to become operational in oocytes at puberty, whereas in the later KISS1R signaling, required for gonadotropins to activate Ntrk2 expression in oocytes at puberty, is only partially reduced.

In the absence of NTRK2.FL receptors gonadotropins are unable to activate the well-established oocyte PI3K/AKT-mediated survival pathway (36). *OoNtrk2*$^{-/-}$ oocytes fail to respond to gonadotropins with phosphorylation of either AKT or the downstream S6K substrate RPS6. Both proteins are essential for oocyte survival as widespread oocyte death occurs in the ovaries of mice lacking *Akt1* or *Rps6* (42, 43). By phosphorylating the downstream substrates GSK3$\beta$, BAD and FOXO3a, AKT reduces the proapoptotic activity of these proteins (36). Consistent with the lack of AKT activation, *OoNtrk2*$^{-/-}$ oocytes fail to respond to gonadotropins with an increase in either GSK3$\beta$, or BAD phosphorylation, but paradoxically showed an increase in phosphorylated FOXO3a. This may be due to activation of a yet-to be defined compensatory mechanism.

Because oocyte survival does not require NTRK2.FL receptors before the first preovulatory surge of gonadotropins, the question arises as to why would a NTRK2-mediated survival signal be needed after a preovulatory gonadotropin surge? A potential explanation is that such a signal may be needed to counteract the loss of cAMP antiapoptotic actions (44) that may result from the decrease in cAMP concentrations induced by the LH surge in oocytes, and that is required for meiotic resumption (45).

The reproductive phenotype of *OoNtrk2*$^{-/-}$ mice characterized by adult onset of infertility, progressive postpubertal depletion of oocytes accompanied by loss of follicular structure is similar to that of women with POF. Interestingly, fast progression of the phenotype in about half of *OoNtrk2*$^{-/-}$ mice results in ovaries devoid of follicles, mimicking the human condition known as afollicular POF (46). On the other hand, the persistence of antral follicles in *OoNtrk2*$^{-/-}$ mice with slow progression of the phenotype yields a condition similar to the POF subtype known as follicular POF (46).

In contrast to humans, circulating plasma FSH levels were not significantly elevated in *OoNtrk2*$^{-/-}$ mice even at 22 weeks of age (the latest age studied). However, this difference may be more apparent than real because dysregulation of FSH secretion may be a late event of the disorder. This possibility is supported by the observation that FSH levels are significantly elevated only by 48 weeks of life in *Kiss1r* hypomorph mice (18). It is plausible, therefore, that an elevation in plasma FSH levels may become discernible in older *OoNtrk2*$^{-/-}$ mice. The lack of an early increase may be related to the abundance of steroidogenically active luteal-like interstitial cells that begin occupying the ovarian parenchyma of *OoNtrk2*$^{-/-}$ mice as early as 8 weeks of life, becoming the predominant feature of these ovaries by 22 weeks of age. Androgens presumably produced by these cells may be able to maintain LH and FSH levels from rising as follicle structure deteriorates.

By defining a novel and entirely unsuspected role for the neurotrophin receptor NTRK2 in the control of oocyte biology, our findings have broad physiological and clinical implications, as they provide a new understanding of the molecular mechanism underlying oocyte survival dur-

ing adult reproductive life. The potential contribution of alterations in NTRK2 signaling to the human disorder is suggested by the detection of sequence variation in the BDNF gene as a significant risk factor for POF (47).

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