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Control of Murine Primordial Follicle Growth Activation by IκB/NFκB Signaling

Clyde J. Wright, M.D.[1,2,*], **Evelyn Llerena Cari, M.S**[1,3], **Jeryl Sandoval., B.S.**[1,2], **Elise Bales, B.S**[1,3], **Peter Ka Sam, B.S.**[1,3], **Miguel A. Zarate, Ph.D.**[1,2], **Alex J. Polotsky, M.D.**[1,3,4], **Amanda Kallen, M.D.**[5], **Joshua Johnson, Ph.D.**[1,3,4,*]

[1]University of Colorado School of Medicine, Aurora, CO, USA

[2]Section of Neonatology, Department of Pediatrics

[3]Department of Obstetrics and Gynecology, Division of Reproductive Sciences

[4]Department of Obstetrics and Gynecology, Division of Reproductive Endocrinology and Infertility

[5]Department of Obstetrics & Gynecology, Yale School of Medicine, New Haven, CT, USA

Abstract

The transcription factor NFκB has been associated with the timing of menopause in a large human genome-wide association study. Furthermore, preclinical studies demonstrate that loss of *Tumor necrosis factor alpha (Tnfα)* or its receptor *Tnfr2* slows primordial follicle growth activation (PFGA). Although Tnfα:receptor signaling stimulates NFκB and may mechanistically link these findings, very little is known about NFκB signaling in PFGA. Because signaling downstream of Tnfα/Tnfr2 ligand/receptor interaction has not been interrogated as relates to PFGA, we evaluated the expression of key NFκB signaling proteins in primordial and growing follicles, as well as during ovarian aging. We show that key members of the NFκB pathway, including subunits, activating kinases and inhibitory proteins, are expressed in the murine ovary. Furthermore, the subunits p65 and p50, and the cytosolic inhibitory proteins IκBα and IκBβ, are present in ovarian follicles, including at the primordial stage. Finally, we assessed PFGA in genetically modified mice (AKBI) previously demonstrated to be resistant to inflammatory stress-induced NFκB activation due to overexpression of the NFκB inhibitory protein IκBβ. Consistent with the hypothesis that NFκB plays a key role in PFGA, AKBI mice exhibit slower PGFA than wild-type (WT) controls, and their ovaries contain nearly twice the number of primordial follicles as WT

[*]For correspondence: clyde.wright@cuanschutz.edu; joshua.2.johnson@cuanschutz.edu.

Author Contributions:

CJW, AJP, AK, JJ conception and design of research; CJW, ELC, JS, EB, PKS, MAZ, JJ performed experiments; CJW, ELC, JS, EB, PKS, MAZ, AK, JJ analyzed data; CJW, ELC, JS, EB, PKS, MAZ, JJ interpreted results of experiments; JJ drafted the manuscript; CJW, ELC, JS, JJ prepared figures; CJW, JJ edited and revised manuscript; CJW, ELC, JS, EB, PKS, MAZ, AJP, AK, JJ approved final version of manuscript.

Joshua Johnson, Ph.D., Assistant Professor, Division of Reproductive Sciences, Department of Obstetrics and Gynecology, University of Colorado Denver (AMC), Building RC2, Room P15 3103, Mail Stop 8613, Aurora, Colorado 80045, Office: 303.724.7281, Mobile: 203.889.8123

Declaration of interest

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both at early and late reproductive ages. These data provide mechanistic insight on the control of PFGA and suggest that targeting NFκB at the level of IκB proteins may be a tractable route to slowing the rate of PFGA in women faced with early ovarian demise.

Keywords

Ovary; Ovarian Aging; NFκB Signaling; Menopause

Introduction

A key determinant of the length of time that ovaries function during adult life is the *rate* at which primordial follicles leave their dormant state and begin to grow (primordial follicle growth activation, PFGA)[1, 2]. The ovaries of women in their thirties usually contain tens of thousands of primordial follicles (e.g., the ovarian "reserve"). A small number of primordial follicles undergoes PFGA daily, supplying the ovary with its population of growing follicles [3, 4]. Once committed to growth, the vast majority of follicles die in a process referred to as atresia [5, 6]. This results in the single dominant follicle (in "monovular" species including humans) or, follicles (in "polyovular" species including mice), that survive to ovulate a mature, fertilization-competent egg(s).

Exhaustion of the primordial reserve results in the cessation of ovarian function, and in humans, this is termed the menopause. The median menopausal age in women is 51 years [7]. When ovaries begin to fail before age 40, it is termed "primary ovarian insufficiency (POI)" [7, 2, 8]. POI affects approximately 1 in 250 women under age 35, and 1% of all women under age 40. Approximately 5% of women experience ovarian failure between ages 40 and 45 years, while outside the definition of POI [8]. Beyond the loss of fertility, ovarian demise results in significant and costly health and well-being compromises. Cardiovascular health [9–11], bone density, muscle mass, body fat composition, and self-reported well-being measures [12] all worsen upon ovarian failure. Specifically with regards to cardiovascular health, lower age of menopause and time since the start of menopause are independently associated with poorer carotid artery thickness and aortic stiffness [10, 11]. If afflicted with POI, women face years and possibly decades of increased risk for these problems and their treatment limitations [13]. A major goal of women's health research is to develop interventions that slow the rate of PFGA and thereby delay or prevent POI.

A key known negative physiological regulator of PFGA is Antimüllerian hormone (Amh), a protein that can also be used to indirectly estimate the ovarian reserve [14, 15]. *Amh* knockout mice [14] were found to display an acceleration of PFGA, characterized by a more rapid decline in primordial follicle number and an increase in growing follicles at different postnatal time points. More recently and conversely, Amh delivery to mice as purified recombinant protein, or, using a gene therapy approach, resulted in reduced PFGA and in the latter case, ovarian "quiescence" [16]. Another negative regulator of PFGA is the lipid phosphatase Phosphatase and tensin homolog deleted on chromosome ten (Pten) [17–19]. Blocking Pten activity accelerates PFGA in mouse models *in vivo* and also in human primordial follicles in experimental clinical interventions [20, 21]. Pten is a key upstream

negative regulator of pro-growth nutrient-responsive Akt/mTOR signaling, which has been shown to regulate PFGA at two levels. First, Akt has been shown to phosphorylate the transcription factor Forkhead box protein O3 (FoxO3), and prevent its nuclear localization [22, 23]. FoxO3 has been shown to be a powerful negative regulator of the rate of PFGA [22], and its inactivation by Akt is thus consistent with growth. Second, mTOR action upon downstream factors 4-EBP and p70S6K result in positive regulation of protein synthesis, and the cell cycle, respectively [17, 23]. Pten's action upstream of each event favors blocked PFGA.

Tnfα signaling through the receptor Tnfr2 has shown to be a key positive regulator of PFGA][24, 25]. While the primordial reserve of follicles did not differ between *Tnfr2* knockout (*Tnfr2KO*) animals and wild-type (WT) controls early in postnatal life, mutant ovaries harbored significantly greater numbers of primordial (and small growing) follicles later in life. That outcome can be interpreted as resulting from a slowed rate of PFGA, revealing the positive regulatory action of Tnfr2 and Tnfα. Since these initial reports, no further mechanistic information about Tnfα:Tnfr2 action has been produced. These data are again relevant due to a recent GWAS analysis of nearly 40,000 patients, where the Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signaling pathway was identified as strongly associated with the timing of menopause in women [26]. Because TNFα is known to stimulate NFκB signaling, and TNFα is a well-recognized NFκB target gene, it is reasonable to hypothesize that NFκB signaling may play a role in PFGA.

The signaling pathway leading to NFκB activation following TNFα has been well characterized. In quiescent cells, members of the IκB family of inhibitory proteins (IκBα and IκBβ bind to NFκB dimers in the cytoplasm, preventing their nuclear translocation and subsequent DNA binding [27]. The expression level of the IκB family of inhibitory proteins is the predominant force preventing NFκB nuclear translocation and subsequent target gene expression. TNFα binding to its receptor leads to the activation of I kappa B kinases (IKK), resulting in IκB phosphorylation [28]. This signaling event inactivates the inhibitory potential of the IκBs by targeting them for degradation, allowing NFκB subunit release to the nucleus and subsequent DNA binding and altered gene transcription [27].

Some information is available about NFκB pathway action in cells of the ovary and in ovarian physiology. In an early study, Xiao et al. showed that NFκB activity, as measured by nuclear NFκB subunit translocation, induced expression of the anti-apoptotic gene Xiap in rat granulosa cells.[29] This pro-survival NFκB response occurred in the presence of Tnfα. Signaling through EGR1 (early growth response 1) and its impact on NFκB activation has also been implicated in granulosa cell survival [30]. Similarly, NFκB activation was again found to regulate cell survival in primary granulosa cells collected from large bovine follicles [31]. In that case, NFκB activity was shown to correspond to the amount of estradiol, where high estradiol was associated with higher amounts of NFκB activity and fewer apoptotic granulosa cells. Further linking sex hormones and ovarian NFκB signaling, repression of estrogen receptor beta gene transactivation by NFκB was reported using granulosa cell lines [32]. To date, NFκB signaling has mostly been evaluated in terms of its action regulating granulosa cell death (and thereby follicle atresia), and regulating gene

expression in coordination with steroid hormones [32, 33, 31] in order to guide follicle development and survival [34].

In this study, we have evaluated the expression of key NFκB pathway members in the mouse ovary, with special emphasis on potential expression within primordial follicles. We show that key NFkB pathway proteins are expressed in primordial and also growing follicles, We have also evaluated the ovarian (and ovarian aging) phenotype of "A Knockout B Knock-In" (AKBI) mice that are resistant to pro-inflammatory stress induced NFκB activation [35, 36]. This included a comparison of the expression of Amh and Pten, between AKBI ovaries and wild-type controls. . Furthermore, we demonstrate that AKBI mice have a similar ovarian phenotype as reported for the *Tnfr2* knockout mouse [24]. This manifests in an increased primordial follicle "reserve" at multiple postnatal time points versus WT controls. Overall, our data fill in gaps in our knowledge of signaling that occurs downstream of Tnfr2 activation that favor PFGA, and suggest that selective modulation of IκB activity may be therapeutically useful in the treatment of POI.

Materials and Methods

Animals and Tissue Collection

ICR mice were purchased from Taconic, and "A knockout/B knock-in" (AKBI) mice, ICR background, were a gift of Richard Cohen (Harvard University). In AKBI mice, the IκBα gene has been replaced by IκBβ cDNA, the expression of which is then driven by the native IκBα promoter [37]. Thus, these mice overexpress IκBβ and do not express IκBα. Animals were handled and euthanasia was performed under an approved animal protocol at University of Colorado (#00457).

Livers and ovaries from female mice were collected at postnatal days 11, 25, 60–65, at mid/late time point in reproductive life (six months) and at 48 weeks. Ovaries were optionally fixed in Dietrich's fixative for histomorphometric analysis of follicle numbers, or, in 4% paraformaldehyde for immunohistochemical staining. Where indicated, ovaries were collected and snap-frozen in liquid nitrogen for later use in gene expression experiments.

Quantitative RT-PCR

30 mg of frozen ovarian tissue was digested using 0.5mm RNase free zirconium oxide beads (Next Advance, ZrOB05) and RNeasy Plus Mini Kit (Qiagen, Cat No 74134 or 74136) according to manufacturer's instructions. Tissue dissection was performed for 3 min at 4°C, speed 8 using bullet blender. RNA was assessed for purity and concentration using a NanoDrop Spectrophotometer. cDNA was synthesized using Verso cDNA Synthesis Kit (Thermo Scientific, Cat No AB-1453/B). Relative mRNA levels were evaluated by quantitative real-time PCR using exon spanning primers normalized to 18S RNA levels using the StepOnePlus Real Time PCR System (Applied Biosystems). Each sample was run as three times providing technical replicates for each sample for each gene. Predesigned TaqMan exon-spanning primers used were nf kbia: Mm00477798_m1, nfkbib: Mm00456849_m1 and tnfa: Mm00443258_m1. Relative quantitation was performed *via* normalization to the endogenous control 18S using the cycle threshold (ΔΔCt) method.

Western Blot Analyses

Protein isolation from whole ovaries was performed by using TPER Tissue Protein Extraction (Thermo Scientific, Prod # 78510). Protease Inhibitor Cocktail (Sigma, P8340–1ML) and Phosphatase Inhibitor Cocktail (Calbiochem, Cat No 524625) were each added in a 1:100 dilution. Tissue digestion was performed by agitation with stainless steel beads (SSB14B-RNA) for 5 min at speed 12 at 4°C in a bullet blender. Supernatant was recovered and the Bradford assay was performed for protein quantification. Supernatants were electrophoresed on a 4–12% polyacrylamide gel (Invitrogen), and proteins were transferred to a HyBond PVDF membrane (Amersham). Membranes were blotted with antibodies against the following proteins: IκBα (Cell Signaling, Cat No 4814), IκBβ (R&D, Cat No AF5225), IκBε, (Cell Signaling, Cat No 9249), p65 subunit (Cell Signaling, Cat No 8242), p50 subunit (Abcam, Cat No ab7971), c-Rel (Cell Signaling, Cat No 12707), Ikkα (Cell Signaling, Cat No 2682), Ikkβ (Cell Signaling, Cat No 2370), Gapdh, (Cell Signaling, Cat No 5174), Tnfα (Abcam, Cat No ab9379), and Amh (Fisher Scientific, Cat No PA5–35851). Blots were imaged using the LiCor Odyssey imaging system and densitometric analysis was performed using ImageStudio (LiCor).

Colorimetric immunohistochemical staining

Ovarian samples were embedded in paraffin. Paraffin cross sections of ovaries were used to localize the expression of IκBα (abcam, Cat No ab32518), IκBβ (abcam, Cat No ab7547), p65 subunit (Cell Signaling, Cat No 8242), p50 subunit (Abcam, Cat No ab7971), Amh (Fisher Scientific, Cat No PA5–35851). Briefly, after deparaffinization and rehydration, antigen retrieval was performed by incubating the sections in 10 mM citrate buffer pH 6 at 120°C for 30 min. Endogenous peroxidase blocking was performed by using 3% hydrogen peroxide (Labchem, Cat # LC154301). Sections were then washed in water, then in PBS containing 0.05 % Tween 20 (0.05% TBST). Tissue sections were blocked in 1% (wt/vol) bovine serum albumin plus 10% normal goat serum at room temperature (RT) for 90 min. After blocking, the tissues were incubated overnight at 4°C with primary antibody in a humid chamber. After three washes 0.05 % TBST, sections were incubated for 30 min goat anti-rabbit (abcam 6721). Peroxidase treatment was performed using ABC Kit (Vectastain, PK6100) for 30 min at RT. For detection, DAB (Vector Labs, SK-4100) treatment was performed. Nuclei were counterstained with Harris hematoxylin for 2 min. Following rinse, dehydration was performed and slides were mounted in Permafluor (Fisher Scientific, Cat No SP15–500). Negative control sections were submitted to the same procedures, except that the first antibody was replaced by blocking solution. Pictures were taken using Olympus microscope (Model: CKX41SF, SN:2B77130)

Histomorphometric analysis of follicle numbers

Ovaries fixed in Dietrich's fixative for a minimum of 12 h at 4°C, then transferred to 70% ethanol. Ovaries were embedded in Formula R (Leica Biosystems, PR 3801470). Serial 8 micron sections were cut using a microtome (Leica, Model: RM2235, SN: 6334/01.2012), and mounted in order onto positively-charged glass slides (Fischer Scientific, Cat No 12-550-15). Sections were melted for 1 h at 62 °C, deparaffinized, dehydrated, stained with Weigert's Iron Hematoxylin and counter-stained with Picric acid/Methyl Blue. Cover slip

(Fisher Scientific, Cat No 12–545-M) was applied using Permount (Fisher, Cat No SP15–500). After specimen blinding as to genotype, every 5th section was evaluated and follicle classes and *corpora lutea* were counted per Uslu et al (2017).

Statistical Analysis

For comparison between treatment groups, statistical analyses were performed using the statistical programming language R [38] or optionally, GraphPad Prism. The null hypothesis that no difference existed between treatment means was tested using Student's *t*-test, with statistical significance defined as $p<0.05$.

Results

Key members of the NFκB signaling cascade are expressed in the mature ovary

Our first objective was to evaluate NFκB pathway expression in adult (60–65 day old) WT ovaries by Western blot (Fig. 1) . We used liver tissue lysates as positive controls. We found that the ovary expressed the NFκB subunits cRel, p65 and p50 at levels similar to or even exceeding hepatic expression. Additionally, the we detected ovarian expression of the activating kinases IKKα and IKKβ, as well as the cytosolic inhibitory protein IκBα, IκBβ, and IκBε. Expression of IKKα was lower in ovarian compared to liver lysates, but longer exposure confirmed presence of IKKα in ovarian lysates (Fig. 1, inset). These results demonstrate that the key components of the NFκB signaling cascade are expressed in the ovary.

Our next objective was to localize NFκB pathway expression in adult (60–65 day old) WT ovaries by colorimetric immunohistochemical staining (IHC). Critically, the key members of NFκB signaling are expressed by primordial oocytes and pregranulosa cells within primordial follicles. (IκBα and IκBβ shown in Fig. 2A, B; p50, p65, shown in 2C, D; negative control where primary antibody was omitted shown in Fig. 2E). Because pregranulosa cells are so closely apposed to their enclosed primordial oocyte, we compared NFκB pathway staining to that of FoxO3, which is oocyte-specific in murine primordial follicles (inset, Fig. 2E) [39]. We found that the nucleo-cytoplasmic signal detected in pregranulosa cells for the NFκB pathway proteins (insets, Fig. 2A–D) was lacking in sections stained for FoxO3 (inset, Fig. 2E), supporting *bona fide* IκBα/β, p65 and p50 expression in those cells as well as primordial oocytes. These results demonstrate that the key components of the NFκB signaling cascade are expressed in follicles, including within arrested primordial follicles.

Ovaries from both WT and AKBI mice express NFκB signaling cascade members

Given our interest in learning more about NFκB signaling in the ovary, we took advantage of the genetically modified AKBI murine model. These mice over-express IκBβ and are known to be resistant to inflammatory stress signaling [35]. Protein expression analysis of WT and AKBI whole-ovary lysates from adults (60–65 day old) (Fig. 3) confirmed that IκBα was absent and IκBβ expression was enhanced in ovaries of the AKBI mutant. We found that the NFκB subunits cRel, p65 and p50, and activating kinases IKKα and IKKβ were all

expressed in the AKBI ovary. These results demonstrate that the key components of the NFκB signaling cascade are maintained in the AKBI ovary.

Ovarian levels of the NFκB inhibitory proteins IκBα and IκBβ decrease with age

Next, we asked if ovarian levels of the key NFκB inhibitory proteins IκBα and IκBβ decreased with age, a finding that would be consistent with increased NFκB activity. We found that in the WT ovary, levels of IκBα (Fig. 4A and C) and IκBβ (Fig. 4A and D) decreased with advancing age. Importantly, while levels of IκBα were significantly lower at days 25 and 60 compared to day 11 ($p<.05$), there was no difference between levels at day 25 and 60 (Fig. 4A and C). In contrast, levels of IκBβ were significantly lower at days 25 and 60 compared to day 11 ($p<.05$), and there were significant differences between levels at day 25 and 60 ($p<.05$), suggesting ongoing and active NFκB signaling (Fig. 4A and D). In the AKBI ovary, levels of IκBβ were significantly lower at days 25 and 60 compared to day 11 ($p<.05$), however levels stabilized between day 25 and 60 (Fig. 4B and D). These results suggest less active NFκB signaling in the AKBI ovary, consistent with their demonstrated reduced NFκB response [35].

Expression of key regulators of PFGA are decreased in AKBI compared to WT ovary

Next, we asked whether expression levels of key regulators of PFGA might be altered in the AKBI ovary versus WT controls (Fig. 5). First, we interrogated expression of levels of the Tnfr2 ligand (and putative positive PFGA regulator) and NFκB target gene Tnfα (*Tnf*).[24] In whole ovary of adults (60–65 days), *Tnf* mRNA did not differ in whole ovaries at any of three postnatal time points (Fig. 5A), but trended lower in AKBI. Importantly, both the precursor and mature secreted forms of TNFα protein were significantly lower ($p<.05$) in AKBI ovaries (Fig. 5B–D). While *Tnf* mRNA trended lower in whole ovaries of AKBI compared to WT mice, the difference did not reach significance (Fig. 5A). However, we did note that TNFα protein was significantly lower ($p<.05$) in AKBI ovaries (Fig. 5B–D). There are multiple reasons that may explain the difference in the mRNA and protein findings, including an underpowered mRNA experiment or decreased translation of *Tnf* mRNA in AKBI. These mechanisms deserve to be interrogated, but importantly expression of the active protein was lower in AKBI ovary, and consistent with decreased TNFα protein expression the mRNA trended lower.

Next, we interrogated PI3K/Pten/Akt/mTOR signaling in ovaries of adult (60–65 days) WT and AKBI mice. Both the ratio of phospho-Akt Ser473 to total Akt (Fig. 6A and B) and total Pten (Fig. 6A and C) protein are diminished in the AKBI ovary compared to controls ($p<.05$). In contrast to these proteins, levels of the negative PFGA regulator AMH did not significantly differ (Fig. 6A and D) [14, 15].

Follicle pool and litter size differ between WT and AKBI mice over aging

After being blinded to specimen genotype, we next quantified numbers of primordial follicles, growing follicles, atretic follicles, and *corpora lutea*. Our initial focus was upon primordial and primary (newly growth-activated) follicles to determine if the rate of PFGA was altered in AKBI animals, in accordance with the gene expression changes detected and as seen previously in the *Tnfr2* knockout [24].

No significant difference was seen in the number of primordial follicles at pn8 (Fig. 7A, left). Primordial follicles were significantly higher in number in AKBI mice at 9.4 weeks of age ($p<.05$) (Fig. 7A, center) and at 24 weeks of age ($p<.05$) (Fig. 7A, right). By 48 weeks the number of primordial follicles was not significantly different. Numbers of primary follicles were not significantly different between AKBI and WT control ovaries at 9.4 weeks (not shown). Accordingly, the ratio of primary to primordial follicles did significantly differ ($p<.05$) (Fig. 7B) with AKBI animals displaying nearly two-thirds lower ratio between these follicle classes at this time point. These data are consistent with a slower rate of PFGA in AKBI mice, as found in the *Tnfr2* knockout mouse [24]. In terms of further follicle development, no differences were detected in the numbers of intact growing secondary, small preantral, or antral follicles, nor was there any difference in the numbers of atretic follicles as determined by the number of pyknotic granulosa cell nuclei and/or visibly degenerating oocytes [40].

Despite the lack of significant differences in the primordial pool at 48 weeks, AKBI mice did demonstrate extended fecundity in retrospective breeding data of animals between 8 weeks and nearly one year of age ($n>=25$ litters per genotype). Anecdotally, WT mice had been identified as producing fewer litters with fewer pups earlier than AKBI females. Stratifying breeding outcomes by maternal age “bins” revealed that prior to 15 weeks of age, AKBI females produced litters of significantly fewer pups than WT females (Fig. 8). Numbers of pups per litter did not differ in subsequent age bins, until animals reached 40 weeks of age ($p<.05$). While zero of four WT ICR mice delivered a litter between 40 and 43 weeks of age, all four AKBI mice delivered pups within that time frame. Remarkably, no significant difference was seen between AKBI litter size in animals less than 15 weeks of age and greater than 40 weeks of age. Overall, these data suggest that enhanced IκBβ activity in the absence of IκBα can slow the rate of PFGA mid-life and extend the duration of fecundity late in life.

Discussion

Key findings about the role of NFκB signaling in early mouse follicle development are summarized as follows. The ovary expresses the key components of the NFκB signaling cascade. Inhibitory proteins IκBα and IκBβ, as well as NFκB subunits p50 and p65 are expressed in pregranulosa cells and primordial oocytes within primordial follicles. The levels of the NFκB inhibitory proteins IκBα and IκBβ are dynamic over life (Fig. 4), decreasing as the animal ages. These changes are blunted in the AKBI mouse ovary, and consistent with decreased NFκB activity we found that Tnfα levels are significantly lower in AKBI ovaries compared to WT controls (Fig. 5). A summary cartoon of these findings is in Fig. 9. In the ovary of AKBI mice, there is relative overexpression of IκBβ when compared to WT control (Fig. 3 and Fig. 9A). This is due to the IκBα gene being replaced by IκBβ cDNA in AKBI mice [37]. Our data suggest that as the WT animal ages, NFkB signaling becomes permissive as total ovarian levels of IκB decrease (Fig. 9B). This is consistent with increased TNFα and accelerated PFGA. In contrast, and due to blunted signaling resulting from IκBβ overexpression, the increase in TNFα in the AKBI ovary is attenuated, leading to delayed PFGA (Fig. 9B). Because NFκB signaling can both drive TNFα expression and can be activated by TNFα[27, 41, 42], these data are also consistent with the phenotype of the

Tnfr2 knockout mouse [24]. Blinded histomorphometric analysis of juvenile, adult, and aged ovaries showed that PFGA is significantly slowed during the reproductive period, at least through 6 months of age (Fig. 7). Together, these data begin to fill in the gap in our understanding of regulation of the rate of PFGA downstream of Tnfα/Tnfr2.

Because Pten has been shown to be an important negative regulator of Akt/mTOR signaling and PFGA [18, 19], we interpret the nearly halved net downstream Akt activity in mutant ovaries (Fig. 6) as the key measure consistent with slowed PFGA. Interestingly, reduced Pten stability has been associated with increased Pten enzyme activity [43], and this may explain when Pten levels are lower in AKBI whole ovaries than WT controls (Fig. 6). Because AMH levels were unchanged in mutant ovaries (Fig. 6), we can attribute the slowed rate of PFGA to signaling independent of, or, downstream of that well-known negative regulator. More work is needed to determine how the involved signals are integrated so that PFGA can occur i) acutely in individual primordial follicles and ii) across the entire population of primordial follicles at a rate that supports ovarian function across a female's reproductive lifespan. In addition, more work is needed to determine why the primordial pool of follicles "normalizes" relative to WT controls at the latest time point evaluated. While the PFGA rate is slowed during mid-life (at least through 6 months), a net gain of follicles later in life was not maintained. Altered NFκB signaling during aging is at least responsible for an altered pattern of ovarian aging, if not an increased pool of primordial follicles after 48 weeks.

While our data are in accordance with human GWAS (genome-wide association study) data that identified NFκB signaling as being involved in ovarian aging [26], and we have shed some light on the role of the IκB proteins in this process in our mouse model, there are some limitations of the work to consider. First, while NFκB subunits p50 and p65 were consistently noted in the nuclei of primordial follicles (both primordial oocytes and pregranulosa cells, Fig. 2), whether these factors engage in their expected action upon gene expression in primordial follicles remains unknown. Indeed, the AKBI ovarian phenotype with increased numbers of primordial follicles does not exactly phenocopy the *Tnfr2* knockout [27], where increased numbers of primordial and small growing follicles were found during adulthood. This may be related to a second limitation of our study, that there are many physiological factors that can stimulate, or, block NFκB activation;[27] Tnfα/Tnfr2 interaction is only one example. The physiological factors that modulate NFκB action and also causally impact PFGA also remain unknown. Third, while the AKBI mouse has a reproducible and significant phenotype, it is unclear whether it is the loss of IκBα or the gain of the additional IκBβ gene (under the control of the IκBα locus), or both, that leads to altered ovarian aging. Last and perhaps most importantly, we did not identify the factor(s) responsible for initiating NFκB signaling. These experiments are in progress. Future experiments using whole body IκBβ knockout mice [37], and eventually, oocyte- or granulosa cell-specific IκB knockout models can be used to help resolve these questions. Despite these limitations, our data add NFκB machinery to the list of gene products that influence the rate of PFGA during reproductive life within the mouse model used. Experiments using human ovarian tissue are needed to determine whether the NFκB pathway functions similarly, and thus test whether these data are relevant to the reproductive aging of women.

PFGA involves the coordination between multiple identified pathways [reviewed in 2]. Our murine data suggest that one tractable intervention that might slow PFGA and extend ovarian function is the stabilization of IκBβ. Excitingly, the NFκB pathway and IκB proteins specifically are increasingly being shown to be clinically "druggable" targets [44, 45, 4], and the effect(s) of IκBβ stabilization can be tested using pharmacological agents in the aforementioned human *in vitro* studies. Increasing numbers of women are learning that they are at increased risk for the very common (above, Introduction) condition POI before their ovaries fail. This is due to their participating in assisted reproduction treatments where indirect measures of ovarian reserve are determined [2, 8]. There is therefore a window of treatment for these women where slowing the rate of PFGA could extend their ovarian function, staving off the mentioned significant health compromises.

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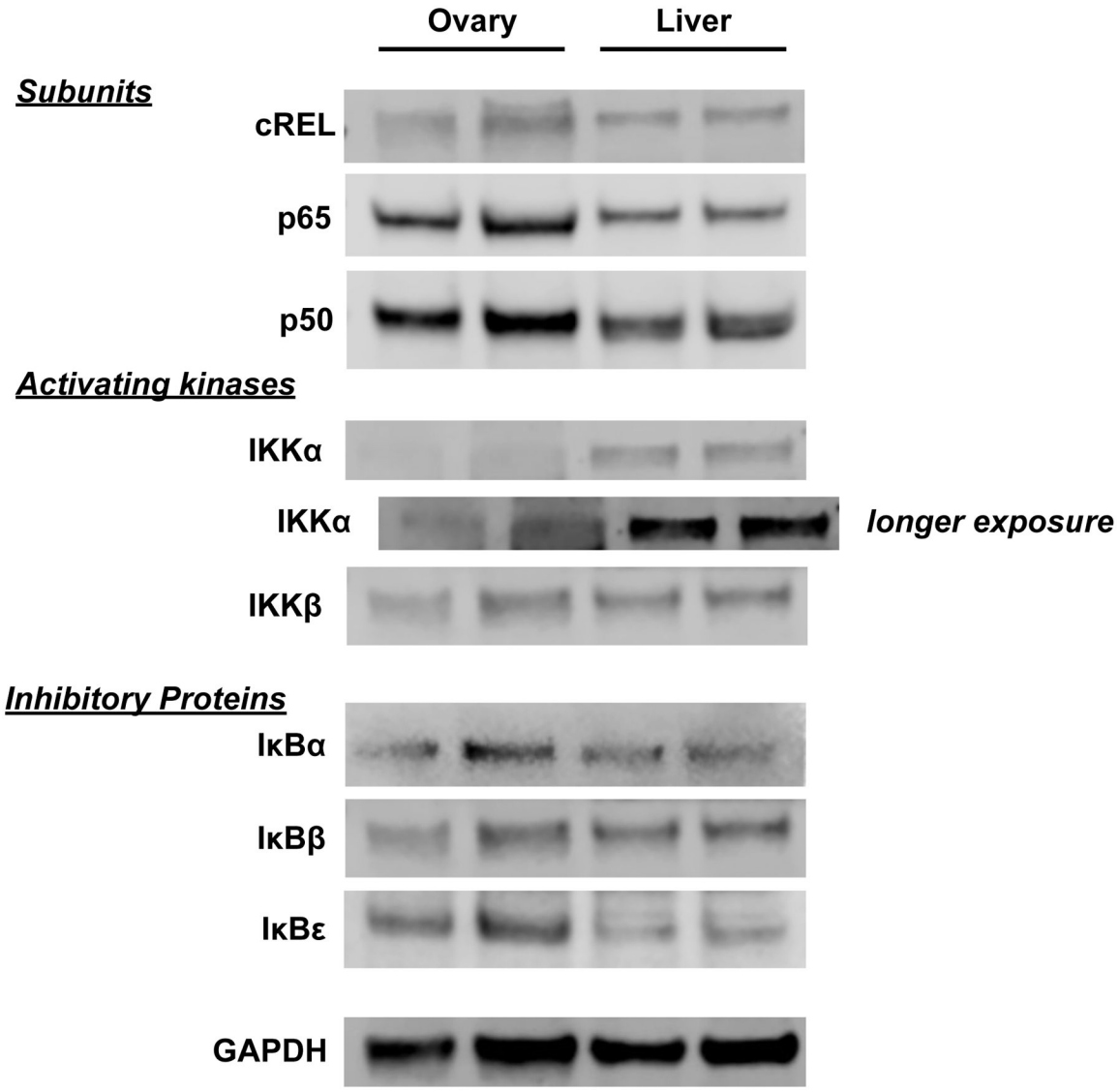

Figure 1. Key members of the NFB signaling cascade are expressed in the ovary.

Representative Western blot showing ovary and liver lysate levels in 60–65 day old female mice of the NFκB subunits c-Rel, p65, p50; the activating kinases IKKα and IKKβ; the cytosolic inhibitory proteins IκBα, IκBβ and IκBε. GAPDH as loading control. A longer exposure of IKKα is provided. Blot is representative of samples from ovarian lysates from 6 separate mice.

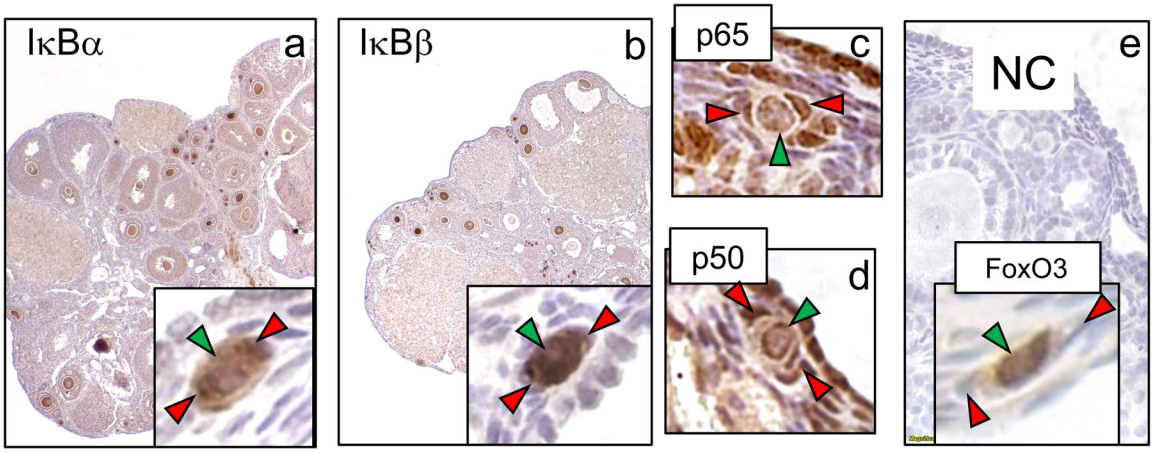

Figure 2. NFκB pathway expression in the mouse ovary.

IκBα (a) and IκBβ (b), as well as activating subunits p65 (c) and p50 (d) are expressed in oocytes, granulosa cells, and luteal cells within adult ovaries (60–65 days). The inhibitory proteins and NFκB proteins were expressed in primordial oocytes (green arrowheads in insets) and pregranulosa cells (red arrowheads) in primordial follicles. NC shows representative signal in a control section where primary antibody was omitted. Foxo3 (immunostaining specificity control, inset panel e) is expressed in primordial oocytes (green arrowheads) but not pregranulosa cells (red arrowheads) and this can be compared to NFκB pathway proteins found in both cell types. Staining is representative samples from 3 separate mice.

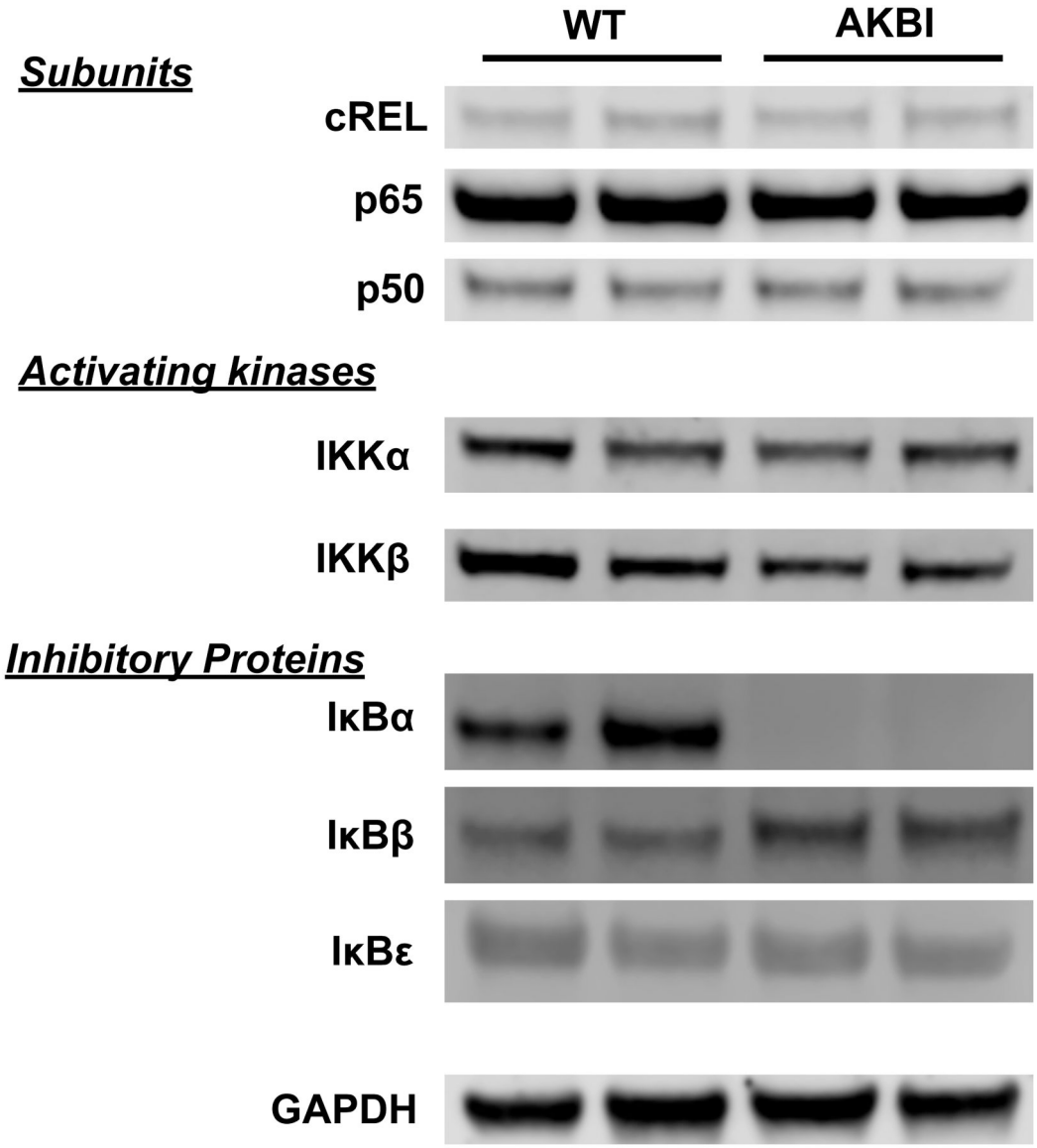

Figure 3. Key members of the NFB signaling cascade are expressed in the AKBI ovary.
Representative Western blot showing WT and AKBI ovary lysate levels of the NFκB subunits c-Rel, p65, p50; the activating kinases IKKα and IKKβ; the cytosolic inhibitory proteins IκBα, IκBβ and IκBε. GAPDH as loading control. Blot is representative of samples from ovarian lysates from from 6 separate mice at 60–65 days old.

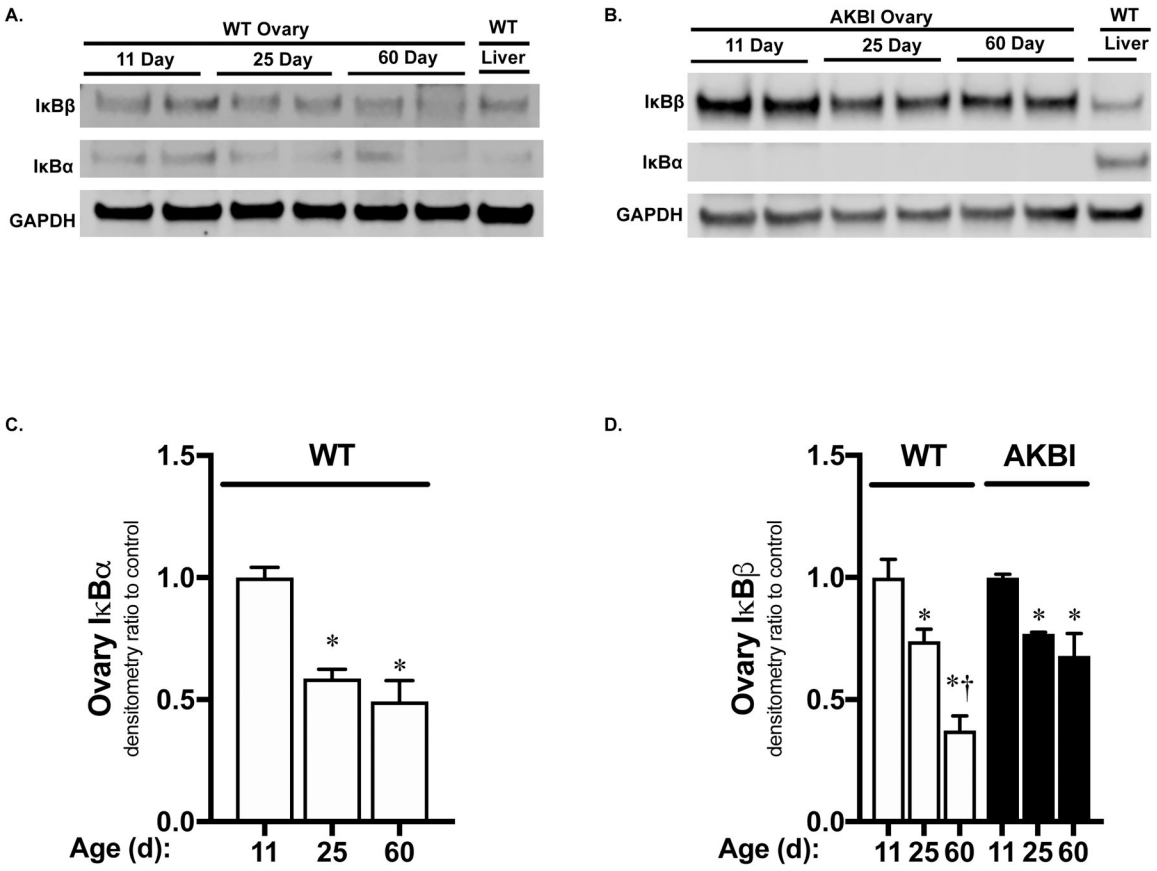

Figure 4. Ovarian levels of the NFκB inhibitory proteins IκBα and IκBβ decrease with age

(A) Representative Western blot showing WT ovary lysate levels of IκBα and IκBβ at day 11, day 25 and day 60. Liver lysate shown as positive control. GAPDH as loading control. (B) Representative Western blot showing AKBI ovary lysate levels of IκBβ at day 11, day 25 and day 60. Liver lysate shown as positive control. GAPDH as loading control. (C) Densitometric analysis of WT ovary IκBα. Data expressed as mean ± SEM (N = 4 per time point). *p < 0.05 vs. day 11. (D) Densitometric analysis of WT and AKBI ovary IκBβ. Data expressed as mean ± SEM (N = 4 per time point). *p < 0.05 vs. day 11. †p < 0.05 vs. day 25.

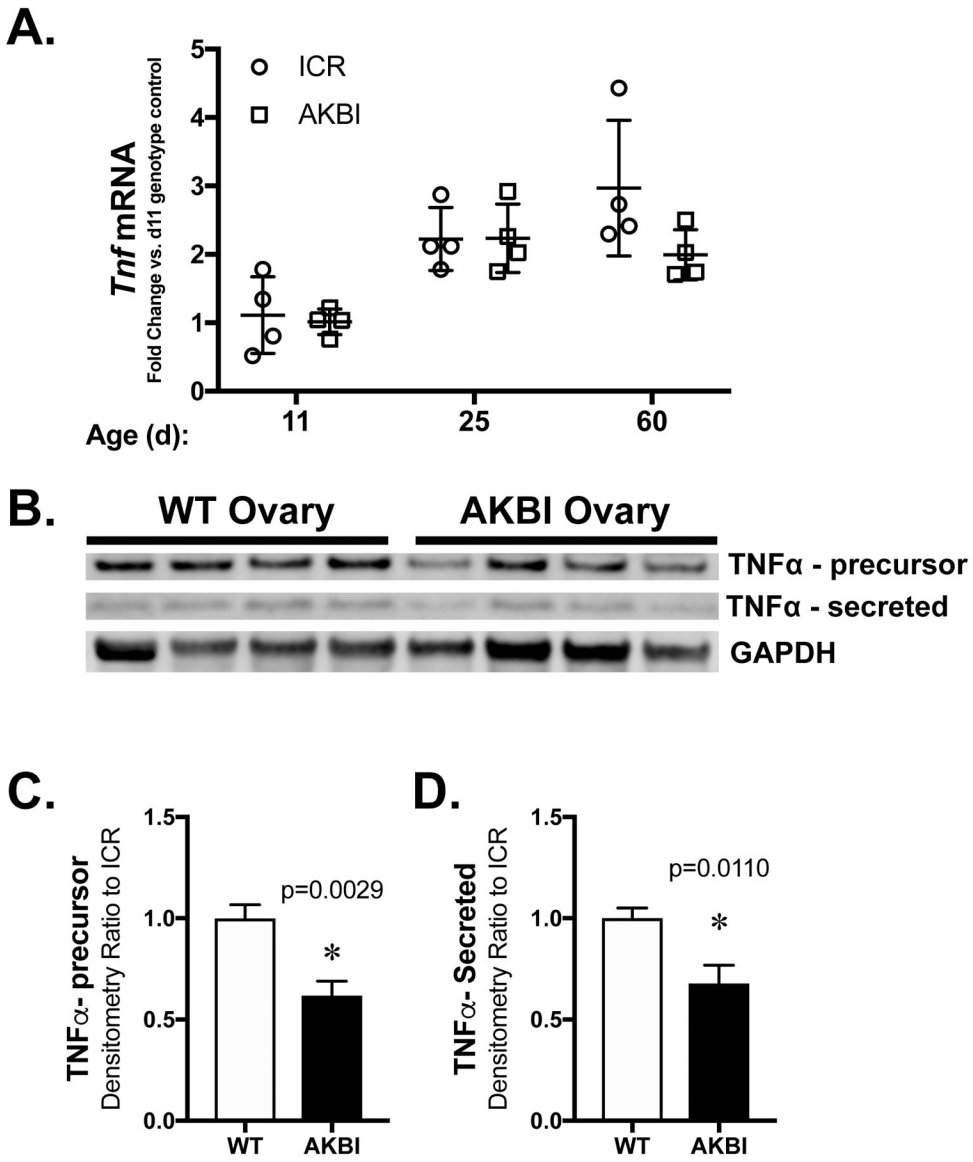

Figure 5. Ovarian levels of the TNFα are decreased in AKBI compared to WT.

(A) Fold change in Tnfr2 ligand *Tnf* mRNA expression at day 25 and day 60 over genotype control level at day 11. Data are expressed as mean ± SEM, N = 4–6 per time point (B) Representative Western blot showing levels of both the TNFα precursor and secreted form in ovarian lysate taken from adult (~60 day) WT and AKBI mice. GAPDH as loading control. (C) Densitometric analysis of WT and AKBI ovary TNFα precursor. Data expressed as mean ± SEM (N = 4 per time point). *p < 0.05 vs. WT. (D) Densitometric analysis of WT and AKBI ovary secreted TNFα. Data expressed as mean ± SEM (N = 4 per time point). *p < 0.05 vs. WT.

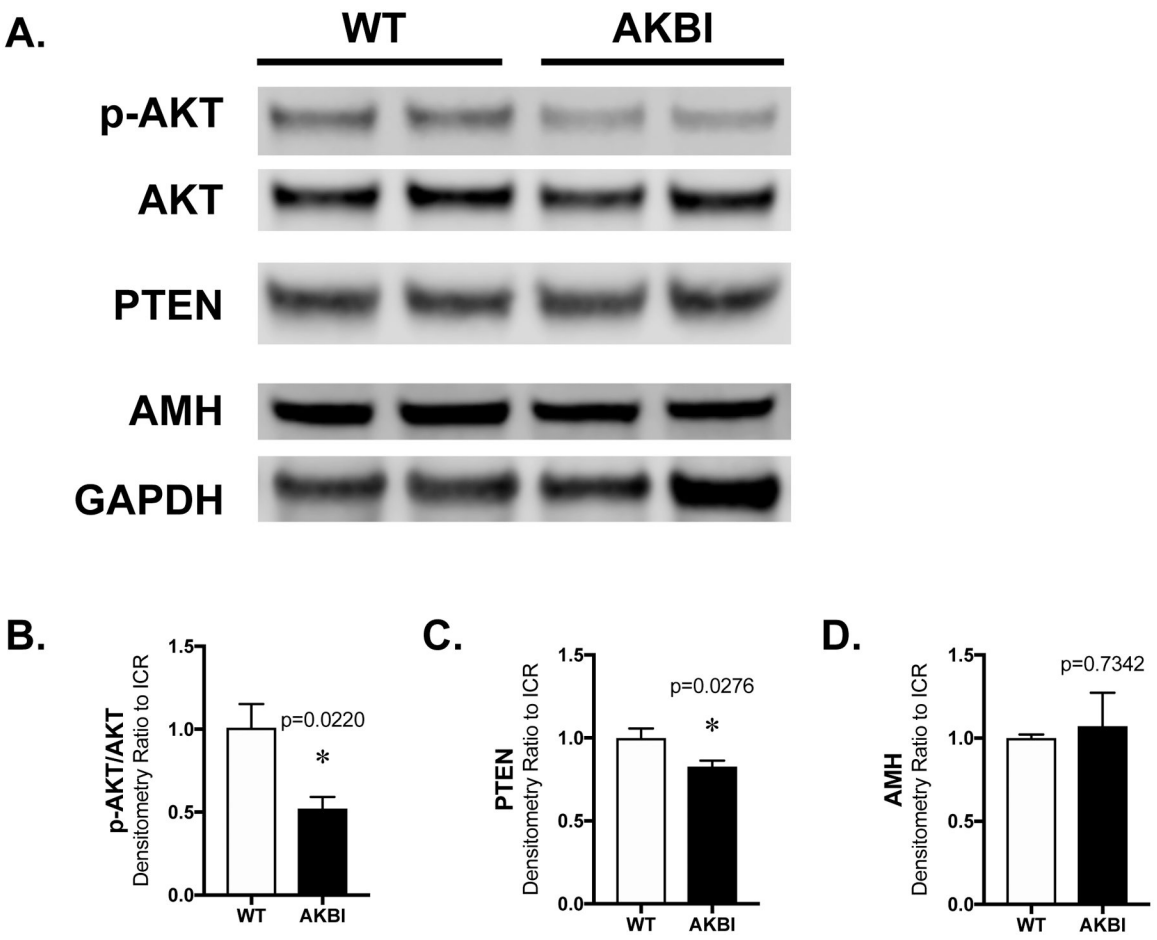

Figure 6. Ovarian levels of the p-AKT/AKT and PTEN are decreased in AKBI compared to WT.

(A) Representative Western blot showing levels of p-AKT, AKT, PTEN, and AMH in ovarian lysate from adult (~60 day) WT and AKBI mice. GAPDH as loading control. (B) Densitometric analysis of WT and AKBI ovary p-AKT/AKT ratio. Data expressed as mean ± SEM (N = 4 per time point). *p < 0.05 vs. WT. (C) Densitometric analysis of WT and AKBI ovary PTEN. Data expressed as mean ± SEM (N = 4 per time point). *p < 0.05 vs. WT. (D) Densitometric analysis of WT and AKBI ovary AMH. Data expressed as mean ± SEM (N = 4 per time point).

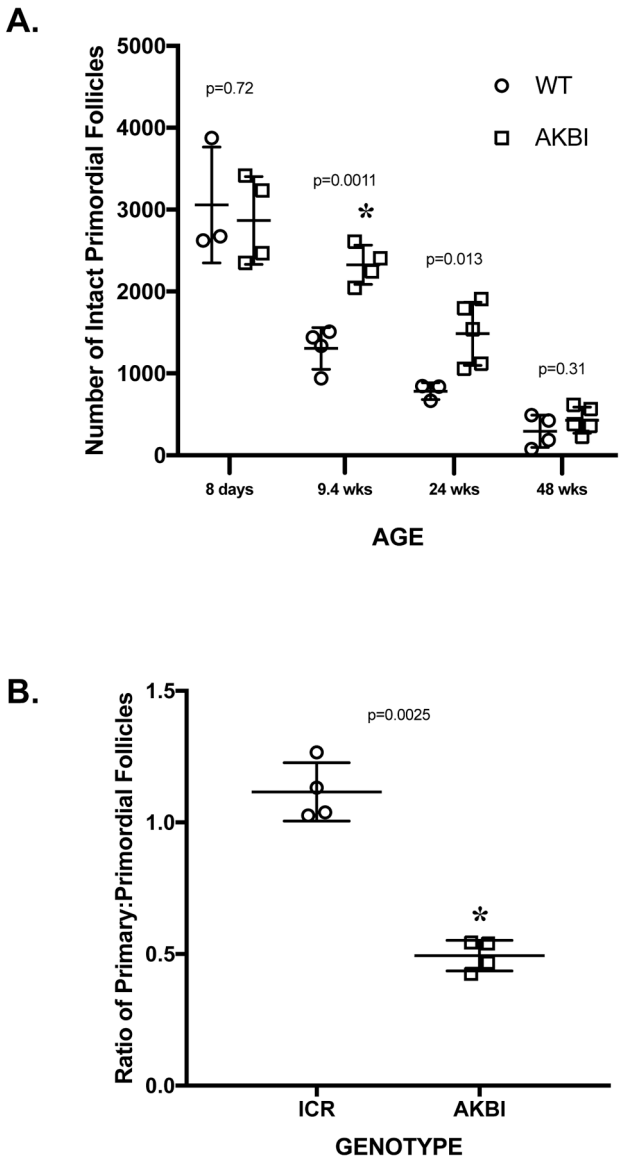

Figure 7. Blinded histomorphometic analyses of immature follicles.

(A) Primordial follicle number in WT and AKBI mice at 8 days, 9.4 weeks, 24 weeks, and 48 weeks. Minimum n=3 ovaries from separate animals counted per time point. Data expressed as mean + SD with individual values shown. p-values calculated by Student's t-test. (B) Ratio of Primary:Primordial follicles at 9.4 weeks in WT and AKBI mice. WT displayed as open circles, AKBI displayed as open squares. Data expressed as mean + SD with individual values shown. P-values calculated by Student's t-test. *$p < 0.05$ vs. WT

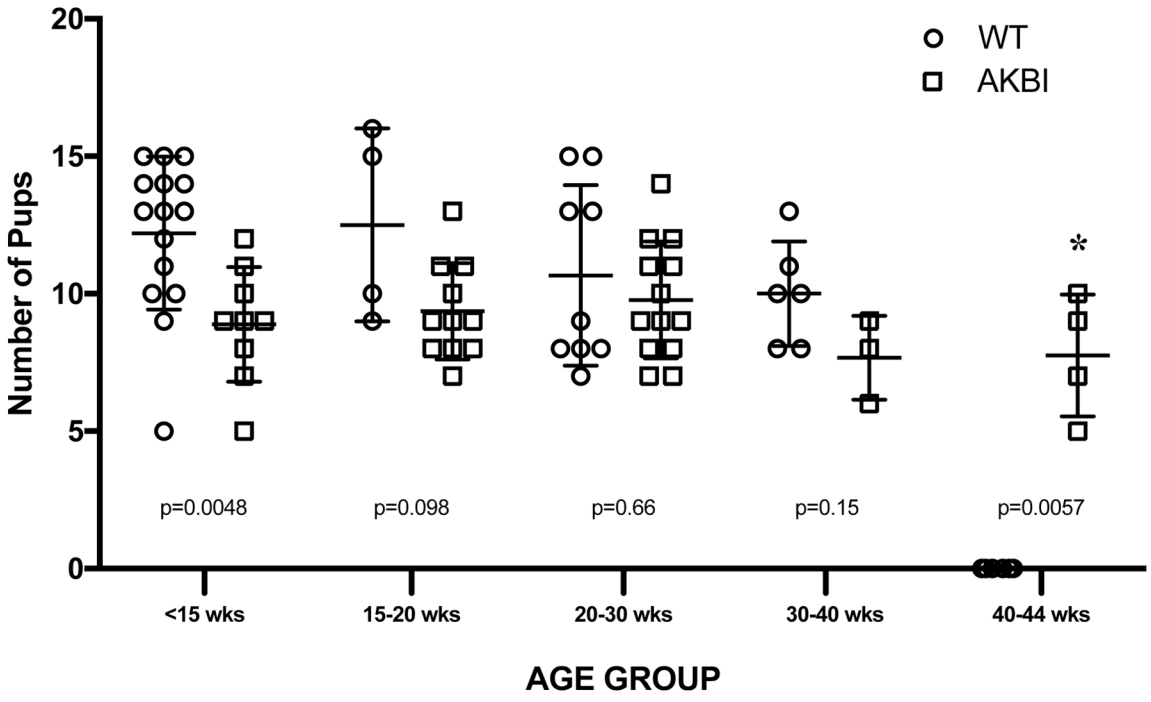

Figure 8. Long-term comparison of litter sizes between AKBI females and WT controls.

Long term breeding records are summarized in the plot, binned into 5 maternal age time periods. Litters from AKBI dams are significantly smaller than in WT controls in animals less than 15 weeks old. No significant difference is then seen in animals up to 40 weeks of age. After 40 weeks of age, AKBI animals continued to produce litters of 5 or more pups, but 0/6 ICR controls delivered pups within 28 days of placement with males of proven fertility. WT displayed as open circles, AKBI displayed as open squares. Data expressed as mean + SD with individual values shown. p-values calculated by Student's t-test. *$p < 0.05$ vs. WT

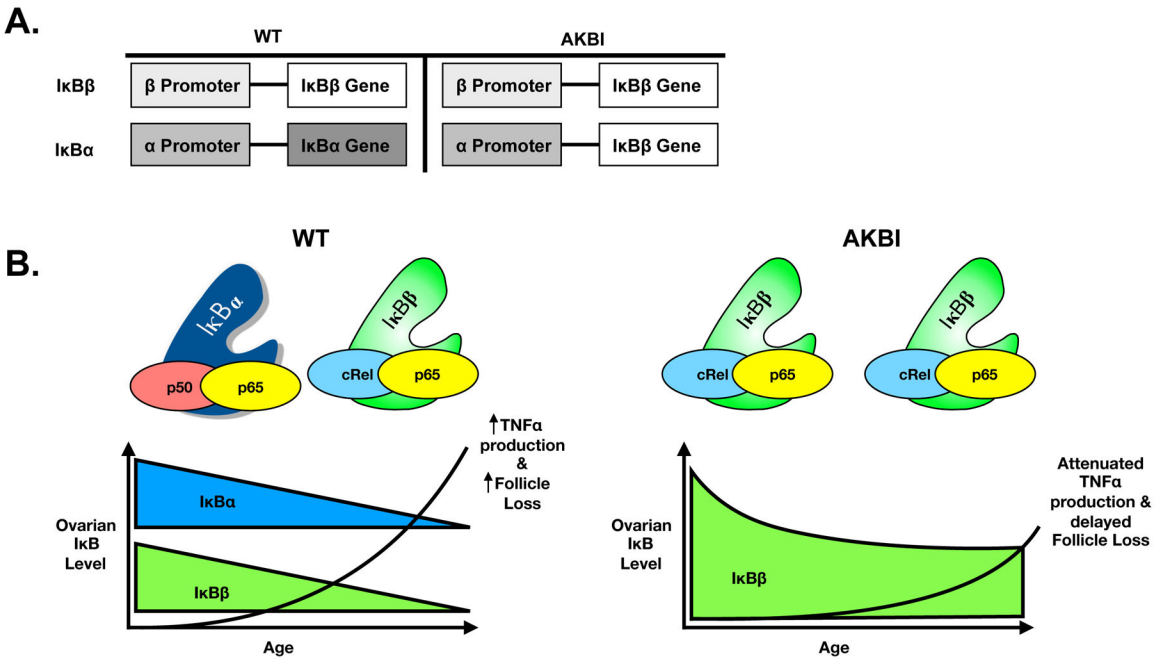

Figure 9. AKBI mouse model and proposed mechanism for delayed PFGA.

(A) Schematic of IκB expression patterns in WT and AKBI mice. The IκBα gene has been replaced by IκBβ cDNA. The IκBα promoter controls the expression of the IκBβ transgenic loci. Thus, AKBI overexpress IκBβ, without expressing IκBα **(B)** Proposed mechanism for delayed primordial follicle growth activation in AKBI compared to WT mice.