Title

c-JUN Dimerization Protein 2 (JDP2) Is a Transcriptional Repressor of Follicle-stimulating Hormone β (FSHβ) and Is Required for Preventing Premature Reproductive Senescence in Female Mice*

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cJun-Dimerization Protein 2 (JDP2) Negative Feedback on Follicle-Stimulating Hormone (FSH) β Expression is Critical for Normal Reproductive Function in Female Mice

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Running title: *JDP2 regulates FSH levels*

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ABSTRACT

Follicle-stimulating hormone (FSH) regulates follicular growth and stimulates estrogen synthesis in the ovaries. FSH is a heterodimer of α subunit, in common with luteinizing hormone, and a unique β subunit, which is regulated at the transcriptional level by GnRH. Since the majority of FSH is secreted in a constitutive manner, tight regulation of transcription is critical for maintaining the hormone level within a narrow physiological range. Previously we identified that GnRH induces FSHβ transcription via induction of cFos and cJun AP1 transcription factors. Herein, we identify a novel negative regulator of FSHβ induction by GnRH, cJun-dimerization protein 2 (JDP2) that binds FSHβ promoter in a complex with cJun. JDP2 exhibits high basal expression and binds to the AP1 site in the absence of stimuli. GnRH treatment induces cFos, which replaces JDP2 as a cJun binding partner to form transcriptionally active AP1 factor, until cFos is degraded due to a short half-life, and JDP2 complex re-forms. *In vivo* studies reveal JDP2 null male mice have normal reproductive function, as expected from a negative regulator. Female JDP2 null mice, however, exhibit early puberty, observed by early vaginal opening, larger litters and early reproductive senescence. JDP2 null females have increased FSH in the circulation and higher expression of FSHβ subunit in the pituitary, which results in elevated estrogen in the serum and higher number of large follicles in the ovary. JDP2, therefore, may contribute to premature ovarian failure and early cessation of reproductive function, a condition associated with elevated FSH in women.

INTRODUCTION

Gonadotrope cells in the anterior pituitary synthesize and secrete LH and FSH in response to pulses of hypothalamic GnRH, which can be modulated by activin/inhibin and steroid feedback from the gonads. Gonadotropin hormones are heterodimers of a common α subunit and a unique β subunit. Gonadotropin levels fluctuate 3-4 fold throughout the menstrual or estrous cycle, preceded by changes in the β subunits transcription (1, 2). Dysregulation of the tightly controlled gonadotropin levels leads to reproductive pathophysiology. Low levels of gonadotropin hormones lead to infertility: low LH leads to hypogonadism and anovulation, while low FSH causes follicular growth arrest (3-5). High levels also lead to reproductive pathologies, particularly in females, such as polycystic ovary syndrome (PCOS) characterized by an increase specifically in LH (6-8), and premature ovarian failure (POF) or primary ovarian insufficiency (POI) that is associated with an increase in FSH (9, 10). Recently, it was determined that increased levels of FSH in the circulation, due to re-trafficking of FSH to the GnRH-regulated secretory pathway, resulted in 5-fold more corpora lutea in the ovaries, implicating higher FSH levels to greater follicle recruitment (11). Furthermore, polymorphism in the FSHβ promoter in women, which regulates β subunit expression, increases level of FSH and has been associated with early menarche and menopause (12). Another genome-wide association study reported that polymorphism 5' of the FSHβ gene, which causes higher level of FSH in the circulation, is also associated with premature puberty, early natural menopause and dizygotic twinning (13).

Normal reproductive function requires pulsatile secretion of GnRH into the hypophysial-portal system, while long-term tonic exposure to GnRH adversely affects the hypothalamic-pituitary-gonadal axis (14-16). GnRH receptors lack the C-terminal tail that normally mediates desensitization through β-arrestin association and encapsulation into clathrin-coated vesicles, rendering the receptor resistant to this regulatory mechanism. Thus, the receptor does not internalize as rapidly as other GPCRs (17). Given that GnRH receptors do not

internalize within the frequency of the pulse, due to the lack of a cytoplasmic tail, other feedback mechanisms must exist within the gonadotrope to shut off the signal and maintain gonadotropin expression within the narrow physiological range. This is especially true for FSH, since once synthesized, the majority of this hormone is constitutively secreted (18). The GnRH receptor primarily couples to Gαq/11 and activates phospholipase Cβ (PLC), causing an increase in inositol 1,4,5-triphosphate (IP3), intracellular calcium and diacylglycerol (DAG) (19). The degree of desensitization that does occur after receptor occupancy may be due to loss of IP3 receptors necessary for propagation of signaling (17, 20, 21).

In addition to desensitization of signaling molecules, such as IP3 receptors, it is likely that mechanisms exist in the nucleus that prevent further transcription of the gonadotropin subunits. There are several possible mechanisms including: dephosphorylation, degradation and deactivation of transcription factors and RNA polymerase II; return of chromatin to a closed conformation by histone modification; or, induction or re-activation of transcriptional repressors. We identified that GnRH induces FSHβ through the AP1 site (22), which is bound by the AP1 transcription factor, comprised of heterodimers of Fos and Jun isoforms. Prototypical members, cFos and cJun, are activated rapidly and transiently, and exhibit a very short half-life in order to bring forth very tight temporal regulation of target genes (23). We also determined that GnRH specifically induces cFos (24) and cJun (25) transcription in the mature gonadotrope cells, while other hormones and stimuli that activate these genes in other cell types are not sufficient to induce AP1 in the gonadotrope. cFos is not present without stimulus in quiescent cells and is more strictly regulated, both at the transcriptional and posttranslational level by GnRH, while cJun has detectable basal expression and is induced 6 fold by GnRH (26). Following cFos induction, it dimerizes with cJun to bind the AP1 site that is currently the only known element to convey GnRH responsiveness of the FSHβ promoter. Negative regulators at the AP1 site or the highly similar CRE element include activating transcription factor 3 (ATF3, (27)), inducible cAMP early repressor (ICER, (28)) and cJun-dimerization protein 2 (JDP2, (29)).

cJun-dimerization protein 2 (JDP2), previously known to form a heterodimer with cJun (29) represses transcription of AP1 target genes. Herein, we determined that JDP2 interacts with cJun at the AP1 site in the FSHβ promoter. Consistent with prior reports that JDP2 can also interact with ATF2 (30), which we previously determined conveys GnRH-induction of cJun in gonadotrope cells (25), JDP2 inhibits induction of cJun mRNA by GnRH. The repressor, JDP2, may function prior to the GnRH signal, and after a wave of transcription to shut off the signal. As many other repressors, JDP2 is known to recruit histone deacetylases (HDACs) to repress transcription through chromatin modification. In particular, JDP2 interacts directly with HDAC3 (31, 32). Previously, it has been shown that HDAC3 dissociates from the FSHβ promoter following GnRH treatment to increase FSHβ transcription (33). Identification of JDP2 provides a mechanism of histone deacetylation in the absence of the GnRH signal.

In this report, we identify a mechanism of negative regulation of FSHβ transcription in the gonadotrope *in vivo* and in the LβT2 model cell line. Previous reports identified that Nab family members antagonize Egr1 and negatively affect GnRH-induction of LHβ (34), while FSHβ induction by activin was negatively regulated by Smad pathway antagonists SKIL and TGIF (35). We identified a member of the AP1 superfamily, JDP2, that serves as a negative regulator of FSHβ induction by GnRH, both directly, by repressing AP1 transcriptional activity, and indirectly, by limiting cJun expression. We further demonstrate that a lack of this repressor *in vivo* leads to early puberty and early cessation of fertility in the null female mice, which may mimic premature ovarian failure in human population. Therefore,

identification of the novel function of JDP2 leads to better understanding of the regulation of FSH physiological levels and pathophysiologies where FSH levels are dysregulated.

RESULTS

JDP2 is a novel negative regulator of FSHβ induction by GnRH - The physiological range and responsiveness of gene expression to subsequent, specific stimuli is tightly controlled by both positive and negative regulators of transcription. This is critical for gonadotropin hormones, whose increase is associated with PCOS or POF in females. Although repressors are relatively more difficult to identify, negative regulators of GnRH induction of LHβ and activin induction of FSHβ have been determined (34, 35). We sought to identify a protein that serves as a transcriptional repressor of GnRH induction of FSHβ transcription, since FSHβ is normally expressed at a low basal level. We first determined the time course of induction of GnRH target genes in LβT2 cells, a model of mature gonadotrope (Fig. 1A). The time course for induction of GnRH target genes is different, but all are biphasic. Similar to previously reported time course for cFos induction (26), the maximal induction of cJun of 15.5 fold is at 1 hour of treatment, after which the message rapidly declines. At 2 hours, fold induction diminished to 5.4 fold of control, 3.8 fold at 6 hours and returned to basal levels at 12 hours of treatment. Compared to our previous report analyzing cFos, the major difference is that cFos is not present at a basal state, without stimuli, while cJun is present at both mRNA (Fig. 1A) and protein levels (26). FSHβ expression is very low at the basal state (Fig. 1A) and maximal induction of 12-fold by GnRH is observed at 2 hours of treatment. After the maximum, the message level diminishes and returns to basal level at 12 hours of treatment. We performed DNA pulldowns followed by mass-spec sequencing and identified cJun-dimerization protein 2 (JDP2) as a novel protein that interacts with the FSHβ promoter at the AP1 site, which was previously known to form a heterodimer with Jun subfamily (29). We then analyzed whether JDP2 is induced by GnRH and determined that JDP2 exists in the cells at a relatively high basal level, and is induced 2-fold at 2 hours of GnRH treatment (Fig. 1B). We also analyzed the expression of a close family member ATF3, which is induced by GnRH (25, 42), and determined that ATF3 mRNA is not found at a basal level, and is induced rapidly by GnRH with a maximal induction of 23 fold at 1 hour, after which the induction diminishes (Figure 1B). Therefore, both ATF3 and JDP2, which can serve as negative regulators of transcription at the AP1 and CRE sites, are induced by GnRH. The difference is that JDP2 is present at the basal level, while ATF3 is non-detectable.

Protein levels of cJun, ATF3 and JDP2 transcription factors correspond to changes in mRNA levels (Fig 1C). cJun protein is induced after 1 hour of GnRH treatment and exhibits maximus at 2 hours, as we determined previously (26). cJun protein level returns to basal after 16 hours of treatment. ATF3 protein induction as determined before (25) is observed after 1 hour of GnRH, exhibits a maximum at 4 hours and returns to basal level by 16 hours. JDP2 protein is present at basal level without GnRH treatment, and the levels change less compared to ATF3 and cJun, with an increase at 2 hours of treatment and return to basal level by 24 hours.

Next, we determined if JDP2 can interfere with GnRH induction of gonadotrope genes. We determined previously that GnRH phosphorylation of ATF2 activates cJun transcription in the gonadotrope (25), and that GnRH induction of cJun and cFos activates FSHβ transcription (22). Since JDP2 in other cells interacts with ATF2 (43) as well as cJun (29), we overexpressed JDP2 with cJun-luciferase (Fig. 2B) and FSHβ-luciferase (Fig. 2E) reporters. In order to determine the specificity of JDP2 function in gene induction by GnRH, we also overexpressed ATF3, which can serve as a repressor and is a close family member of JDP2.

JDP2 can bind at either the TRE/AP1 site (TGAGTCA) where it interacts with cJun, or the CRE site (TGACGTCA) where it interacts with ATF2. The TRE/AP1 half-site conveys GnRH responsiveness of the FSHβ promoter (22), while the CRE site in the cJun promoter is responsible for cJun induction by GnRH (25). Thus, in addition to cJun-luciferase and FSHβ-luciferase, we transfected CRE-luciferase and TRE-luciferase to assay GnRH induction via these sites in the presence of JDP2 or ATF3. These reporters contain four tandem copies of the 8 bp consensus CRE element (Fig. 2A) or of the similar 7 bp consensus TRE multimer, which has 4 tandem copies of the TRE element (Fig. 2D) ligated to the minimal thymidine kinase promoter driving luciferase expression. GnRH induced the CRE site multimer as we have shown previously, while the addition of either ATF3 or JDP2 diminished both basal expression and GnRH induction, indicated with a pound sign (Fig. 2A). Both ATF3 and JDP2 also reduced fold induction of the CRE site by GnRH, indicated with an asterisk. Fold induction was calculated by normalizing samples treated with GnRH to the vehicle-treated samples transfected with the same expression vectors, for easier observation of the induction by GnRH without the effect of basal repression. On the other hand, basal expression and GnRH induction of the cJun promoter, which contains other elements in addition to the CRE site, was reduced by overexpression of JDP2, but not ATF3 (Fig. 2B). The CRE site in the cJun promoter is necessary for the maximal induction of the cJun gene by GnRH, since GnRH induction of cJun is diminished when CRE is mutated (CRE mut, Fig 2C (25)). CRE site mutation prevented further repression of GnRH induction of cJun by JDP2, since the CRE site mutant is induced by GnRH to the same level with and without JDP2 (Fig 2C). This indicates that JDP2 represses cJun promoter via the CRE site. JDP2 overexpression diminished both basal and fold induction of the TRE/AP1 multimer by GnRH, while ATF3 overexpression reduced basal expression and consequently the level of induction by GnRH, but had no effect on fold induction, contrary to the CRE multimer (Fig. 2D). JDP2, but not ATF3, reduced FSHβ fold induction by GnRH (Fig. 2E). As stated previously, GnRH induces FSHβ through TRE/AP1 site, since when this site is mutated, GnRH induction is reduced (TRE mut, Fig 3F (22)). Similarly to cJun promoter, JDP2 does not repress FSHβ induction by GnRH when the TRE site is mutated, indicating that the TRE site is necessary for JDP2 function (Fig. 2F). Therefore, native promoters that contain elements other than GnRH-responsive CRE and/or TRE sites are resistant to the repression by ATF3. However, JDP2 reduces both cJun and FSHβ induction by GnRH via CRE and TRE elements, respectively.

JDP2 binds cJun and FSHβ promoters in complex with ATF2 and cJun respectively - In order to determine if JDP2 can bind FSHβ and cJun promoters, we performed EMSA using nuclear extracts from LβT2 gonadotrope cells following treatment with GnRH. Due to a lack of specific antibodies for JDP2, we were unable to perform a supershift with LβT2 cells extracts. Instead, to determine if JDP2 can bind by itself and/or in combination with other factors known to bind the TRE/AP1 site in the FSHβ promoter and the CRE site in the cJun promoter, we overexpressed proteins of interest in Cos-1 cells: cFos, cJun and JDP2 and their combination for FSHβ promoter analysis (Fig. 3A); and ATF2, ATF3, cJun and JDP2 for cJun promoter analysis (Fig. 3C). We not only monitored their binding, but co-migration with complexes from LβT2 cells as well. Two hour GnRH treatment of LβT2 gonadotrope cells induced two different complexes that bind FSHβ AP1 probe (Fig. 3A), consistent with our previous report (22). The identity of the proteins that comprise these complexes was determined previously by antibody supershift: the higher complex is comprised of cJun and cFos, and the lower complex is comprised of cJun and FosB (22). Here, we show that the higher complex of cJun and

cFos heterodimer comigrates with a cJun and cFos heterodimer overexpressed in Cos-1 cells. These GnRH-induced complexes are not present in the extracts from LβT2 cells at the basal state or following 12 hours of treatment, at which times two other complexes bind that are partially obscured by more abundant GnRH-induced complexes at 2 hours of treatment. Of the two basal complexes, the higher was previously identified as the basal factor NFY, and the lower complex was previously unidentified. Here, this lower complex comigrates with a complex comprised of cJun and JDP2 overexpressed in Cos-1 cells, indicating that a dimer of cJun and JDP2 can bind the FSHβ TRE/AP1 probe. JDP2 can bind the AP1 probe by itself as well, however JDP2-only band from Cos-1 cells does not comigrate with any complex from LβT2 cell extracts, likely because cJun is expressed at a basal level in LβT2 cells and it can heterodimerize with JDP2. When oligonucleotides with a mutation in the TRE/AP1 site were used as competitors with the wild-type probe, competitors were not able to compete for cJun+cFos, cJun+FosB or cJun+JDP2 complexes, indicating that the TRE/AP1 site is necessary for binding of these proteins (right side panel Fig. 3A).

The TRE site in the FSHβ promoter (TTGGTCA) differs slightly from the TRE consensus sequence (TGAGTCA, (22)). Thus, we compared binding to the TRE site in the FSHβ promoter and to the consensus TRE site (Fig. 3B). To easier observe differences in binding intensity, the film was underexposed compared to Fig. 3A. For consistency, all binding reactions contained the same specific activity of FSHβ or consensus 30-bp probe and the same protein amount. Binding of cJun+cFos and cJun+FosB complexes from LβT2 cells following GnRH treatment was stronger when TRE consensus was used as a probe than when FSHβ TRE/AP1 site was used as a probe. Similar results were observed with overexpressed proteins, and binding intensity was stronger to the consensus probe. The core TRE sequence is necessary for binding since neither cJun+cFos or JDP2 bound to either probe when TRE site was mutated (mut, Fig. 3B). The CRE site in the cJun promoter is also necessary for JDP2 and ATF2 binding, since when mutated the JDP2 or ATF2 did not bind, however intensity of binding to the CRE site from the cJun promoter and to the CRE consensus was the same (data not shown).

As determined previously, ATF2 binds the cJun promoter at the CRE site at the basal state (25), since its phosphorylation leads to activation and induction of cJun transcription (Fig. 3C). As shown in Fig. 1, cJun induction diminishes after 1 hour of treatment and previous studies determined that cJun can reduce its own expression in a negative feedback loop manner (44). We determined using overexpression in Cos-1 cells that indeed, cJun can bind its own promoter; not alone, but in a complex with ATF2 and in the complex with JDP2. We further determined, also with overexpression in Cos-1 cells, that JDP2 can dimerize with ATF2 and their complex can bind the cJun probe, in addition to the JDP2 and cJun complex. JDP2-containing complexes comigrate with complexes from LβT2 extracts after 2- and 5-hour GnRH treatment, when cJun transcription is diminished. The CRE site mutant used as a competitor in 200 fold excess, was unable to compete for GnRH-induced complexes, while wild-type competitor successfully competed (right-side panel Fig 3C). Therefore, JDP2 binds both FSHβ and cJun promoters at the TRE and CRE sites, respectively, alone and in complex with cJun and ATF2.

Activation site on JDP2 is required for repressor function - We sought to determine the mechanism of negative regulation of cJun and FSHβ gene expression by JDP2, and the specificity of action, since highly homologous family member ATF3 does not reduce GnRH induction of either gene (Fig. 2B, E). Both the cJun promoter and the FSHβ promoter contain CCAAT element, which binds the NFY basal transcription factor, in close proximity to the GnRH regulated elements, CRE and TRE/AP1, respectively (Fig. 11). To asses if JDP2

is recruited to the promoters via interaction with NFY, we performed GST-pulldown experiments (Fig. 4A). Using GST pulldowns, S^{35}-labeled JDP2, ATF2, ATF3 and cJun are retained in the precipitate with glutathione beads following interaction with GST-NFY, but not with GST control (Fig. 4A). NFY does not interact with cFos, while it interacts with cJun and ATF2, which we have shown previously (22, 25). Both ATF3 and JDP2 can interact with NFY, thus interaction with NFY is not a cause for the lack repression by ATF3 of the cJun and FSHβ promoters and specificity of JDP2 function.

Additionally, to analyze protein in complex with JDP2 we performed immunoprecipitation following overexpression of histidine-tagged JDP2. Flag-tagged cFos served as a positive control since cFos is a known cJun interacting partner. cJun was detected in the immunoprecipitate with both histidine (His) and Flag-tag (Flag) antibodies indicating that cJun can interact with both JDP2 and cFos, as expected (Fig. 4B). JDP2 also precipitated cFos and NFY-A protein that is a part of NFY complex. Since immunoprecipitation will detect all proteins in complex, while GST-pulldowns detect direct protein-protein interactions, taken together these results indicate that JDP2 directly interacts with cJun and NFY-A, and via cJun with cJun+cFos complex.

It was previously demonstrated that although JDP2 and ATF3 exhibit 61% overall homology and 90% homology in their bZIP domains, a major difference in the structures of these highly homologous proteins is the presence of a putative activation site on threonine (T) 148 in JDP2, which is absent in ATF3 (45). Hence, the activation site was mutated to alanine to inactivate it and the effect on cJun and FSHβ induction by GnRH of the mutant (T148A) compared to the effect of the wild-type (JDP2, Fig. 5A, B). Consistent with previous results, wild-type JDP2 repressed basal and GnRH induction of cJun (Fig. 5A), while T148A mutant increased both basal and GnRH induction of cJun, indicating that JDP2 is involved in basal repression of cJun without the stimulus. FSHβ induction by GnRH was diminished with overexpression of wild-type JDP2 as before. T148A mutant not only inhibited the repression observed with wild-type JDP2, but significantly increased the fold induction (Fig. 5B). Thus, threonine 148 is required for repressor function of JDP2. Next, we mutated the same threonine residue to aspartic acid (D) which mimics activation, and determined that this mutant (T148D) repressed GnRH induction of the cJun and FSHβ reporters to the same level as the wild-type (JDP2, Fig. 5A, B). This may indicate that JDP2 in these cells is constitutively activated. Mutation of the DNA-binding domain, DBDm, produced the inactive protein that was unable to repress GnRH induction of either cJun or FSHβ (Fig. 5A, B). Thus, the JDP2 DNA-binding domain is necessary for its repressor function.

JDP2 directly binds histones and recruits histone deacetylases, resulting in the inhibition of histone acetylation (32, 46, 47). In particular, JDP2 interacts with HDAC3 (48), which is the same HDAC that binds FSHβ promoter at the basal state and is dissociated from the promoter following GnRH treatment (33). Again due to the lack of specific antibodies to JDP2, we were not able to analyze JDP2 recruitment to the FSHβ promoter *in vivo*. Thus, we analyzed histone acetylation at the FSHβ promoter following GnRH treatment using chromatin immunoprecipitation (ChIP) with antibodies to acetylated histone H3, expressed as % input, and normalized to the amount of precipitated chromatin with total H3 antibody, also expressed at % input based on the standard curve of input chromatin for each time point. Indeed, 1-hour GnRH treatment resulted in higher level of histone acetylation at the FSHβ promoter (Fig. 6), which may allow for gene activation following dissociation of the JDP2 from the promoter. This is consistent with previous reports that JDP2 inhibits histone acetylation and our hypothesis that GnRH treatment dissociates JDP2 from the FSHβ promoter by inducing cFos as cJun binding partner.

JDP2 regulates reproductive function In Vivo - To determine whether JDP2 plays a role in reproduction *in vivo*, we obtained JDP2 null mice (36) and analyzed their fertility. There is no weight difference between genotypes, and food intake is the same. Male mice had normal sperm count, gonadotropin hormone levels and reproductive function, as expected from a negative regulator. Female mice, on the other hand, exhibited a number of reproductive anomalies (Fig. 7). Female JDP2 null mice exhibited significantly earlier vaginal opening, an external sign of puberty, at postnatal day (p) 24.3 compared to p29.4 for wild-type littermates. At 8 weeks of age, females were paired with a wild-type male of proven fertility for a year; and litter birthdates, numbers of litters and numbers of pups in each litter recorded. Female null mice delivered their 1st litter significantly earlier than wild-type mice. In fertility assessment studies, during which we counted the number of pups, combined number of pups in the first four litters of JDP2 null mice was significantly higher than number of pups in four litters of wild-type female littermates. We also observed an early cessation of fertility in null mice. JDP2 null mice had their last litter at p268, while wild-types on average stopped reproducing at p338. This difference may stem from early depletion of ovarian reserves due to larger litters in younger animals. Thus, female nulls have early vaginal opening, first litter sooner and have larger litters, but stop reproducing at a younger age than wild-types. This early cessation of reproductive function corresponds to our hypothesis that JDP2 nulls may mimic premature ovarian failure in women.

Given that in our model cell line JDP2 negatively regulated FSHβ levels, we first assessed gonadotropin hormone levels in the serum and gene expression in the pituitary (Fig. 8). There is no difference in the LH serum levels (Fig. 8A), but FSH was higher in the diestrus of 8-week old null females compared to the wild-type littermates in diestrus (Fig. 8B). Difference in the expression of the LHβ subunit in the pituitary that confers biological specificity to LH did not reach significance (Fig. 8C), while FSHβ subunit expression was significantly higher in the pituitaries of the null mice (Fig. 8D). On the other hand, the expression of the common α-GSU subunit that heterodimerizes with both LHβ and FSHβ was the same between genotypes (Fig. 8E). Because expression of FSHβ subunit was significantly increased, we assessed whether it stems from an increase in the number of gonadotropes. Pituitary morphology and size, and gonadotrope number are the same between wild-type and null females (Fig 9A-C). Since hypothalamic neuropeptides GnRH and kisspeptin regulate expression and secretion of gonadotropins in the pituitary, we measured the expression of these neurohormones. Expression of GnRH and kisspeptin was the same as well between genotypes (Fig. 9D, E). Therefore, FSHβ expression and FSH hormone levels are higher in the JDP2 null females, which is consistent with our findings in the cell model that JDP2 serves as a negative regulator of FSH synthesis.

Next, we evaluated ovarian function in JDP2 nulls (Fig. 10). In the ovary, Anti-Mullerian hormone (AMH) is produced by the granulosa cells of growing follicles to modulate the recruitment of primordial follicles and the FSH-dependent follicular growth (49, 50). Given that AMH levels may correlate with the increased number of pups and early reproductive senescence, we first analyzed the levels of AMH. AMH levels exhibited a trend towards lower levels in null females ($p=0.11$), although the difference didn't reach significance (Fig. 10A). On the other hand, estradiol levels were significantly higher in JDP2 null proestrus females (Fig. 10B), while there was no change in inhibin A concentration (Fig. 10C). On histological sections, we counted a higher number of large follicles with multilayer granulosa cells in the ovaries of JDP2 null mice (Fig. 10D-E). Therefore, JDP2 null mice demonstrate elevated serum estradiol levels that may stem from higher FSH levels, and increased follicular recruitment to

the growing pool, resulting in the higher number of large follicles in the ovaries and increased number of pups per litter.

DISCUSSION

Notwithstanding extensive studies on regulation of the gonadotropin subunit genes expression and synthesis, little is known about negative regulators that keep the hormone levels within the tight physiological range. Here, we identified a negative regulator of FSHβ synthesis, JDP2, and analyzed its role, first in the model cells line and then *in vivo* using JDP2 null mice. JDP2 binds the FSHβ promoter as a cJun binding partner, and represses GnRH induction of FSHβ transcription. Similarly to the fact that FSHβ null males are not infertile while FSHβ null females are unable to reproduce, JDP2 null males do not exhibit any adverse effects on reproduction, while females have several significant changes. JDP2 null females have higher FSH levels, exhibit earlier vaginal opening, deliver their first litter sooner and have larger litters, but stop reproducing at a younger age than wild-types. The early cessation of reproductive function parallels our hypothesis that JDP2 nulls may mimic premature ovarian failure in women, a condition associated with increased FSH levels (9, 10). Genome-wide association studies (GWAS) in human population identified polymorphisms in the 5' region of the FSHβ gene, which are associated with elevated FSH levels, premature menarche, early natural menopause and dizygotic twinning (12, 13), implicating regulation of FSHβ transcription in the etiology of these reproductive phenotypes.

JDP2 belongs to the AP1 superfamily of transcription factors that bind the AP1/TRE element in the promoter of target genes. Classical AP1 transcription factor is a heterodimer of Fos and Jun immediate-early genes, which are activated rapidly and transiently by various stimuli and growth factors in numerous cell types (23). In turn, AP1 regulates a wide range of cellular processes, including proliferation, death, survival, and differentiation. However, each member of the family, although induced in several tissues, has a cell- and stimuli-specific function, and at time antagonize other family members (51, 52). We previously have shown that cFos has a cell-specific function at each level of the hypothalamic-pituitary-gonadal axis and precise roles in regulation of reproduction (38). We and others have also shown that cFos is rapidly induced by GnRH in the pituitary gonadotrope (53-55), dimerizes with cJun to induce FSHβ expression, and is degraded shortly thereafter. JDP2 was initially described as a repressor of cJun activity, via interaction with cJun and binding to the AP1/TRE site, likely by displacing cFos. Later studies expanded these findings to include repression of ATF2 function on the highly similar CRE element.

JDP2 is expressed at the high basal level and its levels change little with GnRH treatment. GnRH stimulus, however, rapidly increases the transcription of immediate early genes, cFos and cJun, which leads to induction of FSHβ transcription (55). We previously reported that cFos is not present without the stimulus, and after induction diminishes rapidly, due to its short half-life of 10 minutes for mRNA and about 1 hour for protein (26). cJun, on the other hand, is present in cells at the basal state, is further induced by GnRH, and has a longer half-life. Since both JDP2 and cJun are present prior to the stimuli, they form a complex that binds the FSHβ promoter, as we demonstrate here. DNA-binding domain of JDP2 is necessary for its function and JDP2 binds the TRE/AP1 site in the FSHβ promoter. Following GnRH stimulus cFos is induced at a high level and outcompetes JDP2 as a cJun binding partner, which leads to a transcriptionally active AP1 factor. Due to a short half-life, cFos is degraded allowing JDP2 to repress the transcription again until the next stimulus. In part, JDP2 represses transcription via recruitment of HDAC3 that deacetylates the histones (31, 32, 47). Although it is not clear if threonine 148 is necessary for chromatin deacetylation, it is an

activation site that represents a major difference between JDP2 and highly similar ATF3 (56). We establish that threonine 148 is necessary for repressive function of JDP2 at the cJun and FSHβ promoters. We also demonstrate that 1-hour GnRH treatment, coinciding with an increase in AP1 active complex of cJun and cFos formation that replaced the cJun and JDP2 complex, leads to an increase in histone acetylation followed by subsequent increase in FSHβ transcription.

Although the mouse model we used to analyze the *in vivo* role of JDP2 is a complete null, and we cannot exclude potential effects outside the pituitary, expression analysis of relevant hypothalamic neuropeptides, GnRH and kisspeptin, did not reveal any differences. Previous studies that analyzed JDP2 expression determined that although expressed in variety of tissues, JDP2 is highly expressed in female reproductive tissues where it interacts specifically with the progesterone receptor (57). Thus, previous studies postulated a role of JDP2 in female reproduction. We determined that indeed JDP2 controls female reproductive function via regulation of FSH synthesis. We did not observe changes in progesterone. Female null mice had an increase in FSHβ expression in the pituitary and FSH levels in the circulation, which may have caused increase in estrogen and higher number of large follicles.

FSH plays a role in follicular growth during the antral stage and stimulates estrogen synthesis in the granulosa cells (58, 59). Thus, in our mouse model, it is difficult to distinguish effects of the elevated FSH and possible effects of the lack of JDP2 in the ovary. Future studies, afforded by creation of the floxed JDP2 mouse model, may elucidate tissue specific effects of JDP2. Herein, we determined that JDP2 null mice exhibit higher estrogen levels, which may stem from the increase in FSH concentration. Surprisingly, increased estrogen does <u>not</u> exacerbate negative feedback on kisspeptin expression; however, the mix of the persistent positive and negative feedback may cancel each other, resulting in the same level of hypothalamic neuropeptides.

AMH concentration in JDP2 nulls exhibited a trend toward lower level. Effect on AMH levels may correlate with the increased number of pups and early reproductive senescence. In the ovary, AMH is produced by the granulosa cells of growing ovarian follicles, from primary follicle stage after follicular growth activation, to the small antral stage (49, 60). Interestingly, AMH has been shown to inhibit the recruitment of primordial follicles to the growing pool, thereby avoiding the premature exhaustion of the ovarian reserve. The regulation of AMH production in follicles remains poorly understood. While it is not considered that AMH exhibits feedback on the pituitary FSH expression as inhibin does, FSH may regulate AMH levels. The role of FSH in AMH expression in literature is controversial, with one study reporting induction of AMH by FSH (61), while another repression (49). Clinically, AMH serves as a biomarker that accurately reflects ovarian reserve. While relatively stable throughout the menstrual cycle (62), changes in serum AMH levels are associated with ovarian aging (60, 63). More importantly, serum AMH levels in patients with premature ovarian failure are extremely low (64, 65). Therefore, somewhat lower AMH levels may contribute to higher number of growing follicles in the ovary of JDP2 null females and premature cessation of reproduction.

Thus, we postulate that JDP2 serves as negative regulator of FSH to keep this hormone within the tight physiological range (Fig. 11). Without GnRH stimulus, FSHβ transcription (and cJun transcription) is quiescent, due to JDP2 association with the FSHβ promoter and its recruitment of HDAC3 that keeps chromatin, via deacetylation of histone 3, in a closed conformation. Following GnRH stimulus, others have shown that HDAC3 dissociates from the FSHβ promoter and herein we provide a mechanism of increased acetylation. Specifically, GnRH signaling leads to phosphorylation of ATF2, which displaces JDP2 and induces cJun transcription. Increased

expression of cJun together with elevated cFos, which removes JDP2 as a cJun binding partner, induce FSHβ transcription. Following a wave of transcription, ATF2 is dephosphorylated, cFos degraded and JDP2 returns to the cJun and FSHβ promoters to shut down their transcription until the next pulse of GnRH. *In vivo* studies strengthen our hypothesis, since JDP2 null females exhibit higher FSH levels and elevated serum estradiol that may result in higher follicular recruitment to the growing pool; larger number of late secondary and antral follicles in the ovaries leading to larger litters in young animals; and early cessation of reproductive function in older animals. These findings may have provided a candidate gene for premature ovarian failure, a condition associated with increased FSH levels and early loss of ovarian function in women.

EXPERIMENTAL PROCEDURES

JDP2 null mice - The JDP2 null mice were obtained from Dr. Shizuo Akira (WPI Immunology Frontier Research Center, Osaka University, Japan (36)). Animals were maintained under a 12-hour light, 12-hour dark cycle and received food and water ad libitum. All experiments were performed with approval from the University of California, Riverside; Animal Care and Use Committee and in accordance with the National Institutes of Health Animal Care and Use Guidelines. For genotyping, genomic DNA was extracted from toe biopsies and analyzed with PCR. Since male animals did not exhibit differences in hormone levels, sperm count or reproductive capacity, only female animals are analyzed in detail. At least 5 animals per genotype (WT and null), were analyzed, unless otherwise indicated, and differences compared by Student's T-test and Tukey's posthoc test.

Cell culture and transient transfections - The expression vectors for JDP2 and ATF3 were kindly provided by Dr. Ami Aronheim (Technion-Israel Institute of Technology, Haifa, Israel). The mouse FSHβ-luciferase, cJun-luciferase, 4x CRE and 4x TRE multimer reporter vectors were published previously (22,25,37).

LβT2 cells, kindly provided by Pamela Mellon, UCSD, San Diego, CA, were cultured at 37°C in DMEM (Cellgro, Mediatech, Inc., Herndon, VA) containing 10% fetal bovine serum (Omega Scientific Inc., Tarzana, CA) and penicillin. Cells were split into 12-well plates 1 day prior to transfection and transfected using Fugene 6 reagent (Roche Molecular Biochemicals, Indianapolis, IN) in accordance with the manufacturer's protocol. Wells were transfected with 500 ng of reporter plasmid, 100 ng of the β-galactosidase reporter plasmid driven by the Herpes virus thymidine kinase promoter to serve as an internal control for transfection efficiency, and 200 ng of expression vectors, or control, as indicated in the figure legends. Cells were incubated in serum-free DMEM containing 0.1% BSA and antibiotics overnight prior to hormone treatment with 10 nM GnRH (Sigma-Aldrich). Subsequent to treatment, cells were lysed with 60 μL of 0.1 M potassium-phosphate buffer pH 7.8 with 0.2% Triton X-100. A 96-well luminometer plate was loaded with 20 μL of each of the lysates and luciferase activity was measured after injection of a buffer containing 100 mM Tris-HCl with pH 7.8, 15 mM $MgSO_4$, 10 mM ATP, and 65 μM luciferin per well, using a Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, CA). The Galacto-Light Assay (Tropix, Bedford, MA) was performed according to the manufacturer's protocol to measure galactosidase activity. All experiments were performed a minimum of three times and in triplicates within each experiment. Luciferase values were normalized to β-galactosidase for each sample, and results presented as an average of three experiments +/- SEM. Statistical significance was determined with analysis of variance (ANOVA) followed by Tukey's posthoc test and significance was set at $p < 0.05$.

EMSA - Nuclear extracts from GnRH or vehicle treated LβT2 cells or COS-1 cells following overexpression for 48h were obtained by swelling the cells with hypotonic buffer (20 mM Tris pH 7.4, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM PMSF, protease inhibitor cocktail (Sigma-Aldrich), 10 mM NaF, 0.5 mM EDTA, 0.1 mM EGTA). Cells were broken by passing through a $25^5/_8$ G needle, 3 times. Samples were centrifuged at 4000 rpm for 4 min and the nuclear pellets were resuspended in hypertonic buffer (20 mM Hepes pH 7.8, 20% glycerol, 420 mM KCl, 1.5 mM $MgCl_2$, 1 mM PMSF, protease inhibitor cocktail (Sigma-Aldrich), 10 mM NaF, 0.5 mM EDTA, and 0.1 mM EGTA). Protein determination was performed using the Bradford reagent (Bio-Rad, Hercules, CA). The oligonucleotides that were used as 30-bp probes, encompassing the sites of interest from FSHβ and cJun promoters were reported previously (22, 25). *Oligonucleotides* were annealed and labeled with γ^{32}P ATP using T4 Polynucleotide Kinase (New England Biolabs, Inc., Beverly, MA). Binding reactions contained 2 μg of nuclear proteins in a total volume of 20 μl containing the following: 10 mM Hepes pH 7.8, 50 mM KCl, 0.5 mM $MgCl_2$, 10% glycerol, 0.1% NP-40, 0.25 μg dIdC, 5 mM DTT, and 5 fmol of labeled probe. Reactions were loaded onto a 5% nondenaturing polyacrylamide gel and ran in 0.25X Tris-borate-EDTA buffer. Gels were run at 250 V/cm^2 constant voltage and dried. Autoradiography was performed to identify complexes. Experiment was repeated at least three times and representative image is presented.

Western Blotting - Following overnight starvation and hormone treatment, LβT2 cells were rinsed with PBS and lysed with lysis buffer [20 mM Tris-HCl pH 7.4, 140 mM NaCl, 0.5% NP-40, 0.5 mM EDTA, with protease inhibitors (aprotinin, pepstatin, leupeptin at 10 μg/ml each), and 1 mM phenyl-methyl-sulfonyl-fluoride (PMSF)]. Protein concentrations were determined with Bradford reagent (Bio-Rad, Hercules, CA) and an equal amount of protein per sample was loaded on SDS-PAGE gels, or first immunoprecipitated with antibodies to protein tags and the precipitate loaded. After proteins had been resolved by electrophoresis and transferred to a membrane, they were probed with specific antibodies for cJun (sc-1694), ATF3 (sc-188,), or JDP2 (sc-23458) (Santa Cruz Biotechnology, Santa Cruz, CA). The complexes were detected with secondary antibodies linked to horseradish peroxidase and enhanced chemiluminescence reagent (Amersham Pharmacia, Piscataway, NY).

GST Interaction Assay - The Glutathione S-Transferase (GST)-NFY in the pGEX vector was reported previously (22). ^{35}S-labeled proteins were produced using the TnT® T7 Coupled Reticulocyte Lysate System (Promega Corporation, Madison, WI). Bacteria transformed with the pGEX vectors were grown to an OD of 0.6, upon which protein expression was induced by addition of 0.25 mM isopropyl-β-D-thiogalactosidase (IPTG). Bacterial pellets were sonicated in PBS with 5 mM EDTA and 0.1% Triton X-100, centrifuged and the supernatant was bound to glutathione sepharose beads (Amersham Pharmacia, Piscataway, NY). Beads were washed 4 times with sonication buffer followed by equilibration in the binding buffer (below), and split equally between different samples and the control. ^{35}S-labeled proteins were added to the beads and bound for 1 h at 4ºC in 20 mM Hepes (pH 7.8), with 50 mM NaCl, 10 mg/ml BSA, 0.1% NP-40, and 5 mM DTT. After extensive washing, samples were eluted and subjected to SDS-PAGE. Afterwards, the gels were dried and autoradiographed. Experiment was repeated three times and representative images are presented.

Immunohistochemistry - Tissues were fixed in 4% paraformaldehyde overnight at 4°C and dehydrated in ethanol/water washes before embedding in paraffin. Embedded tissues were cut into 14-μm sections with a microtome and floated onto SuperFrost Plus slides (Fisher Scientific, Auburn,

Alabama) and dried overnight at room temperature. Slides were incubated at 60°C for 30 minutes, deparaffinized in xylene washes, and rehydrated in ethanol/water washes. The slides were then used for either for hematoxylin-eosin staining (ovary) or immunohistochemistry (pituitary). For immunohistochemistry, antigen unmasking was performed by heating for 10 minutes in a Tris-EDTA-Tween20 mixture and endogenous peroxidase was quenched by incubating for 10 minutes in 0.3% hydrogen peroxide. After washing in phosphate-buffered saline (PBS), slides were blocked (PBS, 5% goat serum, 0.3% Triton X-100) for 45 minutes, incubated with primary antibodies against LH (1:1000, obtained from National Hormone and Peptide Program, NIDDK) overnight at 4°C. After washing, slides were incubated with biotinylated goat anti-rabbit IgG (1:300, Vector Laboratories) for 30 minutes. The Vectastain ABC elite kit (Vector Laboratories) was used per manufacturer's instructions and incubated for 30 minutes. After washing, the VIP peroxidase kit was used for colorimetric staining for 3 minutes. Slides were dehydrated in an ethyl alcohol series and xylene, and covered using Vectamount (Vector Laboratories). Images were obtained using Leica microscope system.

qPCR analysis - Tissues were dissected, total RNA extracted and reverse transcribed using Superscript III (Invitrogen, CA). qPCR was performed using an iQ SYBR Green supermix and an IQ5 real-time PCR machine (Bio-Rad Laboratories, Hercules, CA), with primers previously reported in (38), under the following conditions: 95 C for 15 min, followed by 40 cycles at 95 C for 20 sec, 56 C for 30 sec, and 72 C for 30 sec. A standard curve with dilutions of 10 pg/well, 1 pg/well, 100 fg/well, and 10 fg/well of a plasmid containing LHβ, or FSHβ, cDNA, was generated in each run with the samples. The amount of the gene of interest was calculated by comparing threshold cycle obtained for each sample with the standard curve generated in the same run. Replicates were averaged and divided by the mean value of the housekeeping gene in the same sample. After each run, a melting curve analysis was performed to confirm that a single amplicon was generated in each reaction. Statistical differences in expression between genotypes were determined using T-test and Tukey's posthoc test using JMP software (SAS Institute; Cary, North Carolina).

Hormone analysis - For serum collection, 8-week old mice were sacrificed by isoflurane inhalation and blood was obtained from the inferior vena cava. The blood was left to coagulate for 15 minutes at room temperature, and then centrifuged at 2000 RCF for 15 minutes for serum separation. Hormone assays were performed by University of Virginia, Ligand Core. The University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core is a fee-for-service core facility and is in part supported by the Eunice Kennedy Shriver NICHD/NIH (SCCPIR) Grant U54-HD28934. LH was analyzed using a sensitive two-site sandwich immunoassay (39), and mouse LH reference prep (AFP5306A; provided by Dr. A.F. Parlow and the National Hormone and Peptide program) was used as standard. FSH was assayed by RIA using reagents provided by Dr. A.F. Parlow and the National Hormone and Peptide Program, as previously described (40). Mouse FSH reference prep AFP5308D was used for assay standards. Estradiol, AMH and Inhibin levels were analyzed using validated commercially available assays, information for which can be found on the core's website:
http://www.medicine.virginia.edu/research/institutes-and-programs/crr/lab-facilities/assay-methods-page and reported in (41). Limits of detection were 0.24 ng/ml for LH, 2.4 ng/ml for FSH, 3 pg/ml for estradiol. Intra- and inter-assay coefficients of variation were 6.4%/8.0%, 6.9%/7.5%, 6.0%/11.4% and 4.4%/6.4% for the LH, FSH, AMH and estrogen (E2), respectively. For the assays used for this manuscript, inter-assay coefficients of variation data are the result of 30

assays. Six animals per group were used for each hormone analysis. Statistical differences in hormone levels between wild-type and null group were determined by Student's T-test, and Tukey-Kramer post hoc HSD for multiple comparisons using JMP software (SAS Institute; Cary, North Carolina).

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CONFLICT OF INTEREST:
The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS:

CRJ performed analysis of JDP2 null animals, including reproductive function, tissue dissection, and histological staining. NML performed experiments for the revision of the manuscript. LLR conducted experiment in gonadotrope cell line, including EMSA and transient transfections. ADW performed analysis of gene expression in tissues and cell line. DC conceived and coordinated the study and wrote the paper. All authors reviewed the results, revised the manuscript and approved the final version of the manuscript.

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FOOTNOTES

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The abbreviations used are: FSH, follicle-stimulating hormone; LH, luteinizing hormone; GnRH, gonadotropin-releasing hormone; JDP2, cJun-dimerization protein 2; AMH, anti-Mullerian hormone; AP1, activating protein 1; CRE, cyclic-AMP response element; TRE, TPA response element; HDAC, histone deacetylase; GWAS, genome-wide association study;

FIGURE LEGENDS

FIGURE 1. Time line of GnRH induction of gonadotrope genes. A, FSHβ (solid line) and cJun (dashed line) mRNA expression was determined by qPCR following 10 nM GnRH treatment of LβT2 cells for the times indicated on X-axis and normalized to the TBP mRNA. B, mRNA of the putative repressors, JDP2 (solid line) and ATF3 (dashed line) was analyzed in the same samples and also normalized to TBP mRNA. Experiment was repeated three times and results presented as an average +/- SEM. C, Protein levels of transcriptional regulators in the whole cell lysates following GnRH treatment for times indicated above each lane, were analyzed by western blots.

FIGURE 2. JDP2 diminishes GnRH induction of cJun and FSHβ. A, Four tandem copies of the CRE element (TGACGTCA) were linked to the minimal heterologous promoter and luciferase reporter in pGL3 backbone and transfected into LβT2 cells with Herpes virus thymidine kinase-driven β-galactosidase gene as an internal control for transfection efficiency; additionally, cells were co-transfected with empty vector control (ctrl) or expression vector for ATF3, or JDP2. After overnight starvation, cells were treated with vehicle or 10 nM GnRH for 5 hours, after which the luciferase and β-galactosidase values were obtained. B-C, cJun-luciferase plasmid (-1000 bp) was used as a reporter. D, Four copies of TRE element (TGAGTCA) served as a reporter. E-F, -1000 bp FSHβ-luciferase was transfected as a reporter. * indicates significant change in fold induction by GnRH, where GnRH-treated luciferase/β-galactosidase ratio was normalized to vehicle treated samples transfected with the same expression vector. # indicates significantly lower luciferase expression from the empty vector control (vehicle-treated samples transfected with overexpression vector compared to vehicle-treated empty vector; and GnRH-treated samples transfected with overexpression compared to GnRH-treated vector control). Vehicle (white bars); GnRH-treated (black bars).

FIGURE 3. JDP2 binds FSHβ (A) and cJun (C) promoters. Nuclear extract from either LβT2 cells treated with GnRH for times indicated above the lanes; or from Cos-1 cells transfected with overexpression vectors for proteins indicated above the corresponding lanes; were incubated with: (A) 30 bp radiolabeled probe that encompasses the AP1/TRE site in the FSHβ promoter, (B) 30 bp radiolabeled probe encompassing AP1/TRE site in the FSHβ promoter (TTGGTCA), or its mutation (TTGaaaA); compared to AP1/TRE consensus (TGAGTCA) or its mutation (TGAaaaA), (C) 30 bp radiolabeled probe encompassing the CRE site in the cJun promoter. EMSA was performed and gels subjected to autoradiography.

FIGURE 4. JDP2 interacts with NFY. A, GST-pull-down assays demonstrate that NFY can interact directly with JDP2, ATF2, ATF3, and cJun, but not cFos. S^{35} labeled proteins, indicated above each panel, were used in the binding assay with GST-NFY fusion protein, or control GST, labeled above each panel.

In the input panel, 10% of the in vitro transcribed and translated labeled proteins that were used in the binding reaction were run on the gel as control for their expression and labeling. B, Cells were transfected with histidine-tag-JDP2 (His) or Flag-tagged-cFos and complexes precipitated with antibodies to His-tag or Flag-tag, run on the gel, transferred and membranes probed with antibodies to cJun, cFos and NFY-A to determined proteins that interact with JDP2.

FIGURE 5. Threonine 148 is necessary for JDP2 repressor activity. cJun-luciferase (A) or FSHβ-luciferase (B) reporter were transfected into LβT2 cells with empty vector control, wild-type JDP2 (JDP2) or JDP2 mutated at the threonine 148 to alanine (T148A), or to glutamic acid (T148D), or with JDP2 with mutated DNA-binding domain (DBDm) and treated with vehicle or GnRH for 5 hours. # indicates statistically significant difference in reporter expression with JDP2 overexpression compared to empty vector control. Vehicle (white bars); GnRH-treated (black bars).

FIGURE 6. Histone 3 acetylation increases following GnRH treatment. Chromatin immunoprecipitation was performed using antibodies against acetylated histone H3 and total histone H3 following LβT2 cell treatment with GnRH for times indicated below each bar. Percent of precipitated chromatin with each antibody at each time point was calculated using serial dilution of total input chromatin, then acetylated H3 was normalized to total H3. # indicates statistically significant increase in acetylated H3 compared to 0 time point.

FIGURE 7. JDP2 null females exhibit reproductive anomalies. White bars, wild type; black bars, JDP2 nulls; 5 per group. Asterisks * indicate significant difference in analyzed reproductive parameters in the female JDP2 null animals from the wild type animals.

FIGURE 8. JDP2 null females have higher levels of FSH and higher expression of FSHβ. A-B, Serum from six 8-week old females per genotype was analyzed by RIA for LH and FSH. C-E, Whole pituitary mRNA was extracted from 6 animals per group and mRNA levels for LHβ (C), FSHβ (D) and common α-GSU (E) was determined by qPCR. White bars, wild type; black bars, JDP2 nulls. Asterisks * indicate significant difference in the JDP2 null animals from the wild type animals.

FIGURE 9. Pituitaries and gonadotrope number are unchanged in JDP2 null females. A, B Pituitaries were stained with antibodies to LH and representative images are shown at low magnification (A) and higher magnification (B, 40x objective, bar illustrates 50 μm). C, Gonadotropes from three different 8-week old females per group were counted and numbers presented per 0.08 mm^2. White bar, wild type; black bar, JDP2 nulls. D, E, Hypothalamus mRNA was extracted from 6 females per genotype, and GnRH and kisspeptin (Kiss1) mRNA analyzed by qPCR. White bars, wild type (WT); black bars, JDP2 nulls (KO).

FIGURE 10. JDP2 null females exhibit elevated estrogen levels and higher number of large follicles in the ovaries. White bars, wild type; black bars, JDP2 nulls. A-C, Serum AMH, estradiol and Inhibin A were analyzed in estrus females by ELISA. D, number of large follicles with multilayer granulosa cells were counted throughout the ovary. Asterisks * indicate significant difference in the JDP2-null animals from the wild type animals. E, Bottom panels, representative image of H&E stain of the wild-type (WT) and null (KO) ovaries.

FIGURE 11. JDP2 repression of cJun and FSHβ gene expression. JDP2 is present in the gonadotrope at the basal level and inhibits cJun and FSHβ transcription, indicated with "X". JDP2 binds cJun and FSHβ promoters at the CRE and TRE elements, respectively, and interacts with basal transcription factor NFY. JDP2 also interacts with ATF2 that binds cJun promoter at the basal state alone and as a heterodimer with JDP2 (top left); and with cJun that is expressed at some basal level and binds FSHβ promoter as a heterodimer with JDP2 (bottom left). GnRH treatment leads to phosphorylation and activation of ATF2 by p38 MAPK, which causes dissociation of JDP2 and induction of cJun transcription, indicated with an arrow (top middle). GnRH treatment also leads to induction of cFos via calcium calmodulin kinase II (CKII) activation, after which cFos displaces JDP2 as a cJun binding partner and activates FSHβ transcription (bottom middle). Dephosphorylation of ATF2 (top right) or degradation of cFos proteins due to their short half-life (bottom right) leads to recruitment of JDP2 and cessation of transcription until the next pulse of GnRH.

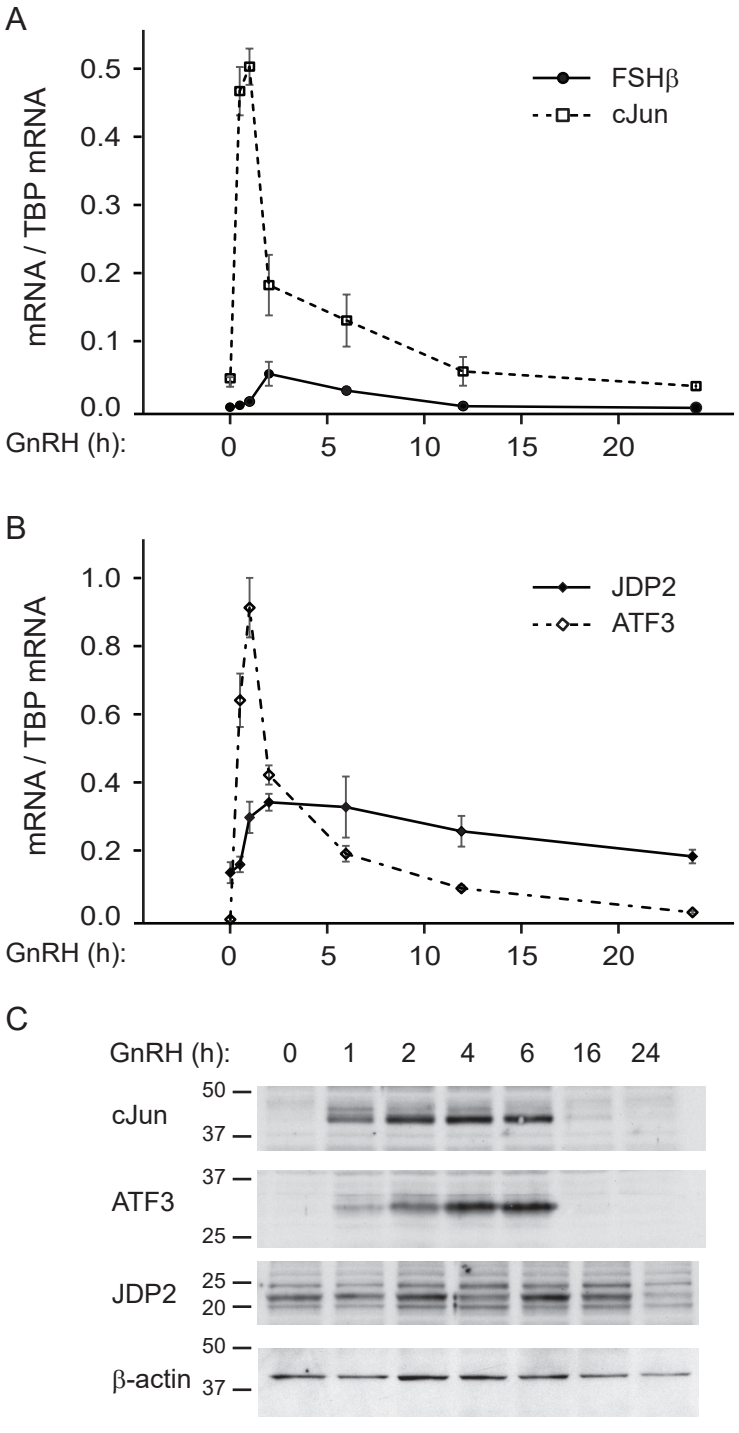
A
mRNA / TBP mRNA
0.5
0.4
0.3
0.2
0.1
0.0
FSHβ
cJun
GnRH (h):
0
5
10
15
20
B
mRNA / TBP mRNA
1.0
0.8
0.6
0.4
0.2
0.0
JDP2
ATF3
GnRH (h):
0
5
10
15
20
C
GnRH (h):
0
1
2
4
6
16
24
cJun
50
37
ATF3
37
25
JDP2
25
20
β-actin
50
37

Figure 1

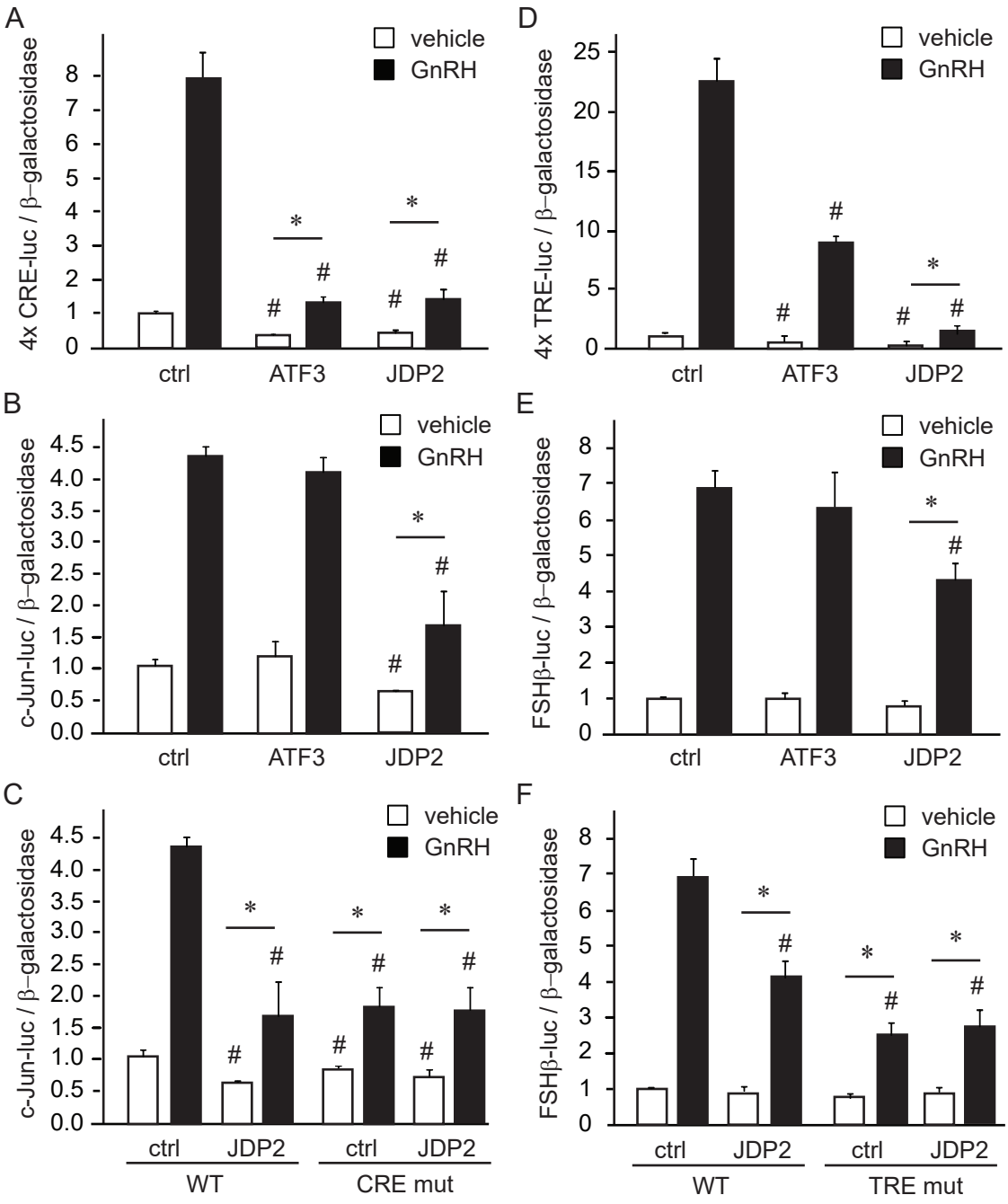
A
vehicle
GnRH
4x CRE-luc / β–galactosidase
ctrl
ATF3
JDP2
D
4x TRE-luc / β–galactosidase
B
c-Jun-luc / β–galactosidase
E
FSHβ-luc / β–galactosidase
C
WT
CRE mut
F
TRE mut

Figure 2

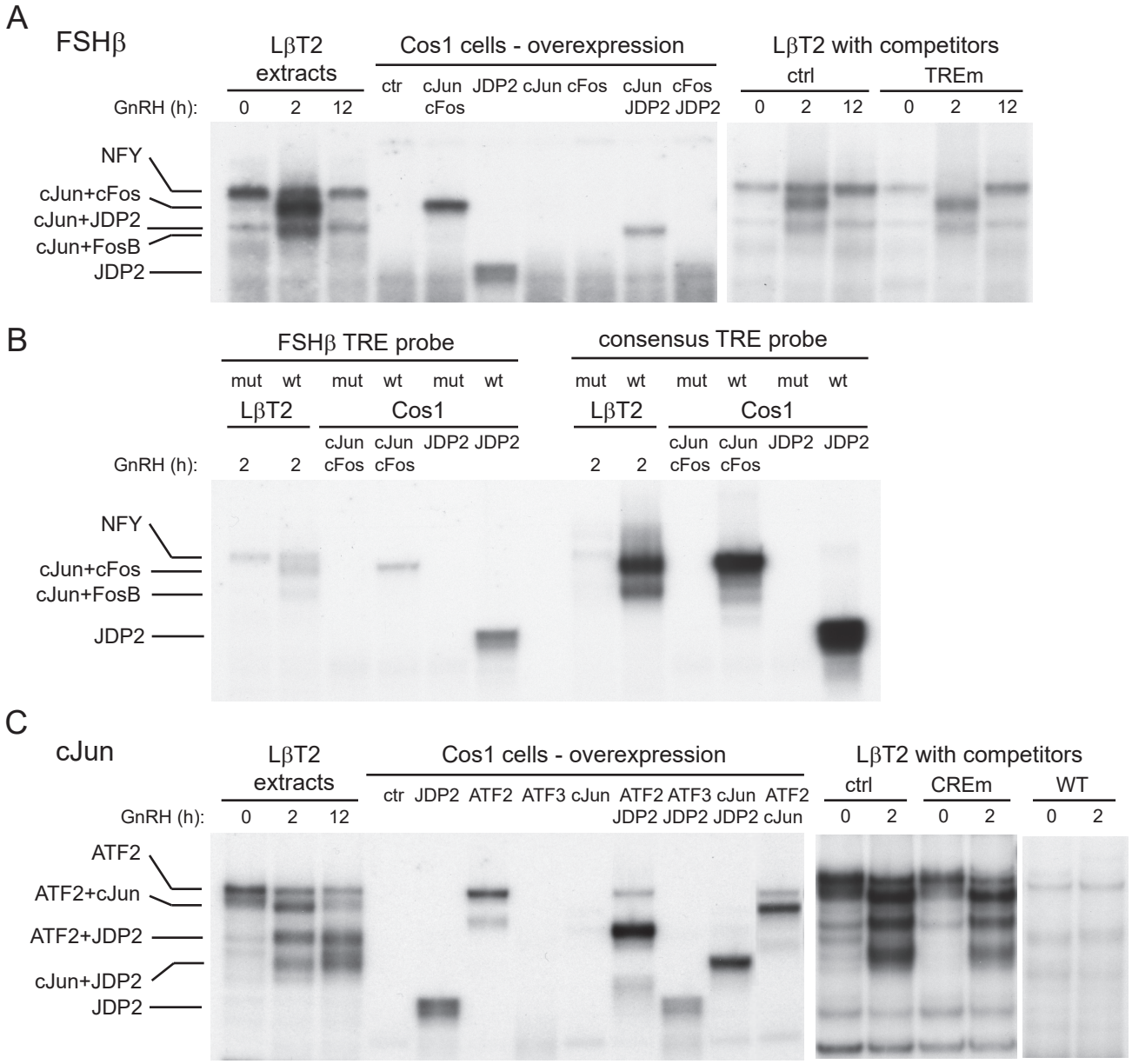
A
FSHβ
LβT2 extracts
Cos1 cells - overexpression
LβT2 with competitors
ctrl
TREm
ctr
cJun
JDP2
cJun cFos
cJun JDP2
cFos JDP2
GnRH (h):
0 2 12
0 2 12 0 2 12
NFY
cJun+cFos
cJun+JDP2
cJun+FosB
JDP2
B
FSHβ TRE probe
consensus TRE probe
mut wt mut wt mut wt
LβT2
Cos1
cJun cFos
JDP2
GnRH (h):
2 2
NFY
cJun+cFos
cJun+FosB
JDP2
C
cJun
LβT2 extracts
Cos1 cells - overexpression
LβT2 with competitors
ctrl
CREm
WT
ctr JDP2 ATF2 ATF3 cJun ATF2 JDP2 ATF3 JDP2 cJun JDP2 ATF2 cJun
GnRH (h):
0 2 12
0 2 0 2 0 2
ATF2
ATF2+cJun
ATF2+JDP2
cJun+JDP2
JDP2

Figure 3

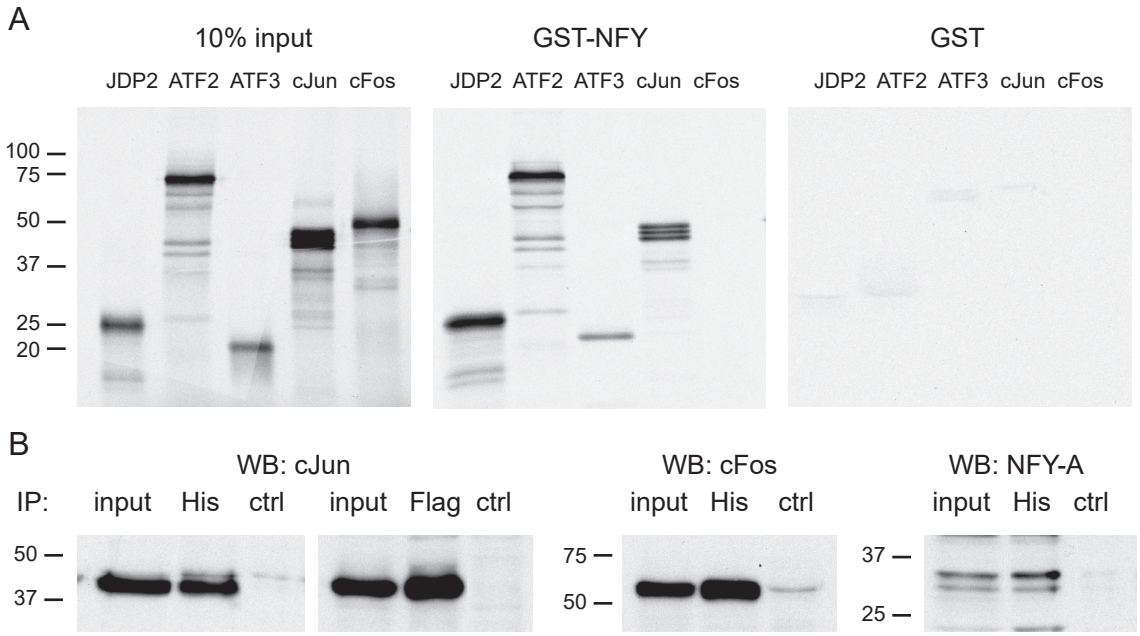
A
10% input
GST-NFY
GST
JDP2 ATF2 ATF3 cJun cFos
JDP2 ATF2 ATF3 cJun cFos
JDP2 ATF2 ATF3 cJun cFos
100
75
50
37
25
20
B
WB: cJun
WB: cFos
WB: NFY-A
IP: input His ctrl input Flag ctrl
input His ctrl
input His ctrl
50
37
75
50
37
25

Figure 4

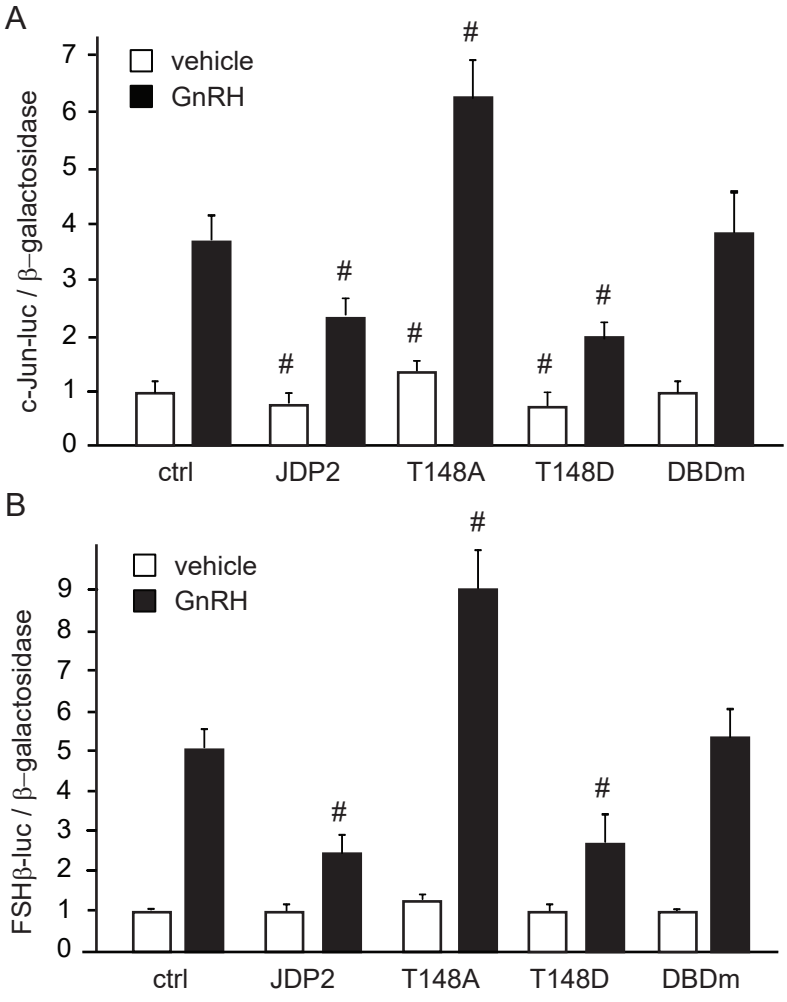
A
vehicle
GnRH
c-Jun-luc / β–galactosidase
0
1
2
3
4
5
6
7
#
ctrl
JDP2
T148A
T148D
DBDm
B
vehicle
GnRH
FSHβ-luc / β–galactosidase
0
1
2
3
4
5
6
7
8
9
#
ctrl
JDP2
T148A
T148D
DBDm

Figure 5

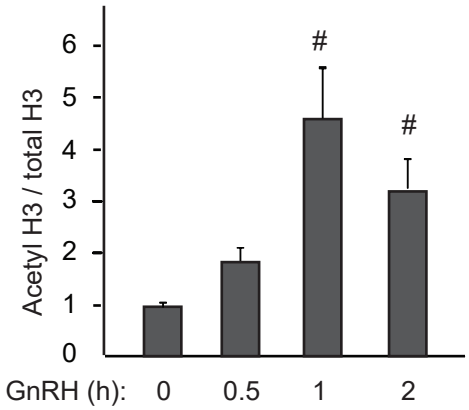

Figure 6

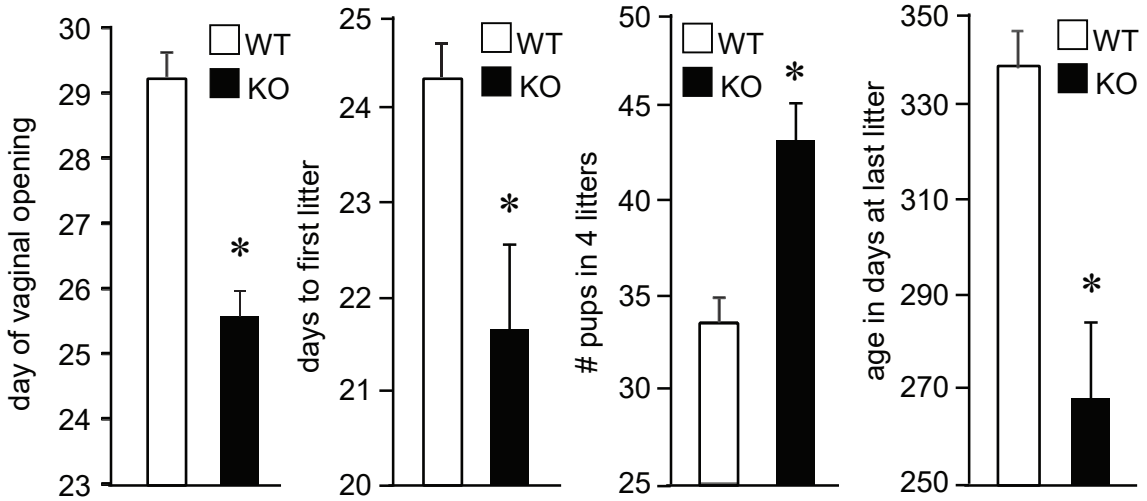
day of vaginal opening
30
29
28
27
26
25
24
23
WT
KO
*
days to first litter
25
24
23
22
21
20
WT
KO
*
pups in 4 litters
50
45
40
35
30
25
WT
KO
*
age in days at last litter
350
330
310
290
270
250
WT
KO
*

Figure 7

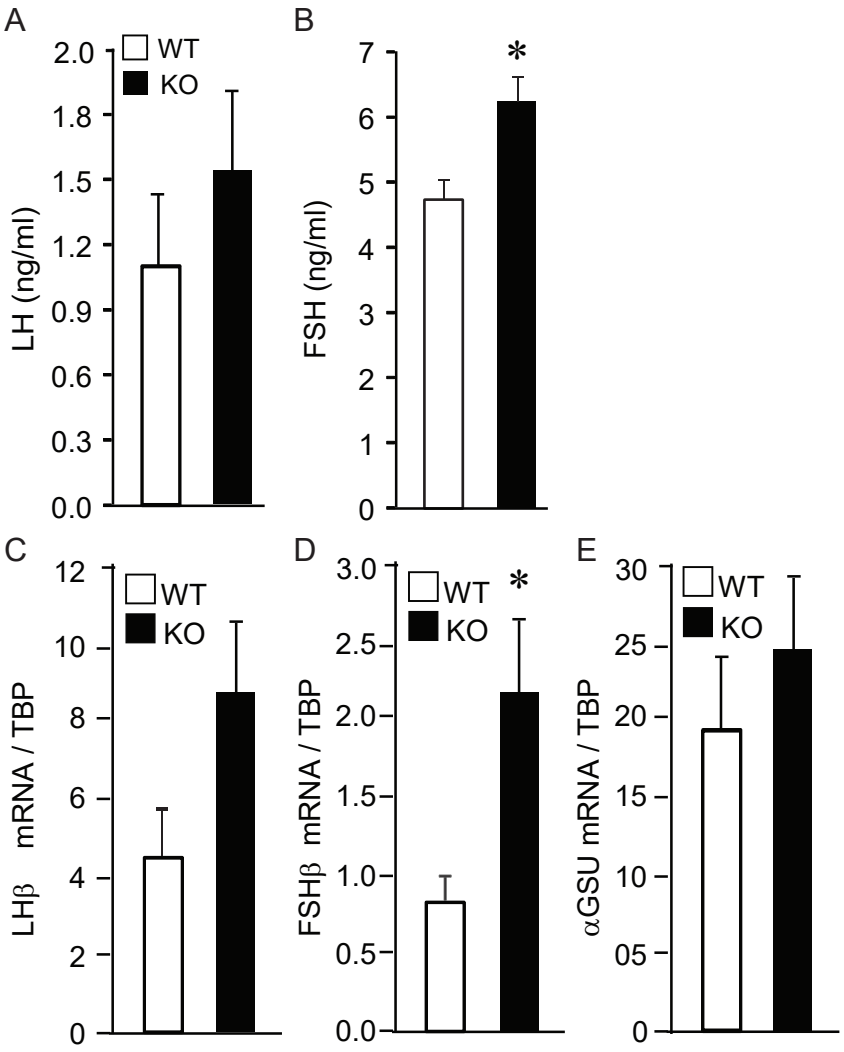
A
WT
KO
LH (ng/ml)
2.0
1.8
1.5
1.2
0.9
0.6
0.3
0.0
B
*
FSH (ng/ml)
7
6
5
4
3
2
1
0
C
WT
KO
LHβ mRNA / TBP
12
10
8
6
4
2
0
D
WT
KO
*
FSHβ mRNA / TBP
3.0
2.5
2.0
1.5
1.0
0.5
0.0
E
WT
KO
αGSU mRNA / TBP
30
25
20
15
10
05
0

Figure 8

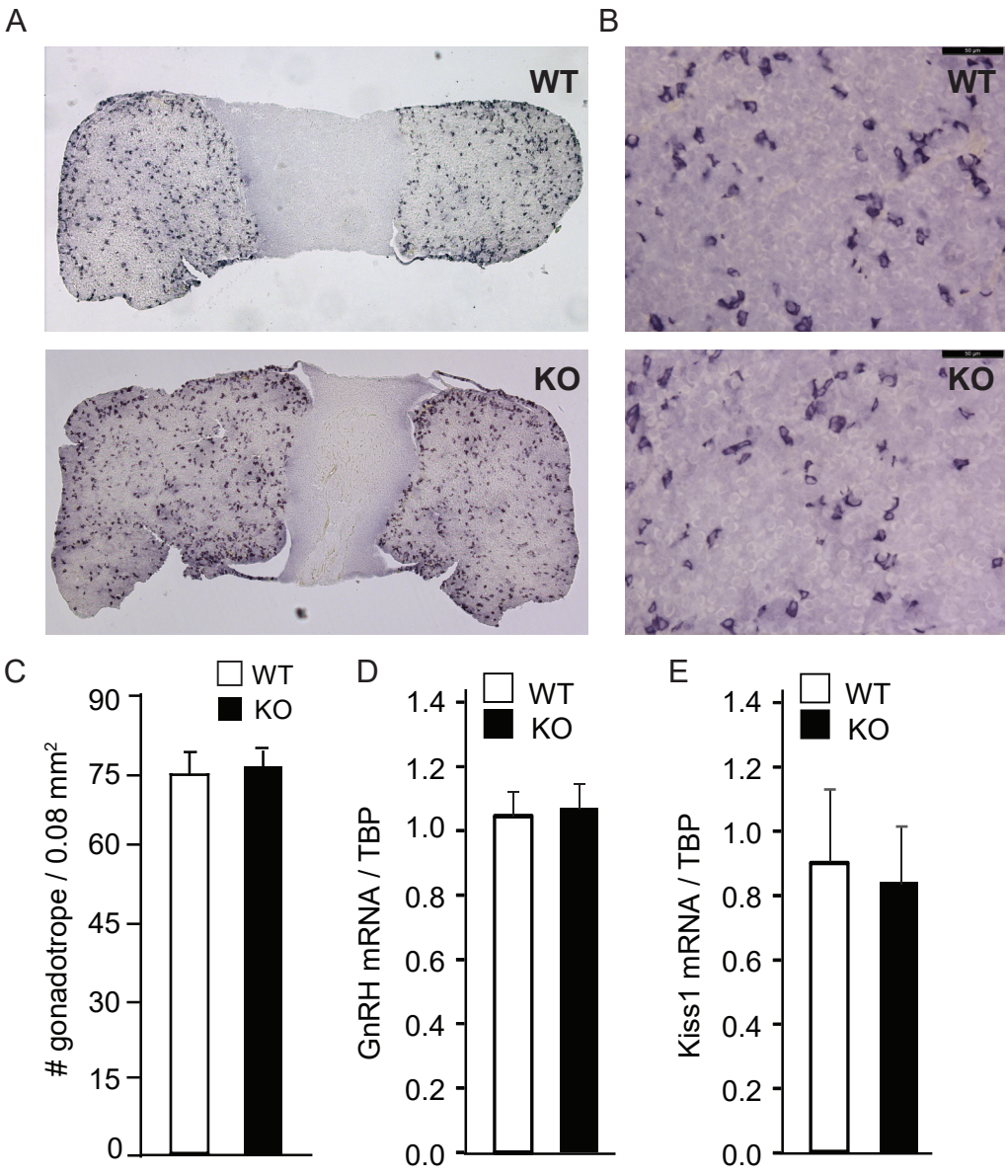
A
B
WT
KO
WT
KO
C
D
E
WT
KO
gonadotrope / 0.08 mm²
90
75
60
45
30
15
0
GnRH mRNA / TBP
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Kiss1 mRNA / TBP

Figure 9

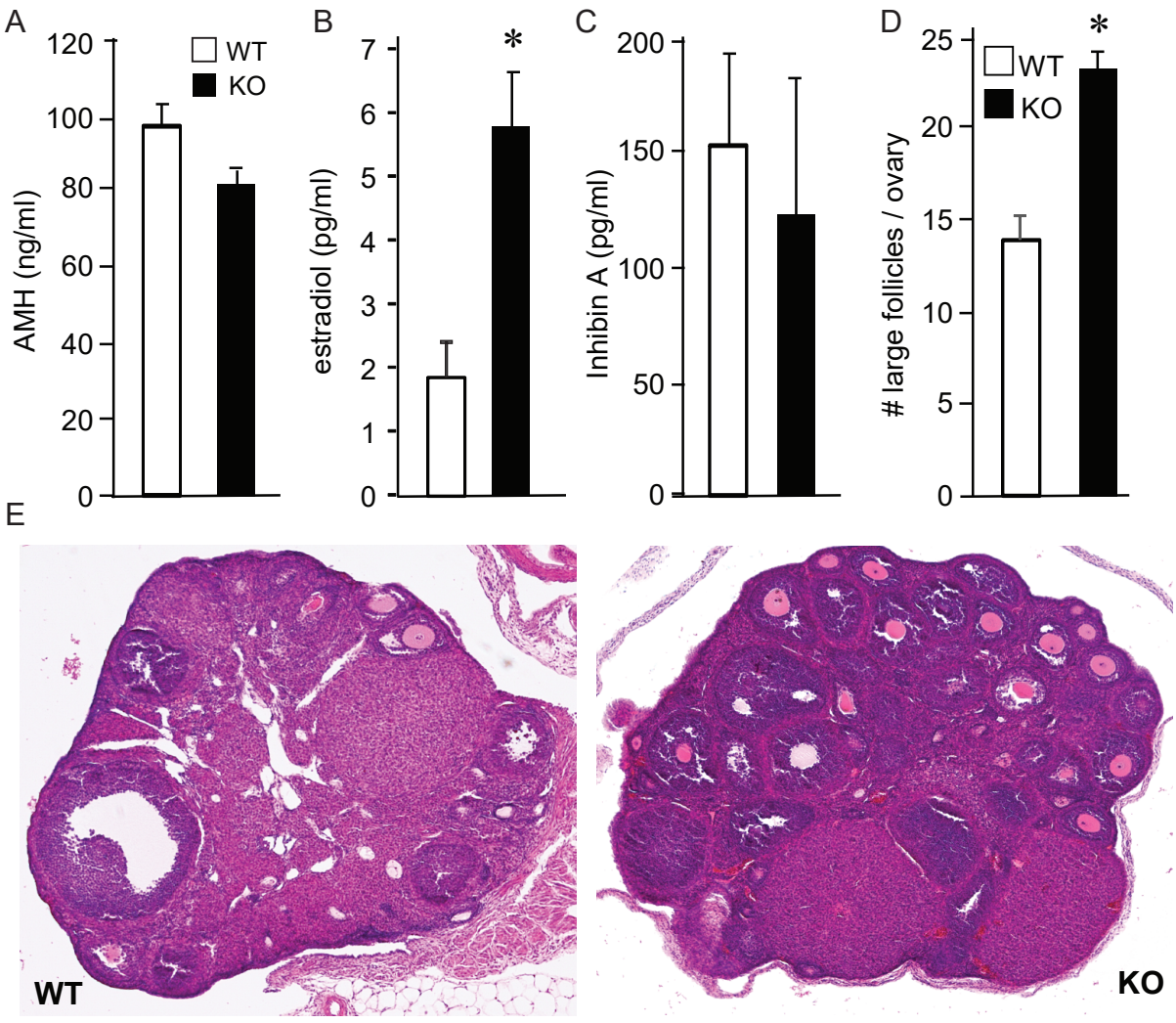
A
AMH (ng/ml)
120
100
80
60
40
20
0
WT
KO
B
estradiol (pg/ml)
7
6
5
4
3
2
1
0
*
C
Inhibin A (pg/ml)
200
150
100
50
0
D
large follicles / ovary
25
20
15
10
5
0
WT
KO
*
E
WT
KO

Figure 10

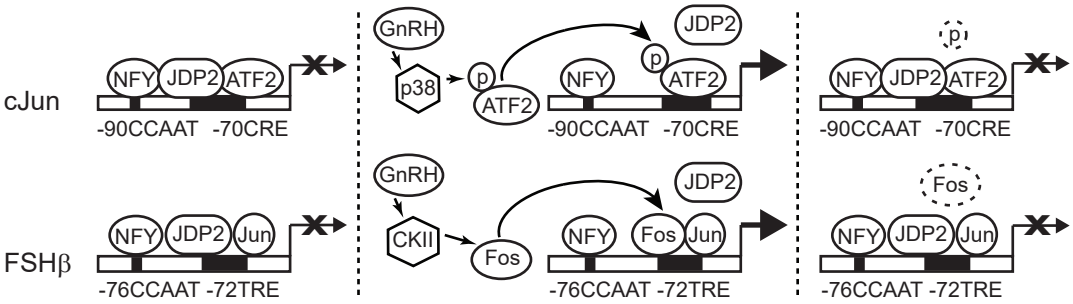
cJun
NFY
JDP2
ATF2
-90CCAAT -70CRE
GnRH
p38
p
ATF2
JDP2
p
NFY
ATF2
-90CCAAT -70CRE
p
NFY
JDP2
ATF2
-90CCAAT -70CRE
FSHβ
NFY
JDP2
Jun
-76CCAAT -72TRE
GnRH
CKII
Fos
JDP2
NFY
Fos
Jun
-76CCAAT -72TRE
Fos
NFY
JDP2
Jun
-76CCAAT -72TRE

Figure 11