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Notch signaling represses GATA4-induced expression of genes involved in steroid biosynthesis

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Abstract

***Notch2* and *Notch3* and genes of the Notch signaling network are dynamically expressed in developing follicles, where they are essential for granulosa cell proliferation and meiotic maturation. Notch receptors, ligands, and downstream effector genes are also expressed in testicular Leydig cells, predicting a potential role in regulating steroidogenesis. In this study, we sought to determine if Notch signaling in small follicles regulates the proliferation response of granulosa cells to FSH and represses the up-regulation steroidogenic gene expression that occurs in response to FSH as the follicle grows. Inhibition of Notch signaling in small preantral follicles led to the up-regulation of the expression of genes in the steroid biosynthetic pathway. Similarly, progesterone secretion by MA-10 Leydig cells was significantly inhibited by constitutively active Notch. Together, these data indicated that Notch signaling inhibits steroidogenesis. GATA4 has been shown to be a positive regulator of steroidogenic genes, including STAR protein, P450 aromatase, and 3B-hydroxysteroid dehydrogenase. We observed that Notch downstream effectors HEY1, HEY2, and HEYL are able to differentially regulate these GATA4-dependent promoters. These data are supported by the presence of HEY/HES binding sites in these promoters. These studies indicate that Notch signaling has a role in the complex regulation of the steroidogenic pathway.**

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Introduction

It is well established that gonadotropins participate in complex feedback loops required for spermatogenesis and folliculogenesis. Follicle-stimulating hormone (FSH) is important for the development of spermatogonia and steroidogenesis in Leydig cells. FSH also regulates the number of Sertoli and Leydig cells (Baker & O'Shaughnessy 2001, Baker *et al*. 2003). During folliculogenesis, granulosa cells respond differently to FSH depending on follicle size. In small preantral follicles, FSH induces granulosa cell proliferation. In larger follicles, however, FSH induces these cells to express the luteinizing hormone receptor and enzymes of the steroid biosynthesis pathway, such as P450 aromatase (*Cyp19a1*), P450 side chain cleavage enzyme (*Cyp11a1*), and STAR protein (*Star*) (Cortvrindt *et al*. 1997, Robker & Richards 1998, Spears *et al*. 1998, Kreeger *et al*. 2005, Kwintkiewicz *et al*. 2007). The mechanism(s) by which small follicle granulosa cells are responsive to only the mitogenic activity of FSH remain poorly understood. In preantral follicles, all granulosa cells have active Notch signaling (Johnson *et al*. 2001, Hahn *et al*. 2005, Vanorny *et al*. 2014), and thus, this pathway may mediate the proliferation response of granulosa cells to FSH in growing follicles by suppressing their differentiation and the expression of genes involved in steroidogenesis.

The evolutionarily conserved Notch signaling pathway has been shown to be required for follicle development and fertility. We, and others, have demonstrated that *Notch2*, *Notch3*, and the ligands Jagged1 (*Jag1*) and *Jag2* are expressed dynamically in the cells of growing follicles (Johnson *et al*. 2001, Vorontchikhina *et al*. 2005, Vanorny *et al*. 2014). The downstream effector genes *Hes1*, *Hes5*, *Hey1*, *Hey2*, and *HeyL* overlap with Notch and its ligands in the granulosa cells (Johnson *et al*. 2001, Hahn *et al*. 2005). Several lines of evidence indicate that Notch signaling in the ovary is necessary for granulosa cell proliferation and normal follicle development. In mice deficient for lunatic fringe (*Lfng*), a modifier of the extracellular domain of the Notch receptors, Notch signaling was

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inhibited in granulosa cells, and these ovaries contained aberrant multi-oocyte follicles (MOFs) that did not undergo meiotic maturation properly (Hahn *et al.* 2005). In *ex vivo* ovary culture, the addition of γ-secretase inhibitors that block Notch signaling resulted in a loss of granulosa cell proliferation (Zhang *et al.* 2011). *Notch2* is necessary for primordial follicle formation (Tromby *et al.* 2008) and *Notch2* deficient ovaries had MOFs that demonstrated a lack of granulosa cell proliferation (Xu & Gridley 2013, Vanorny *et al.* 2014). A similar ovarian phenotype was found in mice with an oocyte-specific mutation of *Jag1* (Vanorny *et al.* 2014).

Notch signaling pathway genes are also expressed in the developing testis and juvenile and adult Leydig cells (Dirami *et al.* 2001, von Schönfeldt *et al.* 2004, Lupien *et al.* 2006, Tang *et al.* 2008, Defalco *et al.* 2013). During gonadogenesis, Notch signaling regulates the differentiation of Leydig cells from progenitors (Tang *et al.* 2008). Inhibiting Notch signaling increased the number of Leydig cells, and conversely, expression of constitutively active Notch resulted in a loss of these cells pre- and postnatally (Tang *et al.* 2008, Defalco *et al.* 2013). Interestingly, gain- or loss-of-function Notch mutations resulted in both aberrant testis cord formation and loss of germ cells (Tang *et al.* 2008).

There are four Notch receptors (*Notch1–4*) in mammals and five ligands: Deltalike1 (*Dll1*), *Dll3*, *Dll4*, *Jag1*, and *Jag2* (Kopan & Ilagan 2009). On ligand activation, the Notch intracellular domain (NOTCHICD) translocates to the nucleus and binds DNA in a complex with an obligate cofactor, RBPJK (Jarriault *et al.* 1995, Lu & Lux 1996, Ong *et al.* 2006), activating the transcription of its downstream target effectors, the HES and HEY proteins. The HEY and HES proteins are two families of basic helix–loop–helix Orange (bHLH-O) transcriptional repressors (Nakagawa *et al.* 2000, Iso *et al.* 2003, Kokubo *et al.* 2005). In this way, Notch signaling can both activate and repress gene transcription.

The HES and HEY proteins are able to repress transcription through several distinct mechanisms. They can form homodimers that bind to E (CANNTG) or N (CACNAG) boxes in target promoters and then recruit transcriptional repressor complexes (Sasai *et al.* 1992, Kageyama & Nakanishi 1997, Takata & Ishikawa 2003, Kokubo *et al.* 2005, Holderfield & Hughes 2008, Niwa *et al.* 2011). Alternatively, they can repress transcription through the formation of heterodimers with ubiquitously expressed class A bHLH factors, such as E12 and E47. The sequestration of these factors in nonfunctional heterodimers prevents the formation of heterodimers with tissue-specific bHLH activators, such as MYOD or PARAXIS (Leimeister *et al.* 2000, Iso *et al.* 2003). The HEY proteins also form complexes with non-bHLH transcription factors and repress their activity. For example, HEY1 binds to the androgen receptor (Belandia *et al.* 2005), and HEY2 binds to the aryl hydrocarbon receptor nuclear translocator (Chin *et al.* 2000). The HEY proteins have also been demonstrated to form complexes with GATA factors and repress expression of genes such as atrial naturietic factor, myosin heavy chain, and Müllerian inhibiting substance through GATA sites (Elagib *et al.* 2004, Kathiriya *et al.* 2004, Fischer *et al.* 2005, Ishiko *et al.* 2005, Martin *et al.* 2005, Shirvani *et al.* 2006, Xiang *et al.* 2006).

Transcription of genes in the steroid biosynthesis pathway is positively regulated in the gonads by steroidogenic factor 1 (referred to as *SF1*, gene symbol *Nr5a1*), a nuclear receptor and CREB/CREM (Ito *et al.* 1997, Gurates *et al.* 2003, Ragazzon *et al.* 2006, Schimmer & White 2010). In contrast, dosage-sensitive sex reversal adrenal hypoplasia critical region on chromosome X (*NrOb1* gene symbol, referred to as *Dax1*) is a nuclear receptor that acts as a pan transcriptional inhibitor of steroid biosynthetic genes (Tremblay & Viger 2001*a*, Wang *et al.* 2001, Lalli & Sassone-Corsi 2003, Jo & Stocco 2004, Manna *et al.* 2009). Notch signaling could regulate steroid biosynthesis by acting on these regulators of steroidogenesis, either by inhibiting the transcription of SF1 or by up-regulating expression of Dax1.

GATA4, a zinc finger domain transcription factor, has a double zinc finger-binding domain that recognizes the GATA motif in promoters (review Tevosian (2014)). GATA4 is expressed in both Leydig and granulosa cells (Heikinheimo *et al.* 1997, Viger *et al.* 1998, 2004, Laitinen *et al.* 2000, Tremblay & Viger 2001*a*,*b*, Martin *et al.* 2005, Padua *et al.* 2014, 2015) and is a key positive transcriptional regulator of genes involved in steroid biosynthesis, including *SF1*, *Cyp19a1*, *Star*, 3B hydroxysteroid dehydrogenase (*Hsd3b1*), and *Cyp11A1* (Wooten-Kee & Clark 2000, Tremblay & Viger 2001*a*,*b*, Tremblay *et al.* 2002, Bouchard *et al.* 2005, Martin *et al.* 2005, Bergeron *et al.* 2015, Schrade *et al.* 2015). Notch signaling has been demonstrated to inhibit GATA factor-mediated transactivation of cardiac, hematopoietic, and skeletal muscle specific promoters (Kathiriya *et al.* 2004, Fischer *et al.* 2005, Ishiko *et al.* 2005, Shirvani *et al.* 2006), indicating that the steroidogenic promoters could also be targets of the Notch pathway.

In the present study, we sought to examine the role of Notch and its HEY downstream effector proteins in the regulation of the expression of genes involved in gonadal steroidogenesis. Our data indicates that Notch signaling inhibits FSH-induced steroidogenic gene expression in small follicles. Also, activated Notch blocked the synthesis of progesterone in cultured MA-10 Leydig cells. A number of genes encoding important enzymes involved in the gonadal steroidogenesis pathway are positively regulated by GATA4 through direct transcriptional activation of the corresponding promoter sequences. We now show that GATA4-dependent activation of these promoters is also a target for

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Notch-mediated repression, further supporting a role for Notch signaling in the control of steroidogenesis.

Materials and methods

Cells

MA-10 cells, a Leydig tumor cell line (a kind gift from Dr Mario Ascoli, University of Iowa), were cultured in RPMI-1640 (Life Technologies) with 15% horse serum (GE Healthcare Hyclone, Logan, UT, USA), 20 mM HEPES (Sigma–Aldrich), 4 mM glutamine (GE Healthcare Hyclone), and 50 μg/ml gentamycin (Mediatech, Herndon, VA, USA) on gelatinized plates as described in Mizutani *et al*. (2006). NIH3T3 cells were purchased from ATCC (Manassas, VA, USA) and cultured in DMEM (Life Technologies) with 10% newborn calf serum (Atlanta Biologicals, Flowery Branch, GA, USA) and Primocin (Invivogen, San Diego, CA, USA).

Plasmids

The GATA4 expression plasmid and promoter–luciferase constructs, mouse *Star* (−902 to +17), *Cyp19a1* (−218 to +44), and human *HSD3B2* (−224 to +53), have been described elsewhere (Tremblay & Viger 2001*a*,*b*, Tremblay *et al*. 2002, Martin *et al*. 2005). The pCS2+ -Notch1ICD and -Notch3ICD constructs were kind gifts of Dr Raphael Kopan (Washington University, St Louis, MO, USA). The mouse Notch2ICD (a kind gift of Dr Kathy Loomes, Children's Hospital of Philadelphia, Philadelphia, PA, USA) was cloned into the pCS2+ backbone. The Hey2 expression plasmid and deletion constructs Hey2-62 and Hey2-121 were cloned into the pCS2+ vector and confirmed by sequencing.

Animals

CD1 mice (*Mus musculus*) were bred and housed in a vivarium at Arizona State University (ASU) on a 10 h light:14 h darkness schedule with access to food and water *ad libitum*. ASU is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AALAC). All procedures were carried out in compliance with the ASU Institutional Animal Care and Use Committee and AALAC under an approved research protocol.

Follicle culture

Ovaries from 14-day-old CD1 mice were harvested and follicles were mechanically isolated using a fine needle. Small preantral follicles that were ∼200 μm in diameter and had attached theca cells were chosen for culture, as described previously (Murray *et al*. 1998, Lenie *et al*. 2004). The follicles were cultured in α-MEM (Mediatech) with 5% FBS (Atlanta Biologicals), insulin, transferrin and selenium (ITS) (Sigma–Aldrich), 50 μg/ml ascorbic acid (Sigma–Aldrich), 0.3% BSA (Sigma–Aldrich), 2 mM glutamine (GE Healthcare HyClone), and penicillin/streptomycin (Mediatech) in the presence of 200 mIU/ml FSH (Sigma–Aldrich) under one of the following conditions: DMSO vehicle only, 25 or 50 μM *N*-(*N*-(3,5-difluorophenacetyl-L-alanyl))-(*S*)-phenylglycine *t*-butyl ester (DAPT), a Notch inhibitor (Sigma–Aldrich). Each treatment well received 20 follicles and each treatment was done in duplicate. After 48 h, total follicle RNA was purified for analysis by quantitative RT-PCR (qRT-PCR). The data are the results of three experiments.

Enzyme immunoassay progesterone assay

MA-10 cells were seeded at 1×10^5 cells/well and cultured as above. These cells were transfected using Fugene6 (Invitrogen) at a 6:2 ratio with a maximum of 2 μg of DNA. Empty vector was used to equalize total DNA concentration across all transfections. At 48 h post-transfection the cells were transferred into serum-free medium and treated with 1 mM dibutyryl cAMP (dbcAMP; Sigma–Aldrich) or vehicle for 6 h. The culture medium was collected for enzyme immunoassay (EIA). The concentration of progesterone in the collected medium was determined using an EIA assay per the manufacturer's protocol (Cayman Chemicals, Ann Arbor, MI, USA). Cells were then lysed and RNA was isolated for qRT-PCR.

Quantitative RT-PCR

MA-10 cells were cultured, transfected, and treated with dbcAMP, as described above. MA-10 cells or intact follicles were lysed in TRIzol (Invitrogen) for RNA isolation, per the manufacturer's protocols. For these studies, three biological replicate experiments were performed. RNA was treated with DNase I and quantified by NanoDrop prior to cDNA synthesis using SuperScriptIII reverse transcriptase (Invitrogen). For each sample, 1 μg of RNA was used for cDNA synthesis. The cDNA was quantified using transcript-specific, intron-spanning primers and real-time PCR with Syber Green (Eurogentec, Fremont, CA, USA) on an ABI 7900 HT thermocycler using a 384-well format in 10 μl reactions. Products from each primer set were sequenced and analyzed by BLAST (National Center for Biotechnology Information (NCBI)) to verify their identity. Primer efficiency was determined using a standard curve. For each transcript, three biological replicates were assayed in triplicate. All samples were normalized to the *Gapdh* transcript and relative gene expression was calculated using $\Delta\Delta Cq$ analysis (Haimes & Kelley 2010). For follicle RNA, the control sample was follicles cultured with FSH and vehicle. For MA-10 cells, the control sample was cells cultured without any treatment. Primer sequences for mouse genes are as follows: *Gapdh*, 5′-GGGAAGCCCATCACCATCTT-3′ and 5′-GCCC-TTCTCCATGGTGGTGAA-3′; *Dax1*, 5′-GCCCTTTTCCTGCT-GAGATTC-3′ and 5′-TCACAGCTTTGCACAGAGCAT-3′; *SF1*, 5′-CGCAACAACCTTCTCATTGAGA-3′ and 5′-TGGA-TCCCTAATGCAAGGAGTCT-3′; *Star*, 5′-AAGCTGTGTGCTG-GAAGCTCCTAT-3′ and 5′-TGCTTCCAGTTGAGAACCA AGCAG-3′; *Hsd3b1* (mouse homolog of human HSD3B2), 5′-ACACAAGGAAGGAATTCTCCAAGCTG-3′ and 5′-CCTCC-AATAGGTTCTGGGTACCTT-3′; *Cyp19a1*, 5′-CAGCAAGT-CCTCAAGCATGTTCCA-3′ and 5′-TTCCACCATTCGAAC AAGACCAGG-3′; *Hes1*, 5′-CAACACGACACCGGACAAAC-CAAA-3′ and 5′-TGGAATGCCGGGAGCTATCTTTCT-3′; and *Notch2*, 5′-TGCTGTGGCTCTGGCTGT-3′ and 5′-TGTGG-TAGGTAACACAGGTCCCT-3′.

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Luciferase assays

NIH3T3 cells were plated at a density of 8×10^4 cells/ml in 24-well plates and cultured as described above. Cells were transfected the next day with Lipofectamine and Plus reagent following the manufacturer's protocol (Invitrogen). Each transfected well received 0.05 μg of GATA4 expression vector, 0.2 μg of steroidogenic gene promoter–luciferase reporter construct, and 0.025 μg of plasmids expressing NotchICDs or Hey proteins. All transfections included 0.05 μg of pCMV–eGFP (Invitrogen). Total plasmid DNA was kept at 0.4 μg/well in all transfections with the addition of empty vector. All assays were done in triplicate on the same plate. The control sample for these assays was transfection of the reporter only. At 48 h post-transfection, cells were lysed in Luciferase Cell Culture Lysis Buffer (Promega). Luciferase activity was measured for each well by reacting 20 μl of cell lysate with 100 μl of Luciferase Assay Buffer (Promega) in 96-well plates, using an FLx800 microplate reader (Biotek Instruments, Winooski, VT, USA). All samples were normalized to GFP expression. All data are the mean±s.d. of three experiments done in triplicate, and statistical significance was determined using a one-way ANOVA.

Bioinformatics techniques

Steroid enzyme promoter–luciferase constructs were sequenced and analyzed for the presence of transcription factor binding sites using the TFsearch database (Heinemeyer *et al*. 1998), which can be found at http://www.cbrc.jp/cbrc-databases.

Statistical analyses

All data are the mean±s.d. of a minimum of triplicate replicates from three biological experiments. Statistical significance was determined using one-way ANOVA except for qRT-PCR that was analyzed using two-way ANOVA, $P<0.05$ for statistical significance at the 95% confidence limit.

Results

FSH induces both proliferation and expression of steroidogenic genes in granulosa cells depending on follicle size (Robker & Richards 1998, Kwintkiewicz *et al*. 2007). *Notch2* and *Notch3* are expressed in all granulosa cells of small growing follicles (Johnson *et al*. 2001), and Notch signaling induces proliferation of many cell types, including granulosa cells (Zhang *et al*. 2011, Vanorny *et al*. 2014), thus, it is possible that Notch acts downstream of FSH. To examine this, we cultured preantral follicles from 14-day-old CD1 mice in the presence or absence of a Notch inhibitor, DAPT, that blocks ligand-mediated activation of Notch and relieves Notch-mediated repression of gene expression (Fig. 1). Follicles were cultured in the presence of FSH under one of the following conditions: DMSO vehicle only or 25 or 50 μM DAPT. After 48 h, total follicle RNA was purified and gene expression was analyzed using qRT-PCR. *Hes1*, whose transcription is up-regulated by active Notch signaling, was significantly inhibited by DAPT indicating that Notch signaling was blocked (Fig. 1). Conversely, *Notch2* expression was increased through a reciprocal signaling pathway, as expected (Vanorny *et al*. 2014). Genes encoding enzymes important for the synthesis of gonadal steroids – *Star*, *Cyp19a1*, and *Hsd3b1* (Payne & Hales 2004) – were significantly up-regulated in follicles cultured in 25 or 50 μM DAPT (Fig. 1). These data indicate that Notch signaling inhibits

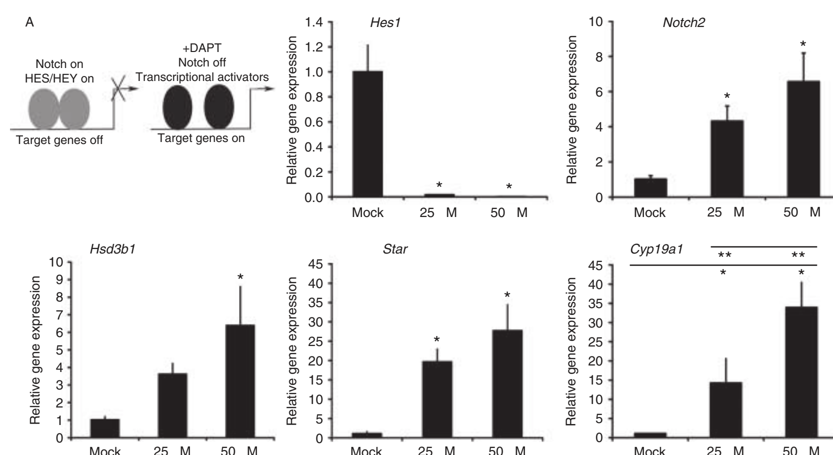

Figure 1 Notch signaling inhibits expression of steroidogenic genes in granulosa cells from preantral follicles. (A) Cartoon describing the assay. Preantral follicles were isolated from 14-day-old CD1 mice and 20 follicles were cultured in the presence of FSH and 0, 25, or 50 μM DAPT to block Notch signaling for 48 h. Gene-specific qRT-PCR was done on total follicle RNA. *Hes1* transcription is repressed and demonstrates that DAPT inhibited Notch signaling. *Notch2* transcription was increased, as expected. *Hsd3b1*, *Star*, and *Cyp19a1* demonstrated increased transcription when cultured with FSH and DAPT. Significance was determined by ANOVA, $P<0.05$, *indicates that the sample is statistically different from the mock sample and **indicates 25 and 50 μm DAPT samples are statistically different from each other. Data are the results of three experiments, each done in triplicate.

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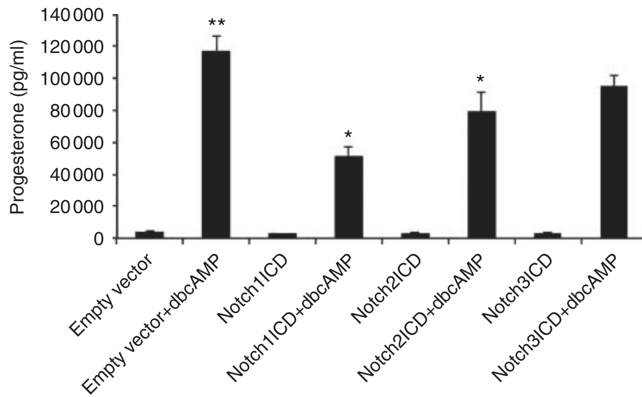

Figure 2 Activated Notch inhibits progesterone secretion in MA-10 cells. MA-10 cells were transfected with a Notch1–3ICD or empty vector. At 48 h post-transfection, cells were stimulated with dbcAMP to induce steroidogenesis and progesterone was detected in cell supernatants by EIA. Cells transfected with empty vector and treated with dbcAMP secreted a significant amount of progesterone compared to empty vector transfected cells (**$P<0.001$). When transfected with Notch1ICD or Notch2ICD, progesterone secretion by dbcAM-treated cells was significantly inhibited, as compared to dbcAMP-treated cells that received empty vector (*$P<0.05$). Notch3ICD decreased progesterone secretion in the presence of dbcAMP, but not significantly. Transfection with Notch1–3ICD without dbcAMP treatment had no effect on progesterone synthesis. Data are the means±S.E.M. of three experiments done in triplicate.

FSH-induced expression of steroidogenic genes in granulosa cells of small preantral follicles.

Because Notch pathway genes are also expressed in Leydig cells, the effect of Notch signaling on steroidogenesis was examined in MA-10 cells, a well-characterized Leydig tumor cell line that synthesizes progesterone when stimulated by hormones or dbcAMP (Ascoli 1981). MA-10 cells were transfected with the constitutively active intracellular domains of *Notch1*, *Notch2*, or *Notch3*. At 48 h post-transfection the cells were either stimulated with dbcAMP or vehicle and the medium was assayed for progesterone by EIA. Treatment with dbcAMP induced a >30-fold increase in progesterone secretion in untransfected cells (Fig. 2). A combination of dbcAMP treatment and transfection of *Notch1ICD* or *Notch2ICD* resulted in a significant reduction of progesterone synthesis (*$P<0.05$; Fig. 2). *Notch3ICD* followed this trend but was not statistically significant. Therefore, Notch signaling can inhibit synthesis of progesterone in MA-10 Leydig cells.

Since *Dax1* and *SF1* are central regulators of steroidogenesis, we next determined if their expression was activated or inhibited, respectively, by Notch signaling. MA-10 cells were transfected with plasmids expressing NOTCH1–3ICD and induced with dbcAMP for 6 h, and RNA was isolated for qRT-PCR. As shown in Fig. 3, *Dax1* mRNA levels decrease in response to dbcAMP, consistent with the previously reported decrease in protein (Jo & Stocco 2004, Manna *et al*. 2009). Transfection with any activated Notch receptor did not block or attenuate this effect. Similarly, neither dbcAMP nor activated Notch receptors had any effect on the expression of *SF1* mRNA (Fig. 3). These data suggested that Notch may regulate the promoters of specific genes necessary for steroid biosynthesis because progesterone synthesis was inhibited (Fig. 2).

Whether Notch could mediate repression of specific steroid biosynthesis genes was tested using the well-characterized promoters of *Star*, *HSD3B2*, and *Cyp19a1 PII*, which are active in granulosa cells and Leydig cells. These promoters are synergistically activated by GATA4 and SF1 (Tremblay & Viger 2001*a*,*b*, Bouchard *et al*. 2005, Martin *et al*. 2005, Kwintkiewicz *et al*. 2007,

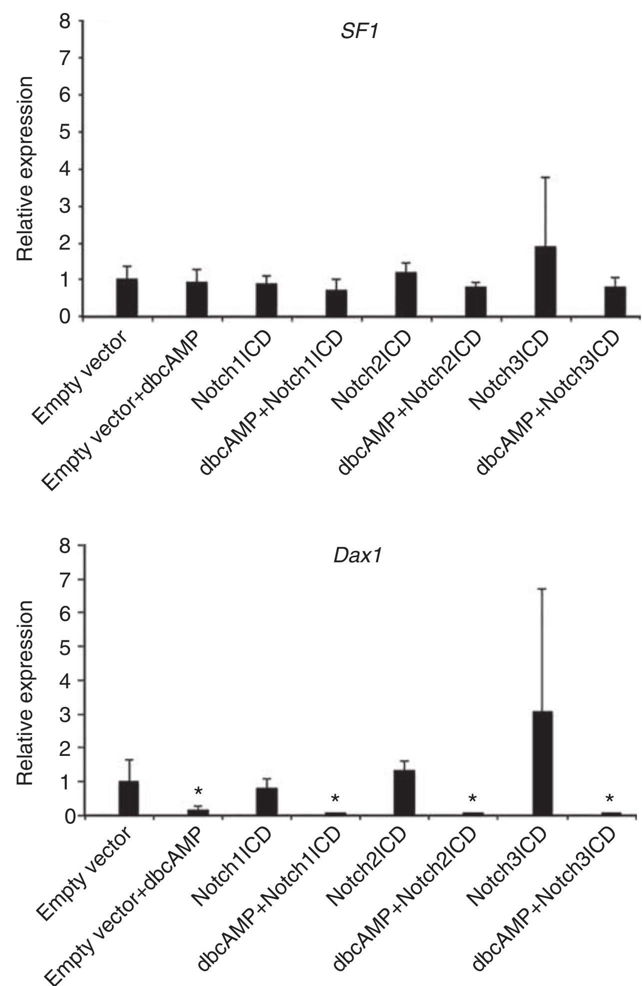

Figure 3 Activated Notch does not inhibit *Dax1* or *SF1* transcription. MA-10 cells were transfected with plasmids expressing Notch1–3ICD or empty vector and cultured in the presence or absence of dbcAMP, and total RNA was harvested for qRT-PCR using gene specific primers. *Dax1* transcripts are decreased by dbcAMP but not by activated Notch. *SF1* transcription was not affected by either dbcAMP or activated Notch. Data presented are the relative expression of three experiments done in triplicate ±S.D. *$P<0.05$ as compared to untreated MA-10 cells.

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Schrade *et al.* 2015, Bergeron *et al.* 2015). Interestingly, GATA4 activity is negatively regulated by HEY2 in the heart and developing cardiovascular system, making it a possible target for Notch signaling in gonadal cells also (Kathiriya *et al.* 2004, Kokubo *et al.* 2005). NIH3T3 cells were used in these studies to reduce background signals due to endogenous GATA4, SF1, and DAX1 activity. Cells that were transfected with a plasmid expressing *Gata4* and a *Star* promoter–luciferase reporter gene demonstrated greater than sixfold up-regulation of luciferase activity over the reporter alone. This activity was significantly inhibited by co-transfection with NOTCH1ICD, NOTCH2ICD, or NOTCH3ICD (Fig. 4A). Similarly, both the −222 to +55 *HSD3B2*- and the −284 to −23 *Cyp19a1 PII*-luciferase reporters were up-regulated 2.2- and fourfold, respectively, by GATA4. NOTCH1ICD, NOTCH2ICD, and NOTCH3ICD significantly inhibited the ability of GATA4 to activate these promoters (Fig. 4B and C). These observations are consistent with the MA-10 cell data and indicate that activated Notch can repress the promoters of specific genes in this pathway.

Direct transcriptional repression via Notch signaling is mediated by the HES and HEY bHLH-O repressor proteins binding to N and E boxes in the promoters of genes (Iso *et al.* 2003, Grogan *et al.* 2008, Heisig *et al.* 2012, Leal *et al.* 2012). Using TFSearch, an algorithm that searches DNA sequences for enhancer binding sites (Heinemeyer *et al.* 1998), the promoter regions of *Star*, *HSD3B2*, and *Cyp19a1 PII* were analyzed for N boxes (CACNAG consensus) and E boxes (CANNTG consensus) that could potentially bind HEY family members. The *Cyp19a1* PII promoter has two E boxes (Fig. 5). The *HSD3B2* promoter has a single N box, CACAAG, but no E boxes. Finally, the *Star* promoter has an N box and three E boxes (Fig. 5). The GATA sites have been confirmed as GATA4-binding sites that activate transcription of these genes in other studies (Tremblay & Viger 2001*a*,*b*, Bouchard *et al.* 2005, Martin *et al.* 2005).

To determine if the HEY bHLH-O repressors could inhibit GATA4, transcriptional activation of the steroidogenesis gene promoters was tested using a similar approach to the above discussion. As can be seen in Fig. 6A, GATA4 highly induced *Star*-luciferase activity when transfected into 3T3 cells, and this activity was significantly inhibited by co-transfection with any of the three HEY proteins. Similarly, the *HSD3B2* reporter demonstrated a >11-fold increase in luciferase activity when co-transfected with GATA4 but was significantly repressed by HEY1 and HEYL only. HEY2 had no effect on the *HSD3B2* promoter. Co-transfection of any of the three HEY proteins with the *Cyp19a1 PII*-luciferase reporter gene and GATA4 showed decreased activity, but only HEY1 inhibited this promoter significantly (Fig. 6C).

HEY proteins also can form complexes with the GATA factors and repress transcription. To determine if HEY2

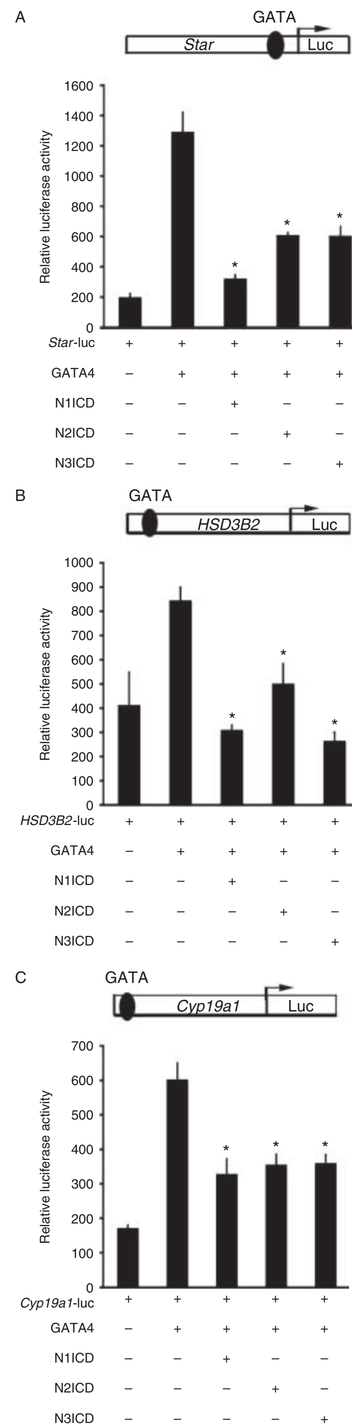

Figure 4 Activated Notch represses GATA4-mediated transcription of steroidogenic enzyme gene promoters. (A, B and C) NIH3T3 cells were transfected with a specific luciferase reporter gene construct, an expression plasmid for GATA4, and a specific activated Notch receptor, as indicated. After 48 h in culture, cells were lysed and luciferase activity was determined. GATA4 binds to the promoter regions of the *Star*, *HSD3B2*, and *Cyp19a1* reporter constructs, inducing transcription. The GATA4-induced luciferase activity of each gene was inhibited significantly by co-transfection of Notch1–3ICD (*$P<0.05$). All data are the results of three experiments done in triplicate ±S.D. Schematics above the graphs depict the reporter constructs.

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(-259 to +2)*Cyp19a1 PII*

GATGATAAGGTTCTATCAGACCAACTGTTGAACAGAACCTGAGCCTCCCAAGGTCATCCTTGTTT
TGACTTGTAACCACAAATTTGTCTTGTCTAAGTGTCCAATCACTATAAAACAAAATGCCCATCTC
TCCAATCCAGCACCCTTCCAAGTGACAGGAGCCACAGCCAAACCGCTGGGTTACGTTCTAACTCC
ACCACTGCTTTCTTCCCATAGAACTTTACTTTTTTTTTTTTTCTTCTGAGGCCAAATAGCGCAAGA
T

(-224 to +53)*HSD3B2*

BCTGTTAAGGCTAAAGCCAAGACTCTTTATCACACTGTGGCCTTAAGATTGGATTTCTCTTCCTG
TTCCTGGGAAGAATTAGAGATATAACCTAAAGGTCACTATTATTCTGAGAAAAGGGATTCTGGAG
GAGGAGGGAGCAATGAGTATGTGGCAGGAGTTCAAGGTAATAAGGGCTGAGACACAAGCCACAGA
GCATAAAGCTCCAGTCCTTCCTCCAGGGATGAGGCAGTAAGGACTTGGACTCCTCTGTCCAGCTT
TTAACAATCTAAGTTACG

(-958 to -39) *Star*

GGTCAGGACCTGTAGCAGGGCAGGCCAGCCTTAGCTGCATGAGGAAAGGGTGAGCTTGCAGGGTG
GAACCCAGGCCTAAGTTACTCTCTGCCTTGAGAGCTTACTGGGTGGGTCATCTCATTTCCAGAGA
GTTCCCAGAATGAGAAGTTAGAGTGGAGTTGGTATCAATGGGTGAGCAAATAAATGAAAACTGAA
CACATGGGGTGCTCAGGAGGCCCCACATAGAAGGAACCAAGGCCAGCTAGAGGACTTGAGCTATT
GTCCTATATTGCCCTAGCCCTGACACTGGCATCCTGTTTGTCTTTGAGCCAGCCATTGATACCCT
CCAGACCTGATGGACGGTCTTGCAGGTTTTGTTTGTTTGTTTGTCTGTTTGTTTGTTCTTTTGAG
ACAGGGTCATTCTGTGTAGCCCTTGTTGTCCTAAACCTTACTATATAGACCAGGCTGGGCTTGAA
CTCACCAGAGATCTACTTGCCTCTGCCAGGGAGAGTATGTGCCACCATGCCTACCTCTGGTCTTG
TTGCAAGAACTCACTCTTTGACTCTTTTACCCTGGCTCCCTCTCTTGGCCAGAGCTTCTATATAC
TTTTTGATGCACCTCAGTTACTGGGCATTTAAGCGGAGGACAGGGCTTGAAGTCTACATTTACAA
CTTTAGAGAAGCTATATATACATATCCTCTGCCCCATCTCCGTGACCCCTGCTTTCCCCTACCTG
CAGAGTCTGGTCCTCCCTTTACACAGTCTGCTCCCTCCCACCTTGGCCAGCACTGCAGGATGAGG
CAATCATTCCATCCTTGACCCTCTGCACAATGACTGATGACTTTTTTATCTCAAGTGATGATGCA
CAGCCTTCCACGGGAAGCATTTAAGGCAGCGCACTTGATCTGCGCCACAGCTGCAGGACTCAGGA
CCTTGAAAG

GATA box (confirmed)
E boxCANNTG (predicted)
N box CACNAG (predicted)

Figure 5 TFSearch analysis of steroidogenic gene promoters. The sequence of the promoters in each luciferase reporter gene is presented. Each promoter was examined using TFsearch for the presence of N and E boxes. All of the GATA sites (blue highlight) have been confirmed in other studies. *Cyp19a1* has two E boxes (green highlight), *HSD3B2* has an N box (red highlight), and *Star* has three E boxes and an N box.

represses the *Star* promoter directly by binding to the E boxes or acts through a complex with GATA4, 3T3 cells were transfected with the *Star*-luciferase reporter, plasmids expressing GATA4, and either full length HEY2 or one of two mutants (Fig. 7A). The first mutant, HEY2-62, lacks the N terminus, including the basic domain, but has an intact HLH domain. This mutant can form a dimer and interact with GATA4 but cannot bind DNA on its own (Kathiriya *et al*. 2004). The second mutant, HEY2-121, lacks both the basic and HLH domains; it can neither dimerize nor bind DNA (Fig. 7A). When co-transfected with GATA4 and the *Star*-luciferase reporter gene, neither HEY2-62 nor HEY2-121 could inhibit GATA4-induced transcription (Fig. 7B). These data indicated that Hey proteins likely regulate activation of steroidogenesis genes through specific sites in this promoter.

Discussion

Follicle maturation and growth includes an increase in the size of the oocyte and the proliferation and differentiation of granulosa cells, leading to antrum formation and increased steroidogenesis. Growth of the follicle is regulated by the interplay of signals between the oocyte and the granulosa cells. A combination of

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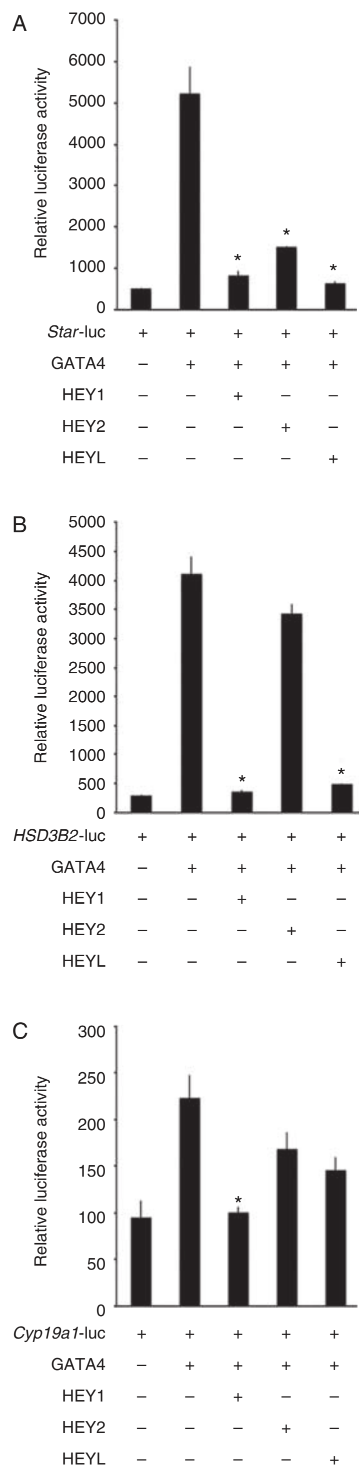

Figure 6 Hey1, Hey2, and Heyl can inhibit promoters of steroid biosynthesis genes. (A, B and C) NIH3T3 cells were transfected with a luciferase reporter gene construct along with expression plasmids for GATA4 and a HEY protein. Cell lysates were made 48 h post-transfection and luciferase activity determined. GATA4 activates each luciferase reporter to high levels and this is significantly inhibited with specific Hey genes (**P*<0.05, over bar). HEY1, HEY2, and HEYL differentially inhibit GATA4 mediated up-regulation of transcription of *Star*, *Cyp19a1*, and *HSD3B1*. All data are the results of three experiments done in triplicate ±S.D.

oocyte factors, FSH, and estradiol are necessary for granulosa cell proliferation in preantral growing follicles (Moor *et al.* 1980, Robker & Richards 1998, Britt *et al.* 2000, Kawashima *et al.* 2008, Murray *et al.* 2008, West-Farrell *et al.* 2009). Notch signaling has been shown to be important for granulosa cell proliferation. Disruption of Notch signaling in conditional mutants of *Notch2* and *Jag1* in the ovary resulted in a lack of granulosa cell proliferation (Zhang *et al.* 2011, Vanorny *et al.* 2014). DAPT, a biomimetic inhibitor that is bound by the presenilin component of γ-secretase, has been used to disrupt Notch signaling *in vitro*. This inhibitor preferentially targets amyloid precursor protein and Notch processing by γ-secretase (Berezovska *et al.* 2000, Hadland *et al.* 2001, Yang *et al.* 2008). Using DAPT to inhibit Notch signaling in *ex vivo* ovary culture inhibited granulosa cell proliferation and folliculogenesis was halted (Zhang *et al.* 2011, Manosalva *et al.* 2013). In our studies, DAPT treatment resulted in the up-regulation of steroid biosynthesis genes in small follicles (Fig. 1).

Based on our data, altered Notch signaling disrupts the normal process of steroid biosynthesis during folliculogenesis, and this is consistent with the defective folliculogenesis noted in $Lfng^{-/-}$ and conditional mutants of *Notch2*, *Jag1*, and *Hes1* (Hahn *et al.* 2005, Manosalva *et al.* 2013, Xu & Gridley 2013, Vanorny *et al.* 2014). The data presented here demonstrates that Notch signaling has a role in the complex regulation of the expression of enzymes of the steroid biosynthesis pathway and likely regulates FSH-induced proliferation of granulosa cells (Fig. 1). This is consistent with the role of Notch signaling in other tissues, promotion of proliferation, and regulation of the timing of the fully differentiated state. We propose that the differential response to FSH, proliferation in small preantral follicles, and increased steroidogenesis in larger preantral and antral follicles is regulated, in part, by Notch signaling.

In gonadal cells DAX1, a repressor, and the activator, SF1, regulate expression of multiple genes involved in steroidogenesis, including *Cyp19a1 PII*, *Star*, *Cyp11A1*, and *HSD3B2* (Tremblay & Viger 2001*a*, Wang *et al.* 2001, Lalli & Sassone-Corsi 2003, Jo & Stocco 2004, Manna *et al.* 2009). Our studies indicated that activated Notch receptors can inhibit steroid synthesis in cAMP-activated MA-10 Leydig cells and in granulosa cells of preantral follicles (Figs 1 and 2), thus it was possible that expression of either factor might be regulated by Notch. For example, *Dax1* transcription could be up-regulated by the Notch-ICD/RBPJK complex, blocking activation of SF1 and steroidogenesis. Alternatively, *SF1* transcription could be inhibited by the HES or HEY repressors, also blocking steroidogenesis. Treatment of MA-10 cells with dbcAMP results in a decrease in DAX1 protein levels, increasing the transcriptional activity of SF1 (Wang *et al.* 2002, Rao *et al.* 2003, Jo & Stocco 2004, Trbovich *et al.* 2004, Manna *et al.* 2009). Our observations indicated that

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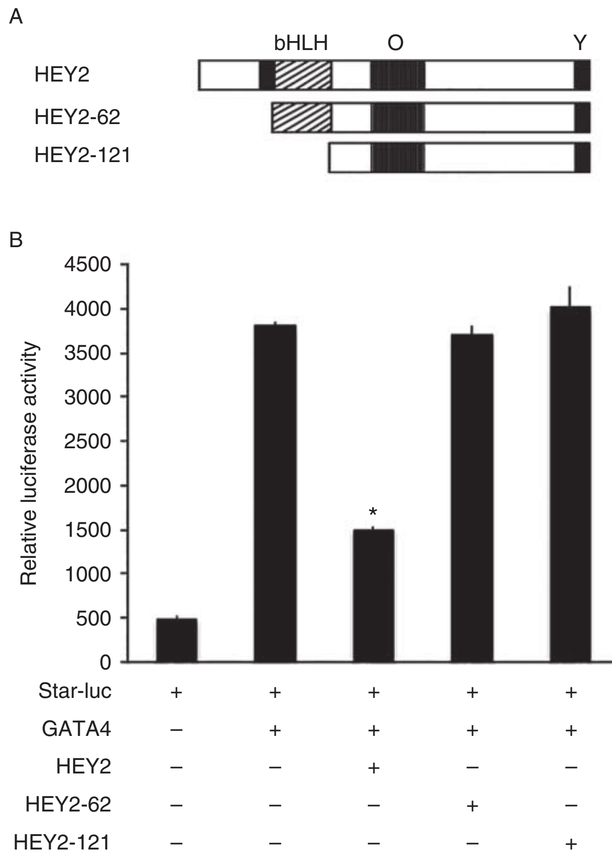

Figure 7 Hey proteins repress gene promoter activity through DNA binding. (A) The truncation mutants HEY2-62 (b) is missing the basic domain only, and HEY2-121 is missing both the basic and helix–loop–helix domains (bHLH). (B) NIH3T3 cells were transfected with the Star-luciferase reporter along with plasmids that express GATA4 and HEY2, HEY2-62, or HEY2-121. HEY2 significantly inhibited GATA4-induced luciferase activity. The loss of either the basic or bHLH domains of Hey2 resulted in a loss of repression of the *Star*-luciferase reporter gene (*$P<0.05$) and indicated that DNA binding was necessary for HEY2-mediated inhibition of this promoter. All data are the results of three experiments done in triplicate ±S.D.

Notch-mediated inhibition of steroid biosynthesis was not achieved by modulating the expression of these two major transcriptional regulators (Fig. 3) but rather by repressing expression of genes that encode important nodal points in gonadal steroid biosynthesis.

Steroidogenesis genes have well-studied promoters that are transactivated by GATA4 (Tremblay & Viger 2001*a*,*b*, Tremblay *et al*. 2002, Viger *et al*. 2004, 2008, Bergeron *et al*. 2015, Schrade *et al*. 2015). All three activated Notch receptors significantly inhibited *HSD3B2*, *Star*, and *Cyp19a1* promoter activity (Fig. 4). This observation is consistent with work by others that showed that the transactivation ability of GATA4 can be inhibited by Notch through HEY2 (Kathiriya *et al*. 2004). We determined that the *Cyp19a1 PII*, *HSD3B2*, and *Star* promoters had E and N boxes that HEY repressors could bind (Fig. 5), and subsequent experiments demonstrated that there was a differential inhibition of the promoters. For example, the *Star* promoter was significantly inhibited by HEY1, HEY2, and HEYL, but the *HSD3B2* promoter, which contains a single N box, was only inhibited by HEY1 and HEYL (Fig. 6). This is consistent with previous data that demonstrated that HEY2 does not bind to N boxes with the sequence CACAAG (Nakagawa *et al*. 2000). Consistently, the *Cyp19a1* promoter was not inhibited by HEY2 or HEYL and the 5′ most E box had a sequence that was not bound by HEY2 in previous studies (Nakagawa *et al*. 2000). The second E box found in this promoter is very similar in sequence and thus is likely a poor target for the HEY proteins also. These data indicate that direct DNA binding to specific sites in the promoters of these genes by the HEY proteins is likely the mechanism for Notch-mediated regulation of steroidogenesis gene transcription. We further ruled out the likelihood of the HEY repressors acting through complex formation with GATA4 by using mutants of *Hey2* (Fig. 7). Our data demonstrated that GATA4-induced luciferase activity was repressed only by full-length HEY2 and not by a mutant that could form a complex with GATA4 but not bind DNA. In previous studies, there were no N or E boxes in the promoters examined, so this might indicate that in the absence of direct binding, the HEY repressors will act through complex formation with GATA factors (Kathiriya *et al*. 2004, Shirvani *et al*. 2006).

Determining that the genes necessary for steroid biosynthesis in developing follicles and Leydig cells are Notch targets is a novel observation with important implications. Notch signaling is important for the development of the male reproductive tract, and Leydig cells particularly (Dirami *et al*. 2001, Tang *et al*. 2008, Hahn *et al*. 2009, Defalco *et al*. 2013). Perturbation of Notch signaling in granulosa cells results in MOFs, defective meiotic maturation, and loss of oocytes (Hahn *et al*. 2005, Trombly *et al*. 2008, Manosalva *et al*. 2013, Xu & Gridley 2013, Vanorny *et al*. 2014). Because *Notch2*, *Notch3*, and *Jag2* are not expressed in the oocyte, it is logical to conclude that these defects arise because altered Notch signaling disrupted the reciprocal communication between the oocyte and granulosa cells. Also, GATA factors have important roles in gonad development and the function and regulation of steroid biosynthetic genes (Viger *et al*. 2008, Padua *et al*. 2014, 2015, Tevosian 2014, Bergeron *et al*. 2015, Schrade *et al*. 2015). Conditional alleles of *Gata4* resulted in impaired fertility in both adult female and male mice, *Gata4*$^{-/-}$ ovaries ovulated fewer oocytes and developed ovarian cysts, and there was decreased *Cyp19a1* expression (Kyrönlahti *et al*. 2011*a*). When *Gata4* and *Gata6* are simultaneously deleted in granulosa cells, there is a complete lack of proliferation and no follicular development in the adult (Padua *et al*. 2014). Knock-down or deletion of *Gata4* in Leydig cells resulted in decreased expression of genes involved in cholesterol synthesis and transport and steroid biosynthesis

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(Bergeron *et al*. 2015, Schrade *et al*. 2015). The disruption of GATA4 function or conditional loss of *Gata4* in Sertoli cells in adult males also resulted in a loss of fertility and repression of steroid biosynthesis genes (Kyrönlahti *et al*. 2011*b*, Schrade *et al*. 2014, Bergeron *et al*. 2015, Padua *et al*. 2015). HEY2 has been demonstrated to be an important factor in the regulation of GATA-mediated transactivation of cardiac specific genes (Kathiriya *et al*. 2004, Fischer *et al*. 2005), and our data implicates the HEY proteins in the regulation of steroidogenesis postnatally. Because Notch genes are expressed in adrenal glands (de Mendonca *et al*. 2014) and the developing pituitary (Goldberg *et al*. 2011), the possibility that alterations to the Notch pathway can affect steroidogenesis and potentially the hypothalamic–pituitary–gonadal axis will be an important future consideration.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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