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Genetics of Sex
Highlighted Article

A fish with no sex: gonadal and adrenal functions partition between zebrafish *NR5A1* co-orthologs

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Abstract

People with *NR5A1* mutations experience testicular dysgenesis, ovotestes, or adrenal insufficiency, but we do not completely understand the origin of this phenotypic diversity. *NR5A1* is expressed in gonadal soma precursor cells before expression of the sex-determining gene *SRY*. Many fish have two co-orthologs of *NR5A1* that likely partitioned ancestral gene subfunctions between them. To explore ancestral roles of *NR5A1*, we knocked out *nr5a1a* and *nr5a1b* in zebrafish. Single-cell RNA-seq identified *nr5a1a*-expressing cells that co-expressed genes for steroid biosynthesis and the chemokine receptor Cxcl12a in 1-day postfertilization (dpf) embryos, as does the mammalian adrenal–gonadal (interrenal-gonadal) primordium. In 2dpf embryos, *nr5a1a* was expressed stronger in the interrenal-gonadal primordium than in the early hypothalamus but *nr5a1b* showed the reverse. Adult Leydig cells expressed both ohnologs and granulosa cells expressed *nr5a1a* stronger than *nr5a1b*. Mutants for *nr5a1a* lacked the interrenal, formed incompletely differentiated testes, had no Leydig cells, and grew far larger than normal fish. Mutants for *nr5a1b* formed a disorganized interrenal and their gonads completely disappeared. All homozygous mutant genotypes lacked secondary sex characteristics, including male breeding tubercles and female sex papillae, and had exceedingly low levels of estradiol, 11-ketotestosterone, and cortisol. RNA-seq showed that at 21dpf, some animals were developing as females and others were not, independent of *nr5a1* genotype. By 35dpf, all mutant genotypes greatly under-expressed ovary-biased genes. Because adult *nr5a1a* mutants form gonads but lack an interrenal and conversely, adult *nr5a1b* mutants lack a gonad but have an interrenal, the adrenal, and gonadal functions of the ancestral *nr5a1* gene partitioned between ohnologs after the teleost genome duplication, likely owing to reciprocal loss of ancestral tissue-specific regulatory elements. Identifying such elements could provide hints to otherwise unexplained cases of Differences in Sex Development.

Keywords: adreno-gonadal primordium; Differences in Sex Development; disorders of sex development; scRNA-seq; SF1; subfunctionalization; Genetics of Sex

Introduction

Sex determination (SD) involves several interacting cell types (germ cells, somatic cells), multiple organs (hypothalamus, pituitary, gonads, and adrenal), and impacts multiple traits, including disease susceptibility. We lack, however, full knowledge of the mechanisms by which genetic and environmental factors interact to establish an individual's sex. In mammals, the Y-chromosome gene SRY generally initiates male development and without SRY, people usually become females (Sinclair *et al.* 1990). Less frequent outcomes, called Differences in Sex Development (DSD, or disorders of sex development), are a pediatric concern, occurring in one in 2000–5000 live births (Sax 2002; Baetens *et al.* 2019). DSDs can arise by mutations in sex determination pathway genes or by environmental factors (Marrocco *et al.* 2015; Baetens *et al.* 2019). Many cases of DSD involve gonadal dysgenesis, but the etiology of most cases is unknown (Rocha *et al.* 2011; Garcia-Acero *et al.* 2020).

The Undiagnosed Diseases Network case UDN365839 presented at the age of 16 years with primary hypogonadism, azoospermia, low serum testosterone (44 ng/dl), clinical obesity, and a 46, XX SRY-negative karyotype with a novel missense mutation in NR5A1 (p.R92W) (Bashamboo *et al.* 2016). NR5A1 [Nuclear Receptor Subfamily 5, Group A, Member 1, also called Steroidogenic Factor 1 (SF1) or AD4BP] is a nuclear receptor with no known ligand; mutations in NR5A1 can cause XX sex reversal (OMIM 617480) and XY sex reversal (OMIM 612965) (Achermann *et al.* 1999; Bashamboo *et al.* 2016), as well as adrenocortical insufficiency (OMIM 612964) (Guran *et al.* 2016) and premature ovarian or spermatogenic failure (OMIM 612964 and 613957). Null alleles of *Nr5a1* in mouse result in the loss of the ventral medial hypothalamus and the adrenal–gonadal primordium (Ikeda *et al.* 1995; Val *et al.* 2003). We do not completely understand, however, what causes different NR5A1 mutations to result in different human

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phenotypes or the regulatory mechanisms that evoke *NR5A1* expression in these organs.

Teleost fishes have two co-orthologs of *NR5A1*, called *nr5a1a* (*ff1b*) and *nr5a1b* (*ff1d*) (von Hofsten *et al.* 2001; Kuo *et al.* 2005), that originated in the teleost genome duplication event (TGD) (Amores *et al.* 1998; Postlethwait *et al.* 1999; Taylor *et al.* 2003; Jaillon *et al.* 2004). Here, we probed the question: What are the roles of the two zebrafish co-orthologs compared to those of the single-copy mammalian *NR5A1* gene? Under one hypothesis (Force *et al.* 1999), the functions of Nr5a1 in the adrenal–gonadal primordium, adrenal, gonad, and hypothalamus were present in the last common ancestor of humans and zebrafish and partitioned between the two fish ohnologs after the TGD. Alternatively, some mammalian functions evolved after the divergence of zebrafish and human lineages and would be absent either from zebrafish or from human. To evaluate these possibilities, we studied single-cell transcriptomes in wild-type embryos and made mutations in both zebrafish *nr5a1* duplicates, studying their gene expression patterns, phenotypes, transcriptomes, and hormone titers. The results showed that *nr5a1a* maintains the interrenal [the teleost adrenal cortex homologue that, along with chromaffin cells, represents the adrenal medulla and lies within the fish kidney (Chester Jones and Mosley 1980)]. Furthermore, without the wild-type allele of *nr5a1b*, the gonad disappeared from adult fish, leading to the loss of secondary sex characteristics. Both organs disappeared in double mutants. These results suggest that the ancestral functions of *Nr5a1* included both adrenal and gonad, but that they partitioned between *nr5a1a* and *nr5a1b* in teleosts. Identification of conserved regulatory elements that specify interrenal *vs.* gonadal expression patterns in zebrafish *NR5A1* ohnologs may help to identify sequences responsible for human DSDs of unknown origin.

Materials and methods

Mutagenesis

CRISPR/Cas9 mutagenesis generated deletions in zebrafish *nr5a1a* (ENSDARG00000103176) and *nr5a1b* (ENSDARG00000023362) (http://ensembl.org), using sites identified by ZiFiT Targeter (http://zifit.partners.org/ZiFiT/). Mutagenesis targeted region in *nr5a1a* exon-4 was: GTGCGTGCAGACCGGATGAG, and in *nr5a1b* exon-4: GTGCGGATAGGATGCGAGG, using gRNAs synthesized from DNA oligomer templates: aattaatacgactcactataGTGCGTGCAGACCGGATGAGgttttagagctagaaatagc for *nr5a1a* and aattaatacgactcactatagGTGCGGATAGGATGCGAGGgttttagagctagaaatagc *nr5a1b* (DT, Coralville, IA). MEGAscript T7 Transcription Kit transcribed gRNA and mMESSAGE mMACHINE T3 Transcription Kit (Thermo Fisher Scientific, Waltham, MA) synthesized *Cas9* mRNA. Approximately, 2 nl of a solution containing 100 ng/µl *Cas9* mRNA and 25 ng/µl of both nr5a1 gRNAs was co-microinjected into one-cell embryos of the AB strain. Genomic DNA from injected embryos at 24 hpf (hour postfertilization) provided template to amplify a 312-bp PCR fragment for *nr5a1a* gene (primers: F-ACACAAATGCATTATTCCTCTCTCT and R-CCTCCAGTTTGAAGCCGCTA); a 375-bp PCR fragment for *nr5a1b* (primers: F-TGGGGAAAAGAATTAACAGGGGT and R-GACGATGTTCGGATGGGTGT). Wild-type alleles have a BstCI recognition site for both *nr5a1a* and *nr5a1b* genes that are disrupted in *nr5a1* mutant alleles. Sanger sequencing (GENEWIZ, Inc., NJ) verified mutations. We established stable lines for two noncomplementing alleles: deletion of 11 nucleotides designated *nr5a1a*(b1388) and a deletion of 8 nucleotides designated *nr5a1b*(b1389) (Figure 2C).

Histology and *in situ* hybridization

In situ hybridization was performed as described previously (Rodriguez-Mari *et al.* 2005) using the probes: *nr5a1a* (ENSDARG00000103176) using a 859-bp fragment including exon-2 to exon-6 (primers F-AAGTGTCCGGTTATCATTACGGCC and R-TGTCTGCAGATGTGATCCAGAAGC) (Yan *et al.* 2019); *nr5a1b* (ENSDARG00000023362) using a 821-bp fragment including partial exon 5 and 3′UTR (primers F-AACTTCTAGTGCTGGACTATGTTGCC and R-CATTTCTTAAGAGGCCACAGAGCGTA); *amh* (ENSDARG00000014357) (Yan *et al.* 2017); *cyp11c1* (ENSDARG00000042014) (Wang and Orban 2007) using a 592-bp fragment located in exons 9–13 (primers F-CAGAGCCAACATCACTGAGCTGAT and R-GAAGGTGATTCTCGGTGGACACTC); *cyp19a1a* (ENSDARG00000041348) (Chiang *et al.* 2001b); *cyp21a2* (ENSDARG00000037550) using a 875-bp fragment including partial exon 1 to exon 8 (F-TGGTCAGTGTTGTGCTATTGCTGT and R-TGAAGAAGTCAGTGTGCCACCTTC); ddx4, (*vasa*) (ENSDARG00000014373) (Yoon *et al.* 1997); *nkx2.1* (ENSDARG00000019835) using a 612-bp fragment including partial exon (F-ACAGAACAATGTCGATGAGCCCTAAG andR-TTGAGTCGGAGTCAAGTGTATCATGC); *nkx2.4a* (ENSDARG00000075107) using a 1023-bp fragment including partial exon 1-2 (F-CCACGAGAACAGAGCTGATACAACAA and R-AGAAGTGTCTATCTGGCCCATACTGT); *nkx2.4b* (ENSDARG00000104107) using a 936-bp fragment including partial exon 1-2 (F-ATCGAGGAGACCTTCAAGAAGTTTGC and R-CACATATCTCCGTCCGTCAGTTGATT).

Histology used paraffin-embedded Bouin's-fixed tissue sectioned at 10 µm and stained with hematoxylin and eosin (H&E) (Rodriguez-Mari *et al.* 2005). The gonadosomatic index was calculated as (gonad weight)(100)/(fish body weight). As a measure of gonad size, we cut cross-sections, counted the number of gonad-containing rows on a slide, identified the middle row, photographed each gonad from two sections in that row for four images, and used ImageJ to measure the area of each gonad twice using the freehand selection tool and calculated the area of the gonad with the measure function.

Transcriptomics

Fish homozygous for either *nr5a1a*(b1388), *nr5a1b*(b1389), double mutants or double wild-type siblings at 21 and 35 dpf were euthanized in Tricaine followed by isolating the gonad-containing trunk from just posterior of the pectoral fin to just anterior to the vent. Trunks from each fish were individually homogenized in 200 µl Trizol. Total RNA was extracted according to Amores *et al.* (2011) and enriched for mRNA using Dynabeads® Oligo(dt)[25] (ThermoFisher). We constructed strand-specific, indexed, cDNA sequencing libraries (NEXTflex™ qRNA-seq kit, BIOO Scientific), quantified libraries by Qubit® fluorometer (Life Technologies), normalized libraries to 2.3 nM, multiplexed libraries, quality-checked libraries (Kapa Library Quantification Kit, Kapa Biosystems), and sequenced libraries in two lanes on an Illumina HiSeq 4000 using paired-end 100 base pair (bp) reads.

For single-cell RNA-seq, embryos from natural crosses were collected at 24, 48, and 120 hpf in two pools of 15 individuals per stage from the genotypes *Tg(olig2: GFP)vu12* or *Tg(elavl3: GCaMP6s)*. Embryos were dissociated and cell suspensions were prepared as described previously (Farnsworth *et al.* 2020). The University of Oregon Genomics and Cell Characterization core facility (https://gc3f.uoregon.edu/) separated cells and prepared libraries on a 10X Chromium platform using 10× v.2 chemistry targeting 10,000 cells. Fifteen cycles of PCR-amplified cDNA

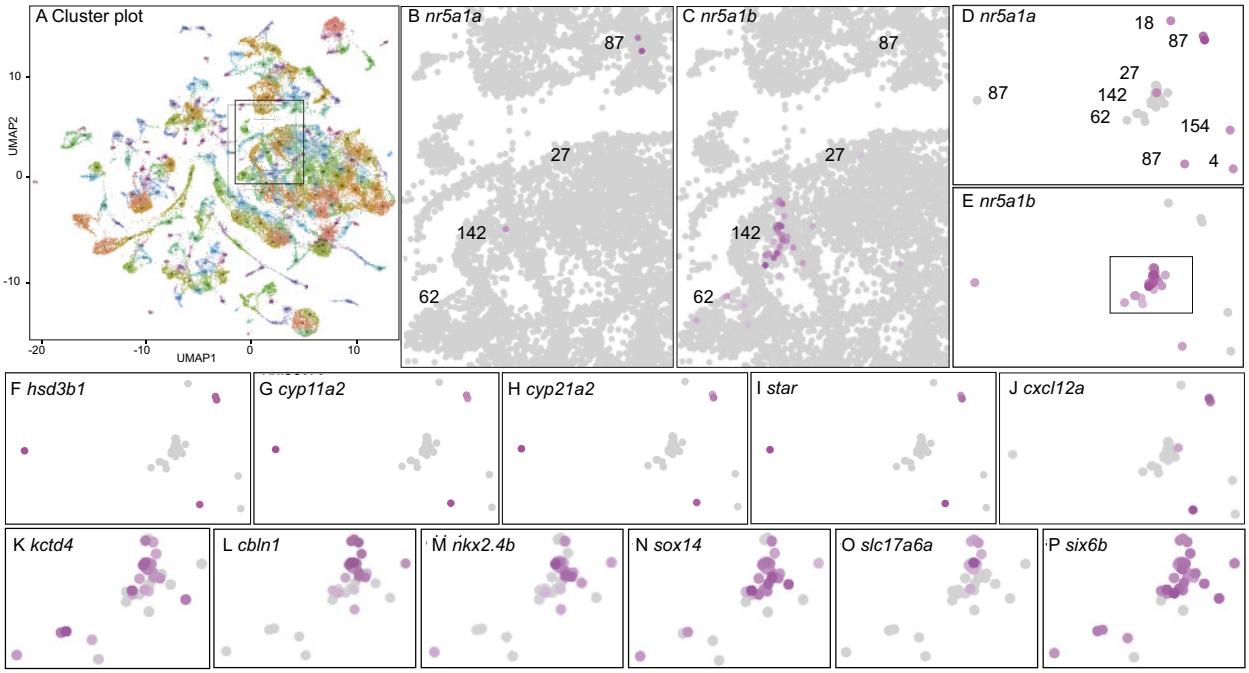

Figure 1 scRNA-seq identification of genes co-expressed with *nr5a1a* and *nr5a1b*. (A) In total, 220 clusters from scRNA-seq of whole animals at 24, 48, and 120 hpf (Farnsworth *et al.* 2020). The box indicates the portion enlarged in (B) and (C). (B) *nr5a1a*-expressing cells; color intensity is proportional to log expression level. (C) *nr5a1b*-expressing cells. (D) *nr5a1a*- and/or *nr5a1b*-expressing cells labeled only for *nr5a1a*-expression, with clusters numbered. (E) *nr5a1a*- and/or *nr5a1b*-expressing cells labeled only for *nr5a1b*-expression. The box indicates the portion enlarged in panels J–O. (F–I) Expression of the steroid biosynthesis genes *hsd3b1*, *cyp11a2*, *cyp21a2*, and *star*. (J) Cluster 87 cells labeled for the cytokine *cxcl12a*. (K–P) Expression of ventromedial hypothalamus genes *kctd4*, *cbln1*, *nkx2.4b*, *sox14*, *slc17a6a*, and *six6b*, respectively, in the boxed region of (E).

libraries that were sequenced on either an Illumina Hiseq (5/6 samples) or an Illumina Next-seq (1/6 samples). Reads were aligned to GRCz11_93 and bioinformatic analyses were done using CellRanger and Seurat as described previously (Farnsworth *et al.* 2020).

RNA-seq libraries were constructed from trunk tissue from eight individuals of 21-dpf wild-type fish, *nr5a1a* mutants, *nr5a1b* mutants, *nr5a1ab* double mutants, and eight individuals of 35-dpf wild-type fish, *nr5a1a* mutants, *nr5a1b* mutants, and *nr5a1ab* double mutants. In a preliminary principal component analysis, one 35-dpf wild-type individual was a substantial outlier and was subsequently ignored. The remaining 63 libraries produced 642,409,629 preprocessed reads, of which 473,982,972 reads aligned to zebrafish GRCz10 genome. Following correction for PCR duplicate reads, 413,607,158 reads remained, and 315,250,017 of these corrected reads aligned to protein-coding regions.

Bioinformatics

We used Dupligänger duplicate removal software (Sydes *et al.* 2019) to preprocess RNA-seq reads, to identify, and to remove BIOO inline unique molecular identifiers (UMIs), to remove read-through adapters (using cutadapt v1.18 (Martin 2011) with command line options: -n 3 -O 1 -m 30 -a AGATCGGAAGAGC-A AGATCGGAAGAGC –too-short-output –too-short-paired-output), and then to remove low-quality sequences at both the 5′-ends and the 3′-ends using Trimmomatic (v0.36) (Bolger *et al.* 2014), with command line options: LEADING : 10 TRAILING : 10 SLIDINGWINDOW : 5:10 MINLEN : 30. Dupligänger tracked the number of nucleotides removed from the 5′-end and deleted reads <30 nt. STAR (version 2.7.0f, command line options: –outFilterMultimapNmax 1 –outSAMtype BAM Unsorted –alignIntronMax 1000000 –alignMatesGapMax 1000000) (Dobin *et al.* 2013) aligned processed PE reads to the zebrafish genome (GRCz11, Ensembl version 96) in a splice-aware manner. Dupligänger then deleted PCR duplicates from the sequence alignment file if (1) the read pair shares with another read its 5′ alignment starts for both R1 and R2 after correcting for 5′ trimming, and (2) the read pair shares the same R1 UMI and R2 UMI. Dupligänger forwarded de-duplicated sequence alignment files to HTSeq-count (Anders *et al.* 2015) (using command line options: -m intersection-strict –stranded=reverse) to obtain per-gene counts for protein-coding genes. DESeq2 (version 1.22.2) provided statistical differential expression analysis (Love *et al.* 2015). Reads are available at the Sequence Read Archive under project accession number PRJNA561212.

Endocrinology

Sex steroids were extracted from zebrafish homogenates according to the flowchart in (Newman et al., 2008). Individual flash-frozen fish were macerated using scissors and a knife on a cold cutting board, transferred to cold glass test tubes, and weighed. Water and HPLC-grade methanol were added to the tissue and this mixture was homogenized and sonicated. Tubes were shaken on a multi-tube vortexer (Glas-Col Large Capacity Mixer, speed set at 500 rpm; Glas-Col, Terre Haute, IN, USA) for 1 h at room temperature and then stored overnight at 4 °C. Before use, tubes were shaken again and then centrifuged at 1500 *g* for 15 min at 4 °C. In brief, 1 ml of supernatant was combined with 10 ml water and then extracted using solid-phase extraction (SPE columns: Agilent Bond Elut-C18 OH, 500 mg 3 ml, cat # 12102046). Eluates were dried in a Savant SpeedVac Concentrator (model

SDP121P; Thermo Fisher Scientific, Waltham, MA, USA) at 35°C and then stored at −80°C. One day prior to assay, samples were resuspended in 500-µl assay buffer (X065 buffer; Arbor Assays, Ann Arbor, MI, USA) and shaken at 500 rpm for 1 h at room temperature. After storage at 4°C overnight, samples were shaken and then assayed. Both male and female extracts were diluted 1:4 for cortisol. Female extracts were diluted 1:2 for both E2 and 11-KT. Male extracts were assayed undiluted for E2 and diluted 1000× for 11-KT. All extracts were run undiluted for both E2 and 11-KT. Commercially available EIAs were used to measure cortisol (Cortisol ELISA Kit #K003, Arbor Assays, Ann Arbor, MI), estradiol (E2; Estradiol ELISA Kit #K030, Arbor Assays, Ann Arbor, MI), and 11-ketotestosterone (11-KT; 11-keto Testosterone ELISA Kit #582751, Cayman Chemical, Ann Arbor, MI), according to the manufacturer's instructions. These kits were validated for zebrafish homogenate extract using tests of parallelism and standard addition (Hunt *et al.* 2017). Any sample that exceeded 10% coefficient of variation between duplicates or was outside the range of the standard curve was reanalyzed. Intra- and inter-assay variation for cortisol was 2.2% and 5.2%, respectively. Intra- and inter-assay variation for E2 was 2.6% and 3.1%, respectively. Intra- and inter-assay variation for 11-KT was 2.5% and 13%, respectively.

Data availability

The Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) contains RNA-seq reads under accession number PRJNA561212. Supplementary Tables list differentially expressed genes. The scRNA-seq data are publicly available at https://cells.ucsc.edu/?ds=zebrafish-dev. Additional data and code relevant to the Atlas can be accessed at: https://www.adammillerlab.com/. Work was performed under the University of Oregon Institutional Animal Care and Use Committee (IACUC) protocol #14-08 R. Mutant strains are available on request. Data should be cited according to the citation of this article.

Supplementary material is available at figshare: https://doi.org/10.25386/genetics.13236983.

Results

Searching for the adrenal–gonadal primordium

In mammals, *NR5A1*-expressing cells give rise to the adrenal and to the gonadal soma before the expression of the sex-determining gene *SRY* (Ikeda *et al.* 1994; Hatano *et al.* 1996; Sekido and Lovell-Badge 2008). To identify the earliest *nr5a1*-expressing cells in the zebrafish embryo and the adrenal (interrenal)-gonadal primordium, we performed single-cell RNA-seq (scRNA-seq) on whole bodies of wild-type animals at 1, 2, and 5 dpf (days post-fertilization) (Farnsworth *et al.* 2020). Analyses identified 220 clusters of transcriptionally related cells in the zebrafish scRNA-seq atlas ("Atlas," Figure 1A), but only four clusters had multiple cells expressing *nr5a1a* (ENSDARG00000103176, *ff1b*), or *nr5a1b* (ENSDARG00000023362, *ff1d*), or both (Figure 1, B and C). We bioinformatically isolated cells expressing *nr5a1* ohnologs (Figure 1, D and E) and identified genes differentially expressed (DE) compared to all other cells in the Atlas.

In nr5a1a-expressing cells, 43 genes were differentially overexpressed (Padj < 0.05), the top six of which were related to steroid biosynthesis (*hsd3b1*, *cyp11a2*, *cyp21a2*, *star*, *fdx1b*, and interestingly, *nr5a1b*), and all were expressed in Cluster 87 (Figure 1, F–I and Supplementary Table S1). The expression of *CYP21A2* (*cyp21a2*) is restricted to the human adrenal cortex and the fish interrenal (Fagerberg *et al.* 2014; Eachus *et al.* 2017), and *FDX1* is expressed almost exclusively in the human adrenal (Fagerberg *et al.* 2014). Human orthologs of the other top six genes are expressed in both adrenal and gonad (Fagerberg *et al.* 2014). This result suggests the hypothesis that *nr5a1a*-expressing cells may mark the zebrafish adrenal (interrenal)–gonadal precursor. Cells in Cluster 87 also significantly differentially expressed *six1b* (Supplementary Table S1), whose mammalian ortholog marks the adrenal–gonadal primordium (Kobayashi *et al.* 2007; Fujimoto *et al.* 2013), and they strongly expressed *cxcl12a*, the ligand that guides migrating primordial germ cells to the gonadal soma in the celomic epithelium (Boldajipour *et al.* 2011) (Figure 1J) although *cxcl12a* was not DE with respect to other cells in the atlas because it also helps guide the migration of vascular, hematopoietic, and neural cells (Aiuti *et al.* 1997; Tachibana *et al.* 1998; Zou *et al.* 1998; Peled *et al.* 1999; Knaut *et al.* 2005; Lieberam *et al.* 2005; Siekmann *et al.* 2009; Walters *et al.* 2010). Cluster 87 cells also differentially expressed *prox1b* (Supplementary Table S1), a proposed coregulator of *nr5a1a* (Liu *et al.* 2003). These scRNA-seq results are predicted by the hypothesis that Cluster 87 contains the adrenal (interrenal)–gonadal primordium and its derivatives.

In nr5a1b-expressing cells, 630 genes were differentially coexpressed by scRNA-seq (Padj < 0.05) (Supplementary Table S2). The 11 most DE genes from the 32 *nr5a1b*-expressing cells in Clusters 27 [1dpf (days postfertilization)], 62 (1, 2, and 5 dpf), and 142 (1 and 2 dpf) included ventromedial hypothalamus (VMH) genes in zebrafish and/or mammals [*kctd4*, *cbln1*, *sox1a*, *nkx2.4b*, *sox14*, *nkx2.1*, *slc17a6a*, *sox1b*, *six6a*, *six6b*, *lhx5* (Karlstrom *et al.* 2003; Segal *et al.* 2005; Kurrasch *et al.* 2007; Toro *et al.* 2009; Appelbaum *et al.* 2010; Larder *et al.* 2011; Machluf *et al.* 2011; Armant *et al.* 2013; Manoli and Driever 2014; Sun *et al.* 2015; Chen *et al.* 2017)] (Figure 1, K–P and Supplementary Table S2). In mouse, *Nr5a1* is the most specific marker for the VMH (Segal *et al.* 2005), but the role of *Nr5a1* in the VMH is not yet understood (Budefeld *et al.* 2012). The *rx3* gene that was shown to interact with *nr5a1b* in zebrafish hindbrain development was strongly DE in *nr5a1b*-expressing cells (Muthu *et al.* 2016), and *Six6* expression in the VMH is necessary for normal fertility in mouse (Xie *et al.* 2015). These results show that the expression of *nr5a1a* and *nr5a1b* already differs at 1 dpf in the hypothalamus. Genes co-expressed with *nr5a1* ohnologs provide a resource to further investigate the roles of Nr5a1 in hypothalamus development.

Mutagenesis to identify functions of *nr5a1* ohnologs

Having identified single-cell transcriptomes in *nr5a1*-expressing cells, we wanted to learn the phenotypes of mutants lacking these gene functions. CRISPR/Cas9 mutagenesis induced premature stop codon alleles in *nr5a1a* and *nr5a1b*. Mutation targets were in the conserved A-box within the DNA-binding domain (Lin and Achermann 2008) near the site of the R92W mutation that results in variable testis development in 46, XX humans (Bashamboo *et al.* 2016; Miyado *et al.* 2016; Werner *et al.* 2017). The amino acid sequence of the human A-box and its surroundings is precisely conserved in both zebrafish ohnologs (Figure 2, A and B). Supplementary Figure S1 shows CRISPR target sites and sequence traces for an 11-base pair (bp) deletion in *nr5a1a* (the $nr5a1a^{-11}$) allele, and an 8 bp deletion in *nr5a1b* (the $nr5a1b^{-8}$ allele). Both deletions should produce an 87-residue polypeptide with two out-of-frame residues in *nr5a1a* and nine in *nr5a1b* (Figure 2C). Predicted proteins disrupt the DNA-binding domain and eliminate the ligand-binding domain and hence should be null activity alleles.

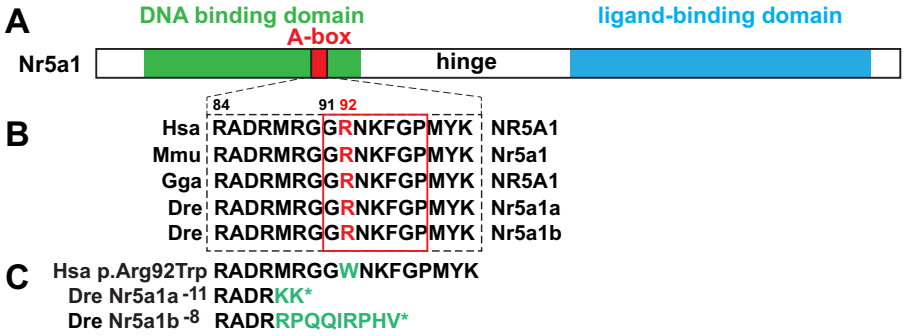

Figure 2 CRISPR/Cas9-induced *nr5a1a* and *nr5a1b* mutants. (A) Consequences of induced mutations on Nr5a1 proteins (DNA-binding domain, green; Abox, red; ligand-binding domain, blue). (B) Amino acid sequences surrounding the zebrafish CRISPR target sites for orthologs in human (Hsa), mouse (Mmu), chicken (Gga), and zebrafish (Dre). The human mutations R84H and G91S are dominant and cause 46, XY sex reversal; R92W is a dominant mutation resulting in variable testis development in 46, XX individuals; and R92Q is a recessive that causes adrenal insufficiency, 46, XX sex reversal, and 46, XY sex reversal (Lin and Achermann 2008; Bashamboo *et al.* 2016; Miyado *et al.* 2016; Werner *et al.* 2017). (C) The zebrafish $nr5a1a^{-11}$ and $nr5a1b^{-8}$ deletion alleles are predicted to result in polypeptides with short out-of-frame sequences (green) that are truncated owing to a premature stop codon (*) before or within the A-box.

Embryonic expression patterns of *nr5a1a* and *nr5a1b*

Gene expression in wild types

To verify scRNA-seq results, we performed *in situ* hybridization experiments in wild types. Results showed that at 48 hpf (hours postfertilization), *nr5a1a* and *nr5a1b* were both expressed in the hypothalamus and in the left-biased interrenal (Figure 3, A and E), confirming previously published results (von Hofsten *et al.* 2001; Chai *et al.* 2003; Hsu *et al.* 2003; Kuo *et al.* 2005; Kurrasch *et al.* 2007; Chai and Chan 2000; von Hofsten *et al.* 2002; Liu and Guo 2006; Wang *et al.* 2007; Chiu *et al.* 2012; Nakamoto *et al.* 2012). The mammalian adrenal enzyme Cyp21a2 converts progesterone to an aldosterone precursor (Miller and Auchus 2011), although teleosts lack aldosterone (Jiang *et al.* 1998; Bridgham *et al.* 2006). Expression of *cyp21a2* in zebrafish (Weger *et al.* 2018) confirmed that the abdominal expression domain of *nr5a1* ohnologs is the interrenal (Figure 3I), consistent with the results of scRNA-seq (Figure 1E). To identify *nr5a1*-expressing cell types in the brain, we studied the hypothalamus marker genes *nkx2.4b* (see also Figure 1M for scRNA-seq), *nkx2.4a*, and *nkx2.1* (Rohr *et al.* 2001; Kurrasch *et al.* 2007, 2009). These *nkx2* paralogs were expressed in domains that encompassed those of *nr5a1* ohnologs (Figure 3M and Supplementary Figure S2), and hence confirm the expression of *nr5a1a* and *nr5a1b* in the hypothalamus. In 48 hpf wild-type embryos, *nr5a1a* expression was more intense in the interrenal than in the hypothalamus, but the reverse was true for *nr5a1b* (Figure 3, A and E).

Gene expression in nr5a1a mutants

Expression of *nr5a1a* transcript virtually disappeared from the interrenal and was far lower in the hypothalamus in *nr5a1a* mutants compared to wild-type siblings hybridized in the same experiment (Figure 3B). This result suggests that *nr5a1a* function is required to maintain *nr5a1a*-expressing cells or that the message is unstable. Transcripts from *nr5a1b* also disappeared from the interrenal in *nr5a1a* mutants but were merely reduced in the hypothalamus (Figure 3F). Greatly decreased expression of the interrenal marker *cyp21a2* confirmed defective interrenal development in *nr5a1a* mutants (Figure 3J). In contrast, *nr5a1a* mutants showed no obvious expression changes for hypothalamus markers (*nkx2.4b*, Figure 3N; *nkx2.4a*, *nkx2.1*, Supplementary Figure S1). We conclude that *nr5a1a* is necessary for normal development of the embryonic interrenal but seems less important for hypothalamus gene expression patterns, confirming morpholino studies (Hsu *et al.* 2003).

Gene expression in nr5a1b mutants

Expression patterns of *nr5a1a*, *nr5a1b*, and *nkx2.4b* in the hypothalamus of 48hpf *nr5a1b* mutants were similar to those in wild types (Figure 3, A, C, E, G, M, and O), providing no evidence that *nr5a1b* strongly affects the development of the hypothalamus despite its expression in the hypothalamus as early as 1 dpf (Figure 1, C, E, and L). In the interrenal, *nr5a1b* mutants had only slightly reduced expression of *nr5a1a*, *nr5a1b*, or *cyp21a2* (Figure 3, A, C, E, G, I, and K). We conclude that *nr5a1b* appears to be less important for interrenal development than *nr5a1a*.

Gene expression in double mutants

In brief, 48 hpf embryos lacking both *nr5a1a* and *nr5a1b* activity (Figure 3, D, H, L, and P) lacked the expression of genes marking interrenal development but had little effect on the expression of *nr5a1a*, *nr5a1b*, or *nkx2.4b* in the hypothalamus (Figure 3, B, F, J, and N). We conclude that at 48 hpf, *nr5a1a* is important for interrenal development and that neither *nr5a1a* nor *nr5a1b* is required for apparently normal expression of examined hypothalamus marker genes (Supplementary Figure S2).

nr5a1 Ohnologs in juvenile gonad development

To learn the roles of *nr5a1* ohnologs in juveniles, we made histological sections of wild types and mutants (Figure 4). Gonads in AB strain wild-type juveniles at 19 dpf are undifferentiated, but they are beginning to transition from the juvenile ovary stage to differentiated gonads (Takahashi 1977; Uchida *et al.* 2002; Rodriguez-Mari *et al.* 2005; Orban *et al.* 2009) (Figure was 4, A and B). Mutant gonads at 19 dpf lacking *nr5a1a* activity (Figure 4, C and D) were somewhat smaller than wild-type gonads and were undifferentiated. Gonads in *nr5a1b* mutants were even smaller (Figure 4, E and F), and gonads in double mutants were smaller still (Figure 4, G and H). These results show that both zebrafish *nr5a1* ohnologs are required for juvenile gonads to reach normal size.

Gonads in postmetamorphic wild types at 35 dpf had differentiated into either ovaries with stage I oocytes (Figure was 4I) or testes with tubules containing developing spermatogonia (Figure 4J). In contrast, gonads in 35dpf *nr5a1a* mutants remained small, and although they formed immature oocytes like juvenile

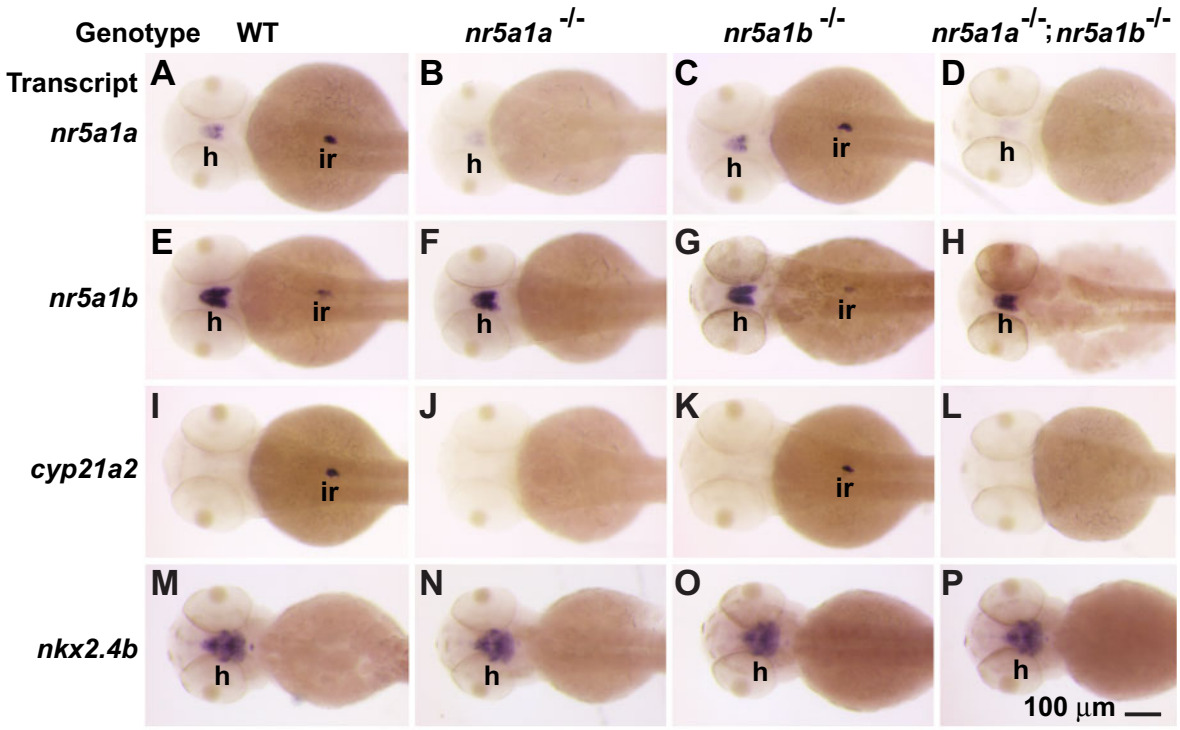

Figure 3 Gene expression patterns in embryos at 48 hpf. (A, E, I, M) Wild-type embryos. (B, F, J, N) *nr5a1a*$^{-11}$ mutant embryos. (C, G, K, O) *nr5a1b*$^{-8}$ mutant embryos. (D, H, L, P) *nr5a1a*$^{-11}$; *nr5a1b*$^{-8}$ double mutant embryos. *In situ* hybridization for *nr5a1a* (A, B, C, D), *nr5a1b* (E, F, G, H), *cyp21a2* (I, J, K, L), *nkx2.4b* (M, N, O, P). interrenal development depends on *nr5a1a*, but hypothalamus expression of nkx2-family marker genes is not strongly affected in either single mutant or double mutants. Based on three clutches, each containing 20–25 wild types, 42–48 heterozygotes, 19–24 *nr5a1a* mutants, 18–27 *nr5a1b* mutants, and 8–12 double mutant embryos. h, hypothalamus; ir, interrenal. Scale bar in P represents 100 μm.

wild types, these oocytes did not mature; *nr5a1a* mutant gonads appeared to be on a delayed pathway to become a small testis (Figure 4, K, I, L, and Q). At 35 dpf, *nr5a1b* mutant gonads remained undifferentiated and were no larger than those in 19 dpf wild types (Figure 4, M and N). Gonads in 35 dpf double-mutant fish were smaller than in either single mutant (Figure 4, O–Q). We conclude that Nr5a1 action is necessary for ovary development and gonad growth. Furthermore, each *nr5a1* ohnolog has independent functions in gonad development because the double-mutant phenotype is more severe than either single-mutant phenotype, and because *nr5a1a* is required to form ovaries but not testes, whereas *nr5a1b* is required for gonad differentiation in either sex-specific direction.

nr5a1 Ohnologs and secondary sex characteristics

To evaluate the roles of *nr5a1* ohnologs in adults, we examined secondary sex characteristics at 8 mpf (months postfertilization). All three mutant genotypes had body shapes more similar to wild-type males than wild-type females, but coloration was not strongly masculine (Figure was 5, A–J). Wild-type males, but not females, had breeding tubercles (Figure 5, F, G, K, and L) (McMillan *et al.* 2015), but mutants for either or both *nr5a1* ohnologs lacked breeding tubercles (Figure was 5, H–J and M–O). Females have a longer genital papilla than males do (Figure 5, U and V) (McMillan *et al.* 2015), but mutants for either or both *nr5a1* ohnologs had genital papillae of intermediate size (Figure 5, W–Y). We conclude that secondary sex characteristics were absent or ambiguous in *nr5a1* mutants.

nr5a1 Ohnologs and body size

By 11 mpf, *nr5a1* mutants were enormous compared to co-housed wild-type siblings. At 8 mpf, *nr5a1a* mutants were three times the mass of wild-type males and 35% longer (Figure 5A vs. C, Supplementary Figure S3). The *nr5a1b* mutants were somewhat larger than normal males, as they were double mutants. Body cavities of *nr5a1b* mutants were filled with fat cells and had abnormal gonads or no gonad at all. It is possible that when free cholesterol is not used for making steroid hormones, the excess becomes esterified and stored in lipid droplets (Hatano *et al.* 2016). The lack of the stress hormone cortisol might also contribute to more efficient calorie usage and hence more growth.

nr5a1 Ohnologs and adult gonadogenesis

Given the ambiguous sex phenotypes of *nr5a1* mutants, we wondered about adult gonads. Adult gonads of 3-mpf *nr5a1a* mutants contained testes tubules with a few spermatocytes, but they lacked mature spermatozoa already possessed by wild-type male siblings (Figure 6, B, C, G, and H). At 8 mpf, *nr5a1a* mutant gonads had still not developed any mature spermatozoa (Figure was 6, L, M, Q, and R). We conclude that *nr5a1a* is necessary for the maintenance of oocytes, for female development, and for sperm maturation.

The gonadal phenotype of *nr5a1b* mutants was more severe than that of *nr5a1a* mutants. At 3 mpf, gonads in *nr5a1b* mutants continued to be small and undifferentiated (Figure 6, D and I) like they were at 35 dpf. By 8 mpf, *nr5a1b* mutants, remarkably, had no gonad at all (Figure 6, N and S). The gonadal phenotype of double mutants was similar to that of *nr5a1b* mutants (Figure 6,

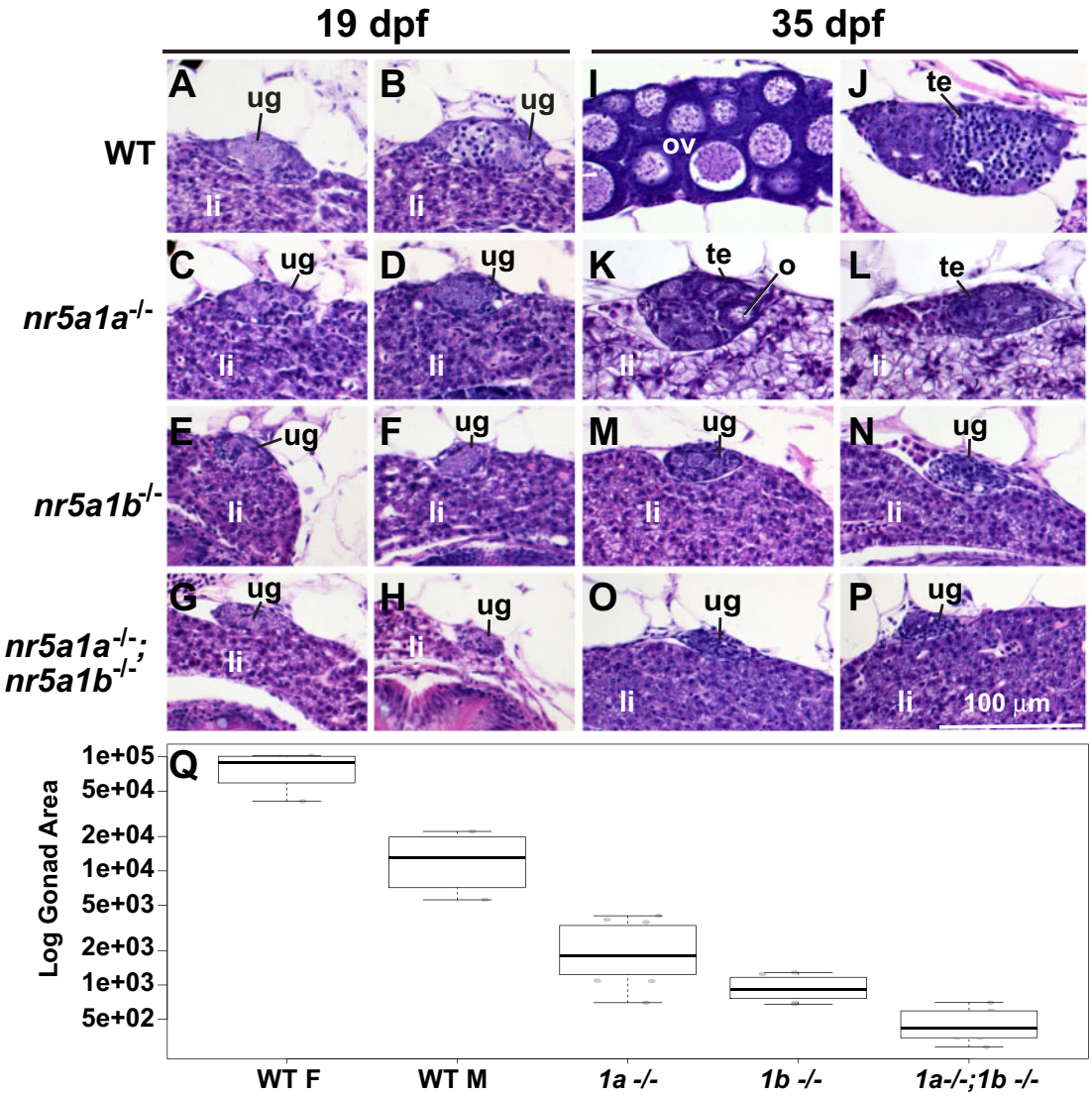

Figure 4 Histology of gonads in 19 and 35 dpf wild-type and *nr5a1*-mutant fish. (A–H) 19 dpf. (I–P) 35 dpf. In 19-dpf juveniles, gonads in all (8/8 fish) wild-type fish (WT) were undifferentiated (*e.g.*, A, B). Gonads in all *nr5a1a*$^{-11}$ mutants (6/6 fish) (*e.g.*, C, D), all *nr5a1b*$^{-8}$ mutants (6/6 fish) (*e.g.*, E, F) and in all (4/4 fish) *nr5a1a*$^{-11}$; *nr5a1b*$^{-8}$ double mutants (*e.g.*, G, H) were undifferentiated like wild-type gonads but all mutant gonads were smaller than wild-type sibling gonads, especially in *nr5a1b*$^{-8}$ mutants and *nr5a1a*$^{-11}$; *nr5a1b*$^{-8}$ double mutants (*e.g.*, E–H). At 35-dpf (I–P), wild-type fish contained gonads that were clearly either ovaries (4/4 fish) (*e.g.*, I) or testis (4/4 fish) (*e.g.*, J). In 35-dpf *nr5a1a*$^{-11}$ mutants, all fish examined had smaller testis (13/13 fish) (*e.g.*, K, L, Q); In 35-dpf *nr5a1b*$^{-8}$ mutants (8/8 fish), gonads were still undifferentiated (*e.g.*, M, N, Q); In 35-dpf *nr5a1a*$^{-11}$; *nr5a1b*$^{-8}$ double mutants, gonads were still undifferentiated and were even smaller than earlier (4/4 fish) (*e.g.*, O, P, Q). (Q), Log cross-sectional area of the gonad in arbitrary units for 35-dpf wild-type females (4/4 fish) and males (4/4 fish); for 35-dpf *nr5a1a*$^{-11}$ mutants (13/13 fish); 35-dpf *nr5a1b*$^{-8}$ mutants (8/8 fish), and 35-dpf *nr5a1a*$^{-11}$; *nr5a1b*$^{-8}$ double mutants (4/4 fish). Gonad sizes were significantly smaller in *nr5a1* mutants compared to wild-type siblings. Outer bars, minimum and maximum; thick bar, median; lower box edge, first quartile; upper box edge third quartile. li, liver; ov, ovary; te, testis; ug, undifferentiated gonad. Statistical significance: different letters (a–e) signify differences at $P < 0.05$. Scale bar in *P* is 100 µm for all panels.

E, J, O, and T). We conclude that *nr5a1b* function is required for gonad differentiation, growth, and maintenance.

To test the fertility of *nr5a1a* mutants, we set up single pair crosses between mutants and wild-type females. All six single-pair control crosses of 4.5 mpf wild-type male siblings mated to AB strain wild-type females produced eggs with an average of 150 ± 114 (SD) viable offspring among 173 ± 121 deposited eggs with 83%±11 (SD) eggs, developing per clutch. In contrast, 14 crosses of 4.5 mpf *nr5a1a* mutants produced no eggs in any cross. Similarly, crosses of 11-mpf wild-type siblings produced an average of 150 ± 35 (SD) viable offspring among 155 ± 36 deposited eggs, with 97%±2 (SD) of the eggs developing; in contrast, 10 crosses of 11-mpf *nr5a1a* mutants to AB females yielded no eggs. These experiments are consistent with the results of histology, which showed that *nr5a1a* mutants lacked mature sperm (Figure 6), and furthermore show that *nr5a1a* mutants did not stimulate wild-type females to lay eggs, consistent with their lack of male-breeding tubercles and other masculine traits (Figure 5).

Gene expression in *nr5a1* mutant adult gonads

To help interpret adult mutant phenotypes, we studied gene expression patterns. In 8-mpf wild-type adults, granulosa and theca cells in ovaries and Leydig cells in testes expressed *nr5a1a* (Figure 7, A and B) and young oocytes and Leydig cells expressed *nr5a1b* (Figure 7, D and E) (Kuo *et al.* 2005; Quek and Chan 2009; Yan *et al.* 2019). Testes in *nr5a1a* mutants did not express *nr5a1a* or *nr5a1b* or the Leydig cell marker *cyp11c1* (Wang and Orban 2007) (Figure , G–I). We conclude that each *nr5a1* ohnolog is

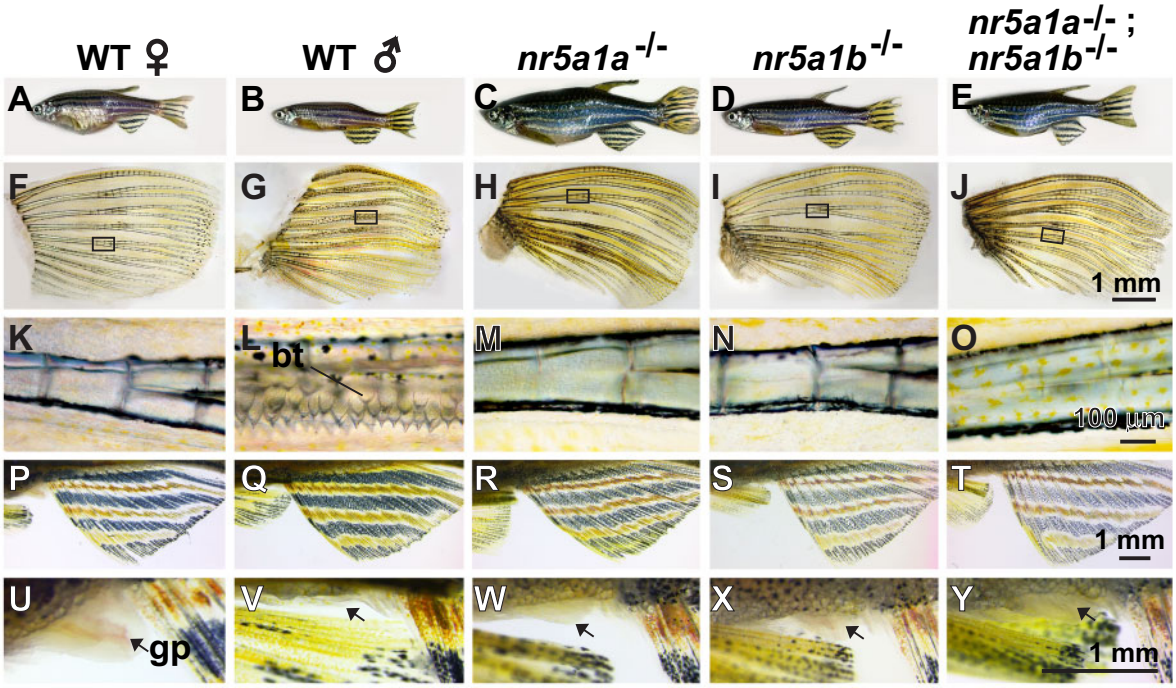

Figure 5 Secondary sex characteristics. (A–E) 8-mpf adult zebrafish. (A) Wild-type female. (B) Wild-type male. (C) *nr5a1a*$^{-11}$ mutant. (D) *nr5a1b*$^{-8}$ mutant. (E) *nr5a1a*$^{-11}$; *nr5a1b*$^{-8}$ double mutant. Among mutants, body shape was male-like but body color was not. (F–O) Pectoral fin of 8-mpf fish. (F) wild-type female, (G) wild-type male, (H) *nr5a1a*$^{-11}$ mutant, (I) *nr5a1b*$^{-8}$ mutant, and (J) *nr5a1a*$^{-11}$; *nr5a1b*$^{-8}$ double mutant. (K–O) Boxed region of pectoral fin in higher magnification, showing breeding tubercles (bt) in (I) wild-type male but not in (K) wild-type female or in any *nr5a1* mutants (M–O). (P–T) Anal fins dissected from (P) wild-type female, (Q) wild-type male, (R) *nr5a1a*$^{-11}$ mutant, (S) *nr5a1b*$^{-8}$ mutant, and (T) *nr5a1a*$^{-11}$; *nr5a1b*$^{-8}$ double mutant. (U–Y) Genital papilla (gp, arrows) located anterior to the anal fin. (U) Long genital papilla in wild-type female compared to (V) wild-type male and (W–Y) *nr5a1* mutants. Based on five fish of each genotype, a total of 25 fish. bt, breeding tubercles; gp, genital papilla. Scale bar in J for F–J: 1 mm; scale bar in O for K–O: 100 µm; scale bar in T for P–T; scale bar in Y for U–Y: 1 mm.

required for Leydig cell development. In wild types, Sertoli cells express *amh* (Rodriguez-Mari *et al.* 2005) (von Hofsten *et al.* 2005a; Schulz *et al.* 2007; Yan *et al.* 2017, 2019) (Figure K). In *nr5a1a* mutant gonads, *amh* expression appeared in fewer, less organized cells than in wild types (Figure 7, K and L), consistent with the loss of normal Leydig cells. The granulosa cell marker *cyp19a1a* is not expressed in normal testes (Chiang *et al.* 2001a,b) and was not expressed in *nr5a1a* mutant gonads (Figure 7, M–O). Expression of the germ cell marker *ddx4* (*vasa*) (Yoon *et al.* 1997) showed that *nr5a1a* mutant testis tubules contained a substantial number of disorganized germ cells. The *nr5a1b* mutants had no gonads at 8 mpf, precluding investigation of gonadal gene expression. These *in situ* hybridization experiments show that *nr5a1a* is necessary for the development of Leydig cells and secondarily for the organization of *amh*-expressing Sertoli cells.

Development of the interrenal in *nr5a1* mutants

Because *in situ* hybridization experiments (Figure 3) showed that *nr5a1* paralogs are expressed in the embryonic interrenal, we investigated this organ in adults. interrenal cells express *nr5a1* paralogs, reside within the head kidney, and occupy a cell layer surrounding the posterior cardinal veins in male and female zebrafish (Figure 8, A, B, F, G, K, and L) (Chester Jones and Mosley 1980; Hsu *et al.* 2003; Menke *et al.* 2011; Eachus *et al.* 2017). The interrenal produces corticosteroids using the interrenal-specific gene *cyp21a2* (Eachus *et al.* 2017) (Figure 11, P and Q). In *nr5a1a* adult mutants, however, histology failed to show any interrenal cells around the cardinal vein (Figure 8, C, H, and M) confirmed by *in situ* hybridization experiments that showed no expression of *cyp21a2* (Figure 8R), mimicking 2-dpf *nr5a1a* mutant embryos (Figure is 3, B, F, and J). In contrast, *nr5a1b* mutants had interrenal cells visible in histological sections and *cyp21a2*-expressing cells surrounding the cardinal vein although they were fewer and more scattered than in wild types (Figure 8, D, I, N, and S). Double mutants, like *nr5a1a* mutants, also appeared to lack an interrenal, (Figure 8, E, J, O, and T). We conclude that *nr5a1a* action is necessary for the development of the interrenal and that *nr5a1b* function is required for fully normal interrenal development.

Endocrinology of *nr5a1* mutants

The analysis of secondary sexual characteristics, gonad development, interrenal morphologies, and gene expression all suggested profoundly aberrant steroid biosynthesis in *nr5a1* mutants. To determine which hormone levels are regulated by which *nr5a1* ohnolog, we assayed estrogen (E2), 11-keto testosterone [11-KT, the primary fish androgen (Borg 1994)], and cortisol [the primary corticosteroid in actinopterygian fishes (Sangalang *et al.* 1971; Idler and Truscott 1972; Hanson and Fleming 1979; Barton *et al.* 1998)]. Tests assayed entire bodies of individual wild-type and mutant adults at 4.5 and 12 mpf. Assays showed that estrogen levels in 4.5-mpf wild-type females were far greater than those in wild-type males, but that estrogen levels in single and double *nr5a1* mutants were substantially lower than in either wild-type sex (Figure 9A). Levels of 11-KT were several hundred times lower in wild-type females than in wild-type males, and interestingly, were still lower in both single-mutant genotypes and in double mutants (Figure 9B). Cortisol concentrations were about 70% higher in wild-type males than in wild-type females and, on average, cortisol levels in single and double *nr5a1* mutants were only

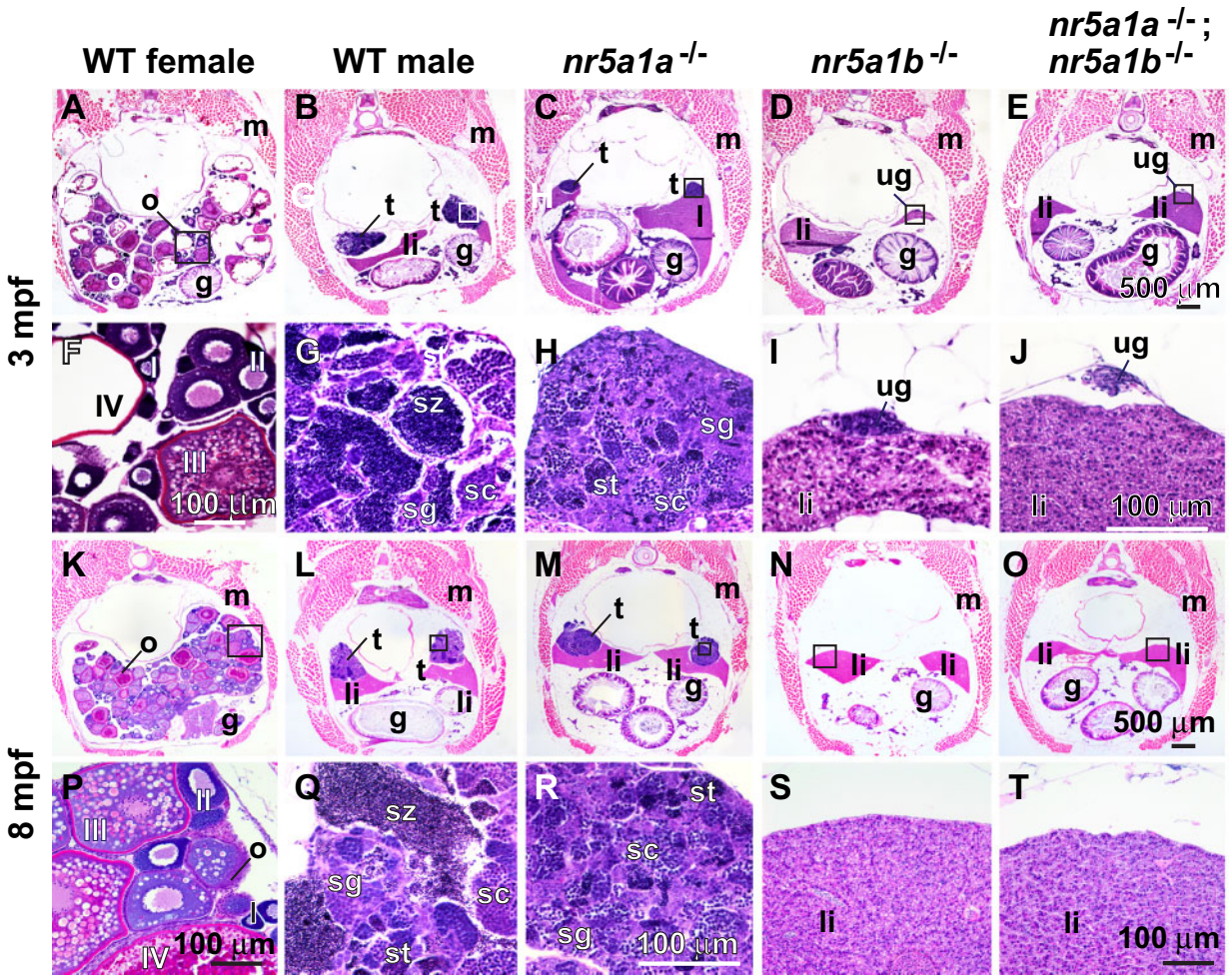

Figure 6 Histology of gonads in adult zebrafish. (A–J) 3-mpf adult zebrafish. (A, F) wild-type female, (B, G) wild-type male. (C, H) *nr5a1a*$^{-11}$ mutant (n.8). (D, I) *nr5a1b*$^{-8}$ mutant (n.5). (E, J) *nr5a1a*$^{-11}$; *nr5a1b*$^{-8}$ double mutant (n.4). (K–T) 8-mpf adult zebrafish. (K, P) Wild-type female, (L, Q) wild-type male, (M, R); *nr5a1a*$^{-11}$ mutant (n.8). (N, S); *nr5a1b*$^{-8}$ mutant (n.8). (O, T) *nr5a1a*$^{-11}$; *nr5a1b*$^{-8}$ double mutant (n.4). (A–E) Low magnification at 3 mpf, (F–J), high magnification of boxed region in AE at 3 mpf (K–O), low magnification at 8 mpf (P–T), high magnification of boxed region at (K–O) at 8 mpf. Crosssections of 3mpf (A, F) and 8-mpf wild-type female siblings (K, P) show maturing (stage-I and -II) and vitellogenic (stage-III and -IV) follicles. Wild-type male siblings at 3mpf (B, G) and 8 mpf (L, Q) had formed all spermatogenic stages. Mutant *nr5a1a* males at 3 mpf at low (C), and high magnification (H) show that older animals had developed more immature sperm and spermatocytes, compared to wild-type siblings (B, C, G, H). I, II, III, IV: ovarian follicle stages 1–4; g, gut; li, liver; o, ovary; s, Sertoli cells; sc, spermatocytes; sg, spermatogonia; st, spermatids; sz, spermatozoa; t, testis. Scale bar in (E) for (A–E): 500 μm; scale bar in (J) for (F–J): 100 mm; scale bar in (O) for (K–O): 500 mm; scale bar in (R) for (P–R): 100 μm; scale bar in (T) for (S) and (T): 100 μm.

about 7% of those in wild-type males, with *nr5a1a* mutants being more strongly affected than *nr5a1b* mutants (Figure 9C). Consistent with *in situ* hybridization and histology data, *nr5a1a* mutants had less than half the concentration of cortisol as found in *nr5a1b* mutants (Figure 9C). The results for 12-mpf fish were similar to those for 4.5-mpf fish (Supplementary Figure S4). We conclude that both zebrafish co-orthologs of *NR5A1* are critical for normal levels of estrogen and 11-keto testosterone, and owing to the low levels of both sex steroids, mutant fish are neither masculinized nor feminized. Cortisol assays showed that both *nr5a1* co-orthologs are important to maintain cortisol levels, but that *nr5a1a* is more important than *nr5a1b* in mature adult zebrafish (Figure 9C).

Transcriptomic analysis of *nr5a1* mutants

To understand how *nr5a1* ohnologs regulate the expression of other genes, we made RNA-seq libraries from fish trunks, which included interrenal, gonad, liver, and other viscera. We made 64 sequencing libraries, encompassing four homozygous genotypes (wild types, *nr5a1a* mutants, *nr5a1b* mutants, *nr5a1ab* double mutants), two ages (21 and 35 dpf), and eight individuals for each time point and genotype although one 35-dpf wild-type sample failed. Two-way similarity clustering by regularized log-transformed Euclidean distances and principal component analysis separated all 21-dpf animals from all 35-dpf samples in the PC1 axis, which explained 37% of the variance (Figure 10A). This result is consistent with the histological analyses (Figure 4), showing substantial changes in gonads during this period. In the PC2 axis, all *nr5a1a* mutants at 35 dpf and all double mutants at 21 dpf were in the lower half of the plot (Figure 10A), suggesting that interrenal functions were driving PC2.

Transcriptomics of 21-dpf zebrafish juveniles

At 21 dpf, principal component analysis separated the 32 samples into approximately four quadrants (Figure 10B).

The PC1 axis separated individuals into two groups: Group21L on the left in Figure 10B, containing wild types plus *nr5a1b* mutants, *vs* Group21R on the right, containing *nr5a1a* mutants

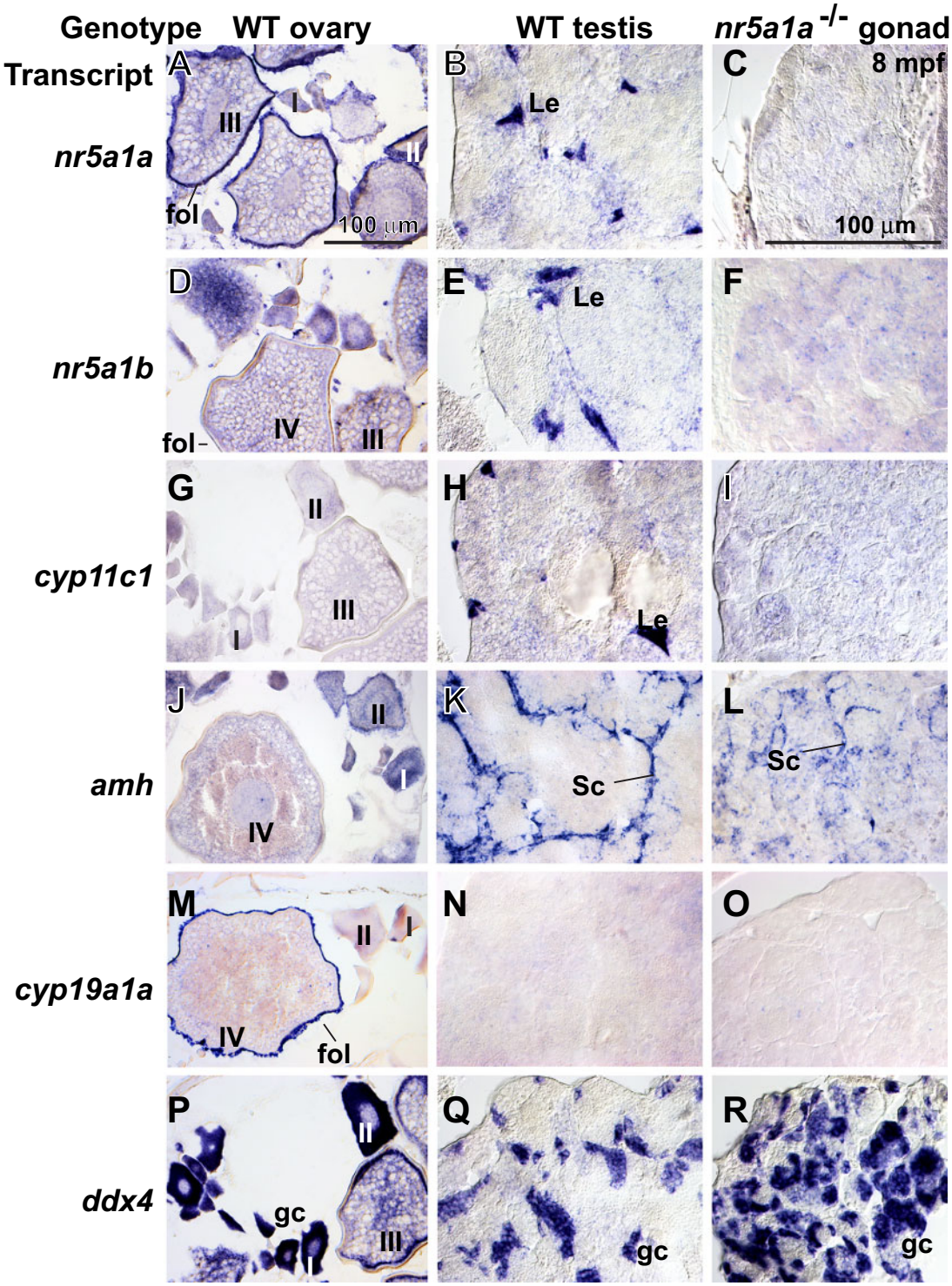

Figure 7 Gene expression patterns in adult gonads at 8 mpf. (A, D, G, J, M, P) Wild-type ovaries. (B, E, H, K, N, Q) Wild-type testis. (C, F, I, L, O, R) *nr5a1a*$^{-11}$ mutant testis. *In situ* hybridization using probes for (A, B, C) *nr5a1a*, (D, E, F) *nr5a1b*, (G, H, I) *cyp11c1*, (J, K, L) *amh*, (M, N, O) *cyp19a1a*, and (P, Q, R) *ddx4*. Scale bar in A (for all wild-type ovaries) and scale bar in C (for all testis panels) represents 100 μm. I, II, III, IV: ovarian follicle stages 1–4; fol, follicle cell; gc, germ cell; Le, Leydig cell; Sc, Sertoli cell.

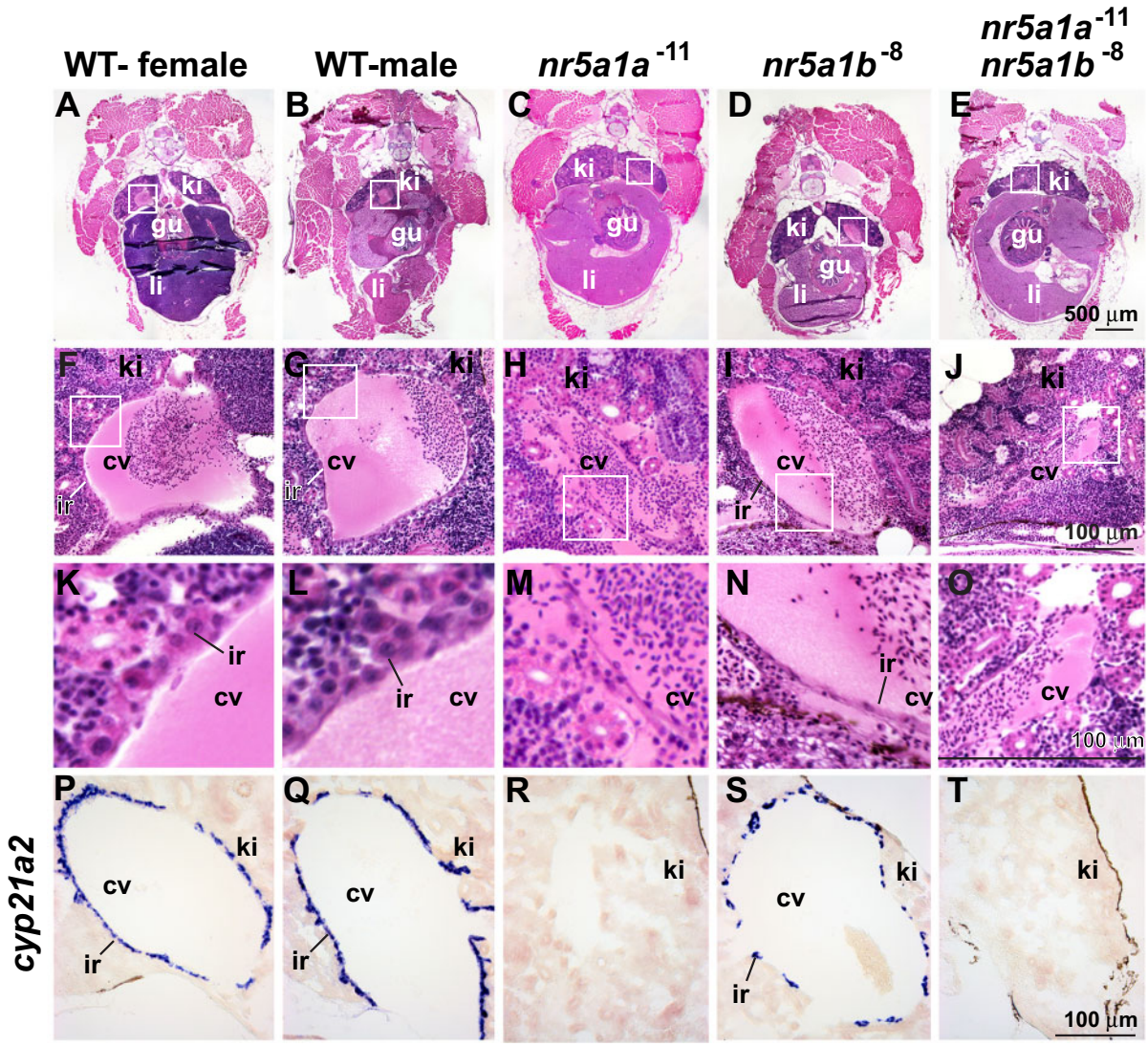

Figure 8 Nr5a1 activity is required for normal interrenal morphology in adult zebrafish. (A–O) Cross-sections of 8-mpf adult zebrafish histology and (P–T) *in situ* hybridization for *cyp21a2*. (A, F, K, P) Wild-type females. (B, G, L, Q) Wild-type males. (C, H, M, R) *nr5a1a*$^{-11}$ mutants. (D, I, N, S) *nr5a1b*$^{-8}$ mutants. (E, J, O, T) *nr5a1a*$^{-11}$; *nr5a1b*$^{-8}$ double mutants. Histological sections of anterior trunk: (A–E) at low magnification; white box expanded in (F–J) medium magnification; and white box expanded in (K–O) high magnification. (P–T) *In situ* hybridization of cyp21a2 to interrenal cells. cv, cardinal vein; gu, gut; ir, interrenal; li, liver; ki, kidney. Scale bar in (E) for (A–E); scale bar in J for F–J; scale bar in (O) for (K–O); scale bar in (T) for (P–T). All scale bars: 100 µm. Based on five fish of each genotype for morphology (25 fish), and four fish of each genotype for gene expression (20 fish).

plus double mutants (Figure 10B). Genes differentially over-expressed in Group21L versus Group21R included the "female" genes *zp2.2* (*zona pellucida*-2.2, 7216-fold up) and *vtg5* (vitellogenin, 21-fold up); the steroid biosynthesis genes *cyp17a1* (13-fold up), *hsd3b1* (12-fold up); the interrenal gene *cyp21a2* (6-fold up); and *nr5a1a* (2.5-fold up). We conclude that: (1) Group21L individuals had more advanced ovary development than Group21R; (2) Group21L juveniles were expressing interrenal steroid biosynthesis genes but Group21R juveniles had not formed a functioning interrenal; (3) the *nr5a1a* phenotype drives the double-mutant phenotype because these two genotypes associated together in Group21R in PC1; and (4) at 21 dpf, the gonadal phenotype of *nr5a1b* in the context of the entire trunk is not distinct enough to distinguish its transcriptome from wild types.

The PC2 axis at 21 dpf also separated samples into two groups, each of which had some representatives from all four genotypes (Figure 10B), suggesting that some factors other than genotype was driving the PC2 axis. The six most over-expressed genes in Group21B (bottom) compared to Group21T (top) were the uncharacterized genes *si:ch211-250e5.16*, *zgc:175135*, *zgc:171977*, *CU467646.2*, *zgc:173544*, plus *zp2.2*, which were over-expressed from 9,005 to 11,957-fold. Because wild-type adult ovaries over-expressed this gene set *vs* wild-type adult testes from 938 to 3,159-fold (Yan *et al.* 2019), we conclude that Group21B animals had initiated development as females but Group21T juveniles had not. This finding reflects a similar situation in a study of *amh* mutants and wild types in which transcriptomes from similar 21-dpf trunks separated samples into developing female *vs* not-female groups, both of which contained both *amh* mutants and wild types (Yan *et al.* 2019). We conclude that in an AB genetic background, some 21-dpf individuals have already embarked on a female developmental pathway without respect to the action of either *nr5a1a* (Figure 0B) or *amh* (Yan *et al.* 2019).

nr5a1a mutants compared to wild-type siblings at 21 dpf had 1071 DE genes (Padj < 0.1, Supplementary Table S3). *Regarding genes over-expressed* in *nr5a1a* mutants, the top five genes all

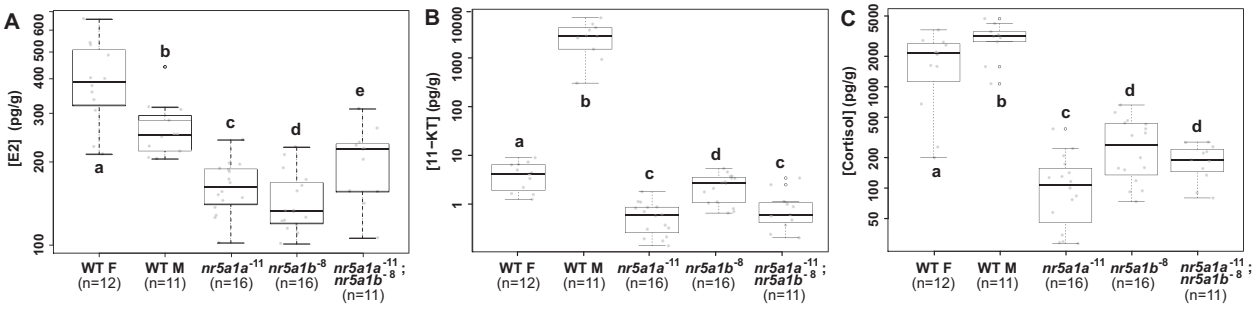

Figure 9 Loss of function of *nr5a1* ohnologs disrupts whole-body concentrations of sex steroids and cortisol at 4.5 mpf (log scale). (A) Estradiol (E2, pg/g wet weight). (B) 11-Keto testosterone (11-KT) (pg/g wet weight). (C) Cortisol (pg/g wet weight). WT F (wild-type females, *n*.12); WT M (wild-type males, *n*.11); *nr5a1a*$^{-11}$ (*n*.11); *nr51b*$^{-8}$ (*n*.16); *nr5a1a*$^{-11}$; *nr5a1b*$^{-8}$ double mutants (*n*.11). Different letters (a–e) signify statistically different groups at $P < 0.05$.

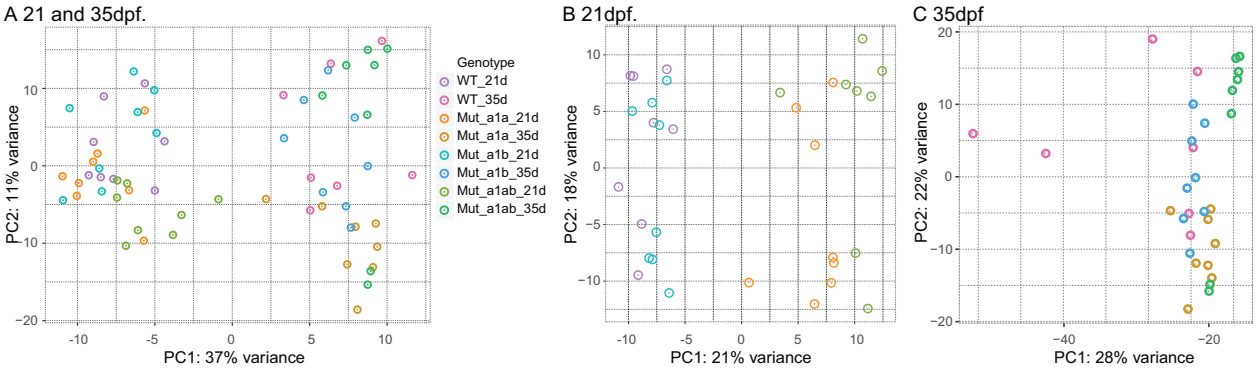

Figure 10 Principal component analysis of RNA-seq data for juvenile and young adult wild-type and *nr5a1* mutants. Each dot represents a different individual fish. (A) Analysis of all samples together. B. Analysis of 21dpf samples. C. Analysis of 35dpf samples. Abbreviations: WT, wild type; a1a, *nr5a1a* mutants; a1b, *nr5a1b* mutants; a1ab, *nr5a1a*; *nr5a1b* double mutants; 21d, 21dpf; 35d, 35dpf.

encode uncharacterized proteins (*zgc:175135*, *si:ch73-160p18.3*, *si:dkeyp-46h3.8*, *zgc:171750*, *si:dkey-229d11.3*; from 48,846- to 94,500-fold up) and were also greatly over-expressed in adult wild-type ovaries versus adult wild-type testes (1,045- to 3,159-fold) (Yan *et al.* 2019), suggesting that some ovary functions are inhibited by the normal allele of *nr5a1a* at 21 dpf. The first over-expressed known gene was *fosb* (23-fold up), and it was paradoxically *under*-expressed (63-fold) in adult wild-type ovaries versus testes (Yan *et al.* 2019), whereas *fosab*, *fosl1a*, and *jdp2b* were also among the first 33 most over-expressed genes. *FOS* family genes help regulate the levels of mRNA for both *Star* and *Cyp19a1* in mammalian ovaries and hence the rate of estrogen production (Beshay *et al.* 2007; Manna *et al.* 2009; Patel *et al.* 2009). Other over-expressed genes included *sox21a*, which is up-regulated 7.9-fold by the oocyte maturation-inducing steroid MIS (Klangnurak and Tokumoto 2017), and metabolism-related genes including the fatty acid elongase gene *elovl7b*, glucokinase *gck*, and the potential diabetes-related hepatic gluconeogenesis regulators *foxq1a*, and *foxq1b* (Cui *et al.* 2016), all among the 25 most over-expressed genes. This result suggests metabolic compensation for altered cortisol and sex steroid metabolism in *nr5a1a* mutants.

Regarding under-expressed genes in 21dpf *nr5a1a* mutants, results identified the ovarian follicle tight junction gene *cldnd* (15,049-fold down) and the retinoic acid signaling gene *retsatl* (6,208-fold down) (Sreenivasan *et al.* 2008), consistent with disrupted ovary organization and retinoic acid control of meiosis. Additional *nr5a1a* under-expressed genes in the top 11 included *star* (54-fold down), whose promotor is enhanced by Nr5a1 in mouse (Yang *et al.* 2010), the steroidogenic enzyme *cyp17a1* (20-fold down), and the estrogen-responsive liver-expressed yolk protein gene *vtg5* (13-fold down). The interrenal gene *cyp21a2* trended downward (6-fold) in *nr5a1a* mutants (Pval = 0.00013). We conclude that at 21 dpf, Nr5a1a is necessary for inhibiting some ovarian developmental steps and for the production of steroid hormones, likely from both the gonad and the interrenal, supporting data from histology and *in situ* hybridization studies (Figures 4 and 8).

Gene Ontology clustering for 21dpf nr5a1a mutants compared to wild-type siblings identified metabolic processes as the top three clusters (glycerol biosynthetic process, cellular glucose homoeostasis, and gluconeogenesis). These clusters included *pck1* and *pck2*, which encode phosphoenolpyruvate carboxykinase enzymes that help control gluconeogenesis. The fourth cluster was "response to glucocorticoid." These findings suggest that disturbed interrenal development in *nr5a1a* mutants greatly altered metabolism throughout the trunk. Continuing alteration of metabolism likely led to the enormous body size and fat cell accumulation in *nr5a1a* mutants at 4.5 and 11 mpf (Supplementary Figure S3).

nr5a1b Mutants vs. wild-type siblings at 21 dpf had 78 DE genes (Supplementary Table S4). *Regarding over-expressed genes*, the second most differentially expressed gene (CABZ01059627.1) was also over-expressed in adult wild-type ovaries versus testes (1,713-fold). The oocyte gene *ca15b* (Wang *et al.* 2013) was massively over-expressed [234,946,505-fold up in *nr5a1b vs* wild types

and 1,337-fold up in wild-type adult ovaries *vs.* wild-type testes (Yan *et al.* 2019)]). The fifth most over-expressed gene encodes an egg coat protein (*zp2.2*, 9,474,925-fold up). As in *nr5a1a* mutants, *fosb* was high on the list of over-expressed genes in *nr5a1b* mutants (10-fold). *Regarding under-expressed genes* in 21-dpf *nr5a1b* mutants, the most under-expressed was the ovary gene *cldnd*, mimicking *nr5a1a* mutants, with *cyp17a1* in seventh place. This mix of ovary genes both over- and under-expressed in *nr5a1b* mutants *vs* wild types suggests that gonads had initiated oocyte development in *nr5a1b* mutants at 21,dpf, but that regulation of oogenesis was greatly disturbed. *Gene Ontology clustering* identified no significant DE clusters, perhaps because of the low number of DE genes.

nr5a1a; nr5a1b Double mutants vs wild types at 21 dpf had 2,218 DE genes (Supplementary Table S5). *Regarding over-expressed genes* in double mutants, 6 of the top 22 were also among the 22 most over-expressed genes comparing *nr5a1a* mutants to wild types (*si:ch73-160p18.3, si:dkeyp-46h3.8, si:ch211-125e6.5, gck, sox21a, zgc:153642*) and seven comparing *nr5a1b* mutants to wild types (*zgc:171446, CABZ01059627.1, ca15b, zgc:175135, si:ch73-160p18.3, si:dkeyp-46h3.8, nr0b2b*). Several of these genes were greatly over-expressed in wild-type ovaries versus wild-type testes (Yan *et al.* 2019), suggesting that at 21 dpf, double mutants had begun to embark on a female pathway. *Under-expressed genes* in double mutants included some female genes, like the yolk protein gene *vtg5* (35-fold down), consistent with low levels of estrogen. Some "male" genes were also under-expressed, including *gsdf* (21-fold down), the male sex determinant in some fish (Myosho *et al.* 2012; Rondeau *et al.* 2013), suggesting disruption of the male developmental pathway. Of the 22 most under-expressed genes in double mutants, 12 (*nr1d2a, si:dkey-242g16.2, cdkn1d, muc5.2, vtg5, zgc:153932, si:ch211-89o9.4, cyp17a1, klf9, cyp2k6, fkbp5, star, cldnd*) were also among the 22 most under-expressed genes in *nr5a1a* mutants, but only one (*cyp17a1*) was also among the 22 most under-expressed in *nr5a1b* mutants, suggesting that *nr5a1a* has a stronger effect in double mutants. *Gene Ontology clustering* for 21-dpf double mutants identified the same four metabolic GO clusters as for *nr5a1a* mutants.

Transcriptomics of 35-dpf zebrafish young adults

Principal component analysis of 35-dpf fish (Figure 10C) showed that in the PC1 axis, three of the seven wild types were further to the left than any other fish; the other four wild types nested with single-mutant individuals in PC1; and six of the eight double mutants clustered at the far right. This result suggests that individuals sorted along PC1 according to the level of gonad maturation and interrenal function. The broad dispersion of wild types along PC1 suggests that different wild-type individuals may have varied greatly in gonadal maturation. Along PC2, all *nr5a1a* mutants were in the lower half of the plot.

nr5a1a mutants at 35 dpf had 1,404 DE genes *vs* wild-type siblings (Supplementary Table S6). *Regarding over-expressed genes*, human orthologs of several highly over-expressed genes (*ly6m6, adm2b, nxph2b*; 8- to 15-fold) have their strongest expression in the human ovary (Fagerberg *et al.* 2014). In contrast, other genes over-expressed in mutants, including the steroid-breakdown gene *cyp2k6* (11-fold) (Wang-Buhler *et al.* 2005), and several unstudied genes (*si:ch211-219a15.4, si:ch211-161h7.5, si:ch211-114l13.3*; 6- to 8-fold up) were under-expressed in wild-type ovaries versus wild-type testes (7- to 11-fold down) (Yan *et al.* 2019). *Regarding under-expressed genes* in 35 dpf *nr5a1a* mutants, the top gene was *zgc:175135*, which was, in contrast, the most over-expressed gene in this genotype at 21 dpf. Because *zgc:175135* is over-expressed (1,081-fold) in normal ovaries versus testes (Yan *et al.* 2019), this finding likely reflects the presence of immature oocytes at 21 dpf that were dying or de-differentiating at 35 dpf. The fourth most under-expressed gene in 35dpf *nr5a1a* mutant trunks was *ccna1*, which in mouse is necessary exclusively for meiosis in male germ cells (Liu *et al.* 1998), suggesting a decrease in male meiosis in 35 dpf *nr5a1a* mutants. Several oocyte genes were also under-expressed (*e.g.*, *bucl2, buc*, and *wee2* (10- to 132-fold), and many *zona pellucida* genes (*e.g.*, *zp3.2*, 66-fold). Many steroid biosynthesis genes were also under-expressed in 35-dpf *nr5a1a* mutants (*cyp11a1, cyp17a1, star, hsd3b1, cyp11c1, cyp11a2, cyp17a2*, 14- to 95-fold down) including the interrenal gene *cyp21a2* (7-fold down). *Gene Ontology clustering* for 35dpf *nr5a1a* mutants *vs* wild types identified 35 functional clusters with under-expression of corticosteroid genes dominating the top three categories. The fourth cluster, "long-chain fatty-acyl-CoA biosynthetic process," contained five genes encoding enzymes that elongate very long fatty acids (Elovl), possibly associated with the accumulation of fat in older *nr5a1a* mutants. We conclude that gene expression patterns in *nr5a1a* mutants showed a chaotic mix of processes involved in shutting down the gonad and interrenal between 21 and 35 dpf in *nr5a1a* mutants and altering energy metabolism.

nr5a1b Mutants at 35 dpf compared to wild types had 260 DE genes. *Regarding over-expressed genes*, the four strongest (*rfesd, mylpfb, oacyl, si:ch211-161h7.5*; 4- to 8-fold up) are also over-expressed in testes relative to ovary [7- to 14-fold up (Yan *et al.* 2019)], suggesting that oocytes were disappearing in *nr5a1b* mutants as gonads became testes. *Regarding under-expressed genes*, fold changes for the lowest 10 for *nr5a1b* mutants versus wild types were massive [log2fold of -26 to -49 for *cldnd*, *zar1l* (*zygote arrest 1 like*), and many uncharacterized genes; Supplementary Table S7]; these genes were all over-expressed in wild-type ovary versus testis by an average of 1737-fold (Yan *et al.* 2019). Additional under-expressed genes in *nr5a1b* mutants included other oocyte-expressed genes (*ddx4, bmp15, dazl, nanog*, and several *zona pellucida* genes, from 37- to 584-fold down), in contrast to under-expressed genes in *nr5a1a* mutants, which included many steroid biosynthesis genes. *Gene Ontology clustering* identified as top clusters several oocyte functions ["binding of sperm to zona pellucida," "positive regulation of acrosome reaction," and "egg coat formation"]. This result is consistent with histology, which suggested that a main role of *nr5a1b* is to support oocyte development rather than interrenal steroid biosynthesis.

Direct comparisons of *nr5a1a* mutants to *nr5a1b* mutants identified over-expressed ovary genes (*e.g. cldnd* and *zar1l*, log2FoldChange = 30.3 and 20.1, respectively), under-expressed steroidogenesis genes (*e.g.*, *vtg5, cyp17a1, star*, fold change 12- to 58-fold), and under-expressed lipid metabolism genes (*klf9, lpin1*, fold change of 7- to 13-fold), verifying the role of *nr5a1a* in interrenal and lipid biology and *nr5a1b* in oocyte development.

nr5a1a; nr5a1b Double mutants at 35 dpf showed 2,219 DE genes with respect to wild-type siblings (Supplementary Table S9). *Regarding over-expressed genes*, the top gene (*si:ch211-253p18.2*) was the third most over-expressed in 35 dpf *nr5a1b* mutants and the third gene (*si:ch211-125e6.5*) was the most over-expressed in 35-dpf *nr5a1a* mutants. Some other genes in the top 10 (*sid1, oacyl, rfesd*) were also in the top 10 over-expressed genes in *nr5a1a* or *nr5a1b vs* wild types, showing that double mutants combined the effects of each single mutant. *Regarding under-expressed genes*, some were massively changed in double mutants at 35 dpf, including the germ cell gene *ca15b* (log2fold change, −37.5), in contrast to 21 dpf, when *ca15b* was massively over-expressed

comparing double mutants to wild types (log2fold change, +28.0). The zona pellucida gene *zp2.2* (log2fold change, −32.8) was also massively under-expressed in 35-dpf double mutants. Other under-expressed genes included the male meiosis gene *ccna1*, several endocrine genes (*cyp17a1, cyp11a1*), and the estrogen-responsive gene *vtg5*. These results suggest that double mutants had more oocytes than wild types at 21 dpf but the situation reversed by 35 dfp. One of the most consistently under-expressed genes in *nr5a1a* mutants and double mutants at both 21 and 35 dpf was *cyp2k1* (in the 93rd to 99th percentile); its apparent human ortholog *CYP2W1* is highly expressed in normal and cancerous adrenal glands (Ronchi *et al.* 2014). This finding is expected by the lack of the interrenal in nr5a1a-containing mutants. *Gene Ontology clustering* identified the top three categories as "C2 steroid hormone metabolic process," "glucocorticoid metabolic process," and "gluconeogenesis," similar to *nr5a1a* single mutants. The fourth category, "binding of sperm to zona pellucida," mimicked *nr5a1b* results, reflecting loss of both genes.

Remarkably large bodies of nr5a1a mutants (Figure 6) might be due to the mis-expression of downstream growth regulating genes. The pituitary hormone somatotropin (growth hormone, GH) regulates growth in many fish species (Canosa *et al.* 2007). GH binds to GH receptors (GHRs) in target cells and can stimulate the liver to produce insulin-like growth factors, which can promote growth (Picha *et al.* 2008; Fuentes *et al.* 2013). Although the large size of *nr5a1* mutants does not appear until later, the trunk transcriptomes of 21 and 35 dpf *nr5a1a* mutants, surprisingly, under-expressed the *igf*-related genes *igf2a, igfals, igfbp7*, and *igf2bp2a* by 1.3- to 2.5-fold down with respect to wild types (Supplementary Tables S3 and S6). Understanding the mechanisms that lead to the continuing growth of *nr5a1a* mutants will require further analysis.

Discussion

Nr5a1 and the adreno-gonadal primordium

In mammals, the gonadal soma and the adrenal cortex arise from the adreno-gonadal primordium as a thickening of the celomic epithelium that expresses *Nr5a1* before the activation of *Sry* (Ikeda *et al.* 1994; Hatano *et al.* 1996). The rostral portion of the adreno-gonadal primordium forms the anlage of the adrenal cortex (Morohashi 1997). In mouse, scRNA-seq of *Nr5a1*-expressing cells showed that they become Sertoli and granulosa cells using a common, non-sex-specific transcriptomic program before they acquire sex-specific identity (Stevant *et al.* 2019). An adreno-gonadal primordium has not been demonstrated in teleosts. Our zebrafish scRNA-seq experiments showed that *nr5a1a*-expressing cells are already specifically expressing gonadal and adrenal cortex genes (*hsd3b1, cyp11a2, cyp21a2, nr5a1b, star, fdx1b*) at 1 dpf, 3 weeks before the acquisition of sex identity. Far fewer *nr5a1b*-expressing cells were in this cluster as verified by *in situ* hybridization experiments on 2-dpf embryos (Chai and Chan 2000; von Hofsten *et al.* 2001; Chai *et al.* 2003; Hsu *et al.* 2003; von Hofsten *et al.* 2005b; Quek and Chan 2009). The mammalian adreno-gonadal primordium expresses *Nr5a1* in the context of *Wt1, Emx2, Six1, Six4, Gata4, Cbx2, Cited2, Odd1*, and *Lhx9* (Kreidberg *et al.* 1993; Birk *et al.* 2000; Schnabel *et al.* 2003; Katoh-Fukui *et al.* 2005; Buaas *et al.* 2009; Fujimoto *et al.* 2013; Hu *et al.* 2013). Among these genes, only *six1b* was DE (Supplementary Table S1) in *nr5a1a*- or in *nr5a1b*-expressing cells in zebrafish embryos, providing little support for a teleost adreno-gonadal primordium because other genes, like *hsd3b1* and *cyp11a2*, are expressed in both organs, so at 5 dpf, *nr5a1a*-expressing cells might be precursors of the interrenal but not the gonad. Arguing against that interpretation is the finding that some Cluster 87 *nr5a1*-expressing cells in zebrafish embryos were co-expressing *cxcl12a*, encoding the ligand secreted by the genital ridge that guides migrating primordial germ cells expressing the receptor *cxcr4b* to the gonadal soma in the celomic epithelium (Boldajipour *et al.* 2011). In the Atlas, *cxcr4b* is expressed in 1-dpf germ cells (Cluster 219) as expected (Farnsworth *et al.* 2020). Future cell-lineage experiments are necessary learn whether the gonad and interrenal share a common origin in zebrafish and the earliest time at which the somatic component of the gonad arises, which is at least by 10 dpf because *gata4* and *nr5a1a* are co-expressed in the somatic gonad at this time (Leerberg *et al.* 2017).

Nr5a1 and the hypothalamus

For *nr5a1a*-expressing cells, the most strongly DE genes in scRNA-seq were related to steroid biogenesis, but for *nr5a1b*-exressing cells, the strongest DE genes were related to the ventromedial hypothalamus (VMH); *in situ* hybridization experiments verified this result. In mouse, *Nr5a1* is expressed both in the adreno-gonadal primordium and in the VMH, and hence these zebrafish experiments show first, that these two roles of Nr5a1 already existed in the last common ancestor of zebrafish and mammals, and second, that after the teleost genome duplication, the two roles partitioned between the two ohnologs, with regulatory elements controlling one expression domain going to *nr5a1a* and the other to *nr5a1b* as predicted by the process of subfunctionalization (Force *et al.* 1999).

nr5a1a mutants and transcriptional adaptation

Some mutations that result in nonsense-mediated transcript decay exhibit genetic compensation by transcriptional adaptation, the upregulation of related genes independent of feedback loops involving proteins (El-Brolosy *et al.* 2019). Our *nr5a1a* mutant allele generated a premature stop codon seven codons prior to the stop codon formed by our *nr5a1b* allele (Figure 2C). *In situ* hybridization experiments showed reduced quantities of *nr5a1a* transcript in *nr5a1a* mutant embryos at 48 hpf (Figure 3B), suggesting transcript instability and thus a potential for transcriptional adaptation that might ameliorate the mutant phenotype owing to the upregulation of a paralog (El-Brolosy *et al.* 2019). RNA-seq analysis confirmed the reduction of *nr5a1a* mutant transcript (3.9- and 6.3-fold at 35 dpf in *nr5a1a* mutants and double mutants, respectively), but showed that transcripts for paralogs that might perform transcriptional adaptation (*nr5a1b, nr5a2, nr6a1b*, and *nr6a1a*) were not differentially expressed in any mutant combination. This observation makes transcriptional adaptation unlikely for our $nr5a1a^{-11}$ allele. The quantity of *nr5a1b* message was not differentially expressed in any mutant genotype relative to wild-type siblings in RNA-seq experiments, as expected if it was not subject to transcriptional adaptation; in addition, none of the paralogs mentioned above were differentially expressed. We conclude that the mutant alleles we studied for both genes are unlikely to have been partially rescued by transcriptional adaptation and thus, owing to the early stops, represent null activity mutations.

Contrasting phenotypes of nr5a1a and nr5a1b mutants

Newborn mice totally lacking *Nr5a1* have no adrenal glands or gonads but do have a uterus, a vagina and oviducts regardless of genetic sex (Luo *et al.* 1994; Sadovsky *et al.* 1995; Shinoda *et al.* 1995). In addition, the VMH of mouse *Nr5a1* mutants is smaller

than normal and disorganized and the pituitary lacks cells that secrete gonadotrophins. If ancestral functions of *Nr5a1* partitioned between the two zebrafish co-orthologs of *Nr5a1a*, we would expect some of these phenotypes to be associated with *nr5a1a* mutants and others to accompany *nr5a1b* mutations. Results indeed showed that *nr5a1a* mutants lost the adrenal and *nr5a1b* mutants lacked gonads. Furthermore, unlike mouse *Nr5a1* mutants, zebrafish *nr5a1b* mutants appeared to lack sex-specific traits, including male sex tubercles and pigmentation and female-specific sex papillae. Coupled with exceedingly low sex steroid titers, *nr5a1b* and double mutant fish seemed to lack both primary and secondary sex characteristics.

Most homozygous null *Nr5a1* mutant mice die shortly after birth owing to adrenal deficiency (Luo *et al.* 1994; Sadovsky *et al.* 1995; Shinoda *et al.* 1995), but tissue-specific knockouts survive longer. *Nr5a1* knockout in Leydig cells led to hypoplastic testes and sterility and knockout in granulosa cells decreased the number of ovarian follicles and resulted in failed ovulation (Jeyasuria *et al.* 2004; Anamthathmakula *et al.* 2019). The knockout of *Nr5a1* specifically in the VMH led to increased anxiety-like behaviors and susceptibility to high-fat diet-induced obesity (Kim *et al.* 2009, 2011). The phenotypes of zebrafish *nr5a1* mutants were a mixture of these phenotypes, with *nr5a1a* mutants, showing enormous growth, accumulation of abdominal fat cells, aberrant gonad differentiation, and loss of the interrenal, whereas *nr5a1b* mutants lost their gonads completely and developed an abnormal interrenal. Zebrafish double mutants combined these phenotypes and mimicked the situation in complete knockout mice. These phenotypes are as predicted by the duplication, degeneration, and complementation hypothesis (Force *et al.* 1999).

Nr5a1 and growth control

In mammals, *NR5A1*-expressing cells in the VMH help regulate food intake and body weight (King 2006). The VMH-specific knockout of *Nr5a1* in mice leads to late-onset obesity, especially when fed a high fat diet (Kim *et al.* 2011). VMH-specific *Nr5a1* knockout mice were not longer than littermate controls, but they were more adipose. Similarly, *nr5a1a* mutant zebrafish were large, some nearly three times the mass of their wild-type siblings; in contrast to *NR5A1* mouse mutants, however, zebrafish *nr5a1a* mutants also grew longer, perhaps related to indeterminant growth in fish. The mechanism for weight gain in *Nr5a1*-mutant mice likely involves leptin, which is expressed in adipocytes in mammals and directly activates leptin receptors on *Nr5a1*-expressing VMH cells: mice lacking leptin receptors on these cells gained body weight and were sensitive to diet-induced obesity (Dhillon *et al.* 2006; Bingham *et al.* 2008). These observations raise the hypothesis that without Nr5a1a activity, cells in the zebrafish VMH do not express leptin receptor and thus cannot detect leptin or satiety, and hence continue to acquire energy from food and grow to the enormous observed sizes. Evidence to evaluate that hypothesis, however, is mixed. Zebrafish and other teleosts generally have one gene encoding the leptin receptor (*lepr*) and two genes encoding leptin (*lepa, lepb*) (Gorissen *et al.* 2009; Liu *et al.* 2010). Arguing against the hypothesis is the finding that zebrafish bearing a premature stop codon in *lepr* were the same size as controls (Michel *et al.* 2016). Similarly, medaka *lepr* mutants were longer and heavier than wild-type controls up to 7 weeks posthatching, but by 9 weeks, the two genotypes were the same size (Chisada *et al.* 2014). On the other hand, zebrafish *lepa* mutants were about 10% longer and 15% heavier than wild-type siblings at 6 mpf (Audira *et al.* 2018), as expected from the hypothesis. Mechanisms leading to the massive increase in mutant size may be related to the observed substantial misregulation of long-chain fatty acid genes and gluconeogenesis genes in *nr5a1a* mutant zebrafish, reflecting the finding that in mouse, nearly all genes in the glycolytic pathway are regulated by Nr5a1 (Baba *et al.* 2014). A further conundrum is that our scRNA-seq and *in situ* hybridization experiments showed that *nr5a1b* is expressed more broadly than *nr5a1a* in the embryonic hypothalamus but *nr5a1b* mutants were of near normal size. We hypothesize that in adults, *nr5a1a* is expressed in hypothalamic cell types that help regulate growth but *nr5a1b* is expressed in hypothalamic cells with different functions. Future work is essential to understand the mechanism that leads to greatly increased body size in zebrafish *nr5a1* mutants.

Nr5a1 and sex steroids

Nr5a1 targets steroidogenesis genes in mammals (Clemens *et al.* 1994; Sugawara *et al.* 1996; Parker *et al.* 2002). Accordingly, in zebrafish adults, *nr5a1a* and *nr5a1b* mutants had greatly reduced titers of both estrogen and 11-ketotestosterone. As a consequence, these mutants lacked secondary sex characteristics mediated by these hormones, including the androgen-induced characters of male coloration and sex tubercles on male pectoral fins (McMillan *et al.* 2015), and the estrogen-induced trait of extended urogenital papillae in females (Brion *et al.* 2004), as if these mutants had no somatic sex. Similarly, expression of the liver-expressed, estrogen-induced (Brion *et al.* 2004; Hao *et al.* 2013) yolk protein gene *vtg5* (Meng *et al.* 2010) was greatly reduced in 21 and 35 dpf *nr5a1a*-bearing mutants, almost 100-fold down in double mutants, consistent with low estrogen titers. Vitellogenin genes were not differentially expressed in *nr5a1b* mutants despite their small gonads at 21 and 35 dpf and low estrogen titer at 4.5 months (Figure 9A), and hence it is possible that the small gonads of *nr5a1b* mutants were producing estrogen in juveniles but not in young adults.

Low levels of sex steroids in *nr5a1* mutants can be explained by downregulation of steroidogenesis genes mimicking mouse *Nr5a1* mutants [*Star* (Caron *et al.* 1997), *Cyp11a1* (Clemens *et al.* 1994), *Cyp17a1* (Park *et al.* 2010), *Cyp19a1* (Lynch *et al.* 1993), *Hsd3b1* (Buaas *et al.* 2012), and *Hsd3b2* (Martin *et al.* 2005)]. The only zebrafish orthologs of these mouse genes that were not DE in *nr5a1a* mutants and double mutants were *hsd3b2* and *cyp19a1a*, both of which had exceptionally low base-mean counts that prevented adequate statistical tests. The *cyp19a1a* gene was also not mentioned to be induced by estrogen treatments in zebrafish larvae or adult males (Brion *et al.* 2004; Hao *et al.* 2013). The depression of steroidogenic gene expression in *nr5a1a* mutants compared to wild types was much stronger at 35 dpf than at 21 dpf, demonstrating the increasing failure of mutants during this critical period for sex determination. In *nr5a1b* mutants, only *cyp11a1, hsd3b1*, and *cyp17a1* among sex steroid genes were DE, and again the effect was stronger at 35 dpf than at 21 dpf.

Nr5a1 and cortisol

The stress hormone cortisol is secreted from the adrenal cortex, which is lacking from *nr5a1a* mutants; correspondingly, wild types had 25-times more cortisol than their *nr5a1a* siblings. The cortisol-synthesizing enzyme gene *cyp21a2* was not DE at 21 dpf in any of the three mutant genotypes but was downregulated about sevenfold at 35 dpf in *nr5a1a* mutants and double mutants, which eventually had no interrenal; it was not DE in *nr5a1b* mutants, which developed a small interrenal. Zebrafish 5-dpf larvae treated with cortisol upregulate a number of genes, including

fkbp5, *tsc22d3*, *socs3a*, and *cyp2k6* (Hartig *et al.* 2016); as expected for cortisol-stimulated genes, these genes were all under-expressed in *nr5a1a* mutants (4- to 99-fold), in accordance with low cortisol levels in these mutants. These cortisol-sensitive genes, as well as *cyp21a2*, were not mis-expressed in *nr5a1b* mutants, which has a small interrenal.

Gonad development in *nr5a1a* mutants

Oocyte development has already begun in 19-dpf zebrafish AB-strain gonads because by this time, they are already expressing zona pellucida genes (*zp2* and *zp3* paralogs) (Liu *et al.* 2006). In *nr5a1a* mutant genotypes, *zp3* genes were not DE in mutants *vs* wild types at 21 dpf, as expected if *nr5a1a* mutants began to develop oocytes like wild types do. In *nr5a1a* mutants, however, many *zp* genes were under-expressed at 35 dp (20- to 698-fold), showing that they failed to maintain oocyte development. Although 35dpf *nr5a1a* mutants under-expressed many *zp3* genes (*zp3a.1, zp3b, zp3c, zp3d.1, zp3d.2, zp3.2*), they did not mis-express the seven *zp2* genes that are strongly over-expressed in adult wild-type ovaries *vs.* testes [*zp2.1, zp2.2, zp2.3, zp2.5, zp2.6, zp2l1, zp2l2* (Yan *et al.* 2019)] or the *zp*-gene regulator *figla* (Qin *et al.* 2018). Although *figla* regulates both *zp2* and *zp3* gene families and estrogen regulates neither (Liu *et al.* 2006), the mechanism that leads to their differential regulation in *nr5a1* mutants remains to be discovered. Like *zp* genes, many meiosis genes (*fanci, sycp1, dazl, mns1, eme1, wee2*) were not mis-expressed in 21dp *nr5a1* mutants but were under-expressed in 35-dpf mutants, as were the oocyte genes *buc* (Marlow and Mullins 2008; Bontems *et al.* 2009) and *bmp15* (Clelland *et al.* 2006; Dranow *et al.* 2016). These results give the general picture that gene expression in *nr5a1a* mutant gonads is not disrupted severely enough to be distinguished from that in wild types at 21 dpf but by 35 dpf, many more mutant fish than wild types were transitioning to male phenotypes.

Loss of *nr5a1a* gene activity affected some gonadal cell types more than others. *In situ* hybridization experiments showed that in adult *nr5a1a* mutants, Leydig cells were missing, which was confirmed by transcriptomic studies, showing that the Leydig cell marker *cyp11c1* (Wang and Orban 2007) was downregulated 16- to 19-fold by 35 dpf in *nr5a1a* mutants. In contrast, *in situ* hybridization studies showed that *amh*-expressing Sertoli cells were merely fewer and less-well organized in *nr5a1a* adult mutants than in wild types, confirmed by RNA-seq (2.3- to 4.4-fold). We conclude that *nr5a1a* is necessary for Leydig cell development and that effects on *amh*-expressing Sertoli cells are likely to be secondary.

Gonad development in nr5a1b *mutants*

Histology showed that *nr5a1b* activity is essential for gonads to move beyond the juvenile ovary stage. This conclusion is supported by RNA-seq, which showed that the top 10 genes over-expressed in 21 dpf *nr5a1b* mutants were also upregulated about a thousand-fold or more in wild-type ovaries *vs* testes (Yan *et al.* 2019), as expected if *nr5a1b* mutants had started making oocytes before wild types do; by 35 dpf, however, most of these genes were among the 20 most downregulated in *nr5a1b* mutants, as expected if early oocytes had disappeared. By 3 mpf, gonads in *nr5a1b* mutants were not much larger than at 35 dpf, and by 8 mpf, gonads were not detected at all, similar to the "vanishing testis syndrome" of some human *NR5A1* alleles (Philibert *et al.* 2007). We conclude that *nr5a1b* action is required for the proliferation and maintenance of the gonad, whereas *nr5a1a* is required for the maintenance of just oocytes. In adult wild types, *nr5a1b* is expressed in Leydig cells, but we do not yet know whether gonad loss in *nr5a1b* mutants is due to cell-autonomous action of *nr5a1b* in the gonad or to a non-cell-autonomous action owing to its role in the hypothalamus. Construction of genetic mosaics would answer that question.

Sex determination and zebrafish nr5a1 *ohnologs*

Results showed that all *nr5a1a* mutants developed a testis, and hence we conclude that *nr5a1a* is required for ovary development in AB strain zebrafish. A male-biased sex ratio also occurs after mutation of a number of other gonadal genes, including homologs of genes in the female branch of the mammalian sex-determination pathway [*e.g.*, *wnt4a* (Kossack *et al.* 2019), *nr0b1* (Chen *et al.* 2016), *foxl2a; foxl2b* double mutants; *cyp19a1a* (Dranow *et al.* 2016; Lau *et al.* 2016; Yin *et al.* 2017)]. In contrast, mutations in zebrafish orthologs of mammalian male-pathway genes provide female-biased sex ratios [*ar*, encoding the androgen receptor (Crowder *et al.* 2018), *dmrt1* (Webster *et al.* 2017), *amh* (Lin *et al.* 2017; Yan *et al.* 2019)]. Mutants for genes necessary for uncommitted germ cell survival or proliferation also tend to develop as males [*nanos3* (Dranow *et al.* 2013), *fgf24* (Leerberg *et al.* 2017), *piwil1* (Houwing *et al.* 2007), *piwil2* (Houwing *et al.* 2008), *ddx4* (Hartung *et al.* 2014), *prmt5* (Zhu *et al.* 2019)]. Male development, sometimes with fertility, can come from mutations in genes that are important for early oocyte survival, [*e.g. bmp15* (Dranow *et al.* 2016), *figla* (Qin *et al.* 2018), and 15 different *Fanconi anemia* genes (Rodriguez-Mari *et al.* 2010; Rodriguez-Mari *et al.* 2011; Botthof *et al.* 2017; Ramanagoudr-Bhojappa *et al.* 2018)]. This observation led to the hypothesis that a large number of germ cells is important for female sex determination in AB strain zebrafish because a proportion of them will form oocytes that enter meiosis and emit a signal to the gonadal soma that maintains female development (Slanchev *et al.* 2005; Siegfried and Nusslein-Volhard 2008; Rodriguez-Mari *et al.* 2010). In line with this hypothesis, the expression of germ-cell marker genes [*e.g. ddx4, piwil1, piwil2, tdrd1*, (Yoon *et al.* 1997; Houwing *et al.* 2007, 2008; Huang *et al.* 2011)], including oocyte-specific genes (*bmp15, buc, dazl, wee2*), were not differentially expressed at 21 dpf, but had drastically reduced expression by 35 dpf in *nr5a1a* and *nr5a1b* mutants (9- and 26-fold, respectively), consistent with germ cell loss, especially oocyte loss, over these 2 weeks as confirmed by histology. Principal component analysis also separated 21-dpf individuals into fish embarking on a female developmental pathway *vs.* those not yet developing as females independent of the action of *nr5a1a*, as was found earlier for *amh* (Yan *et al.* 2019). Oocytes in transitioning gonads die by Tp53-mediated apoptosis (Rodriguez-Mari *et al.* 2010; Rodriguez-Mari and Postlethwait 2011; Sun *et al.* 2013; Miao *et al.* 2017). In accordance with this finding, several apoptosis-related genes (*tp53, casp7, casp6l1*) were upregulated significantly, although to a small degree (1.2- to 1.6-fold). Taken together, these histological and gene expression studies support the hypothesis that zebrafish *nr5a1* mutants begin to develop testes owing to death of oocytes.

Although *nr5a1a* mutants developed testes with developing spermatocytes, they failed to develop male secondary sex characteristics. Gonads in *nr5a1b* mutants failed to increase in size from 21 dpf and eventually disappeared and hence these animals and double mutants appeared to have no sex at all: they had no gonads and no secondary sex characteristics.

Subfunctionalization and Nr5a1

The last common ancestor of mammals and zebrafish had a single *Nr5a1* gene, but the teleost genome duplication produced two

ohnologs, *nr5a1a* and *nr5a1b*, which survive in zebrafish. The duplication, degeneration, complementation hypothesis (Force *et al.* 1999) predicts that one mechanism for the survival of both gene copies after a gene duplication event is the reciprocal partitioning of essential ancestral gene subfunctions between the two duplicates, which can evolve further in diverging lineages (Force *et al.* 1999; Postlethwait *et al.* 2004; Glasauer and Neuhauss 2014; Freeling *et al.* 2015; Braasch *et al.* 2018). Mutations in the single-copy *NR5A1* gene in human cause a large range of gonadal phenotypes, including primary ovarian insufficiency and lack of ovary formation in 46, XX individuals and partial testicular dysgenesis in 46, XY karyotypes leading to being raised as a girl (Achermann *et al.* 1999; Bashamboo *et al.* 2016; Fabbri-Scallet *et al.* 2020), but only infrequently leads to adrenal dysfunction (only 10 of 175 patients tested) (Fabbri-Scallet *et al.* 2020). *Nr5a1* mutations in mouse give gonadal phenotypes similar to *NR5A1* mutations in humans, but have a stronger adrenal phenotype, with death shortly after birth (Luo *et al.* 1994; Shinoda *et al.* 1995; Val *et al.* 2003). The early death of *nr5a1* mouse mutants is likely because they lack the adrenal hormone aldosterone, which is essential for salt and water balance in mammals. Fish, however, achieve salt and water balance without aldosterone, and hence the lack of the adrenal homolog in fish is not lethal. Like mammals, Nile tilapia has a single copy of *nr5a1* whose relationship to zebrafish *nr5a1* ohnologs has not previously been identified; our analysis shows that the tilapia gene is an ortholog of *nr5a1a* in the genomic context *adgrd2 nr5a1a nr6a1a mir-181b-2* and that tilapia lost *nr5a1b* and *nr6a1b* in the ancestral context *nr6a1b nr5a1b psmb7 nek6 lhx2a dennd1a* after the cichlid lineage diverged from the medaka lineage. Nile tilapia individuals developing from zygotes injected with CRISPR/Cas9 reagents targeting *nr5a1a* showed a high rate of mutation associated with gonadal dysgenesis and few steroidogenic cells coupled with feminization of XY fish and sex reversal of XX fish (Xie *et al.* 2016), similar to the zebrafish *nr5a1a* mutants. Given the essential nature of *nr5a1b* in zebrafish, it is difficult to understand how tilapia manages to develop gonads without the function of *nr5a1b*.

The reciprocal loss of the interrenal and the gonad in zebrafish *nr5a1a* and *nr5a1b* mutants, respectively, likely reflects differential roles of *nr5a1a* and *nr5a1b* in the specification of key cell types in the adrenal–gonadal (interrenal-gonadal) primordium at or before the bipotential gonad stage (Kreidberg *et al.* 1993; Birk *et al.* 2000; Schnabel *et al.* 2003; Katoh-Fukui *et al.* 2005; Buaas *et al.* 2009; Fujimoto *et al.* 2013; Hu *et al.* 2013). The milder phenotypes—oocyte loss leading to the development of testes that are abnormal owing to aberrant Leydig cell development in *nr5a1a* mutants and the reciprocal reduction and disorganization of the interrenal in *nr5a1b* mutants—likely come from the later roles of Nr5a1 in the regulation of steroid biosynthesis, which was greatly reduced in both genotypes. These differences are associated with differential expression patterns of the two *nr5a1* ohnologs that may represent the subfunctionalization events that preserved both gene copies. Resolving these issues will require future single-cell transcriptomics experiments and functional analyses of promotors.

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Conflicts of interest

None declared.

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