Title

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Journal

Developmental Dynamics, 246(11)

ISSN

1058-8388

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Publication Date

2017-11-01

DOI

10.1002/dvdy.24579

Peer reviewed

Published in final edited form as:

Dev Dyn. 2017 November ; 246(11): 925–945. doi:10.1002/dvdy.24579.

The gonadal soma controls ovarian follicle proliferation through Gsdf in zebrafish

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Abstract

Background—Aberrant signaling between germ cells and somatic cells can lead to reproductive disease and depends on diffusible signals, including TGFB-family proteins. The TGFB-family protein Gsdf (gonadal soma derived factor) controls sex determination in some fish and is a candidate for mediating germ cell/soma signaling.

Results—Zebrafish expressed *gsdf* in somatic cells of bipotential gonads and expression continued in ovarian granulosa cells and testicular Sertoli cells. Homozygous *gsdf* knockout mutants delayed leaving the bipotential gonad state, but then became a male or a female. Mutant females ovulated a few oocytes, then became sterile, accumulating immature follicles. Female mutants stored excess lipid and down-regulated *aromatase, gata4*, *insulin receptor, estrogen receptor*, and genes for lipid metabolism, vitellogenin, and steroid biosynthesis. Mutant females contained less estrogen and more androgen than wild types. Mutant males were fertile. Genomic analysis suggests that Gsdf, Bmp15, and Gdf9, originated as paralogs in vertebrate genome duplication events.

Conclusions—In zebrafish, *gsdf* regulates ovarian follicle maturation and expression of genes for steroid biosynthesis, obesity, diabetes, and female fertility, leading to ovarian and extra-ovarian phenotypes that mimic human polycystic ovarian syndrome (PCOS), suggesting a role for a related TGFB signaling molecule in the etiology of PCOS.

Keywords

Gonad development; GDF9; BMP15; oogenesis; Polycystic ovarian syndrome (PCOS); gonadal soma germ cell interaction; granulosa cells; Sertoli cells; ovarian follicle; insulin signaling; TGFβ

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INTRODUCTION

Germ cells and somatic cells interact reciprocally to promote gonad development and gamete maturation (Eppig, 2001). In mouse, gonadal soma factors including Bmp7, Wnt4, and Rspo1 (Jeays-Ward et al., 2003; Ross et al., 2007; Chassot et al., 2008) stimulate proliferation of primordial germ cells (PGCs). Reciprocally, germ cells signal the soma because gonads without germ cells develop aberrantly, leading to, for example, sterile testes in zebrafish (Slanchev et al., 2005; Houwing et al., 2007; Kurokawa et al., 2007; Siegfried and Nusslein-Volhard, 2008; Rodriguez-Mari and Postlethwait, 2011; Tzung et al., 2015).

Signals exchanged between germ cells and somatic cells include members of the transforming growth factor-beta (TGFB) pathway, including ligands (Amh, Bmp2, Bmp8b, Bmp15, and Gdf9) receptors (ACVR1); and downstream effectors (Smad1, Smad4, and Smad5) (Chang and Matzuk, 2001; Aubin et al., 2004; Hanrahan et al., 2004; Clelland et al., 2007; Mendis et al., 2011). Decreased levels of the oocyte-secreted TGFB factors GDF9 and BMP15 occur in polycystic ovarian syndrome (PCOS), a condition affecting 6% of reproductive age women and the most frequently diagnosed problem when couples visit fertility clinics (Diamanti-Kandarakis, 2008; Goodarzi et al., 2011; McAllister et al., 2015). PCOS involves at least two of three signs: hyperandrogenism, infrequent or irregular ovulation, and the accumulation of numerous immature antral follicles (Rotterdam, 2004b; Wei et al., 2014). In addition, people with PCOS often show disrupted insulin profiles, altered gonadotrophin signaling (Diamanti-Kandarakis, 2008), and high levels of AMH (Pigny et al., 2003; Diamanti-Kandarakis, 2008), a granulosa cell-expressed TGFB family member (Durlinger et al., 2002; Pellatt et al., 2010; Seifer and Merhi, 2014).

In addition to their roles in mammalian gonad development, TGFB family genes also act in sex determination in some species of fish. In two species of pejerrey, a Y-linked gene encoding Amh (*amhy*) is the primary sex determinant (Hattori et al., 2012; Yamamoto et al., 2014). In fugu pufferfish, the X-linked sex determinant is a hypoactive allele for Amh receptor type II (Amhr2) (Kamiya et al., 2012; Myosho et al., 2012), mimicking male-to-female sex reversal in *amhr2* mutants in Japanese medaka (Matsuda et al., 2002; Nanda et al., 2002; Morinaga et al., 2007). In Luzon ricefish and likely sablefish, the major sex-determining gene is the TGFB family member *gsdfY* (Myosho et al., 2012; Rondeau et al., 2013). Gsdf (gonadal soma derived factor) was first identified in the somatic gonad of rainbow trout, induced when germ cells invade the genital ridge (Sawatari et al., 2007; Yazawa et al., 2010). Granulosa cells express *gsdf* in ovaries and Sertoli cells express *gsdf* in testes (Gautier et al., 2011b). Knockdown experiments show that *gsdf* stimulates PGC proliferation in trout (Sawatari et al., 2007). Gonads in most teleosts express *gsdf* (Luckenbach et al., 2008; Shibata et al., 2010; Yazawa et al., 2010; Gautier et al., 2011a; Crespo et al., 2013; Nagasawa et al., 2014) (Robledo et al., 2015; Jiang et al., 2016). In Japanese medaka (*Oryzias latipes*), *gsdf* is expressed weakly in gonads of both XX and XY embryos, then increases in males in somatic cells that express *dmy/dmrt1by* (Shibata et al., 2010). In Japanese medaka, *gsdf* initiates but does not maintain male fate (Imai et al., 2015; Zhang et al., 2016); in contrast, in tilapia *gsdf* does not initiate, but maintains *dmrt1* expression and testis development (Jiang et al., 2016). In medaka, *dmrt1by* activates *gsdf* (Zhang et al., 2016) and suppresses expression of *rspo1* and the female pathway, and *gsdf*

activity can rescue sex reversal caused by loss of *dmrt1by* (Chakraborty et al., 2016). Another *Oryzias* species eliminates the upstream factor (*dmrt1by*) and uses *gsdf* directly as a sex determinant (Myosho et al., 2012).

Gsdf likely plays a special role during the sex change in hermaphroditic fish species. In the sequentially hermaphroditic protogynous three-spot wrasse, *gsdf* expression turns on as the sex change begins (Horiguchi et al., 2013), while in the protandrous yellowfin sea bream, high *gsdf* expression appears only in the testis part of the ovotestis (Chen et al., 2015). Zebrafish laboratory strains also pass through a hermaphroditic stage as all juveniles form meiotic oocytes that eventually die in individuals that become males and survive in individuals that become females (Takahashi, 1977; Uchida et al., 2002; Rodriguez-Mari and Postlethwait, 2011; Tzung et al., 2015). These considerations raise the hypothesis that in zebrafish, sex determination might work by manipulating *gsdf* expression. Here we show that the juvenile bipotential gonad in zebrafish expresses *gsdf* and confirm that Sertoli cells continue to express *gsdf* but support cells contacting meiotic and post-meiotic germ cells in transitioning individuals down-regulate *gsdf* expression (Yazawa et al., 2010; Gautier et al., 2011b). Consistent with Sertoli cell expression of *gsdf*, immunization of zebrafish against Gsdf protein conjugated to keyhole limpet hemocyanin (KLH) dramatically reduced testis development, although controls were not injected with KLH alone, and all antigens tested had similar effects on the testis (Presslauer et al., 2014). The role that *gsdf* plays in zebrafish sex determination, however, is as yet unknown. To answer this question, we made *gsdf* knockout mutations. Results showed that *gsdf* mutants became either fertile males with enlarged testes or sterile females that accumulated non-vitellogenic follicles. These results rule out the hypothesis that *gsdf* plays a major role in sex determination in laboratory zebrafish, and show that it provides a somatic signal that regulates follicle maturation and prevents the formation of polycystic ovaries. We showed that *gsdf* likely arose in the vertebrate genome duplication events along with paralogs *bmp15* and *gdf9*, and because coelacanth possesses *gsdf* but tetrapods do not (Forconi et al., 2013), the gene was lost in tetrapods; these three paralogs likely share ancestral subfunctions. The ovarian phenotype of zebrafish *gsdf* mutants mimics PCOS, suggesting a model for PCOS that could be used in screens for therapies.

RESULTS

The gonadal soma expresses *gsdf*

The hypothesis that *gsdf* acts in zebrafish sex determination predicts that it should be expressed at or before sex determination, which becomes evident by oocyte death between 19 and 27 days post fertilization (dpf) (Siegfried and Nusslein-Volhard, 2008; Orban et al., 2009). In situ hybridization experiments on tissue sections detected *gsdf* transcripts in gonads but in no other tissues of zebrafish embryos, larvae, juveniles, or adults. In gonads, *gsdf* expression had already begun by 8dpf in gonadal somatic cells, which we identified by staining adjacent sections for *vasa* expression (Yoon et al., 1997) (Fig. 1A, B). In tissue sections prepared under identical conditions, staining of *gsdf* expression was darker by 12dpf (Fig. 1C, D), continued at 25 and 35dpf (Fig. 1E–H), and appeared in Sertoli cells in testes and in granulosa cells in ovaries (Fig. 1I–K). Double *in situ* hybridization experiments

showed that *gsdf* is expressed in somatic cells adjacent to germ cells (Fig. 1K), similar to *amh*-expressing Sertoli cells (Fig. 1L). Adult ovaries expressed *gsdf* in granulosa cells of pre-vitellogenic follicles (stage-I and stage-III, Fig. 1J), which contrasts with medaka, where expression is weak or not detected in genetic females (Shibata et al., 2010). We conclude that *gsdf* expression begins before overt sex becomes evident and continues throughout gonad differentiation; thus, *gsdf* is expressed at a time and place consistent with a role in sex determination in zebrafish.

*Gsdf k*nockouts

To learn the roles of *gsdf*, we used TALENs to induce deletion alleles targeting exon-1, which precedes the conserved pro-peptide cleavage site in exon-3 (Massague, 1998; Bottner et al., 2000; Gordon and Blobe, 2008) (Fig. 2A, B). The TALEN target sequence contains a BslI restriction enzyme recognition site that provides evidence for deletion of the target sequence (Fig. 2B). We isolated stable out-of-frame deletion lines (allele designations b1279 (8-nucleotide deletion), b1280 (1-nucleotide deletion), and b1281 (14-nucleotide deletion), (Fig. 2C). To confirm the transcription of the mutated *gsdf* gene, we made cDNA from the 8 bp deletion, designed primers in exon-1 and exon-2, and sequenced the PCR fragment, predicted to be 275 bp in the wild-type cDNA, 267 bp in the mutant cDNA, and about 1700 bp in the wild-type genomic DNA. Results confirmed the transcription of the -8 mutant gene and verified the sequence of the mutant (Fig. 2C, D). The mutant alleles predict truncated polypeptides of 19 to 39 amino acids including out-of-frame sequences rather than the normal 172 residue Gsdf polypeptide (Fig. 2E). Because these deletion mutants should disrupt the predicted pro-region (Walton et al., 2010) and delete the TGFB domain, they should be *gsdf* null activity alleles. Although we were not able to develop antibodies to zebrafish Gsdf, qPCR provides evidence for nonsense mediated transcript decay (see below). Because phenotypes of all mutant alleles were similar, results presented below focus on the 8-nucleotide deletion allele.

Sex ratios and fertility

Sex ratios in zebrafish *gsdf* mutants were about the same as in wild-type siblings: for example, crossing heterozygotes for the 8-nucleotide deletion allele produced homozygous wild-type offspring with a ratio of 25 males to 33 females (43% males), heterozygotes with a ratio of 54 to 66 (45% males), and homozygous mutants with a ratio of 27 to 33 (45% males) when scored at 4.5 months post fertilization by gonad dissection; other alleles produced similar results. Even old mutants maintained a female phenotype in terms of fin color, body shape, and germ cell development (immature eggs, no sperm). We conclude that in zebrafish, *gsdf* activity is not the primary genetic sex determinant.

Roles of Gsdf in gonad development

To test for roles of *gsdf*, we studied the fertility of mutants homozygous for the -1, -8, and -14 alleles. Results showed that, while most homozygous *gsdf* mutant females at 4.5mpf (months post fertilization) were fertile, by 8mpf, fertility was greatly reduced, and by 18mph, all mutant females tested were sterile (Fig. 3A, B). In males, in contrast, for the -8 allele, for example, 18 of 18 homozygous *gsdf* mutants were fully fertile and similar results

were obtained for other alleles. We conclude that in zebrafish, *gsdf* activity is essential to maintain female, but not male fertility.

To learn how *gsdf* acts to maintain female fertility, we examined gonad histology. Histological sections of 22 wild-type fish at the committed but immature gonad stage (35dpf) identified 19 fish with only testis tissue and the remaining three with only ovary tissue (Fig. 4A, B). In contrast, among 22 mutant fish at 35dpf, ten had only testis and six had only ovary, but the remaining six (27%) had either one gonad developing as an ovary and the other as a testis (one fish in Fig. 4C, D and another in Fig. E, F) or individual gonads that contained both ovarian follicles and testis lobules (one individual in Fig. 4G, H). Sections of older fish (8mpf and 18mpf, 10 male and 10 female fish) showed no evidence of hermaphroditism. We conclude that Gsdf accelerates the juvenile-to-adult gonadal transition.

At 4.5 months post fertilization (mpf), *gsdf* mutant ovaries contained nearly normal follicles from stage-I to stage-IV as in wild-type siblings (Fig. 3C, Fig. 4I–L) (for staging, see (Selman et al., 1993)). Mutant ovaries, however, had a greater number of younger and fewer older stage follicles than wild types (Fig. 3C). At 8mpf, the trend continued (Fig. 3C, Fig. 4M, N, Q, R). At 18mpf, mutant females contained only pre-vitellogenic follicles but no stage-IV follicles (Fig. 3C, Fig. 4P, T), compared to wild types, which had many mature oocytes (Fig. 3C, Fig. 4O, S). We conclude that in fully adult zebrafish, *gsdf* activity is required for follicles to mature beyond stage-III and to prevent the accumulation of young ovarian follicles; the second phenotype might be a consequence of the first.

In contrast to *gsdf* mutant females, *gsdf* mutant males were fertile but developed larger testes than wild types. Histological sections showed that, like wild-type males (Fig. 4U, V), homozygous 8mpf *gsdf* mutant males contained all stages of sperm development, including mature sperm (Fig. 4X, Y), but had about three times more mass than wild-type testes (see also Fig. 6N), due to an increase of lobules of all stages. We conclude that *gsdf* is not required for zebrafish male sex determination or sperm development and maturation, but helps to regulate testis size.

Gene mis-expression in *gsdf* mutant gonads

To investigate the molecular genetic basis for the observed histological phenotypes, we studied gene expression patterns. At eight months post fertilization, *gsdf* mutant females showed less intense *gsdf* expression in somatic cells surrounding oocytes, but greater amounts in oocytes compared to histological sections of wild type ovaries stained under the same conditions (Fig. 5A, A′). To determine whether the lack of *gsdf* expression surrounding oocytes was due to the absence of follicle cells or to their failure to mature, we examined expression of the granulosa cell markers *amh cyp19a1a*, and *gata4* (Rodriguez-Mari et al., 2005; von Hofsten et al., 2005); (Chiang et al., 2001c; Efimenko et al., 2013). Amh inhibits the FSH-induced recruitment of ovarian follicles (Weenen et al., 2004); *cyp19a1* encodes aromatase, the enzyme that converts testosterone to estrogen (Rouiller-Fabre et al., 1998); and Gata4 encourages granulosa cell proliferation and theca cell recruitment (Anttonen et al., 2003). Expresson of none of these markers was detected surrounding oocytes in 8mpf *gsdf* mutant females (Fig. 5B, B′, D, D′, E, E′), despite the clear presence of gonadal somatic cells (Fig. 4R). Although *cyp19a1a* expression was not

detected in 8mpf follicle cells, it must have been produced in younger mutant fish at 4.5mpf that laid eggs because *cyp19a1a* is necessary for gonadal estrogen production. We conclude that in zebrafish, *gsdf* is required for the maintenance or maturation of granulosa cells acting either as an autocrine signal or a paracrine signal to the oocyte, which then might reciprocally support granulosa cells.

In contrast to *gsdf*, which wild types expressed in ovarian follicle cells (Fig. 5A), the *gsdf* paralog *bmp15* was expressed in wild-type oocytes, granulosa cells, and theca cells (Clelland et al., 2006; Dranow et al., 2016), but its expression gradually decreased as oocytes matured (Fig. 5C). In mutant ovaries, *bmp15* expression was strong only in oocytes younger than stage-III (Fig. 5C′). Adult wild-type females at 8mpf expressed the *gsdf* paralog *gdf9* mainly in young oocytes, stronger in stage-I follicles and weaker or diluted as follicles matured (Fig. 5F, F′), confirming previous qPCR evidence (Wang and Ge, 2003; Liu and Ge, 2007; Poon et al., 2009). Expression of *gdf9* was not greatly changed in *gsdf* mutant ovaries (Fig. 5F,F′ and Fig. 7.B3). Expression of other markers, including *sox9b* (Chiang et al., 2001a), *sycp3* (synaptonemal complex protein 3) (Rodriguez-Mari and Postlethwait, 2011), and *vasa* were not dramatically different between wild types and *gsdf* mutants (Fig. 5H–T′). We conclude that expression of gonadal soma genes is greatly disrupted in *gsdf* mutants, but expression of early oocyte genes is nearly normal.

Our results confirm *gsdf* expression in Sertoli cells of wild-type zebrafish males (Gautier et al., 2011a), but show greatly reduced *gsdf* expression in *gsdf* mutant testes (Fig. 5K, K′), as would be expected from nonsense-mediated transcript decay. Expression of the Sertoli cell marker *amh* (Rodriguez-Mari et al., 2005; von Hofsten et al., 2005) appeared to increase in *gsdf* mutant males (Fig. 5L, L′, see also qPCR results in Fig. 7C3). Ovarian follicle cell markers *bmp15, cyp19a1a*, and *gata4*, as well as oocyte markers *gdf9* and *sox9b*, were not strongly expressed in wild-type or *gsdf* mutant testis (Fig. 5M–O′, R, R′); the Sertoli cell marker *sox9a* (Chiang et al., 2001a) and the early meiotic spermatocyte marker *sycp3*, were expressed at about the same level in mutant and wild-type testes (Fig. 5Q, Q′ and S, S′); and the germ cell marker *vasa* appeared to be expressed more extensively in *gsdf* mutant testes than in wild-type testes (Fig. 5T, T′ and Fig. 6C5). These experiments suggest that some activities of Sertoli cells may be up-regulated in zebrafish *gsdf* mutant testes, a conclusion verified below by qPCR experiments.

Pathophysiology of *gsdf* mutants

In PCOS, ovaries accumulate young ovarian follicles as in *gsdf* mutants, but in addition, PCOS often includes obesity, lipid accumulation, type II diabetes, hypertension, and ovarian cancers (Franks, 1995; Daniilidis and Dinas, 2009; van Houten and Visser, 2014). The series of experiments reported in this section test two questions: Do zebrafish *gsdf* mutants show changes in genes relevant to the pathophysiology of PCOS patients? And, how does the knockdown of *gsdf*, which is expressed only in ovarian follicle cells, alter the physiology of non-gonadal organs, including liver and brain?

We found that the body mass of 8mpf zebrafish *gsdf* mutant females was 32% greater than wild-type siblings and the mass of mutant males was 42% greater mass than wild-type siblings (Fig. 6A–D and M), and that only about half of the excess mass was due to gonad

hypertrophy (Fig. 6N). If increased body mass is related to obesity, then lipid metabolism in mutants might be disturbed in *gsdf* mutants as in PCOS (Macut et al., 2006; Wild et al., 2011). At 8mpf, *gsdf* mutant muscle and hepatopancreas (liver) (Fig. 6E–L, O) (also gonads and intestine, not shown) all accumulated substantially more and larger lipid droplets than these organs in wild types when stained with Oil Red O for neutral lipids. Female *gsdf* mutants had 6.2 times more Oil Red area than wild-type females, and mutant males had 9.6 times more. We used qPCR to examine expression of *fabp10a* and *fabp11a*, which encode fatty acid binding proteins (Her et al., 2003; Furuhashi and Hotamisligil, 2008), and *pparg*, which encodes a transcription factor for adipocyte differentiation and insulin sensitivity (Lowell, 1999; Ferre, 2004). At 8mpf, mutant females had greatly reduced expression of all three genes and mutant males tended to have reduced expression of *fabp10a* (Fig. 7A1). We conclude that lipid metabolism is dysregulated in zebrafish *gsdf* mutant livers, likely a secondary effect because *gsdf* is expressed only in gonads.

To test insulin signaling (Dunaif et al., 2001; Vigouroux, 2010), we examined the hepatopancreas for expression of both zebrafish insulin genes (*ins* and *insb*) (Papasani et al., 2006) and both insulin receptor genes (*insra* and *insrb*) (Irwin, 2004; Toyoshima et al., 2008). Results showed that in females, expression of all four genes was dramatically reduced as in human PCOS (Vigouroux, 2010; Carreau and Baillargeon, 2015), and that in males, expression was statistically reduced for *insb* and *insrb* (Fig. 7A2). We conclude that insulin signaling is altered in *gsdf* mutant zebrafish.

PCOS patients have increased levels of testosterone and decreased titers of estrogen (Laven et al., 2002; Escobar-Morreale et al., 2005; Pellatt et al., 2010). A convenient assay for estrogen activity in egg-laying vertebrates is the synthesis of yolk protein (vitellogenin, Vtg) because estrogen binds to hormone receptors encoded by *esr1* and *esr2a* in the hepatopancreas (Tingaud-Sequeira et al., 2004), stimulating synthesis of Vtg, which is secreted into the blood and sequestered into oocytes (Arukwe and Goksoyr, 2003; Hutchinson et al., 2006; Meng et al., 2010). Expression of *vtg1* and the estrogen receptors *esr1* and *esr2a* was significantly reduced to 3.6%, 2.9%, and 14.9% of wild-type levels in *gsdf* mutant females, but was normal in mutant males (Fig. 7A3). These results are consistent with decreased estrogen signaling from mutant ovaries (Heldring et al., 2007).

We used ELISAs to measure estradiol and the primary fish androgen, 11-ketotestosterone (11KT). Female mutants at 4.5mpf had normal amounts of estradiol compared to WT control (Fig. 8A), consistent with their ability to ovulate. In contrast, when fish became older, for example at 11mpf and 20mpf, females had significantly less estradiol than controls (Fig. 8A). Estradiol levels in mutant males did not appear to differ from normal (Fig. 8A). Levels of 11KT in *gsdf* mutant females at 4.5mpf and 20mpf were significantly higher than in wild-type females of the same age, and overall, disregarding age, genotype was highly significant for elevated 11KT in mutant females ($p=0.005$) (Fig. 8B), mimicking high testosterone in PCOS patients. Male *gsdf* mutants also tended to have higher levels of 11KT, as expected from their larger testes, but differences were significant only at 4.5mpf (Fig. 8C).

Mutant ovaries at 8mpf had a significant overall reduction in expression of many steroid production genes including *star*, *cyp11a1, cyp17a1, cyp17a2, cyp19a1a, hsd17b1, hsd17b2, hsd17b3* (Fig. 7B1). Expression of *hsd17b3*, which encodes the enzyme that converts androstenedione to testosterone, was reduced to 9% of normal and *cyp19a1a*, which encodes aromatase, the enzyme that converts testosterone to estrogen, was reduced to about 6% of normal in mutant ovaries (Fig. 7B1 and Fig. 5D, D′). We conclude that in 8mpf mutant females, enzymes that provide estrogen are dramatically reduced, low enough to greatly diminish Vtg production (Fig. 7A3) but not so low that *gsdf* mutants switched sex to become males like *nanos3* and *bmp15* mutants do (Dranow et al., 2013).

Regarding hormone receptors, *gsdf* mutant ovaries down-regulated estrogen receptor genes (Liu et al., 2011; She and Yang, 2014) *esr1* to 10% and *esr2a* to 25% of normal (Fig. 7B2). The *lhcgr* and *fshr* genes (encoding receptors for brain-derived hormones LH and FSH) were both significantly reduced in mutant ovaries (Fig. 7B2). In zebrafish ovaries, *fhsr* and *lhcgr* both increase dramatically when follicles enter vitellogenesis (Kwok et al., 2005). Because *gsdf* mutant ovaries are deficient in vitellogenic stages, follicle cell development must derail in *gsdf* mutants before the stage appropriate for gonadotropin receptor expression.

Among TGFB pathway genes, *gsdf* expression decreased to about 10% of normal in *gsdf* mutant ovaries (Fig. 7B3), either as a result of nonsense-mediated transcript decay (Lykke-Andersen and Jensen, 2015) or failure to maintain *gsdf*-producing cells. Expression of *bmp15* diminished a small but significant amount, and *gdf9* expression was unchanged in *gsdf* mutant ovaries (Fig. 7B3). Mammalian testes secrete Amh, which eliminates female sex ducts, and mammalian ovaries express Amh in secondary, preantral, and small antral follicles, which retards follicular atresia (Josso, 1971; Merhi et al., 2013; Seifer and Merhi, 2014). In zebrafish *gsdf* mutant ovaries, *amh* expression weakened significantly to about a third of normal (Fig. 7B3). Zebrafish granulosa cells express *amh* in Stage-II follicles but not in younger (late stage-IB) or older (early or late stage-III) follicles (Rodriguez-Mari et al., 2005). Thus, although *gsdf* mutants accumulate stage-II follicles, both qPCR (Fig. 7B3) and *in situ* hybridization experiments (Fig. 5B, B′) confirm that granulosa cells do not express *amh* normally, showing that Gsdf positively regulates Amh production in zebrafish ovaries. In many species, Amh acts by binding to the Amhr2 receptor (Morinaga et al. 2007), but zebrafish lacks an *amhr2* gene (Kamiya et al., 2012). Like zebrafish *gsdf* mutants, however, medaka *amhr2* mutants fail to mature late stage oocytes and presumably have greatly reduced Amh signaling, which would mimic the low levels of *amh* transcript in zebrafish *gsdf* mutants (Fig. 7B3). In addition, however, half of medaka *amhr2* mutants that have an XX sex chromosome constitution reverse their sex phenotype and become males (Morinaga et al. 2007); we did not observe sex reversal in zebrafish *gsdf* mutants. Ovaries in *gsdf* mutants expressed 1.7 and 3.4 times as much of the inhibin genes *inhbaa* and *inhbb* as normal (Fig. 7B3), consistent with their high level of expression in normal early stage follicles (Poon et al., 2009), and about a tenth as much *inha* (Fig. 7B3), which is expressed strongly only in full grown follicles (Poon et al., 2009), consistent with accumulation of early follicles and depletion of late-stage follicles.

Smad1 is an intracellular mediator of some TGFB family proteins in mammalian granulosa cells (Massague and Wotton, 2000; Pangas et al., 2008; Richards and Pangas, 2010). In

zebrafish *gsdf* mutant ovaries, *smad1* expression was about 35% of normal (Fig. 7B3), as expected from perturbed TGFB signaling.

In mammals, *Gata4* is expressed in the bipotential genital ridge, continues in Sertoli cells, but down-regulates in granulosa cells (Viger et al., 1998). In zebrafish *gsdf* mutant ovaries, *gata4* further down-regulated to 8% of normal (Fig. 7B4, Fig. 5E, E′). *Sox9* also acts in the mammalian male pathway (Koopman, 1999). Zebrafish express *sox9a* strongly in testes (Chiang et al., 2001a; Yan et al., 2005) and weaker in ovaries. Expression of *sox9a* in *gsdf* mutant ovaries seemed lower, but was not significantly different from, that in wild-type siblings.

Nr0b1(*Dax1*) in mammals acts as a dominant-negative anti-testis gene (Swain and Lovell-Badge, 1999; Meeks et al., 2003) and *Foxl2* represses male differentiation (Ottolenghi et al., 2007). Both genes were strongly down-regulated in *gsdf* mutant ovaries, suggesting that *gsdf* promotes development of ovarian support cells in zebrafish (Fig. 7B4).

Wnt4 is a female-promoting signal expressed by granulosa cells in mammals (Rastetter et al., 2014) and is essential for ovary development in zebrafish (High, 2016). Wnt4 can act by altering the cytoplasmic stability of beta-catenin protein (Bernard et al., 2008), and our studies showed that *ctnnb1* transcript was significantly down-regulated in *gsdf* mutant ovaries (Fig. 7B3).

Tp53 is a transcription factor that induces cell death (Haupt et al., 2003) and Casp3 is a death protease (Boatright and Salvesen, 2003; Kumar, 2007); both were slightly, though significantly reduced in *gsdf* mutant ovaries (Fig. 7B5), suggesting that the enormous accumulation of early stage follicles in *gsdf* mutant ovaries at 8mpf is not associated with enormous amounts of follicle death. Expression of *pcna* (proliferating cell nuclear antigen, a marker of cell proliferation (Korfsmeier, 2002; Leung et al., 2005), showed no statistical change in *gsdf* mutant ovaries (Fig. 7B5), suggesting that ovarian follicles originate at a normal rate in mutants and accumulate simply because they do not disappear by either extensive death or ovulation.

Most steroid biosynthesis genes (*star*, *cyp11c1, cyp17a1, cyp17a2, cyp19a1a, cyp21a2, hsd3b1, hsd17b1, hsd17b2, hsd17b3*) were expressed normally in *gsdf* mutant males (Fig. 7C1). Cyp11a1 is the first, and rate-limiting enzyme in sex steroid biosynthesis; its increased expression in *gsdf* mutant males (Fig. 7C1) suggests a greater capacity for testosterone synthesis in the larger testes of mutants compared to wild types.

Among several hormone receptor genes (*esr1, esr2, ar, lhcgr*, and *fshr*), only *fshr* tended to be upregulated ($p= 0.072$), about twice normal, in *gsdf* mutant testes (Fig. 7C2). This result may reflect the role of Fsh in stimulating spermatogenesis through Fshr on Sertoli cells, which stimulates germ cell proliferation and androgen synthesis (Dierich et al., 1998; O'Shaughnessy et al., 2010).

Among TGFB pathway genes, expression of *gsdf* was 18% of normal in mutant testes and *bmp15* and *gdf9* were unchanged, but *amh* expression was significantly up-regulated nearly two-fold (177%) (Fig. 7C3, Fig. 5L, L′). As in mutant ovaries, *inhbb* was upregulated in

gsdf mutant testes. Consistent with normal differentiation of fertile sperm in *gsdf* mutants, neither of the developmental signaling pathways we checked (*smad1* in the TGFB pathway, and *ctnnb1* in the Wnt pathway) had altered expression levels in mutant testes (Fig. 7C3).

None of the four genes we checked that encode sex-related DNA-binding proteins (*gata4, nr0b1, foxl2b, sox9a*) had significantly altered gene expression in mutant testes (Fig. 7C4).

Expression of *p53* trended upward (p=0.0502) and *casp3a* was significantly up-regulated in mutant testes (Fig. 7C5), suggesting that, while *gsdf* mutants are fertile, cell death may be increased in some testis lobules (Fig. 4U, X). In addition, *pcna* expression was significantly higher in *gsdf* mutant testes (Fig. 7C5), suggesting that increased testis size might be mediated by increased cell proliferation. Expression of *vasa* increased 129% in *gsdf* mutant testis compared to wild-type testes, which is likely related to the enlarged testes in *gsdf* mutants (Fig. 7C5).

The brain, regulated by feedback from gonads, makes hormones that control gonadal functions. Among brain-expressed steroid biosynthesis genes, neither *star* nor brain aromatase (*cyp19a1b*) (Chiang et al., 2001b) were altered in expression in female or male *gsdf* mutants (Fig. 7D1). In contrast, expression of *hsd17b1*, which encodes the enzyme that converts estrone to estradiol, was greatly diminished in *gsdf* female brains, but was not changed in male brains (Fig. 7D1). This result reflects reduced *hsd17b1* expression in mutant ovaries (Fig. 7B1) and decreased *vtg* expression in livers of female *gsdf* mutants (Fig. 6A3).

Gonadotropin releasing hormone (Gnrh2) (Bhattacharya et al., 2011; Biran et al., 2012) is secreted by the hypothalamus, binds to Gnrh receptor (encoded in zebrafish by *gnrhr1* and *gnrhr4*, which are expressed in brain, ovary, and testis (Tello et al., 2008)), and stimulates the pituitary to release LH and FSH, which induce estrogen synthesis in ovaries and testosterone synthesis in testes (Kenealy et al., 2013). Expression of neither *gnrh2* nor *gnrhr1* changed significantly in *gsdf* mutants, but expression of *gnrhr4* increased 3.3-fold in mutant females and decreased to about half of wild-type levels in mutant males (Fig. 7D2). Despite increased *gnrhr4* expression in female mutant brains, expression of *lhb* tended to be lower in female brains (p=0.072), and the lower level of *gnrhr4* expression in male mutant brains did not result in diminished *lhb* expression (Fig. 7D3).

In mammals, Pomc polypeptide is cleaved to make endogenous opioids and peptides that regulate appetite, sexual behavior, and secretion of the stress hormone cortisol from the adrenal cortex. Because *pomca* expression in female or male *gsdf* mutant brains was not statistically different from wild types, loss of *gsdf* activity does not likely result in serious physiological stress.

In brief summary, although *gsdf* is expressed only in gonadal cells, the impact of its loss was apparent throughout the fish, including effects on the liver, brain, and on fat and insulin biology that mimic the effects of PCOS. Because mammals do not have an ortholog of *gsdf* (Sawatari et al., 2007), for connectivity, it is important to understand the evolutionary relationships of *gsdf* to mammalian TGFB genes.

Origin of *gsdf*

Because phylogenetic analyses of TGFB family genes disagree on the affinities of *gsdf* (Sawatari et al., 2007; Shibata et al., 2010; Gautier et al., 2011a; Myosho et al., 2012; Forconi et al., 2013; Horiguchi et al., 2013; Kaneko et al., 2015), we analyzed conserved syntenies to learn which tetrapod TGFB genes are most closely related to those of fishes. Results showed that zebrafish *gsdf* shares conserved syntenies with *gsdf* of spotted gar (Fig. 9A1, A2), whose lineage diverged from that of teleosts before the teleost genome duplication (TGD) (Hoegg et al., 2004; Crow et al., 2006; Amores et al., 2011; Braasch et al., 2016). Coelacanth also has *gsdf* (Gautier et al., 2011a; Forconi et al., 2013), providing evidence that *Gsdf* was present in the last common ancestor of human and zebrafish but was lost from the tetrapod lineage after it diverged from coelacanth.

To clarify the mechanism of *gsdf* loss, we compared gar and human genomes. Results revealed a chromosome rearrangement breakpoint at the location expected for a human ortholog of *gsdf* (Fig. 9B3, B4), as predicted if a chromosome break destroyed *gsdf* after the divergence of coelacanth and tetrapod lineages. Analysis of conserved syntenies showed that the region of gar chromosome 2 (Loc2) surrounding *gsdf* is paralogous to the region of Loc6 surrounding *gdf9*, with paralogs *ccng2/ccng1, ccni/ccni2, wowahb/sowaha, shroom3/shroom1* and *aff1/aff4* in addition to *gsdf/gdf9* (Fig. 9B2, B3). In addition, the gar region around *gdf9* shares almost exact conserved syntenies with the human *GDF9* region (Fig. 9B1, B2). We performed sequence similarity searches of the Elephant shark genome (Venkatesh et al., 2014) and identified the previously unannotated shark sequence LOC103190318, which shares reciprocal best BLAST similarity to zebrafish Gsdf, as expected for orthologs. This result shows that the last common ancestor of all gnathostomes had a copy of *gsdf*. Our results also showed that a chromosome break in the tetrapod lineage occupies the ancestral position of *gsdf;* and that *gsdf* and *gdf9* occupy paralogous segments and hence are likely paralogs from genome duplication events that preceded the vertebrate radiation (Holland, 1999; Dehal and Boore, 2005).

In phylogenies, GDF9 and BMP15 generally group as sister clades variably related to *gsdf* (Sawatari et al., 2007; Shibata et al., 2010; Gautier et al., 2011a; Myosho et al., 2012; Forconi et al., 2013; Horiguchi et al., 2013; Kaneko et al., 2015). Our comparative genomic investigations found that *GDF9*, *BMP15*, and *gsdf*, are all near *Aff* family genes, which occupy paralogous chromosome segments in the human genome (Fig. 9C). These data are as predicted by the hypothesis that *GSDF, GDF9*, and *BMP15* arose as paralogs in the vertebrate genome duplication events; the fourth ohnolog disappeared, but should have been located near *AFF3* on the ancestor of Hsa2 (Fig. 9C). According to the principle of subfunctionalization (Force et al., 1999), these considerations suggest that *gsdf*, *gdf9*, and *bmp15* are likely to share some ancestral gene subfunctions.

DISCUSSION

Gsdf is not a sex determination gene in zebrafish

Gsdf is a male sex determinant in several species of fish. In *O. luzonensis* and sablefish, sex-specific *gsdf* variants are present in genetic males and females (Myosho et al., 2012;

Rondeau et al., 2013), and in Nile tilapia, chromosomally XX fish that are homozygous *gsdf* mutants reverse their sex to develop as males (Jiang et al. 2016). In addition, in two fish (three-spot wrasse and rice field eel) that are sequential protogynous hermaphrodites, *gsdf* expression is up-regulated as ovaries transform to testes (Horiguchi et al. 2013; Zhu et al. 2016). Although *gsdf* is not the primary genetic sex determinant in *O. latipes* (Japanese medaka), forced expression of *gsdf* on an autosome causes XX females to become males (Zhang et al., 2016) and *gsdf* knockout causes XY *O. latipes* to become phenotypic females, demonstrating that *gsdf* is strongly male determining in this species (Imai et al., 2015; Zhang et al., 2016). In contrast to *Oryzias* and several other spiny-rayed fish (Acanthopterygii), we conclude that in zebrafish, *gsdf* is not a strong sex determinant because *gsdf* mutants are about as likely to become males or females as their wild-type siblings. It is a bit surprising that the function of *gsdf* in spiny-rayed fish and zebrafish are so different. These differences might be due to lineage-specific divergence of gene functions in the 290 million years since the spiny-ray fish and zebrafish lineages separated (Steinke et al., 2006). Without knowing the function of *gsdf* in the last common ancestor of zebrafish and spiny-rayed fish, it is not possible to know whether the lack of a strong male-determining role for *gsdf* in zebrafish is due to the loss of that role in the zebrafish lineage or the gain of that role in the spiny-rayed fish. In addition, even in zebrafish, individuals lacking *gsdf* activity attained their definitive sexual phenotype later than wild-type controls (Fig. 4A–H), showing that *gsdf* retains a weak male-determining function even in laboratory strains of zebrafish. Because *gsdf* is not located near any of the sex-linked loci found for a variety of zebrafish strains (Bradley et al., 2011; Anderson et al., 2012; Liew et al., 2012; Howe et al., 2013; Wilson et al., 2014), we conclude that *gsdf* does not play a direct role in zebrafish sex determination.

The role of Gsdf in zebrafish females

Although *gsdf* is not a strong sex determinant in zebrafish, it is nevertheless expressed early in the sex determination process. Somatic cells in zebrafish bipotential gonads already express *gsdf* in the gonadal soma by 8dpf, long before sex differentiation becomes apparent at about 21dpf (Takahashi, 1977; Uchida et al., 2002; Wang et al., 2007; Siegfried and Nusslein-Volhard, 2008; Orban et al., 2009; Rodriguez-Mari and Postlethwait, 2011; Tzung et al., 2015). This result suggests that Gsdf plays an early role in supporting cells of the somatic gonad.

Adult zebrafish express *gsdf* in Sertoli cells and granulosa cells (Gautier et al., 2011b) and our results confirmed that in juveniles, granulosa cells express *gsdf* only in pre-vitellogenic follicles. In contrast, female medaka stop expressing *gsdf* after 6dpf and male medaka up-regulate *gsdf* expression in cells that express *dmrt1by*, the male sex determinant (Shibata et al., 2010). Differences in *gsdf* expression in zebrafish and medaka suggest different functions.

In zebrafish, *gsdf* mutant females are briefly fertile, then become sterile as they accumulate non-vitellogenic follicles. qPCR showed that *vtg* expression in 8mpf *gsdf* mutant females was reduced to 4% of normal, so even if ovarian follicles in *gsdf* mutant females could sequester yolk, vitellogenesis would be greatly impaired. Estrogen induces *vtg* expression in

the liver of zebrafish, other teleosts, and yolk-producing tetrapods (Wallace, 1985; Andersen et al., 2003; Tong et al., 2004; Reyhanian Caspillo et al., 2014). Our finding that the liver in zebrafish *gsdf* mutant females produces little *vtg* transcript would be expected if estrogen is low. Estrogen comes from testosterone that is converted to estrogen by aromatase in theca and granulosa cells (Nakamura et al., 2009; Dranow et al., 2016). These considerations suggest that normal Gsdf activity in granulosa cells is necessary for the maturation of theca and/or granulosa cells to the estrogen-producing stage, and with little estrogen, the liver produces little Vtg, leading to lack of yolky oocytes. Evidence that *gsdf* mutant ovaries have defective theca cells comes from our observation that expression of virtually all steroid-synthesizing enzyme genes is greatly reduced, and that ELISA assays detect reduced estradiol in *gsdf* mutant females.

Gsdf is required for normal granulosa cell maturation in zebrafish, according to our *in situ* hybridization and qPCR experiments that showed that granulosa cells in *gsdf* mutant females failed to express normal levels of *amh, cyp19a1a, gata4*, and *foxl2b*. Similarly, in mice lacking activity of the Gsdf paralog Gdf9, follicular development arrests at the primary follicle stage (Dong et al., 1996). The similarity of the follicle maturation defect in knockout mutations of *gsdf* in zebrafish and *Gdf9* in mouse supports the notion that the original function of the progenitor of this gene family was to coordinate development of oocytes and support cells.

The failure of granulosa cells to mature in *gsdf* mutants could be due either to an autocrine mechanism that normally regulates interactions between granulosa cells, or a paracrine mechanism that normally regulates signalling between granulosa cells and oocytes or between granulosa cells and theca cells. Depressed expression of the TGFB signalling gene *smad1* in *gsdf* mutant ovaries is consistent with either possibility.

In addition to the truncated maturation of granulosa cells and the subsequent reduction of estrogen, *gsdf* mutant ovaries abnormally accumulate hundreds of young ovarian follicles. Gonadotropin signaling from the brain regulates follicle recruitment and the brains of zebrafish *gsdf* mutants express several times more Gnrhr4 transcript than normal, but significantly less transcript for LH than normal. This result is consistent with dysregulation of feedback loops for oocyte maturation in zebrafish *gsdf* mutants, likely due to disrupted estrogen signaling.

The role of Gsdf in zebrafish males

Zebrafish males lacking *gsdf* activity were fertile with enlarged testes but nearly normal expression of almost all steroid biosynthesis genes tested. In addition, *gsdf* mutant males over-expressed *fshr*, *amh*, *inhbb, tp53, casp3a, pcna*, and *vasa*; this result is likely related to the enlarged testis phenotype.

Gsdf as a model for PCOS

The Rotterdam criteria for PCOS diagnosis rely on at least two of the following three features: infrequent or irregular ovulation, the accumulation of numerous immature antral follicles, and hyperandrogenism (Rotterdam, 2004b; Wei et al., 2014). PCOS usually also involves disrupted insulin profiles, altered gonadotrophin signaling (Diamanti-Kandarakis,

2008), and high levels of AMH (Pigny et al., 2003; Diamanti-Kandarakis, 2008). The phenotype of zebrafish *gsdf* mutant females mimics most of these criteria, and differ mainly in features related to differences in reproductive strategies of mammals and zebrafish.

People with PCOS experience oligo-ovulation or anovulation. In contrast to humans, where generally one oocyte matures each month, mature zebrafish continue to produce oocytes nearly daily. We found that zebrafish *gsdf* mutants produce and naturally ovulate when young, but then cease ovulation, mimicking the human oligo-ovulation phenotype.

The second key feature in PCOS is the accumulation of premature ovarian follicles that form ovarian cysts. Zebrafish oocytes do not form cysts, but this feature is related to the mammalian-specific relationship of follicle cells to oocytes with respect to mammalian ovulation rather than basic conserved features of vertebrate follicle development, including the absence of yolk sequestration in eutherian mammals. The accumulation of premature follicles in both syndromes suggests that in humans, proteins related to Gsdf may play a role in ovarian symptoms of PCOS. Candidates for the PCOS-relevant TGFB-family gene in humans include the two *gsdf* paralogs from the vertebrate genome duplications, *BMP15, GDF9*, and in addition, *INHB, INHA*, and *AMH*, all of which have altered expression in human PCOS patients (Eldar-Geva et al., 2001; Fleming et al., 2005; Glister et al., 2005; Welt et al., 2005; Kevenaar et al., 2008; Zhao et al., 2010; Wei et al., 2013; Wei et al., 2014), and all of which (except *gdf9*) are also mis-expressed in zebrafish *gsdf* mutant females.

Although human PCOS patients and zebrafish *gsdf* mutant females both accumulate follicles that fail to mature, the impeded stage of folliculogenesis appears to differ between the two species. In zebrafish, *amh* expression begins in Stage-II follicles, down-regulates in Stage-III, then disappears, and reciprocally, *cyp19a1a* expression up-regulates at Stage-III (Rodriguez-Mari et al., 2005; von Hofsten et al., 2005). Human oogenesis follows the same pattern, as primordial and primary follicles show little *AMH* expression, but secondary and small antral follicles express *AMH* strongly, followed by down-regulation in larger antral follicles (Weenen et al., 2004). While the developmental time course of *amh/AMH* expression is similar in zebrafish and human follicle development, zebrafish *gsdf* mutants had low levels of *amh* transcript in granulosa cells, but human PCOS patients have high levels of *AMH* expression (Durlinger et al., 2002; Pigny et al., 2003; Diamanti-Kandarakis, 2008; Pellatt et al., 2010; Seifer and Merhi, 2014), suggesting that follicle cell development arrests later in PCOS than in zebrafish *gsdf* mutants.

The third defining feature of PCOS is production of excess androgens (Legro et al., 1998; Nelson et al., 1999; Diamanti-Kandarakis, 2008). Likewise, our direct hormone assays showed that zebrafish *gsdf* mutants have low estrogen levels and high androgen levels. This key feature unites the zebrafish and human phenotypes, and may help explain the extra-gonadal phenotypes of PCOS and zebrafish *gsdf* mutants.

PCOS pathophysiology involves phenotypes outside the ovaries. Women with PCOS are often obese (van Houten and Visser, 2014). Accordingly, we found that zebrafish *gsdf* mutants were substantially heavier than normal and accumulated more lipid in their muscles, hepatopancreas, and other tissues. PCOS patients are also insulin resistant with low levels of

insulin receptor (Diamanti-Kandarakis and Dunaif, 2012; van Houten and Visser, 2014; Dehghan et al., 2016). Likewise, we found that the liver in zebrafish *gsdf* mutants, especially females, had greatly reduced expression of genes encoding insulin receptor, which is a risk locus for PCOS in GWASs (Shi et al., 2012) and when mutated, causes insulin resistance in humans (Taylor et al., 1990). In addition, zebrafish *gsdf* mutants have greatly reduced expression of *pparg*, which encodes a nuclear receptor for adipocyte differentiation and diabetes, and highly suppressed expression of at least two fatty acid binding protein genes, especially in females. Because *gsdf* is expressed only in gonads, these extra-gonadal phenotypes in PCOS and in zebrafish *gsdf* mutants must arise secondarily due to altered gonadal signaling, either from altered levels of steroids, altered gonadotropins, abnormal levels of circulating Gsdf, or dysregulation of secreted proteins downstream of Gsdf signaling.

The striking phenotypic parallels of zebrafish *gsdf* mutants and human PCOS patients suggest mechanistically related etiologies. Although humans lack an ortholog of *gsdf*, the last common ancestor of humans and zebrafish had a *gsdf* gene (Sawatari et al., 2007; Forconi et al., 2013) and here we show that the ancestor of all jawed vertebrates had a *gsdf* ortholog. In addition, our comparative genomics showed that *gsdf, gdf9*, and *bmp15* all originated as paralogs (ohnologs) in the second vertebrate genome duplication event, the fourth paralog now missing from all surviving jawed vertebrates. Evolutionary principles (Force et al., 1999) suggest that the pre-duplication ortholog of these three genes would have had some functions that are now distributed among *gsdf, gdf9*, and *bmp15*. Expression of all three genes to varying degrees in oocytes and follicle cells in fish and of *Bmp15* and *Gdf9* in mammals supports this conclusion (Sidis et al., 1998; Duffy, 2003; Silva et al., 2005; Clelland et al., 2006; Liu and Ge, 2007). In addition, Gdf9 and Bmp15 retain partially overlapping functions because the phenotype of homozygous *Bmp15* mutant mice is stronger with one rather than two functional *Gdf9* alleles (Yan et al., 2001; Su et al., 2004). Likewise, sheep with large litters are heterozygous for mutations in BMP15 or GDF9, but homozygous single and double mutants are sterile like *Gdf9* mutant mice (Galloway et al., 2000; Hanrahan et al., 2004). Experiments also show that the GDF9:BMP15 heterodimer is far more potent than either homodimer in stimulating cumulus expansion (Peng et al., 2013). Finally, supporting this hypothesis is the decreased levels of GDF9 and BMP15 in PCOS (Goodarzi et al., 2011; McAllister et al., 2015).

PCOS has a strong familial component (Legro et al., 1998), but genome-wide association studies (GWASs) have not identified *GDF9, BMP15*, or other TGFB-pathway genes as significant candidates (Kevenaar et al., 2008; Chen et al., 2011; Shi et al., 2012). GWASs, however, did associate PCOS with variants in Fibrillin-3, an extracellular matrix protein belonging to a small protein family that can bind TGFB-pathway proteins and regulate their signaling (Urbanek et al., 1999; Urbanek et al., 2005; Stewart et al., 2006; Jordan et al., 2010; Raja-Khan et al., 2014). This consideration raises the hypothesis that in human PCOS, altered signaling by a Gsdf paralog might contribute to the PCOS phenotype via protein-protein interactions with genetic variations in Fibrillin-3 structure and function.

PCOS patients with larger ovaries have more severe phenotypes (Legro et al., 2005), and surgical reduction of ovary size can lead to successful reproduction (Stein and Leventhal,

1935; Donesky and Adashi, 1995). This result would be expected if a diffusible factor secreted by immature follicles contributed to the phenotype and could help explain the extra-ovarian phenotypes shared by PCOS patients and zebrafish *gsdf* mutants. Candidates for the deleterious factor include Gsdf-related molecules, perhaps especially Gdf9 and/or Bmp15, or a steroid that depends on properly functioning theca and granulosa cells, especially relative levels of estrogen and testosterone.

Conclusions

PCOS is defined by polycystic ovaries, ovulation failure, and hyperandrogenism often associated with obesity, infertility, and insulin resistance (Rotterdam, 2004a; van Houten and Visser, 2014). Zebrafish *gsdf* mutants show many of these phenotypes, including gradual loss of ovulation, accumulation of immature follicles, hyperandrogenism, obesity, and dysregulation of insulin-related genes, but differ in aspects related to lineage-specific reproductive physiology. In zebrafish *gsdf* mutants, follicles arrest before granulosa cells begin to express large quantities of *amh*, whereas PCOS patients accumulate follicles at a later stage that expresses substantial levels of *AMH*. In contrast to PCOS, where aromatase is the most strongly reduced steroidogenic gene and *CYP17, CYP11A*, and *HSD3B* are up-regulated (Tamura et al., 1993), zebrafish *gsdf* mutant females depress not only aromatase, but most other sex-steroid biosynthesis genes as well. As in PCOS, zebrafish *gsdf* mutants are obese, accumulate fat, abnormally express lipid genes, and lose fertility, and although we did not experimentally check insulin resistance, the great depression of insulin receptor gene expression suggests that insulin resistance would be part of the zebrafish phenotype. Our results suggest that zebrafish *gsdf* mutants could serve as a model for human PCOS and that supplying a related TGFB molecule to human granulosa cells might provide a therapy for major aspects of the disease.

EXPERIMENTAL PROCEDURES

TALEN-mutagenesis generated *gsdf* mutations based on zebrafish *gsdf* sequence (ENSDARG00000075301, http://ensembl.org) verified by sequencing the target site in AB-strain fish. TALEN sites were designed by “TAL Effector Nucleotide Targeter 2.0” (Doyle et al., 2012) to target the first exon. The TALEN sequence is TCGTCCTGCTGCTGCTGGCcttccctttgggggagatGTTTGTGCTCCATCCGTCA (LEFT TALEN ARM–cleavage site—RIGHT TALEN ARM). A BslI restriction enzyme recognition site in the target identified wild-type alleles. TALEN arms were built using the Golden Gate method (Sanjana et al., 2012). Constructs were linearized with *Sma*I and TALEN mRNAs were synthesized with T7 mMESSAGE mMACHINE Kit (Ambion, Naugatuck, CT, USA). TALEN assembly vectors were from Addgene (Cambridge, MA, USA). 250 pl TALEN mRNA for each arm were co-microinjected into one-cell zebrafish embryos. Genomic DNA extracted from injected embryos at 24 hours post-fertilization provided template to amplify a 251bp PCR fragment including the target using primers: (F): GCCAAGCCTGGCCAGCGTAGATAA and (R1): ACCCACACGATGAACACCTGAGGC. For cDNA sequencing from *gsdf* and WT sibling, primer F above and R2 (CAGCTGGGACAGGGAGTTGCTCGG) were used for PCR to amplify a 275 bp fragment (Fig. 2A, D). Oligonucleotides were purchased from Integrated DNA Technologies (IDT)

Coralville, Iowa USA). Sequencing the 251 bp DNA genomic PCR fragment and the 275 bp cDNA fragment was performed by Sanger sequencing (GENEWIZ, Inc. NJ, USA). We established stable lines for a 1-nucleotide deletion, an 8-nucleotide deletion, and a 14-nucleotide deletion (see Fig. 2) and showed that these alleles failed to complement. To verify the absence of Gsdf, we purchased the synthesis of one anti-gsdf antibody from Alpha Diagnostic Intl. Inc. (ADI) and two anti-gsdf antibodies from Genscript and performed Western blots and histology with all three antibodies under various conditions. None of these antibodies actually recognized Gsdf. Work was performed under IACUC protocol #14-08R.

Histology and *in situ* hybridization

In situ hybridization was performed as described (Rodriguez-Mari et al., 2005). Probes were: a 328 bp *gsdf* fragment including part of exon-5 and the 3′UTR, amplified by primers: F-GACACACTCGACCCCGCAGC and R-CTGCCAGAGCCAAACCCGCA), *amh* (ENSDARG00000014357) (Rodriguez-Mari et al., 2005), *bmp15* (ENSDARG00000037491) (Dranow et al., 2016), *cyp19a1a* (ENSDARG00000041348) (Chiang et al., 2001b), *gata4* (ENSDARG00000098952) using a 763 bp fragment including exon-1 to exon-6 amplified by primers: F-AGCACCGGGCACCATCATTCTCCG and R-GAGCTGGAGGATCCGCTTGGAGGC), *gdf9* (ENSDARG00000003229) using a 979 bp fragment including most of the coding region amplified by primers: F-TGTTGAACCCGACGTGCCCC and R-TGGTGTGCATTGGCGACCCG, *sox9a* (ENSDARG00000003293) (Chiang et al., 2001a), *sox9b* (ENSDARG00000043923) (Chiang et al., 2001a), *sycp3* (ENSDARG00000013438) (Rodriguez-Mari and Postlethwait, 2011) *and vasa* (ENSDARG00000014373) (Yoon et al., 1997). Two color in situ hybridization was as described (Yan et al., 2011). Gonad histology used paraffin-embedded Bouin's fixed tissue sectioned at 10μm stained with hematoxylin and eosin (H&E).

Oil red O staining

Adult fish trunks were embedded in OCT, frozen in liquid nitrogen, cut into 10 μm-thick cryosections, and stained with Oil Red O (Mehlem et al., 2013). Images were quantified from 1–7 images per tissue per individual, converted to 8-bit grayscale in ImageJ, analyzed with the Threshold tool to select Oil Red O stain, and quantified with the Measure tool. Significant differences were identified by the non-parametric Wilcoxon rank sum test under the superiority or inferiority alternatives hypothesis using R v.3.1.2.

qPCR

RNAs were extracted and cDNAs were synthesized as described (Desvignes et al., 2014). Briefly, total RNA was isolated from 3–5 fish using Tri-Reagent® (Molecular Research Center, Cincinnati, OH, USA). Contaminating DNA was removed using the DNA-free RNA kit (Zymo Research, Irvine, CA, USA), and reverse transcription (RT) was performed using 1 μg of RNA for each sample with SuperScript III reverse transcriptase and OligoDT_{20} primers (Thermo Fisher Scientific, Danvers, MA, USA). Control reactions were run without reverse transcriptase. cDNAs were treated with RNaseH before PCR. qPCR was performed using a StepOnePlus q-PCR machine (Applied Biosystems, Waltham, MA, USA) as described (Desvignes et al., 2011). Products of reverse transcription, including control reactions, were diluted 1/25, and a 2 μl aliquot was used for each assay. All qPCR reactions

were performed in triplicate using a real-time PCR kit with Fast-SYBR® Green fluorophore (Kappa Biosystems (Pty) Ltd. Wilmington, MA, USA) and 200 nM of each primer. To avoid genomic DNA, primers occupied exon junctions. Supplementary Table 1 lists primer sequences. The relative abundance of target cDNA within sample sets was calculated from serially diluted cDNA pools (a 7-point standard curve with 1:2 dilution steps) using Applied Biosystem StepOne™ V.2.0 software. Primer concentrations and reaction temperatures were adjusted to obtain efficiencies between 97.5 and 102.5%. A fusion curve validated amplification of each PCR product and verified single transcripts. Control reactions informed background expression and identified levels significantly above background at $p<0.05$ and within the range of the standard curve. Beta-actin *actb1* (ENSDARG00000037746) provided normalization. Significant differences were tested using the non-parametric Wilcoxon rank sum test under the superiority or inferiority alternatives hypothesis using R v.3.1.2.

Hormone assays

Sex steroids were extracted from zebrafish homogenates as described (Newman et al., 2008). Briefly, individual fish were homogenized and aliquots transferred to glass test tubes. Water and HPLC-grade methanol were added and tissues homogenized and sonicated. Tubes were shaken at 500 rpm for 1 h at room temperature and stored overnight at 4C. Tubes were shaken and centrifuged at 1500 g for 15 minutes at 4C. Supernatant (1 mL) was combined with 10 mL water and extracted by solid phase extraction. Eluates were dried under N_2 gas at 38C and stored at 20C. One day prior to assay, samples were resuspended with assay buffer and shaken at 500 rpm for 1h at room temperature. After storage at 4C overnight, samples were shaken and assayed. Estradiol and 11-ketotestosterone were measured using commercially available ELISA (Cayman Chemical, Ann Arbor, MI). Kits were validated for zebrafish using tests of parallelism and standard addition. Intra- and inter-assay variation for E2 were 5.9% and 13.9% respectively and for 11-KT were 5.5% and 11.4%, respectively. Hormone concentrations are expressed as pg/g (pg hormone/g whole body homogenate). Significant differences were identified using the non-parametric Wilcoxon rank sum test under the superiority or inferiority alternatives hypothesis using R v.3.1.2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Research reported in this publication was supported by the National Institute of General Medical Sciences of the National Institutes of Health under Award Number R01GM085318 and by the National Institute of Child Health and Human Development Award Numbers P01HD22486 (JHP) and 1R01HD081551 (BD). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. We thank Jeremy Wegner for helping with TALEN assembly, Sam Peterson for help with initial qPCR analysis, and Trevor Enright for animal care.

Grant Sponsors: NIH grants: R01GM085318 (JHP) P01HD22486 (J. Eisen, and JHP). 1R01HD081551 (BD)

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Bullet points

- In zebrafish, the TGFB factor *gsdf* is expressed in gonadal somatic cells before the time of sex determination, and expression continues in granulosa and Sertoli cells.
- Zebrafish *gsdf* mutant females ovulate only a few oocytes, then become sterile as they accumulate hundreds of immature follicles.
- Mutant fish achieve greater mass and accumulate more lipid than wild types.
- Female mutants down-regulate genes for granulosa cells, lipid and insulin pathways, brain-derived reproductive hormones, and up-regulate androgen.
- Male *gsdf* mutants have normal fertility.
- Gsdf, Gdf9, and Bmp15 arose as paralogs in the vertebrate genome duplication events.
- Zebrafish *gsdf* mutants mimic the ovulation failure, young oocyte accumulation, hyperandrogenism, obesity, and insulin-related phenotypes of human polycystic ovarian syndrome (PCOS).
- Zebrafish *gsdf* mutants provide a model for human PCOS disease.

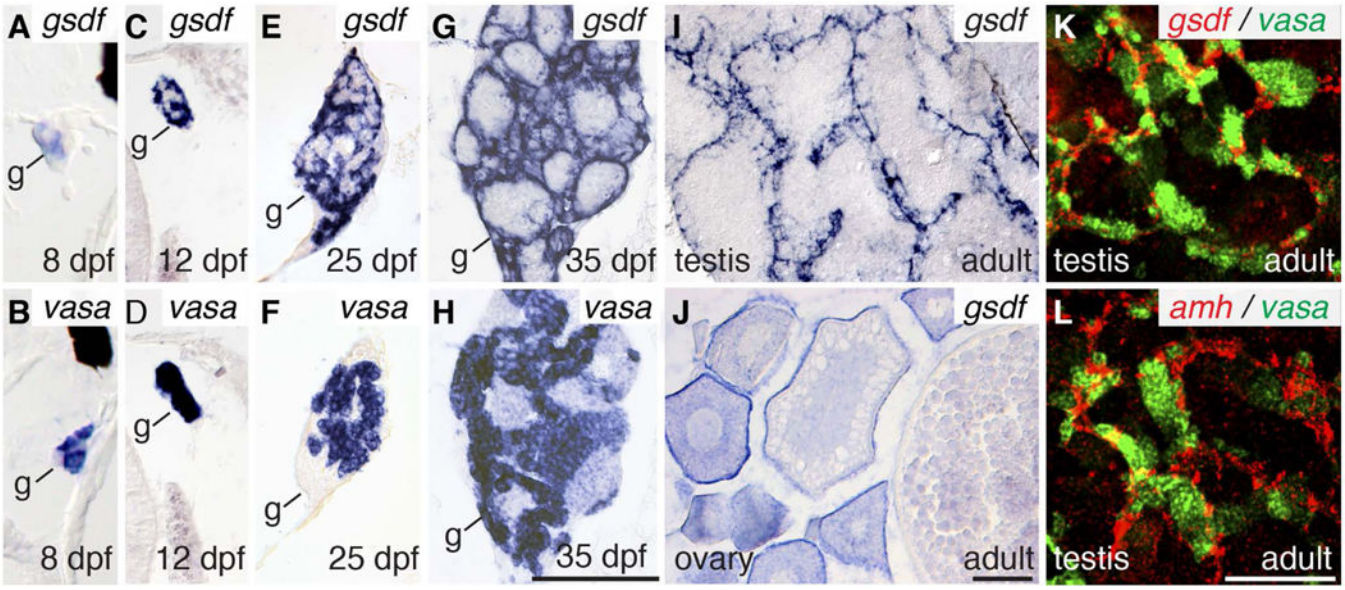

Figure. 1.

Expression of *gsdf*. Expression of *gsdf* in somatic cells (A, C, E, G) and *vasa* in germ cells (B, D, F, H) of 8dpf (A, B), 12dpf (C, D), 25dpf (E, F), and 35dpf (G, H) animals. Expression of *gsdf* in 8mpf adult testis (I) and ovary (J). Two color *in situ* hybridization on adult testis (K) for *gsdf* (red) and *vasa* (green) and (L) *amh* (red) and *vasa* (green). Black scale bar in H for A-H; black scale bar in J for I, J; white scale bar in L for K, L. All scale bars: 100μm.

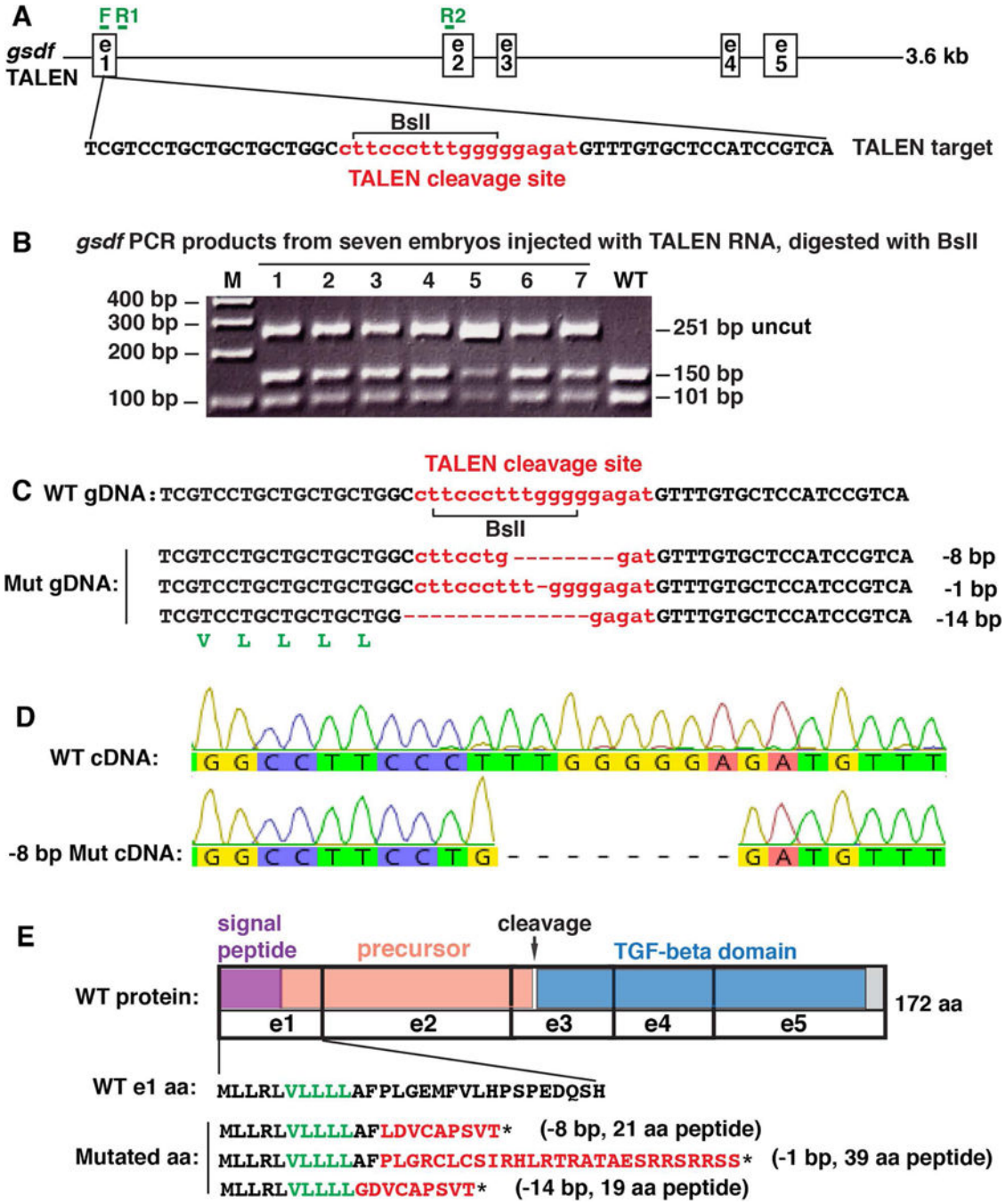

Figure 2.

Mutagenesis. A. A 3.6 kb genomic region of *gsdf* including exons (e) 1–5 with the TALEN target sequence: TALEN cleavage site (small letters in red), TALEN binding sites in black and BslI restriction site (bracket), along with genotyping primer sites (F, R1 and R2 in green). B. PCR analysis of seven G_0 injected embryos at 1dpf using genotyping primers F and R1 shows a 251base pair (bp) fragment in wild types (WT) that digests with BslI to produce fragments of 150bp and 101bp. Seven injected embryos (1–7) contained a substantial load of mutations that destroyed the BslI site, leaving the 251 bp fragment

undigested, in addition to the WT bands. C. Raising injected embryos and subsequent breeding produced stable lines carrying -8 bp, -1 bp and -14 bp deletions. Talen cleavage site shown in red. A translated portion of *gsdf* (in green letters) appears below the -14 bp sequence. D. To verify transcription of the -8 bp deletion, we amplified a 275 bp fragment using primer F in exon-1 and R2 in exon-2 shown in Fig. 2A; results verified the 8 bp deletion, which confirmed the genomic sequence in Fig. 2C. E. TALEN-induced mutations are predicted to result in premature stop codons (*). The translated portion of Gsdf (in black and green letters) indicates the wild-type sequence, while red letters indicate the predicted frame-shifted sequences in *gsdf* mutants. Protein coding domains: purple: signal peptide; salmon: precursor; blue: TGFB-family domain; grey: C-terminus; e: exons; arrow: cleavage site.

A

gsdf allele	Category	4.5mpf WT	4.5mpf Mut	8mpf WT	8mpf Mut	18mpf WT	18mpf Mut
-8bp	% of females that laid eggs	100% (12/12)	67% (8/12)	100% (20/20)	25% (5/20)	75% (9/12)	0% (12/12)
	Number of eggs/cross	159±56	18±15	198±49	4±7	84±61	0
	% of developing eggs/cross	97%±2	62%±46	90%±16	21%±38	72%±43	N/A
	Number of eggs/clutch	159±56	28±9	198±49	14±8	111±41	0
	% of developing eggs/clutch	97%±2	92%±9	90%±16	83%±15	96%±3	N/A
-1bp	% of females that laid eggs	90% (9/10)	70% (7/10)	87% (13/15)	47% (7/15)	80% (8/10)	0% (10/10)
	Number of eggs/cross	159±81	34±27	126±84	11±15	96±67	0
	% of developing eggs/cross	88%±31	66%±46	84%±34	40%±44	79%±41	N/A
	Number of eggs/clutch	177±63	48±17	146±72	24±12	121±49	0
	% of developing eggs/clutch	98%±2	95%±3	97%±3	86%±5	98%±1	N/A
-14bp	% of females that laid eggs	90% (9/10)	70% (7/10)	87% (13/15)	40% (6/15)	70% (7/10)	0% (10/10)
	Number of eggs/cross	154±86	36±32	140±98	8±11	75±62	0
	% of developing eggs/cross	88%±31	67%±46	84%±34	35%±45	68%±47	N/A
	Number of eggs/clutch	171±70	52±25	161±86	19±10	107±41	0
	% of developing eggs/clutch	98%±2	95%±3	97%±3	87%±11	97%±4	N/A

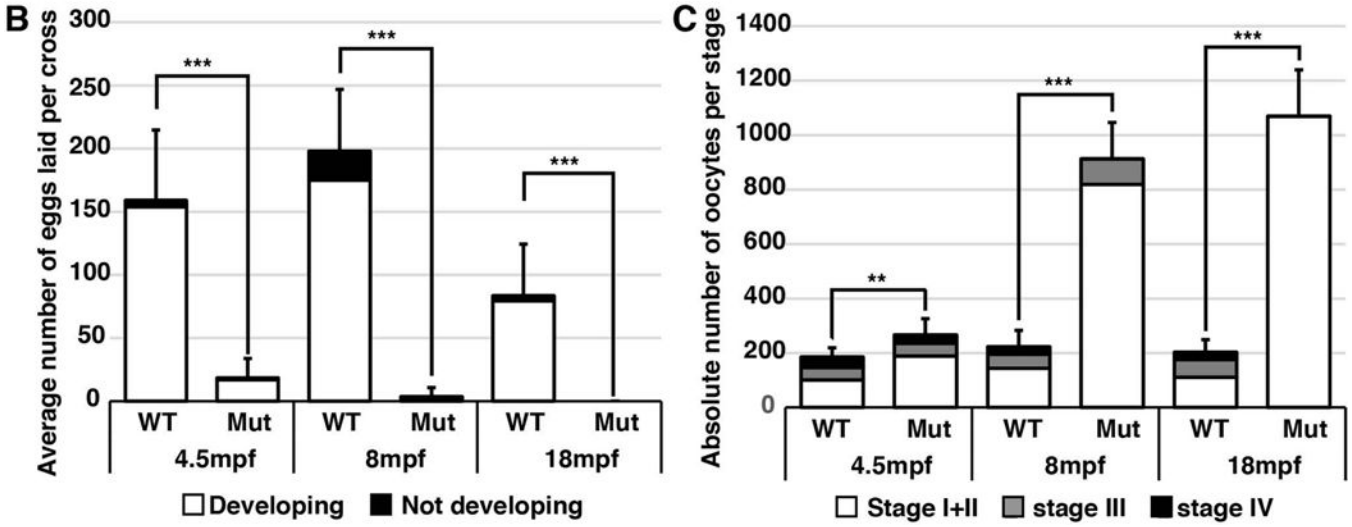

Figure 3.

Fertility in *gsdf* mutant lines. A. Fertility data for the three mutant *gsdf* alleles compared to wild-type siblings. For each allele, the percentage of females that laid eggs is given and in parentheses appears the number of crosses yielding eggs compared to the number of crosses performed. Other quantities and percentages are given with their respective standard deviation. B. Average number of eggs laid per cross of -8bp *gsdf* mutant females compared to wild-type siblings at 4.5, 8 and 18 mpf. For each of 12 to 20 crosses for mutant or wild-type genotypes, one wild-type female or one mutant female was paired with three non-sibling wild-type males. Eggs were collected and counted the following day, and their development was followed for at least three days. Eggs developing normally, not developing, or improperly developing were counted. C. Number of oocytes in histological sections of fish homozygous for the -8bp *gsdf* mutation compared to wild-type siblings at 4.5, 8 and 18 months post fertilization (mpf). Oocytes were categorized in three groups: Stage I + Stage II

(white), Stage III (grey) and Stage IV (black) oocytes. The 18 mpf mutant females had only Stage I + Stage II oocytes. Statistical significance: **, $0.01<p<0.001$ and ***, $p< 0.001$.

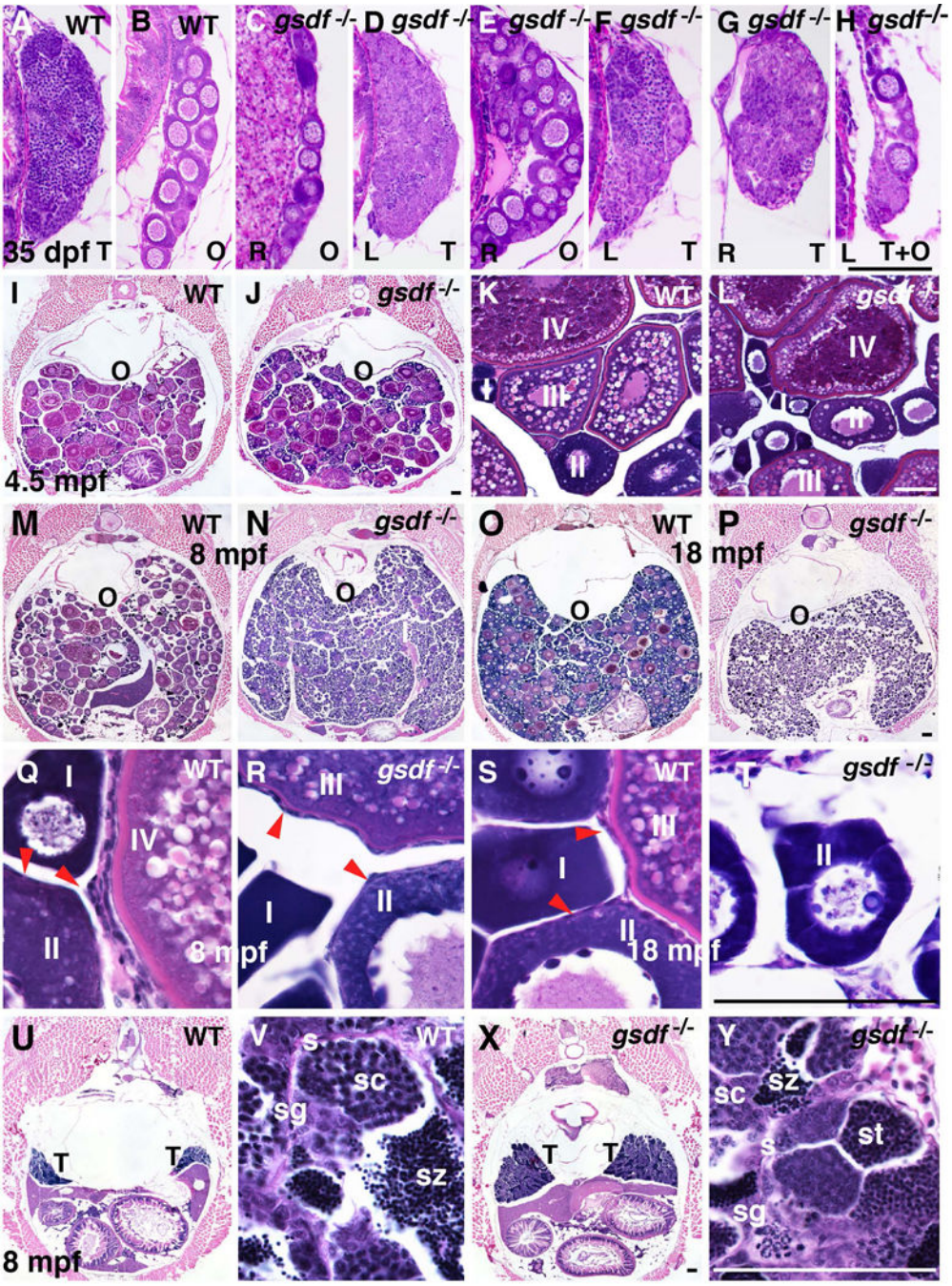

Figure 4.

Gonad histology. A-H. Histological sections of 35dpf wild-type fish showed only ovary or testis (A, B), but six of 22 *gsdf* mutant fish (27%) at 35dpf contained either one ovary and one testis, like the two fish in C-F, or individual gonads containing both ovary and testis like the one fish in G and H. (Abbreviations: O, ovary; T, testis; R, right gonad; L, left gonad). I-T. Adult female ovaries showing low (I, J, M-P), and high (K, L, Q-T) magnification. Cross sections of 4.5mpf of wild types (I, K) and *gsdf* mutants (J, L) that contained maturing (stage-I, II) and vitellogenic (stage-III and -IV) follicles. 8mpf old wild-type females had

gonads filled with maturing (stage-I, -II) and vitellogenic (stage-III and -IV) follicles (M, Q), as well as follicle cells on the surface of oocytes (red arrow heads in panel Q). (N, R) Cross sections of an 8mpf *gsdf* mutant female showed an excess of immature follicles (stage-I and -II), a few early vitellogenic follicles (stage-III), but no late vitellogenic follicles (stage-IV). Follicle cells surround oocytes (red arrow heads, panel R) in mutants as in wild types. (M, Q). Cross section of an old (18mpf) female wild type with many immature follicles, a few normal vitellogenic follicles (O, S), and an 18mpf female *gsdf* mutant female with many young follicles (stage-I and -II) but no mature stages (P, T). U-Y. Cross sections of 8mpf wild-type (U, V) and *gsdf* mutant (X, Y) males at low (U, X) and high magnification (V, Y). Although male *gsdf* mutants had larger testes than wild types, both genotypes formed all spermatogenic stages (Abbreviations: O, ovary; s, sertoli cells; sc, spermatocytes; sg, spermatogonia; st, spermatids; sz, spermatozoa; T, testis). Black scale bar in H for A-H; black scale bar in J for I, J; white scale bar in L for K, L; black scale bar in P for M-P; Black scale bar in T for Q-T; black scale bar in X for U, X; white scale bar in Y for V, Y. All scale bars: 100μm.

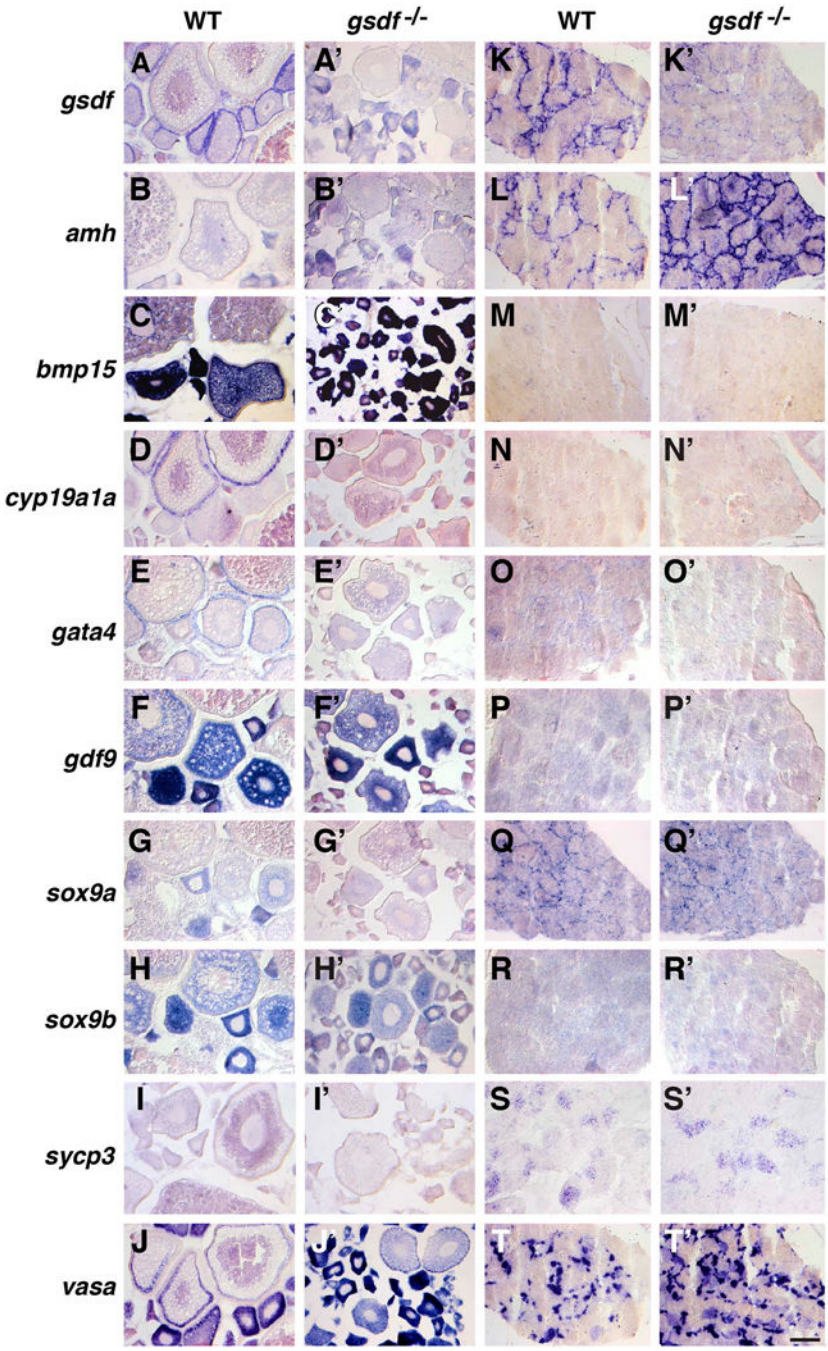

Figure 5.

Gene expression patterns in adult gonads at 8mpf. (A-J) Wild-type ovaries. (A′-J′) *gsdf* mutant ovaries. (K-T) Wild-type testis. (K′-T′) *gsdf* mutant testis. In situ hybridization for *gsdf* (A, A′, K, K′), *amh* (B, B′, L, L′), *bmp15* (C, C′, M, M′), *cyp19a1a* (D, D′, N, N′), *gata4* (E, E′, O, O′), *gdf9* (F, F′, P, P′), *sox9a* (G, G′, Q, Q′), *sox9b* (H, H′, R, R′), *sycp3* (I, I′, S, S′), *vasa* (J, J′, T, T′). Little *gsdf* transcript was apparent in mutant gonads. Transcript of *cyp19a1a* and *gata4* was less abundant in mutant ovarian follicles and *amh* and *vasa* was more abundant in mutant testis. Scale bar in T′: 100μm.

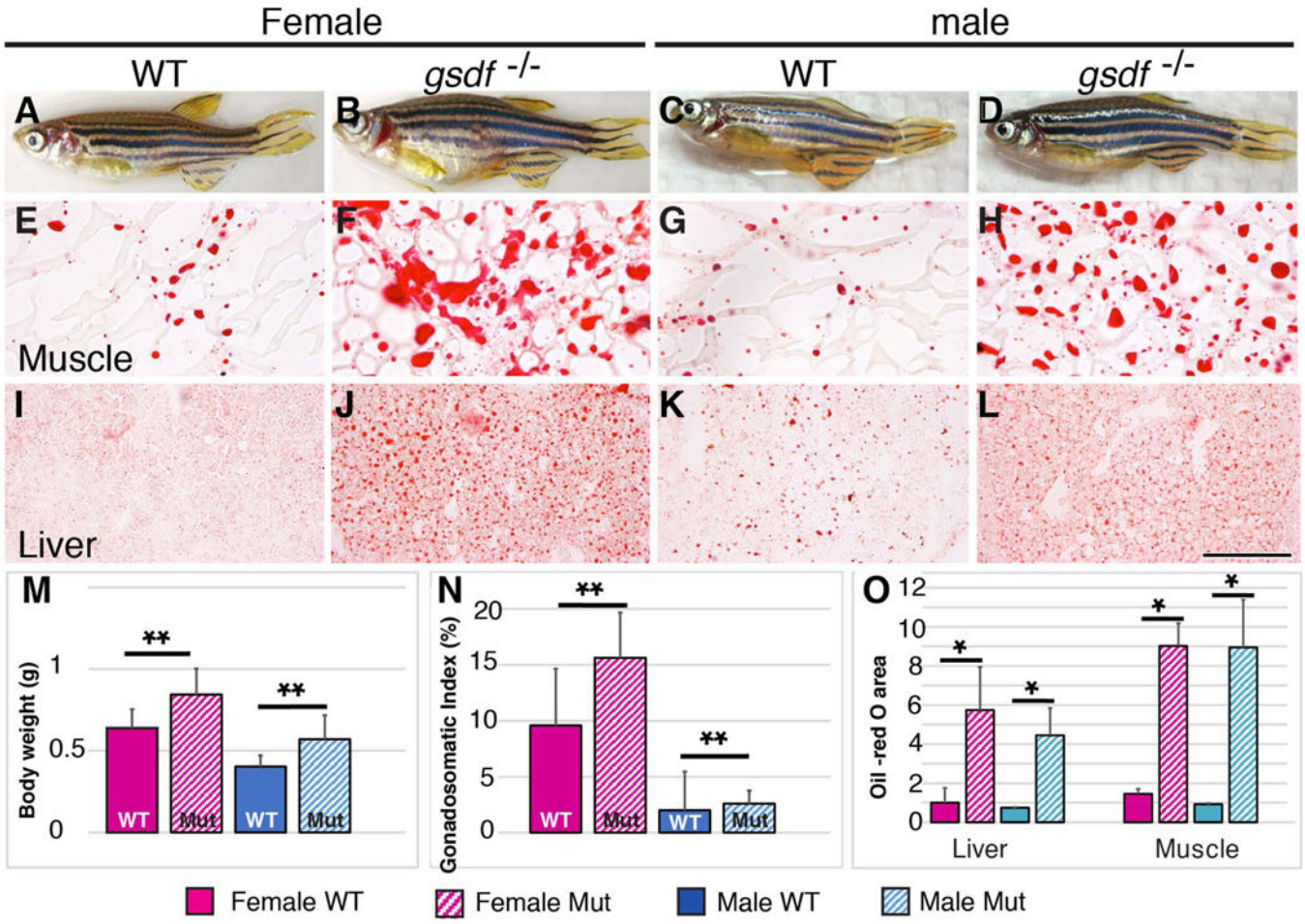

Figure 6.

Body size and lipid homeostasis. (A-D) Adult 8mpf zebrafish: wild types (A, female; C, male) and *gsdf* mutants (B, female; D, male), showing enlarged abdomens in mutants. (E-T) Histological sections showing lipid stained with Oil Red O. (E-H) trunk muscle); (I-L) hepatopancreas (liver). Mutants accumulated more and larger lipid droplets than wild types. (M) Body weight (g); (N) Gonadosomatic Index (%); (O) Oil-red O area. Solid boxes, wild types; striped boxes, mutants; red boxes, females; blue boxes, males. *, 0.05<p<0.01; **, 0.01<p<0.001. Scale bar in L: 100μm.

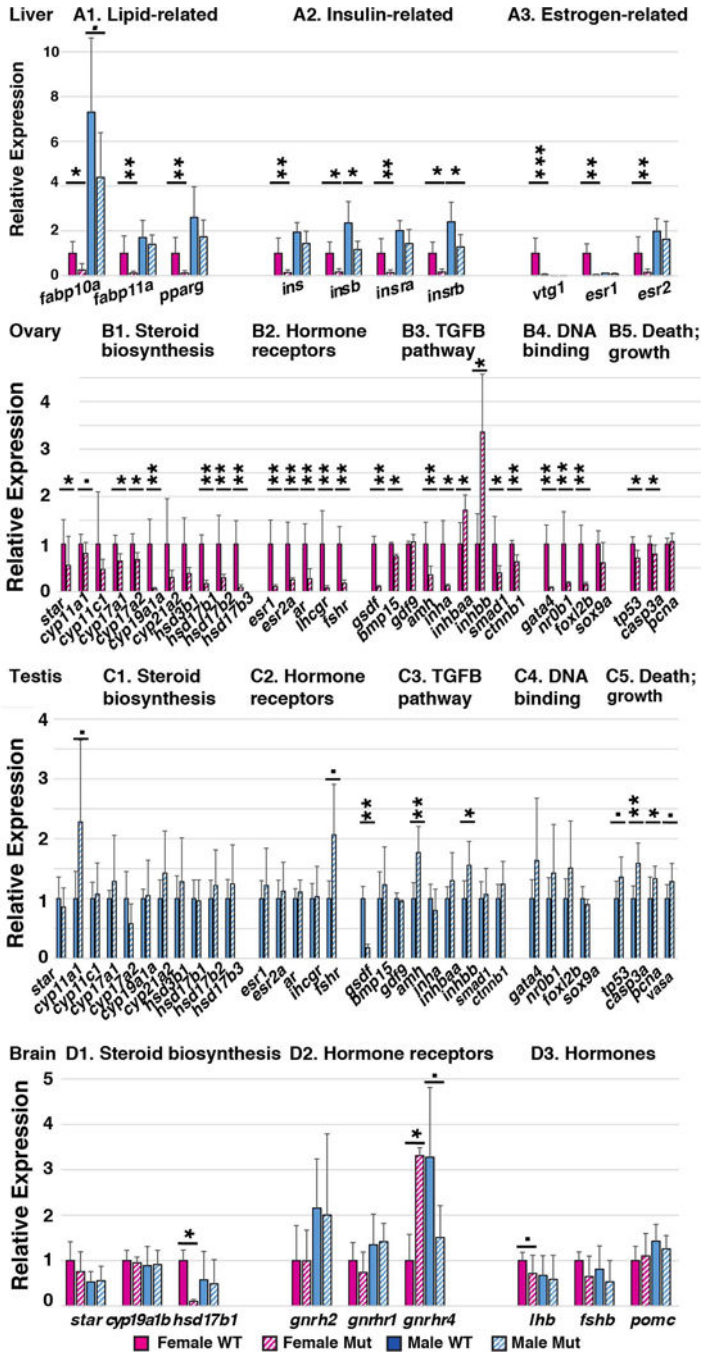

Figure 7.

Loss of *gsdf* function alters gene expression in several organs. mRNA was extracted from *gsdf* mutant and wild-type sibling tissues and assayed by qPCR for (A) hepatopancreas (liver), (B) ovary, (C) testis, and (D) brain. Expression of each gene was compared to expression of beta-actin. The ratio of expression of each gene relative to beta-actin in wild-type females was set to 1.0. Statistical significance: •, 0.05<p<0.1; *, 0.05<p<0.01; **, 0.01<p<0.001; and ***, p< 0.001.

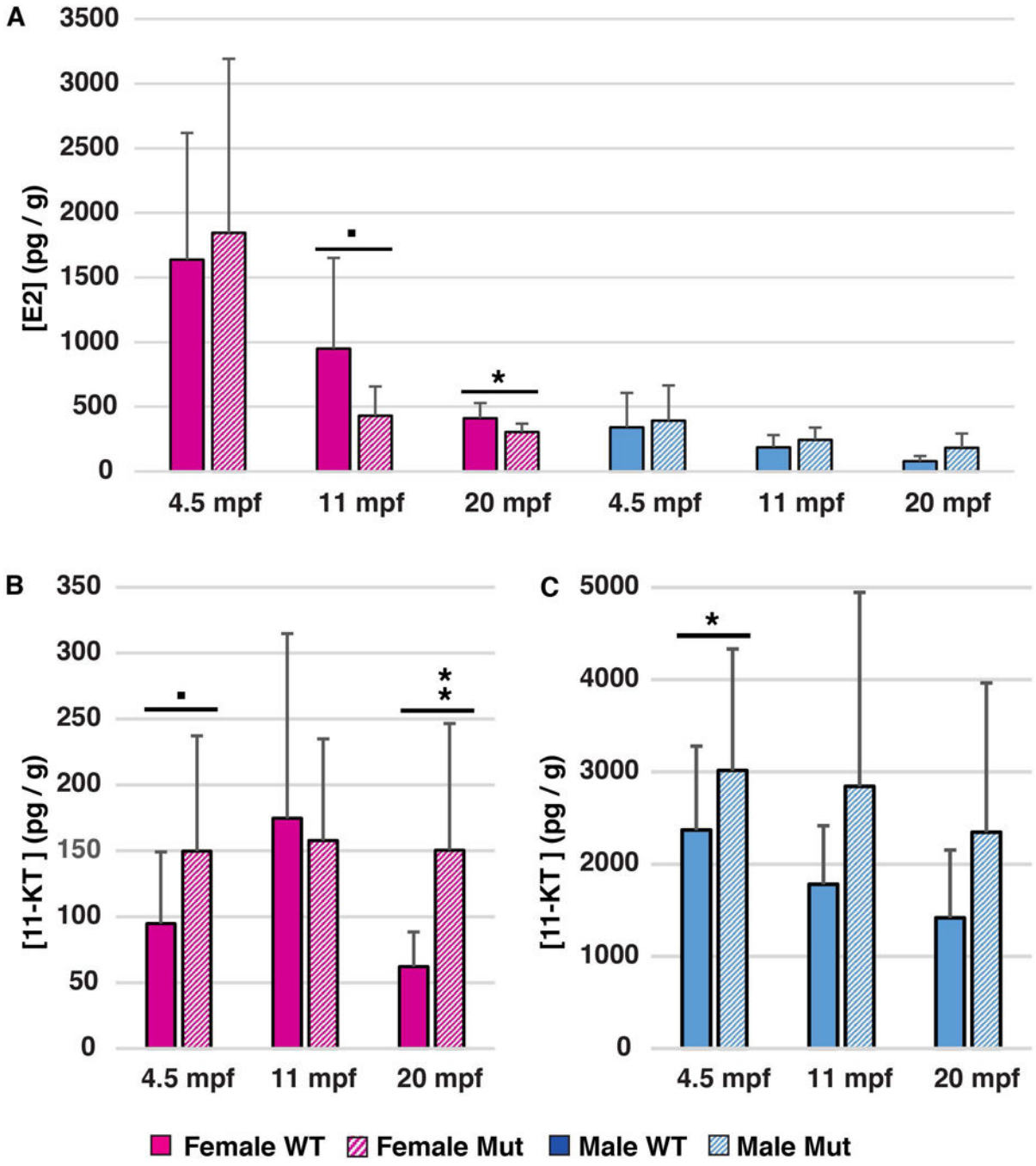

Figure 8.

Assays for estradiol and 11-ketotestosterone (ELISAs). A. Estradiol (E2) levels (pg/g) in female and male *gsdf* mutants and wild-type siblings at 4.5mpf, 11mpf and 20mpf. B, C. 11 KT levels (pg/g) in female (B) and male (C) *gsdf* mutants and wild-type siblings at 4.5mpf, 11mpf, and 20mpf. Statistical significance: •, 0.05<p<0.1; *, 0.05<p<0.01; **, 0.01<p<0.001; and ***, p< 0.001. Solid boxes, wild types; striped boxes, mutants; red boxes, females; blue boxes, males.

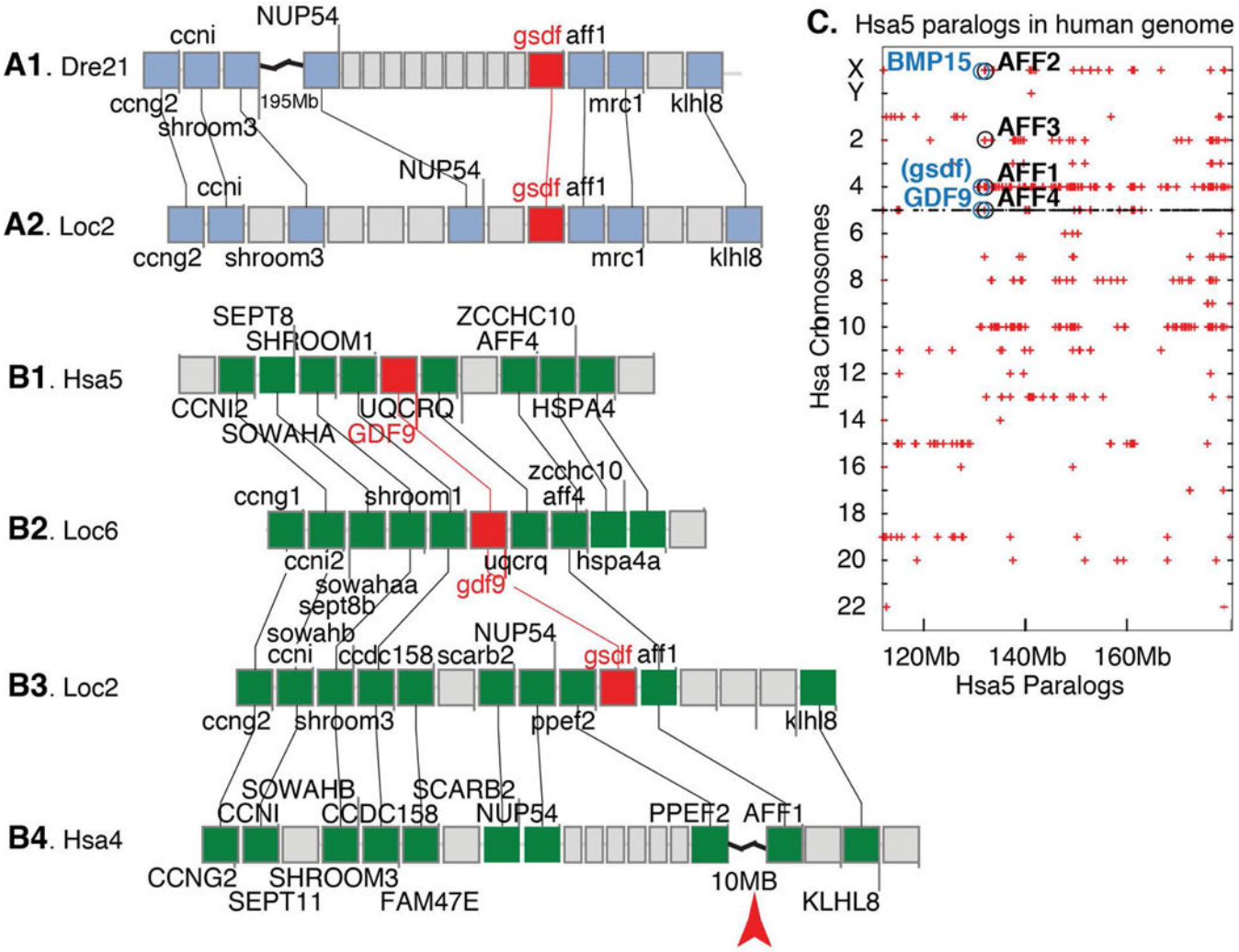

Figure 9.

Conserved syntenies illuminate *gsdf* history. A. Part of zebrafish chromosome Dre21 (A1) compared to spotted gar chromosome Loc2 (A2) shows that genes to the right of *gsdf* in zebrafish and gar are conserved, but an indel to the left of *gsdf* in zebrafish disrupts syntenies. B. Comparing gar Loc2 (B3) to human Hsa4 (B4) shows a break that disrupts the expected location of a human *gsdf* ortholog. The gar *gsdf* region on Loc2 (B3) is paralogous to the *gdf9* region on gar chromosome Loc6 (B2), which in turn is orthologous to the *GDF9* region of human chromosome Hsa5 (B1), as predicted for paralogs. C. A dot plot identifying paralogs of Hsa5 genes on other human chromosomes shows that *AFF* gene family members, which are adjacent or nearly adjacent to *gsdf, bmp15*, and *gdf9*, occupy paralogous human chromosome segments, as expected by ohnologs from the vertebrate genome duplication VGD2.