ADAMTS9 IS CRITICAL FOR THE DEVELOPMENT OF PRIMARY OVARIAN FOLLICLES

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Adamts9 (a disintegrin and metalloprotease with a thrombospondin type 1 motif, member 9) is an extracellular matrix metalloprotease that is highly evolutionarily conserved and critical for development in vertebrates. Knockouts of ADAMTS9 in mice and AdamTS-A in *Drosophila melanogaster* are embryonic lethal; and abnormal ADAMTS9 activity has also been linked to various human disorders including ovarian and uterine disease. Our previous work has demonstrated that Adamts9 is necessary for ovarian development in zebrafish. Adamts9 KO zebrafish had a heavily male-biased sex ratio as 6–7-month-old adults, and female Adamts9 KOs were infertile. Furthermore, a novel intersex phenotype was also discovered in Adamts9 KOs zebrafish. These fish lacked clearly defined ovary or testis structure. To further understand the roles of Adamts9 in ovarian development and maintenance in zebrafish, we investigated Adamts9's expression, role in primordial germ cell (PGC) migration, gonad development, sexual differentiation, and development of primary ovarian follicles in zebrafish. We found *adamts9* was widely expressed in various tissues during embryonic and larval development and transcripts

are also maternally deposited. We found strong expression in the developing retina at 48 hours post fertilization (hpf), that shifted to the ciliary marginal zone at 72hpf. We also found expression in somites surrounding the PGCs during migration, in primary follicles in juveniles, and preovulatory follicular cells in adult ovaries. In contrast to its essential role in PGC migration in invertebrate models, we only observed migration delay of PGCs in Adamts9 KOs. But interestingly, we observed slower and under development of juvenile gonads in Adamts9 KO, and significantly reduced size and number of primary oocytes in Adamts9 KO zebrafish. Surprisingly, Adamts9 KO had a negligible effect on primary sex determination, but in female Adamts9 KOs the ovary remained dramatically underdeveloped compared to wildtype control siblings. Rescuing global growth defects by overfeeding and lower rearing density did increase female percentage but did not rescue the underdeveloped ovary phenotype. Further, follicles in rescued Adamts9 KO females remained at Stage IB and only few follicles could continue development into late stages including mature follicles. As the fish continued to age, the male biased sex ratio continued to increase in mid- or late-juvenile stages and even in adults, indicating that female Adamts9 KOs are sex reversing into males throughout their life. We also found morphological evidence for sex reversal in Adamts9 KO at 90 days old adults, including coexistence of Stage IB oocytes and sperm in the same tissue section. Taken together, we show that Adamts9 is essential for proper ovarian development and maintenance and that loss of Adamts9 leads to folliculogenesis deficiency, follicle arrest, loss of ovarian follicles and eventual female to male sex reversal after primary sex determination in juvenile and adult zebrafish.

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CHAPTER I: Introduction

ADAMTS9 (a disintegrin and metalloprotease with thrombospondin type-1 motifs, member 9) is an extracellular matrix (ECM) metalloprotease that is secreted from cells and is known to cleave the proteoglycans aggrecan and versican, and the glycoprotein fibronectin (Clark et al., 2000; Somerville et al., 2003; Koo et al., 2006; Koo et al., 2007; Wang et al., 2019). Cleavage of proteoglycans and glycoproteins both contributes to ECM turnover and clearage, as well as generates shorter bioactive peptide fragments (Iozzo and Schaefer, 2015; Graae et al., 2019, Fontanil et al., 2021). Several other protein targets for ADAMTS9 have been proposed, but need to be explored further (Tharmarajah et al., 2018). In addition to its primary enzymatic function, ADAMTS9 and its non-mammalian orthologs have been found to have non-enzymatic/non-canonical functions in mediating cell signaling and secretion by protein-protein interactions (Choi et al., 2019; Nandadasa et al., 2019; Du et al., 2013; Chen et al., 2017; Yoshina et al., 2012; Yoshina and Mitani, 2015). Several human health conditions have been linked to mutations in or abnormal expression of ADAMTS9, including Type II diabetes (Simonis-Bik et al., 2010; Kong et al., 2015), nephronophthisis-related ciliopathies (i.e., cystic kidney disease)(Choi et al., 2019), poly-cystic ovarian syndrome (PCOS) (GohariTaban et al., 2019), natural age of menopause (Pyun et al., 2014), primary hyperparathyroidism (Karakose et al., 2016), cognitive aging (Lin et al., 2017), uterine endometriosis (Hismiogullari et al., 2021), arthritis (Juliá et al., 2015; Li et al., 2018), uterine leiomyoma (Gueye et al., 2017), and cardiovascular disease (Karakose et al., 2016; Wei et al., 2020). Further, ADAMTS metalloproteases have critical roles in development and normal functions of the reproductive system and fertility (Russell et al., 2015; Zhu, 2021). However, specific roles for ADAMTS9 in vertebrate sex differentiation and ovarian folliculogenesis are still unknown.

In invertebrates, ADAMTS9 orthologs are indispensable for normal migration of primordial germ cells (PGCs) and gonad development (Blelloch and Kimble, 1999; Blelloch et al., 1999; Ismat et al., 2013; Imanishi et al., 2020). Previous work from our lab has shown that the zebrafish Adamts9 is involved in follicle rupture during ovulation, and is also critical for female gonadal development (Liu et al., 2017; Liu et al., 2018; Carter et al., 2019; Liu et al., 2020). However, involvement and mechanisms for Adamts9 in early ovarian development in zebrafish are still unclear. While ADAMTS9 has been detected in the developing gonads, adult ovary, and ovarian follicular fluid of vertebrates including humans, phenotypic description of *Adamts9* disruptions have not been described previously because of embryonic lethality in ADAMTS9 KO mice (Jungers et al., 2005; Kern et al., 2010; Piprek et al., 2018; Russell et al., 2015; Liu et al., 2017; Zhu, 2021). Our survivable Adamts9 KO zebrafish provides a unique model to study the role(s) of Adamts9 in vertebrate PGC migration, early gonad development, sexual differentiation, and ovarian folliculogenesis (Carter et al., 2019; Carver et al., 2021; Zhu, 2021). Doing so will reveal new evidence of the involvement of vertebrate Adamts9 in normal ovarian physiology and may offer clues to the development of treatments for infertility or ovarian and uterine disease in human patients (Pyun et al., 2014; Gueye et al., 2017; GohariTaban et al., 2019; Tokmak et al., 2019; Hismiogullari et al., 2021).

The aim of this thesis is to determine the cause of male sex bias in Adamts9 KO zebrafish and elucidate roles of Adamts9 in oocyte development and physiology. In Chapter II, I summarize current knowledge about zebrafish gonad development and known roles of metalloproteases in vertebrate gonad development. In chapter III, we demonstrate widespread expression of *adamts9* in various tissues and cell types in developing juvenile and adult zebrafish including in the gonads. In chapter IV, we investigated PGC migration in Adamts9 KO zebrafish

embryos and demonstrated *adamts9* expression in Stage IB (primary) follicles. And in chapter V, we founnd defective folliculogenesis as the primary cause of male sex bias and sex reversal in Adamts9 KO. This is a significant finding that will be further explored in future studies. Finally, a brief summary and perspectives will follow in chapter VI.

CHAPTER II: Metzincin Metalloproteases in Gonadal Sex Differentiation of Zebrafish

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Chapter Summary

Development of a functional gonad includes migration of primordial germ cells (PGCs), formation of primary follicles or spermatogenic cysts with somatic gonadal cells, maturation of gametes, and subsequent release of mature germ cells. These processes require extensive tissue remodeling including cellular differentiation, migration, development, and growth of germ cells and adjacent somatic gonadal cells; as well as broad alterations of the surrounding extracellular matrix (ECM). Metalloproteases, including MMPs (matrix metalloproteases), ADAMs (a disintegrin and metalloproteinases), and ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs), are suggested to have critical roles in the remodeling of the ECM during gonad development. However, few research articles and reviews are available on the functions and mechanisms of metalloproteases in remodeling gonadal ECM, gonadal development, or sex differentiation. Moreover, most studies focused on the roles of transcription and growth factors in early gonad development and sex determination, leaving a significant knowledge gap on how differentially expressed metalloproteases exert effects on the ECM, cell migration, development, and survival of germ cells during the rapid development of gonads. I will focus this chapter on gonad development and available evidence of metalloproteases involvement, with an emphasis on zebrafish as a model for studying gonadal sex differentiation and metalloprotease functions.

Introduction

During development, the undifferentiated gonad undergoes a striking transformation from a primordial gonadal ridge to a functional testis or ovary. Germ cells, somatic gonadal cells, and their extracellular matrix (ECM) must go through dramatic remodeling and differentiation for proper gonad development to occur (Marty et al., 2003; Beckman and Feuston, 2003; Maack and Segner, 2003; King et al., 1968; McLaren, 1991; Yildirim et al., 2020). Disruption of this complex process from either genetic or environmental causes typically leads to disorders of sexual development (DSDs) and infertility in humans (Allen, 2009; Öçal, 2011; Juul et al., 2014; Abrao et al., 2013; Roupa et al., 2009; Ferlin et al., 2006a; Ferlin et al., 2006b; Galani et al., 2008; Zuccarello et al., 2007) but can also lead to complete sex reversal in other animal species, such as in fish (Carter et al., 2019; Dranow et al., 2013; Dranow et al., 2016; Sakae et al., 2020; Baroiller and D'Cotta, 2020). Most studies in gonad development and sex determination were focused on the roles of transcription factors in early sex determination and differentiation (Mamsen et al., 2017; Nagahama, 2005; Hayes, 1998; Devlin and Nagahama, 2002; Nagahama et al., 2021; Wilhem et al., 2007). A few reports are available concerning the roles of ECM components and secreted metalloproteases that are required for cell migration, proliferation, signaling, and survival during gonad development. In this review, we will attempt to fill a knowledge gap by briefly summarizing current evidence of metalloproteases and ECM constituents in gonad development and sex differentiation with a focus on zebrafish, mainly because of availability of literature and genetic evidence. We will include studies using other species when appropriate. We will also review roles of metalloproteases in ovarian folliculogenesis, a critical process for ovarian development, and sex maintenance in zebrafish (Ge, 2005; Zhang et al., 2018; Gershon et al., 2020). Finally, we will speculate on directions for investigating metalloproteases in the development and function of gonads.

Gonadal Development and Sex Differentiation in Zebrafish

The zebrafish is a protogynous species, where the primordial, bipotential gonad develops into an ovary-like structure containing early stages of ovarian follicles prior to transitioning into committed development of testes or ovary in early juveniles (Fig. 2.1)(Takahashi, 1977). The genes and pathways for sex determination in zebrafish have been studied intensively for several decades (Fig. 2.2)(Liew and Orbán, 2013; Kossack and Draper, 2019). Wild caught zebrafish from India were reported to have a ZZ/ZW chromosomal sex determination (CSD) system (Sharma et al., 1998; Mahapatra et al., 2002; Spence et al., 2007), but the major sex-linked locus responsible for CSD is missing in domesticated lab strains Tübingen (TUB) and AB (Anderson et al., 2012; Wilson et al., 2014). CSD loss makes zebrafish a unique model for studying mechanisms of sex determination and differentiation following the loss of a major sex-locus on a sex chromosome, which may occur in humans due to genetic instability of the Y sex chromosome (Perry et al., 2007; Wilson et al., 2020; Graves, 2006; Griffin, 2012). In the absence of a master-switch gene, domesticated zebrafish utilize a polygenic sex determination (PSD) system that involves interactions between multiple minor sex determining genes (Uchida et al., 2002; Tong et al., 2010; Abozaid et al., 2011; Liew et al., 2012; Liew and Orbán, 2013; Kossack and Draper, 2019). Environmental conditions including temperature, stress, endocrine disrupting chemicals (EDCs), nutrition, or rearing density have been demonstrated to affect sex determination and skew sex ratios in zebrafish (Abozad et al., 2011; Uchida et al., 2004; Larsen and Baatrup, 2010; Örn et al., 2003; Ribas et al., 2017a; Ribas et al., 2017b). Zebrafish are gonochoristic once matured, with the exception that adult fish can undergo female-to-male sex reversal if there is extensive loss of germ cells or oocyte follicles from experimental manipulations (Dranow et al., 2013; Dranow et al., 2016; Takatsu et al., 2013; McMenamin,

2016). In sex reversal, the ovarian tissue completely remodels into a functional testis which begins producing sperm and secreting androgens to alter secondary sex characteristics (Dranow et al., 2013; Larsen and Baatrup, 2010). Female zebrafish are typically bigger than males with wider abdomens and silver colored ventral sides with prominent genital papilla. Males are slimmer with yellow ventral coloring and possess breeding tubercles on their pectoral fins (Kang et al., 2013; McMillan et al., 2013). Mating behaviors between adult males and females are well characterized and can be used to assess expression of sex-typical behaviors (Darrow and Harris, 2004; Yong et al., 2017; Spence et al., 2008).

Like in *Drosophila*, *C. elegans*, and *Xenopus*, special granules in developing zebrafish oocytes contain germ plasm, necessary for germline development in succeeding embryos. Germ plasm is a sub-cytoplasmic collection of cellular components collected within a mitochondrial cloud termed the Balbiani body (Selman et al., 1993; Knaut et al., 2000; Dosch et al., 2004; Kosaka et al., 2007; Marlow and Mullins, 2008; Bontems et al., 2009; Gupta et al., 2010; Dosch, 2014; Jamieson-Lucy and Mullins, 2019). The Balbiani body is a conserved structure among *Drosophila*, fish, mouse, human, and *Xenopus* oocytes (Dosch, 2014; Jamieson-Lucy and Mullins, 2019). Balbiani bodies can be identified by their large size and dense composition of RNA, RNA binding proteins (RBPs), ER, and mitochondria (Dosch et al., 2004; Marlow and Mullins, 2008; Zhang et al., 2008). During oocyte maturation, germ plasm remains segregated from the rest of the yolk and collects at the vegetal pole in the oocyte. In the fertilized embryo, precursor cells that inherit germ plasm becomes PGCs and begin expressing PGC markers including *vasa* (Yoon et al., 1997; Braat et al., 1999; Braat et al., 2000), *buc* (Kirshnakumar et al., 2018; Perera et al., 2021), *nanos1/nanos3* (Köprunner et al., 2001), and *dnd1* (Weidinger et al., 2003; Liu and Collodi, 2010; Li et al., 2017). The inheritance nature of PGC specification in

zebrafish contrasts with the inductive method of mammalian PGC specification, in which competent stem cells are induced to PGC fate by paracrine signaling (Tang et al., 2016; Canovas et al., 2017). Nevertheless, genes required for PGC identity including *ddx4*(*vasa*), *nanos3,* and *dnd1* show conserved expression between zebrafish and mammalian PGCs (Yoon et al., 1997; Toyooka et al., 2000; Thisse et al., 2001; Weidinger et al., 2003; Julaton and Pera, 2011; Tang et al., 2015).

Using *vasa* as a marker, PGCs are identifiable as four distinct clusters by the dome stage in zebrafish embryos. During epiboly stage, PGCs migrate from their original position to become two clusters at either side of the midline/notochord (Yoon et al., 1997). The PGCs become morphologically distinct at the 5-somite stage and are positioned in the endodermal layer around the second and third somite (Braat et al., 1999). From this position, the PGCs migrate along the dorsal midline to the gonadal ridge. PGC migration is dependent on germ cells responding to chemokine signals and forming cellular protrusions in the direction of chemical attractants such as Sdf-1a (*cxcl12a*) (Doitsidou et al., 2002; Paksa and Raz, 2015; Aalto et al., 2021). Sdf-1a acts on the Cxcr4 (*cxcr4b*) and Cxcr7 (*askrb*) receptors to direct formation of protrusions in towards higher concentrations of the chemoattractant (Blaser et al., 2006; Boldjaipour et al., 2008). Sdf-1a is present in the presumptive gonadal ridge during PGC migration but disappears shortly after the PGCs have reached the gonads (Paksa et al., 2016).

Zebrafish PGCs normally finish their migration within 24 hours post fertilization (hpf). During the first week of larval development, the PGCs do not proliferate and only migrate slightly around the developing swim bladder and gut (Braat et al., 1999). The PGCs form an aggregate around the gonadal ridge and form increasing amount of contact with somatic cells (Braat et al., 1999; Tzung et al., 2015). Beginning ~7dpf, the somatic cells of the gonad begin

proliferating and differentiating, accompanied by a dramatic proliferation of germ cells (Tzung et al., 2015; Leerberg et al., 2017). The first signs of sexual dimorphism begin at 14dpf, as the number of germ cells becomes more varied. Fish with low or no germ cells either naturally, from manual ablation or genetic mutations tend to form testes, while fish with high numbers of germ cells tend to continually develop as females in zebrafish, Japanese medaka, and Prussian carp (Tzung et al., 2015, Slanchev et al., 2005; Siegfried and Nüsslein-Volhard, 2008; Liu et al., 2015; Kurokawa et al., 2007; Nishimura et al., 2018). Instead of a testis, formation of empty ovarian cavities in PGC depleted goldfish suggests the presence of a different kind of gonadal differentiation mechanism in some fishes (Goto et al., 2012).

Between 21-28dpf, germ cells continue to increase in number and differentiate into oogonia and Stage IA/IB oocytes (IB is equivalent to primary follicles in mammals) (Tong et al., 2010). While the gonad has ovarian morphology, presence of early-stage oocytes, follicles, and *cyp19a1a* expression; it is still bipotential and expresses male sex determining genes like *amh* (Leerberg et al., 2017; Slanchev et al., 2005; Siegfried and Nüsslein-Volhard, 2008; Maack and Segner, 2003; Tong et al., 2010). This stage can last till ~20-30dpf depending on the zebrafish strain and rearing conditions. Soon after, the gonad becomes committed to differentiate into either a testis or ovary. Dimorphic expression of sex-determining genes *sox9a*, *amh*, *dmrt1*, *ar*, *fig α*, and *cyp19a1a/b* can be found between 21-35dpf (Leerberg et al., 2017; Siegfried and Nüsslein-Volhard, 2008; Tong et al., 2010; Jørgensen et al., 2008; Bradley et al., 2011; Guo et al., 2005; Gao et al., 2005; Schulz et al., 2007; Chiang et al., 2001; Lin et al., 2017). Though many genes are involved in zebrafish sex determination, *dmrt1* and *cyp19a1a* seem to have a direct, antagonistic relationship during sex determination and differentiation (Lau et al., 2016; Yin et al., 2017; Webster et al., 2017; Fenske and Segner, 2004). Heavily female-biased sex

ratios and testicular dysgenesis were reported in knockouts of *dmrt1* (Webster et al., 2017; Lin et al., 2017). During primary sex determination and subsequent sexual differentiation, *dmrt1* mutant males had oocytes that persisted long past the bipotential stage while proper testicular tubule structures failed to form (Webster et al., 2017; Lin et al., 2017). Conversely, 100% male progeny was observed in two independent *cyp19a1a* knockout studies (Lau et al., 2016; Yin et al., 2017). *Cyp19a1a*$^{-/-}$ fish had early-stage oocytes that entered meiosis but failed to develop mature follicles or ovaries (Lau et al., 2016; Yin et al., 2017). In wildtype zebrafish, reducing aromatase activity or expression by use of competitive inhibitor fadrozole hydrochloride or synthetic steroid 17α-methyltestosterone (MT) leads to male-biased progeny and sex reversal in adults (Uchida et al., 2004; Takatsu et al., 2013; Fenske and Senger, 2004). Ovary-specific Igf3 may function downstream of Cyp19a1a in sex differentiation. Igf3 KOs had 100% male progeny due to female-to-male sex reversal that could not be rescued by E2 treatment and failed to downregulate *amh* and *dmrt1* in juvenile ovaries (Xie et al., 2020). *dmrt1* and *cyp19a1a* have reciprocal and antagonistic expression during normal sex differentiation of wildtype male and female fish (Wu et al., 2020). Interestingly, double KO *dmrt1*$^{-/-}$*cyp19a1a*$^{-/-}$ fish had a normal sex ratio around maturation, but slowly reverse to majority male with underdeveloped testes (Wu et al., 2020).

For zebrafish females, oocytes are maintained within their follicles and continue to grow. For zebrafish males, the oocytes undergo apoptosis, and the gonad space is filled by stroma, Sertoli cells, and spermatogonia (Takahashi, 1977; Uchida et al., 2002; Maack and Segner, 2003). Suppressing apoptosis through the NF- κB pathway results in a female-biased sex ratio (Pradhan et al., 2012). There is limited evidence that a second population of juveniles never fully develop the bipotential ovary and remain in a mostly undifferentiated state until testes development

(Hsiao and Tsai, 2003; Wang et al., 2007; Luzio et al., 2021). Spermatogonia are not required for testes development nor for normal somatic function of adult testes, the absence of ovarian follicles is seemingly the only requirement for initiating male sex differentiation (Slanchev et al., 2005). Nevertheless, nearly all gonads in zebrafish will have their gonadal sex determined and committed to ovary or testis path of development by ~35dpf.

Oocytes express and secrete signaling molecules, including well studied Gdf9 and Bmp15, to actively reinforce follicular cell function and identity in mammals and fish (Dranow et al., 2016; Wu and Chang, 2013; Dong et al., 1996; Clelland et al., 2006; Otsuka et al., 2010). In *gdf9*$^{-/-}$ mice and *bmp15*$^{-/-}$ zebrafish, primary follicles can be formed but do not progress further (Dranow et al., 2016; Dong et al., 1996). Ablating oocytes in rats causes granulosa cells to begin exhibiting Sertoli cell characteristics, whereas ablation of oocytes in zebrafish causes full sex reversal to testes (Dranow et al., 2013; Dranow et al., 2016; Guigon et al., 2005; McLaren, 1991). Similarly, Sertoli cells suppress oocyte formation and secrete signals for further testes development in mammals and fish. In mammals, Sertoli cell differentiation stimulated by SRY and SOX9 occurs very early in gonad development and is critical for testes cord formation and sequestering of the germ cells (Sinclair et al., 1990; Berta et al., 1990; Koopman et al., 1991; Li et al., 2017; Koopman, 1999; Kashimada and Koopman, 2010). Without Sertoli cells, germ cells proceed into meiosis and transition into oocytes upon retinoic acid (RA) exposure; whereas in the presence of Sertoli cells, germ cells become prespermatogonia and enter mitotic arrest inside testes cords in mice (Adams and McLaren, 2002; MacLean et al., 2007; Trautmann et al., 2008; Ohta et al., 2012). DMRT1 expression maintains Sertoli cell identity and inhibits FOXL2 in controlling somatic cell fate from fish to mammals, including humans (Veitia et al., 1997; Raymond et al., 1998; Raymond et al., 2000; Matsuda et al., 2002; Nanda et al., 2002; Matson et

al., 2011; Uhlenhaut et al., 2009). Though mammals are not able to fully reverse their sex like fishes, oocyte depleted XX gonads began to express testes-like characteristics (Guigon et al., 2005; Koopman, 1999; Kashimada and Koopman, 2010; Lindeman et al., 2015; Veitia, 2010; Jiménez et al., 2021). Given the relatively increased complexity of reproductive systems and more specialized sex chromosomes in mammals compared to fishes, it's likely more difficult for mammals to maintain such a high degree of sexual plasticity in adults (Arnold, 2004; Arnold, 2009; Graves, 2006).

Zebrafish achieve sexual maturation, marked by appearance of mature gametes, around 90dpf or when they reach adult size (SL:20-30mm) (Dai et al., 2021; Lessman and Brantley, 2020; Chen and Ge, 2013; Singleman and Holtzman, 2014). The expression of *vasa* is maintained in most germline cells throughout adult life and is essential for fertility (Hartung et al., 2014). In contrast to mammals, both sexes in zebrafish also retain *nanos2/nanos3* expressing germline stem cells (GSCs) through adult life (Beer and Draper, 2013). Enhanced feeding and/or low rearing density leads to rapid growth of juveniles and causes sexual maturation to happen early, <60dpf (personal observations). Diet can also impact fecundity of the zebrafish (Frederickson et al., 2021).

Metzincins in Facilitating Sexual Differentiation and Development

Several families of the metzincin superfamily, including matrix metalloproteases (MMPs), A disintegrin and metalloprotease (ADAMs), A disintegrin and metalloprotease with thrombospondin type-1 motifs (ADAMTSs/ADAM-TSs), non-enzymatic ADAMTS-like proteins (ADAMTSLs), and endogenous antagonists tissue inhibitors of metalloproteases (TIMPs), all play roles in various cellular processes throughout the body during development or maintaining homeostasis. These metalloproteases utilize a zinc ion in their active sites for

cleaving peptide bonds in proteoglycan chains (Gomis-Rüth, 2009; Tallant et al., 2010). In addition to their primary enzymatic role, ADAM, ADAMTS, and ADAMTSL are large, multidomain proteins that have non-canonical/non-enzymatic functions in the regulation of cellular functions via interactions with other proteins including signaling molecules or enzymes. These protein families are relatively new, having only been discovered in the last fifty years, yet have already been demonstrated to play diverse roles in development and physiology (Gross and Lapiere, 1962; Woessner, 1962; Wolfsberg et al., 1995; Kuno et al., 1997). Within the past two decades, there has been an increased interest in the biological significance of metalloproteases, and we refer the reader to several excellent reviews (Russell et al., 2015; Zhu, 2021; Seals and Courtneidge, 2003; Visse and Nagase, 2003; Nagase et al., 2006; Page-McCaw et al., 2007; Apte, 2009). Briefly, metalloproteases have been shown to be involved in processes including extracellular matrix (ECM) assembly/disassembly, cell migration (Blelloch and Kimble, 1999), regulating cell proliferation (Kasper et al., 2007; Na et al., 2012; Li et al., 2007; Chang and Werb, 2001), signal transduction (Choi et al., 2019; Nanddadasa et al., 2019), ectodomain shedding (Weber and Saftig, 2012), ciliogenesis (Choi et al., 2019; Nandadasa et al., 2019), tumor suppression (Chen et al., 2017; Du et al., 2013), angiogenesis (Quintero-Fabián et al., 2019), follicullogenesis (Richards et al., 2005; Meng et al., 2017; GohariTaban et al., 2019), organ development (Shindo et al., 2000), spermatogenesis (Aydos et al., 2017), and ovulation (Richards et al., 2005; Liu et al., 2017; Liu et al., 2018; Liu et al., 2020) (Fig. 2.3; Fig 2.4.). Various human genetic disorders have also been linked to mutations in ECM metalloproteases (Choi et al., 2019; Lamblin et al., 2002; Cheng et al., 2017; Rim et al., 2020; Duffy et al., 2009; Reiss and Saftig, 2009; Mead and Apte, 2018). Gene knockdown and genetic knockout animal models are powerful tools for teasing out biological roles of metalloproteases in developmental

processes and adult physiology *in vivo*. Zebrafish have a total of 83 metzincin genes in their genome, and four TIMP genes (Huxley-Jones et al., 2007). Here we will focus on involvement of metzincin metalloproteases in sexual differentiation in zebrafish and mammals.

Following establishment of PGCs, the cells must migrate between cells and ECM components to reach the gonadal ridge. During migration, PGCs are found in proximity to and can adhere to collagen IV, fibronectin, and laminin (García-Castro et al., 1997). Metalloproteases are thought to facilitate migration by disassembling ECM fibers in front of migrating cells and untethering the lagging edge of the cell. The *gon-1*, *adamts9* ortholog, in *C. elegans* is necessary for PGC migration and gonad development but did not produce any other significant abnormality when disrupted (Blelloch and Kimble, 1999; Blelloch et al., 1999). In a follow-up study, either *ADAMTS-4* or *ADAMTS-9* from humans can rescue the *gon-1* KO phenotype. Further, GON-1 has an antagonistic role to fibulin in gonad development and fibulin depletion can also rescue the *gon-1* KO phenotype (Hesselson et al., 2004). About the same time of the *gon-1* discovery, a second metalloprotease gene, *mig-17,* which closely aligns to vertebrate *adamts1* was also described to have aberrant migration of distal tip cells (DTCs, leading migrating cells for germ cell migration) when disrupted (Nishiwaki, 1999; Nishiwaki et al., 2000). In *mig-17* mutants, DTCs can migrate but mis-migrate and become detached from the dorsal musculature. In more recent work, *mig-17* and *gon-1* phenotypes could be suppressed by disrupting collagen or fibulin (Imanishi et al., 2020). Similarly, in *AdamTS-A*$^{-/-}$, a *Adamts9* ortholog in *Drosophila,* PGCs mis-migrated and some PGCs did not reach the gonadal ridge (Ismat et al., 2013). In zebrafish, we only found delayed migration in *adamts9*$^{-/-}$ embryos (Carver et al., 2021). In mice, integrin β1 is essential for proper germ cell migration into the gonad ridge and high percentages of integrin β1$^{-/-}$ PGCs were left in the mesentery or hind gut and became ectopic (Anderson et al., 1999).

Motile mouse PGCs express *mmp2*, *mmp9*, *mmp11* and *mt1-mmp* at significantly higher levels than non-motile PGCs (Díez-Torre et al., 2013). Heparan-sulfate proteoglycans (HSPGs) were found by immunohistochemistry near migrating PGCs in zebrafish embryos, and overexpression of *heparanase 1* (*hpse1*) in PGCs resulted in disturbed migration and apoptosis (Wei and Liu, 2014).

Further development of the gonad is preceded by formation of the gonadal ridge, comprised of germ cells, support cells, and stromal cells. Most of our knowledge regarding the formation of the gonadal ridge comes from mouse. In mice, the gonadal ridge originates as a thickening of the coelomic epithelium, with epithelial cells proliferating into a pseudostratified layer. The initial gonad primordium does stain positive for laminin, fibronectin, and collagens but the basement membrane has not been well developed yet (Fröjdman et al., 1989). Once the gonadal ridge has been colonized by PGCs, the basal lamina becomes discontinuous for a brief period allowing for migration of coelomic epithelial cells into the gonadal primordium and these immigrating cells transition into mesenchymal somatic gonadal cell precursors (Karl and Capel, 1998; Kusaka et al., 2010). In XY mice, the coelomic epithelial that invade the testes cord between 10.5-11.5dpc become Sertoli cells (Karl and Capel, 1998). Sertoli cells surround and sequester germ cells in XY mammals, the nascent testes cords are in turn surrounded by collagen type III, IV and V, laminin, fibronectin, and HSPGs (Fröjdman et al., 1989). ECM components are essential for testes cord formation, cultured testes *in vitro* without basement membrane support do not form cords (Hadley et al., 1985; Kanai et al., 1995). In contrast, XX gonads do not show as robust expression of ECM fibers at this stage (Fröjdman et al., 1989).

A few sophisticated studies in *Xenopus* show PGC colonization, envelopment by coelomic epithelium, changes in laminin concentration, and stromal infiltration from the

mesonephros are conserved features of gonadal ridge formation among vertebrates (Piprek et al., 2018a). A similar gonadal ridge structure has been described in teleost fish, but a detailed description in zebrafish embryos is still lacking (Van Winkoop et al., 1992; Nakamura et al., 1998; Nakamura et al., 2006; Devlin and Nagahama, 2002). A study in Medaka showed that *sdf-1a*$^{+}$ somatic gonad cell precursors originate from the lateral plate mesoderm and migrated to form the gonad primordium (Nakamura et al., 2006). Similar studies have not been performed in zebrafish. Nevertheless, it was demonstrated in zebrafish that Wt1 is expressed around the area of the gonadal ridge, and disrupted mesoderm patterning by *hand2*$^{-/-}$ prevented proper localization of PGCs to the gonadal ridge (Weidinger et al., 2002). Significant organization and proliferation of somatic cells, and differentiation of pre-Sertoli and pre-granulosa cells does not begin until ~8dpf, which may mark the establishment of the "true" primordial gonad in zebrafish (Leerberg et al., 2017).

WT1 and GATA4 are two well-studied transcription factors that are essential for establishment of primordial gonads and regulation of differential gene expression during sexual differentiation (Rudigier et al., 2017). Murine GATA4 is necessary for the initial thickening of the coelomic epithelium, as well as subsequent breakdown of basal lamina required for further development (Hu et al., 2013). However, as a transcription factor GATA4 is incapable of causing breakdown of basal lamina by itself. In separate studies of Sertoli and granulosa cell functions, *Gata4* silencing or conditional knockout reduced expression of laminin (*Lamc1)*, collagen (*Col4a1/Col4a5*), *Mmp10, Mmp23, Vcan, Adamts1*, *Adamts2*, and *Timp2*, linking functional GATA4 as necessary for inducing expression of key ECM proteases (Bennett et al., 2013; Schrade et al., 2016). It is likely that GATA4 also regulates metalloprotease expression in the gonad primordium, as dysregulation of MMP activity by exposure to enzyme inhibitors or

activators causes abnormal primordial gonad morphology (Piprek et al., 2019). WT1 binds to the promoter of *adamts16* in mouse gonads and induces expression of ADAMTS16 in kidneys and gonads (Jacobi et al., 2011; Jacobi et al., 2013). In XY animals, strong expression of ADAMTS16 can be found in nascent testis cords between 11.5-14.5dpc whereas females only stained positive for ADAMTS16 on 14.5dpc (Livermore et al., 2019). Two knockout rodent models were used for the examination of the effects of ADAMTS16, one in mouse and the other in rat. Both showed reduced testes weight, but only the rat showed severe cryptorchidism and sterility (Livermore et al., 2019; Abdul-Majeed et al., 2014). In one congenital heart defect study, disruption of the transcription factor *hltf*, which disrupts expression of *wt1* and *gata4*, significantly downregulated expression of *vcan, adam12, adam17, adam19, adamts1* and *adamts9* (Helmers et al., 2013). Taken together, available evidence suggests that GATA4 and WT1 exert their effects on gonad development via regulation of downstream targets and thus expression of ECM fibers and ECM metalloproteases.

Fetal mouse also express *timp1, timp2, timp3* from 14.5dpc onwards and *mmp1,2,3,9,11,12,14* from 11.5dpc onwards. *Timp1* shows differential expression between males and females, with males expressing significantly higher (Guyot et al., 2003). Expression of *adamts9* was also noted around the developing gonad from 13.5-17.5 dpc (Jungers et al., 2005). *Adam3, adam5, adam15* and *adam6* transcripts were detected in fetal rat testes 17dpc, while *adam1* and *adam2* were only found in newborns by postnatal day 2 (Rosselot et al., 2003; Tres and Kierszenbaum, 2005). ADAMTS19 is expressed significantly higher in XX mouse ovaries and is not found in the mouse testes and is expressed several folds higher in male-to-female reversed *sf, dax1*, or *wt1* mutants (Menke and Page, 2002; Bouma et al., 2005; Correa et al., 2012). Specific roles of ADAMTS19 in early ovarian development are unknown, but

ADAMTS19 polymorphism is associated with PCOS and premature ovarian failure in humans (Knauff et al., 2009; Urbanek et al., 2012; Russell et al., 2015).

A recent high throughput transcriptomics study of ovary and testes formation in rats supported earlier findings for ECM and metalloproteases in early gonad formation (Piprek et al., 2018b). Expression of many laminin and collagen genes were distributed around supporting, stromal, and interstitial cells in XX and XY gonads. Versican was also expressed in stromal cells of the ovary, and pre-Sertoli cells and interstitial cells in testes cords. Many *mmp*, *timp*, *adam*, and *adamts* genes are also expressed in the gonads, but only *mmp15*, *adam4,10,17,19,* and *22* are expressed by the germ cells. *Adamts1* and *adamts9* are expressed in all somatic cell types, pre-follicle, ovarian stroma, pre-Sertoli, and interstitial cells. In a knockout study of the tumor suppressing transcription factor p73 (related to p53 and known to play roles in gamete production), mutant testes were found to have defective spermatogenesis and empty seminiferous tubules caused by degenerate Sertoli cells, early dissociation of gametes from testicular epithelium, and increased expression of *adam23*, *mmp23*, and *timp1* (Holembowski et al., 2014). Blocking meiosis-promoting retinoic acid (RA) synthesis by Aldh1a2 antagonism downregulated *sox9a* and *amh*, but increased *dmrt1* and *mmp9* expression in 15-25dpf zebrafish juveniles and disrupts spermatogenesis and oocyte production (Pradhan and Olsson, 2015).

Fetal human testes and ovaries express members of the MMP and TIMP families by 13 weeks of gestation. In prenatal testes, MMP1, MMP2, and MMP9 were found in gonocytes, Sertoli cells, surface epithelium, vascular endothelium, and interstitium. Fetal ovaries expressed MMP1, MMP2, and MMP9 in oocytes, surface epithelium, and vascular endothelium but only MMP1 was found in the ovarian stroma (Robinson et al., 2001). In males, invasion of the primordial gonad by mesonephric interstitial is the major source of fetal Leydig cells (Buehr et

al., 1993; Martineau et al., 1997; Merchant-Larios et al., 1998). In cultured mouse gonads, chemical inhibition of MMPs prevented mesonephric cells from migrating into the gonad. MMP-3 and MMP-10 showed higher activity in male gonads than female gonads in gelatin zymogram (Nishino et al., 2002). Co-cultured Sertoli cells and peritubular myoid cells expressed multiple proteases, including multiple high molecular weight gelatinases (Sang et al., 1990). In the sex-reversing marbled swamp eel, *Synbranchus marmoratus*, *mmp2, mmp9, and mmp14* are all expressed highly by interstitial cells during sex reversal (Mazzoni et al., 2018). The rise of *mmp* activity is concomitant with alterations of basement membrane the germinal epithelium rests upon. At the completion of sex reversal, *mmp* activity lowers in the nascent testes (Mazzoni et al., 2018).

Functions of the metzincin metalloproteases in gonad development have not been well studied in vertebrates including zebrafish. Although multiple research groups have found expression of metalloproteases in the gonads (Liu et al., 2017; Liu et al., 2018; Liu et al., 2020; Brunet al., 2015; Mazzoni and Quagio-Grassiotto, 2021; Liu et al., 2022), few phenotypic characterizations have been reported. The expression of *gata4* in the gonad around 10dpf, before significant development of gonads, has been suggested to be involved in expression of metalloproteases, which likely may facilitate development of gonads in zebrafish (Leerberg et al., 2017). Although early PGCs (<16hpf) did not have positive immuno-staining of *mmp2, mmp9,* or *mmp14,* adjacent somatic cells along the dorsal mesentery did react positively (Mazzoni and Quagio-Grassiotto, 2021). *Mmp* expression remained in the dorsal mesentery at all timepoints, then in appeared in bipotential germ cells of the undifferentiated gonad at 7dpf in zebrafish (Mazzoni and Quagio-Grassiotto, 2021). A basal lamina layer was located around somatic gonad cells at 11dpf (Leerberg et al., 2017).We found low expression of *adamts9* in

PGCs and widespread expression in somites (Carver et al., 2021). This expression was shifted from germ cells to somatic follicular cells, which was dramatically elevated prior to ovulation (Liu et al., 2017; Liu et al., 2018; Liu et al., 2020). New single cell RNA sequence (scRNAseq) data from zebrafish ovaries revealed multiple Mmps, Adams, and Adamts expressed in juvenile ovaries, though their functions remain to be defined (Liu et al., 2022). The next critical step is to find out what are the functional effects of metalloproteases on gonad development.

Metalloproteases in Folliculogenesis

Ovarian follicles express metalloproteases that have roles for continued growth and survival of the follicles (Russell et al., 2015; Zhu, 2021). In mammals, multiple studies show expression and function of ECM fibers and metalloproteases for continued ovarian folliculogenesis (Berkholtz et al., 2006; Ambekar et al., 2013; Heeren et al., 2015; Tomaszewski et al., 2021). Significantly less evidence is currently available in zebrafish despite the progression of folliculogenesis being similar in both mammals and fish. Therefore, we will evaluate evidence of metalloproteases in folliculogenesis mainly from mammalian studies but also include studies of fish species when they are available.

In both fish and mammals, germ cells initially form interconnected cysts of meiotic oocytes (Stage IA in zebrafish) before cyst breakdown and formation of individual follicles (Selman et al., 1993; Nakamura et al., 2010; Tingen et al., 2009; Niu and Spradling, 2022). After the breakdown of oocyte cysts, individual oocytes become encased by somatic pre-granulosa cells to form the primordial follicles (Pepling and Spradling, 2001; Mala,ira et al. 2011; Lechowska et al., 2011; Wang et al., 2015; Qin et al., 2018; Pepling et al., 1999; Pepling, 2006; Sarraj and Drummond, 2012; Elkouby and Mullins, 2017). When the primordial follicle becomes activated, follicle cells change shape and a second layer of somatic cells become recruited as the

primordial follicle transitions into a primary follicle in mammals (Hope, 1965; Fortune, 1994; Fortune et al., 2000). The oocyte secretes paracrine signals to direct functions and signaling of follicular cells (Gilchrist et al., 2004; Doherty et al., 2022). Mammals have two types of granulosa cells, the cumulus granulosa located proximally to the oocyte, and the mural granulosa located between the layers of theca cells and cumulus granulosa cells. In mammals, basement membrane builds up and separates two layers of theca cells, theca interna and theca externa. As follicular fluid is formed and begins to coalesce, the primary follicle becomes a secondary or preantral follicle. The next stage of folliculogenesis is defined by the formation of a large fluid cavity in the follicle, called the antrum. From the antral follicle, the fluid continues to expand until the oocyte is ovulated (Hennet and Combelles, 2012). In zebrafish, the primordial/primary follicle is referred to as Stage IB. Unlike mammals, zebrafish only contain one layer of granulosa, and one layer of theca cells. As the zebrafish oocyte matures, it expands within the follicle and accumulates yolk granules as nutrition storage for the external development of embryo. A fully matured zebrafish oocyte can reach 800μm in diameter (Nagahama and Yamashita, 2008; Lubzens et al., 2010).

Gene expression and proteomic analyses of follicular cells/fluid have uncovered multiple metalloproteases expressed within ovarian follicles in zebrafish, rats, mice, bovines, hamsters, and humans (Shindo et al., 2000; Carver et al., 2021; Zamah et al., 2015; Russell et al., 2015; Zhu, 2021). Expression of metalloprotease activities are differentially regulated during follicle development and in different reproductive cycle stages in mammals and fishes (Shindo et al., 2000; Carver et al., 2021; Zamah et al., 2015; Bagavndoss, 1998; Vrooman and Young, 2010; Smith et al., 2002: Curry and Osteen, 2003; Goldman and Shalev, 2004). *Adam10* is necessary for breakdown of germ cysts and formation of primordial follicles by regulating differentiation

and proliferation of progenitor cells in mice (Feng et al., 2016). Timp2b was found in zebrafish ovarian follicles, and its expression was upregulated during ovulation by hCG (Liu et al., 2020). MMP-2 and MMP-9 were found in bovine preantral follicles and used for predicting follicle health (McCaffery et al., 2000). Blocking metalloprotease activation via oocyte-specific *furin* deletion caused female infertility due to secondary follicle arrest in mice (Meng et al., 2017). Deletion of inhibin-α induces ovarian cancer and abnormal granulosa cell activity in mice associated with abnormal expression of brevican, *mmp2*, *col1a2*, *col4a6*, and *adamtsl2* (Nagaraja, et al., 2010). In rats, Mmp23 was found in granulosa cells of preantral and small antral follicles, but not in large antral follicles (Ohnishi et al., 2001).

ADAMTS1 is one of most studied members of the ADAMTS family in mouse ovaries. Reduced fertility was found in ADAMTS1 KO mice (Shindo et al., 2000; Mittaz et al., 2004). In mice, *adamts1* is essential for folliculogenesis and development of antral and periovulatory follicles was significantly reduced in *adamts1* null mice (Brown et al., 2006). Histological sections revealed multiple abnormal follicles lacking granulosa cell layers (Shozu et al., 2005). Closely related to *adamts1, adamts9* transcripts and proteins were found in ovarian stromal cells and inside ovarian follicles (Carter et al., 2019; Carver et al., 2021; Liu et al., 2022). Adamts9 is essential for ovarian development in zebrafish, *adamts9*$^{-/-}$ have male biased sex ratio and female infertility in adult zebrafish (Carter et al., 2019). Our recent studies suggest progressive loss of follicles after sex determination is the main cause of female to male sex reversal (Carter et al., 2019; Carver et al., 2021). In addition to lowered gonad mass proportional to body mass and underdeveloped ovaries, *adamts9*$^{-/-}$ fish had an accumulation of Stage IB oocytes (equivalent to mammalian primary follicles) that did not progress to later stages of oogenesis (Carter et al., 2019). Because of embryonic lethality in *adamts9* null mice, roles of *adamts9* in murine

folliculogenesis have not been studied extensively (Kern et al., 2010). Zebrafish also express *adamts1* in ovarian follicles, in addition to *adamts9* (Liu et al., 2017). The effects of *adamts1* in zebrafish folliculogenesis are still uncertain, but it appears *adamts1* is not fully able to compensate for *adamts9* loss in zebrafish. Similarly, *adamts9* does not compensate for *adamts1* loss in mice, suggesting non-redundant roles of *adamts9* and *adamts1* in folliculogenesis.

Perspectives and future directions

Much remains to be discovered concerning roles of metalloproteases in the formation of functional gonads. These metalloproteases share redundant functions with each other and co-express in the same cells which makes many single knock-out phenotypes unnoticeable. We propose double, triple, or higher-order number of multiple global or conditional knockouts, or inhibition of enzyme activities, are likely to be more useful in elucidating functions of metalloproteases in gonad development and folliculogenesis. Another exciting development in the field of sex determination and differentiation comes from studies utilizing high throughput transcriptomic analyses including scRNAseq. These approaches yield new insights of genes and pathways differentially regulated between males and females and provide a good framework for future studies. The high throughput and unbiased nature of RNAseq makes it ideal for identifying novel differentially expressed genes. Thirdly, much remains to be discovered about the metalloproteases themselves, particularly ADAMTSs. Due to their large size (Mw>100K Da), it is difficult to generate a full-length recombinant protein for each member of Adamts, and therefore impedes functional analyses of these metalloproteases including studies of substrates, binding partners, and crystal structures etc. These metalloproteases may also behave unexpectedly *in vivo* than those from biochemical studies reported *in vitro*. New approaches and new techniques need to be developed to overcome these challenges. Finally, study on activation

of these metalloproteases is still lacking mainly due to lack of biochemical reagents and/or sensitive bioassays for majority of these metalloproteases. Determining enzymatic activities is more critical than monitoring changes of transcripts and proteins of these metalloprotease enzymes, which are pre-synthesized, pre-secreted, and pre-stored in a latent form. A recent report suggesting on involvements of blood coagulation in the activation of metalloproteinases, and development of both *in vivo* and *in vitro* ovulation bioassays is likely to open a new research area (Jing-Huang et al., 2022). In summary, studying the effects of the ECM and metalloproteases in gonads will likely fill in an important knowledge gap, and cross the bridge from nuclear signaling and activities of transcription factors to cellular signaling and functions, and then cell surface/intracelluar signaling and functions. In turn, knowledge of metalloprotease functions will provide new insights into treatments of fertility or disorders of sex in the future.

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Figure 1.1

Gonad development in zebrafish. In a maternal oocyte, the evolutionarily conserved Balbiani body contains germ plasm, which is segregated in the succeeding embryo. Cells that inherit germ plasm become primordial germ cells (PGCs), which then migrate to the gonadal ridge. Between 7~20 dpf, the germ line and somatic gonad cells rapidly proliferate and develop into pre-granulosa and pre-Sertoli cells. As a protogynous species, zebrafish initially develop into an ovary-like biopotential gonad with the presence of stage IA/IB oocytes. In female fish, these early-stage oocytes persist within their follicles and continue to develop further into late-stage follicles (II, III, and IV). In male fish, oocytes are lost, and the remaining somatic and primordial germ cells transition into cells of the testes.

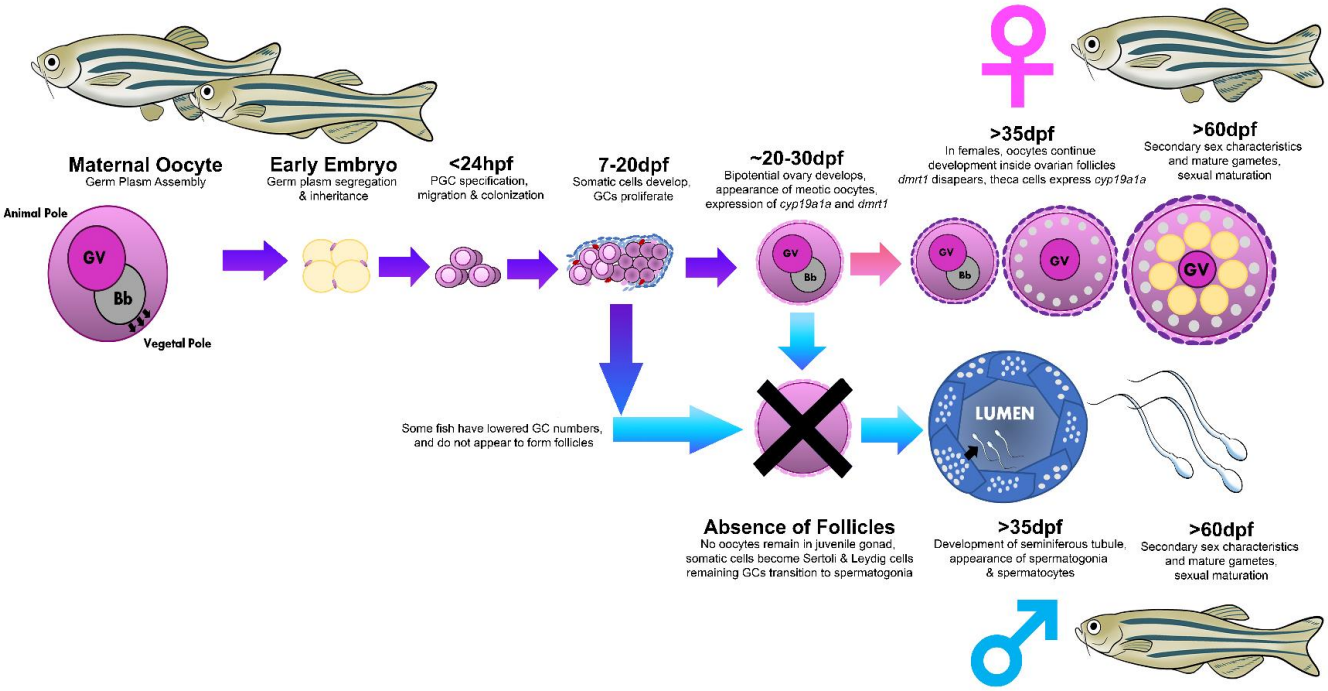


Figure 1.2

Known genes essential for proper gonad development and sex determination in zebrafish. Expression of *fgf24*, *gata4*, *wt1a*, *cxcl12a*, *dnd1*, and *nr5a1a* is essential for germline maintenance and somatic gonad development prior to gonadal differentiation. The bipotential gonad (i.e., ovary-like in zebrafish) co-expresses sex determining genes *dmrt1*, *amh*, *cyp19a1a*, *sox9a*, and *sox9b*. Though the exact interplays between genes during sex determination is still not fully understood in zebrafish, some genes are known to be consistently upregulated in males vs. females, and vice versa. The outcomes of these expression profiles lead to observed changes in cell functions and gonad architecture.

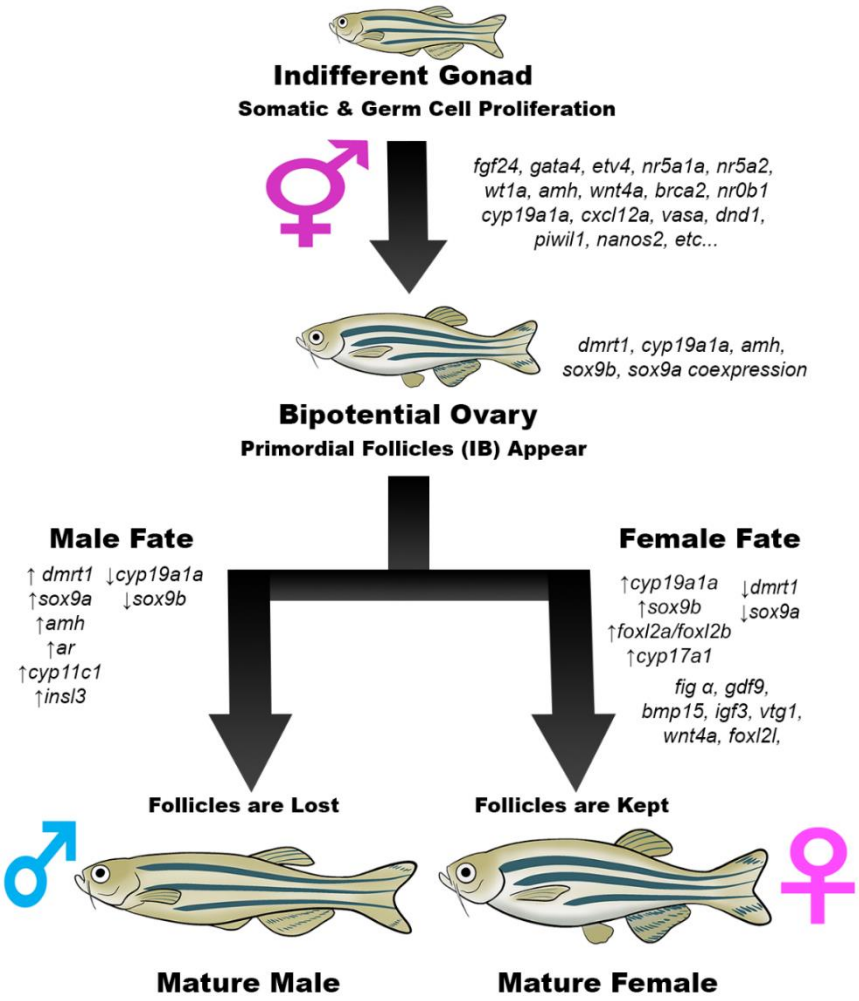


Figure 1.3

Functions of metalloproteases. Left side drawings show that metalloproteases are secreted or anchored onto the cell surface where a convertase, Furin, cleaves off the pro-domain and activates the metalloprotease. The active metalloprotease can be reversibly inhibited by the presence of tissue inhibitors of metalloproteases (Timps). Right side of figure illustrate a few known functions for metalloproteases in cell physiology including degradation of ECM fibers, ectodomain shedding, cell migration, cell proliferation, and signal transduction.

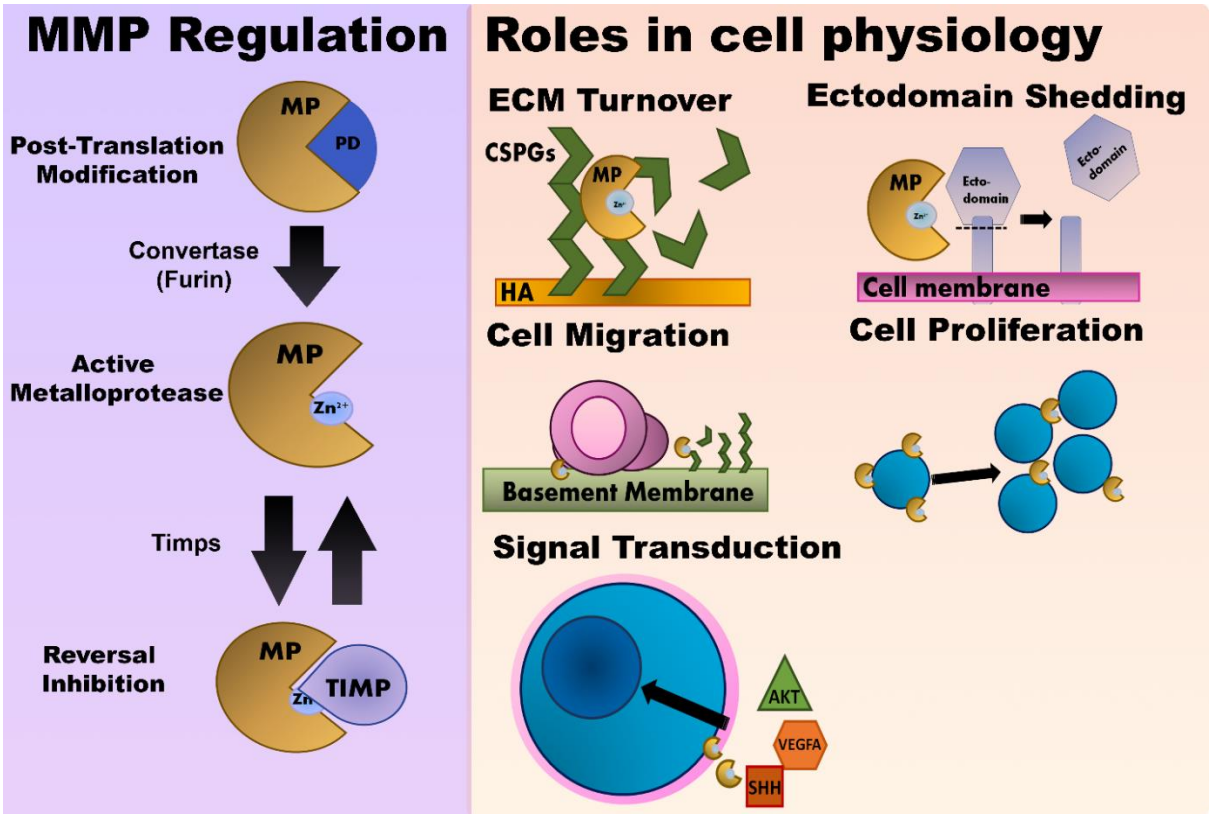
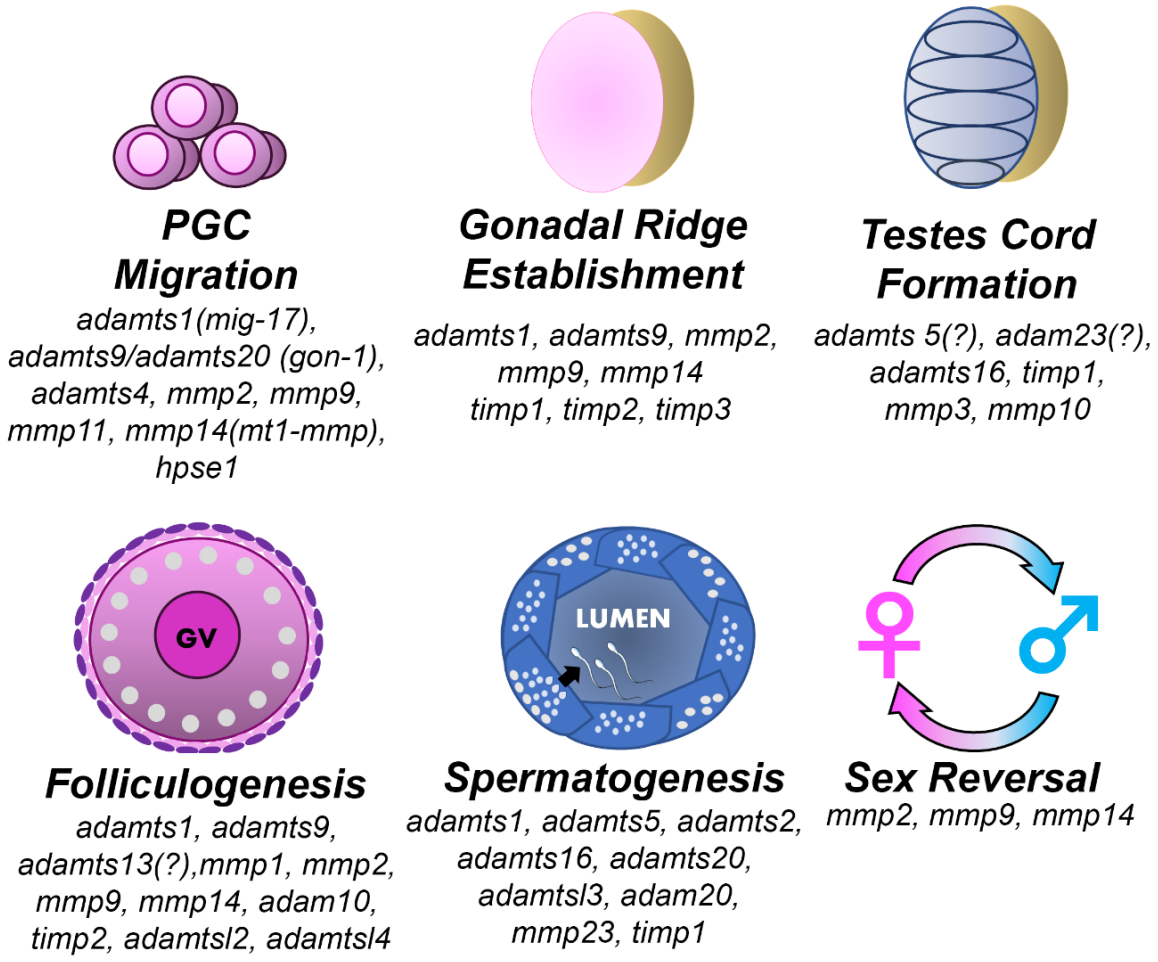


Figure 1.4

Known roles of metalloproteases in gonad development and sex differentiation.

Metalloproteases are involved in diverse processes during gonad development including PGC migration, gonadal ridge establishment, testes cord formation, folliculogenesis, spermatogenesis and sex reversal. *Adamts1, adamts9,* and *mmps* have been demonstrated to be involved in PGC migration. Multiple *mmps, adams,* and *adamts* are expressed and likely play roles during gonadal development. *Adamts16* facilitates testes cord formation. *Adamts1, adamts9, adam10, timp2,* and *mmps* are expressed in ovarian follicles, while *adamts2, adamts16, adamts20 and adamtsl3* have been shown to have roles in spermatogenesis.



CHAPTER III: Widespread and Transient Expression of *adamts9* in Developing and Adult Zebrafish

He, Y., Carver, J.J., Erickson, T.P., Zhu, Y. (*in preparation*). Widespread and transient expression of *adamts9* in developing and adult zebrafish.

Chapter Summary

Our published results have demonstrated that Adamts9 (a disintegrin and metalloprotease with a thrombospondin type-1 motif, member 9) has been shown to be involved in ovulation and the migration of primordial germ cells in zebrafish. In the present study, we determined *adamts9* expression in adult tissues and embryonic cells at high resolution by confocal imaging analyses of a newly generated transgenic zebrafish lines (*Tg(adamts9:EGFP)*) in addition to analyses using real-time PCR and *in situ* hybridization. The expression of *adamts9* was found in wide range of zebrafish adult tissues and cells, including preovulatory follicular cells, oocytes, testis, brain, eye, pectoral fin, kidney, liver, intestine, skin, gill, muscle, bone, and heart. High expression levels were found in the preovulatory follicular cells, retina, kidney cells and testis, while lower expression levels were found in the liver and growing ovarian follicles (stage II and III). Furthermore, mRNA *in situ* hybridization targeting *adamts9* confirmed these expression patterns including high expression of *adamts9* in preovulatory ovarian follicular cells and ciliary marginal cells of eyes at hours post fertilization. In addition, high expression of *Tg(adamts9:EGFP)* in gonads was observed at 4 and 6 wpf (weeks post-fertilization) during primary sex determination and early transition of Stage IA to Stage IB ovarian follicles. Our results indicated that Adamts9 is widely expressed in zebrafish with high expression in preovulatory follicular, ciliary marginal, and kidney cells. Interestingly, Adamts9 expression changes according to developmental stage and physiological status.

Introduction

Metalloproteases play critical roles in morphogenesis, tissue remodeling, and cell migration, all of which are important in physiological processes. The ADAMTS (a disintegrin and metalloprotease domain with thrombospondin type 1 motif) proteases are a family of 19 secreted mammalian metalloproteases (Apte, 2004). From genome-wide association studies (GWAS), *ADAMTS9* polymorphisms in humans are associated with various human diseases, such as diabetes (Apte, 2004; Artunc-Ulkumen et al., 2016; Zillikens, 2017), asthma (Paulissen et al., 2009), arthritis (Yang et al., 2017; Mead and Apte, 2018), artery calcification (Franceschini et al., 2018; Suna et al., 2018), macular degeneration (Singh and Tyagi, 2017), and cognitive aging (Rosenberg, 2017). Previous studies suggested that *ADAMTS9* is expressed in all human fetal tissues examined and most adult tissues by Northern blot and RT-PCR analyses (Clark et al., 2000; Jungers et al., 2005). *Adamts9* is also found to be highly expressed throughout mouse embryo development by RNA in situ hybridization and RT-PCR analysis (Somerville et al., 2003; Jungers et al., 2005). Attempted *Adamts9* knockouts in mice caused death before gastrulation, highlighting the importance of functional ADAMTS9 in vertebrate development (Kern et al., 2010). *Xenopus* models show an evolutionarily conserved expression patterns of *adamts9* compared with mouse, suggesting that the expression and function of *adamts9* during development is conserved among diverse groups of vertebrates (Desanlis et al., 2018).

In our previous study, we found multiple phenotypes caused by Adamts9 KO in zebrafish (Carter et al., 2019). These included delayed growth, spinal deformities, poor survivability, PGC migration delay, female infertility, and heavily male biased sex ratios (Carter et al., 2019; Carver et al., 2021; Carver and Zhu, *in preparation*). A separate group also recently reported a Adamts9 KO line as part of a forward genetic screen, and confirmed spinal and eye deformities, as well as demonstrated that Adamts9 KO had kinky barbels and significantly wider central canal in the

vertebrae (Gray et al., 2020). Despite these striking phenotypes, systematic investigation of *adamts9* adult expression levels and high-resolution cellular expression is currently lacking in zebrafish. Known expression patterns includes Stage IVb follicles determined by qPCR, next-gen sequencing, transgenic reporter, and immunostaining (Liu et al., 2017; Liu et al., 2018; Carter et al., 2019; Carver et al., 2021), brain, ciliary marginal zone, melanocytes, lateral line, cloaca, and fin rays by *in situ* hybridization (Gray et al., 2020); and all adult organs tested by RT-PCR (Brunet et al., 2015). However, expression levels between different organs, different developmental stages, and cellular localization are currently still unclear.

To better determine tissue-, developmental-, and cellular-specific gene expression patterns and changes of *adamts9* in zebrafish, we generated a new transgenic reporter line to levels to visualize spatial and temporal distribution at the cellular level. The Tol2 transposon system was used to generate a transgenic zebrafish reporter line (*Tg(adamts9:EGFP)*), in which a ~4.5 kb proximal promoter region is fused to the coding region of EGFP. The *Tg(adamts9:EGFP)* was then inserted into the zebrafish genome and drove EGFP expression in *adamts9* expressing cells. The advantage of the transgenic reporter approach is high sensitivity, ability to rapidly observe multiple tissues without extensive staining or washing procedures, and spatial and temporal resolution at the cellular level. The cloned promoter region mimicked the expression profile of endogenous *adamts9* transcripts in zebrafish and reliably revealed cells in which *adamts9* was being transcribed in both larvae and adults. Embryonic, larval, and follicular expression was subsequently confirmed by real-time quantitative PCR (qPCR) and mRNA *in situ* hybridization.

Materials and methods

Animal Husbandry

Wildtype AB strain and transgenic zebrafish lines were housed in the zebrafish core facility with a 14-hour(h) light and 10h dark photoperiod, at water temperature of 28.5°C, pH of approximately 7.2, and salinity/conductivity ranging from 500 to 1,200μS in an automatically controlled zebrafish rearing system (Aquatic Habitats Z-Hab Duo systems, Florida, USA). Fish were fed to satiation three times daily with a commercial food (Otohime B2, Reed Mariculture, CA, USA) containing high protein and lipid content, and supplemented with newly hatched brine shrimp *Artemia* (Brine Shrimp Direct, Utah, USA). The Institutional Animal Care and Use Committee (IACUC) at East Carolina University have approved all experimental protocols.

Cellular expression of adamts9 analyzed by adamts9 promoter driven EGFP

Briefly, a 4.5-kb upstream of *adamts9* start codon was cloned into pGEM-T easy vector (Promega), Sanger sequence confirmed and then subcloned into a p5E-mcs entry vector. Next, multisite Gateway cloning (Invitrogen) was conducted with a 5' entry vector containing 4.5 kb *adamts9* promoter sequence, a middle entry vector containing EGFP, a 3'entry vector with stop poly A signal, and a destination vector that expresses a GFP selection marker specifically in the lens of eye (Kurita et al., 2003). In this final vector, the expression of EGFP is controlled by *adamts9* promoters. Transgenic embryos with the insertion could be easily selected by an eye marker that displays green fluorescence at 48 hpf under a fluorescent dissecting microscope. About 500 nl mixture containing 20 ng/ml construct, 20 ng/ml Tol2 transposon, and phenol red indicator were microinjected into the one-cell stage of embryos. Multiple F0 transgenic embryos were selected based on the lens selection marker, raised to adults, crossed with AB wildtype, then produced several F1 lines. Subsequently, we established multiple F2 stable transgenic lines (*Tg* (*adamts9: GFP*)) with stable expression of EGFP in the zebrafish.

Collection of different adult tissues and extraction of total RNA

Different adult tissues (preovulatory stage IVb follicles, ovary, testis, brain, eye, pectoral fin, kidney, liver, intestine, skin, gill, muscle, bone, and heart) were collected from 4-month-old AB wildtype zebrafish. Samples were placed in 1.7 ml RNase-free microcentrifuge tubes (GeneMate) containing 200 μl RNAzol (Molecular Research Center, Inc., OH. Catalog: RN 190) and homogenized immediately. Total RNA was extracted from homogenized solutions according to the manufacturer's protocol. For each sample, cDNAs were synthesized using 2 μg total RNA and a high-capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA, Catalog#4368814) following the manufacturer's instructions.

PCR amplification of adamts9

A set of PCR primer (forward: 5'-GCGGTACGCGTGGTAAAATC-3'; reverse: 5'-AGGCATGTGGACATAACGCA-3') targeting 1181bp of 3'-UTR of adamts9 was used for PCR amplification. PCR amplification was carried out using a Taq DNA polymerase (New England Biolabs, Ipswich, Massachusetts, USA, Catalog#0273) with initial denaturation at 95 °C for 2 minutes followed by 35 cycles of 30 seconds denaturation at 95 °C, 30 seconds annealing at 65 °C, and 60 seconds elongation at 68 °C. Zebrafish *eukaryotic translation elongation factor 1 alpha 1a* (*eef1a1a*) showed stable expression in different tissues and different developmental stages. Therefore, it was used as a housekeeping gene control. A set of PCR primers targeting 242 bp of the coding region of *eef1a1a* (forward: 5'-AGTGTTGCCTTCGTCCCAAT-3'; Reverse:5'-CACACGACCCACAGGTACAG-3') was used for PCR amplification. The efficiency of the PCR and authentic PCR products were confirmed by gel electrophoresis analysis and Sanger DNA sequencing. The concentrations of these plasmids were quantified on

Nanodrop 2000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA), serially diluted and used as DNA templates for generating standard curves described in the following paragraph.

Real-time quantitative PCR (qPCR) amplification of adamts9

The levels of *adamts9* transcripts in different tissues were determined by real-time quantitative PCR (qPCR) using SYBR green dye (Invitrogen) and a CFX Connect real-time thermal cycler (Bio-Rad Laboratories, Hercules, California, USA). The qPCR reaction was conducted with initial denaturation at 95 ˚C for 3 minutes, followed 45 cycles of 30 seconds denaturation at 95 ˚C, 30 seconds annealing at 65 °C, and 30 seconds extension at 72 °C using the specific primers (Forward: 5'- CTGTCTGCGCGGTGATTCTA -3'; Reverse: 5'-CTCTTGCAGGGGCGTGATTA -3') and GoTaq G2 DNA polymerase (Promega, Madison, Wisconsin, USA). Each PCR mixture (15 µl) consisted of 7.795 µl DNase free water, 3 µl 5XGoTaq buffer, 1.5 µl 25 mM $MgCl_2$, 0.3 µl 10 mM dNTP mix, 0.15 µl 10 mM forward or reverse primer, 2 µl 5X diluted cDNA, 0.03 µl 100X SYBR green dye (final concentration 0.2X), and 0.075 µl Taq. The transcript levels, expressed as absolute values (copies/µg total RNA), were determined using Ct values of samples and a standard curve generated from serial known concentrations of plasmid containing the target region of *adamts9*. The efficiency of the PCR and authentic PCR products were further confirmed by analyses of melting curve, gel electrophoresis, and Sanger sequencing.

In situ hybridization

Antisense or sense whole mount in situ hybridization (WISH) probe corresponding an 808-bp segment of the zebrafish *adamts9* was PCR amplified from a pCR 4-TOPO vector (Invitrogen, Carlsbad, CA), in which a fragment of *adamts9* was already cloned using a set of

PCR primers (forward: 5'- ATACAGCGGCTCTGACCATG-3'; reverse: 5'-CGACTCGGGTTTGGATGAGT-3'). A 1181-bp segment of the zebrafish *adamts9* poly(A) tailed was PCR-amplified (forward: 5'-GCGGTACGCGTGGTAAAATC-3'; reverse: 5'-AGGCATGTGGACATAACGCA-3') and ligated into a pCR 4-TOPO vector (Invitrogen, Carlsbad, CA). Specimens were fixed in freshly prepared 4%-PFA, then permeabilized with proteinase K treatment. Samples were submerged in 1ml of the hybridization solution containing 200ng of probe and incubated over night at 70ºC. Samples were subsequently washed and reacted with secondary anti-DIG antibody (Roche Diagnostics, Indianapolis, Indiana). The colorization reaction was allowed to proceed until strong coloration was detected. Samples were then washed with methanol to remove background staining and photographed.

Statistical Analysis

All results were presented as mean ± SEM. One-way ANOVA was used to analyze the effect of genotypes on expected outcomes of gene expression in different tissues. Statistical significance was set at $p < 0.05$. GraphPad Prism 8.0 (GraphPad Prism Software Inc., San Diego, CA, USA) was used to make histograms and to run statistical analyses.

Results

Expression of adamts9 gene in different tissues of adult wildtype zebrafish

Expression of *adamts9* was found in the ovary, testis, brain, eye, pectoral fin, kidney, liver, intestine, skin, gill, muscle, bone, and heart by qPCR (Fig. 3.1). Gene expression of *adamts9* was highest in preovulatory stage IVb follicles (Fig. 3.1). The highest and lowest gene expression of adamts9 were observed in the testis and liver, respectively. The expression of *adamts9* was significantly higher in the testis than in the ovary, liver, intestine, muscle, or bone (ANOVA, $p < 0.05$) (Fig 3.1).

Confocal imaging and analyses of GFP expression in different tissues of F2 transgene (Tg(adamts9:GFP)) embryos during embryonic development

Significant zygotic expression of GFP was observed around the 8-somite stage of embryos 10 hpf (hours post fertilization) in the embryos from crossing of F2 transgenic males (*Tg(adamts9:GFP*)) and non-transgenic AB females (Figs. 3.2c, 3.4, 3.4); however, maternal expression was observed as early as at one cell stage in the embryos from crossing of F2 transgenic females and non-transgenic AB males (Figs. 3.2-3.3). Maternal expression faded away when zygotic expression kicked in. The GFP expression of Adamts9 was widely observed in different adult tissues (Figs. 3.4-3.10). Strong and transit GFP expression driven by *adamts9* promoters were observed in both retina and brain at ~36 hpf (hours post-fertilization), with strongest expression found in the retina at 48 hpf and then gradually decreased at 72 hpf (Figs. 3.4-3.5). Strong GFP expression driven by adamts9 promoters were also observed in notochord, muscle, and pectoral fin of the fish at 48 hpf (Fig. 3.4). The GFP expression was also found in the heart and posterior lateral line primordium (PLLP) of the fish at ~78 and ~60 hpf, respectively (Figs. 3.4, 3.6). The GFP expression in the craniofacial muscle and blood vessels of the fish were also observed at 48 and 72 hpf (Fig. 3.6-3.7).

Confocal imaging and analyses of GFP expression in different tissue of F2 transgene line (Tg(adamts9:GFP)) in adult zebrafish

Strong GFP expression driven by *adamts9* promoters were found in adult testis of zebrafish (Fig. 3.8). In ovaries, the strong GFP expression driven by *adamts9* promoters was observed at both 4 and 6 wpf (weeks post-fertilization). In addition, the GFP expression in stage IV preovulatory follicular cell was among strongest (Fig. 3.9), while weak or no GFP expression was observed in immature female follicles (stage I, III) or ovulated stage V oocytes (Fig.3.2).

Strong GFP expression was also observed in the gill, kidney, pectoral fin, preovulatory follicular cells, ciliary marginal cells of eyes of adult zebrafish (Fig. 3.10). Whole mount mRNA *in situ* hybridization confirmed strong expression of *adamts9* in 24 hpf retina, 72 hpf ciliary marginal zone, brain, and preovulatory follicular cells (Figs. 3.11-.3.13)

Discussion

ADAMTS9 was found to be expressed in all human fetal tissues and some adult tissues examined by Northern blot and RT-PCR analysis (Clark et al., 2000; Jungers et al., 2005). In this study, *adamts9* is observed to be widely expressed in different developing and adult zebrafish tissues, similar to that in mouse and *Xenopus* models (Jungers et al., 2005; Desanlis et al., 2018). Interestingly, the *adamts9* expression level was the highest in the adult testis compared to every other organ. This is surprising with respect to our earlier study, which indicates that adamts9 is necessary for the gonadal development in female zebrafish but not for males (Carter *et al*., 2019). The most likely explanation is that another metalloprotease with redundant function can compensate for Adamts9 loss in testis, but not in ovarain development (Carter *et al*., 2019) due to dramatic expansion of Adamts members in vertebrates (Brunet et al., 2015). The lowest gene expression of *adamts9* was observed in the liver, which was similar to that reported in mouse, (Jungers et al., 2005). This suggests that it is unlikely for disrupted vitellogenin synthesis to be the main cause of the observed deficits in ovarian development and oocyte maturation in Adamts9 KO zebrafish (Carter et al., 2019; Levi et al., 2009; Li and Zhang, 2017).

To further investigate expression patterns of *adamts9* at a high resolution, we used the Tol2 transposon system to generate a transgenic zebrafish strain *Tg(adamts9:GFP)*. In this study, the GFP expression in both the retina and brain were observed at ~36 hpf, and the retina exhibited strong signaling at 24 hpf. In transgenic zebrafish line (*Tg(cyclin B1: GFP)*), the retinal GFP

expression was also found at a similar time point (36 hpf) (Kassen et al., 2008). A recent finding suggests the Adamts9 mutations cause Nephronophthisis-related ciliopathies (NPHP-RC) and that Adamts9 is required for the formation and function of primary cilia (Choi et al., 2019). NPHP-RC is a clinically and genetically heterogeneous disorder that causes dysplastic or degenerative diseases in the retina and central nervous system (Hildebrandt and Zhou, 2007). These results implied that Adamts9 is required for the development and function of the retina and central nervous system. In addition, the GFP expression of Adamts9 observed in the fish's pectoral fin at 48 hpf may suggest role of metalloproteinase in the development of fin as suggest role of Adamts9 in the development of limb in mouse model. In adult transgenic zebrafish, strong GFP expression of Adamts9 was observed in the tissues of gill, kidney, pectoral fin, and eye, which is consistent with results obtained using qPCR.

Strong GFP expression in *Tg(adamts9:GFP)* was also found in notochord at 48 hpf. The notochord is critical for the normal development of vertebrate embryos. Adamts9 was associated first with the initial condensations of mesenchyme that forms the cartilage centers and subsequently with the perichondrium around formed cartilage (Jungers et al., 2005). Perichondrium expression is significant since Adamts9 is capable of degrading versican, an important component of this tissue (Shinomura et al., 1993). In another Adamts9 KO fish, it was reported KOs have significantly wider central canals than wildtype controls (Gray et al., 2020). However, Gray et al. failed to detect adamts9 expression in notochord, likely due to low sensitivity of in situ method they were using. In Choi et al., 2019, human individuals carrying *ADAMTS9* mutations were reported to have macrocephaly, or abnormal accumulation of cerebrospinal fluid (CSF) in the brain.

The circulatory system is one of the first organ systems to begin functioning during vertebrate development, and its proper assembly is critical for embryonic survival (Kamei et al., 2010). Blood vessels innervate all other tissues, supplying them with oxygen, nutrients, hormones, and cellular and humoral immune factors. In this study, the GFP expression of Adamts9 in blood vessels of the fish was observed at 2dpf with strong expression at 3dpf. Intriguingly, we also observed a few GFP flowing cells inside the blood vessels. A previous study reported that some non-granulosa cells locating at the branches of the interstitial blood vessels exhibited strong GFP signals in transgenic zebrafish (*Tg (pgr:eGFP)*) (Huang et al., 2020).

The first skeletal muscle formation in zebrafish occurs in the pairs of somites that form along the animal's trunk (Sanger et al., 2009). In this study, the GFP expression of Adamts9 in the muscle of the fish were observed at 48 hpf and then this expression persisted into adult zebrafish. Using a gon-1 promoter fused to GFP, GFP expression was observed in muscle cells throughout the development of *C. elegans* (Blelloch and Kimble, 1999). By BLAST analysis, metalloprotease domain of zebrafish Adamts9 shares 55% identity with that of gon-1, a ortholog in *C. elegans* in our recent report (Carver et al., 2021). In addition, Adamts9 of this study was also detected in skeletal muscle by RT-PCR and qPCR similar as those found in other vertebrates (Clark et al., 2000; Yaffe et al., 2010). Vertebrate craniofacial muscles are highly conserved during evolution (Noden and Francis-West, 2006). In the current study, the GFP expression of Adamts9 in the craniofacial muscle was found at 2 dpf. GFP signaling got stronger at 3dpf, implying that Adamts9 might play a role in the craniofacial myogenesis of zebrafish. The GFP expression of Adamts9 in PLLP was also observed during embryonic development.

Development of the posterior lateral line system in zebrafish involves cell migration, proliferation, and differentiation of mechanosensory cells (Gallardo et al., 2010).

Zebrafish are a gonochoristic species (two distinct sexes) of teleost that utilize transitory hermaphroditism. Juveniles first form undifferentiated ovaries containing primordial oocytes before either undergoing apoptosis to develop as testes or maintaining the follicles to develop as ovaries (Takahashi, 1977). In this study, the strong GFP expression of adamts9 in ovaries was observed at both 4 and 6 wpf, and testis showed a strong GFP signaling with higher expression of *adamts9*. In addition, GFP expression was observed in preovulatory follicles (stage IVb), which is consistent with our previous report on dramatic increase of adamts9 expression in stage IV preovulatory follicular cells, just prior to occurrence of ovulation (Carver et al., 2021). Because Adamts9 KO caused a male sex bias, and does not seem to affect male fertility, we hypothesize that another Adamts protease that is known to share overlapping enzymatic function with Adamts9 (in particular Adamts1, Adamts5, Adamts8a/Adamts8b or Adamts15a) may be able to compensate for Adamts9 loss in testes development but not in the maintenance of ovarian follicles (Brunet et al., 2015; Liu et al., 2018; Carter et al., 2019; Carver et al., 2021).

In summary, Adamts9 is widely expressed in zebrafish. Analyzing *Tg(adamts9:GFP)* expression in zebrafish provided leads toward new routes to study the role of adamts9 in reproduction. Further studies are required to elucidate the functions and mechanism of Adamts9 in gonad formation in vertebrates.

Figure 3.1

The expression of *adamts9* in different tissues of adult wildtype zebrafish determined by qPCR. Results were presented as mean ± SEM (n = 6). Different letters above the error bars indicate that those groups are significantly different at $p < 0.05$.

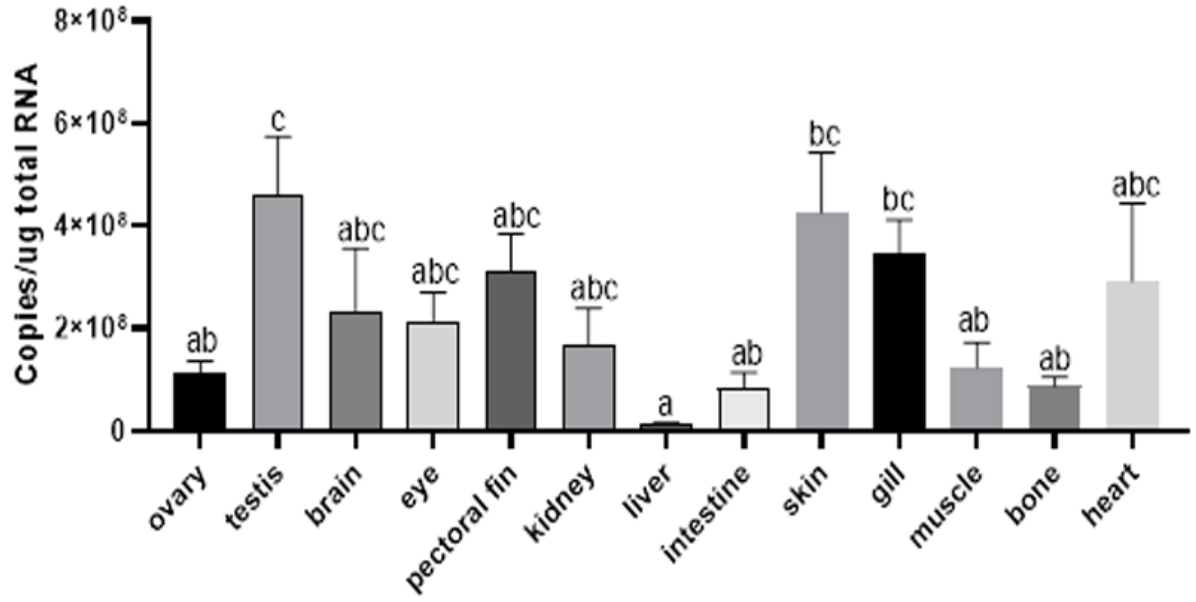


Figure 3.2

Maternal expression of *adamts9* in F2 transgenic (*Tg(adamts9:GFP)*) line during early dividing embrys. The maternal expression of adamts9 was determined by GFP expression driven by *adamts9* promoters located in a 4.5 kb upstream sequence of adamts9 start codon using embryos from crossing F2 transgenic (*Tg(adamts9:GFP*)) and AB strain. Representative confocal z-stack images of various stages of development embryos that were imaged by a confocal microscope under GFP or transmit light (T-PMT) channels. A similar expression was confirmed in multiple F2 embryos from five independent F1 transgenic lines (*Tg(adamts9:GFP)*). In contrast, no GFP expression was detected at same developmental stages in early dividing embryos from crossing of F2 male transgenic line (*Tg(adamts9:EGFP*)) and AB strain. Scale bar: 200 μm.

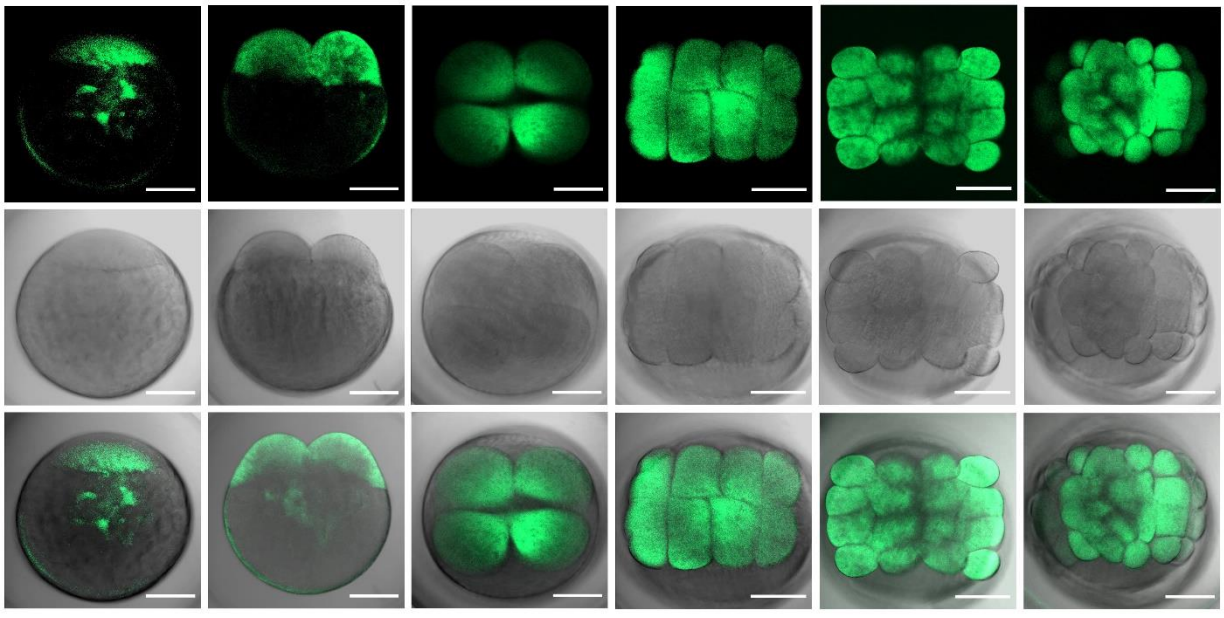


Figure 3.3

8 Cell stage expression of adamts9. The expression of adamts9 was determined by adamts9 promoter driven GFP expression, which was deposited in the oocytes prior to fertilization. Representative confocal z-stack images of various stages of development embryos were from crossing of female transgenic (*Tg(adamts9:GFP)*) and AB strain. In contrast, no GFP expression was detected in early embryos that were from crossing of male transgenic (*Tg(adamts9:GFP*)) and AB strain prior to 24-hour post fertilization (hpf, data not shown). All embryos were imaged by a confocal microscope under GFP or transmit light (T-PMT) channels. A similar expression was confirmed in multiple F2 embryos from five independent F1 transgenic lines (*Tg(adamts9:GFP)*). Scale bar: 200 μm.

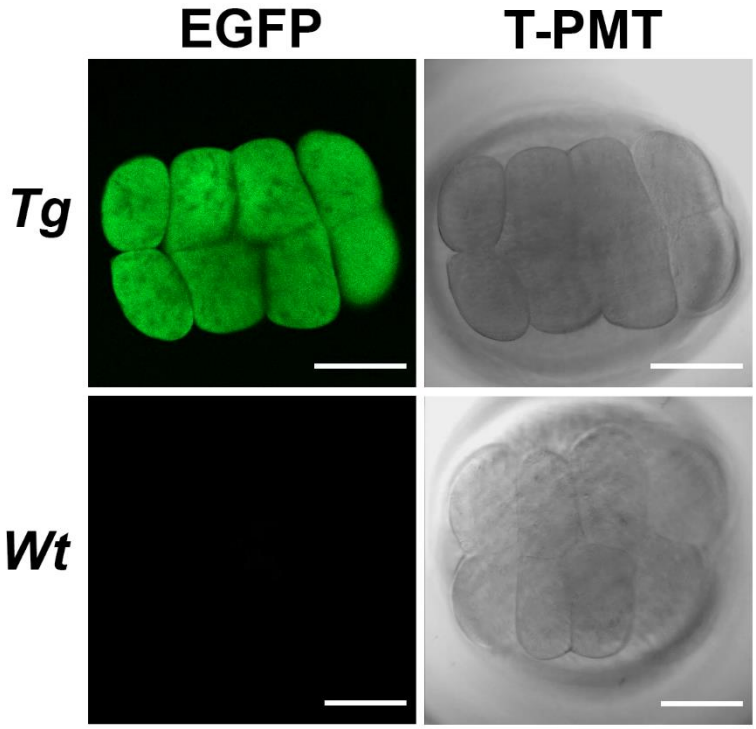


Figure 3.4

Expression of Adamts9 in different tissues in F2 transgene (*Tg(adamts9:EGFP)*) embryos during embryonic development. The expression of adamts9 was determined by EGFP expression driven by *adamts9* promoters located in a 4.5 kb upstream sequence of *adamts9* start codon. Representative confocal z-stack images of different tissues were imaged by a confocal microscope under EGFP or transmit light (T-PMT) channels. A similar expression was confirmed in multiple F2 embryos from five independent F1 transgenic lines (*Tg(adamts9:EGFP)*). Retina and brain EGFP at ~36 hpf (hours post-fertilization); notochord, muscle, and pectoral fin at ~48 hpf; heart at ~78 hpf. In the first, second, and third row, these images represent EGFP, T-PMT, and merged EGFP, respectively. Scale bar: 50 μm.

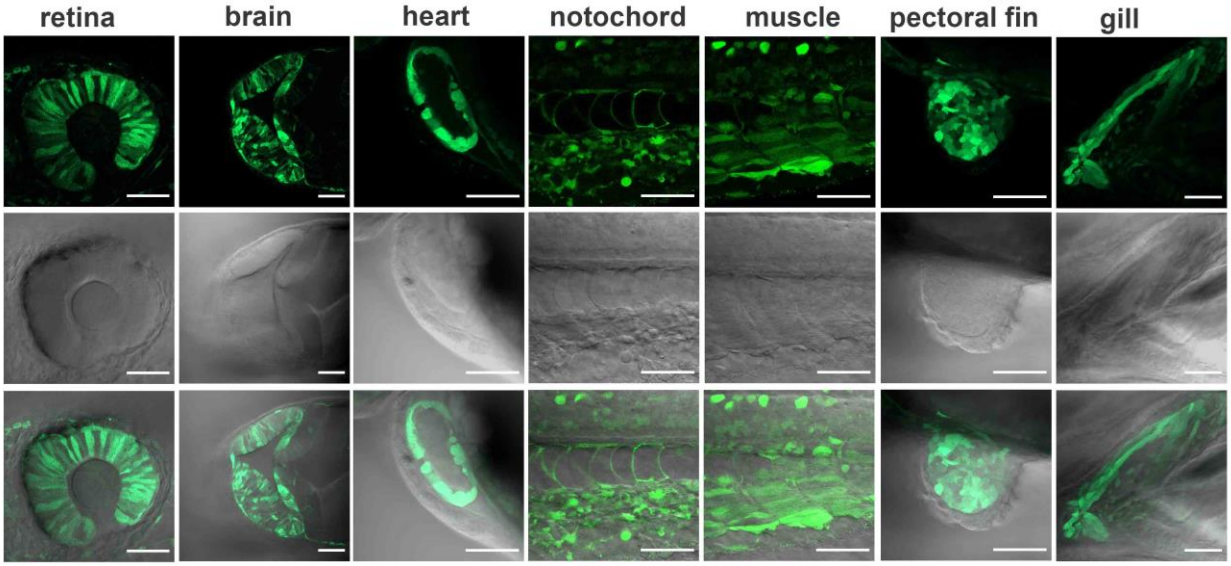


Figure 3.5

Expression of Adamts9 in retina in F2 transgene (*Tg(adamts9:GFP)*) embryos during eye development. The expression of adamts9 was determined by GFP expression driven by *adamts9* promoters located in a 4.5 kb upstream sequence of adamts9 start codon. Representative confocal z-stack images of retina were imaged by a confocal microscope under EGFP, mCherry (to observe autofluorescence), or transmitted light (T-PMT) channels. A similar expression was confirmed in multiple F2 embryos from five independent F1 transgenic lines (*Tg(adamts9:GFP)*). Retina and brain GFP at ~36 hpf (hours post-fertilization); notochord, muscle, and pectoral fin at ~48 hpf; heart at ~78 hpf. In the first, second, and third row, these images represent EGFP, mCherry (autofluorescence), T-PMT, and merged image, respectively. Scale bar: 50 μm.

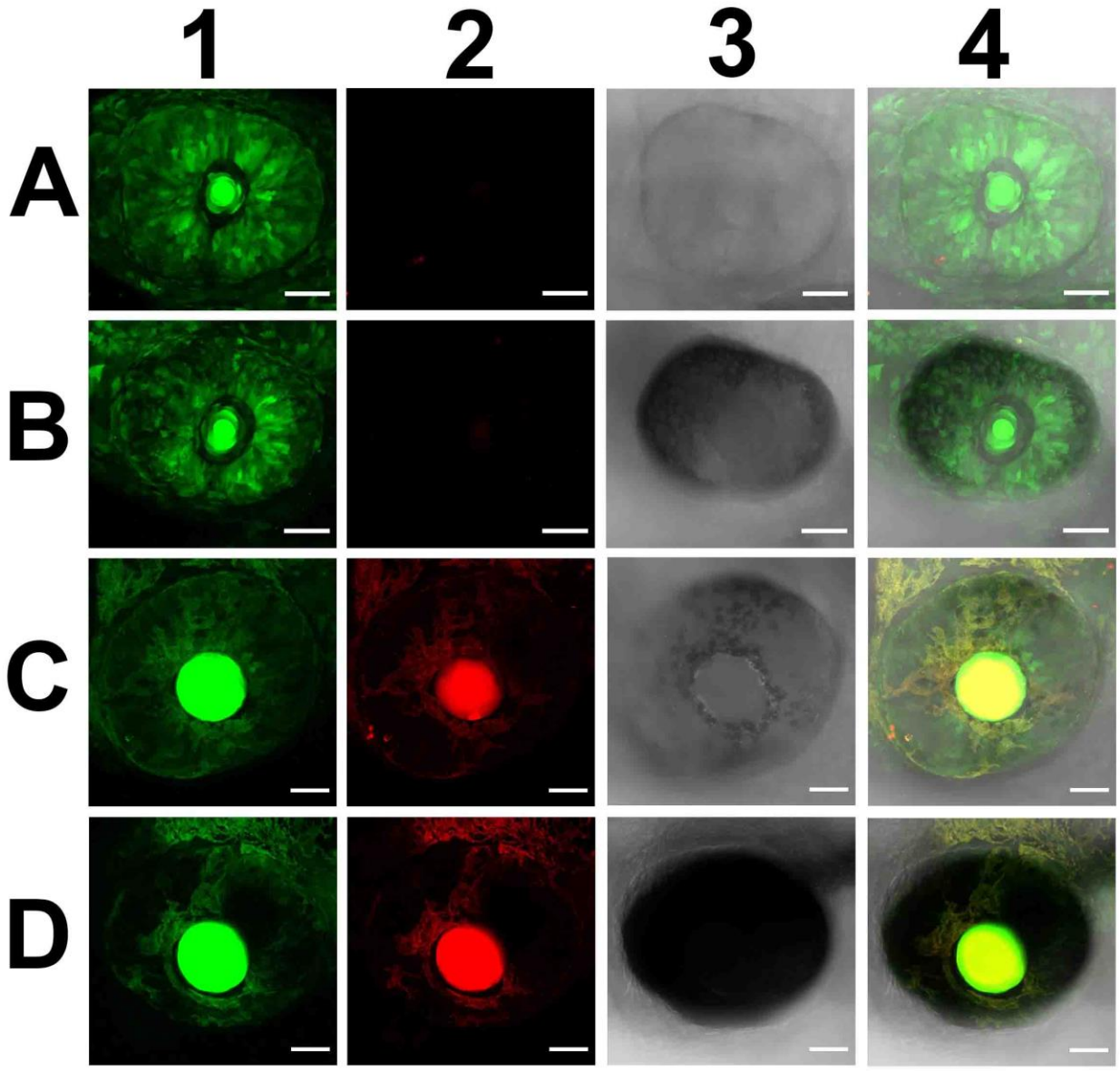

1
2
3
4
A
B
C
D

Figure 3.6

Expression of Adamts9 in posterior lateral line primordium (PLLP, A) and blood vessels (B) in *Tg(adamts9:GFP)* transgene zebrafish. The expression of adamts9 was determined by GFP expression driven by *adamts9* promoters located in a 4.5 kb upstream sequence of adamts9 start codon. Representative confocal z-stack images of PLLP of the fish were imaged by a confocal microscope under GFP or transmit light (T-PMT) channels. A similar expression was confirmed in multiple F2 embryos from five independent F1 transgenic lines (*Tg*(*adamts9:GFP*)). Scale bar: (**A**) 100 μm (top panel), 20 μm (bottom panel); (**B**) 50 μm.

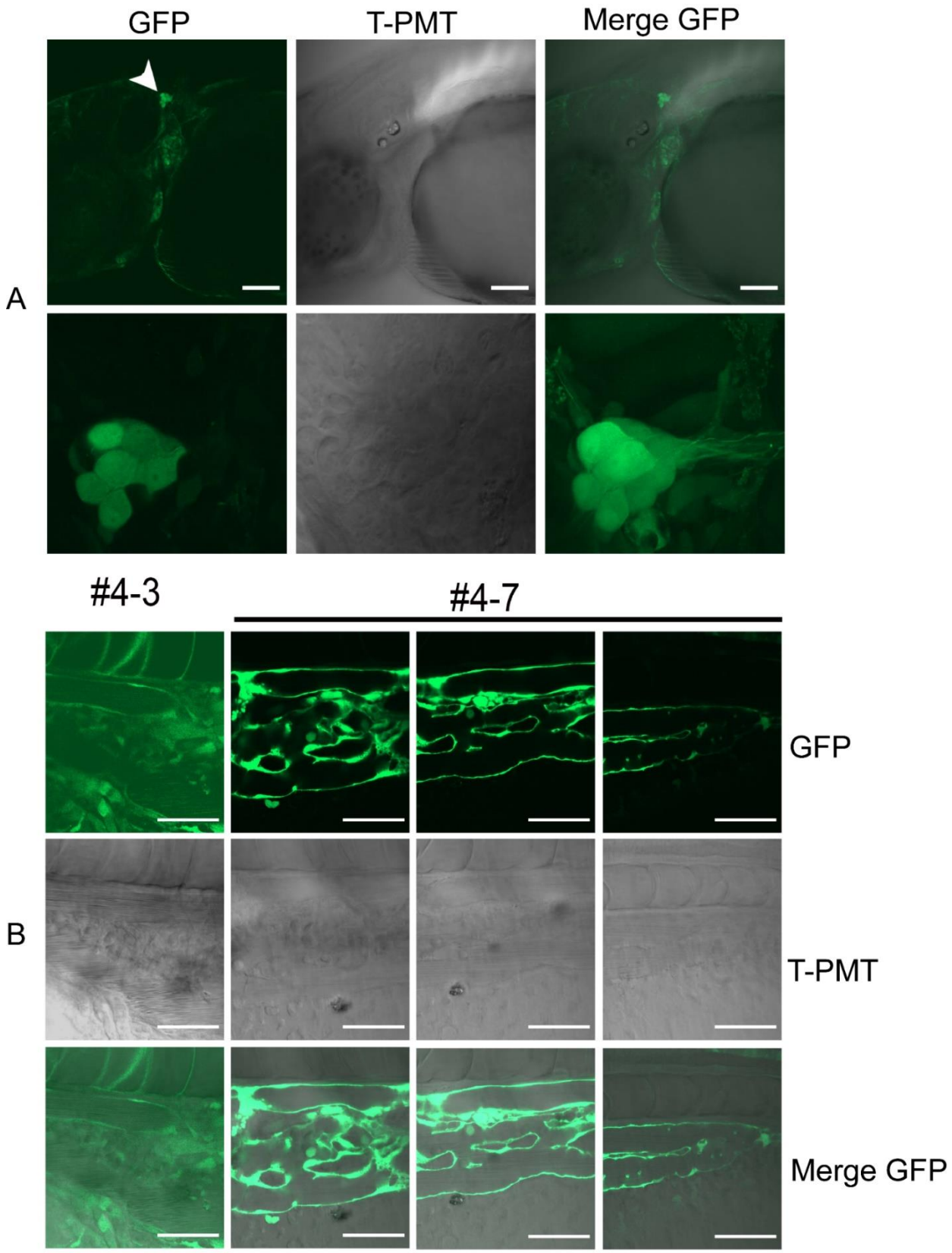

GFP
T-PMT
Merge GFP
A
#4-3
#4-7
GFP
B
T-PMT
Merge GFP

Figure 3.7

Expression of Adamts9 in craniofacial muscle of F2 *Tg(adamts9:EGFP)* transgene zebrafish at both 2 and 3 dpf (days post-fertilization). The expression of adamts9 was determined by EGFP expression driven by *adamts9* promoters located in a 4.5 kb upstream sequence of adamts9 start codon. Representative confocal z-stack images of the craniofacial muscle of the fish were imaged by a confocal microscope under EGFP or transmit light (T-PMT) channels. A similar expression was confirmed in multiple F2 embryos from five independent F1 transgenic lines (*Tg(adamts9:EGFP*)). Scale bar: 50 μm.

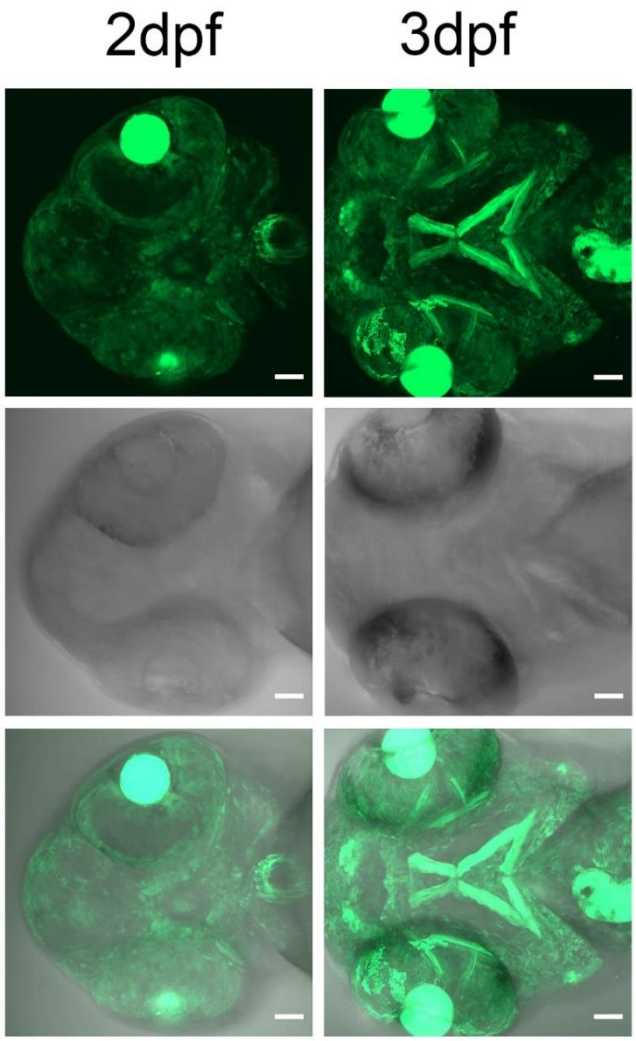


Figure 3.8

Expression of Adamts9 in testes in transgene *Tg(adamts9:GFP)* zebrafish. The expression of Adamts9 was determined by GFP expression driven by *adamts9* promoters located in a 4.5 kb upstream sequence of adamts9 start codon. Representative confocal z-stack images of testis were imaged by a confocal microscope under GFP or transmit light (T-PMT) channels. A similar expression was confirmed in multiple F2 embryos from five independent F1 transgenic lines (*Tg(adamts9:GFP)*). In the first, second, and third columns, these images represent GFP, T-PMT, and merged GFP, respectively. Scale bar: 200 μm (10X, top panel), 100 μm (middle panel), 50 μm (bottom panel).

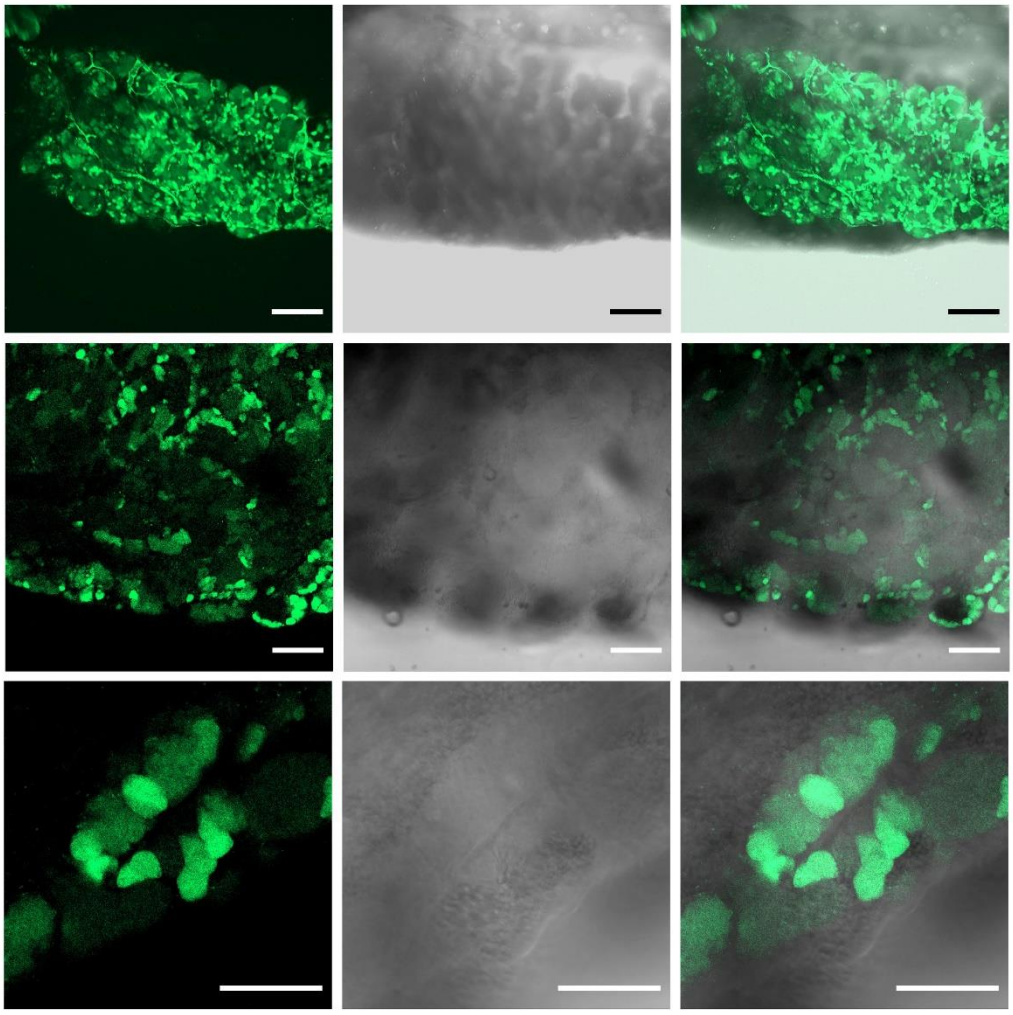


Figure 3.9

Expression of Adamts9 in oocytes in *Tg(adamts9:GFP)* transgene zebrafish. The expression of Adamts9 was determined by GFP expression driven by *adamts9* promoters located in a 4.5 kb upstream sequence of adamts9 start codon. Representative confocal z-stack images of oocytes were imaged by a confocal microscope under GFP or transmit light (T-PMT) channels. A similar expression was confirmed in multiple F2 embryos from five independent F1 transgenic lines (*Tg(adamts9:GFP)*). In the first, second, and third row, these images represent GFP, T-PMT, and merged GFP, respectively. Scale bar: 200 μm (10X; columns 1, 3, 5, 6), 10 μm (40X; columns 2, 4, 7).

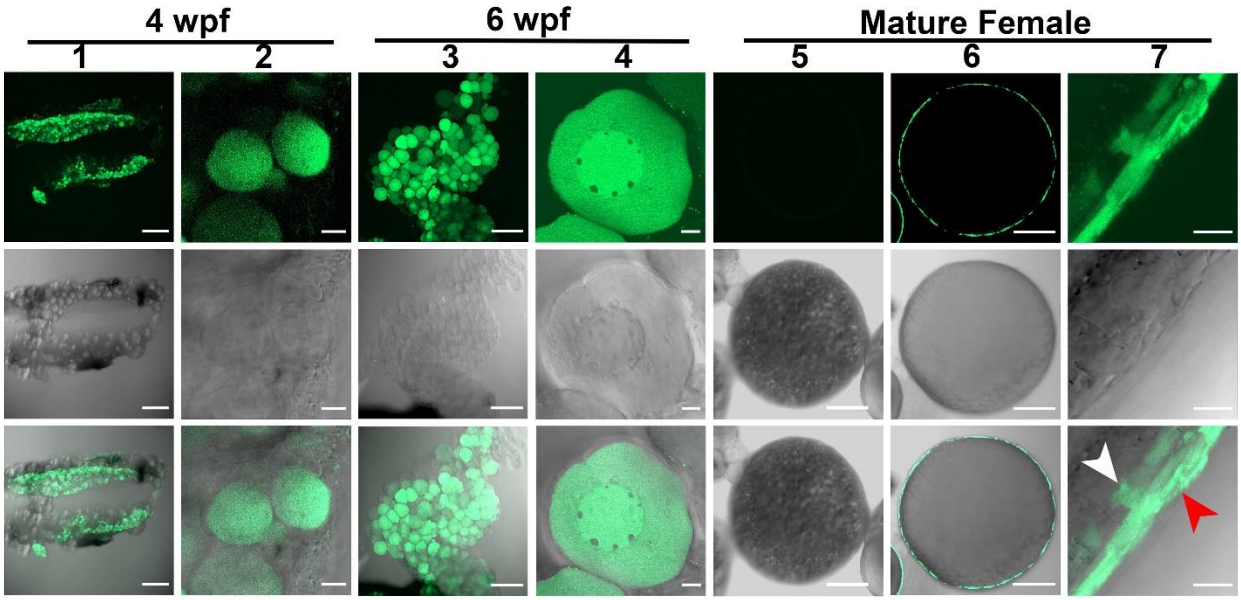


Figure 3.10

Adamts9 expression in adult gills (A), kidney (B), pectoral fin (C), and eye (D) determined by EGFP expression in F2 transgene line (*Tg(adamts9:GFP)*) adult zebrafish. The expression of Adamts9 was determined by GFP expression driven by *adamts9* promoters located in a 4.5 kb upstream sequence of adamts9 start codon. Representative confocal z-stack images of testis were imaged by a confocal microscope under GFP or transmit light (T-PMT) channels. A similar expression was confirmed in multiple F2 embryos from five independent F1 transgenic lines (*Tg(adamts9:GFP)*). Scale bar: (**A**) 200 μm (10X, top panel), 10 μm (40X, bottom panel); (**B**) 10 μm (40X); (**C**) 200 μm (10X, top panel), 10 μm (40X, bottom panel); (**D**) 200 μm (10X).

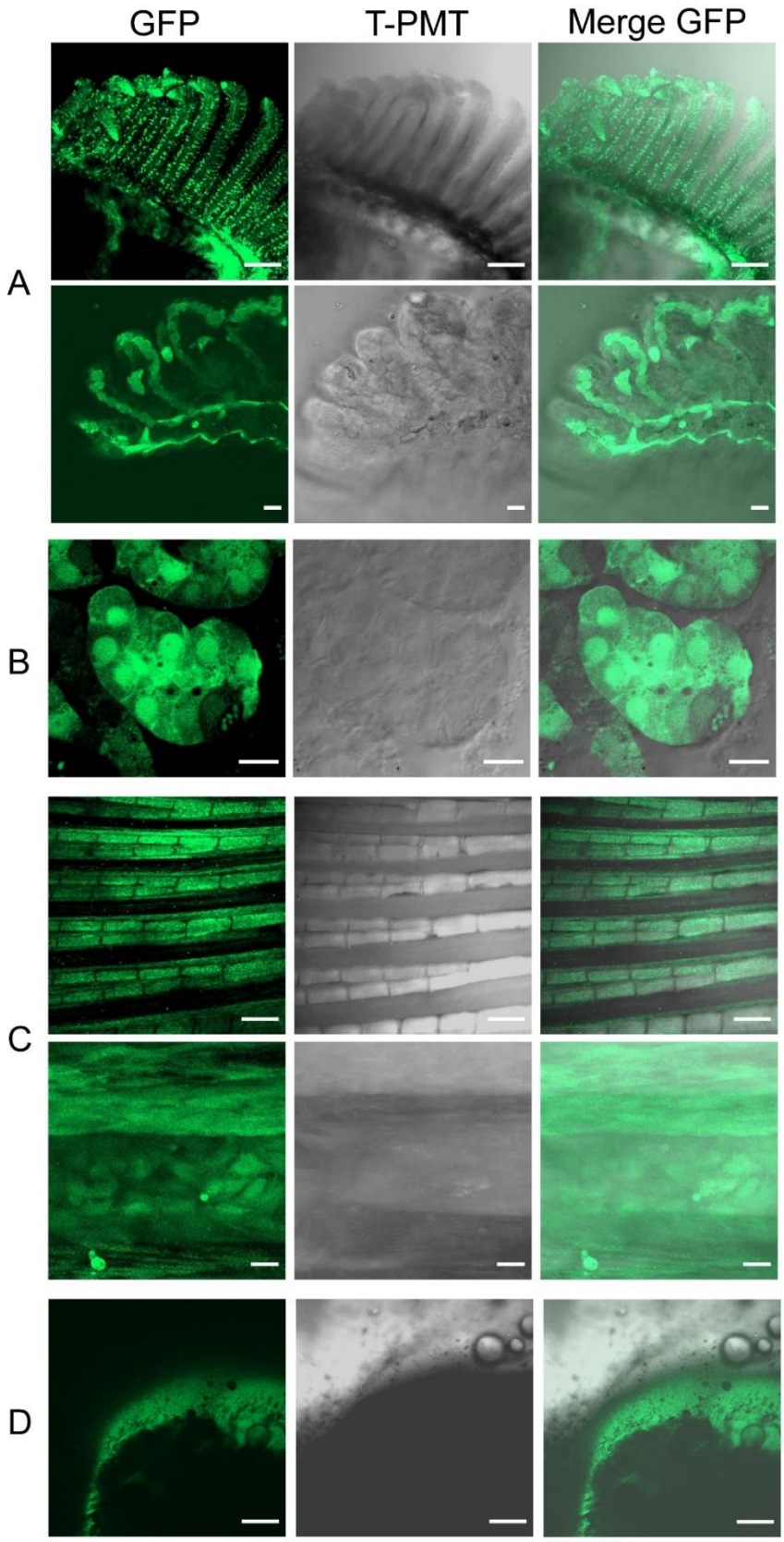

GFP
T-PMT
Merge GFP
A
B
C
D

Figure 3.11

Expression of *adamts9* transcripts in follicular cells of preovulatory follicles determined by mRNA whole mount *in situ* hybridization (WISH). Both antisense, and sense control probes were used WISH for detection of *adamts9* expression in follicular cells. Top row: stereoscope images of follicular cells. Middle row, sectioned oocytes counterstained with eosin. Bottom row, magnified images of follicular cells stained by ISH for *adamts9* with an eosin counterstain.

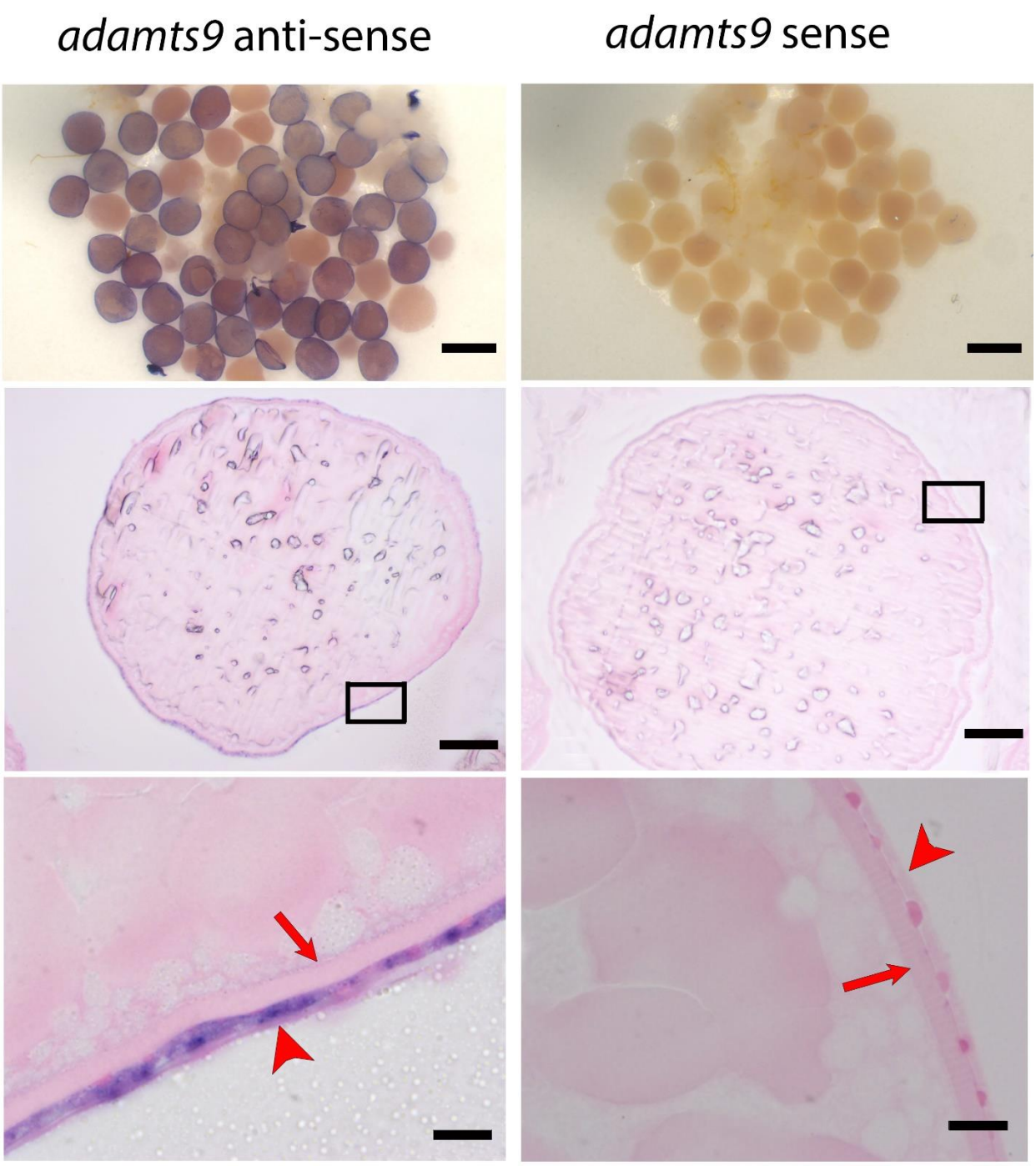

adamts9 anti-sense
adamts9 sense

Figure 3.12

Strong expression of *adamts9* in the retina at 24hpf by WISH. A-C) antisense probe staining *adamts9* expression in whole mount *in situ* hybridization. Red arrowheads point to strong expression along the developing retina. D-F) Control sense probes showing a lack of staining along the retina.

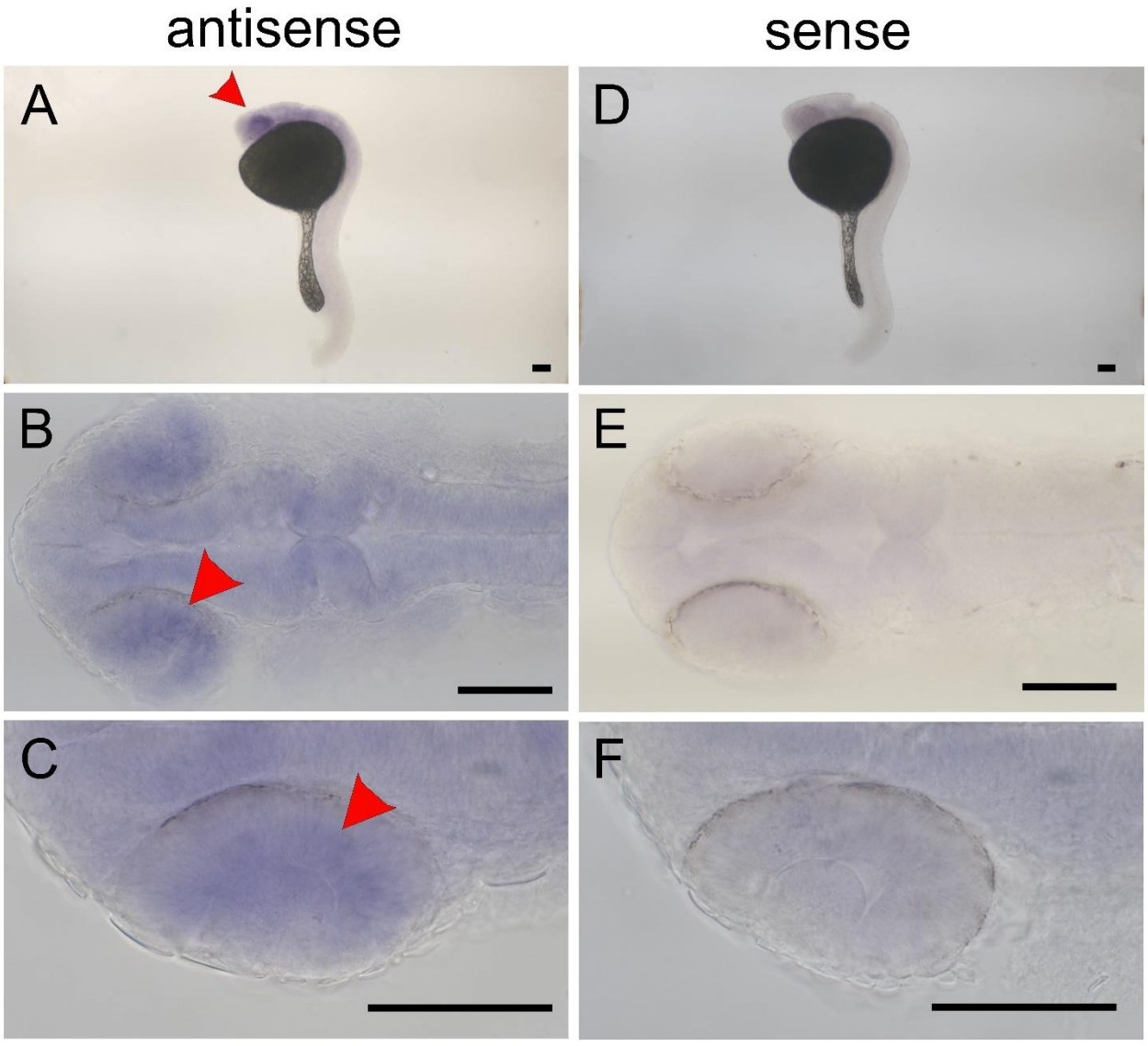
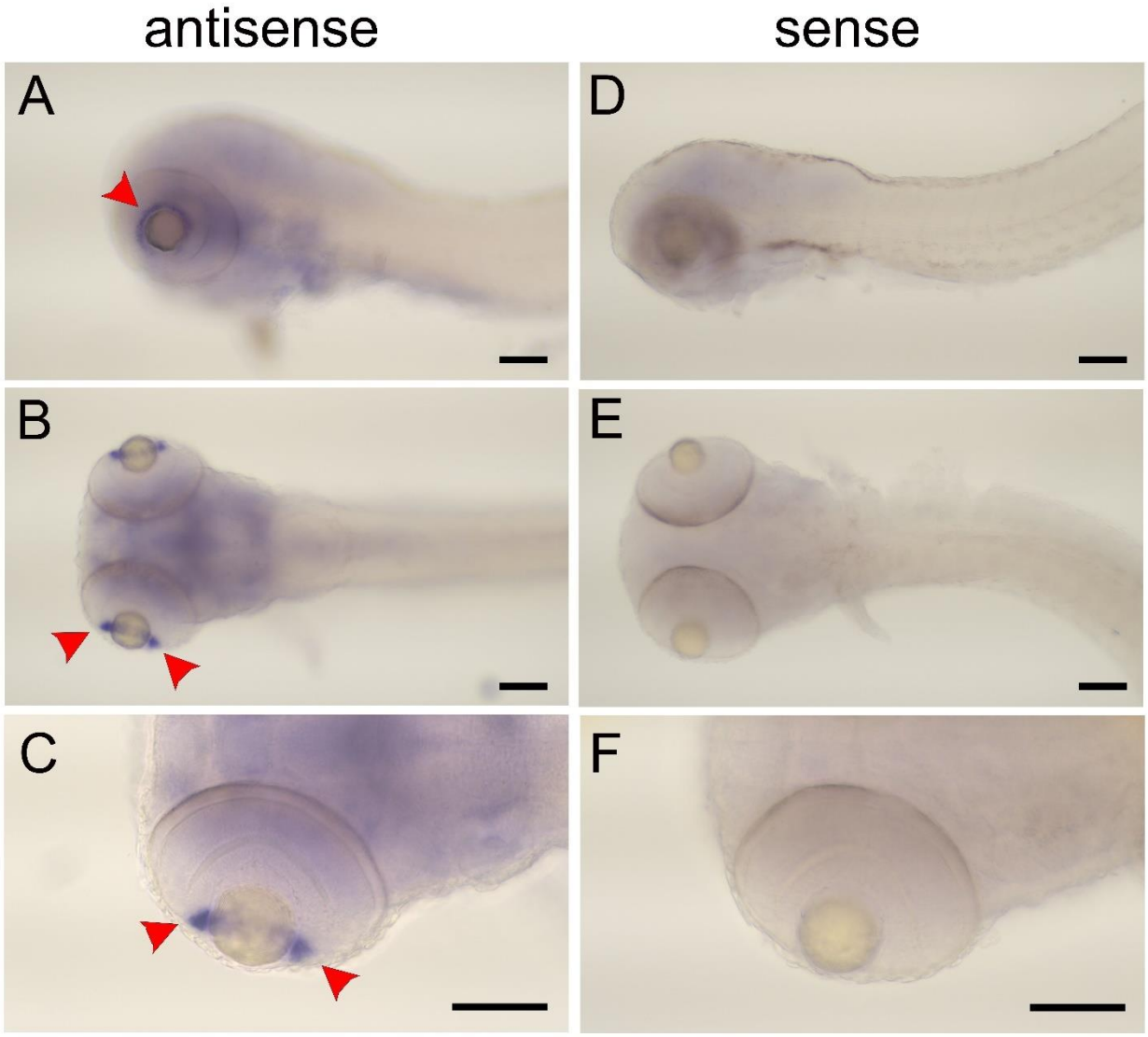


Figure 3.13

Strong expression of *adamts9* in the ciliary marginal zone at 72hpf by WISH. A-C) antisense probe staining *adamts9* expression in whole mount *in situ* hybridization. Red arrowheads point to strong expression along the ciliary marginal zone. D-F) Control sense probes showing a lack of staining along the ciliary marginal zone.



CHAPTER IV: Delay in primordial germ cell migration in adamts9 knockout zebrafish

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Chapter Summary

Adamts9 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 9) is one of a few metalloproteinases structurally conserved from C. elegans to humans and is indispensable in germ cell migration in invertebrates. However, adamts9′s roles in germ cell migration in vertebrates has not been examined. In the present study, we found zygotic expression of adamts9 started around the germ ring stage and reached peak levels at 3 days post fertilization (dpf) in zebrafsh. The migration of primordial germ cells (PGC) was completed within 24 hours (h) in wildtype siblings, while a delay in PGC migration was found at 15 and 24-h post-fertilization (hpf) in the Adamts9 knockout (KO). However, the delayed PGC migration in Adamts9 KO disappeared at 48 hpf. Our study suggests a conserved function of Adamts9 in germ cell migration among invertebrates and vertebrates. In addition, our results also suggest that Adamts9 is not essential for germ cell migration as reported in C. elegans, possibly due to expansion of Adamts family members and compensatory roles from other metalloproteinases in vertebrates. Further studies are required in order to elucidate the functions and mechanisms of metalloproteinases in germ cell migration and gonad formation in vertebrates.

Introduction

Metalloproteinases serve essential roles in morphogenesis, tissue remodeling, and cell migration, all of which are important in normal or disease processes. From genome-wide association studies (GWAS), Adamts9 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 9) is associated with various human diseases, such as

diabetes (Zilikens et al., 2017; Artunc-Ulkumen et al., 2017; Graae et al., 2019), asthma (Paulissen et al., 2009), arthritis (Yang et al., 2017; Mead and Apte, 2018), artery calcification (Suna et al., 2018; Franceschini et al., 2018), macular degeneration (Sign and Tyagi, 2017), and cognitive aging (Rosenberg, 2017). However, Adamts9-dependent physiological processes, adamts9 expression, and in vivo functions are still poorly understood mainly due to the lack of non-lethal vertebrate knockout animal models.

The formation of a functional gonad is essential for animals to reproduce. A functional, adult ovary or testis develops from a juvenile bipotential gonad via several physiological processes that include cell migration, apoptosis, proliferation, and tissue remodeling. These processes are regulated precisely by various cellular signaling molecules and proteinases. Adamts9 is one of few metalloproteinases structurally conserved from C. elegans to humans (Somerville et al., 2003; Clark et al., 2000; Kelwick et al., 2015)(Fig. 1), and it is required in gonad formation in invertebrates (Blelloch and Kimble, 1999; Blelloch et al., 1999; Nishiwaki et al., 2000). In the knockout of Adamts9 ortholog, *gon-1* in *C. elegans*, germ cells do not migrate, and the gonad develops as a disorganized mass of somatic and germ line tissues (Blelloch and Kimble, 1999; Blelloch et al., 1999; Nishiwaki et al., 2000). In *Drosophila*, the knockout of Adamts9 ortholog, AdamtTS-A causes the mis-migration of collective cells, including germ cells (Ismat et al., 2013). However, due to embryonic lethality in the knockout *Drosophila* (Ismat et al., 2013) and mouse models (Kern et al., 2010; Nandadasa et al., 2015), the functions and underlying mechanisms of Adamts9 during gonad development and formation are still unknown. It is also important to note that compared to invertebrates, members of the extracellular matrix (ECM) protein families and ADAMTS family expand dramatically in vertebrates (Brunet et al., 2015), which may lead to loss and gain of functions for Adamts9.

Our previous studies suggest that Adamts9 is critical for normal development of ovaries and ovulation in the zebrafish (Liu et al., 2017; Liu et al., 2018; Liu et al., 2020; Carter et al., 2019). In the present study, we examined expression of Adamts9 in the embryonic development and its roles in germ cell migration. Zygotic expression of Adamts9 started around germ ring stage of embryos in zebrafish development. A delay in the migration of primordial germ cells was found during the gonadal development.

Materials and Methods

Animal husbandry

The AB strain of zebrafsh (Danio rerio) used here originated from the Zebrafsh International Resource Center and then propagated in our lab following previously published guidelines (Zhu et al., 2015). All methods were carried out in accordance with relevant guidelines and regulations. The study was carried out in compliance with the ARRIVE guidelines (Percie et al., 2020). All experimental protocols have been approved by the Institutional Animal Care and Use Committee (IACUC) at East Carolina University

Collection of ovarian follicles and embryos and extraction of total RNA

Various stages of AB wildtype embryos were collected at different times of development. Ovarian follicles were divided into different stages according to follicular size and morphological criteria (Selman et al., 1993) with a slight modification (Liu et al., 2017; Liu et al., 2018; Liu et al., 2020). Stage I, III, IVb and V ovarian follicles were collected from 4-month-old mature female AB wildtype fish between 7:00am and 8:30am (lights on photoperiod 8:30am-10:30 pm). Samples were placed in 1.7 mL RNase-free microcentrifuge tubes (GeneMate) containing 200 µl RNAzol (Molecular Research Center, Inc., OH. Catalog: RN 190) and

homogenized immediately. Total RNA was extracted from homogenized solutions according to the manufacturer's protocol. For each sample, cDNAs was synthesized using 2 μg total RNA and a high-capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA, Catalog#4368814) following the manufacturer's instructions.

RT-PCR amplification of adamts9

A set of PCR primer (forward: 5′-GCGGTACGCGTGGTAAAA TC-3′; reverse: 5′-AGGCATGTGGACATAACGCA-3′) targeting 1181 bp of 3′-UTR of adamts9 was used for RT-PCR amplification. PCR amplification was carried out using a Taq DNA polymerase (New England Biolabs, Ipswich, Massachusetts, USA, Catalog#0273) with initial denaturation at 95 °C for 2 min followed by 35 cycles of 30 s denaturation at 95 °C, 30 s annealing at 65 °C, and 60 s elongation at 68 °C. Zebrafish eukaryotic translation elongation factor 1 alpha 1a (eef1a1a) showed stable expression in different stages of embryos and ovarian follicles, therefore was used as a housekeeping gene control. A set of PCR primers targeting 242 bp of coding region of eef1a1a (forward: 5′-AGTGTTGCCTTCGTCCCAAT-3′; Reverse:5′-CACACGACCCACAGGTAC AG-3′) was used for PCR amplification. The efficiency of the PCR and authentic PCR products was confirmed by gel electrophoresis analysis. The PCR products were also cloned into pGEM-T easy vector and confirmed by Sanger sequencing. The concentrations of these plasmids were quantified on Nanodrop 2000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA), serially diluted and used as DNA templates for generating standard curves described in the following paragraph.

Real-time quantitative PCR (qPCR) amplification of adamts9

The levels of adamts9 transcripts were also determined by quantitative real-time PCR (qPCR) using SYBR green dye (Invitrogen) and a CFX Connect real-time thermal cycler (Bio-Rad Laboratories, Hercules, California, USA). The qPCR reaction was conducted with initial denaturation at 95 °C for 3 min, followed by 45 cycles of 30 s denaturation at 95 °C, 30 s annealing at 65 °C, and 30 s extension at 72 °C using the specific primers (Forward: 5′-CTGTCTGCGCGGTGA TTCTA-3′; Reverse: 5′-CTCTTGCAGGGGCGTGATTA-3′) and GoTaq G2 DNA polymerase (Promega, Madison, Wisconsin, USA). Each PCR mixture (15 µl) consisted of 7.795 µl DNase free water, 3 µl 5XGoTaq buffer, 1.5 µl 25 mM MgCl2, 0.3 µl 10 mM dNTP mix, 0.15 ml 10µM forward or reverse primer, 2 µl 5X diluted cDNA, 0.03 µl 100X SYBR green dye (final concentration 0.2X), and 0.075 µl Taq. The transcript levels, expressed as absolute values (copies/µg total RNA), were determined using Ct values of samples and a standard curve generated from serial known concentrations of plasmid containing the target region of adamts9. The efficiency of the PCR and authentic PCR products was further confirmed by analyses of melting curve, gel electrophoresis, and Sanger sequencing.

Adamts9 expression analyzed by adamts9 promoter driven EGFP

Detailed generation and characterization of EGFP expression driven by *adamts9* promoters in zebrafish will be reported in a separate manuscript. Briefly, a 4.5-kb upstream of adamts9 start codon was cloned into pGEM-T easy vector (Promega), Sanger sequence confirmed, and then subcloned into a p5E-mcs entry vector. Multisite Gateway cloning (Invitrogen) was conducted with a 5′ entry vector containing 4.5 kb *adamts9* promoter sequence, a middle entry vector containing EGFP, a 3′entry vector with stop poly A signal, and a destination vector that expresses a EGFP selection marker specifically in the lens of eye (Kurita et al., 2003). In this final vector, the expression of EGFP is controlled by adamts9 promoters.

Transgenic embryos with the insertion could be easily selected by the eye marker that displays green fluorescence at 48 hpf under a fluorescent dissecting microscope. About 500 nl mixture containing 20 ng/µl construct, 20 ng/µl Tol2 transposon, and phenol red indicator were microinjected into one cell stage of embryos. Multiple F0 transgenic embryos were selected based on the lens selection marker, raised to adults, crossed with AB wildtype, then produced several F1 lines. Subsequently, we established multiple F2 stable transgenic lines (*Tg(adamts9:EGFP)*) with stable expression of EGFP in the zebrafish.

Confocal imaging and analyses of GFP expression, migration, and numbers of PGCs in Adamts9 knockout

Fresh, live follicles collected from adult transgenic females (*Tg(adamts9:EGFP)*) were placed immediately in 15 mM HEPS buffer (pH 7.8) containing 50% L-15 medium (Fisher Scientific, #41-300-039). Follicles were pipetted up and down several times to separate individual follicles. Ten, individual follicles of various developmental stages were placed on a depression glass slide and mounted in 1.2% low melting point agarose and immediately imaged by confocal microscopy.

Live transgenic embryos of various developmental stages were collected by crossing male transgenic fish (*Tg(adamts9:EGFP*)) with AB females. Individual embryos were placed on a depression glass slide, mounted in 1.2% low melting point agarose, and immediately imaged by confocal microscopy. Five independent F1 transgenic lines were used to confirm similar expression among all the transgenic lines. Adamts9 knockout fish were generated and reported in our previous study (Carter et al., 2019). Vasa is an RNA helicase expressed exclusively in primordial germ cells (PGCs)(Hartung et al., 2014). By using vasa promoter to drive EGFP expression, it is possible to visualize PGCs in zebrafish embryos with a laser confocal scanning

microscope. Adamts9 knockout male fish (adamts9−/−) were crossed with *Tg(vasa:EGFP)*(Lau et al., 2016). Embryos were collected, raised to adult, genotyped, and then in-crossed to obtain a transgenic line with all germ cells labeled with EGFP in Adamts9 knockout background (*adamts9+/−; Tg(vasa:GFP)*). Tis transgenic line was used for generating wildtype (+/+), heterozygote (+/–) and homozygote (−/−) embryos for confocal imaging of PGCs. Zebrafish embryos were collected at 15, 24, 48 hpf, fixed in 10% buffered formalin for four hours at room temperature. Embryos were subsequently washed with distilled water, a PBS solution, and then increasing concentrations of methanol, before storage in 100% methanol at − 20 °C until analyses. Individual embryos were mounted onto a depression glass slide in 1.2% low melting point agarose and then imaged by confocal microscopy. Distance between two most distant PGCs was determined as an indicator of PGC migration using Zen 2.6 software. The numbers of germ cells were determined with aid of computer software (Imaris, Bitplane Inc, Zürich, Switzerland).

Statistical analysis

All results were presented as mean±SEM. Two-sample two tailed t-test was used to analyze the effect of genotypes on germ cell migration, One-way ANOVA was used to analyze the gene expression. Statistical significance was set at $p<0.05$. All statistical analysis were conducted using GraphPad Prism.

Results

Expression of Adamts9 in follicular cells and during embryonic development.

Zebrafish Adamts9 (NP_001244125) shares high sequence identities with its orthologs in *C. elegans*, *Drosophila*, mice and human (Fig. 4.1, supplemental Figs.4.1 and 4.2,

supplemental Tables 4.1–4.4). The highest percentages of amino acid sequence identities (40-88%) between C. elegans and human were found in the metalloproteinase domain of Adamts9 (Supplemental Table 4.2), while an amino acid motif (HELGHXXXXXHDD) for the Adamts9 enzymatic activation site was completely conserved and aligned (Supplemental Fig. 4.1). Intriguingly, zebrafish Adamts9 has two propeptide domains, but lacks the GON-1 domain that is conserved from C. elegans to human. Currently, we have no evidence to support that zebrafish Adamts9 was truncated, or the unique GON-1 domain was lost in some vertebrate species (https://www.ncbi.nlm.nih.gov/gene/56999/ortholog/?scope=7776&term=ADAMTS9), even the duplication of propeptide domain might be unique to the zebrafish.

The expression of adamts9 transcript was low in immature oocytes, but dramatically increased in the follicular cells of stage IVb mature and preovulatory ovarian follicles. The expression of adamts9 decreased beneath the detection limit in stage V ovulated oocytes (Fig. 4.2A,B). These results suggest adamts9 is expressed highly in somatic cells adjacent to mature germ cells, but not in the mature germ cells.

Zygotic expression of adamts9′s transcript was under the PCR detection limit between 1 cell and oblong stage of embryos (~4 h post fertilization (hpf)), gradually increased around germ ring stage (~6 hpf) and reached a peak level around 3 days post fertilization (dpf) (Fig. 4.2A,C).

In corresponding to the expression of adamts9 transcripts, strong GFP expression driven by adamts9 promoters was observed in mature/preovulatory stage IVb follicles, while weak or no GFP expression was observed in immature follicles (stage I, III) or ovulated stage V oocytes. (Fig. 4.3). Zygotic expression of GFP was observed around bud stage of embryos, gradually increased and become obvious around 8 somite stages of embryos, and reached peak levels afer 2 dpf (Figs. 4.4 and 4.5).

Delay in primordial germ cell (PGC) migration in Adamts9 knockout (KO).

We generated a zebrafish line with all the germ cells labeled with GFP in Adamts9 KO background (*adamts9+/−; Tg(vasa:GFP)*). We crossed these heterozygotes and obtained wildtype (adamts9+/+), heterozygotes (adamts9+/−), and homozygotes (adamts9−/−) sibling. PGC completed their migration and tightly clustered together at 24hpf in wildtype (adamts9+/+) fish. We found a delay in PGC migration in the Adamts9 KO (adamts9−/−) zebrafish. PGCs spread in wider area in homozygotes than those in wildtype sibling at 15 and 24 hpf, i.e. delay in the migration of PGCs (Fig. 4.6). However, this delayed migration effect disappeared at 48 hpf (Fig. 4.6). In addition, all the PGCs migrated to the gonadal ridge despite the delay of germ cell migration in Adamts9 KO zebrafish between 15 and 24 hpf. There is no significant difference in the numbers of germ cells among different genotypes (Fig. 4.6).

Discussion

Matrix metalloproteinases (MMPs) are well known for their involvements in cell motility such as stem cell migration or cancer cell invasion (Kessenbrock et al., 2010; Golan et al., 2011; Molyneauz et al., 2004; Kucia et al., 2007; Gialeli et al., 2011). ADAMTS is a subgroup of secreted zinc metalloproteases with several distinct domains separated from classical MMPs (Apte, 2004; Porter et al., 2005). Studies of Adamts9 orthologs in C. elegans and Drosophila suggest this proteinase may release a signal or clear a path in order for PGC to migrate appropriately in invertebrates (Blelloch and Kimble, 1999; Blelloch et al., 1999; Nishiwaki et al., 2000; Ismat et al., 2013). However, studies of possible involvements of metalloproteinases in PGC migration is still unknown in vertebrates (Díez-Torre et al., 2013). In present study, we provided first evidence that a metalloproteinase, Adamts9, plays a role in the PGC migration in a vertebrate model. The availability of several transgenic lines that label PGCs with fluorescent

proteins and nearly transparent embryos made zebrafish an excellent choice for studying germ cell migration at a high resolution within a live organism (Raz, 2002; Dumstrei et al., 2004). Transgenic zebrafish lines in a different genetic background, including Adamts9 KO allowed studying the roles of Adamts9 in PGC development starting from the earliest stages of their development. We determined the expression of Adamts9 in early development. We also determined germ cell migration and numbers of germ cells in the homozygous Adamts9 KO in comparison to their wildtype and heterozygous siblings. Our results suggest a conserved function of Adamts9 in germ cell migration among vertebrates and invertebrates (Fig. 4.7). Our results also suggest that Adatms9 is not essential in the germ cell migration as demonstrated in *C. elegans* (Blelloch and Kimble, 1999; Blelloch et al., 1999; Nishiwaki et al., 2000) (Fig. 4.7). It should be noted that zebrafish Adamts9 lacks GON-1 domain. The role of Adamts9 in germ cell migration may be shared by other proteinases due to loss of a function domain in zebrafish. However, studies of Adamts9 ortholog (AdamTS-A) in *Drosophila* also suggest that this enzyme is not essential for germ cell migration as some germ cells migrate appropriately, while others mis-migrate (Ismat et al., 2013). None of previous studies have reported survival or number of germs in the knockouts. In present study, we found the numbers of PGCs were not affected in Adamts9 KO zebrafish embryos. One single gene, the ancestor of Adamts, is found in the sponge corresponding to the origin of multi-cellularity and embryogenesis (Brunet et al., 2015). Up to 8 Adamts members are found in invertebrates. The Adamts family expanded dramatically during Metazoan evolution to 19 genes in vertebrates including zebrafish, mice, and humans (Brunet et al., 2015; Apte, 2004; Porter et al., 2005). The expansion of Adamts members in vertebrates may lead to gain and loss functions for each Adamts family member, which could explain diminished roles of Adamts9 in germ cell migration in vertebrates. On the other hand, heterozygotes

(adamts9+/−) was used to produce wildtype (+/+), heterozygote (+/−) and homozygote (−/−) embryos due to female infertility in homozygous KO. Therefore, we could not completely exclude the possibility that maternally deposited Adamts9 from heterozygous females may have exerted influence on PGC migration. Future studies, such as using conditional knockouts, will exclude such possibilities. However, the expressions of adamts9 in wildtype were under the detection PCR limit in both ovulated oocytes and in early embryos from 1 cell stage to oblong stage. Zygotic expression of Adamts9 began around germ ring stage of embryos (~6 hpf, He et al., in review). Delayed germ cell migration observed between 15 and 24 hpf happened after zygotic expression of Adamts9 in zebrafish embryos, suggesting little or no role of maternal deposited Adamts9 if present in zebrafish. The expression of adamts9 orthologs (GON-1 in C. elegans and AdamTS-A) was found in somatic cells adjacent to germ cells, but not in the germ cells (Blelloch and Kimble, 1999; Blelloch et al., 1999; Ismat et al., 2013). Interestingly, ectopic expression of GON-1 or AdamtTS-A in the germ cells in C. elegans or Drosophila would rescue germ cell migration defects. Adamts9 is expressed in various tissues including ovaries and testes in human and mice according to NIH gene database; however, whether Adamts9 is expressed in the somatic cells or germ cells of the gonads in human or mice is still unknown. Interestingly, we found adamts9 is expressed in early germ cells during early development or in adult gonads, although the expression was low. It is possible that the low expression of adamts9 in the germ cells was missed in previous studies in *C. elegans* and *Drosophila*. Alternatively, the low expression in the germ cells found in zebrafish is related to the dramatic expansion of Adamts members, and thereafter gain of functions in the germ cells in vertebrates. Further studies in other vertebrate species are required to confirm this hypothesis. Intriguingly, adamts9 expression decreased as follicles grow, disappeared in the germ cells then increased dramatically in adjacent

follicular cells in preovulatory follicles (stage IVb, Figs. 2 and 3). The dramatic increase of adamts9 in the follicular cells of preovulatory follicles is due to increased LH and progestin signaling to prepare these follicles for ovulation (Liu et al., 2017; Liu et al., 2018; Liu et al., 2020). However, the regulatory mechanisms, the cause and the effects of adamts9 decrease in growing follicles are still unknown. Adamts9 knockout in mice is lethal to embryos giving evidence of its essential role during development. It is highly expressed in the mouse genital tubercle and ovary (Jungers et al., 2005). However, due to embryonic lethality in the knockout Drosophila and mouse models (Ismat et al., 2013; Kern et al., 2010; Nandadasa et al., 2015), the specific functions and underlying mechanisms of Adamts9 during gonad development, differentiation, and maintenance are still unknown. We generated zebrafish knockouts by targeting a CRISPR site prior to the enzymatic active site of Adamts9 (Supplemental Fig. 1)(Carter et al., 2019). No immune-positive signals were detected in the follicular cells of zebrafish Adamts9 knockout, whereas strong positive signals were detected in the follicular cells of wildtype and heterozygous sibling (Carter et al., 2019). Any residual of truncated proteins of Adamts9, if present, would have no enzymatic activities as it lost its active metalloproteinase sites in our zebrafish knockouts (Carter et al., 2019). It is well established that the number of germ cells is important for gonadal development and sex determination in zebrafish (Dranow et al., 2013; Dai et al., 2015; Tzung et al., 2015). A threshold number of germ cells is required for ovarian development. The depletion of germ cells in development or the adult will lead to the development of males (Dranow et al., 2013; Dai et al., 2015; Tzung et al., 2015). We hypothesized that the unusual development of ovaries and male biased sexual ratio found in Adamts9 KO was due to a defect in germ cell migration, early germ cell survival, and/or proliferation of PGCs. Our results show that removing Adamts9 had no effects in the numbers of

germ cells during early development (15–48 hpf). Further studies are required to elucidate the processes and mechanisms of Adamts9 at late time points that could lead to male biased sex ratio, abnormal ovary, and female infertility reported in Adamts9 KO (Carter et al., 2019).

Figure 4.1

Conserved structure of Adamts9 from C. elegans to human. Different function domains are illustrated in different color. Green triangles show sites for two commercial antibodies (Triple Point Biologics, Inc., OR) generated against 180 peptide sequence of prodomain and metalloproteinase domain of human Adamts9, which share 41% and 86% sequence identity with zebrafish Adamts9, respectively. Red triangle shows CRISPR targeting site for generating zebrafish adamts9 knockout. Two mutant zebrafish lines (Δ10 and Δ11) which generated premature stop codons before the enzymatic active site (at 573aa) were selected and propagated (Carter et al., 2019) (Please see supplemental Figs. 1 and 2, Supplemental Tables 1–4 for detail).

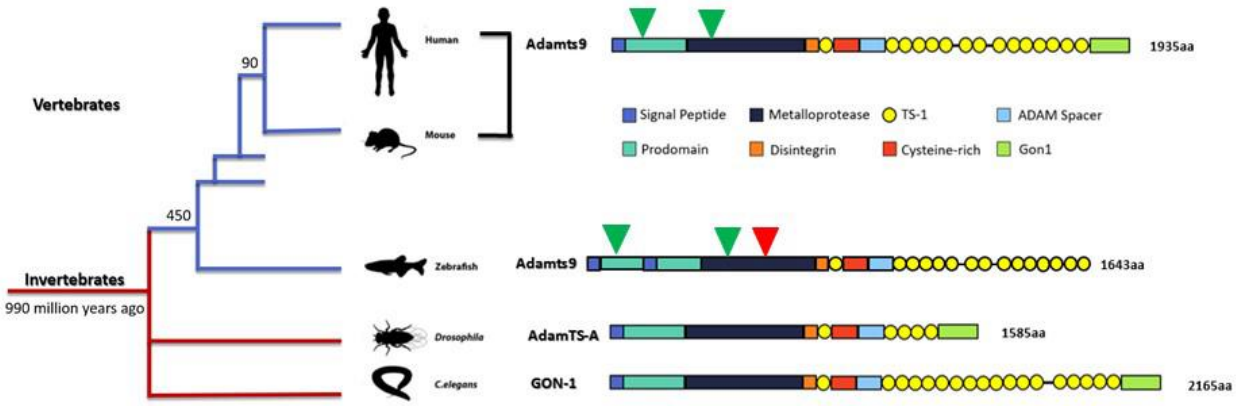


Figure 4.2

Strong adamts9 expression in preovulatory follicular cells and developing embryos. (A) Expression of adamts9 determined by RT-PCR. Top panel is adamts9 transcript analyzed by RT-PCR (35 cycles), bottom panel is expression of a house keep gene, eukaryotic translation elongation factor 1 alpha 1a (eef1a1a). M. NEB 1 kb plus DNA ladder; 1. Ovary (fully grown immature follicles); 2. Preovulatory stage IVb follicles; 3. Ovulated stage V oocytes; 4. One cell stage embryos; 5. Four cell stage embryos; 6. Oblong stage embryos (~3.5 hpf, hours post fertilization); 7. Germ-ring stage embryos (~5.5 hpf); 8. Eight somite stage embryos (~11.5 hpf); 9. 24 hpf (hours post fertilization) embryos; 10. Two dpf (days post fertilization) embryos; 11. Tree dpf embryos; 12. Six dpf embryos; 13. Six wpf (weeks post fertilization) gonad. Please see Supplemental Figs. 3 and 4 for full agarose gel images. (B) Strong adamts9 expression in preovulatory follicles, low or no expression in immature follicles or ovulated oocytes determined by real-time quantitative PCR (qPCR). Stage I and III immature follicles, IVb preovulatory follicular cell enclosed oocytes and stage V ovulated oocytes were collected from mature AB wildtype females between 7–8:30 am (lights on 8:30am-10:30 pm). Results were presented as mean±SEM (n=5). Diferent letters above the error bars indicate that those groups are signifcantly diferent from each other at $p<0.05$.

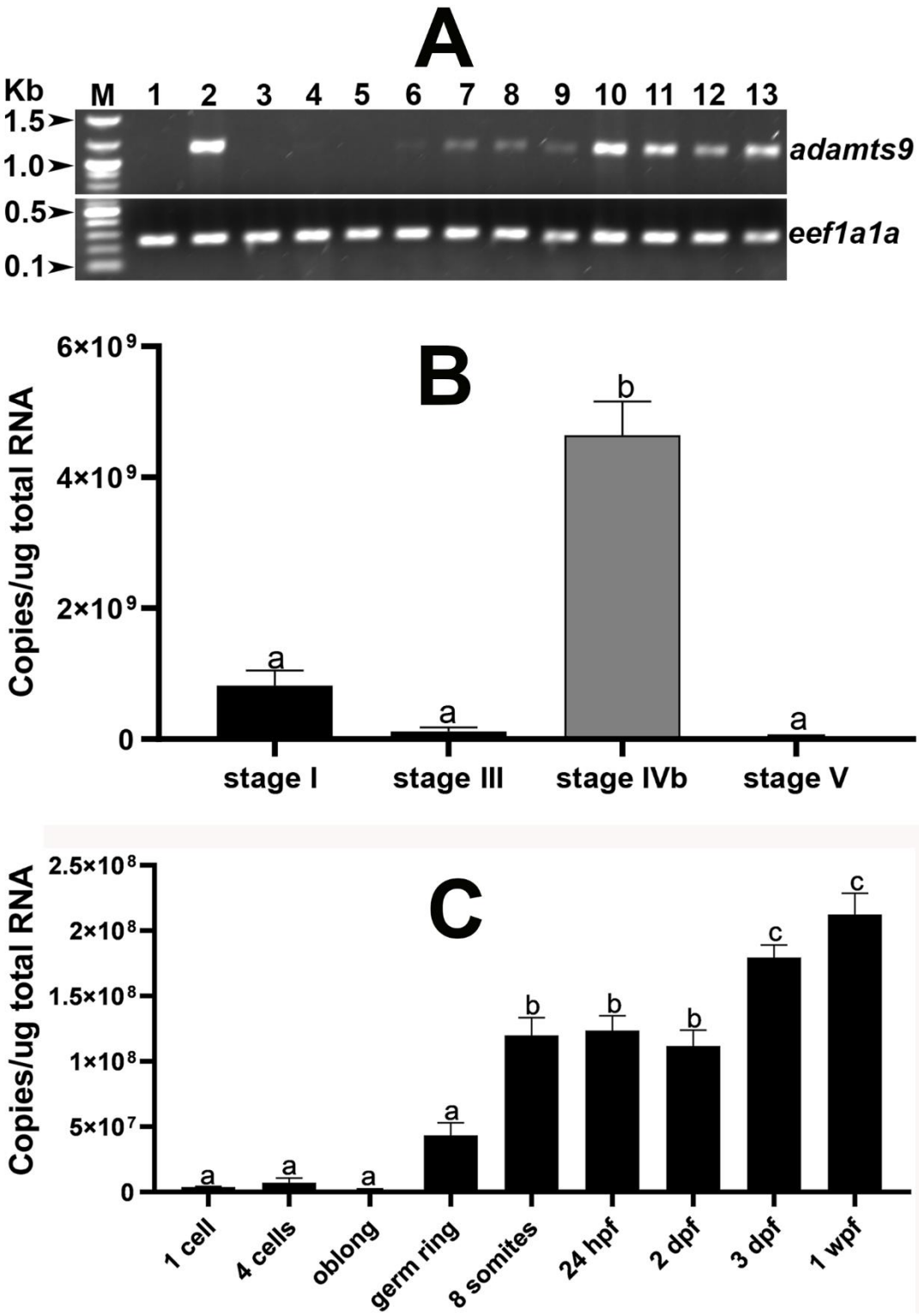

A
Kb
M 1 2 3 4 5 6 7 8 9 10 11 12 13
1.5
1.0
0.5
0.1
adamts9
eef1a1a
B
Copies/ug total RNA
6×10^9
4×10^9
2×10^9
0
a
a
b
a
stage I
stage III
stage IVb
stage V
C
Copies/ug total RNA
2.5×10^8
2×10^8
1.5×10^8
1×10^8
5×10^7
0
a
a
a
a
b
b
b
c
c
1 cell
4 cells
oblong
germ ring
8 somites
24 hpf
2 dpf
3 dpf
1 wpf

Figure 4.3

Strong expression of Adamts9 in preovulatory follicles, weak or no expression in immature ovarian follicles or ovulated oocytes in Adamts9 transgenic lines (*Tg(adamts9:GFP)*). The expression of Adamts9 was determined by EGFP expression driven by adamts9 promoters located in a 4.5 kb upstream sequence of adamts9 start codon. Representative confocal slice images of stage I (immature pre-vitellogenic), III (immature vitellogenic), IVb (mature & preovulatory) follicles and stage V ovulated oocytes were shown. Follicles or oocytes were confocal imaged under EGFP and transmitted light (T-PMT) channels. Similar expression was confrmed in the follicles or oocytes of several F1 and F2 adult females from fve independent transgenic lines. Scale bar: 10 μm (stage I); 100 μm (stages III, IVb, V).

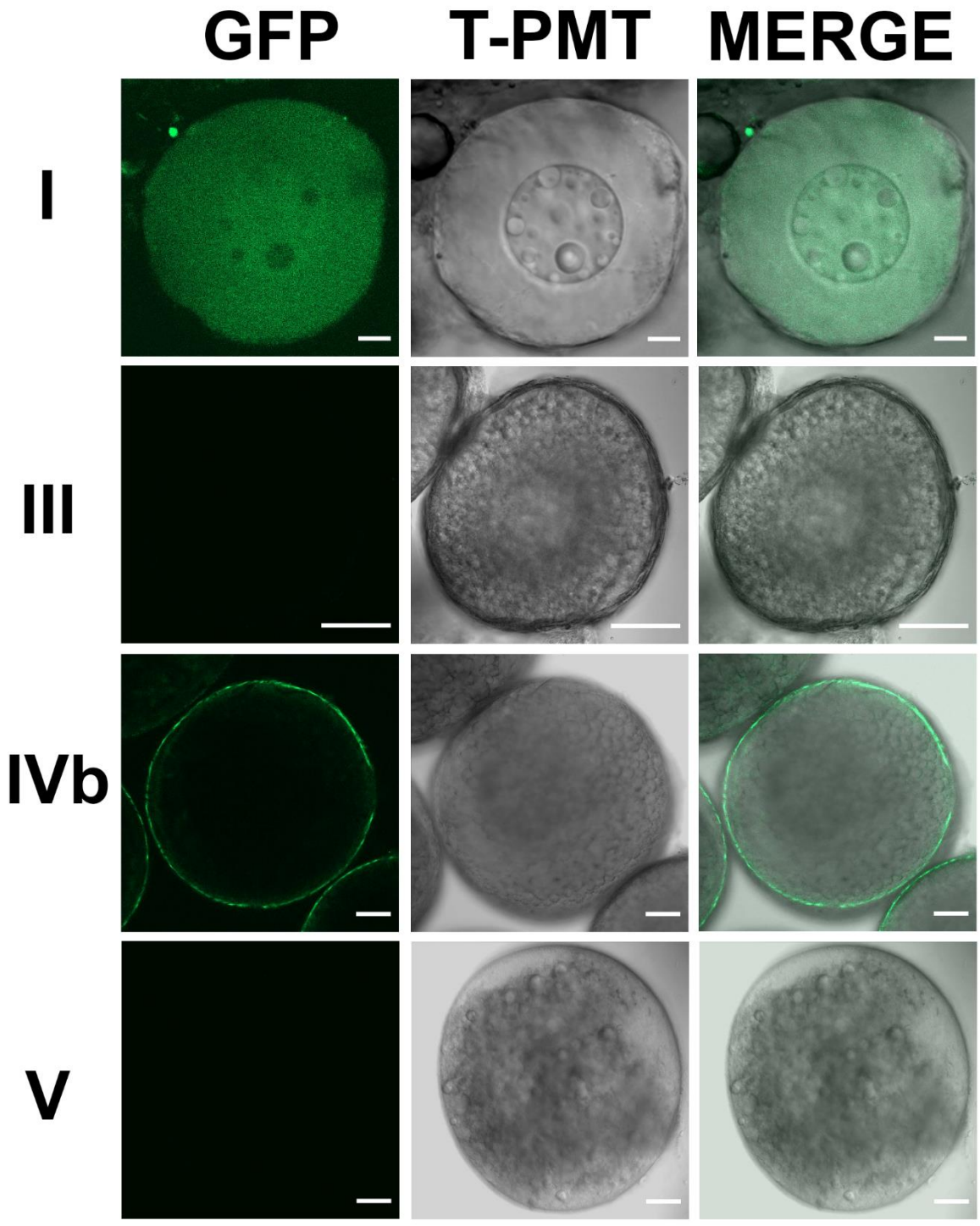

GFP
T-PMT
MERGE
I
III
IVb
V

Figure 4.4

Expression of Adamts9 in F2 transgenic (*Tg(adamts9:GFP)*) embryos during embryonic development. The expression of Adamts9 was determined by EGFP expression driven by adamts9 promoters located in a 4.5 kb upstream sequence of adamts9 start codon. Representative confocal z-stack images of various stages of development embryos were imaged by a confocal microscope under EGFP or transmitted light (T-PMT) channels. Similar expression was confirmed in multiple F2 embryos from five independent F1 transgenic lines (*Tg(adamts9:GFP)*). Scale bar: 200 μm.

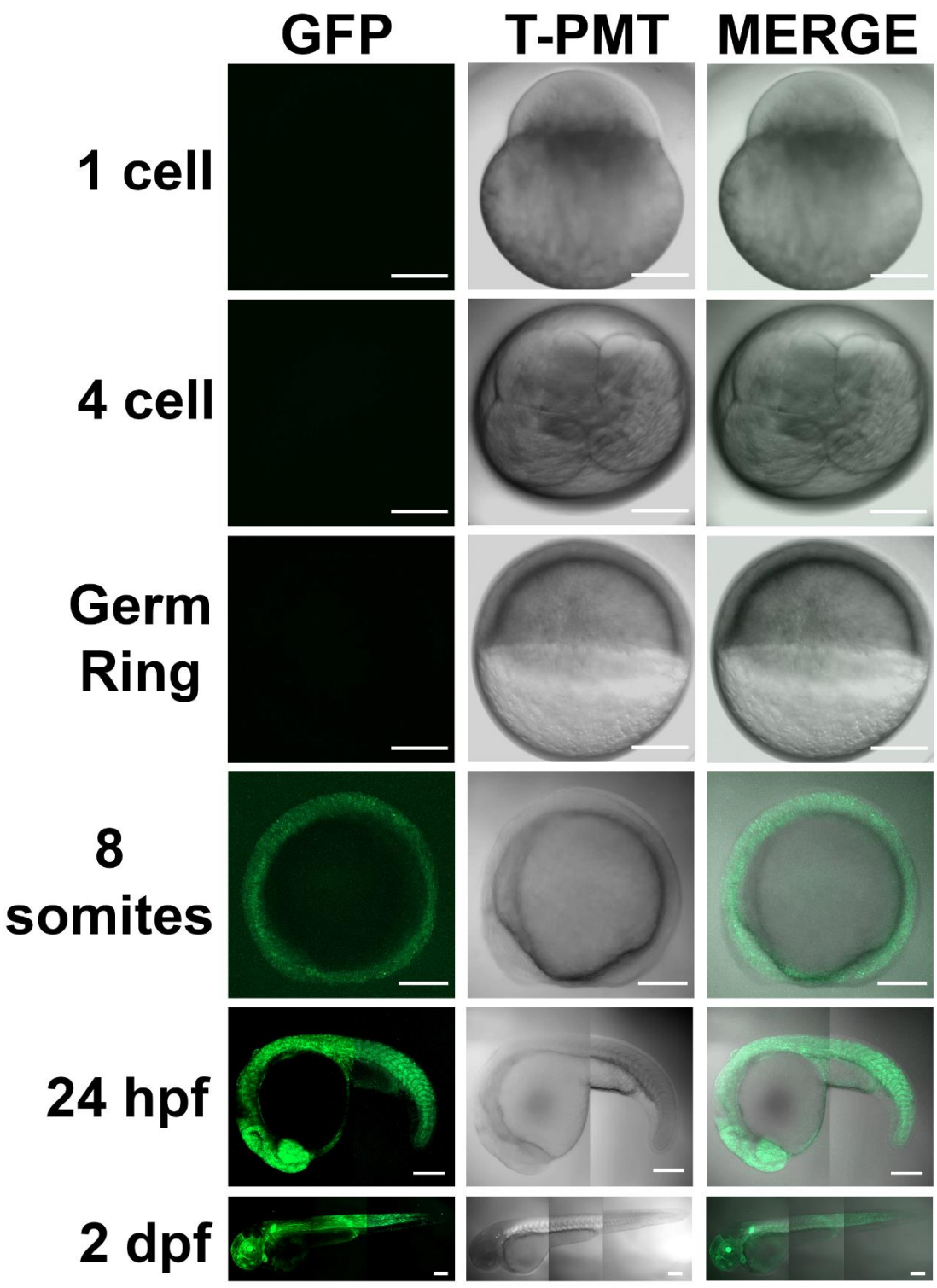

GFP
T-PMT
MERGE
1 cell
4 cell
Germ Ring
8 somites
24 hpf
2 dpf

Figure 4.5

Strong Adamts9 expression in F2 transgenic (*Tg(adamts9:GFP)*) zebrafish embryos. The expression of Adamts9 was determined by EGFP expression driven by adamts9 promoters located in a 4.5 kb upstream sequence of adamts9 start codon. Same conditions were used for imaging embryos under the EGFP, red (for monitoring autofluorescence), and transmitted light (T-PMT) channels. Picture (A) is z-stack confocal images of a representative F2 transgenic embryo at 10 h post fertilization (hpf) from GFP channel; Picture B is a wildtype control embryo imaged under same condition. Pictures (C) or (D) are corresponding images of same embryo from pictures A or B using T-PMT channel. Pictures (E,F) are confocal z-stack images of a representative F2 transgenic embryo at 48 hpf, while pictures (G,H) are confocal z-stack images of a wildtype embryo at 48 hpf. Pictures (E,G) are merged confocal z-stack images from all three channels, while pictures (F,H) were confocal z-stack images from red channel to show background. Scale bar: 100 μm (A–D), 400 μm (E–H).

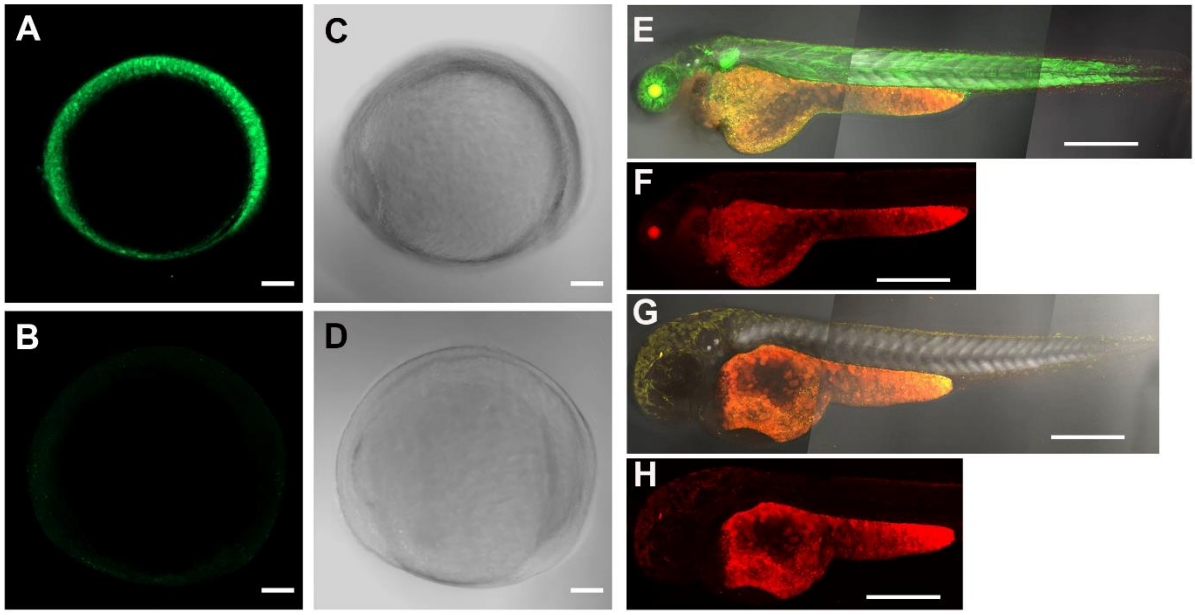


Figure 4.6

Delayed primordial germ cell (PGC) migration in Adamts9 knockout zebrafish during early development. PGC is labeled with GFP by crossing Adamts9 KO with Tg(vasa:GFP). Distance between two most distant PGCs were determined as an indicator of PGC migration (See Fig. 7 for detail). Showing distance, number of PGC, or representative images of zebrafish embryos at 15, 24 or 48 h post fertilization (hpf) in wildtype (+/+), heterozygous (+/–), and homozygous (–/–) Adamts9 KO. Embryos from at least 4 sets of parents were analyzed. The numbers on the left side of the forward slash is the number of embryos analyzed, and the numbers on the right side of the forward slash indicate sets of parents used for producing these embryos. Top panels are representative confocal images showing entire or part of embryos with GFP labeled PGC at a low magnification (scale bar: 200 μm). Bottom row are magnified confocal images of the embryos from the top (scale bar: 50 μm).

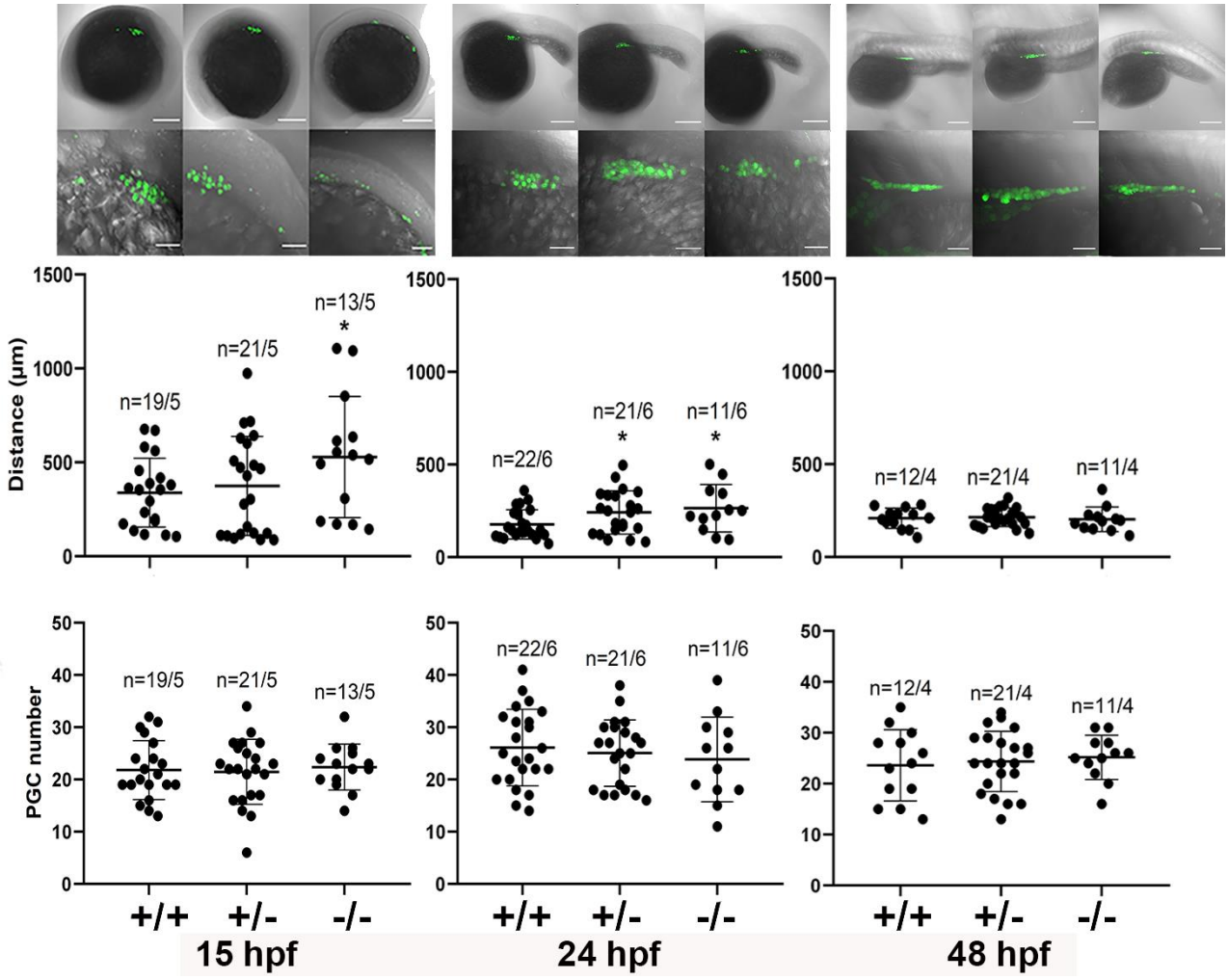
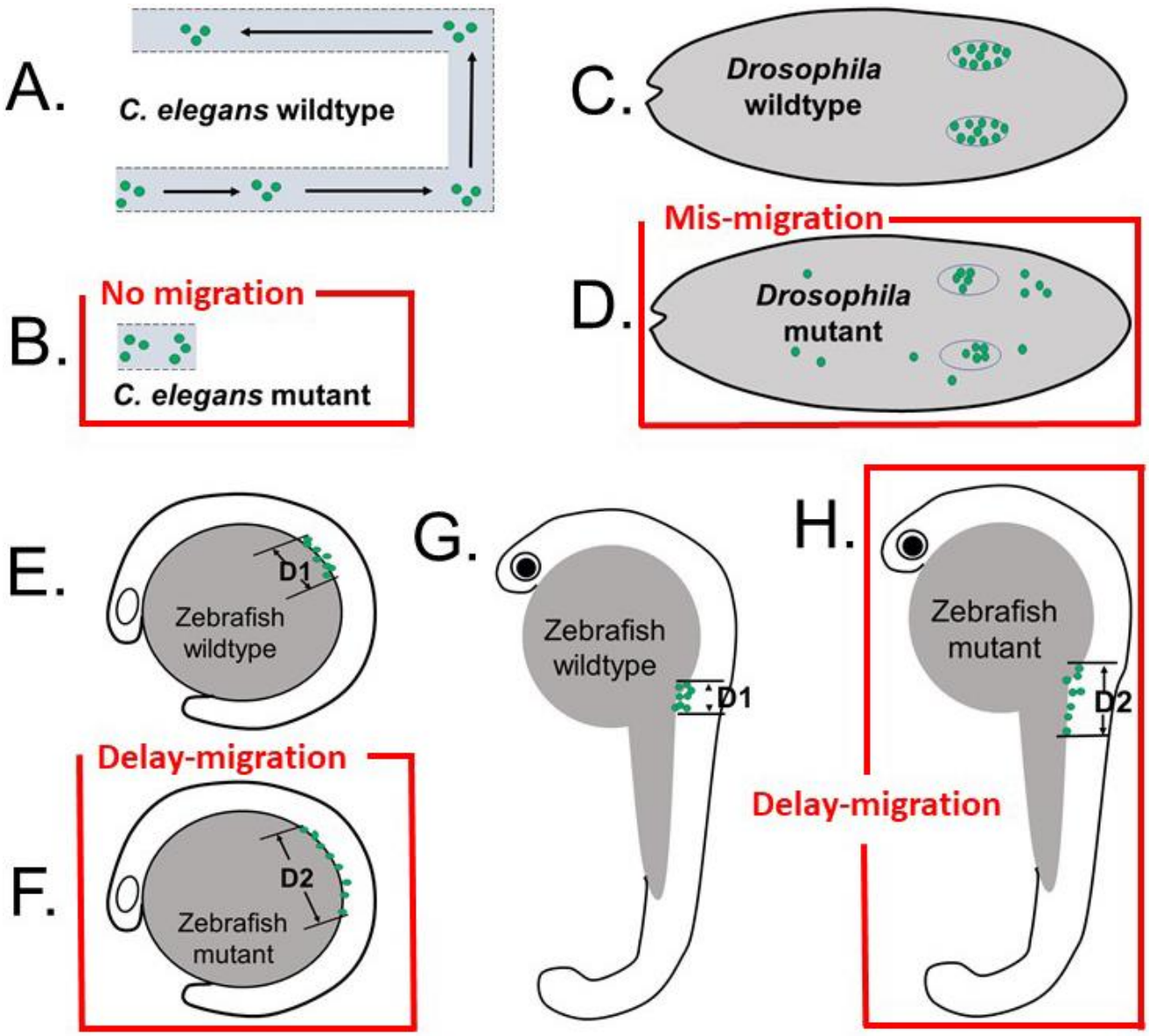

Distance (µm)
PGC number
n=19/5
n=21/5
n=13/5
*
n=22/6
n=21/6
*
n=11/6
*
n=12/4
n=21/4
n=11/4
+/+
+/-
-/-
15 hpf
24 hpf
48 hpf

Figure 4.7

Schematic drawings show difference in the defects of germ cell migration in Adamts9 or its ortholog knockouts in C. elegans, Drosophila, and zebrafish models. Green dots denote primordial germ cells (PGC). Only the posterior gonad arm is shown for C. elegans. (A) PGCs migrate in wildtype C elegans, (B) no migration occurs in gon-1 mutant (modified from Blelloch and Kimble 1999; Blelloch et al., 1999). (C) PGCs migrate correctly in wildtype Drosophila and clustered together at gonadal ridge at stage 16 embryo. (D) PGC mis-migrate in AdamTS-A knockout embryos (modified from Ismat et al. 2013). E–H. All PGC migrate toward gonadal ridge in zebrafish embryos, though these PGC were distributed in wider area (D2>D1) in the Adamts9 knockouts at 15 h post fertilization (hpf, E: wildtype; F: Adamts9 KO) and 24 hpf (G: wildtype; H: Adamts9 KO), i.e. delayed migration. The migration of PGC is completed around 24hpf in wildtype embryos, but 48hpf in Adamts9 KO zebrafish embryos (see Fig. 4.6 for detail).


A.
C. elegans wildtype
C.
Drosophila wildtype
Mis-migration
No migration
B.
C. elegans mutant
D.
Drosophila mutant
E.
D1
Zebrafish wildtype
G.
Zebrafish wildtype
D1
H.
Zebrafish mutant
D2
Delay-migration
Delay-migration
F.
D2
Zebrafish mutant

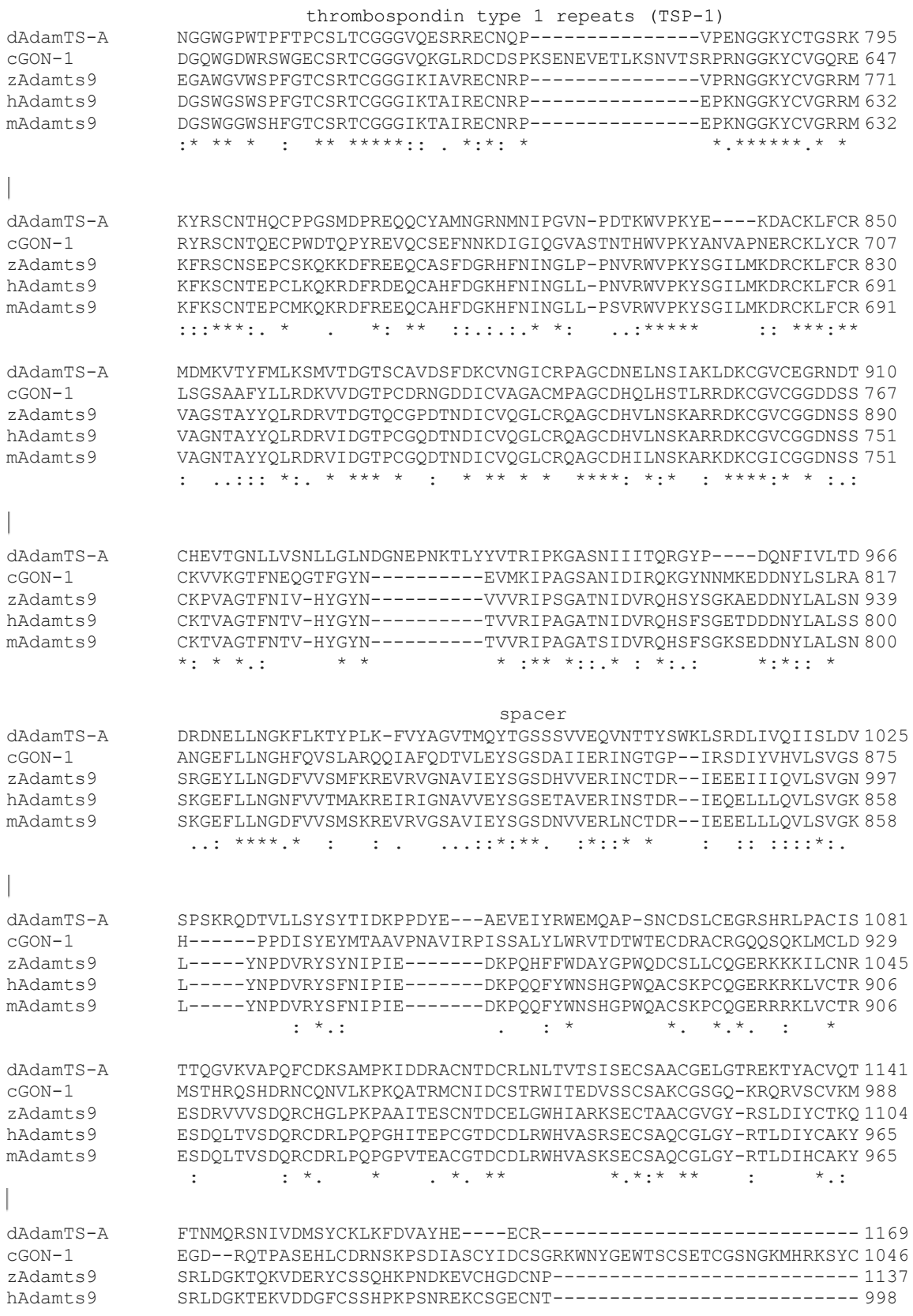
Supplemental Figure S4.1

Amino acid sequence alignments of human ADAMTS9 (hAdamts9, NP_891550), mice ADAMTS9 (mAdamts9, NP_780523), zebrafish Adamts9 (zAdamts9, NP_001244125), their ortholog in *Drosophila melanogaster* (dAdamTS-A, NP_996218) and *C. elegans* (cGON-1,NP_001255448). Metalloproteinase activation sites are highlighted in green color. Red arrow indicates the position of frame shift mutation caused by CRISPR/Cas9 genome editing that led to a premature stop codon in zebrafish Adamts9 (see Carter et al., 2019 for detail).

```
dAdamTS-A      MSMPDAGSLKSPAGGQEVECHRLLSDLPAFAYPSTTL--RSTTSGH--STGTNSTTVCES 56
cGON-1         ------------------------------------------------------------ 0
zAdamts9       ----------------------MLSKLQEFGAYEIVTPARLNEVGEQLPTGVHFKR---- 34
hAdamts9       ------------------------------------------------------------ 0
mAdamts9       ------------------------------------------------------------ 0

dAdamTS-A      PGARDRTSFACSSSCVSSACSATASDDDEDERALERCLR--GTTDDLGESGLRYSLKGPP 114
cGON-1         ---------------------------------------------MRSIGGSFHLLQPV 14
zAdamts9       ---RKRS-------------------TDPTTANISHHWTSPHAYYQISAFGQDYYLNLTL 72
hAdamts9       ------------------------------------------------------------ 0
mAdamts9       ------------------------------------------------------------ 0

dAdamTS-A      PELYSAKKDFISKGPLLGRQLEVK--------KRCEWWCHKYI--QKMSTHWRQNACLYA 164
cGON-1         V-----------------------------------------------------------A 16
zAdamts9       ESGFIAP---VYTVTILGASSEGHNSVEGEEEEDTEYQHCFYKGHVNAGQEH-------T 122
hAdamts9       ----------------------------------MQF----------VSW-------A 7
mAdamts9       ----------------------------------MQL----------VSW-------A 7
                                                                          :
dAdamTS-A      CCIAFLLGMLIMFHLGLRSAHKGQEELPQSTHPLAN-------SPPATPATLHPRRLDND 217
cGON-1         ALILLVVCLVYAL----QSGSGTISEF--SSDVLF--SRAKYSGVP-VHHSRWRQDAGIH 67
zAdamts9       AVISLCSGLLGTF----RSPEGE---F--FVEPLHSYNSEHYEEEHIKPHVVYRKDASKK 173
hAdamts9       TLLTLLVRDLAEM----GSPD-----------------------------------AAAA 28
mAdamts9       TLLTLLVPDLVEM----RSPD-----------------------------------SAAA 28
                 : :    :  :     *
|
dAdamTS-A      TSTDHEPPDGLDDLDEEEHSAFVMPTKVYNYSLSE----ADLIYESKRNSDINSFLKESA 273
cGON-1         VIDSHHIVR-RDSYGRRGKRDVTSTDRRRRLQGVARDCGHACHLRLRSDDAV--Y----I 120
zAdamts9       TVDDSAACETSAKLQEFGAYEIVTP---ARLNEVGEQLPTGVHFKRRKRS----T----D 222
hAdamts9       VRKDRLHPRQVKLLETLSEYEIVSP---IRVNALGEPFPTNVHFKRTRRS----I----N 77
mAdamts9       VRKDRLHPRQVKLLESLSEYEIASP---IRVNPLGEPFPTNVHFKRRRRS----I----N 77
               .  .                ..      . .           .    .

                                        prodomain
dAdamTS-A      SAFAMTGTYRNMSNEIWDPHPQYNLNVFGRQLHLVLRQDASFVHNHSMTHIRILK-EGEE 332
cGON-1         VHLHRWNQIPD---SHNKSVPHFSNSNFAPMVLY-------------------LD--SE- 155
zAdamts9       PTTA------NISHHWTSPHAYYQISAFGQDYYLNLTLESGFIAPVYT--VTILGASSEG 274
hAdamts9       SATDPWPAFASSSSSSTSSQAHYRLSAFGQQFLFNLTANAGFIAPLFT--VTLLGTPGVN 135
mAdamts9       SASDPWPAFASSSSSSTSSQEHYRLSAFGQQFLFNLTAHTGFIAPLFT--VTLLGEPGVN 135
                         .      .    :  . *.                        *   .


dAdamTS-A      HPGPETEAEAEQRHLGCFYSGYVEDDPHSMVSVSLC---GGMTGYIKTSFGALLIQPVNR 389
```

```
cGON-1          ---EEVRGGMSRTDPDCIYRAHVKGVHQHS-IVNLCDSEDGLYGMLALPSGIHTVEPIIS 211
zAdamts9        HNSVEGEEDEDTEYQHCFYKGHVNAGQEHTAVISLC---SGLLGTFRSPEGEFFVEPLHS 331
hAdamts9        QTK--FYSEEEAELKHCFYKGYVNTNSEHTAVISLC---SGMLGTFRSHDGDYFIEPLQS 190
mAdamts9        QTK--LYSEEEMELRHCFYKGHVNTKSEHTAVISLC---SGMMGTFRSHDGDYFIEPLQS 190
                          .     *:* .:*:   .    :.**   .*: * :    *   ::*:

dAdamTS-A       TSSDEV------LHRVFRKSQRN-----ARHAVSKFEL---------------------- 416
cGON-1          GNGTEHDGASRHRQHLVRKFDPMHFKS--------FDHLNSTSVNETETTV--------- 254
zAdamts9        YNSERYEEEHIKPHVVYRKDASKKTV-DDSAACETSGHTEPNRRHRNRL---KRKSPSSM 387
hAdamts9        MDEQEDEEEQNKPHIIYRRSAPQREPSTGRHACDTSEHKNRHSKDKKKTRARKWGERINL 250
mAdamts9        VDEQEDEEEQNKPHIIYRHSTPQREPSTGKHACATSELKNSHSKDKRKIRMRKRRKRNSL 250
                 .  .        : : *:

dAdamTS-A       ---------GLDDFMSKLEQVQEEEQKSKSRKLNRKKRHYADVDNQVYTLEVLIAVDNSM 467
cGON-1          ------------------ATWQDQWEDVI-ERKARSRRA---ANSWDHYVEVLVVADTKM 292
zAdamts9        LSDLETLNSRLFPFS---ENKHNSANESSDSKPHRRSKR---FLSYPRFVEVMVVADSKM 441
hAdamts9        AGDVAALNSGLATEA--FSAYGNKTDNTREKRTHRRTKR---FLSYPRFVEVLVVADNRM 305
mAdamts9        ADDVALLKSGLATKV--LSGYSNQTNSTRDRWNHKRTKR---FLSYPRFVEVMVVADHRM 305
                              :. :.       :  :      .    :**::..*  *

dAdamTS-A       KQFHGEDLQPYILILMSIVSSIFADASIGNSIRILLVRLISLPNINDQT---HSSNEMLK 524
cGON-1          YEYHGRSLEDYVLTLFSTVASIYRHQSLRASINVVVVKLIVLKTENAGPRITQNAQQTLQ 352
zAdamts9        VEHHGSNLQHYILTLMSIVSSIYKDPSIGNLINIVIVKLVIIKNELDGPTISFNAQATLK 501
hAdamts9        VSYHGENLQHYILTLMSIVASIYKDPSIGNLINIVIVNLIVIHNEQDGPSISFNAQTTLK 365
mAdamts9        VLYHGANLQHYILTLMSIVASIYKDSSIGNLINIVIVNLVVIHNEQEGPYINFNAQTTLK 365
                  .** .*: *:* *:* *:**: . *:   *.:::*.*: : .         .::  *:
```

```
                                metalloprotease domain
dAdamTS-A       HFCQFINQSG-------YERDTAMLITREPICGSVPGKICHMLGLAELGTVCSS-SSCSI 576
cGON-1          DFCRWQQYYNDPDDSSVQHHDVAILLTRKDICRSQ--GKCDTLGLAELGTMCDMQKSCAI 410
zAdamts9        NFCIWQQSQNHPDDNHPSHHDTAILITRQDICRAR--DKCDTLGLAELGTVCDPYRSCSI 559
hAdamts9        NFCQWQHSKNSPGG---IHHDTAVLLTRQDICRAH--DKCDTLGLAELGTICDPYRSCSI 420
mAdamts9        NFCQWQHSKNYLGG---IQHDTAVLVTREDICRAQ--DKCDTLGLAELGTICDPYRSCSI 420
                .** : :  .        .:*.*:*:**: ** :     *. ********:*.   **:*

dAdamTS-A       VQDTGLPTAFTMAHELGHILNMNHDDDDKCMPYVTRQNNNKVLHIMSSVMGIHMHPWSWS 636
cGON-1          IEDNGLSAAFTIAHELGHVFSIPHDDERKCSTYMPVNK--NNFHIMAPTLEYNTHPWSWS 468
zAdamts9        NEDNGLSTAFTIAHELGHVFNMPHDDSNKCKEDG--VK--NQQHVMAPTLNYYTNPWMWS 615
hAdamts9        SEDSGLSTAFTIAHELGHVFNMPHDDNNKCKEEG--VK--SPQHVMAPTLNFYTNPWMWS 476
mAdamts9        SEDSGLSTAFTIAHELGHVFNMPHDDSNKCKEEG--VK--SPQHVMAPTLNFYTNPWMWS 476
                 :*.** :***:******::.: ***. **       :  .  *:*: .:    :** **

dAdamTS-A       KCSRHFVSEFLEKT--DKSCLETSVGAHIPY---GTERLPGEIYSLDAQCQLSFGNDFGY 691
cGON-1          PCSAGMLERFLENNRGQTQCLFDQPVERRYYEDVFVRDEPGKKYDAHQQCKFVFGPASEL 528
zAdamts9        KCSRKYITEFLDTG--YGECLLDEPVSRPYSL---SQQLPGQIYSVNKQCELIFGPGTQV 670
hAdamts9        KCSRKYITEFLDTG--YGECLLNEPESRPYPL---PVQLPGILYNVNKQCELIFGPGSQV 531
mAdamts9        KCSRKYITEFLDTG--YGECLLNEPASRTYPL---PSQLPGLLYNVNKQCELIFGPGSQV 531
                 **   : .**:.     .**  .   :           **  *. . **:: **

dAdamTS-A       CPTDEECKRLWCNRTSGNSNEQCASSNLPWADGTPCGSSG-HWCQRGKCVSNKHGYGRQV 750
cGON-1          CPYMPTCRRLWCATFYGS-QMGCRTQHMPWADGTPCDESRSMFCHHGACVRLAPESLTKI 587
zAdamts9        CPYMTQCRRLWCTSPDGV-QRGCRTQHMPWADGTDCAPGK--HCKHGLCIHKEHE-YVPV 726
hAdamts9        CPYMMQCRRLWCNNVNGV-HKGCRTQHTPWADGTECEPGK--HCKYGFCVPKEMD-VPVT 587
mAdamts9        CPYMMQCRRLWCNNVDGA-HKGCRTQHTPWADGTECEPGK--HCKFGFCVPKEME-GPAI 587
                **    *:****    *  :  * :.: ****** *  .    *: * *:
```




```
                        thrombospondin type 1 repeats (TSP-1)
dAdamTS-A       NGGWGPWTPFTPCSLTCGGGVQESRRECNQP---------------VPENGGKYCTGSRK 795
cGON-1          DGQWGDWRSWGECSRTCGGGVQKGLRDCDSPKSENEVETLKSNVTSRPRNGGKYCVGQRE 647
zAdamts9        EGAWGVWSPFGTCSRTCGGGIKIAVRECNRP---------------VPRNGGKYCVGRRM 771
hAdamts9        DGSWGSWSPFGTCSRTCGGGIKTAIRECNRP---------------EPKNGGKYCVGRRM 632
mAdamts9        DGSWGGWSHFGTCSRTCGGGIKTAIRECNRP---------------EPKNGGKYCVGRRM 632
                :* ** *  :  ** *****:: . *:*: *               *.******.* *

dAdamTS-A       KYRSCNTHQCPPGSMDPREQQCYAMNGRNMNIPGVN-PDTKWVPKYE----KDACKLFCR 850
cGON-1          RYRSCNTQECPWDTQPYREVQCSEFNNKDIGIQGVASTNTHWVPKYANVAPNERCKLYCR 707
zAdamts9        KFRSCNSEPCSKQKKDFREEQCASFDGRHFNINGLP-PNVRWVPKYSGILMKDRCKLFCR 830
hAdamts9        KFKSCNTEPCLKQKRDFRDEQCAHFDGKHFNINGLL-PNVRWVPKYSGILMKDRCKLFCR 691
mAdamts9        KFKSCNTEPCMKQKRDFREEQCAHFDGKHFNINGLL-PSVRWVPKYSGILMKDRCKLFCR 691
                :::***:. *   .   *: **  ::.:.:.* *:   ..:*****     :: ***:**

dAdamTS-A       MDMKVTYFMLKSMVTDGTSCAVDSFDKCVNGICRPAGCDNELNSIAKLDKCGVCEGRNDT 910
cGON-1          LSGSAAFYLLRDKVVDGTPCDRNGDDICVAGACMPAGCDHQLHSTLRRDKCGVCGGDDSS 767
zAdamts9        VAGSTAYYQLRDRVTDGTQCGPDTNDICVQGLCRQAGCDHVLNSKARRDKCGVCGGDNSS 890
hAdamts9        VAGNTAYYQLRDRVIDGTPCGQDTNDICVQGLCRQAGCDHVLNSKARRDKCGVCGGDNSS 751
mAdamts9        VAGNTAYYQLRDRVIDGTPCGQDTNDICVQGLCRQAGCDHILNSKARKDKCGICGGDNSS 751
                :  ..::: *:. * *** *  :  * ** * *  ****: *:*  : ****:* * :.:

dAdamTS-A       CHEVTGNLLVSNLLGLNDGNEPNKTLYYVTRIPKGASNIIITQRGYP----DQNFIVLTD 966
cGON-1          CKVVKGTFNEQGTFGYN----------EVMKIPAGSANIDIRQKGYNNMKEDDNYLSLRA 817
zAdamts9        CKPVAGTFNIV-HYGYN----------VVVRIPSGATNIDVRQHSYSGKAEDDNYLALSN 939
hAdamts9        CKTVAGTFNTV-HYGYN----------TVVRIPAGATNIDVRQHSFSGETDDDNYLALSS 800
mAdamts9        CKTVAGTFNTV-HYGYN----------TVVRIPAGATSIDVRQHSFSGKSEDDNYLALSN 800
                *: * *.:      * *           * :** *::.* : *:.:     *:*:: *

                                        spacer
dAdamTS-A       DRDNELLNGKFLKTYPLK-FVYAGVTMQYTGSSSVVEQVNTTYSWKLSRDLIVQIISLDV 1025
cGON-1          ANGEFLLNGHFQVSLARQQIAFQDTVLEYSGSDAIIERINGTGP--IRSDIYVHVLSVGS 875
zAdamts9        SRGEYLLNGDFVVSMFKREVRVGNAVIEYSGSDHVVERINCTDR--IEEEIIIQVLSVGN 997
hAdamts9        SKGEFLLNGNFVVTMAKREIRIGNAVVEYSGSETAVERINSTDR--IEQELLLQVLSVGK 858
mAdamts9        SKGEFLLNGDFVVSMSKREVRVGSAVIEYSGSDNVVERLNCTDR--IEEELLLQVLSVGK 858
                 ..: ****.*  :   : .   ...::*:**.  :*::* *    :  :: ::::*:.

dAdamTS-A       SPSKRQDTVLLSYSYTIDKPPDYE---AEVEIYRWEMQAP-SNCDSLCEGRSHRLPACIS 1081
cGON-1          H------PPDISYEYMTAAVPNAVIRPISSALYLWRVTDTWTECDRACRGQQSQKLMCLD 929
zAdamts9        L-----YNPDVRYSYNIPIE-------DKPQHFFWDAYGPWQDCSLLCQGERKKKILCNR 1045
hAdamts9        L-----YNPDVRYSFNIPIE-------DKPQQFYWNSHGPWQACSKPCQGERKRKLVCTR 906
mAdamts9        L-----YNPDVRYSFNIPIE-------DKPQQFYWNSHGPWQACSKPCQGERRRKLVCTR 906
                          : *.:            .   : *       *.  *.*.  :   *

dAdamTS-A       TTQGVKVAPQFCDKSAMPKIDDRACNTDCRLNLTVTSISECSAACGELGTREKTYACVQT 1141
cGON-1          MSTHRQSHDRNCQNVLKPKQATRMCNIDCSTRWITEDVSSCSAKCGSGQ-KRQRVSCVKM 988
zAdamts9        ESDRVVVSDQRCHGLPKPAAITESCNTDCELGWHIARKSECTAACGVGY-RSLDIYCTKQ 1104
hAdamts9        ESDQLTVSDQRCDRLPQPGHITEPCGTDCDLRWHVASRSECSAQCGLGY-RTLDIYCAKY 965
mAdamts9        ESDQLTVSDQRCDRLPQPGPVTEACGTDCDLRWHVASKSECSAQCGLGY-RTLDIHCAKY 965
                 :       : *.    *    . *. **         *.*:* **    :     *.:

dAdamTS-A       FTNMQRSNIVDMSYCKLKFDVAYHE----ECR---------------------------- 1169
cGON-1          EGD--RQTPASEHLCDRNSKPSDIASCYIDCSGRKWNYGEWTSCSETCGSNGKMHRKSYC 1046
zAdamts9        SRLDGKTQKVDERYCSSQHKPNDKEVCHGDCNP--------------------------- 1137
hAdamts9        SRLDGKTEKVDDGFCSSHPKPSNREKCSGECNT--------------------------- 998
```

```
mAdamts9        SRMDGKTEKVDDSFCSSQPRPSNQEKCSGECST---------------------------998
                     :   ..   *. :           :*

||
                          TSP-1
dAdamTS-A       ------------------------------------------------------------
cGON-1          VDDSNRRVDESLCGREQKEATERECNRIPCPRWVYGHWSECSRSCDGGVKMRHAQCLDAA1106
zAdamts9        ------------------------------------------------------------
hAdamts9        ------------------------------------------------------------
mAdamts9        ------------------------------------------------------------

||

————————                    TSP-1
dAdamTS-A       ------------------------------------------------------------1197
cGON-1          DRETHTSRCGPAQTQEHCNEHACTWWQFGVWSDCSAKCGDGVQYRDANCTDRHRSVLPEH1166
zAdamts9        ----------------------------------------------------1173
hAdamts9        ----------------------------------------------------1035
mAdamts9        ----------------------------------------------------1035


|
                        TSP-1                                TSP-1
dAdamTS-A       ------------------------------------------------------------1197
cGON-1          RCLKMEKIITKPCHRESCPKYKLGEWSQCSVSCEDGWSSRRVSCVSGNGTEVDMSLCGTA 1226
zAdamts9        --------------------GGWEYSSWSECSRSCGGGTRRRNAICGKS-DERDDDSKCN 1176
hAdamts9        --------------------GGWRYSAWTECSKSCDGGTQRRRAICVNTRNDVLDDSKCT 1038
mAdamts9        --------------------GGWRYSAWTECSRSCDGGTQRRRAICVNTRNDVLDDSKCT 1038
||

                                                TSP-1
dAdamTS-A       --------------EGCWVLSEWSTCSKSCGTGSQQREAHCYLHNSRVSDDLCNPRTKPH1215
cGON-1          SDRPASHQTCNLGTCPFWRNTDWSACSVSCGIGHRERTTECIYREQSVDASFCGDTKMPE1286
zAdamts9        PQEKLTAQPCNEFLCPQWKTGDWSECLVTCGKGYKHRQTWCQFGEERLDVRFCDS-SKPE1235
hAdamts9        HQEKVTIQRCSEFPCPQWKSGDWSECLVTCGKGHKHRQVWCQFGEDRLNDRMCDPETKPT1098
mAdamts9        HQEKVVVQSCNEFSCPHWKTGDWSECLVTCGKGHKHRQVWCQFGEDRLSDRMCDPEAKPE1098
                          *   :**.* .:**.*       *   :. :.  :*.    *
||

                                              TSP-1
dAdamTS-A       LNTLI-------------------------------------------------------G1221
cGON-1          TSQTCHLLPCTSWKPSHWSPCSVTCGSGIQTRSVSCTRGSEGTIVDEYFCDRNTRPRLKK1346
zAdamts9        SVQACQQQECASWQVGPWGQCTTTCGPGYQMRAVKCVVGSYGSVMDDTECNAATRPTDTQ1295
hAdamts9        SMQTCQQPECASWQAGPWGQCSVTCGQGYQLRAVKCIIGTYMSVVDDNDCNAATRPTDTQ1158
mAdamts9        PMQTCQQPECAAWQAGPWGQCSVTCGQGYQLRAVKCIMGTYMSVVDDNDCNAATRPTDTQ1158
|

————————|                                                          TSP-1
dAdamTS-A       ICNTESCP-------TYTKSPNALAVSNWVIGEWGECNEWCEK-----------------1257
cGON-1          TCEKDTCDGPRVLQ----KLQADVPPIRWATGPWTACSATCGNGTQRRLLKCRDHVRDLP1402
zAdamts9        DCGLSQCPVTHPVAPEPKVMPHPGHKTQWRFGSWTQCSATCGKGTRMRYVSCRDQQGGVA1355
hAdamts9        DCELPSCH-PPPAAPETRRSTYSAPRTQWRFGSWTPCSATCGKGTRMRYVSCRDENGSVA1217
mAdamts9        DCELASCH-PSILALEPRRNAQSIPRTQWRFGSWTPCSATCGKGTRMRYVSCRDEDGSVA1217
                 *    *                  .*  * *  *.  * :

||                                                      TSP-1
dAdamTS-A       ------------------------------------------------------------1257
cGON-1          DE-YCNHLDKEVSTRNCRLRDCSYWKMAEWEECPATCGTHVQQSRNVTCVSAEDGGRTIL1461
zAdamts9        EESACAHLPKPPASEVCSIVACGQWKVLEWTACSVSCGQGTT-TRQVVCMNISD---QVV1411
hAdamts9        DESACATLPRPVAKEECSVTPCGQWKALDWSSCSVTCGQGRA-TRQVMCVNYSD---HVI1273
mAdamts9        DESACATLPKPVAKEECSVTPCGQWKALDWSSCSVTCGQGKA-TRQVVCVNYSD---HVI1273
```

```
dAdamTS-A       ------------------------TRSVSCSHPYGIGCGSRKPKDVRKCCH --------1284
cGON-1          KDVDCDVQKRPTSARNCRLEPCPKGEE-------------------------------H1489
zAdamts9        ELSECDLDDKPAAEQECAMPQCPSRSSDHGGFS---PNPDFRKKTALPGRTDRNRAGRLQ1468
hAdamts9        DRSECDQDYIPETDQDCSMSPCPQRTPDSGLAQHPFQNEDYRPRSAS-----PSRTHVLG1328
mAdamts9        DRSECDPDYIPETDQDCSMSPCPQWTG----LAHPFQNEDFRPRSDS-----PSRTHVLG1324

                                                TSP-1
dAdamTS-A       ------------------------------------------------------------1284
cGON-1          IGSWIIGDWSKCSASCGGGWRRRSVSCT------SSSCDETRKPKMFDKCNEELCPPLTN1543
zAdamts9        AQQWRTGPWGACSSTCAGGFQRRVVVCQDENGYPASSCDESIQPIEQRSCESGSCP----1524
hAdamts9        GNQWRTGPWGACSSTCAGGSQRRVVVCQDENGYTANDCVERIKPDEQRACESGPCP----1384
mAdamts9        GNQWRTGPWGACSSTCAGGSQRRVVVCQDENGYTANDCVERIKPDEQRACESGPCP----
1380

                                                  TSP-1
dAdamTS-A       -IKYTSDWTDCSVQCGEGVKRKKQSCTRVYKPDVPGTRKRRVY---VDESYCISR-----1335
cGON-1          NSWQISPWTHCSVSCGGGVQRRKIWCEDVLSGRKQDDIECSEIK-PREQRDCEMPPCRSH1602
zAdamts9        -QWFYGSWSECSKSCGGGIKTRLVACQRPN-GERFNDLSCEILDKPPDREQCNTQ-----1577
hAdamts9        -QWAYGNWGECTKLCGGGIRTRLVVCQRSN-GERFPDLSCEILDKPPDREQCNTH-----1437
mAdamts9        -QWAYGSWGECTKLCGGGMRTRLVVCQRAN-GDRFPDLSCEVLDKPTDREQCNTH-----1433
                     . * .*:  ** *:: :   *            .        :.  *

dAdamTS-A       K------------------VHRPKLRTTTKSCRINCKWNASDWRRCPADC----------1367
cGON-1          YHNKTSSASMTSLSSSNSNTTSSASASSLPILPPVVSWQTSAWSACSAKCGRGTKRRVVE1662
zAdamts9        -------------------------------SCSINPHWSTDQWSLCFASCWSLHSSHLDQ1607
hAdamts9        -------------------------------ACPHDAAWSTGPWSSCSVSCGRGHKQRNVY1467
mAdamts9        -------------------------------ACPQDAAWSTGPWSSCSVSCGRGHKHRNVY1463
                                                 *.:. *  * ..*

                          TSP-1
dAdamTS-A       ------------------------------------------------------SEEYQT1373
cGON-1          CVNPS-------LNVTVASTECDQTKKPVEEVRCRTKHCPRWKTTTWSSCSVTCGRGIRR1715
zAdamts9        VGLQISSGESGKGGDEVDRGFFKNKD------MGGGPKETDW------------------1643
hAdamts9        C--------MAKDGSHLESDYCKHLAKPHGHRKCRGGRCPKWKAGAWSQCSVSCGRGVQQ1519
mAdamts9        C--------LAKDGSHLESDNCKHLPKPHGHRRCRGGRCPRWKAGAWSQCSVSCGQGVQQ1515

                               TSP-1
dAdamTS-A       RDVRC--------------------------------------------------------1378
cGON-1          REVQCYRGRKNLVSDSECNPKTKLNSVANCFPVACPAYRWNVTPWSKCKDECARGQKQTR1775
zAdamts9        ------------------------------------------------------------1643
hAdamts9        RHVGCQIGTHKIARETECNPYTRPESERDCQGPRCPLYTWRAEEWQECTKTCGEGSRY-R1578
mAdamts9        RHVGCQIGTHKAARESECSSYSRPESERVCQASPCPLYTWRAEQWQQCTKTCGEGSRY-R
1574

                               TSP-1
dAdamTS-A       ----ESFQGDGVEDKHCDAKKRPSKRRICNNCVRRQSRVI------SQCNCEGVEKRRDF1428
cGON-1          RVHCISTSGKRAAPRMCELARAPTSIRECDTSNCPYEWVPGDWQTCSKSCGEGVQTREVR1835
zAdamts9        ------------------------------------------------------------1643
hAdamts9        KVVCVDDNKNEVHGARCDVSKRPVDRESCSLQPCEYVWITGEWSECSVTCGKGYKQRLVS1638
mAdamts9        QVVCVAEDQSEVHSTHCDSDQRPPDRESCSLQPCEYVWITGEWSECSVTCGKGYRQRLVS1634

                                  TSP-1
dAdamTs-A       CFNSHKGRIACPTRARVERHSCTPPPHCRR------------RS-------AIGS-SISS1468
cGON-1          CRRKINFNSTIPIIFMLEDEPAVPKEKCELFPKPNESQTCELNPCDSEFKWSFGPWGECS1895
```

```
zAdamts9        ------------------------------------------------------------ 1643
hAdamts9        CSEIYTGKENYEYSYQ--TTI-----NCP-GTQPPSVHPCYLRDCPVSATWRVGNWGSCS 1690
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                                TSP-1
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cGON-1          KNCGQGIRRRRVKCVANDGRRVERVKCTTKKPRRTQYC-FERNC-LPSTCQELKSQNVKA 1953
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mAdamts9        VSCGIGVMHRSVQCLTNEDQPSHLCPTDT-KPEERKACRNVYNCELPQNCKEVKKLNSAS 1745


dAdamTs-A       KDGNYQLEVRSRMVHIYCHGMNSRTPQEYVNVD--PQENYSIYYEYRTKQTNSCPPESRG 1564
cGON-1          KDGNYTILLDGFTIEIYCHRMNSTIPKAYLNVN--PRTNFAEVYGKKLIYPHTCPFNGDR 2011
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hAdamts9        EDGEYFLMIRGKLLKIFCAGMHSDHPKEYVTLVHGDSENFSEVYGHRLHNPTECPYNGSR 1809
mAdamts9        VDGEYFLAVRGKPLKVFCAGMNSDYPKEYVTLAHGDSENFSEVYGHRLHNPTECPYNGSR 1805

                                      gon-1 domain
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cGON-1          NDSCHCSEDGDASAGLTRFNKVRIDLLNRKFHLADYTFAKREYGVHVPYGTAGDCYS-MK 2070
zAdamts9        ------------------------------------------------------------ 1643
hAdamts9        RDDCQCRKDY-TAAGFSSFQKIRIDLTSMQIITTDLQFARTSEGHPVPFATAGDCYS-AA 1867
mAdamts9        RDDCHCRKDY-TAAGFSSFQKIRLDLTSMQIITTDLEFARTSEGHPVPFATAGDCYS-AA 1863


dAdamTS-A       QCPQGDFSINMKDTDFSIRPGTVWRMHGQYSVMKRISEFDTTTQMRRGFCGGYCGGCYIA 1678
cGON-1          DCPQGIFSIDLKSAGLKLVDDLNWEDQGHRTSSRIDRFYNNA--KVIGHCGGFCGKCSPE 2128
zAdamts9        ------------------------------------------------------------ 1643
hAdamts9        KCPQGRFSINLYGTGLSLTESARWISQGNYAVSDIKKSPDGT--RVVGKCGGYCGKCTPS 1925
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dAdamTS-A       PDSGLYLDVL---------------------------- 1688
cGON-1          RYKGLIFEVNTKLLNHVKNGGHIDDELDDDGFSGDMD 2165
zAdamts9        ------------------------------------ 1643
hAdamts9        SGTGLEVRVL---------------------------- 1935
mAdamts9        SGTGLEVRVS---------------------------- 1931
```

Supplemental Figure S4.2

Amino acid sequences and functional domains (highlighted in different color) of Adamts9 in human, mouse, zebrafish, and their ortholog in *Drosophila* and *C. elegans.*

Signal peptide, highlighted in red color;
Prodomain, highlighted dark blue color;
Metalloproteinase domain, highlighted in grey color;
Thrombospondin type 1 repeats (TSP-1), highlighted in yellow color;
Spacer, highlighted in light blue color;
GON-1 domain, highlighted in purple color;

>hAdamts9 NP_891550.1 *Homo sapiens* ADAMTS9 amino acid sequence
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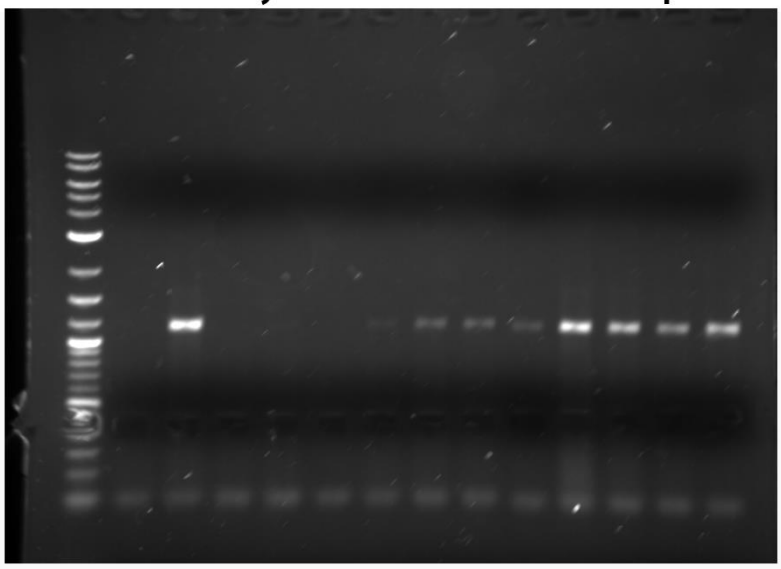
Supplemental Figure S4.3

Supplemental Figure 3

Full agarose gel image analysis of adamts9 expression in zebrafish embryos at different development stages

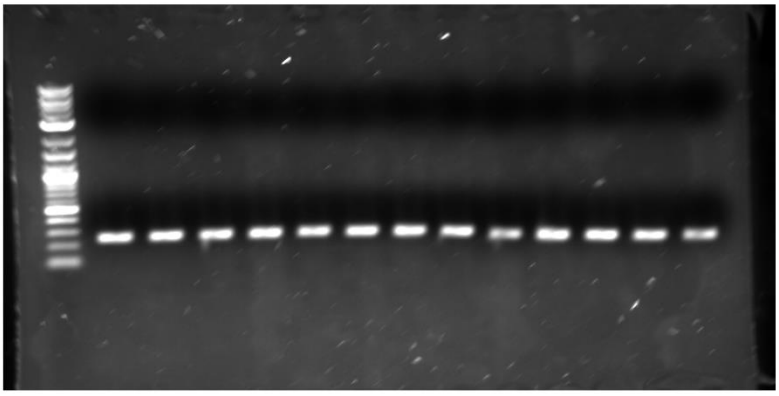


M: NEB 1kb plus DNA ladder; 1: Ovary (fully grown immature follicles); 2: Preovulatory stage IVb follicles; 3: Ovulated stage V oocytes; 4: One cell stage embryos; 5: Four cell stage embryos; 6: Oblong stage embryos (~3.5 hpf, hours post fertilization); 7: Germ-ring stage embryos (~5.5 hpf); 8: Eight somite stage embryos (~11.5 hpf); 9: 24 hpf (hours post fertilization) embryos; 10: Two dpf (days post fertilization) embryos; 11: Three dpf embryos; 12: Six dpf embryos; 13: Six wpf (weeks post fertilization) gonad.

Supplemental Figure S4.4

Supplemental Figure 4

Full agarose gel image analysis of eukaryotic translation elongation factor 1 alpha 1a (eef1a1a) expression in zebrafish embryos at different development stages



M: NEB 1kb plus DNA ladder; 1: Ovary (fully grown immature follicles); 2: Preovulatory stage IVb follicles; 3: Ovulated stage V oocytes; 4: One cell stage embryos; 5: Four cell stage embryos; 6: Oblong stage embryos (~3.5 hpf, hours post fertilization); 7: Germ-ring stage embryos (~5.5 hpf); 8: Eight somite stage embryos (~11.5 hpf); 9: 24 hpf (hours post fertilization) embryos; 10: Two dpf (days post fertilization) embryos; 11: Three dpf embryos; 12: Six dpf embryos; 13: Six wpf (weeks post fertilization) gonad.

Supplemental Table S4.1

Percentage of sequence identities (top right) and similarities (bottom left) of Adamts9 prodomain among representative species. Zebrafish has two prodomins, in which only two amino acids were different. We used first prodomain for comparison.

	human	mice	zebrafish	*Drosophila*	*C. elegans*
human		77	51	29	24
mice	81		43	27	22
zebrafish	60	56		30	29
Drosophila	42	37	42		23
C. elegans	38	28	39	35	

Supplemental Table S4.2

Percentage of sequence identities (top right) and similarities (bottom left) of Adamts9 metalloproteinase domain among 5 distant species.

	human	mice	zebrafish	*Drosophila*	*C. elegans*
human		88	81	45	53
mice	92		77	42	50
zebrafish	91	85		45	55
Drosophila	60	59	60		40
C. elegans	68	65	71	55	

Supplemental Table S4.3

Percentage of sequence identities (top right) and similarities (bottom left) of Adamts9 spacer domain among five distant species.

	human	mice	zebrafish	*Drosophila*	*C. elegans*
human		78	76	31	47
mice	90		78	32	42
zebrafish	91	88		33	46
Drosophila	52	49	52		33
C. elegans	66	59	63	48	

Supplemental Figure S4.4

Percentage of squence identities (top right) and similarities (bottom left) of Adamts9 gon-1 domain among five distant species. Zebrafish Adamts9 lacks gon-1 domain and thus was not included for comparison.

	human	mice	*Drosophila*	*C. elegans*
human		92	34	41
mice	95		35	41
Drosophila	52	50		34
C. elegans	56	56	48	

CHAPTER V: Folliculogenesis Deficiency Causes Follicle Loss and Sex Reversal in Adamts9 KO Zebrafish

Carver, J.J., Zhu, Y. (*in preparation*). Folliculogensis deficiency causes follicle loss and sex reversal in Adamts9 KO zebrafish (*Danio rerio*).

Chapter Summary

Adamts9 (a disintegrin and metalloprotease with thrombospondin type-1 motifs) is an extracellular matrix (ECM) metalloprotease necessary for tissue modeling, development, and ovulation. Limited reports suggest that ADAMTS9 function or expression is affected in human ovarian and uterine diseases and may influence the natural age of menopause. Our previous studies in Adamts9 KO zebrafish found male sex bias and female infertility in adult fish, as well as expression of *adamts9* in juvenile gonads and early-stage ovarian follicles. In the current study, we sought to characterize development of juvenile gonads and sexual differentiation in Adamts9 KO by utilizing *Tg(vasa:GFP)* to visualize early gonad development. Whole-mount confocal microscopy revealed delayed early gonad development, and significantly reduced number and size of Stage IB (primary) follicles in Adamts9 KO juveniles compared to controls. Primary sex determination was not affected in Adamts9 KO, but male sex bias appeared at 90 days post fertilization. During later development at 56-, 70-, or 90-days post fertilization, we found arrested follicles and significantly lower gonadosomatic index ratios in Adamts9 KO females. Because we also discovered persistent, global growth delay in Adamts9 KO, we rescued this phenotype by overfeeding the fish. Overfeeding increased the proportion of female fish but did not rescue defects in ovarian development in Adamts9 KO. Our results indicate that Adamts9 is critical for development of ovarian follicles and female sex maintenance in zebrafish.

Introduction

Formation of a functional gonad is essential for fertility and producing viable offspring across all animal taxa. Genetic or environmental disruption of this complex process in human development can lead to Disorders of Sexual Developments or adult fertility problems such as azoospermia or premature ovarian insufficiency (Shah et al., 2003; Pajkrt and Chitty, 2004; Vidaeff and Sever, 2005; Lee et al., 2006; Allen 2009; Jiao et al., 2021). However, most recent studies into gonad development have focused on the roles of transcription factors and cell signaling pathways (Mansen et al., 2017; Nagahama, 2005; Hayes, 1998; Devlin and Nagahama, 2002; Nagahama et al., 2021; Wilhelm et al., 2007). A significant knowledge gap still exists on the roles of enzymes, particularly extracellular matrix metalloproteases, necessary for tissue remodeling of the developing gonad.

Members of the ADAMTS (A Disintegrin and Metalloprotease with Thrombospondin type-1 motifs) family of extracellular matrix (ECM) metalloproteases regulate gonadogenesis and gonadal function by modification and release of membrane located signaling molecules and ECM components (Blelloch and Kimble, 1999; Nishiwaki et al., 2000; Shindo et al., 2000; Kouno et al., 2002 Apte, 2009; Gomis-Ruth, 2009; Tallant et al., 2010; Aydos et al., 2017; Liu et al., 2017; Liu et al., 2018; Piprek et al., 2018; Russell et al., 2015; Imanishi et al., 2020; Zhu, 2021). The ADAMTS family members are zinc-dependent matrix metalloproteases that facilitate signal transduction, cell migration, matrix remodeling, and tissue morphogenesis in animals during embryonic development and in adults (Porter et al., 2005; Brunet et al., 2012; Kelwick et al., 2015). Adamts9 is a highly conserved ADAMTS-protease and is widely expressed during vertebrate embryo development in mice, *Xenopus*, and zebrafish models (Jungers et al., 2005; Desanlis et al., 2018; Carver et al., 2021; He et al., *in preparation*). Previous studies suggest that Adamts9 is necessary for the migration of primordial germ

cells (PGCs) and gonad formation in invertebrates and the development of functional ovaries in zebrafish (Blelloch and Kimble, 1999; Blelloch et al., 1999; Ismat et al., 2013; Carter et al., 2019; Carver et al., 2021). Limited reports also suggest that ADAMTS9 expression is affected in human ovarian and uterine diseases and may affect the natural age of menopause (Pyun et al., 2014; Gueye et al., 2017; GohariaTaban et al., 2019; Tokmak et al., 2019; Hismiogullari et al., 2021). Despite these reports, specific roles for Adamts9 in vertebrate ovarian development are still lacking.

Zebrafish begin expressing *adamts9* as early as 7.5 hours post fertilization (hpf), or germ ring stage, and can be found in the Stage IB oocytes, follicular cells, muscle, retina, heart, and midbrain-hindbrain boundary in juvenile and adult zebrafish (Carver et al., 2021; Brunet et al., 2015; Liu et al., 2018). Additionally, recent single-cell sequencing found expression of *adamts9* in the ovarian stroma (Liu et al., *in review*). Despite the importance of Adamts9 in developmental and reproductive biology, genetic knockouts were not able to be characterized previously in vertebrates mainly because of embryonic lethality in *ADAMTS9* null mice (Kern et al., 2015). However, *Adamts9* KO zebrafish can survive to adulthood which allows for *in vivo* study (Carter et al., 2019). *Adamts9* KO zebrafish have multiple defects, including delayed growth, ocular lens malformations, poor survivability, and spinal deformities (Carter et al., 2019; Gray et al., 2021). But most surprising was that *Adamts9* KO fish had a severe sex bias towards male in adult fish, with only a few KO females observed in the progeny of heterozygous crosses. *Adamts9* KO females were infertile, had smaller ovaries with more interstitial space, and most oocytes present in ovaries from knockout fish were in stage I, before vitellogenesis (Carter et al., 2019). In contrast, testes development in male KO fish seems to be relatively normal. Currently, it is still unknown what developmental changes in *Adamts9* KO leads to the observed male sex

bias in adults. Understanding how *Adamts9* KO affects zebrafish sex determination and ovarian development will provide more insight on *Adamts9* function in vertebrate gonad formation.

There are several points in zebrafish development where disruption of normal gonad development can cause male sex bias or sex reversal from female to male. Lack of PGCs or germ cells causes sterile testis development, while proliferation and survival of PGCs is required for ovarian development (Slanchev et al., 2005; Siegfried and Nüsslein-Volhard, 2008; Tzung et al. 2015). In zebrafish and medaka (*Oryzias latipes*), depletion of early PGCs causes sterile testes or testes-like organ development and masculinization of the adult fish (Slanchev et al., 2005; Kurokawa et al., 2007; Nishimura et al., 2018). Additionally, removal of germ cells in adult zebrafish caused female fish to sex reverse into males (Dranow et al., 2013). Development and maintenance of meiotic oocytes is critical for female sexual development and sexual phenotype maintenance in zebrafish (Uchida et al., 2002; Slanchev et al., 2005; Dranow et al., 2016). In fish and mammals, oocytes actively reinforce somatic Granulosa cell differentiation and identity through paracrine signaling, and in turn, follicular cells produce steroids and key nutrients to support oocyte growth (Dong et al., 1996; Guigon et al., 2005; Clelland et al., 2006; Dranow et al., 2016). In the absence of oocyte-derived signaling, granulosa cells transition into pre-Sertoli cells (Guigon et al., 2005; Dranow et al., 2016). Sertoli cells begin secreting signals of their own to suppress female pathway genes and prevent germ cells from entering oogenesis.

To determine the primary cause behind male sex bias and female infertility in adult Adamts9 KO zebrafish, we have sampled the Adamts9 KO fish periodically throughout development. We crossed Adamts9 KO zebrafish with another line containing the transgene *Tg(vasa:GFP)* to easily visualize larval gonads and study the effects of Adamts9 KO on early gonad development. Multiple measures were taken at each timepoint to assess growth of

Adamts9 KO fish and gonad development. We found delayed gonad development, deficient folliculogenesis, and underdeveloped in our Adamts9 KO zebrafish. In this study, we provide evidence for Adamts9 involvement in folliculogenesis in the zebrafish model.

Materials and Methods

Animal Husbandry

WT AB strain and mutant zebrafish lines were housed in the zebrafish core facility with a 14-hour(h) light and 10h dark photoperiod, lights on at 8:30am, lights off at 10:30pm; at water temperature of 28.5°C, pH of approximately 7.2, and salinity/conductivity ranging from 500 to 1,200µS in two automatically controlled standalone zebrafish rearing systems (Aquatic Habitats Z-Hab Duo systems, Florida, USA). Fish are fed to satiation three times daily with a commercial food (Otohime B2, Reed Mariculture, CA, USA) containing high protein and lipid content, and supplemented with newly hatched brine shrimp *Artemia* (Brine Shrimp Direct, Utah, USA). The Institutional Animal Care and Use Committee (IACUC) at East Carolina University have approved all experimental protocols.

Genetic Manipulations

Two Adamts9 KO lines were originally created using the CRISPR-Cas9 system as described in Carter et al., 2019. Briefly, a small deletion of either 10(*adamts9*$^{\Delta 10}$) or 11(*adamts9*$^{\Delta 11}$) base pairs caused frameshift mutations and early stop codons to appear in the *adamts9* gene before the metalloprotease active site, which resulted in a complete loss of function mutation. Immunostaining of Adamts9 KO fish failed to detect any Adamts9 protein in high *adamts9* expressing Stage IVb follicular cells. Zebrafish were genotyped as previously described (Carter et al., 2019).

An additional Adamts9 KO zebrafish line was created by crossing *adamts9*$^{\Delta 10-/-}$ zebrafish with *Tg(vasa:GFP)* zebrafish (Lau et al., 2016). Embryos were collected, raised to adult, genotyped, and then in-crossed to obtain a transgenic line with all *vasa*$^{+}$ germ cells labeled with GFP in Adamts9 knockout background (*adamts9*$^{\Delta 10+/-}$;Tg(*vasa:GFP*)). This transgenic line was used for generating wildtype (+/+), heterozygous (+/–), and homozygous Adamts9KO (−/−) larvae and juveniles for whole-mount confocal imaging of *vasa:GFP*$^{+}$ PGCs, oocytes, and juvenile gonads. A third line was created by crossing (*adamts9*$^{\Delta 10+/-}$;*Tg*(*vasa:GFP*)) fish with fish containing a *tp53* null mutation, provided generously by Raman Sood and Blake Carrington (NIH, Maryland). Detailed information on *tp53* mutations and genotyping can be found in Ramanagoudr-Bhojappa et al., 2018. After initial crossing, the (*adamts9*$^{\Delta 10+/-}$;*Tg*(*vasa:GFP*);*tp53*$^{+/-}$) fish were in crossed once more to generate GFP^{+} double mutants for analysis.

Specimen Collection and Imaging

Zebrafish embryos were collected after spawning around noon each day, switched from fish system water into larval E3 media, and then allowed to develop in an incubator at 28°C for the first ten days before being transferred to the automatic fish rearing system. Fish were humanely euthanized by hypothermic shock between 8-11am on the day of sampling before fixation at 7-, 14-, 21-, 28-, 35-, 56-, 70-, or 90-days post fertilization (dpf). The larvae and juveniles were fixed in 10% neutral buffered formalin for four hours at room temperature while gently shaken. For mid-juvenile and adult specimens, to allow for better fixation of the gonad the ventral body cavity was exposed to the formalin solution by severing of the head and creating a lesion in the ventral portion of the abdominal cavity. Specimens were subsequently washed with

distilled water (3x for 10 minutes each) before storage in 100% methanol at −20 °C until imaging.

For confocal imaging of juveniles, whole individual 7-14dpf fish were mounted onto a depression glass slide in 1.2% low melting point agarose. At 21dpf, the body wall of the juvenile fish becomes too thick for the laser to penetrate, therefore gonads were fully dissected out from 21-70dpf fish and agarose mounted alone before imaging. Dissections and agarose embedding were done under Leica Mz75 dissecting microscope (Wetzlar, Germany). Fluorescence imaging was collected using a laser scanning confocal microscope on a Zeiss 800 LSM (Oberkochen, Germany).

Fish had their standard length taken after fixation by use of a digital caliper (General Tools & Instruments, Secaucus, NJ). Body mass and gonad mass were measured using an electronic scale. Gonads were fully removed from the abdominal cavity and separated out from the rest of the visceral organs before measurement. For measuring gonad length, an image was captured under the dissecting microscope and length was measured using ImageJ. Sex was assigned in later juvenile fish based on gross anatomy of the gonad; zebrafish oocytes fixed in 10% formalin are easily visible under dissecting microscope. Finally, gonadosomatic index (GSI) was calculated by the following formula: GSI% = (mass of gonad / mass of body) * 100%. For cellular imaging of adult specimens, hematoxylin & eosin (H&E) staining was favored because mature oocytes stop expressing *Tg*(*vasa:GFP)* and block transmittance of light due to their opacity. Fixed gonads were dehydrated by increasing ethanol and xylene washes and paraffin embedded before sectioning. 10-micron sections were cut and mounted onto glass slides before staining with Mayer's hematoxylin and Eosin-Phloxine. Permount was used to adhere the

cover glass to slides. H&E sections were imaged and photographed under Olympus BX41 microscope (Shinjuku city, Tokyo, Japan).

Image Analyses

The total number of *vasa:GFP*$^{+}$ germ cells and gonadal volume in juvenile fish were determined with aid of computer software (Imaris, Bitplane Inc, Zürich, Switzerland). Germ cells were first auto-counted using the spots function, and then corrected by hand. Gonadal volume was determined by Imaris surface function, corrected manually, and then measured by taking the total (sum) volume of the surface. Gonad length, number of Stage IB oocytes, and oocyte diameter were analyzed in ImageJ. Gonadal length was determined by drawing a line through the center of the gonad from each tip and taking the measurement. Number of Stage IB oocytes were counted manually by scrolling through each slice of the z-stack to identify the large nucleus indicative of these cells. Stage IB oocyte diameter was measured using the procedure detailed in Elkouby and Mullins, 2017. In the slice of the z-stack where the oocyte appears the largest, two intersecting lines were drawn through the longest axes of the oocyte and averaged to get the diameter. 20 oocytes per fish were randomly sampled for diameter measurement. Finally, juvenile fish were classified as either male-like (presumptive testis) or female-like (presumptive ovary) based upon cellular morphology. Fish with highly organized ovarian structures and that contained stage IB oocytes were classified as female-like at 28-35dpf. Fish that had a total absence of stage IB oocytes, no stage IA oocyte cysts, or had already begun developing spermatogenic cyst structures were classified as male-like.

H&E stained adult sampled were photographed under light microscope. Number of follicles was manually counted in ImageJ. Follicle stage was identified based on oocyte morphology. Each fifth section in a 10-micron series was sampled for analysis, up to 250-

microns in depth. Ratio of follicles was calculated by dividing number of follicles at each stage by the total number of follicles present in the ovary.

Nutrition and Rearing Conditions

To test the influence of nutrition and growth on sex determination in Adamts9 KO, fish were raised under either normal rearing conditions or a combination of low rearing density and high feed (overfed) rearing condition. Under normal conditions, fish are housed as 20 fish per tank and fed under normal feeding schedule. Fish under the overfed rearing conditions were kept at 10 fish per tank and given three extra Otohime B2 feedings per day in addition to normal diet and supplemented with 3 mL additional Brine shrimp daily starting at 10dpf. Fish were fed consistently under the same diet until sampling. In both conditions, fish were kept in mixed genotype tanks for the first 3 months. Water conditions were kept constant between the two groups, as they were housed on the same fish rearing system and tanks were checked regularly to remove buildup of excess food. Fish were then sexed at the 3 months mark by their external and gonadal morphology, photographed, and measured before genotyping.

Statistical Analysis

Two-tailed independent t-test was used to assess the pairwise significance of heterozygous and homozygous Adamts9KO fish compared to wildtype. Fisher's exact test was used to compare the sex ratios of wildtype, heterozygous, and homozygous Adamts9KO. MANOVA and linear regression were used to compare significance of genotype along with confounding variables, SL and body mass. All statistical analyses were run through either GraphPad Prism (t-test, Fisher's exact test) or SPSS (MANOVA, linear regression modeling) (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

Results

Early Gonad Development is affected by Adamts9 KO, but does not affect primary sex determination

To visualize early developing gonads in Adamts9 KO fish, gonads were imaged under confocal microscope at 7, 14, 21, 28, and 35dpf (Fig. 5.1; Fig. 5.2). At 7dpf, gonads are a small collection of primordial germ cells in the gonadal ridge (Fig. 5.1). At 14dpf, we observed significant proliferation of the germ cells, and earliest signs of germ cell cysts in agreement with previously published results (Fig. 5.1; Tzung et al., 2015; Leerberg et al., 2017). By 21dpf, germ cell cysts were more common and some of the fish had already began developing Stage IB follicles (Fig. 5.1). At 28dpf, well-developed biopotential ovaries had formed in all three genotypes marked by the presence of Stage IB follicles (Figure 5.2, Figure 5.4). At 35dpf, we observed the fish had gone through primary sex determination as their sex ratios mirrored those of adult fish (Figure 5.2; Figure 5.8). This timeline agrees with previously reported literature (Takahashi, 1977; Maack and Segner, 2003; Leerberg et al., 2017).

We did not find any significant difference in standard length (SL), gonad length (GL), gonad volume (GV), or *Vasa:GFP*$^{+}$ germ cells (GCs) at 7dpf (p>0.05; Figure 5.3A-D). This is not surprising, as little development has happened in the gonad at this timepoint. At 14dpf, we began to see divergence in the SL and GL measures between wildtype and Adamts9 KO individuals (SL: p=0.0265; GL: p=0.0206; Figure 5.3A-B). GL was highly correlated with SL across all time points regardless of genotype or sex in adults (n=350; r=0.8480, R^2=.7190, Y=287.0X-1307, p<0.0001; Suppl. Figure S5.2). We also saw significant decrease in GV and total number of *vasa:GFP*$^{+}$ germ cells in Adamts9 KO compared to wildtype at 14dpf (GV: p=0.0276; GCs: p=0.0205; Figure 5.3C-D).

At 21dpf, the slight difference in SL and GL did not cross the significance threshold (SL: p=0.0913; GL: p=0.4089 Figure 5.3A-B). However, the significant decrease of GV and GCs in Adamts9KO persisted compared to wildtype (GV: p=0.0176; GCs: p=0.0036; Figure 5.3B-C). At this time point, we did not see significant difference in the number or size of the Stage IB follicles that appeared (IB number: p=0.6859; IB diameter: p=0.3449; Figure 5.3D-E).

At 28dpf, the difference in SL, GL, and GV between wildtype and Adamts9 KO became more pronounced (SL: p<0.0001; GL: p<0.0001; GV: p=0.0026; Figure 5.3A-C). But the difference in total number of *vasa:GFP*$^{+}$ failed to cross (GCs: p=0.0521; Figure 5.3D). Interestingly, the difference in IB number and diameter became significant (IB number: p=0.0111; IB diameter: p<0.0001; Figure 5.3D-E). Looking at the trends in the data, it appears that wildtype and heterozygous begin to slow down in proliferating GCs and instead start to develop the primary follicle pool (Figure 5.3). Numerous amounts of Stage IB follicles could be observed in 28dpf wildtype gonads (Figure 5.4).

Much like 28dpf, at 35dpf SL, GL, and GV were still significantly reduced in Adamts9 KO compared to control siblings (SL: p<0.0001; GL: p<0.0001; GV: p=0.0035; Figure 5.3A-C). No difference was detected in the number of *vasa:GFP*$^{+}$ GCs at this timepoint, indicating that Adamts9 KO had caught up in proliferation to wildtype siblings (GCs: p=0.1986; Figure 5.3D). The number and size of follicles in Adamts9 KO were still very significantly reduced (IB number: p<0.0001; IB diameter: p<0.0001; Figure 5.3E-F). Because SL was a potentially significant, confounding variable in our experiments we sought out to mathematically determine whether SL alone could explain the difference in oocyte numbers. From MANOVA, we found that SL was a significant predictor for number of Stage IB follicles but even when factoring in size difference we still saw significant predictor effect of genotype on Stage IB follicle number

(Pillai's Trace SL: $p<0.0001$; Genotype: $p=0.038$; Supplemental Figure S5.3; Table S5.1). Age alone was not a significant predictor for number of oocytes, but we did find significant interaction between age and genotype (Pillai's Trace Age: $p=0.125$; Genotype*Age: $p=0.001$; Supplemental Figure S5.3; Table S5.1). Using linear regression modeling, we were able to predict number of Stage IB follicles when factoring in Age, Genotype, and SL (r=.9330; $R^2=.8706$; $p<0.0001$; Supplemental Figure S5.3; Table S5.2). When SL was set to constant value in the regression model, we still found significant effect of genotype on the number of Stage IB follicles at 28 and 35dpf (28pdf: $p=0.0027$; 35dpf: $p<0.0001$; Supplemental Figure S5.3; Table S5.2). Taken together, the reduction of Stage IB follicles is likely due to two effects: 1) global growth delay in Adamts9 KO impacts ovarian follicle development 2) Adamts9 KO has direct effect on the number of ovarian follicles, even when factoring in SL as a confounding variable (Supplemental Figure S5.3; Table S5.2).

Despite the impact of Adamts9 KO on early gonad development, we were surprised to learn that Adamts9 KO had no effect on primary sex determination. The sex ratios between males and females were indistinguishable between controls and Adamts9 KO at 35dpf (Fisher's exact test: $p>0.9999$; Figure 5.8; Table S5.3). This contrasted with 90dpf old fish, when sex ratios were significantly different between wildtype and Adamts9 KO (Fisher's exact test: $p=0.0028$; Figure 5.8; Table S5.3). Between these time points, we did not observe dead fish in the tanks, thus we rationalized that the Adamts9 KO fish must be undergoing sex reversal between these two time points. Loss of ovarian follicles is a reported cause of sex reversal in zebrafish (Dranow et al., 2016).

Later gonad development is affected by Adamts9 KO, male-skewed sex ratio begins to appear by 90dpf

To investigate our hypothesis of sex reversal in Adamts9 KO, we set out to sample gonads from late juvenile fish at 56dpf, 70dpf, and newly matured adults at 90dpf. (Figures 5.5-5.7). In female fish, Adamts9 KOs were significantly smaller in standard length at 56, 70, and 84dpf (56dpf $p<0.0001$; 70dpf $p=0.003$; 90dpf $p=0.0029$; Fig. 5.5A). Body mass was also significantly reduced in Adamts9 KO at all timepoints (56dpf $p=0.0041$; 70dpf $p=0.0023$; 90dpf $p=0.0091$; Fig. 5.5B). Related to standard length, gonad length was similarly reduced in Adamts9 KO (56dpf $p<0.0001$; 70dpf $p=0.0002$; 90dpf $p<0.0001$; Fig 5.5C). Whereas SL and BM maintained upwards trend in Adamts9 KO, the GL began to plateau in Adamts9 KO. Gonad mass was significantly reduced in Adamts9 KO, and this difference increased as the fish aged (56dpf $p=0.0478$; 70dpf $p=0.0008$; 90dpf $p<0.0001$; Fig. 5.5D). The gonadosomatic index (GSI) was significantly lower in the Adamts9 KO fish at all timepoints (56dpf $p=0.0027$; 70dpf $p=0.0009$; 90dpf $p<0.0001$; Fig 5.5E). Intriguingly, female fish had a significantly longer GL in wildtype and heterozygous females compared to wildtype and heterozygous males (Supplemental Figure, Fig. S5.4).

In male fish, SL was significantly in reduced Adamts9 KO compared to wildtype at all timepoints (56dpf: $p=0.0014$; 70dpf: $p<0.0001$; 90dpf: $p<0.0001$; Fig. 5.6A). Similarly, body mass was significantly reduced at all timepoints in Adamts9 KO as well (56dpf: $p<0.0001$; 70dpf: $p=0.0001$; 90dpf: $p=0.0001$; Fig. 5.6B). The same was true for gonad length (56dpf: $p=0.001$; 70dpf: $p<0.0001$; 90dpf: $p<0.0001$; Fig. 5.6C) and gonad mass (56dpf: $p=0.0077$; 70dpf: $p=0.0013$; 90dpf $p<0.0001$; Fig. 5.6D). However, despite being initially different, GSI% was the same between wildtype and Adamts9 KO males at maturity (56dpf: $p=0.0040$; 70dpf: $p=0.8279$; 90dpf: $p=0.2534$; Fig. 5.6E). These results indicate that testes in Adamts9 KO are still at a healthy size relative to their body size in mature fish. Additionally, histological and

fluorescence microscopy imaging did not show any gross abnormalities in Adamts9 KO males (Carter et al., 2019; data not shown), therefore we focused our efforts on Adamts9 KO females.

Fluorescence images of 56 and 70dpf Adamts9 KO female fish revealed that most follicles were arrested in Stage IB (Fig. 5.7). In wildtype and heterozygous fish, multiple follicles progressed past Stage IB into Stage II (Fig. 5.7A). In contrast, ovaries in Adamts9 KO fish only contained follicles at Stage IB (Fig. 5.7B). Additionally, we found evidence of abnormal oocytes (white arrows; Fig. 5.7C,D) and empty follicles (red arrow; Fig. 5.7C). Follicles continued growing in wildtype and heterozygous siblings (Fig. 5.7E), but most follicles in Adamts9 KO fish remained as Stage IB at 70dpf (Fig. 5.7F-H). Only a few follicles in the Adamts9KO were able to progress into Stage II (Fig. 5.7F).

These results indicate that there is a marked decrease in growth of Adamts9 KO males and females. In zebrafish females we also observed have dramatically underdeveloped ovaries compared to wildtype siblings and follicles that generally do not progress past stage IB in Adamts9 KO compared to wildtype siblings.

Nutrition alone cannot explain Adamts9 phenotype

Because delayed body growth is a potential confounding variable in our analyses, we sought to rescue the growth defects in Adamts9 KO fish. To do this, the rearing density was reduced by half and fish were overfed continuously for 90 days. We found that overfeeding the fish affected sex ratios, and skewed sex ratios towards female in all three genotypes (Fisher's exact test, +/+ vs. +/+OF: p=0.0124; +/+ vs. +/-OF: p<0.0001; +/+ vs. -/- OF: p=0.0166; Fig. 5.9A). In the overfed group, standard length was no longer significantly different between Adamts9 KO females and wildtype fish (Independent t-test, p=0.0554; Fig. 5.9B). Body mass

was still significantly lower in Adamts9 KO females (p=0.0007; Fig. 5.9C), though a significant portion of the difference was due to differences in mass of the ovary (p<0.0001; Fig. 5.9D). The GSI% was still significantly reduced in Adamts9 KO females compared to wildtype and heterozygous overfed siblings (p<0.0001; Fig. 5.9E).

The rescued, overfed Adamts9 KO females did not have significantly different standard length or body mass compared with the age matched, wildtype siblings raised under normal conditions (SL: p=0.1057; BM: p=0.3177; Fig. 5.10A-C). However, the GSI% is still dramatically lower in Adamts9 KO than in wildtype controls (p<0.0001; Fig. 5.10C), indicating that the defects in ovarian development are not caused by a general decrease in body mass. In wildtype females, the ovary continues growing and reaches sizes above 200mg, and a GSI% reaching between 15-20% (Fig. 5.10D,E). In Adamts9 KO, the ovary stops growing past 70dpf (KO GM 70dpf vs. 90dpf: p=0.3022; GSI 70dpf vs. 90dpf: p=0.4885; Fig. 5.10F,G).

Deficient Folliculogenesis in Adamts9 KO gonads

To investigate cellular morphology, dissected ovaries from overfed wildtype (+/+), heterozygous (+/-), and Adamts9 KO (-/-) zebrafish were paraffin embedded and sectioned for H&E staining (Fig. 5.11). Serial sections were cut in 10µm increments and photographed. In wildtype and heterozygous ovaries, multiple maturing follicles (Stage II, III, IV) were seen under section (Fig. 5.11 A-B'). In contrast, the Adamts9 KO ovary contain mostly Stage IB follicles that had not progressed any further (Fig. 5.11 C,C'). Additionally, multiple Adamts9 KO specimens either contained empty follicles (Fig 5.11 D-E) or the coexistence of oocytes and sperm (Fig. 5.11 H-I) indicating the fish are going through sex reversal. The total of number of follicles observed was not significantly different between wildtype and Adamts9 KO siblings (Independent t-test, p=0.8210; Fig. 5.11 J), but the total number of matured Stage IV follicles

was significantly lower in Adamts9 KO (Independent t-test, p=0.0091; Fig. 5.11 K). In comparing the proportions of follicles at each stage, nearly all of the follicles in Adamts9 KO remained at Stage IB (p<0.0001), while a significantly smaller ratio was at Stage II (p=0.0001) or Stage III (p=0.0001)(Fig. 5.11 L). Although the ratio of Stage IV follicles was lower in Adamts9 KO, it did not cross the significance threshold (p=0.12152; Fig. 5.11 L). This was because some ovaries contained very few follicles, one of which had matured but did not ovulate and began regressing like in Fig. 5.11 D. The arrested follicles were likely undergoing apoptosis, as 90dpf Adamts9 KO females had degraded RNA (an early step in apoptosis) and blocking apoptosis with a *tp53*$^{-/-}$ rescue allowed GSI% to recover in overfed 90dpf Adamts9 KO (Supplemental Figure S5.6-5.7).

mRNA in situ hybridization and immunostaining of adamts9 in juvenile zebrafish ovary

To ascertain which ovarian cell types were synthesizing and secreting Adamts9, *in situ* hybridization and immunohistochemistry was used to stain for Adamts9 transcripts and proteins in juvenile ovaries (Fig. 5.12-5.13). mRNA *in situ* hybridization detected strong expression of *adamts9* in the pockets of ovarian cells. Using both anti-sense (column A) and control sense probe (column B), we detected pockets of ovarian somatic cells that strongly expressed *adamts9* (Fig. 5.12). No signal was found in Stage IB follicles. We reasoned that *in situ* hybridization is less sensitive. This result is not against our previous finding of low levels of Adamts9 expression in Stage IB follicles using highly sensitive methods, i.e., qPCR or transgenic reporter (Carver et al., 2021; He et al., in preparation). In contrast, immunostaining using primary antibodies targeting either the pro-domain (RP1) or metalloprotease domain (RP5) of Adamts9 detected protein present within Stage IB follicles in both adult ovary sections and whole-mount juvenile

ovaries (Fig. 5.13). The antibodies were previously shown to be specific to Adamts9 and do not stain ovary samples from Adamts9 KO (Carter et al., 2019).

Discussion

In the present study, we found that the cause of male sex bias is not from changes in primary sex determination but instead sex reversal in late juvenile or adult Adamts9 KO zebrafish (Fig. 5.14). We found deficient folliculogenesis in Adamts9 KO (Fig. 5.3-5.4, 5.7), and most ovarian follicles stayed arrested at Stage IB in rescued, adult Adamts9 KO ovaries (Fig. 5.11). As ovarian tissue fails, the gonad undergoes remodeling and sex reversal (Fig. 5.11) and causes the increase in male sex ratio (Fig. 5.8, 5.14) (Carter et al., 2019).

Persistent growth delay in larval and juvenile Adamts9 KO zebrafish

Starting at 14dpf, we saw the divergence of the growth trajectory of the Adamts9 KO fish from their wildtype siblings raised in the same tank and rearing conditions. This was also reported in Carter et al., 2019 and Gray et al., 2020. Initially, we found standard length to not be significantly different between wildtype and Adamts9 KO fish at 7dpf. Thereafter, the standard length did become significantly different at 14dpf, and the difference between wildtype and Adamts9 KO siblings increased as the fish aged at 28dpf and 35dpf. It should be noted that Adamts9 KO fish did continue to grow in length and mass in late adulthood, at rates comparable to their wildtype siblings.

The cause of the growth delay is not clear in Adamts9 KO. We postulate multiple possibilities. The first is the ability to compete with tankmates to obtain food. Because of spinal deformities and high expression of *adamts9* in muscles, sensory cell precursors, and CNS, it may be more difficult for the Adamts9 KO to detect and swim towards food under flowing water

(Gray et al., 2020; He et al., *in preparation*). As wildtype siblings grew bigger, it would also become easier from them to push the smaller Adamts9 KO siblings out of the way and outcompete them for food. We have also observed abnormal and seizure-like swimming patterns of young Adamts9 KO fish in communal tanks. The second possibility is that Adamts9 KO can obtain adequate amounts of food but have some metabolic dysfunction that prevents nutrients from being utilized efficiently. In humans, *ADAMTS9* polymorphism is associated with insulin resistance and Type-II diabetes risk in European and Chinese populations (Simonis-Bik et al., 2010; Boesgaard et al., 2009; Trombetta et al., 2013; Grarup et al., 2008; Kong et al., 2015). In GON-1 *C. elegans* knockouts, loss of functional GON domain impaired secretion of insulin orthologs and TGFβ and shortened average lifespans (Yoshina and Mitani, 2015). In mice, *Adamts9* regulates insulin sensitivity in skeletal muscles (Graae et al., 2019). We found expression of *adamts9* in the muscle tissue of zebrafish adults as well (He et al., *in preparation*). Leptin, another hormone secreted by adipocytes important for regulating energy balance, can induce ADAMTS9 expression in human chondrocytes *in vitro* (Yaykasli et al., 2015). However, a recent study in zebrafish suggests Leptin signaling is important for final oocyte maturation and ovulation but not folliculogenesis *in vivo* (Tsakoumis et al., 2022).

Though we have attempted to mathematically account and experimentally control for the potential of body size as a confounding variable in our study, we cannot rule out the possibility of a metabolic syndrome or irregular insulin signaling playing an effect on ovarian development in Adamts9 KO. However, we did find very low levels of *adamts9* in the liver, which makes it unlikely for disrupted vitellogenin synthesis to be responsible for the defects in ovarian folliculogenesis (Carver et al., 2021; He et al., *in preparation*). Future work will need to determine the cause of delayed body growth in Adamts9 KO zebrafish, and if any metabolic

syndrome is present in these fish. Conditional knockouts or rescue Adamts9 specific to the ovary will facilitate future investigation and will eliminate this problem.

Early gonad development defects in Adamts9 KO

In the present study, we found that Adamts9 KO caused delayed growth of the early gonad but did not affect primary sex determination in zebrafish juveniles. In the 7dpf samples, no difference in the numbers of primordial germ cells were found between wildtype and Adamts9 KO siblings. In fact, we saw little change of the gonad from the 48hpf sample though the PGCs had shifted position slightly around the developing swim bladder and gut (Carver et al., 2021). However, once the gonad became active again, and had both somatic and germ line cells that were rapidly differentiating and proliferation, we saw a delayed cell proliferation in Adamts9 KO (Leerberg et al., 2017; Tzung et al., 2015; Dai et al., 2015). During this time, the somatic gonadal cells become more differentiated, separate into multiple layers, and there is an accumulation of laminin and epithelial-like cell adhesion molecules (Leerberg et al., 2017). Expression of *gata4* and *wt1a*, transcription factors that have been shown to regulate metalloprotease expression in mice, are also present in 10-11dpf zebrafish gonads (Leerberg et al., 2017; Bennett et al., 2013; Schrade et al., 2016; Jacobi et al., 2011; Jacobi et al., 2013; Helmers et al., 2013). It is reasonable to expect that lack of Adamts9 activity and abnormal ECM composition can disrupt proper gonad development, dysregulating MMP activity in mice causes abnormal primordial gonad morphology (Piprek et al., 2019).

Interestingly, scRNAseq data reveals that *adamts9* has enriched expression in *cxcl12a*$^{+}$ (Sdf-1a^{+}) and *fgf24*$^{+}$ ovarian stroma in addition to the follicular cells (Liu et al., 2022). In the germline, Sdf-1 is well-known for guiding the migration of primordial germ cells in mouse, chicks, *Xenopus*, and zebrafish (Stebler et al., 2004; Doitsidou et al., 2002; Takeuchi et al.,

2010). In addition, Sdf-1 has been demonstrated to stimulate cell proliferation and stem cell maintenance in other tissues and cell types (Lataillade et al., 2000; Cencioni et al., 2012; Yang, Wang et al., 2012; Ratajczak et al., 2006). Unfortunately, *Sdf-1*$^{-/-}$ mice embryos are not viable and *sdf-1a*$^{-/-}$ zebrafish lose PGCs during migration, which prevents studying knockouts in early ovarian development (Tachibana et al., 1998; Gross-Thebing et al., 2014; Paksa et al., 2016). In adult mice, antagonism of the SDF-1 receptor CXCR4 reduced cell proliferation and germline stem cell (GSC) maintenance in males (Yang et al., 2012). Cxcl12a receptors Cxcr4a/Cxcr4b and Ackr3a/Ackr3b (CXCR7) are distributed throughout the juvenile zebrafish ovary, including follicle cells, vasculature, and GSCs (Liu et al., *in review*). Impaired Cxcl12a signaling in Adamts9 KO juvenile gonads is a potential explanation for the reduced number and slower proliferation rate of *vasa:GFP*$^{+}$ germ cells in Adamts9 KO at 14 and 21 dpf, and the slow recovery of GCs back to wildtype levels in Adamts9 KO.

We found that the number and size of Stage IB follicles, is significantly lower and smaller in Adamts9 KOs at 28 and 35dpf (Fig. 5.3-5.4), despite being initially similar between wildtype and Adamts9 KO siblings at 21dpf. We have several hypotheses for reasons why this may be, including: 1) slower rate of meiosis entry in Adamts9 KO, 2) abnormal oocyte cyst progression or excessive apoptosis of Stage IA oocytes, 3) failure to recruit pre-granulosa cells during the transition to Stage IB, and/or 4) abnormal communication or ECM composition between oocytes and granulosa cells leading to delayed follicle growth and loss. Investigating each of these hypotheses is beyond the scope of the current paper and will need to be explored further by future experiments.

Despite the observed phenotypes, Adamts9 KO had negligible effect on primary sex determination based on no significant difference in the male/female sex ratios between Adamts9

KO and their wildtype sibling at 35dpf, when zebrafish gonadal sex is determined (Fig. 5.8). Wild zebrafish were reported to have a ZZ/ZW chromosomal sex determination (CSD) system that was lost in domesticated lab strains Tübingen and AB (Sharma et al., 1998; Mahapatra et al., 2002; Spence et al., 2007; Anderson et al., 2012; Wilson et al., 2014). Though several sex determining genes have been identified and studied, we still do not fully understand the sex determination system of domesticated zebrafish (Liew and Orbán, 2012; Kossack and Draper, 2019). Zebrafish are a protogynous species, in which a bipotential juvenile ovary develops first before transitioning into committed ovary or testes fate (Takahashi, 1977; Maack and Segner, 2003). Presence of germ cells, oocytes, and ovarian follicles are critical for female sex determination and phenotype maintenance in adult zebrafish (Slanchev et al., 2005; Siegfried and Nüsslein-Volhard, 2008; Tzung et al., 2015; Leerberg et al., 2017). Loss of oocytes leads to full female to male sex reversal and appearance of Sertoli cells and sperm in late juvenile or adult fish (Dranow et al., 2013; Takatsu et al., 2013; Dranow et al., 2016; McMenamin et al., 2016).

Although the number of *vasa:GFP*$^{+}$ germ cells was initially lower at 14-21dpf in Adamts9 KO juveniles, many mutant fish were able to initiate ovarian development by 28dpf same as in wildtype and heterozygous siblings (Fig. 5.1-5.5, 5.8). These results suggest functional Adamts9 is not critical for initiation of female sex development in the germ line cells. Because our transgene only labelled the germline, and previous H&E sections from juvenile Adamts9 KO fish at this time were inconclusive (*data not shown*), further investigation is required for elucidation of Adamts9 activities in somatic gonadal cells.

Folliculogenesis Deficiency and Sex-Reversal in Adamts 9 KO Zebrafish

In mammals and fishes, multiple studies show expression and function of ECM components and metalloproteases for continued ovarian folliculogenesis (Berkholtz et al., 2006;

Ambekar et al., 2013; Heeren et al., 2015; Tomaszewski et al., 2021; Russell et al., 2015; Zhu, 2021; Shindo et al., 2000; Zamah et al., 2015; Bagavndoss, 1998; Vrooman and Young, 2010; Smith et al., 2002: Curry and Osteen, 2003; Goldman and Shalev, 2004; McCaffery et al., 2000). In mice, disruption of metalloprotease activity impairs oocyte maturation and can lead to oocyte arrest (Meng et al., 2017). ADAMTS1, which is evolutionarily related to and shares overlapping functions with ADAMTS9, has been particularly well studied in murine folliculogenesis. ADAMTS1 KO mice have reduced fertility, significantly fewer antral and periovulatory follicles, and have abnormal follicles lacking granulosa cell layers (Shindo et al., 2000; Mittaz et al., 2004; Brown et al., 2006; Shozu et al., 2005). Because of lethality in ADAMTS9 KO mice, it has been more difficult to study ADAMTS9 in mammalian folliculogenesis (Jungers et al., 2005; Kern et al., 2010). We were also able to detect *adamts1* transcripts in zebrafish follicles and juvenile gonads at all stages measured (Supplemental Figure S5.8). Because these cells are likely still expressing functional Adamts1 in our Adamts9 KO model, this result tentatively suggests that Adamts1 and Adamts9 may have non-overlapping functions in folliculogenesis in zebrafish. ADAMTS9 has been detected in whole-ovary and ovarian follicle samples in various mammalian and fish species, including humans (Somerville et al., 2003; Rosewell et al., 2015; Clark et al., 2000; GohariTaban et al., 2018; Peluffo et al., 2014; Wu et al., 2017; Tsubota et al., 2015; Khristi et al., 2018; Fan and Chuva de Sousa Lopes, 2021; Russell et al., 2015; Zhu, 2021) In rhesus macaques, ADAMTS9 protein expression was found in granulosa, theca, and stromal cells of ovarian follicles before and after hCG exposure (Peluffo et al., 2014). In the zebrafish ovary, *adamts9* expression has been detected in follicular cells, theca, Stage IB oocytes, and stromal cells (Liu et al., 2022; Liu et al., 2017; Liu et al., 2018; Carver et al., 2021; He et al., *in preparation*, Fig. 5.13).

Folliculogenesis requires the coordination of activity between oocytes, granulosa cells, theca cells, and stromal cells, as well as changes in ECM and passage of secreted signals between the different populations of cells. In mammals and fishes, a thick ECM barrier termed the zona pellucida emerges between the oocyte and somatic support cells and is largely composed of *zp* glycoproteins (Wassarman and Litscher, 2013; Litscher and Wassarman, 2018). However, expression of several other ECM components including laminins, collagens, and known Adamts9 substrates versican and fibronectin have also been detected in mammalian, teleost, and avian follicles (Russell et al., 2003; McArthur et al., 2000; Eriksen et al., 1999; Takahashi et al., 2019; Berkholtz et al., 2006; Rodgers et al., 2003; Asem et al., 1992; Tsuiki et al., 1988; Zhao and Luck, 1995). Sequestration of ECM fibers by addition of heparan sulfate and basement binding proteins improves murine folliculogenesis *in vitro* (Tomaszewski et al., 2021). Granulosa cells utilize specialized filopodia to make physical contact with the oocyte in mice through the dense zona pellucida (Li and Albertilini, 2013; Abbassi et al., 2021; El-Hayek et al., 2018). The signaling communication between the oocyte and granulosa cell is bidirectional in nature. The oocyte secretes signals such as GDF9 and BMP15 to maintain granulosa cell function and identity (Dong et al., 1996; Guigon et al., 2005; Clelland et al., 2006; Mottershead et al., 2012; Dranow et al., 2016; Edson et al., 2009; Doherty et al., 2022). At the same time, granulosa and theca cells supply key nutrients, hormones, and biochemical substrates to support the growth of the oocyte (Edson et al., 2009). Likewise, granulosa and theca cells also are involved in extensive cross-communication necessary to regulate somatic follicle cell functions in various species (Roberts and Skinner, 1990; Nilson and Skinner, 2001; Yada et al., 1999; Liu et al., 2015; Edson et al., 2009). Recent work has also identified the ovarian stroma as having significant importance in ovarian health and egg production (Tagler et al., 2011). The stroma

includes nerves, blood and lymph vessels, immune cells, stem cells, fibroblasts, ECM connective fibers, and somatic cells with poorly defined functions (Kinnear et al., 2020). Proper functioning of all of the previous stated cells types in conjunction with each other is required for proper folliculogenesis and oocyte survival. Abnormal cell functioning can lead to follicle arrest and atresia, like the results we observed in Adamts9 KO females.

Because of widespread expression of Adamts9 in Stage IB oocytes, follicular cells, theca, and stromal cells, it is hard to determine in which cell types Adamts9 expression is critical for continued folliculogenesis. Certainly, disrupted ECM composition of the follicle or abnormal oocyte-somatic cell signaling can lead to failed folliculogenesis and follicle loss. But, disorganized/disrupted ovarian stroma, lack of adequate blood supply, or disrupted stroma-derived signaling may also be responsible for the observed phenotypes in Adamts9 KO zebrafish. Several cell signaling pathways have been shown to be potentially affected or regulated by ADAMTS9 either *in vivo* or *in vitro* including the Akt/mTOR pathway, hedgehog signaling, and VEGFA (Du et al., 2013; Chen et al., 2017; Nandadasa et al., 2019; Choi et al., 2019). Roles of Adamts9 in ECM fiber composition or cell signaling is warranted for future studies. Conditional knockout studies in the future will help to determine in which cells specifically functional Adamts9 expression is critical for continued folliculogenesis.

Conclusion

In the present study, we demonstrated that Adamts9 KO zebrafish have early gonad development delay, persistent global growth delay, and defective folliculogenesis leading to sex reversal in Adamts9 KO females. This is the first-time phenotypic description of Adamts9 KO has been described in vertebrate ovarian follicle development. Future work will determine which

populations of cells are responsible for the phenotype, and molecular mechanisms underlying observed cellular phenotypes.

Figure 5.1

Representative images of 7-21dpf wildtype (+/+) and Adamts9 KO (-/-) juvenile gonads with whole-mount confocal microscopy. Germ line cells were labeled with (*Tg(vasa:GFP)*) and imaged using a laser scanning confocal microscope. At 7dpf, the primordial gonad is a cluster of approximately 30-50 germ cells. By 14dpf, the germ line rapidly proliferates, and the gonad has expanded significantly. At 14dpf, we saw undifferentiated gonocytes and clusters of proliferative cells. At 21dpf, we began to see the appearance of Stage IB primary follicles in all three genotypes. By comparison, Adamts9 KO gonads are much smaller than wildtype and heterozygous siblings. Scale bar = 200 µm.

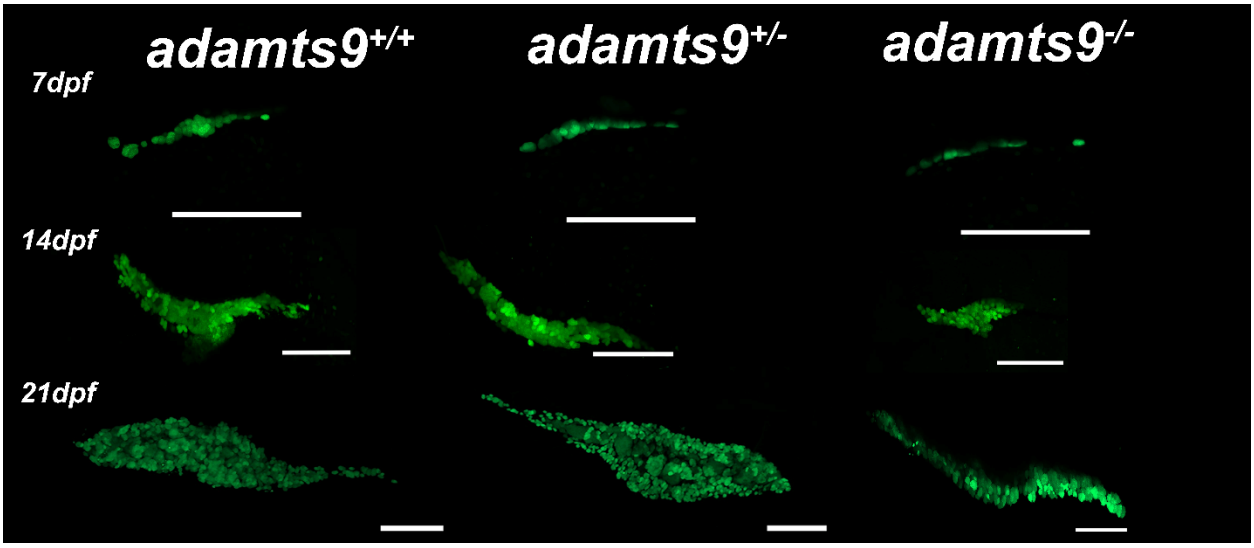


Figure 5.2

Representative images of 28-35dpf ovaries in wildtype and Adamts9 KO juveniles with whole-mount confocal microscopy. Germ line cells were labeled with (*Tg(vasa:GFP)*) and imaged using a laser scanning confocal microscope. At 28dpf, we observed the appearance of well developed, bipotential ovaries containing numerous Stage IB primary follicles. At 35dpf, we saw differentiation of males and females, the ovaries are shown here. The sex ratios of the juveniles matched those of adults (Fig. 8). Adamts9 KO ovaries are significantly smaller and contain fewer and smaller primary follicles (Fig. 3). Scale bar = 200 µm.

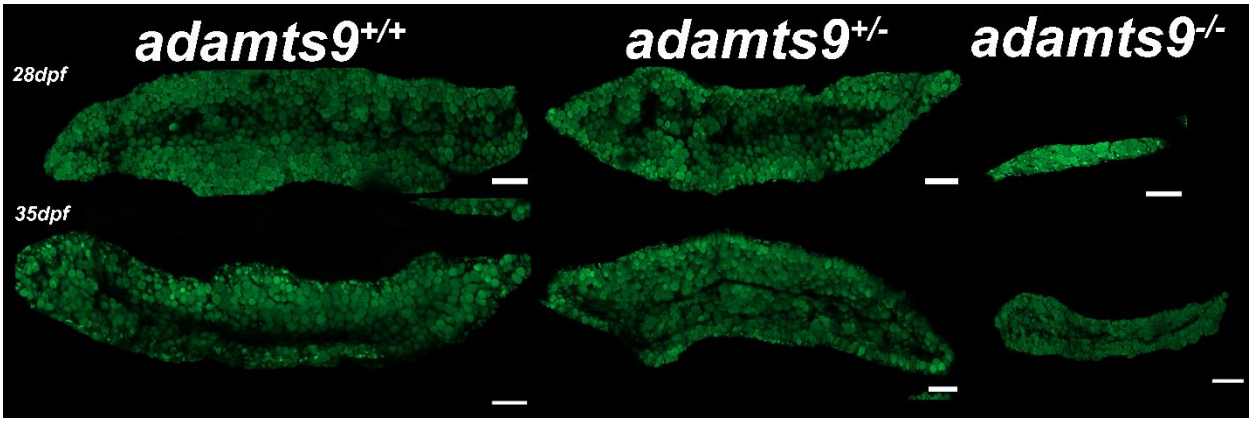


Figure 5.3

Early development of Adamts9 KO juvenile gonads compared to wildtype siblings.

Quantification of Standard Length (SL), Gonad Length (GL), Gonad Volume (GV), *vasa:GFP*$^{+}$ Germ Cells (GCs), Stage IB Oocytes, and Average IB Diameter. **A)** SL was not changed in 7dpf larvae (p>0.05), but wildtype and Adamts9 KO siblings began to diverge at 14dpf (p=0.0265). At 21dpf the difference was small and did not cross significance threshold (p=0.0913), but the difference in SL became dramatic at 28-35dpf (28dpf: p<0.0001; 35dpf: p<0.0001). **B)** GL tended to scale with SL and the most dramatic effect in Adamts9 KO was seen in 28-35dpf (supplemental Fig. 2)(7dpf p>0.05; 14dpf p=0.0206; 21dpf p=0.4089; 28dpf p<0.0001; 35dpf p<0.0001). **C)** GV was not changed in 7dpf (p>0.05) but was significantly smaller in Adamts9 KO in all subsequent timepoints (14dpf p=0.0276; 21dpf p=0.0176; 28dpf p=0.0026; 25dpf p=0.0035). **D)** Numbers of *Vasa:GFP*$^{+}$ germ cells are initially not different in 7dpf larvae (p<0.05). At 14-21dpf, during active proliferation of gonadal cells Adamts9 KO juveniles had significantly fewer *Vasa:GFP*$^{+}$ germ cells than wildtype siblings (14dpf: p=0.0205; 21dpf p=0.0036). However, this difference began to disappear at 28dpf (p=0.0521) and number of GCs became similar at 35dpf (p=0.1986). **E**) Stage IB primary follicles did not appear until 21dpf. At 21dpf, the number of IB follicles was not significantly different between the three genotypes (p=0.6859). At 28dpf, Adamts9 KO contained fewer IB follicles (p=0.0111) and the difference became dramatic in committed ovaries at 35dpf (p<0.0001). **F)** Similar to the number of IB follicles, at 21dpf the size of the follicles were unchanged (p=0.3449) but became significantly different at 28dpf (p=0.0002) and 35dpf (p<0.0001). The follicles appeared to grow at a much smaller rate in Adamts9 KO compared to wildtype siblings. Scatter plots for each timepoint/measurements can be found in Supplemental Fig. 4.1.

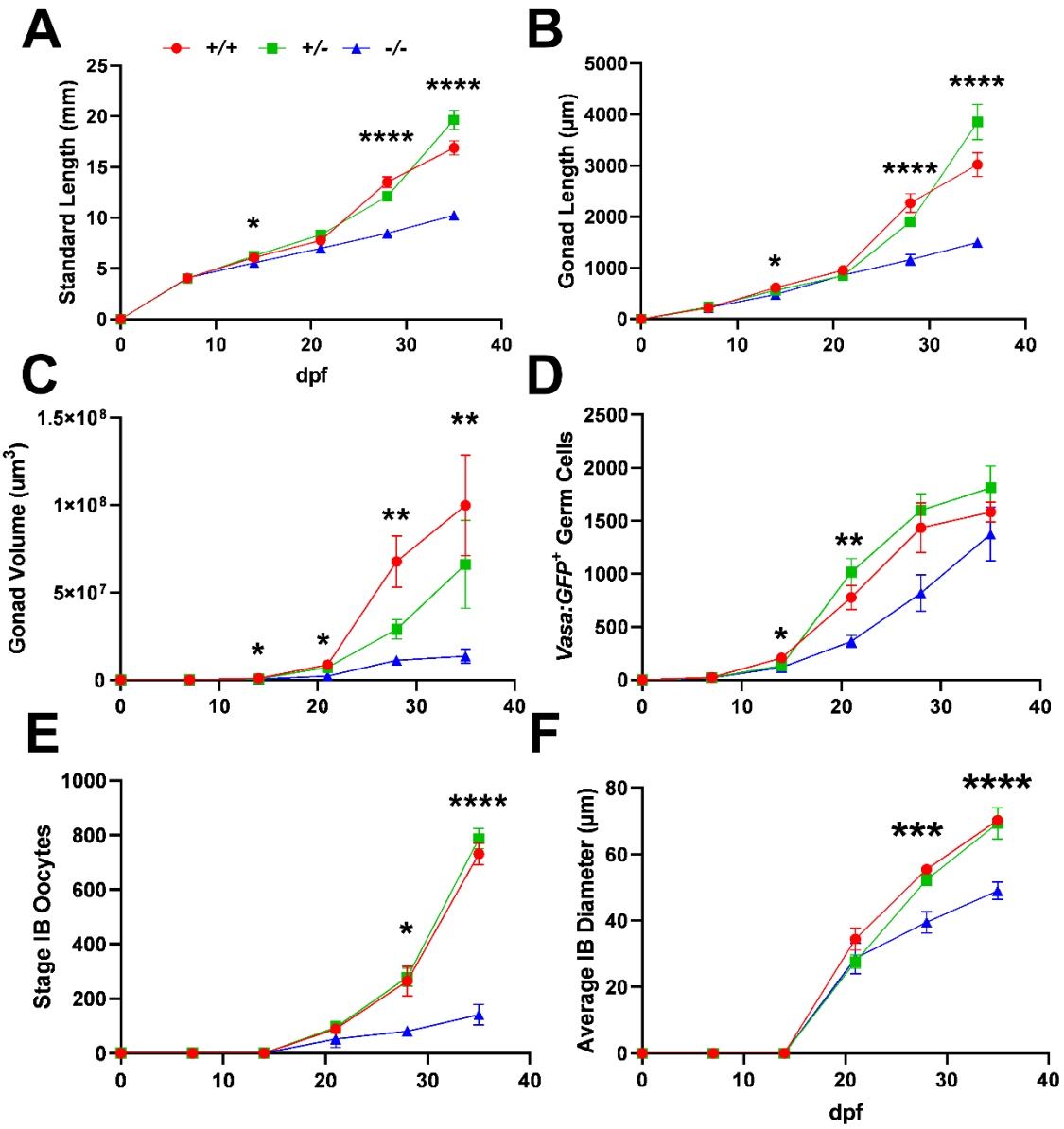

A
+/+
+/-
-/-
Standard Length (mm)
dpf
B
Gonad Length (µm)
C
Gonad Volume (um3)
D
Vasa:GFP+ Germ Cells
E
Stage IB Oocytes
F
Average IB Diameter (µm)
dpf

Figure 5.4

Sexual differentiation and primary follicle formation in wildtype and Adamts9 KO. Germ line cells were labeled with (*Tg(vasa:GFP)*), somatic cells are stained with DAPI, and imaged using a laser scanning confocal microscope. Cellular morphology became sexually distinct beginning at 28dpf. Left column for each genotype corresponds to male fish. Males did not contain any Stage IB follicles and did not have cysts resembling Stage IA oocytes at 28dpf. By 35dpf, some males had formation of more well defined spermatogenic cysts. In contrast, females (right column for each genotype) had numerous Stage IB follicles that grew between 28-35dpf. IB follicles in Adamts9 KO were visually smaller those in wildtype or heterozygous siblings. Scale bar = 50 µm.

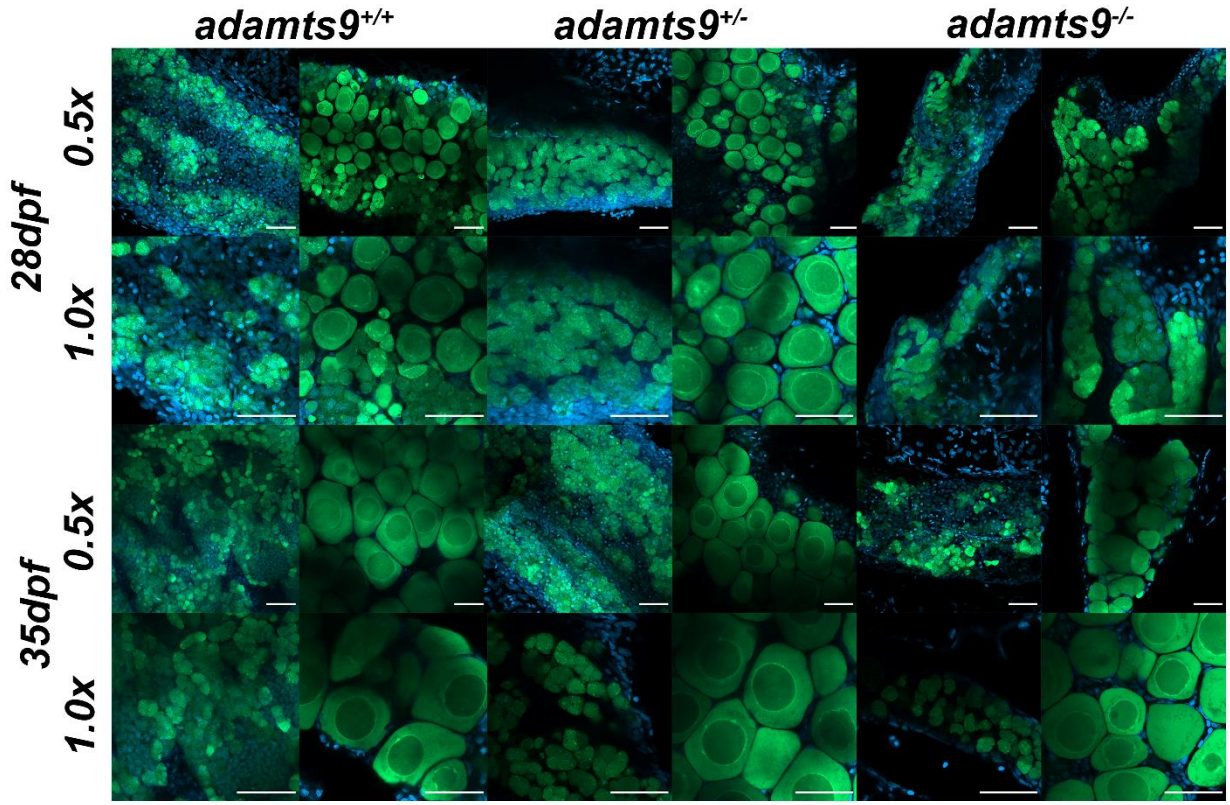


Figure 5.5

Later juvenile ovarian development in wildtype and Adamts9 KO females. Juvenile female wildtype (+/+), heterozygous (+/-), and Adamts9 KO (-/-) zebrafish were dissected and measured for Standard Length (SL), Body Mass (BM), Gonad Length (GL), Gonad Mass (GM), and the gonadosomatic index (GSI%) was calculated. **A)** SL was significantly different between Adamts9 KO juvenile females and WT females at all timepoints (56dpf: $p<0.0001$; 70dpf: $p=0.0003$; 90dpf: $p=0.0029$). Heterozygous siblings were not significantly different from wildtype (all timepoints: $p>0.05$). **B)** BM was significantly reduced in Adamts9 KO compared to wildtype siblings at all timepoints (56dpf: $p=0.0041$; 70dpf $p=0.0023$; 90dpf $p=0.0091$). **C)** Similar to SL, GL was significantly lower in Adamts9 KO at all timepoints measured (56dpf: $p<0.0001$; 70dpf: $p=0.0002$; 90dpf: $p<0.0001$). **D**) GM was significantly decreased at all timepoints, but unlike SL, BM, or GL the trendline for the Adamts9 KO fish did not show an upward trend (56dpf: $p=0.0478$; 70dpf: $p=0.0008$; 90dpf $p<0.0001$). **E)** Because BM kept increasing, but not GM in Adamts9 KO females, GSI% in Adamts9 KO females did not increase and decreased between 70 and 90dpf knockouts. GSI% was significantly lower in Adamts9 KOs at all timepoints (56dpf: $p=0.0027$; 70dpf: $p=0.0009$; 90dpf: $p<0.0001$). Scatter plots for each timepoint/measurements can be found in Supplemental Fig. S4.4.

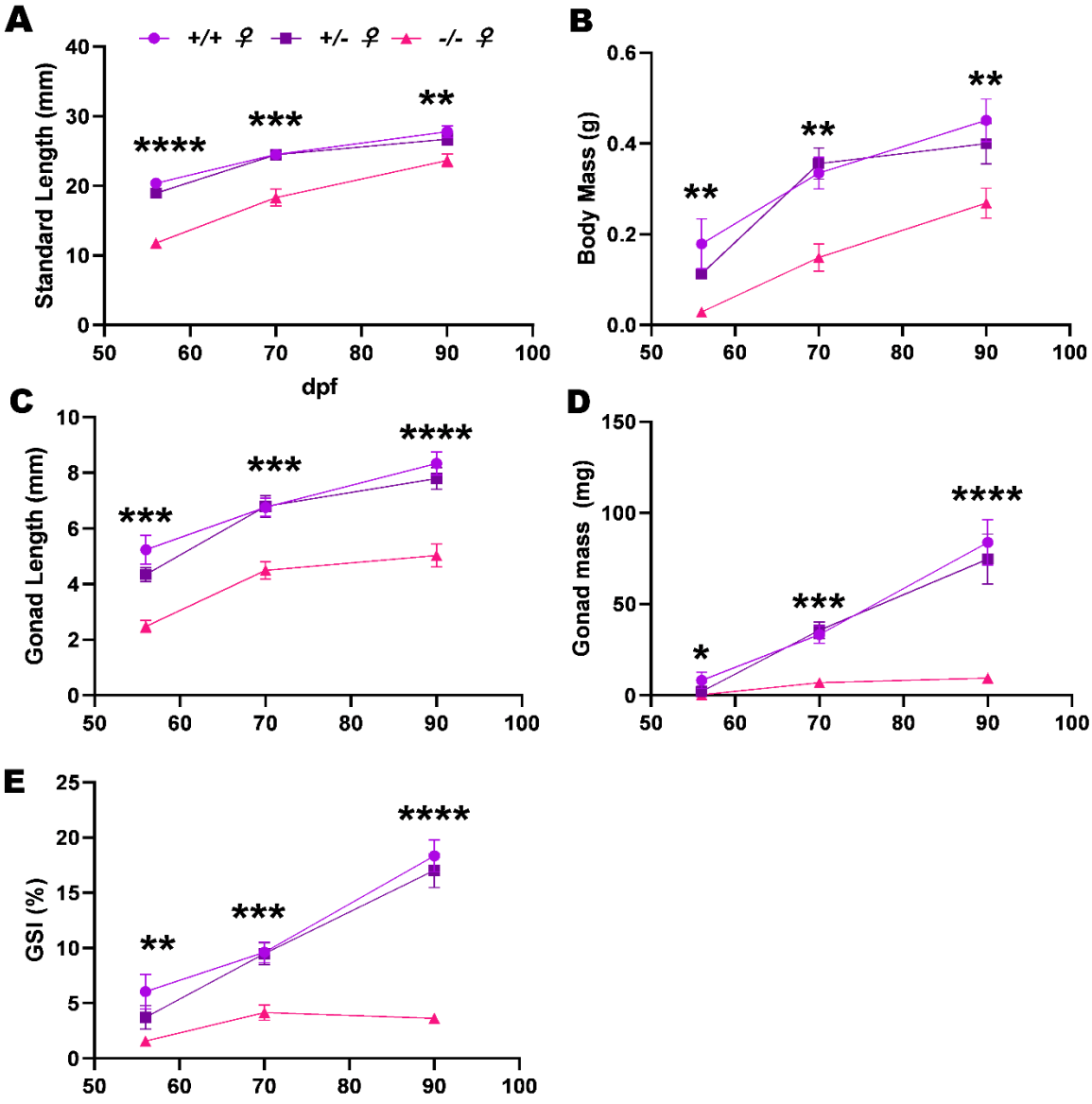

A
+/+ ♀
+/- ♀
-/- ♀
Standard Length (mm)
dpf
****
***
**
B
Body Mass (g)
**
**
**
C
Gonad Length (mm)
***
***
****
D
Gonad mass (mg)
*
***
****
E
GSI (%)
**
***
****

Figure 5.6

Figure 6: Late juvenile testes development in wildtype and Adamts9 KO males. Juvenile male wildtype (+/+), heterozygous (+/-), and Adamts9 KO (-/-) zebrafish were dissected and measured for Standard Length (SL), Body Mass (BM), Gonad Length (GL), Gonad Mass (GM), and the gonadosomatic index (GSI%) was calculated. **A)** SL was significantly different between Adamts9 KO juvenile males and WT males at all timepoints (56dpf: p=0.0014; 70dpf: p<0.0001; 90dpf: p<0.0001). Heterozygous siblings were not significantly different from wildtype (all timepoints: p>0.05). **B)** BM was significantly reduced in Adamts9 KO compared to wildtype siblings at all timepoints (56dpf: p<0.0001; 70dpf p=0.0001; 90dpf p=0.0001). **C)** Similar to SL, GL was significantly lower in Adamts9 KO at all timepoints measured (56dpf: p=0.001; 70dpf: p<0.0001; 90dpf: p<0.0001). **D**) GM was significantly decreased at all timepoints (56dpf: p=0.0077; 70dpf: p=0.0013; 90dpf p<0.0001), but like wildtype and heterozygous siblings, Adamts9 KO males maintained an upward trend of testes growth. **E)** GSI% in Adamts9 KO males was significantly lower in 56dpf males, but not significant at 70dpf or 90dpf (56dpf: p=0.0040; 70dpf: p=0.8279; 90dpf: p=0.2534). Scatter plots for each timepoint/measurements can be found in Supplemental Fig. S4.4

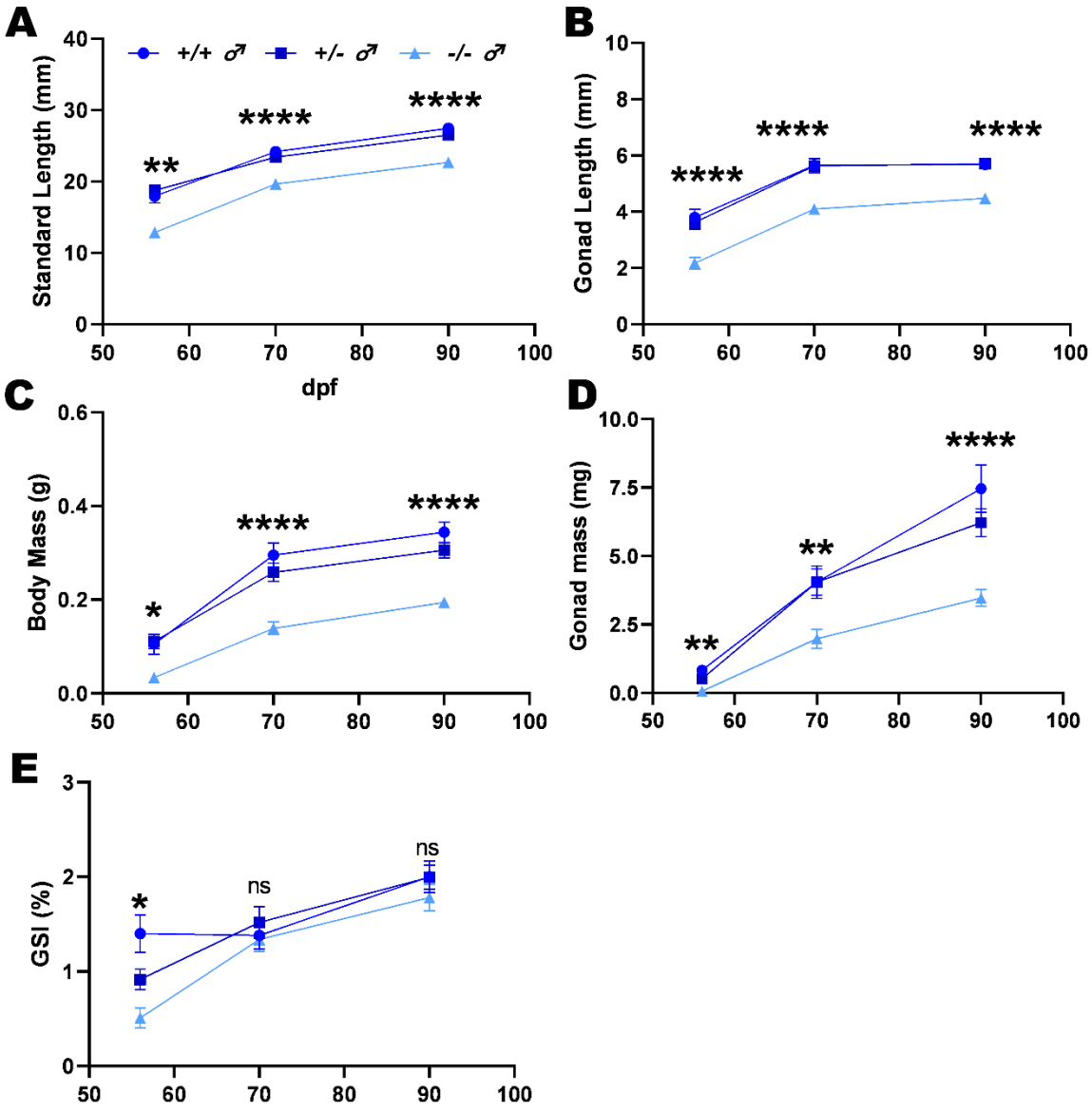

A
+/+ ♂
+/- ♂
-/- ♂
Standard Length (mm)
dpf
**
****
****
B
Gonad Length (mm)
****
****
****
C
Body Mass (g)
*
****
****
D
Gonad mass (mg)
**
**
****
E
GSI (%)
*
ns
ns

Figure 5.7

Confocal Images of 56 and 70dpf juvenile Adamts9 KO ovaries. Dissected ovaries from wildtype and Adamts9 KO gonads were imaged under laser scanning confocal microscopy at 56dpf and 70dpf. Germ cells are labeled with *Tg*(*vasa:GFP*) and somatic cells are labeled with DAPI. **A)** Wildtype fish had multiple follicles that were progressing into later stages of oogenesis (Stage II, Stage III) at 56dpf. **B-D)** By contrast, all follicles observed were observed to still be in Stage IB in Adamts9 KO. Some oocytes had clearly abnormal morphology (white arrows), and some follicles were observed to be empty in Adamts9 KO (red arrow). **E)** Zoomed out imaged showing presence of multiple maturing follicles in wildtype fish at 70dpf. **F-H)** At 70dpf, follicles remained at Stage IB in Adamts9 KO, even though they grew to very large sizes in **G**. Few follicles were observed to move to the next stage of folliculogenesis, Stage II. Scale bars are 50um for all images.

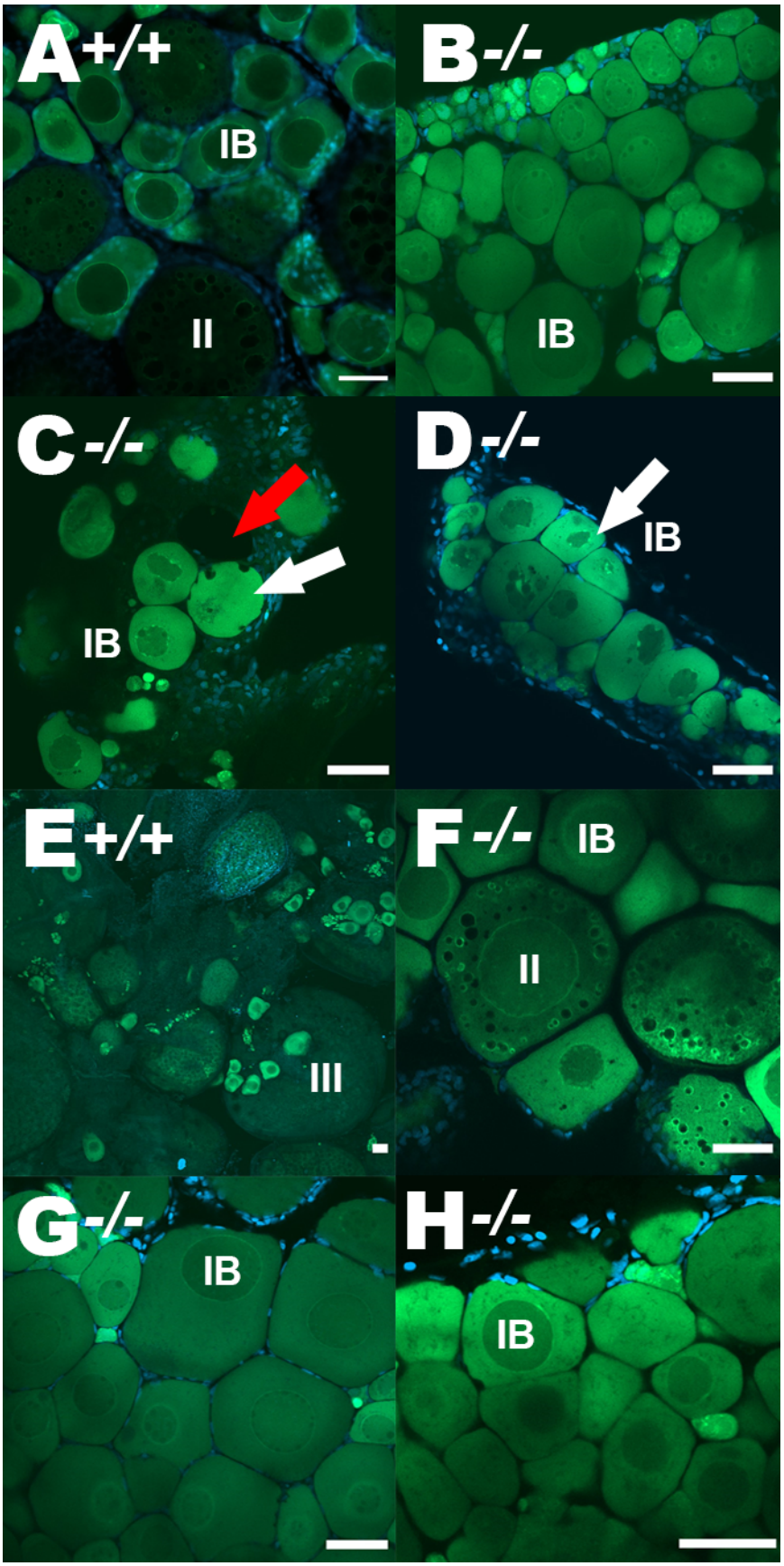

A +/+
IB
II
B -/-
IB
C -/-
IB
D -/-
IB
E +/+
III
F -/-
IB
II
G -/-
IB
H -/-
IB

Figure 5.8

Sex ratios after primary sex determination and maturation in Adamts9 KO. Sex ratios between Adamts9 KO fish, wildtype, and heterozygous siblings. **A)** At 35dpf, right after primary sex determination, Adamts9 KO sex ratio is not significantly different from wildtype siblings (Fisher's exact test, $p>0.9999$). **B)** At 90dpf, once the fish has reached sexual maturity, Adamts9 KO had a significantly male biased sex ratio compared to age-matched wildtype siblings (Fisher's exact test, $p=0.0028$). **C)** As the Adamts9 KO fish age, the sex ratio continues to skew towards males (Carter et al., 2019; 12mpf fish). For raw numbers counted at each time point, see supplemental table S4.3.

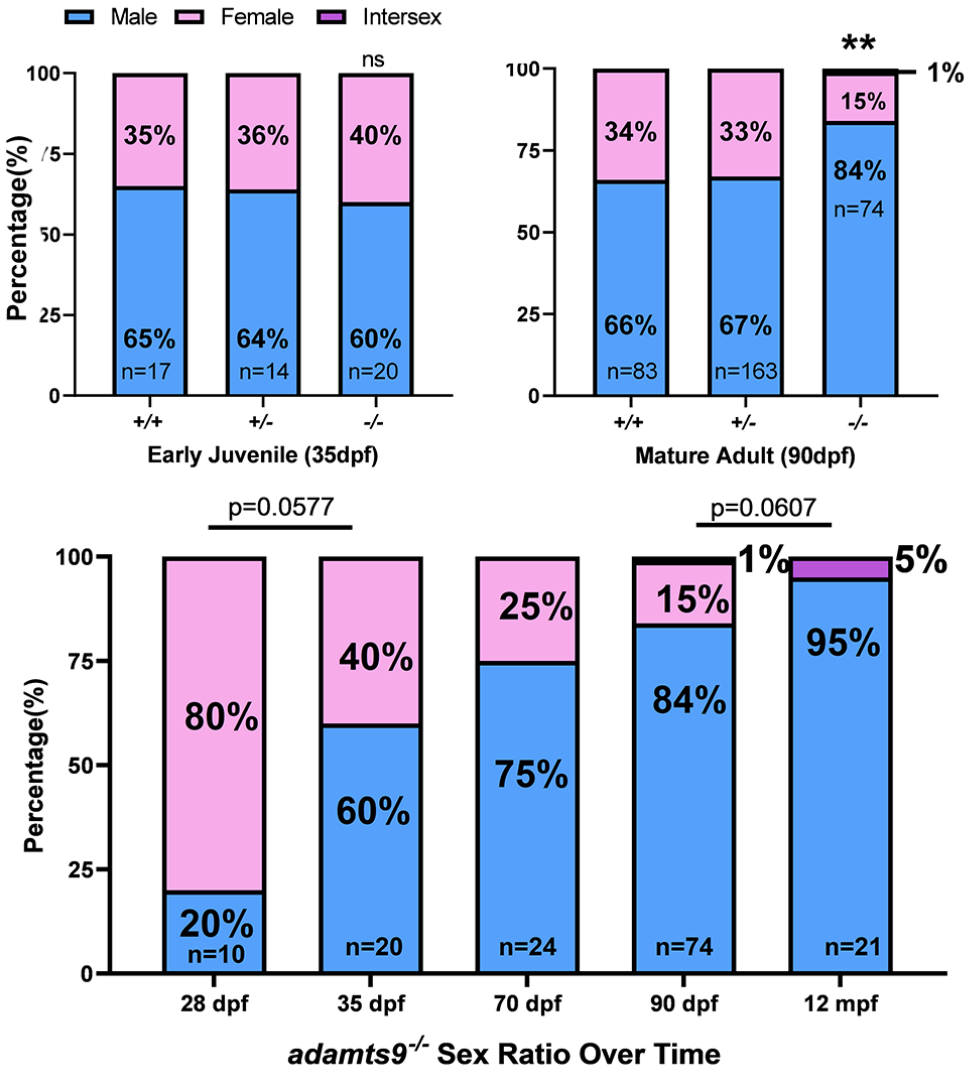


Figure 5.9

Enhanced nutrition alone cannot rescue the Adamts9 KO phenotype. To attempt to rescue the male sex bias in Adamts9 KOs, and to mitigate the effects of global growth delay, we employed a low rearing density and overfeeding regimen. **A)** Under the dissecting microscope, the Adamts9 KO females had dramatically smaller ovaries than wildtype siblings. **B)** Enhanced feeding regimen caused female sex bias in wildtype, heterozygous, and adamts9 KOs (Fisher's exact test, +/+ vs. +/+OF p=0.0124; +/+ vs. +/-OF p<0.0001; +/+ vs. -/-OF p=0.0166). **C)** SL was not significantly different between wildtype and adamts9 KO overfed females (Independent t-test, p=0.0554) or males (p=0.3116). **D)** BM was still significantly smaller in Adamts9 KO compared to wildtype and heterozygous females (Independent t-test, p=0.0007), but not in males (p=0.3745). **D)** Gonad mass is significantly smaller in Adamts9 KO females than wildtype and heterozygous females (Independent t-test, p<0.0001). **E)** GSI% was also significantly reduced in Adamts9 KO rescued females versus wildtype and heterozygous females (Independent t-test, p<0.0001).

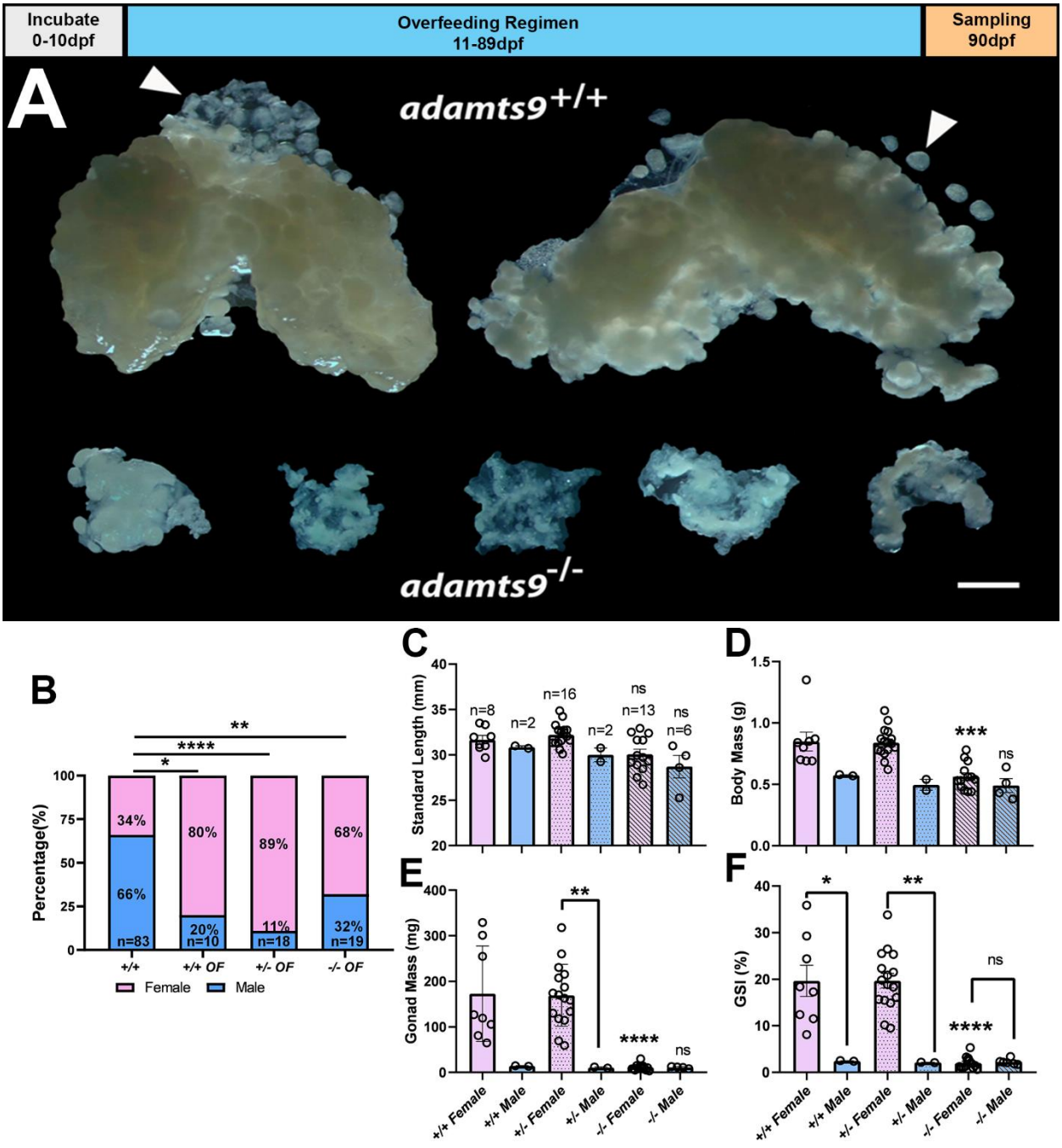

Incubate
0-10dpf
Overfeeding Regimen
11-89dpf
Sampling
90dpf
A
adamts9+/+
adamts9-/-
B
Percentage(%)
34%
66%
n=83
80%
20%
n=10
89%
11%
n=18
68%
32%
n=19
+/+
+/+ OF
+/- OF
-/- OF
Female
Male
C
Standard Length (mm)
n=8
n=2
n=16
n=2
ns
n=13
ns
n=6
D
Body Mass (g)
***
ns
E
Gonad Mass (mg)
**
****
ns
F
GSI (%)
*
**
****
ns
+/+ Female
+/+ Male
+/- Female
+/- Male
-/- Female
-/- Male

Figure 5.10

Comparison of "rescued" Adamts9 KO females vs. normally reared wildtype controls.

(A,B) We found that Adamts9 KO females were did not have significantly different SL or BM compared with age-matched, normally reared wildtype controls (Independent t-test, SL: p=0.1057; BM: p=0.3177). **C)** Therefore, we compared ovarian development between age and size matched sibling and found GSI% was still dramatically different between age and size matched Adamts9 KO and wildtype controls (p<0.0001). **D,E)** Next, we compared the means between different age groups and rearing conditions within genotypes to compare the rates of ovarian growth between wildtype and Adamts9 KO siblings. GM continually increased in wildtype siblings, accompanied with a rise in the GSI% (WT GM 56-70 p=0.0014; 70-90: p=0.0012; 90-90OF: p=0.0137; GSI% 56-70: p=0.0542; 70-90: p=0.0002; 90-90OF: p=0.5331. **F,G)** In Adamts9 KO, GM and GSI did not continue to increase past 70dpf, and GSI actually decreased in overfed condition, suggesting somatic tissue continue to develop but not the gonad (KO GM 56-70: p=0.0006; 70-90: p=0.3022; 90-90OF: p=0.5314; GSI% 56-70: p=0.0003; 70-90: p=0.4885; 90-90OF: p=0.0064).

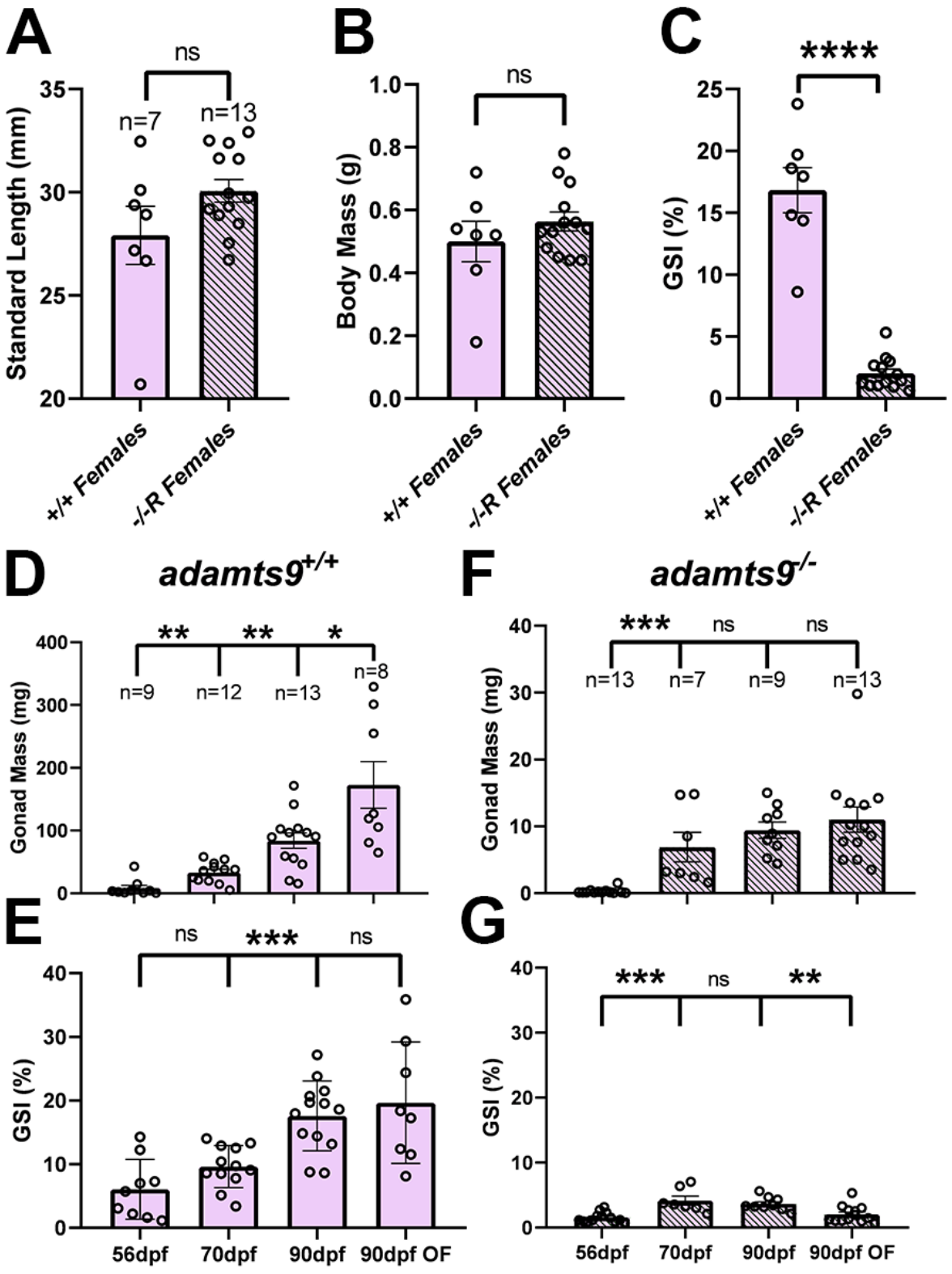

A
ns
n=7
n=13
Standard Length (mm)
35
30
25
20
+/+ Females
-/-R Females
B
ns
Body Mass (g)
1.0
0.8
0.6
0.4
0.2
0.0
+/+ Females
-/-R Females
C
****
GSI (%)
25
20
15
10
5
0
+/+ Females
-/-R Females
D
adamts9+/+
**
**
*
n=9
n=12
n=13
n=8
Gonad Mass (mg)
400
300
200
100
0
E
ns
***
ns
GSI (%)
40
30
20
10
0
56dpf
70dpf
90dpf
90dpf OF
F
adamts9-/-
***
ns
ns
n=13
n=7
n=9
n=13
Gonad Mass (mg)
40
30
20
10
0
G
***
ns
**
GSI (%)
40
30
20
10
0
56dpf
70dpf
90dpf
90dpf OF

Figure 5.11

Accumulation of primary follicles and morphological evidence of sex reversal in Adamts9 KO. To determine cellular differences between wildtype, heterozygous, and Adamts9 KO ovaries, dissected tissues were Paraffin embedded and stained with hematoxylin and eosin. **A,A')** Representative images of wildtype ovaries under two different magnifications. Many maturing follicles (Stage III, IV) could be observed. **B,B')** Representative images of heterozygous ovaries under two different magnifications. Like wildtype, many maturing follicles could be observed. **C,C')** Representative images of Adamts9 KO ovaries under two different magnifications. Unlike wildtype siblings, only primary follicles were common though a few follicles had progressed past the primary stage. **D-I)** Morphological evidence of follicle loss and sex reversal in Adamts9 KO fish. Asterisks mark empty follicles in the somatic gonadal tissue, black arrows point to sperm cells in sex reversing fish. Scale bars are 100µm. **J-L)** Quantification of follicles between Adamts9 KO and wildtype controls. Serial 10µm sections were cut, every 5^{th} section was photographed, and follicles were counted creating a sample that represented 300µm of depth through the ovary. **J)** The total number of follicles was not significantly different between wildtype and Adamts9 KO siblings (Independent t-test, p=0.8210). **K)** The number of matured follicles (Stage IV) was significantly lower (p=0.0094). **L)** Adamts9 KO had a significantly higher proportion of follicles in Stage IB compared to wildtype siblings (p<0.0001), and significantly fewer follicles in Stage II (p=0.0001) and Stage III (p=0.0001). The difference in Stage IV follicle proportions failed to cross the significance threshold (p=0.1215), likely because some Adamts9 KO ovaries had very few follicles, one of which had matured but not ovulated.

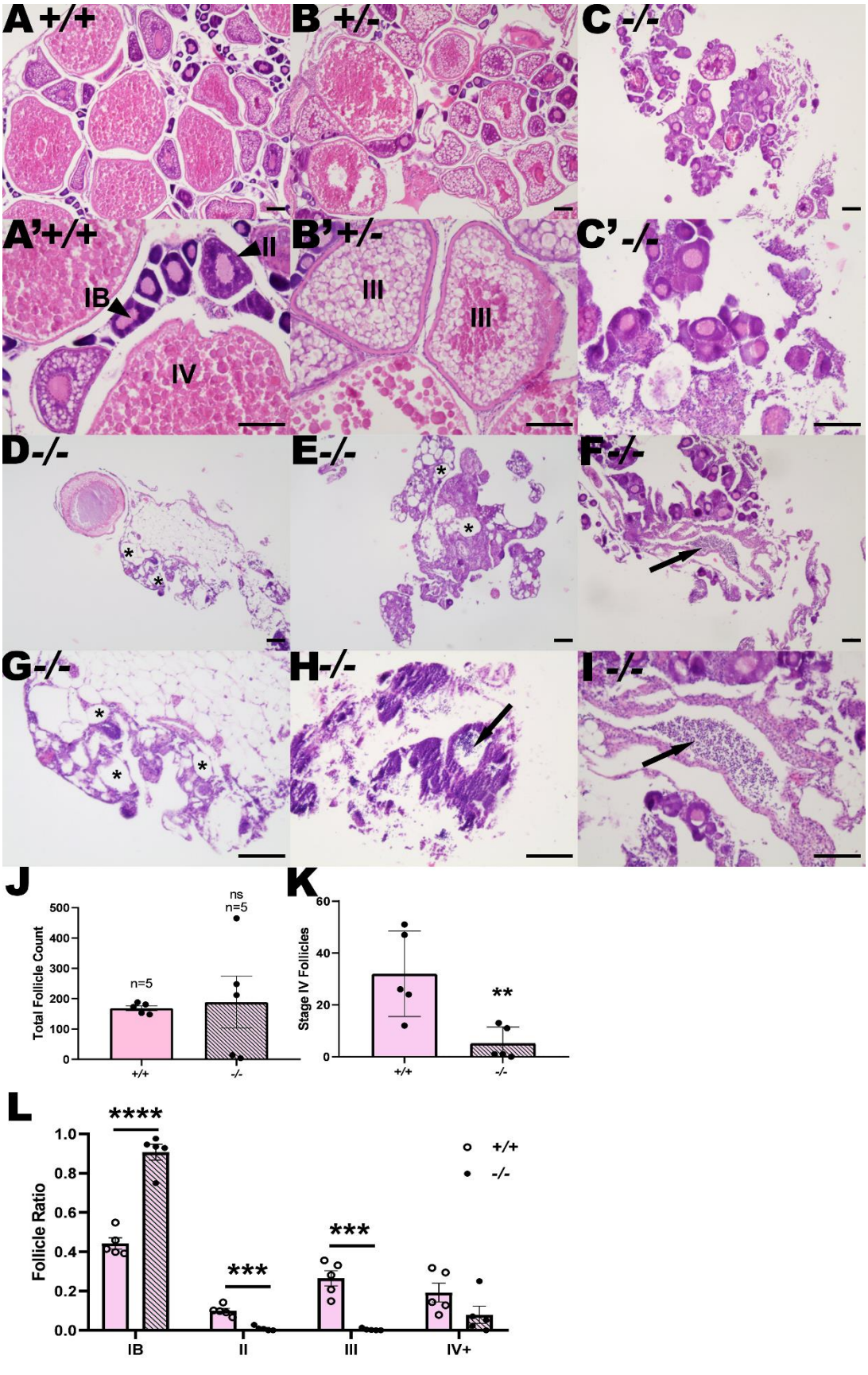

A +/+
B +/-
C -/-
A'+/+
B'+/-
C' -/-
IB
II
III
III
IV
D-/-
E-/-
F-/-
G-/-
H-/-
I -/-
J
Total Follicle Count
500
400
300
200
100
0
n=5
ns
n=5
+/+
-/-
K
Stage IV Follicles
60
40
20
0
**
+/+
-/-
L
Follicle Ratio
1.0
0.8
0.6
0.4
0.2
0.0
****
***
***
+/+
-/-
IB
II
III
IV+

Figure 5.12

***In situ* hybridization of *adamts9* mRNA in ovarian somatic cells.** mRNA *in situ* hybridization detected strong expression of *adamts9* in pockets of ovarian cells. Using both anti-sense (column A) and control sense probe (column B), we detected pockets of ovarian stromal cells that also expressed Adamts9 under brightfield and confocal microscopy (top and bottom panels, respectively). From a scRNAseq data set (Liu et al., 2022), enriched expression of *adamts9* was found in the ovarian stroma, suggesting this is the observed population of cells in the staining.

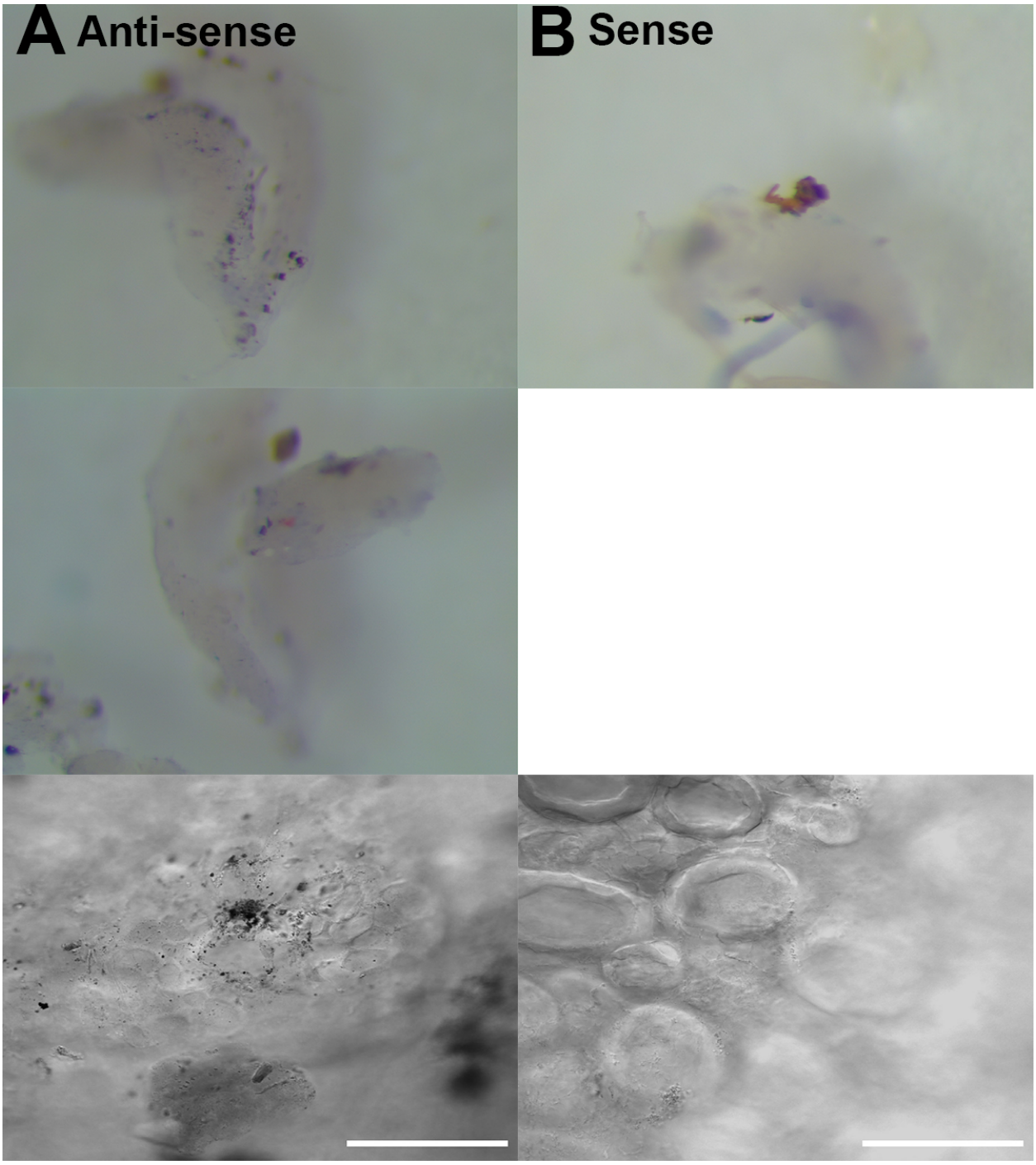

A Anti-sense
B Sense

Figure 5.13

Immunostaining of Adamts9 in primary ovarian follicles. Primary RP5 and RP1 antibodies targeting the metalloprotease and pro- domains respectively (Carter et al., 2019), were used to detect Adamts9 protein expression inside ovarian follicles using either horseradish-peroxidase (HRP) or AlexaFluor 488 (AF488) secondary antibodies. Previous work has demonstrated RP1/RP5 to be specific to zebrafish Adamts9 and does not stain Adamts9 KO samples (Carter et al., 2019). **A)** Detection of Adamts9 in Stage IVB follicular cells and Stage I follicles by RP1, **B)** and RP5 primary antibody from adult tissue sections. **C,D)** Magnified image of antibody staining for Adamts9 in Stage IB follicles by RP1 and RP5 antibodies, respectively. **E,F)** Immunostaining of Adamts9 using RP5 primary antibody in 35dpf juvenile wildtype ovary, at two different magnifications. **G-I)** Immunostaining of Adamts9 using RP5 primary antibody, and AF488 conjugated secondary antibodies. mCherry was used to detect background autofluorescence of fixed tissues. **G-I')** Grayscale images of G-I. Scale bar is 20µm.

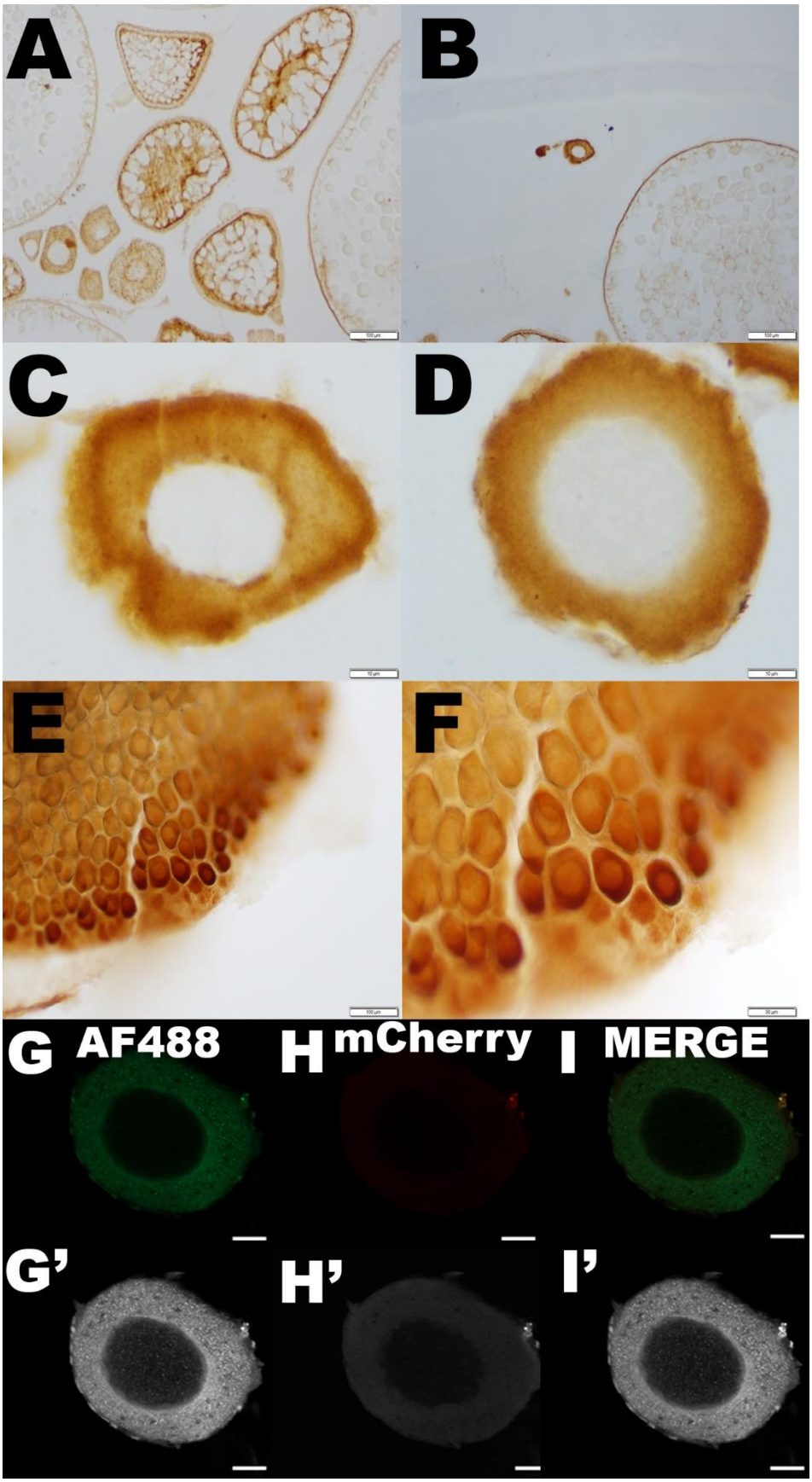
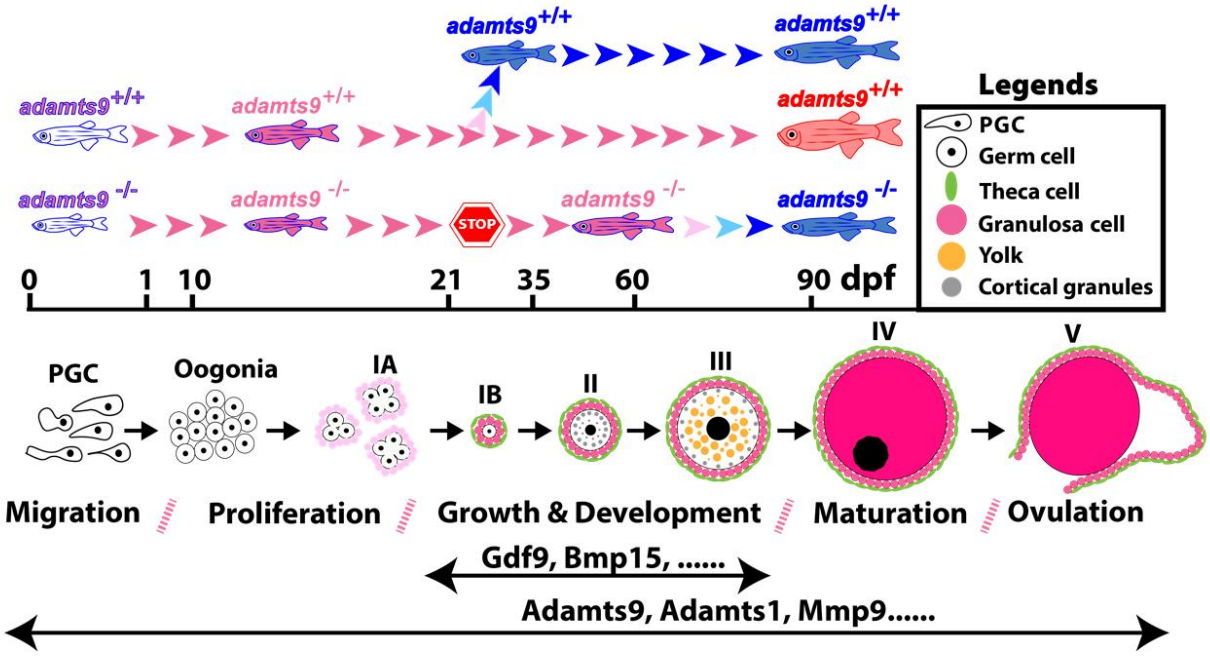

A
B
C
D
E
F
G AF488
H mCherry
I MERGE
G'
H'
I'

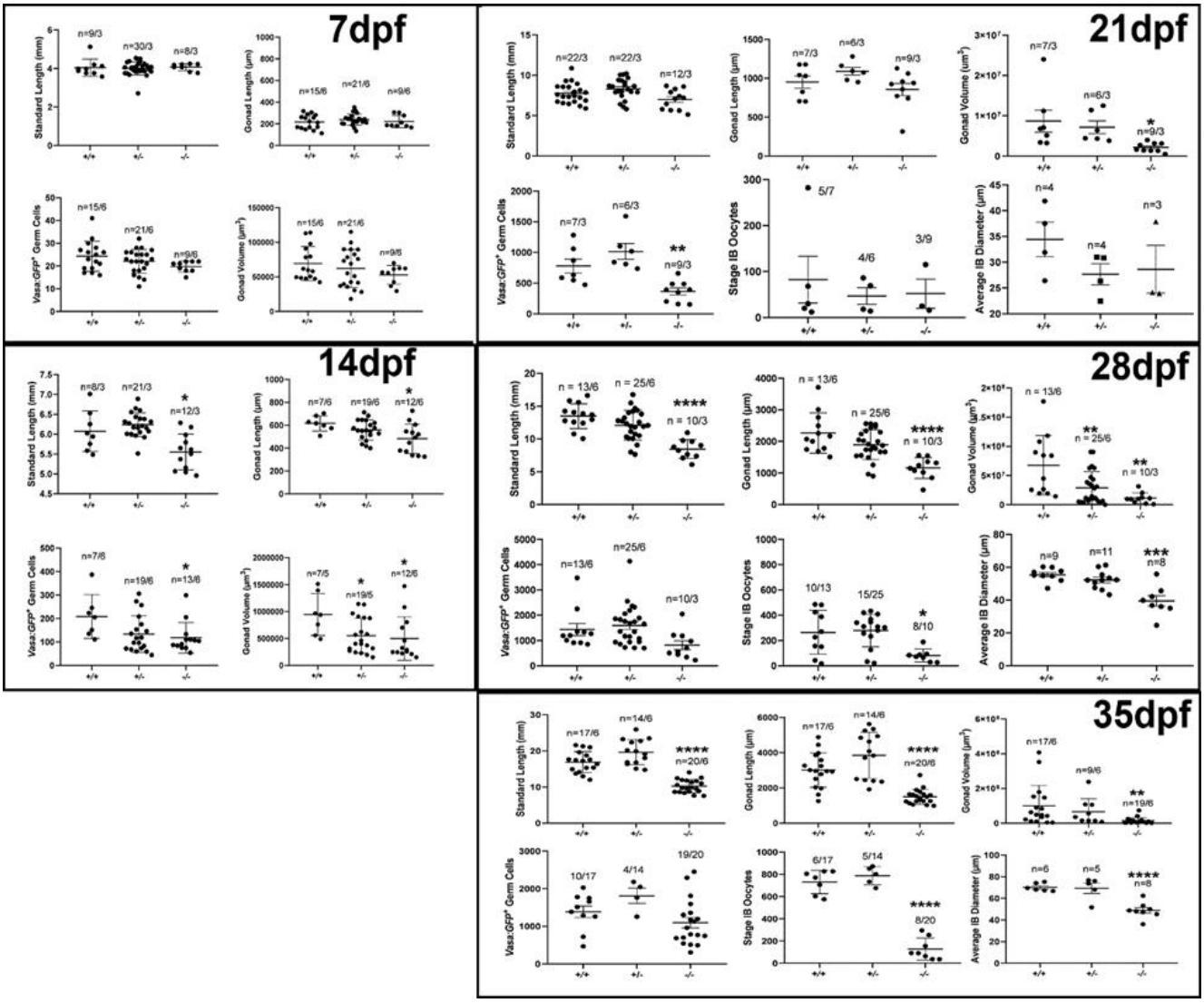
Figure 5.14

Diagram summary of ovary growth and developmental stages in zebrafish. During the first 24hpf, PGCs are specified and migrate to the gonadal ridge (Carver et al., 2021; Carver and Zhu, *in review*). During the first week, somatic and gonad cells begin to rapidly proliferate and begin differentiating. From 21dpf onwards, primary follicles develop and continue maturation within their follicles. Oocytes secrete vital signals for folliculogenesis, including Gdf9 and Bmp15 (Dranow et al., 2016; Chen et al., 2017). Metalloproteases including Adamts1, Adamts9, and Mmp9 are involved in continued folliculogenesis (Shindo et al., 2000; Carter et al., 2019; this paper). In Adamts9 KO zebrafish, primary follicles can be formed but do not progress further, eventually causing sex reversal as the ovarian tissue fails (Carter et al., 2019; this paper).



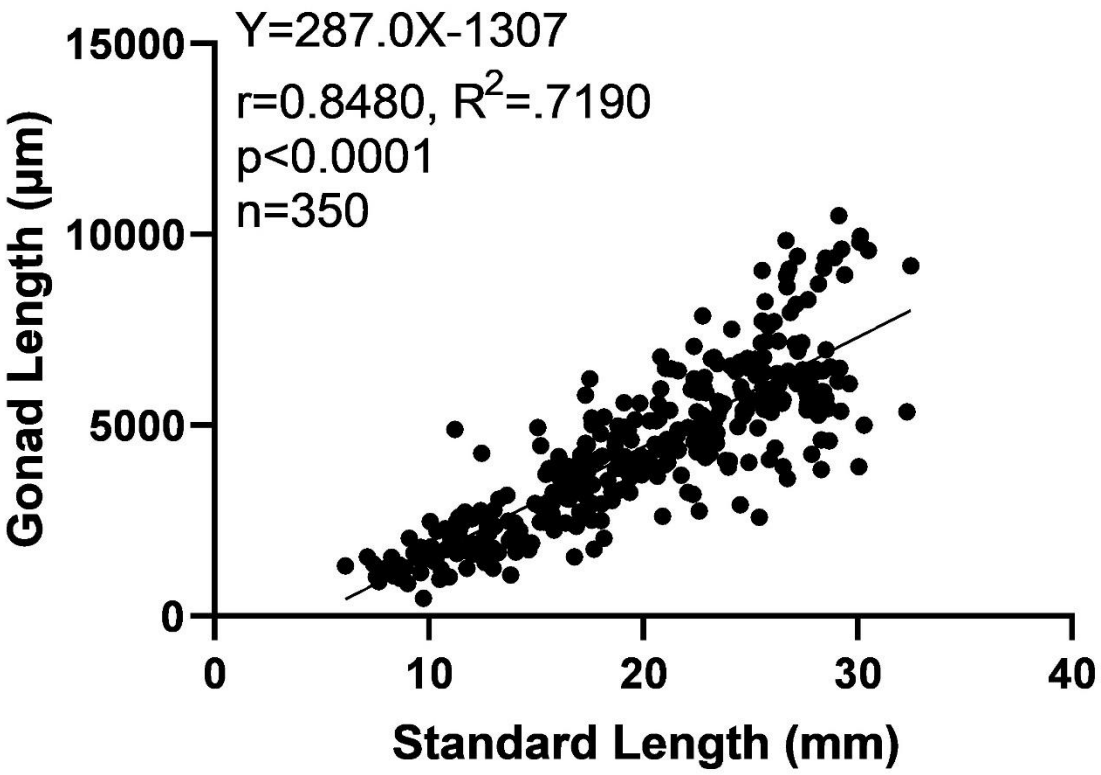
Supplemental Figure S5.1

Scatter dot plots for each timepoint / measurement taken for figure 5.3. For SL, GL, GV, and *vasa:GFP*$^{+}$ GCs: n = #number of inidividuals / #number of parents used to generate embryos. For Stage IB oocytes n = # of individuals with IB oocytes / # individuals total.



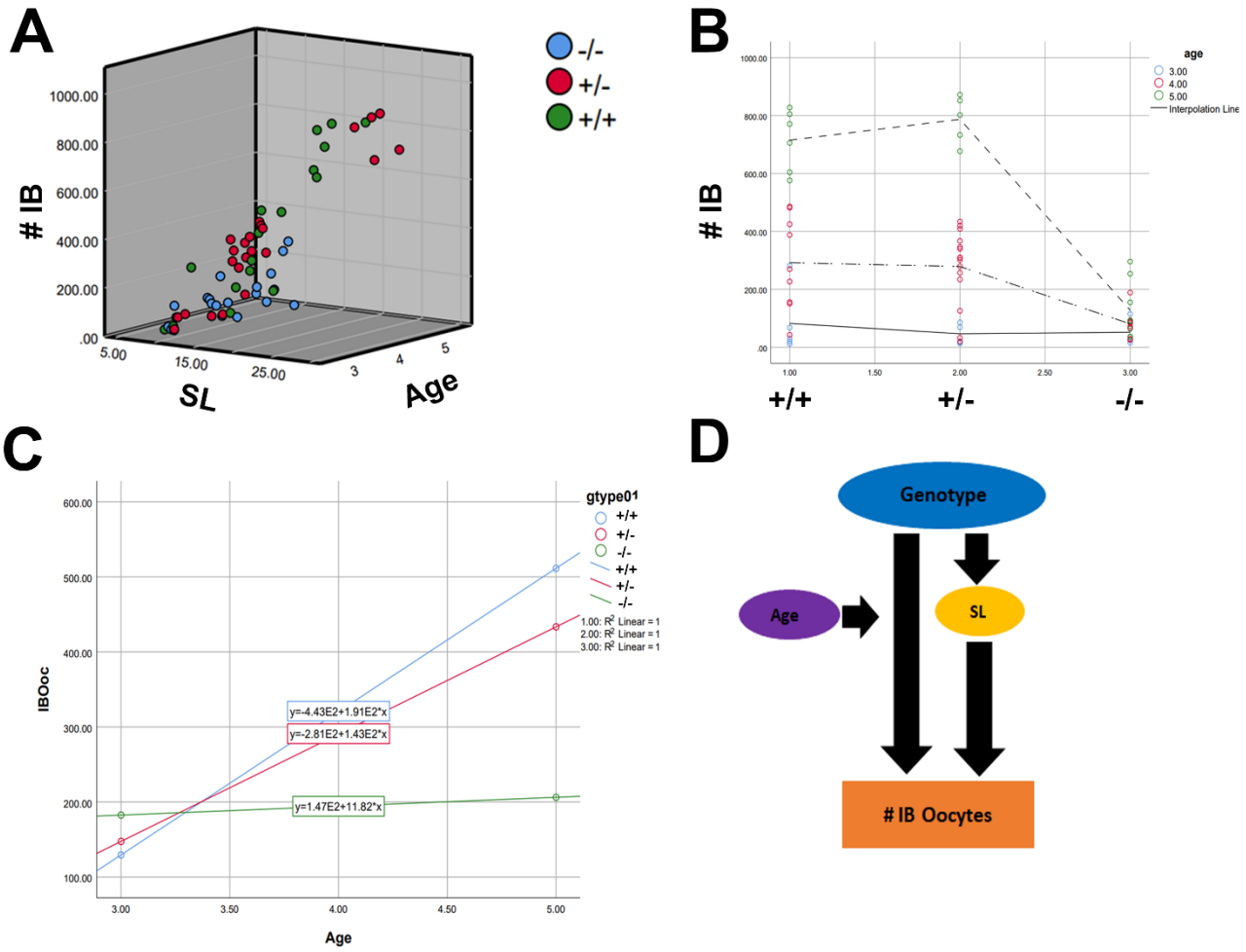
Supplemental Figure S5.2

Linear regression model of gonad length as a function of standard length, without regards to age, genotype, or sex in adults. N = 350. Y=287.0X-1307, r=0.8480, R^2=.7190; p<0.0001.



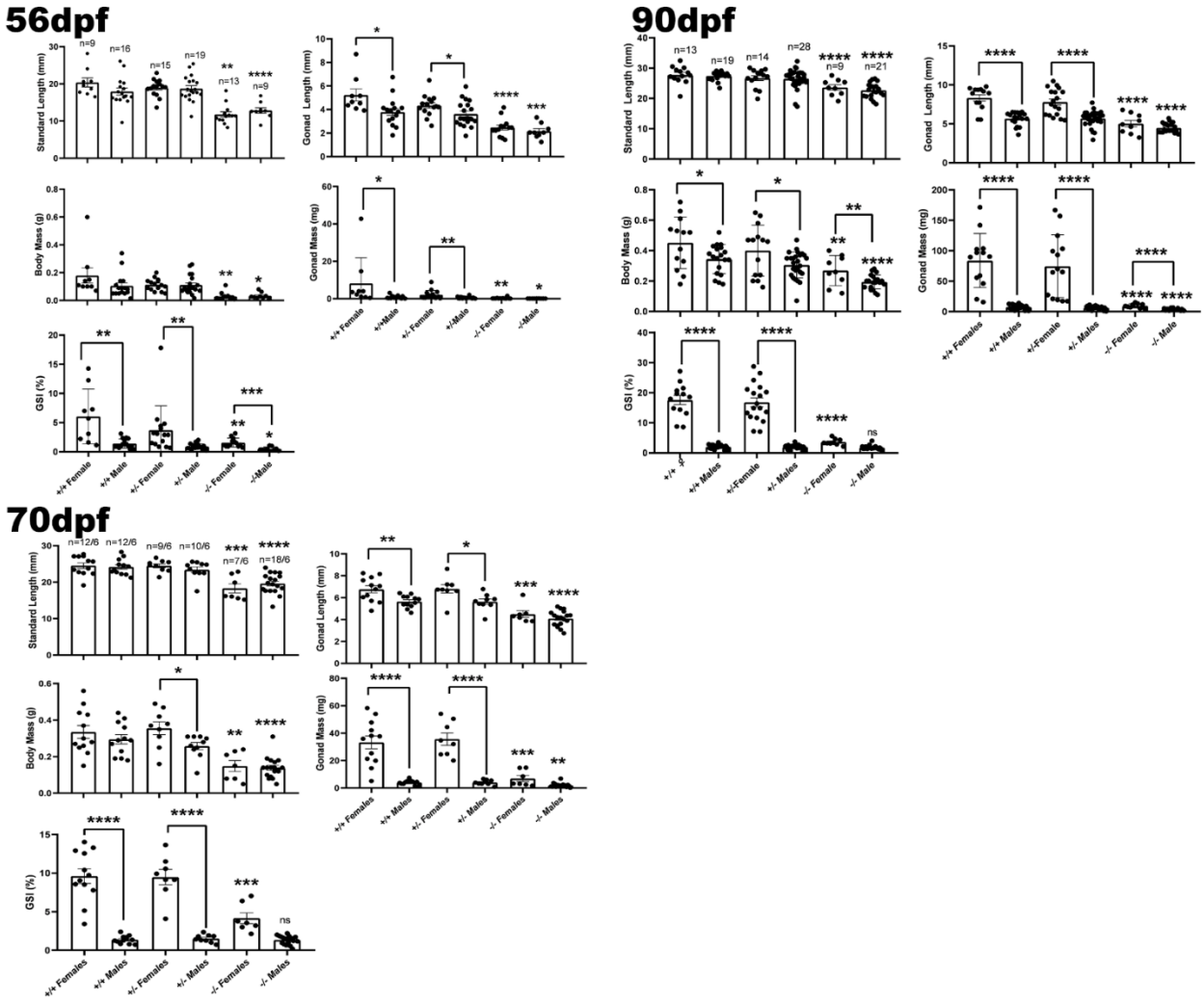
Supplemental Figure S5.3

Statistical modeling of number of Stage IB follicles accounting for genotype, age, and standard length. **A)** 3D scatter plot of number of IB oocytes with age, SL, and genotype as independent variables. **B)** Bivariate scatter plot of age and genotype on the number of Stage IB oocytes. **C)** Linear regression model of genotype on number of Stage IB oocytes, treating age as a moderator variable and standard length as a covariate. **D)** Graphical summary of understood relationship between genotype, age, and standard length on the number of Stage IB oocyte. Adamts9 KO has a direct effect on the Standard Length of the fish, which in turn has a predicted significant effect on the number of Stage IB oocytes. However, even when accounting for SL as a confounding covariate, we still predicted significant effect of genotype on number of Stage IB oocytes. Age was not a significant predictor on the number of Stage IB oocytes but moderated the effect of genotype on Stage IB oocytes. Full SPSS coding and output can be found in supplemental tables S4.1 and S4.2.


A
-/-
+/-
+/+
IB
1000.00
800.00
600.00
400.00
200.00
.00
5.00
15.00
25.00
SL
3
4
5
Age
B
age
3.00
4.00
5.00
Interpolation Line
IB
1000.00
800.00
600.00
400.00
200.00
.00
1.00
1.50
2.00
2.50
3.00
+/+
+/-
-/-
C
gtype01
+/+
+/-
-/-
+/+
+/-
-/-
1.00: R² Linear = 1
2.00: R² Linear = 1
3.00: R² Linear = 1
IBOoc
600.00
500.00
400.00
300.00
200.00
100.00
y=-4.43E2+1.91E2*x
y=-2.81E2+1.43E2*x
y=1.47E2+11.82*x
3.00
3.50
4.00
4.50
5.00
Age
D
Genotype
Age
SL
IB Oocytes

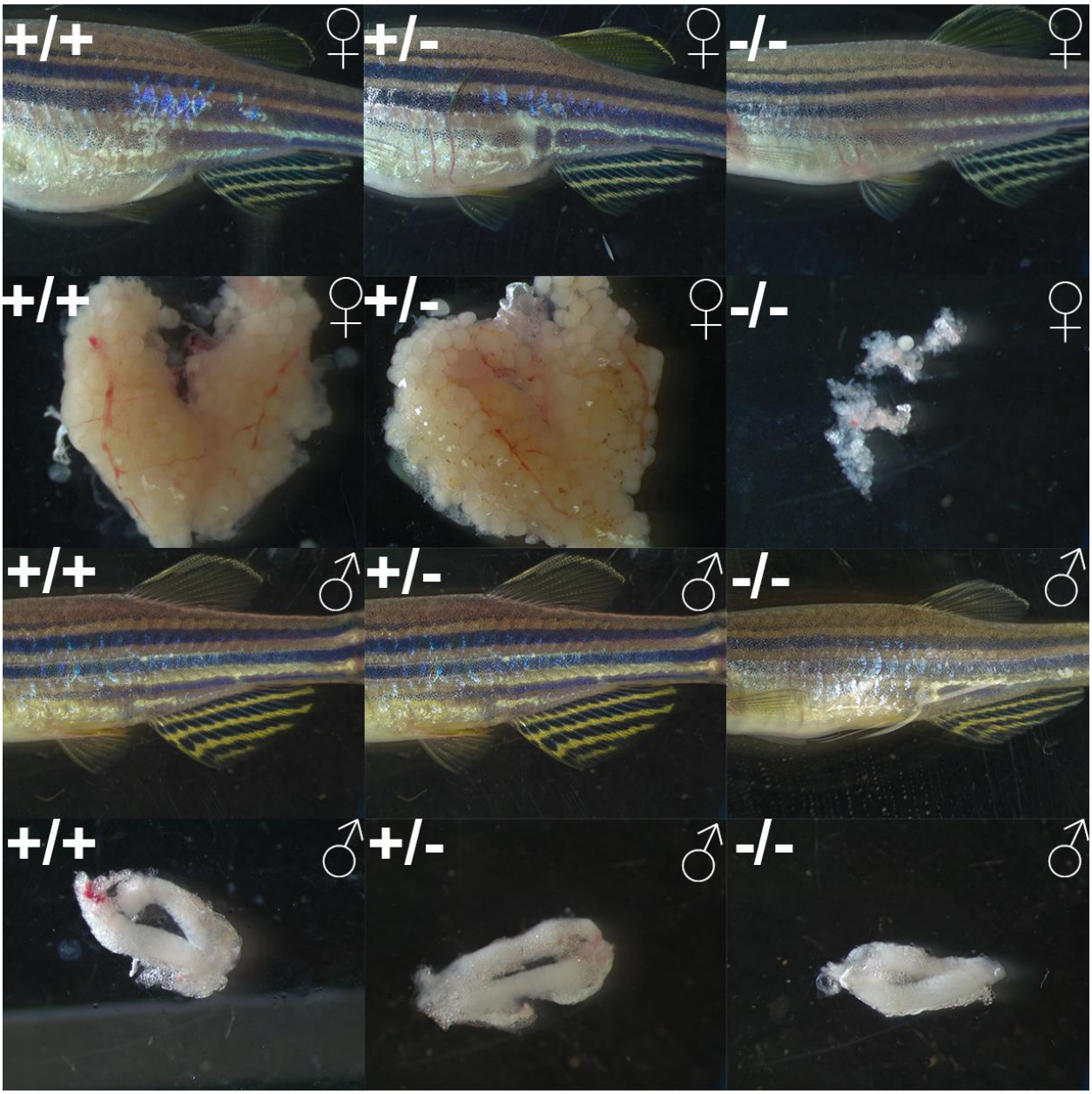
Supplemental Figure S5.4

Scatter dot plots for each timepoint / measurement taken for figures 5.5 and 5.6.



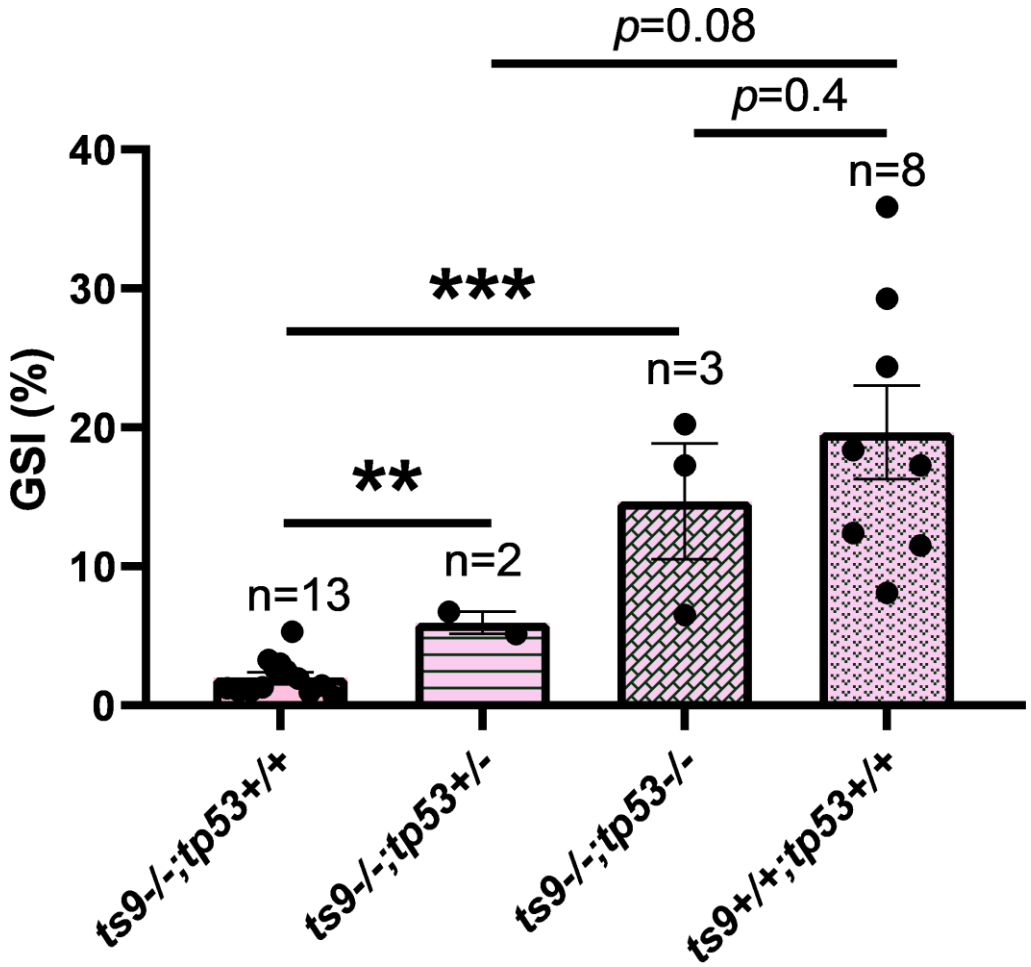
Supplemental Figure S5.5

Gross external and gonad morphology in 90dpf wildtype and Adamts9 KO male and female siblings. Top Row: External morphology of wildtype (+/+), heterozygous (+/-), and Adamts9 KO (-/-) female siblings. Dissected ovary is shown below. Bottom Rows: External morphology of wildtype (+/+), heterozygous (+/-), and Adamts9 KO (-/-) male siblings. Dissected testes are shown below.



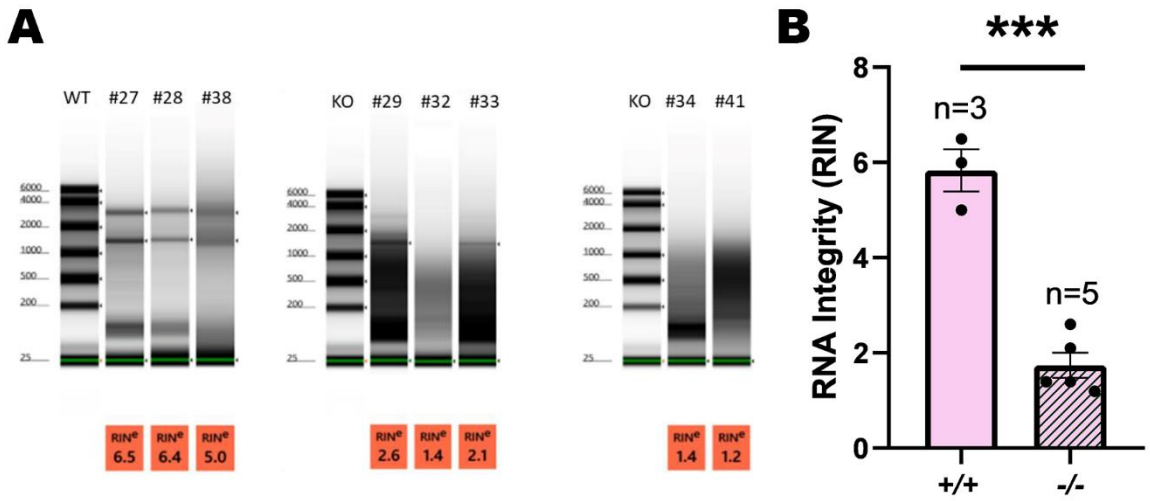
Supplemental Figure S5.6

p53 KO rescued GSI% in Adamts9 KO females. In a second rescue experiment, fish containing the *tp53*$^{-/-}$ null mutation were in crossed with *adamts9*$^{-/-}$ fish to generate double mutants. The double mutants were then overfed rescued and sacrificed at 90dpf. GSI% was significantly higher in *adamts9*$^{-/-}$*tp53*$^{-/-}$ fish than in *adamts9*$^{-/-}$*tp53*$^{+/+}$ fish (Independent t-test, $p<0.0001$).



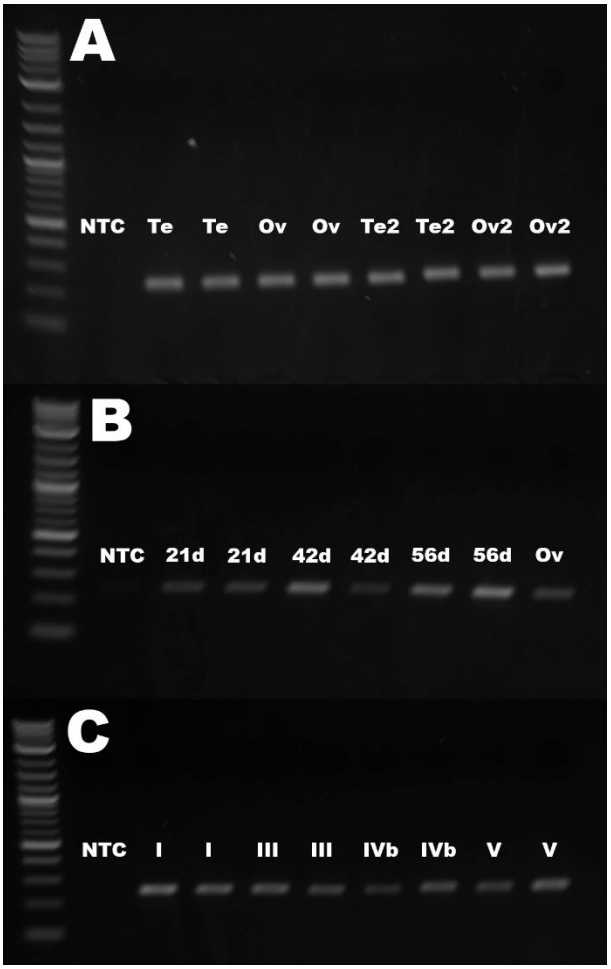
Supplemental Figure S5.7

Degraded RNA in rescued Adamts9 KO females. A) Extracted RNA from 90dpf Adamts9 KO overfed females was analyzed using Agilent RNA Screen Tape Assay. Gel images as well as raw RNA Integrity (RIN) scores are shown below. **B)** Adamts9 KO females had degraded RNA by gel analysis, and statistically significant lower average RIN scores (Independent t-test, p=0.0001). RNA degradation is an early step in apoptosis, indicating the remaining ovarian cells are dying.



Supplemental Figure S5.8

***adamts1* expression in zebrafish follicles and developing gonads.** (A) *adamts1* transcripts were detected in adult zebrafish gonads. Two different primer sets were used to verify expression, PCR reaction was cycled 35 times. NTC: No template control; Te: adult testis; Ov: adult ovary; Te2: 10X dilution of adult testis cDNA; Ov2: 10X dilution of adult ovary cDNA. (B) Detection of *adamts1* in juvenile zebrafish gonads at 21dpf, 42dpf, 56dpf, and in adult ovary. (C) Detection of *adamts1* in all stages of zebrafish follicles.



Supplemental Table S5.1

SPSS MANOVA output using number of Stage IB oocytes as the dependent variable, and SL, Genotype, and Age as independent variables.

Multivariate Tests[a]

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	.112	3.097[b]	2.000	49.000	.054
	Wilks' Lambda	.888	3.097[b]	2.000	49.000	.054
	Hotelling's Trace	.126	3.097[b]	2.000	49.000	.054
	Roy's Largest Root	.126	3.097[b]	2.000	49.000	.054
SL	Pillai's Trace	.512	25.687[b]	2.000	49.000	.000
	Wilks' Lambda	.488	25.687[b]	2.000	49.000	.000
	Hotelling's Trace	1.048	25.687[b]	2.000	49.000	.000
	Roy's Largest Root	1.048	25.687[b]	2.000	49.000	.000
Genotype	Pillai's Trace	.192	2.651	4.000	100.000	.038
	Wilks' Lambda	.809	2.747[b]	4.000	98.000	.033
	Hotelling's Trace	.237	2.838	4.000	96.000	.028
	Roy's Largest Root	.235	5.878[c]	2.000	50.000	.005
Age	Pillai's Trace	.138	1.849	4.000	100.000	.125
	Wilks' Lambda	.865	1.840[b]	4.000	98.000	.127
	Hotelling's Trace	.153	1.830	4.000	96.000	.129
	Roy's Largest Root	.126	3.152[c]	2.000	50.000	.051
Genotype * Age	Pillai's Trace	.468	3.821	8.000	100.000	.001
	Wilks' Lambda	.574	3.918[b]	8.000	98.000	.000
	Hotelling's Trace	.668	4.010	8.000	96.000	.000
	Roy's Largest Root	.529	6.616[c]	4.000	50.000	.000

a. Design: Intercept + SL + Genotype + Age + Genotype * Age

b. Exact statistic

c. The statistic is an upper bound on F that yields a lower bound on the significance level.

Supplemental Table S5.2

SPSS linear regression and PROCESS coding and output

Variable Creation

	Label
GType_1	Genotype=+/+
GType_2	Genotype=+/-
GType_3	Genotype=-/-

```
 RECODE Genotype ('+/+'=1) ('+/-'=2) ('-/-'=3) INTO gtype01.
EXECUTE.
COMPUTE inter1=gtype01 * SL.
EXECUTE.
COMPUTE inter2=gtype01 * Age.
EXECUTE.
REGRESSION
  /MISSING LISTWISE
  /STATISTICS COEFF OUTS CI(95) R ANOVA
  /CRITERIA=PIN(.05) POUT(.10)
  /NOORIGIN
  /DEPENDENT IBOoc
  /METHOD=ENTER GType_1 GType_2 inter1 inter2 SL Age.
```

Variables Entered/Removed[a]

Model	Variables Entered	Variables Removed	Method
1	age, Genotype=+/+ , Genotype=+/-, length, inter1, inter2[b]	.	Enter

a. Dependent Variable: IBOoc

b. All requested variables entered.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.935[a]	.874	.861	96.62170

a. Predictors: (Constant), age, Genotype=+/+, Genotype=+/-, length, inter1, inter2

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	3639555.315	6	606592.553	64.975	.000[b]
	Residual	522802.113	56	9335.752		
	Total	4162357.429	62			

a. Dependent Variable: IBOoc

b. Predictors: (Constant), age, Genotype=+/+, Genotype=+/-, length, inter1, inter2

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients		
		B	Std. Error	Beta	t	Sig.
1	(Constant)	-281.029	126.688		-2.218	.031
	Genotype=+/+	-577.656	183.992	-1.046	-3.140	.003
	Genotype=+/-	-244.176	96.307	-.461	-2.535	.014
	inter1	-7.572	8.683	-.285	-.872	.387
	inter2	-65.949	30.912	-.958	-2.133	.037
	length	51.059	18.141	.863	2.815	.007
	age	225.993	76.325	.609	2.961	.004

Coefficients[a]

Model		95.0% Confidence Interval for B	
		Lower Bound	Upper Bound
1	(Constant)	-534.816	-27.243
	Genotype=+/+	-946.235	-209.076
	Genotype=+/-	-437.102	-51.249
	inter1	-24.966	9.822
	inter2	-127.874	-4.024
	length	14.717	87.401
	age	73.095	378.891

a. Dependent Variable: IBOoc

```
REGRESSION
 /MISSING LISTWISE
 /STATISTICS COEFF OUTS CI(95) R ANOVA
 /CRITERIA=PIN(.05) POUT(.10)
 /NOORIGIN
 /DEPENDENT IBOoc
 /METHOD=ENTER GType_1 GType_2 inter1 inter2 SL Age
 /SAVE PRED.
```

```
Run MATRIX procedure:

***************** PROCESS Procedure for SPSS Version 3.4.1 *****************

          Written by Andrew F. Hayes, Ph.D.       www.afhayes.com
    Documentation available in Hayes (2018). www.guilford.com/p/hayes3

**************************************************************************
Model  : 1
    Y  : IBOoc
    X  : gtype01
    W  : Age

Covariates:
 SL

Sample
Size:  63

Coding of categorical X variable for analysis:
 gtype01      X1      X2
   1.000    .000    .000
   2.000   1.000    .000
   3.000    .000   1.000

**************************************************************************
OUTCOME VARIABLE:
 IBOoc

Model Summary
          R       R-sq        MSE          F        df1        df2          p
      .9351      .8744  9334.5053    64.9851     6.0000    56.0000      .0000

Model
              coeff         se          t          p       LLCI       ULCI
```

```
constant  -842.9568   121.2051    -6.9548      .0000 -1085.7615  -600.1522
X1         161.9178   192.7716      .8399      .4045  -224.2526   548.0881
X2         590.4783   186.0676     3.1735      .0024   217.7377   963.2190
Age        190.9474    36.9020     5.1744      .0000   117.0233   264.8715
Int_1      -47.9886    47.3479    -1.0135      .3152  -142.8384    46.8612
Int_2     -179.1305    46.1340    -3.8828      .0003  -271.5486   -86.7124
SL          32.8688     5.7174     5.7489      .0000    21.4153    44.3222

Product terms key:
 Int_1    :        X1       x        Age
 Int_2    :        X2       x        Age

Test(s) of highest order unconditional interaction(s):
       R2-chng          F        df1        df2          p
X*W      .0338     7.5419     2.0000    56.0000      .0013
----------
    Focal predict: gtype01  (X)
          Mod var: Age      (W)

Conditional effects of the focal predictor at values of the moderator(s):

Moderator value(s):
Age     3.0000

       Effect         se          t          p       LLCI       ULCI
X1    17.9519    56.6721      .3168      .7526   -95.5767   131.4804
X2    53.0869    58.9738      .9002      .3719   -65.0526   171.2265

Test of equality of conditional means
          F        df1        df2          p
      .4104     2.0000    56.0000      .6653

Estimated conditional means being compared:
    gtype01      IBOoc
     1.0000   129.5084
     2.0000   147.4603
     3.0000   182.5953
----------
Moderator value(s):
Age     4.0000

       Effect         se          t          p       LLCI       ULCI
```

```
X1   -30.0368    29.3705    -1.0227      .3109   -88.8733    28.7998
X2  -126.0435    40.1275    -3.1411      .0027  -206.4290   -45.6580

Test of equality of conditional means
          F        df1        df2          p
     4.9408     2.0000    56.0000      .0106

Estimated conditional means being compared:
    gtype01      IBOoc
     1.0000   320.4558
     2.0000   290.4191
     3.0000   194.4123
----------
Moderator value(s):
Age     5.0000

       Effect         se          t          p       LLCI       ULCI
X1   -78.0254    54.7464    -1.4252      .1596  -187.6964    31.6456
X2  -305.1740    63.2393    -4.8257      .0000  -431.8583  -178.4897

Test of equality of conditional means
          F        df1        df2          p
    12.0446     2.0000    56.0000      .0000

Estimated conditional means being compared:
    gtype01      IBOoc
     1.0000   511.4033
     2.0000   433.3779
     3.0000   206.2293

Data for visualizing the conditional effect of the focal predictor:
Paste text below into a SPSS syntax window and execute to produce plot.

DATA LIST FREE/
   gtype01    Age        IBOoc      .
BEGIN DATA.
     1.0000     3.0000   129.5084
     2.0000     3.0000   147.4603
     3.0000     3.0000   182.5953
     1.0000     4.0000   320.4558
     2.0000     4.0000   290.4191
     3.0000     4.0000   194.4123
```

```
     1.0000     5.0000   511.4033
     2.0000     5.0000   433.3779
     3.0000     5.0000   206.2293
END DATA.
GRAPH/SCATTERPLOT=
 Age      WITH     IBOoc    BY       gtype01  .

********************** ANALYSIS NOTES AND ERRORS ************************

Level of confidence for all confidence intervals in output:
  95.0000

W values in conditional tables are the 16th, 50th, and 84th percentiles.

------ END MATRIX -----


 DATA LIST FREE/
   gtype01    Age        IBOoc      .
BEGIN DATA.
     1.0000     3.0000   129.5084
     2.0000     3.0000   147.4603
     3.0000     3.0000   182.5953
     1.0000     4.0000   320.4558
     2.0000     4.0000   290.4191
     3.0000     4.0000   194.4123
     1.0000     5.0000   511.4033
     2.0000     5.0000   433.3779
     3.0000     5.0000   206.2293
END DATA.
GRAPH/SCATTERPLOT=
 Age      WITH     IBOoc    BY       gtype01  .
```

Graph

Supplemental Table S5.3

Raw count data for sex ratios in Figure 5.8. For simplicity, intersex Adamts9 KO fish were tallied in the male column.

	28dpf		
	+/+	+/-	-/-
Male	3	10	2
Female	10	15	8
	35dpf		
	+/+	+/-	-/-
Male	11	9	12
Female	6	5	8
	56dpf		
	+/+	+/-	-/-
Male	16	19	9
Female	9	15	13
	70dpf		
	+/+	+/-	-/-
Male	12	10	18
Female	12	9	7
	90dpf		
	+/+	+/-	-/-
Male	55	108	63
Female	28	51	11
	90dpf Overfed		
	+/+	+/-	-/-
Male	8	16	13
Female	2	2	6
	1 year		
	+/+	-/-	
Male		21	
Female		0	

CHAPTER VI: Summary and Perspectives

In the present work, I have highlighted the importance of ECM metalloproteases in gonad development and folliculogenesis (II), demonstrated widespread expression of *adamts9* in developing and adult zebrafish (III), demonstrated PGC migration delay in Adamts9 KO embryos (IV), and provided evidence that follicle arrest and loss is the primary cause of male sex bias in Adamts9 KO zebrafish (V) (Carter et al., 2019). This is the first genetic Adamts9 model that has been studied extensively in vertebrate ovarian development, yet much remains to be discovered about the molecular mechanisms of Adamts9 underlying the observed phenotypes.

Zebrafish *adamts9* has a widespread expression pattern during early embryonic development. *adamts9* transcripts are maternally deposited and present at the animal pole at the one-cell stage through early embryonic cleavage (He et al., *in preparation*). Zygotic expression of *adamts9* begins around 8hpf when the majority of zygotic gene expression is turned on (Carver et al., 2021). Several other metalloproteases have been shown to be maternally deposited in zebrafish or expressed early in embryonic development, including *mmp9*, *mmp2*, *adamts1*, *mmp14a*, *adam12*, and antagonist *timp2b* (Zhang et al., 2003b; Yoong et al., 2007; Zhang et al., 2003a; Brunet et al., 2015; Thisse et al., 2004; Charlton-Perkins et al., 2019; Yin et al., 2010; Tokumasu et al., 2016). Morpholino knockdown, knockout model, or metalloprotease downregulation/antagonism during zebrafish embryonic and larval development can cause disrupt many different aspects of proper development including cell migration, lymphangiogenesis, craniofacial morphogenesis, and somite differentiation (Lu et al., 2020; Dancevic et al., 2018; Cao et al., 2018; Zhang et al., 2020; Wang et al., 2020; Iida et al., 2010; Zhang et al., 2003a; Leigh et al., 2013; Coyle et al., 2008; Yin et al., 2010). Functional roles for metalloproteases in early embryonic cell cleavage in zebrafish are not reported to the best of my

knowledge and should be investigated further. We found *adamts9* expression in craniofacial muscles, blood vessels, brain, retina, and migration pre-lateral line ganglion (He et al., *in preparation*). Expression of Adamts9 KO phenotypes in these structures will need to be investigated further in our zebrafish model.

Surprisingly, adult testes had the highest overall expression of *adamts9* in zebrafish. However, our Adamts9 KO males have healthy testes and can produce viable offspring when paired with female partners (Carter et al., 2019). Because many metalloproteases share redundant enzymatic function, and multiple Adamts proteases are expressed in adult zebrafish testes including Adamts1, our most probable explanation is that another Adamts protease can effectively compensate for Adamts9 loss in testes and sperm production (Brunet et al., 2015). Spermatogenesis is another complex cellular process that is required for fertility of male animals, and metalloproteases have been demonstrated to be expressed in adult testes. Understanding functional roles of metalloproteases in spermatogenesis can offer new avenues for exploring fertility treatments in males and should be explored further. Unfortunately, because of enzymatic redundancy, double- or triple- knockouts of Adamts paralogs may be necessary to observe a dramatic phenotype.

Despite abundant expression of ADAMTS metalloproteases during gonad development, few functional roles or molecular mechanisms are known for ADAMTS in early gonadogenesis. Among these, murine ADAMTS1 is necessary folliculogenesis in female mice (Shindo et al., 2000; Mittaz et al., 2004) and ADAMTS16 in male mice testes development (Livermore et al., 2019; Abdul-Majeed et al., 2014). ADAMTS19 SNPs are associated with PCOS in human patients, but roles of ADAMTS19 in ovarian development and health are unknown (Knauff et al., 2009; Urbanek et al., 2012; Russell et al., 2015). Multiple ADAMTS proteases have been

shown to be expressed during early gonad development in mice and zebrafish (Zhu, 2021; Carver and Zhu, *in revision*). Invertebrate models show that Adamts9 orthologs are essential for proper germ cell migration and primordial gonad establishment (Blelloch et al., 1999; Ismat et al., 2013). We demonstrated that PGC migration defects are not as severe in zebrafish Adamts9 KO embryos as they are in invertebrate ortholog GON-1 or AdamTS-A knockouts (Blelloch et al., 1999; Ismat et al., 2013). The ADAMTS family is dramatically expanded in vertebrates compared to invertebrates, hypothetically meaning another protease may be able to compensate for Adamts9 loss in vertebrates (Brunet et al., 2015). Because our Adamts9 KO females are infertile, we also cannot rule out the possibility of maternally deposited *adamts9* also mitigating the potential phenotypes.

We are the first to show that Adamts9 plays a significant role in ovarian folliculogenesis, in addition to its previously reported role in ovulation (Liu et al., 2018). In mice and other mammals, most attention has been focused on ADAMTS1 in folliculogenesis, but here we provide evidence that evolutionarily related Adamts9 also plays a significant role in folliculogenesis using a zebrafish model (Mittaz et al., 2004; Shindo et al., 2000; Brown et al., 2006; Shozu et al., 2005; Russell et al., 2015). Studying roles of MMPs, ADAMs, and ADAMTS functions in ovarian physiology will provide significant insight into the clinical understanding and treatment of ovarian infertility and disease (Pyun et al., 2014; GohariTaban et al., 2019).

Much remains to be done to understand the molecular mechanisms of Adamts9 in zebrafish gonad development and folliculogenesis. Answering these questions, although beyond the scope of this thesis, will further elucidate the biological significance of Adamts9 during organ development, sex differentiation, and adult ovarian physiology.

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APPENDIX A: IACUC Approval Letter

ECU

Animal Care and Use Committee
003 Ed Warren Life Sciences Building | East Carolina University | Greenville NC 27834 - 4354
252-744-2436 office | 252-744-2355 fax

October 14, 2021

Yong Zhu, Ph.D.
Department of Biology, ECU

Subject: Protocol D185f, original approval date 02/18/2020

Dear Dr. Zhu:

The amendment#2 to your Animal Use Protocol entitled, "Studies of Hormones and Receptors in Zebrafish" (AUP#D185f) was reviewed by this institution's Animal Care and Use Committee on 10/14/2021. The following action was taken by the Committee:

"Approved as submitted"

Please contact Aaron Hinkle prior to any hazard use

A copy of the protocols is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP/Amendment and are familiar with its contents.

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

JD/GD

enclosure

APPENDIX B: IACUC Approval of Research

EAST CAROLINA UNIVERSITY
ANIMAL USE PROTOCOL (AUP) FORM
LATEST REVISION NOVEMBER, 2017

Project Title:

Studies of Hormones and Receptors in Zebrafish

	Principal Investigator	Secondary Contact
Name	Yong Zhu	Fadi IssaJonathan CarverJonathan Carver
Dept.	Biology	Biology
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For IACUC Use Only

AUP #	Q185f		
New/Renewal	Renewal		
Full Review/Date	DR/Date 2/10/2020		
Approval Date	2/18/2020		
Study Type			
Pain/Distress Category	C		
Surgery	Survival	Multiple	
Prolonged Restraint			
Food/Fluid Regulation			
Other			
Hazard Approval/Dates	Rad	IBC 11/2017	EHS 2/17/2020
OHP Enrollment			
Mandatory Training			
Amendments Approved	#1 06/12/2020 VVC	#2 10/14/2021	#3 2/2/2022

I. Personnel

A. Principal Investigator(s):

Yong Zhu

B. Department(s):

Biology

C. List all personnel (PI's, co-investigators, technicians, students) that will be working with live animals and describe their qualifications and experience with these specific procedures. If people are to be trained, indicate by whom:

Please provide the responsibilities for each person as requested

Name/Degree/Certification	Position/Role(s)/ Responsibilities in this Project	Required Online IACUC Training (Yes/No)	Relevant Animal Experience/Training (include species, procedures, number of years, etc.)
Yong Zhu/PhD	Professor/PI/SupervisorProfessor/PI/Supervisor & leader of all the projects, overall responsible.	YesYes	Have relevant animal experience and training with fish since 1986, know well zebrafish husbandry requirements, spawning protocol, and microinjection procedure etc., responsible for training all collaborators and associates working in the lab
Click here to enter text.	Click here to enter text.		
Myon He Lee/PhD	Associate Professor/Co-Investigator/co-supervisor /collaborators, responsible for the experiments related to his project only (effects on RNA binding protein, especially PUF proteins-Pum1 and Pum2 in zebrafish germline stem cells)	Yes	Five years with zebrafish, trained directly by PI (Zhu), helping training associates
Jonathan Jacob Carver, BS	Graduate student/student/research assistant	Yes	4 years with zebrafish, trained by PI (Zhu)

Joyel Stephen Puthuparampil	Undergraduate student/student/research assistant	Yes	3 year with zebrafish, trained by PI (Zhu)
Andriy Lylyk	Undergraduate student/student/research assistant	Yes	3 year with zebrafish, trained by PI (Zhu)
Susana Contreras-Blanco	Undergraduate research assistant	Yes	Twi years with zebrafish, trained by PI (Zhu)
Alyssa Blackwell	Undergraduate research assistant	Yes	Two years with zebrafish, trained by PI (Zhu)
Caroline Marie Johnston	Undergraduate research assistant	Yes	Two years with zebrafish, trained by PI (Zhu)
Alex Salazar	Undergraduate research assistant	Yes	One year with zebrafish, trained by PI (Zhu)
Clarisse Tata Ginyu, BS	Graduate research assistant	Yes	Two months with zebrafish trained by PI
Tierra N White	Undergraduate research assistant	Yes	Two months with zebrafish trained by PI