UNIVERSITAT DE BARCELONA

Establishment and Remodeling of Sexually Dimorphic Tissues: Immune Cell Contributions

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ESTABLISHMENT AND REMODELING OF SEXUALLY DIMORPHIC TISSUES: IMMUNE CELL CONTRIBUTIONS

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Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.

Marie Curie

Acknowledgments

(EN)

Getting here was not easy, and I didn’t do it alone. Thank you to **each one of you** that helped me along the way, mostly by strongly believing that I could do it. And to the ones that didn’t. You all played an essential role in my journey to become the person I am today.

To **science**, that never stops surprising me.

And to *Danio*.

(ES)

Llegar hasta aquí no ha sido fácil, y no lo he hecho sola. Gracias a **cada uno/a** de los que me habéis ayudado en el camino, sobre todo por creer firmemente que podía conseguirlo. También a quienes no lo hicieron. Todos/as habéis jugado un papel esencial en el camino para convertirme en la persona que soy hoy.

A la **ciencia**, que nunca deja de sorprenderme.

Y a *Danio*.

(CA)

Arribar fins aquí no ha estat fàcil, i no ho he fet sola. Gràcies a **cadascun/a** dels que m'heu ajudat en el camí, sobretot en creure fermament que podia aconseguir-ho. També als que no ho van fer. Tots/es heu jugat un paper essencial en el camí per convertir-me en la persona que sóc avui.

A la **ciència**, que mai no deixa de sorprendre'm.

I a *Danio*.

To *her*

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LIST OF ABBREVIATIONS

ALPM	anterior lateral plate mesoderm
ANOVA	analysis of variance
BABB	benzyl alcohol/ benzyl benzoate
Cce	cerebellum
CNS	central nervous system
DAPI	4',6-diamidino-2-fenilindol
dpf	days post fertilization
DM	double mutant
DMSO	dimethyl sulfoxide
DSD	differences of sexual development
DVA	dorsal ventral aorta
EDTA	ethylenediaminetetraacetic acid
FC	follicle cells
FSH	follicle-stimulating hormone
FIJI	Fiji is just Image J
FISH	fluorescence *in situ* hybridization
GFP	green fluorescence protein
GnRH	gonadotropin-releasing hormone
GSC	germ stem cells
HCR	hybridization chain reaction
hpf	hours post fertilization
HPG	hypothalamic-pituitary-gonadal
HT	hypothalamus
IACUC	Institutional Animal Care and Use Committees
IHC	immunohistochemistry
LH	luteinizing hormone
LSD	least significant difference
MAFC	macrophage activating follicle cells
MG	microglia
MI	Molecular Instruments
MPH	macrophage

NK	neutral killer
NPC	neural precursor cells
OB	olfactory bulb
OEp	olfactory epithelium
OpT	optic tectum
OR	odor receptor
OSN	olfactory sensory neurons
PBS	phosphate-buffered saline
PBST	PBS with Tween/Triton
PCR	polymerase chain reaction
PFA	paraformaldehyde
PGC	primordial germ cells
PGE	prostaglandin E
PGF2a	prostaglandin $F_{2\alpha}$
POF	premature ovarian failure
POI	premature ovary insufficiency
PTU	1-phenyl 2-thiourea
RE	restriction enzyme
RST	reticulospinal tract
TA	transit amplifying
Te	telencephalon
TRM	tissue resident macrophages
TSF	tumor suppressor factors
UMAP	uniform manifold approximation and projection

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Preface

I hope that this thesis makes you wonder. This should be a thesis that awakes a new set of questions and ideas about the things that some of us – many of us – might have been missing when looking at some of our data, our experiments, and our projects. Not everyone works in a field that will be influenced by what I am about to tell you, but I hope that, just as it happened to me, still influences the way that we look at things.

I started this project long before I totally knew what it was going to be about. It was the perfect combination of looking for specific answers to very particular questions and finding very particular answers to questions I didn't plan on asking. Even though I am a biochemist and did not train as an immunologist or a developmental biologist, I had a passion for understanding how different fields and topics influence each other. And anyone that knows me knows one thing about me: I believe communication is essential when doing science. When we share and discuss, things work better, faster, and more efficiently. And that kept me looking for that connection within my own research: we cannot – we should not – account for each thing that we study as independent, because they never are. The body itself is a group of systems that work together to make everything function. And that is what made me fall in love with this project: I wanted to know how things were connected, how they talked and shared information and, most importantly, how that communication shapes and regulates important biological processes like development, growth, remodeling, or disease.

At the beginning, I was obsessed with the brain and I considered myself a *neuroscientist*. But that is not all I am anymore. During the past few years working with the gonad, I fell in love with its biology and functions, and I don't think I will ever be able to separate one from the other again. This is not only a story of who they are and the amazing abilities they hold, but how they can influence each other to make everything work so beautifully and efficiently. The two stories can be understood and enjoyed on their own, but they also make sense together, as part of a bigger picture and a broader question. I hope, as the writer of this thesis, that I can make you see that connection and the beauty of their relationship.

Lastly, I want to share one important lesson that I learned while doing this work: we should always make sure that we are studying something in the right context. We may not make a mistake

if we don't, but there is a lot we could be missing. It was only in the context of disease that I was able to discover the essential role of particular cells in the ovary during remodeling. And it was in the context of development and organization of very specific brain areas that I was able to find what accounts for the fundamental differences that we see in the adult.

Science is about learning new things, pushing the limits of what is known to make that knowledge greater and better. I have tried to keep my eyes and mind opened to new hypothesis, ideas, and questions. And hopefully my own work will one day inspire someone else, so that knowledge can be challenged once again and find the space to grow a little more.

PB

CHAPTER 1

Introduction

Sex-specific differences established in the brain during early critical periods are thought to affect function and behavior later in life (Huang et al., 2024; Sofer et al., 2024; Waters & Simerly, 2009), but the mechanisms and cell types involved in the organization of the brain during development or in states of disease are not fully understood. It is also not known how interactions between the immune, endocrine, and nervous systems influence development, brain function, and behavior, or whether sexual dimorphism in microglia colonization and morphology like that observed in mammals also exists within brain regions of the adult zebrafish nervous system.

Both reproductive and neurological disorders can arise from disruption of sexually dimorphic tissues (reviewed in (Joel et al., 2016; López-Ojeda & Hurley, 2021; McCarthy, 2016)). It is then important to understand how **gonad** differentiation occurs, and to what extent it affects establishment of sex-specific **brain** organization during development, and maintenance in adulthood. Better describing how distinct cell types and their interactions influence a tissue and its function, and how they are altered in reproductive and sex-biased neurological disorders is key to understanding tissue function in health and disease.

Sexual dimorphism in nature and biology

Females and males within species share almost all genetic information and yet they are able to develop considerably different tissues with very distinct functions and morphologies (Lopes-Ramos et al., 2020; Oliva et al., 2020; Yang et al., 2006). This biological condition, not always necessary for reproduction, is known as sexual dimorphism and is driven by presence of cells in

the tissue that are sensitive to and regulated by sex hormones (Andrew et al., 2022). Establishment of sexual dimorphism in any animal involves development of sex-specific fundamental structures, like the ovary in the female and testis in the male, and of other less obvious structures that account for differences in coloration, size, social behaviors or sensory disparities (reviewed in (Mank & Rideout, 2021; McCarthy et al., 2015)) (Figure 1.1). Interestingly, the nervous and immune systems are two of the tissues with highest affinity and sensitivity to sex-specific signals ((Gal-Oz et al., 2019; Gegenhuber et al., 2022; Sciarra et al., 2023; Zhou et al., 2024) and reviewed in (Klein et al., 2016)), which makes them great candidates when investigating the origin and progression of disorders and diseases that show strong sex-bias.

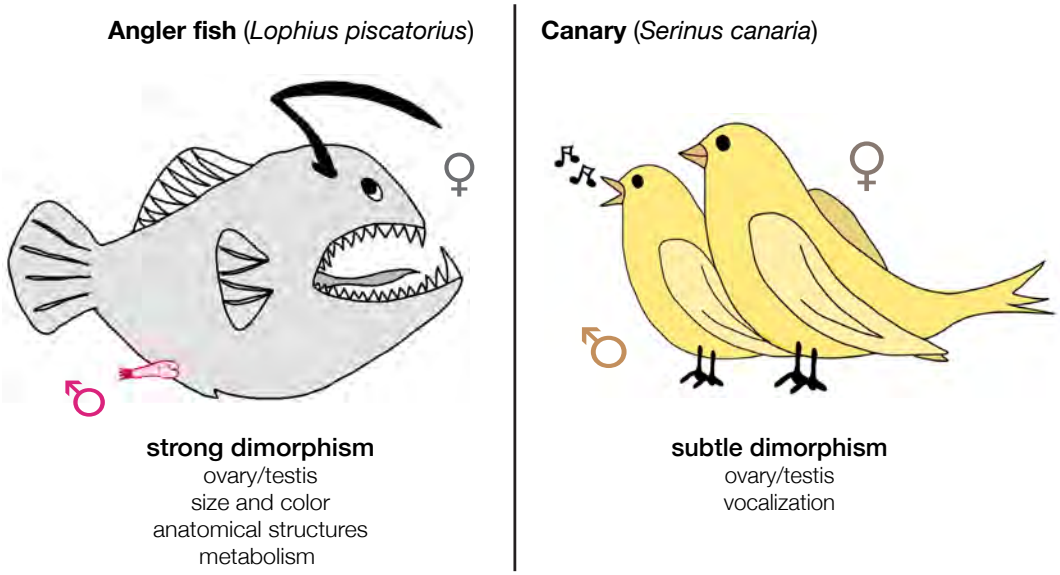


Figure 1.1: **Sexual dimorphism in the animal kingdom.**

- Sexually dimorphic immune system

  It is well known that endocrine and immune systems interact (Bereshchenko, 2018), and sexual dimorphism of immune responses is thought to be governed by sex hormones since, like other steroid hormones, they can modulate development and activity of cells involved in both innate and adaptive immunity (Bereshchenko, 2018; Gilliver, 2010; Klein et al., 2016; Straub, 2007). In humans, females are less likely to suffer from infections by pathogens, and they show higher susceptibility to autoimmune conditions than males (reviewed in (Shepherd et al., 2020)), however, the exact mechanisms on how sex hormones alter immune cell activities is not fully understood. Sex differences in the physiology of immune responses could be driven by expression of immune genes encoded within sex chromosomes (X and Y), or by autosomal genes that are regulated by steroid signaling or

epigenetic changes (Figure 1.2) (reviewed in (Dunn et al., 2023)). Female hormones, estrogen and progesterone, and male androgens, are involved in processes of inflammation and are robust gene regulators, being involved in many biological processes from early development and during adulthood (Bereshchenko, 2018; Klein et al., 2016; Shepherd et al., 2020).

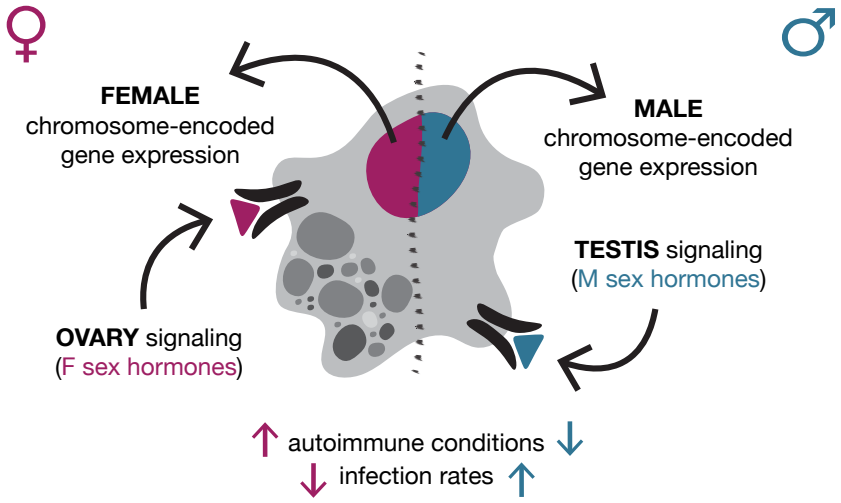


Figure 1.2: **Immune cell susceptibility to biological sex.**

Brain-gonadal axis: the neuroendocrine system

During brain sexual differentiation, the developing nervous system receives signals from the gonad in the form of sexual hormones (Gegenhuber et al., 2022) that are essential for specification of sex-specific structures and functions (Bridget M. Nugent et al., 2015; Oberlander & Woolley, 2016; Tabatadze et al., 2015). After development, and throughout adulthood, sex hormones continue to play an important role in the maintenance of reproductive tissues and behaviors ((Chakravarthi et al., 2021) and reviewed in (Downs & Wise, 2009; Marshall et al., 1991)). One of the first experiments in neuroendocrinology, performed using chicks by Arnold A. Berthold in 1849, aimed to test the idea that male-typical sex characteristics were driven by the presence of intact testes. His findings supported the hypothesis that the testes influence establishment of male morphologies and behaviors and do so by releasing a substance to the bloodstream rather than by direct neural connections (nerves). It was not until 1905 that Ernest Henry Starling termed this substance *hormones* (Starling, 1905), and in 1940 that Eugen Steinach first defined sexual hormones as the factors responsible for – physical, physiological, and behavioral – *sexuality* (Steinach, 1940).

The hypothalamic-pituitary-gonadal (HPG) neuroendocrine axis, that includes the hypothalamus, pituitary, and gonadal glands, plays a critical role during formation and regulation of processes in the reproductive and immune systems (Gal-Oz et al., 2019; Meethal et al., 2009; Sciarra et al., 2023). Specialized neurons in the hypothalamus release gonadotropin-releasing hormone (GnRH) and the pituitary gland secretes luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which stimulate function and maturation of the gonadal tissues (Marshall et al., 1991). In response, the gonads produce sex-steroid hormones, estrogens and testosterone, leading to local effects within the reproductive tissue, as well as systemic effects on target tissues in the body, including skeletal muscle, cardiovascular tissues, bone, and brain (Figure 1.3) ((Martel et al., 1994; Sato et al., 2008) and reviewed in (McEwen & Milner, 2017)).

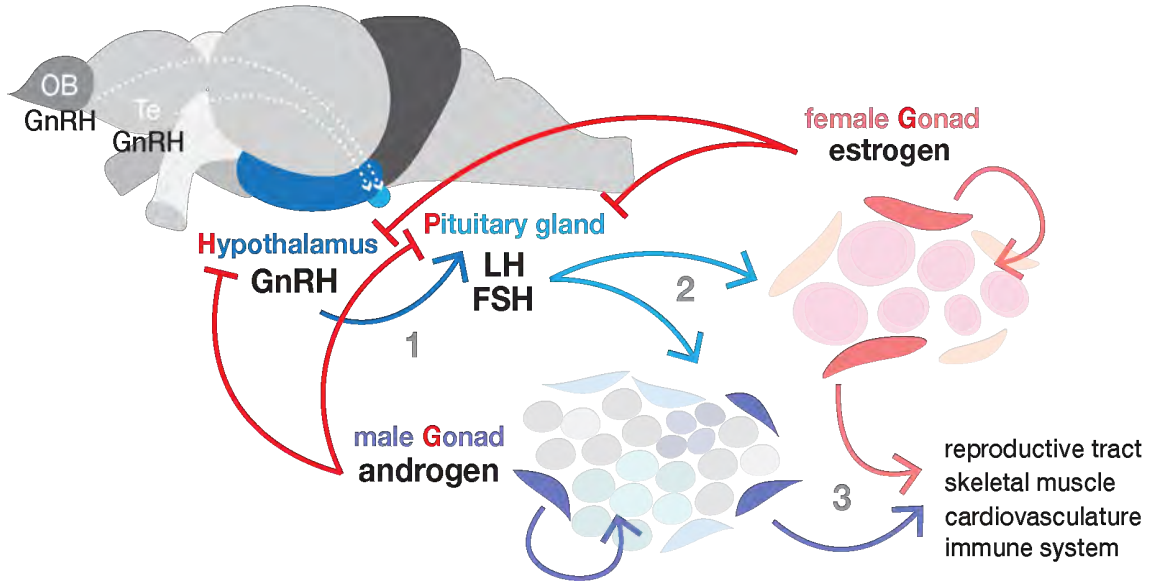


Figure 1.3: **Hypothalamic-pituitary-gonadal axis and main hormonal targets.**

- Brain regions involved in the HPG axis

  - **Hypothalamus**: this brain structure present in all vertebrate animals acts as the link between the endocrine and nervous systems and is essential to keep balance of the chemicals in the body. It aids in the production of hormones that regulate the function of different tissues and organs and maintains homeostasis. In the HPG axis, the hypothalamus plays a crucial role in fertility by modulating promotion and inhibition of gonadal sex hormone secretion (Chakravarthi et al., 2021; Li et al., 2024).
  - **Pituitary gland**: the pituitary gland is a small, conserved structure in the brain of all vertebrate animals. It is considered the *master gland* since it regulates the function of all other glands in the body. Specifically, the adenohypophysis (anterior part of the

pituitary gland) contains endocrine cells that secrete pituitary hormones important for reproduction (Li et al., 2024; Meethal et al., 2009). Neural connections that control the function of the adenohypophysis in teleosts come from the hypophysiotropic nucleus preopticus and the nucleus lateralis tuberis (Fryer & Maler, 1981). Other extra hypothalamic regions also send neuronal projections to the pituitary, mainly from the olfactory system and telencephalon (Anglade & T Zandbergen, 1993).

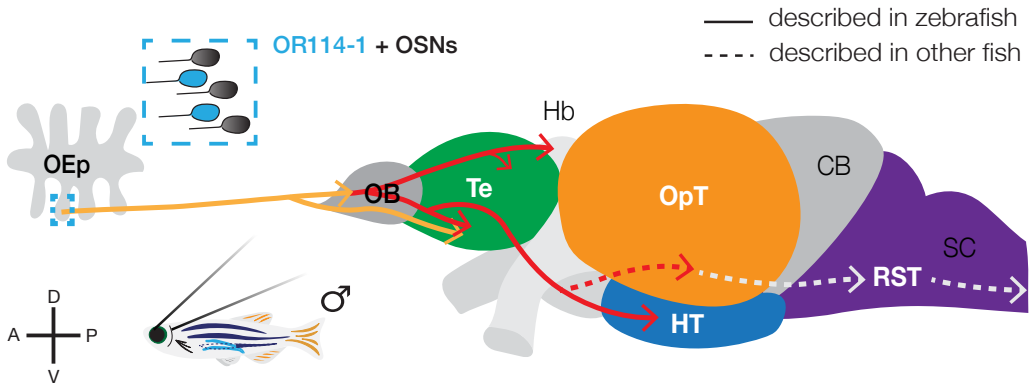
- Principal hormones associated with the HPG axis

  - **GnRH**: peptide secreted by various hypothalamic and extra-hypothalamic nucleus in the brain that stimulates secretion of LH and FSH from the adenohypophysis (Jiang et al., 2012; Marshall et al., 1991; Ulloa-Aguirre et al., 2018). Most vertebrates have two GnRHs, with some species having only one or teleost having up to three (Amano et al., 1997; Somoza et al., 2020). Both the hormone and its receptor, GnRH-R, are also expressed by non-neural tissues, including the gonad (Cheon et al., 2001; Hong et al., 2008; Tang et al., 2023). In the ovary, GnRH/GnRH-R have autocrine and paracrine roles and modulate oocyte maturation and synthesis or steroidogenic hormones by gonadal somatic cells (Metallinou et al., 2007; Silva et al., 2023).
  - **FSH/LH**: their secretion is stimulated by activation of the gonadotropic cells in the anterior pituitary gland by GnRH from the hypothalamus (Ogawa et al., 2021). Low pulse frequencies of GnRH drive synthesis of FSH, high pulse frequencies lead to LH production, and a continuous pulse stops LH/FSH release from the pituitary (reviewed in (Stamatiades & Kaiser, 2018)). Female and male sex hormone secretions by somatic gonadal cells lead to inhibition of FSH through a negative feedback mechanism (Shaw et al., 2010).

Courtship as a well-studied sex-specific behavior

Courtship is one of the animal behaviors with the most extensive research focus, and it remains a favored topic when studying conserved, complex and stereotyped neural pathways. In some animal models, researchers have been able to define the transcriptome, proteome, and connectome of most of the regions, including the molecules and cell types involved. During courtship, females release pheromones that stimulate male specific olfactory receptors to activate the behavioral circuit responsible for male courtship (Kermen et al., 2013; Wright & McCarthy, 2009). These odorant receptors (OR) that are expressed in male olfactory epithelium (OEp) are

essential for responsiveness to female pheromones and male mating behavior (Bowers et al., 2023; Touhara & Vosshall, 2009; Yabuki et al., 2016). In olfactory ciliated sensory neurons (OSN) of male zebrafish, PGF2a secreted by the females activates the specific odor receptor OR114-1 that signals and projects to areas within the olfactory bulb (OB), telencephalon (Te), optic tectum (OpT), and hypothalamus (HT) (Figure 1.4) (Kermen et al., 2013). The resulting attraction and courtship behaviors rely on visual cues as well as olfaction, since fish that cannot smell due to OEp deficits or that lack OR114-1 show impaired courtship and mating behaviors (Yabuki et al., 2016).



CB: cerebellum, Hb: habenula, HT: hypothalamus, OEp: olfactory epithelium, OB: olfactory bulb, OpT: optic tectum, OR: odor receptor, OSN: olfactory sensory neuron, RST: reticulospinal tract, SC: spinal cord, Te: telencephalum

Figure 1.4: **Sex-specific regions in the male brain involved during courtship behavior.** [1]

- Molecules and receptors involved during courtship

  - **Odorant receptors**: also known as olfactory receptors (OR), are present in olfactory sensory neurons and evoke the sense of smell by triggering activation of brain smell pathways in response to an *odorant (Elsaesser et al., 2007)*. In vertebrates, OR are G protein-coupled receptors, which allows for a wide range of activations in response to a combinations of molecule sizes and receptor affinity (Kato & Touhara, 2009). In mammals, OR represent around 3% of the genome, with more than 1,000 different receptors (Axel & Buck, 1991; Lane et al., 2001). They are involved in many biological processes, with activation of behavioral pathways being the most studied.
  - **Prostaglandins**: arachidonic acid derived lipids found in most tissues. Structural differences between prostaglandins together with the specific receptor they bind drive a variety of functions and responses (Ricciotti & FitzGerald, 2011). Most cells produce prostaglandins, and, unlike endocrine hormones, they are not secreted only by specific

[1] Adapted from Kermen et al., 2013.

tissues and regions, but throughout the whole body (Ricciotti & FitzGerald, 2011). Prostaglandins bind G protein-coupled receptors (GPCR), and affect biological processes like inflammation, vasodilation or vasoconstriction, tissue contraction or relaxation, thermoregulation, mating behaviors, and more (Keijzer et al., 2013; Wu et al., 2023). Additionally, prostaglandins can act as sex pheromones in some species, driving reproductive behaviors when secreted into the environment and received by the opposite sex via OR (Bowers et al., 2023; Kimchi et al., 2007; Touhara & Vosshall, 2009; Yabuki et al., 2016).

Sex differences in the vertebrate gonad

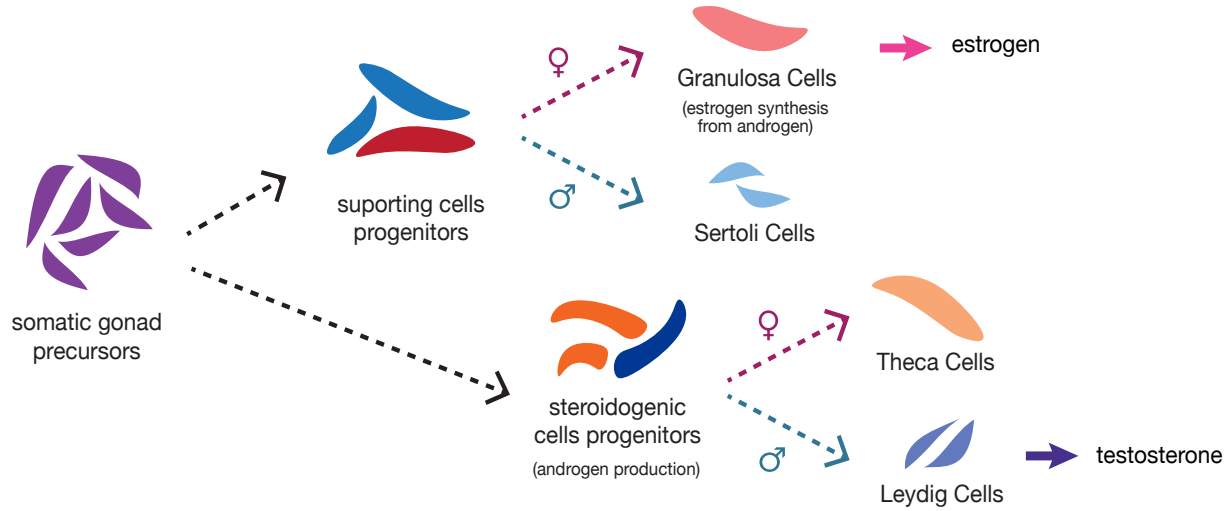


Figure 1.5: **Differentiation of somatic gonadal cell types.**

The gonad of many vertebrate animals, including mammals, initially develops as an indeterminant tissue that is cytologically and molecularly more female-like, and is either maintained in females to form an ovary, or remodeled in males to form a testis (reviewed in (Capel, 2017)). The early gonads consist of sexually indeterminant germline and somatic gonad precursors. The germline gives rise to gametes (eggs or sperm) and the somatic precursors give rise to distinct functional somatic gonadal cell types – support cells and steroidogenic cells (Figure 1.5) (reviewed in (Xie et al., 2022)). Regardless of sex or mechanism of sex determination, early differentiation of the vertebrate gonad involves specification of supporting cell fates (granulosa cells in ovary and Sertoli cells in testis) followed by specification and recruitment of the steroidogenic cells (theca cells in ovary and Leydig cells in testis). In the ovary, theca cells produce androgen that will be aromatized to estrogen in the granulosa cells, whereas in testis, Leydig cells

are the primary source of androgen ((M. A. Estermann et al., 2020; Koopman, 2001) and reviewed in (Xie et al., 2022)).

During the earliest stages and throughout reproductive life, intragonadal signals between germline and somatic gonad guarantee proper function of the reproductive tissue (Figure 1.6). This includes regulation of differentiation, growth, proliferation, and secretion of hormones necessary for proper sexual differentiation of the gonad, for gamete production, and for fertility (Estermann et al., 2020; M. A. Estermann et al., 2020). Regardless of the initial trigger for sex determination, the differentiation pathways converge on conserved factors and mutually antagonistic sex-specific differentiation programs that are activated in the early ovary and testis and maintained throughout reproductive life (Adolfi et al., 2021; Aharon & Marlow, 2021; Capel, 2017; Herpin & Schartl, 2011; Nagahama et al., 2021; Pan et al., 2016; Pan et al., 2021; Yao et al., 2002). Disruption of these processes can cause misalignment of gonad and somatic cell sex, both within the gonad and throughout the body, and differences of sexual development (DSD) (Donohoue, 2020).

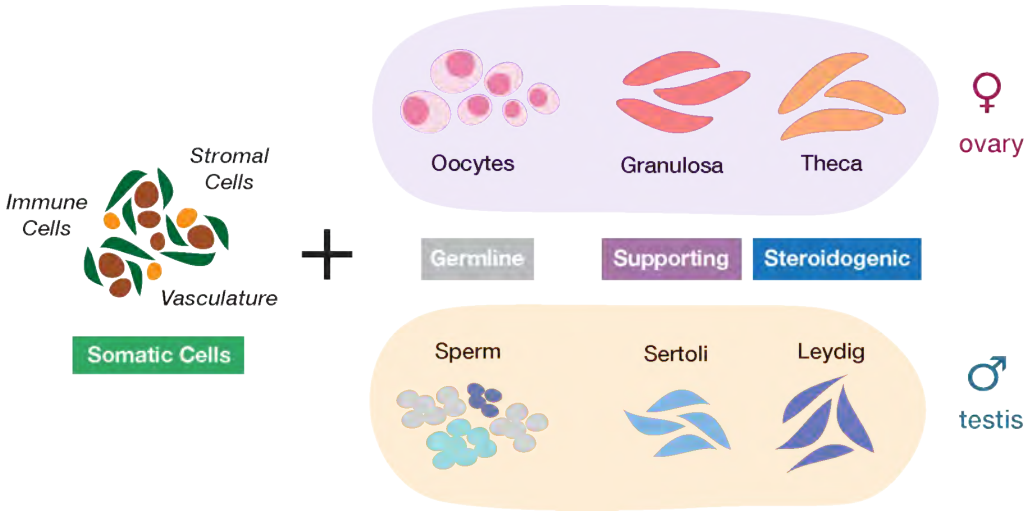
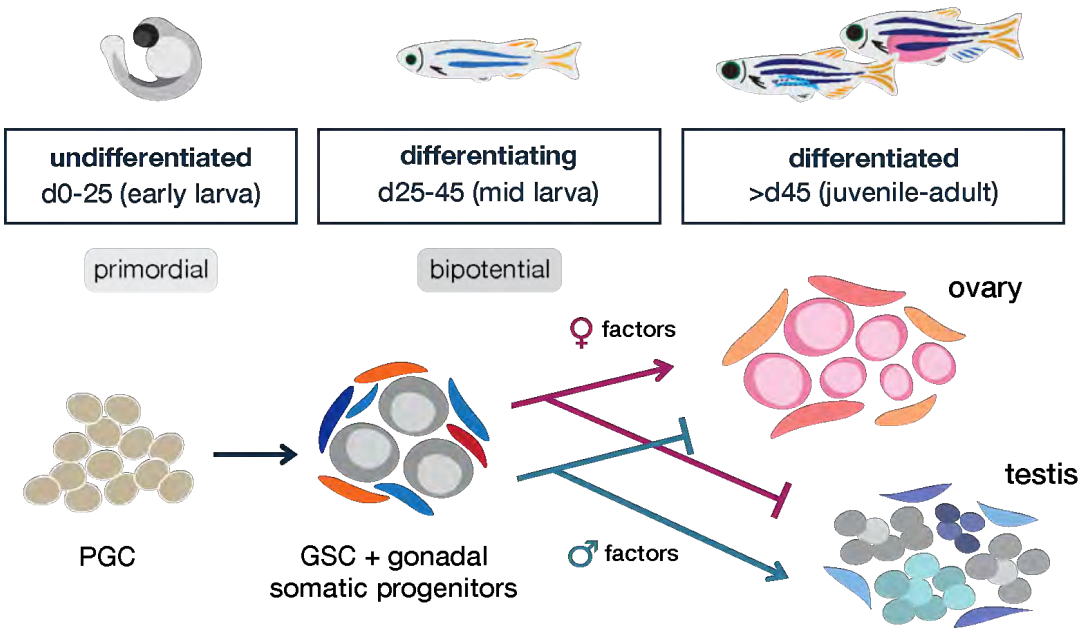


Figure 1.6: **Cells comprising the vertebrate gonad.**

Gonadal sex differentiation and sex reversal in zebrafish

The zebrafish ovary, like ovaries of other vertebrates, is comprised of germline cells and somatic gonadal cells that support differentiation, growth, and maintenance of the germline. In contrast to the XY sex determination system present in most mammals and many other animals, in the wild, zebrafish have a ZW system in which the female is the heterozygote, but this system drifted to a polygenic mechanism in laboratory strains (Anderson et al., 2012; Bradley et al., 2011; King et al.,

2020; Kossack & Draper, 2019; Liew et al., 2012; Nagabhushana & Mishra, 2016). As in mammals, the initial gonads that form are bipotential and can become either ovary or testis, with the nascent gonad being first visible at 5 days post fertilization (dpf) as an elongated primordium (Braat et al., 1999), with the first two populations of early gonadal somatic cells detected by 8-10dpf. In zebrafish, sexual differentiation starts at around 20-25dpf (reviewed in (Aharon & Marlow, 2021)). The molecular trigger initiating sex-specific differentiation in zebrafish remains elusive, but the germline is key to forming a bipotential organ, since gonadal somatic cells differentiate exclusively as testis somatic fates in the absence of germ cells (Campbell et al., 2015; Siegfried & Nusslein-Volhard, 2008; Slanchev et al., 2009; Weidinger et al., 2003). Accordingly, acquisition of testis fate involves low expression of or targeted silencing of factors that promote differentiation into an ovary (Figure 1.7) (Beer & Draper, 2013; Dranow et al., 2016; Dranow et al., 2013; Kossack & Draper, 2019; Kossack et al., 2019; Laing et al., 2018; Li & Liu, 2021; Ortega-Recalde et al., 2019; Rodriguez-Mari & Postlethwait, 2011; Shive et al., 2010; Siegfried & Nusslein-Volhard, 2008).



PGC: primordial germ cells; **GSC:** germline stem cells

Figure 1.7: **Sex differentiation timeline in zebrafish (*Danio rerio*).**

Although the zebrafish ovary is stably maintained in normal conditions, it can be remodeled to form a functional testis when oogenesis is compromised (Anderson et al., 2012; Bradley et al., 2011; Dranow et al., 2016; Dranow et al., 2013; King et al., 2020; Kossack & Draper, 2019; Liew et al., 2012; Nagabhushana & Mishra, 2016). Despite being a major vertebrate developmental model, neither the initial triggers of ovary or testis differentiation nor the ensuing mechanisms that regulate sexual differentiation of the juvenile zebrafish gonad into mature ovary or its

transformation into testis are known (Aharon & Marlow, 2021). Transformation of the juvenile ovary to testis during development and the transition from mature ovary to testis associated with ovarian failure in adult zebrafish both involve elimination of oocytes and ovary tissues and replacement or remodeling of the germline and somatic gonad into testis. Despite these common features, neither the mechanistic basis of ovary to testis transition during development nor transitions resulting from ovarian dysfunction in adults are understood.

- Bmp15 as a conserved regulator of ovarian function

  In humans, *Bone morphogenetic protein 15 (BMP15)* is a key regulator of ovarian development (Di Pasquale et al., 2006; Layman, 2006). BMP15, a conserved ligand produced by early oocytes of vertebrates, is an essential regulator of follicular growth that, in mammals, promotes granulosa cell proliferation (Otsuka et al., 2001; Shimasaki et al., 2004; Zhao et al., 2010). BMP15 secreted by oocytes binds BMPR2 and signals through SMAD1 to activate targets in the somatic gonad. In mouse, ovarian follicles with high BMP15 expression progress while those with low levels undergo atresia (Su et al., 2004). Accordingly, BMP15 has been implicated in the pathophysiology of premature ovarian insufficiency or failure (POI, POF) (Di Pasquale et al., 2006; Rossetti et al., 2009), which can involve a combination of genetic, endocrine, immune, and environmental factors. Variants in the *BMP15* gene are a predominant genetic cause POI, a reproductive disorder caused by genetic and immunity-related disorders that affects oocyte quality and leads to hyperandrogenism and inflammation. POI and POF not only lead to sterility, but more broadly can adversely affect health, including bone, cardiovascular and neurological pathologies (Ferrarini et al., 2021). In zebrafish, Bmp15 is also essential for maintenance of the female germline. *bmp15* mutants initially develop as females but undergo ovarian failure and sex reversal, presumably due to granulosa cell and estrogen deficiencies (Dranow et al., 2016). We showed that loss of oocytes but not follicle progression in *bmp15* mutants is attenuated by eliminating the conserved masculinizing transcription factor, Doublesex and mab-3 related transcription factor 1 (Dmrt1) (Romano et al., 2020). However, the underlying mechanisms and cellular mediators driving ovarian failure and sex reversal are not fully understood.

Tissue resident macrophages

Macrophages are immune cells with essential roles in host defense, clearance of apoptotic or dead cells, injury repair, and tissue development and homeostasis. Tissue resident macrophages (TRM) are specific macrophage populations that colonize body organs and structures and are responsible for its surveillance and maintenance (Lin et al., 2019; Sehgal et al., 2021).

- Origin and tissue colonization

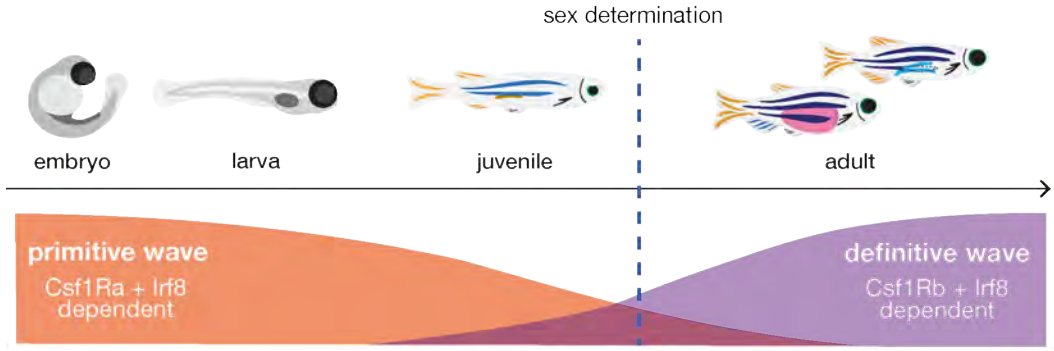


Figure 1.8: **Primitive and definitive waves of TRM colonization**.

In zebrafish, tissue resident macrophages arise in two waves of specification and colonization of somatic organs. The first wave of anterior lateral plate mesoderm (ALPM)-derived macrophages enters organs and brain parenchyma at 48hpf (Figure 1.11) (Herbomel et al., 2001; Rossi et al., 2015; Xu et al., 2015) and a second wave of ventral dorsal aorta (VDA)-derived macrophages contributes to the adult TRM and the microglia pool at 14dpf (Brannstrom et al., 1993). The first wave is dependent on evolutionarily conserved factors, including the transcription factor Irf8 (Li et al., 2011; Shiau et al., 2015), the macrophage receptor Csf1R (Hume et al., 2016), and Csf1a and Il34 ligands (Kuil et al., 2019; Tushinski & Stanley, 1985; Wu et al., 2018). The second wave requires the activities of the duplicated and partially redundant Csf1Ra and Csf1Rb receptors (Hume et al., 2016; Li et al., 2011; Shiau et al., 2015) and was initially thought to be Irf8 independent (Shiau et al., 2015). However, loss of *irf8*, like *csf1rDM* (double mutants lacking both Csf1Rs), causes lifelong loss of both primitive and definitive populations (Figure 1.8) (Ferrero et al., 2020). In zebrafish, Csf1Rs have three ligands encoded by the duplicated *csf1a* and *csf1b* genes and *il-34* (Ferrero et al., 2021; Wang et al., 2008). Overexpression of Csf1a, but not Csf1b or Il-34, promotes proliferation of embryonic macrophages (Hason et al., 2022) and only Csf1a is required for macrophage specification, while Il-34 regulates macrophage and microglia

colonization (Kuil et al., 2019; Wu et al., 2018). After specification, macrophages colonize the different tissues and adopt individual activities dependent on environmental and tissue-derived signals (Bennett & Bennett, 2020; Gosselin et al., 2014; Gosselin et al., 2017; Guilliams et al., 2020; Lavin et al., 2014; Matcovitch-Natan et al., 2016).

- Macrophage colony-stimulating factor (M-CSF) and its receptor (CSF1R)

M-CSF, or CSF1, is a conserved hematopoietic growth factor involved in differentiation of monocytes and macrophages from hematopoietic stem cells into their target tissues or circulation. It is active as a secreted cytokine and forms homodimers to bind its receptor, colony-stimulating factor 1 receptor (CSF1R) (reviewed in (Hu et al., 2021)). CSF1 roles are not limited to development, and macrophage activation through CSF1/CSF1R binding has been shown to modulate processes of immune response, metabolism, and fertility (Jones & Ricardo, 2013). Additionally, CSF1 appears to be implicated in the physiology of many inflammatory diseases (Rajavashisth et al., 1998; Zhang et al., 2012) and cancer (Cannarile et al., 2017; Lin et al., 2019; Nathan et al., 1984), although the mechanisms and specific roles are not well understood yet.

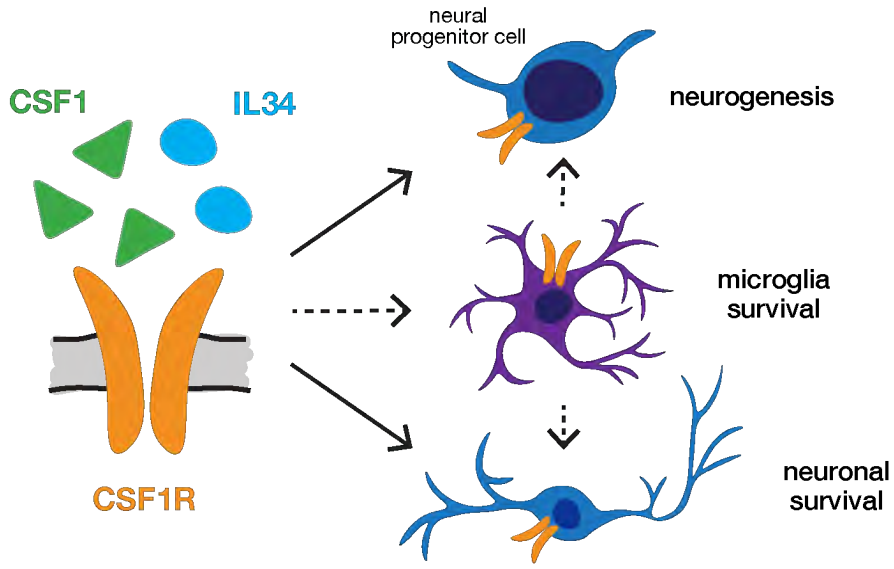


Figure 1.9: CSF1/CSF1R roles in the brain.[2]

CSF1R is a tyrosine kinase transmembrane receptor essential for macrophage and microglia survival (reviewed in (Chitu et al., 2016; Stanley & Chitu, 2014)). In the brain, CSF1R is expressed by microglia and its precursors, neuronal precursor cells (NPC), and some

[2] Adapted from Banglian et al., 2012.

neuronal populations, which makes this receptor a key modulator in processes of neurogenesis and microglia and neuronal survival (Figure 1.9) ((Chitu et al., 2015; Luo et al., 2013; Nandi et al., 2012; Stanley & Chitu, 2014) and reviewed in (Hu et al., 2021)).

- Morphological states and activation

Activation of macrophages occurs through binding of a variety of molecules to specific myeloid receptors. Macrophages are very dynamic and showcase great plasticity in the ways they respond to signals and changes in the environment. This plasticity is in part possible because of the diversity of ligand-receptor pairs, which gives rise to activation of different populations of macrophages with unique characteristics and roles (Mosser et al., 2008). Physiological alterations in the tissue lead to morphological changes and specific responses from macrophages in order to maintain homeostasis, with their principal functions including: immune regulation, defense from pathogens, and healing from injury (reviewed in (Leopold Wager et al., 2014; Mosser et al., 2008)).

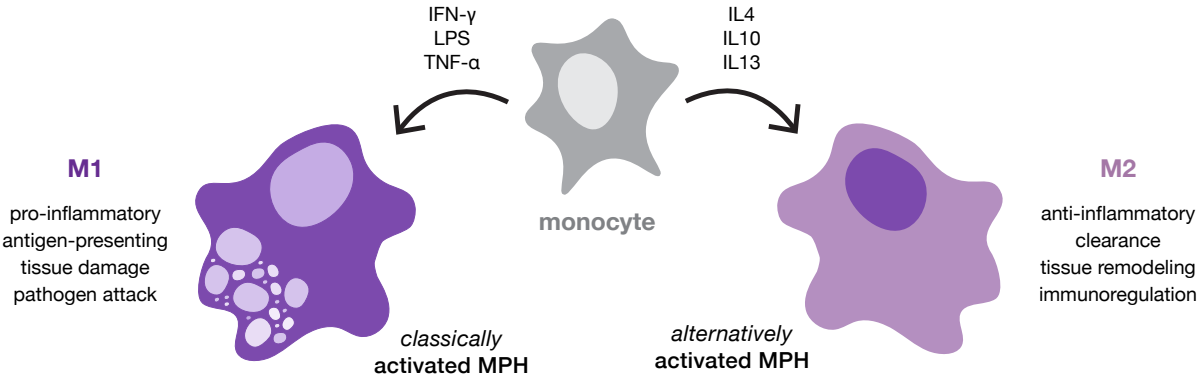


Figure 1.10: **Macrophage activation phenotypes.**

Macrophages have two polarization states (Figure 1.10), known as the *classically activated* (M1) and *alternatively activated* (M2) morphologies. M1 macrophages are involved in the stimulation of the immune system and have inflammatory properties (Leopold Wager et al., 2014). They arise in response to IFN-γ and tumor-necrosis factors produced and secreted by other immune cells like Th lymphocytes, natural killer cells or antigen presenting cells. M2 macrophages have anti-inflammatory features and encourage tissue repair. Their functions include immunity, metabolism, tissue development and repair, and endocrine signaling (Martinez & Gordon, 2014).

Macrophages and gonadal health

Macrophages are phagocytic cells known to target and eliminate foreign substances and dying cells from their host tissues. In addition to their roles in immunity, macrophages are known to contribute to homeostasis of the tissues in which they reside (Guilliams et al., 2020; Watanabe et al., 2019). In the testis, they have been shown to have an essential role during specification of male-gonadal cells and organization of the tissue (DeFalco et al., 2015; Li et al., 2021; Li et al., 2020). Although their function is not fully understood in the mammalian ovary, macrophages are abundant and heterogeneous, showing distinct and ovarian cycle dependent distributions and activation states (Brannstrom et al., 1993; Katabuchi et al., 1997; Nicosia et al., 1992; Petrovska et al., 1996; Varol et al., 2015). In the ovary, inflammation has been increasingly recognized to accompany pathology, such as polycystic ovarian syndrome (PCOS), cancer, and diminished fertility associated with aging. Key to the known and hypothesized contributions of macrophages to ovarian physiology is their ability to both respond to and produce factors that influence the cytokines produced by somatic follicle cells. Apart from their roles during gonadal-tissue development, macrophages may influence both normal physiology and pathogenic states in adulthood, but the mechanisms for their activation and regulation are still not fully understood.

Microglia in neurodevelopment and disease

The development of the brain is largely influenced by gonad function, as nervous tissues are comprised of cell lineages that respond to reproductive organ signals. Notably, the majority of neuroimmune cells are responsive to gonadal and sex hormones (reviewed in (Nelson et al., 2018)). Microglia, as main cells of the neuroimmune system, contribute to normal CNS function and are also associated with pathological states including autoimmune conditions and neurodegeneration, which are often sex-biased disorders (Dou et al., 2024; Forsyth et al., 2024; Geleta et al., 2024). In the brain, microglia are among glial cells of the nervous system and are thought to refine neural connections during development and promote remodeling of neural circuitry, as well as to eliminate synapses (Xavier et al., 2014). Microglia have also emerged as candidates that promote neurogenesis and oligodendrogenesis associated with neurodevelopment (Casano & Peri, 2015; Lenz & Nelson, 2018; Li & Barres, 2018; Lopez-Atalaya et al., 2018; Nelson et al., 2018). Inflammatory events or genetic anomalies during critical periods of brain development cause abnormal activity or numbers of microglia, which could adversely change how the brain governs behavior (reviewed in (Lenz & Nelson, 2018; Li & Barres, 2018;

Salter & Stevens, 2017; Sierra et al., 2010; Villani & Peri, 2019)). Microglia have been associated with neurodegeneration and neurodevelopmental diseases (NDD), particularly those with social and behavioral presentations, including autism, Rett Syndrome, Schizophrenia, and Alzheimer's disease, all neurological disorders that show sex bias (Doorduin et al., 2009; Lenz & Nelson, 2018; Li & Barres, 2018; Morgan et al., 2010; Salter & Stevens, 2017; Steiner et al., 2006; Takano et al., 2010; Tetreault et al., 2012; van Berckel et al., 2008). Additionally, different morphological states of microglia have been suggested to influence brain function and influence or alter disease progression ((Sun et al., 2023) and reviewed in (Gao et al., 2023; Vidal-Itriago et al., 2022)). Still, microglia contributions during the development or remodeling of the brain are not fully known and although sexual dimorphisms in microglia have been documented in other models, both spatially, morphologically, and molecularly with respect to sex hormones (reviewed in (Nelson et al., 2018)), how these differences are established and their potential role in health or disease is still unknown.

- Colonization of the brain

  In vertebrates, microglia arise from the yolk sac and migrate and colonize the brain parenchyma during a very restricted time during embryogenesis, guided by highly regulated molecular processes (Dermitzakis et al., 2023; Kuil et al., 2019; Ranawat & Masai, 2021; Wu et al., 2018). Microglia distribution in the brain from progenitor cells depends on activation of CSF1R, in a mechanism involving the main CSF1R ligands IL34 and CSF1 (Nandi et al., 2012; Stanley & Chitu, 2014; Wu et al., 2018). Once distributed in the brain, microglia stay in a quiescent state and their overall numbers are maintained by self-renewal, mainly by proliferation during cell activation (Lopez-Atalaya et al., 2018; Nandi et al., 2012).

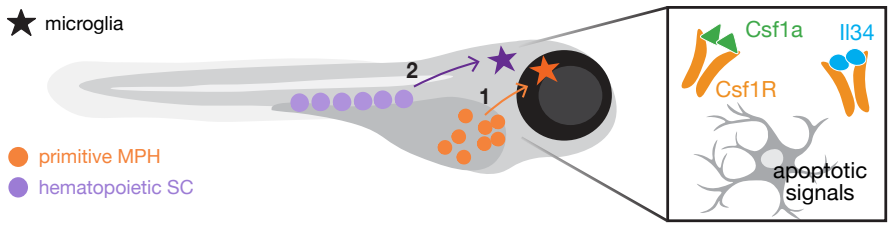


Figure 1.11: **Microglia colonization of the zebrafish brain.**[3]

In zebrafish, microglia start migrating into the cephalic area at around 22hpf, and first colonize the brain retina at 48hpf (Herbomel et al., 2001). Apoptotic signals and activation

[3] Adapted from Ferrero et al., 2018.

of Csf1R by Csf1a and Il34 drive migration of microglia precursors from the yolk sac into the brain (Figure 1.11) (Casano et al., 2016; Kuil et al., 2019; Lou et al., 2022; N. Oosterhof et al., 2018; Wu et al., 2018; Xu et al., 2016).

- Morphological states

Microglia variety of functions and roles in the brain are accomplished by their high plasticity and dynamic morphologies (Figure 1.12). The different phenotypes found in microglia are triggered by environmental changes and the cellular and molecular cues that they receive from their surroundings (Bollinger et al., 2016; Li et al., 2012; Wu et al., 2020).

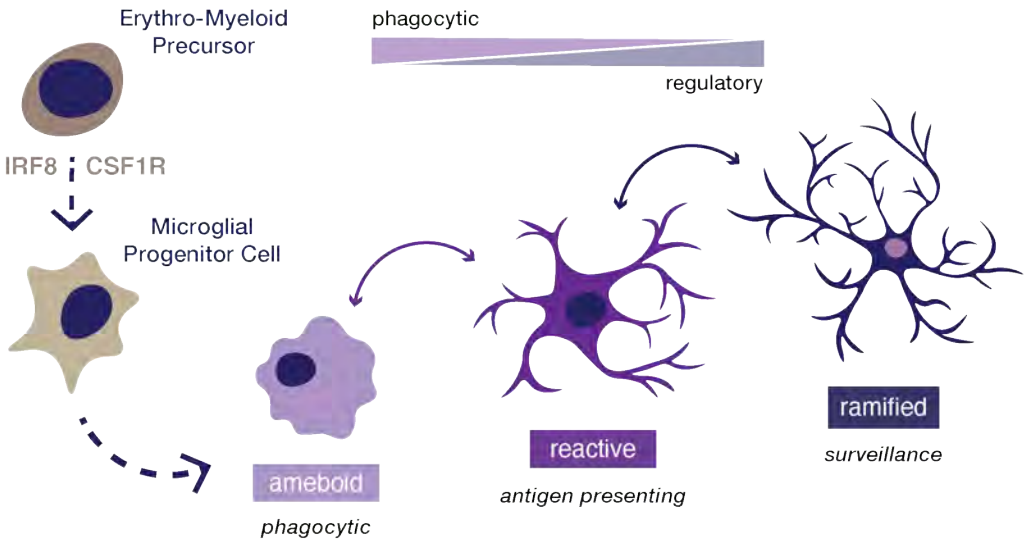


Figure 1.12: **Microglia differentiation and morphological states.**

- **Ameboid**: In this cellular state, microglia show a rounded morphology which allows them to easily move through the tissue eating debris and functioning as phagocytic cells. They do not have an inflammatory role or antigen-presenting capacity in this state, but this morphology is especially important for the clearing of dead cells and during development and remodeling (Silva et al., 2021; Thion et al., 2018).
- **Reactive**: the most immunogenic of all states is the reactive morphology (previously called *activated*), where microglial cells adopt their most phagocytic phenotype and are able to quickly and aggressively evoke an immune response (reviewed in (Bennett & Viaene, 2021)). Mainly by acting as antigen presenter cells and interacting with other glial cells in the brain, like astrocytes, in this morphology, microglia can phagocytose foreign materials and fight infections (Fu et al., 2014).

- **Ramified** (or homeostatic): When microglial cells are in their most relaxed state, they adopt a ramified morphology with branches that allow them to survey and interact with the surrounding cells and molecules in the area (Li et al., 2012; Silva et al., 2021; Svahn et al., 2013; Wu et al., 2020). In this *resting* state, the cell body is reduced to its smallest size and much of the cell surface extends and branches out (Wake et al., 2009). Ramified microglia cells can cover a larger surface area of the tissue by moving their projections around and are able to sense physiological changes to which they are extremely sensitive to (Vidal-Itriago et al., 2022). Although they are not able to phagocytose while in this morphological state, their dynamic capacities allow them to quickly transform into the other phenotypes if needed.

- Microglia and sex differences in the brain

Prominent differences in microglia between females and males have been documented in early development in mammals, beginning with differential colonization of the brain based on estrogen or androgen exposure and extending throughout lifespan (reviewed in (Nelson et al., 2018)). Earlier studies have also identified sexual dimorphism in microglia numbers and regional occupancy between the brains of male and female mice (reviewed in (Condello et al., 2018; C. Y. D. Lee et al., 2018)).

Microglia express prostaglandin receptors (Niraula et al., 2023), produce prostaglandin (Nugent et al., 2009; Wright & McCarthy, 2009; Zhang et al., 2009), and are highly responsive to estrogens (Baker et al., 2004; Loiola et al., 2019; Morale et al., 2006; Perez-Pouchoulen et al., 2019; Wu et al., 2016). The contribution of microglia and prostaglandins to masculinization of the mouse brain has been previously demonstrated by experiments in which a single dose of PGE2 led to dendritic spine density associated with male specific sexual behaviors (Nugent et al., 2009; Wright & McCarthy, 2009). Different studies have also shown that pharmacological inhibition of PGE2 resulted in diminished microglia numbers with a cellular morphology that resembled the patterns observed in females (Lenz et al., 2013), whereas stimulation with PGE2 resulted in females with microglia numbers and cellular morphology similar to males (Minghetti, Polazzi, et al., 1997). Additionally, inhibition of microglia activation in neonatal females exposed to PGE2 prevented establishment of the male pattern and the associated adult male behaviors otherwise observed in exposed females (Minghetti, Polazzi, et al., 1997).

CHAPTER 2

Aims and Hypothesis

A. Investigate if cell death pathways promote ovarian failure and remodeling of the gonad.

Hypothesis: Tumor suppressor factors contribute to sex-reversal by activating cell death pathways in the oocyte during ovarian failure.

B. Examine if sex-reversal and acquisition of male specific traits require activation of macrophages.

Hypothesis: Activation of macrophages by the germline and/or somatic gonadal cells drives remodeling of the ovary.

C. Describe if microglia is necessary for establishment of brain sexual dimorphism during early development.

Hypothesis: Microglia are required for the development of sex-specific differences between the female and male adult brains.

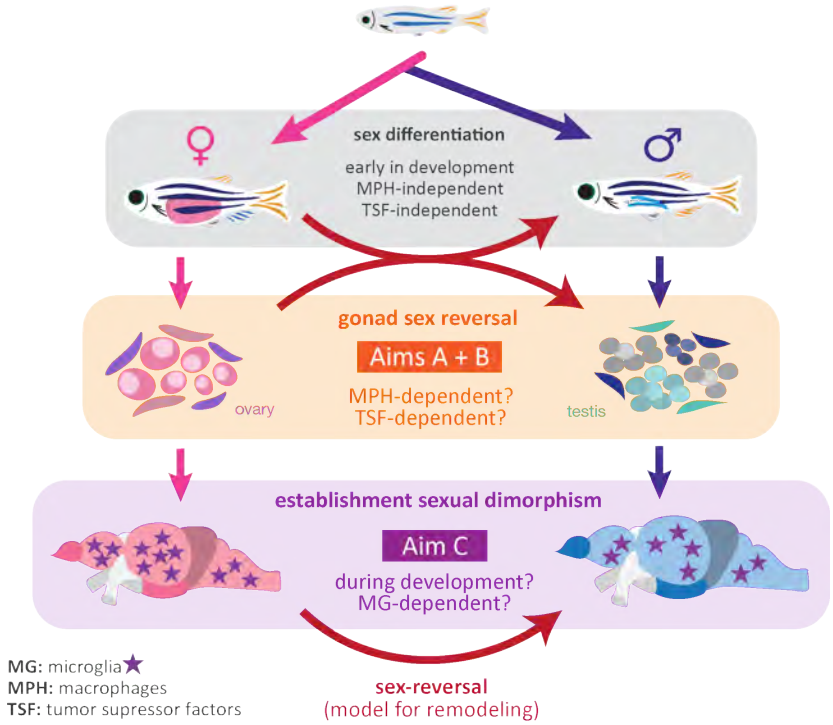
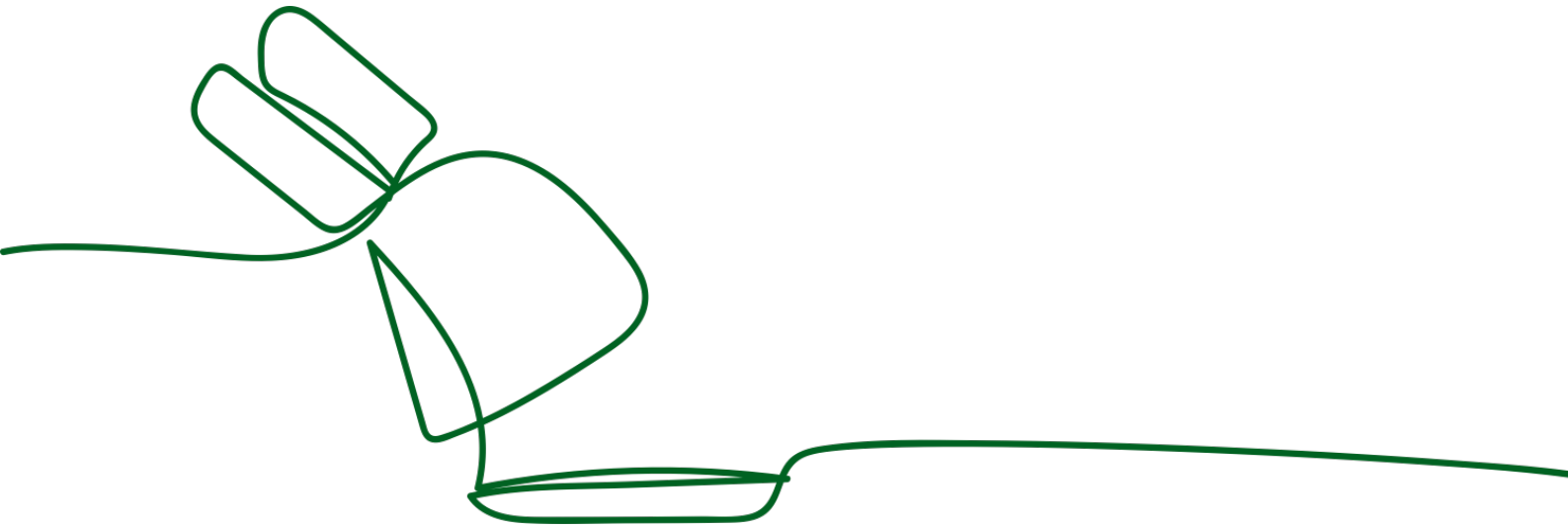


Figure 2.1: **Thesis aims in the context of sex-reversal as a model for tissue remodeling.**



CHAPTER 3

Methodology

Materials

Animals

Mutant zebrafish lines used for this thesis work were: *bmp15*^{uc31}, *chek2*^{sa20350}, *csf1a*^{re05}, *csf1ra*^{j1e4}, *csf1rb*^{re01}, *il34*^{re07}, *irf8*^{st96}, *tp53*^{zdf1}, and *Tg*(*piwil1:eGFP*)^{uc02} (Berghmans et al., 2005; Dranow et al., 2016; Herbomel et al., 2001; Kuil et al., 2020; Kuil et al., 2019; Leu & Draper, 2010; Li et al., 2011; Parichy et al., 2000). Experimental fish referred as "adults" used for whole-mount immunohistochemistry and live tissue images were 3 months old or older. Fish used for scRNAseq libraries and single-molecule whole-mount RNA fluorescence *in situ* hybridization of the ovary were 40 days post fertilization. Juvenile fish used for brain analysis were either 28dpf or 35dpf. Standard conditions were used for maintenance of all zebrafish. All protocols and procedures were performed following guidelines from the National Institutes of Health and approved by the Icahn School of Medicine at Mount Sinai Institutional (ISMMS) Animal Care and Use Committees (IACUC, 2017-0114).

Genotyping

Samples from fin clip, trunk or gonad tissue were lysed in an alkaline lysis buffer (25 mM NaOH and 0.2 mM EDTA pH 12) to obtain genomic DNA (gDNA), heated at 95°C for 20 min, and cooled at 4°C before adding neutralization buffer (20 mM Tris-HCl and 0.1 mM EDTA pH 8.1) (Truett

et al., 2000). gDNA was PCR-amplified, followed when needed by restriction enzyme digestion and then resolved in a 3% 1:1 MetaPhor/agarose gel. All primers and restriction enzymes used for each gene are listed in Table 1 at the end of this chapter. All genotyping assays were confirmed by sequencing.

Technical software

A list of the software used for processing and analysis of data and for the design of figures and illustrations can be found on Table 2 at the end of this chapter.

Imaging

Microscopy images shown in this thesis work were acquired using microscopes in the laboratory of Dr. Florence Marlow or at the Microscopy and Advanced Bioimaging CoRE of the Icahn School of Medicine at Mount Sinai and processed using Fiji/ImageJ or Imaris Viewer. Summarized list of equipment used can be found in Table 3 at the end of this chapter.

Live anatomical tissue images were acquired with a Stemi stereo microscope (ZEISS Microscopy, Germany), while dissected gonads were imaged using a Zoom.V16 microscope (ZEISS Microscopy, Germany). After fluorescence staining, all gonadal samples were imaged with a confocal on a LSM980 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with PMT detectors in confocal mode. Imaging was performed using a water 40x lens (Carl Zeiss Microscopy GmbH, Jena, Germany) and an optical zoom of 1; frame size was set to 1024x1024 pixels or more. Experimental positive controls were used to set the excitation parameters (laser power, detector gain); the parameters were set to provide the best signal to noise ratio for each chromophore, while utilizing the full dynamic range of the detector and avoiding detector saturation and detector gains were kept below 800V to ensure linearity of the response.

Brain images were acquired by using image tiling on a light-sheet UltraMicroscope II (Miltenyi Biotec, Germany) equipped with a Neo 5.5 sCMOS camera (Andor, Ireland). Samples were imaged with Olympus magnification lenses of 4X or 12.6X (1X zoom), using the three light sheet configuration from a single side, with the horizontal focus centered in the middle of the field of view, and a light sheet width of 60–100% (adjusted depending on sample size to ensure even illumination in the y-axis). Spacing of Z slices was 3 or 5 µm. Samples were illuminated with 568nm

and 640nm lasers (Coherent, Germany). The chromatic correction module on the instrument was used to ensure both channels were in focus.

Reagents

All reagents, antibodies, and probes used for experiments for this thesis are listed in Table 4 at the end of this chapter.

Methods: gonads

Dissections and Fixation of the gonads

Fish were anesthetized with a lethal dose of tricaine (MS-22) (400mg/l). Sex was assessed by imaging overall body morphology, tubercles, or lack thereof on pectoral fins, and urogenital papilla morphology.

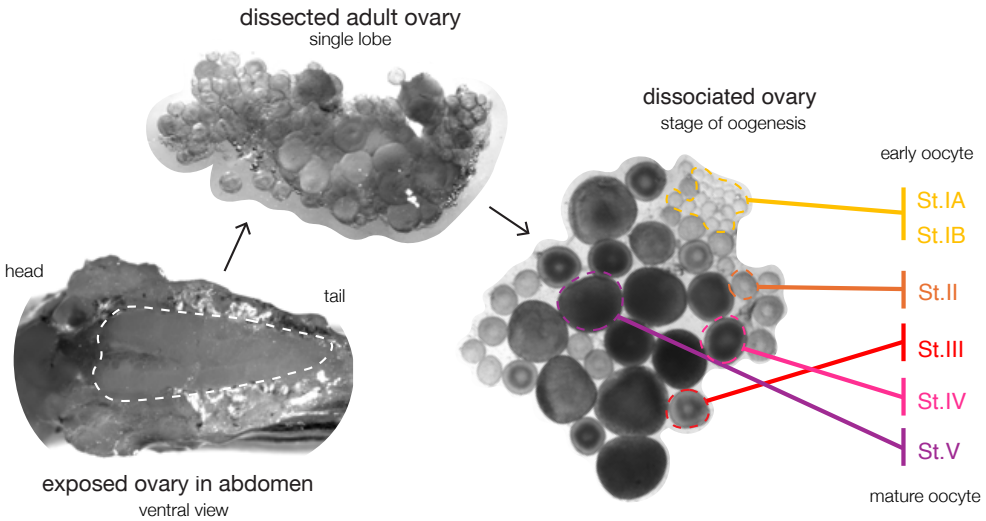


Figure 3.1: **Female gonad dissection and identification of oocyte stages.**

Gonads were dissected prior to or after fixation (Figure 3.1). Briefly, the head was removed with a razorblade, then using forceps, the body was opened along the ventral body wall (anterior to posterior). Gastrointestinal organs and swim bladder were removed to expose the gonad (ovary/testis/indeterminant). Both lobes of the gonad were dissected out of the body cavity

(Figure 3.1). Images were acquired of intact and gently dissociated gonads to confirm the sex of the fish. Dissected gonads were subsequently fixed in 4% paraformaldehyde (PFA) overnight, washed with PBS, and then dehydrated in 100% methanol (MeOH).

Single-molecule whole-mount RNA fluorescence in situ hybridization

After fixation, samples were stored in MeOH at −20°C for at least one night or until use. HCR RNA FISH probes were designed from Molecular Instruments (Table 4). Hybridization was performed following the manufacture's protocol (MI-Protocol-RNA-FISH-Zebrafish) with the following changes: After rehydration in PBS with a series of MeOH>PBS 5 min washes, gonads were dissected and washed 4x 5 min in PBST (PBS + 1% Tween 20). Gonads were permeabilized with proteinase K at 50 µg/ml in PBST for 15 min. After the last manufacture's protocol step, samples were cleared in 1h-incubations of 30, 50, and 70% glycerol/PBS. Lastly, samples were mounted in ProLong Diamond Antifade Mountant with DAPI and imaged with a confocal microscope Zeiss 980 AiryScan2.0 (Table 3) using a 40X water or 63X oil objective, and a 1024x1024 or more pixels format. All pictures were processed using ImageJ/FIJI (Table 2).

Whole-mount immunofluorescence

After fixation, samples were stored in MeOH at −20°C for at least one night or until use. Tissues were rehydrated with several washes of PBS before permeabilization with acetone for 10 minutes at −20°C and then incubated with 1) blocking buffer (5% normal goat serum, and 2% DMSO in 0.1% Tween/PBS) at room temperature for 1 hour or at 4°C overnight, 2) primary antibody overnight at 4°C, 3) washed in PBSTw (0.1% Tween in PBS), 4) incubated in secondary antibody at room temperature for 2 hours or overnight at 4°C, and 5) washed in PBSTw. To label germ cells I used a chicken anti-Ddx4 primary antibody (Blokhina et al., 2019) (Table 4) at a 1:3000 dilution followed by Alexa Fluor 488 or Alexa Fluor 647 secondary antibody (Molecular Probes) diluted 1:500. To label macrophages, I used a rabbit anti-Aif1l or mouse anti-4c4 primary antibody (Rovira et al., 2023; Sasaki et al., 2001) at a 1:200 dilution (Table 4) followed by Alexa Fluor 568 or Alexa Fluor 680 secondary antibody (Molecular Probes) diluted 1:500. Whole mount tissues were mounted on slides using Vectashield with DAPI or ProLong Diamond Antifade Mountant with DAPI (Table 4) and imaged using a Zeiss Axio Observer inverted microscope equipped with Apotome.2 and a charged-coupled device (CCD) camera or a confocal microscope Zeiss LSM980 AiryScan2.0 (Table 3). All pictures were processed using ImageJ/FIJI and Adobe Illustrator (Table 2).

Single-cell RNA sequencing

Single-cell RNA sequencing library expression data were from raw and processed data obtained from (Liu et al., 2022) for the zebrafish ovary, and from (Garcia-Alonso et al., 2022) for the fetal human ovary. UMAPS and graphs were generated using BBrowser3 software and online browsers embedded in the zebrafish (Single Cell Portal) and human (CellxGene) publications, following published analysis parameters (Garcia-Alonso et al., 2022; Liu et al., 2022).

Statistical Analysis

Statistical analysis of sex ratios comparisons of all mutants analyzed were performed by Chi-square test with Bonferroni correction. p-Value comparisons were made between *bmp15* mutant genotypes and *bmp15*$^{+/-}$ genotypes, *P ≤ 0.0125, ***P ≤ 0.0001.

Methods: brains

Dissections and fixation of the brains

Fish were anesthetized with a lethal dose of tricaine (MS-22) (400mg/l). Brains were dissected prior to or after fixation. Briefly, the head was removed with a razorblade and placed upside down, exposing the ventral side of the head. With forceps, soft tissue and jaw were removed until the bones of the skull were visible. Using spring microdissection scissors both optic nerves connecting the eyes to the optic chiasm were severed and the rest of the face bones and eyes were pulled away. Gently, the bones of the skull were removed, starting with in the most anterior area (olfactory bulb) and advancing to the posterior part of the brain until the whole brain was exposed and most brain anatomical structures can be seen (Figure 3.2). Dissected brains were placed in 4% PFA overnight, then washed with PBS, dehydrated in MeOH, and kept at -20°C until use.

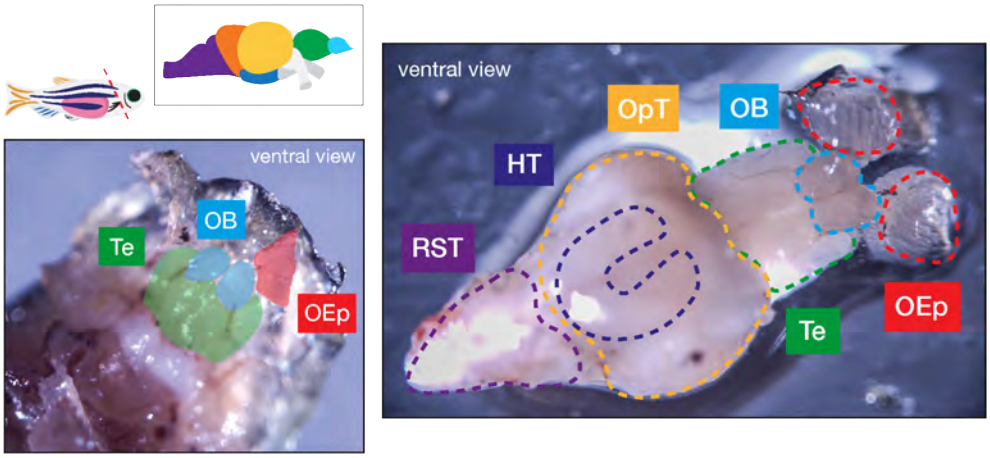


Figure 3.2: **Adult brain during dissection and identification of anatomical areas.**

Whole-mount immunofluorescence, tissue embedding, and clearing

After fixation, samples were stored in MeOH at -20C overnight or until use. Whole tissue staining, embedding, and clearing was performed as described in detail in (Lindsey et al., 2018). All immunofluorescence steps were done in the dark, at 4°C, in a 12-well plate where individual samples were placed in mesh baskets and transferred from one well to the next following the following steps: 1) rehydration with 0.3% PBSTx (0.3% Triton X-100 in PBS), 2) permeabilization with 5% DMSO in 1% PBSTx, 3) incubation overnight in blocking buffer (2% normal goat serum/1% bovine serum albumin in 0.3% PBSTw), 4) primary antibody incubation in blocking buffer for 7 days, 5) wash with 0.3% PBSTx, 8) secondary antibody incubation in 0.3% PBSTx for 7 days, 9) nucleic acid stain (Sytox 1:2000 in 0.3% PBSTx) overnight, and 10) wash in 0.3% PBSTx. Following staining, brains were washed in milliQ water and embedded into 1% low-melting agarose as described in (Lindsey et al., 2018). Briefly, each brain was placed in the center of a well in a 6-well plate filled with filtered 1% low-melting agarose and left to cool down at 4° C for at least an hour before trimming excess. Clearing of the embedded tissues was performed at room temperature in a fume hood and consisted in several continuous incubations in 100% MeOH, followed by incubations in BABB (benzyl alcohol/benzyl benzoate) (Sigma Aldrich). Images of cleared, embedded tissues were acquired using a LaVision Ultramicroscope II light sheet microscope and analyzed/processed using Imaris Viewer (Bitplane).

Brain sex-specific regions analysis

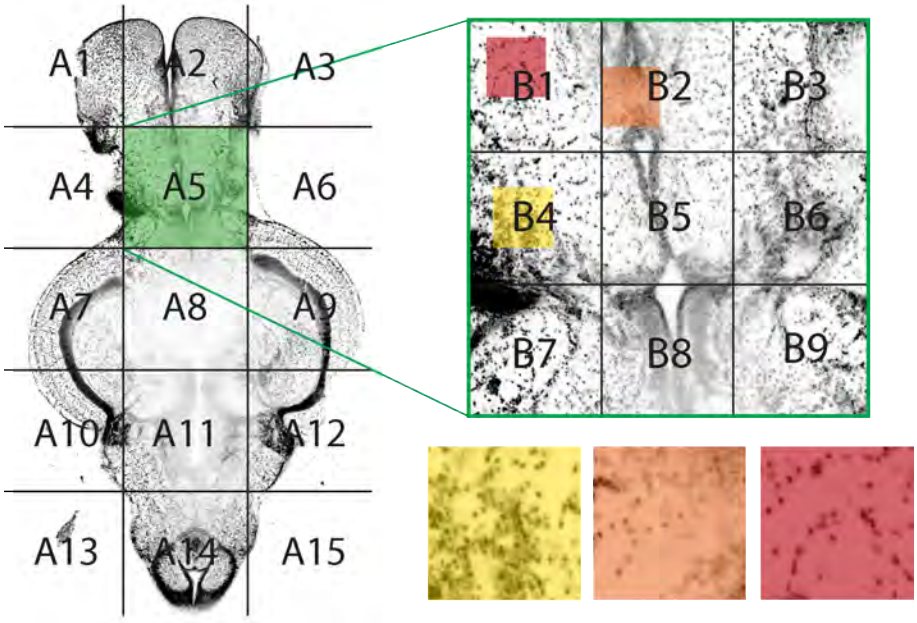


Figure 3.3: **Cell counting strategy.**

Anatomical measurements of the different regions of the brain were done in Imaris Viewer (BitPlane) using the section view with normal and extended crosshair. Analysis of microglia densities was done by manually counting Sytox-positive cells and 4c4-positive cells in 200x200 pixel size squares from 3 difference areas in a 3x3 grid placed over a 5-slices MAX projection (Figure 3.3).

Statistical Analysis

Statistical analysis of adult brain anatomical measurement comparisons was performed using ordinary TWO-way ANOVA with multiple comparisons (uncorrected Fisher LSD) and 95% CI. P-values: ns ≥ 0.05, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. Statistical analysis of microglia cell density comparisons was done by unpaired t-test with Welch's correction and 95% CI. P-values: ns ≥ 0.05, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. Analysis and statistical graphical representations were done in Prism (GraphPad Software, Inc.).

Table 1: **Genotyping assays**

Gene	Forward primer	Reverse primer	Restriction enzyme (cut)
bmp15	AGCCTTTCAGGTGGCACTCG	CCACTGAAAAACACTTTCTCCC	-
chek2	AGCCACACGAAATGCTGAG	CAGACTGAAGACTCCTACTACATTG	HpyCH4III (mut)
csf1a	GCCGGTTGAGCTTCTGAAAAT	GCATTTTGGTTAGGCTGCTG	-
csf1ra	TCTGGGCAAAGAGGACAACATCACAC	CCACAGCTCTGCAAGGTTTG	SpeI (wt)
csf1rb	GGACAGAGTTTTCGCTCCAG	ATTGGACTCCGCTCATGTTC	MspI (wt)
il34	TGGTCTTCGTGATTCCCTTC	TGCTCCTCATTCCTTCAACC	-
irf8	ACATAAGGCGTAGAGATTGGACG	GAAACATAGTGCGGTCCTCATCC	AvaI (wt)
tp53	ACATGAAATTGCCAGAGTATGTGTC	TCGGATAGCCTAGTGCGAGC	-

Table 2: **Software for data processing**

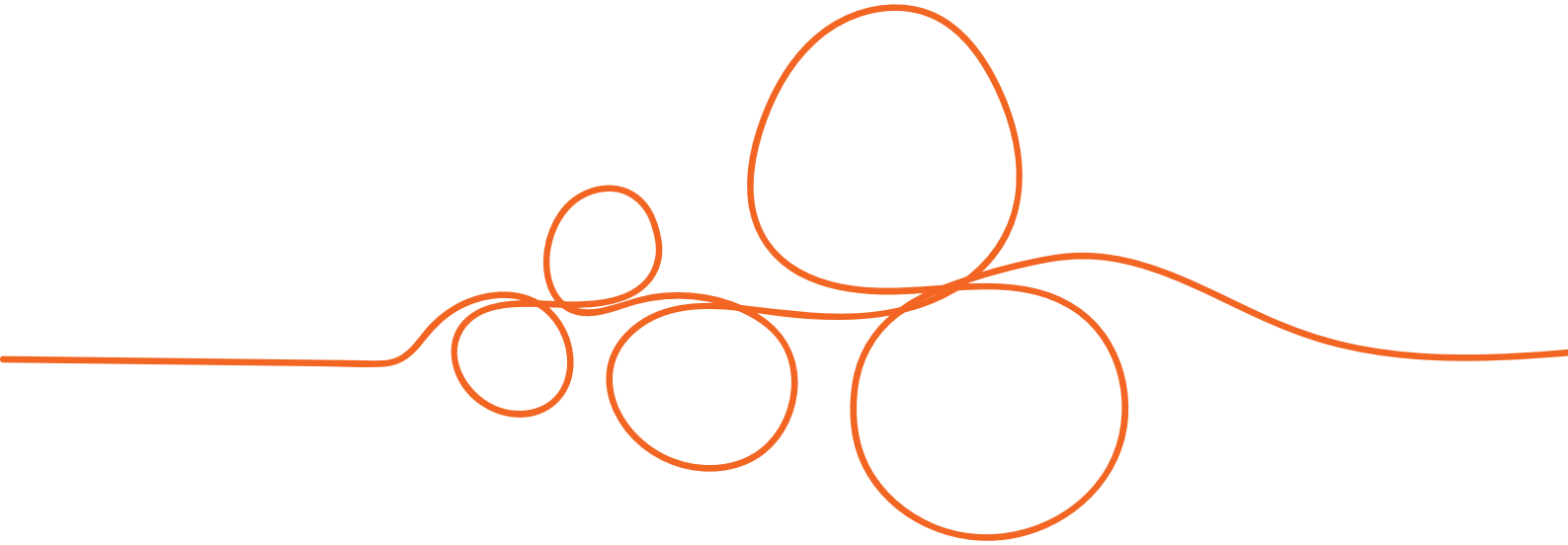
Name	Use	Manufacter
Prism	scientific 2D graphing and statistics	GraphPad Software, Inc.
BBrowser3	single-cell RNA sequencing data visualization	BioTuring, Inc.
MacVector	genetic sequence analysis	MacVector, Inc.
Illustrator	design and editing of figures and illustrations	Adobe
Imaris Viewer	image processing and analysis	Bitplane (Oxford Instruments)
ImageJ/FIJI	image processing and analysis	Wayne Rasband (NIH)
ZEN blue	ZEISS microscopes acquisition software	ZEISS Microscopy, Germany
ImSpectorPro	light sheet microscope acquisition software	Miltenyi Biotec, Germany

Table 3: **Microscopes and equipment**

Microscope	Type	Use
Axio Observer (ZEISS Microscopy, Germany)	widefield	gonads IHC imaging
Zoom.V16 (ZEISS Microscopy, Germany)	widefield	live tissue imaging
Stemi stereo (ZEISS Microscopy, Germany)	widefield	dissections/tissue analysis
LSM 980 AiryScan2.0 (ZEISS Microscopy, Germany)	confocal	fluorescence imaging
LaVision Ultramicroscope II (Miltenyi Biotec, Germany)	light sheet	brains IHC imaging

Table 4: **Reagents, antibodies, and probes**

Name	Type (use)	Concentration	Source (catalog #)
Chicken anti-Ddx4	primary antibody (IHC)	1:3000	PMID: 30653507
Rabbit anti-Aif1l (Iba1)	primary antibody (IHC)	1:200	FUJIFILM Wako Chemicals
Mouse anti-Y14 (4c4)	primary antibody (IHC)	1:200	EMD Millipore Corp (05-1511)
DAPI	DNA dye (IHC/FISH)	1 µg/ml	Invitrogen (62248)
Sytox™ Deep Red	nuclei acid stain (IHC)	0.5 µM	Invitrogen (S11381)
Neutral Red	lysosome stain	4.3g/L	Sigma Aldrich (N2889)
csf1a	probe (FISH)	8nM	Molecular Instruments (custom)
il34	probe (FISH)	8nM	
lhx9	probe (FISH)	8nM	
ProLong Diamond Antifade	mountant (IHC/FISH)	NA	Invitrogen (P36961)
Vectashield® Antifade	mountant (IHC/FISH)	NA	Vector Laboratories (H-1900)
BABB (Benzyl Alcohol/Benzyl Benzoate)	tissue clearing (IHC)	1:2	Sigma Aldrich (W213713/W213810)



CHAPTER 4

Results[4]

Results for Aims A and B

Bmp15 functions in follicle progression, ovary maintenance, and fertility

Although Bmp15 was known to be essential for ovary maintenance, this could be due to failure to promote Bmp15 dependent somatic cell fates, deficits of paracrine or autocrine factors, or a combination of these functions. To determine if Bmp15 promotes follicle survival, we generated double mutants (DM) lacking both Bmp15 and tumor suppressor factors (TSF) Tumor protein 53 (Tp53) or Checkpoint kinase 2 (Chek2), which can suppress cell death and oocyte loss in some infertile zebrafish and mouse mutants (Bolcun-Filas et al., 2014; Rodriguez-Mari & Postlethwait, 2011; Shive et al., 2010). We found that, as in wild type, *bmp15* heterozygotes and *chek2* or *tp53* single mutants showed no sex bias by 85 days post fertilization (dpf), but all *bmp15* homozygous mutants were fertile males (Fig. 4.1, A and C). Similarly, *bmp15* mutants that were also heterozygous for *tp53* or *chek2* and *bmp15;tp53DMs* (n=8) were all male (Fig. 4.1, B and C). In contrast, 2 of 9 *bmp15;chek2DM*s retained oocytes through stage II (previtellogenic) and showed female secondary sex traits, including body coloration, fin morphology, and urogenital papilla structures at 95dpf (Fig. 4.1, B and C). Notably, using previously reported single-cell RNA

[4] Excerpts of this chapter are published in Bravo, P., Liu, Y., Draper, B. W., & Marlow, F. L. (2023). Macrophage activation drives ovarian failure and masculinization in zebrafish. *Sci Adv*, *9*(47), eadg7488. https://doi.org/10.1126/sciadv.adg7488 .

sequencing (scRNA-seq) data (Liu et al., 2022), we found that *chek2* is expressed predominantly in oocytes (Fig. 4.1, D-F). Therefore, we conclude that sex reversal of *bmp15* mutants, including primary and secondary sex traits, can be suppressed by preventing Chek2-mediated cell death of oocytes. However, suppression was only partially penetrant and mutant follicles did not progress developmentally even when oocyte loss and ovary to testis sex reversal were blocked. This result indicates that Bmp15 signaling functions extend beyond survival and include regulating follicle progression. Therefore, Bmp15 has a conserved function in signaling from the oocyte to promote growth, survival, and progression of ovarian follicles, which prevents ovarian failure in zebrafish.

Macrophages and ovary to testis transition

Since preventing oocyte loss only partially suppressed sex reversal, we investigated potential involvement of somatic gonad populations in sex reversal/oocyte maintenance. Because immunity-related disorders are implicated in ovarian failure ovarian failure (Calan et al., 2016; Foley et al., 2021), we investigated the role of macrophages, which are key innate immune cells that eliminate foreign or dying cells from tissues. They regulate cell removal, produce cytokines and factors that recruit and regulate other immune cells, express growth factors to control proliferation and vascularization, and deposit extracellular matrix during wound healing and repair thus contribute to tissue homeostasis (Gordon & Pluddemann, 2017; Watanabe et al., 2019; Wynn & Vannella, 2016). In mammalian ovary, macrophages are abundant and heterogeneous, showing distinct and ovarian cycle dependent distributions and activation states (Katabuchi et al., 1997; Petrovska et al., 1996). Further, they are hypothesized to degrade the follicle by promoting granulosa cell apoptosis (Fukumatsu et al., 1992; Kasuya, 1995, 1997; Kasuya & Kawabuchi, 1998). scRNA-seq of 40dpf ovary confirmed the presence of macrophages based on RNA expression of the zebrafish *colony stimulating factor 1 receptors, csf1ra/b,* and *interferon transcription factor 8* (*irf8)*, a conserved regulator of macrophage differentiation (Fig. 4.2, A-E) (Liu et al., 2022). Analyses of gene expression correlations among these genes and markers of other immune cell types, e.g., natural killer-like cells (NK), confirm that *csf1ra/*b and *irf8* are expressed by macrophages (Fig. 4.2, D-F).

To determine if loss of Bmp15 was associated with an increase in macrophages, we examined the inflammatory macrophage marker *aif1l* (*allograft inflammatory factor 1-like;* formerly called *iba1*) in 40dpf wild-type and *bmp15* mutant ovaries (Imai et al., 1996; Sasaki et al., 2001). Aif1l positive cells included somatic cells and early oocytes in 40dpf wild type (Fig. 4.2, G). Aif1l had a similar distribution in 40dpf *bmp15* mutant ovaries (Fig. 4.2, H). Importantly, 40dpf

juvenile ovaries were chosen because this stage aligns with the scRNA-seq dataset and is a stage before ovary to testis reversal occurs in *bmp15* mutants. Further, 40dpf ovaries with stage II oocytes were selected as juvenile ovaries lacking stage II oocytes have the potential to undergo a late transformation to a testis, and thus may not be future females. To determine if suppression of oocyte loss in *chek2* mutants was due to loss of macrophages, we examined Aif1l, the macrophage marker 4c4 (Rovira et al., 2023) and Ddx4, to mark the germline and confirm that the somatic cells were macrophages, in adult wild-type and *chek2* mutant ovaries (Fig. 4.2, I and J, and Fig. 4.3, A and B). That *chek2* is expressed in oocytes and that macrophages were present in wild-type and *chek2* mutant ovaries suggests that Chek2 loss likely suppresses sex reversal of *bmp15;chek2DMs* by acting in oocytes to prevent their death rather than regulating macrophages. However, based on this analysis alone a potential role for activating or mobilizing macrophages in the absence of Bmp15 cannot be excluded.

To investigate if macrophages contribute to follicle atresia and sex reversal, we genetically ablated macrophages in *bmp15* mutants using two genetic manipulations. In zebrafish, macrophages arise in two waves of specification and colonization. Loss of Colony stimulating factor 1 receptor a (Csf1ra) ablates primitive macrophages, which arise from the anterior lateral plate mesoderm in the early embryo ~16 hours post fertilization. By contrast, *csf1 receptor b (csf1rb)* mutation affects the definitive population that originates from the ventral dorsal aorta at 14dpf (Herbomel et al., 2001; Xu et al., 2015). Despite distinct contributions to the primitive and definitive waves, both *csf1ra* and *csf1rb* single mutants have macrophage deficits as adults and DM fish lacking both receptors (*csf1r*^{DM}) show a lifelong absence of macrophages without affecting sex ratios (Fig. 4.3, C) (Kuil et al., 2020; Oosterhof et al., 2019; N. Oosterhof et al., 2018). Similarly, mutation of *irf8* ablates macrophages without disrupting normal sexual differentiation (Fig. 4.3, C) (Li et al., 2011; Shiau et al., 2015). To study macrophage contributions to follicle atresia and sex reversal, we examined *bmp15* mutants lacking all macrophages (MΦ^{-}): *csf1r*^{DM} or *irf8* mutants (Fig. 4.4, A). Analysis of adult gonads revealed that *bmp15* mutants with macrophages were all fertile males while mutant females lacking macrophages retained ovaries but were sterile. This is because *bmp15* mutant oocytes arrest development prior to vitellogenic stages of oogenesis and eliminating macrophages does not restore deficits in Bmp15-dependent somatic gonadal cell populations or related signaling between germline and soma. Instead, macrophage likely eliminate ovarian tissue and promote or facilitate gonad remodeling and development of testis tissue during ovarian failure. Consequently, suppression of sex reversal is incomplete, and mutant females remain infertile (Fig. 4.4, A and E).

Primitive macrophages are present in the early embryo prior to sex determination, are dependent on Csf1Ra, and are normally still present when ovarian failure occurs in *bmp15* mutants, whereas definitive macrophage populations, which are present during sex determination and through adulthood are dependent on Csf1Rb (Ferrero et al., 2020; Kuil et al., 2020; Oosterhof et al., 2019; N. Oosterhof et al., 2018). Taking advantage of the duplicated Csf1Rs, which are differentially required for primitive and definitive macrophage populations (Fig. 4.3, D), allowed us to assess the contribution of each macrophage population and to effectively deplete or remove macrophages at the onset of ovarian failure in *bmp15* mutants. Thus, we examined mutant fish deficient for primitive and/or definitive macrophages ($M\Phi^{haploinsufficiency}$), *e.g.,* lacking one *csf1* receptor and heterozygous for the other or for *irf8* (Fig. 4.4, B-D, and Fig. 4.5). We found that *csf1ra* mutants heterozygous for *csf1rb*, which eliminates primitive and most definitive macrophages, prevented ovarian failure and sex reversal of *bmp15* mutants (Fig. 4.5, A). Interestingly, double heterozygosity for both *csf1* receptors ($csf1r^{DH}$) or for *irf8* also prevented oocyte loss and sex reversal (Fig. 4.4, B and C). These observations indicate a threshold number or specific macrophage population mediates sex reversal.

To determine if ovarian failure and sex reversal requires a threshold number or a unique subtype of macrophages, we analyzed compound mutants for various combinations of *csf1ra/b* and found that *bmp15* mutants lacking only *csf1ra* were all male as adults (Fig. 4.4, E, and Fig. 4.5, A). Thus, ovarian failure and sex reversal occur in the absence of primitive macrophages. In contrast, loss or haploinsufficiency of definitive macrophages (Fig. 4.5, A and B) suppressed ovarian failure and sex reversal. In addition to macrophages, *irf8* was also expressed in stromal cells and some pre-follicle cells (Fig. 4.6, A, C-E). Nonetheless, we conclude that macrophages are direct cellular mediators of sex reversal in the absence of Bmp15, based on the genetic evidence that loss of *csf1ra/b,* which are highly expressed in macrophages, or loss of *irf8* both block sex reversal. Furthermore, sex reversal requires an activity unique to a specific state or subpopulation of definitive macrophages.

Csf1 and Il34 ligand involvement in masculinization of the gonad

Csf1 receptors on macrophages are activated by Csf1 ligands and Il-34 (Kuil et al., 2019; Wu et al., 2018); therefore, the cells that express these ligands represent candidate triggers of sex reversal. Thus, we determined which cells in the ovary express RNAs coding for Csf1R activating ligands. Analysis of reclustered 40dpf gonadal cell populations, including pre-follicle cell populations, recently defined by scRNA-seq based on their expression of pre-follicle cell genes

including *lim homeobox 9 (lhx9), iroquois3a (irx3a),* and *iroquois5a (irx5a)* (Liu et al., 2022) revealed a unique group of ovarian pre-follicle cells that express *irf8* and *csf1a* ligands (Fig. 4.6, B-E). Among known *csf1r* ligands, reclustered follicle and pre-follicle populations from 40dpf scRNA-seq data indicated that *csf1a* RNA is enriched in pre-follicle cells, *interleukin 34 (il34)* was detected in a distinct population of pre-follicle cells, and *csf1b* was not appreciably expressed (Fig. 4.6, A). Based on its limited expression, we reasoned that *csf1b* was not likely the relevant ligand. Using fluorescence *in situ* hybridization to verify expression profiles and spatial distribution of *lhx9, csf1a,* and *il34* in 40dpf wild-type pre-follicle cells we found that, as indicated by the scRNA-seq data, *csf1a* is expressed in *lhx9*-expressing pre-follicle cells and in *lhx9*-negative cells that are likely stromal cells (Fig. 4.6, F-H, and Fig. 4.7, A, C, and E). In contrast, although detected in distinct follicle cell populations by single cell analysis, *il34* was not detectable in wild-type gonads at this stage (Fig. 4.7, B-G). Based on the single cell data, which indicates *il34* is most highly expressed in immune cells (Fig. 4.6, A), and the correlation data showing that *il34* is only expressed in relatively few follicle cells (Fig. 4.7, D), *il34* expressing cells likely become more prominent in the gonad as it differentiates. Analysis of recently published scRNA-seq data of human embryonic ovary (Garcia-Alonso et al., 2022) indicates the presence of *lhx9, csf1a,* and *irf8* expressing populations in the human early ovary (Fig. 4.8).

To determine if loss of *bmp15* influences the expression of Csf1Rb ligands, we examined *il34* and *csf1a* in *bmp15* mutant ovaries. As in wild type (Fig. 4.6, F, and Fig. 4.7, E), *csf1a* was expressed in distinct subsets of pre-follicle cells, but appeared to be more abundant in *bmp15* mutants (Fig. 4.7, E and F). However, unlike wild-type juvenile ovaries in which *il34* was undetectable, *il34* was highly expressed in the germline and somatic cells of *bmp15* mutants (Fig. 4.7, E and F). Abundant *il34* in *bmp15* mutant oocytes suggests Il34 from oocytes could be a trigger of oocyte loss. Although previously thought to signal primarily through Csf1Ra, which is dispensable for ovarian failure and sex reversal on its own, Il34 was recently shown to also signal through Csf1Rb (Hason et al., 2022). Because Csf1a and Il34 ligands can signal through Csf1Rb and that their transcripts were detected in different populations of pre-follicle cells and were elevated in *bmp15* mutants (Fig. 4.7), it was possible that either ligand could contribute to macrophage activation and ovary to testis transition. To determine whether one or both Csf1Rb ligands were required for ovarian atresia and sex reversal, we examined double mutants lacking *bmp15* and *csf1a* (*bmp15*;*csf1a DM*s) and double mutants lacking *bmp15* and *il34* (*bmp15*;*il34 DM*s). We found that removing Csf1a was sufficient for sustained suppression of ovarian failure and sex reversal of *bmp15* mutants, indicating that Csf1a drives ovary to testis transformation (Fig. 4.9, A and B). Consistent with a role for Il34 in promoting oocyte loss, removal of Il34 suppressed ovarian failure of *bmp15*

mutants, but unlike loss of Csf1a, eliminating Il34 only delayed the ovary to testis transition (Fig. 4.9, A and B). As expected, macrophages marked by Aif1l were still present in *csf1a* single mutants, *il34* single mutants, *bmp15;csf1aDMs,* and *bmp15;il34DMs* (Fig. 4.9, C-F). Our findings identify a previously unknown role for the subpopulation of pre-follicle cells, hereafter called Macrophage-Activating Follicle Cells (MAFCs) based on their expression of *csf1a*, a known ligand for Csf1Rb that is essential for ovary to testis transformation. Further, MAFCs are in direct contact with ovarian follicles, and thus are positioned to sense oocyte cues and release Csf1a to activate ovarian macrophages.

Figure 4.1

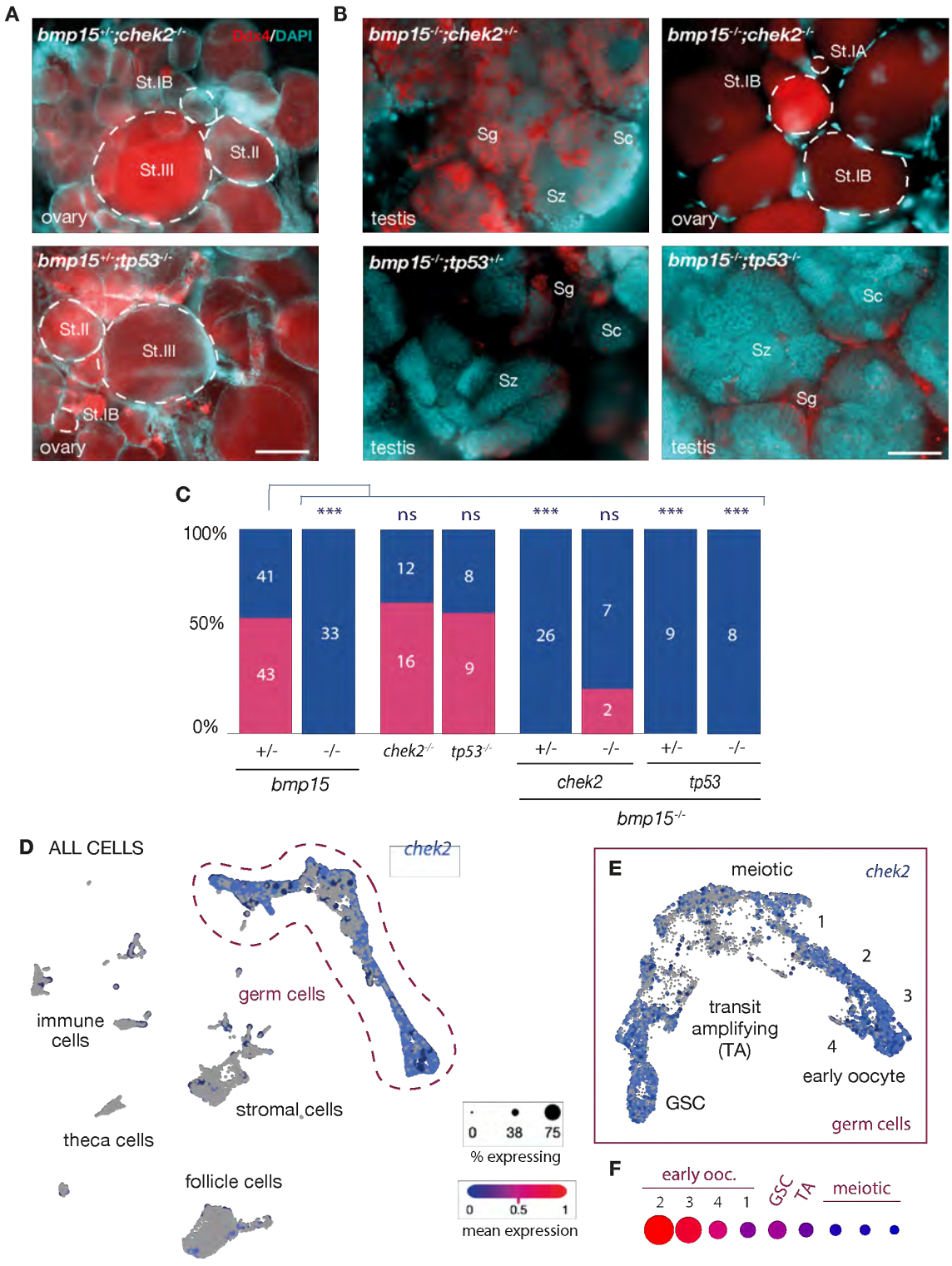


Fig. 4.1: **Loss of Chek2 suppresses oocyte death and sex reversal in the absence of Bmp15.**

(**A** and **B**) Immunostained adult *bmp15;chek2* gonads of indicated genotypes. Ddx4 (red) labels germ cells, and 4',6-diamidino-2-phenylindole (DAPI; cyan) labels DNA. Scale bar, (A)

250 µm, (B) 50 µm. Sg, spermatogonia; Sc, spermatocyte; Sz, spermatozoa; St.IA, stage IA oocyte (prophase I meiotic cells in nests), St.IB, stage IB oocyte (late prophase within definitive follicles); St.II, stage II oocyte (apparent cortical alveoli and a vitelline envelope); St. III*: arrested stage III oocyte (increased size and apparent yolk).

(**C**) Adult sex ratios of indicated genotypes. Female, pink; male, blue. Numbers indicate individuals examined. Statistical analysis: chi-square test with Bonferroni correction; *P* value comparisons to *bmp15*$^{+/-}$, ***$P \leq 0.0001$. ns, not significant.

(**D** and **E**) UMAP plot of *chek2* expression in the 40-dpf ovary in (D) all cells, and (E) reclustered germ cells.

(**F**) Analysis of *chek2* expression profile in specific germ cells subclusters represented by dot plot graph. GSC, germline stem cells; TA, transit amplifying.

Figure 4.2

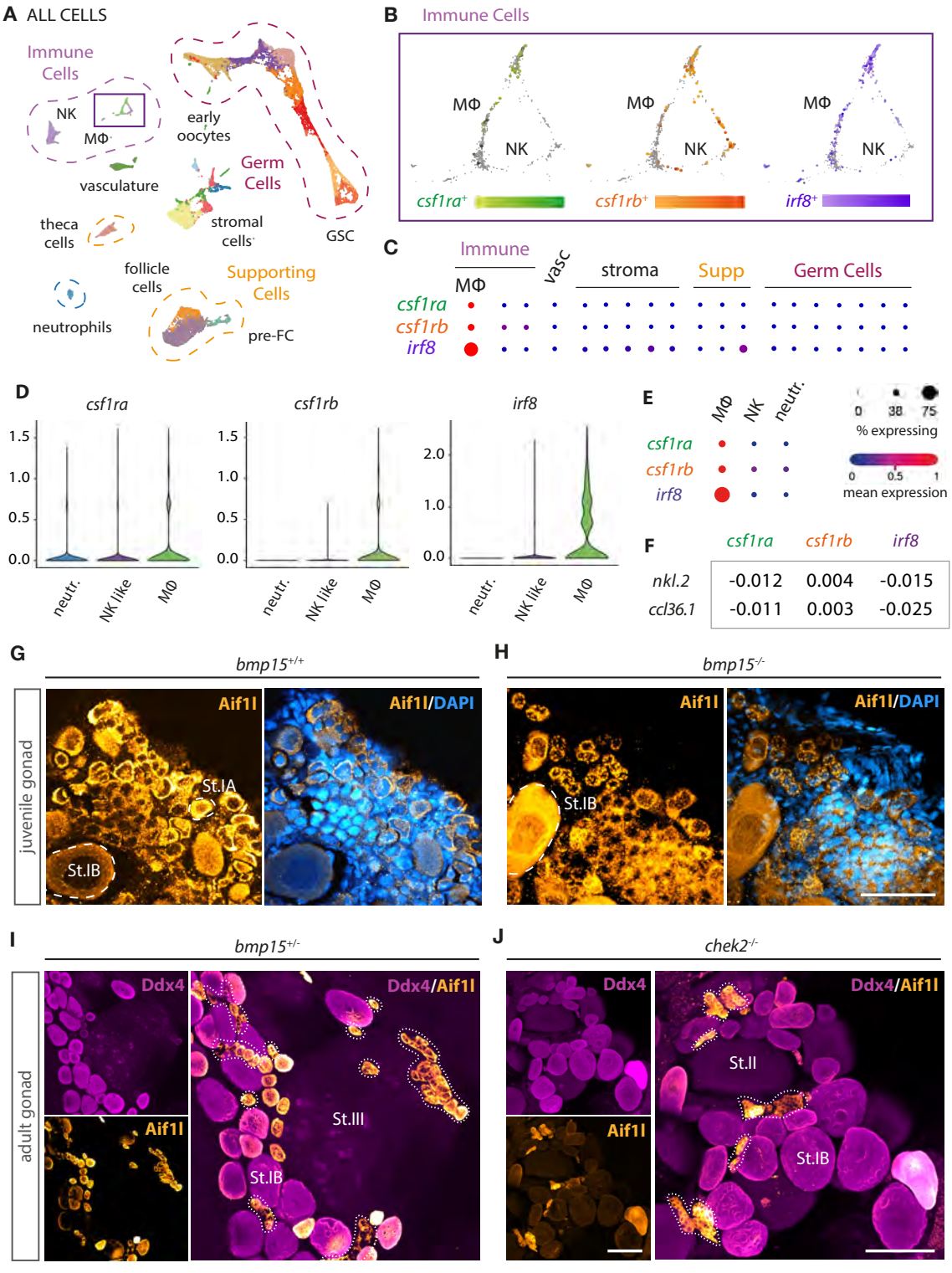


Fig. 4.2: **Macrophages are resident ovary cells in juvenile and adult zebrafish ovaries.**

(**A**) UMAP plot of cell types present in 40-dpf ovary.

(**B**) Magnified view of populations boxed in (A) showing expression of indicated genes. MΦ, macrophages; NK, natural killer–like cells; FC, follicle cells; GSC, germline stem cells; Vasc, vasculature.

(**C**) Analysis of indicated macrophage gene expression profiles in ovarian clusters represented by dot plot graph.

(**D and E**) Expression of indicated genes in different immune cell populations represented by (D) violin and (E) dot plot graphs. MΦ: macrophages, NK: natural killer-like cells, neutr.: neutrophils.

(**F**) Analysis of expression profiles of indicated genes in specified clusters of immune cells represented by Spearman's Rho correlation values.

(**G** to **J**) Immunostained (G) and (H) juvenile and (I) and (J) adult gonads of indicated genotypes. Aif1l (yellow) labels macrophages, Ddx4 (magenta) labels germ cells, and DAPI (blue) labels DNA. Dotted lines mark macrophages. Scale bars, (G) and (H) 50 µm, (I) and (J) 100 µm. St.IA, stage IA oocyte (prophase I meiotic cells in nests), St.IB, stage IB oocyte (late prophase within definitive follicles); St.III, stage III oocyte (increased size and apparent yolk).

Figure 4.3

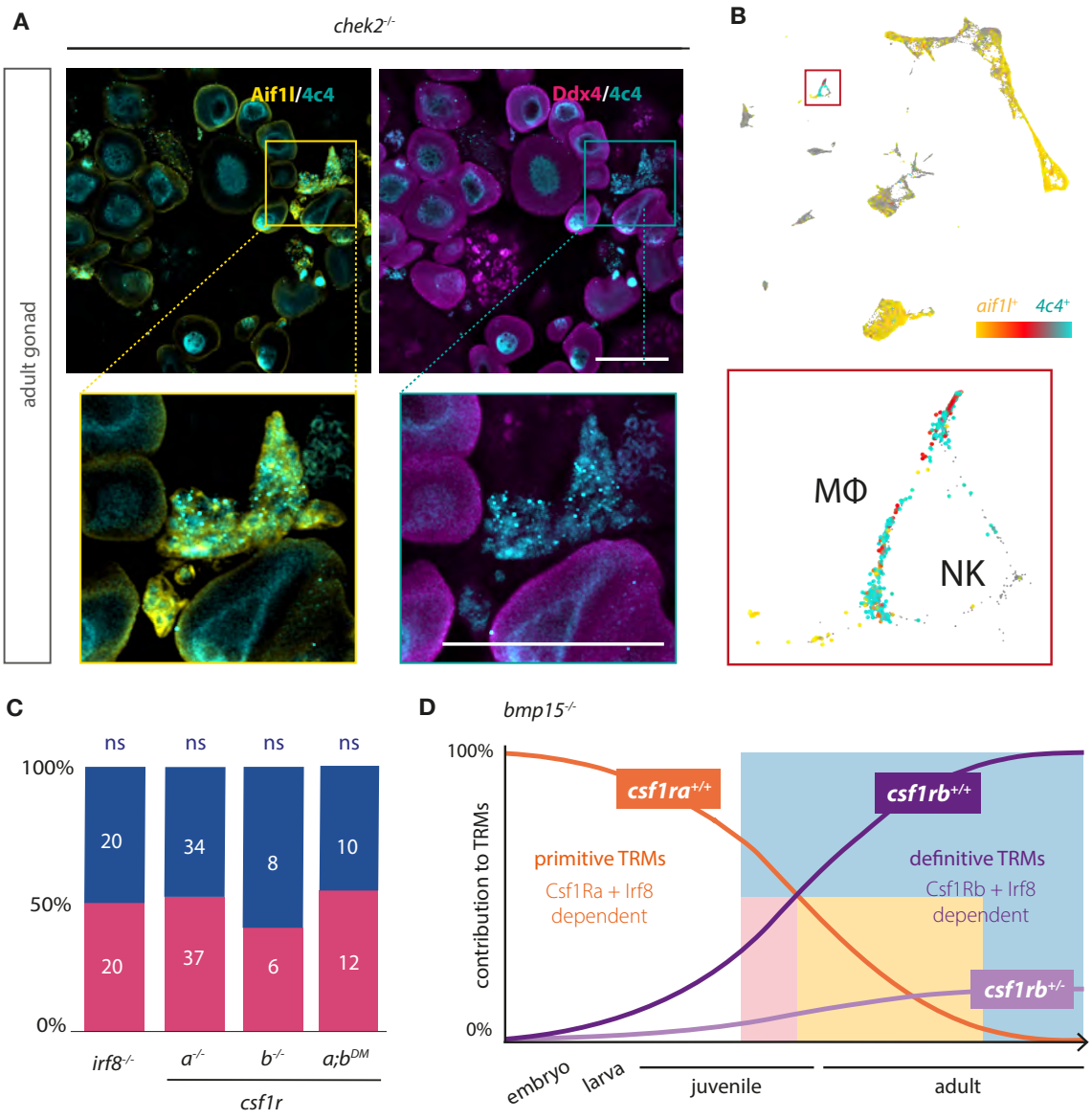


Fig. 4.3: **Macrophages are not required for normal sex determination or differentiation.**

(**A**) Immunostained adult gonads of indicated genotype. Aif1l (yellow) and 4c4 (cyan) label macrophages, Ddx4 (magenta) labels germ cells. Scale bar: 100µm.

(**B**) UMAP plots showing co-expression of indicated genes in the 40dpf ovary. Magnified population of immune cells boxed in red.

(**C**) Adult sex ratios of indicated genotypes. Female (pink), male (blue). Numbers indicate individuals examined. Statistical analysis: Chi-square test with Bonferroni correction; *p-Value* comparisons to *bmp15*$^{+/-}$.

(**D**) Diagram representation of macrophage waves specification and contribution in zebrafish. Sex after differentiation represented by background color: pink, female; blue, male; yellow, sex reversal. X-axis: fish stage, y-axis: percentage of macrophage.

Figure 4.4

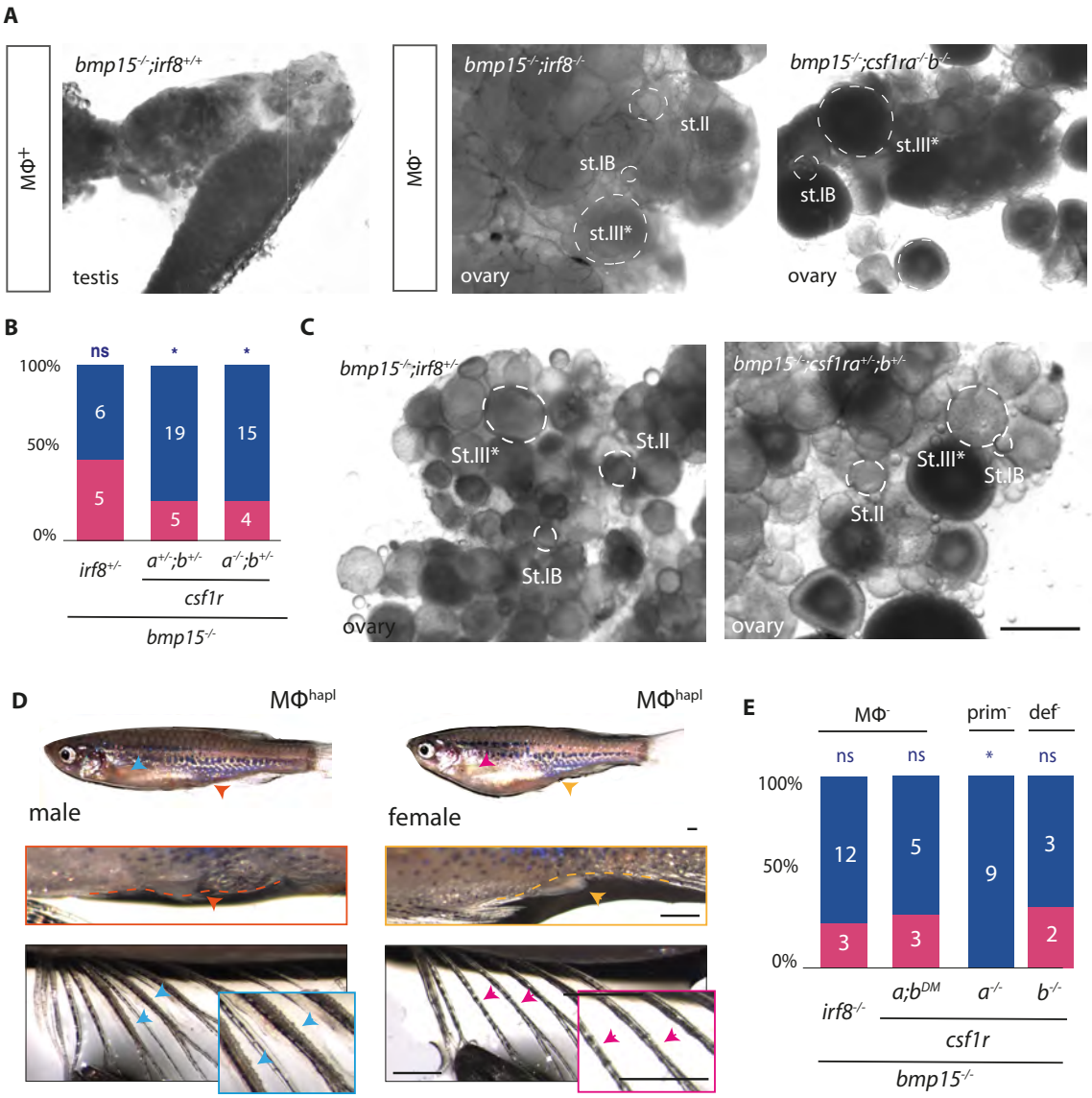


Fig. 4.4: **Macrophages are required for sex reversal of *bmp15* mutants.**

(**A**) Live tissue pictures of adult gonads of *bmp15* mutants when macrophages are present or completely ablated.

(**B** and **E**) Adult sex ratios graph of indicated genotypes. Female (pink); male (blue). Numbers indicate individuals examined. Statistical analysis: Chi-square test with Bonferroni correction; *p-Value* comparisons to $bmp15^{+/-}$, *P ≤ 0.0125. St.IB, stage IB oocyte (late prophase within definitive follicles), St.II, stage II oocyte (apparent cortical alveoli and a vitelline envelope); St. III*: arrested stage III oocyte (increased size and apparent yolk).

(**C**) Live tissue pictures of adult gonads of *bmp15* mutant fish heterozygous for *irf8* or *csf1rs*. Scale bar: 500 µm.

(**D**) Secondary sex traits of adult: male body shape, urogenital papilla (orange dashed line and arrowhead), and lateral fin tubercles (blue arrowheads and box); rounded female body shape, distinct urogenital papilla (yellow dashed line and arrowhead), and absence of tubercles (pink arrowheads and box). Scale bars, 1 mm.

Figure 4.5

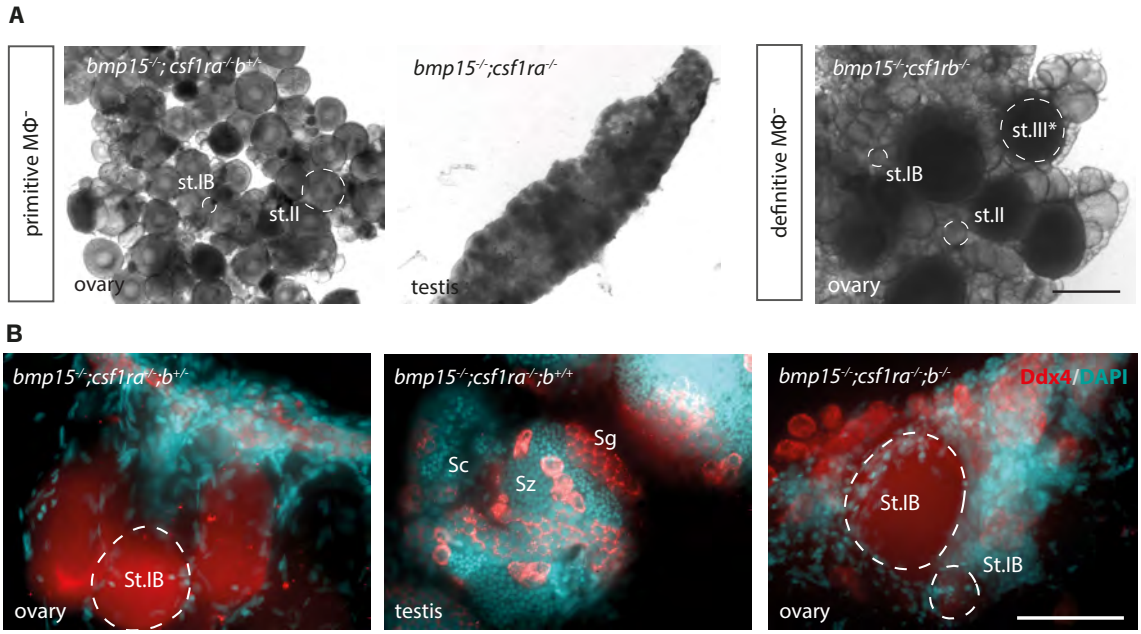


Fig. 4.5: **Lack of definitive macrophages suppresses sex reversal of *bmp15* mutants.**

(**A**) Live tissue pictures of adult gonads of *bmp15* mutants with lack of primitive, or definitive populations. Scale bar, 500 µm. St.III*, arrested stage III oocyte (increased size and apparent yolk).

(**B**) Immunostained adult *bmp15* mutant gonads lacking all, only primitive, or primitive and haploinsufficiency for definitive macrophages. Ddx4 (red) labels germ cells, DAPI (cyan) labels DNA. Scale bar: 50µm. St. IB: stage IB oocyte (late prophase within definitive follicles), St.II: stage II oocyte (apparent cortical alveoli and a vitelline envelope), St. III*: arrested stage III oocyte (increased size and apparent yolk), Sg: spermatogonia, Sc: spermatocyte, Sz: spermatozoa.

Figure 4.6

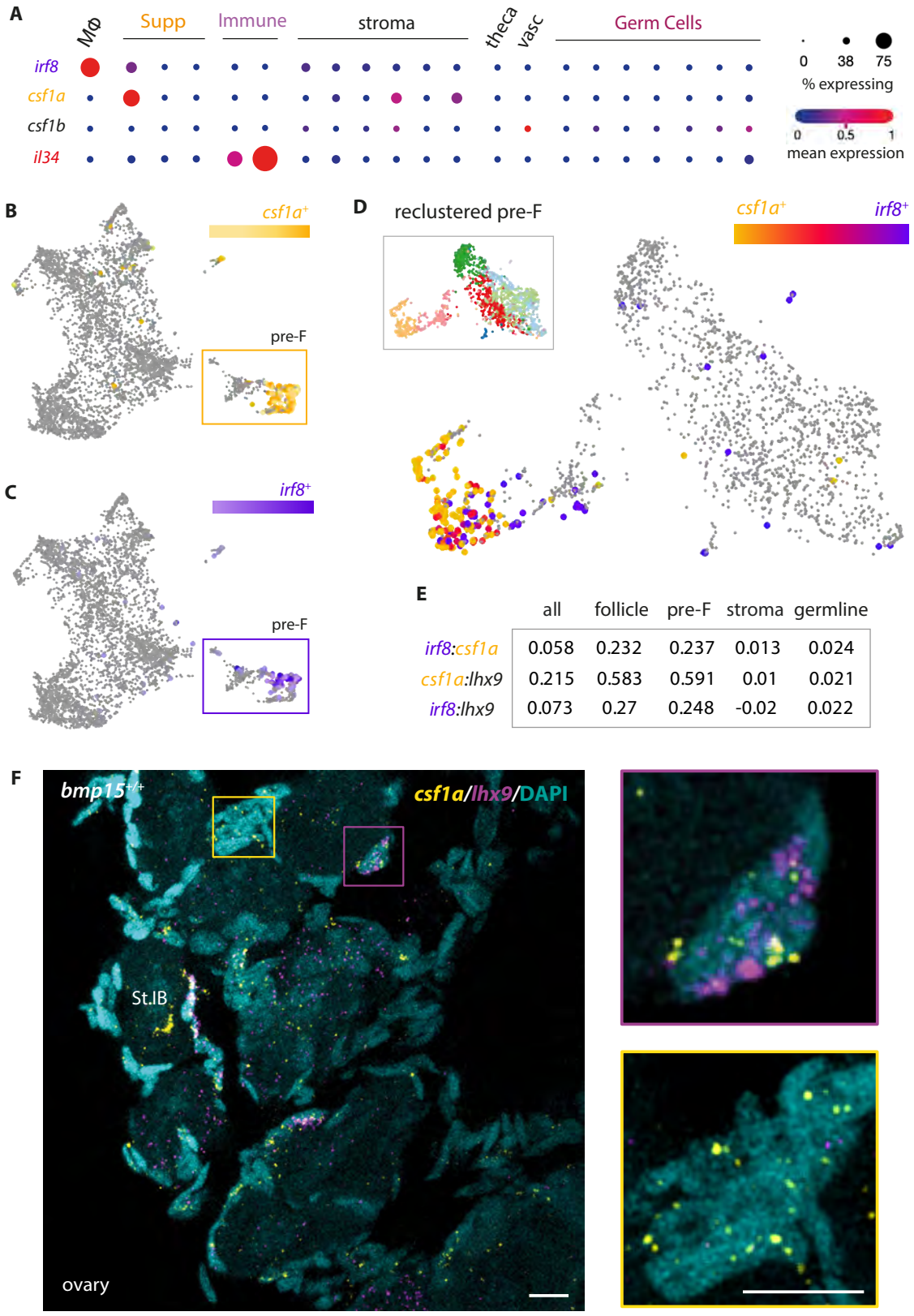

A
Mφ
Supp
Immune
stroma
theca
vasc
Germ Cells
irf8
csf1a
csf1b
il34
0
38
75
% expressing
0
0.5
1
mean expression
B
csf1a+
pre-F
C
irf8+
pre-F
D
reclustered pre-F
csf1a+
irf8+
E
all
follicle
pre-F
stroma
germline
irf8:csf1a 0.058 0.232 0.237 0.013 0.024
csf1a:lhx9 0.215 0.583 0.591 0.01 0.021
irf8:lhx9 0.073 0.27 0.248 -0.02 0.022
F
bmp15+/+
csf1a/lhx9/DAPI
St.IB
ovary

Fig.4.6: *irf8* and *csf1a* are co-expressed by a subpopulation of pre-follicle cells in the early ovary.

(**A**) Analysis of expression profiles of indicated genes in specified clusters of ovarian cells represented by dot plot graph.

(**B**-**D**) UMAP plot of indicated gene's expression in the 40-dpf ovary in (B) and (C) follicle cells, and (D) reclustered subpopulation of pre-follicle cells. Subpopulation of pre-F cells boxed in (B) and (C). UMAP of reclustered pre-F cells legend boxed in (D).

(**E**) Gene expression correlation values of indicated genes in specified clusters of ovarian cells represented by Spearman's Rho correlation analysis.

(**F**) Double HCR RNA FISH confocal images of 40-dpf wild-type ovary. *lhx9*, follicle cells (magenta); *csf1a*, Csf1rs' ligand (yellow), and DAPI, DNA (cyan). Regions boxed in (F) show magnified views of *lhx9/csf1a*-coexpressing MAFCs and *csf1a*-expressing somatic cells. Scale bars, 10 µm. F, follicle cells; Supp, supporting cells. St. IB: stage IB oocyte (late prophase within definitive follicles).

Figure 4.7:

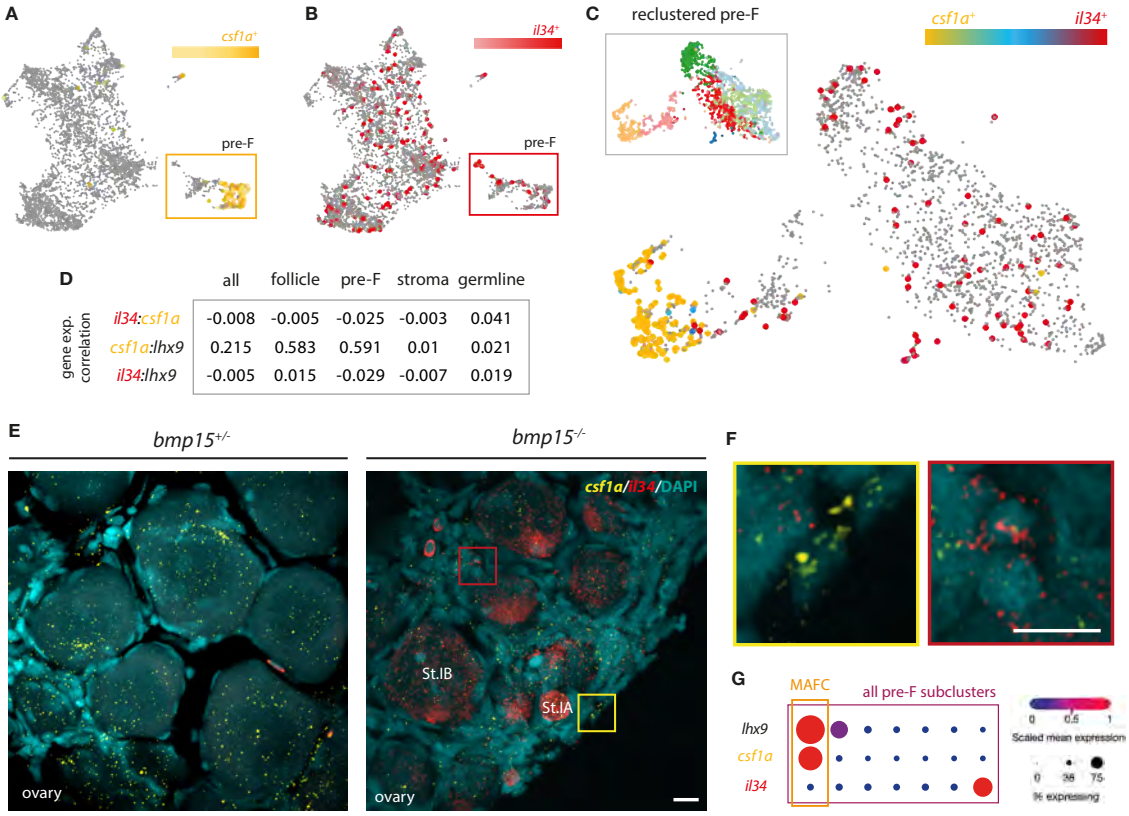


Fig 4.7: ***csf1a*** **and** ***il34*** **are detected in distinct follicle cell populations of the early ovary.**

(**A**-**C**) UMAP plot of indicated gene's expression in the 40-dpf ovary in (A) and (B) follicle cells, and (C) reclustered subpopulation of pre-follicle cells. Subpopulation of pre-F cells boxed in (A) and (B). UMAP of reclustered pre-F cells; legend boxed in (C).

(**D**) Gene expression correlation values of indicated genes in specified clusters of ovarian cells represented by Spearman's Rho correlation analysis.

(**E** and **F**) Double HCR RNA FISH maximum projections of confocal images of 40-dpf *bmp15* heterozygous and mutant ovaries. *il34*, red; *csf1a*, yellow; and DAPI (DNA), cyan. Regions boxed are magnified in (F). Scale bars, 10 µm. St.IA, stage IA oocyte (prophase I meiotic cells in nests), St.IB, stage IB oocyte (late prophase within definitive follicles).

(**G**) Analysis of expression profiles of indicated genes in specified clusters of follicle cells represented by dot plot graph. MAFCs, macrophage-activating follicle cells.

Figure 4.8

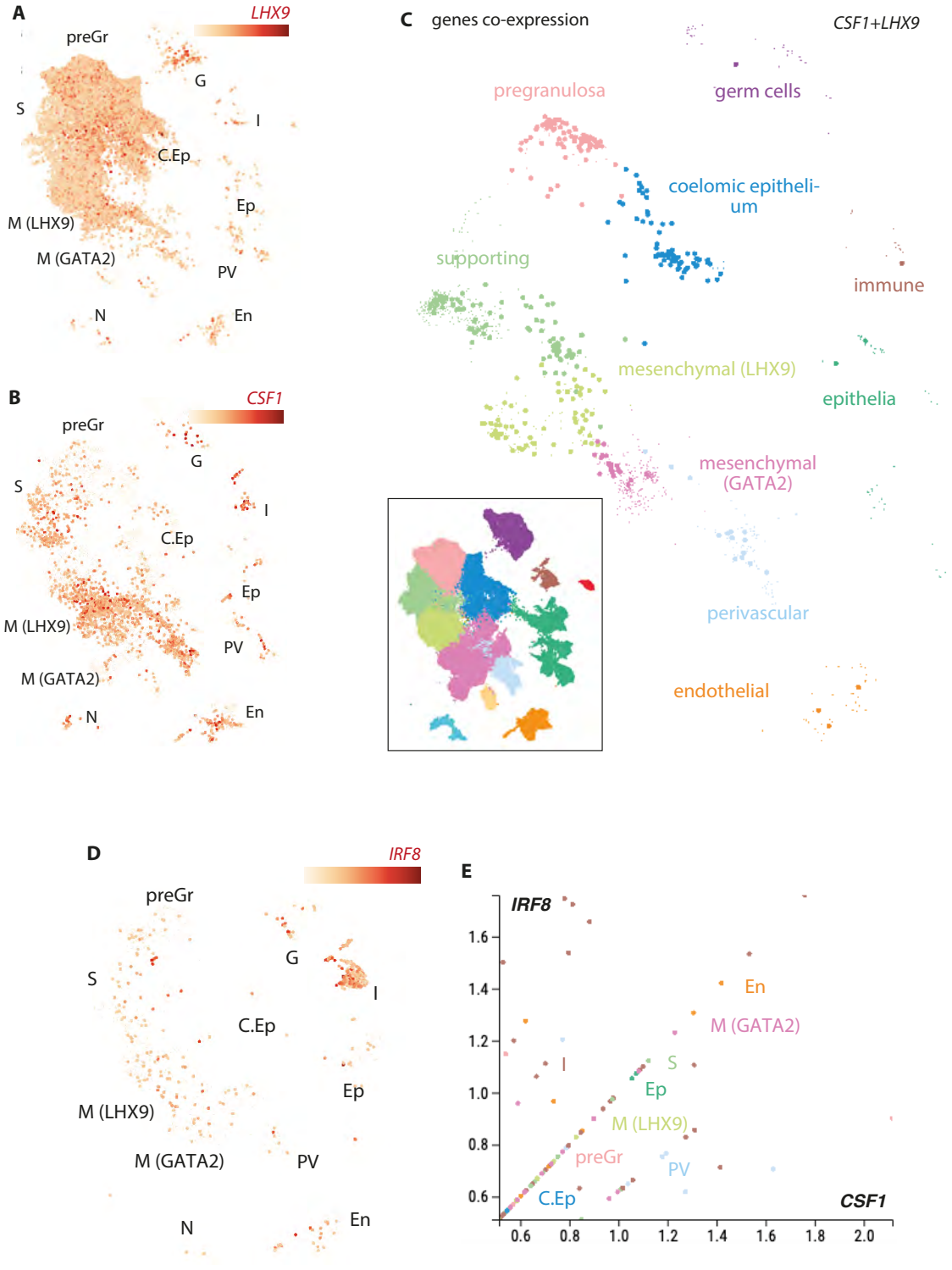


Fig. 4.8: **CSF1 source cells in human fetal ovary.**

(**A-D**) UMAP plots of indicated gene expression profiles in the fetal human ovary showing (A, B, D) overall expression and (C) co-expression. UMAP clusters legend boxed in (C).

(**E**) Scatterplot of cell clusters co-expressing indicated genes. C.Ep: coelomic epithelium, En: endothelial, Ep: epithelia, I: immune, M: mesenchymal, preGr, pregranulosa, PV: perivascular, S: supporting.

Figure 4.9

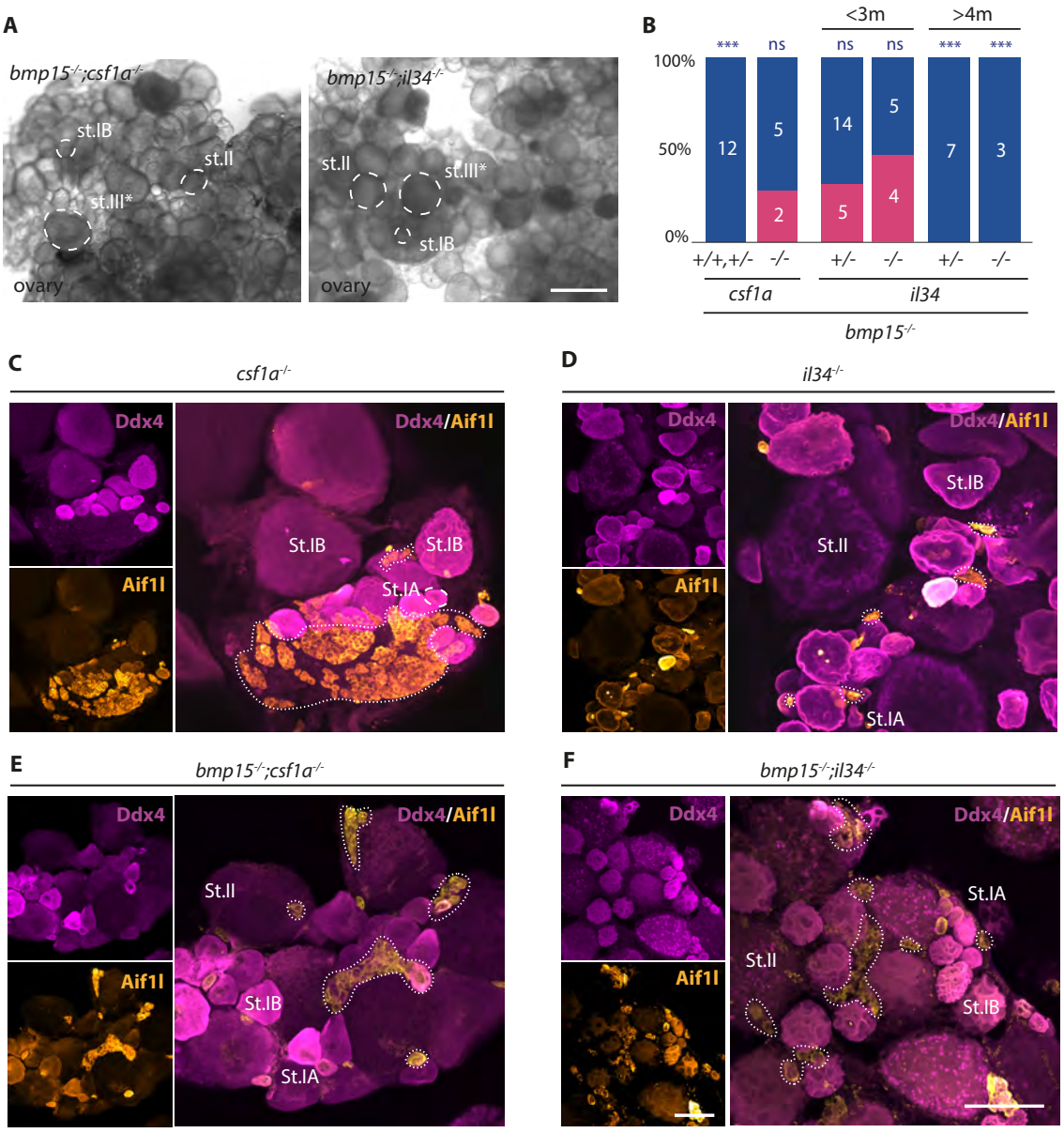


Fig. 4.9: **Il34 and Csf1a differentially contribute to ovarian failure and sex reversal and macrophages persist in *csf1a*- and *il34*-mutant ovaries.**

(**A**) Live tissue images of adult gonads of indicated genotypes. Scale bar, 500 µm.

(**B**) Graph with adult sex ratios of indicated genotypes. Female, pink; male, blue. Numbers indicate individuals examined. Statistical analysis: chi-square test with Bonferroni correction; *P* value comparisons to *bmp15*$^{+/-}$, ***$P \leq 0.0001$. St.IA, stage IA oocyte (prophase I meiotic cells in nests), St.IB, stage IB oocyte (late prophase within definitive follicles); St.II, stage II oocyte (apparent cortical alveoli and a vitelline envelope); St. III*: arrested stage III oocyte (increased size and apparent yolk).

(**C**-**F**) Immunostained adult gonads of (C) and (D) ligand single mutants, and (E) and (F) *bmp15;ligand* DMs of indicated genotypes. Aif1l (yellow) labels macrophages, Ddx4 (magenta) labels germ cells, and DAPI (blue) labels DNA. Dotted lines mark macrophages. Scale bars, 100 µm.

Results for Aim C

Morphological differences in the juvenile and adult zebrafish brain

Courtship in zebrafish is a well-studied behavior with defined specific regions and brain nuclei in the adult brain that influence mating (Kermen et al., 2013; Yabuki et al., 2016). To investigate potential morphological or cellular differences between female and male zebrafish brains, I focused on the brain regions that are known to be part of this behavioral pathway. I performed whole mount immunohistochemistry of juvenile (28dpf and 35dpf), and adult (>3months) zebrafish brains (Figure 4.10 A) to determine if the regions involved during sex-specific behaviors like mating – telencephalon (Te), optic tectum (OpT), and hypothalamus (HT) – showed any differences in size (Kermen et al., 2013; Yabuki et al., 2016). Adult brains represent the developed tissue, after its structures are fully functioning and performing sex-specific roles during courtship. Conversely, juvenile brains at the selected stages are still developing and, more importantly, potentially still undergoing the developmental process of gonadal differentiation (Figure 4.10 B). Analysis of lightsheet microscopy images of these regions indicate that the adult brain structures involved in this sex-specific neural pathway (Te, OpT, and HT) are sexually dimorphic and larger in the male than in the female (Figure 4.10 C). Interestingly, brain structures of sexually immature juvenile fish do not show any differences in volume prior to sex differentiation, indicating that sexual dimorphism arises only after gonadal sex has been fully developed.

The larger size of the Te, OpT, and HT in the male could be a consequence of prolonged growth of the male brain relative to the female brain, or equal growth in both sexes but different rates of cell death. Programmed cell death through apoptosis during development has been extensively studied as a mechanism to refine tissue morphologies ((Kerr et al., 1972) and reviewed in (Ameisen & Ameisen, 2002; Jacobson et al., 1997)), like the loss of epithelial tissue between the digits during hand formation (Mori et al., 1995). Similarly, during nervous system development, half of the neurons that are born ultimately die and are eliminated through clearance processes involving immune cells (reviewed in (Dekkers et al., 2013; Pfisterer et al., 2017)). To better understand if the differences in volumes between the female and male brain regions are due to higher proliferative rates in the males or higher cell death rates in the female, I analyzed brains of both sexes in the absence of Chek2. Chek2 is a tumor suppressor factor with essential roles in the

activation of Tp53- and Tp563-dependent cell death pathways, and its ablation has been previously shown to suppress cell loss and tissue damage phenotypes (Emori et al., 2023; Bolcun-Filas et al., 2014; Bravo et al., 2023). The Te, OpT, and HT of adult zebrafish brains that have been genetically ablated of Chek2 are indistinguishable from each other and lack the sexual dimorphism seen between wildtype female and male brains (Figure 4.11 A). Additionally, both the Te and HT of heterozygous female and males remain sexually dimorphic and are comparable to the wildtype, but the female OpT – instead of the male OpT – appears to be larger (Figure 4.11 A). Although not significant, the brains of both sexes of *chek2* mutant fish also seem to be slightly bigger than WT. Considering the difference in size of the female brain with the loss of one or both copies of the TSFs, these results suggest that cell death pathways affect specific areas of the brain in a sex-specific manner, and that apoptosis most likely contributes to the smaller brain regions in WT females, based on the enlarged brains regions of females when cell death is suppressed.

Microglia contribution to sexual dimorphism

Previous studies have identified sexual dimorphism in microglia numbers and regional colonization between the brains of male and female mice (reviewed in (Lenz & Nelson, 2018; Villa, Della Torre, & Maggi, 2018; Villa, Gelosa, et al., 2018). Moreover, microglia express prostaglandin receptors, produce prostaglandin (Minghetti & Levi, 1998; Minghetti, Nicolini, et al., 1997; Minghetti, Polazzi, et al., 1997), are responsive to estrogens (Morale et al., 2006), and pharmacological inhibition of PGE causes changes in microglia morphology and numbers (Lenz et al., 2013). Similarly, microglia ablation in young rats causes lifelong and sex-specific behavioral deficits (Nelson & Lenz, 2017). To determine if any sex-specific differences in microglia distribution or morphology are apparent in zebrafish, I analyzed microglia density in the Te, HT, and reticulospinal tract (RST) of adult brains. I observed no significant differences in total number of cells between the female and male adult brain areas analyzed (Figure 4.12 A), but the densities of microglia cells were different in some of these regions (Figure 4.12 B). In the HT, an average of 52% of the total adult female cells are $4c4^+$ (microglia), while in males only 16% of the cells were $4c4^+$ (Figure 4.12 B). Similarly, in the RST 31% of total cells were $4c4^+$ in females and only 18% in the male (Figure 4.12 B). This result indicates a much higher density of $4c4^+$ cells in the female than in the male brains, specifically in regions involved in sex-specific behaviors.

In adults, the female Te, OpT, and HT are smaller in size than the male's (Figure 4.10), and some of these regions have higher microglia densities (Figure 4.12 B), which could account for these morphological differences. Since microglia are known to be involved in neurogenesis,

oligodendrogenesis, and cell proliferation (Casano & Peri, 2015; Lenz & Nelson, 2018; Li & Barres, 2018; Lopez-Atalaya et al., 2018; Nelson et al., 2018), they could have a role in shaping sex-specific region differences in the brain during development and/or adulthood. To study if the presence of microglia in the brain contributes to the size differences seen in the Te, OpT, and HT, I analyzed the brains of females and males that lack microglia due to genetic ablation of the transcription factor Irf8 (Figure 4.12 C). Results confirm that, unlike in the wildtype, sexual dimorphism is lost in the absence of microglia and the Te, OpT, and HT of females and males no longer differ in size (Figure 4.12 D). Interestingly, the cellular and structural differences between the two sexes are not necessary for the organization of behavioral pathways, since adult fish lacking microglia show normal mating behaviors. This suggests that microglia play a role in establishing morphological sex-specific differences in the adult wildtype brain but are not required for the cellular or molecular processes that regulate behavioral specification.

Ablation of myeloid cells by mutation of *irf8* has been a useful tool to study changes in colonization and loss of tissue resident macrophages as well as the consequences of their absence during development, immune response, or regeneration (Constanty et al., 2024; Ferrero et al., 2020; Kierdorf et al., 2013; Li et al., 2011; Shiau et al., 2015). But the effects of ablation or changes of TRM density during tissue growth have not been extensively studied. To further investigate if the presence of microglia in the brain could be responsible for the clearance of unnecessary cells and maintenance of brain size, I compared the brains of *irf8* mutant and wild-type fish. One of the main roles attributed to microglia in the nervous system is to remove unwanted synapses (by pruning) and cells (by phagocytosis) as part of the mechanism to conserve tissue homeostasis (Casano & Peri, 2015; Li & Barres, 2018; Matcovitch-Natan et al., 2016), which is also useful during repair or regeneration (Balena et al., 2023; Pfisterer et al., 2017). I found that fish lacking TRM have a greater brain volume-to-body size than WT, meaning that they have larger brains but not bodies (Figure 4.12 E). This result suggests that Irf8-dependent cell lineages are important to maintain the correct balance of cell types and numbers in the brain, potentially by contributing to neuronal death and clearance.

Female-to-male brain organization after remodeling

Sex-reversal in zebrafish can be induced by a variety of environmental or genetic factors (Dranow et al., 2013; Kossack & Draper, 2019). In the absence of Bmp15, a conserved ligand released by the oocyte that is essential for follicle progression, the female zebrafish ovary undergoes oocyte loss, masculinization, and is remodeled into a testis (Figure 4.13 A). Transition

of the female zebrafish into a male due to loss of Bmp15 takes place between day 35 to 85 post fertilization (Dranow et al., 2016) due to a series of cellular and molecular events that involve cell death and activation of macrophages in the gonad by Csf1a-expressing prefollicle cells (Aims A and B/(Bravo et al., 2023)). This process of masculinization involves not only remodeling of the gonad, but also reversal of other secondary sex-specific traits, which include changes in pigmentation, fin morphology, and, most importantly, behavior (Dranow et al., 2016). Presumably, specific behavioral pathways in the brain of female *bmp15* mutants need to be modified to resemble the male patterns necessary for mating, but it is not known whether the female to male sex-reversed brain acquires the morphological features of a direct-differentiating male. I analyzed the size of the Te, OpT, and HT of adult brains in the absence of one or both copies of *bmp15* and compared them to unrelated adult wildtype brains of similar age. As expected, all three structures of female and male heterozygous fish for *bmp15* were sexually dimorphic and, interestingly, the volume-to-body size ratio of the OpT of both females and males was larger (Figure 4.13 B) than in wildtypes of similar age and size. Lastly, the volumes of *bmp15* homozygous mutant male brain regions were comparable to those of heterozygous males but differed greatly from heterozygous females (Figure 4.13 C). This suggests that, in *bmp15* female-to-male mutant fish, areas of the brain that are responsible for sex-specific behaviors (Yabuki et al., 2016) and are sexually dimorphic (Figure 4.10 C and 4.12 B) may undergo growth and/or morphological changes to resemble direct-differentiating male brains. Since the brains analyzed were from adults, and mutant fish had already switched from female to male, it is not known which of the males examined were originally females. As previously described (Dranow et al., 2016), testis of all adult *bmp15* mutant fish studied looked normal and there were no morphological differences suggesting whether they were originally ovaries that got remodeled or direct-differentiating testis. Unlike mammals, zebrafish neurogenesis does not stop after maturation of the nervous system and is maintained throughout their lives (reviewed in (Kizil et al., 2012; Schmidt et al., 2013)), which accounts for the regenerative and remodeling properties seen in teleost fish and could constitute part of the mechanisms that allow for female to male brain transformation. Conversely, suppression of cell death could play a role in masculinization of the brain after or during ovary-to-testis transition. It remains to be studied if microglia are required during this process, similar to what happens during remodeling of the gonad (Bravo et al., 2023), and what other cell types and mechanisms are involved.

Figure 4.10

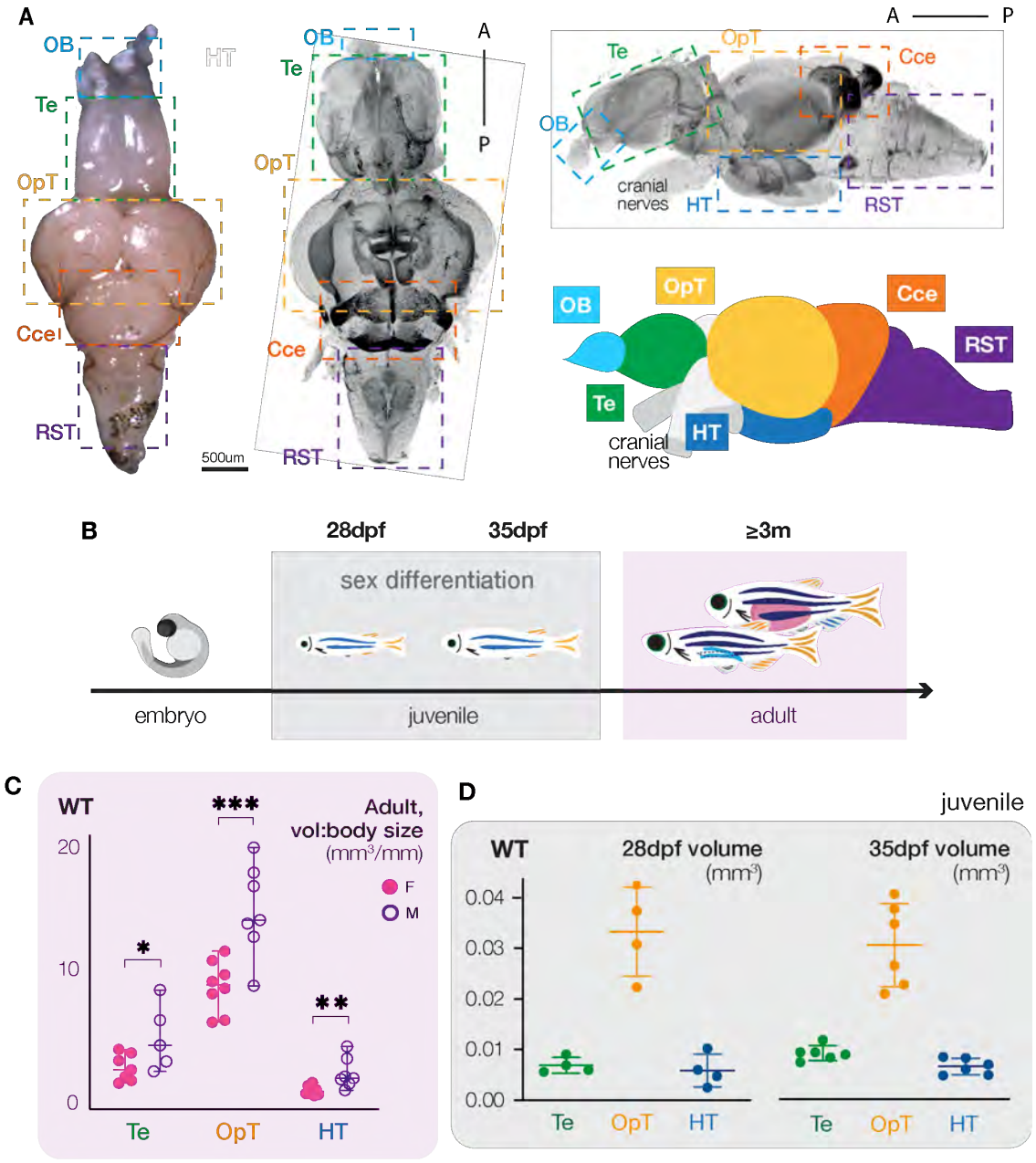


Fig. 4.10: **Morphological differences between female and male adult brains appear after sex determination.**

(**A**) Live tissue, fluorescence images, and representative illustration of the adult dissected brain indicating morphological structures.

(**B**) Zebrafish developmental timeline with experimental time-points for brain staining and analysis.

(**C**) Wild-type brain volumes of indicated regions of the adult female and male.

(**D**) Wild-type brain volumes of indicated regions of the indeterminant juvenile at 28dpf and 35dpf.

OB: olfactory bulb; Te: telencephalon; OpT: optic tectum; Cce: cerebellum; RST: reticulospinal tract; HT: hypothalamus.

Statistical analysis: ordinary TWO-way ANOVA with 95% CI, and Fisher LSD test for multiple comparisons. P-values: ns≥ 0.05, *<0.05, **<0.01, ***<0.001, ****<0.0001.

Figure 4.11

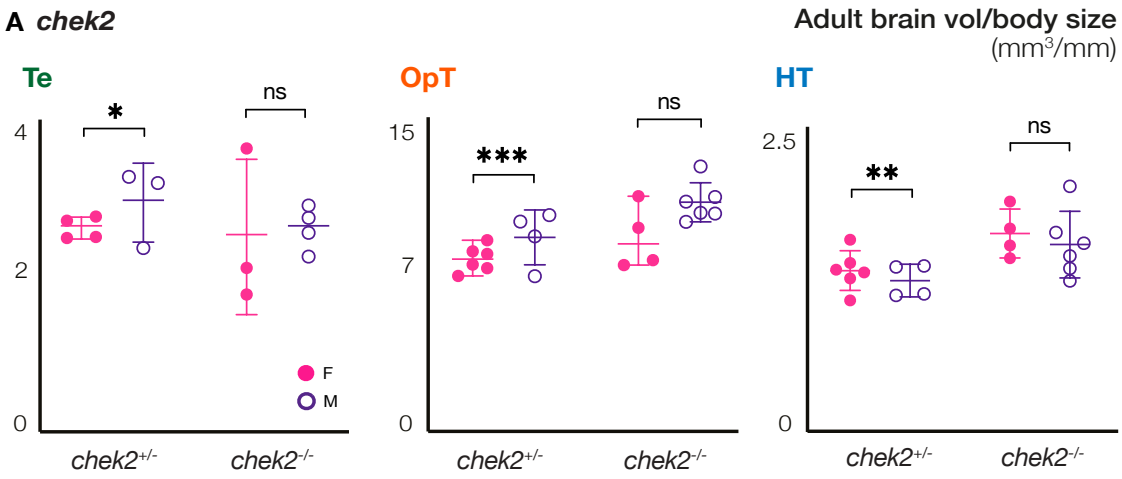


Fig. 4.11: ***chek2*** **contributes to regional size differences between female and male adult brains.**

(**A**) Comparison of *chek2*$^{+/-}$ and *chek2*$^{-/-}$ brain volumes of indicated regions of adult females and males.

Te: telencephalon; OpT: optic tectum; HT: hypothalamus.

Statistical analysis: ordinary TWO-way ANOVA with 95% CI, and Fisher LSD test for multiple comparisons. P-values: ns≥ 0.05, *<0.05, **<0.01, ***<0.001, ****<0.0001.

Figure 4.12

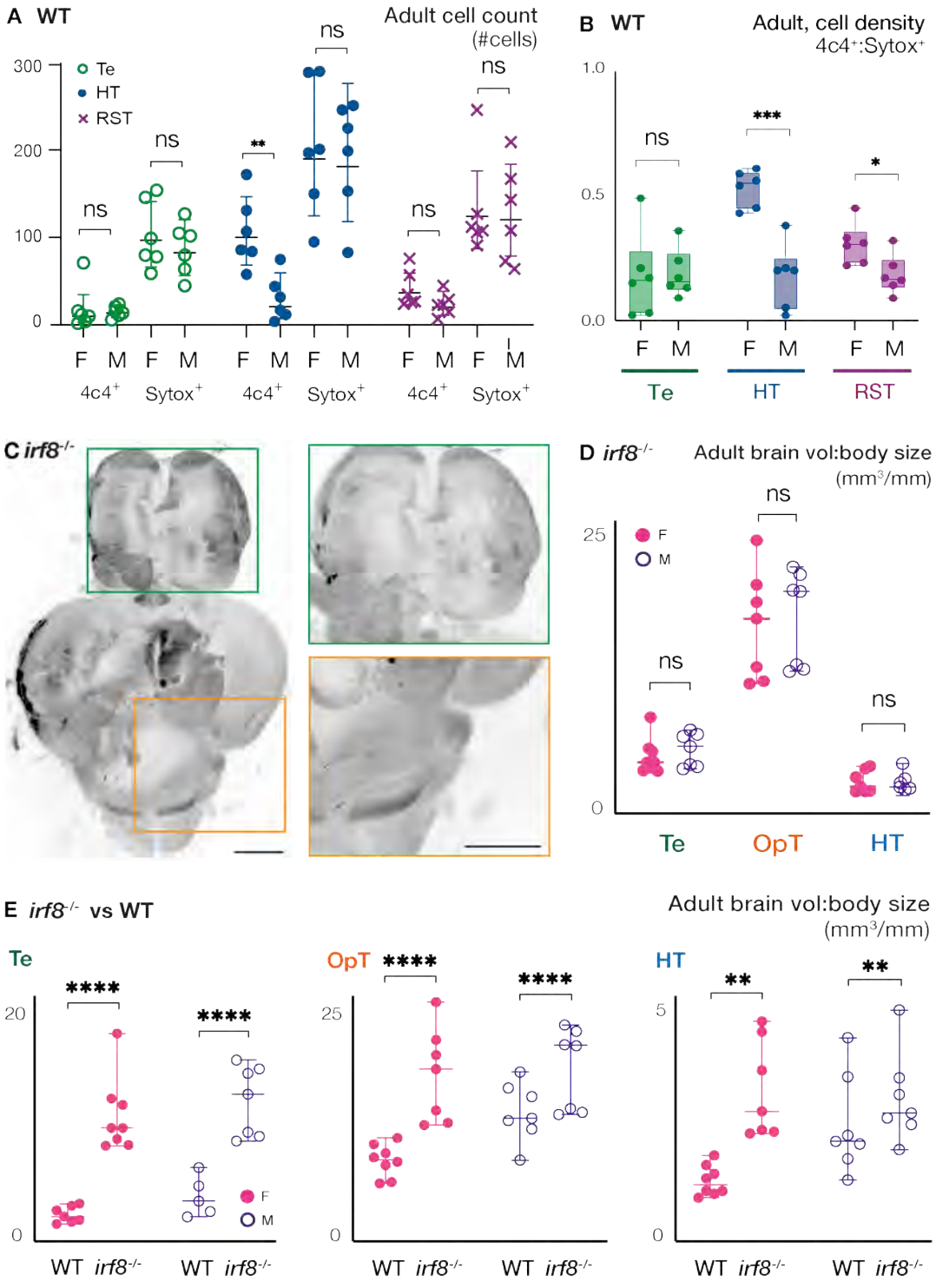


Fig. 4.12: **Microglia density is higher in females and microglia contribute to establishment of sex-specific differences in the adult.**

(**A**) Number of Sytox (all nuclei) and 4c4 (microglia) positive cells in the indicated regions of the adult wild-type brain.

(**B**) Microglia densities in adult female and male brains. Statistics: unpaired t-test with Welch's correction and 95% CI. P-values: ns≥ 0.05, *<0.05), **<0.01), ***<0.001)

(**C**) Adult *irf8* mutant brain stained for 4c4 (microglia). Scale bars: 500um.

(**D**) Region specific volumes of adult brains lacking microglia.

Te: telencephalon; OpT: optic tectum; HT: hypothalamus; RST: reticulospinal tract.

Statistical analysis for cell quantification: unpaired t-test with Welch's correction and 95% CI. P-values: ns≥ 0.05, *<0.05, **<0.01, ***<0.001.

Statistical analysis for volume comparisons: ordinary TWO-way ANOVA with 95% CI, and Fisher LSD test for multiple comparisons. P-values: ns≥ 0.05, *<0.05, **<0.01, ***<0.001, ****<0.0001.

Figure 4.13

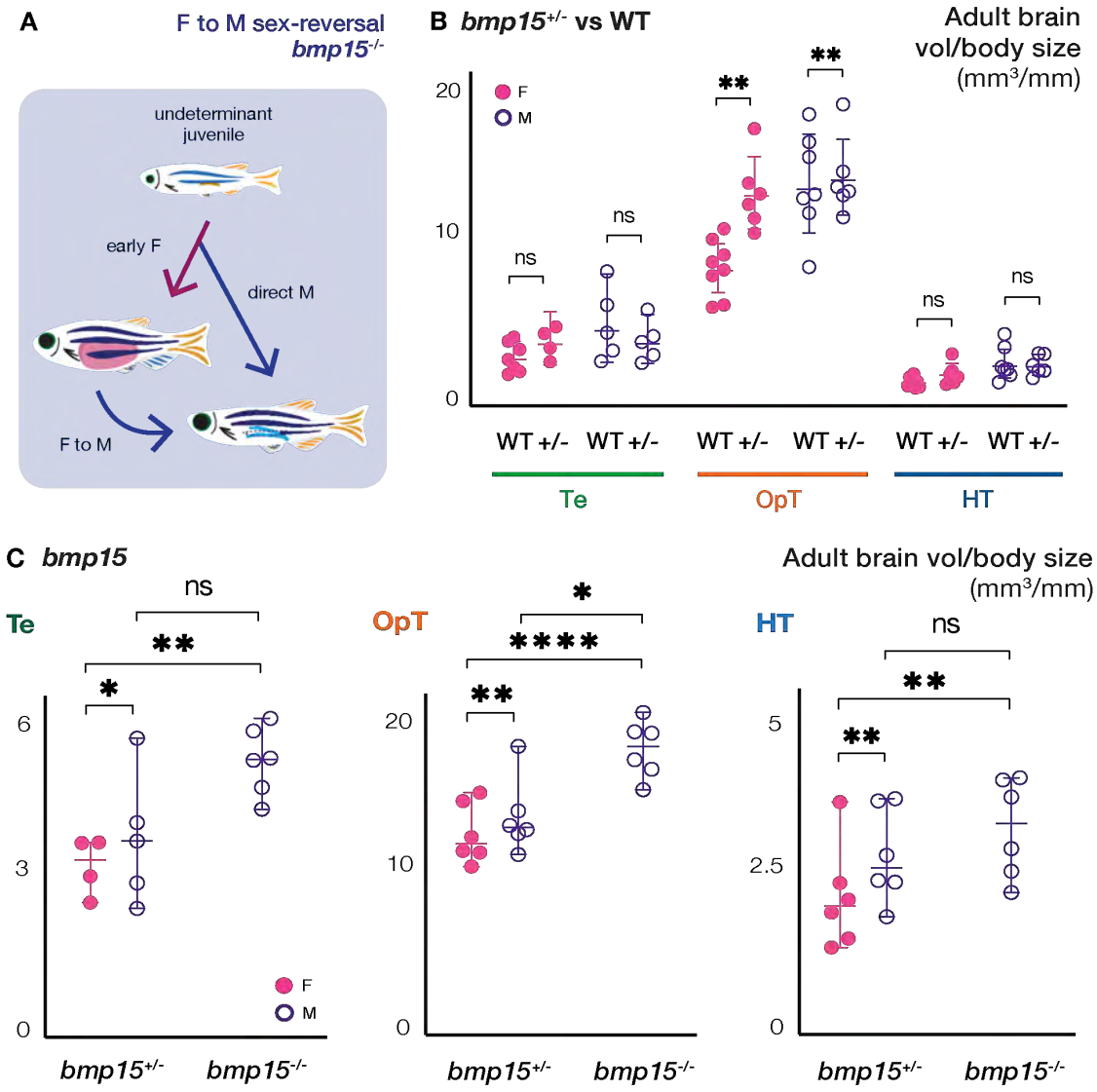


Fig. 4.13: ***bmp15*** **mutant fish acquire direct-differentiating male brain morphologies as adults.**

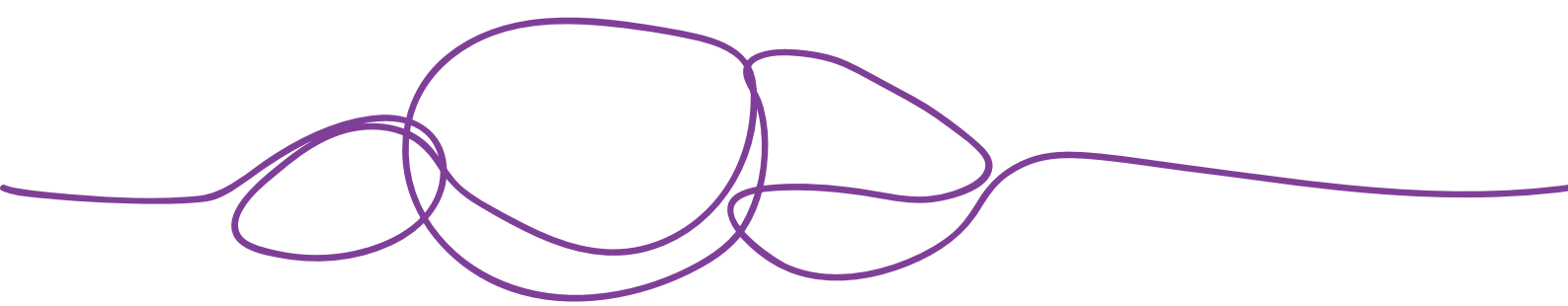
(**A**) *bmp15* mutant fish sex-reversal illustration from indeterminant juvenile to reversed male.

(**B**) Comparison of WT and *bmp15*$^{+/-}$ adult female and male brain volumes of indicated regions.

(**C**) Comparison of indicated brain region volumes of adult male *bmp15*$^{-/-}$ to *bmp15*$^{+/-}$.

Te: telencephalon; OpT: optic tectum; HT: hypothalamus.

Statistical analysis: ordinary TWO-way ANOVA with 95% CI, and Fisher LSD test for multiple comparisons. P-values: ns≥ 0.05, *<0.05, **<0.01, ***<0.001, ****<0.0001.



CHAPTER 5

Discussion[5]

Macrophage activation drives ovarian failure and masculinization in zebrafish.

Although many factors involved in sex determination in zebrafish have been discovered, the molecular trigger initiating sexual differentiation, and the factors involved in remodeling the gonad, from juvenile gonad to ovary or testis during development, or sex reversal of adult females during ovarian failure remain elusive (Aharon & Marlow, 2021). This work identifies macrophages as mediators of sex reversal during ovarian failure but not during differentiation of the juvenile gonad to testis in zebrafish. Specifically, primitive macrophages, which form before sex determination occurs in zebrafish, are dispensable for ovary to testis transition during development and during ovarian failure in adults. In contrast, definitive macrophages peak around the time when sex is determined in zebrafish, suggesting that definitive macrophage development could be influenced by or influence sexual differentiation and the transition from indifferent gonad to ovary or testis during development. However, the observation that adult female to male sex ratios are normal in mutants lacking all macrophages (*csf1r* double mutants and *irf8* mutants) indicates that macrophages are not essential triggers of sexual differentiation of the indeterminant gonad nor for development of the juvenile gonad into an ovary or testis. Instead, definitive macrophages are required after sex has been established for ovary to testis transformation in response to pathological contexts, like ovarian insufficiency or failure due to mutation of *bmp15.* In this context, macrophages, or macrophage activation by MAFCs expressing Csf1a, may be the trigger or respond to the trigger for testis differentiation (Fig. 5.1). This notion is consistent with

[5] Excerpts of this chapter are published in Bravo et al., 2023.

the observation that loss of Csf1a or definitive macrophages preserves oocytes and blocks ovary to testis transition of *bmp15* mutants. Future investigation is needed to determine the developmental roles of MAFCs and how they contribute to testis transformation during ovarian failure in zebrafish.

Ovarian failure in humans has been associated with immunity-related disorders and genetic factors, such as genetic variants in BMP15, but the specific immune cells and mechanisms that drive premature follicle loss, infertility and masculinization were not known. In zebrafish, as in the mammalian ovary, Bmp15 regulates both cell survival and promotes cell fates needed for follicle progression (Otsuka et al., 2001; Su et al., 2004; Zhao et al., 2010). Failure of *bmp15* mutant follicles to progress to vitellogenic stages, even when oocyte loss is suppressed by loss of Chek2, macrophages, or Dmrt1 (Romano et al., 2020), and a recent report showing that loss of Inhibin A (Inha) could both suppress oocyte loss and promote follicle progression through mid-vitellogenic stage, underscore the important role of Bmp15 in follicle progression and preservation (Chen et al., 2022). We propose that Bmp15 promotes follicle development and survival, and silences specialized pre-follicle cells that express *csf1a* (MAFCs) (Fig. 5.1). Further, our data suggest that MAFCs may act as sentinels of oocyte or follicle quality. Accordingly, in response to failed follicle differentiation and loss of oocytes or follicle signals, MAFCs would release Csf1a ligand and signal to ovary macrophages to trigger ovary to testis sex reversal (Fig. 5.1).

Given that follicle turnover is an ongoing and normal process in ovaries, there must be mechanisms to control macrophage activity and responses to prevent widespread ovarian failure. The finding that both Csf1a and Il34 ligands contribute to ovary to testis transformation associated with ovarian failure is exciting because it provides a mechanism for differential macrophage activation that could allow macrophages within the ovary to distinguish between normal homeostatic turnover or “quality control” and catastrophic events within the germline/follicle, such as loss of Bmp15. While Csf1a is required and its loss completely blocks ovary to testis transition, loss of Il34 only delays it, suggesting the two ligands contribute differently and that Csf1a is the main driver of ovary to testis transformation during ovarian failure. That these ligands contribute distinctly is not surprising because single cell and spatial transcript analyses show they are expressed in different subsets of cells within the ovary. Moreover, in other contexts, macrophage stimulation by Csf1a versus Il34 has been shown to have different consequences on macrophage activation; for example, Csf1 has been reported to induce molecular signatures associated with enhanced phagocytic activity, aggregation and migration, while Il34 does not or does so to a lesser degree (Freuchet et al., 2021). Further investigation will be required to

understand the specific roles of Csf1a and Il34 and to determine if the distinct contributions of these ligands is solely due to their expression profiles, or instead reflects differences in their activities, unique roles of their respective pre-follicle cell populations, or a combination of these factors. Conservation of the molecular pathways and their respective reproductive and immune functions suggests that our discoveries and the arising questions may more broadly represent cellular and molecular targets to prevent premature oocyte loss or ameliorate aspects of ovarian insufficiency/failure-related reproductive disorders.

In addition to premature ovarian insufficiency due to genetic factors, POI and permanent infertility are serious side effects of chemotherapy that impact reproductive health and general health more broadly of young cancer survivors, and there is no standard of care to preserve ovarian health after chemotherapy (Koyama et al., 1977). Moreover, Csf1 is elevated in numerous cancers, including reproductive cancers; thus elevated Csf1 and macrophage dysregulation might similarly contribute to adverse effects on fertility in this context (Freuchet et al., 2021). Our finding that eliminating Csf1 allows for sustained maintenance of oocytes and prevents ovarian failure due to genetic factors, raises the possibility that blocking Csf1 might be a strategy to preserve ovarian health during chemotherapy.

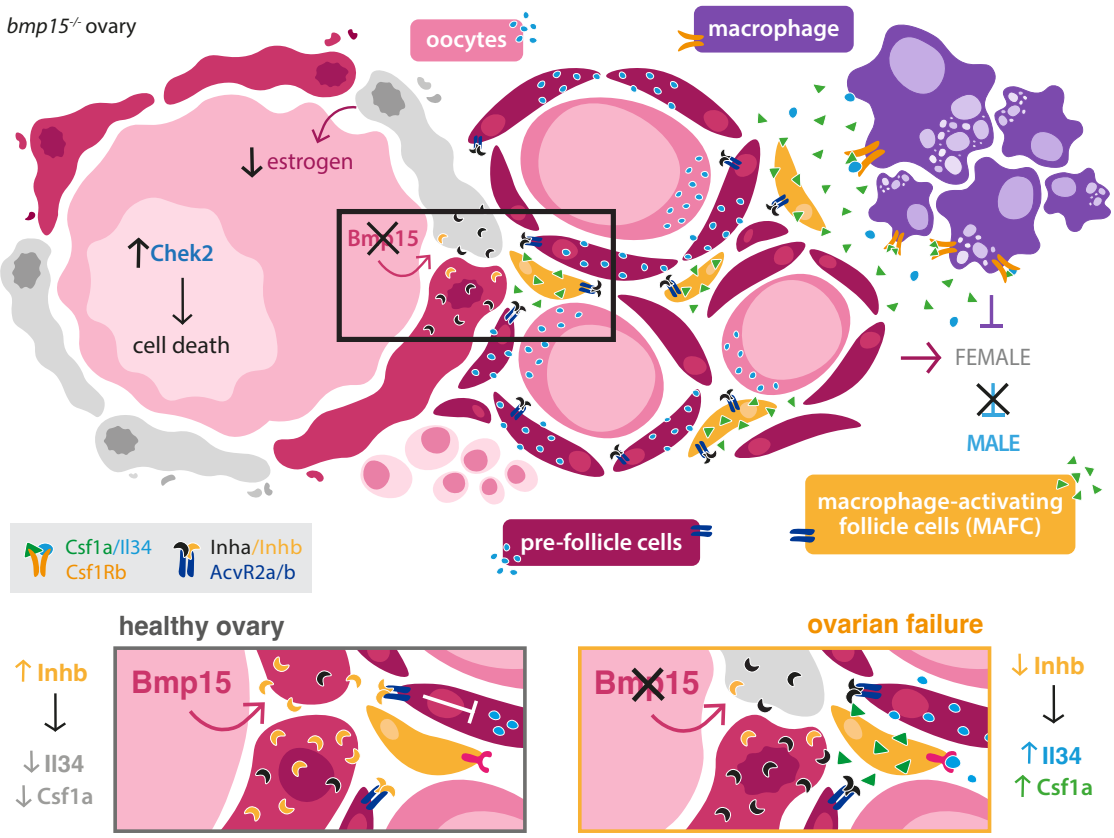


Figure 5.1: **Model of germline-gonadal somatic-immune cell axis in healthy ovary and during ovarian failure.**

Schematic depicts the signals and cellular players in healthy ovary and during ovarian failure. Bmp15 from the oocyte signals to promote somatic follicle fates and survival such that the balance of Activins (Inhb) and Inhibins (Inha) prevents release of Csf1a and Il34 from pre-follicle cells and MAFCs. MAFCs express activin receptors, thereby blocking activation or expansion of macrophages. During ovarian failure caused by loss of Bmp15, this balance tips to favor Inhibin a causing elevated Il34, and MAFCs to release Csf1a. Together, elevated Il34 and Csf1a trigger macrophage activation and activation of programs that regulate ovary-to-testis remodeling and sex reversal in zebrafish.

Chek2 pathways and microglia contribute to sex-specific organization of the adult brain.

Conversely, molecular and cellular differences between the female and male adult zebrafish brain have been previously studied without fully defining the mechanisms and developmental processes involved in their establishment (Ampatzis et al., 2012; Santos et al., 2008; Sreenivasan et al., 2008; Weinhard et al., 2018; Yang et al., 2006). This sexual dimorphism includes differences in modulation of cell types critical for sex-specific neural pathways (S. L. J. Lee et al., 2018; Ogawa

et al., 2021; Takesono et al., 2022), sexually dimorphic expression and regulation of genes with a role in reproductive and endocrine systems (Li et al., 2024; Santos et al., 2008; Sreenivasan et al., 2008), sex-specific mechanisms of recovery from injuries (Das et al., 2019), and differences in cell-proliferation patterns that influence organization of sexually dimorphic brain regions (Ampatzis & Dermon, 2007; Ampatzis et al., 2012). Here, I described morphological differences between females and males in specific regions of the adult brain. These differences were not seen before sex differentiation of the gonad. I defined the time window when sex-specific organization and sexual dimorphism in the adult brain is established. I characterized those brain areas known to have a role and undergo sex-specific activation during mating, which include the telencephalon, optic tectum and hypothalamus (Li et al., 2024; Ogawa et al., 2021; Yabuki et al., 2016). The optic and hypothalamic areas showed the greatest differences in size between the female and male brains, consistent with previous studies showing cellular and gene expression differences in these areas between sexes (Gorelick et al., 2008; Ogawa et al., 2021; Waters & Simerly, 2009). These differences are similar to the sexually dimorphic expression of androgen receptors seen in mice (Shah et al., 2004). This could be explained by the high susceptibility of these regions to sexual hormones and their contribution to sexual behaviors and modulation of the reproductive system. My results identified the tumor suppressor factor Chek2 as one of the contributors to establishment of the differences in volume of these structures. Loss of cells during development has a major role in the organization and function of many tissues, especially the brain (Ampatzis et al., 2012; Casano et al., 2016; Xu et al., 2016). Variations of apoptotic events that result in a smaller sized female brain could be explained by sex-specific regulation of cell death pathways by sex hormones (Ampatzis et al., 2012; Waters & Simerly, 2009). Accordingly, immune cells also contribute to the size differences seen in the adult brain – most likely by active clearing of cells that have undergone apoptosis or that are no longer needed – since microglia are known to phagocyte excess and unwanted live or dead cells to ensure appropriate cell density and function in mammals ((Cunningham et al., 2013; McCarthy et al., 2015; Perez-Pouchoulen et al., 2015) and reviewed in (Márquez-Ropero et al., 2020)). In mice, one of the conserved roles of microglia is to oversee brain homeostasis and help maintain correct cell numbers and interactions in a sex-specific manner (Baker et al., 2004; Loiola et al., 2019; Wu et al., 2016). Similar to what has been observed in mammals, microglia distribution and densities in adult female and male zebrafish brains are also sexually dimorphic. These differences could be driven by a higher colonization of microglia in the female brain earlier in development since, in vertebrates, immune cells are largely influenced by sex hormones and cell death events (Casano et al., 2016; Loiola et al., 2019; Schwarz et al., 2012; Tränkner et al., 2019; Weinhard et al., 2018; Xu et al., 2016). Since Chek2 is

contributing to differences in brain size and microglia are present at higher densities of in some female brain areas, this suggests that both cellular (microglia) and molecular (Chek2-mediated pathways) mechanisms account for the smaller size of the adult female brain and that organization of sex-specific structures happens after sex has been determined. Considering the ability of zebrafish to undergo neurogenesis throughout their lifetimes (Byrd & Brunjes, 1998; Grandel et al., 2006) – a capacity that seems to be restricted to specific regions of the brain (Oehlmann et al., 2004) – remodeling of structures and behavioral pathways could take place at a later stage in development or even adulthood, through mechanisms that may differ from other vertebrates including mammals.

In the context of sex reversal by loss of Bmp15 in the ovary, young mutant female brains may need to undergo the necessary changes that will allow for male-associated behaviors once reversal happens (Dranow et al., 2016). Masculinization of the *bmp15* mutant female could lead to exclusively molecular regulation of behavioral pathways with conservation of all other female brain features, or a broader cellular and morphological remodeling of sex-specific brain areas to achieve male-specific brain features. In the presence of microglia and without alteration of Chek2-dependent pathways, I found that all *bmp15* mutant adult brains resemble non-mutant direct-differentiating male brains, suggesting that sex-specific brain areas of the mutant fish that were initially female had to be remodeled into male-specific organization. Presumably, the shift of ovary to testes signals during reversal could influence brain regions that are susceptible to sexual hormones, affecting their cellular and molecular organization to reach the higher numbers of microglia cells and larger structure sizes observed in direct-differentiating males. However, ovary to testis sex reversal in *bmp15* mutants happens relatively early in the reproductive life of females and oocytes in these ovaries cannot complete oogenesis or reach mature stages (Bravo et al., 2023; Dranow et al., 2016; Zhai et al., 2023). Accordingly, the concentration and/or duration of mutant ovary signals may not be enough to contribute to specification of brain female morphologies and cellular distributions before masculinization occurs. Additionally, Bmp15 deficiency has an indirect effect on estrogen production (Zhai et al., 2023), and estrogens are known to modulate brain growth during development (Ahmed et al., 2008; Bridget M. Nugent et al., 2015). Together with the fact that *bmp15* mutant females change sex before reaching adulthood and therefore cannot be compared to direct-differentiated mutant male or wild-type female brains, it is difficult to establish if they ever develop female-specific morphologies and later need to undergo male-specific changes during reversal. Instead, *bmp15* mutant brains could directly acquire male-like characteristics in response to the switch of hormonal signals that accompanies masculinization of the ovary. It also remains to be elucidated if cell death and

microglia are required to develop male-specific behaviors in sex-reversed *bmp15* mutants – similar to the crucial role of macrophages during masculinization of the ovary (Bravo et al., 2023) – and whether there is an active mechanism of remodeling that takes place.

Interestingly, both Chek2-dependent pathways and microglia are essential for the establishment of sexual dimorphism in the zebrafish brain (differences in size and microglia density) but are not required for proper sex differentiation or reproductive behaviors, since fish lacking Chek2 or microglia develop and behave normally as females or males. This observation suggests that characterization of sex-specific behavioral pathways (e.g. mating) during development is either not regulated by Chek2-dependent pathways or microglia and needs to be specified by other regulators, or is redundantly regulated by Chek2, microglia and other cellular and/or molecular mechanisms. Accordingly, establishment of differences in the brain between the two sexes, which is not necessary for female and male specific behaviors, could drive other processes mediated by specific areas the brain that are susceptible to changes in hormonal signals (Figure 5.2). This dimorphic sensitivity, although not necessary for mating, could positively or negatively influence other physiological processes in the tissue, like responses to injury, inflammation, or aging.

Because zebrafish undergo sex determination independent of sex chromosomes (in laboratory strains) and later in development as compared to mammals (reviewed in (Aharon & Marlow, 2021)), gonads only release sex hormones and ovary/testes signals once sex differentiation starts. In the absence of germ cells, zebrafish gonads differentiate into sterile testis with gonadal supporting cells producing male-specific sex hormones, which then leads to normal development of male secondary sex traits and behaviors (Dai et al., 2023; Slanchev et al., 2005). Conversely, in mammals, development, maintenance, and remodeling of reproductive tissues are strongly influenced by peaks and changes in sex hormones that occur both in women and men at various timepoints throughout their lifetimes ((Ahmed et al., 2008; Mosconi et al., 2024) and reviewed in (Gegenhuber & Tollkuhn, 2020)). These shifts of hormones influence function of many tissues in the body, with the nervous and immune systems being the most susceptible (Bereshchenko, 2018; Klein et al., 2016; McCarthy et al., 2015; Sciarra et al., 2023). Many neuropsychiatric disorders that are sex biased appear or are often diagnosed near the timepoints when hormonal changes, like puberty, pregnancy, or menopause, take place (Craig & Murphy, 2007; He et al., 2024; Mathews et al., 2015). The susceptibility of the brain to hormonal changes (Ahmed et al., 2008; Geleta et al., 2024; McEwen & Milner, 2017; Pradhan & Olsson, 2015) – or to acute loss of signals that are abruptly disrupted (e.g. premature failure of the gonad) – could be

worsening or influencing the onset of sex biased neurological diseases. Understanding the mechanisms and roles of cell death and microglia during development and sex hormone-driven remodeling will help better describe their contributions to the physiology of tissues sensitive to gonadal signals, and their potential implications during other sex-biased biological processes.

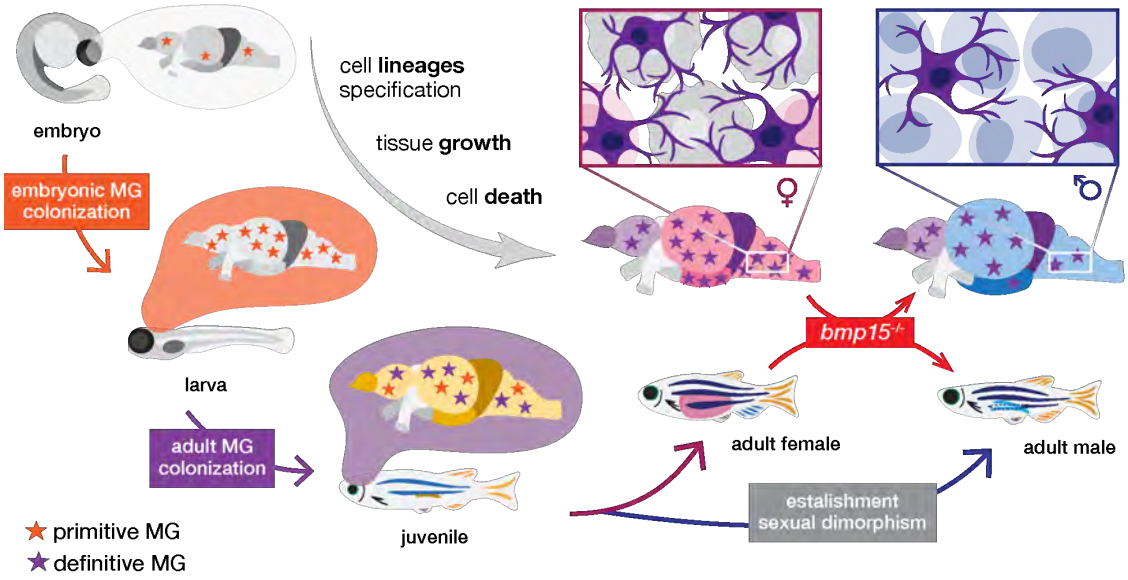


Figure 5.2: **Microglia contributions to the establishment of sexual dimorphism in the adult zebrafish brain.**

Distinct microglia colonization timepoints give rise to embryonic (primitive), and adult (definitive) waves of tissue resident macrophages. During initial developmental steps, the brain is patterned, grows, and different cell lineages are specified. At some point after sex determination takes place in the late juvenile stage (at around 28-35dpf), sex-specific traits in the female and male brains arise. These differences in the adult include variances in microglia density (females have higher density than males), and differences in brain structure sizes (male Te, OpT, and HT are bigger than in female). Contributions from both Chek2 and microglia are required for sexually dimorphic morphological and cellular features of female and male adult brains.

Final Discussion

This thesis aimed to bring together two anatomically and functionally distant, but molecularly connected tissues, and discover and describe some of their shared biological and

physiological roles. Sexual dimorphism is present in a variety of tissues and structures, some of which are strikingly more susceptible to sex hormones than others – the nervous and immune systems being highly influenced by the signals secreted by the gonad (Bereshchenko, 2018; Geleta et al., 2024; McCarthy et al., 2015; McEwen & Milner, 2017; Sciarra et al., 2023). All three players are modulated by each other's cellular and molecular states: the gonad and the brain are in constant communication with each other, and they both regulate and are regulated by the immune system, while immune cells are also responsive to changes in both tissues and can impact their functions (Caldwell et al., 2019; Gu et al., 2023; Lenz & McCarthy, 2015; Lenz & Nelson, 2018; Lenz et al., 2013; Li et al., 2021; Li et al., 2020; Li et al., 2012; Olah et al., 2020). The involvement of immune cells – specifically myeloid-derived lineages like macrophages – to the development and maintenance of the tissues in which they reside has gained interest in more recent studies, focused on describing TRM contributions to tissue organization and homeostasis (reviewed in (Mass et al., 2023; Varol et al., 2015; Wculek et al., 2021)). However, since they were initially defined as mostly immunogenic cells, much of their roles in tissue organization and specification are still not completely understood. How immune cells are regulated and influenced by the tissue, if that regulation may be redundant to other mechanisms to maintain tissue function, or to what extent their responses affect tissue physiology is not known. Understanding mechanisms of signaling between the different systems and describing the cell types and lineages involved in this communication could reveal how their interactions contribute to each other's biological states.

Macrophages in the gonad and microglia in the brain are both shown in this thesis to have essential but slightly different contributions to the tissue they reside in. In the ovary, macrophages are not required during development or organization of the gonad, since animals lacking tissue resident macrophages develop normally and without any sex bias (Li et al., 2011; Nynke Oosterhof et al., 2018). Interestingly, they play an essential and highly regulated role in the process of masculinization, as their activation by cells that express the Csf1R ligand, Csf1a, is required for ovary to testis transition during ovarian failure (Aims A and B/(Bravo et al., 2023)). Similarly, tissue resident macrophages are also dispensable during development and for the organization of behavioral pathways in the brain but, unlike in the gonad, also contribute to establishment of regional differences in brain volume between females and males (Aim C). To understand this distinction in the role of tissue resident macrophages within tissues (ovary/testis/brain) it is important to remember that, although the ovaries and testes are both gonadal tissues and have parallel functions, they are morphologically different. In zebrafish, early differentiation of the female or male gonadal supporting cells drives organization of the tissue (reviewed in (Aharon & Marlow, 2021)), leading to very distinct morphological, cellular (sperm vs oocytes), and molecular

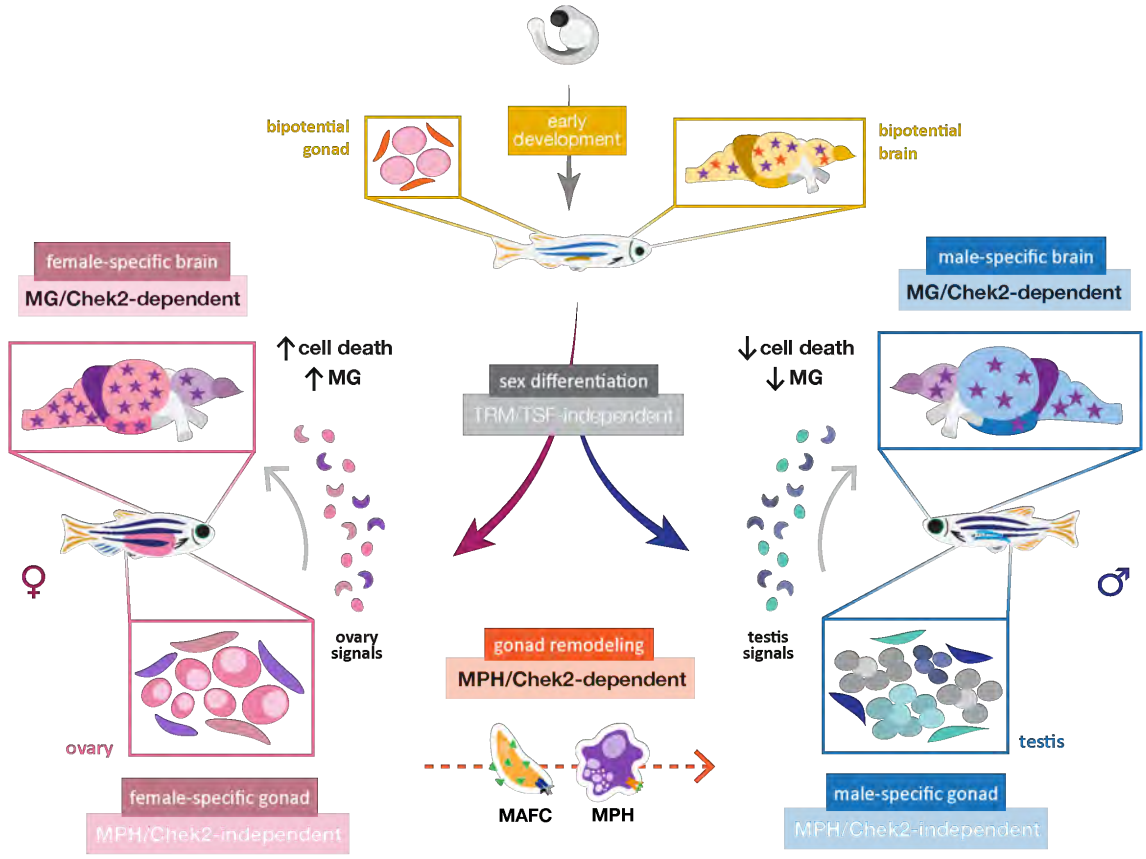
(testosterone vs estrogen synthesis) features. Although ovaries and testes arise from a common bipotential indeterminant tissue, complete specification of the gonad relies on many genetic and molecular factors (Aharon & Marlow, 2021; Kossack & Draper, 2019; Nagahama et al., 2021; Rodriguez-Mari & Postlethwait, 2011; Siegfried & Nusslein-Volhard, 2008). Macrophages are mostly ignored in this context since their ablation does not affect gonad or testis differentiation (Bravo et al., 2023; N. Oosterhof et al., 2018; Shiau et al., 2015). Conversely, the *female* and *male* brain are developmentally and functionally the same tissue and ultimately acquire regional sex-specific differences that drive female and male physiology and behaviors – through unknown mechanisms that my work suggests are dependent on microglia (Aim C). Because TRM are influenced by gonadal signals (Sciarra et al., 2023), formation of the ovary and testis could, via production of sex-specific hormones, trigger establishment of differences in the brain of females and males by modulating microglia's capacities in specific brain regions. Microglia roles in shaping neural synapses and glia-neuron interactions have been studied (Li et al., 2012; Smith & Bilbo, 2019; Thion et al., 2018), and together with recent interest in macrophage involvement during morphogenesis, this thesis work invites further exploration of potential TRM contributions during tissue reorganization. Additionally, definitive macrophages colonize tissues around the time when sex determination takes places, making this window of time uniquely suited to organize microglia distributions in brain areas involved in sex-specific traits/functions. In the gonad, definitive macrophages are first present at this time, with only primitive macrophages accounting for TRM in the tissue before then (N. Oosterhof et al., 2018). This would explain why definitive macrophages have such crucial contribution to the remodeling of the ovary, but not earlier during development of the tissue (Figure 5.3). However, it's still not known how definitive macrophages influence tissue physiology, if variation of their responses is regulated by differences in the signals or cell types, and whether those signals drive only activation or also expansion of macrophages within the tissue. Studying the transcriptional and molecular effects of tissue failure in TRM at a single-cell level could be useful to define the specific mechanisms that are activated during remodeling and if there are specialized subsets of macrophages that are specifically responsible for remodeling tissues.

Tissue resident macrophages are known to have different roles in immune response to infections, inflammatory response after injury, or clearing during tissue regeneration and remodeling (Caldwell et al., 2019; Cavone et al., 2021; Morales & Allende, 2019; Var & Byrd-Jacobs, 2020; Wu & Hirschi, 2021). Many of these biological processes involve cellular and molecular mechanisms, like cytokine signaling or phagocytosis, that are similar to those used to shape and organize a tissue during development (Giugliano et al., 2024; Wang et al., 2015). Additionally, the

highly dynamic states and sensitivity of immune cells to cellular cues and tissue condition make TRM perfect modulators during both specification and maintenance of tissues. Highly involved in the response of immune cells residing in a tissue are the resident cells of the tissue which can send and receive signals to TRM for their activation and modulation. In the ovary, activation of TRM during ovarian failure and masculinization is regulated by somatic gonadal prefollicle cells that expresses the macrophage receptor ligand Csf1a and are essential for the process (Bravo et al., 2023). Similarly, gonadal signals could influence cells in the brain, their distribution and states, overall priming the tissue towards a more female or male function. Conversely, they could act as modulators during specific biological events like inflammation, injury, or cell activation or expansion. This work discovered a previously undescribed germline-somatic-immune cell axis in the gonad with critical functions in the maintenance of ovarian cells fate and during remodeling (Bravo et al., 2023). Oocytes, prefollicle cells, and macrophages were found to be essential during ovary-to-testis transition, and so was communication between all of them, with signals being secreted and received as a way of informing about cellular and molecular states (Aims A and B/(Bravo et al., 2023)). In the brain, tissue-specific cell-types (neurons), supporting cell lineages (glia), and immune cells (microglia), are already known to interact with each other (Cunningham et al., 2013; Li et al., 2012; Wake et al., 2009), but if or how that communication is modulated or influenced by gonadal signals is not known. Hypothetically, if an axis like the one observed in the gonad to preserve sex-specific features is present in the brain, it would involve cell types that express sex hormone receptors. Microglia are perfect candidates since, like macrophages in the gonad, are largely influenced by sex hormones (Osborne et al., 2018; Velez-Perez et al., 2020), sensitive to changes in the tissue environment (Davalos et al., 2005; Svahn et al., 2013), and in contact with all other cell types in the tissue (Wake et al., 2009). Finally, although microglia are the only resident immune cells in nervous tissues, monocyte-derived macrophages are capable of colonize the brain in response to injury or inflammation (Carrier et al., 2024; Shechter & Schwartz, 2013; Sun et al., 2024). Accordingly, it remains to be confirmed whether microglia and/or circulating macrophages are the ones responsible for the establishment of sexually dimorphic features in the female and male brains, and their essential role – if any – during sex reversal. This could be done by ablating only microglia but no other macrophages in the body, or by ablation of all TRM and subsequent re-population of circulating macrophages.

Given their susceptibility to sex hormones (Nelson et al., 2019; Osborne et al., 2018; Velez-Perez et al., 2020), and their ability to regulate tissue homeostasis (Casano & Peri, 2015; Li & Barres, 2018; Matcovitch-Natan et al., 2016), microglia could act as sentinels to preserve proper tissue organization and function or to modulate cellular and molecular changes when needed.

Maintaining a female gonad is an active and continuous process and failure to sustain female-related fates leads to masculinization of the gonad in zebrafish (Dranow et al., 2016; Draper et al., 2007; Kurokawa et al., 2007; Liu et al., 2018; Winship et al., 2024; Zhai et al., 2023). In humans, this happens during aging processes like menopause, or in disorders in which ovarian function is disrupted prematurely (Benetti-Pinto et al., 2020; Burger et al., 2002). The drastic shift in female and male hormone levels affects different tissues in the body and, in zebrafish, results in transformation of the female into a male, independently of developmental stage (Beer & Draper, 2013; Dranow et al., 2016; Dranow et al., 2013). Since the brain is highly receptive to the state of the gonad and involved in many sex-specific functions, it is possible that maintaining a *female* or *male* brain also requires a constant and active input of information regarding the sex of the animal through a mechanism regulated by sex hormones and driven by microglia.

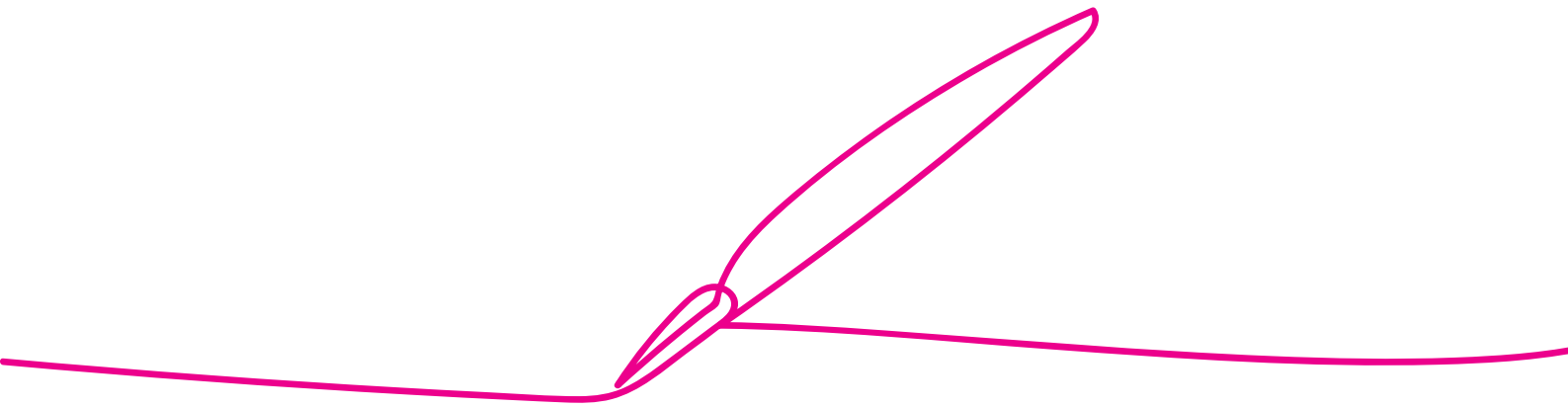


MAFC: macrophage-activating follice cell; **MPH:** macrophage; **MG:** microglia

Figure 5.3: **Model of sexually dimorphic tissues establishment and maintenance.**

With this work, I focused on understanding the mechanisms driving ovarian failure during sex reversal and the organization of the female and male adult brain after sex determination and differentiation, hoping to advance our understanding of the role of sex differences in these tissues in health and disease. I revealed fundamental cellular and molecular mechanisms underlying

remodeling of sexually dimorphic tissues and establishment of sex-specific traits and behaviors and confirmed the essential role of cell death pathways and immune cells in these processes. Correct function and maintenance of reproductive tissues requires proper sex differentiation of the gonad and aligned establishment of sex-specific structures and behavioral pathways in the brain (Lenz et al., 2013; Bridget M Nugent et al., 2015; Nugent et al., 2009; Pradhan & Olsson, 2018; Wright & McCarthy, 2009). For this reason, overall reproductive health depends not only on primary sex organs, e.g. ovary and testis, but also on other sexually dimorphic tissues and organizations that are essential for reproduction. The brain has a key role in regulation and control of the reproductive system (reviewed in (Okafor et al., 2022)), and it is also highly responsive to gonadal signals (McCarthy et al., 2015; McEwen & Milner, 2017; Pradhan & Olsson, 2015). Because of that, disorders in organs and tissues involved in reproduction should be studied under the assumption that sexual dimorphism could play a protective role or promote susceptibility to some diseases. Understanding the differences between female and male reproductive tissues and how they are established can potentially help determine the underlying causes of disorders that show strong sex bias. Additionally, defining the essential factors and players of organ-specific functions during homeostasis could be useful to ameliorate dysfunctional states and disease. Reproduction and behavior are only two of the many roles of sexual dimorphism in the animal body. I aimed to highlight in this thesis the importance of this exceptional feature, and to stress the need to always consider it when studying biological and medical conditions with sex disparities that disproportionately affect one sex or another.



CHAPTER 6

Conclusions

The main conclusions of this thesis work can be summarized as follow:

- Bmp15 promotes follicle survival and cell fates required for follicle development.
- A novel population of ovarian prefollicle cells that express macrophage activating ligands is essential during ovary-to-testis transition.
- Definitive macrophages are key cell types activated by Csf1 ligand-expressing cells during masculinization of the ovary.
- Sex-specific morphologies and structures in the adult brain exist in zebrafish and are organized after gonadal differentiation.
- Sexual dimorphism in the adult brain is in part driven by differences in cell death regulation between sexes.
- Microglia colonization of the female and male zebrafish brains is sexually dimorphic.
- Establishing female and male differences in regions of the brain involved in sex-specific behaviors requires microglia.
- Adult brains of female-to-male reversed fish by loss of Bmp15 likely undergo morphological changes after sex-reversal to resemble direct-differentiating male brain region.

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Illustrations statement

All schematics and illustrations throughout this thesis are original and have been designed and created by the author.

Thesis submission: September 2024