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ISBN

9798247994688

Author

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Publication Date

2026-06-03

Peer reviewed|Thesis/dissertation

Establishing the Fundamental Role of Somatic Cells in Age-Related Reproductive Fitness Decline

by
Emily Allyson Wolfgram

DISSERTATION

Submitted in partial satisfaction of the requirements for degree of
DOCTOR OF PHILOSOPHY

in

Developmental and Stem Cell Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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by

Emily Allyson Wolfgram

Dedication

To Mom and Dad: Thank you for your endless support and for teaching me that I can do anything. I wouldn't be here without you.

Acknowledgements

What an opportunity this has been. I feel incredibly fortunate to have had the ability to do science at an extraordinary institution, surrounded by fantastic mentors, peers, family, and loved ones. Isaac Newton said, “If I have seen further than others, it is by standing on the shoulders of giants.” I have always carried this sentiment with me, reminding myself that so much of where I am now, and where I will continue to go, is because of the giants who raised me, guided me, and supported me. I am deeply grateful for the opportunity to thank all of you. This journey has not been supported by just one village, but by many, many villages from every aspect of my life. It is very hard to hear doubt when you have so many people cheering for you. To each and every one of you, thank you for being the loudest and most wonderful cheer squad.

Dear Dr. Todd Nystul, thank you for your unwavering mentorship over so many years. Thank you for bringing me into the lab through the Summer Research Training Program in 2018 and for continuing to support me in countless ways every day since. You have taught me what being an exceptional mentor looks like. My biggest piece of advice to incoming graduate students is to choose a lab based on mentorship, and I can say without a shadow of a doubt that I received some of the very best mentorship in your lab. I distinctly remember after joining the lab in the first year of this PhD, thinking how it felt a bit daunting that there were so many years ahead. But now as the days in lab are numbered, I am genuinely going to miss seeing the green “Center for Reproductive Sciences” sign that greets me each day. Thank you for providing me with this home to do science. Thank you for the wealth of scientific knowledge you have instilled in me and for constantly challenging me to be the best scientist I can be. Thank you for having so much

faith in me as I took on a completely new direction in my project. You gave me remarkable autonomy to explore what excited me most. You encouraged me to try new things and allowed me tremendous creative freedom, even when you probably knew an experiment wasn't going to pan out. Rather than simply telling me what to do, you taught me how to shape a project by providing guidance, direction, and trust. Thank you for always making me feel heard. Since day one, I have never once felt discounted for asking a question or proposing an idea. The certainty of your belief in me has given me confidence as I move into other scientific spaces, and the value of that cannot be understated. Thank you for supporting every career direction I have considered over the years and for always being ready to prepare me for whatever I wanted to do next. Thank you for teaching me not only how to be a scientist in the lab, but also every other aspect of the scientific career. There is no course on "how to run a lab someday," yet you were always willing to talk openly about funding, mentorship, building a lab, and everything in between. I will carry with me everything you taught me in this lab, about rigorous science, critical thinking, mentorship, and navigating the academic landscape. Thank you for the countless hours of teaching, reading, editing, scientific discourse, and advice. There are truly not enough ways to say thank you for everything I have learned from you.

Dear members of the Nystul lab, both past and present, thank you for the endless support, feedback, and advice throughout this journey. I feel so lucky to have worked in an environment that I truly enjoyed day in and day out, and that is entirely thanks to all of you. To my first Nystul lab mentor, Dr. Katja Rust, thank you for teaching me the joy of studying the *Drosophila* ovary and for sharing the many techniques that carried me through my PhD. You introduced me to the concepts of fly husbandry, dissections, and

sequencing analysis, and I hope I have continued to pass these tools down as thoughtfully as you taught them to me. To Vanessa Huynh, thank you for all of the technical support throughout this project and allowing me the opportunity to be your mentor. To past Nystul lab members Harnoor Virk, Adriana Guajardo, Andreana Gomez, and Lewis Hendrianto, thank you for the role each of you played in helping me grow as both a scientist and a mentor. You each brought such unique perspectives on science and life that I continue to carry with me. To Rhiannon Roarke and Cristy Mendoza, thank you for seeing me through the final stretch of this journey. You cheered me on through every small and big milestone. Thank you for reminding me what belly-aching laughter feels like and bringing me so much joy in the last couple of years. To all of you, thank you for the countless coffee runs, the lab yaps, the in-depth scientific discussions, and all of your support along the way. I look forward to being a front-row cheerleader for all of your successes!

Dear Dr. Nathaniel Paul Meyer, I cannot say thank you enough for everything you have done for me. You have been the most incredible mentor, friend, and confidant throughout this PhD. From being my recruitment host, to helping me build a bookshelf with what felt like 500 IKEA pieces, to inviting me into your DnD group, you made UCSF feel immediately welcoming. Thank you for the many drives back and forth from Parnassus to Tidelands, which were filled with laughs, bonding, scientific discussions, and gentle guidance as I navigated my first year of graduate school. Thank you for always being there, from helping me find a rotation lab at the beginning to navigating career options and assembling this dissertation at the end. It is so rare to find such an all-around incredible mentor and friend. I have no doubt that we will continue to share our journeys with one another as we move forward along our paths in science.

To my committee members, Dr. Diana Laird and Dr. Leanne Jones, thank you for your support and mentorship throughout this journey. Both of you have played pivotal roles in guiding me from my qualifying exam through my dissertation. You have devoted your time to reading my work, listening to my presentations, and providing invaluable insights into my project. My qualifying exam and committee meetings have genuinely been highlights of my PhD. I have learned so much from you both, particularly in the field of aging. Thank you to the additional members of my qualifying exam committee, Dr. Barbara Panning and Dr. Licia Selleri. My qualifying exam closely aligned with the work presented in this dissertation, and I am grateful for your insightful feedback during the early stages of this project, which helped shape its development. Beyond your scientific guidance, you all have been pillars in my career development, and I truly believe I have had the best qualifying exam and thesis committees, and the best mentors here at UCSF.

Thank you to the UCSF Bakar Aging Research Institute, the National Science Foundation, the Eunice Kennedy Shriver National Institute of Child Health and Human Development, and the National Institute of General Medical Sciences for the funding and support that made this research possible.

Dear UCSF, the DSCB program, and the 2020 cohort, thank you for providing a space where I could grow and develop as a scientist. From my time at UCSF in the Summer Research Training Program in 2018, this place has continued to feel like home. The small, student-focused nature of the DSCB program gave me a true platform to thrive and grow. Thank you to all of the DSCB faculty and fellow students for the spontaneous elevator chats and the insightful feedback and discussions at each annual retreat and symposium. To the 2020 cohort, I feel so grateful to be part of such an incredible group

of scientists. I look forward to seeing where we all go from here and feel fortunate to have such a brilliant, supportive network of friends and colleagues.

Dear Dr. Stephanie Gupton, I don't know how to thank you enough for being my introduction to research and for starting me out on this incredible journey. I attribute so much of my foundation as a scientist to you, Dr. Fabio Urbina, and the entire Gupton lab. You challenged me and pushed me to continually do better. You taught me what it means to conduct rigorous and creative science, how to speak the language of science, how to present my work, and how to write my first grant. You encouraged me to explore new ideas and follow my passions. Thank you for challenging me and always believing in my ability to rise to that challenge. Finally, thank you for your continued mentorship beyond my time in the lab. I feel so proud to forever be an alumnus of the Gupton lab.

To my Marine Biological Laboratory (MBL) Physiology family, it is difficult to put into words how much I appreciate all of you. My summer at the MBL in 2024 came at the perfect time to reignite my passion for creative scientific exploration. It was a summer like no other, an experience that can only be fully understood by those who have shared it. My scientific network grew tremendously, but even more so did my joy for exploring the fascinating biology all around us. The Physiology course taught me to “just try it” and to pursue unique results, which often lead to the most interesting discoveries. Thank you to Maya Waarts and Carolin Klose for being the best “troublesome trio” partners, and to Tamas Nagy for encouraging me to explore both in the lab and out on the running trails. Thank you to Dr. Cliff Brangwynne and Dr. Amy Gladfelter for being incredible co-directors for our course. I learned so much from you both, as well as from all of the remarkable speakers and faculty you brought into the program. To Dr. Amy Gladfelter

and Dr. Zach Geisterfer, thank you for allowing me to continue exploring non-model biology in the *Physarum polycephalum* project. You have both taught me to learn from the biological processes of unique species to better understand our world.

Dear Keara McLean, thank you for being you and for being the absolute best friend I could ever dream of. “Best friend” doesn’t even begin to capture what you mean to me. You are sunshine, comfort, laughter, and everything good in the world. I feel so incredibly fortunate that we were placed in the same orientation group before college, and that from that moment onward we have shared so much of our lives together. From late-night talks and study sessions to celebrating milestones and navigating challenges, your presence has been a constant source of joy and strength. Your light and laughter brighten every room, and your empathy and honesty make every conversation meaningful. Thank you for loving and accepting me exactly as I am, for showing me what unconditional love and unwavering support truly feel like. Whether we are talking for hours, sharing our dreams and fears, or sitting in comfortable silence, I always feel understood and at peace. You have cheered me on through every challenge, celebrated every success, and helped me find hope even when things felt overwhelming. You have taught me what true sisterhood feels like. Thank you for your humor, your loyalty, your fierce encouragement, and your ability to make even the hardest days lighter. I cannot imagine navigating college, graduate school, or life without you.

Dear Sarah Stephens, thank you for being my very first friend in college. I am so glad we happened to sit next to each other on a park bench at admitted students’ day, and that I didn’t entirely freak you out as I danced around avoiding a bee. As roommates through freshman year, you saw first-hand how I navigated classes, college life, and my

first research lab experience. Thank you for the countless laughs, long study hours, late nights, and memories we shared. I feel so fortunate that, after all these years, we have remained so close. Thank you for always believing in my dreams and supporting my pursuit of a career in science.

Dear my La Porte family, thank you for the many years of fun and adventures. Thank you for the hundreds of hours out on the lake, the snowmobile rides through the mountains, and the late-night dance parties. La Porte has and always will be my second home, a place to escape from work and the city, to recharge, and to be fully myself. I feel so fortunate to have grown up with such a huge, extended family of friends who have loved and supported me since childhood. You have watched me grow up and cheered me on at every step of my life. I know I can always count on each of you. How rare is that, to have a community like this in your life? There are far too many of you to list, which in itself shows just how lucky I am. Thank you to every one of you who makes up my La Porte family, for the laughter, the adventures, the memories, and for always making me feel at home.

Dear Merri Pittman, thank you for providing me a home in San Francisco, not just four walls, but a place that truly felt like home. Thank you for introducing me to triathlon and for pulling me through so many training days, for the long rides and runs we shared, and for making even the toughest workouts fun. This sport has given me so much, and it is all thanks to you. Thank you for the countless times you checked on and watched Archie, you are definitely his biggest fan! Thank you for all the hallway chats, the errands together, and the moments of comfort and joy that made living in your house feel like a

home. Your generosity, friendship, and support have meant more than words can capture, and I am forever grateful to you for opening your home to me.

Dear Leslie Ausburn, Elisabeth Starr, Joseph Dinh, my coach John Dahlz (JD), and the rest of the San Francisco Triathlon Club, thank you for being the all-around best. You all have supported me in more ways than you know. So many of my core memories of my time in San Francisco come from Tuesday track workouts, Friday swims, and Saturday long rides. This community has given me so much more than just workouts, you have been there to pull me out of work slumps, cheer during the trenches of a race, and yap together to make the longer training days feel much shorter. Thank you for the Type 2 fun we've shared, the tough workouts, the windy rides, and the early mornings that seemed impossible at the time but are now some of my fondest memories. You are what will make saying goodbye to San Francisco so hard. Truly, thank you for giving me that, and for being such an incredible community to grow, push, and laugh with.

Dear my dog, Archie (or Lil Mans or Bubbas), thank you for being the absolute most lovable doofus. I look forward to your excitement every day when I come home, and your wagging tail has gotten me through more stressful experiments and frustrating data analyses than I can count. Thank you for the many evenings you have spent curled up next to me on the couch as I worked, you made even the hardest days feel lighter. Thank you for reminding me to step outside, take a breath, and find joy in the little moments. If anyone were to ask what getting a PhD is like, I think Archie would have the most up-close answer, and I'm so grateful he was there for every part of it. To my first dog, Siller, I know your life was short, but thank you for giving me so much love, comfort, and joy. You were a constant source of companionship during the COVID-filled first year of

graduate school. I hope you are running around puppy paradise with as many toys as your heart desires—you will always have a special place in my heart.

To my big brother, Josh McNeill, and my sister-in-law, Katie McNeill, thank you for always being there for me and for always listening when I need someone to talk to. Josh, your humor and steady support have been a constant source of strength throughout my life and particularly during the ups and downs of graduate school. Katie, your encouragement and your ability to make everyone feel seen has meant so much to me, and I feel incredibly lucky to have you both in my life. I know that no matter where I am, I can always come to you and receive love, laughter, and an expertly curated collection of memes to brighten my day. To my niece, Elliott McNeill, thank you for giving me the joy of watching you grow up. Witnessing your excitement as you learn the scientific principles that govern the world, things I often take for granted, has reminded me to stay curious and appreciate the wonder in the everyday. Your infectious joy and laugh never fail to lift my spirits. The calls and voice texts I receive from you always brighten up my day. Being your auntie is one of my most favorite titles, and I feel so lucky to be part of your life as you grow, explore, and delight in the world around you.

To Linda McNeill (Gramcracker), thank you for your endless support throughout my entire life. Thank you for always playing Dancing Queen when I got into your car and letting me sing at the top of my lungs without hesitation. Thank you for attending nearly every volleyball tournament, sitting on the most uncomfortable chairs for hours on end, and recording my games so I could study and improve. Thank you for always showing up for me, at school activities, graduations, holidays, and every milestone in between. Your encouragement, your pride in my accomplishments, and the love you express so freely

have meant the world to me. Beyond the big moments, thank you for the small ones too: sharing your life stories, telling me about my childhood, and talking about the future. I am so lucky to have you in my life, and your presence has made every part of my journey richer and more joyful.

Dear Steven Howard, thank you for being both my rock and my soft place to land. Thank you for the sense of calm and security you bring, even when I am stressed and frazzled. Hearing your voice and seeing your smile (and dimples) never fails to brighten my day. You are the most driven person I know, and your dedication inspires me to be the best version of myself. Thank you for showing up in all the big ways—like being open to wherever our next chapter takes us so I can pursue this scientific journey—and in the “little” ways that really aren't little at all, like surprising me with coffee before an interview. You have shown up for me every single day, and I cannot thank you enough for that. Your unwavering belief in me has given me the confidence to chase my biggest dreams, knowing that no matter what, I will always have your open arms waiting for me. Thank you for everything that you are. You are my favorite part of every day. It's the everyday moments, the shared laughs, and your thoughtful presence that make being with you so extraordinary. I have come to learn that I have many little quirks, and you somehow seem to know and anticipate every single one of them. I never knew that someone could understand me so completely and support me so wholeheartedly. I am so grateful for the adventures we've already shared and look forward to many years of travel and exploration side by side, with a primary mission of finding the best gluten-free treats wherever we go. I thank the lucky stars, fate, and some incredible reverse-parent trapping that brought us

together. I don't think there will ever be enough days, months, or years with you. I love you.

To my parents, Jim and Julie Wolfgram, thank you. A million times over, thank you. Thank you for creating a home overflowing with love, laughter, and unwavering support. Thank you for giving me unconditional love, and for giving me the freedom to learn, grow, and even fail, knowing that you would always be there to pick me up and say, "try again." From my earliest years, you encouraged me to explore the world, to ask questions about everything around me, and to seek answers, sometimes helping me find those answers, and sometimes nudging me to go look for them myself. That foundation of curiosity and wonder has been at the heart of my drive in science. You have always challenged me to be my best, instilling in me the drive to reach for new heights and pursue my dreams. You didn't just tell me that hard work pays off, you showed it to me through your actions, day in and day out. You raised me to be determined and confident in my abilities, yet humble and always open to learning more. Thank you for never hesitating to encourage my biggest aspirations and for providing every resource I needed to pursue them. You taught me that perseverance is as important as curiosity, that questions are to be celebrated, and that the process of learning is just as meaningful as the result. You also taught me that hard work doesn't mean we can't have fun, and you have filled my life with joy, adventure, and countless memories, from the everyday moments of dinner with sports on, to the weekends in La Porte, to the family vacations (and many games of Brain Quest along the way). Thank you for showing up for me over and over, staying up while I finished homework, traveling countless hours for volleyball or triathlon, and showing up for every other challenge I took on. You have always been my biggest supporters, my #1 fans,

cheering for me proudly at the front of the crowd. I hope you both know that I am who I am because of you. I am so, so proud to be your daughter.

To my dad, thank you for instilling in me a curiosity to figure out how things work, what makes something “tick.” Thank you for going above and beyond for my elementary school science fairs, where building the citrus clock, the water bottle windmill, and the mousetrap race car were my first real science experiments. I carry that same curiosity into the lab every day. Beyond science, you also taught me to approach life with curiosity, to think critically, and to tackle challenges with determination and confidence. You applied that same logical and precise approach to the sports and activities we did together, teaching me to wakeboard, wake surf, and snowmobile with careful strategy, attention to detail, and patience. Those lessons in problem-solving, persistence, and technique extended far beyond the water and snow, shaping the way I approach every challenge in life.

To my mom, thank you for the countless hours you devoted as I navigated school, sports, and every new hobby I decided to pick up. I don’t know how you did it all, but you went above and beyond to make sure I had every tool to succeed in whatever I set my mind to. Thank you for going to my fifth-grade teacher and insisting she couldn’t limit the number of questions I asked in a day. You have always stood up for me, but most importantly, you taught me how to stand up for myself. You have challenged me to be my best, pushing me to grow, to think critically, and to never settle for less than I am capable of. I am endlessly grateful for the countless long conversations we’ve shared, where you listened, gave honest and insightful advice, and encouraged me to consider new perspectives. The bond we share is unique and irreplaceable. I am so grateful not just for

what you have done for me, but for who you are—your strength, your wisdom, your boldness, and your heart. Thank you for being my mom, my mentor, and my forever home.

There will never be enough words to convey how lucky and grateful I am to have such extraordinary parents. Your unwavering support has carried me through my whole life, and through this PhD. Thank you for answering endless phone calls as I navigated this journey, for celebrating my wins, comforting me on the hard days, and continually reminding me that I could do this. I may have left the house when I went off to college, but I have never left home. Home will always be with you both, in the lessons you taught me, the love you gave me, and the example of the people you are. I love you both.

To all the giants in my life, those I have named here and those whose support has shaped me from afar, thank you. Your guidance, love, and support have carried me through every step of this journey, reminding me that no journey is traveled alone.

Contributions

The work in this dissertation was conducted under the supervision of Dr. Todd G. Nystul. Further guidance and insight came from thesis committee members, Dr. Diana Laird and Dr. Leanne Jones.

Chapters 1 and 2 were adapted from the following manuscript available on bioRxiv at the time of this writing:

Emily A. Wolfgram & Todd G. Nystul. (2026). A Decline in Follicle Cell Function is a Major Driver of *Drosophila* Ovarian Aging.

Chapter 3 was written by Emily Allyson Wolfgram.

Establishing the Fundamental Role of Somatic Cells in Age-Related Reproductive Fitness Decline

Emily Allyson Wolfgram

Abstract

Aging is defined by a progressive deterioration of tissue and organ functionality during adulthood. Across species from invertebrates to mammals, the female reproductive system has consistently been found to be one of the first organs to lose functionality with age. While oocytes have long been assumed to be the primary cells affected by aging, growing evidence points to ovarian somatic cells as key contributors, though the molecular mechanisms driving their aging remain poorly understood. In the *Drosophila* ovary, a layer of somatic follicle cells surrounds each germ-cell cyst. These follicle cells are essential for guiding and supporting germ-cell cyst development into a mature oocyte. We found that aging is characterized by an accumulation of phenotypes in the somatic compartment, including failure of the follicle cells to encapsulate germ-cell cysts, an extended S phase, and increased DNA damage. These follicle encapsulation defects are associated with the lack of a germ-cell cyst checkpoint in early oogenesis. Single-cell RNA sequencing revealed that, across all cell types in the ovary, cells in the follicle lineage have the highest number of differentially expressed genes. Overexpression of *Atg8a*, a key autophagy machinery gene homologous to mammalian LC3, specifically in follicle cells prevents age-associated decline in the follicle epithelium

and loss of reproductive capacity. Collectively, these findings demonstrate that genetic manipulation of a small population of ovarian somatic cells is sufficient to improve both cell-autonomous and non-autonomous features of reproductive aging.

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List of Abbreviations

ATG	Autophagy Related Genes
BDSC	Bloomington <i>Drosophila</i> Stock Center
DSHB	Developmental Studies Hybridoma Bank
CC	Cap Cell
DEG	Differentially Expressed Gene
EC	Escort Cell
FC	Follicle Cell
FSC	Follicle Stem Cell
GC	Germ-Cell Cyst
GSC	Germline Stem Cell
MB	Main Body Follicle Cell
pFC	Prefollicle Cell
scRNAseq	Single-Cell RNA Sequencing
TF	Terminal Filament
UMAP	Uniform Manifold Approximation and Projection

Chapter 1: *Drosophila* Ovarian Aging

1.1 *Drosophila* ovary composition

While ovarian morphology varies between species, the mechanisms governing oogenesis are highly conserved. From *Drosophila* to higher-order mammals, oogenesis involves the progression of early germ cells into fully developed eggs [1]. There are broad similarities in organ morphology, with many direct parallels between cell types and stages of oogenesis, as well as several conserved features at the molecular and cellular level. Both *Drosophila* and mammalian ovaries have germ cells that undergo multiple rounds of incomplete division, forming multicellular cysts [2]. In the somatic compartment, the follicle cells in the *Drosophila* ovary functionally parallel the granulosa cells in the mammalian ovary. Both follicle cells and granulosa cells directly contact germ cells and proliferate during oogenesis to maintain a single continuous layer that forms the surface of the growing follicle [3]. In both *Drosophila* and mammalian ovaries, these cells help guide the process of oogenesis via direct cell-cell communication with germ cells through gap junctions [4,5] and the production of endocrine signals that coordinate the progression of germ cell development with the actions of other cell types [6,7].

The *Drosophila* ovary is comprised of ~16 strands of ovarioles, with a germarium at the anterior end of each ovariole. The germarium is where oogenesis begins and is host to both the germline stem cells (GSCs) and follicle stem cells (FSCs). At the anterior tip of the germarium reside the GSCs, which asymmetrically divide to self-renew and produce a germ cell. Each germ cell undergoes four rounds of incomplete mitosis, producing a 16-cell germline cyst that moves posteriorly into the germarium at a position called the Region 2a/2b border. At this position in the germarium, a population of follicle stem cells (FSCs), supported by an escort cell (EC) niche, asymmetrically divide to self-

renew and produce pre-follicle cells (pFCs) [8,9]. These pFCs will continue to divide and differentiate into polar cells, stalk cells, and main body (MB) follicle cells. When a germline cyst reaches region 3 of the germarium, it buds off, encapsulated by MB follicle cells, and is connected to other encapsulated cysts via stalk cells. The germline cyst continues to grow until one cell is selected as the egg, the other 15 cells becoming nurse cells that support the developing egg. Follicle cells continue to divide and grow to maintain encapsulation of the developing cyst, prior to full egg development [10]. There are only 2-4 FSCs per ovariole, and yet each ovariole contains several thousand follicle cells, so FSC daughter cells undergo up to eight transit-amplifying divisions before terminally differentiating to meet this demand [11,12]. Given the continuous demand for follicle cells to encapsulate and guide the germline cyst through oogenesis, FSCs and pFCs are among the most proliferative cells in adult *Drosophila* [11]. Any stressors that impair FSC proliferation and differentiation could have severe consequences for fertility. Indeed, genetic manipulations that disrupt FSC activity often result in significant morphological defects and, in some cases, sterility. This makes the FSC lineage particularly susceptible to replicative stress and may make it more vulnerable to other insults that are known to increase with age including stem cell maintenance, reactive oxygen species production, mitochondrial dynamics, and autophagy regulation [13].

1.2 Reproductive aging

Aging is an inevitable biological process that leads to the decline of cellular functionality [13]. Among the organs affected, the ovary is one of the first to lose function, with human females reaching menopause at an average age of 51 [14]. The average lifespan of wildtype *Drosophila* is approximately 70 days [15], yet fertility begins to

decrease after the first week of adulthood and drops below 10% of the maximum rate by 50 days [16]. The causes of this decrease in fertility are not fully understood but, in *Drosophila*, as in mammals, it involves a decline in germ cell number and germ cell-niche interactions, which are strongly influenced by nutrition and stress signaling pathways, along with marked changes in the transcriptional profile [17–28]. Likewise, emerging evidence from studies of both *Drosophila* and mammals is beginning to elucidate the important role of ovarian somatic cell aging in the loss of fertility [26–30].

Mammalian reproductive aging is associated with a marked loss of primordial follicles. In humans, the initial pool of follicles is estimated to be ~6-7 million during fetal development. Throughout fetal development, there is a massive wave of follicle atresia that decreases the ovarian reserve to ~1-2 million oocytes at birth. At the time of menopause, around age 50, there are ~1000 follicles remaining [31,32]. The loss of the ovarian reserve is also coupled with a progressive decline in follicle integrity across the lifespan, characterized by impaired somatic support, increased atresia, and reduced oocyte quality [32]. In the *Drosophila* ovary, GSCs persist throughout the adult lifespan and retain the capacity to produce germ-cell cysts. However, aging is marked by a progressive decline in GSC-niche interactions, leading to reduced cell division rates and decreased germ-cell cyst production [19,20]. GSCs exhibit decreased adhesion to their supporting niche of cap cells (CCs) and reduced responsiveness to key self-renewal signals, such as decapentaplegic (*dpp*), the fly homolog of BMP [19,33]. This *dpp* signal is produced by the somatic CCs that function as a niche for the GSCs, highlighting how aging in a somatic tissue can directly impact germline function [33]. These age-associated

changes do not result in the complete loss of GSCs but instead leads to a gradual decline in germline output, and ultimately oocyte production.

Initial studies in the field largely focused on germline-intrinsic defects, particularly the dramatic increase in aneuploidy observed in oocytes from aged mothers [34–36]. While these studies established that germ cell quality declines with age, they did not fully account for the coordinated, tissue-level nature of oogenesis. Reproductive aging is characterized by a number of molecular and cellular changes, often described as hallmarks, which include proteostasis decline, genomic instability, mitochondrial dysfunction, and altered intercellular communication [13]. More recently, attention has shifted toward the somatic environment in which oocytes develop, revealing an essential role for somatic support cells in shaping reproductive aging trajectories in both *Drosophila* and mammals [26–28,37].

Advances in single-cell RNA sequencing and ATAC-sequencing have enabled high-resolution analyses of aging ovaries, uncovering extensive transcriptional and chromatin changes across multiple cell types. Notably, these studies have repeatedly identified somatic compartments as exhibiting some of the most pronounced and regulatory age-associated differences [26–28,37]. Rather than acting as passive scaffolds, somatic cells emerge as dynamic regulators of germline development whose functional decline may precede or even drive reproductive failure. These findings have prompted growing interest in targeting somatic compartments as a means to extend reproductive lifespan.

Successful oogenesis requires precise coordination across multiple cell types within the ovary. As germ cells grow in size, differentiate, and move through the tissue,

somatic cells must dynamically remodel, provide metabolic and hormonal support, and relay developmental signals [38–40]. In the mammalian ovary, primordial follicles are encapsulated by granulosa cells, followed by a thecal layer, as it grows into a follicle prior to ovulation [41]. In the *Drosophila* ovary, germ-cell cysts become encapsulated by a single-layer follicle epithelium, with polar cells indicating the A-P axis and stalk cells connecting germ-cell cysts to one another [1]. These processes depend on continuous and bidirectional communication between germline and somatic compartments. Given this extensive interdependence, reproductive aging may reflect a breakdown in cellular dialogue rather than isolated defects within individual cell types. However, it remains unclear how age-associated stressors, such as oxidative stress or environmental insults, are sensed within the ovary and whether damage in one compartment is communicated to, or amplified by, the other. Understanding how these signaling networks deteriorate with age remains a major gap in the field.

Somatic cells are not merely permissive, but active regulators whose aging may define the pace of reproductive decline. In mammals, granulosa cells play a central role in oogenesis by providing metabolic support and producing estrogen, a steroid hormone essential for follicle development and fertility [42,43]. Loss of estrogen production is a key indicator of menopause and is tightly associated with the cessation of reproductive capacity [44,45]. In *Drosophila*, an analogous role is played by follicle cells, which produce the steroid hormone ecdysone [6,46]. While ecdysone is known to influence multiple aspects of oogenesis, how this hormone communicates with the germline and how such signaling changes with age remain poorly understood. Whether age-associated defects

in steroid hormone production, signaling, or responsiveness contribute causally to reproductive aging is an open question.

Beyond fertility itself, reproductive aging has profound implications for overall health. Aging is the strongest risk factor for chronic disease, and earlier entry into menopause is correlated with increased risks of cardiovascular disease and earlier mortality [47,48]. At the same time, demographic trends indicate that women are increasingly delaying pregnancy, intensifying the need to understand and potentially extend reproductive healthspan [49]. Despite this growing need, there are currently no clinical strategies to delay menopause or preserve reproductive function with age. A comprehensive understanding of how the ovary functions as an integrated tissue, comprising both germline and somatic compartments, and how this coordination deteriorates over time is therefore essential. Elucidating the mechanisms by which somatic aging, intercellular communication, and stress responses contribute to reproductive decline may provide critical insights into strategies for delaying reproductive aging and extending reproductive longevity.

1.3 Focus of this dissertation

In this study, we conducted a phenotypic analysis of aged ovaries and identified cellular and tissue-scale aberrations in the typically well-organized layer of follicle cells. In addition, we performed single-cell RNA sequencing and found a particularly high number of transcriptional differences in the FSC lineage between young and aged ovarioles. We identified *Atg8a*, an upstream effector in the autophagy pathway, as a key regulator of follicle cell aging and reproductive capacity. These results highlight a new

avenue for extending reproductive longevity through manipulation of the somatic lineage rather than the germline, which is ultimately passed on to offspring.

**Chapter 2: A Decline in Follicle Cell Function is a Major
Driver of *Drosophila* Ovarian Aging**

2.1 Establishment of follicle cell phenotypes associated with aging.

To establish hallmarks of follicle cell aging, we assayed for phenotypes in the follicle epithelium at weekly intervals from one to six weeks post eclosion in ovarioles from *w1118(x)* flies. In healthy ovarioles, follicles bud from the germarium in a single file, are surrounded by an intact, single-layered follicle epithelium, and are separated from one another by a single row of stalk cells (Figure 2.1A,C). Ovarioles with follicles that deviated from this pattern were scored as having a phenotype. The frequency of ovarioles with these phenotypes progressively increased over the six-week time course (Figure 2.1D-E). We therefore designated the one-week old ovary as the “young” condition and six-week-old ovary as the “aged” condition. Qualitative assessment of these follicle cell phenotypes produced four categories (Figure 2.1F, Supplementary Figure 2.1A-D). The two most prominent categories were epithelial gapping, where follicle cells, identified by the marker Fasciclin3 (Fas3), are absent from the perimeter of the germarium or developing follicle (Figure 2.1D, yellow short-dashed line), and epithelial dysregulation, in which the follicle cells were misshapen or failed to form into a single-layered epithelium (Figure 2.1D, white long-dashed box). We also observed Fas3⁺ cells with a rounded morphology consistent with epithelial cell death (Figure 2.1D, white long-dashed box), and a small number of instances of impaired stalk development (Figure 2.1D’-D’’, white arrows).

The loss of follicle cell integrity in aged germaria prompted us to investigate whether another follicle cell adhesion protein, N-cadherin (N-cad) is also diminished with age. N-cad is highly expressed in prefollicle cells and main body follicle cells from Stage 1 to approximately Stage 5 [10]. N-cad mediates cell adhesion through homotypic

interactions at the apical-lateral surface of the cells [25], creating a characteristic “ring” of N-cad signal between follicle cells and germ cells (Figure 2.1G, G’). We found that this N-cad ring is frequently lost in aged germlaria (Figure 2.1H, H’, I). In contrast, we found no significant difference between young and aged germlaria in the level of Collagen IV (*Vkg*) in the basement membrane (Supplementary Figure 2.2A-C, B’-C’). Likewise, we performed a TUNEL assay to identify apoptotic cells and found that the frequency does not significantly change in aged conditions (Supplementary Figure 2.2C-E, C’-D’, yellow arrows) [26]. However, the overall low rates of apoptosis in this tissue may make it difficult to detect changes. Taken together, these data establish quantitative and qualitative hallmarks of follicle cell aging in the *Drosophila* ovary.

2.2 Characterization of cell cycle regulation in aging follicle cells.

The FSC lineage is highly proliferative, undergoing up to nine rounds of division before exiting the cell cycle at Stage 6 [13]. We therefore investigated whether the cell cycle of follicle cells is altered with age. First, we used an EdU assay to quantify the number of follicle cells per germlarium in S-phase in ovarioles from young and aged flies (Figure 2.2A-B, A’-B’). Indeed, we observed a statistically significant increase in EdU⁺ cells, from 6.5 ± 1.4 cells per germlarium in young flies versus 12.5 ± 2.8 cells per germlarium in aged flies (Figure 2.2C). In contrast, we found no significant difference in the number of follicle cells that were positive for phospho-histone H3 (pHH3), which is a marker of mitosis (Figure 2.2D-F, D’-E’). Together, these results indicate that follicle cells in aged ovarioles are spending a greater fraction of time in S-phase but are not dividing more frequently. We hypothesized that this could be the result of follicle cells in aged germlaria requiring more time to accommodate replication stress and DNA damage.

Consistent with this, we found that the number of follicle cells per germarium that are positive for the DNA damage marker, gH2Av, significantly increased with age from 1.7 ± 0.03 cells per germarium to 3.4 ± 0.6 cells per germarium (Figure 2.2G-I).

2.3 Follicle cells are unable to accommodate germ-cell cysts in aged germaria.

As the appearance of severe defects in follicle formation is not associated with a substantial change in follicle cell proliferation in aged ovarioles, we next considered whether these phenotypes are associated with a loss of coordination between germ cells and follicle cells. The rate of germ-cell cyst progression from Region 2a of the germarium into the follicle epithelium in Region 2b is regulated in part by a nutrition-associated checkpoint. This checkpoint is detectable as an apoptotic cyst in Region 2a, which occurs in approximately 65% of germaria when flies are maintained on standard growth media [27]. The dying cysts can be visualized with Apoptag or TUNEL staining and are likely also the source of LysoTracker⁺ germ cells in Region 2a that were reported separately [18,27,28]. To investigate this checkpoint as a function of age, we first stained young and aged germaria with LysoTracker (Figure 2.3A, A'). We found that a band of LysoTracker⁺ germ cells was present in Region 2a in approximately 50% of germaria from young flies maintained under the growth conditions we used in this study. Though LysoTracker staining was incompatible with the Click-iT TUNEL assay, we found that both methods identify structures with the same frequency, shape, and location (Figure 2.3F-J). In addition, we found that these structures are much larger than typical lysosomes and they do not contain either the canonical lysosome marker Lamp1 or the late endosome marker

Rab7 (Supplementary Figure 2.3A-B). Together, these observations indicate that both LysoTracker and TUNEL staining identify apoptotic cysts in Region 2a. Surprisingly, the frequency of germaria with a band of LysoTracker⁺ germ-cell cysts did not change in the aged condition (Figure 2.3C), indicating that this checkpoint does not decline with age. However, we found that ovarioles from aged flies without LysoTracker⁺ germ-cell cysts in Region 2a were significantly more likely to have one or more age-associated follicle cell phenotypes, whereas no such association was found in ovarioles from young flies (Figure 2.3B-B', D). This suggests that the increase in follicle cell phenotypes with age is linked to a lack of coordination between germ cells and follicle cells.

2.4 Single-cell RNA sequencing of aged *Drosophila* ovaries reveals key transcriptional differences in the follicle cell lineage.

To build a comprehensive understanding of the transcriptional differences between young and aged germaria, we performed single-cell RNA sequencing (scRNA-seq) of one-week and six-week-old ovaries using 10x Genomics system and Illumina sequencing. This produced transcriptional data for 20,818 cells across two timepoints (Table 2.1) after applying standard quality control filters (Supplementary Figure 2.4A-B). Using the standard Seurat workflow, we clustered the cells into nine broad clusters (Figure 2.4A): Germ cells (*vas*⁺), Escort cells (*Wnt4*⁺, *Wnt6*⁺), FSCs + pFCs (*zfh1*⁺, *cas*⁺, *eya*⁺, *Fas3*⁺), Early-Mid FCs (*eya*⁺, *br*⁺, *Fas3*⁺, *mid*⁺), Late FCs (*br*⁺, *mid*⁺, *Yp1*⁺), Muscle (*Mhc*⁺), Immune (*atilla*⁺, *nec*⁺), and Spermatheca (*lz*⁺), and Neurons (*pros*⁺, *nrv3*⁺) (Supplementary Figure 2.5A) [8,50–55]. We mapped the cells from aged ovaries onto the young ovary dataset using the Seurat integration features (Figure 2.4B). Mean silhouette

scores revealed that most ovarian cell types maintained strong transcriptional separability across age, indicating strong clustering identities (Supplementary Figure 2.5B). Notably, we found that the Early-Mid FCs have the highest number of differentially expressed genes, followed by FSCs + pFCs and muscle, with germ cells ranking eighth (Figure 2.4C, Table 2.2). In looking at the whole aged ovary, we found 321 differentially expressed genes (DEGs) between the young and aged condition, and 156 DEGs specifically in the FSCs + pFCs cluster (Figure 2.4D, Supplementary Figure 2.5C, Tables 2.3-2.4). To determine how these transcriptional changes relate to the transcriptional changes in mammalian granulosa cells with age, we merged the FSCs + pFCs cluster with the Early-Mid FCs cluster to create a more “granulosa-like” cell cluster, identified the mammalian orthologs of the DEGs in this merged cluster and cross-referenced them with a list of age-associated DEGs from a recently published study of mouse and human aged granulosa cells [19]. Interestingly, we found several genes that are significantly upregulated in both mouse and human granulosa cells that have homologous genes that are significantly upregulated in our aged *Drosophila* “granulosa-like” cell cluster (Figure 2.4F). This list includes genes involved in signal transduction, such as the PDGF-related factor *Pvf1*, and the JNK-stress pathway effector *Jun-related antigen*; and immune response genes such as the NFKB1 homolog, *Relish*, and an uncharacterized serine hydrolase, CG10472, that is homologous to mammalian CTRB1 and is predicted to be involved in innate immunity.

2.5 Overexpression of *Atg8a* in early follicle cells suppresses age-related follicle cell decline and rescues reproductive capacity.

To identify genetic modifications that could counteract the age-associated decline in fertility, we screened through well-known genes associated with classic hallmarks of aging, such as cell differentiation, response to stress, and epithelium development (Figure 2.4E, Supplementary Figure 2.5D). Interestingly, we noticed that overexpression of *Atg8a::mCherry-GFP* specifically in the early FSC lineage using *109-30-Gal4* produced particularly healthy flies. *Atg8a* is an autophagy gene that is essential for the initiation of an autophagosome and fusion of the autophagosome to a lysosome [56–58] and, while it did not come up as a significant DEG in our single-cell RNA sequencing dataset (Supplementary Figure 2.5E), autophagy defects are highly associated with age-associated defects. We therefore investigated whether upregulating *Atg8a* in follicle cells could improve the age-related decline in ovarian function.

Indeed, we found that there was a significant reduction in the frequency of follicle cell phenotypes relative to an internal control in aged ovaries (Figure 2.5A-C). Interestingly, we observed a slight increase in follicle cell phenotype frequency in young ovaries (Figure 2.5C), suggesting that at least some of the beneficial effects of *Atg8a* overexpression may be specific to the aged condition. In addition, we observed a significant increase in the percentage of aged germaria that have maintained the ring of N-cad upon overexpression of *Atg8a::mCherry-GFP* compared to control (Figure 2.1G', H'). Moreover, we found that overexpression of *Atg8a::mCherry-GFP* decreased the number of highly expressing gH2Av positive follicle cells (Figure 2.5G-H, G'-H', I). Additionally, we found that overexpression of *Atg8::mCherry-GFP* in follicle cells has cell

non-autonomous effects on the aged germline. While overexpression of *Atg8::mCherry-GFP* did not significantly alter the frequency of germaria with LysoTracker⁺ germ-cell cysts in Region 2a, it eliminated the association between the presence of follicle cell phenotypes and the lack a LysoTracker⁺ cyst in Region 2a (Figure 2.6A-D). Finally, we assayed for egg-laying to determine if overexpression of *Atg8a* in follicle cells could rescue age-related decline in reproductive capacity [59]. We found that egg-laying increased from 2 ± 1.5 eggs per female per day in aged control *Drosophila* to 5.9 ± 2.6 eggs per female per day in *Atg8a* overexpression condition, a nearly 2-fold increase in egg-laying (Figure 2.6F). Importantly, egg-laying in aged *Atg8a* overexpression condition was not significantly different from that of young control *Drosophila* (Figure 2.6F). Taken together, these data indicate that overexpression of *Atg8a* improves age-related follicle cell decline in tissue integrity, cell adhesion, DNA damage repair, and reproductive capacity in aged ovaries.

2.6 Discussion

Here, we have identified several key genetic and cellular changes that accumulate with age in the *Drosophila* follicle epithelium and discovered a targeted intervention, overexpression of *Atg8a* specifically in the early FSC lineage, that ameliorates these phenotypes. Several of the defects we identified, such as gaps in the follicle epithelium and the lack of an interfollicular stalk, are incompatible with follicle development and thus likely to be significant causes of reduced fecundity in aged flies. In addition, we identified intracellular phenotypes, including increased DNA damage and a disruption of cellular junctions, that indicate a loss of normal follicle cell function. Moreover, our scRNAseq data provide a comprehensive account of cell type specific changes in gene expression

with age. Interestingly, our analysis predicts that there are more differentially expressed genes in the follicle lineage than any other cell type in the ovary. Additionally, several of the highly upregulated genes in mammalian aged granulosa cells are homologous to significantly upregulated genes in the aged *Drosophila* follicle lineage. Taken together, these observations emphasize the importance of the early FSC lineage in *Drosophila* ovarian aging and provide a baseline description of age-associated changes in this tissue that can be used to evaluate the effects of experimental interventions that target follicle cells. Notably, the follicle cell phenotypes we observed, including DNA damage and cell cycle alterations, are also hallmarks of reproductive aging in mammals [13], highlighting the opportunity to understand conserved mechanisms of reproductive aging through these studies.

We were surprised to find that overexpression of *Atg8a* with *190-30-Gal4* is sufficient to increase fecundity in both young and old flies. *109-30-Gal4* expression is restricted to a relatively small population of somatic cells located in the posterior half of the germarium and the first 2-3 budded follicles, yet genetic manipulation of this small population increased the overall output of the whole ovary. This demonstrates that oogenesis is not operating at maximum capacity under the growth conditions we used, and that the function of these cells in the early FSC lineage is rate-limiting. In addition, though fecundity still declines with age in flies with *Atg8a* overexpression, it is notable that the rate of egg laying in the aged condition is not significantly different from that of young control flies. Thus, despite the impact of other sources of decline that inevitably occur with age, these flies still maintain a youthful level of fecundity in the aged condition. These findings are consistent with emerging evidence that targeting the effects of ovarian

somatic cell aging in mammals may slow reproductive aging. For example, the pro-inflammatory signal, CD38, which is primarily expressed in endothelial and immune cells, increases with age and global inhibition of this increase enhances fertility in aged mice [60]. Likewise, interventions that reduce fibrosis caused by ovarian somatic cells are being considered as possible therapeutic avenues [61]. An advantage of these interventions is that, by focusing on the support cells, they avoid direct manipulation of the germ cells that will contribute to the next generation. The genetic tools available here enabled us to use targeted genetic manipulations to study the role of somatic cells in reproductive aging.

The cells of the early FSC lineage may influence the rate of oogenesis through their direct role in facilitating follicle formation as well as through signaling with germ cells that helps coordinate the supply of follicle cells with demand from newly-produced germ-cell cysts. A key factor in this coordination process is the nutrition dependent checkpoint at Region 2a. This checkpoint and a second one that occurs in mid oogenesis may be orthologous to a process in the mammalian ovary in which granulosa cells secrete signals to induce follicle atresia [62]. We found that the frequency of germaria with a dying cyst in Region 2a did not change between young and old flies or even with the overexpression of *Atg8*. However, the presence of age-associated follicle cell phenotypes is linked to the absence of a dying cyst in Region 2a, raising the interesting possibility that germaria without a recent activation of this checkpoint are more susceptible to a loss of coordination between germ cells and follicle cells. Alternatively, germaria with follicle cell phenotypes may be less likely to activate the checkpoint, possibly leading to further loss of

coordination. Understanding how a loss of coordination at this stage of oogenesis contributes to overall fecundity rates will be an important topic for future studies.

Mechanistically, *Atg8a*, which is homologous to mammalian LC3, is well-studied as a key regulator of autophagy [63,64]. Autophagy is a protective cellular function that promotes the degradation of proteins, organelles, and other cellular components, to mitigate the accumulation of damaged biomolecules over time [64]. Autophagy is regulated by a cascade of signals, with several ATG (autophagy related genes) involved [58,64]. *Atg8a* resides on the membrane of autophagosomes during formation and expansion and is known to increase in expression in response to stress conditions such as starvation and reactive oxygen species accumulation in the *Drosophila* ovary [56–58]. In addition, an upstream regulator, *Atg7*, is required in follicle cells for coordination between germ cells and follicle cells in the progression of oogenesis [57]. In the mammalian ovary, LC3 expression and standard indicators of autophagy activity, such as lysosome number and autolysosome fusion, decline with age, thus reducing the capacity of this protective mechanism [64,65]. Autophagy is critical for DNA damage mediation, where autophagy is promoted by the DNA damage response and repair pathways [66,67]. Additionally autophagy, and *Atg8a* specifically, also works upstream to prevent DNA damage and oxidative stress [68,69]. Consistent with this, we found that overexpression of *Atg8a* decreases the accumulation of DNA damage in follicle cells. Likewise, autophagy machinery is known to traffic E-cadherin to lysosomes [69]. This suggests that similar mechanisms may facilitate the maintenance of proper N-cad localization in ovarioles with *Atg8a* overexpression, as we have shown. These anti-aging effects at the cellular level likely contribute to the improved function of the follicle epithelium at the tissue

level. In summary, this study contributes to a growing body of literature that highlights the importance of ovarian somatic cells in reproductive health and aging.

2.7 Figures

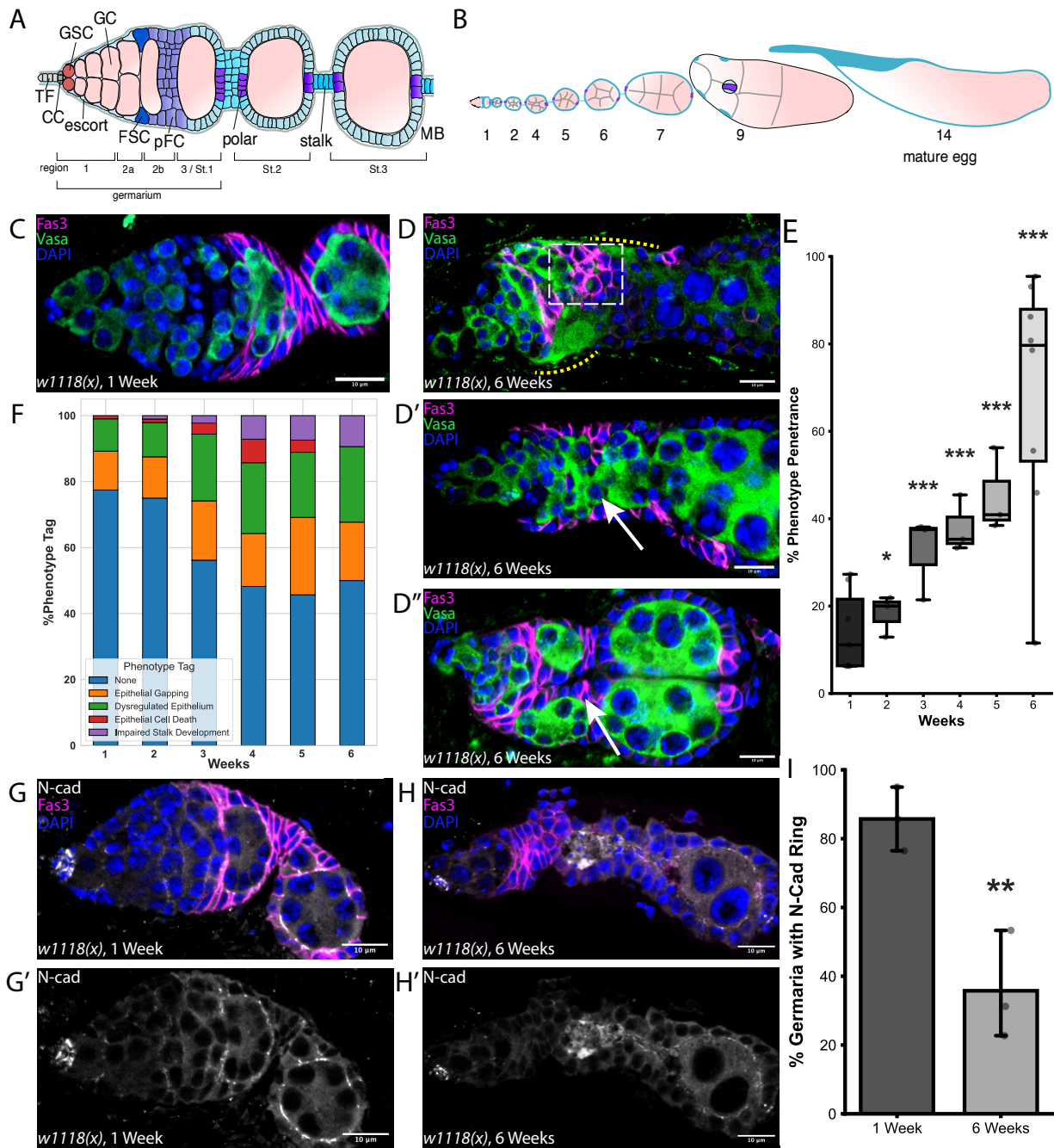


Figure 2.1. Aging leads to increased follicle cell phenotypes in the *Drosophila* ovary.

(A) Schematic of the *Drosophila* ovariole, positioned at the anterior tip of the ovary. TF: terminal filament, GSC: germline stem cell, EC: escort cell, FSC: follicle stem cell, pFC: prefollicle cell, MB: main body. (figure caption continues on next page)

(figure caption continues from previous page) **(B)** Schematic of the ovariole. **(C)** Germarium from a *w11118(x);* fly at 1 week old, stained for Fas3 (magenta), Vasa (green), and DAPI (blue). **(D-D'')** Representative examples of germaria from *w11118(x);* flies at 6 weeks old. Yellow dotted lines show epithelial gapping, white dashed box shows epithelial dysregulation and epithelial cell death, white arrows show impaired stalk development. **(E)** Quantification of the percent of germaria with follicle cell phenotypes at weekly timepoints from 1 to 6 weeks old. n = 298, 88, 73, 43, 64, and 201 germaria, respectively. **(F)** Quantification of follicle cell phenotype tags from 1 to 6 weeks old. n = 98, 88, 73, 43, 64, and 77 germaria, respectively. **(G)** Germarium from a *w11118(x);* fly at 1 week old, stained for N-cad (grey), Fas3 (magenta), and DAPI (blue). **(G')** N-cadherin ring forms around developing germ-cell cysts in 1-week-old germaria. **(H)** Germarium from a *w11118(x);* fly at 6 weeks old, stained for N-cad (grey), Fas3 (magenta), and DAPI (blue). **(H')** Loss of N-cadherin ring around developing germ-cell cysts in a 6-week-old germarium. **(I)** Quantification of the percent of germaria with the N-cadherin ring around germ-cell cysts at 1 and 6 weeks old. n = 58 and 53 germaria, respectively. Scale bars are 10 μ m. ns = not significant, *p<0.05, **p<0.01, ***p<0.001, using Dunnett's Test (E) and Student's t-test (I).

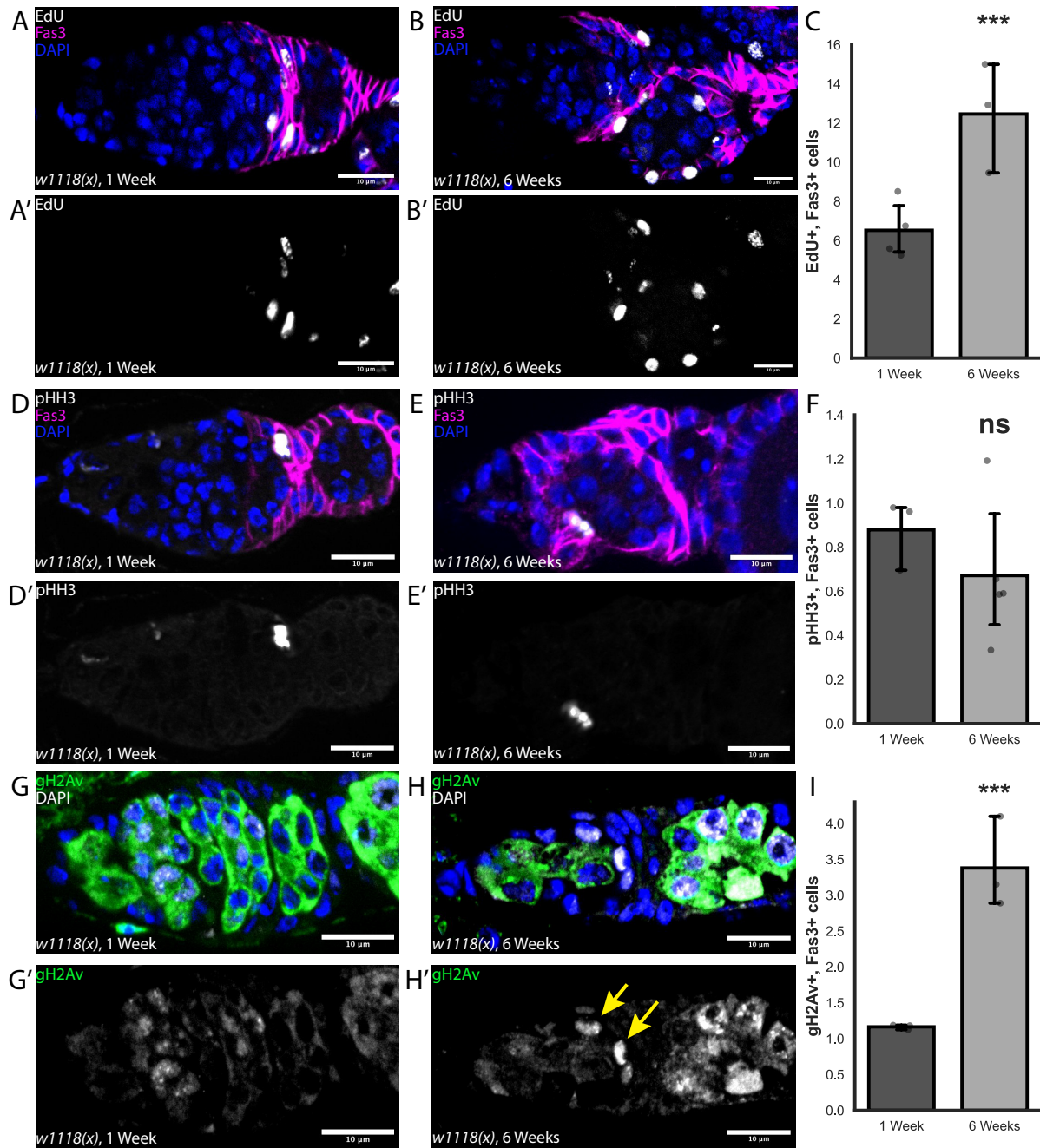


Figure 2.2. Aging leads to an extended S-phase and increased DNA damage in follicle cells.

(A-B) Germarium from *w1118(x);* flies at 1 (A) or 6 (B) weeks old, stained for EdU (grey), Fas3 (magenta), and DAPI (blue). (A'-B') EdU identifies cells that are in or enter into S-phase during the 1-hour incubation prior to fixation. (C) Quantification of the number of EdU+, Fas3+ cells per germarium at 1 and 6 weeks old. $n = 98$ and 65 germaria, respectively. (figure caption continues on next page)

(figure caption continues from previous page) **(D-E)** Germarium from *w1118(x);* flies at 1 (D) or 6 (E) weeks old, stained for pHH3 (grey), Fas3 (magenta), and DAPI (blue). **(D'-E')** pHH3 identifies cells that are in mitosis. **(F)** Quantification of the number of pHH3+, Fas3+ cells per germarium at 1 and 6 weeks old. $n = 200$ and 124 germaria, respectively. **(G-H)** Germarium from *w1118(x);* flies at 1 (G) or 6 (H) weeks old, stained for gH2Av (grey), Fas3 (magenta), and DAPI (blue). **(G'-H')** gH2Av identifies sites of DNA damage. **(I)** Quantification of the number of highly positive gH2Av+, Fas3+ cells per germarium at 1 and 6 weeks old. $n = 35$ and 39 germaria, respectively. Scale bars are $10\ \mu\text{m}$. ns = not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, using Poisson Dispersion Test (C, F, I).

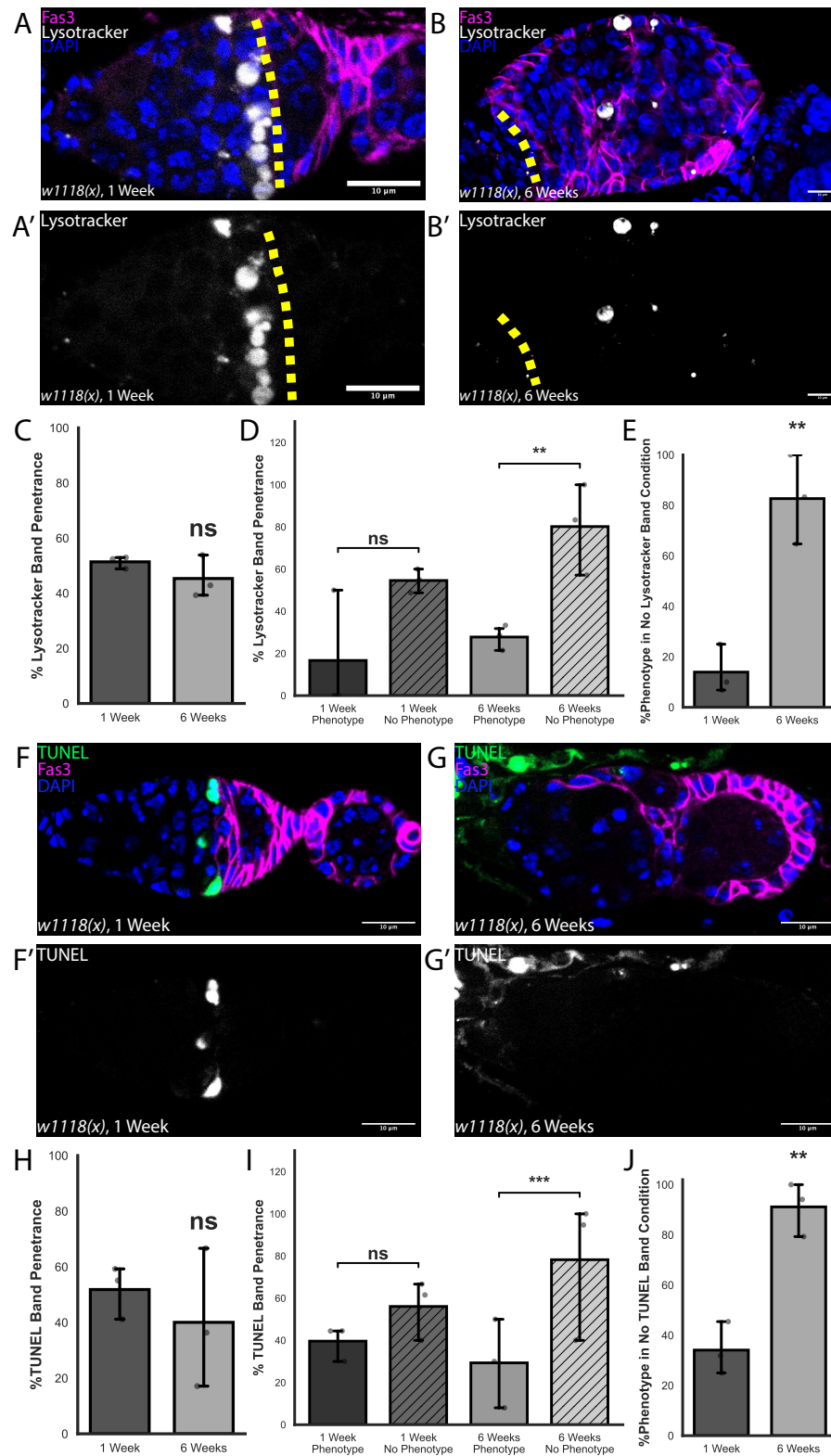


Figure 2.3. Aged ovaries cannot accommodate germ-cell cyst death. (figure caption continues on next page)

(figure caption continues from previous page) **(A-B)** Germarium from *w1118(x)*; flies at 1 (A) and 6 (B) weeks old, stained for Lysotracker (grey), Fas3 (magenta), and DAPI (blue). **(A')** A Lysotracker-positive band may be present anterior to the Region 2a/2b border (yellow dashed line) in 1-week-old germaria. **(B')** Lysotracker-positive bands may be absent anterior to the Region 2a/2b border (yellow dashed line) at 6-weeks old, phenotype-positive germaria. **(C)** Quantification of the percent of germaria with the Lysotracker-positive band at 1 and 6 weeks old. $n = 124$ and 83 germaria for 1- and 6-weeks old. **(D)** Quantification of the percent of germaria at 6 weeks old with the Lysotracker-positive band in phenotype-positive and phenotype-negative germaria. $n = 57$ and 26 germaria, respectively. **(E)** Quantification of phenotype penetrance in germaria in which no Lysotracker-positive band is present at 1 and 6 weeks old. $n = 62$ and 47 , respectively. **(F-G)** Germarium from *w1118(x)*; flies at 1 (A) and 6 (B) weeks old, stained for TUNEL (grey), Fas3 (magenta), and DAPI (blue). **(F')** A TUNEL-positive band is present anterior to the Region 2a/2b border in germaria at 1 week old. **(G')** TUNEL-positive bands may be absent anterior to the Region 2a/2b border in phenotype-positive germaria at 6 weeks old. **(H)** Quantification of the percent of germaria with the TUNEL-positive band at 1 and 6 weeks old. $n = 110$ and 119 germaria, respectively. **(I)** Quantification of the percent of 6-week-old germaria with the TUNEL-positive band in phenotype-positive and phenotype-negative germaria. $n = 87$ and 32 germaria, respectively. **(J)** Quantification of phenotype penetrance in germaria where no TUNEL-positive band is present at 1 and 6 weeks old. $n = 53$ and 67 germaria, respectively. Scale bars are $10\ \mu\text{m}$. ns = not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, using Welch's t-test (C, D, H, J) and Binomial General Linearized Mixed Model (D, I).

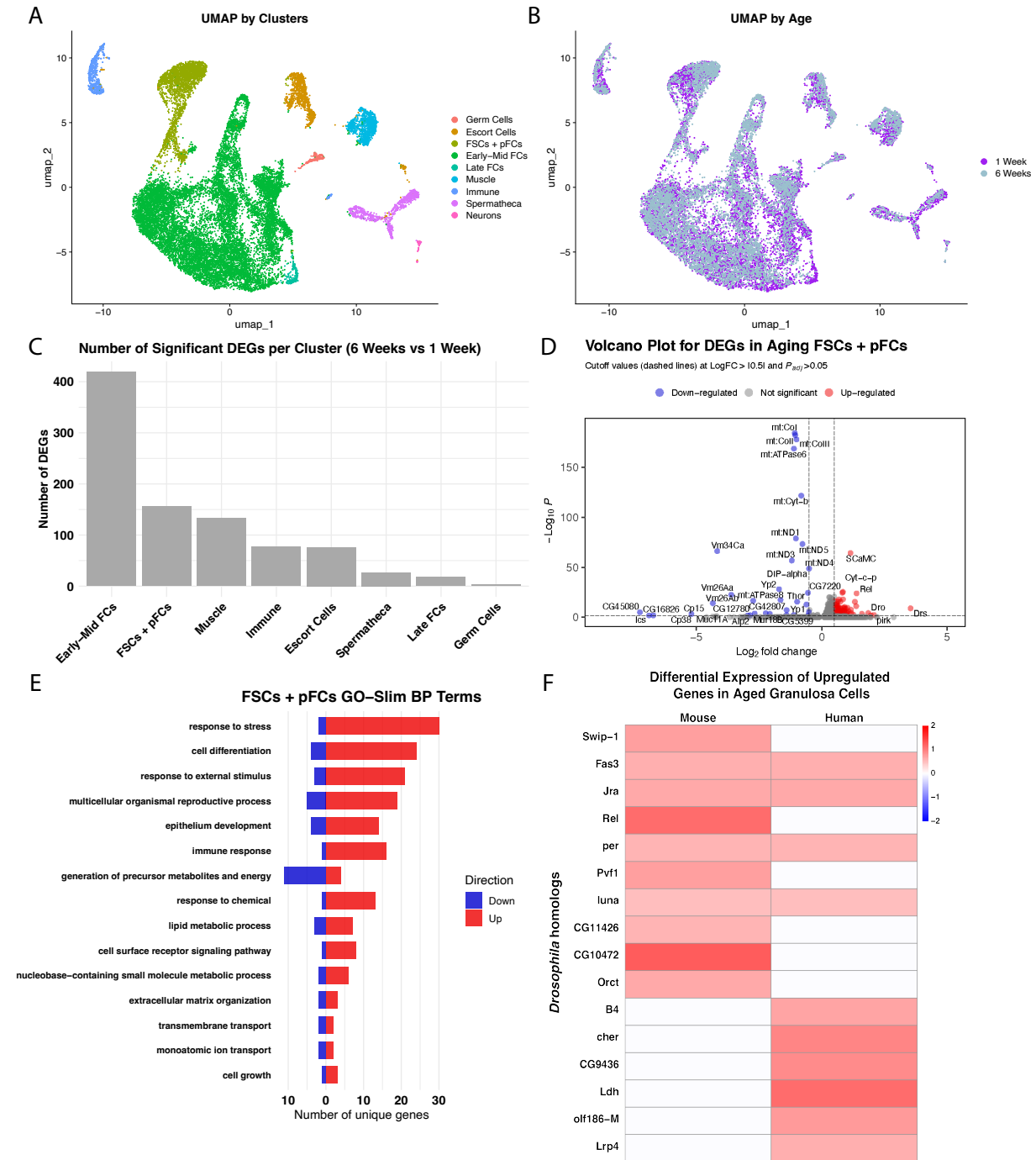


Figure 2.4. Single-cell sequencing of young and aged *Drosophila* ovaries reveals large transcriptional differences in aged follicle cells.

(A) UMAP of the merged dataset, annotated by the 8 clusters. (B) UMAP of the merged dataset, annotated by the 1 and 6-week old datasets. (C) Ranking of the number of significant differentially expressed genes (DEGs) in each cluster, comparing the 6-week to the 1-week old datasets. (figure caption continues on next page)

(figure caption continues from previous page) **(D)** Volcano plot of DEGs from the FSCs + pFCs cluster. Blue dots are down-regulated genes, grey dots are not significant genes, and red dots are upregulated genes. Significance is indicated by an absolute value log fold-change greater than 0.5 and adjusted pvalue greater than 0.05. **(E)** Gene ontology terms, using GO-Slim for the FSCs + pFCs cluster. **(F)** Heatmap of fly genes with mammalian homologs that are up-regulated with age, from Gaylord et al, 2025 [28].

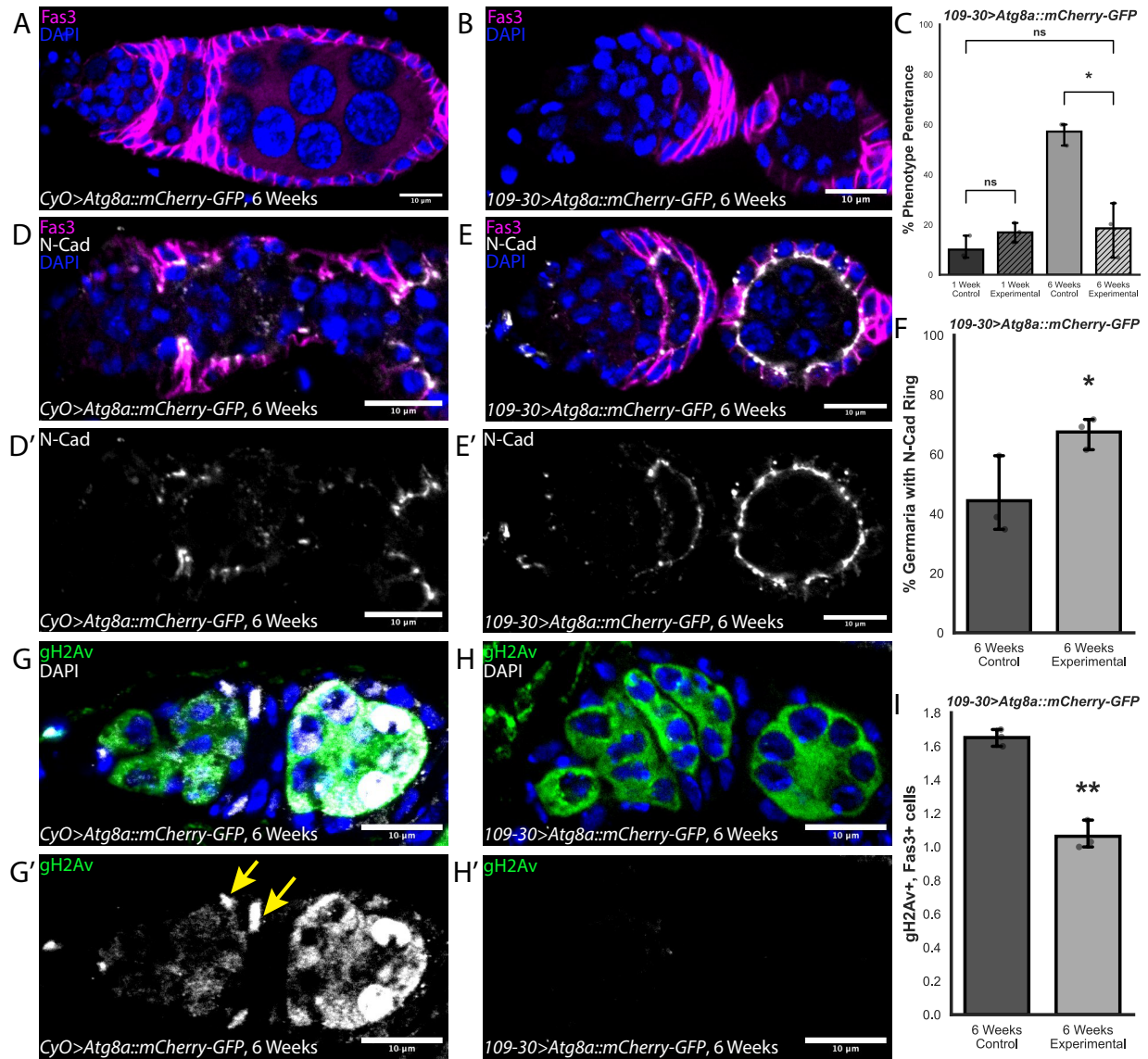


Figure 2.5. Overexpression of *Atg8a* in follicle cells suppresses age-related follicle cell decline.

(A) Germarium from a *CyO>;Atg8a::mCherry-GFP*; control fly at 6 weeks old stained for Fas3 (magenta) and DAPI (blue). (B) Germarium from a *109-30>;Atg8a::mCherry-GFP*; experimental fly stained for Fas3 (magenta) and DAPI (blue). (C) Quantification of percent of germaria with follicle cell phenotypes in 1- and 6-week-old *CyO>;Atg8a::mCherry-GFP*; and *109-30>;Atg8a::mCherry-GFP*; . n = 115 and 235 germaria for 1-week-old *CyO>;Atg8a::mCherry-GFP*; and *109-30>;Atg8a::mCherry-GFP*; , and n = 61 and 140 for 6-weeks-old *CyO>;Atg8a::mCherry-GFP*; and *109-30>;Atg8a::mCherry-GFP*; . (D) Germarium from a 6-week-old, *CyO>;Atg8a::mCherry-GFP*; fly, stained for N-cad (grey), Fas3 (magenta), and DAPI (blue). (figure caption continues on next page)

(figure caption continues from previous page) **(D')** Loss of N-cadherin ring around developing germ-cell cysts in 6-weeks-old ;CyO;>;Atg8a::mCherry-GFP;. **(E)** Germarium from a 6-week-old ;109-30;>;Atg8a::mCherry-GFP; fly, stained for N-cad (grey), Fas3 (magenta), and DAPI (blue). **(E')** Rescue of N-cadherin ring around developing germ-cell cysts in 6-week-old ;10930;>;Atg8a::mCherry-GFP; germaria. **(F)** Quantification of the percent of germaria with the N-cadherin ring around germ-cell cysts in 6-week-old ;CyO;>;Atg8a::mCherry-GFP; and ;109-30;>;Atg8a::mCherry-GFP;. n = 101 and 118 for 6-weeks-old ;CyO;>;Atg8a::mCherry-GFP; and ;109-30;>;Atg8a::mCherry-GFP;. **(G)** Germarium from a 6-week-old ;CyO;>;Atg8a::mCherry-GFP; control fly stained for gH2Av (grey), Fas3 (magenta), and DAPI (blue). **(H)** Germarium from a 6-week-old ;109-30;>;Atg8a::mCherry-GFP; control fly stained for gH2Av (grey), Fas3 (magenta), and DAPI (blue). **(G'H')** gH2Av identifies sites of DNA damage. **(I)** Quantification of the number of highly positive gH2Av+, Fas3+ cells per germarium in 6-week-old ;CyO;>;Atg8a::mCherry-GFP; and ;109-30;>;Atg8a::mCherry-GFP;. n = 77 and 84 for 6weeks-old ;CyO;>;Atg8a::mCherry-GFP; and ;109-30;>;Atg8a::mCherry-GFP;. Scale bars are 10 μ m. ns = not significant, *p<0.05, **p<0.01, ***p<0.001, using Welch's t-test (C), Student's t-test (F), and Poisson Dispersion Test (I).

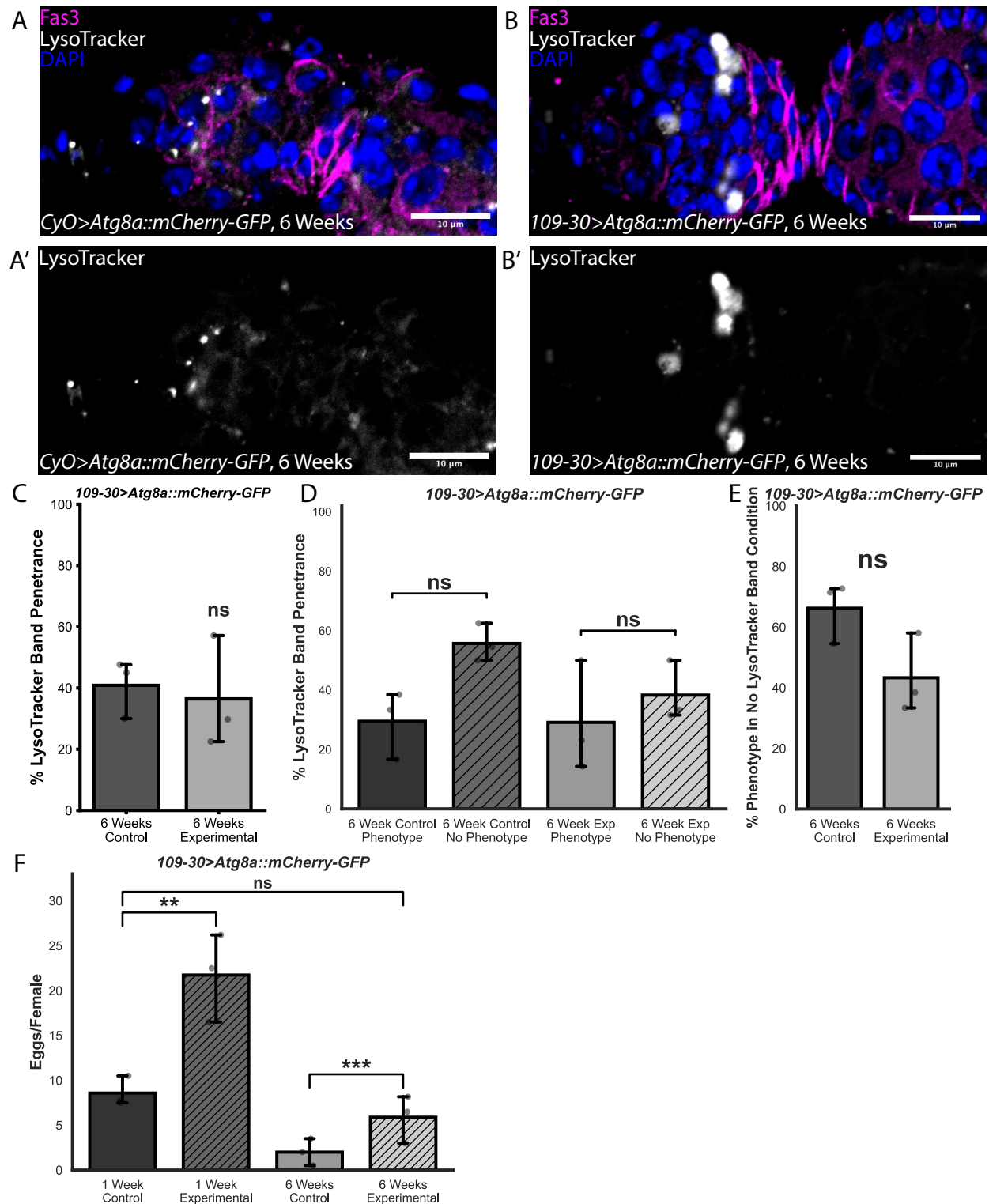
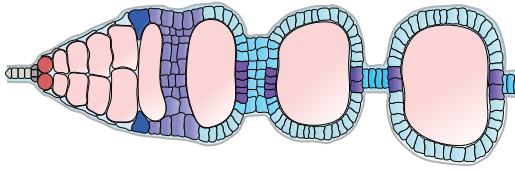


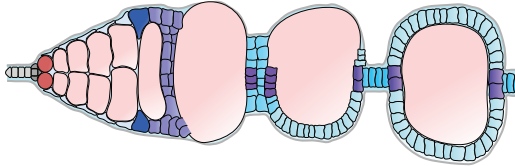
Figure 2.6. Overexpression of *Atg8a* in follicle cells has non-autonomous effect on germ-cell cyst regulation and rescues egg laying. (figure caption continues on next page)

(figure caption continues from previous page) **(A)** Germarium from a 6-week-old ;CyO>;Atg8a::mCherry-GFP; control fly, stained for Lysotracker (grey), Fas3 (magenta), and DAPI (blue). **(A')** A Lysotracker-positive band may be absent anterior to the Region 2a/2b border in phenotype-positive germaria. **(B)** Germarium from a 6-week-old ;109-30>;Atg8a::mCherry-GFP; experimental fly, stained for Lysotracker (grey), Fas3 (magenta), and DAPI (blue). **(B')** Lysotracker-positive bands may be present anterior to the Region 2a/2b border in phenotype-negative germaria. **(C)** Quantification of the percent of germaria with the Lysotracker-positive band in 6-week-old ;CyO>;Atg8a::mCherry-GFP; and ;109-30>;Atg8a::mCherry-GFP; n = 51 and 84 germaria for ;CyO>;Atg8a::mCherry-GFP; and ;109-30>;Atg8a::mCherry-GFP. **(D)** Quantification of the percent of 6-week-old ;CyO>;Atg8a::mCherry-GFP; germaria and percent of 6-week-old ;109-30>;Atg8a::mCherry-GFP; with the Lysotracker-positive band in phenotype-positive and phenotype-negative germaria. n = 28 ;CyO>;Atg8a::mCherry-GFP; phenotype-positive germaria, n = 23 ;CyO>;Atg8a::mCherry-GFP; phenotype-negative germaria, n = 36 ;10930>;Atg8a::mCherry-GFP; phenotype-positive germaria, n = 49 ;109-30>;Atg8a::mCherry-GFP; phenotype negative germaria. **(E)** Quantification of phenotype penetrance in germaria where no Lysotracker-positive band is present for ;CyO>;Atg8a::mCherry-GFP; and ;109-30>;Atg8a::mCherry-GFP. n = 29 and 60 germaria for ;CyO>;Atg8a::mCherry-GFP; and ;109-30>;Atg8a::mCherry-GFP. **(F)** Quantification of eggs laid per female in a 24-hour period for 1- and 6week-old ;CyO>;Atg8a::mCherry-GFP; and ;109-30>;Atg8a::mCherry-GFP flies. n = 10 and 11 vials for 1-week-old ;10930>;CyO; and ;109-30>;Atg8a::mCherry-GFP;, and n = 5 and 8 vials for 6-weeks-old ;CyO>;Atg8a::mCherry-GFP; and ;10930>;Atg8a::mCherry-GFP;. Scale bars are 10 μ m. ns = not significant, *p<0.05, **p<0.01, ***p<0.001, using Welch's t-test (C, E), Binomial General Linearized Mixed Model (D), and Poisson Distribution Test (F).

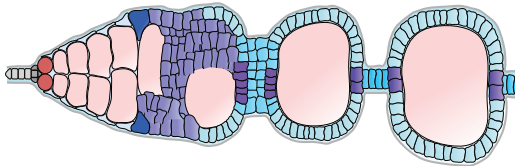
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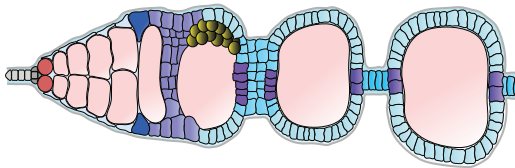
B Epithelial gapping:



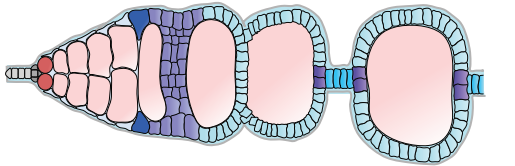
C Epithelial dysregulation:



D Epithelial cell death:

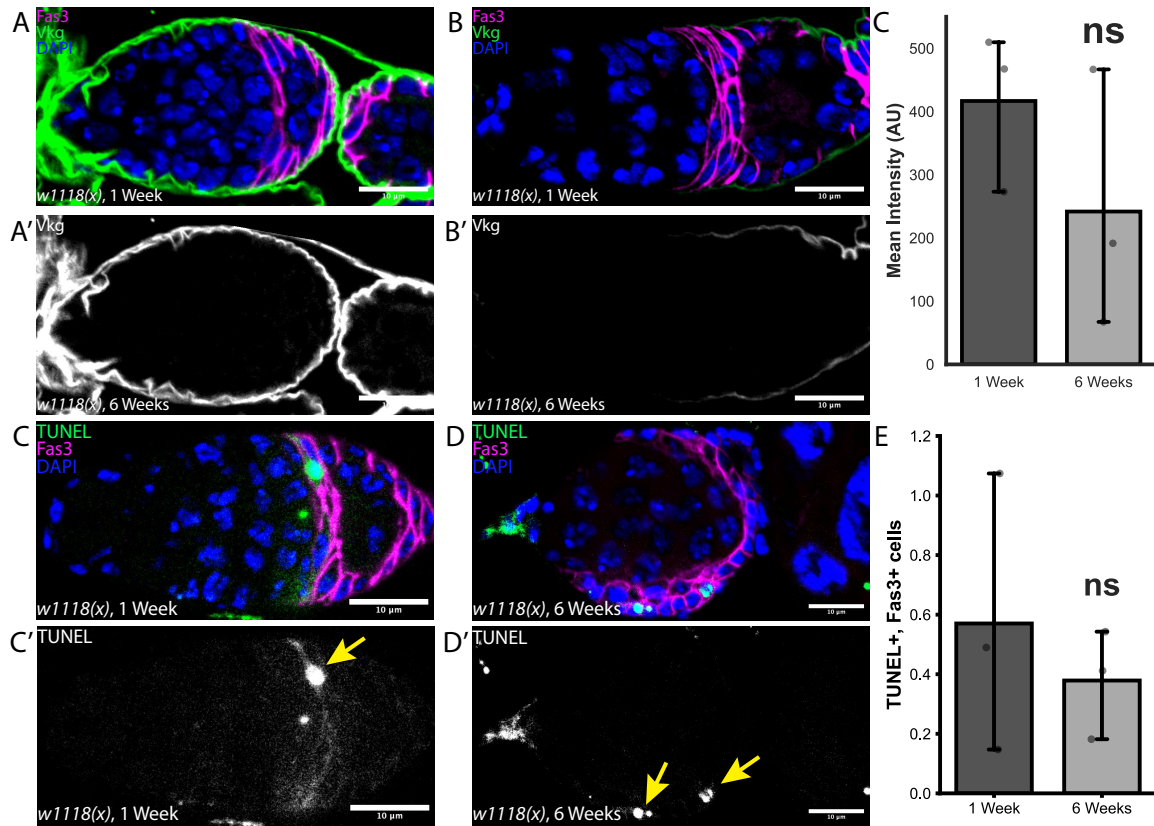


E Improper stalk development:



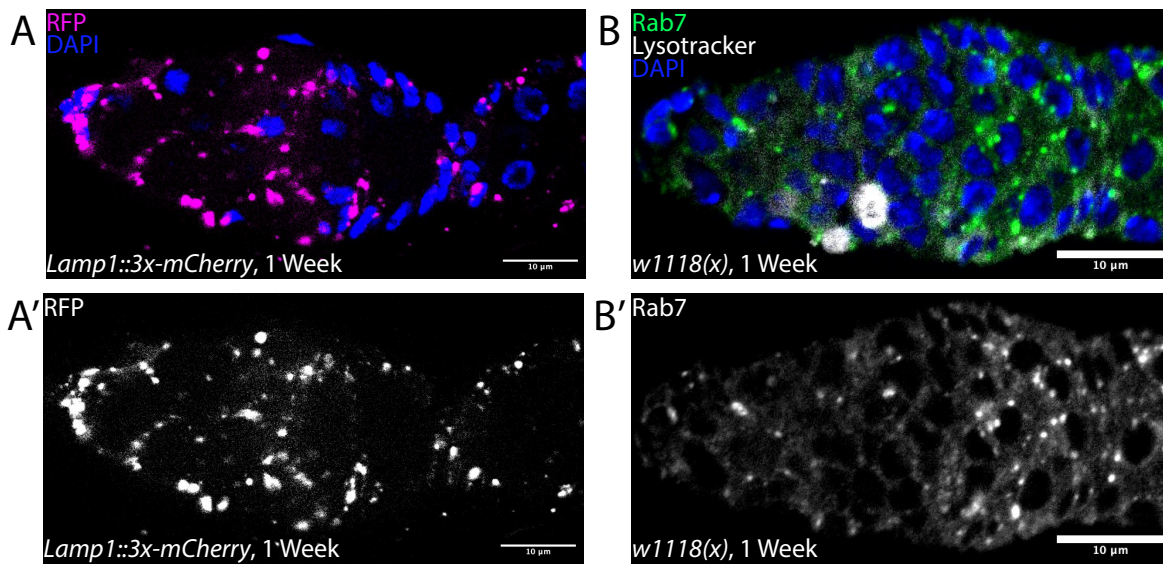
Supplementary Figure 2.1. Schematics of aging phenotypes for characterizations.

(A) Schematic of no phenotype condition. **(B)** Schematic of epithelial gapping phenotype, where germ cysts are not fully encapsulated by early follicle cells. **(C)** Schematic of dysregulated epithelium phenotype, where early follicle cells are not forming a single-layer epithelium. **(D)** Schematic of epithelial cell death phenotype, where large groups of follicle cells appear rounded, rather than cuboidal. **(E)** Schematic of an example of impaired stalk development phenotype, where stalk cells may not be present between developing follicles.



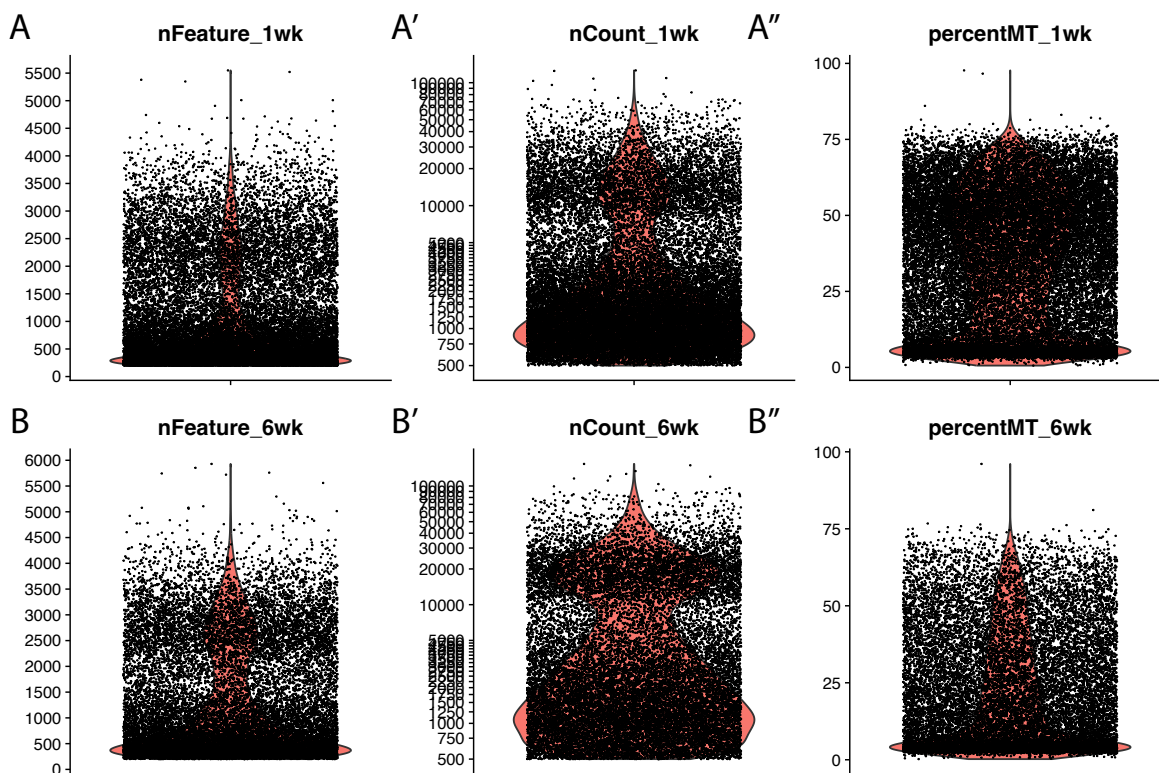
Supplementary Figure 2.2. Further characteristics of the aging ovary.

(A-B) Germarium from a 1-week-old (A) and 6-week-old (B) *w1118(x);* flies, stained for Vkg (green), Fas3 (magenta), and DAPI (blue). **(A'-B')** Vkg stains for collagen IV, which adheres to the perimeter of the germarium. **(C)** Quantification of the average intensity of Vkg around the perimeter of the germarium at 1- and 6-weeks-old. $n = 26$ and 27 germaria for 1- and 6-weeks-old. **(D-E)** Germarium from a 1-week-old (D) and 6-week-old (E) *w1118(x);* flies, stained for TUNEL (green), Fas3 (magenta), and DAPI (blue). **(D'-E')** TUNEL stains sites of DNA fragmentation, indicating final stages of apoptosis. **(F)** Quantification of the average number of TUNEL-positive, Fas3-positive cells per germarium at 1- and 6-weeks-old. $n = 110$ and 119 germaria for 1- and 6-weeks old. Scale bars are $10\ \mu\text{m}$. ns = not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, using Welch's t-test (C) and Poisson Distribution Test (F).



Supplementary Figure 2.3. Large Lysotracker-positive structures in Region 2a are not canonical lysosomes or late endosomes.

(A) Germarium from a 1-week-old *Lamp1::3x-mCherry* fly, stained for RFP (magenta) and DAPI (blue). **(A')** Lamp1 is a membrane protein on lysosomes and does not have the same pattern as the large Lysotracker positive structures seen in Region 2a. **(B)** Germarium from a 1-week-old *w1118(x)* fly, stained for Rab7 (green), Lysotracker (grey), and DAPI (blue). **(B')** Rab7 is a protein associated with late endosomes and does not colocalize with the large Lysotracker-positive structures seen in Region 2a.



Supplementary Figure 2.4. Quality control plots for single-cell RNA sequencing of young and aged ovaries.

(A-B) Violin plots for the number of RNA features by age and replicate. **(A'-B')** Violin plots for the number of RNA counts by age and replicate. **(A''-B'')** Violin plots for the percent mitochondria reads per cell ID by age and replicate.

2.8 Tables

Table 2.1 Cell counts by dataset and cluster from single-cell RNA sequencing.

Dataset	Cluster Name	Cell Count	Percent Cells Per Condition
AgingOvary1wk	Germ Cells	136	1.22%
AgingOvary1wk	Escort Cells	691	6.21%
AgingOvary1wk	FSCs + pFCs	1402	12.60%
AgingOvary1wk	Early-Mid FCs	7099	63.81%
AgingOvary1wk	Late FCs	151	1.36%
AgingOvary1wk	Muscle	566	5.09%
AgingOvary1wk	Immune	438	3.94%
AgingOvary1wk	Spermatheca	593	5.33%
AgingOvary1wk	Neurons	50	0.45%
AgingOvary6wk	Germ Cells	75	0.77%
AgingOvary6wk	Escort Cells	495	5.11%
AgingOvary6wk	FSCs + pFCs	1195	12.33%
AgingOvary6wk	Early-Mid FCs	6764	69.79%
AgingOvary6wk	Late FCs	19	0.20%
AgingOvary6wk	Muscle	507	5.23%
AgingOvary6wk	Immune	350	3.61%
AgingOvary6wk	Spermatheca	260	2.68%
AgingOvary6wk	Neurons	27	0.28%
Total Cells in AgingOvary1wk		11126	
Total Cells in AgingOvary6wk		9692	
Total Cells		20818	

Table 2.2 Number of significantly differentially expressed genes in aged ovaries compared to young by cluster.

Cluster Name	Number of DEGs
Early-Mid FCs	419
FSCs + pFCs	156
Muscle	133
Immune	77
Escort Cells	76
Spermatheca	27
Late FCs	18
Germ Cells	3

Table 2.3 List of top 50 significant DEGs for whole aging ovary.

Gene	avg_log2FC	p_val_adj
Vm34Ca	-4.913168	0
mt:ND1	-0.8208348	0
mt:Col	-0.8746262	0
mt:Coll	-0.8239562	0
mt:ATPase6	-0.8706447	0
mt:ColIII	-0.7670087	0
mt:Cyt-b	-0.5983892	0
SCaMC	0.82450445	1.41E-293
Socs36E	0.61442107	1.00E-278
mt:ND3	-0.8827089	2.33E-265
CG32512	1.43291458	2.65E-257
Ets21C	1.52686839	6.49E-223
Vm26Aa	-4.1635429	4.08E-205
Cp15	-2.3512696	1.04E-170
CG5346	0.6804653	1.34E-169
B4	0.6945107	3.74E-169
Yp2	-1.2292007	2.02E-154
Vm26Ab	-3.4453434	2.47E-152
CG5835	0.9846637	2.10E-136
Swip-1	0.72661221	1.30E-127
Fas3	0.59383414	3.99E-123
Drs	0.64915138	1.30E-122
mthl5	0.648784	3.83E-119
AdamTS-A	0.50412675	2.86E-98
Tsp42Ef	0.53694956	9.42E-97
mt:ATPase8	-1.1323091	9.87E-95
insc	0.54407811	7.65E-91

Gene	avg_log2FC	p_val_adj
CG10098	0.55489741	2.22E-89
Act42A	0.6097591	6.11E-89
Cp36	-5.5608586	2.19E-85
rpr	0.6830347	1.60E-82
per	0.62857037	1.98E-82
CG34417	0.67008264	1.68E-81
prage	0.52025215	1.82E-80
Rel	0.84105422	6.78E-76
SH3PX1	0.66648227	2.24E-73
Orct2	0.54382906	7.46E-69
Mur18B	-3.5600637	1.99E-68
MCU	0.53057536	2.07E-68
CG45080	-5.4739328	3.10E-68
Cp38	-6.7020343	2.15E-65
Yp1	-1.8850369	2.05E-64
cher	0.79795482	4.13E-64
Mmp1	0.76456118	1.96E-63
Myo31DF	0.51093772	6.90E-59
Def	3.84202438	9.66E-57
CG31955	0.53035368	5.97E-56
CG42807	-1.4224872	6.31E-56
CG9967	0.5904776	1.76E-54
Cdc7	0.61186391	3.54E-51

Table 2.4 List of top 50 significant DEGs for aging FSCs + pFCs cluster.

Gene	avg_log2FC	p_val_adj
mt:Col	-1.0772123	2.71E-185
mt:Coll	-1.0577374	3.84E-183
mt:Colll	-1.0068829	8.41E-179
mt:ATPase6	-1.1101679	1.11E-169
mt:Cyt-b	-0.8166569	1.78E-122
mt:ND1	-1.0324517	1.22E-79
mt:ND5	-0.7665004	4.74E-74
Vm34Ca	-4.1725051	6.88E-67
SCaMC	1.1466657	5.54E-65
mt:ND3	-1.1965625	1.60E-57
mt:ND4	-0.5047058	1.83E-49
Yp2	-1.7032002	9.19E-29
Cyt-c-p	0.84614893	4.61E-26
CG7220	0.82334265	3.06E-25
DIP-alpha	-0.5493069	4.22E-25
Rel	1.39028662	1.33E-24
Vm26Aa	-3.6137291	3.05E-23
B4	0.84941263	1.26E-18
alpha-Est9	0.68671614	1.33E-18
mt:ATPase8	-1.6447714	5.49E-18
Vm26Ab	-2.7365454	3.21E-17
Pka-R2	0.50884519	8.35E-17
luna	0.64277445	2.00E-16
CG6231	0.75013091	2.44E-16
Thor	-0.9808571	3.40E-16
CG31183	1.3225148	4.31E-16
Cp15	-4.3476192	1.08E-14

Gene	avg_log2FC	p_val_adj
CG30460	0.60228216	3.43E-14
bdl	0.54656568	2.32E-13
mt:ND2	-0.6122415	2.45E-13
CCT6	0.57819438	3.15E-13
CG32982	0.61699148	8.98E-13
Ets21C	1.3767804	8.06E-12
CG2765	0.65848185	2.38E-11
Gadd45	0.91644129	6.57E-11
Lac	0.55169379	9.66E-11
AdamTS-A	0.58905636	1.55E-10
sty	0.5590999	1.96E-10
CG11050	0.55018535	2.83E-10
RhoL	1.02578019	3.45E-10
olf186-M	0.85498033	4.25E-10
Jra	0.61234689	5.39E-10
loco	0.62075904	5.90E-10
FASN1	0.65104705	6.24E-10
CG33926	0.74863814	8.33E-10
tx	0.71172344	9.83E-10
Hmgcr	0.91183637	1.07E-09
Drs	3.54400032	1.27E-09
Act42A	0.50920478	1.64E-09
CG9436	0.95409407	3.19E-09

Table 2.5 List of antibodies and concentrations used.

Antibody	Source	Catalog ID	Concentration
Fas3	DSHB	7G10 anti-Fasciclin III	1:100
Vasa	DSHB	anti-vas	1:200
CadN	DSHB	DN-Ex #8	1:100
pHH3	Millipore Sigma	H0412-200UL	1:500
gH2Av	DSHB	UNC93-5.2.1	1:100
Vkg	Stephane Noselli	N/A	1:1000
RFP	Chromotek	5f8	1:500
Rab7	DSHB	Rab7	1:100

2.9 Methods

Fly husbandry and aging time course.

Flies were maintained on standard molasses food at 25°C. The following stocks from BDSC were used in this study: *w¹¹¹⁸(x);* (3605), *;109-30-Gal4*; (7032), and *;UAS-Atg8a::mCherry-GFP*; (37749). *;;Lamp1::3x-mCherry* was described in Hegedús et al, 2016 [70]. We used FlyBase to find information on phenotypes/function/stocks/gene expression. For all aging assays, virgin females were collected and paired with males and maintained in standard lab conditions until dissections at weekly timepoints. Age was calculated as weeks post-eclosion.

Immunofluorescence staining.

Drosophila ovaries were dissected in PBS and ovaries fixed in 4% PFA + PBS for 15 minutes. Ovaries were rinsed with PBS before blocking for 1 hour in standard block (PBS + 0.2% Triton X-100 + 0.5% bovine serum albumin. Ovaries were incubated in primary antibodies with block overnight on a nutator at 4°C in dark conditions. The following day, ovaries were rinsed with block for 1 hour before adding secondary antibodies. Ovaries were incubated in secondary antibodies with block overnight on a nutator at 4°C. The following day, ovaries were rinsed three times with PBS for 5 minutes each. Once the PBS was removed, DAPI-Fluoromont G Slide Mounting Media (SouthernBioTech) was added, and ovaries were stored at 4°C in dark conditions until mounted on slides. Ovaries were mounted on glass slides in DAPI-Fluoromont G Slide Mounting Media. Primary antibodies used were: mouse anti-Fas3 7G10 (1:100, DSHB), rat anti-Vasa 46F11 s(1:200, DSHB), rat anti-CadN DN-Ex#8 (1:100, DSHB), rabbit anti-

pHH3 (Ser10, 1:500, Millipore Sigma), mouse anti-UNC93-5.2.1 (gH2Av, 1:100, DSHB), and rabbit anti-Vkg (1:1000, Dr. Stephane Noselli), rat anti-RFP 5F8 (1:500, Chromatek), mouse anti-Rab7 (1:100, DSHB). The following secondary antibodies were used at 1:500: goat anti-mouse 488 (Life Technologies, A11001), goat anti-rabbit 488 (Invitrogen, A11034), goat anti-rat 488 (Invitrogen, A11006), goat anti-mouse 555 (Invitrogen, A21422), goat anti-rat 555 (Invitrogen, A21434), goat anti-mouse 647 (Invitrogen, A21236), goat anti-rat 647 (Invitrogen, A21247). A list of antibodies and concentrations used is also available in Table 2.5.

Imaging.

Images were acquired with a Zeiss M2 Axioimager with an Apotome unit using Zen 3.5 software. Initial image processing was performed in Zen using the Apotome Raw Convert feature. All further image processing was done in FIJI, and figures were prepared in Adobe Illustrator.

Image Analysis.

Analyses for N-cadherin prevalence, pHH3, EdU, gH2Av, TUNEL, and Lysotracker were performed by hand-counting. Vkg intensity was performed by outlining the germarium with a segmented line and taking the intensity of Vkg signal after background subtraction.

EdU staining.

For EdU experiments, ovaries were dissected in PBS and incubated in 0.75 μ L EdU (Click-IT Alexa Fluor 555 Imaging Kit (ThermoFisher Scientific, C10338) with 499.25 μ L PBS at room temperature for 1 hour. Ovaries were then fixed in 4% PFA + PBS for 10

minutes and then rinsed in PBS prior to blocking for 1 hour in PBS + 0.2% Triton X-100 + 0.5% bovine serum albumin. Primary antibodies were then added, and ovaries were stored overnight at 4°C on a nutator in dark conditions. The following day, ovaries were rinsed in PBS twice and incubated in a reaction mix (430µL 1x Click-IT EdU Reaction Buffer, 20µL CuSO₄, 1.2µL Alexa Fluor 555 azide, 50µL 10x Click-IT EdU Buffer Additive in deionized water) for 1 hour at room temperature on a nutator in dark conditions. The ovaries were then rinsed twice with PBS and blocked for 1 hour. Secondary antibodies were added with block and ovaries were stored overnight at 4°C on a nutator in dark conditions. Ovaries were then labeled with secondary antibodies according to the standard Immunofluorescence Staining protocol described above.

LysoTracker staining.

For LysoTracker experiments, ovaries were prepared according to the immunofluorescence staining protocol described above, except that they were incubated in LysoTracker Red DND-99 (ThermoFisher Scientific) at a 1:100 dilution in PBS for 5 minutes prior to fixation, washed with PBS for 5 minutes, and then fixed in 4% PFA + PBS for 10 minutes rather than the standard 15 minutes. Ovaries were then labeled with primary and secondary antibodies according to the standard Immunofluorescence Staining protocol described above.

TUNEL staining.

For TUNEL experiments, the following protocol was adapted from the Click-iT TUNEL Alexa Fluor Imaging Assays 488 kit (ThermoFisher Scientific, C10617). Ovaries were dissected in PBS and fixed in 4% PFA + PBS for 10 minutes and then rinsed in PBS. Cell membranes were permeabilized in PBS + 0.2% Triton X-100 for 15 minutes. Ovaries

were rinsed twice with PBS and then primed with TdT Reaction Buffer for 10 minutes. Ovaries were incubated at 37°C for 1 hour in a TdT reaction cocktail (47µL TdT Reaction Buffer + 1µL EdUTP, and 2µL TdT enzyme). A reaction supermix was prepared and stored at 4°C (2630µL 1x Click-iT Plus TUNEL Reaction Buffer, 67µL copper protectant, and 3.7µL 488 picolyl azide). Ovaries were rinsed twice with PBS and incubated at 37°C for 30 minutes, in a Click-iT reaction cocktail (45µL reaction supermix and 5µL 10x Click-iT Plus TUNEL Reaction Buffer Additive), protected from light. Ovaries were then labeled with primary and secondary antibodies according to the standard Immunofluorescence Staining protocol described above.

Egg Laying Assay.

For egg counts, flies were separated into vials containing two females and one male for each genotype, on standard food at 25°C. After 24 hours, eggs were hand counted through a dissection microscope and recorded. Total egg counts were divided by 2, so that final data represents the number of eggs per female in a 24-hour period.

Single-cell RNA sequencing prep.

Preparation for single-cell RNA sequencing of *w1118(x);* ovaries at 1 and 6 weeks old followed the protocol established from Meyer et al, 2024 [71]. However, we did not enrich for the anterior region, as this caused the too much cell loss in the aged condition. The single-cell RNA sequencing was performed at UCSF CoLabs using the 10X Genomics Single Cell 3' kits (v3.3 and v4). Sequencing was performed on Illumina NovaSeq X at the UCSF Center for Advanced Technology (CAT). CAT is supported by UCSF PBBR, RRP IMIA, and NIH 1S10OD028511-01 grants.

Bioinformatic analysis.

Sequencing reads were aligned to the *Drosophila* reference genome using the 10X Genomics Cloud CLI, which performs Cell Ranger alignment and initial quality filtering. The standard Seurat pipeline (v5) was applied to each individual dataset to filter out low quality cells based on the number of RNA features, number of RNA counts, and percent mitochondria reads (Supplementary Figure 2.4A-D). Each dataset was individually normalized and scaled. The two replicates for the 1-week datasets were merged using the IntegrateData function. The two replicates for the 6-week datasets were merged using the IntegrateData function and then anchors from the 1-week merged dataset were applied to the 6-week merged dataset. Principal components and clustering neighbor parameters were determined by the strength of the silhouette scores and mean distance within a cluster, as well as identification of known transcript expression for clusters. One cluster was removed because it had very few cells that all came from one condition and one replicate, suggesting that it was likely a small contaminating group of cells. This workflow resulted in a single Seurat object with 20,818 high-quality cells, grouped into 8 clusters, identified by known transcripts.

Statistics and data availability.

All analysis for single-cell RNA sequencing (Figure 2.4, Supplementary Figure 2.3, Tables 2.1-2.4) were performed in R. FASTQ are available on GEO (accession # [GSE318563](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE318563)). All other statistical analysis and graph generation was performed in Python. Boxplots show the median and interquartile values, and whiskers show the range. Barplots show the mean and error bars show the standard deviation. All quantification is a result of at least three or more independent replicates. A Levene test was used to test

for equal variance in cases where a Student's t-test was used. Raw data files, code for generating graphs and statistical analysis, and code for building the Seurat object are available via the GitHub repository:

(<https://github.com/NystulLab/WolfgramAgingOvary.git>).

Chapter 3: Future Directions and Closing Remarks

3.1 Future Directions

Further studies into the mechanisms of follicle cell aging and their contribution to the overall decline of ovarian function will be critical to understanding reproductive aging. Emerging evidence suggests that ovarian health not only affects fertility but also has systemic consequences, influencing metabolism, cardiovascular function, and overall organismal health. Our findings provide a foundation for future studies investigating how the state of somatic cells may impact other organ systems and potentially modulate lifespan.

Future directions in ovarian aging research span several interrelated avenues, both in *Drosophila* and mammalian models. These include intrinsic decline within somatic compartments, the breakdown of soma-germline communication, the systemic consequences of ovarian deterioration, and the cumulative effects of environmental factors such as diet, stress, and other external insults accumulated throughout lifespan. Addressing these topics will require integrative approaches that combine cellular, tissue-level, and organismal analyses to understand how aging propagates through the ovary and beyond. Key open ended questions remain such as:

What drives the ovary to be the first organ to lose function with age?

The ovary exhibits a striking vulnerability to aging, failing earlier than other organs, yet the underlying mechanisms remain incompletely understood. Deciphering the molecular and cellular factors that make the ovary particularly sensitive to aging will provide insights into reproductive senescence and organismal aging more broadly. A key question is whether the ovary acts as a driver or a sensor of systemic decline. Somatic

support cells within the ovary, including granulosa and theca cells, play essential roles in nurturing oocytes and coordinating hormone production, and their decline may be a key driver of reproductive aging. The ovary is the primary source of hormones such as ecdysone in *Drosophila* and estrogen and progesterone in mammals, which circulate throughout the body [72–75]. Changes in hormone signaling from ovarian aging could actively influence aging across multiple organ systems. Conversely, emerging evidence highlights the role of intestinal health, nutrition, and metabolic signaling in ovarian function, raising the possibility that age-related changes in nutrient sensing and metabolism may trigger or exacerbate ovarian decline. Understanding these bidirectional relationships between the ovary and organismal physiology will be critical to unraveling how reproductive aging intersects with overall health and longevity.

What is the order of cellular breakdown within the ovary?

Elucidating whether specific somatic cell types fail first, which cellular processes are most vulnerable, and how dysfunction propagates from somatic compartments to the germline will be essential for identifying effective points of intervention. Aging within the ovary occurs across multiple biological scales that collectively drive reproductive decline. In mammals for example, at the molecular level, long-lived maternal mRNAs stored within oocytes progressively degrade over time, potentially compromising developmental competence [76,77]. At the organelle level, aging is associated with increased mitochondrial fragmentation and elevated production of reactive oxygen species, which can induce DNA damage and activate cellular stress response pathways [78,79]. At the cellular and tissue levels, intercellular communication and intracellular signaling pathways deteriorate with age, particularly those mediating interactions between germ cells and

their supporting niche environment [13]. Importantly, these processes are unlikely to occur in isolation. Instead, age-associated defects at one scale may exacerbate dysfunction at others, creating reinforcing negative feedback loops that accelerate ovarian decline. Disentangling the sequence of events and relationships among these inter- and intracellular changes will be critical for understanding how reproductive capacity is progressively lost with age and for identifying mechanisms that may be targeted to preserve ovarian function.

What mechanisms could extend reproductive longevity, and could this also delay menopause and improve overall health?

While rapamycin is currently being evaluated in a clinical trial as a promising strategy to extend reproductive longevity, no widely available interventions for this purpose exist in humans [80]. This highlights both the progress made in translating mechanistic insights into potential therapies and the continued need for additional approaches. Identifying pathways that preserve somatic cell function or restore soma-germline communication could offer strategies to prolong fertility without directly altering the germline that is maternally passed to offspring. Beyond fertility, delaying the onset of menopause may have wide-ranging implications for systemic health. Early menopause is linked not only to decreased fertility but also to elevated risks of cardiovascular disease, osteoporosis, cognitive decline, and other age-related conditions. By developing strategies to postpone menopause, it may be possible to extend reproductive lifespan while simultaneously enhancing overall healthspan. Such interventions could benefit individuals who wish to delay childbearing, while also reducing societal burdens associated with age-related disease.

By addressing these questions, future studies have the potential to broaden our understanding of ovarian aging from a germline-centric perspective to a systems-level view and highlighting the ovary as a potential regulator of organismal health. Such insights could inform interventions that extend reproductive lifespan while also improving overall healthspan.

3.2 Closing Remarks

Thank you for reading this dissertation. Reflecting on my PhD and this body of work, I feel immensely proud of its contributions to the field of reproductive aging. My greatest hope is that fellow scientists engaging with this work will discover new ideas and approaches, not only within reproductive aging but across related fields, that they can integrate into their own research. Science grows through collaboration and curiosity, and I hope this work inspires further exploration, sparks new questions, and adds to the ever-expanding web of collective knowledge. Thank you.

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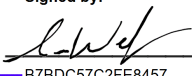
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