Communicating Research to the General Public

At the March 5, 2010 UW-Madison Chemistry Department Colloquium, Prof. Bassam Z. Shakhashiri, the director of the Wisconsin Initiative for Science Literacy (WISL), encouraged all UW-Madison chemistry Ph.D. candidates to include a chapter in their Ph.D. thesis communicating their research to non-specialists. The goal is to explain the candidate's scholarly research and its significance to a wider audience that includes family members, friends, civic groups, newspaper reporters, program officers at appropriate funding agencies, state legislators, and members of the U.S. Congress.

Over 50 Ph.D. degree recipients have successfully completed their theses and included such a chapter.

WISL encourages the inclusion of such chapters in all Ph.D. theses everywhere through the cooperation of Ph.D. candidates and their mentors. WISL is now offering additional awards of $250 for UW-Madison chemistry Ph.D. candidates.

Wisconsin Initiative for Science Literacy

The dual mission of the Wisconsin Initiative for Science Literacy is to promote literacy in science, mathematics and technology among the general public and to attract future generations to careers in research, teaching and public service.

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Structured Aggregation of Germline Determinants in Danionin Embryonic Development

By

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CHAPTER 1

Communicating Research to the General Public:

Wisconsin Initiative for Science Literacy (WISL) Thesis Award Program

Christina L. Hansen

I wrote this chapter in order to describe the context and findings of my doctoral research to a broad, non-specialist audience because I believe that science should be accessible to everyone. I would like to thank the Wisconsin Initiative for Science Literacy (WISL) at UW-Madison for providing this platform, and for sponsoring and supporting the creation of this chapter. I am especially grateful to Professor Bassam Shakhashiri, Elizabeth Reynolds, and Cayce Osborne for their valuable feedback and encouragement, and also to my thesis committee member Professor Dominique Brossard from the Life Sciences Communication department for her helpful suggestions. Conversations with my mom, Kim Hansen, my dad, Larry Hansen, and my husband, Brandon Burkett, provided additional inspiration throughout the writing process. I also offer my immense gratitude to you, the reader, for giving me this opportunity to share my work and ideas.

All up in the germ plasm: A journey with zebrafish embryos, developmental genetics, and primordial germ cells

Christina L. Hansen

A few months after I started my dissertation research in 2016, I joined other graduate students for a lunchtime discussion with a scientist visiting from another university. The speaker asked everyone to briefly introduce themselves and describe a little bit about the research they do – sounds simple enough, but my research topic was still so new to me that I wasn't quite sure how to describe it. When it was my turn, I panicked and inexplicably blurted something out about being "all up in the germ plasm." Well, nearly six years later, I am still very much "all up in the germ plasm" but now I have a lot more to say about it! With that in mind, I have compiled answers to the most "frequently asked questions" I've heard throughout the years and to share the incredible things I have learned along the way.

How does germ plasm research fit into the study of biology in general?

My research is in the field of developmental biology. Developmental biology is the study of how living things grow and/or become more "mature." This can be studied at many different scales of biology, from whole organisms to organ systems to individual cells. For example, a developmental biologist might study how a seedling plant is able to grow leaves, or how baby mice develop lungs while they are still in their mother's uterus. Developmental biologists also study how stem cells (cells that have the potential to mature into many different types of cells) decide upon a specific fate or role. I might be biased, but I think developmental biology, especially embryonic development, which I study, is one of the most awe-inspiring areas of science. There is something

truly incredible about studying how life itself is able to progress. In one way or another, everyone who studies biology is studying life, but it is hard to argue that there is any more authentic study of life than to deeply investigate the very beginning of it.

Every animal on the planet, from humans to fish, starts their journey as a single cell, formed by the union of sperm and egg. That union, called fertilization, involves the blending of DNA from both parents, but the well-being of that future embryo depends on more than DNA alone. The maternal parent contributes much more than genetic information – her large egg cell was also packed full of pre-made molecules, such as RNAs and proteins. RNA molecules can be thought of as copies of specific instructions from the DNA "master document" that tell each cell what materials (proteins) it needs to build in order to function. Making RNAs and proteins takes time and energy, so the maternal parent is giving its offspring a valuable head start on life by pre-packing lots of them into the soon-to-be-fertilized egg! These molecules, collectively termed maternal products, are responsible for everything that a newly formed embryo does until the DNA "wakes up" and starts expressing its own information (Figure 1). In my study organism, zebrafish, the embryonic genome (the full set of DNA in each cell) doesn't fully activate until 3-4 hours after fertilization. However, in those early hours before activation, many extremely important things are already happening thanks to the maternal products.

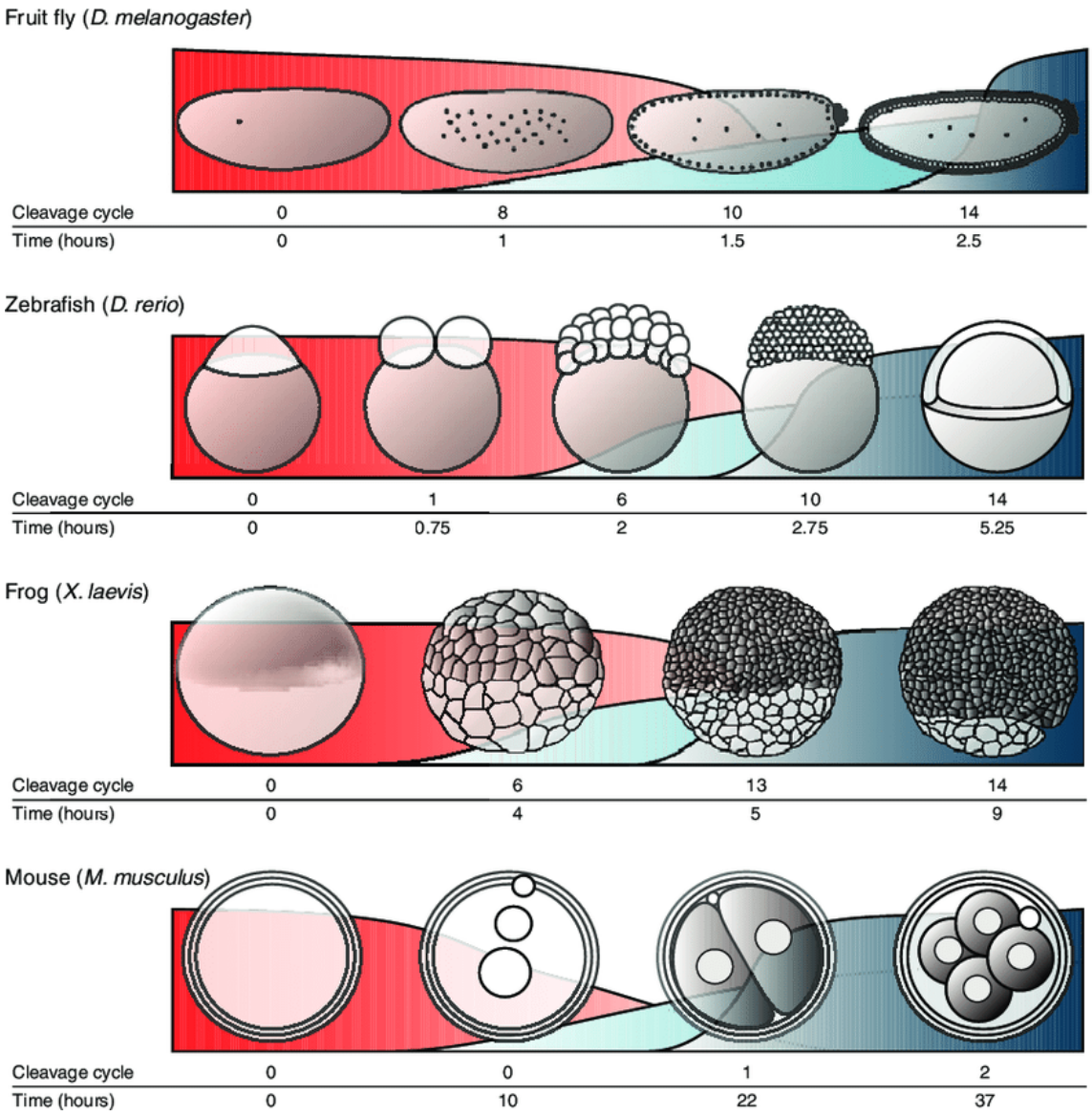

Figure 1. Animal embryos all start life with a period of genomic "silence" – their own DNA is not yet active, so all cellular processes are managed through the activity of maternal RNAs and proteins that were conveniently packed into the egg. The length of the maternally-controlled period (shown in red) varies from species to species before the maternal molecules are gradually degraded and the embryo transitions to making its own RNAs and proteins. This transition is called zygotic activation (shown in teal) and is the rest of development (shown in blue) is controlled by the embryo's own DNA. Figure is adapted from Tadros and Lipshitz 2009.

A four-hour old zebrafish embryo, which started as a single cell, has progressed through enough cycles of DNA replication and cell division to create ~2,000 additional cells. Also, incredibly, even though the embryo looks like nothing more than a ball of cells, the next generation of fish is already being established. This is possible because of a special structure called **germ plasm**, which is made of many gene products (RNAs and proteins) packaged and delivered from the maternal parent. The maternal gene products that comprise germ plasm have unique potential to convert normal embryonic cells into reproductive cells (also known as germ cells, such as sperm and eggs). Only a few cells in an embryo (~4-12) contain this germ plasm and these cells alone will give rise to all the germ cells in this fish for the rest of its life. Due to this special role, germ plasm-containing cells are called primordial germ cells.

Can you tell me more about germ plasm?

Germ plasm is a temporary but extraordinarily important structure inside the embryos of many different animal species. Germ plasm forms when an assortment of RNAs and proteins, all originally produced inside the mother's egg cell, pack together at certain positions within a developing embryo. Germ plasm seems fairly inactive at first, passively residing within just a few cells as the embryo continues to grow, but after a few hours it starts to break apart, flooding each host cell with instructions to commit to a reproductive fate. Only these cells, the ones that contained germ plasm in their first few hours of existence, will be the direct ancestors of every single egg or sperm cell produced by the embryo throughout its lifetime. The many other embryonic cells, which never contained germ plasm, will become other essential parts – perhaps the heart, brain, or eyes. But they will not genetically contribute to the next generation. That is a privilege afforded only to the reproductive or "germline" cells, whose cellular ancestors once contained germ plasm.

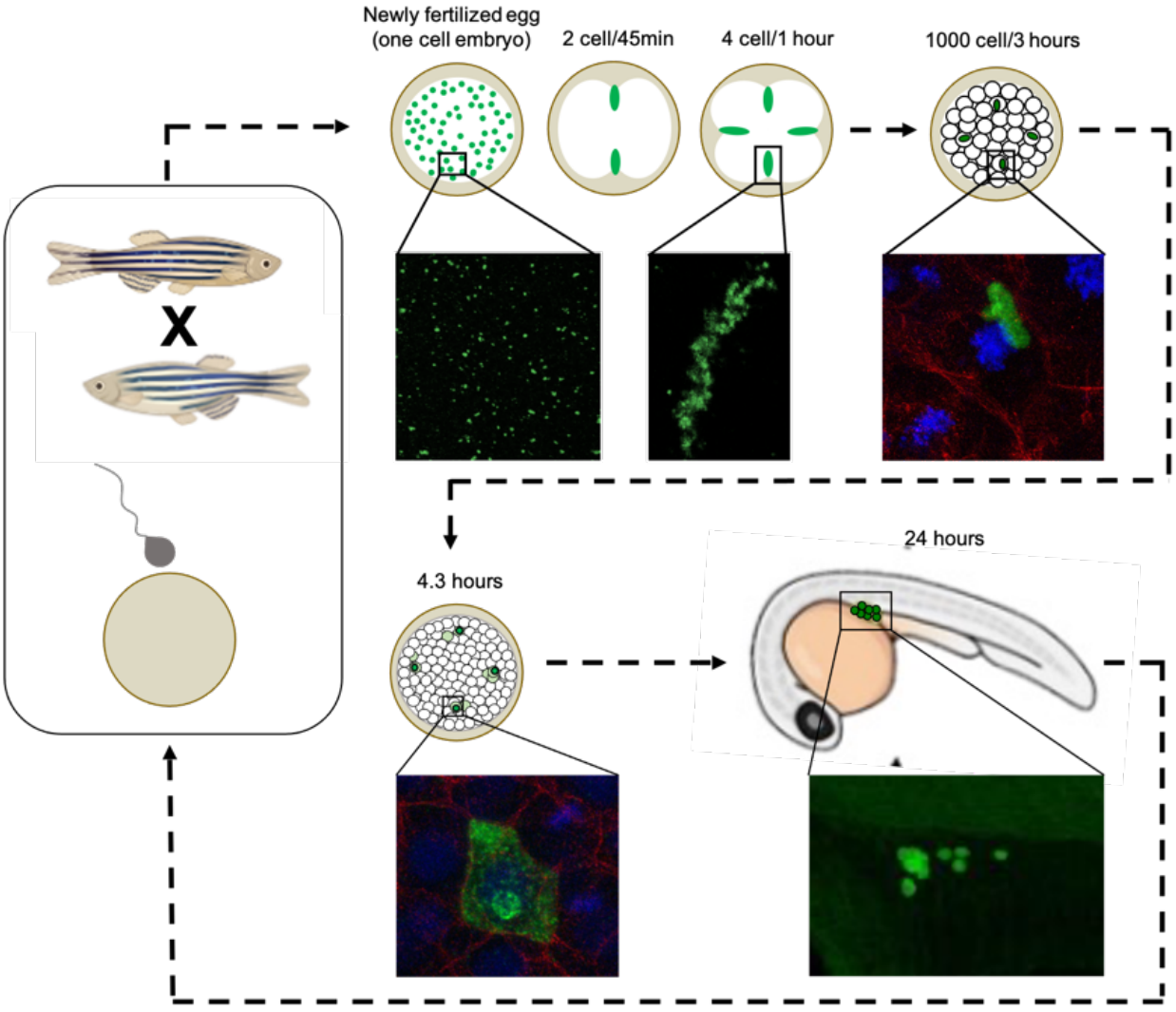

Figure 2. Reproductive cycle of zebrafish: from germ plasm to germ cells and back again. When eggs laid by a female zebrafish are fertilized, pre-existing germline RNAs and proteins (green) start to aggregate together at the ends of cleavage furrows (where the cells are dividing) during the first hour of development. The aggregated RNAs and proteins form germ plasm, which will be transmitted to a small subset of cells (cell boundaries in red, DNA in blue) within the growing embryo until after its own genome/DNA becomes active. At this point, the germ plasm contents leak out and disperse throughout their host cells, known as primordial germ cells (PGCs). The PGCs migrate to form the gonads in larval fish and eventually give rise to all the germ cells (eggs or sperm) of the adult fish.

How do developmental biologists use genetics in their work?

DNA, the instructions for life itself, is at the core of almost every biological process imaginable, and development is no exception. I use genetic tools and thinking to help answer questions. For example, in order to find out if a maternally expressed gene is important for primordial germ cell development, one of the first things I do is search for evidence of its corresponding RNA in embryonic regions where germ plasm is located. I use molecules called probes that stick to the RNA I'm interested in. You might've heard before that DNA is double-stranded, and that the two strands stick together by base-pairing. RNA is single stranded instead, but I can make short stretches of labeled RNA (the probe) that will stick to and make a double-stranded pair with the RNA I want to study. When I look at the fish embryos under a microscope with fluorescent lasers after I add the probes, any place that the probe found a matching RNA to stick to will glow! This technique is called fluorescence *in situ* hybridization.

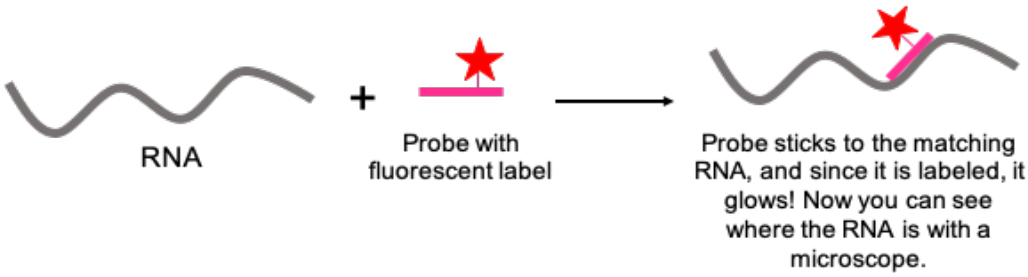

Figure 3. Diagram of fluorescence *in situ* hybridization using RNA probes.

I also directly edit or "mutate" the DNA itself in order to learn more about a gene's role in development. One way to make mutations is by using the CRISPR-Cas9 technique. CRISPR-Cas9 involves injecting zebrafish embryos with a protein which cuts DNA, and a short molecule called a guide that helps the protein find the specific gene sequence that I want it to cut. The cells in the

embryo try to repair the cut in the DNA but usually they make some mistakes. Those mistakes are different from the original DNA sequence, so they make mutated versions of the gene which no longer encode functioning proteins. I carefully observe the embryos with mutated DNA and, if they survive, their future offspring to look for anything that is different from normal fish. For a lot of the genes I study, fish with the mutated gene will grow up to be normal, healthy adults but they are unable to produce healthy babies. Depending on the part of the reproduction process that fails, I can learn more about the gene's normal role.

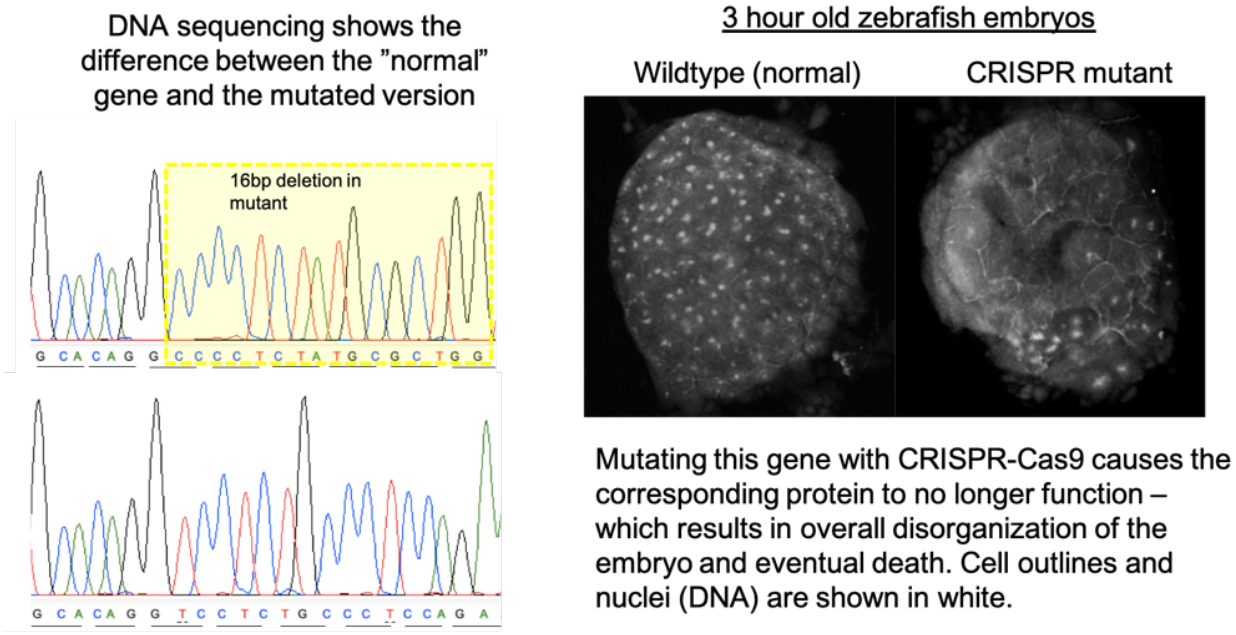

Figure 4. Comparison of the DNA sequence in a short region of a gene in a wildtype (normal) zebrafish and a mutant zebrafish. The mutant gene is missing 16 bases or "letters" of its DNA, which leads to a nonfunctional protein and decreased reproductive ability. Since adult female zebrafish with that mutation can only produce nonfunctional protein in her eggs, after fertilization her embryos show irregular cell sizes and abnormal nuclei compared to a wildtype embryo by the time both are 3 hours old (during the timeframe when all cellular activity is controlled by the pre-packaged maternal products rather than products made by the embryo's own DNA).

What were the main findings from your PhD research?

My dissertation research helped reveal 3 major findings about germ plasm:

1) Germ plasm is made of <u>homotypic RNA particles.</u>

Homotypic means "same" instead of "mixed." Before I started my research, scientists thought that germ plasm was made of molecules that were randomly mixed, with proteins and RNAs from different genes all spread around. We knew that germ plasm formed when many smaller particles containing proteins or RNAs aggregated into larger masses in the embryo, but we did not know much about the particles themselves. Do they fuse when they get very close to each other? How many different molecules made a single particle? Using fluorescence *in situ* hybridization and advanced microscope techniques, which let me see tiny details at very high magnification, I observed something different – the particles that make germ plasm each have their own identity. For example, some germ plasm particles are made of many RNAs from the *nanos* germ plasm gene clustered together, others are made of many RNAs from the *dazl* germ plasm gene clustered together, but none are made of both *nanos* and *dazl* together within the same particle. I was also able to calculate an approximate number of RNA molecules per particle – nine on average.

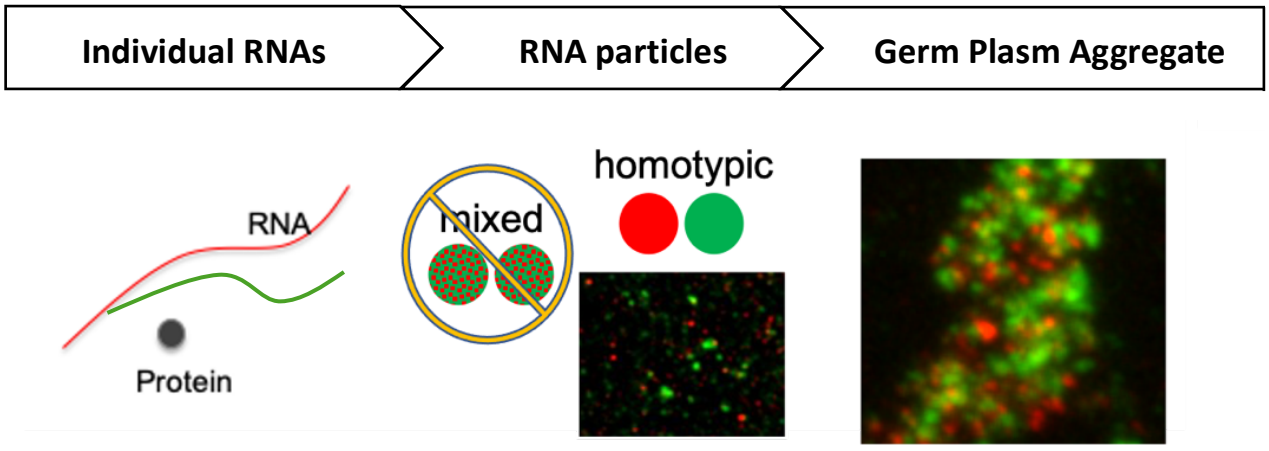

Figure 5. Multiple scales of germ plasm RNA organization. Individual RNAs take up around 50 nanometers (millionths of a millimeter) of space, homotypic particles made of clustered RNAs

range from ~300-600 nanometers, and the average germ plasm aggregate in 4 cell stage embryos are typically ~60 micrometers long by ~10 micrometers wide – less than one thousandth the size of a grain of rice. In these images, two different germ plasm RNAs, *dazl* and *vasa*, are labeled using fluorescence *in situ* hybridization.

2) Germ plasm forms a unique helical structure in young zebrafish embryos.

Most well-known sub-compartments (organelles) of animal cells, like the nucleus or mitochondria, are surrounded by membranes, which form a physical barrier to separate the organelle from the cytoplasm, the gel-like substance that fills the cell. Germ plasm aggregates, unlike mitochondria or nuclei, do not have a membrane to keep it separate from the rest of the cell. It is easy to understand how something with a membrane would stay separated from the outside world, but it is much harder to fathom how something with no physical barrier can remain distinct. But there are many clues in our daily lives to how this occurs. Think of oil and vinegar – both are liquids, but when poured into each other, they do not easily mix. This non-mixing event is called phase separation. Many phase separated structures form round droplets. On a very small scale, this is also true for germ plasm since it is made of many aggregated, round particles. At a larger scale though, the structure of germ plasm was puzzling – it almost never formed a sphere. Depending on the magnification, during the first few hours of life, zebrafish germ plasm can look like a long, thin, often squiggly rod. I investigated this structure in detail and found out that germ plasm often forms a massive helix-like shape (Figure 6).

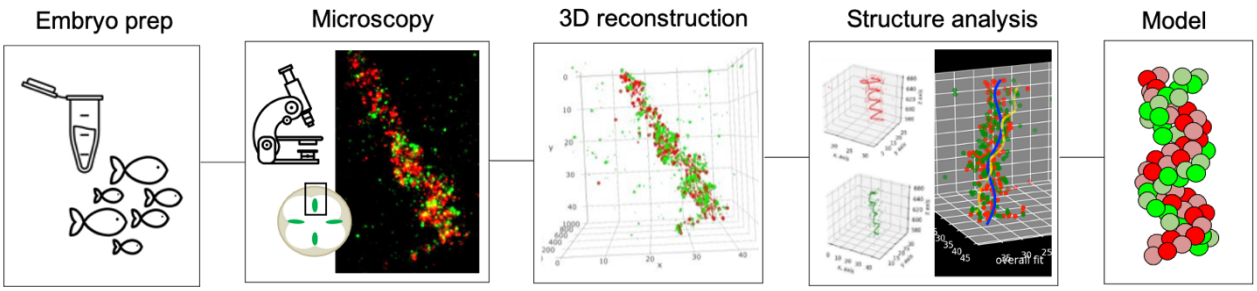

Figure 6. General analysis pipeline for characterizing germ plasm structure. The main steps include 1) embryo collection, preparation, and *in situ* hybridization; 2) acquiring high-resolution images of germ plasm using microscopy – each aggregate is imaged as a "stack" of thin slices that can be used for 3D reconstruction; 3) image processing and computational methods to reconstruct the germ plasm aggregate in a virtual 3D space; 4) curve-fitting and mathematical determination of germ plasm structural parameters based on the 3D reconstruction; 5) model building that incorporates results from the image analysis pipeline.

This finding led to many other questions and possibilities, all with two major themes: **how** and **why** does germ plasm form a helix? The full answers to these questions will likely take decades to uncover but through a lot of careful observation and experimentation I propose that the chemical and physical interactions between different types of RNAs and proteins make the helix structure more energetically favorable than other types of shapes, and that the concentration and timing of each germ plasm molecules' incorporation into the larger aggregate also contribute to the final shape. I explored ways that helical structure could be useful for the embryo – in many biological contexts, helices are stable yet flexible structures (think of the DNA double helix, the strong, ropelike strands of collagen proteins that provide structure to our skin, the spiral of a pea tendril climbing up a pole, etc.). These properties would be necessary in a developing embryo, with its rapid cell divisions and dramatic shape changes. A helix made of many smaller particles can condense those particles into a compact space and keep them together throughout perturbations, while also leaving them accessible for other molecules to act on at the appropriate time.

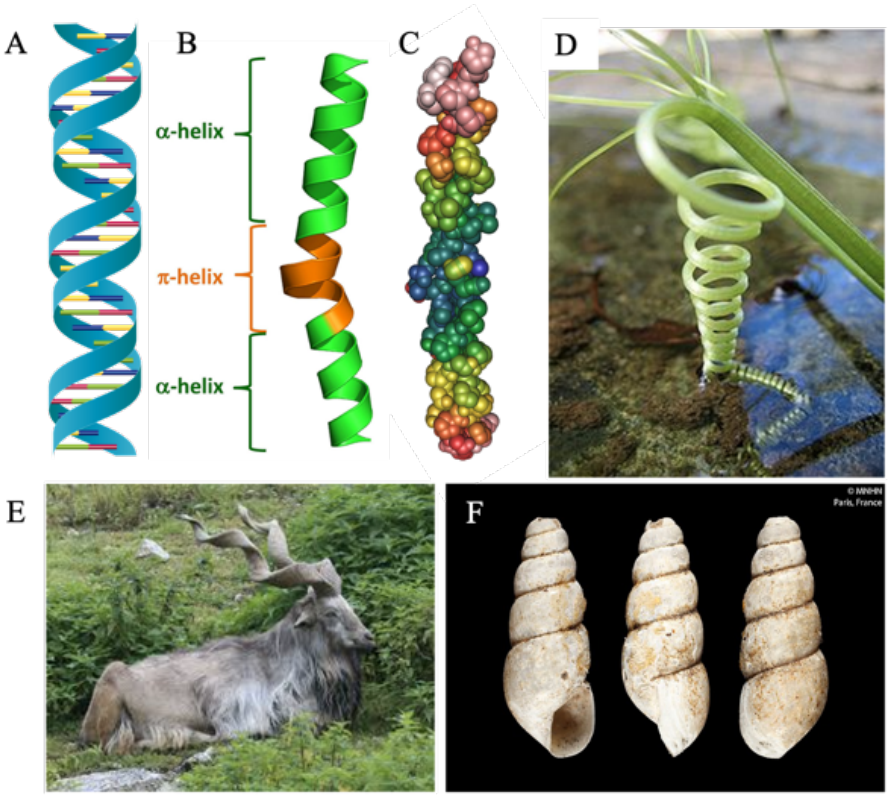

Figure 7. Examples of helical structure in the natural world. A) Double-helix structure of DNA B) Segment of a protein made of alpha- and gamma-helix domain structures C) Atomic model of a triple-helical collagen protein molecule D) Helical growth of a plant tendril E) Helical horns of a male markhor goat F) Shell of a *Helix clavulus* land snail. All images retrieved from Wikimedia Commons.

3) The genes and gene products that make germ plasm in zebrafish are <u>conserved</u> in closely related species.

Midway through my PhD, our lab started collecting other types of fish that are close relatives of zebrafish. Model organisms that are commonly used in scientific research (like zebrafish, mice, fruit flies, etc.) are useful tools but focusing only on those few species misses out on the incredible diversity found in biology. Even though germ plasm has the same general function in all species that use it, from fish to frogs to flies, and often shares several gene products in common, each species studied so far also has some seemingly species-specific molecules (RNAs and proteins) in

its germ plasm. Since these model species are very distantly related, I was curious if close relatives share all of the same germ plasm components or also have differences. I picked three members of the zebrafish family tree to study: *Danio kyathit*, *Danio albolineatus*, and *Devario aequipinnatus*.

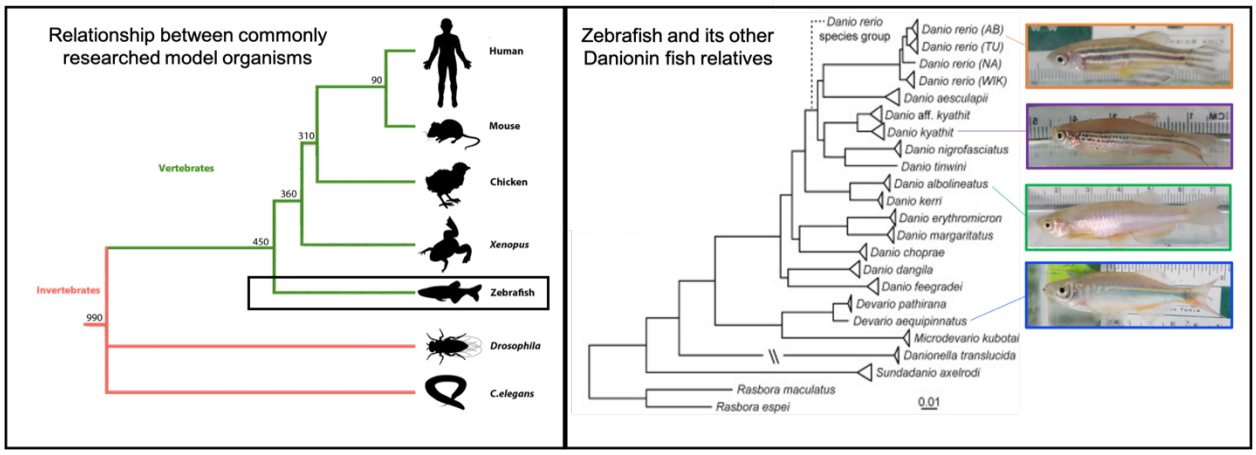

Figure 8. Evolutionary trees depicting the general evolutionary relationships between zebrafish, the other studied fish species, and a selection of commonly used scientific model organisms. A) Vertebrate germ plasm research is typically conducted in zebrafish and *Xenopus* frogs, which most recently shared a common ancestor more than 100 million years ago (MYA). The majority of germ plasm research has taken place using a handful of invertebrate species, particularly *Drosophila* flies and *C. elegans* worms, which last shared a common ancestor with zebrafish more than 990 MYA. B) The Danionin clade of fish, which includes zebrafish (*Danio rerio*) and its closest known relatives, is relatively young in evolutionary terms. Zebrafish (orange) is estimated to have last shared a common ancestor with *Danio kyathit* (purple) 6.5 MYA, with *Danio albolineatus* (green) 9.5 MYA, and with *Devario aequipinnatus* (blue) 13 MYA. This is comparable to the amount of divergence time between humans and chimpanzees (~7 MYA).

I collected embryos from all of these non-zebrafish species and used fluorescence *in situ* hybridization to test them for every single germ plasm RNA (12 total) that has ever been identified in zebrafish. I found that two types of fish, *Danio kyathit* and *Danio albolineatus*, shared all of those 12 RNAs with zebrafish, and the third, *Devario aequipinnatus*, shared 11/12 of the RNAs (Figure 9). The one missing RNA, called *ca15b*, makes a protein in zebrafish that helps signal the start of primordial germ cell migration, which is the process when future germ cells travel through

the embryo until they reach the site where gonads are formed. Defective primordial germ cell migration causes infertility, so we know that *Devario aequipinnatus* embryos can still accomplish migration without germ plasm-localized *ca15b* since they are able to reproduce. This led us to hypothesize that they must be using other molecules or mechanisms to control their primordial germ cell migration process. I am excited to keep exploring this question after my PhD, and also look forward to expanding this type of comparative analysis between additional fish species to gain more insight into which genes are necessary for germ plasm function (and therefore fertility), and characterize the different ways that different species have evolved methods of forming primordial germ cells.

How could society be impacted by your work?

My research is an example of foundational or "basic" science, meaning that it involves the pursuit of knowledge even though there is not yet a clear path to a direct "translational" outcome on human health. However, there are many connections between my research topic and animal reproduction (humans included!). For example, mutations in a gene called "*daz*" in humans are the leading genetic cause of male infertility; a directly related gene called "*daz-like*" is a major component of zebrafish germ plasm, which we know is necessary for fertility in these fish.

Very early embryonic development, when primordial germ cells are formed, can be extremely challenging to study - just thinking about the logistics and ethics involved in trying to study human embryos can be mind-boggling. Unlike humans, zebrafish eggs are fertilized and develop into embryos outside of the mother's body, so they are easily accessible without having to perform any invasive procedures. Zebrafish are also prolific breeders, capable of producing hundreds of embryos each week, making them an ideal animal for my research topic. Since zebrafish share

about 70% of their ~26,000 genes with humans, learning about their genes can help us understand what they do in humans too.

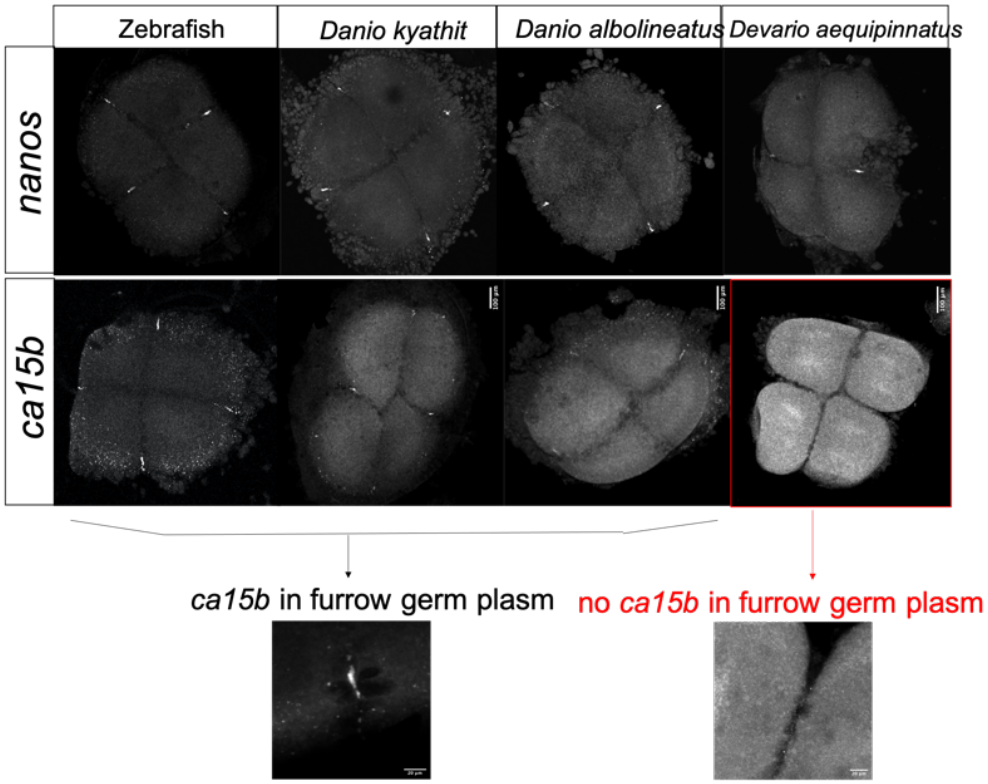

Figure 9. Images of one-hour old Danionin embryos after fluorescence *in situ* hybridization labeling of germ plasm RNAs originally identified in zebrafish. Each image is the top view of a four-cell embryo from either zebrafish, *Danio kyathit*, *Danio albolineatus*, or *Devario aequipinnatus*. The top row shows that *nanos* RNA is in the germ plasm of all four species at the outer edges of cleavage furrows, the deep ridges where the embryonic cells are separating from one another. The same result was seen for an additional 10 germ plasm RNAs. The bottom row shows the sole exception, *ca15b*, is in the germ plasm of zebrafish and it's two *Danio* relatives, but is not in the germ plasm of *Devario aequipinnatus* embryos. Magnified images of the furrow show that instead of distinct, highly-concentrated localization (as in the three *Danio* species), *ca15b* seems to be distributed throughout the entire *Devario aequipinnatus* embryo.

Image attributions

Figure 1. Reproduced from Tadros W, Lipshitz HD. The maternal-to-zygotic transition: a play in two acts. Development. 2009 Sep;136(18):3033-42. doi: 10.1242/dev.033183. PMID: 19700615.

Figure 2. Includes graphic icons from BioRender.com (male and female adult zebrafish) and modified from https://mindthegraph.com/blog/scientific-illustrations-maker/ (24 hpf zebrafish). Micrograph image of Vasa-positive primordial germ cells in a 24hpf zebrafish was reproduced from GeneTex Cat No. GTX128306.

Figure 5. Includes images from C Eno,* CL Hansen,* F Pelegri. (2019). Aggregation, segregation and dispersal of homotypic germ plasm RNPs in the early zebrafish embryo. Developmental Dynamics. doi:10.1002/dydy.18

Figure 6. Depicts analysis pipeline developed in collaboration with Marc Chevrette (UW-Madison; Wisconsin Institutes for Discovery) and Monica Selvaraj (UW-Madison; Whitehead Institute).

Figure 7A. This file is licensed under the Creative Commons Attribution 4.0 International license and retrieved from https://commons.wikimedia.org/wiki/File:201812_DNA_double-strand_B.svg. Source: user To-gopic; https://doi.org/10.7875/togopic.2018.23; DataBase Center for Life Science (DBCLS).

Figure 7B. This file is licensed under the Creative Commons Attribution-Share Alike 3.0 Unported license and retrieved from https://commons.wikimedia.org/wiki/File:Pi-helix_within_an_alpha-helix.jpg. Source: user Rbcooley; own work; structure taken from PBD code 3QHB.

Figure 7C. This file is licensed under the Creative Commons Attribution-Share Alike 3.0 Unported license and retrieved from https://commons.wikimedia.org/wiki/File:1bkv_collagen_02.png. Source: Nevit Dilmen; user Nevit; Self created from PDB entry with Cn3D Data Source: https://www.ncbi.nlm.nih.gov/Structure/

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Figure 7F. This file is licensed under the Creative Commons Attribution 4.0 International license and retrieved from https://commons.wikimedia.org/wiki/File:Helix_clavulus_(MNHN-IM-2000-4725).jpeg. Source: Museum national d'Histoire naturelle (2020). The molluscs collection (IM) of the Muséum national d'Histoire naturelle (MNHN - Paris). https://doi.org/10.15468/xgoxap

Figure 8A. Modified from https://www.genesinspace.org/news/blog/model-organisms-earth-and-space/ and based on figure from Wheeler GN, Brändli AW. Simple vertebrate models for chemical genetics and drug discovery screens: lessons from zebrafish and Xenopus. Dev Dyn. 2009 Jun;238(6):1287-308. doi: 10.1002/dvdy.21967. PMID: 19441060.

Figure 8B. Modified from Braedan M. McCluskey, Ingo Braasch. Chapter 2 - Zebrafish Phylogeny and Taxonomy, Editor(s): Samuel C. Cartner, Judith S. Eisen, Susan C. Farmer, Karen J. Guillemin, Michael L. Kent, George E. Sanders, In American College of Laboratory Animal Medicine, The Zebrafish in Biomedical Research, Academic Press, 2020, Pages 15-24, ISBN 9780128124314, https://doi.org/10.1016/B978-0-12-812431-4.00002-6.