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Methods to study maternal regulation of germ cell specification in zebrafish

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Abstract

The process by which the germ line is specified in the zebrafish embryo is under the control of maternal gene products that were produced during oogenesis. Zebrafish are highly amenable to microscopic observation of the processes governing maternal germ cell specification because early embryos are transparent, and the germ line is specified rapidly (within 4–5 h post fertilization). Advantages of zebrafish over other models used to study vertebrate germ cell formation include their genetic tractability, the large numbers of progeny, and the easily manipulable genome, all of which make zebrafish an ideal system for studying the genetic regulators and cellular basis of germ cell formation and maintenance. Classical molecular biology techniques, including expression analysis through *in situ* hybridization and forward genetic screens, have laid the foundation for our understanding of germ cell development in zebrafish. In this chapter, we discuss some of these classic techniques, as well as recent cutting-edge methodologies that have improved our ability to visualize the process of germ cell specification and differentiation, and the tracking of specific molecules involved in these processes. Additionally, we discuss traditional and novel technologies for manipulating the zebrafish genome to identify new components through loss-of-function studies of putative germ cell regulators. Together with the numerous aforementioned advantages of zebrafish as a genetic model for studying development, we believe these new techniques will continue to advance zebrafish to the forefront for investigation of the molecular regulators of germ cell specification and germ line biology.

INTRODUCTION

To realize the reproductive imperative of the organism, one of the first tasks the developing embryo undertakes is specification of its germ line. Although sexually reproducing organisms universally prioritize germ line formation, there is considerable diversity throughout the animal kingdom in the timing and manner in which germ cells are specified. There are two major modes of germ line formation: the “maternal inheritance” mode utilizes acquisition of maternal products that are pre-localized within the egg to drive germ cell formation in a small number of cells in the embryo, whereas the “inductive mode” utilizes zygotic signals from nearby somatic cells to induce germ cell specification (reviewed in Marlow, 2010; Seervai & Wessel, 2013). Mammals use the inductive mechanism of germ line formation, with the genetic organism *Mus musculus* having contributed significantly to our understanding of this specification mode. On the other hand, a wide range of invertebrate

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and vertebrate species employ the maternal inheritance mode, with *Caenorhabditis elegans*, *Drosophila*, *Xenopus*, and zebrafish serving as the predominant genetic organisms used to study this mechanism (Seervai & Wessel, 2013). Based on these laboratory organisms, it is tempting to speculate that evolutionarily primitive organisms use the maternal inheritance mode, while more complex species use the inductive mode to specify their germ lines. However, a survey of metazoans revealed that the inductive mechanism is actually more prevalent throughout the animal kingdom and likely represents the ancestral mode of germ cell specification (Extavour & Akam, 2003; Johnson et al., 2003). One recent hypothesis is that maternal inheritance is the preferred mode for rapid germ line development because the molecules for specification are prepackaged and ready to go (Evans, Wade, Chapman, Johnson, & Loose, 2014; Extavour & Akam, 2003; Johnson, Richardson, Bachvarova, & Crother, 2011; Wessel, 2014). Accordingly, the mode by which an animal specifies its germ line likely has more to do with speed of specification, such that animals that use the maternal mode specify their germ line earlier than those that use the inductive mode.

Despite differences in specification mode, many basic aspects of germ cell biology and the molecular components that define the germ line are conserved among species (reviewed in Gustafson & Wessel, 2010; Hartung & Marlow, 2014). The zebrafish system has become a key research model for studying conserved and divergent aspects of germ cell biology and germ line molecules because it is a genetically tractable vertebrate organism, with great fecundity, rapid development, and optically transparent embryos. These advantages, in conjunction with classical genetic screens and morpholino technology, have facilitated identification and understanding of crucial regulators of germ cell specification, migration, and development. Lately, new technologies have enabled more directed loss-of-function approaches and have contributed to the generation of more advanced tools for visualization of germ cell components. In this chapter, we will discuss the traditional methods used to study maternal specification in zebrafish and highlight some of the major technological advances that are providing for even more in-depth investigation and understanding of the molecular mechanisms governing this process.

1. STAGES OF MATERNAL GERM PLASM REGULATION AND SPECIFICATION

1.1 RECRUITMENT AND TRANSPORT OF GERM PLASM IN THE OOCYTE

Maternal germ cell specification relies on a form of cytoplasm enriched in RNAs and proteins that determine germ line fate, suitably called the “germ plasm.” Organisms that specify their germ lines through maternal mechanisms first localize the germ plasm in a particular region of the oocyte and eventual egg. When the egg is fertilized, the cells that inherit that specialized cytoplasm become the primordial germ cells (PGCs). Thus the localization of germ plasm in the developing oocyte, and subsequently in the mature egg, is the primary event that precedes maternal germ cell specification in the fertilized embryo.

During the mitotic stages of germ cell development and early oogenesis, germ plasm is symmetrically distributed throughout the cell (Baat, Zandbergen, van de Water, Goos, & Zivkovic, 1999; Selman, Wallace, Sarka, & Qi, 1993). This symmetrical arrangement of

germ plasm is first disrupted in early meiotic stages of oogenesis by the formation of the Balbiani body, a singular large aggregate of germ plasm, that forms adjacent to the nucleus on the future vegetal side of the stage I oocyte (Fig. 1). In addition to germ plasm components, the Balbiani body also contains patterning molecules that will later specify dorsal structures in the embryo, as well as organelles such as endoplasmic reticulum and mitochondria (Kloc, Bilinski, & Etkin, 2004; Kloc et al., 2002; Kloc & Etkin, 1995; reviewed in Marlow, 2010). A zebrafish mutant in a gene required for Balbiani body formation, the *bucky ball* mutant, has provided genetic evidence that formation of the Balbiani body is essential to embryonic patterning and maintenance of germ plasm (Bontems et al., 2009; Dosch et al., 2004; Marlow & Mullins, 2008). Interestingly, the Balbiani body has been observed in primary oocytes of every organism examined, including mammals such as humans and mice which use the inductive mode of specification (Bontems et al., 2009; Kosaka, Kawakami, Sakamoto, & Inoue, 2007; Marinos & Billett, 1981; Marlow & Mullins, 2008; von Wittich, 1845); thus it seems likely that this conserved structure has an important role outside of organizing germ plasm although the exact nature of this role remains elusive.

The Balbiani body exists only transiently in stage I (primary) oocytes and is promptly remodeled in subsequent stages (Kloc et al., 2004; Kosaka et al., 2007). In stage II oocytes, the Balbiani body and its germ plasm components are disassembled and translocated to the vegetal cortex (Fig. 1) (Kloc & Etkin, 1995; Kosaka et al., 2007; Yisraeli, Sokol, & Melton, 1990). This translocation pathway is dependent on the cytoskeleton in zebrafish, as evidenced by analysis of a zebrafish mutant in *microtubule actin crosslinking factor 1* (*macf1*), also called *magellan* (Gupta et al., 2010). Zebrafish mutants for *magellan* are able to assemble the Balbiani body and recruit germ plasm components, but fail to translocate Balbiani body contents to the cortex (Gupta et al., 2010). Zebrafish embryos that lack maternal *magellan* have an apolar phenotype similar to that of *bucky ball* mutants, indicating that translocation of Bb-components is an essential aspect of the vegetal localization pathway. After translocation to the vegetal cortex, germ plasm RNAs are temporarily separated into different expression domains (reviewed in Hartung & Marlow, 2014). For example, in stage III oocytes, the germ plasm RNAs *vasa* and *dazl* remain at the vegetal pole, while *bucky ball* localizes to the animal pole, and *nanos3* is diffusely distributed throughout the cytoplasm (Baat et al., 1999; Kosaka et al., 2007; Marlow & Mullins, 2008; reviewed in Abrams & Mullins, 2009). The reasons behind these differential distribution patterns in late oogenesis are especially perplexing given that upon egg activation, all germ plasm RNAs rejoin at the animal pole (Fig. 1). One hypothesis to explain this unusual pattern is that Balbiani body components are dispersed in late stages of oogenesis to facilitate sorting of germ plasm from other patterning molecules prior to fertilization.

1.2 GERM PLASM LOCALIZATION AND GERM CELL SPECIFICATION IN THE EARLY EMBRYO

The process of reassembling germ plasm at the animal pole upon egg activation (fertilization) also uses more than one pathway. Meticulous tracking of RNA distribution patterns in the freshly laid zebrafish egg has revealed that at least two distinct mechanisms exist in the recruitment of germ plasm RNAs to the animal pole (Theusch, Brown, & Pelegri,

2006). Germ plasm RNAs and proteins are brought to the animal pole of the embryo through nonspecific movement of cytoplasm, as well as microtubule-dependent translocation of specific cargo (Fuentes & Fernandez, 2010; Leung, Webb, & Miller, 2000; Solnica-Krezel & Driever, 1994; and reviewed in Abrams & Mullins, 2009). After reaching the animal pole of the egg, the germ plasm becomes further restricted during cleavage stages by tightly associating with the cleavage furrows of the dividing blastomeres (Fig. 1) (Knaut, Pelegri, Bohmann, Schwarz, & Nusslein-Volhard, 2000; Theusch et al., 2006; Yoon, Kawakami, & Hopkins, 1997). Association with the furrows is maintained for the first two cell divisions, however once the embryo reaches the 8-cell stage, only four cleavage furrows maintain germ plasm (Yoon et al., 1997). Microtubules and microtubule-associated motor proteins (Kinesins) are essential for germ plasm recruitment to the furrow. This has been demonstrated by studies of zebrafish *nebel*/maternal mutants that have a reduced furrow microtubule array and therefore cannot maintain germ plasm within the furrows (Pelegri, Knaut, Maischein, Schulte-Merker, & Nusslein-Volhard, 1999; Theusch et al., 2006), as well as maternal *kinesin Kif5Ba* mutants that cannot localize germ plasm to the furrows despite intact microtubules (Campbell, Heim, Smith, & Marlow, 2015).

During slightly later embryonic cleavage stages, between 16- and 32-cells, the furrow-associated germ plasm becomes sequestered into four PGCs, effectively specifying the germ lineage (Lin, Long, Chen, & Hopkins, 1992; Walker & Streisinger, 1983; reviewed in Raz, 2003). Because zebrafish germ cell specification occurs outside of the future gonad anlage, the PGCs must migrate toward this site during embryogenesis to establish interactions with somatic support cells and differentiate into gametes. Interestingly the germ plasm component Dead end (*Dnd*) is required for migration to the gonad region (Weidinger et al., 2003). Embryos injected with *dnd* morpholino have PGCs that are unable to migrate and eventually cannot be maintained (Weidinger et al., 2003). Similar to other migrating cells, PGCs require chemotactic signaling molecules for their migration, for example the chemokine Stromal cell-derived factor 1 (*Sdf1*) and its receptor *Cxcr4* (Doitsidou et al., 2002; Knaut, Werz, Geisler, Nusslein-Volhard, & Tübingen Screen, 2003; Raz, 2003). Mutation or knockdown of these two gene products through morpholino injection results in various PGC migration defects including mislocalized PGCs. The requirement for a signaling gradient of *Sdf1* also demonstrates that PGC migration partially requires cues from surrounding somatic tissues (Reichman-Fried, Minina, & Raz, 2004). Ultimately the PGCs arrive at and colonize the future gonad where they will proliferate and undergo sexual differentiation (Fig. 1). Here, in the differentiated oocytes of the female fish, the germ plasm life cycle begins again.

2. STUDYING THE DYNAMIC LOCALIZATION OF GERM PLASM RNAs

The easy-to-access and easy-to-manipulate fertilized zebrafish egg has been utilized extensively to examine the repertoire of maternal RNAs, with particular emphasis on those encoding localized patterning molecules such as germ plasm components. This unique category of RNAs has been examined through a variety of techniques ranging from detection of endogenous transcripts to injection of exogenous RNAs and reporters.

2.1 DETECTION OF ENDOGENOUS GERM PLASM RNAs

Whole-mount *in situ* hybridization (WISH) is the gold standard for detection of endogenous RNAs in zebrafish embryonic germ cells and adult tissues such as the ovary. WISH has been performed on nearly the entire library of zebrafish cDNAs using embryos from 1-cell to 5 days old, greatly improving our understanding of which genes are expressed maternally as well as in the germ line (Thisse & Thisse, 2004). As an example, the germ plasm RNA *dead end* was first identified in a large-scale screen for genes expressed in zebrafish PGCs (Weidinger et al., 2003). More recently, microarray-based screens of isolated PGCs have been used to discover germ cell components that were subsequently verified by *in situ* hybridization and other germ plasm assays described below (Strasser et al., 2008).

Conventional *in situ* hybridization makes use of a synthetic antigen-labeled RNA probe complementary to the mRNA whose expression is being detected. The most commonly used methods for WISH to study zebrafish development rely on detection of the labeled probe through antibodies conjugated to enzymes which catalyze a reaction that produces a visible precipitate in the vicinity of the probe (Fig. 2A) (Nusslein-Volhard & Dahm, 2002; Thisse & Thisse, 2004, 1998). The precipitate can either be a dark-colored compound visible by simple light microscopy or a fluorescent compound detectable with fluorescent microscopy (fluorescent *in situ* hybridization, FISH). In both cases, the visible precipitate is not conjugated to the original probe and therefore is diffusible, resulting in limited sensitivity and subcellular resolution due to spreading of the signal and high background.

To address some of these issues, a new form of FISH has been developed that does not employ a diffusible fluorescent molecule. This technology, called RNA-scope, relies on special probes with 5' sequences that are complementary to the target RNA, and 3' tail sequences that serve as scaffolds for amplifier RNA molecules conjugated to fluorescent fluorophores (Wang et al., 2012). RNAscope, therefore, enables specific detection of RNAs, while also restricting the fluorescent signal to the probe–target interaction site. This technique has higher signal specificity and lower background than conventional *in situ* techniques and may even be able to detect single RNA molecules. RNAscope has been adapted for use in zebrafish embryos (Gross-Thebing, Paksa, & Raz, 2014) and has also been used along with dual color FISH to examine the intracellular distribution of germ plasm RNAs within PGCs and oocytes (Campbell, Chao, Singer, & Marlow, 2015; Campbell, Heim, et al., 2015; Gross-Thebing et al., 2014; Welch & Pelegri, 2014).

While these *in situ* hybridization-based techniques remain the only option for detecting endogenous RNAs, there are a few differences between these approaches that one should consider. As previously mentioned, WISH and FISH are less sensitive than the RNAscope technique. Additionally the conventional *in situ* methods are often not compatible with antibody detection of proteins (depending on the antigen); whereas, the RNAscope method is more amenable to simultaneous detection of protein and RNAs (Gross-Thebing et al., 2014). Thus the expression level of your gene, the required level of resolution, and any requirements to examine protein comarkers, should guide your decision in choosing an *in situ* hybridization technique.

2.2 INJECTION OF EXOGENOUS RNAs INTO OOCYTES AND EMBRYOS

Injection of *in vitro*-transcribed RNAs into zebrafish oocytes and 1-cell stage embryos has been widely used in functional studies of germ plasm RNA regulation and maternal-effect mutants (Fig. 2B). This technique offers several advantages including relatively rapid experimental time frames, the ability to visualize translated products with fluorescent protein (FP) fusions, and significant flexibility to modify the transcripts to be injected (for example, mutating putative functional or regulatory domains and creating dominant negative constructs).

The method of injecting oocytes is considerably more technically challenging than injection into embryos. While injection of exogenous RNAs into oocytes has been well established in developmental model organisms such as *Xenopus* and mouse, this technique has only recently been explored in zebrafish due to limitations associated with *in vitro* oocyte culture and maturation competence (Csenki et al., 2010; Nair, Lindeman, & Pelegri, 2013; Nair, Marlow, et al., 2013). Nonetheless, oocyte RNA injection has become a useful tool to assess the translational competence and even functional capacity of maternal molecular factors (Fig. 2B) (Bontems et al., 2009; Nair, Lindeman, et al., 2013; Nair, Marlow, et al., 2013).

Building on previously established methods for culturing zebrafish oocytes (Seki et al., 2008; Selman, Petrino, & Wallace, 1994), the Pelegri group developed a modified oocyte culture and maturation protocol that they then used for *in vitro* injection of RNAs into immature oocytes from stages I through IV (Nair, Lindeman, et al., 2013; Nair, Marlow, et al., 2013). These studies demonstrated that oocytes of all stages are translationally competent when injected with GFP mRNA, with visible detection of GFP requiring approximately 1–2 h. Unfortunately, oocytes from stage I–III could not be matured to fertilization competence. However, stage IV oocytes injected with exogenous RNAs can be matured and fertilized, providing a window of opportunity for assessing (late) maternal contributions of the oocyte to early embryonic development.

Furthermore, injection of RNAs encoding wild-type versions of proteins into oocytes has been demonstrated to rescue aspects of some maternal effect mutant phenotypes—an indication that these exogenous RNAs are not only translated, but are also functional (Bontems et al., 2009; Nair, Lindeman, et al., 2013; Nair, Marlow, et al., 2013). For example, Bontems et al. (2009) demonstrated that stage I oocytes mutant for *bucky ball* recovered aspects of their early oocyte germ plasm phenotypes when injected with wild-type *bucky ball* RNA. Similarly, the early embryonic maternal-effect phenotypes of *aurora kinase B* (*cellular island*) and chromosomal passenger complex protein *birc5b* (*motley*) mutants can be rescued by injection of stage IV mutant oocytes with wild-type RNA (Nair, Lindeman, et al., 2013; Nair, Marlow, et al., 2013).

In contrast to oocyte injection, injection of protein-coding RNAs into 1-celled embryos is an assay often used to identify regulators of germ plasm RNA localization, translation, and stability. For this technique, embryos are injected with a synthetic RNA that contains the candidate mRNA fused in frame to an FP-mRNA. When translated, the FP serves as a reporter, and later embryos are assessed for localization or enrichment of the FP at either the cleavage furrows (8–16 cell stage) or within the PGCs (24–30 hpf) using fluorescent

microscopy detecting the FP directly or by immunohistochemistry (Fig. 2B). The injected molecule can also be evaluated using *ISH* to monitor the nonnative transcript (eg, *gfp*). This assay is particularly useful owing to the easy access to fertilized eggs for injection, as well as the unique property of many germ line RNAs to be specifically enriched and/or translated in the germ plasm and germ cells of the early embryo. Numerous RNAs have been designated as germ line components based on their germ plasm or germ cell localization in this assay; the FP fusions produced by *bucky ball*, *vasa*, *dead end*, *tdrd7*, *granulito*, *dazl*, and *elavl2* RNAs have been visualized in the cleavage furrows or germ cells of early embryos (Bontems et al., 2009; Knaut, Steinbeisser, Schwarz, & Nusslein-Volhard, 2002; Mickoleit, Banisch, & Raz, 2011; Strasser et al., 2008; Takeda, Mishima, Fujiwara, Sakamoto, & Inoue, 2009; Weidinger et al., 2003). Additionally, injection of mutated and truncated

RNAs of zebrafish *vasa* and *dead end* have identified protein domains that are important for localization of these RNA-binding proteins to the germ granules of PGCs (Slanchev et al., 2009; Wolke, Weidinger, Kopranner, & Raz, 2002). RNA injection has also become a valuable tool to test the function of *cis*-acting elements within the 3' untranslated region (UTR) of germ plasm RNAs (Kedde et al., 2007; Mickoleit et al., 2011; Mishima et al., 2006; Wiszniak, Dredge, & Jensen, 2011). For example, systematic mutation of the microRNA miR-430 sites in the 3' UTRs of injected UTR reporters for *nanos3*, *tdrd7*, and *elavl2* have identified a protective role for the RNA-binding protein Dazl (deleted in azoospermia-like) in preventing miR430-mediated degradation of these germ plasm RNAs in the soma (Kedde et al., 2007; Mickoleit et al., 2011; Mishima et al., 2006). Finally the *cis*-acting elements within the 3' UTR of germ plasm RNAs such as those of *nanos3* (Kopranner, Thisse, Thisse, & Raz, 2001) have proven to be a powerful tool to enrich transcripts within germ cells and assess their germ cell-specific functions in PGC migration and survival (Blaser et al., 2006; Ramasamy, Wang, Quach, & Sampath, 2006; Strasser et al., 2008; Xu, Kardash, Chen, Raz, & Lin, 2012).

Despite the utility of RNA injection in mechanistic studies of germ cell development, including maternal inheritance, there are several caveats to consider while interpreting the results of such experiments. Injected in picogram quantities, exogenously provided RNAs are almost always overexpressed relative to endogenous levels. Additionally, injected RNAs are *in vitro* transcribed, usually with the addition of long polyadenylated tails. The length of the polyA tail is a well-known regulatory mechanism of RNA stability and translational capacity (Barkoff, Ballantyne, & Wickens, 1998; Salles, Lieberfarb, Wreden, Gergen, & Strickland, 1994; Subtelny, Eichhorn, Chen, Sive, & Bartel, 2014), especially important during oocyte and egg transitions. Therefore, exogenous injected RNAs likely have artificially high stability and translational capacity. Moreover, because maternal RNAs are produced during oogenesis and are stored, degraded, or translated based on their association with RNA-binding proteins that may or may not be present in eggs, it is unclear how closely metabolism of exogenous RNAs recapitulates the endogenous RNA. Finally, as a result of their exceptionally high abundance and stability, it is important to verify that these RNAs and/or their protein reporters recapitulate the localization of, and the mechanisms used by, their endogenous counterparts.

2.3 LIVE VISUALIZATION OF LABELED RNAs

Given the dynamic nature of germ plasm granules, and the germ plasm translocation events that occur during oocyte development, it's no surprise that researchers have been seeking methods to track RNA particle movement in the germ line of living organisms. Hybridization-based techniques are impossible to apply in live cells of whole organisms because living cells are insufficiently permeable to the probing reagents. Injection of fluorophore-conjugated RNAs, a method widely applied to studies of germ plasm in *Xenopus* (Gagnon & Mowry, 2009, 2010; Lewis, Gagnon, & Mowry, 2008; Messitt et al., 2008), has been utilized to visualize RNA localization and to identify localization motifs and structures (Gilligan et al., 2011); however, thus far, this approach has not been applied to studies of germ plasm RNAs in zebrafish. One limitation to this approach includes variability in incorporating the fluorescent label in the target RNA and the limited window of availability to deliver the labeled RNAs. To address these limitations, the MS2 RNA labeling system, previously employed in several other model organisms (Belaya & St Johnston, 2011; Gagnon, Kreiling, Powrie, Wood, & Mowry, 2013; van Gemert et al., 2009; Park et al., 2014), has been adapted for use in zebrafish (Campbell, Chao, et al., 2015).

This genetically encoded RNA labeling system makes use of two basic components borrowed from the MS2 bacteriophage: the bacteriophage coat protein, and the RNA hairpin to which the coat protein binds. To adapt this system for *in vivo* RNA labeling, researchers have fused the MS2 coat protein (MCP) with an FP, and tethered together several repeats of the RNA hairpin, also called the MS2-binding site (MBS), inserting these MBS repeats into the RNA sequence of interest (Fig. 2C) (Bertrand et al., 1998). In the recently published zebrafish system, an FP-tagged MCP dimer (FP-MCP) (Wu, Chao, & Singer, 2012) and RNA 3'UTR reporters with 24 copies of the MBS, have been engineered into stable zebrafish transgenic lines, for ease of use and detection (Table 1) (Campbell, Chao, et al., 2015).

The zebrafish MS2 system has been successfully used to observe nascent transcription in live embryos (fluorescent nuclear puncta) and to visualize transport of these nascent transcript "particles" out of the nucleus (Campbell, Chao, et al., 2015). Although the system has yet to be used to study the movement of germ plasm RNAs in PGCs or oocytes, MS2-tagged *nanos.3* localizes to the same germ granules as the endogenous *nanos.3* RNA in PGCs (Campbell, Chao, et al., 2015). It is expected to be both interesting and informative to use this system to study germ granule movement during PGC development, as well as germ plasm dynamics in oocytes, although both contexts would require hours-long stretches of imaging live cells, as well as overcoming the technical challenge presented by the optically dense yolk proteins.

One limitation of the MS2 system is that only the 3'UTR sequences of RNAs have been assessed thus far. This has the advantage of monitoring RNA dynamics without the potential complications associated with protein overexpression; however, if coding regions of the RNA are important for RNA metabolism or localization they would be missed. It is also possible that overexpressing a 3'UTR could titrate out RNA-binding proteins (Donnelly et al., 2013, 2011; Garcia-Gras, Chi, & Thompson, 2000; Wang et al., 2015); however, thus far evidence of such an effect in germ cells is lacking. Additionally, another application of this

system would be to immunoprecipitate the tagged MCP proteins in order to identify RNA-binding proteins that associate with one's RNA of interest in a 3'UTR-dependent manner, as has previously been demonstrated for mammalian cell culture and *C. elegans* (Kwak, Wang, Ballantyne, Kimble, & Wickens, 2004; Liu et al., 2015). Finally, using recent advances in targeted mutagenesis such as TALEN or CRISPR-Cas9, along with donor DNA provided for homologous recombination, MBS repeats could conceivably be knocked-in upstream of noncoding 3'UTRs of the zebrafish genome to better understand how these RNAs behave under endogenous circumstances (Auer, Durore, De Cian, Concordet, & Del Bene, 2014; Campbell, Chao, et al., 2015; Hwang et al., 2013; Shin, Chen, & Solnica-Krezel, 2014).

3. STUDYING MOLECULAR REQUIREMENTS THROUGH LOSS-OF-FUNCTION APPROACHES

Most geneticists will agree that the best approach for understanding the potential role of a gene is to eliminate its function. In the zebrafish model, this has been primarily accomplished through two techniques—blocking formation of a functional protein through the use of antisense oligonucleotides (ASOs) known as morpholinos, and genome mutagenesis. The issue of applying loss-of-function approaches to study maternal gene regulation is made more cumbersome by the presence of maternal gene products (RNAs and protein) in early embryos and the requirement to raise genetic mutants to viable adult females. Nonetheless, much progress has been made using both morpholino and mutagenesis approaches, and recent advances in reverse genetic approaches will likely continue to be fruitful in advancing our understanding of maternal-effect gene functions.

3.1 MORPHOLINO TECHNOLOGY IN OOCYTES AND 1-CELLED EMBRYOS

Morpholinos are antisense RNA oligonucleotide analogs synthesized with a morpholine ring in place of the riboside ring (Summerton & Weller, 1997). This artificial construction serves the dual purpose of enhancing morpholino binding to its complementary RNA sequence while resisting endonuclease digestion (Summerton, 1999). Typically, morpholinos (MO) are designed to either target complementary sequences in the 5'UTR, thus preventing translation of the transcript (translation-blocking MO), or to target splice-donor or splice-acceptor sites in pre-mRNA, preventing mRNA maturation and export to the cytoplasm (splice-blocking MO) (reviewed in Eisen & Smith, 2008; Huang, Zhu, Lin, & Zhang, 2012). To assess the functions of noncoding RNAs, a third class of MOs has been developed which are complementary to microRNA sites in the 3'UTRs of miRNA target transcripts (target protector MOs), thus reducing miRNA-mediated RNA decay by preventing association of the miRNAs themselves with the transcript (Choi, Giraldez, & Schier, 2007).

In zebrafish, the three above-mentioned classes of morpholinos have been delivered through injection of 1-celled embryos primarily to investigate zygotic phenotypes or the maternal-to-zygotic transition (reviewed in Huang et al., 2012). In addition to MOs, ASOs, molecules that are analogous to the synthetic RNA–DNA hybrids that are commonly used in *Xenopus* and mouse to promote RNase H–mediated degradation, have been applied to knock down maternal gene products and the essential germ cell factor *dnd* (Pauli, Montague, Lennox, Behlke, & Schier, 2015). However, use of these techniques to assess maternal effect

phenotypes is limited because the preexistence of large quantities of spliced maternal RNA (for splice-blocking MOs) and stable protein (for translation-blocking MOs and ASOs) present in the 1-celled egg render morpholino technology ineffective. To work around this limitation, MO-injection into *in vitro* cultured oocytes has been developed to interfere with maternal gene function before maternal proteins accumulate.

The use of morpholino technology to knock down maternal gene function has largely been limited to proof-of-concept and phenotype validation, as this technique is still in the development stages. Nonetheless, early results indicate that MOs may be a useful tool in assaying some maternal effect phenotypes. As an example, injection of translation-blocking MOs against the membrane-protein Lrmp and the RNA-helicase Dhx16 failed to recapitulate the phenotype of maternal mutant embryos when injected into the 1-celled embryo, presumably because of the existence of wild-type protein preloaded into the egg (Lindeman & Pelegri, 2012; Putiri & Pelegri, 2011). However, injection of these MOs into cultured stage IV oocytes, followed by maturation and fertilization *in vitro*, did phenocopy the defects observed in maternal-effect mutants (Nair, Lindeman, et al., 2013; Nair, Marlow, et al., 2013). In contrast, attempts to phenocopy the maternal-effect mutant disrupting the centriole component Sas6 (Yabe, Ge, & Pelegri, 2007) using a similar approach with a splice-blocking MO was unsuccessful (Nair, Lindeman, et al., 2013; Nair, Marlow, et al., 2013). This likely indicates that accumulation of mature mRNAs happens too early in oogenesis to be a viable target for splice-blocking MO technology, whereas mRNAs that are translated into protein during oocyte maturation or even after fertilization are targetable with translation-blocking MOs using the oocyte injection approach (Nair, Lindeman, et al., 2013; Nair, Marlow, et al., 2013).

Although the use of MOs has proven useful in understanding maternal gene functions that can be targeted in stage IV oogenesis or later, recent studies reinforce the importance of applying this technology with caution due to several limitations of MO technology and the difficulty in interpretation of phenotypes. Off-target effects for morpholinos in zebrafish, mediated by activation of the p53 pathway (Robu et al., 2007), have been well documented. An additional concern is the recently reported poor correlation between previously published MO knockdown phenotypes and genetic mutant phenotypes (Kok et al., 2015). Other reports have highlighted the potential for acute knockdown caused by MO to circumvent longer-acting transcriptional compensatory mechanisms activated with stable genomic mutations, resulting in phenotypes in morphants but not mutants (Rossi et al., 2015). Given these potentially confounding variables, it is likely that future work utilizing MO technology will also include validation of MO-induced phenotypes with genetic mutants.

3.2 GENETIC MUTATIONS TO STUDY MATERNAL FUNCTIONS INCLUDING GERM CELL SPECIFICATION

Aside from the use of morpholino technology, zebrafish genetic research has primarily relied on forward genetic screens, which provide an unbiased means to identify genes that are involved in biological processes. The majority of these screens, which employ irradiation, viral insertion, or chemical DNA alkylating agents to mutate the genome, have focused on identifying mutations associated with phenotypes that are apparent during early development

(reviewed in Patton & Zon, 2001; Sassen & Köster, 2015). More recently, some screens have also concentrated on identifying maternal-effect mutations, which are only apparent in embryos of mutant mothers due to the lack of a maternally provided gene product in the egg (Dosch et al., 2004; Kishimoto, Koshida, Furutani-Seiki, & Kondoh, 2004; Pelegri et al., 2004; Pelegri & Schulte-Merker, 1999; Wagner, Dosch, Mintzer, Wiemelt, & Mullins, 2004; reviewed in Marlow, 2010; Pelegri & Mullins, 2011). Detection and examination of recessive maternal-effect mutations requires one extra generation compared to zygotic mutants and is therefore not routinely done in forward screens. Maternal-effect mutations are broadly considered to be those mutant phenotypes that result in defective egg patterning, fertilization, egg activation, or embryonic development (Marlow, 2010). Although mutations affecting oogenesis that prohibit the laying of mutant eggs (ie, female sterile phenotypes) technically have a “maternal” function, they are not usually classified as such because “maternal-effect” is considered from the point-of-view of the effect of the mutation on the embryo (Marlow, 2010).

A few maternal-effect genes important for egg patterning and early germ plasm segregation have been identified through the use of forward genetic screening, with the largest screens undertaken by the Mullins group (Dosch et al., 2004; Kishimoto et al., 2004; Pelegri et al., 2004; Pelegri & Mullins, 2011; Pelegri & Schulte-Merker, 1999; Wagner et al., 2004). Among these, a maternal-effect mutation in the gene *motley/birc5b* revealed an essential role for Motley/Birc5b as a component of the chromosomal passenger complex, in meiosis and mitosis, as well as an unexpected role in microtubule-dependent cortical microfilament rearrangements and germ plasm RNP multimerization (Nair, Lindeman, et al., 2013; Nair, Marlow, et al., 2013). Maternal-effect screens also revealed roles for the genes *bucky ball* and *magellan (macf-1)* in egg and embryonic patterning (Bontems et al., 2009; Dosch et al., 2004; Gupta et al., 2010; Marlow & Mullins, 2008). As mentioned previously, many patterning RNAs, including germ plasm, are first localized during oogenesis to the Balbiani body and then undergo multiple microtubule-dependent translocation events during oocyte maturation before finally reaching their patterning domains in the early embryo. Mutations in *bucky ball* and *magellan* both result in an unpatterned egg because patterning molecules are either never recruited to the Balbiani body (in *bucky ball*) or are not delivered to the oocyte cortex during oogenesis (in *magellan*) (Gupta et al., 2010; Marlow & Mullins, 2008). Whenever conducting a forward genetic screen, one must consider some of the potential limitations of the screening capabilities. For example, some maternal-effect phenotypes may be missed due to earlier zygotic requirements for the gene, or due to later roles during germ cell development that are only detectable with marker analysis (Campbell, Heim, et al., 2015). Finally, a considerable number of mutations may go undetected due to lack of mutant phenotypes owing to the widespread gene duplication present in the zebrafish genome (reviewed in Meyer & Scharf, 1999).

Targeting-induced local lesions in genomes (TILLING), is a reverse-genetics method used to identify a point mutation in a defined gene by using next-generation sequencing to screen a library of potential mutants generated by UV or chemical mutagenesis (reviewed in Huang et al., 2012). This methodology is expensive and extremely laborious and, therefore, has been mostly employed by large consortia and institutes (Kettleborough et al., 2013; Pan et al., 2015; reviewed in Moens, Donn, Wolf-Saxon, & Ma, 2008). The TILLING technique is

limited in its ability to identify a mutation in a particular gene-of-interest (GOI) by the size of the zebrafish mutant library being screened, as well as the physical size and intron–exon structure of the “target” gene. Because the mutational frequency of ENU mutations is only one in every 100,000 base pairs, genes with small exons are less likely to be mutagenized (reviewed in Huang et al., 2012; Moens et al., 2008). The zebrafish *nanos3* mutation (previously *nanos1*) was identified using a TILLING approach (Draper, McCallum, & Moens, 2007). The *nanos3* gene is required for sustained fertility (mutant females have premature oocyte depletion) and also imparts a maternal-effect phenotype in which germ cells in embryos from *nanos3* mutant mothers are not maintained due to failure of the PGCs to migrate into the gonad anlage (Draper et al., 2007). Since 2013, the TILLING-based Zebrafish Mutation Project undertaken by the Sanger Institute has identified mutations in more than 38% of known protein-coding genes in the zebrafish genome (Kettleborough et al., 2013). A major advantage of this approach is that once a mutation has been detected, it is possible to rigorously examine the mutants for phenotypes using detailed analysis that would not be feasible in a genome-wide phenotype-driven screen. Thus TILLING is a valuable approach for identifying mutations and investigating a candidate gene’s potential role in germ cell development or maternal gene function, provided that your GOI is targetable.

More recently the zebrafish community has welcomed an influx of reverse genetic tools that can be used to create targeted mutations in specific genes. The first of these tools to be applied to zebrafish was the zinc finger nuclease (ZFN) technology. ZFNs are chimeric proteins composed of a DNA-binding zinc finger domain fused to a FokI nonspecific endonuclease (Zhu et al., 2011). The specificity of ZFN targeting results from zinc finger alpha helices that recognize specific three base pair DNA sequences; when several of these helices are tethered together and coupled to the FokI nuclease, DNA double-stranded breaks can be created in a specific sequence of 18–21 bp (Urnov, Rebar, Holmes, Zhang, & Gregory, 2010). The TALEN-targeted mutagenesis technology (transcription activator-like effector nucleases) uses a similar concept to ZFNs with sequence specificity provided by modular protein domains that recognize specific base pair sequences (Huang et al., 2012; reviewed in Hwang, Peterson, & Yeh, 2014). These targeted mutagenesis technologies are also similar in that the double-stranded break which is created is repaired through cellular nonhomologous end joining (NHEJ) mechanisms, resulting in variably sized insertions and deletions (indels), disrupting the genomic sequence of your target GOI.

Recently, however, both ZFNs and TALENs have been overshadowed by the application of CRISPR-Cas9 technology. This targeted mutagenesis approach makes use of a customizable guide RNA that assembles with the Cas9 endonuclease to direct DNA double-stranded breaks, resulting in indels as with the aforementioned approaches (Hwang et al., 2013; Jinek et al., 2012). CRISPR-Cas9 is significantly easier to use than ZFN or TALENS because the gene-specific component is provided by a customized RNA sequence, rather than a custom modular protein, whose coding sequence can require weeks to months of assembly. CRISPR-Cas9 mutagenesis has recently been used to examine the roles of a maternal gene involved in germ cell development. In zebrafish, maternal Kinesin Kif5Ba is required for assembly of germ plasm at the cleavage furrows, and progeny of *kif5Ba* mutant mothers fail to specify PGCs and are infertile (Campbell, Heim, et al., 2015). Given the ease of use of the CRISPR-Cas9 technology, it is becoming the targeted mutagenesis tool of choice for

investigating specific requirements of genes that participate in germ cell specification and maternally regulated embryonic development.

Maternally controlled specification of the germ line and later aspects of PGC development are complex processes that require extensive coordination and multiple levels of posttranscriptional regulation. Yet, forward genetic screens have identified only a few genes required for these processes (Dosch et al., 2004; Wagner et al., 2004), and many of these genes have pleiotropic functions that are not restricted to PGCs (Nair, Lindeman, et al., 2013; Nair, Marlow, et al., 2013; Pelegri et al., 1999; Yabe et al., 2009). It is likely that many maternal genes with functions in germ cell development are required elsewhere in the developing embryo before the mutants reach adulthood. To circumvent this limitation, it is necessary to rescue the lethal phenotypes associated with loss of a particular gene in the soma, while maintaining a mutant germ line. One approach that has been developed to address this is the creation of chimeric embryos using the “germ line replacement” method. This technique was first developed to make maternal zygotic mutations in the *miles apart* gene (Ciruna et al., 2002) and later to examine planar cell polarity mutants (Ciruna, Jenny, Lee, Mlodzik, & Schier, 2006), and the miRNA-processing enzyme *dicer* (Giraldez et al., 2005; Wienholds, Koudijs, van Eeden, Cuppen, & Plasterk, 2003). The germ line replacement technique relies on eradication of germ cells of a wild-type host embryo by injecting *dead end* morpholino, followed by manual replacement of PGCs from mutant donor embryos to produce chimeric animals that have normal somatic cells, but a mutant germ line (Ciruna et al., 2002). While this method has been effectively employed in several circumstances, it is technically very challenging and yields few maternal mutants. This is largely because only one quarter of the chimeras are mutants (from heterozygous in-crosses), and replacement of the germ line is often inefficient, thus frequently resulting in sterile fish or males (further explained below). More efficient and easily applied methods are needed to address this major limitation in the field.

4. UTILITY OF TRANSGENIC FISH TO STUDY GERM CELL DEVELOPMENT

Transgenic zebrafish lines use expression of fluorescent reporter genes to label, quantify, and track specific cell-types-of-interest and cell-lineage relationships. The zebrafish germ cell field has developed transgenic lines to aid in studies of most stages of germ cell development, from germ plasm aggregation in oocytes and at the embryonic cleavage furrows, to PGC specification, germ cell expansion, meiosis, and sexual differentiation (Fig. 3) (Heim et al., 2014; Leu & Draper, 2010; Onichtchouk, Aduroja, Belting, Gnugge, & Driever, 2003; Riemer, Bontems, Krishnakumar, Gomann, & Dosch, 2015; Strasser et al., 2008). In the following section, we summarize these transgenic lines, highlighting their specific utility and potential limitations (see also Table 1).

4.1 TRANSGENIC REPORTER LINES TO EXAMINE STAGE- AND GENDER-SPECIFIC GERM CELLS

Two advantages of the zebrafish model for studying early development are the external fertilization of eggs and the transparency of the developing embryos. These features of zebrafish development have also proven extremely useful in examination of maternal germ

cell specification, PGC migration, and development. To examine germ cells as early as 3 hpf, researchers have used a transgene driven under the maternal *askopos* promoter (Strasser et al., 2008). This transgenic line expresses a simple GFP reporter, or a prenylated GFP that marks germ cell membranes, the protein-coding sequences of which are fused to the *nanos3* 3'UTR to provide transcript stability specifically in germ cells (Strasser et al., 2008). The temporal period during which this transgene is expressed, from PGC specification to approximately 2 dpf when the PGCs have reached the prospective gonad, has made this transgenic line a valuable tool for studies of both germ granule dynamics within developing PGCs as well as PGC migration (Fig. 3) (Strasser et al., 2008). When combined with time lapse or live imaging, the *askopos* transgenics have effectively elucidated several aspects of PGC migration, including roles of the cytoskeleton, cell adhesion molecules, G-proteins, and alkylating enzymes in filopodia formation, and the requirement for several chemokines and chemokine receptors in guiding migrating PGCs to the gonad anlage (reviewed in Paksa & Raz, 2015).

A transgenic line that expresses a GFP-Bucky ball fusion protein (GFP-Buc) under the control of *bucky ball* gene regulatory elements recapitulates endogenous localization of Buc to the germ plasm in early embryos (Riemer et al., 2015). When maternally provided, this fusion protein can be observed in eggs and can be used to trace GFP-Buc as it accumulates within germ plasm at the cleavage furrows and specified PGCs until 2 dpf (Riemer et al., 2015). Because GFP-Buc is detectable in oogenesis and from the very beginning of embryonic development, this transgenic line could prove useful for studies of germ plasm aggregation at the furrows and germ cell specification (Fig. 3) (Riemer et al., 2015; Strasser et al., 2008).

To visualize events occurring after germ cell specification and migration, transgenic lines have been developed to label established germ cells within the gonad. The transgenic GFP reporter driven by the promoter for the *ziwi* gene (*piwi-like 1*) is expressed in germ cells as early as 7 dpf (Leu & Draper, 2010). The *ziwi:GFP* transgene can be used to follow the initial proliferative stages of the indeterminate gonad and continues to label germ cells as they mature, sexually differentiate, and undergo meiotic stages of spermatogenesis or oogenesis (Fig. 3) (Leu & Draper, 2010). When combined with careful examination of nuclear DNA, the *ziwi:GFP* transgene has been utilized to identify oocyte progenitor cells in wild-type fish and defective progenitor numbers and meiosis in *vasa* mutant zebrafish (Draper, 2012; Hartung, Forbes, & Marlow, 2014). This transgene has also been used to study the cell-autonomous requirements of the *nanos3* gene in oogonial stem cell maintenance (Beer & Draper, 2013). This was accomplished by making chimeric adult fish with donor or host germ cells labeled by *ziwi:GFP* and studying the relationship between *nanos3* mutant and wild-type oogonial stem cells cohabiting the same ovary (Beer & Draper, 2013). As demonstrated from the various examples highlighted above, the near ubiquitous expression of the *ziwi* reporter transgene throughout germ cells in the developing gonads has made this an extremely useful tool for studies of the mechanisms that regulate maintenance and differentiation of the zebrafish germ line.

Use of the *ziwi* promoter has not been restricted to labeling experiments but has also been used to specifically express molecules to aid in the selection or ablation of germ cells. For

example, the *ziwi:RFP* transgenic fish have been used to generate *in vitro* cell-culture systems to grow transplantation-competent germ line stem cells (Wong & Collodi, 2013; Wong, Tesfamichael, & Collodi, 2013). To isolate cells with germ cell identity from a primary source of zebrafish ovary or testis, a fluorescent reporter and neomycin were coexpressed under control of the *ziwi* promoter to identify and select-for germ cells, respectively. In another study that aimed to test the requirement of germ cells in the maintenance of adult sexual phenotype, the authors used a transgenic line with *ziwi* driving expression of an FP fusion to bacterial nitro-reductase (NTR) (Dranow, Tucker, & Draper, 2013). Germ cells expressing the NTR enzyme undergo apoptosis when exposed to the prodrug metronidazole; therefore, the authors grew these transgenic fish to adulthood then induced ablation of their germ cells through metronidazole treatment (Dranow et al., 2013). The germ cell ablation of adult females revealed that a threshold number of oocytes are required to maintain female somatic cells of the ovary as well as female secondary sexual characteristics. A drop below the threshold oocyte levels causes the gonad to revert to producing male germ cells with male somatic cells (and male secondary sexual traits), while a complete ablation of germ cells results in sterile males (Dranow et al., 2013).

Although the near-ubiquitous expression of *ziwi:GFP* throughout germ cell development has many advantages, there are some experimental contexts and questions which necessitate distinguishing male and female gonads. Thus, several zebrafish transgenic reporters have been developed that are differentially expressed in testis and ovaries. The first such reporter transgene in zebrafish was built with the promoter of the *vasa* gene (Krovel & Olsen, 2002, 2004). *Vasa* is a universal marker of the germ line that, in zebrafish, is encoded by two major splice isoforms that are differentially expressed between males and females (Bartfai & Orban, 2003; reviewed in Gustafson & Wessel, 2010; Hartung & Marlow, 2014; Raz, 2000; Yajima & Wessel, 2011). The *vasa:eGFP* transgenic line was created by flanking a *GFP* cassette on the 5' end by an 8.5 kb region including the *vasa* promoter, the first three exons and three introns, while on the 3' end of the *GFP* cassette is the *vasa* 3' UTR plus an additional 1.7 kb of downstream sequence (Krovel & Olsen, 2002). The *Vasa-GFP* reporter protein is expressed in male and female zebrafish beginning at 21 dpf and throughout gametogenesis, and (maternal) *GFP* can be observed in early PGCs of embryos from transgenic females (Fig. 3) (Krovel & Olsen, 2002, 2004). Fortuitously, the transgene contains the intron upstream of *vasa* exon 4, which is alternatively spliced out in the *vasa* isoform that is enriched in testis (Bartfai & Orban, 2003; Krovel & Olsen, 2004). *vasa:eGFP* transgenic juvenile fish undergoing sexual maturation can be sorted into two populations—*GFP* “bright,” corresponding to female gonadogenesis and *GFP* “dim,” corresponding to male gonadogenesis (Krovel & Olsen, 2004). The *vasa:eGFP* transgenic line has been used to mark the germ cells of and to reveal defective meiosis and differentiation in *zili* mutants (Houwing, Berezikov, & Ketting, 2008). The sexually dimorphic expression of the *vasa* reporter lines makes this a useful tool to differentially mark the germ cells to study gender-specific aspects of germ line development.

The zebrafish *zona pellucida3* (*zp3* or *zpc*) gene encodes a component of the glycoprotein shell surrounding the oocyte and is involved in sperm binding to the egg and protection of the early embryo (Liu, Li, Emelyanov, Parinov, & Gong, 2008; Liu, Wang, & Gong, 2006; Mold et al., 2001; Onichtchouk et al., 2003). The *zp3:GFP* transgene is expressed

exclusively in oocytes, and thus in a female-specific manner after 30 dpf when sexual differentiation takes place (Fig. 3) (Onichtchouk et al., 2003). Between 22 and 30 dpf, while the juvenile gonad is indeterminate, some GFP expression can be detected in fish that eventually develop as males (Onichtchouk et al., 2003). This is likely because all juvenile gonads contain early oocytes before male-specific differentiation drives elimination of these oocytes (Onichtchouk et al., 2003). Using the NTR enzyme system that was described earlier, zebrafish models of late-stage infertility were established using the *zp3* promoter to ablate differentiated oocytes of females treated with metranodazole (Hu et al., 2010; White, Woods, & Wood, 2011). The *bucky ball* promoter has also been used to generate a transgenic fluorescent reporter line (Heim et al., 2014). Like *zp3*, *buc* expression is restricted to the female germ line and is therefore a useful reporter for monitoring female gonad differentiation or oocyte development (Fig. 3) (Bontems et al., 2009; Dosch et al., 2004; Heim et al., 2014). There are, however, apparent differences in the precise timing of expression between these two transgenes. The Zp3-GFP reporter protein is first detected in stage Ib oocytes and is thereafter continually expressed in oocytes and is maternally inherited; thus eggs and early embryos from *zp3:GFP* transgenic mothers have maternal GFP in their cytoplasm until 24 hpf (Fig. 3) (Onichtchouk et al., 2003). In contrast, the Buc-RFP reporter protein appears earlier in oogenesis, in stage Ia oocytes, but is expressed for only a short period prior to stage III of oogenesis; thus this fluorescent reporter is not detectable in early (maternal) stages of embryogenesis (Heim et al., 2014, and unpublished observations). These differences between the *buc* and *zp3* promoters should be considered before choosing a reporter line for analysis of sexual differentiation, or using the promoter to drive one's own GOI. An advantage of using promoters that are selectively expressed in females is the ability to propagate transgenes that disrupt meiosis or other aspects of germ cell development through males, which are unaffected because they carry but do not express the transgene.

Germ granules are perinuclear structures present in PGCs after they are specified. As a unique and conserved feature of germ cells across species, there is substantial interest in understanding the function and molecular mechanisms regulating the germ granule aggregates of PGCs and oocytes. While not strictly transgenic “reporters,” a few transgenes encoding fluorescent fusion proteins have been used to visualize these germ granule aggregates. An RFP fusion containing the germ granule component Granulito, expressed under the *askopos* promoter, has been used to examine germ granules from their manifestation in PGCs around the time of genome activation until the completion of PGC migration (Strasser et al., 2008). Use of this transgenic line provided a time course of granule formation in zebrafish and demonstrated the involvement of cytoskeletal proteins and the germ cell factor Tudored7 (Tdrd7) in granule morphology and distribution (Strasser et al., 2008). To examine earlier embryonic events, particularly germ plasm recruitment to the cleavage furrows, one could use the MS2 system (Campbell, Chao, et al., 2015) or the GFP-Buc transgenic described earlier (Riemer et al., 2015). Finally, for those interested in the mechanisms that translocate germ plasm components throughout oogenesis, eg, from the Balbiani body to the oocyte vegetal cortex, the Buc-GFP transgenic line could be useful (Riemer et al., 2015). While undertaking these studies, it is important to keep in mind that germ granules are not strictly uniform in their components, with considerable heterogeneity

in RNA and protein components in granules of the same germ cell (Brangwynne et al., 2009; Campbell, Chao, et al., 2015; Knaut et al., 2000; Little, Sinsimer, Lee, Wieschaus, & Gavis, 2015; Trcek et al., 2015; Wang et al., 2014); thus, it is advisable to examine at least two granule components in order to generalize about overall granule formation and morphology. While these transgenics have so far been used in tracking granules to elucidate a specific mechanism or gene function, it is also conceivable that they may be used as reporters of granule formation in forward and reverse genetic screens, thereby potentially identifying novel regulators of germ plasm recruitment to the cleavage furrows of PGCs or oocytes and germ granule formation in PGCs.

4.2 OTHER CONSIDERATIONS FOR CONSTRUCTING TRANSGENIC LINES

The extensive role of maternal factors in determining germ cell fate, along with the inextricable coupling of germ cell specification to events occurring in oogenesis, makes the study of germ line molecules prime targets for analysis using transgenesis. Creating a transgenic line expressing one's germ line GOI is time-consuming, averaging about 3–6 months to generate a stable line, but where reliable antibodies are nonexistent, transgenic lines can be very informative and provide spatial and temporal resolution of the protein of interest in wild-type and mutant contexts. Moreover, transgenic rescue of a mutant phenotype is often necessary to demonstrate specificity and sufficiency of gene function. That being said, there are several basic parameters to consider in the design of a transgenic line for germ cell analysis: (1) use of the native promoter or one of the already-available germ line promoters, (2) use of an FP or antigenic tag to label your GOI protein product, and (3) use of a transgenesis marker to easily identify carrier fish.

While several excellent promoters already exist for transgenic expression (as detailed earlier), these are unlikely to be expressed at the exact levels or spatiotemporal pattern as your GOI. Cloning the putative promoter of your GOI for use in transgenesis may solve this issue but presents the drawbacks of creating additional labor without the guarantee that the promoter will drive expression as desired. Secondly, the zebrafish field is still lacking good antibodies for immunofluorescence of many proteins; in the absence of such a reagent, creation of a transgene harboring a tagged or fluorescent fusion of your GOI may be useful for examination of localization during oogenesis or early embryogenesis (for example, in the germ plasm). Additionally, tagging your transgenic protein may be useful for downstream purposes such as immunoprecipitation. As with any transgenic or fusion protein approach, one should consider that with transgenic expression your GOI may be present at higher or lower than endogenous levels, and the protein tag may interfere with some biological activities. Finally, since most germ line promoters currently used do not produce detectable expression until the zebrafish fry have reached at least 7 dpf (as is the case with *zif*), it is useful to have an independent transgenesis marker in the transgene backbone. For example, the *cardiac myosin light chain 2 (cmcl2)* promoter is turned on in embryonic hearts by 2 dpf and is commonly used to screen for and sort transgene positive embryos prior to raising them for maintenance or analysis (Huang, Tu, Hsiao, Hsieh, & Tsai, 2003; Kwan et al., 2007).

Beyond these basic parameters, there are other elements of the construct to consider when designing a transgene for maternally encoded genes. For the limited number of zebrafish transgenes that have been designed for protein expression in the germ line, there is evidence that extra-coding sequences such as the 5' and 3' UTRs, as well as intronic regions, may be required for proper regulation and translation of the GOI. For example, the zebrafish maternal-effect mutants for the genes *syntabulin*, *Y box 1*, and *bucky ball* require transgenes containing such extra-coding sequences for proper rescue of the mutant phenotype (Heim et al., 2014; Kumari et al., 2013; Nojima et al., 2010). In these cases, injection of mRNA encoding the open reading frame (ORF) (Kumari et al., 2013; Nojima et al., 2010), or even an ORF-containing transgene (Heim et al., 2014), is not sufficient for rescue, presumably because the resulting transcripts are not under proper translational control. There is significantly more evidence for such mechanisms acting in the *Drosophila* germ line, where genes such as *oskar* and *gurken* require elements in the 5' - and 3'UTRs and introns for proper regulation of transcript localization and translation (Hachet & Ephrussi, 2004; Thio, Ray, Barcelo, & Schupbach, 2000; Van Buskirk & Schupbach, 2002). Specific elements within these noncoding regions have been identified as having critical functions in the maturation, transport/localization and translation of these genes, and several RNA-binding proteins, splicing factors, and translational machinery components have been implicated in these roles (reviewed in Lasko, 2012). Thus it may be prudent to include such noncoding elements in the design of a transgene intended for expression in the germ line or maternal stages of embryogenesis.

CONCLUSION

Danio rerio has become a leading vertebrate model for studying maternal germ cell specification due to large embryo clutch sizes, genetic tractability, optical clarity, and rapid germ line formation. The recent advances in techniques for labeling cell lineages and molecules have further rendered this organism as an ideal choice for microscopic examination of germ cell biology. Solid tool-sharing platforms such as the Tol2-based transgenesis plasmids, created with gateway compatibility for simple and efficient cloning, have made it feasible to quickly and easily generate transgenic zebrafish for use in studying your germ cell gene or process of interest (Kwan et al., 2007). Traditional forward-genetics screens have shed light on the importance of maternally provided genes in embryogenesis and gametogenesis (Dosch et al., 2004; Draper et al., 2007; Kishimoto et al., 2004; Pelegri et al., 2004; Pelegri & Schulte-Merker, 1999; Wagner et al., 2004). The publication of the annotated zebrafish genome (Howe et al., 2013), in combination with *in situ* hybridization screens elucidating the expression patterns of genes that are maternally expressed and/or germ cell specific (Thisse & Thisse, 2004) will accelerate discovery of novel regulators of maternal germ cell specification. The combination of forward and reverse genetic tools that we have reviewed here promises to revolutionize our understanding of the molecular factors that regulate germ cell formation, cellular biology, and maternal specification.

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FIGURE 1. Stages of maternal germ plasm localization and germ cell specification

Description begins from the top left of the figure and proceeds clockwise. Early control of germ plasm localization is dependent on maternal products (stages on *pink background* (light gray in print versions)). In the 1-cell embryo, germ plasm accumulates in the animal pole. At early cleavage stages (from 2- to 16-cells), germ plasm becomes aggregated in the cleavage furrows between blastomeres. In high stage embryos, the germ plasm is sequestered into the now-specified four primordial germ cells (PGCs); this is coincident with activation of the zygotic genome (indicated by *yellow stripes* (white in print versions)). In shield stage embryos, PGCs are multiplying and migrating along with many other cells in the gastrula. By 30 hpf, PGCs have reached the location of the prospective gonad, where they will continue to multiply and mature until sexual differentiation. In adult females, germ plasm again becomes localized to the Balbiani body of stage I oocytes. By stage II of oogenesis, germ plasm and other Balbiani body contents are transported to the vegetal cortex of the oocyte. In stage III of oogenesis, numerous localization patterns exist for germ plasm (see text), however the predominant localization pattern is maintenance of germ plasm at the vegetal cortex. Finally, upon ovulation and egg activation, germ plasm is transported from these vegetal domains to the animal pole.

FIGURE 2. Techniques for studying germ plasm RNA localization

(A) In situ hybridization depends on complementary base-pair recognition of target RNAs by labeled RNA probes (*blue lollipops* (gray in print versions)). An antibody recognizing the labeled probe is conjugated to a color-producing enzyme, leaving a colored precipitate in the vicinity of the target RNA. (B) RNA injection can be performed on cultured oocytes, or collected 1-cell embryos. In either case, RNA encoding a fluorescent protein fusion to a germ plasm coding gene is *in vitro* transcribed and then injected. The read-out of such experiments is most often measured as fluorescent expression or localization of fluorescent products (ie, translation products of the RNA) in the oocyte or germ granules of embryonic PGCs. (C) MS2 labeling of transcripts requires special engineering to include MCP-binding site hairpin repeats (MBS), as well as fluorescent fusion with the MCP-binding protein (FP-MCP, see text for further details). When these are introduced, usually in the context of a stable transgenic zebrafish line, germ plasm RNA localization and movement can be tracked through the fluorescent signal of the FP-MCP.

FIGURE 3. Transgenic reporters for studying germ line specification and development

The stages of embryogenesis are delineated from 1-cell to 30 hpf (see Fig. 1 for further details). After the juvenile stage, at approximately 21 dpf, zebrafish gonads undergo sexual differentiation into testis (top), or ovary (bottom). The stages of spermatogenesis are illustrated as spermatogonia (SG), spermatocyte (SC), and spermatozoa (SZ). The stages of oogenesis are illustrated as oogonia (OG), stage I oocyte (st. I), stage II oocyte (st. II), stage III oocyte (st. III), and stage IV oocyte (st. IV). Below these developmental stages (or above in the case of male stages), *colored bars* are drawn across the temporal periods where fluorescent reporters driven under the promoter (labeled in the *bar*) can be observed. Where indicated with an *asterisk* (*), fluorescent protein (FP) expression is maternal, and where indicated with a *degree symbol* (°) the FP contains protein-coding elements from the indicated gene. (See color plate)

Table 1

Resources for Transgenic Zebrafish Lines

Transgenic Line	Expression Domains	Source	References
<i>Tg[βactin:NLS-tdMCP-GFP]</i>	Ubiquitous	<i>Florence L. Marlow</i> , Bronx, NY, USA	Campbell, Heim, et al. (2015)
<i>Tg[kop(askopos): egfp-farnesyl-nos.3-3' UTR]</i>	PGCs	<i>Erez Raz</i> , Münster, Germany	Strasser et al. (2008)
<i>Tg[buc-GFP]</i>	Maternal germ plasm, PGCs, female gonad	<i>Roland Dosch</i> , Göttingen, Germany	Riemer et al. (2015)
<i>Tg[ziwi:GFP]; Tg[ziwi:RFP]; Tg[ziwi: NTR]</i>	PGCs, female gonad, male gonad	<i>Bruce W. Draper</i> , San Diego, CA, USA	Leu and Draper (2010) and Dranow et al. (2013)
<i>Tg[vasa:eGFP]</i>	PGCs, female gonad, male gonad	<i>Lisbeth C. Olsen</i> , Bergen, Norway	Krovel and Olsen (2002)
<i>Tg[zp3:GFP]</i>	Female gonad	<i>Wolfgang Driever</i> , Freiburg, Germany	Onichtchouk et al. (2003)
<i>Tg[zp3:NTR-eGFP]; Tg[zp3:G4VP16/UAS:NTR-mCherry]</i>	Female gonad	<i>Jen-Leih Wu</i> , Taipei, Taiwan; <i>Antony W. Wood</i> , Boston, Ma, USA	Hu et al. (2010) and White et al. (2011)
<i>Tg[buc:RFP]</i>	Female gonad	<i>Florence L. Marlow</i> , Bronx, NY, USA	Heim et al. (2014)
<i>Tg[kop(askopos): granulito-dsRedEx-nos.1-3' UTR]</i>	PGCs	<i>Erez Raz</i> , Münster, Germany	Strasser et al. (2008)

PGC, Primordial germ cell.