Chapter 5

# Germ Cell Determinant Transmission, Segregation, and Function in the Zebrafish Embryo

Celeste Eno and Francisco Pelegri

Additional information is available at the end of the chapter

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**Abstract**

Animals specify primordial germ cells (PGCs) in two alternate modes: preformation and epigenesis. Epigenesis relies on signal transduction from the surrounding tissues to instruct a group of cells to acquire PGC identity. Preformation, thought to be the more derived PGC specification mode, is instead based on the maternal inheritance of germ cell-determining factors. We use the zebrafish as a model system, in which PGCs are specified through maternal inheritance of germ plasm, to study this process in vertebrates. In zebrafish, maternally inherited germ plasm ribonucleoparticles (RNPs) have co-opted the cytoskeletal machinery to reach progressive levels of multimerization, resulting in the formation of four large masses of aggregated germ plasm RNPs. At later stages, germ plasm masses continue to use components of the cell division machinery, such as the spindles, centrosomes, and/or subcellular organelles to segregate asymmetrically during cell division and subsequently induce germ cell fate. This chapter discusses the current knowledge of germ cell specification focusing on the zebrafish as a model system. We also provide a comparative analysis of the mechanism for germ plasm RNP segregation in zebrafish versus other known vertebrate systems of germ cell preformation, such as in amphibian and avian models.

**Keywords:** Germ cells, germ plasm, zebrafish, cell division, cytoskeleton, RNP segregation

## 1. Introduction

### 1.1. Fundamentals of germ cell specification: Epigenesis vs. preformation

One of the most fundamental early cell fate decisions in animal embryos is the specification of primordial germ cells (PGCs) from the somatic tissue. PGCs are the precursors to gametes and thus hold the information to recreate the species in each generation. Consequently, individuals

from every population must employ a robust mechanism for PGC induction. Specification occurs by one of two mechanistic modes: *epigenesis* or *preformation* (Figure 1). Epigenesis refers to a mechanism by which certain cells receive PGC-inducing signals from surrounding tissues. Epigenesis is found in mammals, as well as species in many other clades. Preformation describes a cell autonomous specification of germ cells from maternally inherited germ plasm deposits that specify the PGCs. This mechanism of preformation is used in a large number of organisms distributed in many animal lineages, including major model systems for biological research, such as the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*, amphibians, such as *Xenopus laevis* and *X. tropicalis*, and the chick.

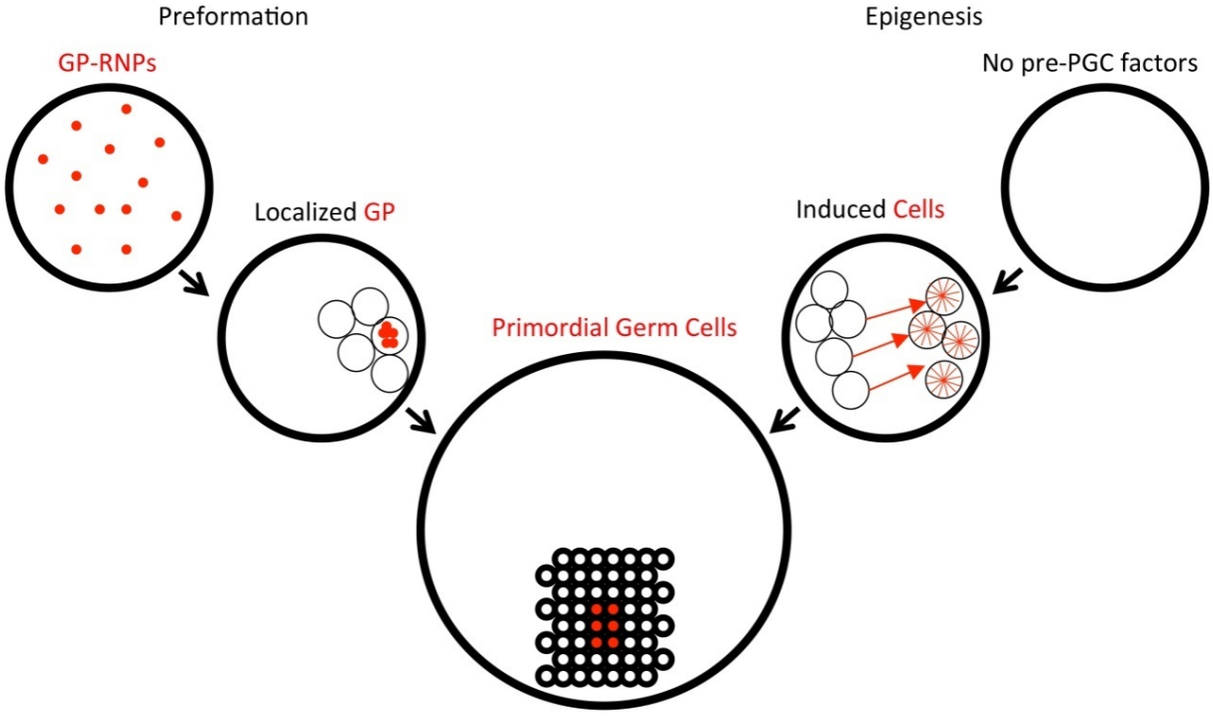


**Figure 1.** Mechanisms of primordial germ cell specification. Species employing preformation deposit germ plasm in oocytes. Throughout development, germ plasm localizes into select cells that eventually become the germ cells. In species employing epigenesis, cells receive inductive signals from neighboring tissue to become the primordial germ cells.

When comparing epigenesis and preformation as PGC-determining mechanisms, it was originally pointed out that epigenetic germ cell determination is an exception, and most animals use germ plasm [1, 2]. Subsequently, the analysis of distribution of these mechanisms within the evolutionary tree has led to the hypothesis that epigenesis is an ancestral PGC-determining mechanism and that preformation has arisen multiple times from this basal mode. Many clades employ both mechanisms, for example, within amphibians axolotls (urodeles) [3] use epigenesis and frogs (anurans) employ preformation [4]. Similarly, species in the group reptilia, such as turtles, use epigenesis [5], and species of the related group aves (birds) use preformation [6].

When protein-coding sequences of vertebrate species using epigenesis are compared with sister taxa employing preformation, genes of the latter evolve more rapidly. For example, when comparing protein-coding sequences from species in the amphibian and actinopterygian (ray-finned fishes) taxons, in all cases, species using preformation grouped further away from mammals than those using epigenesis. Evans et al. [7] found that no biological factor, including genome size, longevity, and generation time, correlates with sequence rate changes as optimally as the mode of PGC induction. This has led to the suggestion that the use of germ plasm relieves constraints on somatic development by dissociating PGC development from somatic development during early embryogenesis. This is because in animals using preformation mechanisms, changes in gene networks involved in somatic tissue development can occur without causing deleterious effects on the germ line, which would cause sterility and would therefore be selected against. This developmental flexibility allows for a faster rate of evolution of developmental programs. For example, ancestral gene regulatory networks (GRNs) for pluripotency and mesoderm specification found in axolotls are conserved through the evolution of mammals, whereas in frogs, pluripotency GRNs have been lost and the number of key regulators (i.e. multiple copies of Nodal and Mix) have increased in the mesoderm specification GRN [2]. Since axolotls specify PGCs through epigenesis within the mesoderm, the GRN is under constraints preventing variation—constraints that are not present in frogs. However, frogs carry preformed germ plasm, whose direct role in PGC induction allows the embryo to undergo drastic changes to the mesoderm specification GRN, such as Nodal expansion [8]. Consistent with this hypothesis, the use of germ plasm coincides with increased morphological variation and enhanced speciation within a clade [7].

Although these two PGC-determining mechanisms differ, many of the factors involved in PGC development are highly conserved regardless of the mechanism employed to differentiate PGCs. For example, the gene product of *vasa* is found in germ cells of a wide range of organisms from planaria [9] to humans [10]. Importantly, both mechanisms, epigenesis and preformation, in addition to promoting germ cell fate in PGCs, must also block somatic fate in this cell type [11].

### 1.1. Epigenesis, a mechanism by which cells receive signals from their surroundings

Most animal clades include lineages that use epigenesis to specify PGCs. Within the amphibian clade, axolotl (salamander) PGC specification occurs in primitive ectoderm (animal cap) cells in response to ventral mesoderm-inducing signals [12], including fibroblast growth factor (FGF) and BMP4 [13]. PGC precursors arise in the ventral marginal zone and migrate over the blastopore during gastrulation and by tailbud stage are detected in the posterior of the dorsal–lateral mesoderm expressing PGC-specific genes, such as *dazl* [3] and *vasa* [14].

All mammals specify PGCs through inductive signaling, and this mechanism is most well studied in mouse. In these organisms, extraembryonic tissue signals the most proximal region of the postimplantation epiblast to become the PGCs, whereas cells that do not receive the signals give rise to somatic tissue [15]. The mouse PGC precursors are detected just prior to primitive streak formation, and by the end of primitive streak formation, a population of around 40 PGCs are generated. Key instructive signals are the bone morphogenic proteins

(BMP2, BMP4, and BMP8B), which act through SMAD1 and SMAD5 [16, 17,18]. The population of PGCs is heavily reliant on BMP dosage as loss of BMP4 reduces their induction [18]. Some RNAs present in the germ plasm in organisms with a preformative mechanism, such as *nanos*, *dead-end* (*dnd*), and *dazl*, also have roles in germ cell development in the mouse [19]. For example, mouse Dazl protein has a complex role in PGC development by targeting mRNAs for translational inhibition, thus preventing somatic differentiation, pluripotency, and apoptotic death [20]. In addition, mouse Dazl also has a role at later stages of germ cell development, during spermatogonia differentiation [21]. This latter role is likely conserved in humans, having led to the original identification of this gene (DAZ or "deleted in azoospermia") as being affected in 13% of human male infertility cases [22, 23].

### 1.2. Preformation: Germ plasm deposits determine the germ cell fate

Preformation describes a mechanism whereby differentiation as germ cells is decided by the acquisition of maternally inherited determinants following fertilization [24]. The common term to describe such determinants is germ plasm, which is considered as a specialized cytoplasm enriched for factors that function in PGC determination. Germ plasm contains RNAs (both coding and noncoding) and proteins, which in many organisms appear to assemble together in punctate structures known as ribonucleoparticles (RNPs). In many organisms, germ plasm closely associates with the cytoskeleton and/or mitochondria. Proposed functions of germ plasm include the translational regulation of germ plasm RNAs, the establishment of a partially repressive chromatin in the germ line and the prevention of activation of somatic development by repressing mRNA transcription (reviewed in [25, 26]).

In *Drosophila*, arguably one of the most well-characterized species that use germ plasm as a means of PGC determination, the progenitors of the germ line are four to five pole cells found at the posterior pole of the embryo before blastoderm formation [27]. Pole cells acquire PGC identity through inheritance of the germ plasm (reviewed in [28]) called pole plasm in this organism [29, 30]. Polar granules appear at the posterior tip during mid-oogenesis, coincident with the time when vitellogenesis occurs. Toward the end of oogenesis, the polar granules associate with other germ plasm-associated structures (i.e. mitochondria) [31]. Germ plasm components are released from cortical actin at the posterior of the oocyte, and are transported via dynein motors on astral microtubules to centrosomes associated with nuclei in posterior regions of the early embryo [32]. These posterior nuclei subsequently separate from the syncytial mass through cell cleavage to form the PGC precursors or pole cells (reviewed in [28]). Embryological manipulations confirm that pole plasm is a true germ cell determinant as it can recreate pole cells when transplanted ectopically, for example, in the anterior region of the embryo. Interestingly, pole cell number relies on the dosage of a single RNA, *oskar* [33], which is required for proper induction of germ cells. However, the function of *oskar* in PGC determination is restricted to dipterans [34], so that other factors must take its role in other organisms.

P-granules are another type of RNA-rich cytoplasm structures involved in the specification of the *C. elegans* germ line. These granules are scattered evenly throughout the cytoplasm before and just after fertilization [35]. At a time coincident with pronuclear fusion, under the influence

of cell polarity factors (PAR factors) and implemented by microfilament-dependent cytoplasmic streaming [36, 37], P-granules translocate to and accumulate at the posterior of the early embryo. As a result of asymmetric positioning of the spindle during cell divisions, each division generates daughters of unequal size, with the smaller daughter inheriting most of the P-granules asymmetrically until the generation of the $P_4$ cell, after four cell divisions. However, the minority of P-granules remaining at the anterior end or in non-$P_4$ cells is degraded [38, 39]. Interestingly, P-granule localization during these early divisions (which generate $P_2$, $P_3$, and $P_4$ cells) relies on attachment to the nuclear periphery coupled to centrosome rotation, which leads to asymmetric placement of the P-granules with respect to the nucleus, specifically at the ventral side of the P cell [38]. Although "local gathering mechanisms" that each of the P cells use to deliver P-granules to the proper location appear to vary, asymmetric cell division and directional P-granule segregation occur through the first four cell cycles, generating P-granule-containing cell $P_4$. This cell type, whose nucleus is surrounded by large, coalesced P-granules, is the first cell whose descendants are restricted to the germ line [40, 41] and whose ablation results in sterility [42]. The $P_4$ cell divides symmetrically during embryogenesis resulting in Z2 and Z3, which after the first larval stage continue to divide symmetrically to produce about 1000 germ cells in the adult gonad [43]. P-granules continue to associate with the nuclear envelope of germ cells throughout embryonic and larval divisions and only detach from this structure during gamete maturation. However, the significance of this dynamic association remains poorly understood.

Although research on PGC specification is sparse for colonial ascidians, studies of a single species, *Botryllus schlosseri*, describe aggregation and ventral localization of maternally inherited germ plasm RNAs, in particular *vasa* mRNA [44]. The solitary ascidian (*Ciona intestinalis*) also uses a specialized cytoplasm (myoplasm) containing maternally inherited determinants of germ cells [45]. The first pre-PGCs form at the posterior end of the early embryo [46] and express conserved germ line gene, such as *vasa* [47]. Interestingly, when the PGCs are ablated, a new collection of *vasa*-expressing cells appears in the juvenile after metamorphosis. Because these cells did not inherit myoplasm, this suggests an alternative, compensatory mechanism for PGC specification in this organism, possibly similar to an inductive mode of PGC specification [48], which may be occurring as a safe–fail mechanism in this organism. Other well-studied systems that use germ plasm to specify PGCs, now within vertebrate lineages, include zebrafish, which is the focus of this chapter, *Xenopus* and chicken (see below).

## 2. Germ cell determinant segregation in zebrafish

### 2.1. Maternal inheritance of germ plasm RNPs

The fish model, *Danio rerio*, employs the preformative model of germ cell specification wherein the mother deposits factors important for PGC specification into the oocytes, which are transmitted to the embryo. RNPs containing RNAs for all known germ plasm factors in this organism originally localize to the same structure during oogenesis. This is the mitochondrial

cloud or Balbiani body (Bb), a large aggregate of organelles found in the oocytes of many species, that translocates from a location near the nucleus to the vegetal pole during oogenesis. Germ plasm RNA and mitochondrial enrichment to the Bb depend on the functional product of *buckyball* (*buc*), a novel vertebrate-specific protein that is the first gene identified as being required for the formation of this structure [49]. The Bb disassembles by stage II oogenesis, when the germ plasm RNAs localize to the vegetal pole via a pathway similar to the messenger transport organizer (METRO) pathway originally described in *Xenopus*. Subsequently, different germ plasm RNAs undergo different patterns of distribution in the oocyte: *vasa* becomes redistributed along the cortex, *nanos* becomes evenly distributed in the oocyte and *deleted in azoospermia* (*dazl*) remains localized to the vegetal pole (reviewed in [50]; see also [51]). These various patterns of redistribution during oogenesis result in the creation of two types of localized germ plasm RNPs, those present in the forming blastodisc at the animal region (animal germ plasm RNPs) and those remaining in the vegetal region (vegetal germ plasm RNPs).

### *2.1.1. Animal region germ plasm factors*

Known animal region germ plasm factors include *vasa,* a gene whose RNA or gene product is one of the most widely used markers for germ cells or germ plasm in metazoans [52]. As mentioned above, *vasa*-containing germ plasm RNPs localize along the cortical region of the oocyte at stage II and after egg activation are rapidly transported to the animal pole, where they are found dispersed evenly around the blastodisc. The protein product for this gene is a DEAD-box ATP-dependent helicase involved in RNA metabolism that facilitates interactions between RNA–RNA and RNA–protein [53]. Loss of function mutations in *vasa* lead to reduction in number or functionality of germ cells in almost all organisms [54–58]. In zebrafish, *vasa* morphant embryos (knock down of protein using a morpholino-conjugated oligonucleotide (MO)) do not exhibit defects in the establishment of the germ line [59]. Moreover, injection of *vasa* RNA into one-cell zebrafish embryos does not lead to an increase in PGCs; instead this RNA is degraded, suggesting that endogenous *vasa* RNA levels are carefully controlled by an intrinsic cellular mechanism [60]. These studies suggest that *vasa* is generally required, but is not sufficient, for germ cell development. However, a more rigorous set of tests using a knockdown or ectopic expression initiated during oogenesis, or *vasa*/*ddx4* maternal mutants, has not yet been reported.

Another animal germ plasm RNA is that of the gene *nanos*. Before egg activation, *nanos* mRNA is not localized; however, shortly after activation *nanos* is found to colocalize with *vasa* RNA. *nanos* RNA and/or gene product are also commonly associated with germ cell development and present in germ plasm. First described in *Drosophila*, *nanos* encodes a RNA-binding zinc finger protein. In *Drosophila*, *nanos* is not generally required for PGC formation; however, *Drosophila* PGCs deficient in *nanos* activity have abnormal development, including failure to migrate to the gonad, reduction in egg number, early activation of germ cell genes, deregulated expression of somatic mRNAs, and irregular morphology [61–63]. In zebrafish, knockdown of *nanos1* using antisense morpholinos injected at the one-cell stage proves that it is essential for

germ cell development [64]. In these morphants, PGCs are specified, yet these undergo aberrant migration and exhibit reduced number.

Other germline-specific RNAs found at the animal pole include *dnd*, *askopos* (*kop*), and *TDRD7*. *dnd* encodes a RNA-binding protein that blocks negative regulation through mRNA target degradation caused by binding of several microRNAs (miRNAs). Dnd protein achieves this protective function against miRNA-mediated mRNA degradation by binding mRNAs at miRNA target sites contained within these transcripts, thus blocking miRNA binding. This mechanism is used in the zebrafish germ line by PGC factor Dnd1 to protect several mRNAs, including *nanos* and *TDRD7*, from miR-430 repression, which is achieved through Dnd binding to U-rich regions in mRNA targets [65, 66]. Similar to *nanos1*, knockdown knockdown of *dnd* in morphants results in defects in PGC migration exhibits defects in PGC migration and viability [67].

*kop* mRNA was found to localize to zebrafish germ plasm and encodes a novel nuclear protein [68] that is not yet well studied. Some tudor domain-containing genes are expressed in germ cells, *RNF17*, *TDRD1*, *TDRD6*, and *TDRD7*. The latter (*TDRD7*) also localizes to germ plasm as it aggregates to the forming furrows, and TDRD7 protein colocalizes with Vasa in PGCs [69]. In *Drosophila*, homologous tudor proteins have been proposed to serve as a platform for polar granule assembly [70], a function that is consistent with the presence of TDRD7 protein in the zebrafish germ plasm. Zebrafish *TDRD7* morphants lack normal germ cell granule integrity at late somite stage [69]. Interestingly, TDRD7 is highly expressed in the developing lens, and mutations of TDRD7 in human, mouse, and chick lead to cataracts [71].

For tested animal germ plasm RNAs (*vasa*, *nanos*, and *dnd*), animal RNPs appear to colocalize and exhibit a similar size [72]. These observations suggest that germ plasm RNPs may be composites containing a set of germ plasm factors essential for germ plasm determination.

### *2.1.2. Vegetal region germ plasm factors*

Vegetally localized germ plasm RNPs contain RNAs for the genes *deleted in azoospermia-like* (*dazl*) and *bruno-like* (*brul*). These germ plasm RNPs are initially localized to the vegetal pole of the egg and upon egg activation translocate toward the blastomeres forming at the animal pole [73–75]. Both *dazl* and *brul* encode RNA-binding proteins present in germ plasm across a wide variety of animal lineages. Although Dazl protein is not on its own sufficient to promote translation, this factor promotes RNA polyadenylation to enhance the stability of PGC transcripts, such as those for *TDRD7* and *dazl*. Thus, Dnd1 and Dazl may work additively in this fashion to protect RNAs from miRNA repression [76]. Studies focusing on *dazl* RNA aggregates show that these are restricted to the vegetal cortex in the mature egg and move animally after egg activation, so that 45 minutes post fertilization *dazl* RNP aggregates begin to be observed at the site of germ plasm accumulation at the first cleavage furrow [75, 77]. During animally directed movement, aggregates do not appear to be of uniform size and may translocate using two pathways: an ooplasmic streaming pathway mediated through axial streamers in internal regions of the zygote and a cortical pathway along meridional streamers in more superficial regions [73, 75, 77]. During furrow formation, vegetally derived germ plasm RNPs associate with large germ plasm masses of animal germ plasm RNPs forming at the

furrows, in a cortical location near the distal-most end of the furrow and close to the yolk membrane cortex (see below). This location suggests that the animally directed cortical pathway may be the primary pathway used by vegetal germ plasm RNPs fated to become a part of a reconstituted and functional germ plasm mass.

Recent studies have shown that vegetal RNPs appear to be differentially distributed in the vegetal cortex, specifically at a cortical depth that is deeper than that of factors that also localize to the animal pole but are required for axis induction [78]. This differential distribution along cortical depth, with germ plasm RNPs at deeper levels and dorsal factors at more superficial ones, likely facilitates the symmetric animally directed movement of germ plasm RNPs [75, 79] in spite of the asymmetric movement of the bulk of the cortex required for axis specification [80, 81].

### 2.2. Repackaging germ plasm: Gradual multimerization and recruitment

As mentioned above, after egg activation and prior to the first cell division, animally localized RNPs are present in single particles, which we refer to as singletons, spread throughout the developing blastodisc. Germ plasm RNP singletons aggregate in a wave-like fashion, where the wave of aggregation emanates from the center of the blastodisc outwards, toward the edge of the blastodisc.

Germ plasm RNP multimerization at this stage depends on the interplay between microtubule and microfilament networks during blastomere cell division (Figure 2A). After fertilization in zebrafish, paternally derived centrioles act as a template to reconstruct the centrosome using maternally derived components. This newly formed centrosome nucleates a sperm aster or monoaster prior to initiation of the first cell division cycle. In this structure, plus ends of the astral microtubules interact with germ plasm RNPs at the cortex and help direct germ plasm RNP multimerization in a process of **pre-aggregation** (the prefix of this term indicates that this process occurs prior to furrow formation). The growing tips of astral microtubules thus generate an aggregation front of multimerizing RNPs, initiating near the center of the blastodisc and moving outward. The inner rim of RNP aggregates remains surrounded by a field of RNPs that have not yet been influenced by astral microtubules and therefore remains as singletons until the front arrives [72, 75]. As this process ensues, central regions of the blastodisc become depleted of germ plasm RNPs, generating an outwardly expanding RNP-free zone (Figure 2B). When embryos are treated with the microtubule inhibitor nocodazole, germ plasm RNPs do not undergo multimerization and instead remain dispersed throughout the cortex. This effect is also observed in embryos deficient for the Chromosomal Passenger Complex protein, Birc5b/Survivin, encoded by the gene *motley* [72]. In wild-type embryos, Birc5b protein localizes to the tips of astral microtubules (plus ends) and interacts with germ plasm RNPs. In motley-mutant embryos, mutated Birc5b/Survivin product localizes to germ plasm RNPs but not to microtubule plus ends, demonstrating Birc5b is an important linker between the two and explaining the *motley*-mutant phenotype. The fact that in these mutants germ plasm RNPs do not multimerize indicates that this linkage is necessary for the aggregation process [72].

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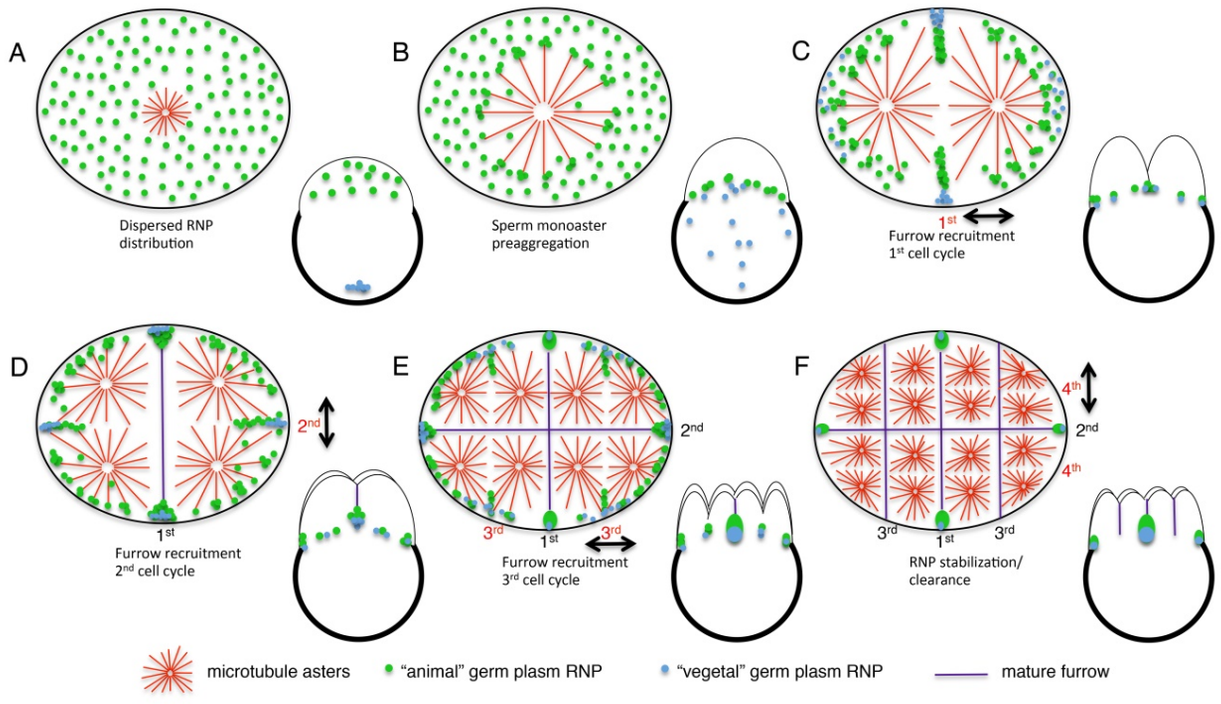


**Figure 2.** Germ plasm accumulation during the first zebrafish cell cycles. A) In the egg, animal pole (AP) germ plasm RNPs are dispersed at the blastodisc cortex, whereas vegetal pole (VP) germ plasm is localized at the vegetal pole. B) Prior to the initiation of cell cycling, during pronuclear fusion, astral microtubule growth moves AP germ plasm RNPs outward, whereas VP germ plasm begins to travel toward the AP. During the next several cell cycles, aggregated RNPs are collected in the (C) first furrow, (D) second furrow and (E) third furrow. F) Germ plasm RNPs in the first and second furrows are stabilized, whereas all other RNPs are cleared. Figure adapted from [86].

Another maternal factor important for germ plasm RNP recruitment during these early stages is Buc. In addition to a role for Buc to assemble the Bb during oogenesis, this factor is also required for recruitment of germ plasm during early embryogenesis, and its overexpression in the early embryo leads to an increase in PGCs [49]. Recently, a microtubule motor protein, Kif5Ba (kinesin), was found to bind to Buc and mediate its recruitment (thereby recruitment of other germ plasm molecules) to the cleavage furrow. Germ plasm in *kif5Ba* mutants is spread throughout the blastomeres, and Kif5Ba is required for the excess of PGCs that form when Buc is overexpressed [82]. This is reminiscent of the role of kinesins in germ plasm movement in *Xenopus*, where Xklp1 (kinesin-like protein) has also been shown to be required for germ plasm localization, and depletion of Xklp1 leads to arrest of germ plasm aggregation [83]. In *Xenopus*, another kinesin has been shown to have a role in later PGC development: KIF13B functions in migration and polarity of germ cells, as manipulations of xKIF13B result in erroneous PGC migration and reduced numbers [84].

Unlike the first cell cycle, which contains a monoaster involved in pronuclear fusion, blastomere cell cycle divisions contain bipolar microtubule asters nucleated by the pair of centrosomes at spindle poles. When spindle microtubules originating from opposing asters overlap at the spindle midzone, they signal furrow formation along the length of the blastomere. The furrow initiates as a microtubule-free zone that forms at the region of overlap between asters

from opposite sides of the furrow. During this process, germ plasm RNP multimers and associated F-actin continue their outward movement, which in the midzone between spindle poles coincides with the microtubule-free zone at the site of furrow formation. This movement results in the accumulation of both F-actin and germ plasm RNPs along the forming furrow, the latter forming a rod-shaped structure [72, 79, 85, 86] composed of individual multimeric groups of RNPs. We refer to this accumulation of germ plasm RNPs at the furrow as the process of **recruitment** (Figure 2C).

During cell division associated with the first several cell cycles, recruitment of germ plasm RNPs to the forming furrow occurs from both sides due to the bipolar nature of the asters. In the furrow, germ plasm RNPs are still connected to the tips microtubules, which form parallel to one another and perpendicular to the furrow forming a structure known as the furrow microtubule array (FMA). Both pre-aggregation and recruitment may facilitate germ plasm RNP multimerization using the same basic mechanism: in both cases, radially expanding microtubule growth (from a monoaster during the first cell cycle and bipolar asters in subsequent cell cycles) facilitates germ plasm RNP multimerization. Because of their intrinsic arrangements, the monoaster does not result in furrow formation and therefore can only contribute to pre-aggregation, whereas asters from bipolar spindles contribute to both continued pre-aggregation and implement recruitment. Thus, germ plasm furrow recruitment employs the normal cell division machinery, in particular astral microtubules, to mediate the aggregation and local gathering of RNP multimers to the forming furrow [86]. This simple mechanism normally couples furrow induction and germ plasm RNP furrow recruitment. Under certain mutant conditions, however, germ plasm RNP furrow recruitment is partially dissociated from furrow formation, as occurs in mutant embryos that fail to initiate a furrow and that nevertheless show *vasa* RNA accumulation at the presumptive furrow site [87, 88].

### 2.3. Insuring a tight fit: High-order RNP multimerization

#### *2.3.1. Compaction of germ plasm in a modified midbody*

As the contractile ring forms and leads to the division of cytokinesis, the FMA tilts distally and moves to the edge of the blastodisc [89, 90]. During this process, the rod-shaped RNP arrangement compacts into tight masses at the edges of the blastodisc, in a process of **distal compaction** (Figure 2D) [85, 91]. Electron microscopy analysis of the distal cleavage furrow shows electron-dense germinal granule-like structures, in which *vasa* RNA is present [92]. Ablation of this area leads to PGC loss later in development [77], providing evidence for these accumulated masses as PGC determinants. Analysis of both *nebel* (maternal-effect mutant) and microtubule-inhibited embryos argues that the dynamic nature of the FMA is needed for proper distal compaction of *vasa* RNA [93]. In addition, non-muscle myosin II function is required in this process, as inhibition of this motor protein leads to FMA stabilization and defects in germ plasm RNP distal compaction [91].

Through distal compaction, animal germ plasm RNPs acquire a distal position at the furrow, and their aggregate is transformed from a rod-like structure to a round and more compact mass, possibly driven by an increase in neighbor-to-neighbor RNP contact and concomitant

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reduction in germ plasm mass volume. The extent by which this process is driven through cytoskeletal rearrangements, RNP–RNP interactions, or both remains to be determined. The subcellular cues that result in the redistribution of FMA microtubules and germ plasm RNPs to the distal end of the furrow also remain largely unknown, although, as mentioned above, a reduction in myosin activity results in FMA stabilization and a lack of proximodistal reorganization [91]. Analysis of *nebel* mutants [85], which exhibit defects in the distal placement of germ plasm RNP aggregates, may help identify the source of this signal.

The process of aggregation, recruitment, and distal compaction of germ plasm RNPs that occurs in the furrow for the first cell cycle becomes repeated during furrow formation for the second and third cell cycles (Figure 2E,F). The observed pattern of germ plasm RNP recruitment supports a model in which astral microtubules of the spindle apparatus mediate the local gathering of cortical germ plasm RNPs. Since in each subsequent spindle apparatus covers half the cortex as in the previous one, this predicts the accumulation of germ plasm RNPs of about half each subsequent cell cycle. This prediction is indeed observed during the first three cell cycles [86]. Thus, local germ plasm RNP furrow recruitment, coupled to the alternating (by 90 degrees) cleavage orientation pattern, gradually allows the gathering of germ plasm RNPs from the blastodisc cortex into the forming furrows. The adaptation of the cell division machinery for germ plasm RNP recruitment constitutes a simple and effective system to amass inherited single germ plasm RNP aggregates.

A consequence of this mechanism is that germ plasm continues to undergo recruitment to forming furrows for as long as there are germ plasm RNP aggregates in the cortex. This manifests in recruitment of germ plasm RNPs to the third furrow, temporarily generating embryos with eight visible germ plasm masses. However, the four aggregates collected during the third cell cycle do not undergo the subsequent step of ingression and instead appear to become degraded. At the same time, the outward movement of germ plasm RNPs remaining at the cortex that do not become recruited to the furrows, which is also mediated by the cycles of growing astral microtubules, result in the accumulation of these RNPs to the periphery of the blastodisc, where they similarly appear to become degraded. After the first several cycles, only the four larger germ plasm masses remain, corresponding to those recruited during the first and second cell cycles and which encompassed larger regions of the cortex and therefore amassed the largest numbers of germ plasm RNPs. The underlying basis for the selective stabilization of the first four aggregates is not known, but it is possible that these aggregates contain an amount of germ plasm RNPs above a certain threshold that allows their stabilization or further routing into the germ plasm segregation pathway [86].

Studies have shown that when RNA constructs containing a green fluorescent protein (GFP)-coding region coupled to a *nanos1* 3′ untranslated region (GFP-*nanos1* 3′UTR) are injected into the embryo, the GFP degrades quickly in somatic tissues while being stabilized in PGCs. Thus, one can hypothesize that if *nanos* RNA is not packaged properly in one of the four large aggregates, it will be degraded efficiently and the degradation information is in its 3′UTR [64]. These control mechanisms appear to be conserved in *Drosophila*, as non-localized *nanos* RNA also undergoes degradation [94].

*2.3.2. Animal meet vegetal RNPs: Generating a full-complement of zebrafish germ plasm*

During formation of the first furrows, only animal pole germ plasm RNPs are found in the forming germ plasm masses; however, at the end of the distal compaction phase as the first furrow is completed, vegetally localized RNPs, such as *dazl1* and *bruno-like*, which have translocated to the animal pole, become attached to the compacted mass of animal germ plasm RNPs [75]. At the distal end of the furrow, animal germ plasm RNPs, containing RNAs for *vasa*, *nanos*, and *dnd*, appear in a single, fully overlapping, RNA localization domain. Localized *dazl* RNA, however, occupies a partially overlapping yet distinct domain, situated in the distal-most region of the germ plasm aggregate. The localization of *dazl* RNA in the distal-most region of the furrow and at the blastomere–yolk cell boundary suggests that *dazl* RNPs that join the germ plasm travel animally along meridional arcs. This idea is consistent with the observation of tracks of *dazl* RNA along meridional arcs of the yolk cell cortex. This germ plasm organization, with animal and vegetal RNPs occupying distinct domains and with vegetal RNAs in a more distal location of the blastodisc, is maintained at least until the 64-cell stage [75]. Further research will be required to determine why germ plasm RNPs use two separate pathways of recruitment, one for animal germ plasm RNPs and one for vegetal ones, as well as distinct domains of localization within the final germ plasm mass. Some precedent exists with regards to germ plasm subcompartmentalization in other systems. In *Drosophila,* noncoding RNAs reside in the polar granules, whereas other RNAs localize to the matrix in which these are embedded [95]. In *Xenopus,* some germ plasm RNAs, such as *Xcat2,* are present in germinal granules corresponding to the germ plasm, others, such as *Deadsouth* and *Xpat*, associate closely with these granules and still others, such as *Xdazl*, localize to the surrounding matrix [96]. The conservation of germ plasm subcompartmentalization across species suggests a functional role for such substructure, a role that remains to be determined.

## 2.4. Maintaining germ plasm potential during cell division

*2.4.1. Ingression into cells and asymmetric segregation*

At about the 16-cell stage, the four germ plasm masses, which formed during the first two cell cycles and do not undergo degradation, translocate from their location at the blastomere–yolk cell boundary in each of four corners of the blastodisc into four cells [75, 79, 85, 92], a process that roughly coincides with cellularization of the blastomeres (Figure 3). Although this process of germ plasm ingression has not been yet characterized in the zebrafish, one might hypothesize that it is similar to *Xenopus* germ plasm ingression, which depends on intact microtubules and microfilaments [97].

Once the germ plasm has ingressed into four PGCs, these cells continue to divide and during the cell division process their germ plasm segregates asymmetrically (Figure 3). Although the mechanism by which asymmetric segregation of germ plasm occurs is not completely understood, the germ plasm aggregates form a cup-shape structure that associates with one of the two spindle poles [92, 98], suggesting that segregation might rely on the spindle apparatus as proposed in *Xenopus* germ plasm segregation [92, 99], as well as the asymmetric segregation of a maternally inherited mRNA in mollusc embryos [100]. This pattern of subcellularly

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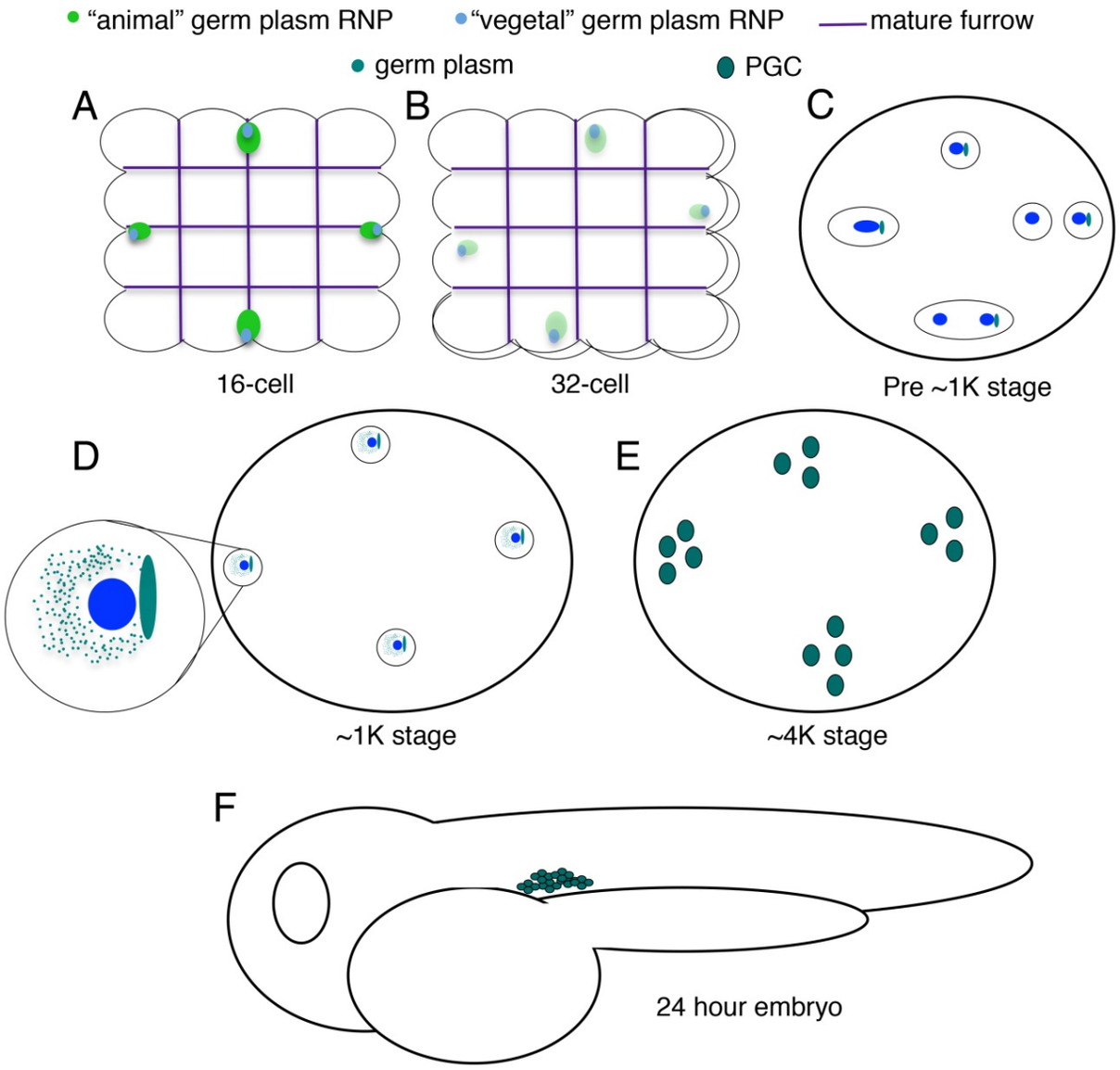


**Figure 3.** Germ plasm to primordial germ cell transition in zebrafish. A) Compact, yet separate animal and vegetal germ plasm aggregates at 16-cell stage are found at the edge of the first two furrows. B) At approximately the 32-cell stage, germ plasm aggregates ingress into four cells (aggregates are depicted with a lighter color to indicate that they are likely in bottom tier blastomeres, closest to their original location at the blastodisc–yolk cell boundary). C) Before PGC-specific expression, germ plasm asymmetrically segregates so that only one cell receives the aggregate (germ plasm masses are diagrammed at the various stages of the cell cycle that is represented as proceeding counterclockwise, indicating association of the germ plasm mass to one of the spindle poles to generate two asymmetric daughter cells, one with and one without the germ plasm mass—in this diagram, the counterclockwise progression is for the representation of the cell cycle only, and in reality each PGC is undergoing cell cycling independently). In (C) and (D), blue color depicts DNA. D) At approximately the 1K-cell stage, germ plasm aggregates break down into smaller particles, and daughter cells inherit these particles symmetrically. E) Cells from these previous founders begin to exhibit PGC-specific expression. F) After migration in the 24-hour embryo, PGCs accumulate in the prospective gonad just above the yolk.

localized, asymmetric segregation continues until about the 1000-cell stage, roughly coinciding with the activation of the genome at the midblastula transition (MBT).

During late blastula stage, the pattern of germ plasm segregation changes: now germ plasm distributes in a perinuclear arrangement and is inherited by both daughter cells [92]. At the same time, zygotic expression of the germ line-specific gene *vasa* begins. Due to the symmetric segregation of germ line RNAs during cell division, the number of PGCs increases, such that by gastrulation the number of germ plasm RNA-positive cells increases to around 30 cells [92].

Interestingly, DNA replication-inhibited embryos display a transition between asymmetric and symmetric segregation patterns that occurs at a developmental time similar to that in control embryos. This suggests that this transition in modes of germ cell determinant segregation does not rely on nucleo/cytoplasmic ratio, which has been proposed to regulate transcriptional activation at MBT [101, 102] or zygotic transcription initiation itself, but instead relies on a DNA-independent maternal temporal mechanism, possibly an intrinsic developmental timer or, alternatively, the counting of cell divisions [92].

*2.4.2. Activation of the germ cell program*

Little is known about the activation of PGC program in zebrafish. In mouse, activation of the PGC program involves the expression of three interdependent proteins: *Prdm1*/BLIMP1, *Tcfap2c*/AP2gamma and PRDM14 (reviewed in [103]). BLIMP1 induction of *Tcfap2c* allows for AP2gamma to initiate the expression of PGC-specific genes (*dnd1* and *nanos3*). In zebrafish, BLIMP1 activates *Tfap2a* (which encodes AP2alpha) during neural crest cell specification, but a role of these factors in PGC development has not been reported. Ziwi (zebrafish Piwi) protein, which also localizes to germ plasm in the cleavage furrows, is also expressed in PGCs in 24-hour embryos [104]. Piwi proteins act within mRNA storage particles involved in the translational control of mRNAs [105].

Even though Vasa protein does not colocalize to the germ plasm during the early cleavage period [92, 106, 107], it is found in perinuclear patches around the germinal vesicle during oogenesis and is uniformly distributed in all embryonic cells prior to MBT [92, 107]. At around 3-4 hours post-fertilization (hpf), when the zygotic genome is activated, Vasa protein levels increase [92]. The bulk of this increase in Vasa protein is dependent on the presence of a nucleus in the PGCs, suggesting that a large part of translated Vasa is derived from new zygotic expression. However, a small amount of Vasa does accumulate in embryos whose cells lack a nucleus, suggestive of translation of Vasa protein from maternal transcripts. This finding has led to the hypothesis that maternally inherited *vasa* mRNA in PGCs results in newly translated Vasa protein, which in turn triggers the activation of a PGC-specific gene expression program that includes zygotic *vasa*. After MBT, Vasa protein resumes perinuclear localization in PGCs. Interestingly, at this stage, Vasa protein does not colocalize with clusters of *vasa* RNA [92], suggesting that the bulk of the protein is not involved in the regulation of its own transcript.

An important hallmark of the activation of the germ cell program in animal systems is their subsequent migration (reviewed in [108, 109]). Two components found in the zebrafish germ plasm are required for PGC migration and maintenance in this organism: *nanos* [64] and *dnd* [67]. Morpholino knockdown of *dnd* illustrates a role of the protein product in the polarization and migration of PGCs, as morphants do not downregulate E-cadherin in PGCs, and cells remain in close contact with one another [67]. These results show that downregulation of E-

cadherin levels in PGCs allows these cells to become motile in order to receive and interpret guidance cues for PGC migration to the gonad [110]. The reader is referred to Raz [111] and Paksa and Raz [109] for in-depth reviews of zebrafish PGC migration.

## 3. Comparative analysis of germ plasm aggregation in vertebrates: Independent yet similar solutions

### 3.1. Other fish species

The mode of PGC induction within teleost fish is not fully conserved. Fish in the ostariophysan lineage, such as carp, Fegrade's danio, tetra, and zebrafish, localize *vasa* RNA at the furrow. On the other hand, euteleost species, such as medaka, rainbow fish, and trout, lack *vasa* RNA localization, exhibiting instead diffusely distributed cytoplasmic *vasa* RNA [112, 113]. In the more basal teleost lineage containing butterfly fish, *vasa* RNA is localized [112]. These patterns of *vasa* RNA localization suggest that germ plasm-mediated PGC determination is an ancestral feature of the teleost lineage, which subsequently became lost in euteleosts. Consistent with a role of the 3'UTR in RNA localization, species exhibiting *vasa* RNA localization have highly conserved 3'UTRs in this mRNA compared to species with diffuse *vasa* mRNA distribution [112].

Embryos from sturgeon species, considered a primitive fish that acts as a basal outgroup for the teleost lineage, show many similarities to anurans including holoblastic cleavage, forming a distinct blastocoel and archenteron and undergoing primary neurulation. Two studies had varying conclusions on whether sturgeon embryos employ epigenesis or preformation [8, 114]. One group found that *vasa* and *dazl* RNAs failed to localize in oocytes [8], whereas the other group argued that sturgeon PGCs are specified in the vegetal hemisphere around the vegetal pole using a maternally derived germ plasm, as is the case in anurans. Interestingly, transplanting a single PGC from sturgeon to goldfish resulted in correct translocation of PGCs to the gonadal ridge [114], indicating that the mechanism for PGC migration is conserved across species in divergent lineages.

### 3.2. Amphibians (*X. laevis*)

*Xenopus* uses maternally inherited germ plasm for PGC determination and exhibits a number of other similarities to zebrafish. During oogenesis, the mitochondrial cloud in *Xenopus* embryos appears homologous to the zebrafish Bb, as these two structures are rich in mitochondria and have a role in germ plasm segregation [115]. *Xenopus* germ plasm contains homologs to many of the germ plasm components in zebrafish, including *Xdazl* and the *nanos* homolog *Xcat2*. In both *Xenopus* and zebrafish, germ plasm components originally localize to the vegetal pole of the egg during oogenesis in small islets (Figure 4). These two RNAs accumulate in the mitochondrial cloud in early oogenesis using a mitochondrial cloud localization element (MCLE) in their 3'UTR and subsequently disperse among the islets. Interestingly, germ line RNAs (*Xcat2*), injected after the time the early transport (METRO)

mechanism is active, are able to localize to the vegetal pole using the late transport pathway. However, these RNAs do not enter the germ plasm [116], suggesting the presence of steps early in the RNP packaging process for these RNAs that are crucial for proper transcript localization.

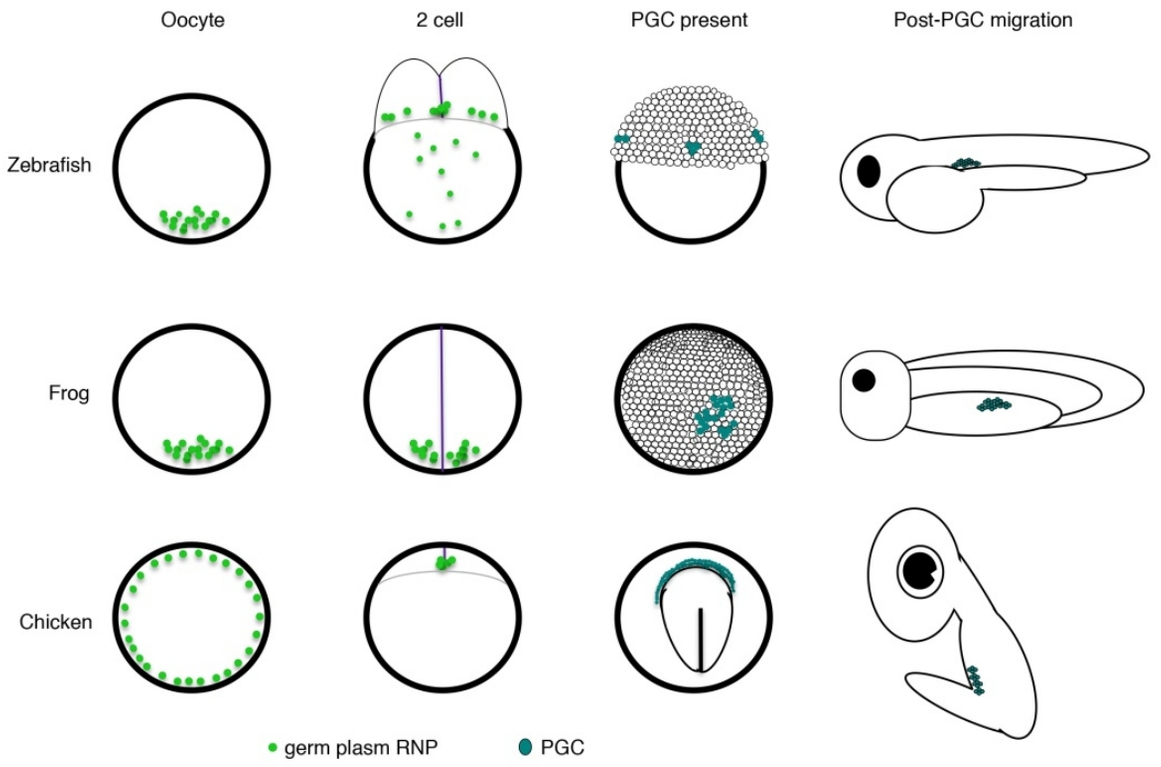


**Figure 4.** Comparison of vertebrate species employing germ plasm. All three species, zebrafish, frog and chicken, localize germ plasm during oogenesis. At the two-cell stage, two sites of localization of germ plasm (animal and vegetal) are found in zebrafish, only one vegetal site in frog and one site localizing at the basement of the first cleavage furrow in the chick. Both zebrafish and frog embryos have been shown to multimerize germ plasm RNPs into four large aggregates during the first several cycles, which during early embryogenesis are inherited by four separate sets of PGCs in zebrafish and one main PGC cluster in frogs. Chick PGCs are found in a crescent-shaped distribution at the anterior edge of the primitive streak. After PGC migration, each organism contains bilaterally situated groupings of PGCs at the site of the prospective gonads.

While the early/METRO pathway does not involve microtubules [117, 118], *Xenopus* germ plasm undergoes a second movement after fertilization, which relies on microtubules. Like zebrafish, *Xenopus* germ plasm undergoes local aggregation to multimerize islets into large patches of germ plasm in the first round of aggregation. The second aggregation process involves periodic surface contraction waves (SCWs) directing the germ plasm to the vegetal cortex to form large patches (Figure 4). SCWs can be inhibited by ultraviolet radiation at the vegetal pole [97] and require the kinesin family motor Xklp1 [119]. These aggregation waves result in the concentration of germ plasm at the cortex, forming four large aggregates that, as in zebrafish, are associated with the microtubule network, localize to cleavage furrows [83] and are later inherited by only four blastomeres [120].

The segregation of germ plasm in *Xenopus* continues to have parallels with that in zebrafish during the cleavage stages. During this period, germ plasm associates with the plasma membrane; during mitosis, it localizes to one of the spindle poles leading to asymmetric segregation until the gastrulation stages, when germ plasm acquires a perinuclear localization; and during cell division, symmetric expression leading to an increase in the number of germ plasm containing cells [121]. Although some experiments show that implanted PGCs can form functional gametes in *Xenopus* [122], others show explanted pre-PGCs, as well as isolated migrating PGCs, do not form germ cells when placed in an ectopic location, differentiating instead as somatic cells [123]. These observations suggest that, unlike *Drosophila* [124], *Xenopus* germ plasm specifies, but does not irreversibly determine, the germline.

Although there are many similarities in the pathways of germ plasm segregation in *Xenopus* and zebrafish, a major difference between the two species is that in zebrafish embryos germ plasm RNPs employ two distinct modes of transport within the early embryo: for animal and vegetal germ plasm RNPs; whereas in *Xenopus*, all germ plasm RNPs are located at the vegetal pole. The difference might be explained by the method each species employs for cellular cleavage. Teleost embryos undergo segregation of ooplasm away from the yolk followed by meroblastic cleavages only at one pole of the embryo. In *Xenopus* embryos, however, the yolk does not segregate away from dividing blastomeres, which as opposed to teleosts undergo holoblastic cleavage involving division across the entire embryo.

### 3.3. Other vertebrate species (chick)

Until relatively recently, studies suggested that the chick used epigenesis [125, 126], although these studies relied on in vitro culture [127]. However, Vasa protein (CVH in chick) was found to accumulate at the base of the membrane furrows in the early cleavage stage chick embryo, a location strikingly similar to that for *vasa* RNA localization in zebrafish embryos (Figure 4). CVH localizes to globular structures in chicken oocytes, which also contained mitochondrial cloud materials, suggesting preformation [6]. Considering the proposed independent appearance of germ plasm in various vertebrate lineages [8], it is possible that the parallels observed in teleosts, amphibians, and the chick reflect the cell division apparatus as a pre-existing intrinsic cellular mechanism that is readily co-opted for the local gathering and segregation in the embryo of maternally inherited germ plasm. Further analysis, involving common cellular and developmental themes in various animal lineages, will be required to better understand germ plasm evolution and its relationship with the basic embryonic cellular apparatus.

## 4. Conclusions

Epigenesis describes an inductive mechanism used notably by mammals in which tissues signal for a set of cells to become the PGCs. Preformation describes a mechanism using germ granules placed in the oocyte, which are collected into a set of cells to become the PGCs. Even though mechanisms differ, many of the RNAs and proteins that specify the germ line are conserved between animals using preformation and epigenesis.

Developmental biology studies have focused on genetic models to decipher the molecules and mechanisms for germ line establishment. Zebrafish use preformed germ granules known as germ plasm RNPs, which aggregate together, recruit to the furrow and distally compact into tight masses that ingress into only four cells. Throughout the remainder of maternal stage cell divisions, these four cells asymmetrically segregate the germ plasm aggregate so as only one of the dividing cells keeps the mass. When the zygotic genome is activated, these cells divide and generate the PGC population. It is tempting to speculate that the maternal process of germ plasm inheritance is designed to optimize the gathering of germ plasm material into large masses capable of influencing cell fate and that their subsequent asymmetric segregation during the cleavage stages preserves their full inductive potential until activation of the zygotic genome.

Understanding the mechanisms of germ cell determination will contribute to our ability to interpret cases of impaired fertility and will facilitate the promotion of healthy reproduction and assisted reproductive methods. In addition, recent studies in various biological systems have identified common links between germ cell gene expression programs, and those of stem and cancer cells [128–131], suggesting that a better understanding of germ cell biology will also contribute to the fields of regenerative and cancer biology. The zebrafish model system provides a tractable experimental system to gain mechanistic insights into these important topics relevant to human and animal health.

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## Author details

Celeste Eno and Francisco Pelegri*

*Address all correspondence to: fjpelegri@wisc.edu

Laboratory of Genetics, University of Wisconsin – Madison, Madison, WI, USA

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