Conserved germ plasm characteristics across the *Danio* and *Devario* lineages

Christina L. Hansen, **Trevor J. Chamberlain**, **Ryan L. Trevena**, **Jacob E. Kurek**, **Francisco Pelegri**

Laboratory of Genetics, University of Wisconsin - Madison, 425-G Henry Mall, Madison, WI 53706

Abstract

In many animal species, germ cell specification requires the inheritance of germ plasm, a biomolecular condensate containing maternally-derived RNAs and proteins. Most studies of germ plasm composition and function have been performed in widely evolutionarily divergent model organisms, such as *C. elegans*, *Drosophila*, *Xenopus laevis*, and *Danio rerio* (zebrafish). In zebrafish, 12 RNAs localize to germ plasm at the furrows of the early embryo. Here, we tested for the presence of these RNAs in three additional species within the Danionin clade: *Danio kyathit*, *Danio albolineatus*, and *Devario aequipinnatus*. By visualizing *nanos* RNA, we find that germ plasm segregation patterns during early embryogenesis are conserved across these species. Ten additional germ plasm RNAs exhibit localization at the furrows of early embryos in all three non-zebrafish Danionin species, consistent with germ plasm localization. One component of zebrafish germ plasm, *ca15b*, lacked specific localization in embryos of the more distantly related *Devario aequipinnatus*. Our findings show that within a subset of closely related Danionin species, the vast majority of germ plasm RNA components are conserved. At the same time, the lack of *ca15b* localization in *Devario aequipinnatus* germ plasm highlights the potential for the divergence of germ plasm composition across a restricted phylogenetic space.

Introduction

Germ line specification begins with the establishment of primordial germ cells, a small subset of embryonic cells that will give rise to the germline of the newly developing organism. This critical developmental process is achieved through two general pathways: induction and preformation (Bertocchini & Chuva de Sousa Lopes, 2016; Extavour & Akam, 2003; Magnúsdóttir & Surani, 2014; Swartz & Wessel, 2015; Wylie, 1999). Some animals, such as eutherian mammals and urodele amphibians, specify primordial germ cells (PGCs) as a result of an inductive mechanism involving cell-cell signaling driven by zygotic genes in a tissue-specific context (Lawson et al., 1999; Ying et al., 2001). Other animals, such as teleost fish, anuran amphibians, insects, and some birds, rely on a mechanism of preformation, in which PGCs are specified through the germ plasm, a membraneless cytoplasmic structure that contains maternally-inherited ribonucleoparticles (RNPs) and becomes incorporated into PGCs (Bontems et al., 2009; Eddy, 1975; Hashimoto

Corresponding author: Francisco Pelegri, fjpelegri@wisc.edu.

et al., 2004; Miranda-Rodríguez et al., 2017; Strome & Updike, 2015; Tada et al., 2012). During *Danio rerio* (zebrafish) embryonic development, germ plasm RNPs proceed through a series of relatively well-characterized stages of localization and behavior prior to germ cell differentiation. First, as a newly fertilized zygote begins to proceed through its initial cell cycles, maternally-derived germ plasm RNPs progressively aggregate into the embryonic cleavage furrows and compact toward the blastodisc periphery (Eno et al., 2018; Miranda-Rodríguez et al., 2017; Pelegri et al., 1999). This process, largely driven through interactions with dynamic cytoskeletal elements (reviewed in (Moravec & Pelegri, 2020)), results in four stable germ plasm masses at the distal ends of each of the first two cleavage furrows. At the 16-32 cell stage, as the embryo begins the process of cellularization, these four germ plasm aggregates become internalized into newly cellularized PGCs and are subsequently asymmetrically transmitted during each successive cell division into a single daughter cell (Braat et al., 1999; Knaut et al., 2000; Pelegri et al., 1999; Yoon et al., 1997). These first four germ plasm aggregates are maintained largely intact throughout the late cleavage, blastula, and early gastrula stages, resulting in embryos with a small subset of cells containing germ plasm (approximately ~4-12 out of ~4000-8000 total embryonic cells during the late blastula period) (Braat et al., 1999; Eno et al., 2019; Knaut et al., 2000; Yoon et al., 1997). Beginning at dome stage (4.3 hours post-fertilization at 28.5°C) in zebrafish embryos, RNAs within the intact germ plasm aggregates initiate dispersal, a process during which they appear to be released from the phase-separated mass into the host cells' cytoplasm (Braat et al., 1999; D'Orazio et al., 2021; Knaut et al., 2000; Yoon et al., 1997). The occurrence of germ plasm RNP cytoplasmic dispersal, which roughly coincides with the bulk of zygotic genome activation in the embryo, is closely followed by the appearance of perinuclear Vasa protein-containing germinal granules (Hartwig et al., 2014; Houwing et al., 2007; Knaut et al., 2000; Strasser et al., 2008). During this period PGCs are also thought to initiate the germ cell-specific gene expression program and continue to proliferate and undergo directed migratory movements towards the prospective gonadal region (Blaser et al., 2005; Bontems et al., 2009).

In zebrafish, 12 RNAs (11 transcripts and one microRNA) have been identified as components of the germ plasm. These include *askopos* (*kop*) (Blaser et al., 2005), *carbonic anhydrase 15b* (*ca15b*) (Hartwig et al., 2014), *celf-1/bruno-like* (*celf1*) (Hashimoto et al., 2004), *deleted in azoospermia-like* (*dazl*) (Hashimoto et al., 2004), *dead end (dnd)* (Weidinger et al., 2003), *granulito* (*gran*) (Strasser et al., 2008), *hook microtubule-tethering protein 2* (*hook2*) (Roovers et al., 2018), *microRNA 202-5p* (*miR-202-5p*) (Zhang et al., 2017), *nanos3* (*nos3*) (Köprunner et al., 2001), *regulator of G-protein signaling 14a* (*rgs14a*) (Hartwig et al., 2014), *tudor domain protein 7* (*tdrd7a*) (Mishima et al., 2006), and *vasa/ DEADbox polypeptide 4* (*vasa/ddx4*) (Yoon et al., 1997) (Table 1).

The composition of germ plasm appears to vary across animal species, as indicated by studies in other model organisms, such as *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Xenopus laevis*, with a majority of germ plasm RNAs present in some lineages but not others (Houston & King, 2000; Ikenishi, 1998). Only one RNA, *nanos*, is known to be a conserved germ plasm RNA component across these broadly divergent species (Köprunner et al., 2001; Subramaniam & Seydoux, 1999). Within vertebrate lineages, zebrafish germ plasm shares four RNAs, *dazl, nanos, hook2*, and *dnd*, with germ plasm in *Xenopus*

laevis (Horvay et al., 2006; Houston et al., 1998; MacArthur et al., 1999; Owens et al., 2017) whereas the remaining nine zebrafish germ plasm RNAs are apparently absent in *Xenopus laevis* germ plasm (Butler et al., 2018; Karimi et al., 2018; Owens et al., 2017). Conversely, some RNA components found in the germ plasm of *Xenopus laevis*, such as *pgat/xpat*, *germes/uncharacterized LOC779566*, and *ddx25*, appear to be absent in its zebrafish counterpart (Berekelya et al., 2003; Hudson & Woodland, 1998; MacArthur et al., 2000).

Within fishes and consistent with their greater phylogenetic relatedness, RNA components of germ plasm appear to be more robustly conserved. At least four RNAs identified as zebrafish germ plasm components (*dnd*, *nanos*, *vasa/ddx4*, and *dazl*) have also been reported to localize to germ plasm in one or more additional teleost fish species, such as *Scophthalmus maximus* (turbot), *Gadus morhua* (Atlantic cod), or *Salmo salmar* (Atlantic salmon) (Lin et al., 2012; Nagasawa et al., 2013; Presslauer et al., 2012). In these fish species, germ plasm appears to exhibit a conserved pattern of aggregation in the forming furrows for the first several cell cycles. However, there is also evidence of germ plasm heterogeneity amongst fish, as demonstrated by the lack of furrow localization of maternally-inherited *vasa/ddx4* RNA in early medaka and seabream embryos (Herpin et al., 2007; Shinomiya et al., 2000; Zhou et al., 2020).

To date, no study has determined if within a more restricted phylogenetic space, germ plasm RNA components are broadly conserved as a core set, or whether some RNAs but not others are conserved within related yet distinct sets of varying composition. To begin to gain some insight into this question, we use the *Danio* genus as a "model genus" (Irion & Nüsslein-Volhard, 2019; McCluskey & Postlethwait, 2015a), additionally taking advantage of the limited set of RNAs evidently present in the germline of this species (Eno et al., 2019). We systematically test for the presence of known zebrafish RNAs in three other species that span the phylogenetic depth of the genus *Danio*, with the orange-finned danio, *D. kyathit*, being most closely related to zebrafish (most common recent ancestor (MRCA) ca. 6.5 MYA), the pearl danio, *D. albolineatus*, as an intermediate relative (MRCA ca. 9.5 MYA), and the giant danio, *Devario aequipinnatus*, as a representative of the sister genus *Devario* (MRCA ca. 13 MYA) (McCluskey & Postlethwait, 2015a; Rüber et al., 2007) (Sup. Fig. 1). We find that 11 of 12 germ plasm RNAs in zebrafish are also germ plasm components in all three of the tested non-zebrafish Danionin species, *Danio kyathit*, *Danio albolineatus*, and *Devario aequipinnatus*, indicating a high level of conservation of germ plasm components at this phylogenetic scale. The sole exception, *ca15b*, is not observed at the furrows of *Devario aequipinnatus* and therefore is unlikely to be a germ plasm component in this species. Our data also indicate that the patterns of germ plasm localization, and likely the underlying cellular mechanisms for germ plasm aggregation and segregation, are conserved across these species.

Results

Identification of presumptive germ plasm in three non-zebrafish Danionin species

We used transcripts of the zebrafish *nanos3* (previously known as *nanos1*, the ortholog of germline-associated *nanos* in other species) (Köprunner et al., 2001) gene as a marker

of germ plasm at selected developmental stages in embryos from the Danionin species, *Danio kyathit*, *Danio albolineatus*, and *Devario aequipinnatus*, which as a set spans the evolutionary depth of the *Danio* genus and its closest sister genus *Devario* (Fig. 1A, Fig. 2, Sup. Fig. 1) (McCluskey & Braasch, 2020; McCluskey & Postlethwait, 2015b). The developmental stages were chosen to be representative of major steps in the germ plasm life cycle established in zebrafish, including aggregation at furrow distal tips during cleavage, asymmetric segregation into a subset of cells during blastula stages, and cytoplasmic dispersal into host PGCs. Due to the lack of reference genomic sequence information for many non-zebrafish species, and as an initial test of the extent of sequence conservation of germline-associated genes within the Danionin subfamily, we used a hapten-labeled RNA probe designed to complement zebrafish *nanos3* (Fig. 2A) for the *in situ* hybridization analysis. We predicted that a probe targeting zebrafish *nanos3* could potentially allow detection of the homologous gene in other *Danio* species and closely related genera such as *Devario* through cross-hybridization due to the broadly conserved nature of the *nanos3* gene across animal lineages, including relatively high conservation at the sequence level across cyprinid fishes (Sup. Fig. 2) (Vallée et al., 2006). Indeed, we found that the zebrafish *nanos3* probe detected transcripts in embryonic regions consistent with germ plasm aggregation, suggesting that the zebrafish probe successfully bound homologous transcripts in all three non-zebrafish species. Specifically, we found that at the four-cell stage of each species tested, *nanos3*-containing RNA particles form large aggregates at the distal ends of both cleavage furrows (Fig. 2A). In all species and similar to zebrafish (Eno et al., 2018; Miranda-Rodríguez et al., 2017; Pelegri et al., 1999; Yoon et al., 1997) the compacted RNAs that form the aggregates appear to form distinctive cylindrical masses within cleavage furrows, which begin as extended structures but gradually compact toward the distal ends. This suggests that the spatiotemporal pattern of germ plasm localization during the initial aggregation period as originally determined in the zebrafish model system is conserved in multiple species across the *Danio* and *Devario* genera.

Conservation of dynamic germ plasm localization patterns throughout embryogenesis

We also assessed germ plasm localization using *nanos3* as a marker at later stages of embryogenesis, including stages that in zebrafish comprise the asymmetric segregation (128-cell to sphere) and RNA dispersal (dome to mid-epiboly) periods of germ plasm dynamics (Eno et al., 2019; Knaut et al., 2000). In order to distinguish individual cells at these later stages of embryogenesis, visualization of DAPI-stained DNA and cell boundaries labeled with anti-ß-catenin antibodies was carried out simultaneously with fluorescent *in situ* hybridization (Figure 3). As with the results at the four-cell stage, signal detection at the sphere stage of embryogenesis from the species *Danio kyathit*, *Danio albolineatus*, and *Devario aequipinnatus* revealed similar patterns to what has been previously established for segregating germ plasm in zebrafish. In each species, the putative germ plasm aggregates appear as intact, phase-separated cytoplasmic masses that occupy a substantial portion of host cells, often near the nucleus. At the dome stage in each species, which occurs post-zygotic genome activation in zebrafish (Aanes et al., 2011; Harvey et al., 2013; Heyn et al., 2014; Kane & Kimmel, 1993; Lee et al., 2014; Mathavan et al., 2005) and can be easily distinguished by the bulging appearance of the yolk cell under a concave blastoderm, the germ plasm undergoes a dramatic morphological transition from intact mass to "leaky"

appearance, with the RNA particles dispersing to fill the cytoplasm (Figure 3) (D'Orazio et al., 2021). Another hallmark of this transition in zebrafish is the subsequent appearance of perinuclear Vasa protein-containing germ granules, which are retained throughout PGC migration to the genital ridge during somitogenesis (Blaser et al., 2005; Knaut et al., 2000; Strasser et al., 2008). Further confirming our identification of germ plasm in these non-zebrafish Danionin species, we observed that *nanos3* RNA-containing cells after the dome stage also exhibited the tell-tale perinuclear Vasa protein granules in *Danio kyathit*, *Danio albolineatus*, and *Devario aequipinnatus* (Figure 4). Together, the observed localization patterns of *nanos3* and Vasa suggest that both maternally-derived germ plasm (labeled by *nanos3*) and germ granules at later stages of development (labeled by Vasa) are expressed and localized consistently across multiple species in the *Danio* and *Devario* genera.

Most zebrafish germ plasm RNAs are localized to the cleavage furrows in *Danio kyathit*, *Danio albolineatus*, and *Devario aequipinnatus*

Since there was no published reference genome for other *Danio* and *Devario* species at the time of this work, we expanded a similar cross-hybridization approach in select species to also assess the presence and localization of all other germ plasm RNAs as originally identified in zebrafish. Using RNA probes against the zebrafish gene sequence, we performed fluorescent *in situ* hybridization at the four-cell stage of embryonic development (approximately one-hour post-fertilization at 28.5°C for all species) in *Danio kyathit, Danio albolineatus*, and *Devario aequipinnatus*. At this developmental stage in zebrafish, germ plasm RNAs and associated proteins have compacted into four distinct aggregates at the distal ends of cleavage furrows and are readily identifiable at low magnification (Fig. 2), making it a convenient timepoint to assess germ plasm localization across the selected species. In addition to *nanos3*, the set of tested germ plasm RNAs included *askopos (kop)* (Blaser et al., 2005), *carbonic anhydrase 15b (ca15b)* (Hartwig et al., 2014), *celf-1/bruno-like (celf1)* (Hashimoto et al., 2004), *deleted in azoospermia-like (dazl)* (Hashimoto et al., 2004), *dead end (dnd)* (Weidinger et al., 2003), *granulito (gran)* (Strasser et al., 2008), *hook microtubule-tethering protein 2 (hook2)* (Roovers et al., 2018), *microRNA 202-5p* (Zhang et al., 2017), *regulator of G-protein signaling 14a (rgs14a)* (Hartwig et al., 2014), *tudor domain protein 7 (tdrd7a)* (Mishima et al., 2006), and *vasa/DEADbox polypeptide 4 (vasa/ddx4)* (Yoon et al., 1997). We first verified that, as with *nanos3* (Fig. 2-3), our other 11 RNA probes (see Methods and Materials) recapitulated germ plasm localization for all established germ plasm RNAs in zebrafish embryos (Figure 5). After verifying the probes in zebrafish, we proceeded to test them in the other three Danionin species. Probes for each of the 12 zebrafish germ plasm RNAs hybridized to the target RNA in germ plasm at the furrow region of four-cell stage *Danio kyathit* and *Danio albolineatus* embryos, while we observed furrow localization of all but one RNA (*ca15b*) in *Devario aequipinnatus* embryos (Figure 5, Figure 6). Another RNA, *dazl*, was difficult to observe in low magnification images of *Devario aequipinnatus* embryos, but germ plasm localization in the distal furrow region was confirmed with increased magnification. Since *dazl* appeared faint even at this increased magnification (perhaps due to interspecies probe mismatch), we also checked blastula-stage embryos and found *dazl*-positive germ plasm in putative PGCs (Sup. Fig. 3). This result underscored that, like *nanos3*, the majority of germ plasm genes originally identified in

zebrafish are also maternally-expressed and, furthermore, germ plasm-localized, in closely related fish species.

ca15b mRNA is not a germ plasm component in the giant danio *Devario aequipinnatus*

Animal genomes, including fish, often encode multiple carbonic anhydrases (Gilmour & Perry, 2009; Howe et al., 2021; Tashian, 1989) (Sup. Fig. 4); however, *ca15b* is the sole member of the carbonic anhydrase family known to localize to germ plasm in any species, as reported in zebrafish (Wang et al., 2013), *Danio kyathit*, and *D. albolineatus* (this report). The apparent lack of germ plasm-specific *ca15b* localization in *Devario aequipinnatus* embryos, in contrast to its presence in at least three *Danio* species, suggested that either the zebrafish and *D. aequipinnatus ca15b* mRNA sequences have diverged enough to prevent interspecies probe hybridization or that *ca15b* is not a component of *D. aequipinnatus* germ plasm. We identified the putative *ca15b* sequence in *Devario aequipinnatus* from data contributed to the NCBI Sequence Read Archive by the Vertebrate Genomes Project (Leinonen et al., 2011; Rhie et al., 2021) and, in addition to confirming that it shares a closer evolutionary history with zebrafish *ca15b* than other members of the carbonic anhydrase IV/XV family (Figure 6C, Sup. File 1), used the sequence information to repeat the fluorescent *in situ* labeling after amplifying exonic template DNA for RNA probe synthesis directly from *Devario aequipinnatus*, rather than zebrafish cDNA (Sup. Fig. 5). Even with a probe that is precisely homologous to *D. aequipinnatus ca15b* and as in our initial experiments, *ca15b* was not observed in germ plasm and instead appeared to distribute uniformly throughout the blastodisc. We extracted total RNA at various times during early embryonic development in *Devario aequipinnatus* embryos and performed RT-PCR to assess for *ca15b* expression. We found that *Devario aequipinnatus ca15b* transcripts are present at the 2-cell (45 mpf) and 1000-cell (3 hpf) but greatly reduced at dome stage (4.3 hpf), suggestive of maternal-specific expression for this gene and supporting our conclusion that *ca15b* transcripts are present in the early embryo but not specifically-localized during this time. Together with the previous expression data, this demonstrates that the RNA composition of germ plasm, as it is currently known, is largely but not completely shared across multiple species spanning the Danionin phylogenetic clade.

Discussion

Stages of germ plasm localization are conserved in multiple Danionin species

Our findings suggest that prior to PGC proliferation and migration to the presumptive gonads, patterns of germ plasm localization and morphology are robustly conserved across the Danionin lineage. Using a probe designed to target zebrafish *nanos3*, we first tracked germ plasm localization at characteristic stages of development in *Danio rerio*, *D. kyathit*, *D. albolineatus*, and *Devario aequipinnatus* embryos with the finding that germ plasm segregation behavior is consistent throughout these Danionin species. In each species, the germ plasm morphology progressed from four, rodlike aggregates to irregular, compact masses, albeit with discrete borders, before dispersing into submicron-units that flood the cytoplasm of the host cell. Moreover, the developmental timing of germ plasm dispersal is tightly conserved across species, with each species initiating dispersal approximately at dome stage. Also, as visualized using combined fluorescent *in situ* hybridization and

immunolabeling, germ plasm-containing cells in zebrafish and each of the three tested non-zebrafish species acquire perinuclear Vasa protein granules soon after germ plasm dispersal.

All RNA germ plasm components previously identified in zebrafish are also present in the germ plasm of *Danio kyathit* and *Danio albolineatus*

The full set of 12 known germ plasm components previously characterized in zebrafish are present and conserved in two other *Danio* species. Probes designed for zebrafish were sufficient in both *Danio kyathit* and *Danio albolineatus*, suggesting a high degree of sequence conservation at the nucleotide level. Mismatch tolerance/specificity of ribonucleotide probes depends on many variables, such as hybridization conditions, probe length, and transcript accessibility, but some estimates suggest that probes generally require at least 85% complementarity between oligonucleotide probe and target sequence (He et al., 2005). The conserved nature of this set of germ plasm RNAs across species and genera indicates the importance of these factors in early development and germline differentiation, and could potentially suggest a conserved core set of RNA components required for PGC specification among Danionin fish. Due to the similarities in localization patterns between species, we also predict that the mechanisms underlying germ plasm aggregation, such as interactions with the cytoskeleton and cell division apparatus, are also shared between these species.

Although reference genomes are not yet available for most non-zebrafish Danionin species, including the three tested in this work, several are expected to be included in the Vertebrate Genomes Project (there is now an assembled version of the *Danio kyathit* genome and raw reads of *Danio albolineatus* and *Devario aequipinnatus* available in the NCBI Sequence Read Archive). We anticipate that reference genome availability will open up additional resources and investigative paths, such as determining the precise extent of sequence conservation between germ plasm genes and if any are present as duplicate copies in the genome.

Divergence of germ plasm RNA composition between *Danio* and *Devario* genera

We found that mRNA encoded by the carbonic anhydrase gene *ca15b* is located in the germ plasm of three *Danio* species but not in *Devario aequipinnatus*, a member of the sister genus. Carbonic anhydrase genes encode enzymes that reversibly catalyze the hydration of carbon dioxide into bicarbonate and a proton in most living organisms, including animals, plants, bacteria, and archaea, thus mediating CO_2 transport, ion regulation, and acid-base homeostasis (Henry, 1996; Hewett-Emmett & Tashian, 1996). Carbonic anhydrases have nearly maximal catalytic efficiencies that approach the diffusion limit, with different isozymes exhibiting different kinetic properties (Boone et al., 2013; Silverman & Lindskog, 1988). Functional specialization in regard to subcellular localization, tissue-type localization (Hewett-Emmett & Tashian, 1996), and even during life mode transitions such as in lamprey metamorphosis (Ferreira-Martins et al., 2016) may have driven the appearance of a large number (greater than 3,200 in vertebrates according to the Ensembl genome database) of carbonic anhydrase genes.

Vertebrate carbonic anhydrase genes can be categorized according to subcellular localization, such as cytosolic, mitochondrial, membrane associated and secreted (Frost, 2014). Bony fish encode seven subgroups of carbonic anhydrase isoenzymes with predicted transmembrane or extracellular function: ca4, ca6, ca9, ca12, ca14, ca15, and ca16 (Gilmour & Perry, 2009), with gene members from each of these subgroups broadly present across vertebrate lineages. Interestingly, the ca15 subgroup, including the *ca15b* gene, though also widely distributed across vertebrates, is present in egg-laying mammals (monotremes) but absent in other mammals (marsupials and placentals) (Sup. Fig. 4) (Howe et al., 2021). The association of *ca15* genes with egg-laying may be indicative of a role for Ca15b in oogenesis and/or embryonic development specifically in egg-laying animals. Indeed, in the zebrafish Ca15b protein localizes to the plasma membrane of oocytes (Wang et al., 2013), suggesting a likely function during oogenesis. Despite the broad distribution of genes from the ca15 subgroup across egg-laying vertebrates, not all egg-laying vertebrates have a *ca15* gene (Sup. Fig. 4) and the presence of a *ca15* gene, including *ca15b*, additionally does not precisely correspond to the use of germ plasm for germ cell induction found in vertebrate phylogeny (Hansen & Pelegri, 2021b; A. D. Johnson et al., 2011). Phylogenetic analysis of the *ca15b* gene thus suggests a maternal function, such as during oogenesis and/or early embryonic development in some egg-laying species, that is broadly distributed but not universal within vertebrates. In the *Danio* lineage, this gene's maternal function may have been co-opted to incorporate its transcripts as a germ plasm component to facilitate primordial gem cell developmental processes, such as migration to the prospective gonad (see below). Further analysis of the distribution of *ca15b* and other germ plasm components across phylogenetic space will provide further insights into the function and evolutionary history of this gene.

In addition to a potential function in oogenesis, zebrafish Ca15b has been proposed to play a role in primordial germ cell migration by establishing a pH gradient within PGCs as a response to a chemokine-sensitive signaling cascade and is thought to promote actin polymerization at the leading edge of migrating cells (Tarbashevich et al., 2015). In zebrafish, Ca15b knockdown impaired the ability of PGCs to migrate to the prospective gonad. It is unclear if Ca15b protein is able to be restricted to the PGC lineage in *Devario aequipinnatus* and similarly function in PGC migration despite the absence of *ca15b* transcripts in germ plasm. Alternatively, *Devario aequipinnatus* maternal *ca15b* may not function in in germ cell migration, and other cellular components or pathways could perform a redundant role. We also note that prior to this study zebrafish was the only known species with germ plasm-localized *ca15b*; therefore, other than species within the *Danio* genus as shown in this work, other animal species may be able to achieve PGC migration and establish their germline without it. Generally speaking, migrating cells, particularly bleb-forming migratory cells, rely on polarization, often generated by concentration gradients of various molecules, including proteins or ions (Martin et al., 2010; Richardson & Lehmann, 2010). It is possible that PGC migration in *Devario aequipinnatus* employs a different cell polarization mechanism, and/or that the Ca15b-mediated pH gradient in embryos of zebrafish and presumably other *Danios* represents an evolutionarily novel (potentially more efficient) means to achieve PGC migration.

In conclusion, we identify shared dynamics of germ plasm segregation and a set of a largely, but not absolutely, conserved germ plasm RNAs within the relatively narrow phylogenetic confines of the related *Danio* and *Devario* genera. By leveraging multiple species within the Danionin clade, this study laid the groundwork for further investigation into the function of individual molecular components of germ plasm in specific species and the processes through which germ plasm composition is adaptable across phylogeny.

Methods

Fish Maintenance

Stocks of wild-type AB *Danio rerio* fish were raised and maintained in standard conditions at 28.5°C (Brand et al., 2002). Stocks of the other Danionin fish species (*Danio kyathit*, *Danio albolineatus*, and *Devario aequipinnatus*) were also raised and maintained in standard conditions at 28.5°C. All procedures involving live fish were conducted according to the University of Wisconsin-Madison and Institutional Animal Care and Use Committee (IACUC) guidelines.

Embryo Collection and Fixation

Embryos from all species were collected and developed in E3 embryonic medium and were staged according to age and morphological characteristics corresponding to those in zebrafish (Kimmel et al., 1995). Embryos were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) while still in their chorions for at least 4 hours at room temperature to overnight at 4°C. All experiments involved mixed clutches of embryos from between 2 and 5 females per experiment for both zebrafish and non-zebrafish species.

RNA extraction and cDNA synthesis

A pool of embryos was collected from three *Devario aequipinnatus* clutches. Total RNA was extracted from 50 dechorionated embryos at the two-cell stage (45 mpf), the 1,000-cell stage (3 hpf), and the dome stage (4.3 hpf) using the standard TRizol (Invitrogen) protocol. Isolated total RNA was quantified using a Nandrop 2000 (Thermo Scientific). Samples (~10 ng total RNA each) were reverse transcribed into first-strand cDNA using SuperScript III RNase H- reverse transcriptase and oligo dT primers (SuperScript III First-Strand Synthesis SuperMix, Invitrogen).

Reverse transcription-PCR

Reverse transcription PCR (RT-PCR) was used to assay maternal gene expression at multiple stages of early development in *Devario aequipinnatus* embryos. Samples of first-strand cDNA synthesized from total RNA were used as templates for RT-PCR. Reaction mixes containing cDNA template, 150nM each primer, and EconoTaq DNA Polymerase ran with the following thermocycler parameters: 55°C for 5min; 95°C for 2min; 40 cycles of 95°C for 15s, 55°C for 15s, and 72°C for 30s. Results were visualized by running the RT-PCR products on 1.7% agarose gels via gel electrophoresis.

Primers used for *Devario aequipinnatus* RT-PCR:

ca15b_F: CATGGCCCATCATTGCAGAA

ca15b_R: GAAATTAATACGACTCACTATAGGGCCAACGTATGTGAAAGAGGT

beta-actin_F: GCCCATCTATGAGGGTTACG

beta-actin_R: AGGAAGGAAGGCTGGAAGAG

Fluorescent *in situ* hybridization (FISH)

Antisense probes targeting germ plasm mRNAs were synthesized using linearized DNA purified from plasmids established for use in zebrafish or amplified from cDNA made from 4 cell stage *Danio rerio* embryos (except where noted). Template DNA for the probes targeting *askopos* (Blaser et al., 2005), *dazl* (Maegawa et al., 1999), *dnd* (Weidinger et al., 2003), *nanos3* (Köprunner et al., 2001), and *vasa* (Yoon et al., 1997) was linearized from plasmids as previously described. DNA templates for *dnd*, *ca15b*, *celf1*, *gran*, *hook2*, *rgs14a*, and *tdrd7a* were produced from 4-cell stage zebrafish cDNA and PCR amplified using a primer that included the T7 RNA polymerase promoter sequence, then transcribed into labeled RNA probes using T7 and hapten-conjugated uridine triphosphates (UTPs) in the reaction mixture. As noted in the text and in the table below, two additional *ca15b* probes were synthesized in this way, although using *Devario aequipinnatus* template DNA instead of zebrafish cDNA. The template for *miR-202-5p*, based off of the zebrafish sequence, was ordered as a DNA oligonucleotide with a T7 RNA polymerase promoter sequence from IDT (GAAATTAATACGACTCACTATAGGTTCCATGCATATACCTCTTTG) and transcribed into antisense labeled RNA probes using T7 and hapten-conjugated UTPs. Primers used for *in situ* probe synthesis listed in Table 2.

Fixed embryos were permeabilized overnight with 100% methanol at –20°C prior to treatment with prehybridization buffer for at least 4 hours at 69°C. Embryos were allowed to hybridize overnight at 69°C with RNA probes labeled with either fluorescein- (FLU-), digoxigenin- (DIG-), or dinitrophenol- (DNP-) conjugated UTPs. FLU, DIG, and DNP haptens were detected and fluorescent signal was developed using the TSA Plus Fluorescein/Cy3/Cy5 system (PerkinElmer) following a modified "Triple fluorescent in situ" protocol (Hansen & Pelegri, 2021a). Following completion of the FISH procedure, embryos were stained to detect DNA with 1:200 dilution of 100 ug/ml DAPI in PBS for 10 minutes followed by PBS washes. All embryos were deyolked after labeling and mounted flat (blastodiscs facing up) in 50% glycerol:PBS on slides for imaging.

Combined FISH and immunofluorescence for simultaneous RNA and protein visualization

For combined fluorescent *in situ* hybridization (FISH) and protein immunofluorescence, embryos were collected, staged at the selected developmental time points, and fixed as described above. FISH was performed first with a fluorescein-labeled antisense *nanos3* probe. At the end of the FISH protocol and prior to embryo deyolking and DAPI labeling, the embryos were washed in PBS-Tween (Tween 0.1%) 3 × 10 minutes and then incubated at room temperature for at least 2 hours in casein blocking reagent. Embryos were then incubated with primary antibody solution (rabbit anti-Vasa, 1:200, GeneTex, and mouse anti-β-catenin, 1:1,000, in blocking reagent) overnight at 4°C, washed with PBS-Tween

the following day and incubated with secondary antibodies overnight at 4°C. After PBS-Tween washes, the embryos were deyolked, DAPI-labeled to detect DNA (1:200 dilution of 100ug/ml DAPI in PBS for 10 minutes followed by PBS washes), and mounted flat as described above.

Microscopy and Image Analysis

Embryos were imaged using a Zeiss LSM710 or LSM780 confocal microscope and analyzed using ImageJ (Schneider et al., 2012). Unless otherwise indicated in figure legends, standard image processing in ImageJ included maximum 2D Z-projection of multiple slices from 3D stacks, signal smoothing, and adjustments to brightness and contrast (applied across entire image) as appropriate.

Phylogenetic and sequence analysis

Devario aequipinnatus raw sequence data was downloaded from NCBI Sequence Read Archive (acc. ERR3332370 and ERR3332374; Vertebrate Genomes Project). Quality was assessed with FastQC (Andrews, 2010) and reads were trimmed with fastp (Chen et al., 2018). Trimmed reads files were concatenated and gene assemblies were performed with HybPiper (M. G. Johnson et al., 2016; Tange, 2011) and SPAdes v3.14.1 (Prjibelski et al., 2020) using the zebrafish Ca15b protein sequence (NCBI RefSeq acc. XP_005169499.1) as bait. Coding regions were annotated and spliced using Exonerate (Slater & Birney, 2005).

The *ca15b* gene tree was built using the Maximum Likelihood method and Tamura-Nei model (Tamura & Nei, 1993). The tree with the highest log likelihood (−16318.87) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 8 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 2960 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018; Stecher et al., 2020).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

References

Aanes H, Winata CL, Lin CH, Chen JP, Srinivasan KG, Lee SGP, Lim AYM, Hajan HS, Collas P, Bourque G, Gong Z, Korzh V, Aleström P, & Mathavan S (2011). Zebrafish mRNA sequencing deciphers novelties in transcriptome dynamics during maternal to zygotic transition. Genome Research, 21(8), 1328–1338. 10.1101/gr.116012.110 [PubMed: 21555364]

Andrews S (2010). FastQC: A Quality Control Tool for High Throughput Sequence Data [Online]. http//:www.bioinformatics.babraham.ac.uk/projects/fastqc/.

Berekelya LA, Ponomarev MB, Luchinskaya NN, & Belyavsky AV (2003). Xenopus Germes encodes a novel germ plasm-associated transcript. Gene Expression Patterns: GEP, 3(4), 521–524. 10.1016/s1567-133x(03)00055-3 [PubMed: 12915321]

Bertocchini F, & Chuva de Sousa Lopes SM (2016). Germline development in amniotes: A paradigm shift in primordial germ cell specification. Bioessays, 38(8), 791–800. 10.1002/bies.201600025 [PubMed: 27273724]

Blaser H, Eisenbeiss S, Neumann M, Reichman-Fried M, Thisse B, Thisse C, & Raz E (2005). Transition from non-motile behaviour to directed migration during early PGC development in zebrafish. Journal of Cell Science, 118(Pt 17), 4027–4038. 10.1242/jcs.02522 [PubMed: 16129886]

Bontems F, Stein A, Marlow F, Lyautey J, Gupta T, Mullins MC, & Dosch R (2009). Bucky ball organizes germ plasm assembly in zebrafish. Current Biology: CB, 19(5), 414–422. 10.1016/j.cub.2009.01.038 [PubMed: 19249209]

Boone CD, Gill S, Habibzadegan A, & McKenna R (2013). Carbonic Anhydrase: An Efficient Enzyme with Possible Global Implications. International Journal of Chemical Engineering, 2013, e813931. 10.1155/2013/813931

Braat AK, Zandbergen T, Water SVD, Goos HJT, & Zivkovic D (1999). Characterization of zebrafish primordial germ cells: Morphology and early distribution of vasa RNA. Developmental Dynamics, 216(2), 153–167. 10.1002/(SICI)1097-0177(199910)216:2<153::AID-DVDY6>3.0.CO;2-1 [PubMed: 10536055]

Brand M, Granato M, & Nüsslein-Volhard C (2002). Keeping and raising zebrafish. Zebrafish (2002).

Butler AM, Owens DA, Wang L, & King ML (2018). A novel role for sox7 in Xenopus early primordial germ cell development: Mining the PGC transcriptome. Development, 145(dev155978). 10.1242/dev.155978 [PubMed: 29158442]

Chen S, Zhou Y, Chen Y, & Gu J (2018). fastp: An ultra-fast all-in-one FASTQ preprocessor. Bioinformatics (Oxford, England), 34(17), i884–i890. 10.1093/bioinformatics/bty560

De Bie T, Cristianini N, Demuth JP, & Hahn MW (2006). CAFE: A computational tool for the study of gene family evolution. Bioinformatics, 22(10), 1269–1271. 10.1093/bioinformatics/btl097 [PubMed: 16543274]

D'Orazio FM, Balwierz PJ, González AJ, Guo Y, Hernández-Rodríguez B, Wheatley L, Jasiulewicz A, Hadzhiev Y, Vaquerizas JM, Cairns B, Lenhard B, & Müller F (2021). Germ cell differentiation requires Tdrd7-dependent chromatin and transcriptome reprogramming marked by germ plasm relocalization. Developmental Cell. 10.1016/j.devcel.2021.02.007

Eddy EM (1975). Germ plasm and the differentiation of the germ cell line. International Review of Cytology, 43, 229–280. 10.1016/s0074-7696(08)60070-4 [PubMed: 770367]

Eno C, Gomez T, Slusarski DC, & Pelegri F (2018). Slow calcium waves mediate furrow microtubule reorganization and germ plasm compaction in the early zebrafish embryo. Development (Cambridge, England), 145(10). 10.1242/dev.156604

Eno C, Hansen CL, & Pelegri F (2019). Aggregation, segregation, and dispersal of homotypic germ plasm RNPs in the early zebrafish embryo. Developmental Dynamics: An Official Publication of the American Association of Anatomists, 248(4), 306–318. 10.1002/dvdy.18 [PubMed: 30741457]

Extavour CG, & Akam M (2003). Mechanisms of germ cell specification across the metazoans: Epigenesis and preformation. Development (Cambridge, England), 130(24), 5869–5884. 10.1242/dev.00804

Ferreira-Martins D, McCormick SD, Campos A, Lopes-Marques M, Osório H, Coimbra J, Castro LFC, & Wilson JM (2016). A cytosolic carbonic anhydrase molecular switch occurs in the

gills of metamorphic sea lamprey. Scientific Reports, 6(1), 33954. 10.1038/srep33954 [PubMed: 27703170]

Frost SC (2014). Physiological Functions of the Alpha Class of Carbonic Anhydrases. In Frost SC & McKenna R (Eds.), Carbonic Anhydrase: Mechanism, Regulation, Links to Disease, and Industrial Applications (pp. 9–30). Springer Netherlands. 10.1007/978-94-007-7359-2_2

Gilmour KM, & Perry SF (2009). Carbonic anhydrase and acid–base regulation in fish. Journal of Experimental Biology, 212(11), 1647–1661. 10.1242/jeb.029181

Hansen CL, & Pelegri F (2021a). Methods for Visualization of RNA and Cytoskeletal Elements in the Early Zebrafish Embryo. Methods in Molecular Biology (Clifton, N.J.), 2218, 219–244. 10.1007/978-1-0716-0970-5_18

Hansen CL, & Pelegri F (2021b). Primordial germ cell specification in vertebrate embryos: Phylogenetic distribution and conserved molecular features of preformation and induction. Frontiers in Cell and Developmental Biology. 10.3389/fcell.2021.730332

Hartwig J, Tarbashevich K, Seggewiß J, Stehling M, Bandemer J, Grimaldi C, Paksa A, Groß-Thebing T, Meyen D, & Raz E (2014). Temporal control over the initiation of cell motility by a regulator of G-protein signaling. Proceedings of the National Academy of Sciences, 111(31), 11389–11394. 10.1073/pnas.1400043111

Harvey SA, Sealy I, Kettleborough R, Fenyes F, White R, Stemple D, & Smith JC (2013). Identification of the zebrafish maternal and paternal transcriptomes. Development (Cambridge, England), 140(13), 2703–2710. 10.1242/dev.095091

Hashimoto Y, Maegawa S, Nagai T, Yamaha E, Suzuki H, Yasuda K, & Inoue K (2004). Localized maternal factors are required for zebrafish germ cell formation. Developmental Biology, 268(1), 152–161. 10.1016/j.ydbio.2003.12.013 [PubMed: 15031112]

He Z, Wu L, Li X, Fields MW, & Zhou J (2005). Empirical Establishment of Oligonucleotide Probe Design Criteria. Applied and Environmental Microbiology, 71(7), 3753–3760. 10.1128/AEM.71.7.3753-3760.2005 [PubMed: 16000786]

Henry RP (1996). Multiple Roles of Carbonic Anhydrase in Cellular Transport and Metabolism. Annual Review of Physiology, 58(1), 523–538. 10.1146/annurev.ph.58.030196.002515

Herpin A, Rohr S, Riedel D, Kluever N, Raz E, & Schartl M (2007). Specification of primordial germ cells in medaka (Oryzias latipes). BMC Developmental Biology, 7(1), 3. 10.1186/1471-213X-7-3 [PubMed: 17217535]

Hewett-Emmett D, & Tashian RE (1996). Functional diversity, conservation, and convergence in the evolution of the alpha-, beta-, and gamma-carbonic anhydrase gene families. Molecular Phylogenetics and Evolution, 5(1), 50–77. 10.1006/mpev.1996.0006 [PubMed: 8673298]

Heyn P, Kircher M, Dahl A, Kelso J, Tomancak P, Kalinka AT, & Neugebauer KM (2014). The earliest transcribed zygotic genes are short, newly evolved, and different across species. Cell Reports, 6(2), 285–292. 10.1016/j.celrep.2013.12.030 [PubMed: 24440719]

Horvay K, Claußen M, Katzer M, Landgrebe J, & Pieler T (2006). Xenopus Dead End mRNA Is a Localized Maternal Determinant that Serves a Conserved Function in Germ Cell Development. Developmental Biology, 291, 1–11. 10.1016/j.ydbio.2005.06.013 [PubMed: 16448642]

Houston DW, & King ML (2000). A critical role for Xdazl, a germ plasm-localized RNA, in the differentiation of primordial germ cells in Xenopus. Development (Cambridge, England), 127(3), 447–456.

Houston DW, Zhang J, Maines JZ, Wasserman SA, & King ML (1998). A Xenopus DAZ-like gene encodes an RNA component of germ plasm and is a functional homologue of Drosophila boule. Development (Cambridge, England), 125(2), 171–180.

Houwing S, Kamminga LM, Berezikov E, Cronembold D, Girard A, van den Elst H, Filippov DV, Blaser H, Raz E, Moens CB, Plasterk RHA, Hannon GJ, Draper BW, & Ketting RF (2007). A role for Piwi and piRNAs in germ cell maintenance and transposon silencing in Zebrafish. Cell, 129(1), 69–82. 10.1016/j.cell.2007.03.026 [PubMed: 17418787]

Howe KL, Achuthan P, Allen J, Allen J, Alvarez-Jarreta J, Amode MR, Armean IM, Azov AG, Bennett R, Bhai J, Billis K, Boddu S, Charkhchi M, Cummins C, Da Rin Fioretto L, Davidson C, Dodiya K, El Houdaigui B, Fatima R, … Flicek P (2021). Ensembl 2021. Nucleic Acids Research, 49(D1), D884–D891. 10.1093/nar/gkaa942 [PubMed: 33137190]

Hudson C, & Woodland HR (1998). Xpat, a gene expressed specifically in germ plasm and primordial germ cells of Xenopus laevis. Mechanisms of Development, 73(2), 159–168. 10.1016/s0925-4773(98)00047-1 [PubMed: 9622619]

Ikenishi K (1998). Germ plasm in Caenorhabditis elegans, Drosophila and Xenopus. Development, Growth & Differentiation, 40(1), 1–10. 10.1046/j.1440-169X.1998.t01-4-00001.x

Irion U, & Nüsslein-Volhard C (2019). The identification of genes involved in the evolution of color patterns in fish. Current Opinion in Genetics & Development, 57, 31–38. 10.1016/j.gde.2019.07.002 [PubMed: 31421397]

Johnson AD, Richardson E, Bachvarova RF, & Crother BI (2011). Evolution of the germ line-soma relationship in vertebrate embryos. Reproduction (Cambridge, England), 141(3), 291–300. 10.1530/REP-10-0474

Johnson MG, Gardner EM, Liu Y, Medina R, Goffinet B, Shaw AJ, Zerega NJC, & Wickett NJ (2016). HybPiper: Extracting coding sequence and introns for phylogenetics from high-throughput sequencing reads using target enrichment1. Applications in Plant Sciences, 4(7). 10.3732/apps.1600016

Kane DA, & Kimmel CB (1993). The zebrafish midblastula transition. Development (Cambridge, England), 119(2), 447–456.

Karimi K, Fortriede JD, Lotay VS, Burns KA, Wang DZ, Fisher ME, Pells TJ, James-Zorn C, Wang Y, Ponferrada VG, Chu S, Chaturvedi P, Zorn AM, & Vize PD (2018). Xenbase: A genomic, epigenomic and transcriptomic model organism database. Nucleic Acids Research, 46(D1), D861–D868. 10.1093/nar/gkx936 [PubMed: 29059324]

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, & Schilling TF (1995). Stages of embryonic development of the zebrafish. Developmental Dynamics: An Official Publication of the American Association of Anatomists, 203(3), 253–310. 10.1002/aja.1002030302 [PubMed: 8589427]

Knaut H, Pelegri F, Bohmann K, Schwarz H, & Nüsslein-Volhard C (2000). Zebrafish vasa RNA but Not Its Protein Is a Component of the Germ Plasm and Segregates Asymmetrically before Germline Specification. The Journal of Cell Biology, 149(4), 875–888. 10.1083/jcb.149.4.875 [PubMed: 10811828]

Köprunner M, Thisse C, Thisse B, & Raz E (2001). A zebrafish nanos-related gene is essential for the development of primordial germ cells. Genes & Development, 15(21), 2877–2885. 10.1101/gad.212401 [PubMed: 11691838]

Kumar S, Stecher G, Li M, Knyaz C, & Tamura K (2018). MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Molecular Biology and Evolution, 35(6), 1547–1549. 10.1093/molbev/msy096 [PubMed: 29722887]

Lawson KA, Dunn NR, Roelen BA, Zeinstra LM, Davis AM, Wright CV, Korving JP, & Hogan BL (1999). Bmp4 is required for the generation of primordial germ cells in the mouse embryo. Genes & Development, 13(4), 424–436. 10.1101/gad.13.4.424 [PubMed: 10049358]

Lee MT, Bonneau AR, & Giraldez AJ (2014). Zygotic genome activation during the maternal-to-zygotic transition. Annual Review of Cell and Developmental Biology, 30, 581–613. 10.1146/annurev-cellbio-100913-013027

Leinonen R, Sugawara H, & Shumway M (2011). The Sequence Read Archive. Nucleic Acids Research, 39(Database issue), D19–D21. 10.1093/nar/gkq1019 [PubMed: 21062823]

Lin F, Xu S, Ma D, Xiao Z, Zhao C, Xiao Y, Chi L, Liu Q, & Li J (2012). Germ line specific expression of a vasa homologue gene in turbot (Scophthalmus maximus): Evidence for vasa localization at cleavage furrows in euteleostei. Molecular Reproduction and Development, 79(11), 803–813. 10.1002/mrd.22120 [PubMed: 23124920]

MacArthur H, Bubunenko M, Houston DW, & King ML (1999). Xcat2 RNA is a translationally sequestered germ plasm component in Xenopus. Mechanisms of Development, 84(1–2), 75–88. 10.1016/s0925-4773(99)00075-1 [PubMed: 10473122]

MacArthur H, Houston DW, Bubunenko M, Mosquera L, & King ML (2000). DEADSouth is a germ plasm specific DEAD-box RNA helicase in Xenopus related to eIF4A. Mechanisms of Development, 95(1–2), 291–295. 10.1016/s0925-4773(00)00357-9 [PubMed: 10906480]

Madeira F, Park Y. mi, Lee J, Buso N, Gur T, Madhusoodanan N, Basutkar P, Tivey ARN, Potter SC, Finn RD, & Lopez R (2019). The EMBL-EBI search and sequence analysis tools APIs in 2019. Nucleic Acids Research, 47(W1), W636–W641. 10.1093/nar/gkz268 [PubMed: 30976793]

Maegawa S, Yasuda K, & Inoue K (1999). Maternal mRNA localization of zebrafish DAZ-like gene. Mechanisms of Development, 81(1–2), 223–226. 10.1016/s0925-4773(98)00242-1 [PubMed: 10330505]

Magnúsdóttir E, & Surani MA (2014). How to make a primordial germ cell. Development (Cambridge, England), 141(2), 245–252. 10.1242/dev.098269

Martin C, Pedersen SF, Schwab A, & Stock C (2010). Intracellular pH gradients in migrating cells. American Journal of Physiology-Cell Physiology, 300(3), C490–C495. 10.1152/ajpcell.00280.2010 [PubMed: 21148407]

Mathavan S, Lee SGP, Mak A, Miller LD, Murthy KRK, Govindarajan KR, Tong Y, Wu YL, Lam SH, Yang H, Ruan Y, Korzh V, Gong Z, Liu ET, & Lufkin T (2005). Transcriptome analysis of zebrafish embryogenesis using microarrays. PLoS Genetics, 1(2), 260–276. 10.1371/journal.pgen.0010029 [PubMed: 16132083]

McCluskey BM, & Braasch I (2020). Chapter 2—Zebrafish Phylogeny and Taxonomy. In Cartner SC, Eisen JS, Farmer SC, Guillemin KJ, Kent ML, & Sanders GE (Eds.), The Zebrafish in Biomedical Research (pp. 15–24). Academic Press. 10.1016/B978-0-12-812431-4.00002-6

McCluskey BM, & Postlethwait JH (2015a). Phylogeny of Zebrafish, a "Model Species," within Danio, a "Model Genus." Molecular Biology and Evolution, 32(3), 635–652. 10.1093/molbev/msu325 [PubMed: 25415969]

McCluskey BM, & Postlethwait JH (2015b). Phylogeny of zebrafish, a "model species," within Danio, a "model genus." Molecular Biology and Evolution, 32(3), 635–652. 10.1093/molbev/msu325 [PubMed: 25415969]

Miranda-Rodríguez JR, Salas-Vidal E, Lomelí H, Zurita M, & Schnabel D (2017). RhoA/ROCK pathway activity is essential for the correct localization of the germ plasm mRNAs in zebrafish embryos. Developmental Biology, 421(1), 27–42. 10.1016/j.ydbio.2016.11.002 [PubMed: 27836552]

Mishima Y, Giraldez AJ, Takeda Y, Fujiwara T, Sakamoto H, Schier AF, & Inoue K (2006). Differential regulation of germline mRNAs in soma and germ cells by zebrafish miR-430. Current Biology: CB, 16(21), 2135–2142. 10.1016/j.cub.2006.08.086 [PubMed: 17084698]

Moravec CE, & Pelegri F (2020). The role of the cytoskeleton in germ plasm aggregation and compaction in the zebrafish embryo. Current Topics in Developmental Biology, 140, 145–179. 10.1016/bs.ctdb.2020.02.001 [PubMed: 32591073]

Nagasawa K, Fernandes JMO, Yoshizaki G, Miwa M, & Babiak I (2013). Identification and migration of primordial germ cells in Atlantic salmon, Salmo salar: Characterization of Vasa, Dead End, and Lymphocyte antigen 75 genes. Molecular Reproduction and Development, 80(2), 118–131. 10.1002/mrd.22142 [PubMed: 23239145]

Owens DA, Butler AM, Aguero TH, Newman KM, Van Booven D, & King ML (2017). High-throughput analysis reveals novel maternal germline RNAs crucial for primordial germ cell preservation and proper migration. Development (Cambridge, England), 144(2), 292–304. 10.1242/dev.139220

Pelegri F, Knaut H, Maischein HM, Schulte-Merker S, & Nüsslein-Volhard C (1999). A mutation in the zebrafish maternal-effect gene nebel affects furrow formation and vasa RNA localization. Current Biology: CB, 9(24), 1431–1440. 10.1016/s0960-9822(00)80112-8 [PubMed: 10607587]

Presslauer C, Nagasawa K, Fernandes JMO, & Babiak I (2012). Expression of vasa and nanos3 during primordial germ cell formation and migration in Atlantic cod (Gadus morhua L.). Theriogenology, 78(6), 1262–1277. 10.1016/j.theriogenology.2012.05.022 [PubMed: 22898013]

Prjibelski A, Antipov D, Meleshko D, Lapidus A, & Korobeynikov A (2020). Using SPAdes De Novo Assembler. Current Protocols in Bioinformatics, 70(1), e102. 10.1002/cpbi.102 [PubMed: 32559359]

Rhie A, McCarthy SA, Fedrigo O, Damas J, Formenti G, Koren S, Uliano-Silva M, Chow W, Fungtammasan A, Kim J, Lee C, Ko BJ, Chaisson M, Gedman GL, Cantin LJ, Thibaud-Nissen F, Haggerty L, Bista I, Smith M, … Jarvis ED (2021). Towards complete and error-free genome

assemblies of all vertebrate species. Nature, 592(7856), 737–746. 10.1038/s41586-021-03451-0 [PubMed: 33911273]

Richardson BE, & Lehmann R (2010). Mechanisms guiding primordial germ cell migration: Strategies from different organisms. Nature Reviews. Molecular Cell Biology, 11(1), 37–49. 10.1038/nrm2815 [PubMed: 20027186]

Roovers EF, Kaaij LJT, Redl S, Bronkhorst AW, Wiebrands K, de Jesus Domingues AM, Huang H-Y, Han C-T, Riemer S, Dosch R, Salvenmoser W, Grün D, Butter F, van Oudenaarden A, & Ketting RF (2018). Tdrd6a Regulates the Aggregation of Buc into Functional Subcellular Compartments that Drive Germ Cell Specification. Developmental Cell, 46(3), 285–301.e9. 10.1016/j.devcel.2018.07.009 [PubMed: 30086300]

Rüber L, Kottelat M, Tan HH, Ng PKL, & Britz R (2007). Evolution of miniaturization and the phylogenetic position of Paedocypris, comprising the world's smallest vertebrate. BMC Evolutionary Biology, 7, 38. 10.1186/1471-2148-7-38 [PubMed: 17355618]

Schneider CA, Rasband WS, & Eliceiri KW (2012). NIH Image to ImageJ: 25 years of image analysis. Nature Methods, 9(7), 671–675. 10.1038/nmeth.2089 [PubMed: 22930834]

Shinomiya A, Tanaka M, Kobayashi T, Nagahama Y, & Hamaguchi S (2000). The vasa-like gene, olvas, identifies the migration path of primordial germ cells during embryonic body formation stage in the medaka, Oryzias latipes. Development, Growth & Differentiation, 42(4), 317–326. 10.1046/j.1440-169x.2000.00521.x

Silverman DN, & Lindskog S (1988). The catalytic mechanism of carbonic anhydrase: Implications of a rate-limiting protolysis of water. Accounts of Chemical Research, 21(1), 30–36. 10.1021/ar00145a005

Slater GSC, & Birney E (2005). Automated generation of heuristics for biological sequence comparison. BMC Bioinformatics, 6, 31. 10.1186/1471-2105-6-31 [PubMed: 15713233]

Stecher G, Tamura K, & Kumar S (2020). Molecular Evolutionary Genetics Analysis (MEGA) for macOS. Molecular Biology and Evolution, 37(4), 1237–1239. 10.1093/molbev/msz312 [PubMed: 31904846]

Strasser MJ, Mackenzie NC, Dumstrei K, Nakkrasae L-I, Stebler J, & Raz E (2008). Control over the morphology and segregation of Zebrafish germ cell granules during embryonic development. BMC Developmental Biology, 8, 58. 10.1186/1471-213X-8-58 [PubMed: 18507824]

Strome S, & Updike D (2015). Specifying and protecting germ cell fate. Nature Reviews. Molecular Cell Biology, 16(7), 406–416. 10.1038/nrm4009 [PubMed: 26122616]

Subramaniam K, & Seydoux G (1999). Nos-1 and nos-2, two genes related to Drosophila nanos, regulate primordial germ cell development and survival in Caenorhabditis elegans. Development (Cambridge, England), 126(21), 4861–4871.

Swartz SZ, & Wessel GM (2015). Germ Line Versus Soma in the Transition from Egg to Embryo. Current Topics in Developmental Biology, 113, 149–190. 10.1016/bs.ctdb.2015.06.003 [PubMed: 26358873]

Tada H, Mochii M, Orii H, & Watanabe K (2012). Ectopic formation of primordial germ cells by transplantation of the germ plasm: Direct evidence for germ cell determinant in Xenopus. Developmental Biology, 371(1), 86–93. 10.1016/j.ydbio.2012.08.014 [PubMed: 23046626]

Tamura K, & Nei M (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution, 10(3), 512–526. 10.1093/oxfordjournals.molbev.a040023 [PubMed: 8336541]

Tange O (2011). Gnu parallel-the command-line power tool. The USENIX Magazine, 36(1), 42–47.

Tarbashevich K, Reichman-Fried M, Grimaldi C, & Raz E (2015). Chemokine-Dependent pH Elevation at the Cell Front Sustains Polarity in Directionally Migrating Zebrafish Germ Cells. Current Biology, 25(8), 1096–1103. 10.1016/j.cub.2015.02.071 [PubMed: 25843033]

Tashian RE (1989). The carbonic anhydrases: Widening perspectives on their evolution, expression and function. BioEssays: News and Reviews in Molecular, Cellular and Developmental Biology, 10(6), 186–192. 10.1002/bies.950100603

Vallée M, Robert C, Méthot S, Palin M-F, & Sirard M-A (2006). Cross-species hybridizations on a multi-species cDNA microarray to identify evolutionarily conserved genes expressed in oocytes. BMC Genomics, 7, 113. 10.1186/1471-2164-7-113 [PubMed: 16686947]

Wang H, Teng Y, Xie Y, Wang B, Leng Y, Shu H, & Deng F (2013). Characterization of the carbonic anhydrases 15b expressed in PGCs during early zebrafish development. Theriogenology, 79(3), 443–452. 10.1016/j.theriogenology.2012.10.016 [PubMed: 23174774]

Weidinger G, Stebler J, Slanchev K, Dumstrei K, Wise C, Lovell-Badge R, Thisse C, Thisse B, & Raz E (2003). Dead end, a novel vertebrate germ plasm component, is required for zebrafish primordial germ cell migration and survival. Current Biology: CB, 13(16), 1429–1434. 10.1016/s0960-9822(03)00537-2 [PubMed: 12932328]

Wheeler GN, & Brändli AW (2009). Simple vertebrate models for chemical genetics and drug discovery screens: Lessons from zebrafish and Xenopus. Developmental Dynamics: An Official Publication of the American Association of Anatomists, 238(6), 1287–1308. 10.1002/dvdy.21967 [PubMed: 19441060]

Wylie C (1999). Germ cells. Cell, 96(2), 165–174. 10.1016/s0092-8674(00)80557-7 [PubMed: 9988212]

Ying Y, Qi X, & Zhao GQ (2001). Induction of primordial germ cells from murine epiblasts by synergistic action of BMP4 and BMP8B signaling pathways. Proceedings of the National Academy of Sciences of the United States of America, 98(14), 7858–7862. 10.1073/pnas.151242798 [PubMed: 11427739]

Yoon C, Kawakami K, & Hopkins N (1997). Zebrafish vasa homologue RNA is localized to the cleavage planes of 2- and 4-cell-stage embryos and is expressed in the primordial germ cells. Development (Cambridge, England), 124(16), 3157–3165.

Zhang J, Liu W, Jin Y, Jia P, Jia K, & Yi M (2017). MiR-202-5p is a novel germ plasm-specific microRNA in zebrafish. Scientific Reports, 7(1), 7055. 10.1038/s41598-017-07675-x [PubMed: 28765643]

Zhou L, Wang X, Du S, Wang Y, Zhao H, Du T, Yu J, Wu L, Song Z, Liu Q, & Li J (2020). Germline Specific Expression of a vasa Homologue Gene in the Viviparous Fish Black Rockfish (Sebastes schlegelii) and Functional Analysis of the vasa 3′ Untranslated Region. Frontiers in Cell and Developmental Biology, 8. 10.3389/fcell.2020.575788
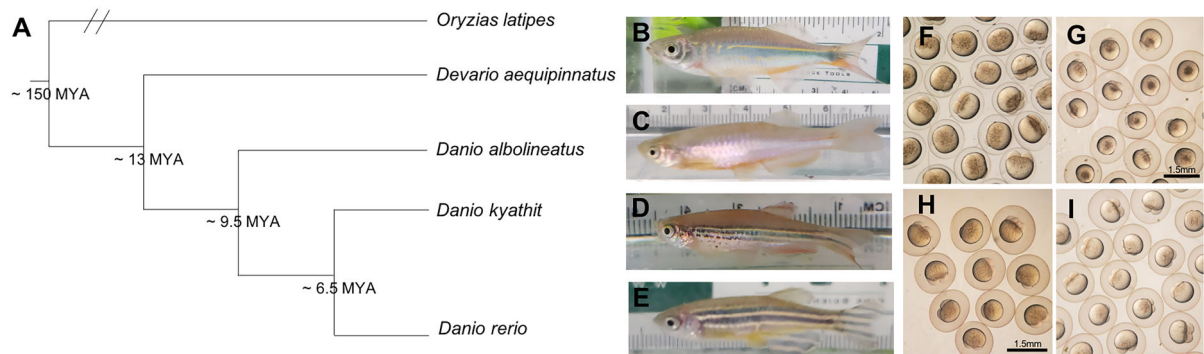
Figure 1.

Phylogeny and morphology of four Danionin species. A) Relationship between *Danio* and *Devario* species used in this study, with the Japanese rice fish, medaka (*Oryzias latipes*), as an outgroup. Simplified and redrawn from: (McCluskey & Postlethwait, 2015a). Branch lengths are arbitrary. See Supplementary Figure 1 for relationship to other lineages and expanded Danionin phylogeny. B-E) Representative adult male fish from each species in our laboratory colonies used in this study, including B) *Devario aequipinnatus* (giant danio), C) *Danio albolineatus* (pearl danio), D) *Danio kyathit* (orange-finned danio), and E) *Danio rerio* (zebrafish). F-I: Live cleavage stage embryos from each of the four species of interest, including F) *Devario aequipinnatus*, G) *Danio albolineatus*, H) *Danio kyathit*, and I) *Danio rerio*.
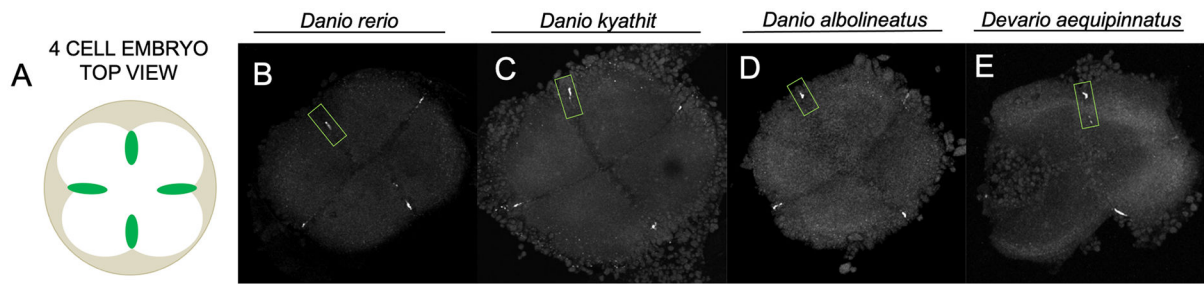
Figure 2.
Identification of furrow-localized germ plasm marker *nanos3* in multiple Danionin species. Diagram (A) and micrographs (B-E) visualizing top view (blastodisc) of 4-cell Danionin fish embryos with *nanos3* mRNA labeled via fluorescence *in situ* hybridization. The general appearance and localization pattern of the germ plasm marker *nanos3* at the distal end of the first two cleavage furrows is consistent across all four species, including *Danio rerio* (B), *Danio kyathit* (C), *Danio albolineatus* (D), and *Devario aequipinnatus* (E). A *nanos3* germ plasm aggregate in each embryo is highlighted by a green box outline. Micrographs are 2D maximum projections of confocal Z-stacks.
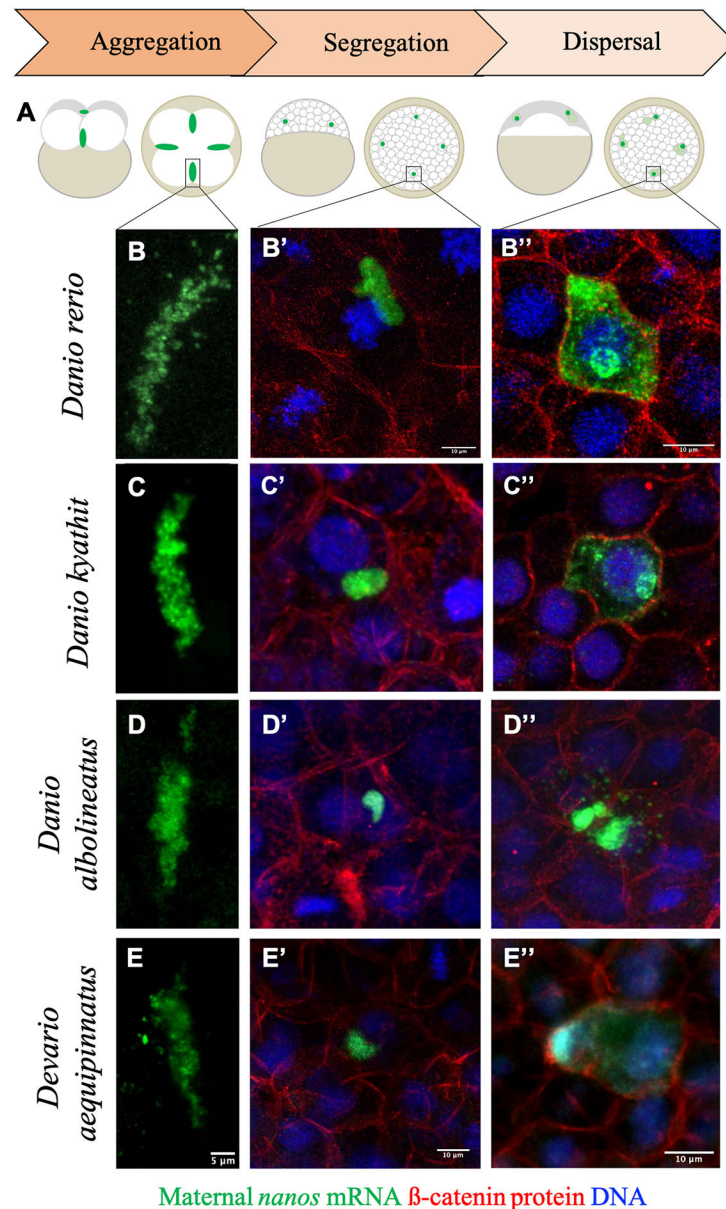
Figure 3.
Key stages of germ plasm localization dynamics across Danionin species. A) Diagram depicting an overview of germ plasm localization during early embryogenesis in zebrafish. Germ plasm ribonucleoparticles (green) in zebrafish form extended aggregates at the distal end of the first two embryonic cleavage furrows (1 hpf; aggregation; left), which become cellularized and segregate asymmetrically during cell divisions throughout the late cleavage through mid-blastula period of development (~1.5 hpf – 4 hpf; asymmetric segregation; middle), before dispersing to fill the cytoplasm of PGCs beginning at the dome stage through early gastrulation (~4.3 hpf – 6 hpf; dispersal; right). B-B") Micrographs of germ plasm localization and morphology in zebrafish (*Danio rerio*) embryos during each of the highlighted stages, represented by the germ plasm marker *nanos* RNA (green) visualized via fluorescence *in situ* hybridization and confocal microscopy. B' and B" also depict cell

boundaries with beta catenin immunolabeling (red) and nuclei with DAPI stain (blue). The general appearance and localization patterns of germ plasm at these stages are consistent across species, including *Danio kyathit* (C-C"), *Danio albolineatus* (D-D"), and *Devario aequipinnatus* (E-E").
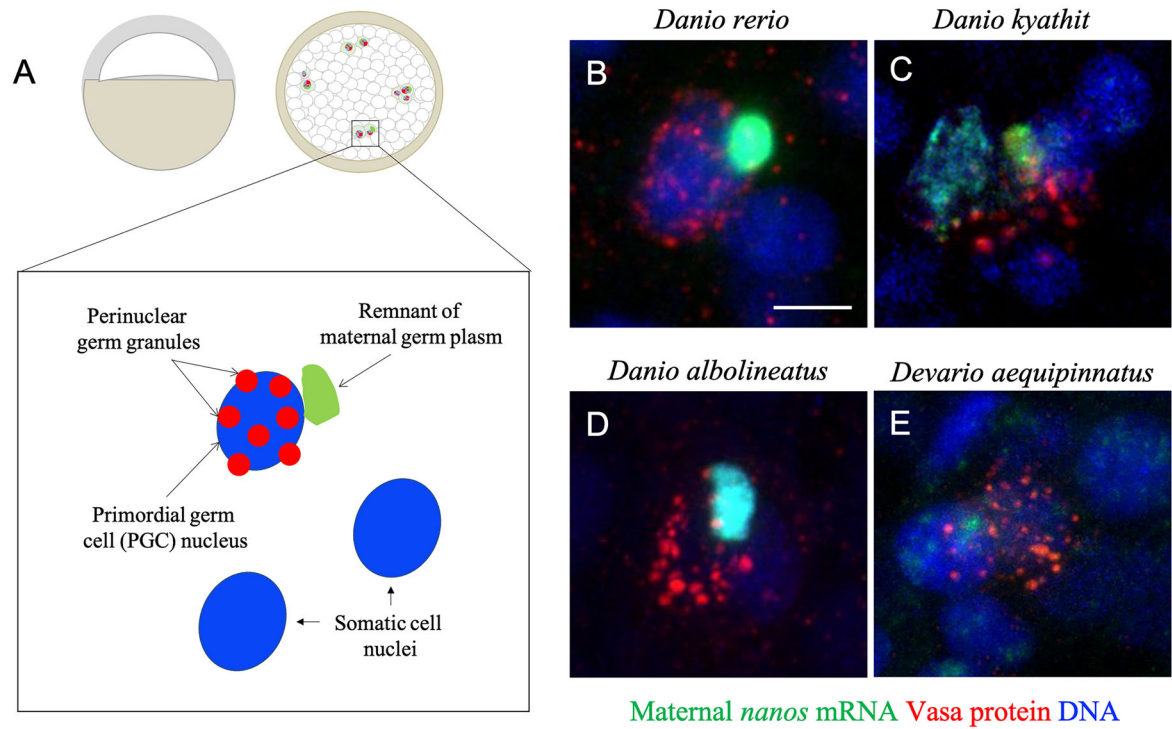
Figure 4.
Co-occurrence of maternally inherited germ plasm RNA and germline protein granules.
Diagram (A) and micrographs (B-E) of germ plasm RNA and perinuclear Vasa granules
during epiboly in four Danionin species. Remnants of maternally inherited germ plasm RNA
(*nanos* RNA: green), and newly formed perinuclear germ granules (Vasa protein: red; nuclei:
blue) co-exist in primordial germ cells during early epiboly/gastrulation in (B) zebrafish, (C)
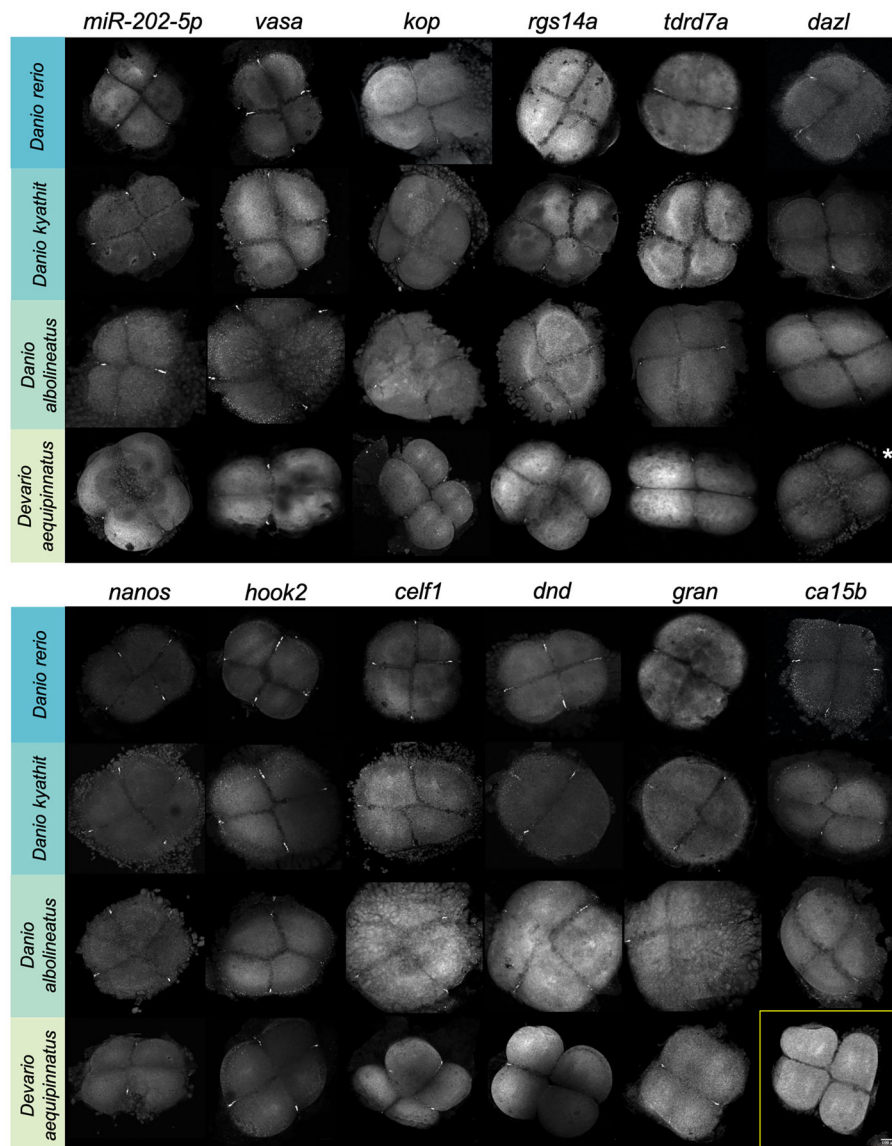*Danio kyathit*, (D) *Danio albolineatus*, and (E) *Devario aequipinnatus* embryos. Scale bar:
10 μm.

Figure 5.
RNA localization at the distal ends of cleavage furrows in four cell stage *Danio rerio, D. kyathit, D. albolineatus*, and *Devario aequipinnatus* embryos as visualized by fluorescence *in situ* hybridization and confocal microscopy. All 12 known RNA components of zebrafish germ plasm are also germ plasm localized in *Danio kyathit* and *Danio albolineatus*, while all except *ca15b* are germ plasm localized in *Devario aequipinnatus* (yellow box; see also Figure 6). Furrow localization and/or labeling of *dazl* RNA in *Devario aequipinnatus* during the early cleavage stages was weak and difficult to visualize at 10x magnification (indicated by white asterisk) but could be observed at higher magnification (Sup. Fig. 3). All micrographs are 2D maximum projections of confocal stacks.
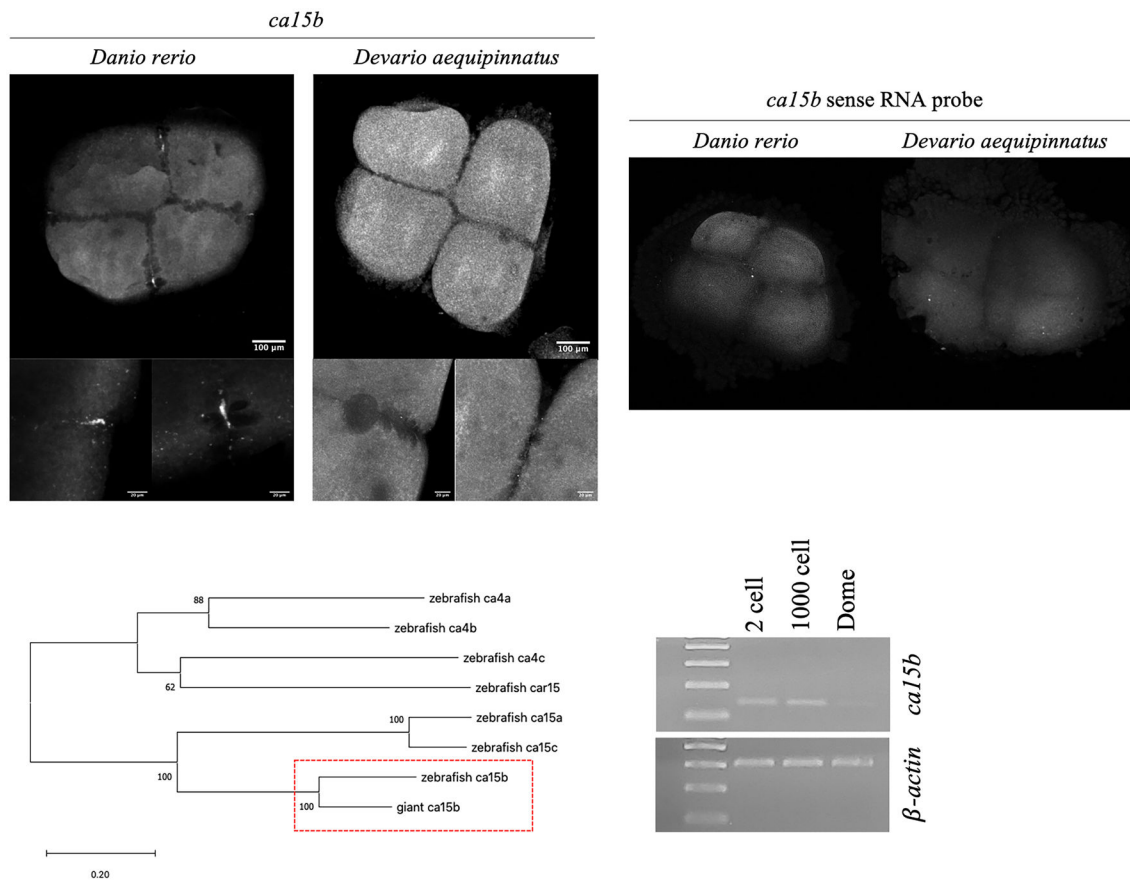
Figure 6.

ca15b RNA is maternally expressed but not germ plasm-localized in *Devario aequipinnatus* embryos. A) *ca15b* RNA localization in zebrafish (*Danio rerio*) and *Devario aequipinnatus* four-cell embryos as visualized by fluorescence *in situ* hybridization and confocal microscopy. In zebrafish, *ca15b* is specifically localized to the germ plasm aggregates at the distal ends of cleavage furrows. In contrast, *ca15b* does not localize to germ plasm in *Devario aequipinnatus* cleavage furrows, and instead appears to be distributed throughout the blastodisc. This is in contrast to the generalized background fluorescence exhibited when using a sense RNA probe for *ca15b* (B). All micrographs are maximum projections of three-dimensional stacks, with lower insets showing increased magnification of furrow regions. C) A gene tree depicting the evolutionary relationship between the putative *Devario aequipinnatus ca15b* sequence and all known members of the carbonic anhydrase 4/15 family in the zebrafish genome. The tree was built using the Maximum Likelihood method and Tamura-Nei model (Tamura & Nei, 1993) with evolutionary analysis conducted in MEGA X (Kumar et al., 2018; Stecher et al., 2020) (also see Methods). D) Expression of *ca15b* during the 2-cell (45 mpf), 1000 cell (3 hpf), and dome (4.3 hpf) stages of *Devario aequipinnatus* as assayed by reverse-transcriptase PCR. Transcripts of *ca15b* are maternally expressed, with positive signal at the 2-cell and 1000 cell stages and decreased signal at the dome stage. *Beta-actin* was used as a control.

Table 1.

RNA components of germ plasm in zebrafish

RNA	Product	Functions	References
ca15b	Carbonic anhydrase 15b	Elevates pH to maintain polarity of migrating PGCs	(Hartwig et al., 2014; Wang et al., 2013)
dnd1	DND microRNA-mediated repression inhibitor 1	Binds target mRNA to prevent miRNA-mediated repression; controls actomyosin contractility, cell cortex rigidity, & cell-cell adhesion for PGC migration; inhibits miR-430 and somatic differentiation	(Goudarzi et al., 2012; Gross-Thebing et al., 2017; Weidinger et al., 2003)
gra	Granulito	Unknown; no knockdown phenotype; predicted negative regulator of translation initiation	(Strasser et al., 2008)
hook2	Hook microtubule-tethering protein 2	Binds to & promotes dynein-dynactin assembly during mitosis; regulates microtubule nucleation at the centrosome	(Dwivedi et al., 2019; Roovers et al., 2018)
miR-202-5p	microRNA 202-5p	PGC migration; KO phenotype in Medaka = significantly decreased fertility, dysregulation of key ovarian genes, & impaired egg production	(Gay et al., 2018; Jin et al., 2020; Zhang et al., 2017)
nanos3	Nanos homolog 3	RNA-binding zinc finger protein; migration & survival of PGCs; maintenance of germline stem cells	(Beer & Draper, 2013; Köprunner et al., 2001)
kop	Askopos	unknown; no knockdown phenotype; potential member of S100 protein-binding protein family	(Blaser et al., 2005)
rgs14a	Regulator of G-protein signaling 14a	Controls onset of PGC migration, polar protrusion formation, & E-cadherin expression	(Hartwig et al., 2014)
tdrd7a	Tudor domain containing 7a	Impacts germ granule size; germ cell differentiation; predicted RNA binding & translation regulator; predicted necessary for spermatogenesis	(D'Orazio et al., 2021; Mishima et al., 2006)
vasa/ddx4	Dead box polypeptide 4	Likely ATP-dependent DEAD box helicase; germ cell differentiation & maintenance	(Hartung et al., 2014; Olsen et al., 1997; Yoon et al., 1997)
celf1/brul	Cugbp, Elav-like family member 1; bruno-like	RNA-binding protein; post-transcriptionally regulates genes necessary for embryonic patterning; necessary for organogenesis of endoderm-derived tissues	(Hashimoto et al., 2004; Suzuki et al., 2000)
dazl	Deleted in azoospermia-like	RNA-binding protein (GUUC); controls polyA tail length; predicted association with polysomes & translation promotion; germline stem cell specification	(Bertho et al., 2021; Hashimoto et al., 2004; Maegawa et al., 2002; Takeda et al., 2009)

Table 2.

Primers used for in situ probe synthesis with T7 RNAP promoter underlined

Probe target	Forward primer	Reverse primer	Source of template
*ca15b*_exon8-9	TGCAATGAAGGTGTGATCTGG	<u>GAAATTAATACGACTCACTATAGG</u>ATTGACTGGCTGAACGGATC	*D. rerio* cDNA (4 cell)
*ca15b*_exon8-9 *sense probe	<u>GAAATTAATACGACTCACTATAGG</u>TGCAATGAAGGTGTGATCTGG	TTGACTGGCTGAACGGATC	*D. rerio* cDNA (4 cell)
*ca15b*_exon4 (*Devario*)	CATGGCCCATCATTGCAGAA	<u>GAAATTAATACGACTCACTATAGG</u>GCCAACGTATGTGAAAGAGGT	*Devario* DNA (adult fin clip)
*ca15b*_exon8-9 (*Devario*)	TGCAATGAAGGTGTGATCTGG	<u>GAAATTAATACGACTCACTATAGG</u>ATTGACTGGCTGAACGGATC	*Devario* cDNA (2 cell)
celf1	GACCAGCCCGACATTGATTC	<u>GAAATTAATACGACTCACTATAGG</u>TCTGTGACTGGTGCATGGAT	*D. rerio* cDNA (4 cell)
granulito	GACCAATAGAAAAACCCGACAAC	<u>GAAATTAATACGACTCACTATAGG</u>TGCCATCTTAACTAGGAAATCCC	*D. rerio* cDNA (4 cell)
hook2	CGCAGACAAAACACACTCGA	<u>GAAATTAATACGACTCACTATAGG</u>TCTTTCTCCGACAGCTGGTT	*D. rerio* cDNA (4 cell)
rgs14a	ATCAGACAGCAAGACCAGC	<u>GAAATTAATACGACTCACTATAGG</u>GATCCAGACACCAACTCCAG	*D. rerio* cDNA (4 cell)
tdrd7a	AATCACCAGTGCCTGAGAAG	<u>GAAATTAATACGACTCACTATAGG</u>ATTCGACCCTGAACCAACTC	*D. rerio* cDNA (4 cell)