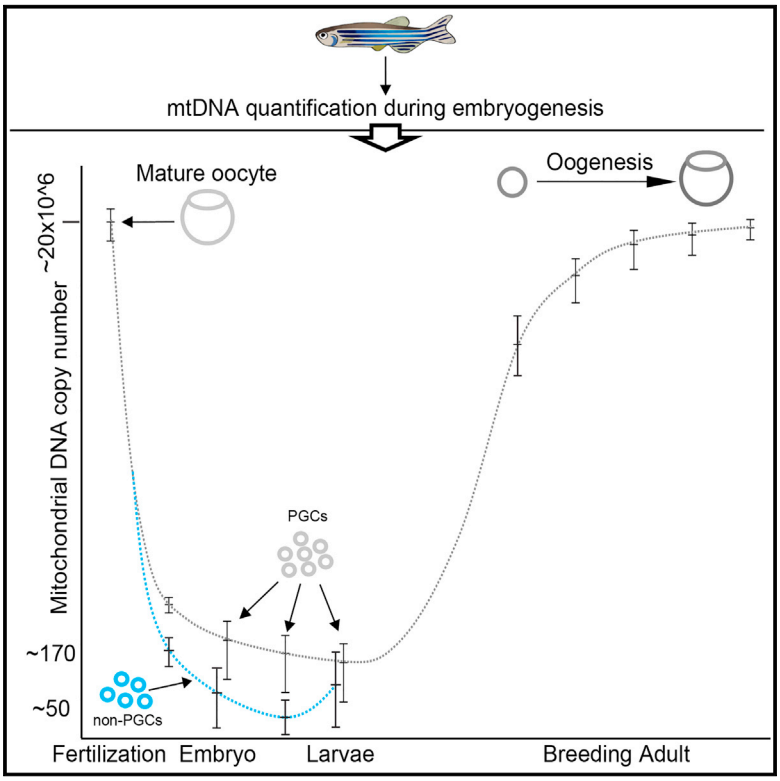
Report

Cell Reports

Differences in Strength and Timing of the mtDNA Bottleneck between Zebrafish Germline and Non-germline Cells

Graphical Abstract



Highlights

- The zebrafish model is highly suitable to study the mtDNA bottleneck mechanism
- Zebrafish oocytes have a high mtDNA number with large intra-individual variation
- Size and timing of the bottleneck differ between germline and non-germline cells
- Low mtDNA amounts in germ cells can explain the occurrence of de novo mutations

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In Brief

Otten et al. describe the mtDNA bottleneck in zebrafish. Oocytes have a high and variable mtDNA copy number, dictating the variation in the mtDNA bottleneck size. Differences in size and timing of the bottleneck between germline and non-germline cells suggest differences in segregation and sensitivity to de novo mtDNA mutations.

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Report

Differences in Strength and Timing of the mtDNA Bottleneck between Zebrafish Germline and Non-germline Cells

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SUMMARY

We studied the mtDNA bottleneck in zebrafish to elucidate size, timing, and variation in germline and non-germline cells. Mature zebrafish oocytes contain, on average, 19.0 × 10^6 mtDNA molecules with high variation between oocytes. During embryogenesis, the mtDNA copy number decreases to ~170 mtDNA molecules per primordial germ cell (PGC), a number similar to that in mammals, and to ~50 per non-PGC. These occur at the same developmental stage, implying considerable variation in mtDNA copy number in (non-)PGCs of the same female, dictated by variation in the mature oocyte. The presence of oocytes with low mtDNA numbers, if similar in humans, could explain how (de novo) mutations can reach high mutation loads within a single generation. High mtDNA copy numbers in mature oocytes are established by mtDNA replication during oocyte development. Bottleneck differences between germline and non-germline cells, due to early differentiation of PGCs, may account for different distribution patterns of familial mutations.

INTRODUCTION

Various mechanisms have evolved to manage the high mtDNA mutation rate (Lynch et al., 2006). In animals, a high mtDNA copy number in cells dilutes the effect of mtDNA mutations (Otten and Smeets, 2015). Heteroplasmic mutations can only manifest above a tissue- and mutation-specific threshold. Another mechanism is the mtDNA bottleneck during maternal inheritance: a limited amount of mtDNA of the oocytes repopulates the cells of the next generation, thereby filtering out low-level mtDNA mutations. As a result, individuals are usually homoplasmic (Lee et al., 2012), which is the healthiest situation (Sharpley et al., 2012). However, in the case of familial pathogenic mutations, the bottleneck can cause high and unpredictable shifts in the mtDNA mutation load and disease risks in the offspring (Howell et al., 1992).

Despite extensive research, the mtDNA bottleneck is still not fully understood. Data from Holstein cows (Hauswirth and Laipis, 1982) indicated that the bottleneck was caused by a sharp decrease in mtDNA copy number during early development, most likely, followed by a clonal expansion of these founder molecules during oogenesis. In mice, a similar decrease in the mtDNA copy number between oocytes (range of 10^5; Cree et al., 2008; Wai et al., 2008) and primordial germ cells (PGCs) was reported, with ~200 mtDNA molecules in a single PGC at the bottom of the bottleneck, in line with previous estimates (185; Jenuth et al., 1996). In contrast, in another study, ~2,000 mtDNA molecules were measured in a single PGC (Cao et al., 2007), and a small effective number of segregational units was proposed to explain the rapid segregation, either by assembly of mtDNA molecules into nucleoids or due to preferential replication of a subpopulation of the mtDNA genome (Cao et al., 2007; Wai et al., 2008). For salmons, it was reported that the bottleneck occurred during oogenesis, with a size of about 85 mtDNA copies (Wolff et al., 2011). In humans, indirect estimations of the bottleneck size have been described, ranging from only 1–5 (Marchington et al., 1997) and 30–35 (Rebolledo-Jaramillo et al., 2014) to ~90 (Pallotti et al., 2014) and 180 (Howell et al., 1992) copies.

We measured mtDNA copy number and variation in zebrafish oocytes and in larval germline and non-germline cells during embryonic development. The zebrafish model allowed relatively easy collection of mature oocytes from individual female fish, allowing assessment of the individual variation. Furthermore, PGCs were specifically visualized with GFP, followed by fluorescence-activated cell sorting (FACS) to isolate both PGCs and

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non-PGCs during embryogenesis (Goto-Kazeto et al., 2010). Lastly, oocytes from different stages of development were isolated. In this way, we characterized the mtDNA bottleneck in zebrafish in germline and non-germline cells, providing a better understanding of the underlying mechanism and possible consequences.

RESULTS

mtDNA Copy Number and Variation in Mature Zebrafish Oocytes

The average number of mtDNA molecules in 186 individual mature oocytes (Figure 1A; Figure S1) was 19.0 × 10^6 (range = 3.3 × 10^6–42.2 × 10^6; Table S1), but the distribution was non-Gaussian (Figure 1A; p = 0.014). We excluded polymorphisms in the primer binding sites as a cause of these variations by sequencing these sites in eight female zebrafish and 10–15 of their oocytes in the *AB* strain used (Table S2) (A.B.C.O. Alphons P.M. Stassen, Michiel Adriaens, M.G., Richard G.J. Dohmen, Adriana J. Timmer, Sabina J.V. Vanherle, Rick Kamps, I.B.W.B., J.M.V, M.M., H.J.M.S, unpublished data). Among these oocytes, 137 were collected from only eight different females (a single batch with ages at both the start [3–6 months] and end [18–24 months] of the fertile period), and the mean values among these females were statistically similar (all ps > 0.05; Figure 1B), with all mean values ~20 × 10^6, implying no differences in oocytes derived from females from the same batch, early (fish A–D) and late (fish E–H) in their fertile period. The individual frequency distribution of the mtDNA content in oocytes fits a Gaussian distribution (ps > 0.05). High variation in the mtDNA copy number exists within the oocytes from a single female fish (highest value is three to seven times the lowest value; Table S1).

mtDNA Copy Number during Zebrafish Embryonic Development

To assess the mtDNA copy number during development, we collected embryos and larvae from different stages from one batch of eggs from five to ten individual females. In the cleavage and early blastula stages, an embryo consists of a fixed number of cells, and absolute copy numbers per cell were assessed (one-cell stage [mature oocyte] to 256-cell stage; Figure 1C). The mtDNA copy numbers of the one-cell stage (mature oocytes) are at the lower bound of the distribution of the mtDNA copy number in all oocytes (Figure 1A). As the different isolation methods used gave comparable results (Figure S1), this most likely reflects biological differences between the female fish (from different batches). Dividing the mtDNA copy number by the cell number shows that the average mtDNA copy number per cell was halved every cell division, indicating that the total number of mtDNA is stable and that mtDNA replication is absent.

From the 512-cell stage until the prim-22 stage, we measured the mtDNA copy number relative to a nuclear gene (*mt-nd1/b2m*; Figures 1D and 1E). The decrease in mtDNA load continued during late blastula and early gastrula stages (Figure 1D) but stopped during segmentation (Figure 1E). Based on the relative and absolute quantification performed at the 256-cell stage, we estimated the amount of mtDNA at this point to be 12–56 mtDNA copies per cell (Table S1).

Isolation of Germline Cells during Zebrafish Embryogenesis

PGCs were visualized by injection of EGFP-*nanos3*-3′UTR mRNA and by generating a transgenic line expressing EGFP-*nanos3*-3′UTR (Figure 2A). Both methods generated similar *GFP* expression patterns and a specific and representative staining of PGCs, but injected embryos had higher rates of ectopic GFP expression (e.g., in the brain; excluded from further analysis). After enzymatic dissociation, cell suspensions were FACS analyzed (gating as in Goto-Kazeto et al., 2010) and sorted based on their morphology and GFP intensity. After removal of aggregates (enrichment run), cells displaying the highest GFP fluorescence were all located in a narrow range on the forward scatter (FSC) channel (purity run), indicating a morphologically homogeneous cell population. Microscopic analysis confirmed that >95% of these isolated PGCs presented high GFP intensity (Figure S1).

The Germline and Non-germline mtDNA Bottleneck Size during Zebrafish Embryogenesis

PGCs from embryos 8, 24, 48, and 72 hr post-fertilization (hpf) had a significantly lower mtDNA copy number, compared to the oocytes (all ps < 0.001; Figure 2B). Although no significant differences were observed at 8–72 hpf (all ps > 0.05), a correlation analysis revealed a significant downward trend (p = 0.0009) in mtDNA copy number in migratory PGCs, following their arrival at the region where the gonad develops (Figure 2D) (Paksa and Raz, 2015). The mean values, SD, range, and CVs (Table S1) indicated that the lowest mtDNA copy number in a single PGC was ~170 mtDNA molecules at 72 hpf. The variation was high during all stages of development (Table S1). Correlation analysis also revealed a downward trend in the mtDNA copy number in non-PGCs during development (p = 0.0097; Figure 2C). The lowest mtDNA load observed in single non-PGCs (~50; Table S1) was not significantly different from the value measured for the PGCs; however, both slopes (PGCs versus non-PGCs) differed statistically from each other (p < 0.0001). The value of ~50 is similar to the estimated non-germline bottleneck size in whole embryos. Again, variation in bottleneck size is large in non-PGCs from all developmental stages (Table S1). Non-linear regression analysis, based on the assumption that the mtDNA load is equally divided among the daughter cells during development, fits the measured mtDNA copy values best (R^2s = 0.75 for PGCs and 0.55 for non-PGCs). We estimated the plateau value (≈bottleneck size) for PGCs at 193.5 (SE = 56.3) mtDNA copies and for non-PGCs at 89.4 (SE = 16.3) mtDNA copies, which is close to the actual lowest mtDNA counts measured in this study. One-phase decay equations (Figure 2D) indicated that the plateau value is reached earlier in non-PGCs than in PGCs, which is corroborated by the differences in the half-times (6.9 hpf for PGCs and 3.4 hpf for non-PGCs).

mtDNA Copy Number during Oocyte Development

Stage I oocytes, the primary growth phase, had an average diameter of 115 μm and possessed ~840,000 mtDNA molecules (Figure 3A). This number was significantly higher in stage II oocytes, the cortical alveolus stage, ranging from 5.0 × 10^6 in small stage II oocytes (II_{small}; average diameter, 191 μm) to 11.1 × 10^6

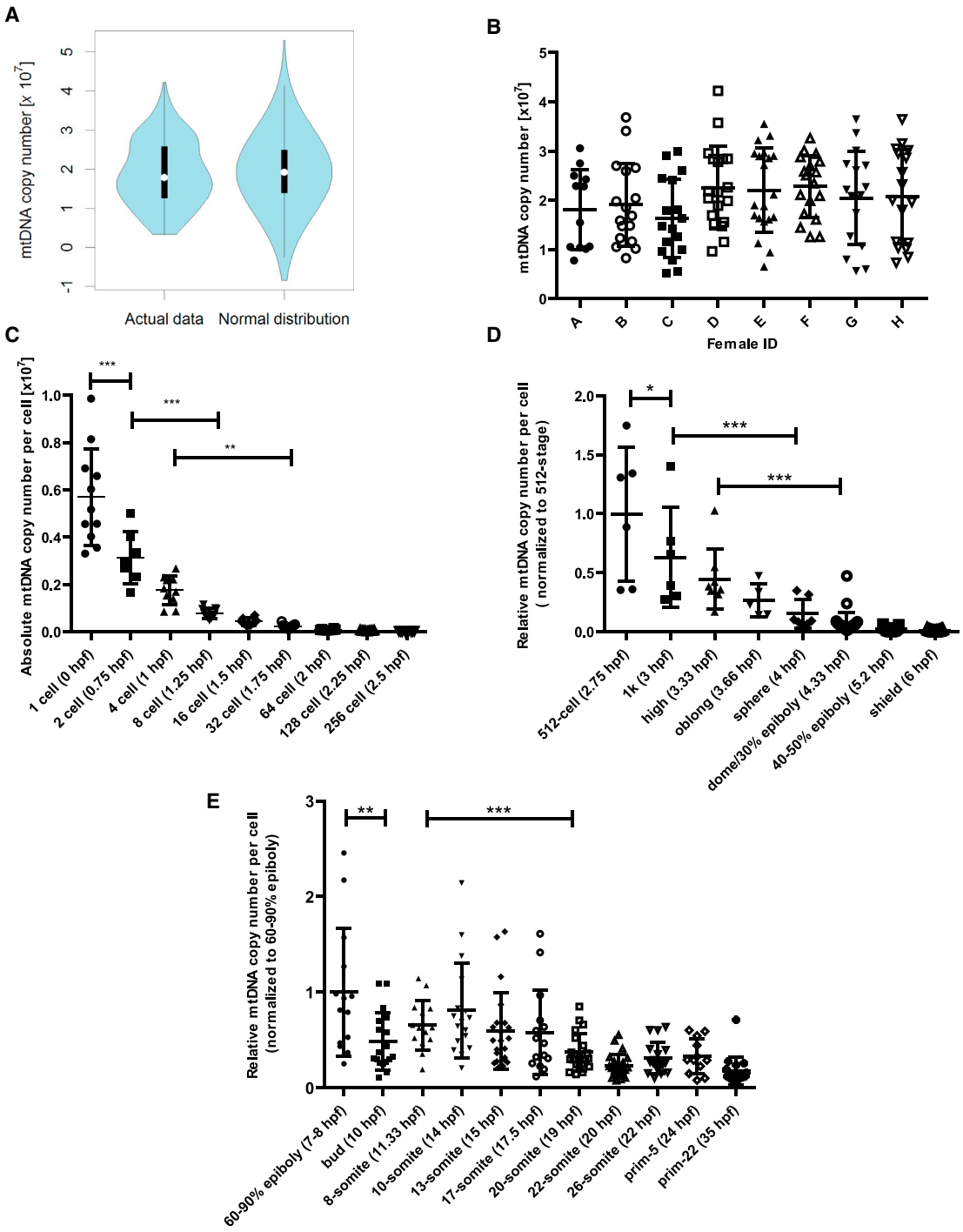

Figure 1. The mtDNA Copy Number in Zebrafish Mature Oocytes and during Embryonic Development

(A) Violin plot of absolute mtDNA copy number in 186 oocytes (left), compared with that of a Gaussian distribution (with same SD). White dot corresponds to the median value, and the black bar corresponds to the 25th (bottom) and 75th (top) percentile values.

(B) Absolute oocyte mtDNA copy number in the 137 oocytes from eight females; each symbol represents a single oocyte. Fish A–D were at the start of the fertile period, and fish E–H were at the end.

(C) Absolute quantification of the mtDNA copy number per cell in single embryos at the 1- to 256-cell stage.

(D and E) Relative quantification (*mt-nd1/b2m*) of the mtDNA copy number (per cell) in single embryos at (D) the 512-cell stage until shield, normalized to the 512-cell stage, and (E) 60%–90% epiboly until prim-22, normalized to the 60%–90% epiboly stage.

*p < 0.05; **p < 0.01; ***p < 0.001, after one-way ANOVA. Horizontal lines indicate the mean value with SD.

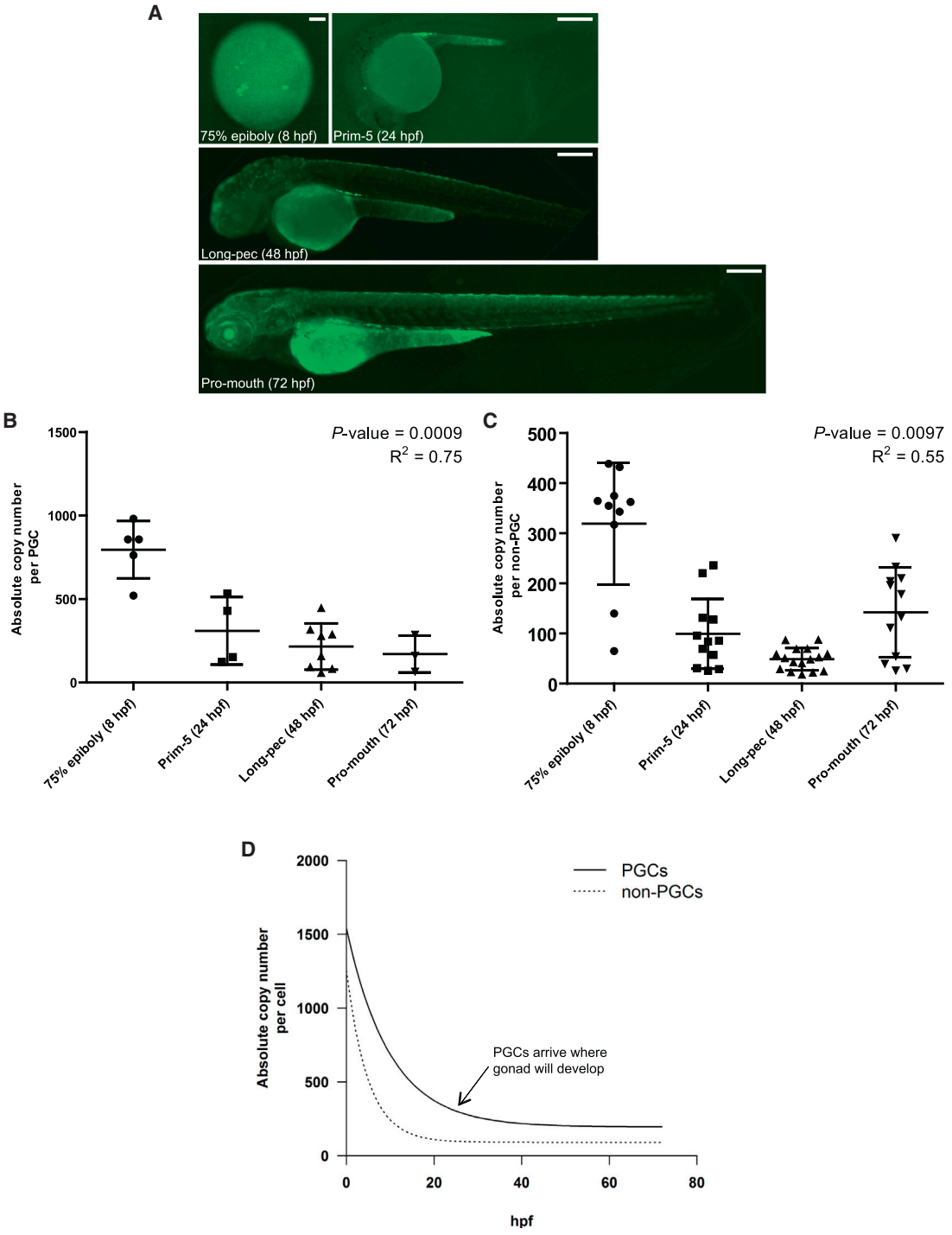

Figure 2. The mtDNA Copy Number in FACS-Isolated PGCs and Non-PGCs from Zebrafish Embryos

(A) GFP expression in transgenic zebrafish embryos (EGFP-*nanos3* 3′ UTR). (A) Embryos were imaged using an Axioplan M1 Zeiss microscope. The images represent a combination of pictures captured along the anterior-posterior axis of the embryo, using specific focal planes. Scale bars represent 100 μm for 8 hpf and 200 μm for 24, 48, and 72 hpf.

(B and C) Absolute mtDNA copy number in (B) PGCs and (C) non-PGCs from various stages of development. Every symbol represents one group of 80 cells. Horizontal lines indicate mean with SD. The p values are generated by Spearman's rank correlation test and indicate a downward trend when <0.05. R^2 indicates fitness of a non-linear one-phase decay exponential equation.

(D) Plot of the non-linear one-phase decay equation for PGCs (derived from the graph in B) and non-PGCs (derived from C). The half-life was 6.9 hpf for PGCs and 3.4 hpf for non-PGCs.

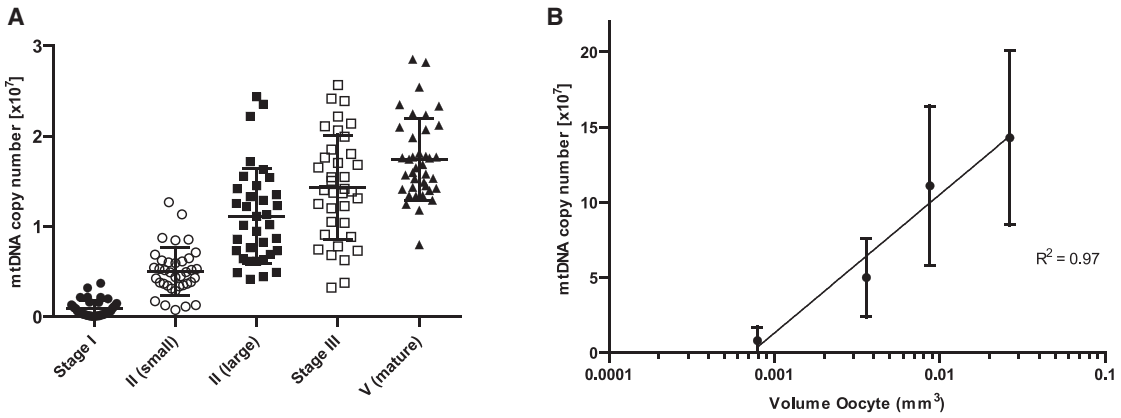

Figure 3. The mtDNA Copy Number Correlates to an Increased Volume during Oocyte Maturation

(A) Mature and immature oocytes from different developmental stages were isolated from four different females and mtDNA copy number was measured. Each symbol represents a single (immature) oocyte. Horizontal lines indicate mean with SD. All groups were statistically different from each other.

(B) Relation between the oocyte volume, assuming a sphere, and the mtDNA amount in oocytes stages I–III. Dots indicate mean with SD. R^2 values indicate fitness after a linear regression analysis between the volume (logaritmic) and the mtDNA copy number.

in large stage II oocytes (II_{large}; average diameter, 256 μm). During stage III, the vitellogenesis stage, the mtDNA copy number per oocyte increased to 14.3 × 10^6 (average diameter, 370 μm). Eventually, stage V (mature) oocytes (average diameter, 750 μm) contained 17.4 × 10^6 mtDNA copies. The variation was higher in stage I oocytes (CV: 106%), compared to the other stages (CVs: 40%–52%; Table S1). We observed a tight, positive logarithmic correlation between the volume of the oocytes at stages I–III (assuming a sphere) and the mtDNA content (Figure 3B).

DISCUSSION

High and Variable mtDNA Copy Number in Mature Zebrafish Oocytes

The mtDNA load in mature zebrafish oocytes was, on average, 19.0 × 10^6 mtDNA copies per cell (Figures 1A and 1B). The distribution of all oocytes indicated a high, non-Gaussian variation (range = 3.3 × 10^6–42.2 × 10^6), with a sharp lower boundary value of 3.3 × 10^6, while the limitation on high mtDNA loads seems to be more moderate (up to 42.2 × 10^6). The mean oocyte mtDNA copy number among the eight different female fish was statistically similar (Figure 1B), suggesting low inter-individual variation. We observed variation in the DNA copy number among different batches of fish. Batch effects have been reported in zebrafish, for example, in transcriptomics (Wang et al., 2014). Our data are comparable with the reported copy number in single zebrafish blastomeres of a four-cell stage (14 × 10^6 copies per cell) (Artuso et al., 2012), corresponding to an mtDNA load of 56 × 10^6 in oocytes, which, although in the same order of magnitude, again indicates biological variation among batches. Oocytes from salmon, another teleost species, contain about 3.2 × 10^9 mtDNA copies (Wolff and Gemmell, 2008). Large differences between fish were also reported for salmon, ranging from 1.1 × 10^9 to 7.0 × 10^9 (factor 7; Otten and Smeets, 2015).

In most mammals, mature oocytes contain fewer than 1.0 × 10^6 mtDNA copies, ranging from 110,000 in *R. Norvegicus* (Zeng et al., 2009) to 807,794 in *B. Taurus* (Iwata et al., 2011), with substantial variation within species (Otten and Smeets, 2015); e.g., in humans, ranging from 143,000 (Duran et al., 2011) to 697,176 (Murakoshi et al., 2013). For humans, mice, and cows, the average reported mtDNA load in the oocytes is below 300,000 copies (Otten and Smeets, 2015), suggesting that zebrafish contain about a hundred times more mtDNA in their oocytes than mammals. This difference is most likely due to differences in implantation and in oxidative activity needed to satisfy the higher energetic demands in teleost compared to mammals, due to a dependency on fatty acid oxidation (Wolff and Gemmell, 2008). Differences in the mtDNA load of oocytes are also reflected by differences in the size of the oocytes, which are smallest in mammals (<0.15 mm; human oocytes, 0.1 mm), moderate in zebrafish (0.75 mm; Kimmel et al., 1995; Selman et al., 1993), and largest in salmon (>4.5 mm; Wolff and Gemmell, 2008). Based on these volumes, we estimated that the mtDNA copy number per unit of volume is fairly equal across species. This is corroborated by a study in ovine oocytes, where the mtDNA copy number per unit oocyte volume showed only little variation, much lower compared to the variation in the mtDNA copy number in the whole oocyte (Cotterill et al., 2013).

A selection against oocytes with low mtDNA load seems to occur (Figure 1A). The non-Gaussian distribution of the mtDNA copy number is most likely due to a sharp boundary at the lowest mtDNA copy number. No oocytes with fewer than 3.3 × 10^6 mtDNA copies have been observed. Mitochondrial function and copy number are important for successful fertilization (Ebert et al., 1988). Low mitochondrial content has been demonstrated to negatively influence the fertilizability of eggs, probably due to inadequate mitochondrial biogenesis or cytoplasmic maturation (Reynier et al., 2001), which has been demonstrated by low mtDNA counts in degenerate oocytes (Santos et al., 2006), in oocytes with fertilization failure (Reynier et al., 2001), or during ovarian insufficiency (May-Panloup et al., 2005). This was confirmed by studies in mice (Wai et al., 2010). In contrast, an upper mtDNA threshold has been proposed in human blastocysts,

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above which implantation was never observed and aneuploidy was more frequent (Fragouli et al., 2015).

For each individual female fish, the inter-oocyte variation in mtDNA copy number was high (3- to 8-fold; Figure 1B). As we collected unfertilized stage V (mature) oocytes (Selman et al., 1993) by squeezing the abdomens of female fish after they were kept with a male overnight, we could exclude variation in maturation of oocytes (Jansen and de Boer, 1998). Furthermore, we excluded that the variation could be due to polymorphisms in the PCR primers used (Table S2). The considerable variation among 137 oocytes from the same eight females (Figure 1B) indicated that it was independent of the nuclear genome, and we conclude that the variation in mtDNA copy number among oocytes is individual stochastic variation rather than genetic variation. Such variation within an individual has also been reported in human (Reynier et al., 2001) and bovine oocytes (Cree et al., 2015), which implies that every individual can generate oocytes with either a very low or a very high mtDNA copy number.

mtDNA Copy Number per Cell Decreases during Embryonic Development

Until the 256-cell stage, the mtDNA copy number per cell in complete zebrafish embryos halved during every cell cycle, while the total amount did not change, consistent with a lack of mtDNA replication and degradation. The decrease in mtDNA content per cell continued until early segmentation, and at these stages, about 16–54 mtDNA molecules were present per cell. This is in line with findings in mice, in which no changes in total mtDNA copy number were observed until the blastocyst stage (~128 cells; Ebert et al., 1988). When the gastrulation stages pass into the segmentation stages, at which primary organogenesis occurs and the first muscle-driven movements can be observed (Kimmel et al., 1995), mtDNA replication is initiated. During this period, zebrafish embryos display maximum proton leak, which might reflect elevated mitochondrial function (Stackley et al., 2011). The onset of mtDNA replication most likely reflects a switch to oxidative phosphorylation (OXPHOS) as the main energy source, while the large energy requirement during the first developmental stage is provided by glycolysis (Facucho-Oliveira and St John, 2009), a faster energy source (Stackley et al., 2011).

High Variation in the Germline mtDNA Bottleneck Size

In PGCs, we found a negative correlation between mtDNA copy number and developmental time, with a minimal mean mtDNA copy number of 171 (an estimated 193.5 [±56.3] following non-linear regression analysis) and an SD of 111. Since we quantified the mtDNA amount for groups of 80 PGCs, the actual variation among individual cells might be even higher. This high variation in PGCs from 8 hpf to 72 hpf is comparable to that in mature oocytes, and the bottom of the bottleneck seems to be defined by developmental stage and not by the mtDNA copy number. Given the high variation of mtDNA copies in the oocyte, each female can produce PGCs, which, by chance, possess at the bottom only a few mtDNA molecules. At this stage, the risk that de novo mtDNA mutations (Thorburn, 2004) reach functional significance will be highest.

The germline bottleneck size of 170–200 mtDNA molecules is close to the values reported in mice of ~185 (based on mtDNA heteroplasmy segregation between several generations in the BALB/NZB mouse; Jenuth et al., 1996) and ~200 (based on direct measurements; Cree et al., 2008; Wai et al., 2008). In humans, the bottleneck size has been estimated to be ~173, based on the heteroplasmy distribution among 82 single primary oocytes from a woman carrying the 3243A > G mtDNA mutation (Brown et al., 2001). The bottleneck size in zebrafish also resembles the size in cows (65–163; Rand and Harrison, 1986), salmon (80–88; Wolff et al., 2011), and even crickets (87–395; Rand and Harrison, 1986). Some studies in humans estimated a lower bottleneck size; for instance, a bottleneck size of ~90 (Pallotti et al., 2014), only 30–35 (Rebolledo-Jaramillo et al., 2014), or even 1–5 (Marchington et al., 1997). These were calculated from segregation patterns of variants that might not be neutral, and selection events could apply (Stewart et al., 2008). Since most models assume genetic drift only (Wonnapinij et al., 2008), this would lead to an underestimation of the mtDNA bottleneck size. Nevertheless, the different reported values on the bottleneck size could also reflect large biological variation. Flexibility in the decrease of mtDNA copy number during the bottleneck is in line with a mathematical model for the bottleneck (Johnston et al., 2015).

Increased mtDNA Copy Number during Oocyte Maturation

The mtDNA copy number increased rapidly during oocyte development, with a 20-fold difference between stage I oocytes and mature oocytes, indicating that mtDNA replication is a prerequisite for oocyte maturation. Substantial increase in the mtDNA copy number during early oocyte maturation has also been observed in mice (Cao et al., 2007). The correlation between the size of the cells and the mtDNA copy number (Figure 3B) showed that growth and mtDNA replication were closely connected. During oogenesis, a PGC of ~7 μm develops into an oocyte, which grows and reaches a diameter of 140 μm at the end of stage I (Selman et al., 1993). At this stage, variation in mtDNA copy numbers between cells was highest (ranging from ~20.000 to 3.7 × 10^6), and no fixed oocyte volume per mtDNA could be observed. Probably, at this stage, cell growth precedes and might trigger mtDNA replication, whereas during the further developmental stages, it is less clear whether cell growth drives mtDNA replication or vice versa. We failed to measure an increase in mtDNA copy number in PGCs that arrived at the region where the gonads develop, as has been described in mice (Cree et al., 2008; Wai et al., 2008). This discrepancy might arise because fish oogonia, in contrast to mammals, keep constantly renewing the stocks of young oocytes and follicles. In fish, mitosis in oogonia (the starting point of oogenesis) is activated after ovulation in adult zebrafish (Jalabert, 2005), while human oogonia undergo mitosis already during weeks 9–22 of embryonic development (Jones, 1997). Although we were not able to isolate oogonia and the earliest and smallest primary oocytes, the high mtDNA copy numbers in stage I oocytes suggests that the mtDNA replication necessary to achieve high oocyte mtDNA loads occurred later in zebrafish life (in adults) than in mammals (during embryonic development).

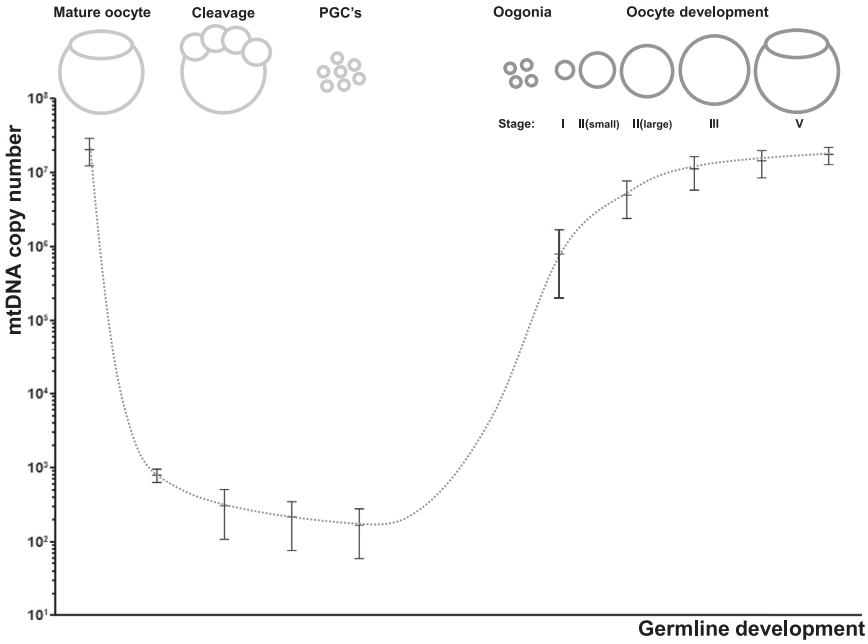

Figure 4. Summary of the Germline mtDNA Bottleneck

The germline mtDNA bottleneck consists of (1) random partitioning of oocyte mtDNA during the early developmental cell divisions, leading to variable mtDNA bottleneck sizes, and (2) extensive mtDNA replication during oogenesis. Horizontal lines indicate mean with SD.

A Small Non-germline mtDNA Bottleneck Size

The bottleneck size in non-germline zebrafish cells (non-PGCs) is ~50 mtDNA molecules (an estimated 89.4 ± 16.3, following non-linear regression analysis) per cell, with a downward trend in the mtDNA copy number during development. As non-PGCs can be any cell from the developing embryo, the bottleneck size derived from the whole-embryo analysis (16–54 mtDNA copies) confirms this number. The decrease in mtDNA copy number has been suggested to establish a set point during early embryogenesis, which increases later during development in order to satisfy cell-specific ATP requirements through OXPHOS (Facucho-Oliveira et al., 2007). During the first 48 hpf, we did not observe an increase in the mtDNA copy number of the non-germline cells (Figure 1), while after 72 hpf, a trend toward higher mtDNA amounts was observed (Figure 2C). The latter observation could indicate that mtDNA amounts go up once the larvae use their skeletal muscle for free swimming and hunting (Kimmel et al., 1995).

The Germline and the Non-germline mtDNA Bottleneck

In line with our observation in zebrafish, murine somatic cells have highly variable mtDNA copy numbers during early embryogenesis, and the mtDNA content was downregulated from 8 days post-coitum (dpc) onward (Cao et al., 2007). Furthermore, as in the present study, the mtDNA copy number in murine non-germline cells was lower than in germline cells (Cao et al., 2007). Although mtDNA copy numbers between PGCs and non-PGCs were not statistically different, correlation and non-linear regression analysis (Figure 2C) indicated a difference in the course or timing of the mtDNA copy number over development. The increase in mtDNA content in germline cells occurs during oogenesis in adult zebrafish (Figure 4), long after the formation of the organ systems (non-germline cells) during zebrafish embryogenesis. This difference in timing, if comparable in humans, could explain differences in segregation of mtDNA heteroplasmic mutations among germline and somatic tissues in humans (Wonnapinij et al., 2008). Especially in the case of familial mtDNA mutations (Otten and Smeets, 2015), these segregation and replication mechanisms could explain the large tissue-specific differences in mutation load (St John, 2012). This is corroborated by the observation that HIV interventions, which reduce mtDNA copy number, can induce a somatic bottleneck and can result in age-related mitochondrial disease due to clonal expansion of preexisting mtDNA mutations (Payne et al., 2011).

We have quantified the whole germline mtDNA bottleneck in a single model (Figure 4). Mechanistically, both the germline and the non-germline bottlenecks rely on the same principle: division of the mtDNA over the daughter cells during early embryogenesis in the absence of replication, until increased energy requirements during development or oogenesis activate mtDNA replication. However, as the bottleneck in non-germline cells only affects the individual, while segregation in PGCs affects future generations, the germline bottleneck is of higher evolutionary importance. Nevertheless, an understanding of the difference between the bottlenecks would allow a better prediction of the risk an individual has for developing an mtDNA disorder, which is determined by both the germline and the non-germline bottlenecks. Due to a clear difference in somatic and germline specification in zebrafish, as well as fast development and easy access to the organs, zebrafish are an attractive model for studying these possible tissue-specific mtDNA bottleneck effects in further detail.

EXPERIMENTAL PROCEDURES

Full experimental procedures can be found in the Supplemental Information.

Zebrafish Embryos and Oocytes

AB zebrafish embryos were raised, housed, and staged according to standard procedures (Kimmel et al., 1995) at Liège University, where local ethical approval by the committee of animal research was obtained. Mature and immature oocytes were collected manually as described previously (Selman et al., 1993).

Isolation of PGCs

PGCs were identified by both mRNA injections and by a transgenic line. Artificial mRNA constructs with the GFP open reading frame fused to the 3′ UTR of zebrafish *nanos3* was prepared as described previously (Goto-Kazeto

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et al., 2010). For the transgenic line, the *kop-EGFP-F'-nos3-'UTR-cry-DsRed* transgene cassette was cloned into the pTolDest vector. Injected or transgenic animals were selected per 100 on the basis of showing similar GFP intensity and no ectopic GFP expression. Embryos were completely disaggregated, and cells were filtered. Subsequently, FACS sorting (BD Biosciences FACSAria II) was performed as described previously (Goto-Kazeto et al., 2010).

Quantification of mtDNA Copy Number

After DNA extraction, the mtDNA copy number was measured. Absolute mtDNA copy number quantification was performed by real-time qPCR of the zebrafish *mt-nd1* (*NADH dehydrogenase 1, mitochondrial*) gene. Amplification results were converted to absolute mtDNA copy number using a standard curve. Relative quantification of the mtDNA copy number was performed by qPCR measurements of the steady-state amounts of both *mt-nd1* and *b2m* (nuclear gene *beta-2-microglobulin*). For the 256-cell stage, we measured the mtDNA copy number both absolutely and relatively, allowing an estimation of the absolute mtDNA copy numbers in embryos staged 512 cells or older (Table S1).

Next-Generation Sequencing

Next-generation sequencing (NGS) was performed on an Illumina HiSeq 2000. Heteroplasmy values were calculated as the ration of the nucleotides over the total count of any nucleotide at a position.

Statistical Analysis

Statistical analyses were carried out using GraphPad Prism Version 5.02 software. Gaussian distribution was tested using the D'Agostino-Pearson omnibus normality test. One-way ANOVA and the Bonferroni multiple comparison test were used for comparing multiple groups. Spearman's rank correlation coefficient was used for analyzing trends. Non-linear regression analysis was performed using the one-phase decay exponential equation. Calculated p values were considered significant at <0.05. Coefficients of variation (CVs) were calculated as the ratio between the SD and the mean.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, one figure, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.06.023.

AUTHOR CONTRIBUTIONS

Conceptualization, A.B.C.O., J.M.V., B.J.C.v.d.B., M.M., and H.J.M.S.; Methodology, A.B.C.O., T.E.J.T., J.G.D., K.T., E.R., M.G., M.M., and H.J.M.S.; Formal Analysis, A.B.C.O., T.E.J.T., M.G., and M.M.; Investigation, A.B.C.O., T.E.J.T., J.G.D., E.H.L., I.B.W.B., A.P.A.v.M., and M.W.; Resources, K.T., E.R., M.M., and H.J.M.S., Writing – original draft, A.B.C.O., and H.J.M.S.; Writing – Review & Editing, A.B.C.O., T.E.J.T., E.R., J.M.V., B.J.C.v.d.B., M.M., and H.J.M.S.; Visualization, A.B.C.O.; Supervision, J.M.V., B.J.C.v.d.B., M.M., and H.J.M.S.; Funding Acquisition, M.M. and H.J.M.S.

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