Transient translational quiescence in primordial germ cells

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ABSTRACT
Stem cells in animals often exhibit a slow cell cycle and/or low transcriptional activity referred to as quiescence. Here, we report that the translational activity in the primordial germ cells (PGCs) of the sea urchin embryo (Strongylocentrotus purpuratus) is quiescent. We measured new protein synthesis with O-propargyl-puromycin and L-homopropargylglycine Click-IT technologies, and determined that these cells synthesize protein at only 6% the level of their adjacent somatic cells. Knockdown of translation of the RNA-binding protein Nanos2 by morpholino antisense oligonucleotides, or knockout of the Nanos2 gene by CRISPR/Cas9 resulted in a significant, but partial, increase (47%) in general translation specifically in the PGCs. We found that the mRNA of the translation factor eEF1A is excluded from the PGCs in a Nanos2-dependent manner, a consequence of a Nanos/Pumilio response element (PRE) in its 3′ UTR. In addition to eEF1A, the cytoplasmic pH of the PGCs appears to repress translation and simply increasing the pH also significantly restores translation selectively in the PGCs. We conclude that the PGCs of this sea urchin institute parallel pathways to quiesce translation thoroughly but transiently.

KEY WORDS: Metabolism, Primordial germ cells, Sea urchin, Translation

INTRODUCTION
Primordial germ cells (PGCs) are the newly formed germ line of the embryo. These cells are specified during early development, migrate to the gonad and then proliferate to form the gonial stem cells of the future eggs and sperm of the adult. In animals for which the PGCs form early in development, usually by acquired maternal determinants (e.g. flies, nematodes, zebrafish), their specification precedes gastrulation and the PGCs become quiescent in terms of cell division and transcription (Williamson and Lehmann, 1996). These cells remain less active than their somatic siblings until tissue rearrangements and organogenesis begin, when they then migrate to the gonad and increase cell division and transcriptional activity.

Echinoderms are sister to the Chordates; in the sea urchin Strongylocentrotus purpuratus, the primordial germ cells originate at the 5th cell division (32 cell stage) by two sequential asymmetric divisions of the small micromeres. This early segregation from a somatic fate creates a unique challenge to the embryo: it is one of the earliest germ lines to form in terms of cell cycle number, and how it deals with this disparity in timing of development is of logistical concern here that the PGCs also quiesce their protein synthesis and do so transiently in part due to the selective expression of the RNA binding protein Nanos2. We have thus named this process transient translational quiescence (TTQ).

Nanos was first identified in Drosophila as a translational repressor (Cho et al., 2006; Irish et al., 1989). It functions through its interaction with Pumilio, which binds RNAs containing a conserved motif usually found in the 3′ UTR of mRNAs; this motif is referred to as the Pumilio response element (PRE) (Sonoda and Wharton, 1999; Wharton and Struhl, 1991). Only a few genes have been identified as Nanos/Pumilio targets: cyclin B (Asaoka-Taguchi et al., 1999; Dalby and Glover, 1993; Kadyrova et al., 2007; Lai et al., 2011), hid (Hayashi et al., 2004; Sato et al., 2007), hunchback (Murata and Wharton, 1995; Wreden et al., 1997), fem3 (Ahringer and Kimble, 1991; Zhang et al., 1997), VegT (Lai et al., 2012) and CNOT6 (Swartz et al., 2014). In the sea urchin Strongylocentrotus purpuratus (the purple sea urchin), three nanos orthologs are present in its genome, but SppNanos2 (mRNA and protein) is the only Nanos that accumulates specifically in the PGCs at the blastula stage when the quiescence phenotype is detected (Juliano et al., 2010). Our data show that Nanos2, by regulating the mRNA coding for eEF1A, is responsible in part for the dramatic translational quiescence seen in these early born PGCs.

RESULTS
Protein synthesis is transiently quiescent in the PGCs
In contrast to radioactive amino acids or specific antibodies against translational factors that limit a direct and specific measurement of the translational activity throughout the development, modified chemistries now enable direct imaging and quantification of protein synthesis at high resolution in situ. We tested both a methionine analog, HPG (homopropargylglycine), and a modified translational inhibitor, the OPP (O-propargyl-puromycin) (Starck et al., 2004), and found highly compatible results in both. Both reagents are taken up by cells and are incorporated into the nascent peptide chain for covalent labeling, even after fixation of the embryo. In the sea urchin, the overall rate of protein synthesis is low in unfertilized eggs due to a low cytoplasmic pH but is stimulated rapidly following fertilization, independent of mRNA transcription and ribosome biogenesis (Epel, 1967; Cormier et al., 2001; Brandhorst, 1976). This nearly instantaneous activation can be mimicked by increasing the cytoplasmic pH of the egg from pH 6.8 to pH 7.3 with ammonium chloride (Epel, 1967). As expected, protein synthesis was not detectable in unfertilized eggs by OPP incorporation, but the embryos rapidly revealed robust activity (Fig. S1). This
synthetic activity was sensitive to protein synthesis inhibitors, demonstrating the specificity of this component to measure translation in situ in this embryo. To test the translational activity of the PGCs throughout development, these cells were co-labeled with a Vasa antibody to definitely identify the PGCs. Translational activity in the PGCs was found to be significantly reduced (6%±2.7) relative to its sibling somatic cells in the animal pole, and is transient – these cells return to normal levels of translational output following gastrulation (i.e. comparable to its precursor siblings and to neighboring cells) within 72 h post-fertilization, demonstrating a transient quiescent activity (Figs 1 and 2). HPG yields similar results (Fig. S2) and, importantly, these in situ results are concordant with the use of radioactive amino acid reagents in this animal (Karp and Weems, 1975). Thus, three different chemistries yield the same biological result. In early dividing cells of the embryo, newly synthesized protein accumulated robustly in the nuclei, a consequence of the significant early stage synthesis of histone proteins (Davidson, 1976). They are translated and incorporate HPG or OPP in the cytoplasm, and then shuttle rapidly to the nucleus, leading to a high nuclear signal (Fig. 1).

Nanos2 is required for the transient translational quiescence in the PGCs
The Nanos2 protein is only detectable by immunolocalization in the PGCs at blastula and gastrula stages (Juliano et al., 2010; Fig. S3), which correlates precisely with the transient translational quiescence (TTQ) phenotype. To test the function of Nanos2 in TTQ, we knocked-down Nanos, using a previously characterized Nanos morpholino to reduce Nanos protein to undetectable amounts (Juliano et al., 2010) and learned that Nanos2 does not influence translation in somatic cells, but dramatically increased the translational activity in the PGCs to 47% that of the somatic cells (Fig. 2). Importantly, vegetal plate cells adjacent to the PGCs also have reduced translational activity relative to other somatic cells (50%), but they maintain their level of translation independently of Nanos protein. This Nanos-independent mechanism observed in the vegetal cells is currently under investigation.

Similar results were obtained when Nanos2 function was inactivated by using the CRISPR/Cas9 approach (Fig. 3). To first test the efficiency of this CRISPR/Cas9 method, we targeted genes of the pigment cell pathway, resulting in albinism in the larvae in over 95% of the embryos (Oulhen and Wessel, 2016; and data not shown). We then designed multiple gRNAs targeting the single exon Nanos2 gene and monitored efficiency of Nanos2 inactivation functionally, by the accumulation of Vasa protein in the PGCs in gastrulae (Juliano et al., 2010). The CRISPR/Cas9 directed Nanos2 inactivation led to high-efficiency mutations (80% of the embryos had 100% of their nuclei mutated) and loss of Vasa expression in the PGCs (Fig. 3). Importantly, targeted gene inactivation of Nanos2 also functionally increased protein synthesis specifically in the PGCs (Fig. 3J). Altogether, these data indicate that Nanos2 is an essential, though not complete, regulator of the TTQ phenotype.

Nanos2 targets the essential translation factor eEF1A to cause low translation in the PGCs
To identify potential mechanistic candidates involved in the TTQ, we used a previously published transcriptomic dataset (Swartz et al., 2014). eEF1A mRNA, which codes for a translation elongation factor, was identified as a transcript that was downregulated in the PGCs (Swartz et al., 2014). When bound to GTP, the protein eEF1A delivers the aminoacylated-tRNA to the A site of the ribosome (Merrick, 2000). Two orthologs of eEF1A exist in mammals, although only one is present in the Sp genome (SPU 000595) (Morales et al., 2006), making it an essential translation factor. By fluorescence in situ hybridization, eEF1A mRNA is found at detectable levels throughout early development (data not shown), but is depleted from the PGCs at blastula and gastrula stages (Fig. 4). The protein is also present ubiquitously in early stages of development, but is rapidly excluded from the PGCs between blastula and early gastrula (Fig. S4). Of significance, we learned that
the morpholino targeting Nanos2 mRNA resulted in the accumulation of eeF1A mRNA specifically in the PGCs (Fig. 4), coincident with the increased translational activity. The 3′ UTR of eeF1A contains a putative PRE sequence (TGTAAAT), suggesting that it is a Nanos/Pumilio target. To test whether the Nanos2-dependent repression of eeF1A mRNA accumulation relied on this element, a morpholino complementary to the eeF1A PRE was injected to block its interaction with the Nanos/Pumilio complex (Fig. 5), an approach used effectively for other mRNAs containing PREs (Swartz et al., 2014). The results show that the PRE is required to exclude eeF1A mRNA from the PGCs; in the presence of the PRE-blocking morpholino, a nearly fourfold increase in protein synthesis occurred specifically in the PGC (Fig. 5). Of note, even though eeF1A mRNA is present throughout the embryo, and the morpholino was injected in the egg, eeF1A mRNA was not significantly affected in the somatic cells (100% in the control morpholino, versus 94% in the PRE morpholino). Thus, exclusion of eeF1A from the PGCs seems dependent upon the presence of Nanos.

To test the function of eeF1A in the TTQ, we ectopically expressed the protein throughout the embryo (Fig. S5). Overexpression of eeF1A in the blastula [using a 3′ UTR lacking the PRE, and thus insensitive to Nanos2 (Oulhen et al., 2013)] does not affect the level of protein synthesis in the somatic cells (Fig. 6). However, the translational activity in the PGCs increased significantly (to 17%) and specifically in the PGCs. Altogether, these data indicate that regulating eeF1A expression, through Nanos2, is essential to maintain the TTQ in the PGCs. Clearly, though, eeF1A by itself is not the sole linchpin regulating TTQ in a Nanos2-dependent fashion. Indeed, scanning the transcripts depleted from the PGCs during TTQ (Swartz et al., 2014) reveals several other candidates that may be regulated similarly to eeF1A and contribute to the TTQ phenotype.

Both cytoplasmic pH and mitochondrial activity are also reduced in the PGCs
Nanos2 is an essential, but not the sole, regulator of TTQ. Indeed, protein synthesis in the PGCs is decreased relative to its neighboring somatic cells even before the exclusion of eeF1A (Fig. 7). Moreover, the mRNA coding for the ADP/ATP translocase 1 was also identified as downregulated in the PGCs (Swartz et al., 2014) and fluorescence in situ hybridization shows a depletion of this RNA in the PGCs, during gastrulation (Fig. 8). We note that the 3′ UTR in the mRNA of this essential mitochondrial factor does not contain a consensus PRE, suggesting a Nanos/Pumilio-independent mechanism of mRNA exclusion from the PGCs.

Furthermore, the activity of the mitochondria is also transiently reduced in the PGCs in late blastula and gastrula (Fig. 8). The mitochondria in the PGCs have only 1.7% (±2.3%) the activity of the adjacent somatic cells. This activity is reduced due to a decrease in the number and a decrease in the activity of mitochondria in the PGCs (Fig. S6). Inactivation of the mitochondria could lead to a shift in metabolism from oxidative phosphorylation to a glycolytic dependence, resulting in acidification of the cytoplasm. Sea urchin
eggs have a slightly acidic cytoplasm (pH 6.8) that represses translation (Epel, 1967), so we used the same strategy used in eggs to determine whether the proposed acidification was a functional contributor to translational quiescence in the PGCs. We incubated blastulae with cell-permeant ammonium chloride, which is known to activate protein synthesis in a pH-dependent process in the egg; this treatment significantly increased the translational activity in the PGCs, compared with the somatic cells, without affecting the level of eEF1A mRNA in the PGCs (Fig. S7). Thus, the TTQ mechanism in this embryo appears to be regulated by at least two parallel pathways: a Nanos2-dependent transcript degradation of essential translation factor(s); and a metabolic shift leading to changes in cellular physiology in the PGCs.

**DISCUSSION**

Quiescence is a shared character of many stem cells. The paradigmatic quiescent stem cell is the hematopoietic lineage, which is maintained at a low cell cycle, but can be stimulated to form new blood cells in response to signaling (Valcourt et al., 2012; Nakamura-Ishizu et al., 2014). Recent research has documented that many tissues in human have quiescent stem cells (Rezza et al., 2014), including skeletal muscle (Boonsanay et al., 2016; Fukada et al., 2007), the intestine (Richmond et al., 2015a,b) and even the central nervous system (Cheung and Rando, 2013; Webb et al., 2013; Gilboa and Lehmann, 2004). Unfortunately, quiescent stem cells are also common in cancer and are the bane of chemotherapy; a quiescent cancer stem cell escapes many of the cancer treatments intending to kill the rapidly proliferating cells (Cheung and Rando, 2013; Nakamura-Ishizu et al., 2014). The quiescent cancer stem cells can eventually transition out of quiescence leading to cancer recurrence (Epel, 1967; Pattabiraman and Weinberg, 2014; Tanaka and Dan, 1991). Quiescence in these examples though usually means a slow cell cycle, and sometimes a restriction in transcription. The work presented here though is the first we know of to find quiescence in protein synthesis and in mitochondrial activity in situ.

In concert with the other quiescent phenotypes found in this same PGC of the sea urchin – cell cycle, transcription, mRNA turnover...
and migration – it serves as an excellent model for how broad cellular activities can be targeted for activation or quiescence with relatively few targets.

The decrease observed here in mitochondrial activity is particularly curious and recently it was found that Hif1α mRNA is present selectively in the PGCs of this animal at the time of TTQ (Ben-Tabou de-Leon et al., 2013). Hif1α activity is normally associated with a transition to glycolysis and although it is not yet known what function, if any, this transcription factor may have in the quiescence phenotype, it is important to consider that its presence in the PGCs is consistent with a shift in metabolism from oxidative phosphorylation towards glycolysis, consistent with the lack of mitochondrial activity in the PGCs; yet this expression is within a normoxic environment. This protein could help explain the

![Fig. 4. eEF1A mRNA depletion in the PGCs requires Nanos.](image)

(A-P) Fertilized eggs were injected with either a control morpholino or Nanos morpholino, and fixed at blastula stage for eEF1A in situ hybridization (red) followed by an immunofluorescence using Vasa antibody (green). DNA was stained with Hoechst (blue). Images are magnified in the area surrounding the PGCs (E-H for the control morpholino; M-P for Nanos morpholino). (Q) The ratio of eEF1A mRNA present in the PGCs versus the somatic cells was quantified using 13 blastulae for the control morpholino and 11 for the Nanos morpholino, and are presented as percentages. The significance of the differences between the control and the Nanos morpholino was assessed using Student’s t-test (*P<0.001). (A-P) Arrows indicate the PGCs. Approximately 100 embryos were visualized and representative embryos are presented. Scale bars: 20 µm.

![Fig. 5. eEF1A mRNA depletion in the PGCs depends on its PRE.](image)

(A-D) Fertilized eggs were injected with either a control morpholino or a morpholino targeting the eEF1A PRE. Embryos were fixed at blastula stage for eEF1A in situ hybridization (red) followed by an immunofluorescence using Vasa antibody (green). Approximately 100 embryos were visualized and representative embryos are presented. Arrows indicate the PGCs. Scale bar: 20 µm. (E) The ratio of eEF1A mRNA present in the PGCs versus the somatic cells was quantified using 10 blastulae for the control morpholino and 12 for the PRE morpholino, and presented as percentages. Significance was assessed between the control and the PRE morpholino using Student’s t-test (*P<0.005).
acidification of the PGC by glycolytic activity and perhaps even its developmental fate (López-Iglesias et al., 2015).

The quiescence phenotype of the sea urchin PGCs is transient and, once the PGCs end their quiescence, the cellular activity of these cells appears to start up where they left off. In this animal, the timing of quiescence is from about 6 h post-fertilization, when quiescence begins, to about 72 h, when the PGCs leave quiescence and return to cellular activities that approximate their neighbors to restore transcription, translation, mRNA turnover, protein synthesis, cell cycle and mitochondrial activity. Importantly, the cellular activity and developmental fate of these cells is restored by, and maybe even dependent on, this suspended animation.

How do these PGCs transition out of quiescence? Currently it is not clear, but the turnover of Nanos2 protein and mRNA from the PGC occurs at a time when cellular activity is restored. Therefore, it could be a natural turnover of Nanos2 that restores the cellular activity, but we do not know whether any new transcription or translation of Nanos2 occurs following its initial burst shortly after PGC formation. We are currently working on methods to extend the life of Nanos2 in the PGCs to determine whether the quiescence phenotype is prolonged, and if such persistence has any effect on the cell fate of the PGCs, but we do know that removal of Nanos2 is eventually lethal to the PGCs. Perhaps elevated protein synthesis in the PGCs uses up stored resources generally or the aberrantly translated protein includes overexpression of apoptotic machinery that tips the balance towards cell death. It needs to be kept in mind that Nanos is not the only regulator of the phenotype, so that even removal of Nanos2 and an increase in protein synthesis (to 47% that of the somatic cells) is sufficient to induce lethality.

Downregulation of general translation machinery is a new mechanism that helps us to understand how PGCs function in this animal, and how quiescence may be manifest. Transcriptome analyses (Swartz et al., 2014) indicate that several RNAs coding for ribosomal proteins are also depleted from the PGCs, which may contribute to the overall 94% decrease in protein synthesis in the PGCs. The 17% increase in newly synthesized proteins when eEF1A is restored suggests that several other factors involved in protein synthesis are also depleted or changed in the PGCs.

**MATERIALS AND METHODS**

**Animals**

Strongylocentrotus purpuratus adults were housed in aquaria with artificial seawater (ASW) at 16°C (Coral Life Scientific Grade Marine Salt). Gametes were acquired by either 0.5 M KCl injection or by shaking. Eggs were collected in ASW or filtered seawater and sperm was collected dry. Embryos were cultured in filtered seawater at 16°C.

**Nascent protein labeling**

Protein synthesis was visualized using the Click-iT protein synthesis assay kit (Life Technologies) with either OPP, O-propargyl-puromycin (C10457) or HPG, L-homopropargylglycine (C10428). Briefly, the embryos were incubated for 30 min with the OPP used at 1:4000, or HPG used at 1:2000, and fixed with PFA 4% in ASW. The 17% increase in newly synthesized proteins when eEF1A is restored suggests that several other factors involved in protein synthesis are also depleted or changed in the PGCs.
being imaged, or incubated in the blocking buffer for 1 hour at room temperature, and then incubated with a primary antibody overnight at 4°C for immunofluorescence. Images were captured using a LSM 510 laser scanning confocal microscope (Carl Zeiss) and fluorescence was quantified using Metamorph. For each embryo, three cells were quantified per cell type and averaged into a value representing the protein synthesis per cell type and per embryo. Box and whisker plots of statistical analysis were obtained with Excel (using the minimal, the quartile 1, the median, the quartile 3 and the maximal values).

**EdU labeling**
The Click-iT EdU Imaging Kit (C10340) was used to label the PGCs (Life Technologies). The modified thymidine analogue EdU is efficiently incorporated into newly synthesized DNA and subsequently fluorescently labeled. Briefly, embryos were soaked in 10 μM EdU in ASW from fertilization until first cleavage, washed six times with ASW and allowed to develop until the desired stage.

**Immunofluorescence**
Embryos were cultured as described above and samples were collected at indicated stages of development for whole-mount antibody labeling. Immunofluorescence was carried out as described previously (Juliano et al., 2010). For the Nanos2 antibody (as used by Juliano et al., 2010), embryos were fixed in 4% paraformaldehyde (Electron Microscopy Sciences)/ASW for 10 min at room temperature, extracted in 100% methanol (at −20°C) for 1 min, washed three times with PBS-Tween, and stored at 4°C. For Vasa and eEF1A antibodies (as used by Voronina et al., 2008), embryos were fixed in 4% paraformaldehyde/ASW overnight at 4°C, washed three times with PBS-Tween, and stored at 4°C. Embryos were blocked for 1 h in PBS-Tween, 4% sheep serum and incubated overnight at 4°C with the primary antibody. The Sp nanos2 affinity-purified antibody (as used by Juliano et al., 2010) was diluted 1:500. The Sp Vasa affinity-purified antibody (as used by Voronina et al., 2008) directed against its N-terminal domain was diluted to 1:200 (Voronina et al., 2008). eEF1A1 antibody (Abcam, ab175274) was used at a dilution of 1:500. For each immunofluorescence, an anti-rabbit Alexa Fluor 488, an anti-rabbit Alexa Fluor 405 or an anti-rabbit Rhodamine was used as the secondary conjugated antibody (Life Technologies), diluted by 1:500 in blocking buffer, for 2 h at room temperature. Images were captured using a LSM 510 laser scanning confocal microscope (Carl Zeiss). Fluorescence was quantified using Metamorph.

**Western blot**
To test the eEF1A1 antibody, 50 embryos were pelleted for each time point, resuspended in the loading buffer and DTT (Roche; Indianapolis, IN) was added at a final concentration of 5 mM. Samples were incubated at 100°C for 5 min, spun at 14,000 g for 2 min, and then loaded onto Tris-glycine, 4-20% gradient gels (Invitrogen). After transfer to nitrocellulose (Pall Corporation), blots were probed with eEF1A1 antibody diluted to 1:2000 in PBS/0.05%Tween/2%BSA. For visualization, blots were probed with an anti-rabbit-HRP secondary antibody diluted 1:5000 in Blotto (Jackson ImmunoResearch Laboratories) and visualized by standard ECL.

**Whole-mount in situ hybridization**
Whole-mount in situ hybridization was performed as described previously (Juliano et al., 2006). Approximately 1 kb antisense probe template was PCR amplified from cDNA using a reverse primer tailing with the T7 promoter (lowercase letters in primer sequence). The template for eEF1A probe was amplified using the following primers: F, GGTTCTCGAAGCCTGAAGG; R, tautagactcactatagggAGGGAATCGTTTGGCAATG. The template for
the ADP/ATP translocase 1 was amplified using: F, ATGGGCATCGATCAGGAAGTCGTC; R, taatacgactcactatagggTTAAAATACAAGGAGATTCTTG. Digoxygenin-labeled antisense probes were transcribed using the Roche DIG RNA labeling kit according to the manufacturer’s instructions.Embryos were fixed with MOPS-buffered PFA and hybridized for at least 5 days at 50°C with 70% formamide and 0.5 ng/µl probe. Hybridization was then visualized using tyramide fluorescence amplification (TSA plus system, PerkinElmer). A non-specific DIG-labeled RNA probe complementary to neomycin-resistance gene (Roche) was used as a negative control.

Microinjections Microinjections of zygotes were performed as previously described (Cheers and Ettensohn, 2004). In brief, eggs were de-jellied with acidic sea water (pH 5.0) for 10 min, washed with filtered sea water three times, lined up with a mouth pipette onto protamine sulfate-coated 60×15 mm petri dishes, fertilized in the presence of 1 mM 3-AT and injected using the Femto Jet injection system (Eppendorf). Glass capillaries (1×90 mm) with filaments (Narishige; Tokyo, Japan) were pulled on a vertical needle puller for injections (Narishige; Tokyo, Japan). Injected embryos were cultured in ASW at 16°C.

Plasmid constructs and RNA in vitro synthesis For the GFP Sp eEF1A construct, Sp eEF1A ORF was amplified using the following primers surrounded by the SpeI restriction site: F1, 5’-cgactagttcacttctttgc-cagccttctggg-3’; R1, 5’-cgactagttcacttctttgc-cagccttctggg-3’. After digestion with SpeI, this ORF was cloned into a GFP plasmid (Oulhen et al., 2013) containing Sp nanos 5’UTR, GFP ORF followed by Sp nanos 3’UTR ΔGNARLE (enabling the accumulation of the RNA in every cell of the blastulae). Sp eEF1A ORF was fused to the C-terminal of the GFP ORF. Capped sense RNAs were synthesized using the mMessage mMachine T7 Kit (Ambion) yielding RNA concentrations between 0.5 and 2 µg/µl. Injection solutions contained 20% glycerol with 1×10¹² copies of a GFP RNA. Approximately 2 pl of each RNA mixture (~5% of egg volume) was injected into each fertilized egg.

Morpholino approach Morpholinos against Sp nanos2 (gtgactaaagtgcgtggaaactcga) (Juliano et al., 2010) and against the PRE of Sp eEF1A (catacacttgtttccatttacatac) were purchased from Gene Tools. Sp Nanos2 morpholino was injected at 500 µM stock concentration and Sp eEF1A PRE morpholino was injected at 1 mM stock concentration. Morpholino injection solutions include 20% glycerol and 1 mM 10,000 MW dextran conjugated to Texas Red (Life Technologies). A non-relevant morpholino against the sea star Patiria miniata dysferlin (5’-cgacacaatcatacagacat-3’) was used as a control.

CRISPR/Cas9 approach The plasmid pCS2-3xFLAG-NLS-SpCas9-NLS (a gift from Yonglong Chen, Chinese Academy of Sciences; Addgene plasmid #51307) was linearized with NdeI and transcribed with SP6. Four gRNA templates directed against Sp Nanos2 were designed according to CRISPRscan priorities (CRISPRscan.org): Sp nanos2.190 (taatacgactcactataGGTGACTGGCTCGTCGAGACgttttagagctagaa), Sp nanos2.250 (taatacgactcactataGGGATCTCAGCGATGTTCAGgttttagagctagaa), Sp nanos2.295 (taatacgactcactataGGAGGAAGGCAGCCAACAAgttttagagctagaa) and Sp nanos2.319 (taatacgactcactataGGAGGTGGTGCTACGGGTGTgttttagagctagaa). The gRNAs were synthesized by T7 RNA polymerase using the MegaShortScript T7 transcription kit (AM1354, ThermoFisher) and purified using the miRNeasy mini kit (217004) (Qiagen). These RNAs were mixed
(400 ng/μl of each gRNA and 500 ng/μl of Cas9 mRNA), injected into freshly fertilized eggs and cultured as described previously (Cheers and Ettenson, 2004). The genomic DNA of injected eggs was extracted with 10 μl of QuickExtract DNA Extraction Solution (http://www.epibio.com/), according to the manufacturer’s instructions.

Mitochondria analysis
The abundance of the mitochondria was tested using the MitoTracker Green FM (M7514, ThermoFisher Scientific): a green-fluorescent mitochondrial stain that appears to localize to mitochondria regardless of mitochondrial membrane potential. The mitochondrial activity was observed using the MitoTracker Red CMXRos (M7512, ThermoFisher Scientific): a red fluorescent dye, the accumulation of which depends upon the membrane potential. For each stage, embryos were incubated with 200 nM of MitoTracker green or MitoTracker Red for 15 min at 16°C.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
N.O. and G.M.W. designed the experiments and wrote the paper. N.O. injected morpholinos and RNAs, measured translational activity with OPP and HPG, and tested the mitochondrial activity. S.Z.S. and J.L. did the in situ hybridization for eEF1A RNA. J.L. tested the eEF1A antibody. A.M. helped to test and quantify the quiescence in the PGCs.

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