Received: 21 November 2022 | Accepted: 18 March 2023

DOI: 10.1002/mrd.23684

RESEARCH ARTICLE

Molecular Reproduction & Development

# Functional annotation of a hugely expanded nanos repertoire in *Lytechinus variegatus*, the green sea urchin

**Cosmo Pieplow** | **Gary Wessel**

MCB Department, Division of Biomedicine, Brown University, Providence, Rhode Island, USA

**Correspondence**
Gary Wessel, MCB Department, Division of Biomedicine, Brown University, Providence RI 02912, USA.
Email: rhet@brown.edu

**Funding information**
National Institutes of Health, Grant/Award Number: 1R35GM140897

## Abstract

Nanos genes encode essential RNA-binding proteins involved in germline determination and germline stem cell maintenance. When examining diverse classes of echinoderms, typically three, sometimes four, nanos genes are present. In this analysis, we identify and annotate nine nanos orthologs in the green sea urchin, *Lytechinus variegatus* (Lv). All nine genes are transcribed and grouped into three distinct classes. Class one includes the germline Nanos, with one member: Nanos2. Class two includes Nanos3-like genes, with significant sequence similarity to Nanos3 in the purple sea urchin, *Strongylocentrotus purpuratus* (Sp), but with wildly variable expression patterns. The third class includes several previously undescribed nanos zinc-finger genes that may be the result of duplications of Nanos2. All nine nanos transcripts occupy unique genomic loci and are expressed with unique temporal profiles during development. Importantly, here we describe and characterize the unique genomic location, conservation, and phylogeny of the Lv ortholog of the well-studied Sp Nanos2. However, in addition to the conserved germline functioning Nanos2, the green sea urchin appears to be an outlier in the echinoderm phyla with eight additional nanos genes. We hypothesize that this expansion of nanos gene members may be the result of a previously uncharacterized L1-class transposon encoded on the opposite strand of a nanos2 pseudogene present on chromosome 12 in this species. The expansion of nanos genes described here represents intriguing insights into germline specification and nanos evolution in this species of sea urchin.

**KEYWORDS**
germ line, Nanos, sea urchin

## 1 | INTRODUCTION

Germline specification mechanisms are of great interest to fields stretching from reproduction and development to cancer biology (Ilaslan et al., 2022; Oulhen et al., 2022; Tsuda et al., 2003; Zhang et al., 2020). A paradigm of germline formation is tight translational regulation of somatic genes within the specified primordial germ cells (Oulhen et al., 2017; Venkatarama et al., 2010). One of the most well-known and conserved germline regulators is the Nanos protein (Bhat, 1999; Hansen & Pelegri, 2021; Lai et al., 2012; Oulhen & Wessel, 2014; Ponz-Segrelles et al., 2018). Nanos proteins are a group of well-characterized zinc-finger domain RNA-binding proteins that function in translational repression of specific messenger RNA (mRNA) transcripts (Hansen & Pelegri, 2021; Oulhen & Wessel, 2014). Nanos proteins regulate the translation of target mRNAs through interaction with its binding partner, pumilio, which functions in concert to bind to sequence-specific elements of somatic cell mRNAs (Dahanukar et al., 1999; Deshpande et al., 2012; Jaruzelska

et al., 2003; Parisi & Lin, 2000; Sonoda & Wharton, 1999). The nanos/pumilio complex recruits proteins to either degrade the target mRNA or to repress translation of that target mRNA bearing an NRE (Nanos response element) in the 3′-untranslated region (3′-UTR), protecting the germline fate of the aforementioned cell (Jaruzelska et al., 2003; Kadyrova et al., 2007; Lai & King, 2013; Parisi & Lin, 2000; Sano et al., 2001; Sonoda & Wharton, 1999). Thus, Nanos genes largely, but not uniquely, function only within the germline, often a result of tight posttranscriptional regulation (Oulhen et al., 2022). The fidelity of this tight posttranscriptional regulation of Nanos is critical for events including early development (Irish et al., 1989), germline formation (Juliano et al., 2010), and even tumor formation (Ilaslan et al., 2022; Janic et al., 2010; Zhang et al., 2020).

For such critical regulatory impact, the size of the nanos gene family is often relatively small, with Drosophila only having one nanos gene (Kobayashi et al., 1996). While insects typically have a single nanos gene, the silkworm moth has four, more resembling deuterostome clades in this regard (Nakao et al., 2008) normally with three or four nanos genes, and deployed in a tissue-, time-, or sex-specific manner. Zebrafish have three nanos genes (Beer & Draper, 2013; Köprunner et al., 2001), the purple sea urchin has three (Juliano et al., 2006, 2010), and humans (Julaton & Reijo Pera, 2011) and mice also have three members: Nanos1, Nanos2, and Nanos3, respectively (Haraguchi et al., 2003; Jaruzelska et al., 2003; Julaton & Reijo Pera, 2011; Kusz et al., 2009; Lolicato et al., 2008). Finally, we come to *Lytechinus variegatus* (Lv) the green sea urchin, which appears to be an anomaly with nine nanos members. Most intriguing is the timing and pattern of expression of these nanos genes, which appear to have tight transcriptional and posttranscriptional regulation essential to prevent aberrant nanos activity, which may be lethal (Lai & King, 2013; Lai et al., 2012).

## 2 | MATERIALS AND METHODS

### 2.1 | RNA-seq expression analysis

RNA-sequencing (RNA-seq) analysis was performed previously on the gonads of adult Lv urchins of both sexes, three ovaries and three testes were dissected and lysed in Trizol. RNA extracts were sent for poly-A selection, library preparation, and sequencing through Novogene (Supporting Information: Figure S1). Primary gene expression analysis was performed using the DESEQ2 pipeline (Liu et al., 2021; Love et al., 2014).

For time-course analyses of *L. variegatus* through development, RNA-seq data sets from Hogan et al. (2020) were accessed from and used in a secondary DESEQ2 analysis. 2 analysis (data not shown in this publication). All data were accessed via ENA Browser, Project: PRJNA554218 (Hogan et al., 2020). Differential expression analysis was performed as described above, and the results produced a matrix of expression profiles for each stage. For graphs of normalized gene expression across developmental time, we compared discrete developmental stages: early cleavage, zygotic genome activation, gastrulation, larval development, larval growth, and metamorphosis and plotted normalized counts corresponding to these developmental time points.

### 2.2 | Identification of nanos orthologs

Ten nanos orthologs were first identified through a scan of an RNA-seq gene expression data, using the Greg Wray (2020) Lv Genome rerelease (Davidson et al., 2020). Upon searching for Nanos2 within the data set, 10 genes were returned, all named "Sp-Nanos," with both "zinc-finger nanos" UniProt IDs and "RNA binding" as associated gene ontology terms. Each transcript was then blasted to the genome and its chromosomal position was noted (Supporting Information: Figure S4). Of the 10 originally identified, 9 returned unique genomic loci. The two sharing a single genomic location were LV_19334 and LV_19343, one transcribed in the (+) orientation and the other (−) orientation, respectively.

After identification and genomic location mapping, each of the 10 transcripts was compared to the Nanos genes of related echinoderm species using multiple sequence alignments (PRALINE, Clustal Omega), protein structure predictions, and closest-neighbor phylogenetic tree analyses. Naming conventions follow those used in *Strongylocentrotus purpruatus*, as referenced on Echinobase (https://www.echinobase.org/).

### 2.3 | Orthology analysis

All transcripts were first translated into associated peptide sequences with ExpasY translate (https://web.expasy.org/translate/). Amino acid (AA) sequences were then aligned using PRALINE multiple sequence alignment software (https://www.ibi.vu.nl/programs/pralinewww/) to gather AA identity when compared with the three known Nanos genes in *S. purpuratus*.

PRALINE parameters are as follows: 3 PSI-blast iterations, BLOSUM62 Matrix for AA substitution, PSIpred secondary structure prediction, define secondary structure of proteins (Kabsch and Sander, 1983; Touw et al., 2013)-defined secondary structure search enabled, and 0.01 *E*-value cutoff. AA substitution scores are presented as a color scale with each figure, where red = no change or perfect substitution score (5–11) and blue = worst possible substitution score (−4). Conservative AA substitutions are shown in yellow, orange, or red coloration, while nonconservative mutations appear green or blue.

For mRNA sequence alignments, ClustalOmega software was used to align sequences for alignments of possible 5′- and 3′-UTRs. (https://www.ebi.ac.uk/Tools/msa/clustalo/).

### 2.4 | Closest-neighbor phylogenetic tree of Sp and Lv nanos orthologs

Closest-neighbor clustering was performed on peptide sequences of all Lv nanos genes using A-la-Carte methods (https://ngphylogeny.fr/).

First, peptide sequences were aligned using Clustal Omega, alignments were curated further by block mapping and gathering with entropy (BMGE) processing, phylogenetic tree was constructed with PhyML software to generate a Newick tree of closest-neighbor proteins, and was rendered using Newick display. Scalebar represents arbitrary units of sequence divergence used to generate the branch lengths. *Patiria miniata* nanos sequences gave sufficient evolutionary distance to predict a root, which is shown with a dotted line.

### 2.5 | Protein structure prediction

AA sequences were first generated by translating mRNA using Expasy translate. AA sequences were annotated using InterProScan software (Mulder & Apweiler, 2007). Significant protein domain scan results were generated into figures using Adobe Illustrator. All peptides are drawn to scale.

### 2.6 | qPCR analysis for gonads

For each quantitative polymerase chain reaction (qPCR) of gonads, $N = 4$ testes and $N = 8$ ovaries were dissected and lysed in Trizol for RNA extraction. $N = 2$ ovaries corresponding to each stage: stage I, stage II, stage III, and stage IV were selected as there is great variability in gene expression in ovaries depending on the stage. $N = 2$ nongravid testes (stages I and II) and $N = 2$ gravid (stages III and IV) testes were selected for RNA extraction due to the lack of variability in testes expression samples as observed in previous RNA-seq experiments (data not shown). Complementary DNA was generated from 800 ng of DNAse-treated RNA, and qPCR was performed as published previously (Pieplow et al., 2021). For gene expression normalization: expression was first normalized to a ubiquitin expression control, and then differential cycle thresshold (DCT) values were then normalized across samples. Each plot point represents expression normalized to ubiquitin of each sample, normalized to the total relative amount of transcript detected in all gonads, and averaged across ($N = 4$) testes or ($N = 8$) ovaries. For Nanos1b expression, where large variation was observed, individual ($N = 2$) points are plotted for the ovary samples according to the stage.

### 2.7 | qPCR for embryos

For each qPCR time-course experiment, multiple time points across normal development were selected; all protocols were used as previously published (Pieplow et al., 2021). $N = 600$ embryos were lysed, RNA was extracted, and cDNA was generated to 300 ng of isolated RNA for each time point of interest: oocyte, morula, mesenchyme blastula, gastrula, prism, pluteus larva, ovary, and testis. For a direct comparison to *S. purpuratus* normal Nanos expression, stages were selected which corresponded to major developmental events, rather than hours postfertilization. For gene expression normalization, the expression was first normalized to a ubiquitin expression control, and thenDCT values were normalized across developmental time (Supporting Information: Table S1). Expression is plotted as a percentage of total expression where timepoints with more abundance of transcripts are a larger proportion of the *Y*-axis than those expressed at lower levels.

### 2.8 | Whole-mount in situ hybridization

Due to the lack of sequence difference between Lv Nanos1 and Nanos2 orthologous transcripts, the generation of sufficiently large and unique WMISH probes proved challenging. For this reason, a probe to the unique region of Lv_05060 (Nanos2), Lv_19628 (Nanos3-like), and Lv_19334 (Transposase) was generated (Supporting Information: Table S2), as these transcripts had the largest spans of unique sequences, and would, therefore, yield unique probes for hybridization. Hybridizations were performed to clarify expression patterns at three stages of development: early, mid, and late gastrula stage embryos. Embryos were fixed and hybridized as described previously (Arenas-Mena et al., 1998). The signal was developed with anti-digoxigenin AP fab fragments with NBT/BCIP. A table of WMISH primers used is supplied in the supplement.

## 3 | RESULTS

While analyzing germline gene expression in the gonads of sexually mature Lv sea urchins, we were puzzled to obtain 10 transcripts with the associated gene name "nanos" (Davidson et al., 2020) after filtering for germline transcript enrichment (Supporting Information: Figure S1). From our RNA-seq results, we then characterized these 10 transcripts, initially annotated as "zinc-finger nanos." Our first finding was that 9 of the 10 returned a bona fide Nanos protein after translation and protein structure prediction analysis (Supporting Information: Data). When examining all the echinoderm species that have been sequenced thus far, most have three, at most four, nanos genes in their genome, making the existence of nine in Lv a striking anominally (Figure 1). Currently, all identified nanos genes in echinoderms fall under two classes: Nanos1 and Nanos2 genes, which are nearly identical in sequence and structure, but have different expression patterns; and Nanos3 genes, with significant sequence divergence. The Japanese urchin *Hemicentrotus pulcherrimus* (Hp), and the purple sea urchin, *Strongylocentrotus purpuratus* (Sp), two sea urchin species of the class Echinoidea, each having three nanos genes: Nanos1, Nanos2, and Nanos3. Lv represents a significant divergence from this norm with nine (Figure 1).

### 3.1 | Nine nanos transcripts occupy unique genomic positions

We first asked whether the transcripts all mapped to a single locus in the genome, pointing to simple splice variants, or if they each

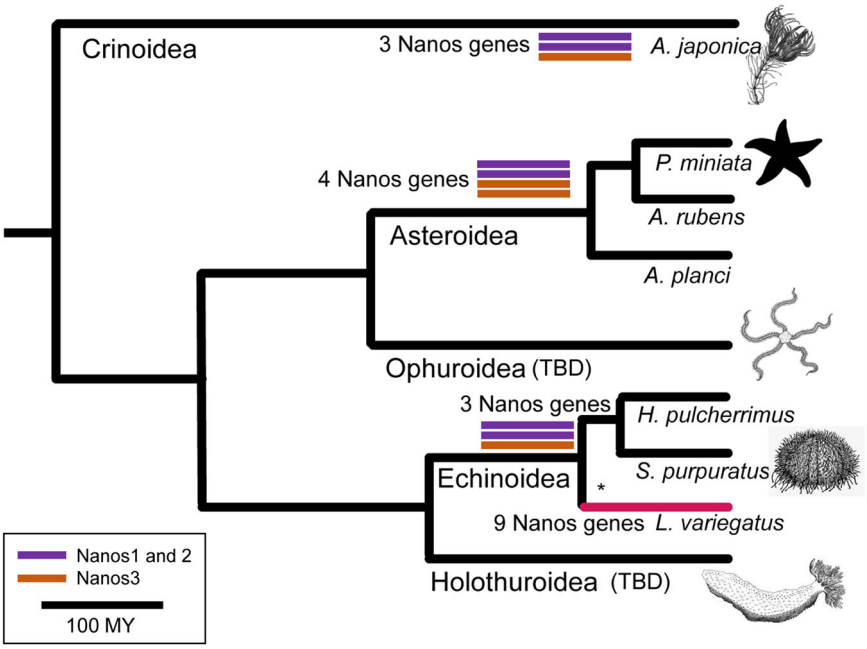



**FIGURE 1** Phylogenetic tree of echinoderm Nanos genes. All extant species of echinoderms with available and annotated genomes were searched for Nanos gene loci. Scale of phylogenetic tree = evolutionary distance, MYA. Orange = Nanos1 or Nanos2 gene; purple = Nanos3 gene. Crinoids have three nanos genes. Asteroids: Pm, Ar, and Ap all have four nanos genes, see Supporting Information: Data for expansion of identification of Pm Nanos. *Hemicentrotus pulcherrimus* (Hp) and *Strongylocentrotus purpuratus* (Sp) sea urchins have three nanos genes. *Lytechinus variegatus* (Lv) represents an outlier with nine nanos genes (pink line). TBD; yet to be determined.

occupied unique positions in the genome, requiring a deeper explanation for the putative duplications. Each Lv nanos transcript encodes a relatively small protein (108–297 AA), as is normal for these proteins. While Sp Nanos genes are known to be single-exon genes, splice variations posed a simple explanation for the nine transcripts (Oulhen et al., 2017). Unlike their Sp orthologs, the Lv nanos genes were not exclusively single exon genes and they had some variations in intron–exon boundaries across each genomic position. However, the genomic positions of each of these exons and introns were unique for each associated transcript ID (with one exception), meaning they were unique gene products and not simply an abundance of splice variants (Supporting Information: Data). Unsurprising, then, was the finding that each of the nine nanos transcripts mapped to unique genomic loci, often on entirely different chromosomes (Figure 2a).

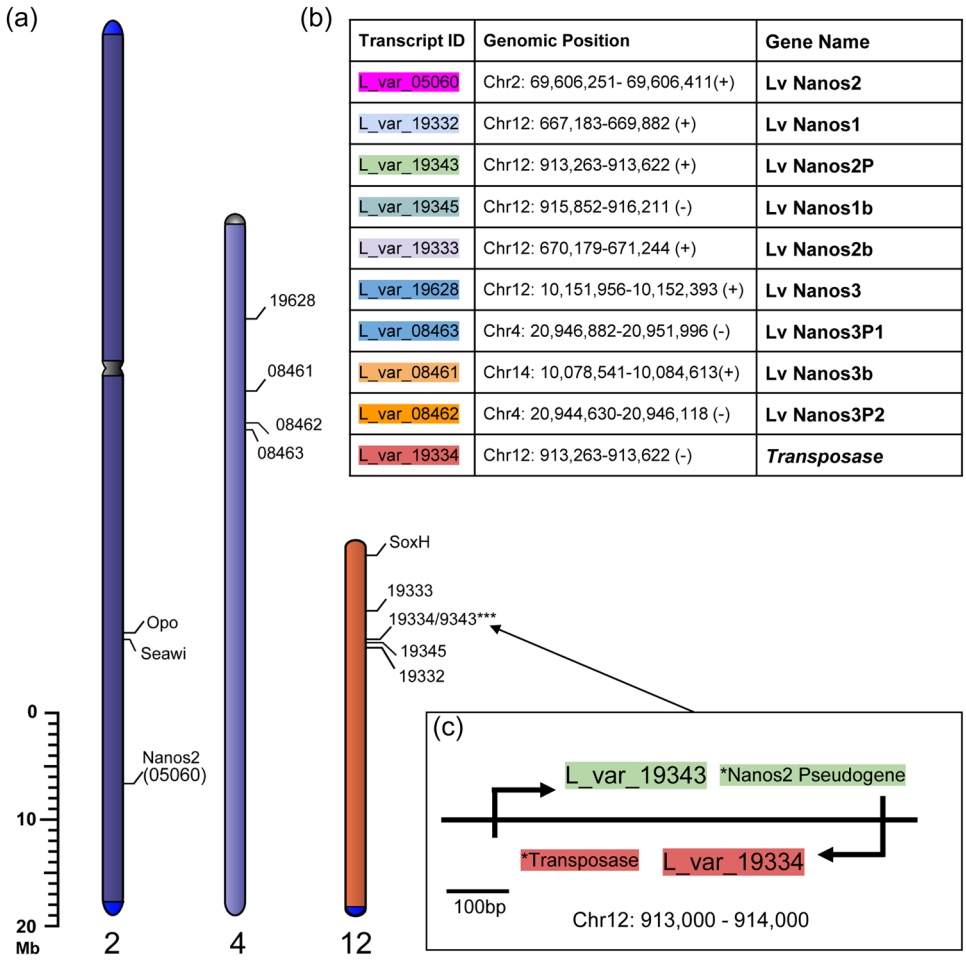
The Nanos2 homolog (Lv_05060) occupies a unique locus on chromosome 2, the same chromosome containing the genomic loci of other germline genes such as ovoperoxidase (Deits et al., 1984) and seawi (Rodriguez et al., 2005). Several of the nanos transcripts (Lv_19628, Lv_08461, Lv_08462, and Lv_08463) are spread across chromosome 4, with 08462 and 08463 occupying very small regions, tightly clustered together (Figure 2a). The remaining nanos genes (Lv_19333, Lv_19345, and Lv_19332) all occupy a similar region within chromosome 12 (Figure 2a). For clarity, multiple sequence alignment data has been integrated comparing all these transcripts to their orthologs, and a table summarizing their gene names and associated unique genomic positions has been provided (Figure 2b). Nanos genes thought to be pseudogenes, or simply a by-product of duplications or rearrangements, have been given the naming convention "P."

The one exception to this finding was the 10th transcript, an L1 transposase (Lv_19334), which occupies the exact same genomic locus as the nanos gene Nanos2P (Lv_19343) on chromosome 12, but in an antisense orientation (Figure 2c). It appears that depending on RNA polymerase II activity, this gene locus can produce a zinc-finger nanos transcript in (+)-orientation, or a transposase if transcribed antisense. It is also important to note that this transposase, but not Nanos2P, maps to three other locations in the Lv genome, on entirely different chromosomes (Supporting Information: Data).

## 3.2 | Nine nanos structure, orthology, and naming

After mapping all nine transcripts across the genome, we performed multiple sequence alignments (Supporting Information: Data) and protein domain prediction (Figure 3) for all nine Lv nanos genes. These protein predictions were juxtaposed with three well-characterized Nanos proteins from a closely related sea urchin species, Sp for comparison. The resultant alignments and predictions were grouped into three clusters: Nanos1 and Nanos2, Nanos3, and possible pseudogenes (Figure 3).

The first cluster contains the Sp Nanos1 and Nanos2 proteins, which are the essential germline fate genes normally expressed in early oocytes within the ovary and early embryos, respectively (Juliano et al., 2010). While it is significantly smaller than Sp Nanos2, the direct homolog is thought to be Lv Nanos2 or Lv_05060, based on three observations: sequence identity, protein structure, and domain similarity, and 3′-UTR conservation (Figure 3 and Supporting Information: Data) (Oulhen et al., 2022; Satoh et al., 2022; Wessel et al., 2021). Also in this first cluster are Lv Nanos2b, Lv Nanos1, and Lv Nanos1b (Figure 3a). These three Nanos2 and Nanos1 orthologs each have unique zinc-finger duplications and structural rearrangements that differentiate them, but they are named as such because they still maintain the most sequence similarity to Sp Nanos1 and Nanos2.




| Transcript ID | Genomic Position | Gene Name |
|---|---|---|
| L_var_05060 | Chr2: 69,606,251- 69,606,411(+) | **Lv Nanos2** |
| L_var_19332 | Chr12: 667,183-669,882 (+) | **Lv Nanos1** |
| L_var_19343 | Chr12: 913,263-913,622 (+) | **Lv Nanos2P** |
| L_var_19345 | Chr12: 915,852-916,211 (-) | **Lv Nanos1b** |
| L_var_19333 | Chr12: 670,179-671,244 (+) | **Lv Nanos2b** |
| L_var_19628 | Chr12: 10,151,956-10,152,393 (+) | **Lv Nanos3** |
| L_var_08463 | Chr4: 20,946,882-20,951,996 (-) | **Lv Nanos3P1** |
| L_var_08461 | Chr14: 10,078,541-10,084,613(+) | **Lv Nanos3b** |
| L_var_08462 | Chr4: 20,944,630-20,946,118 (-) | **Lv Nanos3P2** |
| L_var_19334 | Chr12: 913,263-913,622 (-) | ***Transposase*** |

**FIGURE 2** Genomic loci and chromosome maps of the Nanos genes in *Lytechinus variegatus* (Lv). Ten transcripts in the genome of Lv with the annotation "Nanos." (a) Chromosome maps displaying transcript ID and relative location of each gene, Asterisks represent location with two transcripts at the same location. (b) Table summarizing transcript ID, unique genomic position, and new gene name following naming conventions for echinoderms. Note that the transposase and Nanos2P occupy the same genomic location, but in antisense orientation. (c) Zoom-in of the genomic locus of Nanos2P and L1 transposase with associated transcript ID's. Scale is given in bp.

The second cluster of Lv nanos transcripts shares the most sequence identity with Sp Nanos3, a Nanos gene that is most abundantly expressed in the testes of Sp (Figure 3). Other investigators report evidence that they may play a role in the identity of spermatogonial stem cells (Lolicato et al., 2008). However, in Lv, we hesitate to classify these as the orthologs of the Sp "testes" genes, as there are significant differences in layout and structure. Lv Nanos3-like and Nanos3b have been so named because they share the most significant sequence similarity (Supporting Information: Data) to Sp Nanos3, but both have protein profiles more like an inverted Nanos2. In this same group are possible pseudogenes: Nanos3P1 and Nanos3P2, with duplicated zinc fingers and confusing peptide prediction results, imbuing Nanos3P1 with a possible signal peptide domain, neither of which are observed in the Sp Nanos3 protein (Figure 3b).

The final nanos grouping includes the Nanos2P pseudogene, and it is associated with L1 transposase, which occupies the same genomic position in the (−)-orientation. Interestingly, it was impossible to generate even small product-producing qPCR primers to Nanos2P as its mRNA sequence did not have any regions with significant sequence dissimilarity to the other eight transcripts. For this reason, it appears that Nanos2P is a pseudogene by-product of Nanos2, with tripled zinc-finger motifs as shown (Figure 3c). The transposase is also shown here for reference, with coiled-coil and RRM domains, typical of an L1 transposase.

## 3.3 | Evolutionary analysis of sequences

To determine the sequence divergence of Lv Nanos proteins as they relate to other species, a phylogenetic analysis was pursued comparing the Lv Nanos peptides to the protein sequences of Nanos genes from *S. pururatus* and *H. pulcherrimus*, with those from *P. miniata* as the most divergent (Fresques et al., 2016;

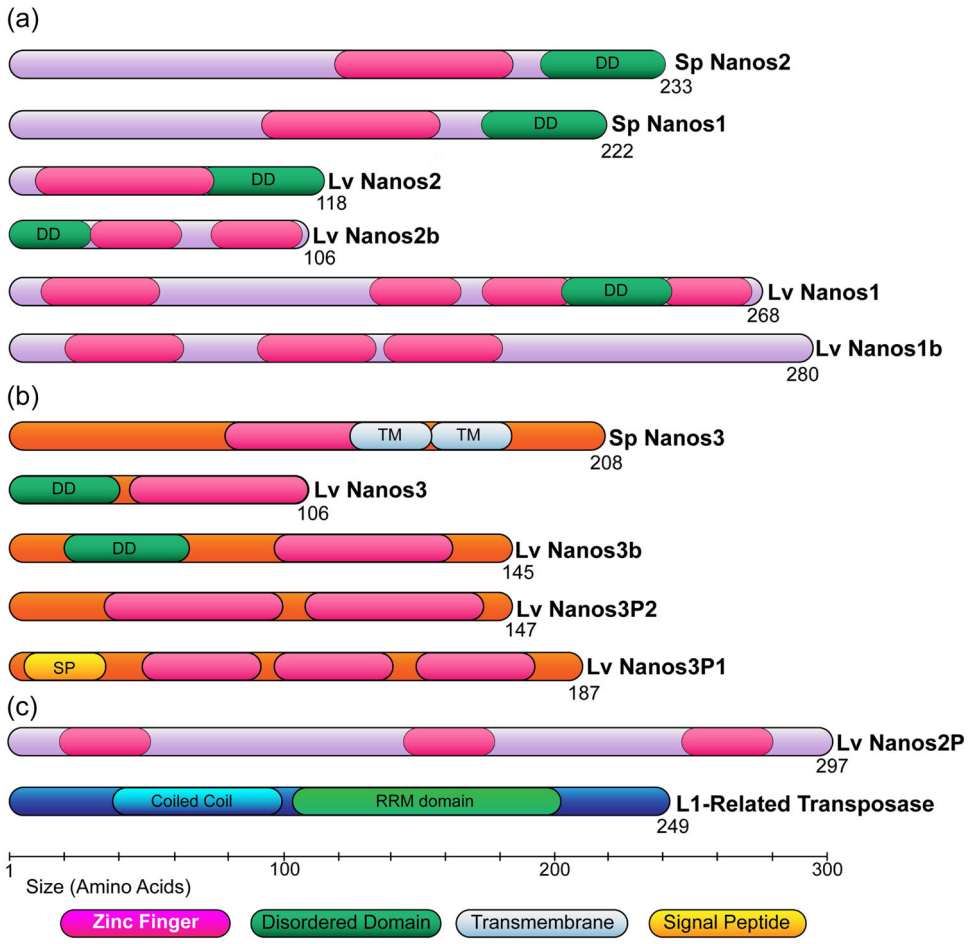



**FIGURE 3** Nanos peptide annotations and structure prediction. Note that a nanos zinc-finger domain and disordered domains are typical features of all the nanos genes in both species of sea urchin. (a) Cluster one of Nanos peptides, *Strongylocentrotus purpuratus* (Sp) Nanos2 (embryonic and ovarian expression) and Nanos1 (Only ovarian expression), *Lytechinus variegatus* (Lv) Nanos2 (both embryonic and ovarian expression), Lv Nanos2b, Lv Nanos1, and Lv Nanos1b. (b) Cluster two of nanos genes, Sp Nanos3 (expressed in the testis), Lv Nanos3-like, and Lv Nanos3b. Two possible pseudogenes: Lv Nanos3P2 and Lv Nanos3P1. Note that the Lv Nanos3 orthologs do not have the transmembrane domains and note that Lv Nanos-like and Lv Nanos3b both resemble Nanos2, but in an inverted configuration. This cluster shared the most significant sequence similarity with Sp Nanos3 and was named as thus. (c) Nanos 2P, a possible pseudogene, with L1 transposase shown for reference. (Pink) Nanos zinc finger, (green) disordered domain, (pale blue) transmembrane, and (yellow) signal peptide. Amino acid length scale at the bottom.

Juliano et al., 2006). The Nanos phylogeny produced two significant gene clusters in Lv, which echo those observed in the peptide alignments (Figure 4). The first cluster includes the Lv Nanos-3-like genes and possible Nanos3 pseudogenes, which cluster with Sp Nanos3 as being significantly divergent from other nanos orthologs in other species (Figure 4a). Lv Nanos2 maintains a unique position, apart from the other Nanos1- and Nanos2-like genes in Lv, as well as those in Sp and Hp. A second cluster: Lv Nanos1 and Lv Nanos1b and Lv Nanos2b and Lv Nanos2P, all cluster in a sister group to the Hp and Sp Nanos1 and Nanos2 genes. Pm Nanos genes represent enough evolutionary divergence to allow for a predicted root, as shown by a dotted line. Overall, these representative sequences are representing 500 MY of Nanos protein evolution (Figure 4).

## 3.4 | Nine nanos transcripts are expressed in gonads

Because these nanos genes were identified during analyses of gonad transcripts, we then validated their expression in gonads with qPCR. First, we compared the expression of the Nanos2 and Nanos1 orthologs between ovaries and testes ($N = 8$, $N = 4$, respectively). We found that the Nanos2 orthologs: Nanos2 and Nanos2b were detectable in gonads, though there were no differences based on sex (Figure 5a). Nanos1 and Nanos1b were most abundant in ovaries, with Nanos1b having significant variation in expression based on the stage of the ovary analyzed. Nanos1b expression levels are low in stage I ovaries that have little to no oocytes, and reached its peak in stage IV ovaries, which contain the most mature eggs and least

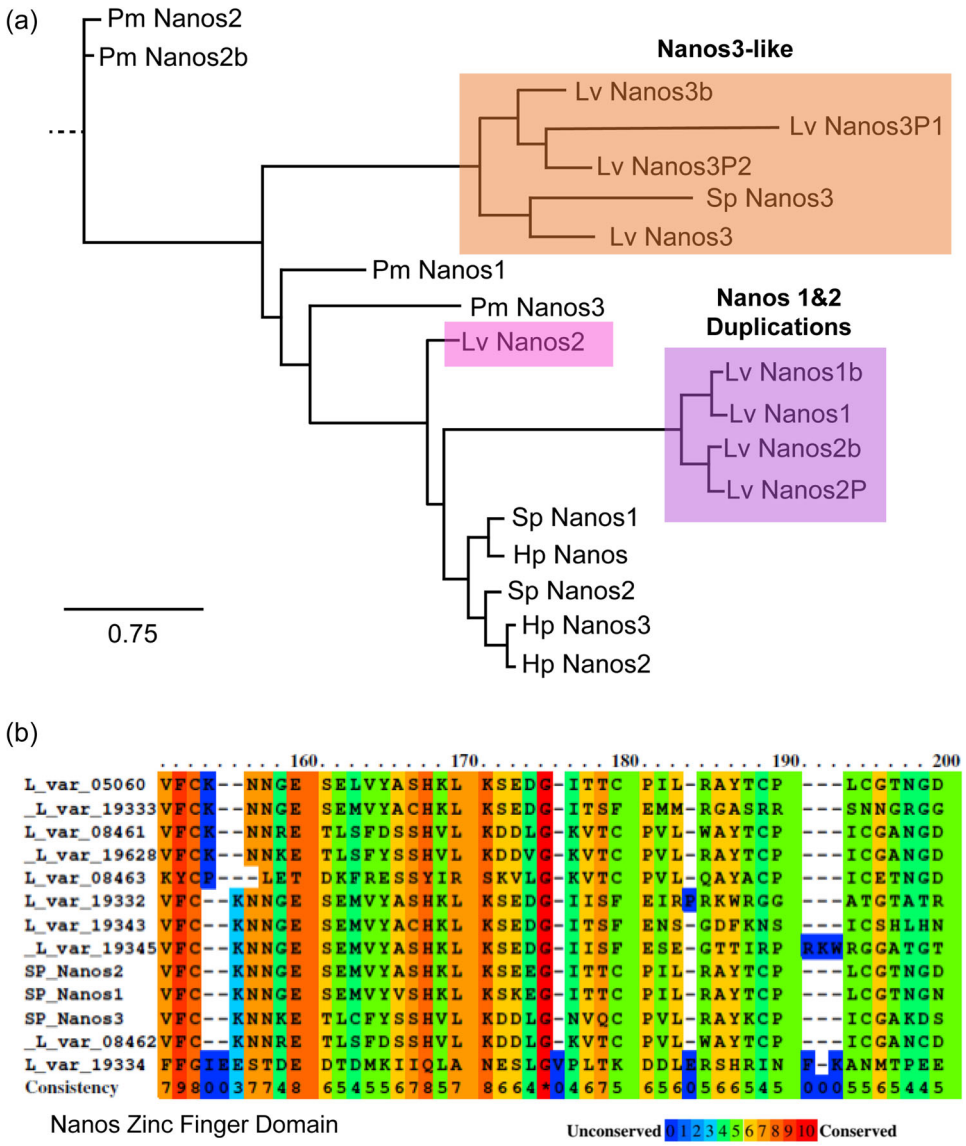



**FIGURE 4** *Lytechinus variegatus* (Lv) Nanos sequence conservation with *Strongylocentrotus purpuratus* (Sp) orthologs. (a) Closest-neighbor phylogenetic tree of Sp, *Hemicentrotus pulcherrimus* (Hp), Pm and Lv nanos orthologs. Predicted root is shown with a dotted line; scale represents sequence divergence (arbitrary units) to construct branch length. (b) Peptide alignment scores within the nanos zinc-finger domains of the Lv and*Strongylocentrotus purpuratus* (Sp) nanos genes.

immature oocytes (Figure 5b). These data support the contention that the Lv Nanos2 and Nanos1 orthologs are similar in expression as well as sequence. Note that the Lv Nanos2P did not have significant mRNA sequence divergence to generate qPCR primers and was not assayed in these analyses.

The expression patterns of the Nanos3 genes were surprising. While they were more abundantly expressed in testes than the Lv Nanos1 and Nanos2 genes (as is reported for the Nanos1 and Nanos2 orthologs in Sp as well), they were most abundantly expressed in ovaries regardless of the stage (Figure 5c). Finally, the Nanos3P genes were analyzed across ($N = 3$) individual gonad samples, as there was wide variation in their expression between individual gonads (Figure 5d). While there is a detectable expression of these Nanos "P" genes, we find it neither consistent nor abundant in the gonads, regardless of sex, and therefore supports our idea that these are likely pseudogenes.

## 3.5 | L1 transposase is expressed at the same time as Nanos2, but in different cells

After assaying gonad expression, we further validated the embryonic and larval expression of all Lv nanos transcripts. The strange antisense L1 transposase only registers as a single point of expression in RNA-seq data at the thickened vegetal plate/late blastula stage (Supporting Information: Figure S7), but due to its significant sequence dissimilarity, we sought to resolve its temporal and spatial expression with qPCR as well as whole-mount in situ hybridizations (WMISH) for two reasons:

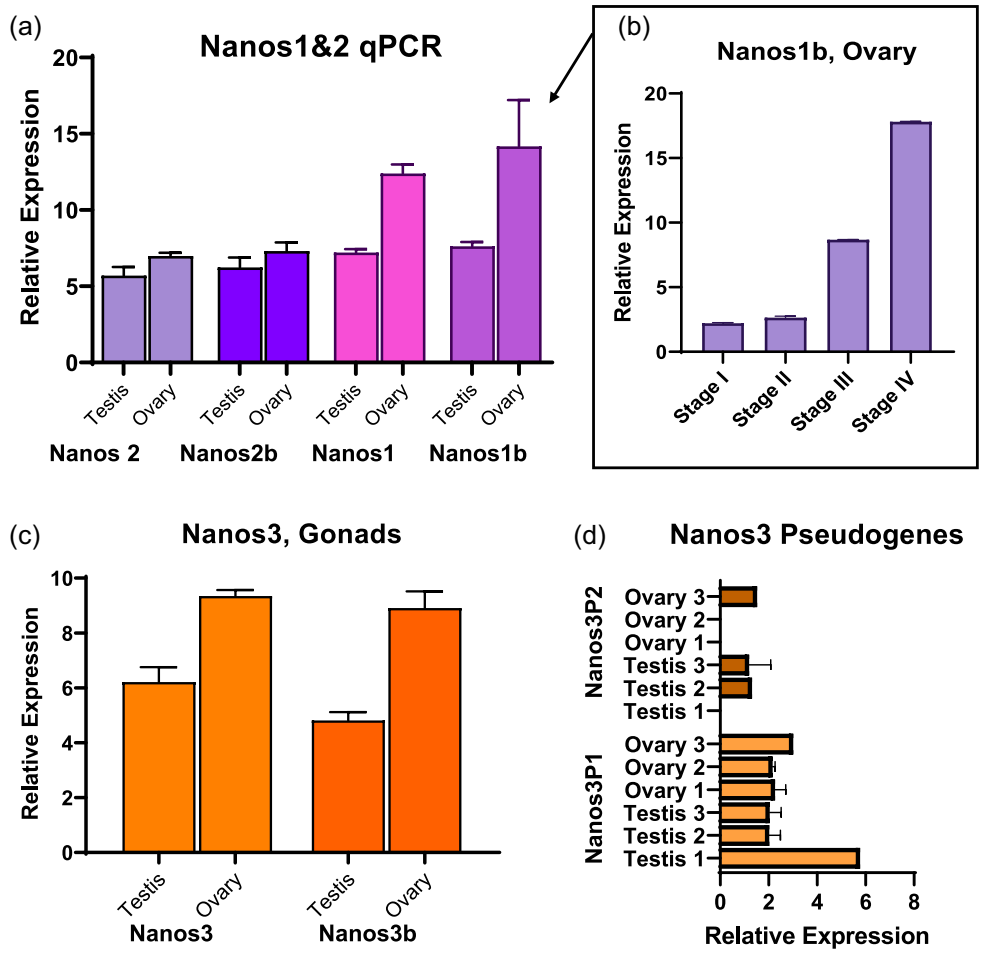



**FIGURE 5** Gonadal expression of *Lytechinus variegatus* (Lv) Nanos genes. All normalized to ubiquitin control. (a) Expression plots of Nanos2 and Nanos1 genes from $N = 4$ testes and $N = 8$ ovaries. Nanos 1b has a significant variation across ovary samples. (b) Expression of Nanos1b at each ovarian stage, $N = 2$ ovaries per stage. (c) Nanos3 expression in gonads; note that both Nanos3 orthologs are more abundantly expressed in ovaries, as is not the case for *Strongylocentrotus purpuratus* (Sp) Nanos3. (d) Possible pseudogenes Nanos3P2 and Nanos3P1 show variable expression across multiple ovary and testis samples. qPCR, quantitative polymerase chain reaction.

First, the occupation of a single locus by two very different gene products (a zinc-finger germline granule component, and a transposase) is something very rarely observed in animals (Canapa et al., 2015). The overlapping gene expression, both temporally and spatially, implicates evidence of a possible gene duplication through transposable element activity on the chromosomes of Lv.

Second, while it is bizarre, it is not outside the realm of possibility that an L1 transposase, observed three times on three separate chromosomes, may have copied a fragment of the Nanos gene into a nonfunctional genomic locus several times (Khazina & Weichenrieder, 2009). To date, no other sea urchin species have been observed to have an associated transposon occupying the same locus as a nanos gene.

When examining the expression of the L1 transposase with qPCR, we not only found it to be expressed embryonically but also found its expression pattern to be like that observed for Nanos2, with the exception that it is not maternally loaded (Figure 6a). As the transition from blastula to larva (gastrulation) is the time that Nanos2 expression is most tightly regulated, and the times Nanos2 expression is most abundant—we performed WMISH using an antisense probe to the transposase at these same time points. We found it to be abundantly expressed, vegetally enriched, and expressed in nonskeletogenic cells within the blastocoel at these stages (Figure 6b). Nanos2 in Lv has identical expression timing and abundance to that of Sp-Nanos2, with the exception that it is most abundantly expressed in the ovaries (Figure 6c). A WMISH of gastrula transitioning embryos showed normal Nanos 2 expression patterns, with a significant overlap to the transposase at the early gastrula stage (Figure 6d).

## 3.6 | Developmental expression of nanos orthologs

Finally, we validated the temporal expression profiles of the remaining nanos genes in embryos using qPCR. Nanos2b resembles no Sp Nanos gene in temporal or tissue expression, with the most

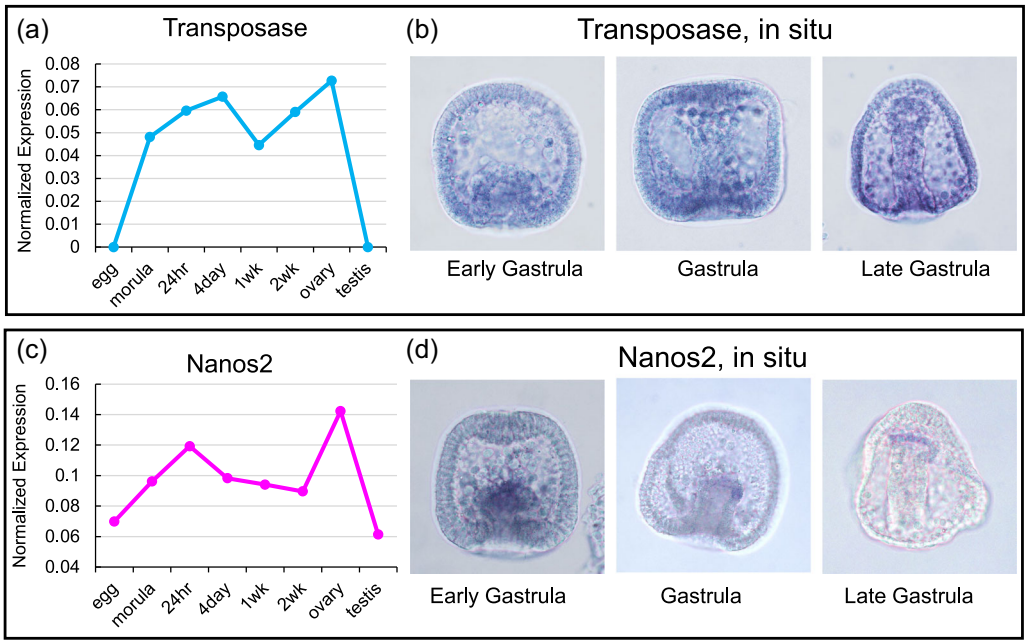



**FIGURE 6** Transposase is expressed at the same time as Nanos2. Quantitative PCR (qPCR) normalized to ubiquitin control. Dig-antisense probes. (a) qPCR assay of expression over developmental time. (b) Whole-mount in situ hybridizations (WMISH) of expression through the process of gastrulation. (c) qPCR assay of expression over developmental time. (d) WMISH of expression through the process of gastrulation.

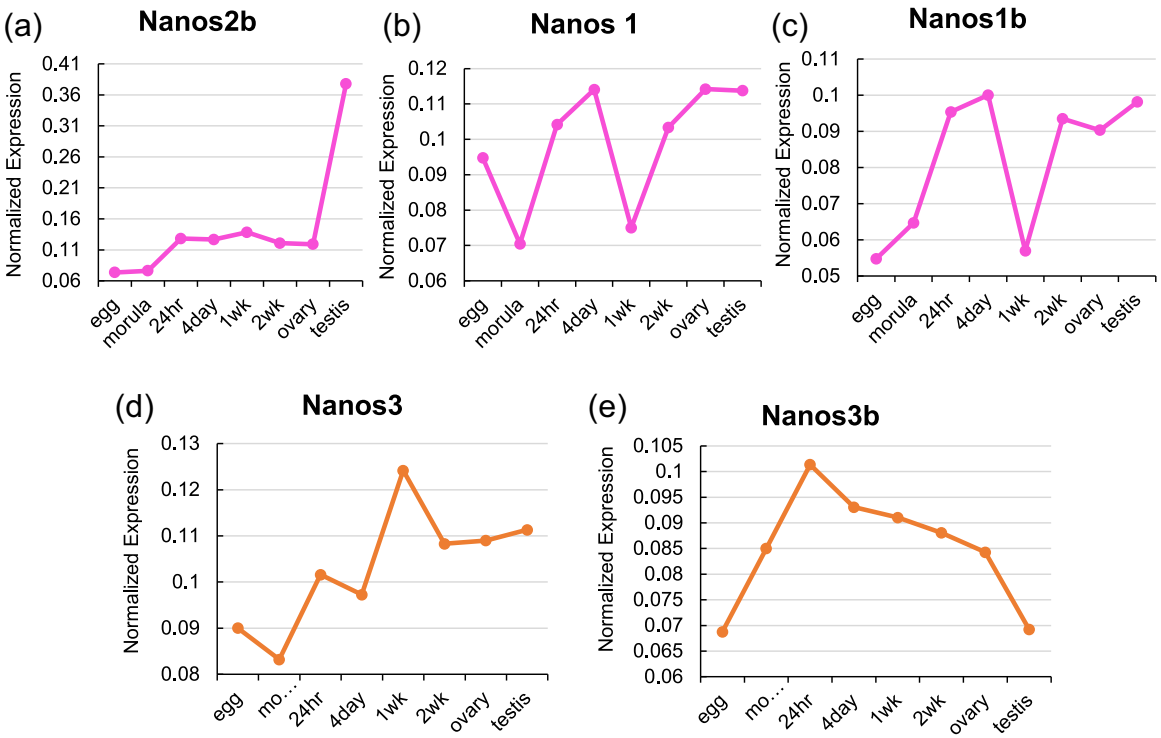



**FIGURE 7** Embryonic Expression of *Lytechinus variegatus* (Lv) Nanos genes. All plots are of individual quantitative PCR (qPCR) data, performed in triplicate, and all expression values are normalized to a ubiquitin control from the same sample, the normalized expression values are comparable across all nine plots. (a) Nanos2b expression, similar to Nanos1 expression, with the exception of the highest abundance detected in the testes. (b) Nanos1 expression (c) Nanos1b expression, comparable to Nanos1 expression. (d) Nanos3 expression, reaches it's highest abundance during week 1 larval growth. (e) Nanos3b expression, similar to that observed for Nanos2, however it is not abundantly expressed in the ovary.

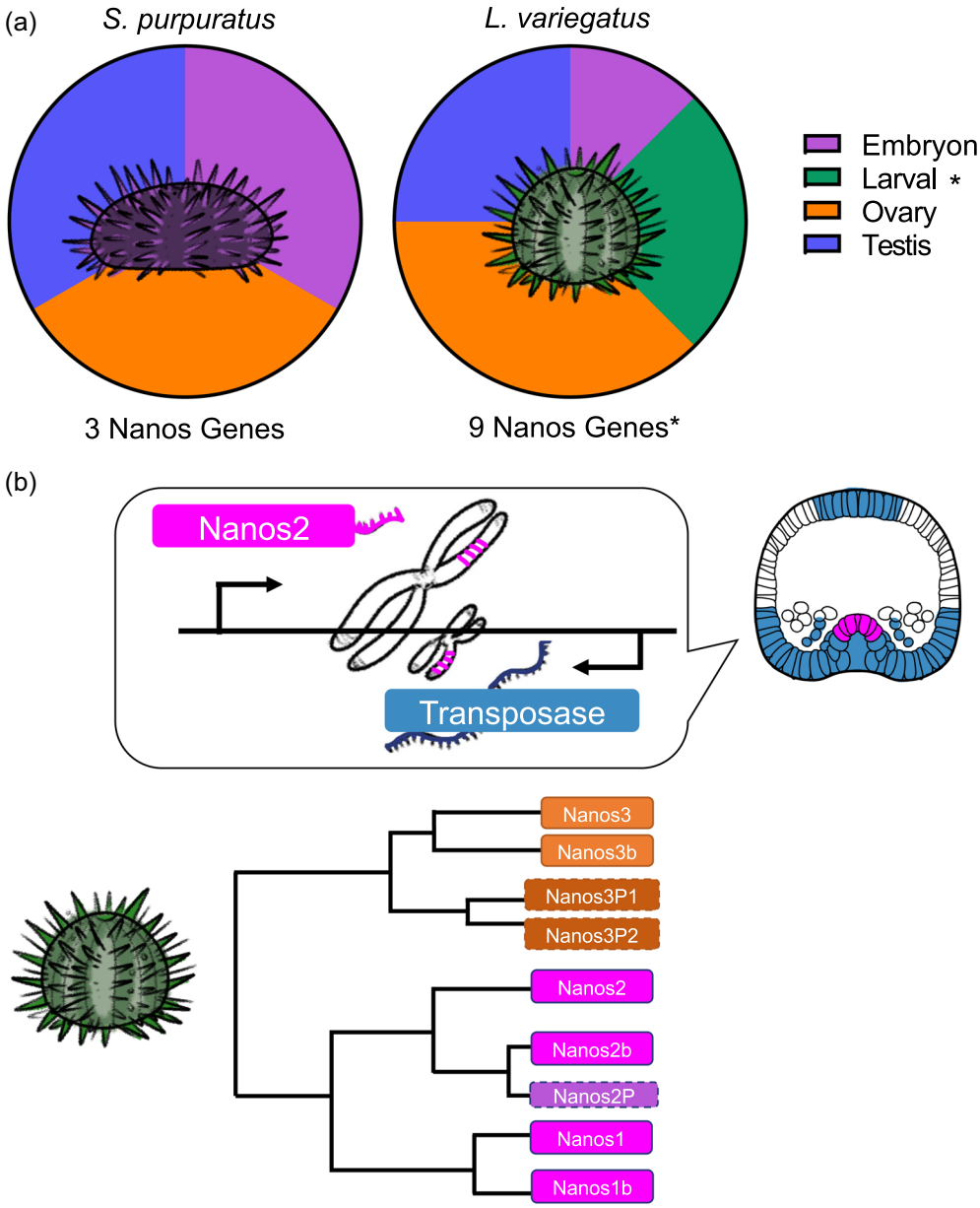



**FIGURE 8** (a) Pie chart summary of characterized nanos genes in *Strongylocentrotus purpuratus* (Sp) and *Lytechinus variegatus* (Lv) sea urchins. Sp has three nanos genes, with one being most abundant in embryos (Nanos2), one being most abundant in the ovary (Nanos1), and the other being most abundant in testes (Nanos3). Nine nanos genes in Lv are most abundant in early embryonic development ($N = 1$). Larval growth ($N = 3$), ovary ($N = 4$), and testes ($N = 1$). (b) Hypothesis for Nanos gene duplications and a family tree of Lv Nanos genes. Lv Nanos2P and an L1 transposase both occupy the same genomic locus and are expressed in overlapping spatial patterns during development, could this be a result of or the reason for such a great expansion and duplication of nanos genes in this species? We summarize them with a tree of the Lv Nanos genes; branches and branch lengths represent estimated sequence divergence. Nanos pseudogenes are in darker colors with a dotted border.

abundant expression level being in the testes, and little-to-no expression detected across developmental time (Figure 7a). Nanos1 orthologs have similar temporal expression patterns, with the transcripts being most abundant during larval development, dropping off at the 1-week stage of larval growth, and then reaching another peak in gonads, especially the ovary (Figure 7b,c). The Nanos3 genes are interesting, as they are not most abundantly expressed in testes like Sp Nanos3, rather they are maternally loaded, and both are expressed at relatively high levels. Nanos3 is most abundant at the 1-week stage of larval growth (Figure 7d), and Nanos3b is most abundant during late gastrulation (24 h), as would be normally observed for a Nanos2 gene (Figure 7e). Overall, each nanos ortholog has very distinct expression patterns, with Nanos3P1 and Nanos3P2 being expressed at barely detectable levels (Supporting Information: Figure S6), supporting our ideas that they are possible pseudogenes.

## 4 | DISCUSSION

Nanos genes represent a "double-edged sword" of sorts when it comes to germline establishment. The Nanos protein itself can be toxic to the embryo by posttranscriptionally inactivating somatic genes important for viability if expressed in the incorrect cell lineages (Lai & King, 2013). Indeed, one target of Nanos in many organisms is cyclin B (Kadyrova et al., 2007), and inactivation of this transcript abruptly halts cell cycling and induces apoptosis in a normal cell (Kadyrova et al., 2007). Therefore, tight regulation and low abundance expression of nanos are important criteria for deploying nanos functionality. As a result of this, overexpression of Nanos in the purple sea urchin is not possible because both its mRNA and protein have degradation elements that keep Nanos undetectable in the somatic cells (Oulhen & Wessel, 2016; Oulhen et al., 2013).

We first hypothesize that the expansion of nanos genes represents a different modality of Nanos protein deployment in this sea urchin species. Nanos2, needed in early embryonic development for germline determination, occupies a unique genomic locus and is under very tight transcriptional and posttranscriptional control similar to that observed in Sp. The expanded members (Lv Nanos3 and others) are then deployed across larval development, or only expressed in mature gonads (Figure 8a). It is important to note that there is no "larval stage-specific" Nanos gene observed in Sp, while a third of the Lv nanos orthologs reach their peak mRNA expression levels at pluteus and later larval stages of development (Figure 8a). The Lv orthologs of Nanos3 also have their own unique and divergent qualities. First, the Lv Nanos3 ortholog shares great mRNA and protein sequences with that of Sp Nanos3, while its expression pattern resembles none of the Sp Nanos genes. Because nearly all these transcripts are expressed during development, we wonder whether they have been selected for enhancing germ cell fate specification early, or for germ cell maintenance later on.

We must remember, however, that an expansion and diversification of nanos genes is not unique to the green sea urchin. Across diverse species in different taxa, expansion and diversification of the nanos family of genes have already been reported (Beer & Draper, 2013; Haraguchi et al., 2003; Julaton & Reijo Pera, 2011; Köprunner et al., 2001; Sun et al., 2017; Tsuda et al., 2003). In mice, nanos genes may not entirely be germline-specific, as the mouse nanos1 gene is expressed in the nervous system and appears to be dispensable for normal development (Haraguchi et al., 2003). While many fish have only two or three nanos genes, *Epinephelus coioides*, a hermaphroditic fish, has four separate nanos genes all expressed differentially, and together play a role in germline establishment (Sun et al., 2017). While most protostomes have a single germline Nanos, the silkworm moth also has four times the nanos genes as many of its insect relatives, and they are each expressed in a tissue- and time-specific manner (Nakao et al., 2008). This widespread occurance of nanos expansion makes the present result interesting, but not surprising considering the evolutionary context.

Finally, we must consider both the L1 transposase and the Nanos2 pseudogene at the same genomic locus. The origin of the expansion in nanos genes and the reason for these two occupying the exact same region on chromosome 4 remains elusive. We hypothesize that perhaps the overlapping temporal and spatial expression of the L1 transposase and Nanos2 led to gene duplication in this species (Figure 8b), although this would more likely have resulted in the generation of only Nanos2P and not the two additional Nanos3P genes, also thought to be pseudogenes (Figure 8b). Almost a third of the expanded nanos genes represent possible pseudogenes, or by-products of gene duplication, with Nanos2P, Nanos3P1, and Nanos3P2 sharing little to no sequence or expression similarities with any of their other gene family members (Figure 8). Further to this point, the nine nanos genes in Lv are expressed at times and in tissues not typically observed in the nanos orthologs studied in *S. purpuratus* (Fresques et al., 2016; Juliano et al., 2006). We believe these phenomena warrant further study to uncover their mechanism of action, whether germ cell-specific or not, using Crispr-Cas9 or MO approaches.

### AUTHOR CONTRIBUTIONS

**Cosmo Pieplow**: Conceptualization; investigation; writing—original draft; methodology; validation; writing—review and editing; software; formal analysis. **Gary Wessel**: Resources; supervision; project administration; writing—original draft; writing—review and editing; funding acquisition; conceptualization.

### ACKNOWLEDGMENTS

We first thank Dr. Professor Nathalie Oulhen. Although the world doesn't orbit Nanos (yet), we know with her contributions, it will soon. We also thank members of Greg Wray's team for assembling a green sea urchin genome of outstanding quality; as well as Cyndi Bradham's team for their work sequencing developmental time points and making them accessible as a resource to the community. We are grateful to the National Institutes of Health for research support (Grant 1R35GM140897 to GMW).

### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

### ORCID

*Gary Wessel* http://orcid.org/0000-0002-1210-9279

### REFERENCES

Arenas-Mena, C., Martinez, P., Cameron, R. A., & Davidson, E. H. (1998). Expression of the Hox gene complex in the indirect development of a sea urchin. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(22), 13062–13067. https://doi.org/10.1073/pnas.95.22.13062

Beer, R. L., & Draper, B. W. (2013). nanos3 maintains germline stem cells and expression of the conserved germline stem cell gene nanos2 in the zebrafish ovary. *Developmental Biology*, *374*(2), 308–318. https://doi.org/10.1016/j.ydbio.2012.12.003

Bhat, K. M. (1999). The posterior determinant gene nanos is required for the maintenance of the adult germline stem cells during Drosophila oogenesis. *Genetics*, *151*(4), 1479–1492. https://doi.org/10.1093/genetics/151.4.1479

Canapa, A., Barucca, M., Biscotti, M. A., Forconi, M., & Olmo, E. (2015). Transposons, genome size, and evolutionary insights in animals. *Cytogenetic and Genome Research*, *147*(4), 217–239. https://doi.org/10.1159/000444429

Dahanukar, A., Walker, J. A., & Wharton, R. P. (1999). Smaug, a novel RNA-binding protein that operates a translational switch in Drosophila. *Molecular Cell*, *4*(2), 209–218. https://doi.org/10.1016/s1097-2765(00)80368-8

Davidson, P. L., Guo, H., Wang, L., Wang, L., Berrio, A., Zhang, H., Chang, Y., Soborowski, A. L., McClay, D. R., Fan, G., & Wray, G. A. (2020). Chromosomal-Level genome assembly of the sea urchin lytechinus variegatus substantially improves functional genomic analyses. *Genome Biology and Evolution*, *12*(7), 1080–1086. https://doi.org/10.1093/gbe/evaa101

Deits, T., Farrance, M., Kay, E. S., Medill, L., Turner, E. E., Weidman, P. J., & Shapiro, B. M. (1984). Purification and properties of ovoperoxidase, the enzyme responsible for hardening the fertilization membrane of the sea urchin egg. *Journal of Biological Chemistry*, *259*(21), 13525–13533. https://www.ncbi.nlm.nih.gov/pubmed/6490663

Deshpande, G., Spady, E., Goodhouse, J., & Schedl, P. (2012). Maintaining sufficient nanos is a critical function for polar granule component in the specification of primordial germ cells. *G3: Genes|Genomes|Genetics*, *2*(11), 1397–1403. https://doi.org/10.1534/g3.112.004192

Fresques, T., Swartz, S. Z., Juliano, C., Morino, Y., Kikuchi, M., Akasaka, K., Wada, H., Yajima, M., & Wessel, G. M. (2016). The diversity of nanos expression in echinoderm embryos supports different mechanisms in germ cell specification. *Evolution & Development*, *18*(4), 267–278. https://doi.org/10.1111/ede.12197

Hansen, C. L., & Pelegri, F. (2021). Primordial germ cell specification in vertebrate embryos: Phylogenetic distribution and conserved molecular features of preformation and induction. *Frontiers in Cell and Developmental Biology*, *9*, 730332. https://doi.org/10.3389/fcell.2021.730332

Haraguchi, S., Tsuda, M., Kitajima, S., Sasaoka, Y., Nomura-Kitabayashid, A., Kurokawa, K., & Saga, Y. (2003). nanos1: A mouse nanos gene expressed in the central nervous system is dispensable for normal development. *Mechanisms of Development*, *120*(6), 721–731. https://doi.org/10.1016/s0925-4773(03)00043-1

Hogan, J. D., Keenan, J. L., Luo, L., Ibn-Salem, J., Lamba, A., Schatzberg, D., Piacentino, M. L., Zuch, D. T., Core, A. B., Blumberg, C., Timmermann, B., Grau, J. H., Speranza, E., Andrade-Navarro, M. A., Irie, N., Poustka, A. J., & Bradham, C. A. (2020). The developmental transcriptome for *Lytechinus variegatus* exhibits temporally punctuated gene expression changes. *Developmental Biology*, *460*(2), 139–154. https://doi.org/10.1016/j.ydbio.2019.12.002

Ilaslan, E., Sajek, M. P., Jaruzelska, J., & Kusz-Zamelczyk, K. (2022). Emerging roles of NANOS RNA-binding proteins in cancer. *International Journal of Molecular Sciences*, *23*(16), 9408. https://doi.org/10.3390/ijms23169408

Irish, V., Lehmann, R., & Akam, M. (1989). The Drosophila posterior-group gene nanos functions by repressing hunchback activity. *Nature*, *338*(6217), 646–648. https://doi.org/10.1038/338646a0

Janic, A., Mendizabal, L., Llamazares, S., Rossell, D., & Gonzalez, C. (2010). Ectopic expression of germline genes drives malignant brain tumor growth in Drosophila. *Science*, *330*(6012), 1824–1827. https://doi.org/10.1126/science.1195481

Jaruzelska, J., Kotecki, M., Kusz, K., Spik, A., Firpo, M., & Reijo Pera, R. A. (2003). Conservation of a Pumilio-Nanos complex from Drosophila germ plasm to human germ cells. *Development Genes and Evolution*, *213*(3), 120–126. https://doi.org/10.1007/s00427-003-0303-2

Julaton, V. T. A., & Reijo Pera, R. A. (2011). NANOS3 function in human germ cell development. *Human Molecular Genetics*, *20*(11), 2238–2250. https://doi.org/10.1093/hmg/ddr114

Juliano, C. E., Voronina, E., Stack, C., Aldrich, M., Cameron, A. R., & Wessel, G. M. (2006). Germ line determinants are not localized early in sea urchin development, but do accumulate in the small micromere lineage. *Developmental Biology*, *300*(1), 406–415. https://doi.org/10.1016/j.ydbio.2006.07.035

Juliano, C. E., Yajima, M., & Wessel, G. M. (2010). Nanos functions to maintain the fate of the small micromere lineage in the sea urchin embryo. *Developmental Biology*, *337*(2), 220–232. https://doi.org/10.1016/j.ydbio.2009.10.030

Kabsch, W., & Sander, C. (1983). How good are predictions of protein secondary structure. *FEBS Letters*, *155*(2), 179–82. https://doi.org/10.1016/0014-5793(82)80597-8

Kadyrova, L. Y., Habara, Y., Lee, T. H., & Wharton, R. P. (2007). Translational control of maternal cyclin B mRNA by Nanos in the Drosophila germline. *Development*, *134*(8), 1519–1527. https://doi.org/10.1242/dev.002212

Khazina, E., & Weichenrieder, O. (2009). Non-LTR retrotransposons encode noncanonical RRM domains in their first open reading frame. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(3), 731–736. https://doi.org/10.1073/pnas.0809964106

Kobayashi, S., Yamada, M., Asaoka, M., & Kitamura, T. (1996). Essential role of the posterior morphogen nanos for germline development in Drosophila. *Nature*, Apr 25 *380*(6576), 708–711. https://doi.org/10.1038/380708a0

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. https://doi.org/10.1101/gad.212401

Kusz, K. M., Tomczyk, L., Sajek, M., Spik, A., Latos-Bielenska, A., Jedrzejczak, P., Pawelczyk, L., & Jaruzelska, J. (2009). The highly conserved NANOS2 protein: Testis-specific expression and significance for the human male reproduction. *Molecular Human Reproduction*, *15*(3), 165–171. https://doi.org/10.1093/molehr/gap003

Lai, F., & King, M. L. (2013). Repressive translational control in germ cells. *Molecular Reproduction and Development*, *80*(8), 665–676. https://doi.org/10.1002/mrd.22161

Lai, F., Singh, A., & King, M. L. (2012). Xenopus Nanos1 is required to prevent endoderm gene expression and apoptosis in primordial germ cells. *Development*, *139*(8), 1476–1486. https://doi.org/10.1242/dev.079608

Liu, S., Wang, Z., Zhu, R., Wang, F., Cheng, Y., & Liu, Y. (2021). Three differential expression analysis methods for RNA sequencing: limma, EdgeR, DESeq. 2. *Journal of Visualized Experiments: JoVE*, (175), e62528. https://doi.org/10.3791/62528

Lolicato, F., Marino, R., Paronetto, M. P., Pellegrini, M., Dolci, S., Geremia, R., & Grimaldi, P. (2008). Potential role of Nanos3 in maintaining the undifferentiated spermatogonia population. *Developmental Biology*, *313*(2), 725–738. https://doi.org/10.1016/j.ydbio.2007.11.011

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq. 2. *Genome Biology*, *15*(12), 550. https://doi.org/10.1186/s13059-014-0550-8

Mulder, N., & Apweiler, R. (2007). InterPro and InterProScan: Tools for protein sequence classification and comparison. *Methods in Molecular Biology*, *396*, 59–70. https://doi.org/10.1007/978-1-59745-515-2_5

Nakao, H., Matsumoto, T., Oba, Y., Niimi, T., & Yaginuma, T. (2008). Germ cell specification and early embryonic patterning in *Bombyx mori* as revealed by nanos orthologues. *Evolution & Development*, *10*(5), 546–554. https://doi.org/10.1111/j.1525-142X.2008.00270.x

Oulhen, N., Morita, S., & Wessel, G. M. (2022). Post-transcriptional regulation of factors important for the germ line. *Current Topics in*

*Developmental Biology*, *146*, 49–78. https://doi.org/10.1016/bs.ctdb.2021.10.003

Oulhen, N., Swartz, S. Z., Laird, J., Mascaro, A., & Wessel, G. M. (2017). Transient translational quiescence in primordial germ cells. *Development (Cambridge, England)*, *144*(7), 1201–1210. https://doi.org/10.1242/dev.144170

Oulhen, N., & Wessel, G. M. (2014). Every which way—Nanos gene regulation in echinoderms. *Genesis*, *52*(3), 279–286. https://doi.org/10.1002/dvg.22737

Oulhen, N., & Wessel, G. M. (2016). Differential Nanos 2 protein stability results in selective germ cell accumulation in the sea urchin. *Developmental Biology*, *418*(1), 146–156. https://doi.org/10.1016/j.ydbio.2016.07.007

Oulhen, N., Yoshida, T., Yajima, M., Song, J. L., Sakuma, T., Sakamoto, N., Yamamoto, T., & Wessel, G. M. (2013). The 3′UTR of nanos2 directs enrichment in the germ cell lineage of the sea urchin. *Developmental Biology*, *377*(1), 275–283. https://doi.org/10.1016/j.ydbio.2013.01.019

Parisi, M., & Lin, H. (2000). Translational repression: A duet of Nanos and Pumilio. *Current Biology*, *10*(2), R81–R83. https://doi.org/10.1016/s0960-9822(00)00283-9

Pieplow, A., Dastaw, M., Sakuma, T., Sakamoto, N., Yamamoto, T., Yajima, M., Oulhen, N., & Wessel, G. M. (2021). CRISPR-Cas9 editing of non-coding genomic loci as a means of controlling gene expression in the sea urchin. *Developmental Biology*, *472*, 85–97. https://doi.org/10.1016/j.ydbio.2021.01.003

Ponz-Segrelles, G., Bleidorn, C., & Aguado, M. T. (2018). Expression of vasa, piwi, and nanos during gametogenesis in *Typosyllis antoni* (Annelida, Syllidae). *Evolution & Development*, *20*(5), 132–145. https://doi.org/10.1111/ede.12263

Rodriguez, A. J., Seipel, S. A., Hamill, D. R., Romancino, D. P., DI CARLO, M., Suprenant, K. A., & Bonder, E. M. (2005). Seawi—A sea urchin piwi/argonaute family member is a component of MT-RNP complexes. *RNA*, *11*(5), 646–656. https://doi.org/10.1261/rna.7198205

Sano, H., Mukai, M., & Kobayashi, S. (2001). Maternal Nanos and Pumilio regulate zygotic vasa expression autonomously in the germ-line progenitors of *Drosophila melanogaster* embryos. *Development, Growth and Differentiation*, *43*(5), 545–552. https://doi.org/10.1046/j.1440-169x.2001.00593.x

Satoh, N., Hisata, K., Foster, S., Morita, S., Nishitsuji, K., Oulhen, N., Tominaga, H., & Wessel, G. M. (2022). A single-cell RNA-seq analysis of Brachyury-expressing cell clusters suggests a morphogenesis-associated signal center of oral ectoderm in sea urchin embryos. *Developmental Biology*, *483*, 128–142. https://doi.org/10.1016/j.ydbio.2022.01.005

Sonoda, J., & Wharton, R. P. (1999). Recruitment of Nanos to hunchback mRNA by Pumilio. *Genes & Development*, *13*(20), 2704–2712. https://doi.org/10.1101/gad.13.20.2704

Sun, Z. H., Wang, Y., Lu, W. J., Li, Z., Liu, X. C., Li, S. S., Zhou, L., & Gui, J. F. (2017). Divergent expression patterns and function implications of four nanos genes in a hermaphroditic fish, *Epinephelus coioides*. *International Journal of Molecular Sciences*, *18*(4), 685. https://doi.org/10.3390/ijms18040685

Touw, W. G., Bayjanov, J. R., Overmars, L., Backus, L., Boekhorst, J., Wels, M., & van Hijum, S. A. (2013). Data mining in the life sciences with random forest: A walk in the park or lost in the jungle. *Briefings in Bioinformatics*, *14*(3), 315–26. https://doi.org/10.1093/bib/bbs034

Tsuda, M., Sasaoka, Y., Kiso, M., Abe, K., Haraguchi, S., Kobayashi, S., & Saga, Y. (2003). Conserved role of nanos proteins in germ cell development. *Science*, *301*(5637), 1239–1241. https://doi.org/10.1126/science.1085222

Venkatarama, T., Lai, F., Luo, X., Zhou, Y., Newman, K., & King, M. L. (2010). Repression of zygotic gene expression in the *Xenopus* germline. *Development*, *137*(4), 651–660. https://doi.org/10.1242/dev.038554

Wessel, G. M., Morita, S., & Oulhen, N. (2021). Somatic cell conversion to a germ cell lineage: A violation or a revelation? *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, *336*(8), 666–679. https://doi.org/10.1002/jez.b.22952

Zhang, F., Liu, R., Liu, C., Zhang, H., & Lu, Y. (2020). Nanos3, a cancer-germline gene, promotes cell proliferation, migration, chemoresistance, and invasion of human glioblastoma. *Cancer Cell International*, *20*, 197. https://doi.org/10.1186/s12935-020-01272-1

## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Pieplow, C., Wessel, G. (2023). Functional annotation of a hugely expanded nanos repertoire in *Lytechinus variegatus*, the green sea urchin. *Molecular Reproduction and Development*, 90, 310–322. https://doi.org/10.1002/mrd.23684