Manuscript File Click here to view linked References

An RNA interference approach for functional studies in the sea urchin and its use in analysis of Nodal signaling gradients.

by

Keen Wilson*, Carl Manner*, Esther Miranda, Alejandro Berrio, Greg Wray, and David R McClay§

Department of Biology, Duke University, Durham, NC USA

*Co-first authors
§Corresponding author

Address of corresponding author:

David R. McClay
Department of Biology, Box 90338
Duke University
Durham, NC 27708

dmcclay@duke.edu

Office: (919)613-8188

Keywords: Sea urchin, development, DsiRNA, Nodal, Lefty, Dorsal-Ventral.

Highlights: DsiRNA provides an RNAi approach for perturbation of sea urchin embryos. A dilution series of DsiRNA oligos reveals properties of the Nodal gradient in establishing the Dorsal-Ventral axis.

Abstract

We report the first RNAi-based gene knockdowns in a sea urchin. Dicer-Substrate interfering RNAs (DsiRNAs) successfully disrupted expression of multiple transcripts in embryos of the sea urchin *Lytechinus variagatus,* phenocopying known morpholino and inhibitor knockdowns. We describe our approach for the design of DsiRNAs most likely to target mRNAs of interest. An examination of DsiRNAs directed against *pks1*, an enzyme necessary for pigment production, reveal destruction of the *pks1* mRNA without destroying the now-albino pigment cells. Application of DsiRNA on Nodal-Lefty signaling in this species confirms well-known functional studies. DsiRNA-based knockdowns of *nodal* and *lefty* phenocopy morpholino- and drug-based inhibition of expression of these genes. A dilution series of DsiRNAs directed against *nodal* and *lefty* reveals new diffusion properties of these signals in specifying dorsal vs ventral mesoderm.

Introduction

Our knowledge of the gene regulatory mechanisms governing development has greatly expanded over the last two decades largely due to targeted perturbation of gene expression in model developmental systems. Experience has shown that the more approaches available, the better. Model systems that utilize forward genetics as a tool have played a leading role in these advances. The sea urchin is one of several excellent model systems for the study of early development that are not yet tractable for forward genetics. Nonetheless, labs leveraging the developing urchin embryo as a model have benefitted from perturbation approaches using reverse genetics. Those approaches uncovered an extensive gene regulatory network (GRN) governing early specification and morphogenetic events up to the larval stage (Davidson et al., 2002; Davidson and Erwin, 2006; McClay, 2011; Peter and Davidson, 2015; Martik et al., 2016; McClay et al., 2021; Massri et al., 2023). While much has been learned about GRNs and other governing events in development, the diversity of perturbing reagents available historically has been limited in this system. Translation blocking morpholino antisense oligos (MASOs) are a widely used method of gene knockdown in many embryos, including the developing sea urchin embryo (Summerton and Weller, 1997; Heasman et al., 2000; Howard et al., 2001; Heasman, 2002). While current MASO-based knockdown approaches in sea urchins are thought to be robust and reproducible, MASO use is not without its challenges. There are issues with MASOs precipitating out of solution, clogging injection needles, and needing extreme measures for resuspension such as autoclaving. These issues make it a challenge to repeat experiments since effective concentrations of a MASO stock solution change over time. Ideally, MASOs completely block the translation of an mRNA, but too often antibodies are not available to determine the actual level of the block. Therefore, the current "gold standard" for validation of a MASO-based knockdown is rescue of the translation block by expression of a construct that is unaffected by the MASO. Rescue by overexpression generally recapitulates neither the tightly controlled spatiotemporal patterns of expression of the endogenous gene, nor the endogenous level of activity, complicating interpretation of the rescue. Measuring the level of translational inhibition produced by a MASO is often prohibitively expensive in non-model systems, demanding the generation and validation of custom antibodies. Additionally, published concerns suggest that off-target effects of the MASOs can lead to incorrect interpretation (Schulte-Merker and Stainier, 2014). To be fair, morpholinos are not the only perturbation approach that is subject to possible off-target effects. To varying degrees, all perturbation approaches, including

mutations, have the potential for off-target or misleading effects. To counter these concerns each animal system benefits from multiple perturbation approaches.

Recent studies have enhanced the toolkit available for genetic perturbation in the developing urchin embryo. CRISPR/Cas9 technology for genome modification (Lin and Su, 2016) has been increasingly used in sea urchins with positive results, including the first instances of directed mutagenesis of adult sea urchins and successful rearing of F2 mutagenized animals (Oulhen and Wessel, 2016, Liu et al, 2019, Yaguchi et al, 2020). While a powerful tool, CRISPR/Cas9 is not without its challenges either. Problems of penetrance, off target effects, high reagent cost, mosaicism, and lack of spatiotemporal control are barriers to wide deployment of this technology for use in the developing sea urchin. Other recent additions of tools for understanding gene function include conditional knockdowns with photo-caged MASOs (Bardhan et al., 2021), and a Tet-On system for conditional control of expression (Khor and Ettensohn, 2023). Each of these advances expands the diversity of experimental possibility, and the capacity to validate results via independent approaches.

One technology that has not been realized in sea urchins is RNA interference (RNAi). This technology rapidly expanded beginning in 1990 after RNAi mediated knockdown was first observed (Napoli et al., 1990; Fire et al., 1991; Lee et al., 1993; Guo 1995), and double stranded RNA was found to be responsible (Fire et al., 1998). Over the last two decades the use of RNAi has been a valuable tool for functional studies in many model embryo systems. Early attempts at RNAi knockdown in sea urchins did not seem to yield specific phenotypes, and failed trials (including in the labs of two coauthors in this paper) were not published. With the successful adaptation of MASOs for perturbations in the sea urchin (Howard et al, 2001), the pursuit of RNAi-based gene knockdown was largely put aside in favor of MASO technology.

Since then, much has been learned about the mechanism of action and the molecular components of RNAi (Hammond et al., 2001; Sharp, 2001; Agrawal et al., 2003). RNAi is induced on detection of dsRNA by Dicer, which cleaves the dsRNA into 21-26 bp pieces (Zamore et al., 2000; Hannon, 2002; Carmell and Hannon, 2004) called small interfering RNAs (siRNAs). Dicer then associates with Argonaut, TRBP, and other proteins to form the RNA-Induced Silencing Complex (RISC) (Chendrimada et al., 2005). A single strand from the siRNA guides the RISC complex by sequence complementarity to target mRNAs, where the RISC mediates the mRNA destruction. The machinery responsible for RNAi is ubiquitous and ancient, present in archaea, fungi, plants, and animals (Shabalina and Koonin, 2008). The molecular components necessary for RNAi are also present in sea urchins (Song and Wessel, 2007). These were initially understood for their role in microRNA (miRNA) regulation, a process that uses the same molecular machinery as transcriptional blocking RNAi, but for regulation of translation. MiRNA regulation in sea urchin development has been studied extensively indicating that all the required proteins for building a RISC complex are present in the embryo (Song et al., 2012; Sampilo et al., 2018; Konrad et al., 2023).

Experiments aimed at improving the efficiency and stability of RNAi reagents in a variety of organisms led to the commercial introduction of a double stranded RNA with a 2-base 3' DNA nucleotide overhang on one strand (Kim et al., 2005; Amarzguioui et al., 2006). The availability of these DsiRNA products prompted us to investigate the possibility of using these reagents for RNAi gene knockdown in the developing sea urchin embryo. DsiRNAs consist of a

25bp RNA-DNA hybrid sense strand where two dNTPs make up the last two bases of the 3' end, and a 27bp RNA antisense strand that overhangs the 5' of the sense strand by two bases. The modification of the sense strand provides stability to the complex and helps bias against sense strand integration into RISC. The length ensures that processing and loading into the RISC complex includes Dicer (Kim et al., 2005; Amarzguioui et al., 2006).

We report the effectiveness of DsiRNA in perturbation experiments with sea urchin embryos in three sets of experiments. A visual experiment reports the efficacy of DsiRNA in initiating transcript degradation using an exogenously supplied GFP mRNA. With knowledge that the Dsi approach functions in sea urchins, Pks1, an enzyme necessary for pigment production in the embryo, was tested and *pks1* mRNA was destroyed by injection of targeted DsiRNA, confirming that endogenous genes can be targeted. DsiRNAs targeted to two well-known signaling pathways for further testing efficacy on perturbing endogenous genes. Nodal and Lefty are well documented as essential for establishing of the dorsal-ventral axis in the sea urchin embryo (Duboc et al., 2004; Duboc et al., 2005; Range et al., 2007; Nam et al., 2007; Duboc et al., 2008; Su et al., 2009; Bolouri and Davidson, 2009; Yaguchi et al., 2010; Duboc et al., 2010; Molina et al., 2013; Materna et al., 2013; Coffman et al., 2014; Piacentino et al., 2015; Floc'hlay et al., 2021; Pieplow et al., 2021). Nodal is expressed on one side of the ectoderm beginning early in cleavage. The side expressing Nodal becomes ventral and much has been learned about the sequence of molecular events initiated by expression of this signal (Molina and Lepage, 2020). Among the first consequences of Nodal expression is activation (through Nodal signaling) of *lefty* expression which antagonizes the function of the Nodal signal (Oulad-Abdelghani et al., 1998; Duboc et al., 2008). Experimentally, if Nodal expression is knocked down by a MASO or with an inhibitor, the embryo fails to establish the dorsal-ventral axis, and without any asymmetry the embryo adopts a radialized phenotype. If, on the other hand, Lefty is knocked down, Nodal function expands throughout and is said to radialize because now the embryo is entirely ventral. *Nodal* and *lefty* Dsi knockdowns matched the known effects of perturbing expression of these genes with their well-known phenotypes. Further, a dilution series showed that the DsiRNAs elicit a graded response. Thus, DsiRNA functions as a perturbation reagent in three sets of experiments, and results are consistent with well-studied genes. The approach is now a routine protocol in the lab with many additional genes tested further confirming MASO data in many additional direct comparisons.

Results.

DsiRNA-based knockdown is effective in sea urchin embryos.

With knowledge that the sea urchin embryo expresses Dicer (Song and Wessel, 2007), and with commercial availability of relatively stable DsiRNA oligomers, we tested an artificial system in the embryo. An mRNA encoding a transmembrane sequence fused to *gfp* was injected into the zygote and the membrane anchored GFP fluorescently labeled all cell membranes (**Fig. 1A)** (Saunders and McClay, 2014**).** At the 2-cell stage of another embryo, one of the two blastomeres was injected with DsiRNA targeting GFP (**Fig. 1B).** By late blastula stage most fluorescence was absent in half the embryo indicating early destruction of the *gfp* mRNA. Whether the *gfp* mRNA is destroyed by this time cannot be determined by this experiment alone, but the diminished amount of fluorescence indicates that the RISC complex has been functioning during cleavage. The experiment was repeated, and this time one blastomere at the 2-cell stage was injected with DsiRNA targeting *membrane-gfp,* and with Rhodamine isothocyanate (RITC)-

dextran to identify the DsiRNA-injected blastomere and its progeny. Embryos at the prism stage were imaged to determine GFP fluorescence. As shown in **Fig. 1, C1 and C2,** GFP expression in the DsiRNA-injected half-embryo is reduced to background indicating complete RNAi-mediated destruction of the *gfp* mRNA. And, since the added *membrane-gfp* mRNA was at a high concentration relative to endogenous transcript concentrations, the DsiRNA knockdown capability is substantial. The GFP protein is known to turn over relatively slowly in sea urchin embryos (Arnone et al., 1997), and the high level of introduced *membrane-gfp* mRNA in this artificial system makes it difficult to know how quickly the introduced DsiRNA destroys the mRNA. However, the data indicates it functions during cleavage and continues to function for many hours after that. Further, the absence of GFP fluorescence appears complete in the RITC labeled half indicating a lack of mosaicism. Meanwhile the control half of the embryo displayed bright GFP fluorescence. We conclude that DsiRNA functions in sea urchin embryos and can generate fully penetrant knockdowns of even highly abundant transcripts.

Optimization of endogenous mRNA knockdown with DsiRNA.

P*ks1*, a gene encoding an enzyme in the pigment production pathway (Calestani et al., 2003; Calestani and Rogers, 2010), was chosen for the initial endogenous test of DsiRNA function. Experiments with MASOs showed that knockdown of *pks1* translation results in albino embryos (Calestani et al., 2003). Crispr-Cas9 experiments also showed that knockout of the *pks1* gene results in albino embryos (Oulhen and Wessel, 2016). Here, a DsiRNA was chosen using the selection criteria described in the methods. These were tested by injection and initially scored based on pigmentation of larvae. **Fig. 2A, B** shows that injection of the chosen DsiRNA led to development of albino larvae. By in situ analysis, a probe to *pks1* indicated the presence of *pks1*mRNA in pigment cells of control embryos, but absent in the DsiRNA-injected embryos (**Fig. 2C, D).** In multiple experiments more than 90% of injected embryos were devoid of *pks1* signal while the remaining 10-15% of the injected embryos displayed a low level of *pks1* expression relative to controls (**Fig. S1),** indicating that the knockdown is occasionally incomplete. Some of the embryos were further tested with a probe to *acaca*, (acetyl CoA-carboxylase), a gene that is expressed almost exclusively in pigment cells (**Fig. 2E, F).** Knockdown of *pks1* has no effect on expression of *acaca* indicating that pigment cells are specified in the knockdowns, but these cells are specifically missing expressed *pks1* mRNA.

Nodal and Lefty DsiRNA knockdowns phenocopy knockdowns using MASOs or drug inhibitors.

We next explored DsiRNA knockdowns of *nodal* and *lefty* because of the well-known functions of these genes in the sea urchin embryo. Many laboratories have repeated and confirmed functions of these signals originally described by the Lepage group. Nodal functions as a crucial signal that establishes dorsal-ventral and left-right axes in sea urchin development (Duboc et al., 2004; Duboc et al., 2005; Duboc et al., 2008; Duboc et al., 2010; Floc'hlay et al., 2021). *Nodal* is expressed by ventral ectoderm and establishes a “community effect” signaling center during cleavage (Gurdon, 1988; Bolouri and Davidson, 2009). Among the genes activated downstream of the Nodal signal, Lefty is an anti-Nodal signal that restricts the reach of the Nodal signal to the ventral ectoderm, ventral mesoderm and ventral endoderm (Duboc et al., 2004; Duboc et al., 2008; Duboc et al., 2010). Inhibition of *nodal* with a MASO or a specific

drug inhibitor radializes the embryo because ventral genes fail to be activated. Inhibition of *lefty* with a MASO allows Nodal signaling to expand its reach into the presumptive dorsal territory and activate ventral-specific genes in cells that would otherwise be dorsal, resulting in a radialized embryo that is entirely ventral in identity. The expression of BMP2/4 is also downstream and controlled by Nodal. *Bmp2/4* expression occurs in the ventral ectoderm, the protein is transported to the Dorsal half of the embryo, possibly using Chordin as a chaperone (Madamanchi et al., 2021), and likely employing Glypican5 to facilitate that transport (Lapraz, 2009). The BMP2/4 signal is activated in the Dorsal half via a tolloid-like protease that removes Chordin from the BMP2/4 ligand which then operates in establishing the Dorsal GRN (Duboc et al., 2004; Duboc et al., 2008; Duboc et al., 2010).

Figs. 3A an B illustrate the phenotypic outcome to mesoderm specification following injection of a dilution series of MASOs to *nodal* and *lefty*. The experiments score the expression of two mesodermal genes. *Irf4* is a transcription factor expressed by ventrally specified blastocoelar cells (Hibino et al., 2006; Allen et al., 2022), with the Nodal signal required for its expression **(Fig. 3A).** Pigment cells arise from dorsal mesoderm. Inhibition of Lefty results in excess Nodal reaching the presumptive dorsal mesoderm leading to an inhibition of *pks1* expression **(Fig 3B)**. As can be seen in **Fig. 3B**, a *lefty* MASO efficiently blocks translation of Lefty protein since a 1:36 dilution of MASO still results in insufficient Lefty protein needed to block Nodal diffusion to the dorsal mesoderm. Dilution of the *nodal* MASO shows that ventral mesoderm specification is sensitive to Nodal concentration, i.e. the number of blastocoelar cells increases from total absence to control levels over about a 10-fold dilution range of the morpholino. This sensitivity is more complicated than the Lefty knockdown since Nodal also controls the expression of *lefty* and *BMP2/4.* Overexpression of BMP2/4, as shown by the Lepage group (Duboc et al., 2004; Duboc et al., 2008; Lapraz, 2009; Duboc et al., 2010), results in a dorsal radialized embryo **(Fig. 3C)**.

With the above as a control series in *Lytechinus variegatus*, we tested three DsiRNA reagents each for blocking expression of *nodal* or *lefty*. One DsiRNA reagent for each was most effective in matching the MASO outcomes (**Fig. 4)**. Each of the six DsiRNAs were also tested by in situ hybridization to determine whether they knocked down expression of *nodal* or *lefty.* **Fig. 4A** shows that DsiRNAs that gave a radialized phenotype for each (Nodal DsiRNA 3, and Lefty DsiRNA 3) were largely devoid of mRNA expression as seen by in situ, while the DsiRNAs that did not produce a visual phenotype were positive for *nodal* or *lefty* expression. Lefty DsiRNA 1 gave a weak phenotype and its expression by in situ was reduced relative to controls. **Fig. 4B** shows the phenotypic outcome of the Nodal and Lefty DsiRNA inhibition. On the top row of **Fig. 4B** each embryo is stained by Hybidization Chain Reaction (HCR) with probes to a dorsal mesoderm marker (Pks1, red), Ventral mesoderm marker (Astacin4, yellow), and a skeletogenic cell marker (Msp130, green). The dorsal marker is missing in the *lefty* Dsi knockdown, and the ventral marker is missing in the *nodal* Dsi knockdown. In the lower row radial phenotypes are seen in Dsi knockdowns of *lefty* and *nodal* and the *nodal* knockdown has extra pigment cells while the *lefty* knockdown is albino.

The *nodal* and *lefty* DsiRNAs that gave phenotypes and were effective in reducing expression were further studied. A dilution series indicated a wide range of concentrations were effective with no signs of toxicity, and no signs of off-target effects. The value in this outcome is

useful because some MASOs are effective only at a high concentration, and that concentration changes over time because the solubility of the MASO changes, becoming more difficult to resuspend as time of storage increases. The DsiRNA reagents retain their efficacy over an extended storage time at -80°C. They also survive repeated freeze-thaw cycles.

To further characterize the *nodal* and *lefty* DsiRNA results, the three HCR probes enabled us to quantify Dorsal-Ventral signaling outcomes of three mesoderm cell types in the same embryo. In the experiment a 20-fold dilution series of the DsiRNA probes to *nodal* or *lefty* are quantified (**Figs. 4B, 5).** We counted the number of dorsal pigment cells (red), ventral blastocoelar cells (yellow) and skeletogenic cells (green) in the confocal-stacked images. In normal development 64 skeletogenic cells are present in the blastocoel following an epithelial-to-mesenchymal transition (EMT) at the mesenchyme blastula stage. As expected, the number of skeletogenic cells remains at a similar level throughout indicating that their number is unaffected by Nodal or Lefty concentration changes (the number is usually an undercount because the fluorescent signal is diminished toward the bottom of the stack, but this affects all three cell types equally). In the Dsi dilution series of *Lefty* (more Nodal reaching dorsal), at higher concentrations there are zero to very few pigment cells down to a 100 μM injected concentration and further dilution indicates a graded response. The same embryos developed an excess number of blastocoelar cells. The signaling response showed a concentration dependency over a 20-fold dilution range. The *Nodal* DsiRNA dilution series also displayed a quantitative sensitivity though less dramatic than the *Lefty* knockdown.

Discussion.

DsiRNAs trigger destruction of targeted transcripts in the sea urchin. That is shown with data from the visual GFP construct, knockdown of *pks1*, and knockdowns of *nodal* and *lefty* affecting acquisition of the Dorsal-Ventral axis. Each of these knockdowns demonstrated that the DsiRNA approach results in phenotypes, and molecular changes that are consistent with MASO and inhibitor studies reported elsewhere in several species of sea urchin. A dilution series of the DsiRNAs to *nodal* and *lefty* indicated that the outcome is a quantitative measure of Dorsal or Ventral signaling perturbations in that the number of pigment cells or blastocoelar cells produced reflected the level of inhibition. Toxic effects were absent or undetected over a 20-fold range in Dsi concentrations. There were no developmental delays or abnormal effects on skeletogenic cell number. The maximal effect of the DsiRNA reagents was achieved at a level 4-fold below the maximum concentration tested.

An experimental advantage of DsiRNA reagents over MASOs is the way the inhibition is achieved. MASOs block translation but have no direct effect on transcription. DsiRNAs, by contrast, destroy the transcript. That makes it possible for in situ analysis to provide strong evidence that the mRNA in question is destroyed in the experiment. While in situ analysis is only semi-quantitative, it supplies critical experimental information determining whether the mRNA in question is significantly reduced, and in the expected location in the embryo. The technique doesn't rule out possible off-target effects, but knowledge of an *on-target* effect is valuable.

Throughout the course of this study, our design process for generating DsiRNAs continually evolved, and our current selection protocol (see Materials and Methods) should be viewed in that light. We arrived at the current approach through some trial and error, with reasoning behind each step provided below. Our initial approach involved two major steps. First, we used the DsiRNA design tool provided by Integrated DNA technologies (https://www.idtdna.com/site/order/tool/index/DSIRNA_CUSTOM) to generate candidate sequences against the full cDNA sequence of a given target gene. This tool produces a maximum of 50 possible DsiRNA designs per input sequence. Secondly, we used NCBI's BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to check each candidate for possible off-target hits against both the genomic and non-redundant databases for *Lytechinus variegatus* (see Materials and Methods for details). This step is particularly important in sea urchins, whose genomes exhibit a high level (about 1 in 20 bases) of polymorphism (Britten et al., 1978; Sodergren et al., 2006). This initial process resulted in multiple functional DsiRNA designs, but with only ~1 out of 3 designs producing a phenotype for each gene tested. As the algorithm used in the IDT DsiRNA design tool is proprietary, we began checking designs against the more transparent shRNA design tool found on the Genomic Perturbation Platform provided by the RNAi consortium at the Broad Institute (GPP, available at https://portals.broadinstitute.org/gpp/public/seq/search) in hopes of generating a higher success rate with that tool's known parameters. The algorithm employed in this tool is designed to score 21-mers while DsiRNAs are 27-mers. As a result, output from the tool provides multiple intrinsic activity scores to evaluate for each design entered (see Materials and Methods). We also removed UTRs from consideration due to their higher rate of polymorphism, using only the CDS from each gene in DsiRNA design. Incorporating all these elements together has resulted in a high success rate (better than 1 in 3) for more than 20 additional genes in ongoing projects in the lab. It is important to note that even with all these design elements considered, there is no guarantee of each reagent producing a highly penetrant knockdown for the gene in question. We still design multiple DsiRNAs for each targeted gene.

Still concerning is the question of off-target effects. The investigator must use caution because these are not necessarily eliminated by the DsiRNA approach. Off-target effect possibilities are a given in experimental biology. Our approach to this concern has always been of two sorts: we attempt to address the question with multiple approaches that should complement each other. The addition of DsiRNA as a tool augments the ability to approach a question in multiple ways, thereby increasing confidence in a result. Also, since in situ analysis can record reduced levels of mRNA targeted by the DsiRNA, and consequently those same cells now display an altered function, an **on**-target effect has been detected. There still could be off-target effects in the system, but that likelihood is much reduced relative to most MASO experiments where the investigator is blind as to whether the intended knockdown of the targeted translation has occurred. Knowledge of an on-target effect thus increases confidence that an experiment is interpreted correctly.

In summary, the limited availability of reliable reagents for genetic perturbation has been a major barrier to work done in the sea urchin embryo. The availability of a simple, inexpensive, and reliable method for generating knockouts in this system is a boon for investigators using this model and offers an avenue for similar advances in other non-model organisms where few or no options for genetic perturbation are available.

Materials and Methods

Animals and embryos.

Adult *Lytechinus variagatus* (*Lv)* animals were obtained either from the Duke Marine Laboratory, Beaufort, NC or from Reeftopia.Com in Florida. Gametes were obtained by injection of 0.5M KCl. Fertilization and culture of embryos was in artificial sea water (ASW) at 23°C.

Selection of DsiRNA sequences

All DsiRNAs used in this study were obtained from Integrated DNA Technologies (IDT, www.idtdna.com). Candidate DsiRNAs are generated by entering the CDS for the gene of choice into the IDT DsiRNA design tool to obtain initial candidates for the gene in question (https://www.idtdna.com/site/order/tool/index/DSIRNA_CUSTOM). BLAST is used to check each candidate for any possible off target matches at both the genomic and transcript level (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Candidates with E-values for any off-target hits >= 1.0, with perfect off-target alignments to another transcript >= 14 bases, and candidates with any alignment of >= 15 bases with <= 2 mismatches to an off-target transcript are eliminated from further consideration. As the study progressed, we added additional selection criteria to increase the likelihood of finding functional DsiRNAs. Following the Blast screen, surviving candidate DsiRNAs are entered into the Broad Institute's GPP shRNA picking tool (Genomic Perturbation Platform available at https://portals.broadinstitute.org/gpp/public/seq/search), to retrieve intrinsic activity scores for each 27-mer using the reverse complement of the antisense strand as input. Candidates with the highest intrinsic activity scores are selected with special emphasis on scores at positions 1, 5, and 4 of the candidate sequence. From screened acceptable candidates, 3 DsiRNAs per gene complementary to distinct, non-overlapping regions of the transcript are ordered and tested for specific knockdown activity. In some cases, if there is a concern for possible polymorphisms in the sequence, we add another screen. The sea urchin genome is known to be highly polymorphic, i.e. on average about 1 of every 20 bases is polymorphic (Britten et al., 1978; Sodergren et al., 2006). To test for this possibility, the sequence of the gene of interest is aligned with orthologs from five species with sequenced genomes (*Strongylocentrotus purpuratus, Heliocidaris erythrogramma, Heliocidaris tuburculata, Lytechinus pictus and Lytechinus variagatus* (Sodergren et al., 2006; Davidson et al., 2020; Warner et al., 2021; Davidson et al., 2022). Candidates with low genetic variation are favored in the design of DsiRNAs. We also verified the lack of genetic variation in candidates tested based on genomes of *L. variegatus* collected from distinct gographical locations, one from animals collected at the coast of North Carolina two others from animals collected in the Florida Keys. This latter test is to determine whether the targeted sequence is identical in animals we use in different seasons of the year.

Sequences of DsiRNA and MASOs used in this study.

The sequences targeted in this study are:

Membrane-GFP: 5'GCAAGCTGACCCTGAAGTTCATCTGCA
LvPks1: 5'GATTCACTGACTACAACA3'
LvNodal:

5’CATGTTCATGGTGGAATTGTTTGAATC3’
5’ACGAGTGGATCATCTACCCAAAGCAGT3’
5’CCGTGTAAATTATGAATTCAGAGAATA3’
LvLefty:
5’CTGATTTTGACTTACAAATGGATATTC3’
5’TTGACTTACAAATGGATATTCTTGATA3’
5’CTGGAAGGTCTTTGACATCACCAACAC3’

GFP reporter experiment.

An mRNA containing a transmembrane sequence in frame with-*gfp* (Saunders and McClay, 2014) was injected into freshly fertilized zygotes. A DsiRNA directed against the *membrane-gfp* transcript was injected into one blastomere at the two-cell stage alone, or along with RITC-dextran to mark the injected cell and its progeny. Injected embryos were imaged at multiple timepoints during development.

Controls for non-specific effects of the presence of DsiRNA included a scrambled control designed to the *nodal* sequence used for morpholino knockdowns. Also, a traditional RNAi 18-mer designed to the successful *nodal* DsiRNA was injected to ask if that would work as well (it did not).

Experimental approach with DsiRNAs against endogenous genes.

Freshly fertilized embryos are injected with a mixture of DsiRNA + FITC-dextran. DsiRNAs are designed and ordered from IDT with design considerations carefully considered as above. DsiRNAs are reconstituted in RNase free water to a concentration of 100 uM, aliquoted into single use volumes and stored at -80°C until use. (the dilution series reconstituted the DsiRNAs to 400 µM). Aliquots are thawed on ice. Injections are performed with final DsiRNA concentrations of 25,50 and 75 uM to optimize concentration for each design, with co-injection of FITC-dextran in 30% glycerol as a tracer. Eggs are injected using established methods (Cheers and Ettensohn, 2004).

Fluorescent embryos (containing FITC-dextran) are selected and cultured to timepoints where a known, or suspected phenotype should be present if the DsiRNA functions as expected. Some injected embryos are fixed for in situ hybridization to determine if there is a visible reduction in RNA relative to control embryos at that stage in the cells of interest. We find that in situ analysis is sufficient and report those data here. In the experiments, in situ analysis for each gene tested revealed visual evidence of targeted mRNA knockdown in the expected location in the embryo.

In situ analysis

Standard protocols for in situ analysis were used with digoxigenin-11-UTP (Roche)- labeled probes (Croce et al., 2003). Hybridization chain reaction (HCR) in situs were performed using probes obtained from Molecular Instruments Corp. and used according to their protocol.

Acknowledgements.

We thank other members of our labs for their support and input, and we thank Brennan McDonald and Zach Pracher, enthusiastic undergraduates, for their many questions and encouragement as this project progressed.

Funding support: Support for the project was from NIH RO1HD14483 (to DRM) and NSF Division of Integrative Organismal Systems, 1929934. (to GAW).

References

Agrawal, N., Dasaradhi, P.V., Mohmmed, A., Malhotra, P., Bhatnagar, R.K., Mukherjee, S.K., 2003.

RNA interference: biology, mechanism, and applications. Microbiol Mol Biol Rev 67, 657-685.

Allen, R.L., George, A.N., Miranda, E., Phillips, T.M., Crawford, J.M., Kiehart, D.P., McClay, D.R., 2022. Wound repair in sea urchin larvae involves pigment cells and blastocoelar cells. Dev Biol 491, 56-65.

Amarzguioui, M., Lundberg, P., Cantin, E., Hagstrom, J., Behlke, M.A., Rossi, J.J., 2006. Rational design and in vitro and in vivo delivery of Dicer substrate siRNA. Nat Protoc 1, 508-517.

Arnone, M.I., Bogarad, L.D., Collazo, A., Kirchhamer, C.V., Cameron, R.A., Rast, J.P., Gregorians, A., Davidson, E.H., 1997. Green Fluorescent Protein in the sea urchin: new experimental approaches to transcriptional regulatory analysis in embryos and larvae. Development 124, 4649-4659.

Bardhan, A., Deiters, A., Ettensohn, C.A., 2021. Conditional gene knockdowns in sea urchins using caged morpholinos. Dev Biol 475, 21-29.

Bolouri, H., Davidson, E.H., 2009. The gene regulatory network basis of the "community effect," and analysis of a sea urchin embryo example. Dev Biol 340, 170-178.

Britten, R.J., Cetta, A., Davidson, E.H., 1978. The single-copy DNA sequence polymorphism of the sea urchin Strongylocentrotus purpuratus. Cell 15, 1175-1186.

Calestani, C., Rast, J.P., Davidson, E.H., 2003. Isolation of pigment cell specific genes in the sea urchin embryo by differential macroarray screening. Development 130, 4587-4596.

Calestani, C., Rogers, D.J., 2010. Cis-regulatory analysis of the sea urchin pigment cell gene polyketide synthase. Dev Biol 340, 249-255.

Carmell, M.A., Hannon, G.J., 2004. RNase III enzymes and the initiation of gene silencing. Nat Struct Mol Biol 11, 214-218.

Cheers, M.S., Ettensohn, C.A., 2004. Rapid microinjection of fertilized eggs. Methods Cell Biol 74, 287-310.

Chendrimada, T.P., Gregory, R.I., Kumaraswamy, E., Norman, J., Cooch, N., Nishikura, K., Shiekhattar, R., 2005. TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. Nature 436, 740-744.

Coffman, J.A., Wessels, A., Deschiffart, C., Rydlizky, K., 2014. Oral-aboral axis specification in the sea urchin embryo, IV: Hypoxia radializes embryos by preventing the initial spatialization of nodal activity. Dev Biol 386, 302-307.

Croce, J., Lhomond, G., Gache, C., 2003. Coquillette, a sea urchin T-box gene of the Tbx2 subfamily, is expressed asymmetrically along the oral-aboral axis of the embryo and is involved in skeletogenesis. Mech Dev 120, 561-572.

Davidson, E.H., Erwin, D.H., 2006. Gene regulatory networks and the evolution of animal body plans. Science 311, 796-800.

Davidson, E.H., Rast, J.P., Oliveri, P., Ransick, A., Calestani, C., Yuh, C.H., Minokawa, T., Amore, G., Hinman, V., Arenas-Mena, C., et al., 2002. A provisional regulatory gene network for specification of endomesoderm in the sea urchin embryo. Dev Biol 246, 162-190.

Davidson, P.L., Guo, H., Swart, J.S., Massri, A.J., Edgar, A., Wang, L., Berrio, A., Devens, H.R., Koop, D., Cisternas, P., et al., 2022. Recent reconfiguration of an ancient developmental gene regulatory network in Heliocidaris sea urchins. Nat Ecol Evol 6, 1907-1920.

Davidson, P.L., Guo, H., 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 Biol Evol 12, 1080-1086.

Duboc, V., Lapraz, F., Besnardeau, L., Lepage, T., 2008. Lefty acts as an essential modulator of Nodal activity during sea urchin oral-aboral axis formation. Dev Biol 320, 49-59.

Duboc, V., Lapraz, F., Saudemont, A., Bessodes, N., Mekpoh, F., Haillot, E., Quirin, M., Lepage, T., 2010. Nodal and BMP2/4 pattern the mesoderm and endoderm during development of the sea urchin embryo. Development 137, 223-235.

Duboc, V., Rottinger, E., Besnardeau, L., Lepage, T., 2004. Nodal and BMP2/4 signaling organizes the oral-aboral axis of the sea urchin embryo. Dev Cell 6, 397-410.

Duboc, V., Rottinger, E., Lapraz, F., Besnardeau, L., Lepage, T., 2005. Left-right asymmetry in the sea urchin embryo is regulated by nodal signaling on the right side. Dev Cell 9, 147-158.

Fire, A., Albertson, D., Harrison, S.W., Moerman, D.G., 1991. Production of antisense RNA leads to effective and specific inhibition of gene expression in C. elegans muscle. Development 113, 503-514.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C., 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391, 806-811.

Floc'hlay, S., Molina, M.D., Hernandez, C., Haillot, E., Thomas-Chollier, M., Lepage, T., Thieffry, D., 2021. Deciphering and modelling the TGF-beta signalling interplays specifying the dorsal-ventral axis of the sea urchin embryo. Development 148.

Gurdon, J.B., 1988. A community effect in animal development. Nature 336, 772-774.

Hammond, S.M., Caudy, A.A., Hannon, G.J., 2001. Post-transcriptional gene silencing by double-stranded RNA. Nat Rev Genet 2, 110-119.

Hannon, G.J., 2002. RNA interference. Nature 418, 244-251.

Heasman, J., 2002. Morpholino oligos: making sense of antisense? Dev Biol 243, 209-214.

Heasman, J., Kofron, M., Wylie, C., 2000. Beta-catenin signaling activity dissected in the early Xenopus embryo: a novel antisense approach. Dev Biol 222, 124-134.

Hibino, T., Loza-Coll, M., Messier, C., Majeske, A.J., Cohen, A.H., Terwilliger, D.P., Buckley, K.M., Brockton, V., Nair, S.V., Berney, K., et al., 2006. The immune gene repertoire encoded in the purple sea urchin genome. Dev Biol 300, 349-365.

Howard, E.W., Newman, L.A., Oleksyn, D.W., Angerer, R.C., Angerer, L.M., 2001. SpKrl: a direct target of beta-catenin regulation required for endoderm differentiation in sea urchin embryos. Development 128, 365-375.

Khor, J.M., Ettensohn, C.A., 2023. An optimized Tet-On system for conditional control of gene expression in sea urchins. Development 150.

Kim, D.H., Behlke, M.A., Rose, S.D., Chang, M.S., Choi, S., Rossi, J.J., 2005. Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. Nat Biotechnol 23, 222-226.

Konrad, K.D., Arnott, M., Testa, M., Suarez, S., Song, J.L., 2023. microRNA-124 directly suppresses Nodal and Notch to regulate mesodermal development. Dev Biol 502, 50-62.

Lapraz, F., Besnardeau, L., Lepage, T., 2009. Patterning of the dorsal-ventral axis in echinoderms: insights into the evolution of the BMP-chordin signaling network. . PLoS Biol. 7, e1000248.

Lee, R.C., Feinbaum, R.L., Ambros, V., 1993. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75, 843-854.

Madamanchi, A., Mullins, M.C., Umulis, D.M., 2021. Diversity and robustness of bone morphogenetic protein pattern formation. Development 148.

Martik, M.L., Lyons, D.C., McClay, D.R., 2016. Developmental gene regulatory networks in sea urchins and what we can learn from them. F1000Res 5.

Massri, A.J., McDonald, B., Wray, G.A., McClay, D.R., 2023. Feedback circuits are numerous in embryonic gene regulatory networks and offer a stabilizing influence on evolution of those networks. Evodevo 14, 10.

Materna, S.C., Ransick, A., Li, E., Davidson, E.H., 2013. Diversification of oral and aboral mesodermal regulatory states in pregastrular sea urchin embryos. Dev Biol 375, 92-104.

McClay, D.R., 2011. Evolutionary crossroads in developmental biology: sea urchins. Development 138, 2639-2648.

McClay, D.R., Croce, J.C., Warner, J.F., 2021. Conditional specification of endomesoderm. Cells Dev 167, 203716.

Molina, M.D., de Croze, N., Haillot, E., Lepage, T., 2013. Nodal: master and commander of the dorsal-ventral and left-right axes in the sea urchin embryo. Curr Opin Genet Dev 23, 445-453.

Molina, M.D., Lepage, T., 2020. Maternal factors regulating symmetry breaking and dorsal-ventral axis formation in the sea urchin embryo. Curr Top Dev Biol 140, 283-316.

Nam, J., Su, Y.H., Lee, P.Y., Robertson, A.J., Coffman, J.A., Davidson, E.H., 2007. Cis-regulatory control of the nodal gene, initiator of the sea urchin oral ectoderm gene network. Dev Biol 306, 860-869.

Napoli, C., Lemieux, C., Jorgensen, R., 1990. Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. Plant Cell 2, 279-289.

Oulad-Abdelghani, M., Chazaud, C., Bouillet, P., Mattei, M.G., Dolle, P., Chambon, P., 1998. Stra3/lefty, a retinoic acid-inducible novel member of the transforming growth factor-beta superfamily. Int J Dev Biol 42, 23-32.

Oulhen, N., Wessel, G.M., 2016. Albinism as a visual, in vivo guide for CRISPR/Cas9 functionality in the sea urchin embryo. Molecular Reproduction and Development.

Peter, I.S., Davidson, E.H., 2015. Genomic Control Process. Development and Evolution. Academic Press, San Diego.

Piacentino, M.L., Ramachandran, J., Bradham, C.A., 2015. Late Alk4/5/7 signaling is required for anterior skeletal patterning in sea urchin embryos. Development 142, 943-952.

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. Dev Biol 472, 85-97.

Range, R., Lapraz, F., Quirin, M., Marro, S., Besnardeau, L., Lepage, T., 2007. Cis-regulatory analysis of nodal and maternal control of dorsal-ventral axis formation by Univin, a TGF-beta related to Vg1. Development 134, 3649-3664.

Sampilo, N.F., Stepicheva, N.A., Zaidi, S.A.M., Wang, L., Wu, W., Wikramanayake, A., Song, J.L., 2018. Inhibition of microRNA suppression of Dishevelled results in Wnt pathway-associated developmental defects in sea urchin. Development 145.

Saunders, L.R., McClay, D.R., 2014. Sub-circuits of a gene regulatory network control a developmental epithelial-mesenchymal transition. Development 141, 1503-1513.

Schulte-Merker, S., Stainier, D.Y., 2014. Out with the old, in with the new: reassessing morpholino knockdowns in light of genome editing technology. Development 141, 3103-3104.

Shabalina, S.A., Koonin, E.V., 2008. Origins and evolution of eukaryotic RNA interference. Trends Ecol Evol 23, 578-587.

Sharp, P.A., 2001. RNA interference--2001. Genes Dev 15, 485-490.

Sodergren, E., Weinstock, G.M., Davidson, E.H., Cameron, R.A., Gibbs, R.A., Angerer, R.C., Angerer, L.M., Arnone, M.I., Burgess, D.R., Burke, R.D., et al., 2006. The genome of the sea urchin Strongylocentrotus purpuratus. Science 314, 941-952.

Song, J.L., Stoeckius, M., Maaskola, J., Friedlander, M., Stepicheva, N., Juliano, C., Lebedeva, S., Thompson, W., Rajewsky, N., Wessel, G.M., 2012. Select microRNAs are essential for early development in the sea urchin. Dev Biol 362, 104-113.

Song, J.L., Wessel, G.M., 2007. Genes involved in the RNA interference pathway are differentially expressed during sea urchin development. Dev Dyn 236, 3180-3190.

Su, Y.H., Li, E., Geiss, G.K., Longabaugh, W.J., Kramer, A., Davidson, E.H., 2009. A perturbation model of the gene regulatory network for oral and aboral ectoderm specification in the sea urchin embryo. Dev Biol 329, 410-421.

Summerton, J., Weller, D., 1997. Morpholino antisense oligomers: design, preparation, and properties. Antisense Nucleic Acid Drug Dev 7, 187-195.

Warner, J.F., Lord, J.W., Schreiter, S.A., Nesbit, K.T., Hamdoun, A., Lyons, D.C., 2021. Chromosomal-Level Genome Assembly of the Painted Sea Urchin Lytechinus pictus: A

Genetically Enabled Model System for Cell Biology and Embryonic Development. Genome Biol Evol 13.

Yaguchi, S., Yaguchi, J., Angerer, R.C., Angerer, L.M., Burke, R.D., 2010. TGFbeta signaling positions the ciliary band and patterns neurons in the sea urchin embryo. Dev Biol 347, 71-81.

Zamore, P.D., Tuschl, T., Sharp, P.A., Bartel, D.P., 2000. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell 101, 25-33.

Figure legends.

Fig. 1. DsiRNA to *gfp* destroys a *transmembrane sequence-gfp* mRNA. (A) Sea urchin eggs were injected with an mRNA expressing a transmembrane sequence in frame with *gfp*. At prism stage (20 hpf) all cells of the embryo are shown to express membrane bound GFP. (**B)** Zygotes were injected with the *membrane gfp* mRNA, then at the two-cell stage one of the two blastomeres was injected with a DsiRNA to *gfp*. A late blastula is shown with half the embryo displaying significantly less fluorescence than the other half. **(C1 and C2)** Zygotes were injected with the *membrane-gfp* mRNA and at the two-cell stage one of the two blastomeres was injected with DsiRNA to *gfp* plus RITC-dextran to mark the injected blastomere. The embryos were imaged at prism stage. Bars = 25 µm.

Fig. 2 DsiRNA to *pks1* eliminates expression of the mRNA for this enzyme. (A,B) 48 hpf larvae with pigment cells in red in the control **(A),** and absent in the larva injected with DsiRNA to *pks1* **(B). (C,D)** In situ of *pks1* at 20 hpf showing presence of mRNA to *pks1* in the control **(C),** but absent from the embryo injected with DsiRNA to *pks1* **(D). (E,F)** In situ of *acaca,* a gene expressed specifically in pigment cells showing its presence in both control and in embryos injected with DsiRNA to *pks1*. Thus, the DsiRNA removes the mRNA to *pks1*, but does not eliminate presumptive pigment cells. The embryo in **B** was injected with a DsiRNA containing a locked nucleic acid at the 3' ends, while the embryos in **D and F** were injected with the same DsiRNA sequence without the locked nucleic acid modifications. Both are effective in destroying the targeted mRNA. Bars = 25µm.

Fig. 3. Nodal, Lefty, and BMP signaling establish Dorsal-Ventral axis. (A) A MASO to Nodal was injected into zygotes in a dilution series beginning with a concentration of 0.75mM. At 20 hpf embryos were fixed and in in situs performed with a dorsal mesoderm marker (Pks1), ventral mesoderm marker (Irf4), and a dorsal ectoderm marker (Tbx2/3). Over the entire dilution range of the *nodal* MASO, excess dorsal pigment cells are specified, and no ventral mesoderm cells are specified until the 1:36 dilution of the MASO. The dorsal ectoderm marker is present throughout. **(B)** A MASO to Lefty was injected into zygotes in a dilution series beginning with a concentration of 0.75mM. Normally, Lefty knockdown results in ventralization of the embryo because too much Nodal reaches the otherwise dorsal half of the mesoderm resulting in absence of pigment cells (Pks1 marker). Even at a 1/36 dilution of the Lefty MASO there is insufficient Lefty produced to block this ventralization. The ventral marker is present throughout as expected. The ectoderm is less sensitive to excess Nodal in that Tbx2/3 returns at a Lefty MASO dilution of 1/24. (**C**). Overexpression of BMP2/4 results in dorsal radialization of the embryo producing pigment cells around the entire embryo (red), a radial arrangement of skeletogenic

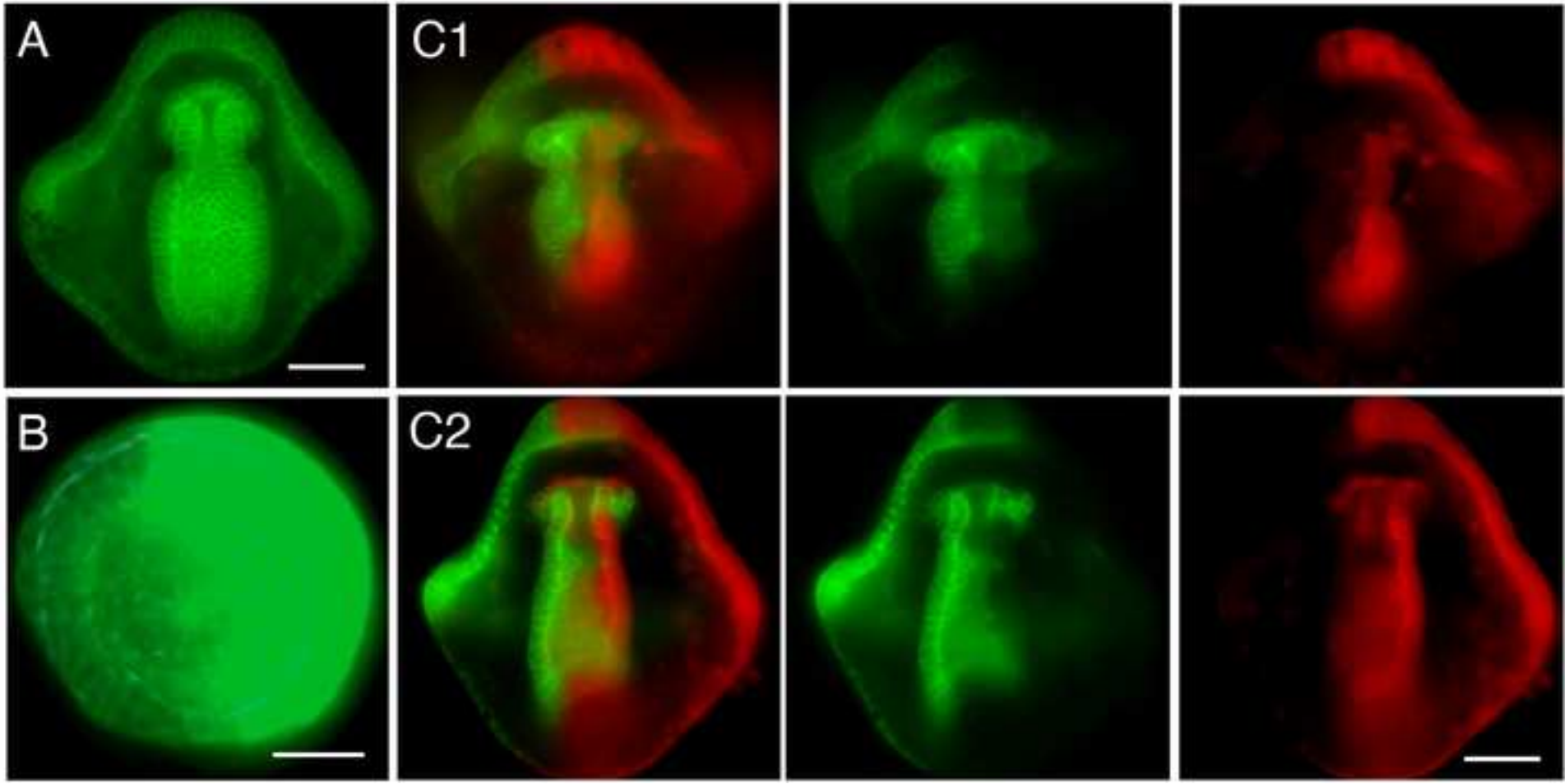
cells (green) and an absence of a ventral mesoderm marker (yellow – Astacin4 was probed but not detected). Bar = 25µm.

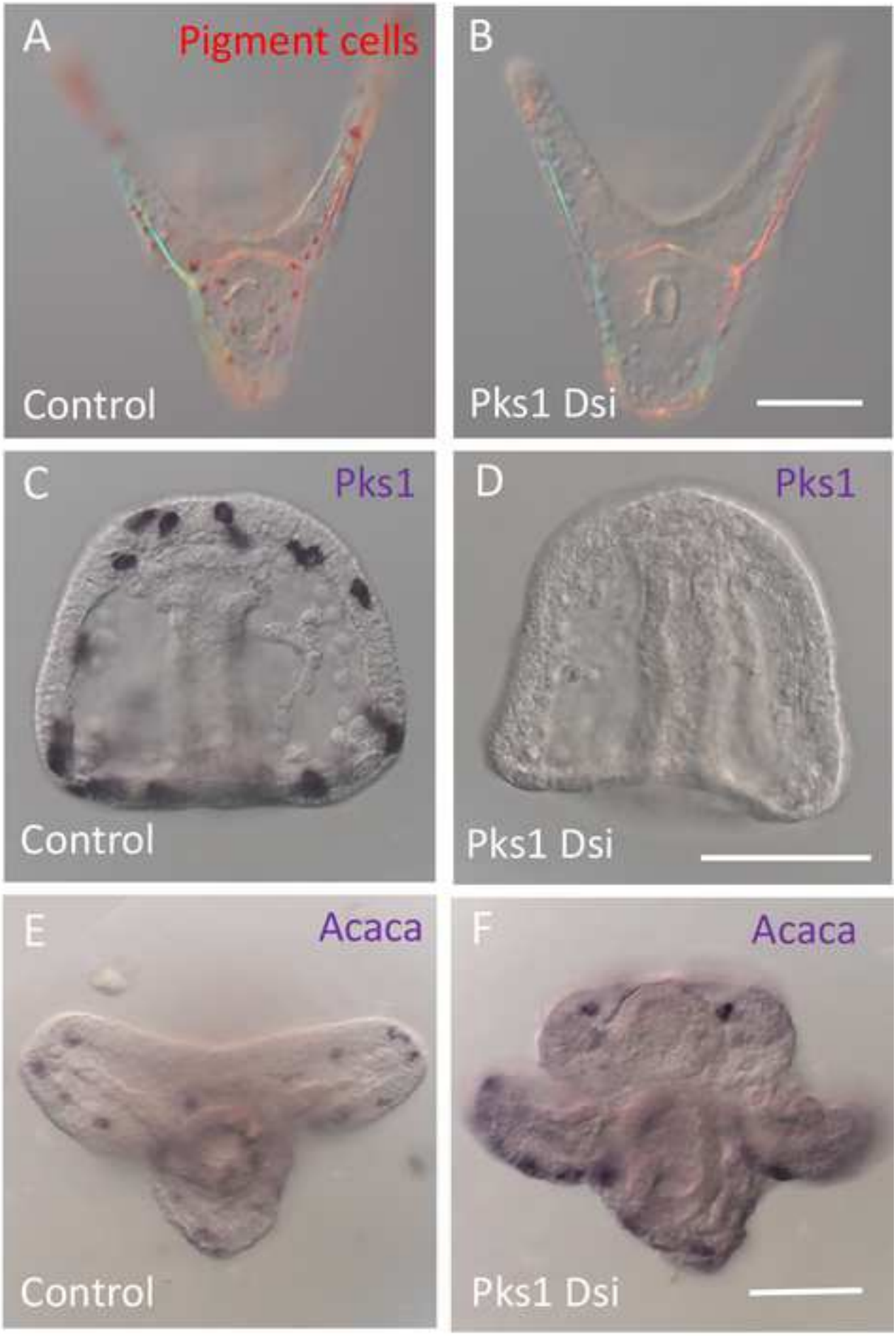
Fig. 4. (A). DsiRNA to *nodal* and *lefty*: Functional DsiRNAs destroy mRNA. Three DsiRNA oligos each were designed for *nodal* and *lefty.* One of the three in each case gave a radialized phenotype (*nodal* DsiRNA 3 and *lefty* DsiRNA 3). By in situ hybridization these embryos lacked expressed *nodal* or *lefty* mRNA. The DsiRNAs that failed to give phenotypes exhibited expressed mRNA as seen by in situ. *Lefty* DsiRNA 1 exhibited a low in situ signal and had a partial penetrance toward radialization. **(B) Phenotypes of DsiRNA knockdowns of *nodal* and *lefty.*** In the top row in situ by HCR (hybridization chain reaction) simultaneously records ventral mesoderm (astacin4, yellow), dorsal mesoderm (Pks1, red), and skeletogenic cells (Msp130, green). Nuclei are stained blue with Hoechst dye. In *Lefty* knockdowns there are no pigment cells and in *nodal* knockdowns there are excess pigment cells and few yellow blastocoelar cells. The lower row shows the overt radial phenotypes of the knockdowns compared to controls.

Fig. 5. A quantitative assessment of *nodal* and *lefty* knockdowns over a dilution range of DsiRNA. Blastocoelar, pigment and skeletogenic cells were quantified over a 20-fold dilution range of *nodal* and *lefty* DsiRNA beginning at 400 µM. Cells were counted from confocal image stacks (7 embryos per concentration) of DsiRNA with embryos fixed at 20 hpf (hours past fertilization).

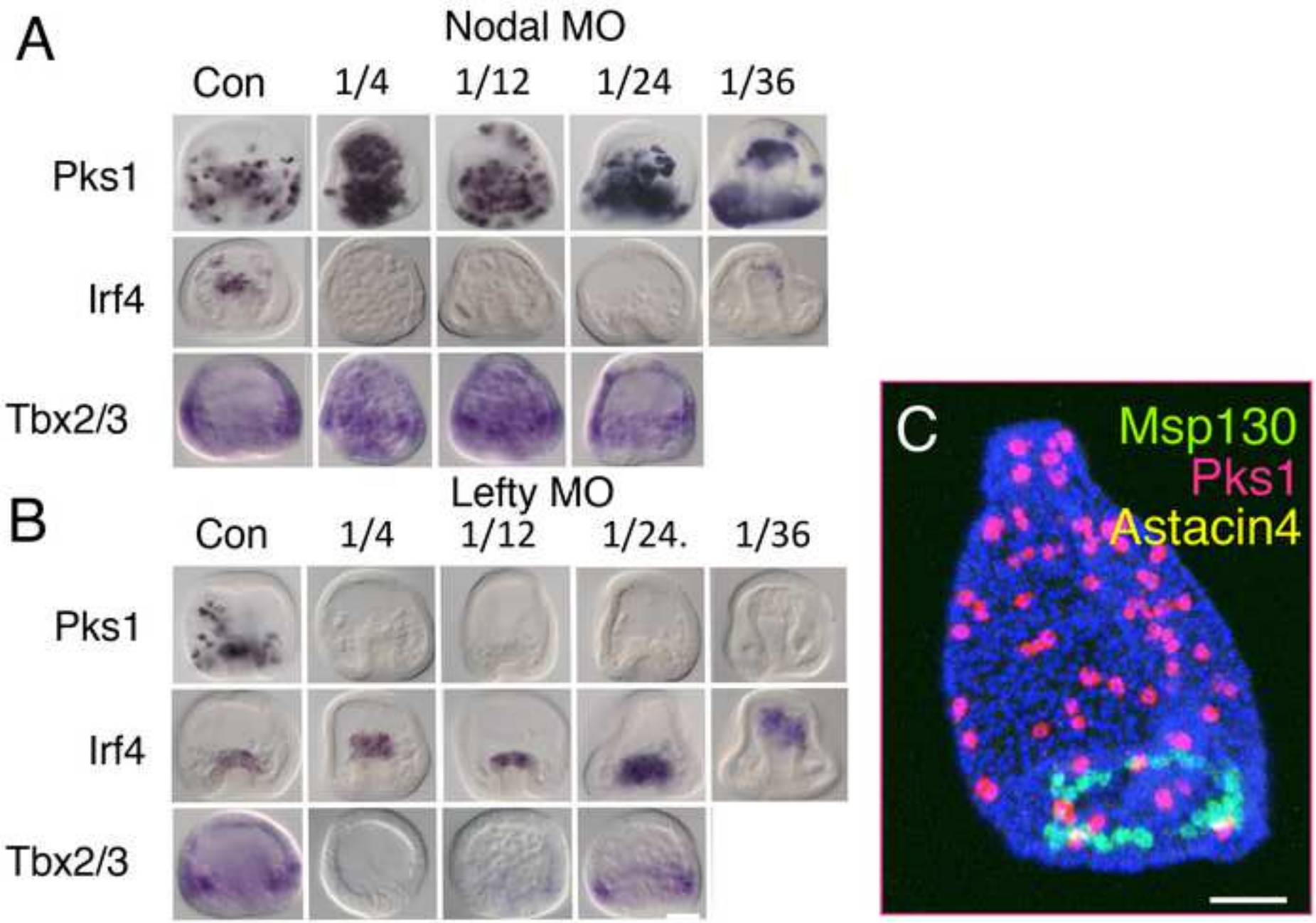
Supplementary Fig. S1. **Incomplete penetrance of DsiRNA.** In situ analysis of DsiRNA results indicate on-target destruction of the mRNA. Though in situ analysis is not quantitative, it also reveals when partial destruction occurs. In (**B**), some *pks1* expression is present (arrows), though in the same experiment, control embryos express far more *pks1* (**A**). In this experiment 85-90% of the embryos imaged exhibited no *pks1* expression as in **Fig. 2D.** 10-15% exhibited a low level of *pks1* as shown here (**B**).

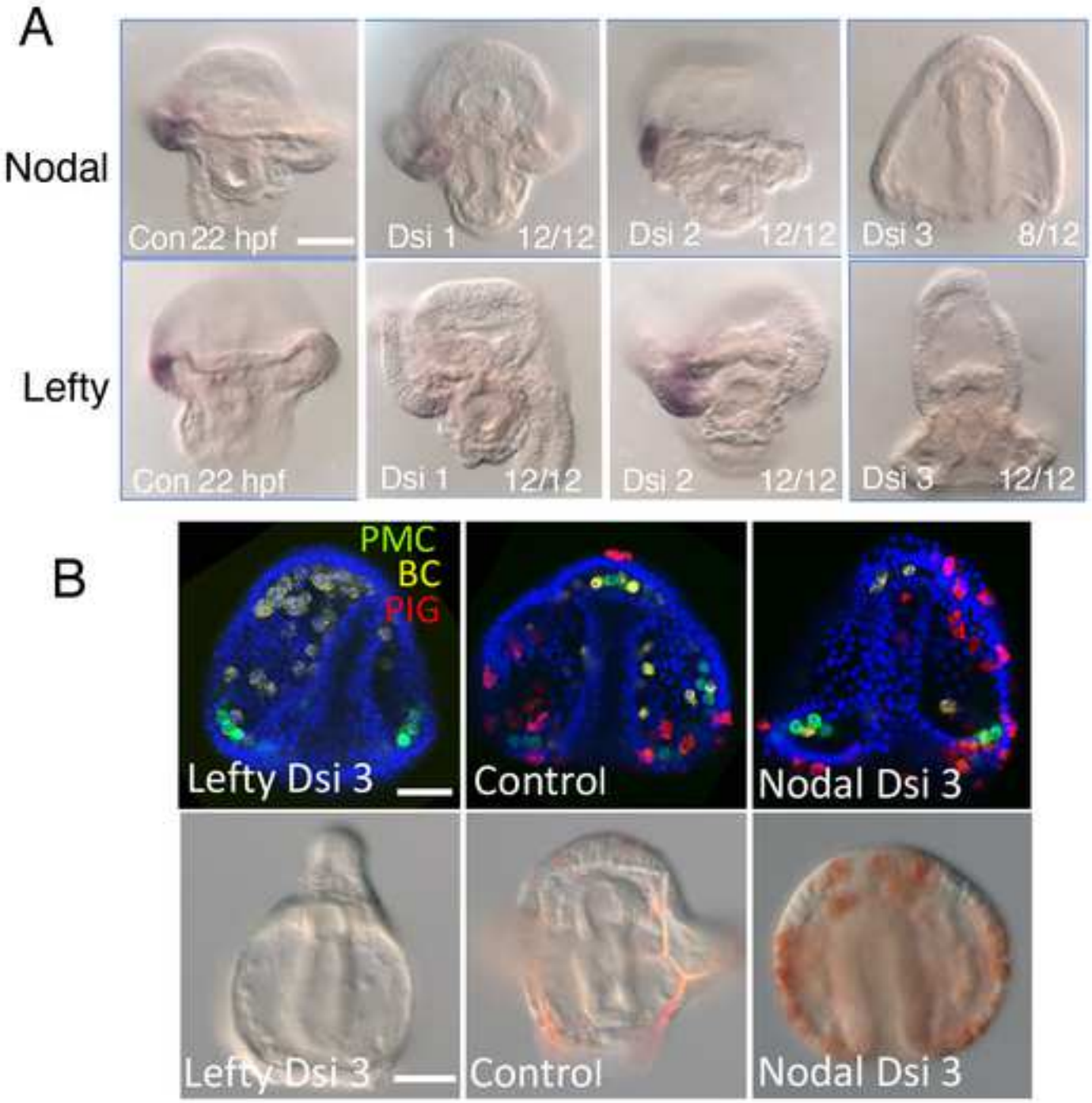
Click here to access/download;Figure;Fig. 1.tif




A
Pigment cells
Control
B
Pks1 Dsi
C
Pks1
Control
D
Pks1
Pks1 Dsi
E
Acaca
Control
F
Acaca
Pks1 Dsi

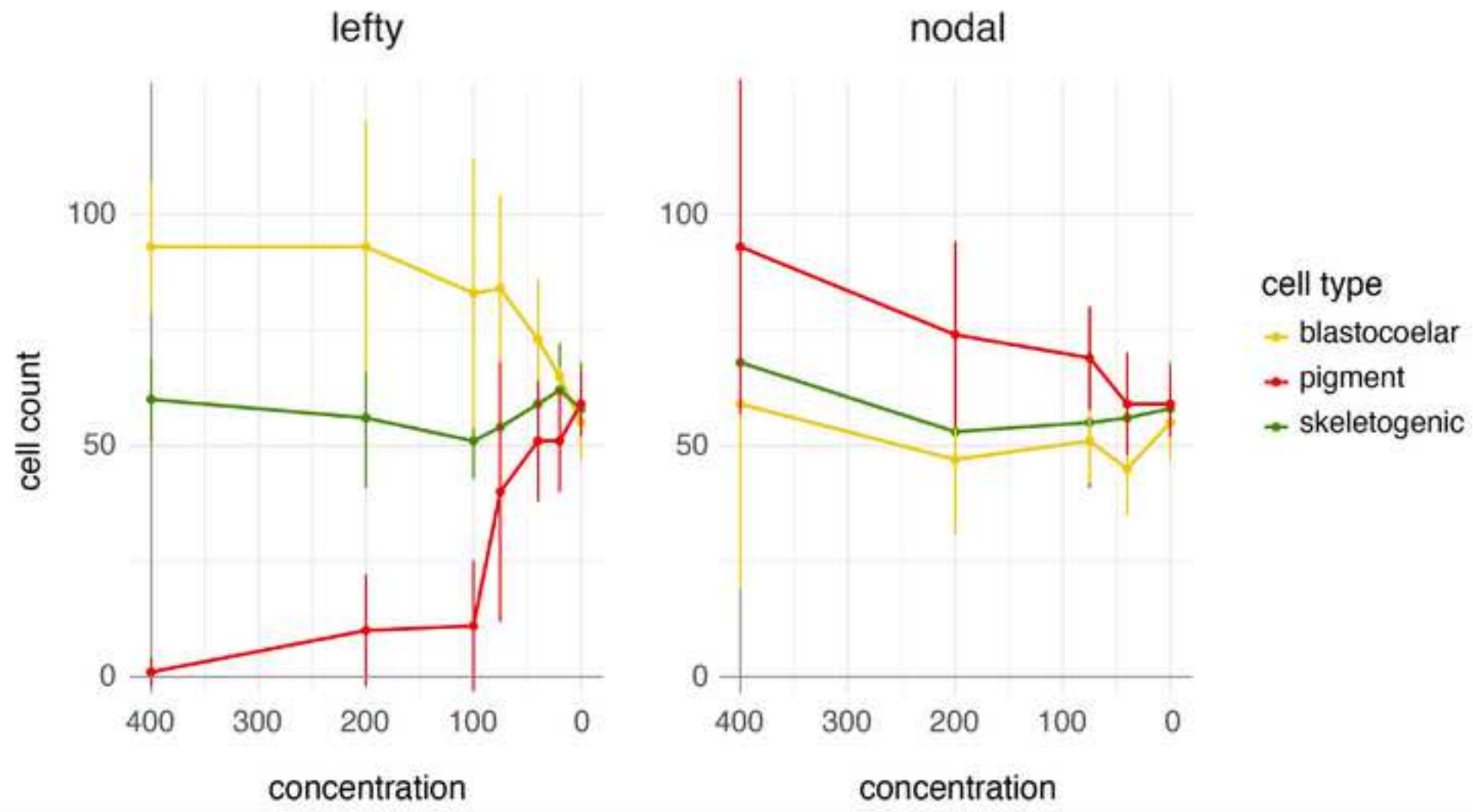
Click here to access/download;Figure;Fig. 3.tif




A
Nodal
Lefty
Con 22 hpf
Dsi 1 12/12
Dsi 2 12/12
Dsi 3 8/12
Con 22 hpf
Dsi 1 12/12
Dsi 2 12/12
Dsi 3 12/12
B
PMC
BC
PIG
Lefty Dsi 3
Control
Nodal Dsi 3
Lefty Dsi 3
Control
Nodal Dsi 3

Figure

Click here to access/download;Figure;Fig. 5.tif



Click here to access/download
Supplementary Material
Fig. S1.tif