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The temporal control and activity of maternal *zsquildlike*-A/*hnrnpaba* during zebrafish embryogenesis indicate a role in early pattern formation

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Research Article

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Abstract

During embryogenesis in *Danio rerio* (zebrafish), the earliest morphological patterning events are dependent on the precise temporal translation and/or localization of specific maternal mRNAs/proteins. Dorsoventral patterning in particular requires the translocation of maternal factors that are present in the Balbiani Body from the vegetal region of the unfertilized egg to the future dorsal side of the embryo (Fuentes *et al*., 2020), leading to the localized activation of the β-catenin pathway in the cells in that region. Since zebrafish are chordates, this dorsoventral patterning then leads to the formation of neural tissue on the dorsal side of the embryo. What is not yet clear is the identity of all maternal and zygotic factors that first establish dorsoventral patterning, and which factors lead to the establishment of neural versus non-neural tissue. Taking an evolutionary approach to this question, we investigated a gene in zebrafish, *zsquidlike-A* (*hnrnpaba*), that is homologous to a key dorsoventral patterning gene in fruit flies (*Drosophila melanogaster*) called *squid* (Kelley, 1993). While dorsoventral patterning in flies and fish looks quite different both morphologically and at the molecular level, we demonstrate that not only has a key dorsoventral patterning gene in flies been conserved in fish, maternal fish zsquidlike-A protein is synthesized precisely as dorsoventral patterning is unfolding in fish embryos, and in its absence, dorsoventral patterning is severely disrupted.

Introduction

During embryogenesis in both vertebrates, such as *Danio rerio* (zebrafish), and invertebrates such as *Drosophila melanogaster* (fruit fly), the initial radial symmetry of the embryos is broken and bilateral symmetry is established. This feature places both of the larger taxonomic groups to which they belong (chordates, which are deuterostomes, and arthropods, which are protostomes) in the bilaterian. The result is that in adult chordates and arthropods, the *overall* body orientation of the dorsal/ventral axis relative to anterior/posterior and left/right axes is the same. In addition, during embryogenesis in both organisms, the molecular pre-pattern for bilateral symmetry begins with the localization of maternal factors present in the unfertilized egg and is realized by localized activation of zygotic genes. While dorsoventral patterning in these two species looks quite different, investigation of the subsequent process of neurulation at the molecular level in bilaterians from all taxonomic groups has revealed remarkable conservation of molecular pathways. In all cases studied to date, establishment of the neural/non-neural axis relies on opposing gradients of the conserved ligand Bone Morphogenic Protein (BMP) (in fish; *decapentaplegic* in flies), which is high on the non-neural side, and BMP antagonists such as chordin (in fish; *short gastrulation* in flies) which are high on the neural side (for review see Yan and Wang, 2021).

In spite of their shared body plan and the conservation of the molecular regulation of neurulation, the initial establishment of bilateral symmetry and early dorsoventral patterning look quite different in fish and flies. In zebrafish, fertilization itself breaks the radial symmetry of the egg, which results in a shift of dorsal determinants that were localized to the vegetal cytoplasm in a non-membrane bound structure called the Balbiani body (Fuentes *et al*., 2020; Houston, 2017). This leads to the localized activation of a central player in dorsoventral patterning in vertebrates, the maternal transcription factor β-catenin, in a subset of dorsal cells called the "organizer" (for review see Abrams and Mullins, 2009; Jones and Mullins, 2022). Among the targets of β-catenin are the zygotic genes *goosecoid*, which codes for a transcription factor, and *chordin* and *noggin*, whose protein products are then secreted by the organizer cells and operate by antagonizing a maternally encoded signalling protein called BMP, a TGF-β family member (Pomreinke *et al*., 2017) that is the central player in the formation of ventral tissue. This antagonism by noggin and chordin is critical, for when these organizer-specific

secreted proteins are removed from the embryo, BMP is capable of producing an embryo with no dorsal side (or nervous system) that is thus 'ventralized'. In vertebrates such as zebrafish, two opposing gradients of chordin/noggin and BMP are therefore established such that BMP is highest on the future ventral side, and this is where non-neural structures form, while on the future dorsal side the organizer proteins chordin and noggin are highest, ultimately leading to the formation of neural tissue (De Robertis *et al.*, 2017; Mizutani and Beir, 2008).

In flies, the prepatterns for both anterior/posterior and dorsal/ventral are not dependent on fertilization. In fact, both are established before fertilization by localizing maternal factors in regions of the egg cytoplasm relative simply to the position of the egg in its egg chamber in the ovary (Stein and Stevens, 2014). Additionally, in flies, there is a different molecular system that establishes the dorsal side of the embryo. This system requires the activity of the product of maternal *gurken* (*grk*) mRNA, a TGF-β-like growth factor that is secreted by the egg only on the future dorsal side of the embryo. The Gurken protein operates by binding to its receptor, the Torpedo protein (a homolog of vertebrate Epidermal Growth Factor Receptor [EGFR]), present on surrounding follicle cells (for review see Stein and Stevens, 2014). A downstream effect of restricting Gurken activity to the dorsal side is that on the opposite side of the embryo, in the absence of Gurken signalling, a pathway is activated that ultimately allows a ventral-specifying transcription factor to enter nuclei on the future ventral side. In a fascinating evolutionary twist however, in a fly embryo – after the dorsal and ventral sides have been established – it is on the ventral side of the fly embryo that the nervous system (the nerve cord) then forms (Stein and Stevens, 2014).

In spite of the significant molecular and morphological differences in fish and fly dorsoventral patterning, and the opposite orientation of neural tissue along the dorsoventral axis, the molecular regulation that establishes neural versus non-neural appears to be remarkably conserved (Schloop *et al.*, 2020). Not only are there fly homologues of many components of the BMP and chordin pathways, but they are also maternally expressed in the fly embryo, and two opposing gradients of these pathways are established in the fly. Furthermore, signalling by the fly homolog of BMP (*decapentaplegic*, *dpp*) is again gradually limited to the non-neural – in this case dorsal – side of the embryo by factors such as the fly homolog of chordin (called *short gastrulation*, or *sog*), which are expressed at their highest levels on ventral side of the embryo where neural tissue differentiates (for review see Mizutani and Beir, 2008; Beir, 2011). As would be predicted by the flipped orientation of neural/non-neural relative to dorsoventral in the two organisms, when the molecular BMP story is mapped onto the morphological patterning in zebrafish versus fruit flies, the orientation of the BMP/chordin gradients is also flipped 180^0. This is why in flies the earliest patterning events somehow result in BMP being high on the *dorsal* side of the embryo; therefore neural, tissue can only form on the *ventral* side of the embryo. Unlike the situation in vertebrates, the molecular players that connect dorsoventral patterning in flies, initiated by Gurken, to non-neural on the dorsal side versus neural on the ventral side via the gradients of BMP and its antagonists, has not been thoroughly elucidated. There is evidence for a second, separate role for Gurken in regulating BMP signalling (Carneiro *et al.*, Carneiro *et al.*, 2006), but the system is clearly distinct from what is seen in zebrafish.

Collectively, the observations about the establishment of bilateral symmetry in different species of bilaterian continues to raise the question regarding what has been evolutionarily conserved versus what are the points of divergence. To date, there is no evidence that a functional homolog of *gurken* is present in zebrafish. The vertebrate *gurken* homolog belongs to the neuregulin family, and there is no evidence of a role of a neuregulin protein during the establishment of bilateral symmetry in the fish embryo (Pu *et al.*, 2017). However, the localization and translation of maternal *gurken* mRNA is regulated in part by a second maternal factor, an hnRNP protein called Squid. The role of Squid in dorsoventral patterning was discovered based on the phenotype of *squid* mutants: females carrying weak *squid* alleles have embryos that are dorsalized (Kelley, 1993). Additional studies demonstrated that the Squid protein is required for the proper localization and translational control of maternal *gurken* mRNA (Norvell *et al.*, 1999), in part because the Squid protein binds to any mis-localized *gurken* mRNA and represses its translation (Clouse *et al.*, 2008). Previously we identified a potential homolog of *squid* in zebrafish (O'Connell *et al.*, 2014), an hnRNP protein called *hnrnpaba/zsquidl-A*, which is one of four members of a monophyletic clade of genes that share significant sequence homology with the single fly *squid* gene, called the hnRNP D subfamily in fish (Akindahunsi *et al.*, 2005).

In the current studies, we confirm that the four zebrafish genes form a single clade that likely resulted from both gene duplication and genome duplication. We also demonstrate that in the case of *zsquidl-A,* the mRNA present throughout early development is entirely maternally provided and that this population of maternal *zsquidl-A* mRNAs is subjected to cytoplasmic polyadenylation at the 64–128 cell stages. Together these data indicate that the zsquidl-A protein first becomes available to the embryo just as maternal and zygotic factors are combining their efforts to generate dorsoventral patterning (Pelegri, 2003). In addition, *zsquidl-A* morphants display pronounced dorsal/ventral patterning defects in a dose-dependent manner. Therefore our results suggest that one of the gene products that is required to establish the dorsoventral body axis of the embryo is conserved between a protostome and a deuterostome, and is the product of the *zsquidl-A*/*squid* gene.

Materials and methods

Fish husbandry

Wild-type zebrafish (*Danio rerio*) were obtained from a local pet shop and housed in fish water (Westerfield, 1993) at 28^0C, in a room with a 14/10 hour light/dark cycle. Prior to embryo collection marbled containers were placed in the tanks, and within 30 minutes of the lights coming on ('dawn') on Day 1, embryos were collected and placed in fish water (Westerfield, 1993) at 28ºC. The protocol for fish handling (#07089-001) was approved by the TCNJ IACUC (Institutional Animal Care and Use Committee).

Bioinformatic analysis

An NCBI BLAST search (www.ncbi.nlm.nih.gov) conducted using the amino acid sequences of the *Drosophila* SquidA and S isoforms as the driver identified the four genes in the *D. rerio* hnRNP D subfamily as the closest homologues. Further information was obtained from ensembl (www.ensembl.org), NCBI and uniprot (www.uniprot.org). Additional information specific to *D. rerio* was obtained from ZFIN (www.zfin.org), and *D. melanogaster* was obtained from flybase (www.flybase.org). The sequence IDs of the genes used in the phylogenetic analysis are as follows: Hnrnpa0a (NP_997810.2), Hnrnpa0l (NP_001268650.1), Hnrnpa0b (NP_999871.1), Hnrnpa3 (NP_001315077.1), Hnrnpa1b (NP_

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956398.1), Hnrnpa1a (NP_001349307.1), Hnrnpaba (NP_997752.2), Hnrnpabb (NP_998467.1), Hnrnpd0 (NP_001103930.1), Hnrnpdl (NP_001315423.1), Hnrnpm (NP_001243560.1), Hnrnpr (NP_998591.1), Hnrnpua (XP_694691.5), Hnrnpub (NP_001028767.2), Hnrnpul (NP_998436.1), Hnrnpul1l (XP_003198760.1), Hnrnph1l (NP_997754.2), Hnrnph1 (NP_991247.1), hnrnph3 (NP_001314853.1), hnrnpk (NP_998159.2), Hnrnpc (NP_998244.1), Hnrnpl2 (NP_998548.1), Hnrnpl (NP_957393.1), Hnrnpi (NP_001018313.1) sqd (NP_731825.1), hnRNPAB (NP_112556.2), hnRNPD0 (NP_112738.1), hnRNPDl (NP_112740.1), hnRNPA2/B1 (NP_002128.1), hnRNPA1 (NP_002127.1).

Phylogenetic analysis

Neighbour-joining phylogenies were constructed to assess parology of the zebrafish hnRNP genes and orthology of the hnRNP D subfamily. Unaligned FASTA (FAST-All) files were imported into MEGA 11 and were aligned via the MUSCLE algorithm before tree construction. 100 bootstrap replicates were performed. The trees were drawn to scale, with branch lengths indicating the evolutionary distances used to infer the phylogenetic tree, which were computed using the Poisson correction method and are in units of the number of amino acid substitutions per site. All ambiguous positions were removed for each sequence pair by the pairwise delete option. To assess amino acid sequence similarity, ClustalO was used to perform multiple alignments of the gene products and their conserved domains and generate percent identity matrices. ClustalO formatted sequences were exported with character counts, and conserved domains were identified on NCBI and confirmed with Prosite analysis.

Synthesis of polyA+ cDNA

Total RNA was isolated from 30 to 50 embryos/stage at indicated stages using Trizol Reagent (Ambion). First-strand polyA+ cDNA was synthesized and subjected to PCR using the PAT assay developed by Salles and Strickland (1995). With this protocol a first-strand cDNA library is created that contains complements of mRNAs with their full poly(A)tails. Briefly, prior to the reverse transcription reaction, 100–500 ng total RNA is incubated with oligo(dT) and T4 DNA ligase at 42° for 30 minutes. An oligonucleotide is then added that has a stretch of 12 Ts followed by a G/C-rich "anchor" sequence (anchorT) for PCR; this hybridizes to the last 8–10 As at the 3' end of the polyA tails of mRNAs. This mixture is then subjected to reverse transcription with Stratascript RT (Stratagene) to generate polyA+ cDNA.

Polymerae chain reaction (PCR)

Standard PCR reactions were performed with GoTaq green (Promega), cDNA from each staged cDNA library (generated from equivalent numbers of embryos), and forward and reverse primers. The primers used for the two controls were: *zef1α*, F:GGC TGACTGTGCTGTGCTGATTG, R:CTTGTCGGTGGGACGGCT AGG; and *Id1*, F:ACCGACCAACAAGAAAGCCA, R:GG TCCATCCATCGGGGTTTG. The primer pairs for the four zebrafish *squidlike* genes were: *zsquidl-A* expression, F:CCCCCTAG TGTCACCTGTGC, R: CACGATGGGAGGAAGTCTCC, and nested PCR F:GGCTGTGTCTATCACATCTAGGCTC; *zsquidl-B*, F:GCCACATTGAGGTTCGGGAGC, R:GAGCGGACACTTCC AGTCAC; *zsquidl-C*, F:CAGTACTACACYTTGGTCGCC, R:CG TTTACATGACTGTAGCG; *zsquidl-D*, 161F:CTTTAGCACCG ATGAGTTTCC, 748R:CTCGTCCYCCYCCGAAGCCCC, 3'UTR-F:GCTCCTCGGAGGCAGCTTGTG, 3'UTR-R:CCCTTTATCCAA AAACGTCAGG. The polyadenylation status of a particular mRNA was assayed by performing PCR on cDNA samples using a gene-specific forward primer and the anchorT reverse primer (anchorT: $GCGAGCTCCGCGGCCGCGT_{12}$). The standard PCR reaction was 95°/60°/72° for 35 cycles (Salles and Strickland, 1995). Amplified products were then either immediately separated on a 1.2% or 3% agarose gel, or used as the template for a second PCR reaction ("nested PCR") using primers internal to those used for the first PCR, before being separated to electrophoresis on agarose gels.

Sucrose pad centrifugation

Embryos were dechorionated and collected in Polysome Extract Buffer (PEB, 0.02M Tris-HCl pH = 7.4, 0.2M KCl, 5 mM$MgCl_2$, 0.5 mM DTT). To ensure that intact RNA was present total RNA was purified from a portion of each extract using Trizol Reagent (Ambion), and used as a template for RT-PCR with *zef1*α primers. To pellet the polysomes, sucrose pads were prepared with 1.75 M sucrose and 4.4 ml 0.5M sucrose in PEB, each with freshly added cycloheximide, heparin and 2-mercapto-ethanol (Masek *et al.*, 2011). Equal quantities of extract were layered on top of the sucrose solution and the tubes were balanced with additional PEB. Centrifugation was performed at 38,500 rpm, 4^0C, for 4 hours. Pellets were re-suspended in Trizol Reagent (Ambion), and RNA was purified. Densitometry of bands produced via RT-PCR was performed using ImageJ.

Embryo microinjection

To generate morphants, all morpholinos were purchased from GeneTools, and in each case, the morpholino was designed so that it would hybridize with the region of the target mRNA that contains the AUG, so as to get maximal translational repression (GeneTools). The morpholino sequences are: *zsquidl-A*, AC TGCTGCTCGGCGTCTGACATGGT; *zsquidl-B*, TCTCCAT GACATGCTCGTCGGCCAT; *zsquidl-C*, TCTCCATGAACT GCT. Embryos were injected in the yolk at the 1–8 cell stage to ensure distribution of morpholino or α-amanatin to all embryonic cells, and then incubated in fish water at 28^0C.

Whole mount *RNA* In Situ *hybridization*

RNA ISH was performed as described (Pelliccia *et al.*, 2017) using a probe for *goosecoid* (*gsc*) (Stachel *et al.*, 1993).

Results

An NCBI blast search using the *Drosophila* Squid protein sequence as the driver resulted in the identification of four genes in zebrafish that are closely related to the fly *squid* gene (between 42 and 44% identity). Two separate neighbour-joining tree algorithms were conducted, the first constructed using the amino acid sequences of the two RNA Recognition Motifs (RRMs) present in all family members (Akindahunsi *et al.*, 2005), and the second based on the amino acid sequences of the longest isoform of each of the four zebrafish proteins. Both indicate that the four zebrafish genes form a monophyletic clade within the entire zebrafish hnRNP gene family (Figure 1). This same result was obtained when the DNA sequences of all zebrafish hnRNP genes were used to generate a phylogeny (data not shown). Therefore we will continue to refer to them as *zsquidl-A-D* (O'Connell *et al.*, 2014). Comparison with homologues in other vertebrate species indicates that the *zsquidl-A*

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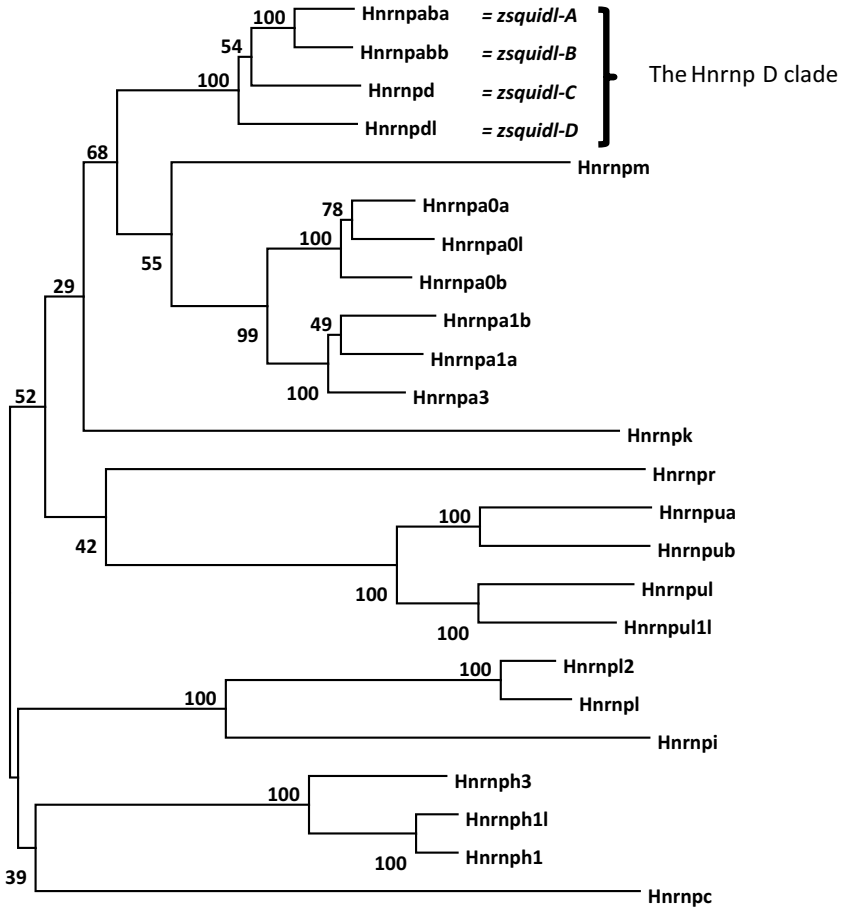

Figure 1. Comparison of the amino acid sequences of all hnRNP proteins in zebrafish in an unrooted neighbour-joining phylogenetic tree. 100 bootstrap replicates were performed, and their values are indicated at the nodes. The bracket indicates the D clade of zebrafish hnRNP proteins.

(*hnrnpaba*) and *zsqdl-B* (*hnrnpabb*) genes, which are on different chromosomes, likely arose from the ancestral zebrafish whole genome duplication event (Taylor *et al.*, 2003). The *zsquidl-C* (*hnrnpd*) and *zsquidl-D* (*hnrnpdl*) genes are adjacent to one another on chromosome 10 (www.zfin.org), suggesting that they arose from an ectopic recombination event leading to gene duplication. A ClustalO alignment of the four zebrafish proteins and the two isoforms of *Drosophila* Squid (SquidA and S) that produce 100% rescue of the dorsalized phenotype in flies (Norvell *et al.*, 1999) revealed that outside of the two RNA-binding domains, there are no remarkable regions of homology between the *Drosophila Squid* proteins and the four zebrafish proteins. In light of the fact that one distinguishing feature between the fly Squid isoforms that mediate *gurken* mRNA nuclear export versus its translation is the presence of an M9 nuclear localization signal, there is a sequence that shares a loose homology with the conical M9 nuclear localization signal in the C terminal regions of zsquidl-A and B, but not zsquidl-C and D.

As reported previously, both *zsquidl-A* and *B* are expressed maternally, and while *zsquidl-B* has a long polyA tail throughout embryogenesis, *zsquidl-A* shows a dynamic pattern of polyadenylation consistent with its being regulated by cytoplasmic polyadenylation (O'Connell *et al.*, 2014). In order to perform an analysis of all four members of this group and determine whether any of them are synthesized at a time when they might play a regulatory role in patterning during zebrafish embryogenesis, we first investigated the expression and polyadenylation status (as an indication of temporal translational control) of *zsquidl-C* and *zsquidl-D* mRNAs via PCR of polyA$^+$ cDNA (Salles and Strickland, 1995) prepared from staged embryos. As seen in Figure 2, *zsquidl-C* transcripts are also present during zebrafish embryogenesis, though they display a dynamic pattern of expression. Significant levels of *zsquidl-C* mRNA are present maternally (see 1-cell stage, Figure 2A), but then disappear. Zygotic expression appears several hours later, well after the Mid-Blastula Transition (MBT) in the mid-epiboly stages (Figure 2A). This pattern of expression was seen with 3 separate pairs of *zsquidl*-C primers, and the identity of the PCR products was confirmed by restriction digest (data not shown). Since the cDNA libraries were constructed by amplifying the entire polyA tail, a PAT-PCR analysis could be performed using the anchorT reverse primer. This revealed that when present, both maternal and zygotic *zsquidl-C* transcripts are polyadenylated throughout the time periods followed (data not shown).

An analysis of *zsquidl-D* gene expression demonstrates that, like *zsquidl-B* mRNA, *zsquidl-D* mRNA is expressed both maternally and throughout early embryogenesis at high levels (four representative stages are shown in Figure 3A, "expression"; the identity of the *squidl-D* PCR products was again confirmed by restriction digest, data not shown). In order to determine the polyA status of *zsquidl-D* transcripts, a PAT-PCR assay was performed (Figure 3A, "polyadenylation"). At all four stages, a faint and heterogeneous series of PCR products is seen that are larger than the distance between the primer and the end of the 3'UTR. This pattern is what is seen when the transcripts of a particular gene are polyadenylated throughout development. This contrasts with what we observed for *zsquidl-A* transcripts, where the entire population of transcripts has a short polyA tail just after fertilization, which then lengthens by several hundred nucleotides to a relatively

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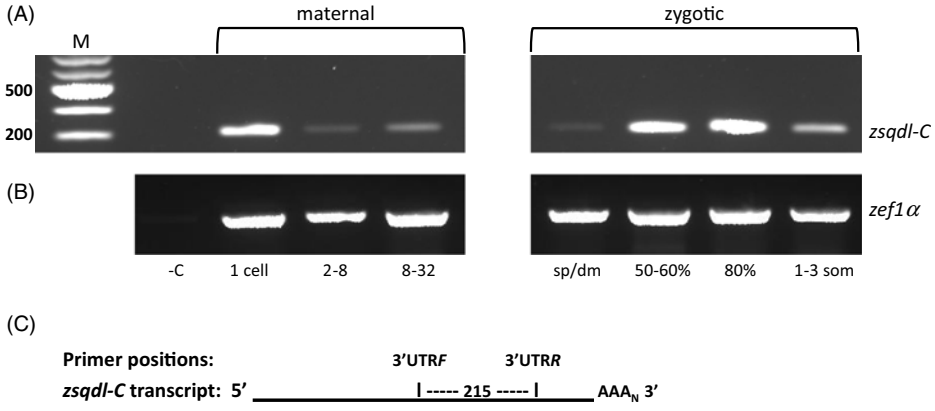

Figure 2. Expression of the maternal and zygotic *zsquidl-C* mRNAs during embryogenesis. Total RNA was purified from the stages indicated and polyA$^+$ cDNA was prepared and diluted 1:10. Equal amounts of cDNA (corresponding to equal numbers of embryo equivalents) were subjected to PCR and products separated by agarose gel electrophoresis. (**A**) PCR performed with primers for *zsquidl*-C with an expected product of 276 bp using cDNA template from the stages indicted, M = 100 bp ladder (**B**) PCR performed with primers for *zef1α*. (**C**) Position of primers in the *zsquidl*-C sequence.

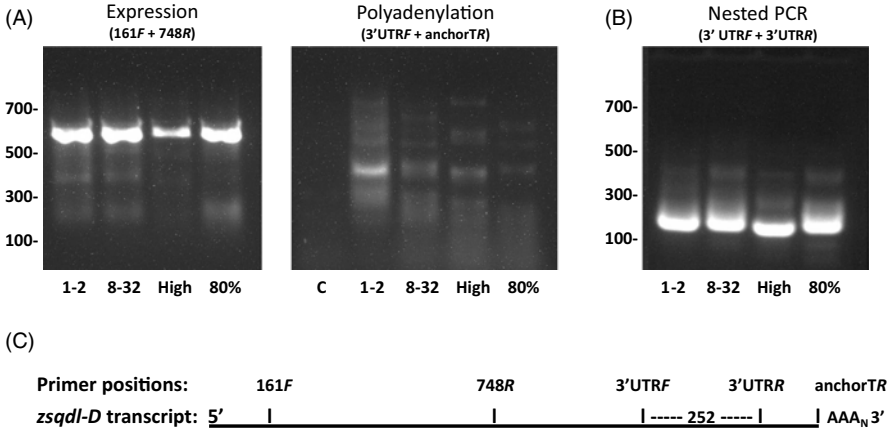

Figure 3. *zsquidl*-D mRNA is expressed and polyadenylated throughout embryogenesis. Total RNA was purified from the stages indicated and polyA$^+$ cDNA was prepared. (**A**) PCR was performed either with primers for *zsquidl*-D that produce a band of 587 nt ("expression"), or a *zsquidl*-D forward primer that anneals in the 3'UTR at position 1248 (3'UTR*F*) and the anchorT reverse primer ("polyadenylation"). (**B**) Nested PCR was performed using the PCR products from B as the template DNA and internal primers for *zsquidl*-D, 3'UTR*F* and 3'UTR*R* (position 1500) (**C**) Position of primers in the *zsquidl*-D sequence.

uniform size by 30% epiboly (O'Connell *et al.*, 2014). The proposed polyadenylated products of *zsquidl-D* of this first round PCR-PAT assay were difficult to detect because, while the reverse anchorT primer preferentially anneals to the 3' end of the polyA tail, it can anneal all along the polyA tail, resulting in a highly diffuse array of PCR products of different sizes. Therefore a second, nested PCR was performed on the first-round PAT-PCR products to confirm that *zsquidl-D* transcripts are all still present and simply have long polyA tails (Figure 3A, "nested PCR"). The presence of equally strong bands at all four stages after nested PCR indicates that throughout embryogenesis, the *zsquidl-D* transcripts are present and polyadenylated.

The expression and polyadenylation results indicate that the protein products of all four *squid* homologues are potentially available in the early zebrafish embryo. However, *zsquidl-A* is the single-family member whose transcripts appear to be subjected to translational control via cytoplasmic polyadenylation. Furthermore, the timing of its regulated translation, which is between the 64–128 cell stages and therefore not only precisely when the transition occurs from maternal to zygotic regulation of embryogenesis, but just as the earliest dorsal regulators appear – would mean that the zsquidl-A protein was first made available to the embryo at a key stage for dorsoventral patterning. To confirm that maternal *zsquidl*-A is regulated by this mechanism, we first had to determine that it is maternal *zsquidl-A* mRNA that is polyadenylated in the cytoplasm during early embryogenesis, and not zygotic *zsquidl-A* transcripts appearing with a long polyA tail due to nuclear polyadenylation. To do so zygotic transcription was prevented in embryos by injecting them with the transcriptional inhibitor α–amanitin at the 1–2 cell stage, and embryos were incubated until the dome stage. cDNA was prepared from total RNA in embryos at the dome stage, and PCR was performed on cDNA samples to assay for both the presence and the polyadenylation status of *zsuiqdl-A*. To confirm that transcription had been inhibited in the injected embryos, PCR was performed for transcripts of one of the first zygotic genes expressed in zebrafish, *Id1* (Sawai and Campos-Ortega, 1997), in both injected and uninjected embryos. As shown in Figure 4A, in the absence of α–amanitin *Id1* transcripts are present at the dome stage. However, when zygotic transcription is inhibited, the *Id1* transcripts are undetectable at the dome stage (Figure 4A). In contrast, analysis of *zsquidl-A* expression indicates that essentially equal levels of *zsquidl-A* transcripts are present in both the presence or absence of the drug and hence in the absence of zygotic transcription. Therefore, the majority (if not all) of the *zsquidl-A* transcripts present at the dome stage are maternal mRNAs.

To confirm that maternal *zsquidl-A* mRNA is polyadenylated while in the egg cytoplasm (which would indicate that the translation of *zsquidl-A* mRNA was regulated by cytoplasmic polyadenylation), the experiment was repeated and two PCR reactions were performed on staged embryonic cDNA in order to first detect the *zsquidl-A* mRNA (standard PCR), and then detect polyadenylated *zsquidl-A* mRNA. As seen in Figure 4B, when PCR amplification was first performed with the forward and reverse primers that amplify a portion of the *zsquidl-A* 3'UTR (Figure 4B, "3'UTR") on cDNA from 2 to 4 cell embryos, or dome-stage cDNA

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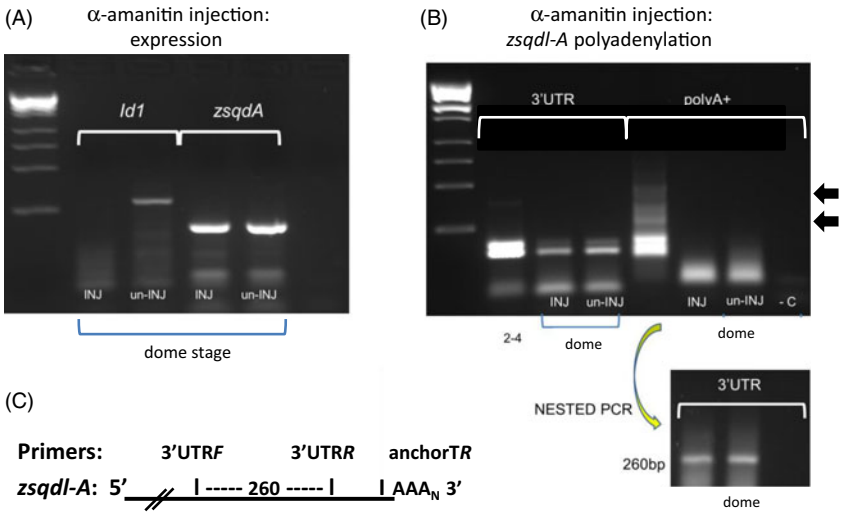

Figure 4. Maternal *zsquidl*-A mRNA is polyadenylated during the blastula stages. Zygotic transcription was inhibited via injection of alpha-amanitin at the 1–2 cell stage, and embryos were collected for RNA purification and polyA$^+$ cDNA synthesis at the dome stage. **(A)** Expression of *Id1* and *zsquidl-A* assayed by PCR of cDNA prepared from RNA purified from embryos collected at the dome stage. (**B)** Expression (3'UTR) and polyadenylation status (polyA+) of maternal *zsquidl-A* were assayed at the 2–4 cells stage and at the dome stage. The solid arrow indicates the absence of detectable PCR products of the correct length. (**C)** Nested PCR using the PCR products from B as template was performed using a nested reverse *zsquidl-A* primers. Filled arrows indicate where polyadenylated transcripts would be visible. **(C)** Position of primers in the *zsquidl*-A sequence.

from either un-injected embryos or embryos injected with α-amanitin at the 1–2 cell stage, a band of strong intensity is see at the 2–4 cell stage, and a band of lesser, but equal intensity is observed in both α–amanitin-injected and un-injected embryos at the dome stage. To detect polyadenylated maternal *zsquidl-A* mRNA this same cDNA was subjected to PAT-PCR assay with the forward *zsquidl-A* primer and anchorT reverse primer (Figure 4B, "polyA+"). While a strong band appears for 2–4 cell cDNA, indicating that at this stage the majority of *zsquidl-A* transcripts have a relatively equally sized and short polyA tail, much fainter, shorter PCR products are seen in the two dome-stage samples. This indicates that, as was seen with *zsquidl-D* (Figure 3A), the PAT-PCR assay for *zsquidl-A* failed to generate detectable bands of polyadenylated *zsquidl*-A mRNA. In order to determine whether the *zsquidl*-A RNA was in fact present and polyadenylated, but simply not detectable, nested PCR was performed on the products of the PAT-PCR assay. The results of the nested PCR re-amplification (Figure 4B, nested PCR) show bands of equal intensity in both lanes with *zsquidl*-A primers, indicating that the *zsquidl*-A maternal transcripts are present, and polyadenylated. The lack of visible bands in the polyA+ lanes simply indicates that, at the dome stage, the polyA tail lengths were longer but heterogeneous in length, and therefore not detected. Taken together, these results indicate that the maternal *zsquidl-A* mRNA is stored with a short polyA tail at the earliest stages of embryogenesis (O'Connell *et al.*, 2014), but then, as had been proposed in O'Connell *et al.* (2014) maternal *zsquidl-A* transcripts are polyadenylated in the cytoplasm, beginning between the 64 and 1K cell stages. Furthermore, the data demonstrate that no zygotic factors are required for the regulated polyadenylation of *zsquidl-A*.

Cytoplasmic polyadenylation is a well-known mechanism of translational control, particularly during embryogenesis. According to the paradigm for this mechanism the polyadenylation of the *zsquidl-A* mRNA would lead to its being loaded onto polysomes beginning at approximately the 1K cell stage and therefore translated at this stage of embryogenesis. To determine whether this is the case, a sucrose pad analysis was performed to separate the RNA found in the pellets – containing the polysomes – from the rest of the RNA in an embryo extract at two stages, either before the MBT (64-cell stage), or after the MBT (50% epiboly). To confirm the overall effectiveness and consistency in the separation of polysomes from total RNA in the sucrose pads, the amount of RNA in the pellets was measured relative to the total amount of RNA in each extract. In every case, the percentages of RNA in the pellets was ~3% of the total RNA in the original loaded extract, which is exactly as expected using the sucrose pad method (Masek *et al.*, 2011). RT-PCR was then performed on the total RNA purified from the pellets at the two stages to determine the amounts of individual RNAs in polysome pellets. As shown in Figure 5, panels A and B (lanes labelled '*zef1α*'), when RT-PCR was performed to detect the control transcripts of *zebrafish EF1α* (*zef1α*) the same pattern was seen every time. When quantitated via densitometry, there was an increase in the amount of *zef1α* mRNA in the 50% epiboly extracts (which was set in both cases to 100%, Figure 5, panels C and D). This is consistent with what would be expected of a housekeeping gene since most of the mRNA should be on polysomes, and before the MBT is all maternal mRNA, while after the MBT it is a combination of both maternal and zygotic mRNA. RT-PCR was then performed with the RNA from the same pellets to detect *zsquidl*-A (Figure 5A) and the amounts in each band normalized by calculating them as a percentage of the *zef1α* positive control at 50% epiboly. Only a very faint band was detected for *zsquidl-A* at the 64-cell stage, while at 50% epiboly, there was an increase of approximately 90% (Figure 5C). Since we had demonstrated that essentially all *zsquidl-A* transcripts are maternal (see Figure 4), this supports the conclusion that less than 10% of maternal *zquidl-A* mRNA is loaded on polysomes before the 64-cell stage, and the vast majority of maternal *zsquidl-A* mRNA is loaded on polysomes after the 64-cell stage. This indicates that the majority of maternal *zquidl-A* mRNA is stored and not translated in the early embryo, and only loaded onto polysomes during the blastula stages. As seen in Figure 5B, the exact opposite pattern is observed for *zsquidl-B*; a much larger percent of *zsquidl*-B transcripts are associated with polysomes before the MBT at the 64-cell stage than after, at 50% epiboly (Figure 5D, relative to *zef1α* there is a 74% *decrease* of *zsquidl-B* in the polysome pellets at 50% epiboly). This is remarkably consistent with the results seen in O'Connell *et al.* (2014), for while *zsquidl-B* mRNA is easily detected throughout early embryogenesis, its levels do begin to decrease at approximately 50% epiboly. Consistent results were obtained after four replicates of this experiment such that after the MBT there was an average increase of 82% in *zsquidl*-A mRNA associated with polysomes and an average decrease of 65% in *zsquidl*-B products. Taken together these results indicate that,

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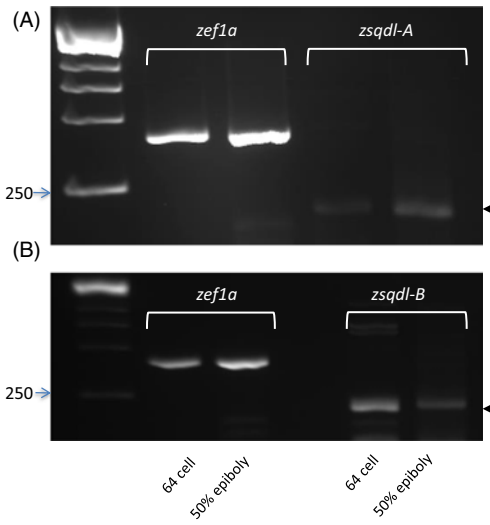

(C)

Percent RNA in pellet			
	preMBT	postMBT	+/-
zef1	84	100	+19%
zsqdl-A	10	19	**+90%**

(D)

Percent RNA in pellet			
	preMBT	postMBT	+/-
zef1	51	100	+96%
zsqdl-B	68	34	**-50%**

Figure 5. Maternal *zsquidl-A* mRNA preferentially associates with polysomes after the MBT. Specific maternal mRNAs were detected via RT-PCR of cDNA prepared from total RNA present in the polysome pellet after sucrose pad analysis. (**A**) Levels of *zef1alpha* mRNA and *zsquidl-A* mRNA in pellets pre- (64-cell stage) and post- (50% epiboly) the MBT. Arrow indicates expected position of *zsquidl-A* PCR products. (**B**) Quantitation of the abundance of *zsquidl-A* products relative to those of *zef1α* as determined by densitometry using ImageJ. (**C**) Levels of the *zef1alpha* and *zsquidl-B* mRNAs in pellets at both stages. Arrow indicates expected position of *zsquidl*-B PCR products. (**D**) Quantitation of the abundance of *zsquidl-B* products relative to those of *zef1alpha* as determined by densitometry using ImageJ.

unlike what was seen with *zsquidl-A*, the expression and polyadenylation status of *zsquidl-B* follows the pattern seen for a housekeeping gene whose mRNA is maternally provided. It is transcribed and polyadenylated in the oocyte and then the early embryo, loaded on polysomes, and translated, until it begins to be degraded at approximately 50% epiboly. This degradation explains the significant decrease in *zsquidl-B* PCR products in the pellet of embryos collected at the 50% epiboly stage. In addition, these results support the conclusion that the translation of maternal *zsquidl*-A mRNA is regulated by cytoplasmic polyadenylation, such that it is stored with a short polyA tail in the egg and embryo until at least the 64-cell stage, after which the transcripts are polyadenylated in the cytoplasm and therefore loaded onto polysomes.

The translational control of zsquidl-A protein synthesis suggests that its function is required at a key time for the molecular regulation of embryogenesis, and may be one of the many factors that combine their efforts to direct dorsoventral patterning. To analyze the function of the protein products of this family of proteins, morpholinos (MOs) were designed against *zsquidl-A*, *B*, and *C* (*zsquidl-D* knockdown experiments have been reported previously, Vieira *et al.,* 2014) and used for microinjection experiments. An extensive analysis of morpholino injection has confirmed that this is an extremely reliable method for the translational inhibition of specific maternal mRNAs in zebrafish, especially if a dose response can be demonstrated, and the morphants phenocopy existing dorsoventral mutants (Stanier *et al.*, 2017). One of the earliest and most reliable indicators of early disruption in dorsoventral pattering is a disruption in the expression of the organizer-specific transcription factor *goosecoid* (Shulte-Merker *et al.,* 1994). Therefore embryos were injected at the 1–4 cell stage with 1.0 mM *zsquidl-A* MO, fixed at 50% epiboly, and processed for *in situ* hybridization with a probe for *goosecoid* (Figure 6A). In contrast with water-injected embryos, nearly a quarter of the *zsquidl-A* MO-injected embryos (22%) showed a dramatically expanded, nearly doubled region of *gsc* expression, as illustrated by the embryo in Figure 6A. This suggests that a much larger percentage of *zsquidl-A* MO-injected embryos have expanded *gsc*-expression domains, with a range of sizes between normal and nearly double in size. If this dramatic expansion in *goosecoid* expression is biologically significant, then a disruption in dorsal patterning should be evident when *squidl-A* morphants

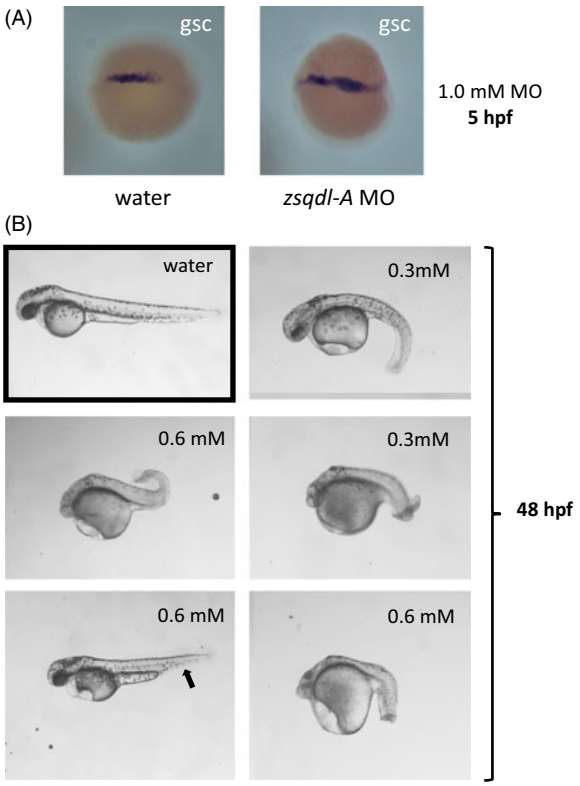

Figure 6. *zsquidl*-A morphants are dorsalized. (**A**) Embryos were collected, and injected with either water, or 1.0 mM morpholino against *zsqdl-A* between the 1–4 cell stages ($n = 30$). At 5 hpf embryos were fixed and prepared for *in situ* hybridization with a *goosecoid* probe. The images of both embryos were taken from a dorsal view. The *zsquidl*-A morpholino-injected embryo shows the greatly expanded *goosecoid* staining that was evident in 22% of injected embryos. (**B**) Embryos were injected with either water, 0.1 mM or 0.6 mM *zsqdl*-A morpholino and fixed at 48 hpf. Images represent the range of phenotypes seen in 100% of surviving embryos. Arrow shows the absence of a ventral fin.

have developed to the small fry stage, when the morphological outcome of dorsoventral patterning is complete. To address this question *zsquidl-A* MO-injected embryos were analyzed morphologically overall, as well as for the appearance of 5 developmental landmarks (eyes, neural tube, notochord, somites, ventral fin), over

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Table 1. Morpholino knockdown of *zsquidl*-A produces a dose response in the dorsalized phenotype. A. Embryos were injected with the indicated morpholino at the 1–8 cell stages and then assayed for phenotype at the stages indicated, and categorized by degree of dorsalization. Percent survival is the average obtained in four separate experiments with >30 embryos per category. Embryos scored as mild dorsal had a slight/moderate curve of the tail (Class 1), while embryos scored as severe dorsal had an enlarged shield at 60% epiboly, and displayed the Class 4 dorsalized phenotype at 24 hpf (Mullins *et al.*, 1995). B. Embryos co-injected with 1.0 mM *zsqdl*-A MO and *in vitro* transcribed *zqsuidl-A* RNA for the rescue experiment

A	Stage	% survival	% mild dorsal	% severe dorsal
un-injected *(n = 110 embryos)*	80% epiboly	100	0	0
	prim 5–10	90	0	0
0.1 mM zsqdl-A MO *(n = 125 embryos)*	60% epiboly	80	0	0
	prim 5–10	68	33	0
0.6 mM zsqdl-A MO *(n = 125 embryos)*	60% epiboly	72	**0**	**57**
	prim 5–10	64	**53**	**47**
1.0 mM zsqdl-C MO *(n = 150 embryos)*	60% epiboly	82	0	0
	prim 5–10	63	0	0
1.0 mM control MO *(n = 80 embryos)*	60% epiboly	78	0	0
	prim 5–10	63	0	0
B				
1.0 mM zsqdl-A MO + zsqdl-A mRNA *(n = 45)*	prim 5–10	56	8	0

a 48-hour time period. As seen in Figure 6B, at 48 hpf embryos injected with 0.3 mM or 0.6 mM *zsquidl-A* morpholino ($n = 125$) show a range of dorsalized phenotypes from mild (Class 1 dorsalized phenotype, Mullins *et al.*, 1995) as indicated by a slightly ventrally-curved tail and reduced ventral fin, to severe dorsalization (Class 4 out of, which phenocopy dorsalized mutant swirl embryos, Mullins *et al.*, 1995), with 57% showing an enlarged shield at 60% epiboly (data not shown) and nearly all embryos showing moderate to hyper-dorsalization at 48 hours (Figure 6B showing five representative embryos). The morphant phenotype could be rescued by co-injection of 1.0 mM *zsquidl-A* morpholino and *in vitro* transcribed *zsquidl-A* RNA (see Table 1), which resulted in nearly all embryos developing normal dorsoventral patterning. Furthermore, fewer than 5% of the embryos showed signs of cell death in the brain area, or other indicators of off-target effects.

To determine whether inhibition of *zsquidl-A* translation was unique among its family members in producing severe defects in patterning and whether a dose response to the loss of zsquidl-A could be detected, additional injection experiments were performed. As seen in Table 1, when embryos were injected with 1.0 mM of either the *zsquidl-A* or *zsquidl-C* morpholino there was a similar level of survival in both groups of injected embryos (approximately 80%). However, morphologically embryos injected with z*squidl-C* morpholino ($n = 150$ embryos) were indistinguishable from those injected with a control morpholino ($n = 80$ embryos); in both cases, all surviving embryos appeared normal through the prim 5–10 stage. This is in stark contrast to what was observed with embryos injected with *zsquidl-A* morpholino, where dorsoventral defects were again evident. Furthermore, since difference in dosage of *zsquidl-A* MO was more pronounced in this experiment (0.1 mM versus 0.6 mM), it was possible to see a dose response to the absence of zsquidl-A. Roughly a third of the embryos injected with a low dose of *zsquidl-A* MO (0.1 mM) were slightly dorsalized, and nearly 100% of embryos injected with the higher dose (0.6 mM) were moderately to severely dorsalized. Again almost none of the injected embryos displayed any of the typical deformities associated with off-target effects (Stanier *et al.*, 2017). One final control for the dorsalizing effect of injecting a morpholino against *zsquidl-A* was to inject embryos in the yolk at the 16–32 cell stage – after cell membranes have been laid down between the yolk and the embryonic cells. In 100% of injected embryos, the embryos appeared completely normal throughout the 48-hour period ($n = 50$ embryos, data not shown). Therefore this confirms that the morpholino must be present in embryonic cells where it has access to the *zsquidl-A mRNA* in order to disrupt dorsoventral patterning.

Take together the expression studies combined with the results of the morpholino injections indicate that the precise temporal control of *zsquidl-A* expression, which of the four family members shows the highest sequence conservation with fly *squid*, is required for proper embryonic patterning.

Discussion

Numerous insights into the molecular regulation of the earliest events of embryogenesis in metazoans have come from evolutionary studies, which often reveal deep homology in molecular mechanisms as well as tremendous plasticity in the diversity of outcomes that stem from this homology. In the case of the *zsquidl-like* clade in zebrafish, a group of four genes that are homologous to the dorsoventral and anterior/posterior patterning gene *squid* in *Drosophila*, we provide evidence that one of them, *zsquidl-A*, plays a role in embryonic patterning in zebrafish. First, we show that the translation of maternal *zsquidl-A* mRNA is temporally regulated by cytoplasmic polyadenylation, which results in the zsquidl-A protein first becoming available to the embryo between the 64-cell and 1K-cell stages, precisely during the MBT. This is notable first because several studies have shown that cytoplasmic polyadenylation-mediated translational control of maternal mRNAs directs the maternal-to-zygotic transition in zebrafish (Winata and Korzh, 2018; Lieberfarb *et al.*, 1996). In fact, cytoplasmic polyadenylation is required during the equivalent stage of embryogenesis in flies (Salles *et al.*, 1994). Additionally, the temporal control of zsquidl-A translation guarantees that the protein will be available to the embryo just as the molecular pre-pattern for dorsoventral patterning in particular is being finalized.

We also demonstrate that the translation of maternal *zsquidl-A* mRNA is required for proper dorsoventral patterning in zebrafish. When *zsquidl-A* translation is prevented via morpholino injection at the 1–8 cell stage (which is ideal for preventing the translation of an mRNA that is not yet loaded onto ribosomes, Stanier *et al.*, 2017), a near doubling of the domain of *goosecoid* (*gsc*) mRNA expression is seen at 50% epiboly in 22% of the embryos, suggesting that a much larger percentage of the embryos had at least a moderately enlarged *gsc* domain. None of the *zsqdl*-A morphants had a reduced level of *gsc* expression, suggesting that the embryos were not simply failing to develop normally overall. In light of the fact that *gsc* is one of the first dorsal-organizer-specific transcription factors expressed at the onset of dorsoventral patterning (Shulte-Merker *et al.*, 1994), these results suggest that in the

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absence of *zsqdl-A* protein, a larger number of cells express *gsc* than is seen in a normal embryo. This would be expected to result in a disruption in dorsoventral patterning, and this is precisely what we have demonstrated; *zsqdl-A* morphants show a range of dorsalized phenotypes from mild to severe by 48 hours. The *zsquidlike*-A morphants phenocopy the dorsalized appearance of fish carrying mutations in genes involved in BMP signalling (Nguyen *et al.*, 1998, Tucker *et al.*, 2008). In addition, the *zsquidl-A* morphants are indistinguishable from embryos injected with morpholinos against several different genes required to establish ventral cell fates by restricting the action of dorsal determinants (for review see Fuentes *et al.*, 2020). For example, Kapp *et al.* (2013) report that maternal-effect mutant embryos lacking Integrator Complex subunit 6 (Ints6), a protein required to establish ventral cell fates, display strongly dorsalized phenotypes, and this phenotype can be rescued by either expressing BMP or suppressing the function of dorsal organizer genes. Interestingly, like *Drosophila* Squid protein, Inst6 functions by restricting the ventral expansion of dorsal signals. Therefore, we will be pursuing an analysis of any interaction between zsquidlike-A, an RNA-binding protein, and Inst6, which is also part of a complex that interacts with mRNAs (Ezzeddine *et al.*, 2011; Tatomer *et al.*, 2019). Additional proteins that have been identified as playing a role in dorsoventral patterning based on dorsalized morphant phenotypes are a collection of proteins that directly bind to maternal β-catenin and promote ventral cell fates, including Leucine zipper tumour suppressor 2, or Lzts2 (Li *et al.*, 2011), Forkhead boxO transcription factor 3, or Foxo3b (Xie *et al.*, 2011), and ELL associated factor 1 and 2 (Eaf1 and Eaf2) (Liu *et al.*, 2018). While zsquidlike-A is an RNA-binding protein, like any regulatory protein, it must also interact with other proteins to carry out its function.

The dorsalizing effect seen in fly embryos lacking Squid and in zebrafish embryos lacking zsquidl-A suggest at least some level of conservation of function between these proteins in the two bilaterian organisms. This is surprising as the two organisms use two different molecular pathways to direct dorsoventral patterning. In zebrafish, dorsal tissue formation requires the activation of the β-catenin pathway, while in flies dorsal is determined by localized synthesis of the Gurken protein. Furthermore, one of the central outcomes of dorsoventral patterning – the formation of neural tissue – occurs on opposite sides of these two embryos (see De Robertis and Sasai, 1996). In zebrafish, neural tissue forms on the dorsal side of the embryo; in flies, the nerve cord forms on the ventral side (for an excellent discussion of this topic, and diagrams of the two species, see De Robertis and Tajede-Munoz, 2022). Our results suggest that, if there is homologous function between *Drosophila squid* and zebrafish *zsquidl-A*, this function would be required as the dorsoventral axis is being established, when the radial symmetry around the A/P axis is first broken, and notably before neural tissue is differentiating. While the establishment of the A/P axis itself in fish and flies is the result of entirely different processes, it has been reported that in fly embryos, Squid is not only required for dorsal patterning but also for establishment of the A/P axis (Norvell *et al.*, 2005, Steinhauer and Kalderon, 2005). In addition, in both organisms, the establishment of dorsal is dependent on microtubule arrays whose orientation is shifted upon fertilization (for review see Houston, 2017). In zebrafish, the microtubules shift the location of the Balbiani body that contains the dorsal determinants from the vegetal cytoplasm to the future dorsal side (Fuentes *et al.*, 2020).

It is possible that these proteins are part of the mechanism in bilaterian embryos that establishes the orientation of the dorsal and ventral sides of the body relative to the substratum – the rule being that in both cases ventral is the side of the organism that faces the substratum (Arendt and Nubler-Jung, 1997) resulting in both cases in appendages that bend ventrally and a mouth faces the substratum. This model would predict that there was evolutionary divergence in the coordination between the dorsoventral orientation of the body and the position of the neural tissue. Hypotheses to explain this evolutionary divergence date back to 1882, Geoffroy St. Hilaire proposed that the common ancestor of both – called the "urbilaterian" by De Robertis (De Robertis and Sasai, 1996; De Robertis *et al.*, 2017) – had a ventral nervous system that has been retained in all bilaterians except the chordates, in which this orientation has been flipped. If this is the case, the direct induction of neural tissue by factors secreted by newly forming dorsal cells that occurs in a fish embryo would be the derived trait that evolved more recently in the chordate lineage. A comparison of protostomes and the earliest deuterostomes does support the flipping model for the orientation of the nervous system relative to the dorsoventral orientation of the body; however, more recent phylogenetic analyses of organism that lie along the lineage from first bilaterian to the proposed urbilaterian itself suggest that the picture may not be so simple and that the basal characteristic for the nervous system was actually an anteriorly located neural centre (Hejnol and Martindale, 2008). In fact, there is now phylogenetic evidence that indicates that the existence of the BMP pathway itself actually predates the emergence of bilaterians (Bier, 2011), and that asymmetric patterns of BMP signaling are seen in radially symmetric organisms such as cnidarians (Technau and Steele, 2011).

In light of the ambiguity regarding the shared evolutionary history of these two groups of bilaterians, it is critical to trace the similarities at the level of individual molecules and their partners, and how they function during embryogenesis, while also delineating the differences, in order to ultimately piece together what has led to the diversity observed in the adults. An analysis of the primary structure of the Squid/zsquildl-A proteins themselves reveal that the only regions of the fly and fish proteins that share a high degree of homology are the two RNA-binding domains (or RNA Recognition motifs, RRMs). This suggests that any shared function would involve one or more of their target RNAs. It is unlikely that there is a direct parallel with the *gurken* story since the *gurken* homolog (neuregulin) plays no role in zebrafish embryogenesis, and zebrafish Epidermal Growth Factor (EGF) itself is expressed by the oocyte during oogenesis but its levels decrease before fertilization (Wang and Ge, 2004). While there is a direct homolog of the EGF receptor, and in zebrafish the EGFR is present throughout oogenesis, several studies indicate that its role (and that of its unidentified ligand) is limited to regulating folliculogenesis, and in fact, Crispr mutants of EGFR develop normally (Song *et al.*, 2022). This suggests that the role of the Gurken/Torpedo receptor/ligand pair in regulating dorsal fates in flies is not conserved in vertebrates. It is also unlikely that there is direct homology in the connection of dorsoventral patterning to neural/non-neural patterning since the only report connecting BMP to Squid in the fly ovary indicates that Squid activity may be downstream of BMP signalling in the maintenance of the undifferentiated germ cell lineage. However, this is a separate and much earlier role for Squid than its role in dorsoventral patterning (Finger *et al.*, 2023).

While a great deal of progress has been made in identifying the cascade of molecular players in zebrafish that result in the localization and translation of β-catenin on the future dorsal side, the identity of the initial dorsal determinants themselves remains a mystery. In fact, a new potential player in this scheme was recently

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identified: a gene called *Huluwa* (Yan *et al.*, 2018) is required for proper dorsoventral patterning but operates independently of the Wnt cascade. It is possible that the maternal *zsquidl*-A mRNA that is present in the oocyte is one of the dorsal determinants, and the result of shifting of the location of the maternal mRNA leads to localization of the translation of the zsquidl-A protein later, when it is translated and could now bind to components of the machinery that activates the β-catenin cascade. While the large-scale *in situ* screen by Thisse and Thisse, (2004) indicated that *zsquidl*-A mRNA is not localized in the embryo, the localization of RNAs in the oocyte has not been investigated.

What is clear is that there are abundant opportunities for *zsquidl-A* to play a role in regulating dorsoventral patterning in zebrafish. RNA-binding proteins as a group are involved in essentially every step of gene regulation (Gehring *et al.*, 2017). This is particularly critical during oogenesis and early embryogenesis in zebrafish when there is no transcription so the translation of maternal mRNAs is the only source of new gene products (for review, Chan *et al.*, 2009). The Balbiani body itself is rich in RNA-binding proteins, and recent studies indicate that two of them, called Cirbpa and Cirbpb, are required for germ cell formation (Jamieson-Lucy *et al.*, 2022). An early role for hnRNPI in egg activation, cytoplasmic segregation, and cell cleavage has been reported (Mei *et al.*, 2009), and Alexander *et al.*, (2021) used morpholino knockdown experiments to demonstrate a role for hnRNPL and hnRNPL2 in myogenic differentiation. Finally, a recent study by Blackwell *et al.* (2022) identified hnRNPUL1 as having a role as a transcriptional regulator and alternate splicing during the patterning of fins and limbs in vertebrates. In light of the myriad requirements for regulating gene expression via interactions with RNAs during embryogenesis, the limited number of identified examples makes it likely that there are many more yet to be discovered.

An obvious avenue for future investigation would rely on a direct comparison between the two zebrafish proteins zsquidl-A and B, which are closely related but do not share a role in dorsal/ventral patterning. While the zsquidl-A and B proteins do share tremendous homology (82% identity overall), there is significant difference in their N-terminal Core binding factor N-terminal (CBFNT) domains, and an additional eight amino acids in the second RNA-binding domain in zsquidl-A. The CBFNT domain has been found in other hnRNPs and in these proteins, it does not play an apparent role in RNA binding (Wang *et al.*, 2018). This is consistent with a model whereby the CBFNT domain of zsquidl-A, and not B, mediates the interaction of zsquidl-A with other protein binding partners required for its role in regulating dorsoventral patterning. Identification of these partners will lead us closer to answering the question of whether fly Squid and zebrafish zsquidl-A are in fact functionally homologous, or whether an inherited molecular tool is actually used in significantly different ways in the two different organisms.

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Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

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