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Functional Divergence of Former Alleles in an Ancient Asexual Invertebrate

Natalia N. Pouchkina-Stantcheva, *et al.*

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in plant signaling and alteration of gene expression has only been demonstrated for phosphatidic acid, which is produced through the activity of phospholipase D (*21*). Therefore, further research on LPC generation and signaling can hopefully tell us more about the evolution of response regulation in plants and mammals, including that in the development of the AM symbiosis.

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Functional Divergence of Former Alleles in an Ancient Asexual Invertebrate

Natalia N. Pouchkina-Stantcheva,[1]* Brian M. McGee,[1] Chiara Boschetti,[1] Dimitri Tolleter,[2] Sohini Chakrabortee,[1] Antoaneta V. Popova,[3]† Filip Meersman,[4]‡ David Macherel,[2] Dirk K. Hincha,[3] Alan Tunnacliffe[1]§

Theory suggests it should be difficult for asexual organisms to adapt to a changing environment because genetic diversity can only arise from mutations accumulating within direct antecedents and not through sexual exchange. In an asexual microinvertebrate, the bdelloid rotifer, we have observed a mechanism by which such organisms could acquire the diversity needed for adaptation. Gene copies most likely representing former alleles have diverged in function so that the proteins they encode play complementary roles in survival of dry conditions. One protein prevents desiccation-sensitive enzymes from aggregating during drying, whereas its counterpart does not have this activity, but is able to associate with phospholipid bilayers and is potentially involved in maintenance of membrane integrity. The functional divergence of former alleles observed here suggests that adoption of asexual reproduction could itself be an evolutionary mechanism for the generation of diversity.

Bdelloid rotifers (Rotifera, Bdelloidea) have survived for tens of millions of years without sexual reproduction and meiotic recombination (*1*–*4*). Male bdelloid rotifers have never been observed, and the genetic evidence is consistent with fully asexual reproduction by thelytoky. Long-lasting asexual lineages are thought to be rare because their apomictic nature does not allow the accumulation of favorable, or the elimination of detrimental, mutations through genetic exchange (*5*–*7*). However, one consequence of apomixis is that the sequence homogeneity of gene copies that previously were alleles in sexual ancestors is no longer maintained by recombination. This allows the former alleles to accumulate mutations and become divergent—a phenomenon referred to as the Meselson effect (*8*). Thus, in sexually reproducing monogonont rotifers (Rotifera, Monogononta) alleles differ very little from each other at synonymous sites (by up to 2.4% for *hsp82*), but corresponding gene copies in individual bdelloid clones can differ by as much as 49% (*1*). In principle, this effect should allow independent evolution of former alleles through which they can acquire different functions.

We looked for evidence of functional divergence among former alleles in a gene set associated with desiccation tolerance in bdelloid rotifers (*9*, *10*). cDNAs representing ~100 dehydration-induced genes from the bdelloid rotifer *Adineta ricciae* were identified, one of which encoded a polypeptide related to the group 3 late embryogenesis abundant (LEA) proteins characterized in plant seeds. LEA proteins are linked with desiccation tolerance in plants, invertebrates, and microorganisms (*11*). We identified two similar but distinct sequences and named them *Ar-lea-1A* and *Ar-lea-1B*. Both genes contain nine small introns (Fig. 1A), although there is a major structural difference in exon 2, which in *Ar-lea-1A* contains a 132–base pair (bp) segment with no counterpart in *Ar-lea-1B*. Aligned coding sequences show 13.5% synonymous site divergence (K_s) over the whole gene. This divergence is much greater than that observed between alleles of sexual animals, but is within the range of values observed in bdelloids for former allele pairs (*1*, *3*, *4*, *8*).

To confirm the presence of two *lea* gene copies in the *A. ricciae* genome, Southern hybridization experiments were performed with probes from both the 5′ and 3′ ends of *Ar-lea-1B*, which cross-hybridize to the corresponding regions of *Ar-lea-1A* (Fig. 1B). Both genes reside on ~5.0-kb Dra I genome fragments, but these could be distinguished by double digestion with either Eco RI or Nde I; a restriction map of each gene was constructed accordingly (Fig. 1, A and B). As further confirmation of *lea* gene copy number, fluorescence in situ hybridization (FISH) was carried out on *A. ricciae* embryo nuclei. Cytogenetic analysis shows 12 chromosomes in this species (Fig. 1C, left), as in the related species, *A. vaga* (*12*). Hybridization with a fluorescent probe corresponding to the whole of *Ar-lea-1A* produced two signals in interphase nuclei, consistent with detection of *lea* genes on two separate chromosomes (Fig. 1C, right). Our cloning and hybridization data show two related, but divergent, *lea* genes on different chromosomes in *A. ricciae*, and we interpret these to be former alleles that have diverged by the Meselson effect. Other interpretations are possible, for

[1]Institute of Biotechnology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QT, UK. [2]UMR 1191 Physiologie Moléculaire des Semences, Université d'Angers/INH/INRA, 49045 Angers, France. [3]Max-Planck-Institute of Molecular Plant Physiology, Am Mühlenberg 1, D-14476 Potsdam, Germany. [4]Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, UK.

*Present address: Department of Biology and Environmental Sciences, University of Sussex, Brighton BN1 9QG, UK.
†Present address: Institute of Biophysics, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria.
‡Present address: Department of Chemistry, Katholieke Universiteit Leuven, Celestijnenlaan 200 F, B-3001 Leuven, Belgium.
§To whom correspondence should be addressed. E-mail: at10004@biotech.cam.ac.uk

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example, that the ancestral bdelloid was the result of a hybridization event between species with unusually similar *lea* genes, and that one copy of one *lea* gene from both parents was subsequently lost. However, the simplest interpretation, consistent with the current understanding of bdelloid genome structure and evolution (*1*, *4*, *8*), is that the two *lea* genes are divergent former alleles. Recent studies suggest that bdelloid rotifers have four copies of some genes located on separate chromosomes, which may indicate that they are ancestrally tetraploids (*1*, *2*), in which the four copies are two gene pairs that correspond to two pairs of former alleles (*3*). We have not obtained evidence to date for a second *lea* gene pair, although we cannot rule out that they are present in the genome but too divergent for us to detect with *Ar-lea-1A* sequences.

Expression of the *lea* genes was shown by quantitative polymerase chain reaction (PCR) to increase about sevenfold over 24 hours of drying (fig. S1 and table S1). A similar pattern of expression of *lea* genes during dehydration has been observed in other anhydrobiotic invertebrates (*11*) and is consistent with a role in desiccation tolerance.

The predicted protein sequences of ArLEA1A and ArLEA1B are very similar, differing only at 12 amino acid sites of 376 aligned positions; ArLEA1A is longer by 44 amino acids because of the 132-bp indel in exon 2 (Fig. 2A). Both sequences have at least four variants of the loosely conserved 11–amino acid motif characteristic of group 3 LEA proteins (*11*, *13*) (Fig. 2A and fig. S2A), although positions 4 and 5 are more likely to be apolar. The ArLEA1A and ArLEA1B proteins both have a 19-residue hydrophobic sequence at the N terminus, revealed by a hydropathy plot (*14*), and a putative variant endoplasmic reticulum (ER) retention signal, ATEL, at the C terminus (Fig. 2A and fig. S2). This suggests that these proteins are localized to or transported through the ER. Most group 3 LEA proteins are highly hydrophilic, with a mean hydropathy (GRAVY) score of –0.97 [SD 0.30; n = 30; dataset of (*15*)], but both bdelloid proteins score –0.46, similar to moderately hydrophilic proteins, such as bovine serum albumin (BSA) (GRAVY: –0.43). This reduced hydrophilicity of the bdelloid LEA proteins is unusual and may impact their structure.

Group 3 LEA proteins are largely unstructured in solution, probably because their extreme hydrophilicity favors interaction with water over intrachain binding, but they show increased folding when dried or associated with phospholipid bilayers (*11*, *16*). Secondary structures of recombinant forms of ArLEA1A and ArLEA1B, without putative N-terminal signal peptides, were examined by far-ultraviolet (far-UV) circular dichroism (CD) spectroscopy in hydrated and dry states. CD spectroscopy of ArLEA1A gave a solution spectrum with a single minimum at ~200 nm and low ellipticity at 222 nm, consistent with a disordered structure. However, when dried, its spectrum changed markedly, showing minima near 208 nm and 222 nm, indicative of an α-helix (Fig. 2B). In contrast, ArLEA1B has an α-helical structure in the hydrated state, which does not change appreciably on drying (Fig. 2C). Secondary structure content calculated from CD spectra showed that in ArLEA1A the proportion of α-helix increased from 29 to 84% on drying, whereas ArLEA1B was 82% α-helix in solution, increasing slightly to 87% when dry. Protein denaturation analysis was performed by monitoring unfolding at 222 nm on exposure to urea at a range of concentrations from 0 to 6 M. For a typical globular protein, unfolding is cooperative and yields a sigmoidal curve. However, structure in ArLEA1B was lost linearly with increasing urea concentration (fig. S2E), which suggests that it exists as a premolten globule without significant tertiary structure in solution (*17*). The relatively small differences in primary structure of the bdelloid LEA proteins are therefore responsible for markedly different secondary structure.

We tested whether the structural differences between ArLEA1A and ArLEA1B are reflected in functional divergence. LEA proteins preserve the activity of desiccation-sensitive enzymes

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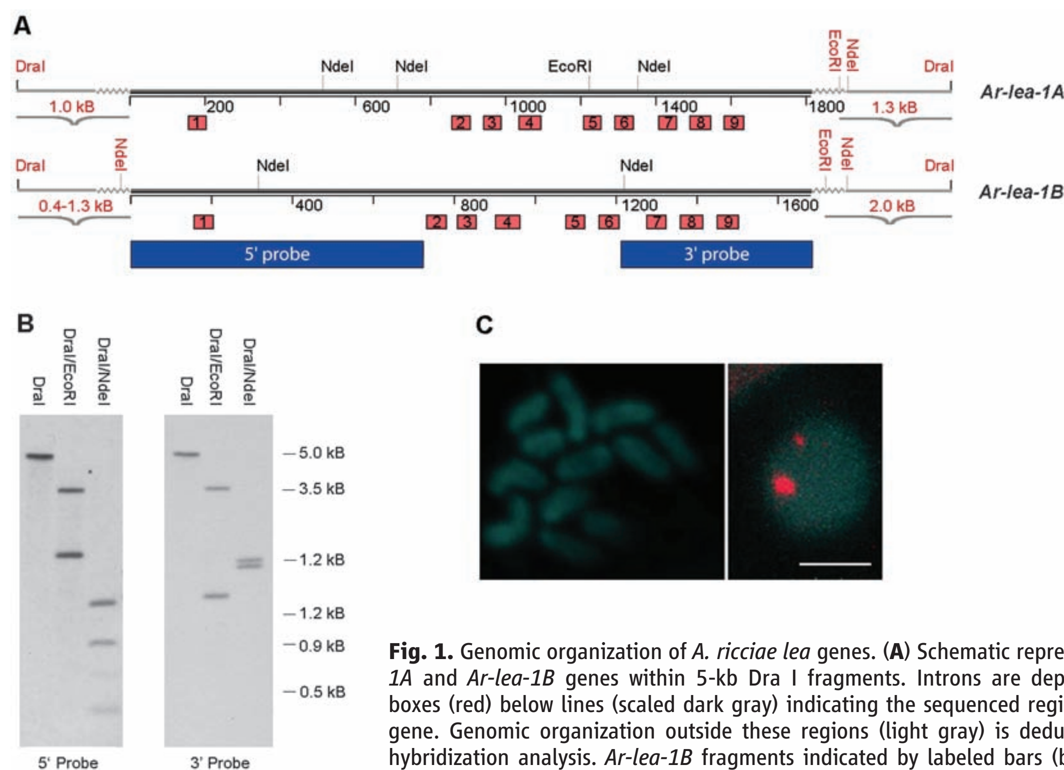

Fig. 1. Genomic organization of *A. ricciae lea* genes. (**A**) Schematic representation of *Ar-lea-1A* and *Ar-lea-1B* genes within 5-kb Dra I fragments. Introns are depicted as numbered boxes (red) below lines (scaled dark gray) indicating the sequenced region containing each gene. Genomic organization outside these regions (light gray) is deduced from Southern hybridization analysis. *Ar-lea-1B* fragments indicated by labeled bars (blue) correspond to probes used in Southern hybridizations. (**B**) Southern hybridization of *A. ricciae* genomic DNA with *lea* gene probes. Each panel contains genomic restriction digests with Dra I, Dra I/Eco RI, and Dra I/Nde I, respectively. Size marker positions are indicated. (**C**) *A. ricciae* karyotype and FISH with *lea* gene probe. (Left) The 12 chromosomes of *A. ricciae* in a single mitotic nucleus from an embryo stained with 4′,6′-diamidino-2-phenylindole (DAPI). (Right) Interphase nucleus hybridized at high stringency to *Ar-lea-1A* probe labeled with Alexa 488. Red (superimposed, false color): fluorescent signals; blue: DAPI-labeled DNA. Scale bar: 2 μm.

during drying (*18–20*), at least partly through prevention of aggregation, in what is called molecular shield activity (*21*). We investigated the ability of both bdelloid LEA proteins to behave as molecular shields by inhibiting desiccation-induced aggregation of citrate synthase (CS). When subjected to drying and rehydration, CS partially denatures and forms particulate aggregates; however, when dried in the presence of a group 3 LEA protein, such as AavLEA1 from the nematode *Aphelenchus avenae* (*22*), CS aggregation is suppressed (Fig. 3). Other proteins, such as BSA, are not effective. ArLEA1A was found to reduce CS aggregation as expected, although to a lesser extent than AavLEA1, perhaps because of the lower hydrophilicity of ArLEA1A compared with the nematode protein. However, ArLEA1B behaved differently, and drying of CS in its presence resulted in increased aggregation compared with CS dried alone. Indeed, ArLEA1B itself is prone to aggregation (Fig. 3), which ArLEA1A and AavLEA1 are not, possibly because of its more structured nature. Thus, ArLEA1A shows molecular shield activity in common with other group 3 LEA pro-

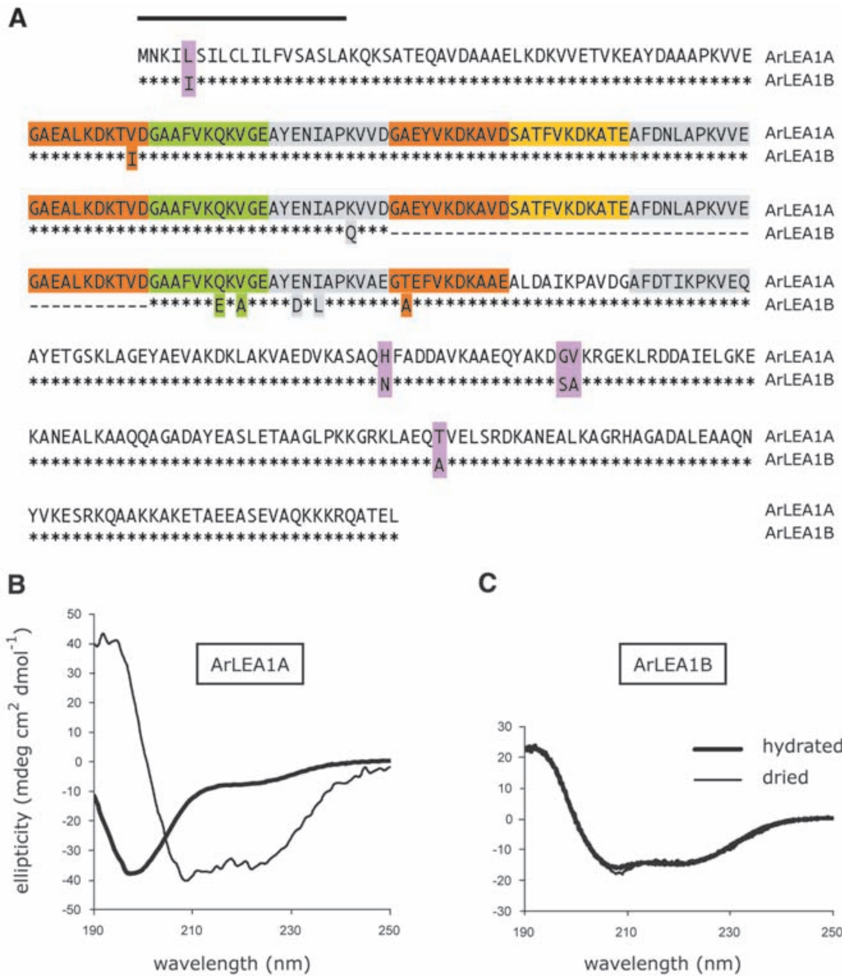

Fig. 2. Primary and secondary structure of *A. ricciae* LEA proteins. (**A**) Alignment of ArLEA1A and ArLEA1B protein sequences showing repeated 11-oligomer motifs. ArLEA1A is 420 residues long with a (predicted) molecular mass of 44.5 kD, whereas ArLEA1B extends for 376 residues with a (predicted) molecular mass of 39.8 kD. Near canonical motifs are orange, green, and yellow; degenerate motifs are gray; highlighted residues differ between the two proteins. The 44-residue indel, shown by dashes, is identical to a more N-terminal sequence whose 11-oligomer motifs are also highlighted orange-yellow-gray-orange. A putative signal peptide is overlined at the N terminus. (**B** and **C**) Far-UV CD spectroscopy of ArLEA1A and ArLEA1B in solution and dry state.

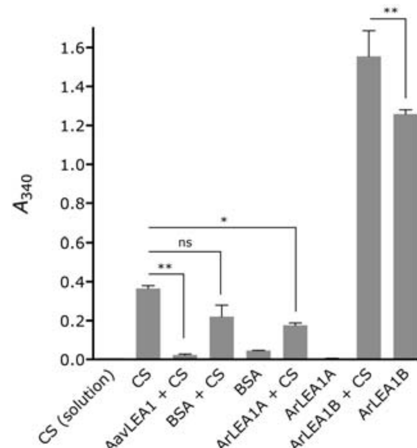

Fig. 3. Bdelloid LEA protein antiaggregation assay. Citrate synthase (CS), with or without LEA proteins or BSA, and the latter proteins alone where indicated, were subjected to two cycles of vacuum drying and rehydration. Light scattering by protein particulates was measured by apparent absorption at 340 nm in the spectrophotometer. Error bars show standard deviation (n = 3); ns, not significantly different ($P > 0.05$); significant values $*P < 0.05$ or $**P < 0.001$.

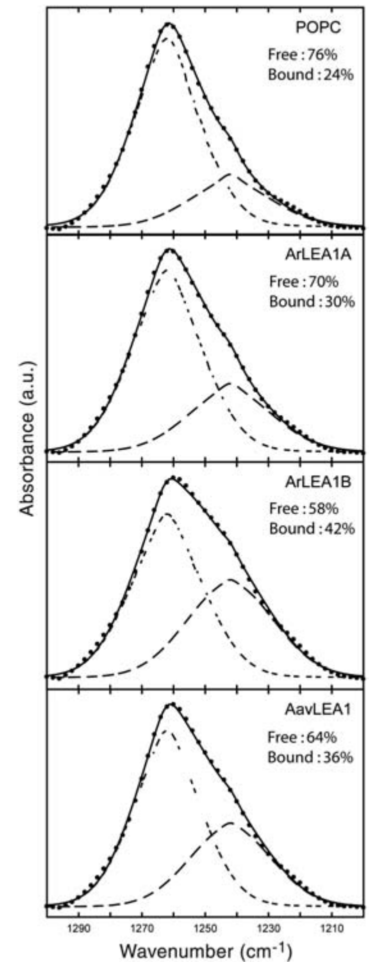

Fig. 4. Bdelloid LEA protein membrane association. Infrared spectra of the asymmetric phosphate stretching region of POPC liposomes dried alone or in the presence of ArLEA1A, ArLEA1B, or AavLEA1. Spectra were recorded at 78°C (liquid-crystalline phase). The solid curve comprises both the measured (dots) and fitted absorbance curves. Normalized peaks were fitted into two bands with maxima at 1262 and 1242 cm^{-1} corresponding respectively to $\nu P{=}Oas_{free}$ (short dashes) and $\nu P{=}Oas_{bound}$ (long dashes).

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teins, but ArLEA1B does not and is itself sensitive to desiccation.

Some LEA proteins have a capacity to associate with and stabilize phospholipid bilayers on dehydration (*11*, *16*, *23*). Membrane interaction was assessed with Fourier transform infrared spectroscopy of liposomes dried in the presence of the bdelloid LEA proteins or AavLEA1. The gel-to–liquid crystalline phase-transition temperature (T_m) of dried palmitoyl oleoyl phosphatidylcholine (POPC) vesicles (59.8° ± 1.2°C) was not affected by the presence of ArLEA1A (58.2° ± 1.1°C) or AavLEA1 (61.9° ± 5.3°C). However, ArLEA1B significantly decreased T_m to 51.8° ± 2.9°C, which indicates that it interacts with lipids. Further examination of the spectra in the asymmetric phosphate-stretching region revealed a distinct effect of ArLEA1B with a marked shoulder at 1242 cm^{-1} (Fig. 4). The peaks were resolved into two components attributed to νP=Oas$_{free}$ (1262 cm^{-1}) and νP=Oas H-bonded (1242 cm^{-1}) (*24*), similar to the effect of water and sugar (*25*). The correlation coefficients for the fitted curves were higher than 0.999. The small bonded P=O population in the absence of protein is because of interlipid charge-pair interactions between P=O and choline groups, whereas the separation of the two P=O populations is probably because ArLEA1B was only in contact with the outer monolayer of the liposomes (*26*). Clearly, a greater proportion of P=O groups are H-bonded in the presence of ArLEA1B compared with ArLEA1A (42% as opposed to 30%), whereas AavLEA1 has an intermediate value (36%). These results show that ArLEA1B has a stronger propensity to interact with dry phospholipid membranes than ArLEA1A and AavLEA1.

In summary, the bdelloid LEA proteins, encoded by gene copies representing former alleles, have different structures and functions. These functional differences are likely to be adaptive, because prevention of protein aggregation and protection of cellular membranes are essential for survival of desiccation (*10*, *27*). The presence of complementary activities in a single gene pair of a desiccation-tolerant bdelloid rotifer illustrates the potential for functional diversity resulting from divergence of former alleles. The process of abandoning sexual reproduction and meiosis, and the resulting sequence homogenization of homologous chromosomes, is similar to genome duplication, which is a major evolutionary force (*28*, *29*) that results in orthologous genes evolving relatively independently. Similarly, apomixis could drive evolutionary change by allowing former alleles to diversify in function and may partly explain how bdelloid rotifers have, without genetic exchange, diversified into almost 400 taxonomic species (*30*, *31*).

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Target Protectors Reveal Dampening and Balancing of Nodal Agonist and Antagonist by miR-430

Wen-Yee Choi,[1,2] Antonio J. Giraldez,[1,3]* Alexander F. Schier[1]*

MicroRNAs (miRNAs) repress hundreds of target messenger RNAs (mRNAs), but the physiological roles of specific miRNA-mRNA interactions remain largely elusive. We report that zebrafish microRNA-430 (miR-430) dampens and balances the expression of the transforming growth factor–β (TGF-β) Nodal agonist *squint* and the TGF-β Nodal antagonist *lefty*. To disrupt the interaction of specific miRNA-mRNA pairs, we developed target protector morpholinos complementary to miRNA binding sites in target mRNAs. Protection of *squint* or *lefty* mRNAs from miR-430 resulted in enhanced or reduced Nodal signaling, respectively. Simultaneous protection of *squint* and *lefty* or absence of miR-430 caused an imbalance and reduction in Nodal signaling. These findings establish an approach to analyze the in vivo roles of specific miRNA-mRNA pairs and reveal a requirement for miRNAs in dampening and balancing agonist/antagonist pairs.

MicroRNAs (miRNAs) are small RNA molecules ~22 nucleotides long and function to block the translation and enhance the decay of target mRNAs (*1*). Recent studies have uncovered activities of specific miRNA families and have identified hundreds of putative target mRNAs (*1*–*3*). However, the physiological roles of specific miRNA-mRNA pairs are largely unknown (*1*, *2*). To develop a method to disrupt specific miRNA-mRNA pairs, we focused on the zebrafish microRNA-430 (miR-430) family. This miRNA family is highly expressed during early zebrafish development, targets hundreds of mRNAs, and is required for embryonic morphogenesis and clearance of maternal mRNAs (*4*, *5*). Analysis of 3′ untranslated regions (3′UTRs) with sites complementary to miR-430 identified *squint* (*sqt*), a member of the Nodal family of transforming growth factor–β (TGF-β) signals, and *lft1* and *lft2*, members of the Lefty family of TGF-β signals (fig. S1). Nodals are the key regulators of mesendoderm induction and left-right axis formation, whereas Leftys act as antagonists of Nodal signaling (*6*, *7*). The balance between Nodals and Leftys

[1]Department of Molecular and Cellular Biology, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138, USA. [2]Developmental Genetics Program, New York University School of Medicine, New York, NY 10016, USA. [3]Department of Genetics, Yale University School of Medicine, New Haven, CT 06510, USA.

*To whom correspondence should be addressed. E-mail: schier@fas.harvard.edu; antonio.giraldez@yale.edu