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Correspondence and requests for materials should be addressed to P.D.C. (e-mail: petec@hgu.mrc.ac.uk).

Conservation of the sequence and temporal expression of *let-7* heterochronic regulatory RNA

Amy E. Pasquinelli*†, Brenda J. Reinhart*†, Frank Slack‡, Mark Q. Martindale§, Mitzi I. Kuroda‖, Betsy Maller‡, David C. Hayward¶, Eldon E. Ball¶, Bernard Degnan#, Peter Müller☆, Jürg Spring☆, Ashok Srinivasan, Mark Fishman**, John Finnerty††, Joseph Corbo‡‡, Michael Levine‡‡, Patrick Leahy§§, Eric Davidson§§ & Gary Ruvkun***

* *Department of Molecular Biology, Massachusetts General Hospital, and Department of Genetics, Harvard Medical School, Boston, Massachusetts 02114, USA*
‡ *Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, Connecticut 06520, USA*
§ *Kewalo Marine Lab, Pacific Biomedical Research Center, University of Hawaii, Honolulu, Hawaii 96813, USA*
‖ *Howard Hughes Medical Institute, Baylor College of Medicine, Houston, Texas 77030, USA*
¶ *Research School of Biological Sciences, PO Box 475, Australian National University, Canberra, ACT 2601, Australia*
# *Department of Zoology and Entomology, University of Queensland, Brisbane, Queensland 4072 Australia*
☆ *Institute of Zoology, University of Basel, CH-4051 Basel, Switzerland*
** *Cardiovascular Research Center, Massachusetts General Hospital, Charlestown, Massachusetts 02129, USA*
†† *Department of Biology, Boston University, Boston, Massachusetts 02215, USA*
‡‡ *Department of Molecular and Cell Biology, University of California, Berkeley, California 94720, USA*
§§ *Division of Biology 156-29, California Institute of Technology, Pasadena, California 91125, USA*
† *These authors contributed equally to this work*

Two small RNAs regulate the timing of *Caenorhabditis elegans* development[1,2]. Transition from the first to the second larval stage fates requires the 22-nucleotide *lin-4* RNA[1,3,4], and transition from late larval to adult cell fates requires the 21-nucleotide *let-7* RNA[2]. The *lin-4* and *let-7* RNA genes are not homologous to each other, but are each complementary to sequences in the 3′ untranslated regions of a set of protein-coding target genes that are normally negatively regulated by the RNAs[1,2,5,6]. Here we have detected *let-7* RNAs of ~21 nucleotides in samples from a wide range of animal species, including vertebrate, ascidian, hemichordate, mollusc, annelid and arthropod, but not in RNAs from several cnidarian and poriferan species, *Saccharomyces cerevisiae*, *Escherichia coli* or *Arabidopsis*. We did not detect *lin-4* RNA in these species. We found that *let-7* temporal regulation is also conserved: *let-7* RNA expression is first detected at late larval stages in *C. elegans* and *Drosophila*, at 48 hours after fertilization in zebrafish, and in adult stages of annelids and molluscs. The *let-7* regulatory RNA may control late temporal transitions during development across animal phylogeny.

The sequence and function of both the *lin-4* and *let-7* small RNAs are conserved in the nematode *Caenorhabditis briggsae*[1,2]. BLASTN searches reveal one DNA segment from the *Drosophila melanogaster* genome sequence, three segments from the human genome sequence on chromosomes 9, 11 and 22 bearing exact sequence matches, and two other human segments on chromosomes 9 and 21 with 20/21 matches to the *let-7* RNA (Fig. 1a). Database searches did not detect potential *lin-4* homologues in any genus except the *Caenorhabditae*. Similar stem-loop secondary structures are predicted for precursor transcripts of *Caenorhabditae*, *Drosophila* and human *let-7* RNAs (Fig. 1a). The mature 21-nucleotide (nt) *let-7* RNA may be efficiently processed from this putative precursor because only the mature RNA is detected in *C. elegans* and most

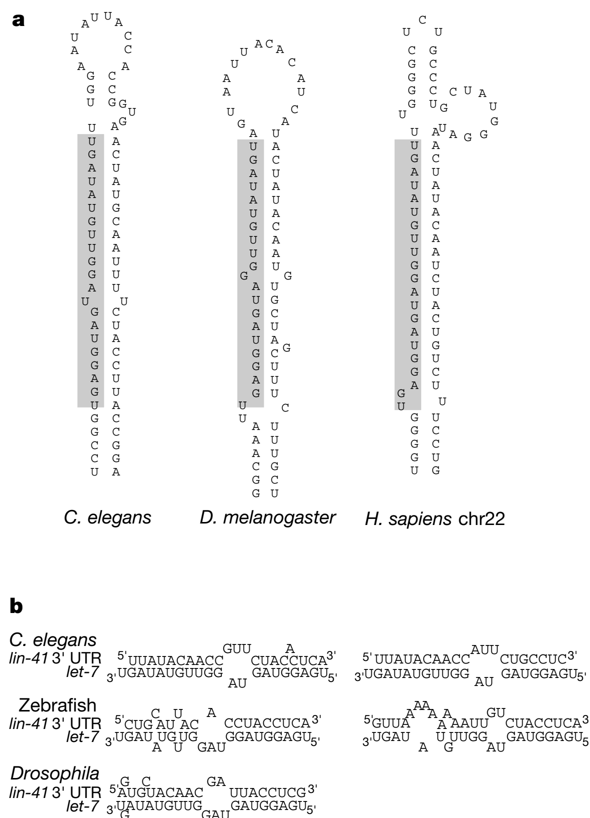

Figure 1 *let-7* gene sequences. **a**, Stem-loop structures of *C. elegans*, *D. melanogaster* and *Homo sapiens let-7* theoretical longer transcripts. The 21-nt *let-7* region is shaded. *let-7* genomic regions from *C. elegans* (Z70203), *D. melanogaster* (AE003659) and *H. sapiens* chromosome 22 (AL049853). The two human *let-7* homologous genes on chromosome 9 are tandemly arranged and separated by 369 base pairs; clustered with the human chromosome 22 *let-7* exact match is an 18/21 match to the *let-7* RNA. **b**, The 3′ UTRs of the *D. melanogaster* (AA3990768) and *Danio rerio lin-41* (AI794385) cDNAs contain *let-7* complementary sites.

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other species (see below). A similarly structured larger transcript is also predicted for the *lin-4* RNA, and rare transcripts that may correspond to it have been detected in *C. elegans*[1].

The *let-7* RNA regulates late developmental events in *C. elegans* by downregulating *lin-41* and perhaps other genes that contain sequences complementary to the small RNA in their 3′ untranslated regions (UTRs)[2,6]. *lin-41* encodes an RBCC protein that has *Drosophila* and vertebrate orthologues[6]. *let-7* complementary sites are present in the 3′ UTRs of both the *Drosophila* and zebrafish *lin-41* complementary DNAs (Fig. 1b).

let-7 RNA transcripts of ~21 nt are expressed in human tissues and *Drosophila*. (Fig. 2). As in *C. elegans*, only RNAs in the ~21-nt range were detected. The expression levels of the human *let-7* RNA varied among tissues, indicating possible cell-type regulation of *let-7* expression. As *let-7* is expressed late in animal development, it may be significant that the lowest level of human *let-7* is observed in bone marrow, which consists of a large proportion of immature cells. The *let-7* RNA from each human tissue could be the product of any or all of the several human *let-7*-like genes.

The *C. elegans* and human *let-7* RNAs were not detected with a sense strand probe and are RNase A sensitive (data not shown). Consistent with the RNA predicted by genome analysis, the sequences of the human and fly *let-7* RNAs appear identical to the *C. elegans let-7* RNA as assayed by S1 analyses (Fig. 2c). However, a single mismatch between the RNA and oligonucleotide could be tolerated in both S1 and northern analyses. Also consistent with the database searches, we did not detect *lin-4* RNA in non-nematode species (Figs 2 and 3).

The expression and function of the *let-7* RNA in *C. elegans* begins during the third larval stage, when the gene specifies a transition from late larval to adult cell fates, and continues at all subsequent stages[2]. Expression of *Drosophila let-7* is also temporally regulated: *let-7* RNA is absent until the late third instar, just before metamorphosis (Fig. 2b). At the early pupal stage, there is at least a 10-fold increase in *let-7* RNA expression that is sustained to adulthood. Thus, temporal regulation of *let-7* is similar in *Drosophila* and *C. elegans*, which are distantly related members of the ecdysozoan clade[7].

The size and temporal regulation of *let-7* are also conserved in the more distantly related lophotrochozoan clade, members of which do not moult but have larval and adult stages. For example, in two mollusc species and in a polychaete annelid, *let-7* RNA is expressed

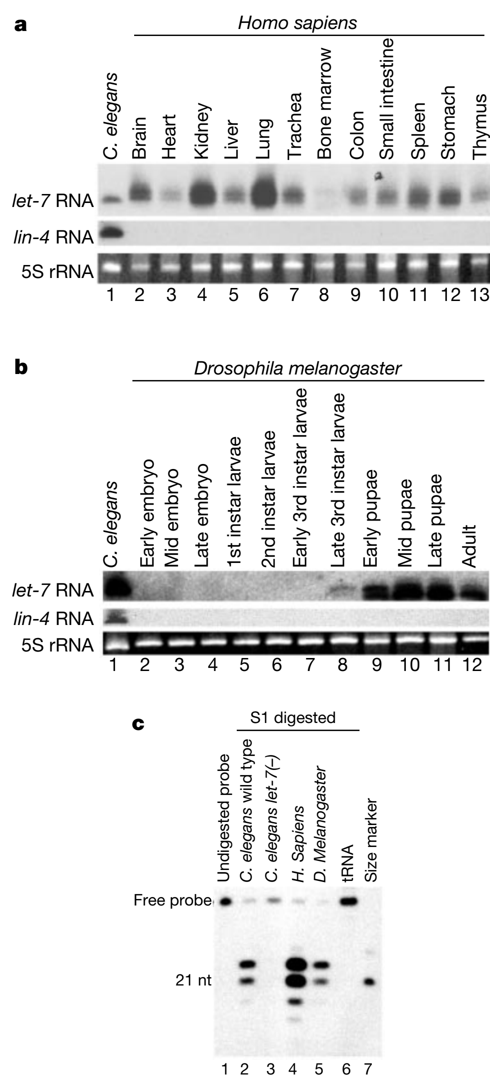

Figure 2 Expression of *let-7* RNA in human and *Drosophila*. **a**, Northern blot of total RNA from mixed stage *C. elegans* (lane 1) and human tissues (lanes 2–13) probed for *let-7* RNA and then stripped and re-probed for *lin-4* RNA. 5S rRNA serves as a loading control. **b**, Northern blot of total RNA from mixed stage *C. elegans* (lane 1) and *D. melanogaster* developmental stages (lanes 2–12), probed as in (**a**). **c**, S1 nuclease mapping detects similar transcripts in *C. elegans*, *Drosophila* and human total RNA. A 5′-end-labelled antisense strand probe undigested (lane 1) and digested after hybridization to RNA from wild-type *C. elegans* (lane 2), *C. elegans let-7(mn112)* null mutant (lane 3), human adult lung tissue (lane 4), *Drosophila* late pupal stage (lane 5) or transfer RNA (lane 6).

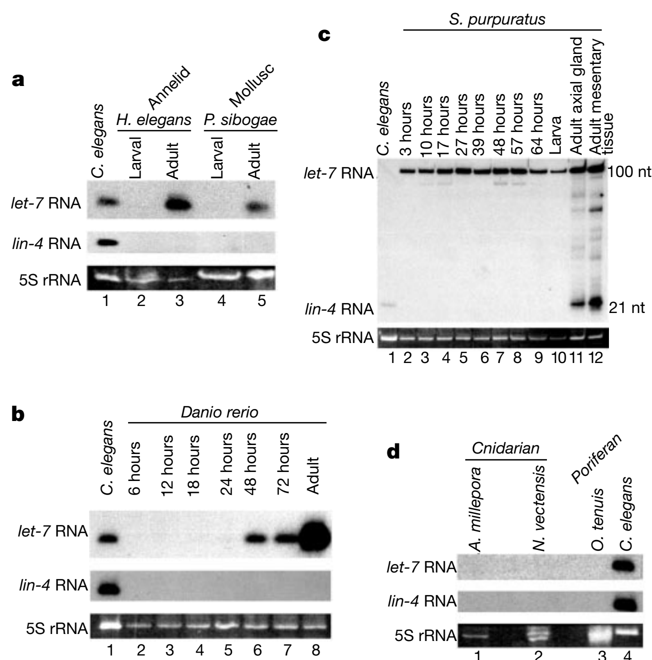

Figure 3 Expression of *let-7* RNA is developmentally regulated in lophotrochozoans and deuterostomes. **a**, Northern blot of total RNA from mixed stage *C. elegans* (lane 1), *Hydroides elegans* trocophore larvae (lane 2) and adults (lane 3), *Phestilla sibogae* veliger larvae (lane 4) and adults (lane 5), probed as in Fig. 2a. **b**, Northern blot of total RNA from mixed stage *C. elegans* (lane 1) and *D. rerio* developmental stages (lanes 2–8), probed as in Fig. 2a. **c**, Northern blot of total RNA from mixed stage *C. elegans* (lane 1) and *Strongylocentrotus purpuratus* embryonic stages 3–64 hours after fertilization (lanes 2–9), pluteus larvae (lane 10) and adult tissues (lanes 11–12), probed as in Fig. 2a. **d**, Northern blot of total RNA from adult *Acropora millepora* (lane 1), adult *Nematostella vectensis* (lane 2), adult *Ophlitaspongia tenuis* (lane 3) and mixed stage *C. elegans* (lane 4), probed as in Fig. 2a. We detected a very weak 21-nt signal in a planula stage *Acropora millepora* but it was probably caused by contamination from a bilaterian predator because these animals are collected from open sea cultures and we could not repeat this detection in an independent planula sample or in embryonic stage *A. millepora*.

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at the adult stage but not at larval stages (Fig. 3a). Thus, *let-7* may function at later stages of these species to regulate developmental progression to the adult.

Vertebrates do not develop through larval stages. But in zebrafish expression of *let-7* RNA is also temporally regulated: expression commences in the embryo between 24 and 48 hours after fertilization and continues with strong expression at the adult stage (Fig. 3b). Analyses of other deuterostomes shows expression of a 21-nt *let-7* RNA in other vertebrates, as well as in urochordate ascidians, and in a hemichordate (Fig. 4). In the echinoderm *Strongylocentrotus purpuratus*, *let-7* may be regulated at the level of precursor RNA processing rather than transcription; a ~100-nt *let-7* RNA is detected during embryonic and early larval development, but at the adult stage the 21-nt *let-7* RNA and possible processing intermediates appear (Fig. 3c).

We did not detect convincing *let-7* RNA in anthozoan and hydrozoan cnidarian species nor in two poriferan species (Figs 3d and 4). Plant and unicellular organisms also failed to show *let-7* RNA expression, consistent with database searches for those species that have been completely sequenced.

Because all three main clades of bilaterian animals express a *let-7* RNA that is temporally regulated, but cnidarian, poriferan and all non-animal species that we analysed do not express a detectable *let-7* RNA, we propose that the gene evolved after the divergence of diploblastic and bilaterian animals (Fig. 4). However, the lack of detection of *let-7* RNA in the simpler animal and non-animal species might also reflect sequence divergence or loss of the gene in the particular species tested, rather than lack of these genes in a common ancestor.

Although the observation that *let-7* homologous RNAs across phylogeny are temporally regulated does not prove that these RNAs actually specify temporal patterning in this broad range of species, the conservation of sequence, of a longer structured precursor, of the 21-nt length, of temporal regulation and of complementary sites in the *lin-41* target in two ecdysosoan and one chordate species, are strong evidence of a conserved function. There are candidate mutations that map to the location of the *Drosophila let-7* and *lin-41* genes which may provide a way to test the function of these genes in another ecdysozoan.

The *let-7* RNA is conserved across bilaterian phylogeny but the earlier acting *lin-4* RNA is not. Consistent with this, the *let-7* target *lin-41* is conserved in *Drosophila* and vertebrates[6], whereas the *lin-4* target *lin-14* appears to be unique to nematodes[5]. The *let-7*-regulated late larval transition in *C. elegans* may be ancestrally related to late transitions in other species, for example from larval to reproductive forms, whereas the earlier *lin-4/lin-14*-regulated transition may be a recent invention of the nematode phylum. Alternatively, *lin-4* and *lin-14* may evolve more quickly.

The 21-nt length of the *let-7* RNA is highly conserved, indicating that this size is central to its function. It may be significant that this length is similar to the 21–25-nt RNAs observed during RNA interference (RNAi)-directed downregulation of target messenger RNAs[8,9]. However, there are differences between the mechanisms: *let-7* is encoded and only the sense strand is expressed, whereas the sense and antisense small RNAs involved in RNAi are processed from exogenously supplied double-stranded RNAs that are hundreds of base pairs long[8–12]. RNAi degrades target mRNA[8–12], whereas the heterochronic *lin-4* RNA affects the translation but not the stability of its target mRNAs[5,13]. Also, mutations in *C. elegans* genes that confer resistance to RNAi[14] do not have heterochronic phenotypes indicative of a defect in *lin-4* or *let-7* regulation of target genes (C. Mello, personal communication). One common feature of RNAi and the putative precursors of the heterochronic RNAs is longer regions of duplex RNA (Fig. 1a), suggesting that similar processing and amplification pathways could generate these 21-nt RNAs.

Two ~21-nt RNAs regulate *C. elegans* temporal development, and we argue that one of these RNAs is likely to regulate developmental timing in bilaterian animals. We propose that these types of RNAs be called small temporal RNAs (stRNAs). Genome sequence comparisons and expression analyses among bilaterian animals may reveal additional stRNAs that regulate other developmental transitions. □

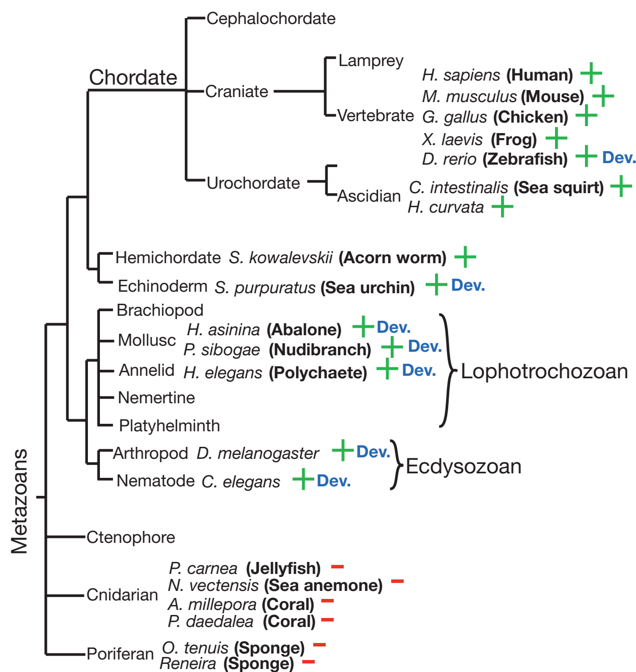

Figure 4 Phylogenetic comparison of *let-7* RNA expression. Phylogenetic tree showing species that do (+) and do not (–) express *let-7* RNA. Species in which we detect the conserved developmental pattern of *let-7* RNA expression (no *let-7* RNA in early stages but *let-7* expression by adulthood) are indicated by 'Dev.'. The full genus and species names are as follows: *Homo sapiens*, *Mus musculus*, *Gallus gallus*, *Xenopus laevis*, *Danio rerio*, *Ciona intestinalis*, *Herdmania curvata*, *Saccoglossus kowalevskii*, *Strongylocentrotus purpuratus*, *Haliotis asinina*, *Phestilla sibogae*, *Hydroides elegans*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Podocoryne carnea*, *Nematostella vectensis*, *Acropora millepora*, *Platygyra daedalea*, *Ophlitaspongia tenuis* and *Reneira* sp.

Methods

Human total RNA samples were purchased from Clontech. Total RNA preparation, northern analysis and S1 nuclease protection assays were performed as described[2]. Oligonucleotides used as northern probes were *let-7* 5′-AACTATACAACCTACTACCT-CACCGGATCC-3′ and *lin-4* 5′-ATAGTACACTCACACTTGAGGTCTCAGGG-3′. 5S rRNA was detected by ethidium bromide staining of the polyacrylamide gels before transfer. Oligonucleotides used for S1 nuclease analyses were 5′-CTATACAACCTAC-TACCTCACCGGAT-3′ (3′ end match to *C. elegans* genomic region), 5′-ACTATA-CAACCTACTACCTCACCCCA-3′ (3′ end match to human genomic region) and 5′-ACTATACAACCTACTACCTCAATTTGC-3′ (3′ match to *Drosophila* genomic region).

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Correspondence and requests for materials should be addressed to G.R.

Polarization of the anterior–posterior axis of *C. elegans* is a microtubule-directed process

Matthew R. Wallenfang & Geraldine Seydoux

Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, USA

In *Caenorhabditis elegans*, polarity along the anterior–posterior (A/P) axis is established shortly after fertilization and is determined by the sperm, whose position specifies the posterior end of the embryo[1]. Although many factors required for the establishment of A/P polarity have been described[2,3], the nature of the spatial cue provided by the sperm remains unknown. Here we show that a microtubule-organizing centre is necessary and sufficient to establish several aspects of A/P polarity. In wild-type embryos, appearance of the first molecular asymmetries along the A/P axis correlates with and requires nucleation of microtubules by the sperm-derived centrosomes (sperm asters). In mutant embryos arrested in meiosis, sperm asters fail to form, and posterior is defined by the position of the persistent meiotic spindle rather than by the position of the sperm. Together, our data indicate that the primary spatial cue for A/P polarity in *C. elegans* derives from microtubules emanating from the sperm asters. Our findings support a parallel[4–7] between *C. elegans* zygotes and other cells, such as *Drosophila* oocytes, which rely on microtubules to regulate polarity.

Caenorhabditis elegans oocytes appear to have no predetermined A/P polarity[1]. The first manifestations of polarity are seen roughly 30 min following fertilization, after the oocyte chromatin, which had been arrested in prophase of meiosis I, completes meiosis. At that time, several proteins become localized asymmetrically along the long axis of the embryo: in particular, PAR-2 localizes to the cortex nearest the sperm pronucleus (future posterior end), and PAR-3 localizes in a reciprocal pattern on the cortex opposite the sperm pronucleus[2] (future anterior end; Fig. 1a). Each of these is required to localize the PAR-1 kinase to the posterior cortex. PAR-1, in turn, is required for the asymmetric segregation of several factors in the cytoplasm, including PIE-1 and the germline-specific P granules which localize to the posterior[2] (Fig. 1a). Segregation of P granules has been correlated with an internal flow of cytoplasm directed towards the sperm pronucleus, which also occurs during this period[8,9] (Fig. 1a). The sperm pronucleus itself is dispensable for polarity[10], leaving open the question of which sperm-derived component is responsible for polarizing the embryo.

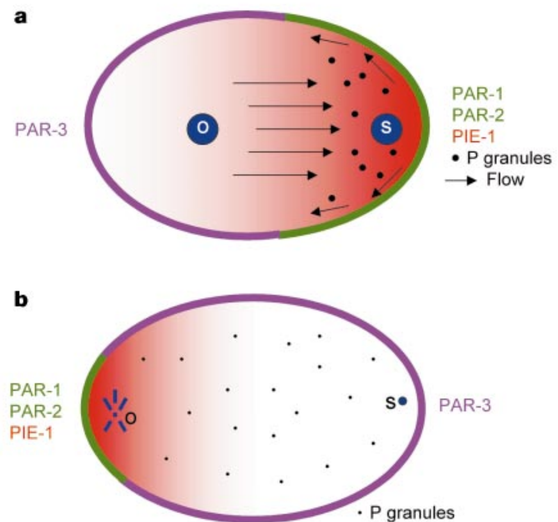

Figure 1 Establishment of A/P polarity in *C. elegans*. **a**, Polarity in wild-type embryos. Embryo is shown in first mitotic prophase during pronuclear migration. In most fertilization events, sperm entry occurs on the side opposite the oocyte nucleus[1]. In this and Figs 2 and 3, the position of the oocyte chromatin is indicated by an 'O' and that of the sperm chromatin by an 'S' (in some figures, the sperm chromatin is not visible in the focal plane shown). Arrows indicate cytoplasmic flow, which flows towards the sperm pronucleus in the interior of the embryo and away from it along the cortex. Shown are the asymmetric distributions of PAR-1 and PAR-2 (green), PAR-3 (magenta), PIE-1 (red) and P granules (dots). **b**, Polarity in *mat* mutant embryos. The oocyte chromatin is arrested in metaphase of meiosis I and the sperm chromatin remains condensed.

To address this question, we have analysed a collection of temperature sensitive mutants (*mat-1*, *mat-2*, *mat-3*, *emb-27*, *emb-30)* that arrest shortly after fertilization while the oocyte chromatin is in metaphase of meiosis I (A. Golden, P. Sadler, M.R.W., G.S. and D. Shakes, manuscript in preparation). Consistent with their metaphase arrest phenotype, *mat-1* and *emb-30* encode *C. elegans* homologues of the anaphase-promoting complex (APC) components Cdc27 (J. Schumacher, D. Shakes and A. Golden, personal communication) and APC4/Lid1 (ref. 11), respectively.

Because fertilization usually occurs opposite the oocyte nucleus, mutant embryos in this collection arrest with the oocyte chromatin in meiosis at one end of the embryo and condensed sperm chromatin at the other end (Fig. 1b). To determine whether A/P polarity can be established under these conditions, we determined the distribution of PIE-1, which in wild type segregates towards the sperm pronucleus after meiosis is completed. We observed asymmetric PIE-1 in 48% of *mat-1* mutant embryos (Table 1). Notably, when asymmetric, PIE-1 always was enriched on the side of the oocyte chromatin rather than on the sperm side (Fig. 2a). Similar results were obtained with the other mutants (Table 1). Examination of live embryos expressing a PIE-1–GFP (green fluorescent protein) fusion showed that PIE-1 becomes asymmetric in all *mat-1* embryos (Fig. 2b); however, this asymmetry is transient, causing it to be observed in only 48% of embryos in asynchronous populations (Table 1).

Immunostaining with PAR-1, PAR-2 and PAR-3 antibodies showed that PAR asymmetry is also 'reversed' in *mat-1* mutants: PAR-1 and PAR-2, which in wild type localize to the cortex nearest the sperm, were on the cortex nearest the oocyte chromatin in *mat-1*

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